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**The effects of bleomycin, mitomycin C, and  
cytoskeletal-disrupting drugs on angiogenesis *in vitro*  
and haemangioma development *in vivo***

by

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## Curriculum Vitae

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Peaceful Mabeta was born in Boksburg. She obtained a BSc degree (Biochemistry and Physiology) from Medunsa. She obtained a masters degree in Physiology in 2002 from the University of Pretoria, where she currently holds a lectureship position.

Her thesis is entitled **The effects of bleomycin, mitomycin C, and cytoskeletal-disrupting drugs on angiogenesis *in vitro* and haemangioma development *in vivo***. Haemangiomas are tumours of the vasculature commonly encountered in pediatrics. The treatment of these tumours has over the years remained unsatisfactory. Recently in South Africa, intralesional bleomycin therapy has been used to treat haemangiomas with promising success. However, there is very little understanding of its mechanism of action. The candidate developed a rapid and sensitive HPLC method for the measurement of bleomycin in human plasma, and demonstrated a negligible systemic spill-over of the drug following intralesional therapy in haemangioma patients. In her thesis, Ms Mabeta showed that bleomycin inhibits haemangioma growth in part by inhibiting angiogenesis. The candidate also showed that cytoskeletal-disrupting drugs with antiangiogenic activity effectively inhibit haemangioma growth in a syngeneic mouse model, thereby supporting the notion that these drugs have potential in the treatment of these tumours. Further investigation of the therapeutic potential of these drugs in the treatment of pediatric haemangiomas is underway. The examiners allocated a mark of more than 80% to the thesis.

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## Summary

Angiogenesis, the process of new vessel formation, appears to be a central mechanism that underlies the development of haemangiomas. Recently, intralesional bleomycin injection was used to treat paediatric haemangiomas with very good results. The purpose of this study was to determine whether there was significant systemic circulatory spill-over of bleomycin in haemangioma patients treated with intralesional bleomycin to determine safety of use. Furthermore, in order to elucidate bleomycin's mechanism of action in inducing haemangioma regression, this study aimed at determining the effects of bleomycin on aspects of angiogenesis, namely, endothelial cell migration, growth and apoptosis, and comparing these effects with those of drugs previously reported to inhibit various aspects of the angiogenic process (mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D). Lastly, the effects of bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D were studied in an animal haemangioma model.

A rapid and highly sensitive high performance liquid chromatographic (HPLC) method was developed. Blood samples were collected from four haemangioma patients before and after (over a 24 hour period) intralesional bleomycin (IB) therapy. As a control, blood samples were also collected at identical time intervals from four patients undergoing intravenous (IV) bleomycin chemotherapy for various malignant tumours. The HPLC method was used to quantitate bleomycin fractions in patient samples. The mean bleomycin concentration detected in plasma samples obtained from IB treated patients was 0.00  $\mu\text{g/ml}$  for both bleomycin A<sub>2</sub> and B<sub>2</sub> over the 24-hour period following therapy. Plasma bleomycin A<sub>2</sub> and B<sub>2</sub> levels of 360.79 and 158.85  $\mu\text{g/ml}$  respectively were detected in samples obtained from cancer patients treated with bleomycin IV. These findings indicate that the low levels detected may translate to a significantly lesser risk of pulmonary fibrosis following IBI.

The effect of drugs on endothelial cell migration was analyzed by wounding a confluent monolayer of cells and determining the number of cells that had migrated from the

wound edge. Endothelial cell growth was determined in cells treated with various drug concentrations while apoptosis was examined using hematoxylin and eosin staining, DNA fragmentation assay and acridine orange staining.

The effect of test drugs on *in vitro* angiogenesis was determined on endothelial cells induced to form capillary-like tubes in collagen gel. Test drugs were then evaluated for antitumour activity in an animal haemangioma model.

Data demonstrated that test drugs inhibited endothelial cell migration, with the exception of mitomycin C. All test drugs induced a reduction in the percentage of viable endothelial cell in a dose-dependant manner, and also induced endothelial cell apoptosis. The drugs inhibited angiogenesis *in vitro* and inhibited tumour development *in vivo* with varying potency.

In general, results from this study indicated that there was negligible systemic spill-over of bleomycin following IB administration in patients with haemangiomas, suggesting a much lesser risk of developing bleomycin-induced pulmonary fibrosis. This study also showed that test drugs inhibited angiogenesis *in vitro* and haemangioma development *in vivo* in a mouse model. Taken together, these observations demonstrate that bleomycin may inhibit haemangioma growth by inhibiting angiogenesis. In addition, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D may have potential in the treatment of haemangiomas of infancy, and should be investigated further in a murine haemangioma model to determine effective dose schedules.

Keywords: bleomycin; cytoskeletal-disrupting agents; angiogenesis; haemangioma; endothelial cells; cell growth; cell migration; vascular endothelial growth factor; basic fibroblast growth factor; polyoma middle T oncogene; vascular tumour.



## Opsomming

Angiogenese, die proses waarby nuwe bloedvate gevorm word, blyk om die sentrale meganisme onderliggend tot die vorming van hemangiomas te wees. Onlangs is bleomisien binne-letsels ingespuut om pediatriese hemangiomas te behandel, met baie goeie resultate. Die doel van hierdie studie was om te bepaal of daar 'n noemenswaardige oorvloed van bleomisien in die sirkulatoriese stelsel van pasiënte waar daar bleomisien binne-in die letsel gespuut is, was, en om sodoende die veiligheid daarvan te kan bepaal. Verder, om die meganisme van aksie van bleomisien op hemangioma regressie te verduidelik indien die studie ook die effekte van bleomisien op endoteelselmigrasie, groei en apoptose met betrekking tot angiogenese, met die effek van middels wat al voorheen beskryf is, vergelyk (mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, cochisine, nocodazole and cytochalasin D). Ten laaste, die effekte van bogenoemde middels is ook in 'n hemangioma diere (muis) model bestudeer.

'n Vinnige en hoogs sensitiewe HPLC metode is ontwikkel. Bloed monsters is van pasiënte met hemangiomas geneem, beide voor en na (oor 'n 24 uur periode) binne-letsel inspuiting van bleomisien (IB). As kontrole, is bloedmonsters van pasiënte wat intraveneuse (IV) sistemiese bleomisien chemoterapie ontvang het vir kwaadaardige tumors, geneem. Die HPLC metode is gebruik om die vlak van bleomisien in die monsters van pasiënte te bepaal. Die gemiddelde bleomisien konsentrasie van die plasma monsters van IB behandelde pasiënte was 0.00  $\mu\text{g/ml}$  bleomisien A<sub>2</sub> en B<sub>2</sub> onderskeidelik oor die 24 uur periode na behandeling. Plasma bleomisien A<sub>2</sub> en B<sub>2</sub> vlakke van 360.79 en 158.85  $\mu\text{g/ml}$  onderskeidelik, is in monsters van kanker pasiënte met bleomisien IV behandel, gevind.

Die effekte van die verskillende middels op die migrasie van endoteelselle is bepaal deur 'n aaneenlopende enkellaagselle te wond en dan die aantal selle wat weg beweeg vanaf die wond te bepaal. Die groei van endoteelselle is bepaal met behulp van selle wat met verskillende konsentrasies van die middels behandel is. Apoptose is ondersoek met behulp van weefselkleuring (hematoxylin en eosin), DNA fragmenteringsbepaling en akridien oranje kleuring.

Endoteelselle wat geïnduseer is om kapillêragtige buise in 'n kollageenjel te vorm, is gebruik vir die toetsing van die middels se effek op *in vitro* angiogenese. Die middels is dan ge-evalueer met betrekking tot die anti-tumor effek met die hemangioma muis-model.



Data het aangetoon dat die toetsmiddels endoteelselmigrasie onderdruk het, maar mytomyacin C was die uitsondering hier en het nie die effek gehad nie. Al die middels het 'n afname in lewensvatbare endoteelselle tot gevolg gehad en die afname was afhanklik van die dosis gebruik. Almal het ook endoteelselapoptose veroorsaak. Die middels het *in vitro* angiogenese onderdruk en het ook tumorontwikkeling *in vivo* tot 'n meerder of minder mate onderdruk.

In die algemeen toon die resultate van hierdie studie dat daar nie 'n noemenswaardige oorfloei van bleomisien in pasiënte met IB behandeling vir hemangiomas was nie en kan dus hier 'n kleiner risiko vir die ontwikkeling van bleomisien-geïnduseerde pulmonêre fibrose wees.

Die studie het ook getoon dat die toetsmiddels angiogenese *in vitro* onderdruk asook hemangioma ontwikkeling in die *in vivo* muis-model. Hierdie opmerkings saam dui aan dat bleomisien hemangioma mag onderdruk deurdat angiogenese onderdruk word. Bykomstig, mitomyacin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, cochisine, nocodazole and cytochalisin D kan ook 'n potensiële rol speel by die behandeling van hemangiomas en behoort met behulp van die *in vivo* hemangioma muis-model ondersoek word om die effektiewe dosisse en skedules te bepaal.

Sleutelwoorde: bleomisien, sitoskelet-ontwrigtingsagente, angiogenese, hemangioma, endoteelselle, selgroeï, selmigrasie, vaskulêr endoteel groeifaktor, basiese fibroblast groeifaktor, poliomamiddel T onkogeen, vaskulêre tumor.



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## LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
bFGF	Basic fibroblast growth factor
BLM	Bleomycin
BME cells	Bovine microvascular endothelial cells
BBCE	Bovine brain capillary endothelial cells
Caspases	CysteinyI aspartate-specific proteases
C18 column	18-Caron reverse phase silica gel column
DdH <sub>2</sub> O	Deionised distilled water
MCF-7	Human breast carcinoma cell line
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DCS	Donor calf serum
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
H&E	Hematoxylin and Eosin stain
HPLC	High performance liquid chromatography
HMEC-1	Human microvascular endothelial cells
IC <sub>50</sub>	Inhibitory concentration for 50% of cells
IH	Infantile haemangiomas
MEM- $\alpha$	Alpha-modified Eagle's minimum essential medium
MMC	Mitomycin C
mRNA	Messenger RNA
NaHS	Sodium heptane Sulphonate
ND	Not detected
PBS	Phosphate buffered saline
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
tRNA	Transfer RNA
UV	Ultra violet



VBL	Vinblastine
VCR	Vincristine
VEGF	Vascular endothelial growth factor
vWF	von Willebrand Factor