UNIVERSITI PUTRA MALAYSIA

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IB 2014 17

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By

AMINU UMAR KURA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Doctor of Philosophy

October 2014

DEDICATION

This thesis is dedicated to my beloved mother Khadijat Muhammad Kura and my father Umar Alhassan Kura.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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October 2014

Chairperson: Sharida Fakurazi, PhD

Institute: Bioscience

Levodopa is the drug of choice in the treatment of Parkinson's disease (PD), a neurodegenerative disorder with no direct fatal outcome. However, peripheral metabolism and poor brain delivery when given alone is a setback of levodopa in PD management. Layered double hydroxide (LDH) is an inorganic nanocomposite that harbors drug between its two layered sheets. It has sustained, continuous and slow release ability, proven to be biocompatible and less toxic in most cases than conventional drug systems. Here, an organic–inorganic nanocomposite material containing levodopa was synthesized to evaluate for a sustain release and decrease toxicity potential. The resulting nanocomposite was composed of the organic moiety, levodopa, sandwiched between Zn/Al-LDH inorganic interlayers. The basal spacing of resulting nanocomposite was 10.9 Å. Estimated loading of levodopa in the nanocomposite was approximately 16% (w/w). A Fourier transform infrared study showed that the absorption bands of the nanocomposite were characteristic of both levodopa and Zn/Al-LDH, and that the intercalated organic moiety in the nanocomposite was more thermally stable than free levodopa. The resulting nanocomposite showed sustained-release properties, caused better viability of fibroblast (3T3) cells than pure levodopa after 72h of exposure.

Further coating of Tween-80 of the levodopa-LDH nanocomposite was achieved through the oxygen of C=O group of Tween-80 with the layered of levodopa-LDH nanocomposite. The X-ray diffraction technique indicates that the Tween-levodopa-LDH nanocomposite was an aggregated structure. From the thermogravimetric analysis data, the loading of Tween-80 coating on the surface of levodopa-LDH nanocomposite was 5.4%. The release of levodopa from Tween-levodopa-LDH nanocomposite was slower compared to that from levodopa-LDH nanocomposite, presumably due to the retarding and shielding effect. A dopaminergic cell line (PC12) showed improved viability with Tween-80 coated levodopa-LDH nanocomposite treatment by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Levodopa-LDH nanocomposite demonstrated lesser dose and time-dependent toxicity on a dopaminergic cell (PC12) compared to pristine levodopa. The cytoskeletal structure of PC 12 was preserved at the IC_{50} concentration of the nanocomposite 178.67±2.6 µg/mL and pure levodopa 49.37±1.2 µg/mL. Metabolism of the nanocomposite was shown via levodopa metabolite (HVA) release from the treated neuronal cell (PC12).

Acute oral toxicity study of nanocomposite on Sprague Dawley rats at a dose of 2000 mg/kg produced neither mortality nor toxicity after 14 days of treatment. Animal treated with nanocomposite gained weight ($p<0.05$). Biochemical analysis of renal and liver functions showed no significant difference between rats treated with nanocomposite and the controls. There was neither any gross lesion nor histo-pathological change observed in various organs.

Repeated dose study with nanocomposite at 5 mg/kg and 500 mg/kg for 28 days showed no sign or symptom of toxicity. Body weight gain, feeding, water intake, general survival, and organosomatic index were not significantly different between control and treatment groups. The differences in AST/ALT of 500 mg/kg levodopa-nanocomposite (0.32±0.12) and 500 mg/kg LDH-nanocomposite treated rats (0.34±0.12) were statistically significant ($p<0.05$) compared to the control (0.51±0.07). The histology of liver, spleen and brain were found to be of similar in morphology in both control and experimental groups. The kidneys of 500 mg/kg treated rats treated with 500 mg/kg body weight of levodopa-nanocomposite or LDH- nanocomposite were found to have slight inflammatory changes, notably leukocyte infiltration around the glomeruli. The ultra-structure of the neurons from the substantia nigra of nanocomposite-treated rats was similar to those receiving only normal saline.

An anti-Parkinsonian drug (levodopa) was successfully intercalated into the inter-layers of zinc aluminium nanodelivery system via co-precipitation method. The nanocomposite was shown to be safe in animal at single 2000 mg/kg dose taken orally, but some changes were noted in the kidney and liver after repeated dose treatment with 500 mg/kg body weight of the nanocomposites. Further assessment through chronic toxicity study is needed to determine the safety profile of long term treatment with the nanocomposite.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENCIRIAN DAN KETOKSIKAN NANOKOMPOSIT ZINK ALUMINIUM HIDROKSIDA BERLAPIS BERGANDA-LEVODOPA

Oleh

AMINU UMAR KURA

October 2014

Pengerusi: Sharida Fakurazi, PhD

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Levodopa adalah ubat pilihan terbaik dalam rawatan penyakit Parkinson (PD) yang merupakan penyakit neurodegenerasi yang tidak membawa maut ini. Walau bagaimanapun, antara halangan yang menyebabkan levodopa sukar untuk di uruskan adalah akibat metabolisme periferal dan penghantaran levodopa yang sangat sedikit apabila diberikan di dalam kuantiti yang tinggi. Hidroksida berlapis berganda (LDH) adalah nanokomposit bukan organik yang mana dadah berada di antara dua lapisan. Ia memiliki keupayaan untutk melepaskan dadah dengan kadar yang berterusan dan perlahan, yang mana akan meningkatkan penyerapan dadah oleh sel, terbukti memiliki kesesuaian biologi dan kurang toksik dalam kebanyakan kes berbanding sistem dadah konvensional. Di sini, satu bahan organik-tak organik nanokomposit yang mengandungi levodopa telah disintesis dengan menggunakan kaedah langsung. Nanocomposit yang terhasil adalah terdiri daripada moieti organik, levodopa, yang diapit di antara lapisan tak organik Zn/Al-LDH. Jarak basal nanocomposit yang terhasil adalah 10.9 Å. Anggaran muatan levodopa dalam nanocomposit adalah kira-kira 16% (w/w). Kajian menggunakan Fourier Transformasi Inframerah menunjukkan bahawa jalur penyerapan nanocomposit itu adalah terdiri daripada ciri kedua-dua levodopa dan Zn/Al-LDH, dan moieti organik terinterkalasi dalam nanokomposit itu lebih stabil secara termal berbanding levodopa yang tidak diinterkalasi. Nanocomposit yang terhasil menunjukkan pelepasan dadah dengan kadar yang berterusan dan perlahan, peningkatan viabiliti sel-sel fibroblast (3T3) berbanding dengan yang terdedah kepada levodopa selama 72 jam.

Seterusnya, ledopa-LDH nanocomposit yang disalut oleh Tween-80 dipermukaan luar telah diperolehi melalui oksigen dari kumpulan C=O dengan lapisan nanokomposit dopa-LDH. Teknik pembelauan sinar-X menunjukkan bahawa nanokomposit Tween-dopa-LDH ialah struktur beraggregasi. Dari analisis termogravimetrik, muatan lapisan Tween-80 pada permukaan nanokomposit dopa-LDH adalah sebanyak 5.4%. Pembebasan levodopa dari pada nanokomposit Tween-dopa-LDH menunjukkan pembebasan lebih perlahan berbanding dengan pelepasan dari nanokomposit dopa-LDH, akibat kesan perlindungan. Sel dopaminergic (PC12) menunjukkan viabiliti yang lebih baik apabila dirawat dengan nanokomposit dopa-LDH yang disalut Tween-80 seperti yang dikaji oleh aktiviti dehidrogenase mitokondria (MTT assay).

Nanokomposit Levodopa-LDH menunjukkan kurang ketoksikan yang bergantung pada dos dan masa ke atas sel dopaminergic (PC12) berbanding levodopa. Struktur sitoskeletal PC 12 adalah tidak berubah pada kepekatan IC_{50} nanokomposit dan levodopa. Penerimaan sel dan metabolisme nanokomposit adalah melalui metabolit levodopa (HVA) yang dilepaskan daripada sel neuron yang dirawat (PC12).

Setelah 14 hari pemerhatian, ketoksikan oral akut nanokomposit pada tikus Sprague Dawley pada dos had 2000 mg/kg tidak mengakibatkan kematian mahupun tanda ketoksikan. Haiwan yang dirawat dengan nanokomposit mengalami penambahan berat badan secara berterusan sepanjang tempoh kajian, terbukti jauh lebih tinggi daripada berat badan haiwan pada awal kajian ($p < 0.05$). Analisis biokimia untuk fungsi buah pinggang dan hati tidak menunjukkan perbezaan yang signifikan antara tikus yang dirawat dengan nanokomposit dan kawalan. Tidak wujud luka mahupun perubahan histo-patologi yang dapat diperhatikan pada organ-organ.

Nanokomposit dengan dos berulang pada dos 5 mg/kg dan 500 mg/kg selama 28 hari tidak menyebabkan sebarang tanda atau gejala ketoksikan. Penambahan berat badan, makan, pengambilan air, kelangsungan hidup umum, dan indeks organosomatik tidak menunjukkan perbezaan yang nyata antara haiwan kawalan dan yang menerima rawatan. Aspartate aminotransferase (AST) dalam 500 mg/kg nanokomposit-levodopa (169 ± 30 U/L), 5 mg/kg nanokomposit-levodopa (172 ± 49 U/L) dan 500 mg/kg nanokomposit-LDH (175 ± 25 U/L) telah meningkat terutamanya berbanding dengan kawalan (143 ±5 U/L), tetapi perbezaan ini adalah tidak signifikan ($p > 0.05$). Walau bagaimanapun, perbezaan nisbah AST/ALT 500 mg/kg nanokomposit-levodopa (0.32 ± 0.12) dan 500 mg/kg nanokomposit-LDH (0.34 ± 0.12) adalah signifikan secara statistik ($p < 0.05$) berbanding dengan kawalan (0.51 ± 0.07).

Histologi hati, limpa dan otak didapati bahawa kedua-dua kumpulan eksperimen dan kawalan memiliki morfologi yang sama. Tikus yang dirawat menggunakan 500 mg/kg nanokomposit-levodopa dan nanokomposit-LDH didapati mengalami sedikit perubahan di bahagian ginjal terutama infiltrasi leukosit di sekitar glomeruli. Ultra struktur-neuron dari substantia nigra daripada kumpulan yang dirawat dengan nanokomposit adalah sama dengan kumpulan yang hanya menerima air garam.

ACKNOWLEDGEMENTS

All gratitude is to Allah, whose guidance has made it possible for me to come this far in my academic pursuit. I also appreciated and thank everyone whose support has made this journey possible and easy.

My deepest gratitude goes to my supervisor Prof. Madya. Dr. Sharida Fakurazi, for all the guidance, encouragement and timely support, throughout the period of my study. To my co-supervisors, Prof. Dr. M. Zobir H and Dr. Cheah-Pike See, I am very grateful for their invaluable support and mentorship.

Thank you to members of the nanodelivery research team in the Institute of Advance Technology and Institute of Biosians, Laboratory of Vaccine and Immunotherapeutic Universiti Putra Malaysia for all their hard work and support. Lastly, I would like to thank my mother Khadijat Muhammad Kura and my wife Hafsat Y. Bashir, my family members too numerous to mention and my friends for their constant support, motivation and faith.

I must also acknowledge the financial support for this project from the Ministry of Science, Technology, and Innovation Malaysia for project funding under nanofund NND/NA/(I) TD11-010 and Universiti Putra Malaysia.

I certify that a Thesis Examination Committee has met on 24th October 2014 to conduct the final examination of Mr. Aminu Umar Kura on his thesis entitled "Characterization and Toxicity of Zinc-Aluminium Layered Double Hydroxide-Levodopa Nanocomposite" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of **Doctor of Philosophy**. The members of the Supervisory Committee were as follows

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LIST OF ABBREVIATIONS

AO	Acridine orange
ATP	Adenosine triphosphate
ALT	Alanine aminotransferase
ATCC	American Type Culture Collection
AST	Aspartate aminotransferase
BBB	Blood brain barrier
CHNS	Carbon-Hydrogen-Nitrogen-Sulphur analysis
CNS	Central nervous system
CETN	Cetirizine nanocomposite
CK	Creatine kinase
Cl^-	Chloride
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
OECD	Economic Co-operation and Development
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FTIR	Fourier transform infrared spectroscopy
GGT	Gamma-glutamyl transferase
GSH	Glutathione assay
GNP	Gold nanocomposite
HAN	Hippuric acid or its nanocomposite
H & E	Haematoxylin-eosin
HVA	Homovallinic acid
IACUC	Institutional Animal Care and Use Committee
ION	Iron oxide nanoparticle
LDH	Layered double hydroxide
LD	Levodopa
LID	Levodopa-induced dyskinesia
LD50	Lethal dose 50
MRI	Magnetic resonance image
MAOs	Monoamine oxidases
nm	Nanometer
NGF	Nerve growth factor
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
PD	Parkinson's disease
PI	Propidium Iodide
K^+	Potassium
RES	Reticular endothelial system
SEM	Scanning electron microscopy
SD	Standard deviations
NaOH	Sodium hydroxide
Na^+	Sodium
TGA	Thermogravimetric analysis
TEM	Transmission electron microscopy

Tween-ZA	Tween-80 zinc aluminium nanocomposite
Tween-dopa	Tween-80 zinc aluminium levodopa nanocomposite
UV-vis	Ultraviolet-visible spectrophotometry
UPM	Universiti Putra Malaysia
ANOVA	Analysis of variance
PPT	Paclitaxel
PD	Parkinson's disease
PASA	Para-amino salicylic acid
PE	Perindopril erbumine
PBS	Phosphate-buffered saline solution
PEI	Polyethyleneimine
ROS	Reactive oxygen specie
rpm	Rotation per minute
SZNs	Salicylate-zinc layered hydroxide nanohybrids
X-RD	X-ray diffraction technique
ZLH	Zinc layered hydroxide
ZnO	Zinc oxide
ZAL	Zinc aluminium levodopa nanocomposite
ZAL	Zinc aluminium nanocomposite
MTT	3-(4,5- dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide

CHAPTER 1

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by impairment/death and degeneration of dopaminergic nerve in the sustantia nigra region of the brain leading to decreased dopamine in circulation (1, 2). The loss of dopamine causes the nerve cells of the striatum to fire out of control, leaving patients unable to direct or control their movements in a normal manner. A balance between dopamine and acetylcholine (chemical transmitters) is essential in controlling muscules movement, as dopamine is excitatory, while acetylcholine is inhibitory (3). This disease usually progresses to severe incapacitation within 10 - 20 years after onset, especially in the most elderly patient. It can seriously impair quality of life in any age group affected. The patient becomes increasingly dependent on family support. The physical and emotional burden of this disease on family members cannot be underestimated (1). In the United Kingdom, Parkinson's disease affects more than 1:1,000 of the general population rising to one per cent of the elderly population, and two per cent over the age of 80 years (2). The four cardinal features of PD are; tremor at rest described as pill rolling, cogwheel rigidity, especially at elbow joint, akinesia/bradykinesia and postural instability.

Additional motor symptoms are, flexed posture and freezing (motor blocks), while non-motor symptoms include autonomic dysfunction, neuropsychiatric problems in the form of mood disturbances, cognition, behavior or thought alterations, sensory and sleep difficulties, are also seen in some patients (4). There is no definitive cure for Parkinson's disease, treatment generally aimed at reducing the symptoms, and thus, the treatment plan is individualized based on presentation at time of diagnosis. Treatment is recommended as soon as symptoms are interfering with daily life.

Medications, surgery, and lifestyle modification alone or in combination, is applied for the treatment of Parkinson’s disease. Medications used in the treatment of Parkinson's disease aimed at increasing dopamine levels in the brain or mimic the action of dopamine (5). A lining covering the brain and isolating it from the rest of the body, the blood brain barrier (BBB), prevents the entrance of dopamine into the brain. A pro-drug called levodopa capable of crossing this barrier is given usually in combination with carboxylase inhibitors like carbidopa to prevent its peripheral metabolism. The carboxylase inhibitors minimize the peripheral breakdown of levodopa and are responsible for decreasing the doses needed before levodopa reaches the brain (6, 7). Other medications for PD include bromocriptine, pramipexole, and ropinirole. These are dopamine agonist also acting on dopaminergic receptors. Other, medications in used include anticholinergic agents (e.g., benztropine), monoamine oxidase B inhibitors (e.g., selegeline), and amantadine (8). Despite the barrage of side-effects like nausea, dyskinesia and the development of response fluctuation, levodopa in combination with a decarboxylase inhibitor (carbidopa) remains the best agent in the symptomatic management of the disease (7).

Prior to the introduction of levodopa, PD caused severe disability or death in 25% of patients within 5 years of onset, and 65% in the next 5 years, and in 89% of those who

survived for 15 years. The mortality rate from PD is 3 times that of the general population matched for age, sex, and racial origin. With the introduction of levodopa, the mortality rate dropped approximately 50%, and longevity was extended by several years. This change in prognosis was thought to be due to the symptomatic effects of levodopa as no clear evidence suggests that levodopa stems the progressive nature of the disease (8, 9).

Problem Statement

Levodopa (LD) is still the drug of choice in the symptomatic treatment of Parkinson's disease. However, long-term treatment with LD is, often complicated by the development of various types of motor response as well as drug-induced dyskinesias. It is widely believed that reducing pulsatile stimulation of dopaminergic neurons will reduce the risk of levodopa-induced dyskinesia (LID). Crossing the brain blood barrier (BBB) by levodopa is another hurdle. Currently it is used in combination with another agent (carbidopa) to aid in crossing the BBB and decreases its peripheral metabolism.

Justification

Layered double hydroxide (LDH) is a nanodelivery system that is generally biocompatible, making them an acceptable alternative drug delivery system. They possess a high intrinsic pharmacological activity compared with conventional drugs and local sustained release property, transcytosis of drugs across tight epithelial and endothelial barriers including the blood brain barrier. Layered double hydroxide can deliver macromolecular drugs to intracellular sites of action, and it is relatively easy to synthezise and manipulate as drug delivery material. Nano-biotechnology in drug delivery is encouraging, particularly in the area of brain drug delivery, local sustained release; improved delivery of poorly water-soluble drugs, targeted delivery of drugs in a cell- or tissue-specific manner.

General Objective:

- To assess the toxicity potential of zinc aluminuim LDH intercalated with levodopa *in vitro* and *in vivo* model.

Specific Objectives

1. To synthesize and characterize zinc-aluminum nanocomposite containing levodopa
2. To modify the synthesized nanocomposite using a surfactant for possible brain delivery

3. To determine the cytotoxicity potential of ZnAl nano-composite containing levodopa on a fibroblast (3T3) and dopaminergic cell line (PC12).
4. To study the biochemical and pathological effects of nanocomposite containing levodopa following acute and sub-acute rat model.

Hypotheses of the study were;

1. A zinc aluminium nanocomposite intercalated with levodopa will have a sustained, control release ability.
2. A nanocomposite containing levodopa will have higher thermal stability, decreses toxicity on cells and animal models.
3. Surface coating of zinc aluminium nanocomposite with tween-80 will increase the chance of levodopa delibery across the blood brain barrier.

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