



**A CELLULAR MODEL FOR AROMATIC L-AMINO ACID
DECARBOXYLASE DEFICIENCY AND NEUROTRANSMITTER
ANALYSIS OF AVAILABLE THERAPEUTICS USING A
NEUROBLASTOMA CELL CULTURE**

By

MELATI KHALID

**Thesis Submitted to the School of Graduate Studies,
Universiti Putra Malaysia, in Fulfilment of the
Requirements for the Degree of Doctor of Philosophy**

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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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Aromatic L-amino acid decarboxylase (AADC) deficiency is a rare pediatric neurotransmitter disease. It is an autosomal recessive genetic disorder affecting to date, less than 100 children worldwide. AADC is the key enzyme required for the synthesis of neurotransmitters in the catecholaminergic and serotonergic pathways. Various mutations at position 11 on the short arm of chromosome 7 are responsible for this condition. To date, no apparent correlation can be identified to link mutations with disease outcome. Disease severity can span the spectrum from mild to complete global disability. Generally, affected children fail to achieve any developmental milestones. Movement disorder resulting from dopamine and serotonin deficiency produces characteristic dystonia and oculogyric crises that occur at regular intervals every 3 to 8 days, each episode lasting on average 6 hours. Autonomic dysregulation in the form of temperature instability and susceptibility to metabolic crisis involving sudden dramatic, life-threatening blood glucose level drop and breathing depression are also hallmarks of AADC deficiency. Current treatment intervention involves bolstering endogenous dopamine levels using MAO inhibitors with concurrent administration of dopamine agonists. Various SSRI are also used to maintain endogenous serotonin levels. Anti-cholinergic agents are also used with various outcomes. More recently, the AADC enzyme co-factor PLP and folinate supplementation has shown some promise in improving general disease outcome. However, the overall response to treatment has been dismal. Due to its rare nature, basic research into the disease is scant. Therapeutic tactics has been dependent on the

Parkinson's approach as both conditions share a dopamine deficiency. However, the dopamine deficiency of Parkinson's is due to nigrostriatal dopaminergic neuron death with a late onset while children with AADC deficiency survive entirely without developmental influences from the neurotransmitter. This key difference is believed to be the reason for poor dopamine transmission in AADC deficiency using anti-Parkinson's drugs. This study sought to create a cell model of AADC deficiency to quantify the dopamine synthesis potential of several such drugs. Building on the success of a working cell model, this study also then tried to identify competitive amino acid inhibitors of dopamine. Treatment of SH-SY5Y cells with L-DOPA coaxed dopamine production to levels detectable by HPLC. Subsequent administration of the AADC inhibitor benserazide quelled this effect. Previous efforts to transform immature neuroblastoma cells to a dopaminergic phenotype using all-trans-retinoic acid failed. No detectable level of dopamine was seen via HPLC. Using this model of L-DOPA with concomitant benserazide inhibition to model AADC deficient states, Parnate, bromocriptine, Artane and PLP were tested for their dopamine enhancing capabilities. Parnate, bromocriptine and Artane all produced detectable levels of dopamine but did so only minimally and after a long incubation time. PLP did not induce any dopamine synthesis. This suggests that Parnate, bromocriptine and Artane promotes some dopamine synthesis in this model of AADC deficiency. This mirrors the small gains accorded from their use in AADC deficiency treatment. Unfortunately it also reflects their ineffectiveness. Tryptophan, phenylalanine and tyramine were tested for competitive inhibition of dopamine. Serotonin, its precursor 5-HTP and the catecholamine noradrenaline were also tested for this possible action. 5-HTP was the only one found to inhibit dopamine production. The findings of this study are hoped to fuel research interest into a condition with minimal monetary gains potential. Most importantly, it is hoped to improve treatment outcome and quality of life for AADC deficiency sufferers towards an ultimate cure.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra
Malaysia
Sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**MODEL SEL NEUROBLASTOMA BAGI PENYAKIT KEKURANGAN
ASID AROMATIK L-AMINO DEKARBOKSILASE DAN ANALISA
KADAR NEUROTRANSMITER DENGAN RAWATAN UBATAN SEDIA
ADA**

Oleh

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Gejala kekurangan enzim asid aromatik L-amino dekarboksilase adalah suatu penyakit jarang jumpa. Keadaan yang disebabkan autosom resesif ini menyerang kurang daripada 100 orang kanak-kanak dalam dunia. AADC merupakan enzim utama dalam pembentukan neurotransmitter katekolamin dan serotonin. Pelbagai mutasi pada kedudukan 11 lengan pendek kromosom 7 berupaya menghasilkan gangguan ini. Sehingga kini, belum ada korelasi dapat mengkaitkan lokasi mutasi dengan tahap serius penyakit. Penghidap boleh tergolong dari keadaan yang tidak serius hinggalah yang kurang upaya sepenuhnya. Lazimnya, kanak-kanak sebegini langsung tidak mencapai sebarang perkembangan kebolehan. Kekurangan dopamin dan serotonin mengakibatkan penyakit gangguan pergerakan. Ia penyebab berlakunya distonia dan krisis okulogirik dengan kerap selang 3 hingga 8 hari. Keadaan ini biasanya berlarutan purata 6 jam setiap kali. Kecelaruhan autonomik berupa ketidakstabilan suhu badan dan kecenderungan menjurus krisis metabolik boleh berlaku. Ia mengakibatkan paras gula dalam darah menjunam mendadak dan gangguan proses pernafasan yang amat serius. Kecenderungan terhadap kecelaruhan autonomik adalah salah satu ciri utama penyakit kekurangan enzim ini. Intervensi ubatan melibatkan usaha meningkatkan kadar dopamin sedia ada dalam badan pesakit menggunakan pengekang monoamine oksidase (MAO-I) dan agonis dopamin. Beberapa jenis SSRI juga diguna bagi mengekalkan tahap serotonin sedia ada. Ubat anti-kolinergik turut diguna pakai dengan hasil yang berbeza-beza. Kajian terbaharu menunjukkan potensi penggunaan kofaktor enzim AADC iaitu PLP dan folinat sebagai zat suplemen bagi meringankan impak penyakit secara amnya.

Hakikatnya, keberkesanan ubat sedia ada sekadar hambar sahaja. Disebabkan penyakit ini amat jarang dijumpai, sukar menjana minat menjalankan kajian asas terhadapnya. Taktik mengubatnya juga banyak menumpang pendekatan penyakit Parkinson. Ini kerana keduanya mengakibatkan kekurangan dopamin. Akan tetapi, kekurangan dopamin Parkinson disebabkan kematian neuron dopaminergik di nigrostriata. Ia lazimnya berlaku pada usia lanjut. Sebaliknya, kanak-kanak kekurangan enzim AADC lahir dan membesar tanpa pengaruh perkembangan yang diurus neurotransmitter tersebut. Perbezaan utama ini punca mengapa ubatan Parkinson tidak berjaya menjana dopamin dalam situasi kekurangan enzim AADC. Maka, kajian ini cuba menghasilkan model penyakit kekurangan enzim AADC dari sel agar dapat mengukur keberhasilan beberapa ubatan menjana dopamin. Dengan adanya model sel, kajian ini dapat meneliti sifat pengekang bersaing pada beberapa asid amino dalam Jaluran Katekolamin dan serotonergik terhadap dopamin. Proses pempotensi mengaruh sel SH-SY5Y hasilkan dopamin hingga dapat dikesan menggunakan teknik HPLC. Kemudian, apabila sel dirawat dengan penghalang enzim AADC benserazide, dopamin hilang semula. Usaha awal mentransformasi sel neuroblastoma yang dianggap tidak matang ini menggunakan asid retinoic semua-trans tidak berhasil menjana dopamin. Kaedah menggunakan L-DOPA dan benserazide berhasil membentuk model sel penyakit kekurangan enzim AADC. Ini membolehkan kami mengukur keberkesanan ubat Parnate, bromocriptine, Artane dan PLP. Parnate, bromocriptine dan Artane berupaya menghasilkan dopamin tetapi memerlukan masa yang agak lama. PLP pula langsung tidak membuahkan hasil. Pengamatan ini menyamai hasil penggunaan ubatan tersebut dalam merawat kekurangan AADC. Ada sedikit keberkesanan tetapi tidak cukup untuk melegakan simptom. Tryptophan, phenilalanin dan tiramin kemudiannya dianalisa untuk sifat penghalang bersaing terhadap dopamin. Serotonin, pemulanya 5-HTP dan noradrenalin yang juga katekolamin turut dianalisis untuk sifat pengekang bersaing. Hasil kajian ini diharap dapat menjana minat dalam bidang yang tidak menjanjikan pulangan kewangan. Yang paling utama, ia diharap dapat membantu menambahbaik kualiti hidup pesakit kekurangan AADC dan seterusnya membolehkan mereka sembuh sepenuhnya.

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Verily for every disease there is a cure.
Subhanallah.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment 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

AADC	aromatic L-amino acid decarboxylase
AAV2	adeno-associated virus, serotype 2
AC	adenylyl cyclase
ACN	acetonitrile
APS	ammonium persulfate
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factors
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CATs	catecholamines
cDNA	complementary DNA
CNS	central nervous system
COMT	catechol-ortho-methyltransferase
CSF	cerebrospinal fluid
CT	computed tomography
DA	dopamine (3,4-dihydroxyphenylethylamine)
DBH	dopamine β -hydroxylase
DDC	dopa decarboxylase
DHBA	dihydroxybenzylamine
dH ₂ O	distilled water
DMSO	dimethyl sulfoxide
DOPAC	3,4-dihydroxyphenylacetic acid
L-DOPA	L- β -3,4-dihydroxyphenyl alanine

EPI / AD	epinephrine / adrenaline
GABA	γ -amino butyrate
GAP-43	growth-associated protein 43
GCH	GTP cyclohydrolase I
GPCR	G-protein coupled receptors
GPL	lateral globus pallidus
GPM	medial globus pallidus
5-HIAA	5-hydroxyindoleacetic acid
HIOMT	hydroxyindole-O-methyltransferase
HPLC-ED	high performance liquid chromatography with electrochemical detection
HRP	horseradish peroxidase
5-HTP	5-hydroxytryptophan
HVA	homovanillic acid / homovanillate
IC ₅₀	inhibition constant at 50%
IP ₃	inositol triphosphate
kDA	kilo Dalton
MAO-B	monoamine oxidase B
MAO-I	monoamine oxidase inhibitor
MAPT	microtubule-associated protein tau
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	magnetic resonance imaging
mRNA	messenger RNA
NE / NA	norepinephrine / noradrenaline
3-O-MD	3-ortho-methyldopa

PBS	phosphate buffer saline
PI	phosphatidylinositol
PIP ₂	phosphatidylinositol bisphosphate
PKA	protein kinase A
PLC	phospholipase C
PLP	pyridoxal phosphate
PND	paediatric neurotransmitter diseases
PNMT	phenylethanolamine N-methyltransferase
P-5-P	pyridoxal 5'-phosphate
RA / ATRA	retinoic acid / all-trans retinoic acid
RBFOX3	RNA binding protein, fox-1 homolog 3 (homolog of neuronal nuclei, NeuN)
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SAM	S-adenosylmethionine
SCN	suprachiasmatic nucleus
SDS	sodium dodecyl sulphate
SMA	supplementary motor area
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
SOS	sodium octyl sulphate
SSRI	selective serotonin reuptake inhibitor
STN	subthalamic nucleus
SYP	synaptophysin
TH	tyroxine hydroxylase

TPA	phorbol ester 12-O-tetradecanoylphorbol-13-acetate
VLN	ventral lateral nucleus
VMA	vanilmalonic acid / vanillylmandelate (3-methoxy-4-hydroxymandelate)
VTA	ventral tegmental area



CHAPTER 1

INTRODUCTION

1.1 Introduction of study

Aromatic L-amino acid decarboxylase deficiency (AADC) is a rare autosomal recessive paediatric neurotransmitter disease. It is caused by a mutation in the AADC gene resulting in a deficiency of the AADC enzyme required to convert L-DOPA to dopamine and 5-HTP to serotonin. The resultant dopamine, serotonin and all downstream metabolite deficit manifests in a debilitating disease that appears in infancy. Hyland and Clayton first characterized AADC deficiency in 1990 and to date it remains a poorly understood disease (Hyland & Clayton, 1990). Awareness of this disease is relatively low among physicians and patients are frequently initially misdiagnosed as suffering from cerebral palsy, epilepsy or gastric reflux. Worldwide, 74 patients have been identified as suffering from AADC (http://www.biopku.org/BioPKU_databasesJAKE.asp). In Malaysia, 5 children are correctly diagnosed with the disease. Taiwan appears to have the largest population of diagnosed AADC children (Lee et al., 2008). Active awareness creation, diagnosis and research in Taiwan may to a certain extent account for this prevalence but a true prevalence may also be the case. Elsewhere, AADC has been diagnosed in the United States, Europe, Israel, Japan and Singapore.

1.2 Problem Statement

AADC deficiency needs a model system in which to study its pathological idiosyncrasies. To date, relatively little research has been carried out to elucidate this disease. A mouse animal model is still being perfected (Hwu et. al., 2013). A zebrafish AADC model had been in use earlier but for obvious reasons not entirely practical (Shih et. al., 2013). This study set out to design a cell culture model for AADC. The neuroblastoma cell culture SH-SY5Y, a line frequently used to model dopaminergic systems in Parkinson's disease was chosen. Preliminary Western Blotting screening revealed it to express key proteins of the dopaminergic pathway and circadian rhythm machinery, thus validating our choice of model system. AADC deficiency symptoms fluctuate with the time of day with a worsening as the day progresses hinting at a strong circadian rhythmicity. The circadian rhythm proteins Period 1, Cryptochrome 1 and 2, CLOCK and BMAL were also detected in the cells via Western Blot. Next, L-DOPA treatment led the cells to produce detectable amounts of dopamine on HPLC-ECD before this *de novo* synthesis is inhibited with

the use of benserazide. Our cell model of AADC deficiency was thus created. The cells were shown to express markers of mature neurons via RT-PCR. Disease-specific medications are desperately needed to address the symptoms of AADC deficiency. Currently, available therapies are based on anti-Parkinson's medication with a generally poor treatment outcome. Due to the lack of alternative treatment, in the short term it is advantageous to assess the dopaminergic synthesis potential of these medications in an AADC context. Thus, this study also set out to test the effects of anti-Parkinson's medications on the cell culture AADC model. Artane, Bromocriptine and Parnate promote cells to synthesize dopamine after more than 4 hours of exposure. The catecholamine and serotonin pathways are made up of biogenic amines sharing a conserved benzene ring structure. Substances with a similar structure can compete for binding at a substrate's receptor binding site. This gives rise to our second parameter of interest involving the possibility of competitive inhibition of dopamine synthesis by another substrate. Testing on our AADC deficiency cell model revealed 5-HTP the precursor to serotonin, to compete with dopamine synthesis.

1.3 Significance of study

Findings from this study will add to the basic knowledge of a very rare pediatric neurotransmitter disease. Due to its rare nature, there is little chance for research funding by large pharmaceuticals as the prospect of monetary gains are next to none. It is hoped that research interest can be generated purely on the basis of scientific and humanitarian interest.

1.4 Objectives of study

1.4.1 General objective

1. To contribute to the limited body of knowledge on the pathology of AADC deficiency.

1.4.2 Specific objectives:

1. To create a cell model of AADC deficiency

2. To test the dopamine synthesis capabilities of anti-Parkinson's drugs used for treatment of AADC deficiency.
3. To determine if any of the biogenic amines are competitively inhibiting dopamine / L-DOPA

1.5 Study hypotheses:

1. A cellular model of AADC deficiency can be created from SH-SY5Y cells.
2. The dopamine synthesis potential of various anti-Parkinson's drugs will be represented by the level of dopamine produced in the AADC deficient cell model.
3. Biogenic amines of the catecholaminergic and serotonergic pathways competitively inhibit dopamine synthesis.

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