A Pulmonary Formulation of L-Dopa Enhances its Effectiveness in a Rat Model of Parkinson’s Disease

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Abbreviations: AUC, Area under the curve; C\textsubscript{max}, Maximal plasma concentration; DOPAC, Dihydroxyphenylacetic acid; L-Dopa, L-Dihydroxyphenylalanine; 6-OHDA, 6-Hydroxydopamine; PD, Parkinson’s disease; T\textsubscript{max}, Time of maximal plasma concentration.
ABSTRACT

The efficacy of oral L-Dopa becomes problematic with the progression of Parkinson’s disease, due in large part to a lost ability to accommodate L-Dopa’s inherently poor pharmacokinetics. Pulmonary delivery represents a novel approach to reducing this problem. L-Dopa was formulated into inhalable (AIR™) particles and its pharmacokinetics and pharmacodynamics compared to those of an oral formulation. Pulmonary administration of L-Dopa (2mg) to rats resulted in a rapid elevation of plasma levels ($C_{\text{max}} = 4.8 \pm 1.10 \mu g/ml$ at 2 min), while oral administration of L-Dopa produced a much delayed and lower $C_{\text{max}} (1.8\pm 0.40 \mu g/ml$ at 30 minutes). In a rat model of Parkinson’s disease (unilateral 6-OHDA lesion), the pulmonary formulation of L-Dopa (0.5-2.0 mg) yielded more rapid and robust elevations in striatal L-Dopa, dopamine and DOPAC levels, as well as 2.5 to 3.7 times as many c-fos-expressing striatal neurons. Moreover, motor function was significantly improved by 10 minutes after administration, with peak improvements occurring within 15 to 30 minutes. In contrast, considerably higher doses (6.8 to 10mg) of orally administered L-Dopa took over three times longer to produce similar effects. These results suggest that an inhalable formulation of L-Dopa has superior pharmacokinetic properties and may provide patients with a more effective form of rescue therapy as well as being a reliable adjuvant or replacement for first-line oral therapy.
INTRODUCTION

Over 2.1 million people worldwide suffer from Parkinson’s disease (PD), a neurological syndrome characterized by bradykinesia, postural instability, rigidity and involuntary tremor (Lanza and Nagle, 2002). These motor symptoms result from the progressive degeneration of dopaminergic neurons in the pars compacta of the substantia nigra, which project to the striatum (Dunnett and Bjorklund, 1999). Administration of the dopamine precursor L-Dopa (Cotzias et al., 1967) combined with the dopamine decarboxylase inhibitor carbidopa (Dunner et al., 1971) help control the neurological manifestations of PD by enhancing dopamine levels in the surviving dopaminergic neurons of the substantia nigra, thereby restoring neurotransmission.

L-Dopa provides a clear therapeutic benefit for most early-stage PD patients, where the duration of its pharmacodynamic effects far exceeds its plasma half-life. This reflects the ability of dopaminergic neurons to decarboxylate L-Dopa to dopamine and then store sufficient amounts of dopamine to maintain adequate motor function long after plasma L-Dopa levels have fallen. This storage capacity buffers the fluctuations in plasma L-Dopa levels that result from its variable oral bioavailability (Rivera-Calimlim et al., 1971, Djaldetti et al., 1996), rapid metabolism and inherently poor pharmacokinetic profile (Mouradian et al., 1988, Nutt and Holford, 1996). However, the benefits of L-Dopa therapy decline as PD progresses (Barbeau, 1974, Marsden and Parkes, 1976). This decrease in the duration of responsiveness to L-Dopa is related to the progressive degeneration of nigral dopaminergic neurons (Diamond et al., 1987) and the subsequent loss of dopamine buffering. The resulting decline in pharmacodynamic efficacy (“wearing-off” phenomena, Fabbrini et al., 1988) mirrors the transient
pharmacokinetic profile of L-Dopa, resulting in the rapid return of PD symptoms consistent with its rapid metabolism ($t_{1/2} \approx 90$ min) of L-Dopa (Marsden and Parkes, 1976, Nutt, 1987). As nigral neurodegeneration becomes more severe, “on-off” fluctuations in motor function, characterized by sudden, unpredictable cycling between states of over-treatment and sub-optimal therapy (Mouradian et al., 1988) begin to occur more frequently.

Whether the patient suffers from motor fluctuations of the “wearing-off” or “on-off” variety, they often find themselves suddenly incapable of effective movement. An emerging treatment modality for dealing with the sudden loss of motor function and improved control of symptoms in patients with moderate PD is referred to as rescue therapy (Tolosa et al., 1994). The intent of this paradigm is to rapidly re-establish dopaminergic activity and motor function by administering an additional, non-scheduled dose of L-Dopa when the patient does not respond to the scheduled dose, or when its effects have worn off prior to the next scheduled dose. Nonetheless, significant problems with the use of oral L-Dopa for rescue therapy remain, including variable gastrointestinal absorption and a relatively long time (30-45 min) before PD symptoms are controlled (Melamed et al., 1999). This delay not only requires that patients suffer while waiting for the rescue dose to take effect, but the excursions in plasma concentrations may contribute to the development of serious motor complications, such as dyskinesias sufficiently severe to require hospitalization (Olanow and Obeso, 2000).

The need for rapid “rescue” of patients with PD in the face of declining efficacy of L-Dopa, complicated by the variable bioavailability and poor pharmacokinetics of oral formulations have led us to develop a pulmonary formulation of L-Dopa. This was
accomplished using a novel pulmonary delivery technology (Alkermes AIR™) involving the formulation of L-Dopa into aerodynamic, biocompatible microparticles (Edwards et al., 1997) and their delivery deep into the lungs using a robust, yet compact inhalation device (Dunbar et al., 2002). Unlike aerosols and large particles, AIR™ particles have aerodynamic properties that allow for their dispersal deep into the lungs where they are deposited upon the alveolar membranes. This allows the particles to rapidly deliver the active principle into the systemic circulation. Drugs absorbed through the lungs then enter the heart via the pulmonary artery and are transported directly to the brain via the ascending aorta and carotid arteries. Thus, the pulmonary route may provide a means for the rapid and efficient delivery of L-Dopa to the brain, avoiding the gut and its associated problems of erratic absorption and extensive metabolism.

The following investigations compare the pharmacokinetic, neurochemical and behavioral characteristics of L-Dopa administered using oral and pulmonary formulations delivered to a rodent model of PD. These studies demonstrate several advantages of pulmonary delivery of L-Dopa over the oral route, and establish the concept that an inhalable formulation of L-Dopa may provide a relatively simple and effective means for improving the treatment of PD patients.
METHODS

Animals

Male Sprague-Dawley rats (450 ± 50 grams; Taconic Farms, Germantown, NY) were used in all studies. Rats were pair-housed in polypropylene cages with free access to food and water. The vivarium was maintained on a 12-hour light:dark cycle with a room temperature of 22 ± 1°C and relative humidity level of 50 ± 5%. All studies were approved by Alkermes Institutional Animal Care and Use Committee and were conducted in adherence with the NIH “Guide for the Care and Use of Laboratory Animals”.

Preparation of Inhalable L-Dopa

Inhalable L-Dopa particles were prepared using proprietary techniques developed by Advanced Inhalation Research (AIR). L-Dopa and excipients (9 g/l) were mixed into a spray drying solution with ethanol/water 70/30 (v/v) as the solvent. The solution was then introduced into a NIRO spray dryer at 40 ml/min and atomized into droplets with a rotary atomizer at 20,000 rpm. The droplets contact the drying gas and the dry particles were collected with a 6” cyclone. The final loading density of L-Dopa in the particles is 20%.

L-Dopa Pharmacokinetics

Determinations of L-Dopa plasma levels were made following oral gavage or insufflation into the lungs. Rats received an intraperitoneal (i.p.) injection of the peripheral decarboxylase inhibitor carbidopa (200 mg/kg) 1-hour before administration of L-Dopa. Under ketamine/xylazine/acepromazine (33/1.7/10 mg/kg) anesthesia, the animals received femoral artery catheters for blood sampling, and were subsequently
were divided into 2 groups. The first group of animals was orally administered L-Dopa (2 mg) suspended in 1 ml of saline containing 3% carboxymethylcellulose and 1% ascorbic acid. The second group was administered AIR-L-Dopa particles (20% loading density) via pulmonary insufflation. A laryngoscope was used to visualize the rat’s epiglottis and a blunt-tip insufflator (PennCentury Insufflation powder delivery device) inserted into the airway. A bolus (3 ml) of air from an attached syringe was used to deliver the pre-loaded powder from the chamber of the insufflator into the rat’s lungs. No more than a total of 10 mg of powder (2 mg L-Dopa) was delivered. Blood samples (500 µl) were drawn from each animal at the femoral cannula at the following time points: 0 (immediately prior to L-Dopa administration), 2, 5, 15, 30, 60, 120, and 240 minutes following L-Dopa administration, and placed into heparinized tubes containing 20 µl of 1 M sodium metabisulfite. Following each blood sample, an equivalent volume of isotonic saline was injected through the cannula to maintain circulating fluid volume. Plasma (≈ 200 µl) was obtained from the blood samples by centrifugation at 1000-x g for 15 minutes, then stored at -80° until analyzed. Striata were disrupted using a probe sonicator (10 sec) in diluent containing 200 mM HClO₄, 0.05%, Na₂S₂O₅ and 0.05% Na₂ EDTA, centrifuged at 20,000 x g for 20 min, and the supernatant retained and stored at -70° C until analysis.

**Catecholamine Quantitation**

Samples were loaded into an auto-injector (6º) and 5-50 µl injected onto a Supelcosil LC-18 column (4.6 mm x 150 cm, 5 µm particle size) maintained at 37° C.

The mobile phase consisted of 0.1 M KH₂PO₄, 80 mM acetic acid, 0.1% Na₂EDTA, 0.4% Na heptanesulfonic acid, and 1.75% acetonitrile, pH 3.1, running at a
flow rate of 2 ml/min. Catecholamine peaks were determined using electrochemical detection (Coulochem II, ESA, oxidation cell +250 mV; screening cell +75 mV/100 nA), and reduction cell –375 mV/20 nA. Total running time was 40 min, with the following retention times: DOPAC 6.3 min; L-Dopa 7.3 min, DHBA (internal standard) 13 min; and dopamine 23.5 min.

**c-Fos Immunohistochemistry**

Sixty minutes after administration of L-Dopa, 6-OHDA lesioned rats were deeply anesthetized and transcardially perfused with 200 mls of heparinized PBS, then 500 mls of 4% Zamboni’s fixative. The brains were removed from euthanized subjects, fixed over-night in Zamboni’s fixative and then placed in 30% sucrose/PBS for cryoprotection. Brains were frozen and cut on a sliding knife microtome at 40 µm.

Sections were first washed in a Tris-buffered saline solution with TritonX-100. Endogenous peroxidase elements were removed with a 20 min incubation in 0.1M sodium peroxide. Background staining was suppressed by incubating the sections for 1 hour in a Tris-buffered saline solution containing 0.1% Triton-X and 3% normal goat serum. Sections were incubated for 24-hours in primary antibody (1:2500; Santa Cruz SC7202), washed and then incubated for 1 hour in biotinylated goat anti-rabbit secondary antibody (1:200; Vector Labs). After washing, the processing was completed by incubating sections for 75 minutes in avidin-biotin complex (1:500; Elite, Vector Labs), using a chromogen consisting of 2.5% nickel II sulfate, 0.05% diaminobenzidine, and 0.005% hydrogen peroxide. Control tissue was treated in the same manner minus the primary antibody, and no staining was observed. All the sections were mounted, dehydrated, and coverslipped for imaging.
The 6-hydroxydopamine (6-OHDA) treated Rat Model of Parkinson’s Disease:

Pharmacodynamic Testing

Rats were anesthetized with ketamine/xylazine/acepromazine (33/1.7/10 mg/kg) and positioned in a stereotaxic instrument. A total of 12 µg of 6-OHDA in 6 µl of 0.9% saline/ascorbic acid (0.2 µg/µl) was unilaterally infused into the medial forebrain bundle (4.2 mm posterior and 1 mm lateral to bregma, 7.4 mm ventral to dura) at a rate of 1 µl/min, then allowed to diffuse for 5 min before the infusion cannula was slowly retracted (Lindner et al., 1996). Beginning two weeks after surgery, animals were tested for apomorphine-induced rotation behavior every week for three weeks (Ungerstedt, 1971). For this test, animals received an i.p injection of apomorphine (0.25 mg/kg for the first test and 0.1 mg/kg for the following two tests) and were placed into a cylindrical Plexiglas bucket. Each 360-degree rotation was counted for 30 minutes and only those animals exhibiting >200 rotations/30 minutes were used in behavioral testing. Twelve of thirty animals passed this objective, a priori test.

Animals passing the apomorphine challenge were subsequently tested in a series of motor function tasks known to be sensitive to nigra-striatal neurodegeneration (Schallert and Tillerson, 2000). These tests allow multiple neurological evaluations in the same animal, thus permitting each animal to be used as its own control when comparing the efficacy of pulmonary versus oral L-Dopa in enhancing motor function. For drug delivery prior to behavioral testing, animals were briefly anesthetized using 1% isoflurane. Individual test sessions were separated by one week.
Placing Task: The “placing task” requires rats to make a directed forelimb movement in response to sensory stimuli. Rats were held so that their limbs were hanging unsupported. They were then raised to the side of a table so that their bodies were parallel to the edge of the table. Each rat received 10 consecutive trials with each forelimb and the total number of times the rat placed its forelimb on the top of the table was recorded. Prior to each test day, animals received an IP injection of the peripheral decarboxylase inhibitor carbidopa (200 mg/kg) as described above. Animals then received L-Dopa orally (6.8 or 10 mg) or by inhalation (0.5, 1.0 or 2.0 mg) and were tested 15, 30, 60 and 120 minutes later. Throughout testing with oral and pulmonary delivery of L-Dopa, each animal received every possible drug combination in a randomized fashion.

Bracing Task: This test was performed using the same animals immediately after placement testing. Rats were placed on a smooth stainless steel surface and gently pushed laterally 90 cm at approximately 20 cm/second. The number of steps the rat took with the forelimb on the side in which the rat was moving was recorded. Each trial included moving the rat 2 times in each direction.

Akinesia Task: The akinesia test was performed immediately after the placement and bracing tasks. In this test, the subject was supported on one forelimb and allowed to move on its own. The number of steps taken with the forelimb upon which the rat was standing was recorded during separate, 30-second trials for each forelimb.
RESULTS

L-Dopa Pharmacokinetics

Plasma levels of L-Dopa were elevated faster following pulmonary delivery of L-Dopa (2 mg) than after oral administration (2 mg, Figure 1, Table 1). Following insufflation, peak plasma levels ($T_{\text{max}}$) of L-Dopa were reached at the earliest time point measured (2 min), decreasing within 15 minutes of administration while remaining elevated for over 120 minutes (Figure 1, Table 1). In contrast, oral administration of L-Dopa resulted in a more gradual increase in plasma L-Dopa levels, peaking 15-30 minutes following administration, then decreasing gradually over the next 1-2 hours.

The oral and pulmonary routes of L-Dopa delivery had a significant impact on striatal levels of L-Dopa, dopamine and DOPAC in rats with unilateral 6-OHDA lesions (Figure 2). Lesioned animals administered L-Dopa by inhalation had significantly (>4-fold) higher L-Dopa levels on both sides of the striatum 5 minutes after administration than rats receiving oral L-Dopa ($p<0.01$, Figure 2A). Striatal L-Dopa levels in rats receiving pulmonary L-Dopa remained elevated by approximately 2-fold above those in orally administered rats out to 30 minutes. L-Dopa levels on the lesioned and normal sides of the striatum were not significantly different from each other, consistent with equivalent access of L-Dopa to these regions through the vasculature. In contrast to L-Dopa levels, dopamine levels in the unlesioned side were significantly higher than in the lesioned side, for both treatment groups. However, pulmonary delivery of L-Dopa produced significantly greater dopamine levels in the striatum both ipsi- and contralateral to the lesion. Dopamine levels at 15 and 30 minutes after pulmonary delivery were more than 2-fold higher than after oral administration ($p<0.01$, Figure 2B).
Note the time differential between the increase in striatal L-Dopa and dopamine levels in the pulmonary delivery group, consistent with a temporal lag associated with the conversion of L-Dopa to dopamine. Finally, DOPAC levels in the lesioned striata of the pulmonary delivery group were significantly elevated above those of the oral L-Dopa treated animals at both 15 and 30 minute time points (p<0.01, Figure 2C). This is consistent with a selective increase of approximately 2.5-fold in the turnover of dopamine released from synaptic terminals arising from neurons residing in the substantia nigra on the lesioned side following pulmonary L-Dopa administration.

**c-fos Expression**

The number of c-fos expressing neurons was generally higher in the striatum ipsilateral to the nigral lesion following L-Dopa administration (Treatment x side interaction, F(3,1)= 15.5, P<0.05, two-way ANOVA). Oral administration of 2 mg of L-Dopa had no significant effect on the number of c-fos expressing neurons in the striatum on either the lesioned or unlesioned side (Figures 3 A, B, 4) relative to vehicle treatment. Only after the oral administration of 10 mg of L-Dopa was a significant increase in the numbers of c-fos expressing neurons observed. In contrast, the number of neurons expressing c-fos mRNA following inhalation of 2 mg of L-DOPA was 5-30 times higher than in vehicle treated animals, and 2-3 times higher than the 2 mg oral L-Dopa groups (Figures 3C, D, 4).
Motor Function

**General**: Pulmonary delivery of L-Dopa was well tolerated and there were no obvious deleterious effects of delivering L-Dopa directly to the lungs either acutely or over repeated sessions. All animals rapidly recovered from isoflurane anesthesia performance with use of the unimpaired limb returning to pre-anesthesia levels in all animals in less than 10 minutes. No animal exhibited any distress from pulmonary L-Dopa during motor testing. Although formal toxicological tests were not conducted in these studies, it is worth mentioning that in pilot studies a qualitative examination of the lungs by a trained pathologist established that delivery of the pulmonary L-Dopa formulation did not produce any gross damage to the lungs. This is consistent with more formal toxicology tests performed with other AIR formulations that failed to show significant toxicological effects in several species, even with chronic administration (Alkermes, unpublished regulatory documents).

**Placing Task**: At baseline (T=0; immediately prior to L-Dopa), the subjects performed nearly perfectly on this task using the limb on the unlesioned side, making greater than 9/10 appropriate placements. In contrast, the animals were markedly impaired in their ability to perform the same task when using the limb on the lesioned side, making approximately 1 response over the 10 trials. Oral L-Dopa dose-dependently improved the performance of the impaired limb (Figure 5A). At the highest dose tested (10 mg), motor performance significantly improved within 30 minutes (p<0.05, 2-way ANOVA and Tukey’s test) and peaked between 1-2 hours after drug administration. The lower dose of oral L-Dopa (6.8 mg) also improved performance
slightly, with maximal effects at 60 minutes and stable performance thereafter. No changes were noted following administration of saline.

Performance of the placing task improved rapidly following pulmonary delivery of L-Dopa (Figure 5B). At the highest dose tested (2 mg), significant improvements occurred within 10 minutes, and peak benefits were reached within 15-30 minutes (as opposed to 1-2 hours with oral administration). These effects were dose-related, with significant improvements seen using doses as low as 0.5 mg of L-Dopa after 1 hour. Not only did the behavioral improvements occur more rapidly following pulmonary delivery of L-Dopa, but also markedly lower total doses were required. For example, the extent of recovery with 10 mg of L-Dopa given orally was comparable to the recovery seen with 1 mg (≈ 3mg/kg) of L-Dopa given by the pulmonary route.

Accordingly, when the L-Dopa doses are normalized by body weight, this represents a 10-fold difference in the dose of drug required to produce equivalent peak efficacy. Moreover, the duration of motor improvement is comparable using either delivery route.

**Bracing Task:** Rats with 6-OHDA lesions of the substantia nigra manifest a profound impairment in their ability to perform this task with the limb on the lesioned side, making approximately 3 responses compared to approximately 7 braces with the unaffected limb. Again, oral L-Dopa administration improved performance of this task in a dose-related manner (Figure 6A), with 10 mg L-Dopa significantly improving motor performance by 30 minutes. Maximal effects were reached within 60 minutes and remained consistently elevated thereafter. A lower dose of oral L-Dopa (6.8 mg) did not significantly improve performance. Vehicle administration had no effect on performance of the affected limb.
In contrast to oral L-Dopa, bracing performance was rapidly improved following pulmonary administration of L-Dopa (Figure 6B). Significant enhancements were seen within 10 minutes of delivery, with peak benefits observed within 15-30 minutes (as opposed to 30-60 minutes after oral administration). These effects were dose-related, with statistically significant improvements seen with 1 mg L-Dopa.

**Akinesia Task:** As was seen with the placing and bracing tests, 6-OHDA treated rats were profoundly impaired in their ability to perform the akinesia task using the limb on the lesioned side. While the animals made approximately 17 steps with the normal limb, they made less than half this number with the impaired limb (range = 0-10 steps). Oral L-Dopa improved performance on this task in a dose-related manner. Administration of 10 mg L-Dopa significantly improved performance within 60 minutes (Figure 7A). A lower oral dose of L-Dopa (6.8 mg) produced the same pattern of recovery although the absolute magnitude of improvement was slightly less than that seen with the higher dose of L-Dopa. Treatment with saline vehicle did not affect performance.

Performance of the akinesia task was rapidly improved following pulmonary administration of L-Dopa, as opposed to oral administration. Significant improvements in function were seen within 10 minutes of pulmonary delivery of 2 mg L-Dopa, with peak benefits observed within 15-30 minutes (Figure 7B). These effects were dose-related, with modest, but statistically significant (p<0.05) improvements seen after 1.0 mg (120 min).

**DISCUSSION**
The current basis for effective symptomatic treatment of PD requires a sustained stimulation of striatal dopaminergic receptors as a replacement for lost dopaminergic input from neurons in the substantia nigra pars compacta (Nutt and Holford, 1996). Currently, oral administration of the dopamine precursor L-Dopa, in combination with the aromatic amino acid decarboxylase inhibitor carbidopa (Dunner et al., 1971), remains an important component of the therapeutic regimen for treating PD.

Nonetheless, serious fluctuations in motor response gradually develop with continued use of oral L-Dopa. These fluctuations in efficacy include a decay in therapeutic effect (“wearing-off” phenomena), unpredictable variations (“on-off” phenomena), and serious motor defects (e.g., dyskinesias) correlated with high plasma L-Dopa levels. These motor irregularities result from a combination of factors, including extreme fluctuations in plasma levels of L-Dopa associated with the oral route of administration. The involvement of a pharmacokinetic component in the development of these motor abnormalities is indicated by their reversal following careful control of plasma L-Dopa levels, either through continuous intravenous (Fabbrini et al., 1988) or intraduodenal L-Dopa infusions (Sage et al., 1988, Kurth et al., 1993). The data presented in this manuscript suggest that a novel, non-invasive mechanism for delivering L-Dopa through the pulmonary route may offer significant pharmacokinetic, and thus, therapeutic advantages over oral L-Dopa therapy for PD patients.

Insufflating rats with a formulation of aerodynamic L-Dopa particles increased plasma L-Dopa levels 5 times faster than an equivalent dose of orally administered L-Dopa in solution ($T_{\text{max}} = 4.7$ vs. 24 min, respectively). In addition, the total exposure of the subjects to L-Dopa is significantly higher after pulmonary than oral administration.
(AUC: 570 vs. 255 µg ml\(^{-1}\) min, P=0.01, suggesting that inhalable L-Dopa has enhanced bioavailability. The impact of improved L-Dopa delivery through the lungs was subsequently manifested at the biochemical, cellular and whole animal levels. L-Dopa levels in both lesioned and unlesioned striata increased 2- to 5-fold above those observed following oral L-Dopa administration as early as 5 minutes after delivery. Similarly, striatal dopamine levels in rats treated with the pulmonary L-Dopa formulation were significantly higher than the oral treatment group by 15 minutes after administration. Furthermore, the rate of dopamine turnover in the lesioned striatum was enhanced significantly more rapidly in the inhalable L-Dopa group.

Concurrent with the increase in striatal levels of L-Dopa and dopamine was an increase in neuronal dopamine release and striatal neuron activity. This was substantiated by elevations in striatal DOPAC and the expression of the intermediate early gene, c-fos (Robertson et al., 1989, Morelli et al., 1993) in striatal neurons, particularly those ipsilateral to the lesioned nigra. Enhanced neuronal c-fos expression in the striatum on the lesioned side likely results from L-Dopa-related activation of postsynaptic D1 and/or D2 receptors, which are present in elevated density due to denervation supersensitivity (Gerfen et al., 1990, Paul et al., 1992). While the presence of L-Dopa increased the number of c-fos expressing neurons throughout the striata regardless of treatment modality, this effect was particularly clear in the lesioned striata of rats insufflated with L-Dopa. Moreover, insufflated L-Dopa (2 mg) was 5 times more potent, and almost 4 times more efficacious than oral L-Dopa (2 mg) in inducing expression of c-fos by 60 minutes after administration.
While the pharmacokinetic, neurochemical and histological evidence for enhanced rapidity of onset and potency of inhaled L-Dopa is compelling, the ability to improve motor function remains the ultimate goal of any therapeutic modality for PD. Using spontaneous (i.e., non-drug-induced) measures of parkinsonian symptoms in rats with unilateral nigral lesions (Lindner et al., 1996, Schallert and Tillerson, 2000), we found that insufflated L-Dopa provided a consistently faster onset of action as well as increased potency. In every motor task investigated (placing, bracing and akinesia tasks), pulmonary delivery of 2 mg of L-Dopa significantly improved motor function at the earliest time point measured (5 minutes post-administration). Restoration of movement was completed by no later than 30 minutes after pulmonary administration and was sustained throughout the 2-hour test period. In contrast, oral administration required over 5 times the amount of L-Dopa to achieve substantial, albeit incomplete, restoration of motor function. Moreover, these improvements required at least 60 minutes to become fully manifested after oral administration.

Collectively, these data suggest that administration of L-Dopa via the pulmonary route shortens the time of therapeutic onset while being minimally invasive. Thus, this pulmonary formulation may provide significant benefits for the treatment of PD, particularly as it relates to improving the reliability and efficacy of rescue therapy and potentially suppressing the development of motor irregularities linked to long-term, oral L-Dopa use, as discussed below.

Rescue therapy (Tolosa et al., 1994) consists of the administration of an unscheduled oral dose of L-Dopa or dopamine receptor agonist in order to re-establish motor function in PD patients suffering from wearing-off phenomena. The use of oral L-
Dopa as rescue therapy remains problematic due to variable uptake from the gut and the long interval before restoration of motor function (Melamed et al., 1999). The data provided in this manuscript suggest that a pulmonary formulation of L-Dopa might overcome the most serious deficiencies associated with oral L-Dopa rescue therapy. While the plasma level of L-Dopa required for successful rescue therapy is unclear, effective oral doses of L-Dopa for this purpose most often range between 50 to 100 mg. Given that the bioavailability of L-Dopa administered by the pulmonary route is superior to that of the oral route, a sufficient quantity of L-Dopa should be deliverable through the lungs to constitute a novel and effective means of providing genuine rescue therapy, if this dose-by-route differential is maintained in humans. This could be accomplished using a robust, small inhaler containing AIR™ L-Dopa particles that can easily be carried in a shirt pocket (Dunbar et al., 2003).

In addition to improving upon the effectiveness of rescue therapy, the present results raise the possibility that a pulmonary form of L-Dopa might provide enhanced reliability and control in delivering L-Dopa for daily, first-line therapy. A delivery modality for L-Dopa that avoids the vagaries of oral absorption and gut metabolism should provide greater consistency in plasma levels, resulting in more reliable and effective treatment of PD patients.

Finally, a major limitation of the effectiveness of L-Dopa is the gradual development of motor complications occurring with long-term use. Compelling evidence links the development of these complications to the chronic, pulsatile stimulation of dopamine receptors occurring with oral L-Dopa administration (Olanow and Obeso, 2000). The pulsatile stimulation occurs because the short half-life of L-Dopa and
delayed and variable gastric emptying create wide fluctuations in plasma levels. These wide fluctuations in plasma levels are believed to initiate a cascade of events that lead to L-Dopa-induced dyskinesias. First, they produce significant fluctuations in brain dopamine (especially in moderate to severe patients), which, in turn, causes pulsatile stimulation of the dopamine receptors. It has been hypothesized that the pulsatile stimulation of dopamine receptors alters post-synaptic gene transcription and protein expression, which, in turn, modifies neurotransmission patterns, leading to the emergence of dyskinesias (Mouradian and Chase, 1988). In any case, the link between variable pharmacokinetics and these motor irregularities is so compelling, that delayed gastric emptying is considered a pathogenic factor in non-responding PD patients (Djaldetti et al., 1996). A pulmonary formulation of L-Dopa can improve upon the highly variable time of onset and unpredictable bioavailability associated with each dose of oral L-Dopa (Cedarbaum, 1987, Nutt and Holford, 1996) by removing the vagaries of gastric absorption. Given the rapid elevation in plasma L-Dopa levels observed in the present study, a pulmonary form of L-Dopa might have to be administered at lower, more frequent doses throughout the day, modulating the peaks while eliminating the troughs characteristic of the oral route. If this could be accomplished in humans with pulmonary delivery of L-Dopa, the debilitating dyskinesias that emerge with long-term L-Dopa use might be significantly reduced and/or retarded. This concept requires further investigation in animal models of L-Dopa-related dyskinesias (DiMonte et al., 2000), which may then justify further long-term testing in humans.

In summary, pulmonary delivery of L-Dopa provided significant pharmacokinetic advantages over oral administration in an animal model of PD. This was manifested as
a more rapid and reliable time of onset and a three- to five-fold dose advantage. A pulmonary formulation of L-Dopa could clearly benefit patients in need of rapidly acting rescue therapy, and the dose advantage, if verified in humans, raises the possibility of delivering sufficient L-Dopa via the pulmonary route to either improve upon or replace the standard, but problematic, oral form of L-Dopa therapy. Finally, by avoiding problems of gastric emptying and other sources of variability characteristic of the oral route, dose-to-dose variations in plasma L-Dopa levels should be tempered, possibly slowing the development of motor irregularities linked to intermittent dopamine receptor stimulation.
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REFERENCES


Table 1. Summary of the plasma pharmacokinetics of oral and pulmonary formulations of L-Dopa.

<table>
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<tr>
<th>Route of Administration</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (min)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</th>
<th>AUC&lt;sub&gt;0-240&lt;/sub&gt; (µg min&lt;sup&gt;-1&lt;/sup&gt; ml)</th>
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</tbody>
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Values represent the mean ±SEM of observations derived from measures of the plasma concentrations of L-Dopa at 2, 5, 15, 30, 60, 120, and 240 minutes after administration<sup>a,b</sup>. Significantly different from oral administration, unpaired t-test, P=0.0017, 0.0197, 0.010, respectively. T<sub>max</sub>: time to maximum plasma levels. C<sub>max</sub>: maximum plasma concentration. AUC<sub>0-240</sub>: area under the plasma concentration vs time curve between 0 and 240 minutes.
Figure Legends

Figure 1. Main graph: Plasma pharmacokinetics of L-Dopa administered by intravenous, oral and pulmonary routes. Rats were administered 2 mg of L-Dopa either by insufflation of an AIR™ formulation of L-Dopa containing particles (open squares) or by oral gavage of a suspension of L-Dopa in carboxymethylcellulose (closed squares). Pulmonary delivery of L-Dopa yielded plasma L-Dopa concentrations that were significantly higher than corresponding levels following oral L-Dopa administration. Each symbol represents the mean ± SEM of data from 7 subjects in the oral group, 6 subjects in the AIR L-Dopa group. (*, **: p<0.05, 0.01, respectively, 2-way ANOVA and Tukey’s post-hoc analysis matrix.) Insert: Expanded graph to permit comparison of differences in slope of curves during initial 15min following pulmonary versus oral delivery of L-Dopa.

Figure 2. Striatal levels of L-Dopa, dopamine and DOPAC after L-Dopa administration by pulmonary or oral routes. Rats receiving unilateral 6-OHDA lesions were administered 2 mg of L-Dopa either by inhaling an AIR formulation of L-Dopa containing particles or by oral gavage of a suspension of L-Dopa in carboxymethylcellulose (CMC). Striatal L-Dopa levels (A) was significantly higher after pulmonary (open symbols) than oral delivery (closed symbols) between 5 and 30 minutes after administration, regardless of whether the striatum was on the lesioned (circles) or unlesioned (squares) side. Similarly, dopamine (B) levels in both the lesioned and unlesioned striata were significantly higher following pulmonary than oral administration. The rate of dopamine turnover, as indicated by the levels of the dopamine metabolite DOPAC (C), was
significantly higher following inhalation of L-Dopa than oral administration. However, this difference was only observed in lesioned striata. Each symbol represents the mean ± SEM of data from 6 subjects per group. *,**: Significantly different from corresponding oral administration groups, p<0.05, 0.01, respectively, multi-way ANOVA and Tukey's post-hoc analysis. a: Significantly different from oral groups in both lesioned and unlesioned striata, p<0.01, multi-way ANOVA and Tukey's post-hoc analysis.

Figure 3. Histological evidence of a significant increase in striatal neuron expression of c-fos message following pulmonary administration of L-Dopa. Rats with unilateral 6-OHDA lesions were administered 2 mg of L-Dopa either by inhalation of AIR L-Dopa particles or oral gavage of a suspension of L-Dopa in CMC. Each panel represents a striatal section (40 x magnification) from 6-OHDA-lesioned rats administered L-Dopa by oral (A, B) or pulmonary (C, D) routes and sacrificed 60 minutes later. Sections were subsequently subjected to hybridization for c-fos message. The number of neurons expressing c-fos in the lesioned striatum was substantially increased 60 min after pulmonary L-Dopa administration (D) compared to rats orally administered L-Dopa (B). (See Figure 4 for quantification of this phenomenon.)

Figure 4. The number of striatal neurons expressing c-fos following inhalation of L-Dopa is significantly higher than after oral administration. Rats with unilateral 6-OHDA-lesions were administered L-Dopa either by inhaling an AIR formulation of L-Dopa (2 mg) containing particles or by oral gavage of a suspension of L-Dopa (2 or 10 mg) in CMC and sacrificed 60 minutes later. Each column represents the mean ± SEM of data from
8 subjects per group. Overall, the number of neurons expressing c-fos was higher in striata from the lesioned compared to the unlesioned side (p<0.01, 2-way ANOVA). In addition, the number of c-fos expressing neurons following pulmonary administration of 2 mg of L-Dopa was significantly higher than either vehicle or 2 mg oral L-Dopa groups, independent of striatum treatment (p<0.01, ANOVA and Tukey’s post-hoc analysis).

Figure 5. Pulmonary delivery of L-Dopa improves the motor performance of 6-OHDA lesioned rats more rapidly and potently than orally administered L-Dopa: Placement test. Rats with unilateral 6-OHDA-lesions were administered L-Dopa either by inhaling an AIR formulation of L-Dopa (0.5, 1, or 2 mg) containing particles or by oral gavage of a suspension of L-Dopa (6.8 or 10 mg) in CMC. Vehicle consisted of placebo AIR particles or CMC vehicle alone. Significant increases in placing behavior were observed beginning 30 minutes after the oral administration of 10 mg L-Dopa relative to vehicle (A). In contrast, pulmonary delivery of 2 mg L-Dopa significantly increased the number of placements by 10 minutes (B). Each symbol represents the mean ± SEM of data from 13 rats treated with AIR L-Dopa and 12 rats treated orally. *, **: Significantly different from corresponding vehicle treated group, p<0.05, 0.01, respectively; a: performance values are not significantly different, unaffected limb vs. 2 mg pulmonary or 10 mg oral L-Dopa, 2-way ANOVA and Tukey’s post-hoc analysis.

Figure 6. Pulmonary delivery of L-Dopa improves the motor performance of 6-OHDA lesioned rats more rapidly and potently than orally administered L-Dopa: Bracing test. Rats with unilateral 6-OHDA-lesions were administered L-Dopa either by inhaling an
AIR formulation of L-Dopa (0.5, 1, or 2 mg) containing particles or by oral gavage of a suspension of L-Dopa (6.8 or 10 mg) in CMC. Significant increases in the number of braces per 90 cm were observed beginning 30 minutes after the oral administration of 10 mg L-Dopa relative to vehicle (A). In contrast, pulmonary delivery of 2 mg L-Dopa significantly increased the number of placements by 10 minutes (B). Each symbol represents the mean $\pm$ SEM of data from 13 rats treated with AIR L-Dopa and 12 rats treated orally. *, **: Significantly different from corresponding vehicle treated group, p<0.05, 0.01, respectively; a: performance values are not significantly different, unaffected limb vs. 2 mg pulmonary or 10 mg oral L-Dopa, 2-way ANOVA and Tukey’s post-hoc analysis.

Figure 7. Pulmonary delivery of L-Dopa improves the motor performance of 6-OHDA lesioned rats more rapidly and potently than orally administered L-Dopa: Akinesia test. Rats with unilateral 6-OHDA-lesions were administered L-Dopa either by inhaling an AIR formulation of L-Dopa (0.5, 1, or 2 mg) containing particles or by oral gavage of a suspension of L-Dopa (6.8 or 10 mg) in CMC. Significant increases in placing behavior were observed beginning 60 minutes after the oral administration of either 6.8 or 10 mg L-Dopa relative to vehicle (A). In contrast, pulmonary delivery of 2 mg L-Dopa significantly increased the number of placements after 15 minutes (B). Each symbol represents the mean $\pm$ SEM of data from 13 rats treated with AIR L-Dopa and 12 rats treated orally. *, **: Significantly different from corresponding vehicle treated group, p<0.05, 0.01, respectively; a: performance values are not significantly different, unaffected limb vs. 2 mg pulmonary or 10 mg oral L-Dopa; b: performance values are
not significantly different, unaffected limb vs. 1, 2 mg pulmonary or 6.8, 10 mg oral L-Dopa 2-way ANOVA and Tukey’s post-hoc analysis.
Figure 1

[Graph showing plasma L-Dopa levels over time after administration. The graph compares pulmonary and oral administration, with significant differences indicated by asterisks.]
Figure 2

A  
**Striatal L-Dopa Levels**

- Inhaled, Lesion
- Inhaled, Normal
- Oral, Lesion
- Oral, Normal

Time after Administration, (min)

B  
**Striatal Dopamine Levels**

- Inhaled, Normal
- Oral, Normal
- Inhaled, Lesion
- Oral, Lesion

Time after Administration, (min)

C  
**Striatal DOPAC Levels**

- Inhaled, Normal
- Oral, Normal
- Inhaled, Lesion
- Oral, Lesion

Time after Administration, (min)
Figure 3

A

B

C

D
**Figure 4**

![Graph showing the number of c-Fos positive nuclei/mm² for different treatments on unlesioned and lesioned sides.](image-url)
Figure 5

A
 Placement
 Oral L-Dopa

Unaffected Limb
L-Dopa (10 mg)
L-Dopa (6.8 mg)
Vehicle

Placements (#/10 Trials)

Time after Administration, (min)

B
 Pulmonary L-Dopa

Unaffected Limb
L-Dopa (2 mg)
L-Dopa (1 mg)
L-Dopa (0.5 mg)
Vehicle

Placements (#/10 Trials)

Time after Administration, (min)
Figure 6

A

Akinesia
Oral L-Dopa

![Graph showing the effects of oral L-Dopa on akinesia]

B

Pulmonary L-Dopa

![Graph showing the effects of pulmonary L-Dopa on akinesia]
Figure 7

A

Akinesia

Oral L-Dopa

Steps, (#/30 sec)

0  5  10  15  20  25

0  30  60  90  120

Time after Administration, (min)

Unaffected Limb
L-Dopa (10 mg)
L-Dopa (6.8 mg)
Vehicle

B

Pulmonary L-Dopa

Steps, (#/30 sec)

0  5  10  15  20  25

0  30  60  90  120

Time after Administration, (min)

Unaffected Limb
L-Dopa (2 mg)
L-Dopa (1 mg)
L-Dopa (0.5 mg)
Vehicle