SUBSTRATE DEPRIVATION AS A NOVEL THERAPY FOR THE MUCOPOLYSACCHARIDOSES

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in

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Addenda

Chapter 1

p10, line 18: Insert words "and produce" after breed (add and produce)
p12, line 5: Change "relative permeable" to "relatively permeable"
p14, line 1: Remove comma after blood-brain barrier
p16, line 18: Insert after co-administration of antihistamine... "or by prolonging the infusion time or by a range of other drugs including those with anti-inflammatory and immunosuppressive properties such as corticosteroids (Schiffman and Brady 2002, Drugs; 62(5):733-742; Beck *et al.* 2004, Eur. J. Clin. Invest. 34:838-844).
p17, line 6: Add word "function" before reference.
p18, line 13: Replace words "Recent evidence has" with "It has been"
p19, line 14: Remove the word "outcome"
p21, line 19: Insert underlined words "In another study of MPS mice were treated... and 12 month..."
p22, line 21: Insert "and cross-correction via mannose-6-phosphate mediated uptake following cell engraftment" at end of sentence.
p23, line 4: Replace word "production" with "localisation"
p24/25, Figure 1.7: Figure 1.7 A-D have been replaced. Legend line 9: replace "though" with "thought"
p26, line 1-3: Sentence should read "These patients showed a reduction of mean liver and spleen volumes and also an increased..."
p28, line 5 and line 8: Replace "Twitcher" with "*twitcher*" to indicate strain of mice
p28, last line: Replace sentence with "These compounds can be formulated to be administered orally, which is a major advantage"
p30, line 3: Add word "white" after "New Zealand"
p32, line 12: Add comma after "isoflavones"
p33, line 24: Replace "beta-glucuronidase" with "beta-glucosidase"
p34, lines 3, 4, 5, 8: Replace "beta-glucuronidase" with "beta-glucosidase"
p34, line 15: Insert word "the" after "stop codons in"
p34, line 20: After "preclinical studies" insert "in MPS I"
Figure 1.9 legend, line 15 Replace "structure" with "structures"
p40, line 11: Replace "2-3" with "two to three"
p41, line 6: Replace "uncovered a role in TGF-β regulation in adult cells via protein-protein interaction" with "uncovered a role of syndecan-2 and its direct binding to TGF-β which regulates TGF-β signalling in adult cells"

Chapter 2

p71, lines 4-6: Replace sentence with "If a mouse failed to reach the escape platform within the allocated 60 seconds, the mouse was place manually onto the platform"

Chapter 3

p85, line 11 and Figure 3.5: Insert: "Female mice included in this study were not used for colony breeding and therefore were not pregnant or had litters." A power analysis to determine how many female mice would be needed to confirm the difference in bodyweight was conducted and to achieve a power analysis of 80% with a difference in weight of 15% a sample size of 4 mice would be needed and 2-5 mice were analysed for each group at 4 months of treatment.
Table 3.1: Insert "(n=3 per group)" after "normal treated mice" in table legend.
p86, line 22: Insert "A reduction in total gag was seen in MPS IIIA treated mice, which on average was reduced to 74% of MPS IIIA untreated total gag levels in the liver (Figure 3.8A). This would incorporate total gag including a lysosomal component, and this decrease is likely to be due to reduced lysosomal gag. To confirm this, lysosomal gag was also analysed. Analysis of lysosomal

gag showed a greater reduction compared to total gag levels, with MPS IIIA treated mice on average reduced to 60% of MPS IIIA untreated lysosomal gag levels (Figure 3.8B). This was expected since MPS IIIA is a lysosomal storage disorder and most of the gag reduction was lysosomal gag."
p91, line 2: "si" should read "is"
p91, line 11: Add word "been" after "has not"

Chapter 4
p94, line 1: Change "Neurological pathology" to "Neural pathology"
p98, bottom line: Change "showed an increased trend..." to "showed a trend to increased..." (reverse order)
p103, 2nd last line of 2nd last paragraph: Missing full stop at end of the sentence.
p114, insert before 3rd paragraph: "Qualitative changes in lysosomal storage in the brain or somatic tissues have not been observed in either study reported in chapters 3 and 4 using light microscopy analysis. However, a small decrease in brain lysosomal gag was seen when a quantitative assay was employed. The discrepancy between the biochemical measurement and visual assessment of storage may be due to the relative insensitivity of light microscopy to detect small changes in lysosomal storage. SDT acts to slow down the accumulation of gag substrate in the lysosome, providing the opportunity for residual lysosomal enzyme activity to slowly turn over resident and newly acquired substrate. Thus conceptually SDT will not have the dramatic effect on gag storage observed with other types of treatment such as enzyme replacement therapy."
p115, last line: Replace "4" with "four"

Chapter 5
p121, line 5: Insert sentences "These stains were chosen as good representative stains which are useful for detecting abnormalities in liver sections. H&E was used to detect general changes in morphology. Perl staining was used to stain iron and determine iron load, which is a marker of hepatic injury. Sirius Red staining was used to detect liver fibrosis and PAS staining is good stain for glycogen and mucins and is useful for outlining tissue structures such as basement membranes, capsules and blood vessels.
p123, line 22: Insert full stop at end of sentence.
p124, line 11: Replace "not" with "no"
p124, line 22: Replace "distal convoluted tubule" with "proximal convoluted tubule"
Figure 5.9 legend, line 4: Replace "distal convoluted tubule" with "proximal convoluted tubule"
Figure 5.9 legend, line 5: Replace "proximal convoluted tubule" with "distal convoluted tubule"
p127, insert before 3rd paragraph: "The colony was regularly checked every three months for a battery of pathogens including salmonella, enteric pathogens, respiratory pathogens including *Mycoplasma spp*, ectoparasite examination, burrowing mites, helminths, protozoa mucosal scrapings and pinworm testing. No pathogens were observed in mice from the multi-generational study, therefore it is unlikely that pathogens would have been an influence of the visceral and neural pathology. All mice included in the multi-generational study were healthy and the only abnormalities were those associated with MPS IIIA disease progression in affected animals."
p128, line 12: Remove "had" after "Kupffer cells"
p129, line 24: Replace "natural level" with "minimal level"
p130, line 18: Remove "was" after "a toxicity study"

Chapter 6
p132, 2nd paragraph: Reword first sentence to clarify: "Even though ERT has been used to treat a large number of patients with non-neurological MPS pathology, there are still a number of problems associated with this type of therapy. Firstly, targeting...."

Thesis summary

Reduction of an enzyme required for the lysosomal degradation of glycosaminoglycan (gag) chains will result in a mucopolysaccharidosis (MPS) disorder. Substrate deprivation therapy (SDT), a new therapy option for MPS, aims to reduce the synthesis of gag chains, the natural substrate for the deficient enzyme. Reduced substrate levels would balance the reduced level of enzyme in patient cells resulting in normalised gag turnover. Rhodamine B, a non-specific inhibitor, reduced gag synthesis in a range of normal and MPS cells and also decreased lysosomal storage of gag in MPS VI (72%) and MPS IIIA (60%) cells. This positive response *in vitro* was extended to an *in vivo* therapy trial in the MPS IIIA mouse. Bodyweight gain of male MPS IIIA mice treated with 1 mg/kg rhodamine B was reduced compared to untreated MPS IIIA mice and was indistinguishable to that of normal mice. Liver size, total gag content and lysosomal gag was reduced in treated MPS IIIA animals as was urinary gag excretion. The alteration in MPS IIIA clinical pathology by rhodamine B combined with the observation that treatment had no effect on the health of normal animals demonstrates the potential for this type of therapy for MPS disorders. The water cross maze was found to be the only learning and memory test capable of detecting differences in learning behaviour in MPS IIIA and normal untreated mice. MPS IIIA mice treated with SDT rhodamine B showed an improved outcome with better learning capabilities than MPS IIIA untreated mice observed using this test. This means that rhodamine B is likely to cross the blood-brain barrier. These results are the first evidence of a positive response by the CNS to a systemic therapy for MPS IIIA. Rhodamine B administration over 4 generations did not produce any deleterious side effects in MPS IIIA. *In utero* therapy over four generations did not cause a reduction in litter size or bodyweight profile demonstrating that reduction of gag over a combined timeframe of two years was safe. A higher dose of 5 mg/kg rhodamine B did not produce any additional benefits on MPS IIIA pathology and no signs of hepatoxicity were noted. Rhodamine B proved to be a

general inhibitor of gag synthesis and had a positive outcome on a number of clinical parameters in MPS IIIA mice. SDT in MPS IIIA mice improved learning capabilities as detected by the water cross maze which has not been previously reported. This provides evidence that small molecules such as rhodamine B, that are able to cross the blood-brain barrier, can have some effect on neurological pathology. This proof of principle study showed that SDT can be used to have a positive outcome on MPS pathology. Additional inhibitors of gag synthesis can also be investigated before this type of therapy can be translated into clinical use in MPS patients. Although it may be feasible to use rhodamine B as a SDT agent *in vivo*, other inhibitors may be more practical.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for photocopying and loan if accepted for the award of the degree

SIGNED:..... DATE:.....

Acknowledgements

The list is long, but I have to thank all these people for helping me over the last 4 years.

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Well statistics is not my forte, so thank you to Janine Jones and particularly Kate Dowling at Biometrics SA for their help with statistical analysis for the bodyweight profiles, RAPC and cross maze data. Thanks for also answering my endless questions and narrowing the gap in my statistical understanding.

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The auditory evoked brainstem response analysis in the RAPC study would not have been possible without Dr Paul Weston from the Neurology department, CYWHS who helped set up the equipment and methodology and also to evaluate the hearing threshold in MPS IIIA and normal mice.

Thanks to my family, who have always been there and made sure that I had every opportunity to have the best start in life. They have always supported me through my studies and research and still don't' understand what MPS is after all these years.

Last but not least, to my fiancé Simon. Thanks for all your support, words of wisdom and encouragement over the years. This would not be possible without someone to share the anger when the experiments don't work.

Abbreviations

AAV	adeno-associated virus
ABR	auditory evoked brainstem response
APES	3-aminopropyltriethoxysilane
BBB	blood-brain barrier
BMT	bone marrow transplantation
bp, kb	base pairs, kilobase pairs
BSA	bovine serum albumin
cDNA	complementary DNA
CNS	central nervous system
cpm	counts per minute
CS	chondroitin sulphate
°C	degrees Celsius
dB	decibel(s)
DGJ	1-deoxy-galactonorjirimycin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
dNTP	deoxyribonucleotide 5'triphosphate
DS	dermatan sulphate
dH_20	deionised water
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
EM	electron microscopy
ER	endoplasmic reticulum
ERT	enzyme replacement therapy
EXTL	exostosin-like gene family
FCS	foetal calf serum
FDA	Food and Drug Administration
gag(s)	glycosaminoglycan(s)
Gen	generation
GET IT	gene-expression targeted isoflavone therapy
GFAP	glial fibrillary associated protein
GPI	glycosylphosphatidylinositol
GSL	glycosphingolipid storage disorders

HA	hyaluronan/hyaluronic acid
HCl	hydrochloric acid
HS	heparan sulphate
HSpg	heparan sulphate proteoglycan
hr	hour(s)
IMVS	Institute of Medical and Veterinary Science
kg, g, mg, µg, ng	kilogram, gram, milligram, microgram, nanogram
kDa, Da	kilodalton, dalton
KS	keratan sulphate
LSD	lysosomal storage disorder
LiCl	lithium chloride
L, ml, µl	litre, millilitre, microlitre
m, cm, mm, µm	metre, centimetre, millimetre, micrometre
mCi, µCi	milli Curie, micro Curie
min	minute(s)
M, mM, µM, pM	moles per litre, millimoles per litre, micromoles per litre, picomoles per litre
$MgCl_2$	magnesium chloride
MGS	multi-generational study
MPS	mucopolysaccharidosis
MPS IIIA	mucopolysaccharidosis type IIIA
mRNA	messenger RNA
MWM	Morris water maze
$Na^{35}SO_4$	radioactive sodium sulphate
*N*B-DNJ	*N*-butyldeoxynorjirimycin
*N*B-DGJ	*N*-butyldeoxygalactonorjirimycin
*N*N-DNJ	*N*-(*n*-nonyl) deoxynojirimycin
NDST	*N*-acetylglucosamine *N*-deacetylase/*N*-sulphotransferase
NS	sulphamidase
OD	optical density
OGT 918	*N*-butyldeoxynorjirimycin
PAPS	3'-phosphoadenosine 5'-phosphosulphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P/S	penicillin/streptomycin

pH	hydrogen ion concentration
% (w/v)	percent weight per volume
% (v/v)	percent volume per volume
RA	repeated acquisition
RAPC	repeated acquisition and performance chamber
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNase	ribonuclease
RNAi	RNA interference
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SDT	substrate deprivation therapy
sec	second(s)
siRNA	short interfering RNA
Taq	*Thermus aquaticus*
Tris	Tris (hydroxymethyl) amino methane
U	units
UV	ultra violet
V	volts