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Biodegradable Microspheres Loaded with an Anti-Parkinson Prodrug: An *in Vivo* Pharmacokinetic Study

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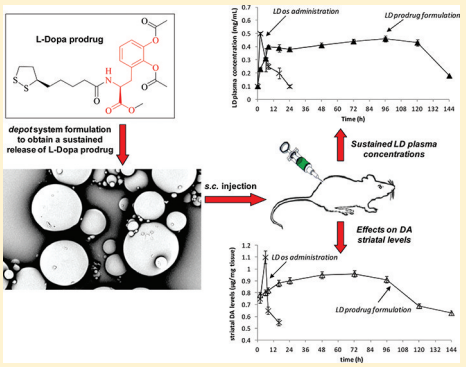
ABSTRACT: During chronic treatment with L-dopa (LD), Parkinsonian patients often experience uncontrolled motor complications due to fluctuations of the plasmatic levels of LD that result in pulsatile dopaminergic stimulation. To overcome these plasmatic fluctuations, a novel prodrug of LD, L-dopa-α-lipoic acid (LD-LA), has been proposed as a tool for achieving continuous dopaminergic stimulation. Due to slower susceptibility toward enzymatic conversion by LD-degrading enzymes (such as catechol-*O*-methyltransferase and monoamine oxidase), the plasma half-life of this prodrug is longer than that of LD. Moreover, the higher lipophilicity of LD-LA over LD promotes its delivery to the CNS, where the resulting levels of dopamine (DA) are kept high for a longer time than after equimolar administration of LD. To further reduce fluctuations in plasma levels of LD, LD-LA has been entrapped into biodegradable polymeric microspheres to be used as a *depot* system with the aim to prevent prodrug degradation and to obtain a sustained release of the intact compound. In the present work, a formulation of LD-LA loaded microspheres (characterized for drug loading, size, morphology, thermal properties, and *in vitro* prodrug release) has been administered subcutaneously to rats, and the resulting levels of LD and DA in plasma and striatal tissue, respectively, have been monitored. A good correlation between the *in vitro* release kinetics and the time range during which the formulation alters the LD/DA tissue levels *in vivo* was observed, suggesting that the polymeric microsphere matrix protects the loaded prodrug from chemical and enzymatic degradation and controls its release. Interestingly, LD-LA microspheres provided sustained levels of DA neurotransmitter in the striatum nucleus for up to 4 days after a single administration. In conclusion, a polymeric microsphere formulation of LD-LA is an attractive medicine for treating Parkinson's disease (PD) symptoms, avoiding motor complications.



KEYWORDS: *Parkinson's disease, L-dopa-α-lipoic acid, PLGA microspheres, depot system*

INTRODUCTION

L-Dopa (LD), the direct precursor of dopamine (DA), is the treatment of choice in the advanced stages of Parkinson's disease (PD).[1,2] However, even in patients who initially respond well to LD, the control of motor symptoms gradually diminishes during treatment.[3] The progressive loss of dopaminergic neurons and the alteration of DA turnover are likely contributing to dyskinesia, a side effect that is most evident at the onset or offset of LD peak plasma levels after oral administration.[4] It is thought that dyskinesia arises from the nonphysiological pulsatile stimulation of postsynaptic DA receptors and that it is connected to the rapid metabolism of LD and its short plasma half-life.[5,6] Hence, modern treatments aim to provide continuous dopaminergic stimulation (CDS), for instance, by fractionation of the LD daily dose or use of continuous intravenous or duodenal LD infusions together with dopaminergic agonists.[7−9] DA agonists or LD formulations resulting in a long plasma half-life are intended for achieving CDS to prevent unwanted side effects related to fluctuations in brain and plasma drug levels.[10] Although each approach appears to provide some benefits, none of them combines convincing features of practicability with unequivocal efficacy.[11]

In recent studies, we showed that prodrug strategies were partially successful in overcoming the problems of erratic absorption, rapid metabolism, and short half-life of LD.[12−14] A novel multifunctional prodrug, LD-LA (Figure 1), was synthesized in our laboratories by coupling (*R*)-α-lipoic acid (LA) to (*O*,*O*-diacetyl)-L-dopa-methylester. After oral administration, this prodrug increased and prolonged the plasmatic

Received: July 4, 2011
Revised: October 10, 2011
Accepted: October 20, 2011
Published: October 20, 2011

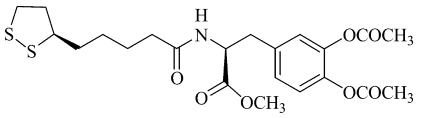


Figure 1. Chemical structure of the anti-Parkinson LD-LA prodrug (methyl *O*-acetyl-3-(acetyloxy)-*N*-{5-[(3*R*)-1,2-dithiolan-3-yl]-pentanoyl}-L-tyrosinate).

levels of LD, thus substantially ameliorating LD pharmacokinetics.[15] This multifunctional compound adds to LD the targeting and neuroprotective properties of the LA moiety because it crosses the blood−brain barrier (BBB), subsequently accumulating in neurons, and can act as an antioxidant as well as a metal chelator.[16−18] Due to these properties, it is active in reducing the auto-oxidation of catecholamines (catalyzed by transition metals) and in scavenging reactive oxygen species generated by LD metabolism, thus affecting several factors involved in PD progression.[19,20] LD-LA activity against the disease symptoms and its antioxidant efficiency have been demonstrated using both *in vitro* and *in vivo* studies.[15] However, the catechol esters and amide bond present in LD-LA are susceptible to rapid chemical and enzymatic hydrolysis, respectively. Therefore, multiple administrations per day are required to keep effective brain levels of LD.[21] In our previous work, the labile prodrug LD-LA was loaded in biodegradable poly(lactic-co-glycolic acid) (PLGA) microspheres to protect this prodrug from enzymatic and chemical degradation and to provide a sustained release of LD-LA. It was shown that the polymeric matrix indeed protected the prodrug against chemical hydrolysis, and it was released via Fickian diffusion up to more than one week; it was also demonstrated that the rate of drug release from microspheres can be tailored by the formulation and processing parameters.[22]

The aim of the present study was to investigate the correlation between the *in vitro* prodrug release and the resulting LD/DA pharmacokinetics of a PLGA microsphere formulation of LD-LA. The microsphere formulation was optimized in terms of drug loading, size, morphology, thermal properties, and *in vitro* prodrug release. In animal experiments, after subcutaneous (sc) injection of the prodrug-loaded microsphere formulation, the resulting levels of LD and DA in rat plasma and striatal tissue, respectively, were measured. Finally, the *in vitro−in vivo* correlation (IVIVC) was investigated.

EXPERIMENTAL SECTION

Chemicals. LD-LA was synthesized in our laboratories as described previously.[15] Benserazide hydrochloride, LD, polyvinyl alcohol (PVA, degree of hydrolysis 88%, molecular weight ranging from 13 000 to 23 000), *n*-octyl sodium sulfate, dimethyl sulfoxide (DMSO), sodium acetate anhydrous, perchloric acid (ACS reagent), and disodium ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich. All other chemicals used were of the highest purity commercially available. PLGA 50:50 Purasorb 5004A (intrinsic viscosity 0.41 dL/g) with free carboxyl end groups was obtained from Purac Biomaterials. High-performance liquid chromatography (HPLC) grade dichloromethane (DCM) and methanol were obtained from Biosolve BV. Formic acid, acetic acid anhydrous, and NaOH (all pro-analysis grade) were obtained from Merck.

Microsphere Preparation. LD-LA loaded microspheres (LDLAMS's) were prepared by an oil-in-water emulsion/solvent evaporation (o/w ESE) method with some modifications; the effect of formulation parameters (polymer concentration in the organic solvent, volume ratio of the phases, rate of the organic solvent evaporation) on microsphere characteristics (size, loading efficiency, and morphology) was previously evaluated.[22] Briefly, 400 mg of PLGA and 80 mg of LD-LA were dissolved in 1 mL of DCM. This oil phase was then homogenized (room temperature (r.t.), 30 s, 25 000 min^{-1}) by means of an IKA UltraTurrax T10 basic (S10N-10G tip) with 5 mL of an aqueous phase consisting of 2% (w/w) PVA in pH 4.5 buffer (formic acid/NaOH) saturated with DCM. The resulting emulsion was subsequently added dropwise to 5 mL of a hardening bath (PVA, 0.5% w/w) in double-distilled water, and the DCM was allowed to evaporate under magnetic stirring for 2.5 h. The solid microspheres were collected by centrifugation (r.t., 2 min, 2500 g), washed twice with 12 mL of double-distilled water, flash frozen with liquid nitrogen, and freeze-dried overnight. A reference sample of empty microspheres (EMS's) was prepared analogously. Several batches of LDLAMS's were prepared and pooled (total yield of 6.2 g).

Microsphere Characterization. The pooled batch of LDLAMS's was characterized for effective drug loading, entrapment efficiency, microsphere yield, size, morphology, thermal properties, and *in vitro* prodrug release. The solubility and diffusibility of the prodrug in the PLGA matrix were discussed in the above-mentioned manuscript.[22]

The loading efficiency (LE, expressed as the encapsulated amount of LD-LA divided by the total amount of LD-LA used for encapsulation) and effective drug loading (EDL, the encapsulated amount of LD-LA divided by the total dry weight of the microspheres) of the formulation were determined by HPLC quantification of the prodrug extracted from the microspheres.[22] Briefly, 5 mg of dried microspheres were dissolved in 0.5 mL of DMSO, followed by the addition of 1 mL of cold methanol to precipitate the PLGA. Next, the samples were centrifuged (30 min, 20 000 g, 4 °C), the supernatant was filtered, and methanol was evaporated under vacuum to obtain a DMSO solution of LD-LA. The prodrug concentration in the DMSO solution was measured using a Waters 2695 separations module equipped with a Waters 2487 UV detector and a Waters SunFire C18 RP column (4.6 × 150 mm, 5 μm). A mixture of methanol and pH 4.5 formiate buffer (60:40) was used as eluent (flow rate 0.5 mL/min, detection wavelength 230 nm). Calibration was performed employing LD-LA stock solutions in DMSO. The microsphere yield was determined by weighting the freeze-dried final batch.

The particle size distribution and mean volume-based diameter of the formulation were determined by means of an Accusizer 780 optical particle sizer (Santa Barbara, CA). The surface morphology of the freeze-dried LDLAMS's was investigated by scanning electron microscopy (SEM). The samples for SEM analysis were prepared by sprinkling a small amount of powdered and freeze-dried formulation onto a double-sided adhesive tape attached to an aluminum stub and subsequently metalized with gold using a sputter coater (Emitech K 550, Emitech Ltd., Ashford, Kent, UK) and observed with a LEO 435 Vp (LEO Electron Microscopy Ltd., Cambridge, UK) microscope.

Temperature-modulated differential scanning calorimetry (TMDSC) analyses were performed on LD-LA, on EMS's, and on LDLAMS's by means of a TA Instruments DSC

Q2000.[23] Briefly, about 5 mg of material were equilibrated at −20 °C for 5 min, heated from −20 to 120 °C (at a heating rate of 1 °C/min), subsequently cooled to −20 °C (5 °C/min), and heated again to 120 °C (1 °C/min). The glass transition temperature (T_g) was obtained using the thermogram recorded during the second heating cycle.

In vitro prodrug release from microspheres was monitored for 144 h at 37 °C. Dried microspheres (5 mg) were suspended in 4.0 mL of pH 4.5 acetate buffer (0.02 M acetic acid, 0.012 M NaOH) and incubated while gently shaken. After centrifugation (30 °C, 3 min, 3000 g) the buffer solution was removed completely at preset sampling times (2, 5, 7, 16, 24, 32, 48, 56, 72, 96, 120, and 144 h) and replaced with fresh solution. The release buffer was filtered through 0.45 μm filters, and the amount of prodrug released was determined by HPLC using a Waters 1525 binary pump equipped with a Waters 2996 photodiode array detector and a Waters XTerra C8 RP column (3.0 × 150 mm, 5 μm). A mixture of methanol and acetate buffer pH 4.5 (60:40) was used as eluent (flow rate 0.5 mL/min, detection wavelength 230 nm). Calibration was done with LD-LA dissolved in pH 4.5 acetate buffer. The release experiment was performed in triplicate.

Animals. Male Wistar rats (Harlan, UD, Italy) that weighed 200−225 g at the beginning of the experiments were used. All procedures were conducted in agreement with the European Community Council Directive for Care and Use of Laboratory Animals.

Drug Administration. Benserazide hydrochloride, a peripheral dopa-decarboxylase inhibitor, was dissolved in sterile saline, whereas the LD and LD-LA were dissolved in sterile saline containing 15% of DMSO. The microspheres were dispersed in sterile saline. All animals received a sc dose of benserazide (1.64 mg/kg) combined with a sc dose of LD (6.5 mg/kg; 33 μmol/kg), LD-LA (16.0 mg/kg; 33 μmol/kg), or LDLAMS's (400 mg/kg, corresponding with a dose of 56 mg/kg LD-LA (115.5 μmol/kg)). In view of the expected sustained release of LD-LA from the microspheres during 3−4 days, the LDLAMS's formulation was given at a 3.5 times higher dose than the prodrug in its free form. All drugs were given at a volume of 4 mL/kg. Two control groups received sterile saline and LD orally administered (6.5 mg/kg), respectively.

HPLC Analysis. Owing to the rapid *in vivo* cleavage of the catechol esters and amide bond in LD-LA, pharmacokinetic analyses were performed by quantifying the plasma concentrations of the formed biodegradation products (LD and DA).[15] After slight anesthesia of the rats with carbon monoxide, blood samples were drawn by cardiac puncture and transferred into vials containing heparin (250 IU) for the determination of LD and DA. The blood samples were taken at 2, 6, 8, 16, 24, 48, 72, 96, 120, and 144 h after administrations of LDLAMS's, LD-LA, and LD (either dissolved in sterile saline or as PLGA microsphere formulation). The blood samples were centrifuged (2000 g, 10 min, 4 °C) and the obtained plasma samples were kept at −80 °C until analysis. Aliquots (400 μL) of the samples were deproteinized by mixing with 40 μL of 4 M perchloric acid and centrifugation (2000 g, 10 min, 4 °C). The supernatants were filtered (Millipore 0.45 μm) and chromatographed by HPLC (10 μL). All analyses were carried out on a Waters 600 controller pump, a Rheodyne 7295 injector with a 10 μL loop, and an Antec Leyden Decade II detector (the operating potential was 0.75 V). Separation was achieved on a Waters Symmetry RP-C18 column (4.6 mm × 150 mm, 5 μm). The mobile phase consisted of 13.61 g/L sodium acetate, 6.0% methanol, 16−19 mg/L *n*-octyl sodium sulfate, and 13 mg/L disodium EDTA dissolved in Milli-Q water and adjusted to pH 4.1 with glacial acetic acid.

For the measurements of the striatal DA levels, the striatum of each animal was homogenized for 2 min with a Dyna-Mix homogenizer (Fisher Scientific) in 500 μL of 0.05 N perchloric acid solution containing 0.064% (w/v) 1-octanesulfonic acid sodium salt, 0.060% (w/v) heptanesulfonic acid sodium salt, 0.004% (w/v) disodium EDTA, 0.010% (w/v) sodium metabisulfite, and 25 ng/mL DHBA as an internal standard. The whole procedure was carried out on ice.[14] The resulting homogenate was centrifuged (4500 g, 10 min, 4 °C), and the supernatant was filtered using 0.45 μm Millipore filters. The filtrate was set in a low volume insert vial and a sample of 10 μL was analyzed by HPLC. The HPLC system consisted of a PU-2080 Plus pump (Jasco), a Rheodyne 7295 injector with a 10 μL loop, and an ESA Coulochem III detector. Separation was achieved on a Waters Symmetry RP-C18 column (4.6 mm × 150 mm, 5 μm). The mobile phase consisted of 0.045 M monobasic sodium phosphate, 0.001 M 1-octanesulfonic acid sodium salt, 0.006% triethylamine, 0.015% 100 μM disodium EDTA, and 6% acetonitrile. The pH of the mobile phase was adjusted to 3.0 with *o*-phosphoric acid. The flow rate was 1 mL/min. The electrochemical detection system included a high-sensitivity dual detector analytical cell (detector 1 set at +350 mV and detector 2 set at −180 mV). The signal was recorded by use of the response from detector 1.[24]

Statistical Analysis. The experimental data are expressed as mean values ± SE (standard error) of three rats used for each time point. The data represent the means of each group and have been analyzed by ANOVA followed by the Newman-Keuls test. P values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

In the present study, we prepared a formulation of the anti-Parkinson prodrug LD-LA entrapped in biodegradable PLGA microspheres which can provide stable plasma levels of LD for an extended period of time. The biocompatibility of PLGA microspheres has been widely studied;[25] the biodegradation of PLA and PLGA microspheres occurs through a homogeneous hydrolytic chain cleavage mechanism where the rates of polymer degradation are similar for both the surface and the bulk of the microspheres. During the time frame of the experiment (i.e., 4 days), erosion of the surface of the microspheres will be minimal, and changes in volume or surface are not to be expected; in other words, during this time frame, the polymeric microspheres can be considered as a nondegrading implant from which drug is released via diffusion.

Characterization of Microspheres. The characteristics of the prepared LDLAMS's formulation as well as those of the reference samples EMS's are summarized in Table 1.

Table 1. Characteristics of the EMS's and of the LDLAMS's[a]

formulation	LE %[b]	EDL %[c]	yield	mean diameter (μm)	T_g (°C)[d]
LDLAMS's	56 ± 5	14 ± 3	68.5%	30 ± 16	40.7
EMS's			70.2%	32 ± 15	47.5

[a]Data are expressed as mean ± SD (n = 3). [b]LE expressed as mass ratio (in %) of drug entrapped in microspheres and drug used to prepare them. [c]EDL expressed as the mass ratio between effectively entrapped drug and microspheres. [d]LD-LA has a T_g of 1.5 °C.[22]

The drug was entrapped with a good efficiency (~70%) in the microspheres, and the drug loading was high (14%), in

agreement with our previous publication. The particles had a mean size of 30 μm which was confirmed by SEM analyses (Figure 2). Furthermore, SEM showed that the LDLAMS's had

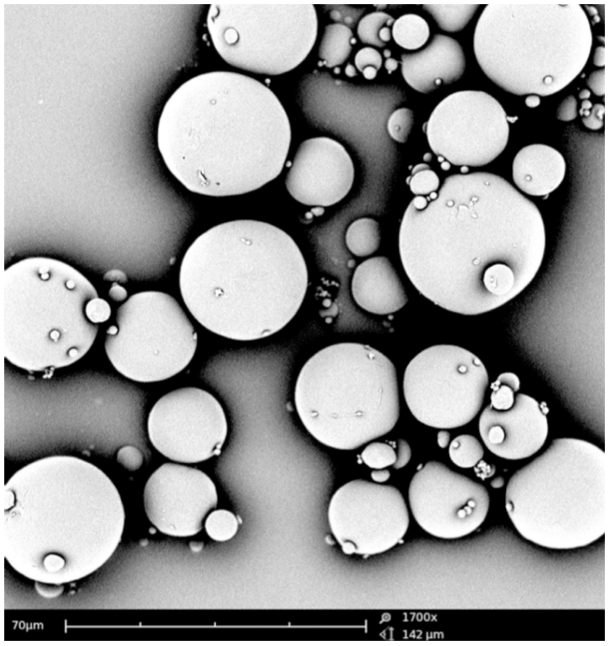


Figure 2. SEM image of LDLAMS's.

a spherical shape and a smooth surface (Figure 2). These results are in agreement with our previous study of the effects of formulation and processing parameters on the properties of similar LDLAMS's.[22]

The thermogram of the LDLAMS's (Figure 3) shows a single T_g (40.7 °C) between that of the EMS's (47.5 °C) and that of

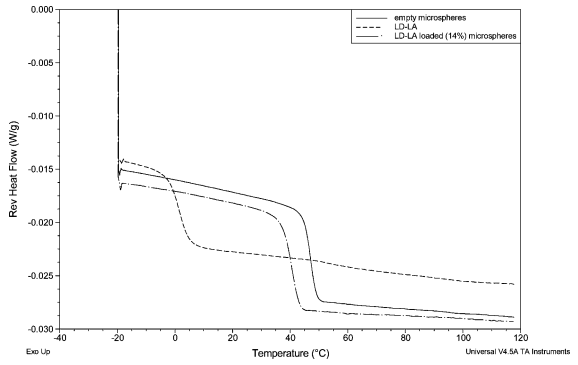


Figure 3. Reverse heat capacity curve from TMDSC analysis of the LDLAMS's.

LD-LA (1.5 °C). Using Fox's equation a T_g of 40.2 °C can be calculated for PLGA microspheres loaded with 14% of LD-LA.[26] The excellent agreement with the found T_g means that LD-LA is molecularly dispersed in the PLGA matrix.

***In Vitro* Release Profile.** It was previously demonstrated that LD-LA undergoes chemical hydrolysis at pH 7.4, while it has a good stability at pH 4.5.[15,22] Importantly, we also demonstrated that LD-LA entrapped in PLGA microspheres is not degraded when the particles are incubated at 37 °C in an aqueous buffer of pH 7.4. Furthermore, it was shown that there is not significant difference in LD-LA release from these formulations between pH 7.4 and pH 4.5.[22] Considering these data, in the present work the *in vitro* release of LD-LA from the microsphere formulation was monitored in pH 4.5 acetate buffer, which ensures the stability of the prodrug after its release in the incubation medium. Figure 4 shows that the

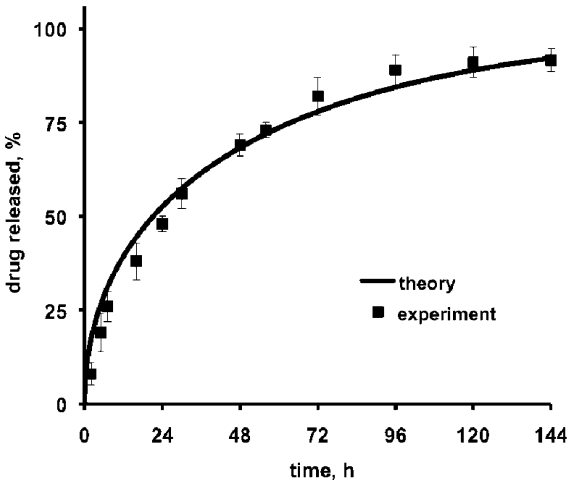


Figure 4. LD-LA *in vitro* release kinetics from the microsphere formulation: experiment (symbols) and theory (curve, eq 1).

PLGA microspheres released LD-LA in a sustained-release for 3−4 days.

The experimental data showed a good fit with the following analytical solution of Fick's second law of diffusion:

$$\frac{M_\infty - M_t}{M_\infty} = \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(-\frac{n^2\pi^2}{R^2}Dt\right) \quad (1)$$

where M_∞ and M_t denote the absolute cumulative amounts of drug released at infinite time and time t, respectively; R represents the radius of the microspheres and D the *apparent* diffusion coefficient of the drug in the PLGA matrix.[27−29] This model is based on the assumption that drug release is controlled by diffusion only and assumes a spherical geometry of the particles, a homogeneous initial drug distribution, perfect sink conditions during release, and that LD-LA is molecularly distributed within the polymeric matrix (monolithic system).

It can be concluded that the prodrug release from this microsphere system is basically governed by Fickian diffusion, and the computed value of the *apparent* diffusivity D is 0.90×10^{-12} cm^2/s ($R^2 = 0.99$).

Pharmacokinetic Investigation. Pharmacological studies were performed using rats to test the ability of LDLAMS's to produce sustained LD plasma levels and to increase the DA concentration in the striatal tissue. At the start of the experiments, rats were treated with a single dose of benserazide, a peripheral carboxylase inhibitor. Co-administration with benserazide avoided the appearance of possible side effects such as nausea, vomiting, and cardiovascular problems due to excessive LD peripheral bioconversion to DA which would affect the pharmacokinetic investigation.[30] The combination of LD with benserazide is also given clinically (Madopar).

Pharmacokinetic parameters following sc administration of LDLAMS's in rats were determined from the plasma concentration/time data and analyzed using a noncompartimental pharmacokinetic model.[31] The calculated parameters (C_{max}, T_{max}, AUC, AUMC, and MRT) are presented in Table 2.

dx.doi.org/10.1021/mp200337h | *Mol. Pharmaceutics* 2011, 8, 2408−2415

Table 2. Noncompartmental Pharmacokinetic Parameters[a]

LD					
formulation	T_{max} (h)	C_{max} (μg/mL)	AUC (μg/(mL h))	AUMC (μg /(mL h^2))	MRT (h)
LD os	1.50 (±0.06)	0.54 (±0.23)	11.80 (±3.54)	108.03 (±32.40)	9.15 (±2.74)
LD sc	6.05 (±0.31)	0.29 (±0.06)	5.68 (±0.28)	36.88 (±1.84)	6.49 (±1.30)
LD-LA sc	6.15 (±0.25)	0.33 (±0.01)	36.2 (±1.30)	1161.00 (±41.80)	32.07 (±1.19)
LDLAMS's sc	96.03 (±3.56)	0.46 (±0.02)	112.94 (±6.57)	8116.80 (±471.91)	71.87 (±3.44)
DA					
formulation	T_{max} (h)	C_{max} (μg/mL)	AUC (μg/(mL h))	AUMC (μg /(mL h^2))	MRT (h)
LD os	2.01 (±0.06)	0.59 (±0.10)	36.10 (±2.01)	275.52 (±15.31)	7.63 (±0.42)
LD sc	6.27 (±0.10)	0.29 (±0.02)	5.68 (±0.31)	40.28 (±2.24)	7.09 (±0.49)
LD-LA sc	6.48 (±0.22)	0.38 (±0.06)	18.55 (±0.71)	490.55 (±18.87)	26.45 (±1.02)
LDLAMS's sc	96.79 (±4.10)	0.31 (±0.04)	60.36 (±0.50)	4256.40 (±35.47)	70.52 (±0.59)

[a]Data are expressed as mean ± SE for four rats. Each experiment was performed in triplicate; standard deviation is given in brackets. In view of the prolonged release, the LDLAMS's have been dosed with a higher total dose which could provide sustained drug levels for 3.5 days. For comparing bioavailability, all AUCs have been normalized to the given doses.

As shown in Figure 5, sc injections of LD resulted in LD levels that were slightly above baseline levels, confirming the impracticality of this administration route for LD; furthermore oral administration of LD produced high fluctuations in LD plasma levels (Figure 5), which is known to contribute to motor complications experienced by patients during the DA restoring therapy. The goal of our formulation is to provide smooth plasma levels and prolonged half-life of the prodrug generating the desired therapeutic goals such as CDS.

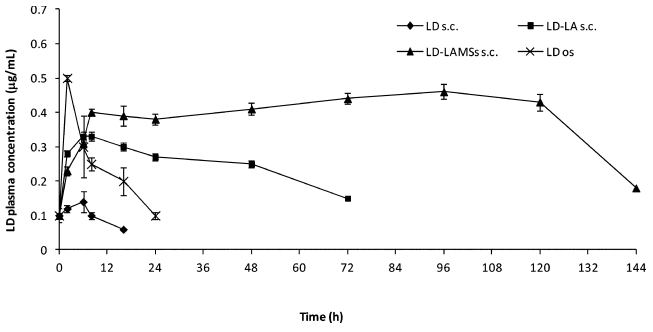


Figure 5. LD plasma concentration in rats after administration of LDLAMS's (400 mg/kg corresponding to LD-LA dose of 56 mg/kg), LD-LA (16 mg/kg), or LD (6.5 mg/kg). Data are expressed as mean ± SE for four rats. Each experiment was performed in triplicate.

Figure 6 shows the plasma concentration−time profiles of DA, after administration of LDLAMS's, LD-LA, and LD;

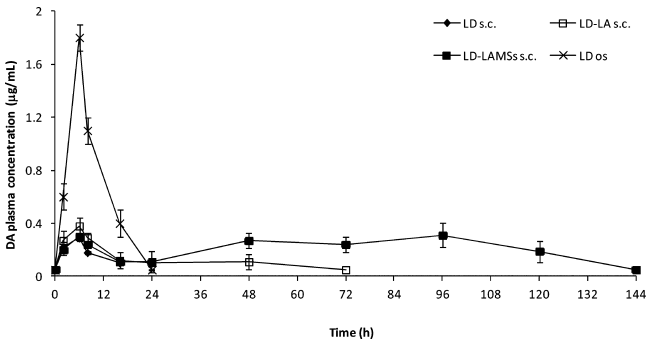


Figure 6. DA plasma concentration after administration of LDLAMS's (400 mg/kg corresponding to LD-LA dose of 56 mg/kg), LD-LA (16 mg/kg), or LD (6.5 mg/kg). Data are expressed as mean ± SE for four rats. Each experiment was performed in triplicate.

Figure 7 reports the DA striatal levels. Due to its rapid conversion into LD, we detected only LD and not the intact LD-DA prodrug.

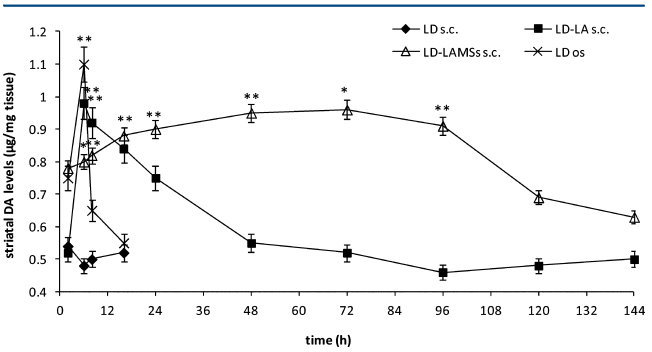


Figure 7. Striatal levels of DA after rat administration of LDLAMS's (400 mg/kg corresponding to LD-LA dose of 56 mg/kg), LD-LA (16 mg/kg), or LD (6.5 mg/kg). Data are expressed as mean ± SE for four rats. Each experiment was performed in triplicate.

Two hours after sc administration of LD-LA, the mean LD plasma concentration was 0.28 μg/mL (Figure 5). As expected, sc administration of LD-LA resulted in prolonged LD blood levels and avoided unwanted initial LD peaks.[32] The initial (1.5 h post administration) mean plasma LD levels after sc administration of LDLAMS's were lower than those measured after the oral administration of LD. Interestingly, soon after the drug administration, LD levels achieved by sc LDLAMS's were comparable to those resulting from sc free LD-LA, for which a slow decrease of LD levels was observed 8 h post administration, reaching baseline levels after 72 h. The LD plasma concentration 16 h after administration of LDLAMS's reached a maximum of 0.40 μg/mL and remained remarkably constant for 120 h. During this five-day period the sustained release of LD-LA from microsphere LDLAMS's compensates for the clearance of LD from circulation inducing minimal fluctuation in LD levels despite the absence of comedication of benserazide during days 2−6. The DA plasma concentrations after administration of LD sc, LD os, LD-LA, and LDLAMS's sc are shown in Figure 6. With the exception of oral LD, relatively low DA plasma concentrations were detected, which is in agreement with the effect of benserazide in inhibiting peripheral LD conversion. Oral LD, instead, resulted in a sharp DA plasma peak, which might be ascribed to the incomplete onset of the

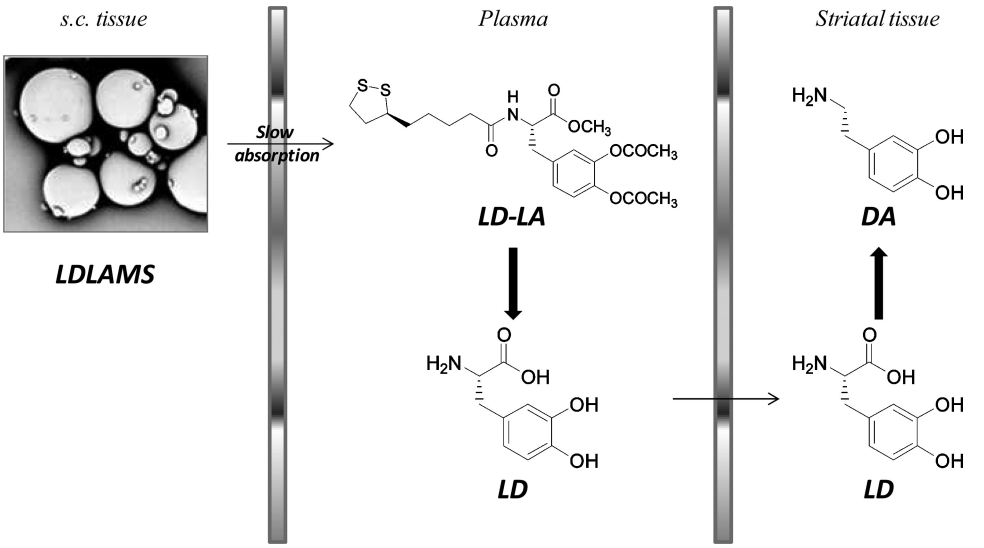


Figure 8. Bioconversion pathways from LDLAMS's sc injection to the delivery of DA in the striatal tissue.

benserazide effect at the early stage of the experiment. As shown in Figure 6 the slow absorption of LD-LA prodrug after sc administration, together with the bioconversion steps into LD, resulted in a DA peak about 10-fold lower if compared with LD oral dosage.

Finally, we evaluated the DA levels in the striatum as a marker for the eventual biological activity of the DA restoring therapies. The PLGA formulation resulted in sustained levels of DA in rat striatum when compared with both LD-DA and an oral dose of LD. In fact, striatal levels of DA were still elevated (about 0.8 μg/mg tissue) 96 h post administration of LDLAMS's.

As previously mentioned, Figure 7 represents the DA striatal levels after administration of LDLAMS's, LD-LA, and LD. Considering the basal levels of DA (0.50 μg/mg tissue), LDLAMS's provided a prolonged DA bioavailability in comparison with that induced by LD ($P < 0.05$) or by free LD-LA. In the light of such persistent high plasma concentrations of LD, together with the high DA levels in the striatal nucleus even 96 h after sc administration, it can be concluded that LDLAMS's is a useful drug formulation for avoiding LD plasma fluctuations, which are thought to cause variations in motor responses, LD-induced dyskinesia, and end-of-dose deterioration in chronic treatments of PD.[33] A schematic representation of bioconversion pathways from LDLAMS's into DA after sc injection is reported in Figure 8.

***In Vitro/in Vivo* Correlation (IVIVC).** To relate LD-LA *in vitro* release kinetics from the microsphere formulation to *in vivo* pharmacokinetic data, an IVIVC analysis was performed. While the FDA IVIVC is applicable only to oral dosage forms, the principles of this guidance can be used for developing *in vitro* drug release test that correlates with the *in vivo* product performance for nonoral products.

It is widely accepted that drug release from biodegradable delivery systems occurs by a combined mechanism of drug diffusion and polymer degradation or bioerosion.[34] The large number of parameters potentially affecting drug release *in vivo* and *in vitro* for this class of formulations leads to difficulties in establishing IVIVC. Some examples are reported in literature where an *in vitro* method predicts the *in vivo* release profile for parenteral biodegradable depot systems.[35−38]

To perform a conversion of measurable pharmacokinetic data to release characteristics of the drug from the delivery systems, a number of mathematical models have been reported.[39−41]

Depending on the method used to correlate the data, the United States Pharmacopeia (USP) established different levels of correlation, designated as A, B, and C, in decreasing order of preferences and acceptability.[42] Among these three, level A is generally viewed as the best method, since level A represents a point-to-point correlation between the *in vivo* absorption profile and the *in vitro* release profile.

Therefore, level A of *in vitro*−*in vivo* correlation (USP XXVI ed.) was studied. Utilizing the intravenous (iv) pharmacokinetics parameters obtained after a single iv administration of LD-LA prodrug, the *in vivo* percent of drug absorbed after sc administration at each time period was estimated by the Wagner−Nelson method.[39] When LD-LA was administered iv (16.0 mg/kg), after a rapid bioconversion in LD (first ascending tract of the curve), the drug was rapidly eliminated with a k_e of 1.2×10^{-2} and fitted to a two-compartment model with a k_{12} and k_{21} of 2.8×10^{-3} and 2.2×10^{-1}, respectively (Figure 9).

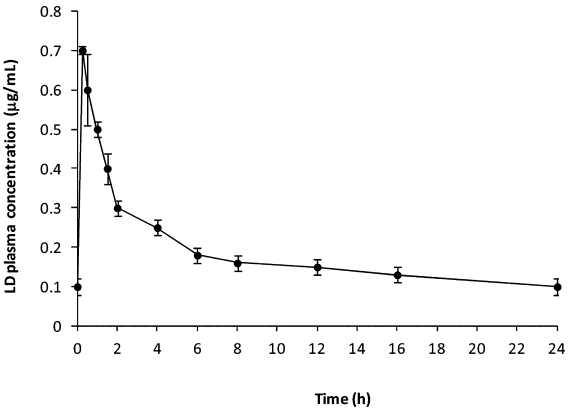


Figure 9. LD plasma concentration in rats after a single iv administration of LD-LA (16 mg/kg). Data are expressed as mean ± SE for four rats.

The LD cumulative percent $AUC_{0-\infty}$ was used to establish IVIVC of the LDLAMS's with the *in vitro* cumulative release.

dx.doi.org/10.1021/mp200337h | *Mol. Pharmaceutics* 2011, 8, 2408−2415

In this study we reported the percentage of drug absorbed up to time plotted versus the amount of drug released *in vitro* and the time *in vivo* from 0 to 90% absorbed plotted versus the time for *in vitro* release of the same amounts of prodrug (Levy plot)[43] (Figure 10).

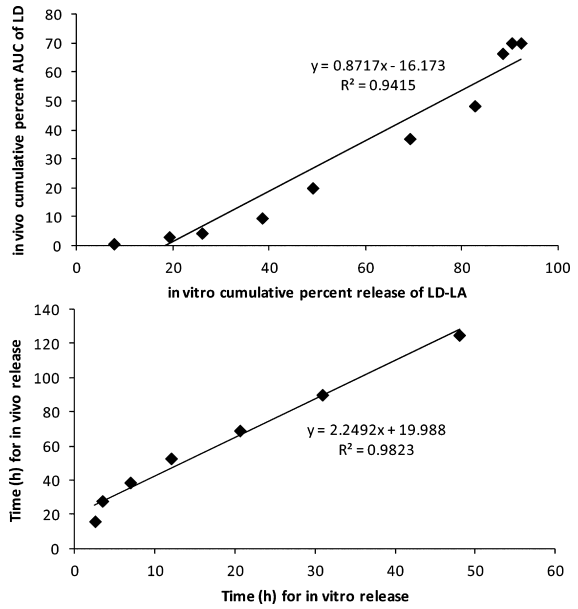


Figure 10. Establishment of IVIVC correlation between the *in vitro* release of LD-LA from microparticles formulation and the LD plasma concentration in rats. (a) Relationship between the percentage of dose absorbed *in vivo* of prodrug and percent release *in vitro*. (b) Levy plot obtained from release times of 0−90% *in vitro* LD-LA and 0−90% *in vivo* LD.

A visual inspection of the plots suggests a good correlation of the release profiles. The *in vivo* LD-LA release from PLGA microspheres induces LD plasma levels consistent with the *in vitro* prodrug release profile; however, the best correlation coefficient (R^2 = 0.9823) was obtained when using the Levy plot. The relationship obtained from the Levy plot indicates that the curves are not superimposable; the different slope and an intercept different from zero is attributable to the LD-DA bioconversion into LD, and from the intercept, the lag time *in vivo* was estimated to be 22 min.

The good IVIVC between the *in vitro* release (acetate buffer at 37 °C) and the cumulative fraction absorbed suggests that the proposed *in vitro* release model of LDLAMS's is well-correlated to *in vivo* performance and may be used to further develop MS's formulations for the effective management of Parkinson's disease.

CONCLUSIONS

By means of an o/w ESE method, the prodrug LD-LA was efficiently entrapped into PLGA microspheres with 14% EDL. An investigation of the *in vitro* release showed that the formulation slowly released LD-LA by Fickian diffusion up to 3−4 days. Importantly, the formulation released the prodrug over nearly the same period when sc administered to rats as demonstrated by the LD and DA plasma levels and DA levels in striatal tissue. The IVIVC studies showed a good linear regression relationship between the percent *in vitro* release in acetate buffer at 37 °C and the percent of AUC. Very interestingly, sc administration of the LDLAMS's resulted in sustained LD concentrations in the blood above baseline and high levels of DA neurotransmitter at the site of action, the *striatum*, for 4 days after its administration. The *depot* formulation of LDLAMS's has a number of advantages: (i) it reduces the required prodrug dosing frequency; (ii) it is useful for optimizing the LD pharmacokinetic profile, avoiding dangerous peaks in LD blood levels and for increasing the efficacy of LD-based PD treatment potentially limiting the associated motor syndrome side effect. Thus, on the basis of the results collected, this new formulation of the LD-LA prodrug merits further investigation for the possibility that it offers to develop a biocompatible delivery system able to ensure CDS.

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ACKNOWLEDGMENTS

Financial support from Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) is gratefully acknowledged.

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