

Optimised topical delivery of 5-fluorouracil

Tawona Nyasha Chinembiri

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Supervisor: Prof. J. du Plessis

Co-supervisor: Dr M. Gerber

Assistant supervisor: Dr L. du Plessis

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TABLE OF CONTENTS

List of figures	xiii
List of tables	xvi
Acknowledgements	xviii
Abstract	xx
Uittreksel	xxiii
Foreword	xxvi
CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT	1
REFERENCES	4
CHAPTER 2 TOPICAL DELIVERY OF 5-FLUOROURACIL FOR THE TREATMENT OF SKIN CANCERS	6
2.1 Introduction	6
2.2 Skin cancer	7
2.2.1 Basal cell carcinoma	10
2.2.2 Squamous cell carcinoma	11
2.2.3 Actinic keratoses	12
2.2.4 Bowen's disease	15
2.2.5 Cutaneous malignant melanoma	15
2.3 5-fluorouracil	16
2.3.1 Mechanism of action of 5-fluorouracil	17
2.3.2 Pharmacokinetics of 5-fluorouracil	19
2.3.3 Clinical uses of 5-fluorouracil	20
2.3.3.1 Systemic uses of 5-fluorouracil	20

2.3.3.2	Topical uses of 5-fluorouracil	20
2.3.4	Adverse effects of 5-fluorouracil	20
2.3.5	Advice on use of 5-fluorouracil	21
2.4	The skin and transdermal drug delivery	22
2.4.1	The epidermis	23
2.4.1.1	Stratum basale	23
2.4.1.2	Stratum spinosum	24
2.4.1.3	Stratum granulosum	24
2.4.1.4	Stratum corneum	24
2.4.2	The dermis and hypodermis	24
2.4.3	Advantages and limitations of topical and transdermal drug delivery	25
2.4.3.1	Advantages of topical and transdermal drug delivery	25
2.4.3.2	Limitations of topical and transdermal drug delivery	25
2.4.4	Mechanisms of skin permeation	26
2.4.4.1	Intercellular route	26
2.4.4.2	Transcellular route	27
2.4.4.3	Transappendageal route	27
2.4.5	Mathematical models of skin permeation	28
2.4.6	Factors influencing skin permeation	29
2.4.6.1	Biological factors	29
2.4.6.2	Physicochemical factors	29
2.4.6.2.1	Skin hydration	30
2.4.6.2.2	Drug concentration	30
2.4.6.2.3	Temperature and pH	30

2.4.6.2.4	Molecular size and shape	30
2.4.6.2.5	Diffusion coefficient	31
2.4.6.2.6	Partition coefficient	31
2.4.6.2.7	Solubility	32
2.4.6.3	Ideal physicochemical properties for passive transdermal delivery	33
2.5	Selected methods for the enhancement of skin penetration	33
2.5.1	Chemical penetration enhancement	34
2.5.2	Fatty acids	35
2.5.3	Drug delivery vehicles	36
2.5.4	Pheroid™ technology	37
2.5.4.1	Advantages of the Pheroid system as a delivery system for 5-fluorouracil	37
2.5.4.2	Structural characteristics of Pheroid™	38
2.5.4.3	Functional characteristics of Pheroid™	40
2.5.4.3.1	Pliable system design and versatility	40
2.5.4.3.2	Entrapment efficiency	40
2.5.4.3.3	Penetration efficiency	40
2.5.4.3.4	Uptake of Pheroid™ and entrapped compounds by cells	41
2.5.4.3.5	Metabolism, targeting and distribution	41
2.5.4.4	Inherent therapeutic effect of the essential fatty acids of Pheroid™	41
2.6	<i>In vitro</i> drug efficacy testing	42
2.7	Prior studies on transdermal and topical delivery of 5-fluorouracil	44
	REFERENCES	46

CHAPTER 3: ARTICLE FOR PUBLICATION IN JOURNAL OF PHARMACEUTICAL SCIENCES	56
TITLE PAGE	57
ABSTRACT	58
INTRODUCTION	59
MATERIALS AND METHODS	61
Materials	61
Methods	61
Formulation of semi-solid dosage forms	61
Cell cultivation	61
Determination of cell viability	62
Optimization of lotion concentration	62
Apoptosis analytical assay	62
High performance liquid chromatography analytical method	63
Preparation of phosphate buffer solution at pH 7.4	63
Aqueous solubility determination	63
Octanol-buffer distribution coefficient (log D) determination	63
Preparation of skin for the skin diffusion experiments	64
Franz cell diffusion studies	64
Tape-stripping procedure	65
Statistical analysis	65
RESULTS AND DISCUSSION	66
Optimization of lotion concentration	66
Drug efficacy testing	66

Aqueous solubility	68
Octanol-buffer distribution coefficient (log D)	68
The release of 5-fluorouracil from formulations	68
Skin diffusion studies	69
Results of the tape-stripping procedure	70
CONCLUSION AND FUTURE RECOMMENDATIONS	71
ACKNOWLEDGEMENTS	73
REFERENCES	74
Figure legend	77
Tables	78
Figures	80
CHAPTER 4: FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS	82
REFERENCES	86
APPENDIX A: VALIDATION OF THE HPLC ANALYTICAL METHOD FOR 5-FLUOROURACIL ANALYSIS	87
A.1 Introduction	87
A.2 Chromatographic conditions	87
A.3 Preparation of standard	88
A.4 Validation parameters	88
A.4.1 Limit of detection and lower limit of quantification	87
A.4.2 Linearity	89
A.4.3 Accuracy	90
A.4.4 Precision	91
A.4.3.1 Intra-day precision	91

A.4.3.2	Inter-day precision	92
A.4.5	Stability	92
A.4.6	System repeatability	94
A.4.7	Specificity	94
A.4.8	System suitability	95
A.5	Conclusion	95
REFERENCES		96
APPENDIX B: FORMULATION OF TOPICAL SEMI-SOLID PRODUCTS WITH 5-FLUOROURACIL AS THE ACTIVE PHARMACEUTICAL INGREDIENT		97
B.1	Introduction	97
B.2	Formulation of semi-solid topical products	98
B.2.1	Preformulation	99
B.2.2	Early formulation	99
B.2.3	Final formulation	100
B.3	Formulation of a lotion	100
B.3.1	Applications of a lotion	101
B.3.2	Stability and storage of a lotion	101
B.3.3	Preservation of a lotion	102
B.3.4	General method for the formulation of a lotion	103
B.4	Formulation of 5-fluorouracil lotions in this study	103
B.4.1	Ingredients used in the formulations	103
B.4.1.1	Cetyl alcohol	104
B.4.1.2	<i>Dl-α</i>-tocopherol	104
B.4.1.3	Ethanol	104

B.4.1.4	Liquid paraffin	105
B.4.1.5	Span 60	105
B.4.1.6	Tween 80	105
B.4.2	Formulation of 5-fluorouracil lotions (non-Pheroid™)	106
B.4.2.1	Method used to formulate 5-fluorouracil non-Pheroid™ lotions	106
B.4.2.2	Outcome	106
B.4.3	Formulation of 5-fluorouracil Pheroid™ lotions	107
B.4.3.1	Outcome	107
B.5	Conclusion	107
REFERENCES		108
APPENDIX C: <i>IN VITRO</i> DETERMINATION OF 5-FLUOROURACIL CYTOTOXIC EFFICACY AGAINST HUMAN MELANOMA CELLS USING FLOW CYTOMETRY		110
C.1	Introduction	110
C.1.1	Flow cytometry analytical method for <i>in vitro</i> drug efficacy testing	112
C.2	Selection of an appropriate cell line	113
C.3	Selection of appropriate drug concentrations for use	113
C.4	Non-assay experimental procedures	114
C.4.1	Materials	114
C.4.2	Cultivation of cells	114
C.4.3	Determination of cell viability	115
C.4.4	Procedure for preparing and treating the cells	116
C.4.5	Optimisation of the formulation concentration	116
C.4.5.1	Day one	116
C.4.5.2	Day two	116

C.4.5.3	Day three	117
C.4.5.4	Results and discussion of the optimisation	117
C.5	Apoptosis analytical method	118
C.5.1	Apoptosis assay method	118
C.5.1.1	Day one	118
C.5.1.2	Day two	118
C.5.1.3	Day three	119
C.5.2	Assay results and discussion	122
C.6	Statistical analysis	128
C.6.1	Statistical methods	128
C.6.2	Statistical analysis of results	129
C.7	Conclusion	130
REFERENCES		132
APPENDIX D: FRANZ CELL DIFFUSION STUDIES OF FORMULATIONS CONTAINING 5-FLUOROURACIL		134
D.1	Introduction	134
D.2	Methods	134
D.2.1	High performance liquid chromatography analytical method	134
D.2.2	Preparation of phosphate buffer solution	135
D.2.3	Aqueous solubility determination	136
D.2.4	<i>n</i> -Octanol-buffer distribution coefficient (log D)	136
D.2.5	Preparation of skin	136
D.2.6	Franz cell diffusion studies	137
D.2.6.1	<i>In vitro</i> drug release studies	138

D.2.6.2	<i>In vitro</i> skin diffusion studies	139
D.2.7	Tape-stripping technique	139
D.2.8	Transdermal and statistical data analysis	139
D.3	Results and discussion	140
D.3.1	Aqueous solubility	140
D.3.2	<i>n</i> -Octanol-buffer distribution coefficient (log D)	141
D.3.3	<i>In vitro</i> drug release studies	141
D.3.4	<i>In vitro</i> skin diffusion studies	142
D.3.4.1	Discussion of <i>in vitro</i> skin diffusion results	142
D.3.5	Tape-stripping	151
D.3.5.1	Concentration in the epidermis	151
D.3.5.2	Concentration in the dermis	152
D.3.6	Statistical analysis of results	152
D.4	Conclusion	156
	REFERENCES	158
	APPENDIX E: JOURNAL OF PHARMACEUTICAL SCIENCES: GUIDELINES FOR AUTHORS	160
E.1	General Guidelines	160
E.1.1	Online submission and peer review	160
E.1.2	Text	161
E.1.3	Tables	161
E.1.4	Figures	161
E.1.4.1	Color figures	161
E.1.4.2	Unacceptable figure formats	161

E.2	Scope of the Journal of Pharmaceutical Sciences	161
E.3	Types of Manuscripts	162
E.4	Preparation of Manuscripts	164
E.4.1	General Considerations	164
E.4.2	Suggested Reviewers	164
E.4.3	Title	164
E.4.4	Abstract	164
E.4.5	Keywords	165
E.4.6	Abbreviations	165
E.4.7	QSAR/QSPR	165
E.4.8	Experimental Section	165
E.4.9	Results	166
E.4.10	Discussion	166
E.4.11	References and Notes	166
E.4.12	Supporting Information	167
E.4.13	Acknowledgements	167
E.4.14	Spectral Data	167
E.4.15	Experimental Data	167
E.4.16	Tables	169
E.4.17	Illustrations	169
E.4.18	Nomenclature	170
E.4.19	Analyses	170
E.4.20	Hazardous Materials	171
E.5	Proofs and Reprints	171

E.6	Publication Online	171
E.7	Corrections	171
E.8	Copyright Transfer Agreements (CTA) must accompany manuscripts when they are accepted	171
E.9	Confirmation of manuscript content must accompany initial submission	172
E.10	Scientific Misconduct Issues	172
E.11	Conflicts of Interest Guidelines: Authors	172
E.12	Conflicts of Interest Guidelines: Reviewers	173
	APPENDIX F: PHOTOGRAPHS OF EQUIPMENT USED IN THE DRUG DELIVERY AND DRUG EFFICACY STUDIES	174

LIST OF FIGURES

CHAPTER 2

Figure 2.1	Comparative histology of normal cells, precancerous cells and cancer cells showing the deep penetrating nature of cancer cells	7
Figure 2.2	Hierarchy of the types of skin cancer	9
Figure 2.3	Treatment algorithm for actinic keratoses showing the treatment options for multiple lesions and solitary lesions	14
Figure 2.4	Chemical structure of 5-fluorouracil with fluorine at the C-5 position	17
Figure 2.5	Mechanism of action of 5-fluorouracil showing DNA and RNA damage as the end points	18
Figure 2.6	Possible side-effects of topical 5-fluorouracil	21
Figure 2.7	Structure of the skin showing the different layers of skin and appendages	23
Figure 2.8	Structure of the skin showing routes of permeation	28

CHAPTER 3

Figure 1:	Line graph showing comparisons between the %MFI induced in the A375 cells treated with 1) the lotion; 2) the API solution and 3) Pheroid™ lotion (n=3)	80
Figure 2:	Comparisons of the average amount of 5-fluorouracil (in µg/cm ²) that diffused through human skin after 12 h from each lotion with the amounts diffused from the respective Pheroid™ lotion of the same concentration	81

APPENDIX A

Figure A.1:	Linear regression curve of 5-fluorouracil	90
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APPENDIX C

Figure C.1:	Basics of flow cytometry	112
Figure C.2:	Generalised haemocytometer slide	115
Figure C.3:	Representative dot plot of the A375 cells in PBS illustrating the gated cell population of interest	122
Figure C.4:	Representative histograms (FL1-H) of the A375 cells in PBS	123
Figure C.5:	A representative overlay histogram (FL1-H) of the A375 cells with the control treatments	124
Figure C.6:	Overlay histogram (FL1-H) with representations of the A375 cells treated with the 13.33 µg/ml lotion, Pheroid™ lotion and 5-fluorouracil solution	125
Figure C.7:	Line graph showing comparisons between the %MFI induced in the A375 cells treated with 1) the non-Pheroid™ lotion; 2) the API solution and 3) Pheroid™ lotion	127

APPENDIX D

Figure D.1:	Amount of 5-fluorouracil that diffused through human skin from the 0.5% lotion (1) after 12 h	144
Figure D.2:	Amount of 5-fluorouracil that diffused through human skin from the 0.5% Pheroid™ lotion (2) after 12 h	144
Figure D.3:	Amount of 5-fluorouracil that diffused through human skin from the 1.0% lotion (3) after 12 h	145
Figure D.4:	Amount of 5-fluorouracil that diffused through human skin from the 1.0% Pheroid™ lotion (4) after 12 h	145
Figure D.5:	Amount of 5-fluorouracil that diffused through human skin from the 2.0% lotion (5) after 12 h	146
Figure D.6:	Amount of 5-fluorouracil that diffused through human skin from the 2.0% Pheroid™ lotion after (6) 12 h	146
Figure D.7:	Amount of 5-fluorouracil that diffused through human skin from the 4.0% lotion (7) after 12 h	147

Figure D.8:	Amount of 5-fluorouracil that diffused through human skin from the 4.0% Pheroid™ lotion (8) after 12 h	147
Figure D.9:	Comparison of the total amounts of 5-fluorouracil that diffused through human skin per unit area from the lotions of different 5-fluorouracil concentrations, (1); (3); (5) and (7)	148
Figure D.10:	Comparisons of the total amount of 5-fluorouracil diffused through human skin per unit area from the Pheroid™ lotions of different 5-fluorouracil concentrations, (2); (4); (6) and (8)	149
Figure D.11:	Comparisons of the amount of 5-fluorouracil that diffused through human skin from each lotion with the amounts diffused from the respective Pheroid™ lotion of the same concentration	150
APPENDIX F		
Figure F.1:	The apparatus used during the drug efficacy and drug delivery testing	175

LIST OF TABLES

CHAPTER 2

Table 2.1	Classification of cutaneous malignant melanoma with the respective presentations and predilection sites	16
Table 2.2	Physicochemical properties of 5-fluorouracil	32
Table 2.3	A comparison between the ideal physicochemical properties for transdermal delivery and the physicochemical properties of 5-fluorouracil	33

CHAPTER 3

Table 1:	Viability of cells after treatment with dilutions of the placebo Pheroid™ and non-Pheroid™ lotions for the purpose of optimization	78
Table 2:	Average concentrations of 5-fluorouracil that remained in the epidermis and dermis after the 12 hour diffusion studies	79

APPENDIX A

Table A.1:	Results for limit of detection and lower limit of quantification	88
Table A.2:	Results for linearity of 5-fluorouracil	89
Table A.3:	Results for accuracy of 5-fluorouracil	90
Table A.4:	Results for intra-day precision of 5-fluorouracil	91
Table A.5:	Results for inter-day precision of 5-fluorouracil	92
Table A.6:	Results for stability of 5-fluorouracil	93
Table A.7:	Results for system repeatability of 5-fluorouracil	94

APPENDIX B

Table B.1:	Ingredients used during formulation of the lotions	104
Table B.2:	Formula for 5-fluorouracil lotions formulated in this study	106

APPENDIX C

Table C.1:	Materials that were used during the in vitro drug efficacy study	114
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Table C.2:	Results for optimisation of the lotion concentrations	117
Table C.3:	Concise summary of methods followed during the formulation optimisation experiments and apoptosis assay	121
Table C.4:	The average MFI expressed as a percentage of the positive control and the standard deviation	125
Table C.5:	Descriptive statistics of the measured MFI	129
Table C.6:	Results of the Games-Howell test and effect size determinations for the drug efficacy tests	130

APPENDIX D

Table D.1:	HPLC analytical conditions for the determination of 5-fluorouracil concentrations in the skin layers and receptor phase	135
Table D.2:	Percentage of 5-fluorouracil released and average cumulative amount of 5-fluorouracil that diffused per unit area after the 6 h drug release studies	141
Table D.3:	Percentage diffused and average cumulative concentration of 5-fluorouracil that diffused per unit area after the 12 h skin diffusion studies	142
Table D.4:	Average concentrations of 5-fluorouracil that remained in the epidermis and dermis after the 12 h diffusion studies	151
Table D.5:	Results of the Games-Howell test and effect size determinations for the skin diffusion studies	155

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ABSTRACT

Skin cancer is the most widely diagnosed form of cancer and it is split in to non-melanoma skin cancer (NMSC) and cutaneous malignant melanoma (CMM). Cutaneous melanoma has a high propensity for malignancy and it has the highest mortality rate of all skin cancers (de Gruijl, 1999:2004). The first line of treatment for most skin cancers is surgical excision but instances do arise in which surgery is not feasible due to the health of the patient or the location of the lesion. Therefore, viable alternatives are necessary in cases where surgery is not possible (Telfer *et al.*, 2008:36). The skin is readily available for delivery of cytotoxic drugs to treat carcinomas and melanomas so the topical delivery of 5-fluorouracil was investigated in this study.

5-Fluorouracil is a pyrimidine anti-metabolite which interferes with deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis by inhibiting the nucleotide synthetic enzyme thymidylate synthase (TS) and by becoming misincorporated into RNA and DNA. Thymidylate is essential for replication as well as repair of DNA, in the event of TS inhibition thymidylate is not formed and “thymineless deaths” of cells occur (Chu & Sartorelli, 2009:935; Longley *et al.*, 2003:330). This active pharmaceutical ingredient (API) causes death of atypical and rapidly dividing cells (Tsuji & Karasek, 1986:474). The intravenous and topical routes are approved for 5-fluorouracil and in the case of skin cancer the obvious choice would be topical application (Chu & Sartorelli, 2009:935). Topical application of 5-fluorouracil results in the occurrence of terrible side effects such as severe inflammation, stomatitis, photosensitivity and dermatitis. A reduction in side effects would reduce the stigma associated with topical 5-fluorouracil and in turn increase patient compliance.

Topical drug delivery entails the delivery of an API onto or into the various layers of the skin (Flynn & Weiner, 1993:33) in order to treat conditions on or within the skin. Topical application of APIs is non-invasive, painless and simple plus the target site is readily accessible for topical therapy, thus the API is delivered directly to the site of action (Naik *et al.*, 2000:318). In the case of skin cancer, 5-fluorouracil should be able to reach the epidermis because NMSC originates from the keratinocytes (Marks & Hanson, 2010:305) and CMM from melanocytes (de Gruijl, 1999:2004) which are both found in the epidermis. The barrier function of the skin limits the penetration of molecules into the skin and the rate-limiting step is usually penetration into the stratum corneum (Foldvari, 2000:418).

The aim of this study was to investigate the diffusion of 5-fluorouracil from formulations into and through the skin. Two physico-chemical properties of 5-fluorouracil that influence skin permeation were determined (aqueous solubility and *n*-octanol-buffer partition coefficient (log D)). The Pheroid™ drug delivery system was used to enhance the delivery of

5-fluorouracil (Grobler *et al.*, 2008:284). Pheroid™ is a novel technology that is used in the delivery of APIs in pharmaceutical products. It enhances the efficacy of delivered compounds while allowing for the reduction of unwanted adverse effects (Grobler *et al.*, 2008:284). Franz cell skin diffusion studies and tape-stripping were conducted with Pheroid™ and non-Pheroid™ formulations to allow for comparison and determination of the effect of Pheroid™. The *in vitro* efficacy of 5-fluorouracil in inducing apoptosis of human melanoma cells was investigated using a flow cytometric apoptosis assay. Different concentrations of 5-fluorouracil in formulation were utilised in the experiments so as to observe the cytotoxic effect of 5-fluorouracil. The effect of the drug delivery vehicle on the efficacy of 5-fluorouracil was investigated by utilising API solutions in addition to Pheroid™ and non-Pheroid™ formulations in the experiments.

Relatively high concentrations of 5-fluorouracil diffused into and through the skin with Pheroid™ formulations resulting in a greatly enhanced *in vitro* skin permeation of 5-fluorouracil. The tape-stripping revealed that the Pheroid™ lotions resulted in higher concentrations of 5-fluorouracil in the epidermis and dermis after 12 h as compared to the lotions. There was no deducible trend with respect to the distribution of 5-fluorouracil between the epidermis and dermis. Subsequent to the apoptosis assay it was found that 5-fluorouracil was able to induce apoptosis in A375 cells after a 24 h incubation period. The Pheroid™ treatment of cells resulted in a greater response (mean fluorescence intensity) as compared to treatments with the other drug delivery vehicles at three of the four concentrations. This showed that the drug delivery vehicle played a role in the *in vitro* efficacy of 5-fluorouracil.

Further research must be done in order to combine these results. Optimum and highly effective topical formulations with low doses of 5-fluorouracil must be formulated for the purpose of treating cutaneous cancers with a reduced incidence of side effects.

Keywords: skin cancer; 5-fluorouracil; Pheroid™; A375 cells; cell culture.

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UITTREKSEL

Velkanker is die algemeenste gediagnoseerde vorm van kanker, Dit word verdeel in nie-melanoom-velkanker (NMSC) en kwaadaardige melanoom (CMM). 'n Velmelanoom het 'n hoë geneigdheid tot kwaadaardigheid en dit het die hoogste sterftesyfer van alle velkankers (de Gruijl, 1999:2004). Die eerste linie van behandeling van die meeste velkankers is sjirurgiese verwydering, maar gevalle kom voor waar sjirurgie nie doenlik is nie as gevolg van die gesondheid van die pasiënt of die lokaliteit van die letsel. Dus is lewensvatbare alternatiewe noodsaaklik waar sjirurgie nie moontlik is nie.(Telfer *et al.*, 2008:36). Die vel is geredelik beskikbaar vir die aflewering van sitotoksiese geneesmiddels om karsinome en melanome te behandel dus is die topikale aflewering van 5-fluorourasiel ondersoek in hierdie studie.

5-Fluorourasiel is 'n pirimidien-anti-metaboliet wat inmeng met die deoksiribonukleïensuur-(DNA) and ribonukleïensuur-(RNA) sintese deur die nukleotied sintetiese ensiemtimidilaatsintase (TS) te verhinder deur verkeerdelik geïnkorporeer word in die RNA en DNA. Timidilaat is noodsaaklik vir die replikasie sowel as die herstel van DNA, in die geval van TS-verhindering word timidilaat nie gevorm nie en "timienlose sterftes" van selle kom voor (Chu & Sartorelli, 2009:935; Longley *et al.*, 2003:330). Hierdie aktiewe farmaseutiese bestanddeel (API) veroorsaak die afsterwe van atipiese en snelverdelende selle (Tsuji & Karasek, 1986:474). Die binnearse en topikale roetes is goedgekeur vir 5-fluorourasiel en in die geval van velkanker sal die voor die hand liggende keuse topikale aanwending wees (Chu & Sartorelli, 2009:935). Topikale aanwending van 5-fluorourasiel lewer aaklige newe-effekte soos ernstige inflammasie, stomatitis, fotosensitiviteit en dermatitis. 'n Vermindering van die newe-effekte sal die stigma wat geassosieer is met topikale 5-fluorourasiel verminder en terselfdertyd die pasiënt se inwilliging vermeerder.

Topikale aflewering van 'n geneesmiddel behels die aflewering van 'n API op of in die verskillende lae van die vel (Flynn & Weiner, 1993:33) om kondisies te behandel op of in die vel. Topikale aanwending van APIs is nie-aanvallend, pynloos en eenvoudig. Verder is die teikenarea geredelik bereikbaar vir topikale terapie sodat die API direk afgelewer word aan die area onder behandeling (Naik *et al.*, 2000:318). In die geval van velkanker, moet 5-fluorourasiel die epidermis kan bereik aangesien NMSC ontstaan uit die keratinosiete (Marks & Hanson, 2010:305) en CMM uit die melanosiete (de Gruijl, 1999:2004) wat beide in die epidermis gevind word. Die skeidingsfunksie van die vel beperk die penetrasie van die molekule in die vel in en die snelheidsbeperkende stap is gewoonlik die penetrasie in die stratum corneum in (Foldvari, 2000:418).

Die doel van hierdie studie was om die diffusie van 5-fluorourasiel uit formulerings in die vel in en deur die vel te ondersoek. Twee fisies-chemiese eienskappe van 5-fluorourasiel wat

velpermeasie beïnvloed, is bepaal (wateroplosbaarheid en die *n*-oktanol-buffer-verdelingskoëffisiënt (log *d*)). Die Pheroid™-geneesmiddel-aflerweringssisteem is gebruik om die topikale aflerwing van 5-fluorourasiel te verhoog (Grobler *et al.*, 2008:284). Pheroid™ is 'n nuwe tegnologie wat gebruik word in die aflerwing van API's in farmaseutiese produkte. Dit verhoog die doeltreffendheid van afgelewerde verbindings terwyl ongevraagde slegte newe-effekte verminder word (Grobler *et al.*, 2008:284). Franz sel veldiffusiestudies en "tape stripping" is uitgevoer met Pheroid™- en nie-Pheroid™-formulerings om 'n vergelyking te tref en om die effek van Pheroid™ te bepaal. Die *in vitro* doeltreffendheid van 5-fluorourasiel in die induksie van apoptose van menslike melanoomA-selle is ondersoek deur van 'n vloeisitometriese apoptoseanalise gebruik te maak. Verskillende konsentrasies van 5-fluorourasiel in formulering is gebruik in eksperimente om die sitotoksiese effek van 5-fluorourasiel waar te neem. Die effek van die geneesmiddelafleweringsdraer op die doeltreffendheid van 5-fluorourasiel . is ondersoek deur API-oplossings te gebruik bo en behalwe Pheroid™- en nie-Pheroid™-formulerings in die eksperimente.

Relatiewe hoë konsentrasies van 5-fluorourasiel het in en deur die vel met Pheroid™-formulerings gediffundeer in 'n sterk vergrote *in vitro* velpermeasie van 5-fluorourasiel. Die "tape stripping" van Pheroid™ velaanwendings het die gevolg dat hoër konsentrasies van 5-fluorourasiel in die epidermis en dermis na 12 h in vergelyking met die velaanwendings verkry word. Daar was geen afleibare neiging met betrekking tot verspreiding van 5-fluorourasiel tussen die epidermis en dermis nie. Na die apoptoseanalise is gevind dat 5-fluorourasiel in staat was om apoptose in A375-selle te induseer na 'n inkubasieperiode van 24 uur. Die Pheroid™-behandeling van selle het 'n groter responsie (gemiddelde fluoressensie-intensiteit) gelever in vergelyking met behandelings met ander geneesmiddeldraers by drie of vier konsentrasies. Dit het getoon dat die geneesmiddelafleweringsdraer 'n rol speel in die *in vitro* doeltreffendheid van 5-fluorourasiel.

Verdere navorsing moet gedoen word om die geneesmiddeldoeltreffendheid- en geneesmiddelaflewingresultate te kombineer om 'n optimale formulering te verkry. Optimum and hoogs effektiewe topikale formulering met lae dosisse 5-fluorourasiel moet geformuleer word met die oog op die behandeling van velkankers met 'n verminderde voorkoms van newe-effekte.

Sleutelwoorde: velkanker; 5-fluorourasiel; Pheroid™; A375-selle, selkultuur.

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FOREWORD

This study aimed to investigate the topical delivery of 5-fluorouracil for the purpose of treating skin cancer and related ailments. Skin cancer poses a serious public health problem because the skin barrier is the body's first line of defence against harmful exogenous substances, therefore removal of a skin tumour can be tricky especially in immune-compromised patients. Alternatives to surgical removal of the tumour are highly valuable for treatment in special cases. Different concentrations of 5-fluorouracil were incorporated into a lotion with and without the use of Pheroid™ technology and utilised in the experiments. The cytotoxic efficacy of 5-fluorouracil against human melanoma cells (A375) was also determined using flow cytometry.

This dissertation is compiled in the article format which consists of introductory chapters, a full length article (Chapter 3), a concluding chapter and appendices. The experimental methods and data that were used and obtained are attached in Appendices A to D. The article in the dissertation is for publication in the Journal of Pharmaceutical Sciences and the authors' guideline has been attached as Appendix E.

During the course of my Masters degree, I learnt that nothing comes easy and hard work pays off. I came to fully realise that to cope with the unpredictable nature of research I had to love what I do, enjoy it, persevere and learn to be patient. Most of all, I learnt to truly rely on my ultimate source – God.

CHAPTER ONE

INTRODUCTION AND PROBLEM STATEMENT

According to Erb *et al.* (2005:68) skin cancer is the most frequently diagnosed cancer in Caucasians worldwide and the incidence keeps increasing due to increased exposure to ultra-violet (UV) radiation. Skin cancer arises from cells within the epidermis of the skin; the keratinocytes (non-melanoma skin cancer) and the melanocytes (cutaneous melanoma). Therefore development of a formulation containing a cytotoxic agent that targets the epidermis would be ideal in the treatment of skin cancer. The risk of developing a cutaneous neoplasm is high in Caucasians with skin type I or II (easily sunburns, suntans poorly; freckles with sun exposure), blue eyes, a fair complexion, red hair or blonde (Diepgen & Mahler, 2002:3). Clearly skin cancers pose a serious public health problem in these populations. Prompt detection and treatment is important when it comes to skin cancer because this drastically improves prognosis and in turn results in reduced skin cancer mortalities (Diepgen & Mahler, 2002:1; Marks, 1995:607).

The first line of treatment of skin cancer is surgical excision but options such as cryosurgery, curettage, chemotherapy and radiation can also be used if viable for the type of cancer (Conroy *et al.*, 2010:455). Chemotherapy often aims to restore or invert the apoptosis imbalance and it uses the apoptotic program to destroy the tumour (Lippens *et al.*, 2011:329). Topical chemotherapy is preferable if effective for the type of cancer because it can be used in situations where surgery is not feasible e.g. due to the patients health or location of the tumour. According to Flynn and Weiner (1993:33) topical therapy is when a formula containing an active pharmaceutical ingredient (API) is applied to the skin so as to treat a superficial condition on or within the skin. The advantages of topical treatment in the treatment of skin cancer are: it is non-invasive and rarely results in pain or scarring which increases its acceptance by the patients (Naik *et al.*, 2000:319); it is relatively painless and simple thus eliminating specialised healthcare staff which may lower treatment costs (Cleary, 1993:19) and the target site is directly accessible for topical therapy so the API is delivered directly to the site of action (Naik *et al.*, 2000:319).

The skin is composed of three distinct layers which are the epidermis, dermis and the subcutaneous fatty layer. Diffusion of substances into and through the skin is mostly limited by the stratum corneum. The stratum corneum is the thin, hydrophobic, outermost layer of the skin which is most resistant to permeation (Foldvari, 2000:418). For an API to effortlessly permeate through the skin it must comply with particular physico-chemical parameters. An aqueous solubility above 1 mg/ml, a melting point below 200 °C, a molecular weight below 500 Da and a log P value between 1 and 3 are ideal for skin permeation (Naik *et al.*, 2000:319).

5-Fluorouracil is a pyrimidine anti-metabolite which acts by inhibiting the formation of thymidylate in cells thus resulting in 'thymine-less' deaths of cells (Chu & Sartorelli, 2009:935). The DNA of the cell is damaged by the misincorporation of 5-fluorouracil and the cell dies. Chemotherapeutic agents such as 5-fluorouracil cause intracellular cell damage that acts as a signal for the induction of apoptosis (Pollard *et al.*, 2008:839). 5-Fluorouracil mainly induces cell death of atypical and rapidly proliferating cells such as neoplasms (Robertson & Maibach, 2009:1047). By inducing cell death 5-fluorouracil may also damage the surrounding healthy skin regardless of its relative selectivity. This results in stomatitis, dermatitis, photosensitivity and severe inflammatory reactions at the site. In order to reach the desired outcome, 5-fluorouracil may cause these unwanted and unsightly side effects. The eradication of these adverse effects will possibly reduce the stigma associated with topical 5-fluorouracil.

The physico-chemical properties of 5-fluorouracil comply with the molecular weight and aqueous solubility ideals with values of 130.08 Da (Rudy & Senkowski, 1973:223) and 12.5 mg/ml (Troy, 2005:1573), respectively. The melting point of 5-fluorouracil is relatively high, 282-283 °C (Rudy & Senkowski, 1973:228), which is above the limit stipulated by Naik *et al.* (2000:319). The log P value of 5-fluorouracil is -0.83 (Buur *et al.*, 1985:55) which indicates that the API is very hydrophilic. Therefore, it was predicted that the chances of 5-fluorouracil passing through the skins lipophilic barrier was low. Due to the effects of the different properties, 5-fluorouracil barely permeates the skin without assistance. Consequently, a delivery system, i.e. Pheroid™ technology, was used in order to deliver the API to the target site (the epidermis).

The Pheroid™ delivery system enhances the efficacy of delivered compounds while allowing for a reduction in dose and in turn reduction of unwanted adverse effects (Grobler *et al.*, 2008:284). The reduction of adverse effects is important in the use of 5-fluorouracil because the side effects (suppuration, pain, tenderness, burning sensations and pruritis, to mention a few) are the main limitations in the use of this API. The API is encapsulated in the Pheroid™ vesicles and this may protect the healthy skin from the caustic effects of the API. The overall advantage of using Pheroid™ as a delivery system is that it creates a safer and more effective formulation (Grobler, 2004:4).

During the course of this project, *in vitro* cell culture studies were utilised to determine whether the 5-fluorouracil in the formulations exerted an effect. This is important because the API must undoubtedly still be able to exert an effect after being incorporated into formulations. A possible limitation with 5-fluorouracil is the development of drug resistance but Pheroid™ technology also reduces or eliminates drug resistance, which makes it very valuable in the treatment of skin cancer (Grobler, 2004:4). The aim of this study was to prepare 5-fluorouracil semi-solid formulations with and without Pheroid™ and to determine if 5-fluorouracil is effective as an anti-

cancer agent after being incorporated into the formulations. In order to achieve these aims, the following objectives were set:

- ❖ development of lotions (with and without Pheroid™) that contain varying concentrations of 5-fluorouracil;
- ❖ performing an apoptotic assay to determine the *in vitro* efficacy of 5-fluorouracil and the influence of the drug delivery vehicle on the efficacy of 5-fluorouracil;
- ❖ development and validation of a high performance liquid chromatography (HPLC) method for analysis of samples from the diffusion studies;
- ❖ performing drug release studies to determine whether 5-fluorouracil is released from the formulations;
- ❖ performing *in vitro* skin diffusion studies to investigate the diffusion of 5-fluorouracil into and through the skin;
- ❖ using the tape-stripping technique to determine the concentration of 5-fluorouracil within the stratum corneum-epidermis and in turn in the epidermis-dermis after 12 hour skin diffusion and
- ❖ determining the influence of Pheroid™ on the transdermal and topical delivery of 5-fluorouracil.

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2.1 Introduction

The incidence and mortality rates of skin cancers are on the rise in the countries in which such tumours are recorded and it is estimated that skin cancer is the most common form of cancer in the USA (U.S. Cancer Statistics Working Group, 2012). According to Erb *et al.* (2005:68) skin cancers are the most frequently diagnosed malignancies in Caucasians worldwide and their incidence keeps increasing due to increased exposure to ultra-violet (UV) radiation. Clearly skin cancers pose a serious public health problem. Early detection and treatment are thus recommended because this improves prognosis substantially in turn resulting in a reduction of number of deaths caused by skin cancer. Skin cancers can be easily spotted early in their development because they occur on the body surface, so people must be alert when it comes to any abnormal lesions on their skin (Diepgen & Mahler, 2002:1; Marks, 1995:607). There are various treatment options for skin cancers which include, but are not limited to, cryosurgery, curettage, chemotherapy and radiation (Conroy *et al.*, 2010:455). Chemotherapy often aims to restore or invert the apoptosis imbalance in order to use the apoptotic program to kill the tumour (Lippens *et al.*, 2011:329). Topical treatment is preferable if effective for the type of cancer because it has good cosmesis whereas the surgical procedures may disfigure the patient or result in scarring (Mangas *et al.*, 2010:134).

According to Flynn and Weiner (1993:35) topical treatment is applying a formula which contains a relevant active agent to the skin, so as to treat a superficial condition on the skin or within the skin. In ancient times, inelegant medicated applications would be placed on the skin for such medicinal purposes. This concept became modernised in the early 1970's when scientists began to understand disease processes more clearly and has since continued to expand (Flynn & Weiner, 1993:33). Gels, ointments, creams, lotions, foams, etc. can be formulated. Topical treatment has become common as it avoids the first-pass metabolism and is a non-invasive method therefore resulting in higher bioavailability for some active pharmaceutical ingredients (APIs) and higher patient compliance, respectively (Naik *et al.*, 2000:319). However, not all APIs are suitable for topical or transdermal drug delivery. The skin is relatively impermeable to 5-fluorouracil, but techniques have been investigated and used so as to enhance the penetration of 5-fluorouracil through the skin's impervious barrier.

2.2 Skin cancer

Cancer of the skin is characterised by an imbalance toward too little apoptosis or too much cell survival in the epidermis (Lippens *et al.*, 2011:329). Most cancer cells develop ways to evade apoptosis or exhibit defective apoptosis mechanisms thus allowing uncontrollable cell development (Erb *et al.*, 2005:69). Figure 2.1 shows the histology of cancer cells in the skin next to precancerous and normal cells. The tumour is clearly seen in the diagram as a mass of deep penetrating cells.

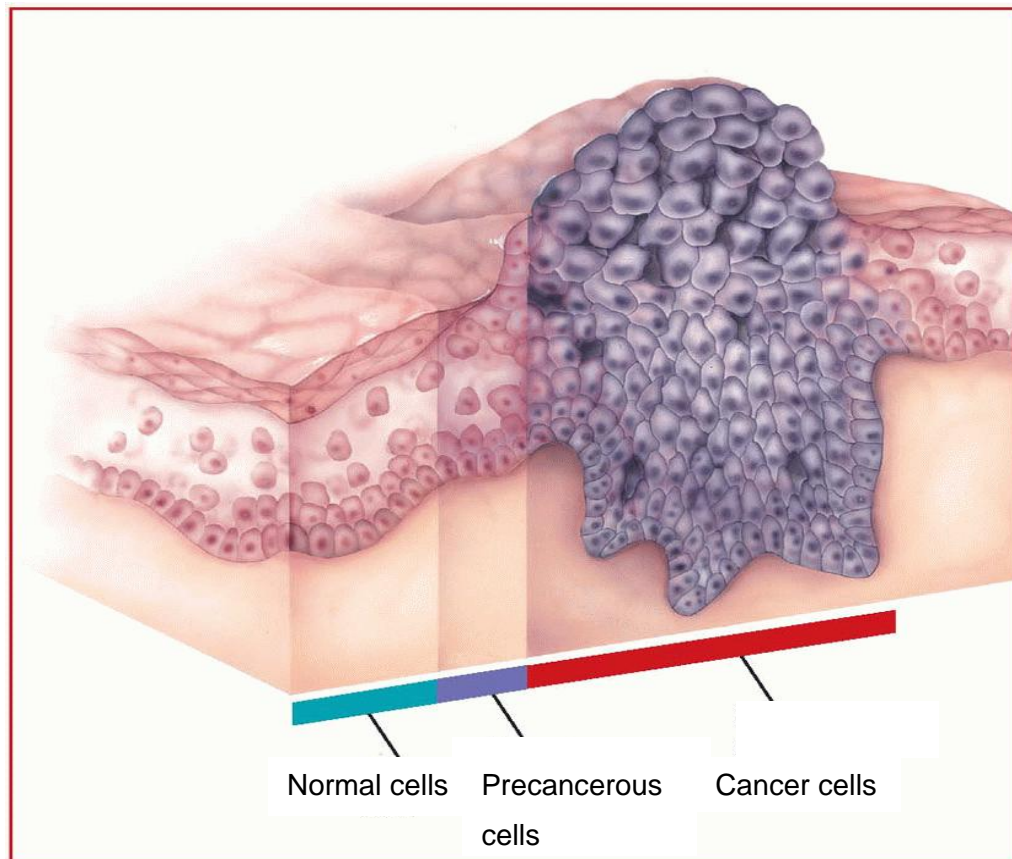


Figure 2.1: Comparative histology of normal cells, precancerous cells and cancer cells showing the deep penetrating nature of cancer cells (Adapted from Conroy *et al.*, 2010:8).

Skin cancer is also referred to as cutaneous cancer and is split into melanoma and non-melanoma skin cancer (NMSC). The common skin cancers include basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and cutaneous malignant melanoma (CMM). Actinic keratoses and Bowen's disease are also considered in discussions to do with skin cancer, although they are not true invasive tumours, because of their relationship to true skin cancers (Marks, 1995:607). Basal cell carcinoma and SCC are the NMSCs and they both arise from cutaneous keratinocytes, the cells that form the epidermal layer of the skin (Marks & Hanson, 2010:305) whereas CMM arises from melanocytes (de Grujil, 1999:2004). The risk of developing NMSC is

high in white populations with skin type I and II (easily sunburns, suntans poorly; freckles with sun exposure), blue eyes, a fair complexion, red hair or blonde hair (Diepgen & Mahler, 2002:3). Cutaneous malignant melanoma has a low incidence rate but a high mortality rate because it is the most aggressive type of skin cancer and can metastasise rapidly thus leading to a poor prognosis. The NMSCs are less aggressive but if they are neglected they may grow invasively and SCC may metastasise (de Gruijl, 1999:2004). The hierarchy of the skin cancers from less severe to more severe is illustrated in Figure 2.2 together with the characteristics of each type of cancer. Sections 2.2.1 – 2.2.5 provide more insight into the different types of skin cancer.

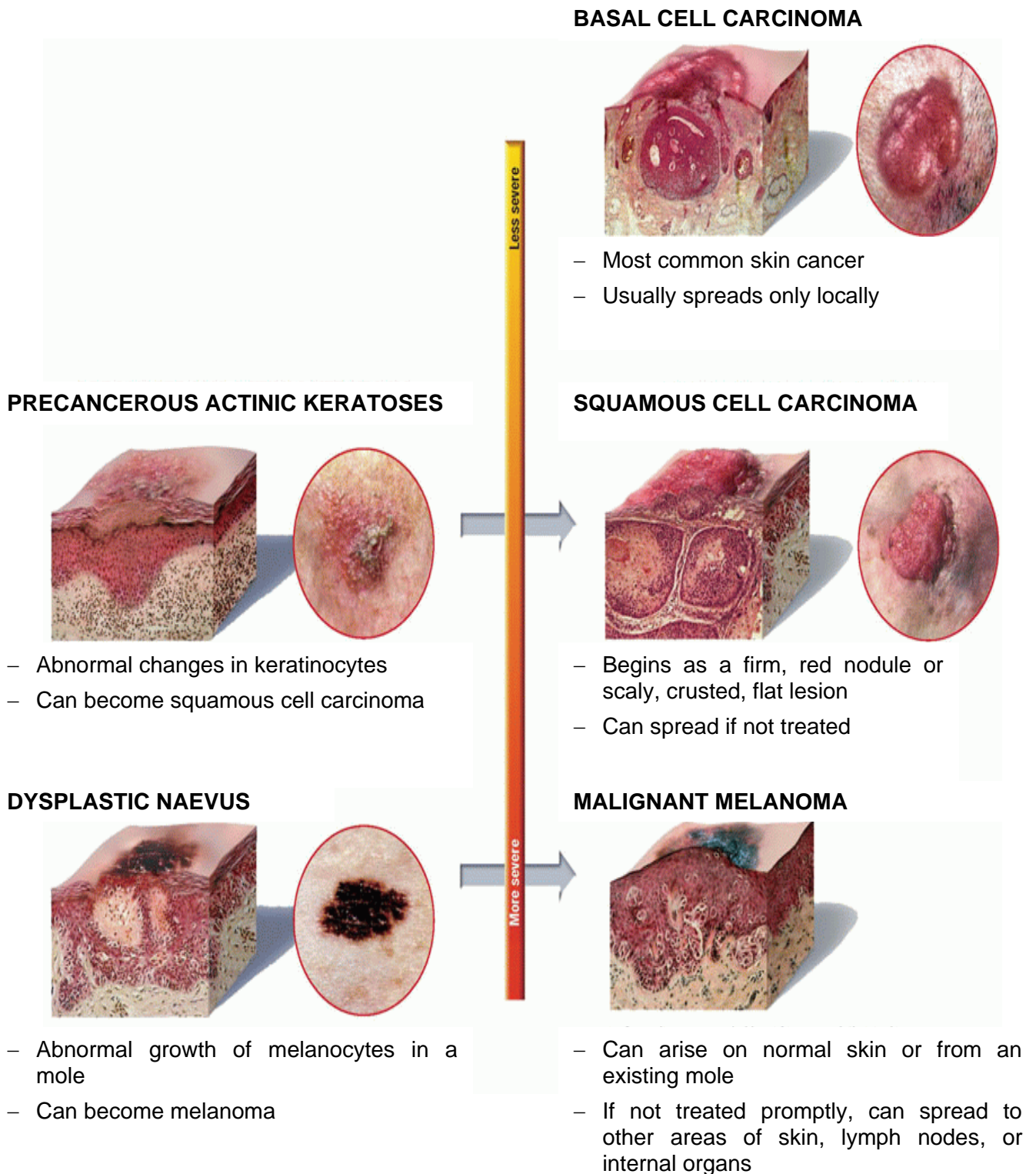


Figure 2.2: Hierarchy of the types of skin cancer from less severe (top) to more severe (bottom). The development from a “precancerous” lesion to cancer is shown by the horizontal arrow (Adapted from Conroy *et al.*, 2010:393).

2.2.1 Basal cell carcinoma

Basal cell carcinoma is the most common skin malignancy in humans and it represents about 70% of diagnosed skin cancers. It is a locally invasive malignant epidermal skin tumour that grows slowly and presents as a red papule or a small crusted area that tends to bleed and not heal (Marks & Hanson, 2010:305). Clinical differential diagnosis of BCC includes SCC, CMM, adnexal or follicular neoplasms, benign fibrous growths and scars. Metastasis rarely occurs in BCC unless the lesions are neglected for a long time. Negligence results in local skin destruction; invasion of surrounding skin, bone, cartilage and other structures (Marks & Hanson, 2010:306). Basal cell carcinoma is classified according to the histological sub-types to give nodular BCC, superficial BCC, infiltrative BCC and micronodular BCC.

There are certain factors that increase a person's predisposition to develop BCC, these include but are not limited to, prolonged sun exposure (most common), extensive sunburns or sun exposure in childhood, arsenic, radiation, burns, immunosuppression and previous X-ray therapy for acne (Conroy *et al.*, 2010:400). Usually BCCs occur on areas that acquire the most sun exposure such as the head and neck, but BCC may occur on any skin surface even those with minimal skin exposure (Marks & Hanson, 2010:307). Effective methods of preventing BCC are:

- ❖ avoiding the peak hours of UV transmissibility;
- ❖ applying sunscreen and sun-block to the skin when exposure is necessary and
- ❖ wearing clothing that protects the skin from the harmful effects of the sun, e.g. broad-brimmed hats and long-sleeved clothing.

The chosen treatment for BCC depends on the histological subtype, size and location of the tumour. Other factors which must be considered are the general fitness and health of the patient, coexisting serious medical conditions, the age of the patient (e.g. very elderly) and the use of anti-coagulant medication. The treatment of BCC is split into surgical and non-surgical techniques. Surgical techniques include excision, curettage and electrodesiccation, Mohs micrographic surgery (MMS), cryosurgery and carbon dioxide laser, while the non-surgical techniques include topical treatment, photodynamic therapy and radiotherapy (Raasch, 2009:66). Infiltrative and micronodular BCC are highly aggressive and surgical removal is the main recommendation for these sub-types (Raasch, 2009:65). Low-risk and asymptomatic lesions should be treated conservatively to avoid causing more problems than the lesion itself. The development of more effective topical and non-surgical therapies increases the options for many low-risk lesions (Telfer *et al.*, 2008:36). Presently 5-fluorouracil and imiquimod are used in the topical treatment of superficial BCC (Conroy *et al.*, 2010:400; Raasch, 2009:68; Sweetman, 2011).

2.2.2 Squamous cell carcinoma

Squamous cell carcinoma is the second most prevalent form of skin cancer and it has a higher propensity for metastasis than BCC (de Gruijl, 1999:2004; Erb *et al.*, 2005:69). It initially presents as a fast growing scaly papule that can become inflamed or indurated and heaped up with mounds of scale on the surface. Bleeding does not occur in SCC as readily as with BCC (Marks & Hanson, 2010:308) but eventual ulceration and invasion of underlying tissues does occur. Actinic keratoses and Bowen's disease (SCC *in situ*) are precursors to SCC (2010:308) and approximately 60% of SCCs arise from actinic keratoses (Glogau, 2000:s23). Therefore it is recommended that the precursor lesions should be detected and treated before progression to SCC.

The pathogenesis of SCC is similar to that of BCC in that they are both pathogenically linked to UV exposure. However, in darkly pigmented patients there is a higher chance of developing SCC rather than BCC. This may be due to underlying chronic scarring conditions or ulcers. The general factors that may increase the chances of developing SCC are (Conroy *et al.*, 2010:400):

- ❖ overexposure to the sun's ultraviolet rays;
- ❖ premalignant lesions, such as actinic keratoses or leukoplakia;
- ❖ x-ray therapy;
- ❖ ingested herbicides, medications, or waxes containing arsenic;
- ❖ chronic skin irritation and inflammation;
- ❖ local carcinogens (e.g. tar and oil) and
- ❖ hereditary diseases, such as xeroderma pigmentosum and albinism.

The same prevention guidelines for BCC are relevant for preventing SCC. In the case of SCC immune-suppressed patients must be fully educated on protecting themselves from the sun and they must have regular dermatologic examinations because they are at high risk of developing aggressive metastatic tumours (Marks & Hanson, 2010:311).

The treatment options for SCC are the same as those for BCC but the high metastasis potential of SCC must be taken into consideration when deciding on treatment. Excision, curettage and electrodesiccation are recommended for low risk SCC (less than 1 cm in diameter) while recurrent tumours, large tumours, aggressive tumours, tumours of the face and other critical structures and ill-defined tumours are preferably managed by MMS. Squamous cell carcinoma *in situ* is managed by curettage and topical modalities such as imiquimod and 5-fluorouracil (Marks & Hanson, 2010:311).

2.2.3 Actinic keratoses

Actinic keratoses are part of a continuum that ultimately leads to SCCs, but not all actinic keratoses result in SCCs (Ko, 2010:250). Approximately 60% of SCCs arise from actinic keratoses, but only 0.025 –16.000% of actinic keratoses progress to SCCs per year (Glogau, 2000:23). Actinic keratoses (also known as solar keratoses) are described as solar induced cutaneous neoplasms that may progress to malignancy or regress. This is consistent with the animal studies on skin carcinogenesis that have been done. These studies reveal that a permanent mutation can be initiated by sunlight on ras proto-oncogenes or p53 tumour suppression genes within the keratinocytes. After repetitive subjection to solar radiation papillomas are formed, which in humans we refer to as neoplasms or actinic keratoses. If the lesions are not further exposed to genotoxic agents they may regress or continue as benign lesions but, if exposed to genotoxic agents (e.g. solar radiation) they may progress to malignancy. These studies show the role of solar radiation as an initiator and promoter of the formation of these neoplasms and subsequent malignant lesions (Callen *et al.*, 1997:651). There has been an ongoing debate since the nineteenth century on the description of actinic keratoses as 'pre-malignant' lesions. Person (2003:637) says actinic keratoses are neither 'pre-malignant' nor 'malignant' but are initiated tumours. On this note, Yantsos *et al.* (1999:13) also aborted this definition and proposed that actinic keratoses be defined and described as keratinocytic intraepidermal neoplasia (KIN), which are classified according to the extent of epidermal involvement (Ko, 2010:250).

Actinic keratoses present on surfaces commonly exposed to the sun such as the head, face, neck, arms, hands and legs. It is usually easier to palpate than see the actinic keratoses as the colours may vary from flesh coloured, red or deeply pigmented like a tan. The lesions appear as irregular scaling papules or plaques from as small as 1 – 2 mm papules to 2 – 6 mm plaques. They rarely exceed 1 cm in size but the lesions may run into each other at the margins and form a continuous mass (Callen *et al.*, 1997:652). On progression to SCC the lesion may harden, erode, bleed, turn red or increase in size (Drake *et al.*, 1995:96).

The main risk factors for developing SCC apply to actinic keratoses and in essence these are:

- ❖ cumulative or long-term exposure to UV radiation e.g. sun, tanning beds, sunbelts, artificial light sources (Callen *et al.*, 1997:650; Salasche, 2000:S4);
- ❖ immunosuppression (Frost & Green, 1994:460); and
- ❖ albinism, Bloom's syndrome, Cockayne's syndrome, Rothmund-Thomson syndrome and xeroderma pigmentosum ((Frost & Green, 1994:459; Holmes *et al.*, 2007:67).

Actinic keratoses are mainly prevented by avoiding sun exposure just as for BCC and SCC. An actinic keratosis is recognised as a biological marker of risk for invasive SCC in patients. It is

recommended to treat actinic keratoses because they have the potential to progress to invasive SCCs. The factors that come into play when deciding on a treatment option are disease-related factors (duration, size, number of lesions, progression of disease etc), the patient profile (age, health status, other risk factors etc), the cost, patient's preference and the ability of the physician to carry it out. The patient's opinion is important because most of the treatment options may cause pain, inflammation or result in scarring which is generally not appealing to the patient (Stockfleth *et al.*, 2008:654). Actinic keratoses can be removed by curettage and electrodesiccation, cryosurgery, excision, dermabrasion and topical therapy. The APIs used for topical therapy include but are not limited to imiquimod, aminolevulinic acid (photodynamic therapy), retinoids, diclofenac and 5-fluorouracil. Figure 2.3 illustrates an algorithm with guidelines on treating actinic keratoses (Stockfleth *et al.*, 2008:654).

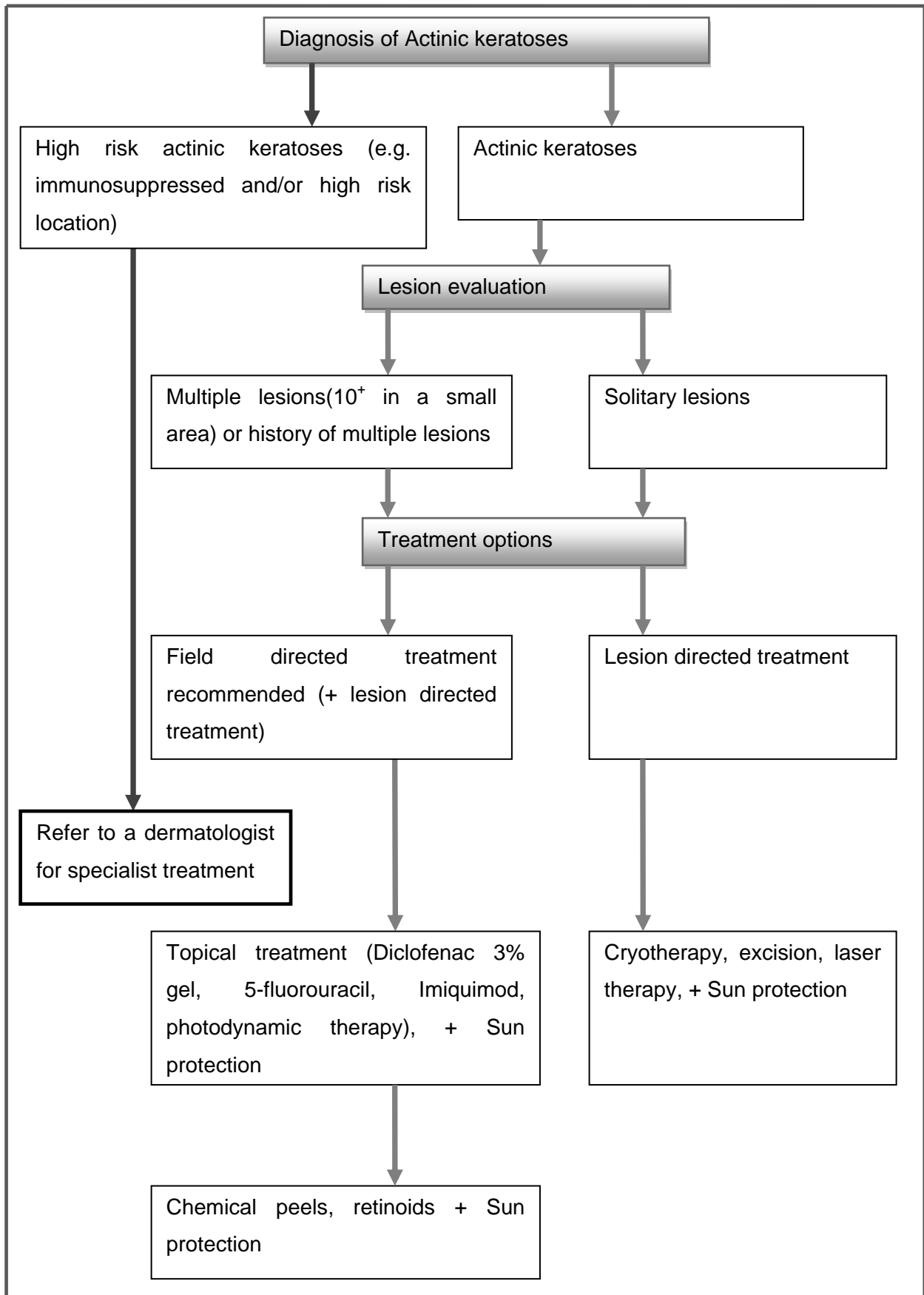


Figure 2.3: Treatment algorithm for actinic keratosis showing the treatment options for multiple lesions and solitary lesions (Adapted from Stockfleth *et al.*, 2008:653).

2.2.4 Bowen's disease

Bowen's disease is also known as SCC *in situ* and it presents as flat, pink to red scaling plaques that resemble eczema, psoriasis or superficial BCC. It occurs on sun-exposed and non-sun-exposed skin. Bowen's disease in the non-sun-exposed areas such as genitalia usually has well distributed and large lesions (Marks & Hanson, 2010:308). Progression to invasive SCC occurs in 4 – 5% of the cases of Bowen's disease (Ishida *et al.*, 2001:178) and therefore it should be treated promptly before the potential progression occurs.

Treatment options for Bowen's disease include surgical excision, cryotherapy, topical therapy, curettage, photodynamic therapy, radiotherapy and carbon dioxide laser. The topical treatment modalities in use are 5-fluorouracil and imiquimod (Sotiriou *et al.*, 2011:164).

2.2.5 Cutaneous malignant melanoma

Cutaneous malignant melanoma is a malignant skin tumour that originates from melanocytes, the pigment producing cells of the skin (Dewar & Powell, 2002:145). Skin cancer accounts for a large fraction of all cancer and majority of skin cancer deaths are attributable to melanoma (Weiner & Yoon, 2010:313). Therefore melanoma is a highly dangerous form of skin cancer which should be detected and treated as early as possible to avoid the dreadful outcome of its progression.

There are certain clinical features that are suggestive of cutaneous malignancy, the major signs are change in size, shape or colour and the minor signs are itching, bleeding or inflammation. Lesions that meet the ABCDE criteria of asymmetry, border irregularity, colour variegation, diameter >6 mm and evolving are high-risk lesions and must be treated as such (Mangas *et al.*, 2010:130; Weiner & Yoon, 2010:314). The factors that increase an individual's likelihood of developing CMM include, but are not limited to: family history (including personal), complexion, presence of naevi, sunlight exposure, occupational factors, protective factors and genetic implications (Rossi *et al.*, 1997:2303). Atypical moles, giant melanocytic naevi, actinic keratoses and lentigo maligna are premalignant lesions whose presence may reflect a predisposition to develop CMM (Dewar & Powell, 2002:145).

Primary CMM is classified into superficial spreading melanoma (70%), nodular melanoma (15%), acral lentiginous melanoma (10%) and lentigo maligna melanoma (5%) (Weiner & Yoon, 2010:313). The presentation and sites of predilection of the different classes of CMM are presented in Table 2.1.

Table 2.1 Classification of cutaneous malignant melanoma with the respective presentations and predilection sites (Adapted from Weiner & Yoon, 2010:313)

Classification	Presentation	Predilection sites
Superficial spreading melanoma	– papule or nodule	– backs of men, backs and legs of women
Nodular melanoma	– dark blue-black papule or nodule that develops rapidly	– the trunk, head and neck. (more frequently seen in men)
Acral lentiginous melanoma	– brown to black macules with irregular borders and variations in colour – papules and nodules may be present	– palms, soles or beneath the nail plate – common type in dark complexed individuals
Lentigo maligna melanoma	– large, irregularly shaped macules or patches with variations of tan, brown or black pigment – may eventually develop a papular or nodular component	– sun-damaged skin especially the forearms and face – typically occurs in the elderly

The first line of treatment of CMM is wide local excision of the tumour (Bichakjian *et al.*, 2011:1039; Mangas *et al.*, 2010:134). Non-surgical methods of treatment such as radiotherapy, topical imiquimod, cryotherapy and observation are only used in select clinical circumstances where excision is not possible. Excision is not feasible in inoperable patients and in patients to whom the excision will be highly disfiguring e.g. a lentigo maligna on the face (Bichakjian *et al.*, 2011:1041; Mangas *et al.*, 2010:134). Caution must be taken with non-surgical treatment because of the risk of missing and under-treating invasive melanoma, higher local recurrence rates and the absence of long-term, randomised, controlled comparative studies. Currently imiquimod is the only API that is topically used for treatment of CMM. In this study the main focus was on the topical use of 5-fluorouracil in skin cancer. The following section (Section 2.3) is a discussion of 5-fluorouracil as an anti-cancer chemotherapy agent.

2.3 5-Fluorouracil

5-Fluorouracil is a uracil analogue with a fluorine atom in place of the hydrogen atom at position C-5. It is a pyrimidine anti-metabolite which is used topically to treat cancer-related ailments. According to Sturm as quoted by (Moyer, 1995:49) topical 5-fluorouracil is an “effective, convenient and inexpensive” treatment option which does away with the probability of multiple treatment sessions in the case of multiple actinic keratoses. It has been observed that a patient can be clear of actinic keratoses for up to 8 or 10 years if 5-fluorouracil is used for 6 weeks (Moyer, 1995:49).

Intravenous (IV), oral or topical administration is possible with 5-fluorouracil. Intravenous administration of 5-fluorouracil results in rapid elimination of the API and a number of severe side effects, it is preferable to avoid these effects. Oral administration on the other hand results in erratic and unpredictable absorption patterns (Chu & Sartorelli, 2009:935). Due to the flaws of these routes, the topical route is a viable alternative route in the treatment of cutaneous conditions such as actinic keratoses. In the case of superficial conditions topical treatment is preferable because the API is in direct contact with the target site. Figure 2.4 illustrates the structure of 5-fluorouracil.

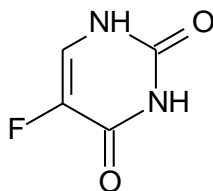


Figure 2.4: Chemical structure of 5-fluorouracil with fluorine at the C-5 position

2.3.1 Mechanism of action of 5-fluorouracil

This pyrimidine anti-metabolite interferes with deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis by inhibiting the nucleotide synthetic enzyme thymidylate synthase (TS) and by becoming misincorporated into RNA and DNA. Thymidylate is essential for replication as well as repair of DNA, in the event of TS inhibition thymidylate is not formed and “thymineless deaths” of cells occur (Chu & Sartorelli, 2009:935). 5-Fluorouracil causes intracellular cell damage which in turn acts as a signal for the induction of apoptosis in cells (Pollard *et al.*, 2008:839).

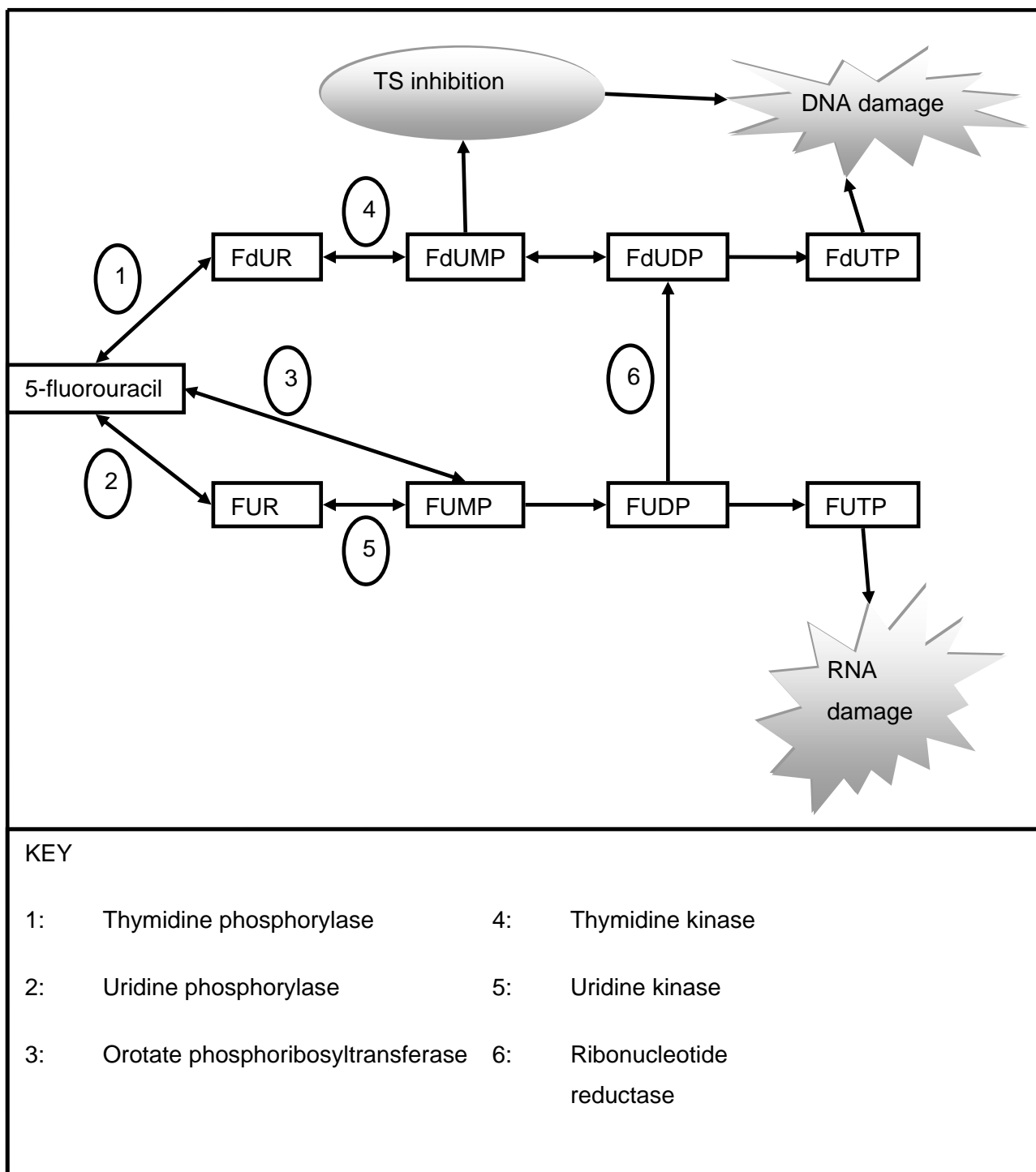


Figure 2.5: Mechanism of action of 5-fluorouracil showing DNA and RNA damage as the end points (Adapted from Longley *et al.*, 2003:331)

The events preceding TS inhibition are shown in Figure 2.5 and are as follows: 5-fluorouracil is taken up into the cell via the same pathway as uracil and is converted to fluorodeoxyuridine (FdUR) by thymidine phosphorylase (1). Fluorodeoxyuridine is further converted by thymidine kinase (4) to fluorodeoxyuridine monophosphate (FdUMP) which forms a covalent ternary complex with TS. Deoxyuridine phosphate (dUMP), the normal TS substrate is hindered from binding to TS and thus thymidine is not synthesised. Besides inhibiting TS function, FdUMP is

metabolised to form fluorodeoxyuridine triphosphate (FdUTP) which is incorporated into DNA and results in inhibition of DNA synthesis or malfunctioning DNA (Longley *et al.*, 2003:330).

The important mechanism of 5-fluorouracil action, however, is via the formation of fluorouridine monophosphate (FUMP). 5-Fluorouracil can be transformed to FUMP by means of two pathways. In the first pathway, direct conversion to FUMP by orotate phosphoribosyltransferase (3) with phosphoribosyl pyrophosphate (PRPP) acting as a co-factor. The indirect pathway entails conversion to fluorouridine (FUR) with uridine phosphorylase (2) as the responsible enzyme. Fluorouridine is further metabolised via uridine kinase (5) to form FUMP. Fluorouridine monophosphate is phosphorylated to an active metabolite, fluorouridine triphosphate (FUTP) which results in damaged RNA. On the other hand FUMP can be phosphorylated to fluorouridine diphosphate (FUDP) then reduced to fluorodeoxyuridine diphosphate (FdUDP). The FdUDP is phosphorylated or dephosphorylated and this results in the formation of fluorodeoxyuridine triphosphate (FdUTP) or FdUMP, respectively. Fluorodeoxyuridine triphosphate then goes further to cause RNA damage (Longley *et al.*, 2003:331). To sum it all up 5-fluorouracil may cause cell death of atypical and rapidly dividing cells.

2.3.2 Pharmacokinetics of 5-fluorouracil

Distribution and plasma clearance of 5-fluorouracil are relatively rapid with a plasma clearance of more or less 3 h. Six hours after administration approximately 15% of an IV dose is excreted unchanged in the urine while a larger fraction is excreted from the lungs as carbon dioxide (Lacy *et al.*, 2001:495). Absorption of 5-fluorouracil from the gastrointestinal tract (GIT) is unpredictable and therefore the oral route of administration is not used. Rather, the IV and topical routes are approved for this API. When applied topically approximately 6% of the dose is absorbed systemically (Robertson & Maibach, 2009:1047). To exert an effect in either route 5-fluorouracil must be metabolised to its active metabolites (FdUMP, FdUTP and FUTP). Besides these metabolites 80% of 5-fluorouracil can be metabolised by dihydropyrimidine dehydrogenase (DPD) in the liver to form dihydrofluorouracil (DHFU). Dihydropyrimidine dehydrogenase is also found in intestinal mucosa, tumour cells and other tissues, thus in hepatic dysfunction metabolism still occurs in the extra-hepatic sites (Chabner *et al.*, 2011). Other end products of the metabolism are carbon dioxide, urea and α -fluoro- β -alanine (Lacy *et al.*, 2001:495).

2.3.3 Clinical uses of 5-fluorouracil

2.3.3.1 Systemic uses of 5-fluorouracil

5-Fluorouracil is used systemically in the treatment of various types of cancers. Its main use is in colorectal cancer, but it is also effective against solid tumours of the neck, head, breast, stomach, pancreas, oesophagus, anus and liver (Chu & Sartorelli, 2009:947). It can be used alone or in combination with leucovorin. Leucovorin is a folic acid derivative and it works synergistically with 5-fluorouracil by enhancing the stability of the FdUMP-TS moiety thus enhancing the action of 5-fluorouracil (Lacy *et al.*, 2001:667). The effect of 5-fluorouracil is not strictly localised to tumour cells so it may have an effect on white blood cells and this effect should be closely monitored. Cyclophosphamide and methotrexate, oxaliplatin or doxorubicin can also be combined with 5-fluorouracil. This regimen is usually used in the adjuvant treatment of breast cancer (Sweetman, 2011).

2.3.3.2 Topical uses of 5-fluorouracil

Topically 5-fluorouracil is used for superficial tumours and premalignant skin conditions such as Bowen's disease, actinic keratoses and superficial BCCs (Sweetman, 2011). Concentrations of 0.5%, 1.0%, 2.0% and 5.0% are used for actinic keratoses, with a twice daily regimen. The healing process follows a particular sequence of events which starts with "erythema and progress through vesiculation, erosion, superficial ulceration, necrosis and finally reepitheliazation". Treatment should be continued until ulceration and necrosis occur. This happens approximately in the third week of treatment, thereafter treatment should be discontinued. Healing continues for up to two months (Robertson & Maibach, 2009:1062). Combination therapy is also possible in this instance, with tretinoin or corticosteroids for speedy recovery. Superficial BCC on the other hand requires 5-fluorouracil with a 5.0% strength only. It is applied twice daily for 3 – 6 weeks on average but treatment may go on for up to 12 weeks (Sweetman, 2011). Recent studies have shown that 5-fluorouracil cream is effective in the treatment of cholesteatomas (Takahashi *et al.*, 2005:356). However, more research is still to be done on the use of 5-fluorouracil in this condition.

2.3.4 Adverse effects of 5-fluorouracil

The adverse effects of 5-fluorouracil differ according to the route of administration. Topically applied 5-fluorouracil mainly has side effects at the site of application while IV 5-fluorouracil presents with gastrointestinal, hematologic, cardiovascular, ocular and dermatologic side effects (McEvoy, 2011).

Topical 5-fluorouracil causes stomatitis, dermatitis, photosensitivity and inflammatory reactions at the site. Inflammatory reactions mentioned include suppuration, pain, tenderness, burning

sensations and pruritis, to mention a few. If an occlusive dressing is applied or a 5-fluorouracil solution containing propylene glycol is used, the incidence of local side effects increases. Other adverse effects associated with topical application are a medicinal taste in the mouth; the patient is irritable and suffers from insomnia. Lacrimation and telangiectasia also occur (McEvoy, 2011).



Figure 2.6: Possible side-effects of topical 5-fluorouracil

Gastro-intestinal side effects include stomatitis, diarrhoea and nausea. The nausea is alleviated by anti-emetics and it generally subsides within 2 – 3 days of onset of treatment whereas the diarrhoea can be severe, requiring dose reduction to avoid serious complications. 5-Fluorouracil also affects the blood cells in its action and results in leucopenia, thrombocytopenia and anaemia. The white blood cell count must be closely monitored during the treatment period. The loss of hair and nails may occur due to 5-fluorouracil, which is a big issue for patients concerned with their appearances. The side effects of systemic 5-fluorouracil also include dermatologic eruptions such as a desquamative skin rash on the hands and feet, redness and scaling of the skin and dry skin (McEvoy, 2011).

2.3.5 Advice on use of 5-fluorouracil

Due to the unwanted effects of 5-fluorouracil several precautions need to be taken when using this API. All patients on 5-fluorouracil treatment should be furnished with the relevant advice as it may differ in topical and systemic treatment. Nonetheless, systemic toxicity is possible on topical application due to percutaneous absorption. In both treatment routes photosensitivity

occurs so the patient should protect them self from UV rays of the sun and other sources by avoiding the sun, covering their bodies or using sun-block. 5-Fluorouracil should not be used in pregnant women and safety in lactation has not been established, so patients should not go on 5-fluorouracil therapy (topical or systemic) if pregnant, breast-feeding or planning on getting pregnant. Male counter-parts may be unable to impregnate during use of 5-fluorouracil (McEvoy, 2011). Use of 5-fluorouracil increases susceptibility to infection and any signs thereof e.g. fever, chills, nausea, painful urination, etc., should be reported to a physician together with any other side effects.

The inevitable inflammatory reaction that occurs on topical use of 5-fluorouracil should be made known to the patient on onset of treatment, so that they do not discontinue treatment when it occurs. If severe and unbearable the patient is advised to contact the prescriber. Care must be taken to ensure that topical 5-fluorouracil never gets in contact with mucocutaneous zones of the body. After application, hands should be washed thoroughly to avoid accidental contact with eyes, nose or mouth and to avoid the occurrence of side effects on the hands (Sweetman, 2008).

2.4 The skin and transdermal drug delivery

The average human skin is approximately 2 m² in surface area and accounts for 10 – 20% of total body weight (Robinson, 2005:25). This makes skin the largest and heaviest organ of the body. In early days scientists thought the skin was completely impermeable but according to Bos and Meinardi (2000:169), the skin can be permeated by exogenous molecules that are smaller than 500 Da. It is a fairly permeable organ, which plays an important role as the outer barrier between our bodies and the hostile external environment (Patzelt *et al.*, 2008:e229). Without this efficient barrier the body would rapidly dehydrate (Potts *et al.*, 1992:14).

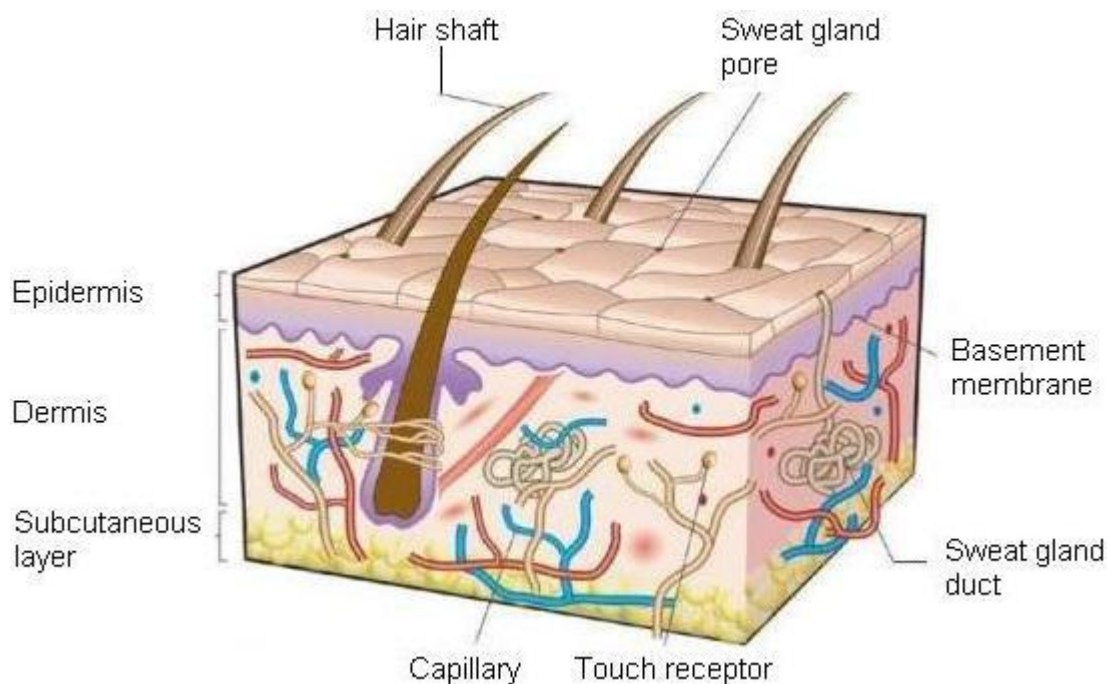


Figure 2.7: Structure of the skin showing the different layers of skin and appendages (Adapted from skininfo.org, 2010).

Human skin is on average 0.05 – 2.00 mm thick (Foldvari, 2000:417). Different regions of the body have differing skin thicknesses, which in turn have an effect on the absorptive characteristics of the area (Potts *et al.*, 1992:14). The skin consists of the epidermis (top layer), the dermis (middle layer) and the hypodermis (subcutaneous fatty layer). These are the three main layers, which are further sub-divided (Eckert, 1992:4). Throughout the skin we also find skin appendages such as hair follicles, sebaceous glands and sweat glands. For a topical effect to become apparent the API must be able to permeate in to the epidermis and dermis or exert a direct local effect. Whereas on percutaneous use the API must permeate through the skin to reach the blood vessels and in turn exert an effect.

2.4.1 The epidermis

The epidermis mainly consists of keratinocytes, which are cells that synthesise keratin. Other cells in the epidermis are Langerhans cells, Merkel cells and melanocytes (Kanitakis, 2002:26 May 2011). The layers of the epidermis are the stratum basale, stratum spinosum, stratum granulosum and the stratum corneum (Venus *et al.*, 2010:469).

2.4.1.1 Stratum basale

This basal cell layer is adjacent to the dermis and it has a one-cell thickness. Keratinocytes are the main cell types and they can be dividing or non-dividing. The other cells present are melanocytes (Venus *et al.*, 2010:469).

2.4.1.2 Stratum spinosum

There are five to fifteen layers of polyhedral cells with desmosomes connecting the cells. In this layer we also find the Langerhans cells. When viewed under the microscope the layer has a 'prickly' or 'spiny' appearance due to the density of desmosomes (Venus *et al.*, 2010:469).

2.4.1.3 Stratum granulosum

The keratinocytes in the stratum granulosum contain granules of keratohyalin and in the cytoplasm there are granules called Odland bodies (Kanitakis, 2002:26 May 2011). This is why it is called the stratum granulosum, which is also referred to as the granular layer. The intercellular spaces are filled with lipid components of surrounding cells and these lipids have a barrier function (Venus *et al.*, 2010:469).

2.4.1.4 Stratum corneum

The stratum corneum is the hydrophobic, thin (10 – 20 µm), outermost layer of the skin which is most resistant to permeation. The main cells are the corneocytes, which are surrounded by a tight lipophilic matrix. Corneocytes are flattened cells that have migrated from the stratum granulosum. They contain keratin but have no nuclei and cytoplasmic organelles. There are ten to fifteen layers of corneocytes in the stratum corneum. This layering can be described by the 'brick and mortar model' where the 'brick' is the intracellular protein (keratin) and the 'mortar' is the lipid composition of ceramides, cholesterol, fatty acids and cholesteryl esters. There are no phospholipids as compared to other biological membranes. The specific ratio of the various lipids seems to determine the barrier function of the skin so if the skin is impaired, active processes go underway to restore these vital skin lipids (Foldvari, 2000:418).

Penetration of the stratum corneum is the rate-limiting step in the absorption of substances through the skin. The outer lipids of the stratum corneum form the main barrier to penetration. A noteworthy fact is that the lipids form a continuous phase from the surface of the skin to the base of the stratum corneum. Research has shown that particles mainly move through the stratum corneum via the lipid based "mortar" than through the cells (Potts *et al.*, 1992:22). The diffusion pathway through the stratum corneum is therefore tortuous because of the staggered layering of corneocytes within the lipid matrix. This enhances the barrier function of skin (Naik *et al.*, 2000:318).

2.4.2 The dermis and hypodermis

The dermis is the layer which is adjacent to the epidermis. It is composed of collagen fibres, elastic tissue, ground substance, fibroblasts, histiocytes, mast cells, plasma cells and other cellular components. All these components have important functions e.g. mast cells regulate

the release of chemo-transmitters in an allergic reaction and fibroblasts secrete collagens, elastic fibres and ground substance (Lai-Cheong & McGrath, 2009:224). The dermis is tough and resilient and therefore has a protective function in the skin (Venus *et al.*, 2010:469). On top of all this there are sweat glands, sebaceous glands, networks of blood vessels, lymphatic vessels and nerve endings in the dermis. The hypodermis on the other hand is mainly the fatty tissue of the skin and is composed of lipocytes (Foldvari, 2000:418).

2.4.3 Advantages and limitations of topical and transdermal drug delivery

Transdermal drug delivery (TTD) is a relatively new and innovative way of administering APIs in to the body. Like any method there are pros and cons to this mode of delivery but the pros seem to outweigh the cons. Penetrating the skins excellent barrier function is a challenge but once penetrated the benefit is invaluable.

2.4.3.1 Advantages of topical and transdermal drug delivery

The advantages of topical and transdermal drug delivery are:

- ❖ the skin has a large surface area (1 – 2 m²) available for absorption of APIs (Naik *et al.*, 2000:319);
- ❖ the skin is readily accessible for API delivery (Naik *et al.*, 2000:319);
- ❖ transdermal drug delivery is non-invasive, so it enhances patient compliance (Naik *et al.*, 2000:319);
- ❖ sustained and controlled delivery is possible with transdermal drug delivery systems, especially for APIs with short half-lives (Naik *et al.*, 2000:319);
- ❖ this form of drug administration is painless and simple thus specialised healthcare staff is not necessary which may lower treatment costs (Cleary, 1993:19);
- ❖ the gastrointestinal tract is avoided so first-pass metabolism and the effect of gastric pH is avoided (Cleary, 1993:19);
- ❖ systemic side effects are reduced (Guy, 1996:1766) and
- ❖ the target site is directly accessible when treating skin conditions.

2.4.3.2 Limitations of topical and transdermal drug delivery

The limitations of topical and transdermal drug delivery are outlined below:

- ❖ diffusion across the stratum corneum is difficult for molecules larger than 500 Da because diffusivity is inversely related to size (Bos & Meinardi, 2000:169);

- ❖ an aqueous solubility greater than 1 mg/ml and an oil-water partition coefficient ($K_{o/w}$) between 10 and 1000 is recommended for ease of permeation through the stratum corneum (Naik *et al.*, 2000:319);
- ❖ the skin may become sensitised or irritated on application (Murphy & Carmichael, 2000:366);
- ❖ this route is only possible for very potent APIs that require minute concentrations (Cleary, 1993:20);
- ❖ there are inter and intra-variations in the permeability of healthy and diseased skin. This implies that absorption profiles may differ (fast, medium and slow absorption) resulting in varying physiological responses (Cleary, 1993:20);
- ❖ the melting point of the API should be below 200 °C (Finnin & Morgan, 1999:955);
- ❖ the API should have at most two hydrogen bonding groups (Finnin & Morgan, 1999:955) and
- ❖ the API should not be directly irritating to the skin and it should not induce an immune reaction in the skin (Finnin & Morgan, 1999:955).

2.4.4 Mechanisms of skin permeation

Molecules penetrate the excellent barrier of the skin via three pathways. The main pathways used by particular API molecules differ with respect to their physico-chemical properties. Movement across the stratum corneum, however, is by a combination of the three routes. The pathways are: 1) through the intercellular lipids; 2) by a transcellular route; or 3) via the skin appendages (Morrow *et al.*, 2007:38). Other scientists have argued for the existence of a 'polar' route and a 'non-polar' route. To the contrary there is evidence proving that diffusion path lengths of molecules with varying physico-chemical properties are significantly greater than the thickness of the stratum corneum. This greatly implicates a tortuous (intercellular) route (Potts *et al.*, 1992:22).

2.4.4.1 Intercellular route

This pathway is whereby the API diffuses through the continuous lipid matrix which surrounds the cells. The interdigitated corneocytes as described by the 'brick and mortar' model result in a very tortuous route for the API (Morrow *et al.*, 2007:38). The API molecule must also partition into and diffuse through the aqueous and lipid components of the bilayers in the matrix. Small and uncharged molecules are said to penetrate the skin via this route (Ghosh & Pfister, 1997:6).

2.4.4.2 Transcellular route

The transcellular route is basically travelling straight across the stratum corneum and it entails passing through the corneocytes and the lipid bilayers repeatedly. Molecules using this route must be able to partition into and diffuse through the hydrophilic environment of the corneocytes and further partition into the surrounding lipid envelope (Morrow *et al.*, 2007:38). Transport through the lipid envelope entails moving through the aqueous and lipid components of the bilayer just as in the intercellular route. This is the shortest route for the API and it is commonly used by highly hydrophilic APIs during steady-state flux (Ghosh & Pfister, 1997:6).

2.4.4.3 Transappendageal route

A continuous permeation route which goes directly across the stratum corneum is via the skin appendages. The skin appendages involved include hair follicles, sweat ducts and sebaceous glands. The limitations of this route are: 1) there is a small surface area (approximately 0.1% of skin surface area) available for permeation of the API molecules; 2) sweat glands have limited permeation during the secretion of sweat as sweat moves in the direction opposite to that of the permeant and 3) sebaceous glands are lipophilic because they contain lipid-rich sebum which may hinder penetration of hydrophilic molecules (Morrow *et al.*, 2007:38). Regardless of the limitations, the appendageal route plays a major role immediately after application, before a steady state is reached. It is important for penetration of large molecules, polar molecules and ions (Ghosh & Pfister, 1997:6).

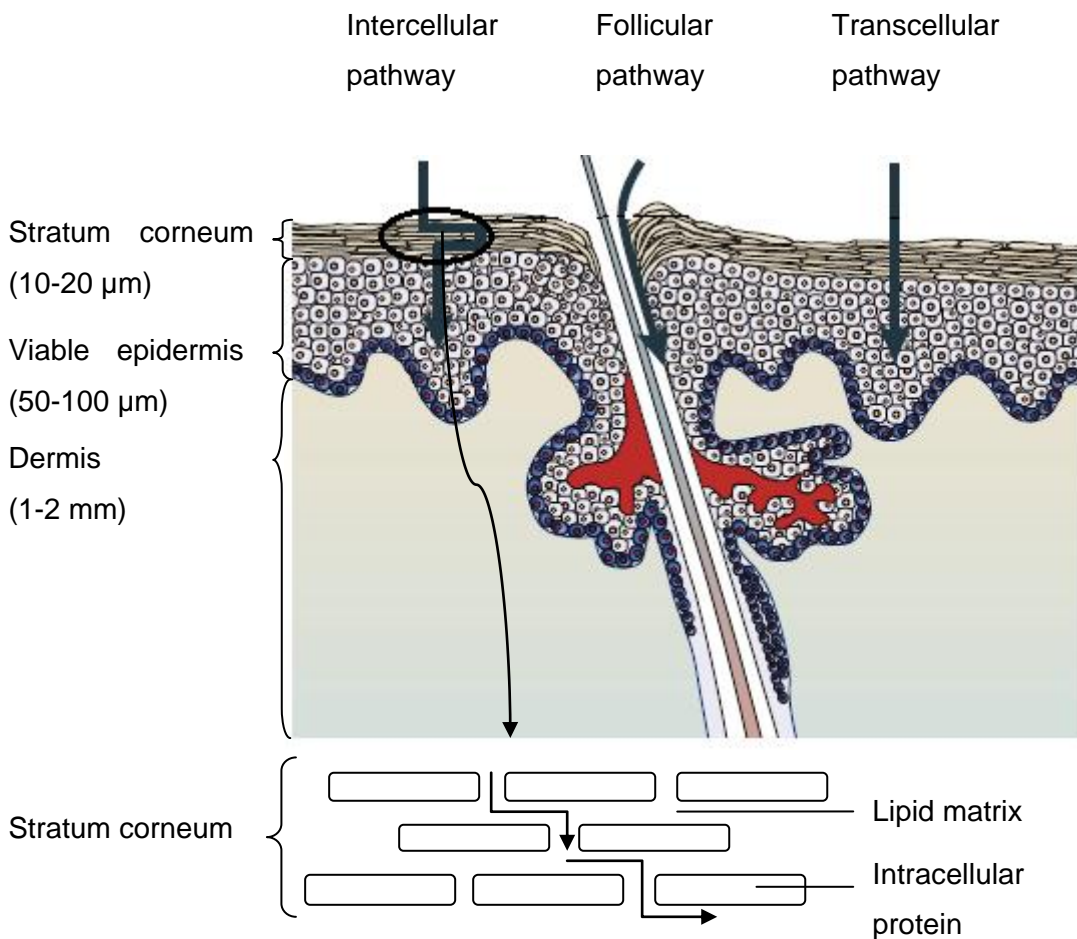


Figure 2.8: Structure of the skin showing routes of permeation (Adapted from Prausnitz *et al.*, 2004:119).

2.4.5 Mathematical models of skin permeation

A basic understanding of the permeation of molecules through a membrane is necessary for transdermal studies. To date, no active processes have been identified as being responsible for skin penetration so it has been concluded that simple passive diffusion is the underlying transport process (Hadgraft, 2001:1). Diffusion across the stratum corneum is described by Fick's laws of diffusion. This is not entirely accurate *in vivo* because a very long time is taken before steady-state conditions are reached *in vivo* and the equation is only valid in steady-state conditions (Guy & Hadgraft, 1989:15). The diffusion of a variety of molecules across the skin is difficult to describe accurately by a Fickian relationship because of the existence of polar and non-polar pathways for penetration. This results in differing permeation rates in the hydrophobic stratum corneum and in the hydrophilic viable tissue (Godin & Touitou, 2007:1158). Regardless of the above-mentioned irregularities Fick's first law of diffusion is commonly used to describe the diffusion process across the skin.

$$J = \frac{dC}{dt} = \frac{DK_p C_0}{h}$$

Equation 2.1

Where:

J = flux

dC/dt = rate of skin penetration (flux)

D = effective diffusion coefficient of API in stratum corneum

K_p = partition coefficient of API between skin and vehicle

C₀ = concentration of API in vehicle/donor compartment

h = effective diffusion path length through the skin barrier

2.4.6 Factors influencing skin permeation

There are many factors that influence the permeation of molecules through the skin. These factors can be classified as biological or physico-chemical. The main factors that were considered in this study are the physico-chemical factors. Some of the factors have a direct effect on Fick's first law (Equation 2.1). An important thing to remember is that as one factor changes it may affect permeation in more than one way (Barry, 2007:565).

2.4.6.1 Biological factors

The biological factors have more to do with the skin itself and as a result they differ from patient to patient. These factors affect skin permeation but very little can be done to alter them in order to enhance permeation as compared to the physico-chemical properties. The biological factors are: skin condition, skin age, skin metabolism, skin site, blood circulation, race differences and species differences.

2.4.6.2 Physico-chemical factors

The physico-chemical properties affecting skin permeation have a lot to do with the API. With respect to Fick's first law (Equation 2.1) the chemical properties influence the concentration difference within the membrane while the physical properties influence the diffusivity (Smith, 1990:25). Below are some of the physico-chemical properties that influence the permeation of an API through the skin. Table 2.2 shows the physico-chemical properties of 5-fluorouracil.

2.4.6.2.1 Skin hydration

When the skin is hydrated it swells, softens and wrinkles, and the skin permeability markedly increases. Hydration occurs when water from perspiration is trapped by an occlusive agent or when water diffuses from the underlying epidermal layers (Barry, 2007:576).

2.4.6.2.2 Drug concentration

According to Ficks law the flux (J) of an API across the stratum corneum is directly proportional to the API concentration (C_0). A high API concentration results in a steep diffusion gradient which enhances the diffusion of the API molecules across the skin. To obtain maximum flux in a thermodynamically stable situation the donor must be fully saturated. Various factors such as pH, presence of micelles or surfactants, complex formation etc are able to alter the thermodynamic stability of a solution (Barry, 2007:577).

2.4.6.2.3 Temperature and pH

The temperature of the stratum corneum is in the range of 30 – 37 °C (Potts *et al.*, 1992:24). As temperature increases the diffusion coefficient also increases so a large temperature variation can change flux by 10-fold. The pH-partition hypothesis states that unionised molecules pass readily across lipid membranes. The fraction of unionised molecules in the medium determines the effective membrane gradient. Due to this, the pH of the medium influences flux of acids and bases by influencing the dissociation. Ionised molecules, however, do pass through the stratum corneum, but to a limited extent (Barry, 2007:576). In this study a pH of 7.4 was used. According to a form of the Henderson-Hasselbach equation (Equation 2.2) 20.1% of 5-fluorouracil was ionised at this pH therefore resulting in 79.90% of unionised API. To enhance permeation of ionised molecules the pH may be altered but a non-physiological pH may affect solubility, partitioning or binding, in turn altering skin permeation (Smith, 1990:27).

$$\% \text{ ionised} = \frac{100}{1 + \text{anti- log} (\text{pKa-pH})}$$

Equation 2.2

2.4.6.2.4 Molecular size and shape

An inverse relationship exists between molecular weight and flux; the smaller the molecule the faster the absorption (Barry, 2007:578). Molecules with low molecular weights (below 600 Da) can diffuse through the skin (Barry, 2007:579). The molecular weight of 5-fluorouracil is 130.08 Da (Rudy & Senkowski, 1973:221), which is in compliance with the recommendations stated above. Due to this, we can possibly predict that 5-fluorouracil is able to permeate through the skin. However, the effects of molecular size on penetration are much less significant than the effects of partitioning and solubility (Hadgraft & Wolff, 1993:164).

2.4.6.2.5 Diffusion coefficient

On application of a topical dosage form to the skin API molecules in the donor vehicle partition into and concentrate in the first lamina of the membrane. A concentration gradient is established which is the driving force for diffusion across the membrane. Molecules in the donor solution diffuse to the interface so as to replace the API partitioning into the skin. Therefore the API must be able to diffuse through the donor vehicle and through the solvent system of the skin. The diffusion coefficient (D) is a measure of the ease with which a specific molecule type is able to diffuse through a medium (Smith & Surber, 2000:29).

The speed of diffusion of a molecule is determined by the state of matter of the medium. Diffusion coefficients are larger in gases as compared to liquids. In skin, diffusion coefficients reach their lowest levels in the compact matrix of the stratum corneum. At a constant temperature, the diffusion coefficient of an API in topical vehicle depends on the API properties, the diffusion medium and the interaction between the two (Barry, 2007:577). The diffusion medium and the diffusant should be as dissimilar as possible to lower the possibility of strong bonding interactions between the two during diffusion. Hence, formulation in a relatively hostile medium will favour the release of the API to the solvent system of the skin. Excipients that penetrate the stratum corneum may alter the fluidity or solubility characteristics of the membrane thus enhancing or hindering absorption (Smith & Surber, 2000:30).

2.4.6.2.6 Partition coefficient

It is apparent in Ficks first law that a high partition coefficient (K_p) results in increased flux (J) through the stratum corneum. The relative affinities of the API for the vehicle and for the secondary environment (the stratum corneum) determine the APIs partitioning behaviour. The value of the partition coefficient is very important when the major diffusional resistance is due to the stratum corneum. It determines the initial concentration of diffusant in the first layer of the membrane (Barry, 2007:578).

Compounds with high partition coefficient values have high lipid solubility. As a result, they easily partition into the stratum corneum but they do not readily pass into the water rich viable tissue from lipid rich stratum corneum (Barry, 2007:578).

As the solubility of the API in the vehicle increases the partition coefficient decreases because the API molecules have a greater affinity for the vehicle than for the stratum corneum (Smith & Surber, 2000:28). The oil-water partition coefficient that is ideal for skin permeation should be between 10 and 1000, which translates to a log P value between 1 and 3 (Naik *et al.*, 2000:319). This sheds some light on the permeation characteristics of 5-fluorouracil. 5-Fluorouracils partition coefficient of -0.83 is low and it reflects the affinity of 5-fluorouracil for

the aqueous phase as compared to the oil phase. Therefore, regardless of its small size 5-fluorouracil barely passes through the stratum corneum in the absence of permeation enhancers.

2.4.6.2.7 Solubility

Following release from the vehicle the API must partition into the stratum corneum and the extent of partitioning is in part controlled by the solubility limit of the API in the stratum corneum (Hadgraft & Wolff, 1993:162). Subsequent to this the API must progress through hydrophilic cell contents and into the surrounding tissue to exert an effect. Once released from the formulation the API traverses hydrophilic and lipophilic environments. Thus for optimum skin permeability an API should have an aqueous solubility larger than 1 mg/ml but it should also be relatively oil soluble (Naik *et al.*, 2000:319). Unionised molecules are able to move through the lipid-rich stratum corneum more easily than ionised molecules. Most APIs are either weak acids or bases so their solubility in the aqueous phase of a particular vehicle is determined by their ionisation state in the medium. A change in pH may increase the solubility of the API in the medium but this may in turn result in a reduction in the number of unionised molecules. This shows that enhancing solubility does not necessarily enhance the overall diffusion process through the skin. It must always be taken into account that the pH of optimum API solubility does not always correspond with the pH of maximum stability so a balance must be obtained (Smith & Surber, 2000:27).

Materials with low melting points tend to have higher absolute solubilities in non-polar media as less energy per mole is required for dissolution in the stratum corneum lipids, this in turn results in increased skin permeation (Flynn & Weiner, 1993:44). This explains how melting point plays a role in the skin permeation of API molecules.

Table 2.2 Physico-chemical properties of 5-fluorouracil

Molecular formula	C ₄ H ₃ FN ₂ O ₂ (Rudy & Senkowski, 1973:223)
Molecular weight	130.08 Da (Rudy & Senkowski, 1973:223)
Appearance	Slightly white crystalline powder (Rudy & Senkowski, 1973:223)
Log D	-0.83 (Buur <i>et al.</i> , 1985:55)
Melting point	282 °C – 283 °C (Rudy & Senkowski, 1973:228)
Stability	Stable in solutions with pH below 9 (Rudy & Senkowski, 1973:234)
Solubility	Sparingly soluble in water – 1 g in 80 ml (Troy, 2005:1573) Slightly soluble in alcohol – 1 g in 170 ml (Troy, 2005:1573)

2.4.6.3 Ideal physico-chemical properties for passive transdermal delivery

Guy (1996:1766), states that compounds that have relatively low molecular weights (below 500 Da), relatively low melting points and octanol-water partition coefficients ($K_{o/w}$) between 10 and 1000 are likely to display optimum passive skin permeation. Examples of such APIs are nitroglycerine and nicotine. 5-Fluorouracil has a relatively high melting point and a low octanol-water partition coefficient which leads to the prediction that it is unlikely to penetrate and diffuse through the skin. These are some of the obstacles in the transdermal delivery of 5-fluorouracil.

Table 2.3 shows a comparison of the properties of 5-fluorouracil with the properties that are ideal for skin permeation. From the table we see that 5-fluorouracil complies with the solubility and molecular weight parameters but lies outside the range for melting point and lipophilicity. It is not known which properties carry the greatest weight in determining the possibility of transdermal delivery but it seems that lipophilicity has a great role to play. 5-Fluorouracil is a polar API while the stratum corneum is lipid rich. The probability of 5-fluorouracil partitioning from the drug delivery vehicle into the lipophilic stratum corneum is therefore low.

Table 2.3 A comparison between the ideal physico-chemical properties for transdermal delivery and the physico-chemical properties of 5-fluorouracil (Adapted from Naik *et al.*, 2000:319).

Physico-chemical property	Ideal limit(s)	5-fluorouracil value
Aqueous solubility	> 1 mg/ml	1 g in 80 ml = 12.5 mg/ml (Troy, 2005:1573)
Lipophilicity	$10 < K_{o/w} < 1000$; ($1 < \log P < 3$)	$\log P = -0.83$ (Buur <i>et al.</i> , 1985:55)
Molecular weight	< 500 Da	130.08 Da (Rudy & Senkowski, 1973:223)
Melting point	< 200 °C	282 – 283 °C (Rudy & Senkowski, 1973:228)

2.5 Selected methods for the enhancement of skin penetration

As mentioned in Section 2.4, the skin has excellent barrier properties and this makes it difficult for molecules to permeate to the underlying structures of skin. Permeation through the stratum corneum is enhanced using physical and chemical strategies. Supersaturation also enhances permeation as supersaturated systems have unusually high thermodynamic potentials and maximum skin penetration rates are obtained when APIs are at their highest thermodynamic potentials (Benson, 2005:26).

Ficks first law gives us some insight on what can be done to enhance skin permeation. From Equation 2.1 it is clear that increasing the diffusion coefficient (D), partition coefficient (K_p) and

the effective concentration (C_0) results in an increased flux (J), while increasing the path length (h) results in decreased flux (J). Respectively, an effective permeation enhancer may disturb the barrier nature of the stratum corneum, improve partitioning into the tissue by altering the solvent nature of the skin membrane, act as an anti-solvent or provide a permeation “shortcut” thus decreasing the path length (Williams & Barry, 2004:605).

According to Foldvari (2000:419) there are three mechanisms of permeation enhancement and these include: 1) disrupting the structure of intercellular lipid channels which is highly ordered; 2) interacting with the components of corneocyte intracellular proteins and 3) enhancing partitioning of the API regardless of the presence or absence of the enhancer. The last mechanism is described by the ‘lipid protein partitioning (LPP) theory’ (Goodman & Barry, 1989:29).

2.5.1 Chemical penetration enhancement

Chemical penetration enhancement is widely researched and it entails reversibly altering the barrier function of the skin using chemicals. Molecules which otherwise would not permeate are given an opportunity to pass through the stratum corneum. Chemicals usually used as skin penetration enhancers include but are not limited to alcohols, amines, amides, fatty acids, terpenes, alkanes, esters, pyrrolidones and surfactants (Naik *et al.*, 2000:321).

A lot of literature has been published regarding the ideal properties of permeation enhancers. Enhancers should adhere to the following ideal properties (Buyuktimkin *et al.*, 1997:359; Finnin & Morgan, 1999:956):

- ❖ the enhancer should have no pharmacological activity;
- ❖ the material must be non-toxic, non-allergenic and non-irritating;
- ❖ the compound must be chemically and physically compatible with the delivery system;
- ❖ the compound must be chemically and physically stable;
- ❖ the compound should be easily incorporated into the delivery system;
- ❖ the enhancer should have a known mechanism of action and metabolism;
- ❖ the enhancer must reduce the barrier function of the skin in one direction only (there should be no efflux of endogenous substances);
- ❖ the effects of the enhancer must be immediate and predictable;
- ❖ there must be complete recovery of the stratum corneum immediately after removal of the enhancer (reversible);

- ❖ the compound should be inexpensive and cosmetically acceptable (colourless, odourless and tasteless) and
- ❖ the API should be released from the formulation in the presence of the enhancer.

These are very high standards to adhere to and thus most permeation enhancers up to date only have some of the required properties. Problems have, however, been encountered with the chemical permeation enhancers even when they adhere to the above-mentioned properties. Predicting the *in vivo* behaviour of the enhancers after *in vitro* experimentation is difficult due to the differing conditions. Another problem encountered is balancing the characteristics of the formulation with the enhancer in order to maintain the tendency of the API to partition into the skin. The future of transdermal drug delivery hinges on overcoming these setbacks (Thomas & Finnin, 2004:700). Only chemical penetration enhancers applicable to the current study have been discussed in Sections 2.5.2 – 2.5.3.

2.5.2 Fatty acids

Lauric acid, linoleic acid and oleic acid (OA) are examples of fatty acids that are commonly employed as permeation enhancers. An advantage of using fatty acids is that they are endogenous components of human skin so the chances of them causing adverse effects are low. Fatty acids work by inserting themselves between the hydrophobic tails of the stratum corneum lipid bilayer. This disturbs the lipid packing arrangement, increases the membrane fluidity and in turn decreases the resistance of the stratum corneum to permeant diffusion (Cheong-Weon Cho *et al.*, 2009:232).

Unsaturated fatty acids have been said to work better as permeation enhancers and their effect is determined by the number, position and type of double bonds. Fatty acids with the *cis*-conformation are more effective because they have a 'kink' in their structure which disrupts the lipid bilayer more than the straight structure in the *trans*-conformation. The intercellular lipid packing is disrupted and this facilitates enhanced API mobility (Morrow *et al.*, 2007:45).

Chain length also influences the extent of the enhancing action of a fatty acid. Saturated fatty acids with a chain length of C₁₀ – C₁₂ attached to a polar head are potent enhancers. On the other hand unsaturated fatty acids are potent enhancers when they have a chain length of C₁₈ (Williams & Barry, 2004:609). Various studies have been done on the use of OA in the transdermal delivery of 5-fluorouracil and according to studies done by Goodman and Barry (1988:325), OA enhances 5-fluorouracil delivery by 16.2-fold in fully hydrated tissue. The skin is relatively impermeable to 5-fluorouracil but in the presence of a fatty acid, in this instance OA, the permeability of the skin is increased.

2.5.3 Drug delivery vehicles

Common types of drug delivery systems are vesicles. Vesicles are generally water-filled colloidal particles that are composed of a bilayer of amphiphilic molecules. Hydrophilic APIs can be entrapped within the internal aqueous compartment while hydrophobic APIs can become associated with the bilayer via hydrophobic interactions or electrostatic interactions (Honeywell-Nguyen & Bouwstra, 2005:67). The physico-chemical properties of the vesicles (e.g. size, charge, thermodynamic phase etc.) determine their behaviour in the skin and thus their effectiveness as drug delivery vehicles (Honeywell-Nguyen & Bouwstra, 2005:68).

The advantages of using vesicles as drug delivery systems as stated by Honeywell-Nguyen and Bouwstra (2005:68) include:

- ❖ vesicles can act as API carriers that deliver the APIs within the skin or carry them across the skin;
- ❖ vesicles can enhance skin penetration because of lipid components of the vesicles that easily penetrate into the stratum corneum and thereafter alter stratum corneum lipid lamellae;
- ❖ vesicles can act as reservoirs for sustained release of compounds acting within the skin and
- ❖ vesicles can act as rate-limiting membrane barriers so as to modulate systemic absorption and this results in a controlled release transdermal delivery system.

The drug delivery systems that are composed of phospholipids are known as liposomes while those composed of non-ionic surfactants are niosomes or non-ionic surfactant vesicles (Honeywell-Nguyen & Bouwstra, 2005:68). Niosomes are advantageous in terms of cost and stability as compared to the typical liposomes (Morrow *et al.*, 2007:42).

A newer type of drug delivery system is the transfersome. Transfersomes are like liposomes; the difference is that, in addition to the phospholipids they contain 10 – 25% surfactant. The surfactant is called an “edge-activator”. The surfactant molecules destabilise the lipid bilayers which makes the vesicles more flexible and in turn lowers the energy required during a change of shape or conformation (Morrow *et al.*, 2007:42). The conferred flexibility enables these vesicles to squeeze through the pores of the stratum corneum thus allowing for delivery of large molecules into the body via the skin. Transfersomes result in a faster onset of API action, longer duration of action and allow for a reduction in the dosage necessary for a therapeutic effect (Choi & Maibach, 2006:177).

Liposomes can also be modified to form ethosomes by addition of high amounts of ethanol. Ethosomes contain 20 – 45% ethanol (Morrow *et al.*, 2007:43). They are soft, malleable

vesicles that are tailored to increase delivery of APIs to and through the skin. Their exact mode of action is not known but it is suspected that the ethanol disrupts the lipid bilayer and gives way for penetration of the ethosomes (Morrow *et al.*, 2007:43).

2.5.4 Pheroid™ technology

Pheroid™ technology combines the use of fatty acids and vesicles, together with other techniques in order to result in penetration enhancement of APIs. The basis of Pheroid™ technology comes from Emzaloid™ technology which was founded by Piet Meyer and Steven Zall of MeyerZall Laboratories (Grobler, 2009:117). Manufacturing conditions are the differentiating factor between Pheroid™ and Emzaloid™. The term Pheroid™ is used to describe the formulations that are manufactured according to the manufacturing protocol used in the research laboratories and pilot plant. Emzaloid™ technology is still being used by MeyerZall Laboratories in manufacturing of their various topical commercial products. All the intellectual property regarding Emzaloid™ technology was procured from MeyerZall (Pty) Ltd, by the North-West University, South Africa in December 2003 (Grobler, 2009:118).

Pheroid™ technology is used as a drug delivery vehicle in pharmaceutical products. In comparison to other delivery systems Pheroid™ improves the control of size, charge and the hydrophilic-lipophilic characteristics of therapies by a larger margin. Therefore the Pheroid™ delivery system enhances the efficacy of delivered compounds while allowing for the reduction of unwanted adverse effects (Grobler *et al.*, 2008:284). The API in this study (5-fluorouracil) barely permeates the skin on its own so it was delivered using Pheroid™ technology.

2.5.4.1 Advantages of the Pheroid system as a delivery system for 5-fluorouracil

The delivery of hydrophilic APIs, such as 5-fluorouracil, via the skin is difficult due to the hydrophobic nature of the stratum corneum. Permeation to the underlying layers of the skin is hindered and thus the expected effect may not be exerted. A high concentration of API may be needed to exert a small therapeutic effect and this results in unwanted effects. Regardless of such challenges, advancements in technology make it possible to deliver 5-fluorouracil via the transdermal pathway. Topical creams and topical solutions of 5-fluorouracil are already on the market but further investigations are still being made to improve current preparations. Mechanisms such as the use of Pheroid™ technology have been employed to enhance the delivery of 5-fluorouracil. According to Van Dyk (2008:67) a lower concentration of 5-fluorouracil can be used when applying Pheroid™ technology as a drug delivery vehicle thus lowering the incidence of side effects. Here are some of the advantages of Pheroid™ as a transdermal drug delivery system as written by Grobler (2004:6):

- ❖ it consists mainly of essential fatty acids (EFAs) which are natural and essential ingredients of the body;

- ❖ cytokine studies showed that Pheroid™ does not cause immune responses in man;
- ❖ the size, charge, lipid composition and membrane packing of the Pheroid™ can be manipulated to suit the active compound and drug indication;
- ❖ it assists with maintenance of the cell membrane and causes no cytotoxicity;
- ❖ it causes a reduction or elimination in drug resistance which is important in the use of 5-fluorouracil;
- ❖ it is polyphilic so APIs with different solubilities and insoluble APIs can be entrapped;
- ❖ it is ideal for quick release but still maintains the possibility of sustained release via pro- Pheroid™;
- ❖ it protects APIs from metabolism, opsonisation and inactivation before reaching the target site;
- ❖ it enhances oral, buccal and topical administration of active compounds. It also enhances absorption;
- ❖ it creates a safer and more effective formulation (Grobler, 2004:4);
- ❖ it reduces the minimal effective concentration which in turn results in reduced side effects (Grobler, 2004:3);
- ❖ it increases the delivery of active agents and it is able to target the treatment sites (Grobler, 2004:3) and
- ❖ it is stable in terms of shelf-life and in body fluids (Grobler, 2009:102).

2.5.4.2 Structural characteristics of Pheroid™

Grobler *et al.* (2008:284) state that the Pheroid™ delivery system is a unique colloidal system. Pheroid™ consist of unique and stable lipid-based submicron and micron-sized structures. They are dispersed in a dispersion medium that is adaptable according to the indication. Furthermore it is possible to manipulate the morphology, size, structure and function of the dispersed structures so as to suit the intended application. Typical colloids have dispersed particles that are between 1 – 100 nm in diameter, while various types of Pheroid™ can have a diameter between 200 nm and 2 µm. The required capacity, rate of delivery and the administration route must be taken into consideration when deciding on the type and diameter of the Pheroid™ (Grobler *et al.*, 2008:285).

The main constituents in Pheroid™ are ethylated and pegylated polyunsaturated fatty acids. These include the omega-3 and omega-6 fatty acids but exclude arachidonic acid. The fatty acids are compatible with the orientation of fatty acids in man as they are in the *cis*-formation (Grobler *et al.*, 2008:285). Nitrous oxide gas (N₂O) is also a component of Pheroid™ which

thus adds another dimension to the basic Pheroid™. N₂O is a gas which is soluble in both water and oil; this gives it the ability to move freely within the dispersed and continuous phase (Grobler *et al.*, 2008:289). According to Grobler *et al.* (2008:289) N₂O has three distinct functions in Pheroid™; it contributes to the:

- ❖ miscibility of the fatty acids in the dispersal medium;
- ❖ self-assembly process of the Pheroid™ and
- ❖ stability of the formed Pheroid™.

The solubility characteristics of N₂O also enable the gas to move freely through the epidermal and dermal layers of the skin but the N₂O mainly accumulates in the lipid-rich membrane. The accumulated N₂O and unsaturated fatty acids result in an increase in membrane fluidity. This increases the movement of hydrophobic molecules or hydrophilic molecules in association with EFAs. The interaction between the fatty acids and the nitrous oxide results in a matrix of stable vesicular Pheroid™ structures. The N₂O EFA (NOEFA) matrix provides a functional model for the transportation of hydrophilic and hydrophobic APIs (Grobler *et al.*, 2008:289).

Various ways of manipulating the structural and functional characteristics of Pheroid™ have been identified in prior investigations (Grobler *et al.*, 2008:292). Pheroid™ can be manipulated by:

- ❖ a change in the fatty acid composition or concentrations;
- ❖ adding non-fatty acids or phospholipids such as cholesterol;
- ❖ adding cryo-protectants;
- ❖ adding charge-inducing agents;
- ❖ a change in the hydration medium (ionic strength, pH);
- ❖ a change in the method of preparation;
- ❖ a change in the character and the concentration of the active compound or
- ❖ adding sunscreen formulations.

The topical formulations based on Pheroid™ technology all contain tocopherol or tocopherol based molecules (vitamin E and vitamin E derivatives). These molecules are fat soluble and function as anti-oxidants and emulsion stabilisers. Vitamin E prevents the oxidation of unsaturated fatty acyl residues of membrane lipids. It scavenges lipid peroxy radicals and becomes oxidised which breaks the oxidation chain. Another function of vitamin E is stabilising the membrane by forming complexes with the products of hydrolysis of lipid membranes (Grobler *et al.*, 2008:293). Vitamin E is said to treat some skin conditions and, to have sun-

protection effects and anti-ageing effects (healthy-skincare.com, 2011). These effects of vitamin E have not been proven but will prove to be very useful if found to be scientifically valid.

2.5.4.3 Functional characteristics of Pheroid™

The efficacy of delivery of a product to the skin is controlled by the manufacturer/designer and by the physiological and biochemical processes of the skin and target cells. The manufacturer/designer must have thorough knowledge of the above-mentioned process so as to optimise the drug delivery process. The contribution of the carrier or delivery vehicle must be investigated (Grobler *et al.*, 2008:293).

2.5.4.3.1 Pliable system design and versatility

This delivery system is very versatile. It increases the efficacy of a variety of compounds, can be used in various routes of administration and can be prepared in different dosage forms. The N₂O gas and the pegylated tails added to the fatty acids render the system more pliable. Pheroid™ are thus extremely elastic and do not shatter under moderate pressure or extravasation (Grobler *et al.*, 2008:294).

2.5.4.3.2 Entrapment efficiency

The entrapment efficiency (EE) of Pheroid™-based products is calculated as a percentage of the amount of entrapped compound over the initial amount of compound added to the formulation. The aim is for all products in development to have an EE of at least 90%. More than one molecule of active compound can be entrapped within one Pheroid™ vesicle; the number of molecules per Pheroid™ vesicle depends on the size, charge and solubility of the active compound (Grobler *et al.*, 2008:294).

2.5.4.3.3 Penetration efficiency

Penetration is in part determined by the physico-chemical properties of the API substance as described in Section 2.4.6.2. Comparative studies between Pheroid™ entrapped products and comparable commercial products have shown that Pheroid™ entrapment enhances the skin penetration of APIs e.g. acyclovir and miconazole (Grobler *et al.*, 2008:296).

2.5.4.3.4 Uptake of Pheroid™ and entrapped compounds by cells

The uptake of Pheroid™ by cells is possibly influenced by the Pheroid™ formulation and by the mechanism of uptake by the cells (Grobler *et al.*, 2008:297). The process has not been fully elucidated but it is suspected that the uptake is actively facilitated by fatty acid membrane-binding proteins that are present within the lipid rafts in the cell membrane (Grobler *et al.*, 2008:299). Permeation of the Pheroid™ formulation is determined by the:

- ❖ size of the Pheroid™;
- ❖ morphology of the Pheroid™;
- ❖ molecular geometry of the fatty acids themselves;
- ❖ concentration and ratios of the various fatty acids;
- ❖ pH of the preparation;
- ❖ presence of charge-changing molecules;
- ❖ presence of molecules that influence the electrostatic milieu;
- ❖ character and concentration of the API and
- ❖ state of the Pheroid™ (i.e. either gel state or fluid state or in between).

2.5.4.3.5 Metabolism, targeting and distribution

The distribution of Pheroid™ can be influenced by the type and extent of fatty acid modifications. Interactions exist between the fatty acids and fatty acid binding proteins (FABPs) in the cell membrane and between the Pheroid™ and the lipid rafts in the cell membrane. Metabolism occurs in the mitochondria or in the peroxisomes of the cell and in turn results in release of the active compound (Grobler *et al.*, 2008:300).

2.5.4.4 Inherent therapeutic effect of the essential fatty acids of Pheroid™

As mentioned in Section 2.4.1.4 intercellular lipids form the only continuous phase in the stratum corneum. This continuous phase is very crucial to the barrier function of the skin. Non-polar solvents should be avoided in any cosmetic preparation because they disturb the intercellular lipids and reduce the barrier function of the skin which results in trans-epidermal water loss (TEWL) and skin dehydration. These intercellular lipids include phospholipids, free sterols, cholesterol sulphate, acylceramides and acylglucoceramides (Grobler *et al.*, 2008:304).

Most EFAs are generated naturally in the body. Linoleic acid and linolenic acid are the only EFAs that must be obtained from nutrition for lipid synthesis to occur. Linolenic acid has a role in restoring the barrier function of skin and normalising skin hydration. Essential fatty acid deficiency results in dry scaly skin, ageing skin and extreme water loss from the epidermis. The barrier function of the skin is also lost (Grobler *et al.*, 2008:305).

Slightly modified EFAs are the main fatty acids used in Pheroid™. Linoleic acid and linolenic acid are the essential components of Pheroid™ and the primary constituents in all formulations. These two are present in the skin in its normal state so the immune system recognises them with no apparent immunological implications (Grobler *et al.*, 2008:305). The selected fatty acids maintain the intercellular lipid layer of the skin thus normalising the physiological

micro-environment. A resultant anti-inflammatory effect occurs together with suppression of epidermal hyper-proliferation and normalisation of the water barrier of skin (Grobler *et al.*, 2008:306).

Delivery in the skin is fast and efficient as a result of binding to the fatty acid binding proteins that are in the cell membranes. The Pheroid™ delivery system is one of the most effective, versatile, inexpensive and safe delivery systems used commercially to date (Grobler *et al.*, 2008:308).

2.6 *In vitro* drug efficacy testing

Pre-clinical screening of chemicals for specific and non-specific cytotoxicity against many types of cells is essential in all drug development programmes. The same is applicable to the screening of anti-cancer APIs in dosage forms. Cell culture and animal studies are critical in determining the efficacy, pharmacodynamics, and mechanism of action of novel anti-cancer APIs prior to human clinical trials (HogenEsch & Nikitin, 2012?). Animal models play an important role in indicating the potential therapeutic target and safety evaluation but there is increasing pressure for a more comprehensive adoption of *in vitro* testing in safety and efficacy evaluations. The shift from animal models to *in vitro* cell culture is driven by (Wilson, 2000:175):

- ❖ the financial implications – *in vitro* tests have considerable economic advantages over *in vivo* tests;
- ❖ an increase in the awareness of the limitations of animal models in relation to human metabolism and
- ❖ the ethical concerns with respect to animal experimentation.

Animal experimentation has its advantages so, it will continue to be used as long as it can be scientifically justified and the quantity of animals plus the amount of pain and distress the animals go through are kept to a minimum (Ninomiya & Inomata, 1998:220). Human tumour cell lines or primary tumour fragments can be injected or implanted into immunodeficient mice and these are called xenograft models (HogenEsch & Nikitin, 2012?). Subcutaneous implantation of primary human tumour fragments results in xenografts that retain the DNA methylation pattern of the original tumour and may more faithfully mimic the response of human tumours to anti-cancer drugs. The disadvantage of xenografts lies in that the mice lack a functional immune system and the tumour does not get to develop a normal relationship with the microenvironment. In light of this, *in vivo* xenograft models pose a limited advantage over *in vitro* cell culture models. An alternative to cell culture and xenograft models is the induction of cancer in mice using chemical, irradiation, hormonal or genetic approaches. The mouse models of cancer are advantageous because they can recapitulate human cancer more closely but the

time involved developing the tumours, the variability in time, number of tumours and progression to malignancy may limit the use of the mouse models (HogenEsch & Nikitin, 2012?).

Human tumour cell lines allow *in vitro* investigation of test compounds under highly controlled and reproducible conditions but the culture conditions do not mimic the complexity of tumours in patients. *In vitro* tests also have limitations because tumours are composed of cells with a high degree of genetic instability and phenotypic variability. In addition to this the host environment is genetically diverse and the environmental stimuli also differ so tumours behave and evolve differently in different patients. The ability to model these sources of variation *in vitro* will go a long way in the cell culture testing of the efficacy of anti-cancer APIs (HogenEsch & Nikitin, 2012?).

The cell culture model was utilised in this study to determine the efficacy of 5-fluorouracil against human melanoma cells *in vitro*. The human melanoma cell line (A375) that was used in this study has been utilised in culture with arbutin (Nawarak *et al.*, 2009:159), *Polygonatum cyrtonema* lectin (Liu *et al.*, 2009:54), cyclooxygenase inhibitors (Chiu *et al.*, 2005:S293), cerulenin and C75 (Ho *et al.*, 2007:578) test substances. Apoptosis is the main end-point that has been investigated with the A375 cells because the unrestrained division of cancer cells is linked to insufficient apoptosis so an increase in apoptosis should reflect anti-tumour activity.

The induction of apoptosis by 5-fluorouracil has been successfully tested both *in vivo* (Eichhorst *et al.*, 2001:243) and *in vitro* on different types of human cancer cells including, but not limited to, melanoma cells (Tsuji & Karasek, 1986:474), colon cancer cells (Tokunaga *et al.*, 2000:1998; Wiebke *et al.*, 2003:63) and oral cancer cells (Tong *et al.*, 2000:237). The ultimate goals of cell culture models in cancer chemotherapy are to (Wilson, 2000:175):

- ❖ identify potentially active compounds;
- ❖ identify the mechanism of action of cytotoxic agents;
- ❖ predict anti-cancer activity;
- ❖ identify potential target cell populations;
- ❖ identify the toxic or effective concentration ranges and
- ❖ determine the relationship between concentration and exposure time.

A combination of *in vitro* and *in vivo* techniques, however, is usually used for the preclinical testing of anti-cancer drugs because there is no method that can single-handedly predict the efficacy in humans (HogenEsch & Nikitin, 2012?). Subsequent to successful *in vitro* studies *in vivo* studies must be performed to confirm the *in vitro* results.

2.7 Prior studies on transdermal and topical delivery of 5-fluorouracil

Various studies have been carried out in the past on the delivery of 5-fluorouracil via the skin. There is a series of studies on 5-fluorouracil that have been done at the North-West University. In 2003, Steenekamp (2003:63) did a research on the delivery of 5-fluorouracil in the presence of terpenes. In this study it was revealed that 5-fluorouracil flux through the skin is enhanced in the presence of menthol, isomenthol and 1,8-cineole. The transdermal delivery of 5-fluorouracil was further investigated by Killian (2004:78), who found that Emzaloid[®] formulation (on which Pheroid[™] technology is based) results in an increased flux of 5-fluorouracil with a possible allowance for reduction in amount of active used without a decrease in efficiency. This current research is thus based on Pheroid[™] technology as it has already been established that it is effective in enhancing 5-fluorouracil transdermal and topical delivery. The efficacy of water-based and phosphate buffer solution-based Pheroid[™] in the transdermal delivery of 5-fluorouracil was later investigated (Van Dyk, 2008:61). This study confirmed the findings by Killian (2004:78) and revealed that a 0.5% solution can be used instead of a 1.0% solution because there are no significant differences between their transdermal permeation. The transdermal delivery of 0.5% 5-fluorouracil incorporated into various topical formulations was investigated by Vermaas (2010:174). After incorporation into emulgels, creams and lotions with and without Pheroid[™] it was found that the presence of Pheroid[™] did not necessarily increase the diffusion of 5-fluorouracil in topical dosage form into or through the skin. A comparison of 5-fluorouracil skin diffusion from 5.0% (market product) and the 0.5% topical formulations revealed that the 5.0% 5-fluorouracil market product did not necessarily result in a higher concentration of 5-fluorouracil diffusing into or through the skin (Vermaas, 2010:79). The above-mentioned studies were all done at the North-West University, Potchefstroom, South Africa and the current study intended to build on this knowledge. In the current study, 5-fluorouracil was incorporated into a topical dosage form of choice (with and without Pheroid[™]) at different concentration levels and skin diffusion studies were performed. The results obtained were then compared with the results from prior studies on 5-fluorouracil.

A clinical study was conducted by Levy *et al.* (2001:918) which led them to believe that a 0.5% 5-fluorouracil formulation would lead to less systemic exposure and thus less systemic side effects. With respect to topical side effects they stated that patients exposed to 5.0% 5-fluorouracil experienced facial irritation whereas the patients exposed to the 0.5% formulation had limited facial irritation. Kaur *et al.* (2010:268) state that information from the above-mentioned study should be carefully evaluated because the patients exposed to the 5.0% 5-fluorouracil had a twice daily regimen while the patients exposed to 0.5% 5-fluorouracil had a once daily regimen. The frequency of exposure probably plays a role in the tolerability of the formulation. The findings by Levy *et al.* (2010:918) do not take into consideration the effects of frequency of exposure yet it is probable that increased exposure may exacerbate the side

effects. Regardless, it is apparent from the study by Levy *et al.* (2001:918) that the once daily 0.5% 5-fluorouracil regimen also clears actinic keratoses though to a lesser extent than the 5.0% regimen.

Taking into consideration past findings, this current study is focusing on a range of concentrations for formulations of 5-fluorouracil. The ultimate intention is to prepare semi-solid formulations for topical delivery with low 5-fluorouracil concentrations as there are advantages related to a lower API concentration. It is possible that the use of Pheroid™ in the current study may enhance the action of 5-fluorouracil by increasing the API concentration that reaches the target site. Further research is still being carried out to build on the existing knowledge on topical delivery of 5-fluorouracil. There are some blind spots that still need to be uncovered so as to maximise the delivery of 5-fluorouracil while reducing the costs and side effects.

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CHAPTER THREE

ARTICLE FOR PUBLICATION IN JOURNAL OF PHARMACEUTICAL SCIENCES

This dissertation is written in the article format so Chapter 3 is the article that has been written for publication in the Journal of Pharmaceutical Sciences. The guidelines for authors' are included in the dissertation as Appendix E. The writing style that was used in Chapter 3 is in accordance with the authors' guidelines and therefore written in concise US English. However, for ease of reading, the stipulated word limit of 5 500 words has not been adhered to in the dissertation for examination purposes.

TOPICAL DELIVERY OF 5-FLUOROURACIL AND ITS *IN VITRO* EFFICACY AGAINST HUMAN MELANOMA

Tawona N. Chinembiri, Minja Gerber, Lissinda du Plessis, Jan L. du Preez and Jeanetta du Plessis*.

Unit for Drug Research and Development, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa.

* Corresponding author. Tel.: +2718 299 2274; Fax: +2787 231 5432. E-mail address: Jeanetta.duPlessis@nwu.ac.za

ABSTRACT

Skin cancer is the most widely diagnosed form of cancer in Caucasians worldwide and its first line of treatment is surgical excision, which may not be feasible in very large or facial lesions. There are few viable alternatives to surgical excision in the treatment of skin cancer. The aim of this study was to make headway in the development of topical alternative treatments with 5-fluorouracil as the active pharmaceutical ingredient. Lotions with different concentrations of 5-fluorouracil were formulated then used in Franz cell skin diffusion studies and tape-stripping. Pheroid™ technology was utilized in order to enhance 5-fluorouracil penetration through the skin. The *in vitro* effect of 5-fluorouracil against human melanoma cells (A375) was investigated using a flow cytometric apoptosis assay. Relatively high concentrations of 5-fluorouracil diffused into and through the skin with Pheroid™ formulations resulting in an enhanced *in vitro* skin permeation of 5-fluorouracil. Subsequent to the apoptosis assay it was found that 5-fluorouracil induced apoptosis in A375 cells after a 24 hour incubation period. The results obtained also suggested that the Pheroid™ drug delivery system somehow enhances the apoptosis inducing effect of 5-fluorouracil. Therefore, using Pheroid™ could possibly be advantageous with respect to both drug delivery and efficacy.

Keywords: 5-Fluorouracil; Permeation enhancer; Pheroid™; Percutaneous; Cancer; Transdermal drug delivery; Topical; Cell culture; A375 cells

INTRODUCTION

There are two categories of skin cancer which are melanoma and non-melanoma skin cancer (NMSC). Non-melanoma skin cancer is the most frequently diagnosed form of cancer but it has the lower mortality rate while melanoma though infrequently diagnosed has a high mortality rate.¹ Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the NMSCs and they originate from epidermal keratinocytes.² Cutaneous malignant melanoma (CMM) originates from melanoma cells, which are also found in the epidermis.¹ Actinic keratoses and Bowen's disease though not true invasive carcinomas are usually included in discussions about skin cancer because of their relationship with skin cancers.³ Early detection of skin cancer is advocated for because neglected NMSCs may grow invasively and SCC may metastasise.¹ Prompt detection will lead to early treatment and in turn improved prognoses.³ Currently, surgical treatment is the main recommendation for the treatment of skin cancer, however, cases do exist where surgery is not feasible and an alternative method must be used. Seeing as NMSC and CMM occur within the skin it falls within reason that effective topical medicaments can be viable alternatives.

Cancer is characterized by evasion of apoptosis or defective apoptosis mechanisms which in turn allow uncontrollable development of cells.⁴ Chemotherapy of cancer is thus aimed at restoring the apoptosis balance or eliminating the rapidly dividing cells.⁵ 5-Fluorouracil is a pyrimidine antimetabolite which acts by inhibiting thymidylate synthase (TS). Thymidylate synthase is essential for the replication and repair of deoxyribonucleic acid (DNA) and its inhibition causes the induction of apoptosis and cell death.⁶ The unrestrained division of cancer cells is linked to insufficient apoptosis so an increase in apoptosis should reflect anti-tumor activity.

Tsuji and Karasek⁷ investigated the effect of 5-fluorouracil on human melanocytes and malignant melanoma cells, where they discovered that concentrations of 1.92×10^{-5} - 3.84×10^{-4} M (2.50 - 49.95 μ g/ml) resulted in cell death after five weeks. This concentration range was used as a guideline. To determine the concentrations of 5-fluorouracil that result in the required response within 24 h *in vitro* three concentrations within the lower end of the range by Tsuji and Karasek and one concentration below the minimum concentration were used. Concentrations of 1.67; 3.33; 6.67 and 13.3 μ g/ml 5-fluorouracil were deemed as appropriate. The drug efficacy tests were conducted utilizing the different active pharmaceutical ingredient (API) concentrations in solution, non-Pheroid™ and Pheroid™ lotions in order to also determine variations due to the differing drug delivery systems.

In order to target tumorigenic cells within the skin and minimize systemic adverse effects, 5-fluorouracil must penetrate into the skin layers and exert its effects within the skin. The available routes for administration of 5-fluorouracil are the intravenous and topical routes,⁶ in

this study the focus was on the topical route of administration. Formulation of topical treatment modalities is challenging due to the relatively impermeable nature and barrier properties of the skin.⁸ The outer-most layer of the skin (the stratum corneum) is lipophilic so it hinders the permeation of hydrophilic drugs such as 5-fluorouracil through the skin. The physico-chemical properties of an API play a huge role in determining whether or not it will diffuse into and through the skin. According to Naik *et al.*⁹ in order to allow for skin permeation an API must have an aqueous solubility of at least 1 mg/ml; a molecular weight below 500 Da; a melting point below 200 °C and a partition coefficient (Log P) between 1 and 3.⁹ The log D (at 22 °C; pH 7.4 – acetate buffer) value of 5-fluorouracil (-0.98)¹⁰ does not lie within the required range and neither does the melting point of 282 °C.¹¹ It is not known which physico-chemical properties have a greater influence on skin diffusion so it is not possible to fully predict skin diffusion by the use of a few physico-chemical properties. However, in light of the literature on hand it was tentatively predicted that 5-fluorouracil would not easily penetrate the skin barrier. A novel and patented form of technology that is used in the delivery of pharmaceutical actives, the Pheroid™ drug delivery system,¹² was used to aid the delivery of 5-fluorouracil into the skin. Pheroid™ is composed of stable lipid-based submicron and micron-sized structures that form a unique colloidal system in a dispersion medium.¹² The entrapment of APIs in Pheroid™ improves the control of size, charge and the hydrophilic-lipophilic characteristics of APIs by a large margin thus altering their ability to permeate through membranes. The effect of Pheroid™ on topical and transdermal drug delivery has been investigated in the past by Vermaas *et al.*¹³ and this study served to corroborate the past findings.

The study partly aimed to determine the topical and transdermal delivery of 5-fluorouracil, investigate the influence of Pheroid™ technology and the influence of a change in API concentration on the delivery of the drug into and through the skin. Skin diffusion studies were conducted with Pheroid™ and non-Pheroid™ formulations at four different concentrations of 5-fluorouracil. The drug efficacy tests aimed to determine the *in vitro* efficacy of 5-fluorouracil on human melanoma cells and to investigate the influence of drug delivery vehicles on the API efficacy. Cells were subjected to various concentrations of 5-fluorouracil in three different vehicles (lotion, Pheroid™ lotion and phosphate buffered saline (PBS) 0.0067M) and analyzed for occurrence of apoptosis using flow cytometry. Therefore the effects of concentration and Pheroid™ technology on topical and transdermal drug delivery and on the *in vitro* efficacy of 5-fluorouracil were investigated.

MATERIALS AND METHODS

Materials

The API, 5-fluorouracil, was obtained from DB Fine Chemicals (Johannesburg, South Africa). Cetyl alcohol (Merck Chemicals, Johannesburg, South Africa), liquid paraffin (Merck Chemicals, Johannesburg, South Africa), span 60 (Merck Chemicals, Johannesburg, South Africa), tween 80 (Merck Chemicals, Johannesburg, South Africa), 96% ethanol (Rochelle Chemicals, Johannesburg, South Africa) and *dl*- α -tocopherol (Chempure, Pretoria, South Africa) are the excipients that were used during formulation. The potassium dihydrogen orthophosphate (KH_2PO_4) and the sodium hydroxide (NaOH) that were used in the preparation of phosphate buffer solution, pH 7.4, were both purchased from Merck Chemicals (Johannesburg, South Africa). Deionized HPLC-grade water was used throughout the whole study.

The human melanoma cells (A375) for cell culture were obtained from American Type Culture Collection (Manassas, USA). The culture media for cell cultivation consisted of Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and Pen-Strep, which were all obtained from Sigma Aldrich, Germany. Other materials that were used for the cell cultivation and drug efficacy tests are trypsin-EDTA 25% (Gibco, USA), PBS 0.0067M (Thermo-Scientific, USA), dimethylsulphoxide (DMSO) obtained from Merck, Germany and 96% ethanol from Merck, South Africa. The Single Channel Annexin V/ Dead Cell Apoptosis kit with Alexa Fluor[®] 488 annexin V and SYTOX[®] Green for Flow Cytometry (Invitrogen, USA) was used for the assay to determine the efficacy of 5-fluorouracil.

Methods

Caution: 5-Fluorouracil is a hazardous cytotoxic drug and it should be handled carefully – avoid direct contact with skin or mucocutaneous zones.

Formulation of semi-solid dosage forms

Lotions were formulated with and without Pheroid[™], containing 5-fluorouracil concentrations of 0.5; 1.0; 2.0 and 4.0% w/w. Eight formulations were thus used in the *in vitro* drug efficacy tests and skin diffusion studies.

Cell cultivation

A cell culture model was utilized in this study to determine the efficacy of 5-fluorouracil in inducing apoptosis in human melanoma cells (A375) *in vitro*. The A375 cells were cultivated in 75 cm² flasks; the culture media consisted of 10% FBS and 1% Pen-Strep in DMEM. The cells were fed with fresh media everyday and split when ~80% confluency was reached.¹⁴

Determination of cell viability

Cell viability was determined with a Countess automated cell counter (hemocytometer). A uniform cell suspension (10 μ l) was mixed with 10 μ l of 0.4% trypan blue.¹⁵ The cells were left for ~3 min for the cells to stain and loaded onto a chamber of a Countess slide for examination. The total number of cells, viable cells, dead cells and the percentage viability was then determined.

Optimization of lotion concentration

The lotion and Pheroid™ lotion both had oil components among other things which could have been potentially harmful to the cells and in turn resulted in cell death (the terms lotion and non-Pheroid™ lotion have been used interchangeably in the text). It was decided to dilute the placebo lotion and the placebo Pheroid™ lotion (0.1; 1.0; 12.5; 25.0; 50.0 and 100%) with PBS and note the effects of the different placebo dilutions on cell viability. An appropriately selected dilution of the lotions together with API was utilized in the assays to rule out cell death due to the effects of the lotion components and not the API.

On the first day 2 ml each of a 1×10^5 cells/ml cell suspension were seeded into 48 wells of 6-well multiwell culture plates. After a 24 h incubation period (37 °C and 5.0% CO₂) 1 ml of a test solution or control (PBS, culture media, ethanol or 0.5% 5-fluorouracil solution) was added to three wells each. On the third day the cells were detached, the trypan blue test was performed and cell viability was determined using a hemocytometer.

Analytical assay

A cell suspension (500 μ l) containing 1×10^5 cells/ml was seeded into 48 wells of 24-well multiwell culture plates. The cells were incubated for 24 h at a temperature of 37 °C and in an atmosphere of 5.0% CO₂. Formulated lotions were diluted by 1000 (dilution factor was determined by the optimization) to give final concentrations of 5.0; 10.0; 20.0 and 40.0 μ g/ml 5-fluorouracil. Each lotion dilution (250 μ l) was added to 3 wells each and the cells incubated for another 24 h. The final API concentrations in the wells became 1.67; 3.33; 6.67 and 13.30 μ g/ml 5-fluorouracil due to the dilution rendered by the 500 μ l of cell suspension already present. The Single Channel Annexin V/ Dead Cell Apoptosis assay with Alexa Fluor® 488 annexin V and SYTOX® Green for Flow Cytometry (Invitrogen, USA) was performed according to the instructions provided for the kit.

High performance liquid chromatography analytical method

An Agilent® 1100 series (Agilent Technologies, Palo Alto, CA) which consists of a quaternary pump, a degasser, an auto-sampler injection mechanism and a diode array detector was used with a Phenomenex® Synergi Fusion Reversed Phase column (4.6 mm x 250.0 mm), with a 4 µm particle size (Phenomenex®, Torrance, CA). The mobile phase (4% acetonitrile) consisted of 40 ml acetonitrile, 1 ml orthophosphoric acid and HPLC-grade water to make it 1000 ml. The analysis was done at a wavelength of 266 nm with a flow rate of 1 ml/min and an injection volume of 50 µl. The API eluted at approximately 4.6 min so the stop time was set to 6 min. Data acquisition and analysis was done with Chemstation Rev. A.10.02 software.

Preparation of phosphate buffer solution at pH 7.4

The phosphate buffer solution was prepared by weighing 3.15 g and 13.62 g of NaOH and KH₂PO₄ respectively. The NaOH (3.147 g) was diluted to 786.8 ml with HPLC-grade water and the KH₂PO₄ was diluted to 500 ml with HPLC-grade water. The two solutions were mixed together to make the phosphate buffer solution and the pH was measured with a Mettler Toledo pH meter (Switzerland). The pH was adjusted to 7.4 with either 10% NaOH or 10% orthophosphoric acid. This buffer was used in the aqueous solubility determination, log D determination, standard preparations and as the receptor phase during the Franz cell diffusion studies.

Aqueous solubility determination

An excess of 5-fluorouracil was added to 5 ml of phosphate buffer solution (pH 7.4) in order to make a super-saturated solution. The super-saturated solution was constantly stirred by a magnetic stirrer and maintained at 32 °C (temperature at the surface of the skin) using a JB Grant® constant temperature water-bath.¹⁶ After 24 h the supersaturated sample was filtered, diluted and analyzed in duplicate by HPLC. The experiment was conducted in triplicate.

Octanol-buffer distribution coefficient (log D) determination

Pre-saturated *n*-octanol and buffer were prepared by mixing equal volumes of phosphate buffer solution (pH 7.4) and *n*-octanol. The mixture was stirred vigorously and left to equilibrate for 24 h. The two layers were separated and 5 mg of 5-fluorouracil were dissolved in a tube with 5 ml of pre-saturated *n*-octanol. Pre-saturated buffer (5 ml) was then added to the 5-fluorouracil in *n*-octanol. The tube was placed in a temperature controlled (32 °C) auto-rotator for 24 h. The solution was centrifuged for 15 min at 5000 rpm with an Eppendorf Centrifuge 5804 R. The whole experiment was done in triplicate. Aqueous phases were then extracted from each tube and analyzed in duplicate using HPLC. The log D value was calculated as the logarithmic ratio

of the concentration of 5-fluorouracil in the *n*-octanol phase relative to the concentration in the buffer phase.

Preparation of skin for the skin diffusion experiments

The *in vitro* skin diffusion studies made use of female abdominal human skin obtained from Caucasian cosmetic abdominoplasty surgery patients. Ethical approval for the obtaining, preparing and using of human skin in the research was obtained from the Research Ethics Committee of the North-West University (Ethical approval number - NWU-00114-11-A5). Permission was obtained from the surgeons and informed consent from the participating anonymous patients, allowing the use of the excised skin in the research. The excised skin was prepared and then frozen within 24 h of the surgery. The skin samples were prepared using an electric Zimmer[®] dermatome (Zimmer, Ohio, USA) at a thickness of 400 µm. This thickness included the epidermis and part of the dermis. The dermatomed skin was placed on top of Whatman[®] filter paper and circles with a diameter of approximately 15 mm were punched into the skin. It was ensured that each circle of skin on the filter paper was big enough to cover the diffusion area. The skin circles were wrapped in foil, placed in a Ziploc[®] bag and then frozen at -20 °C until needed.

Franz cell diffusion studies

Vertical Franz cells were used for the diffusion studies. The Franz cells had a receptor volume capacity of approximately 2 ml and a diffusion area of 1.075 cm². Twelve Franz cells were used for each experiment, ten with the test formulation and two control cells. The buffer (pH 7.4) was pre-warmed to 37 °C (physiological temperature) in a water bath an hour before the experiment commenced. The donor phase formulation was placed in a 32 °C (temperature at the skin surface)¹⁶ water bath for at least an hour. The donor and receptor compartments of a Franz cell were greased with Dow Corning[®] vacuum grease and a magnetic stirring rod was placed into the receptor compartment. A circle of skin (stratum corneum facing up towards the donor compartment) or a polytetrafluoroethylene (PTFE) membrane was placed on to the lower half (receptor) of the Franz cell. The PTFE membrane filters were used in the drug release studies as the aim was to merely determine whether 5-fluorouracil was released from the formulations. Skin samples were used in the skin diffusion studies as the aim was now to determine the diffusion of 5-fluorouracil into and through the skin. The two compartments of the Franz cell were placed together, sealed with vacuum grease to prevent leakage and secured together with a horseshoe clamp. Phosphate buffer solution (2 ml), pH 7.4, was added to the receptor compartment and 1 ml of the semi-solid formulation under investigation was placed into the donor phase. The donor compartment was covered to avoid the loss of constituents by evaporation. The assembled Franz cells were placed on a Franz cell stand and placed in a water bath with a Variomag[®] magnetic stirrer (mixed receptor phase contents to maintain

homogeneity) ensuring that only the receptor compartment of the Franz cell was immersed. This was recorded as time 0.0 h. The entire receptor buffer phase was removed at predetermined time intervals and replaced with fresh buffer to maintain sink conditions. The extracted samples were immediately analyzed by HPLC.

In vitro drug release studies were conducted with 0.45 µm PTFE membrane filters to determine if 5-fluorouracil was released from the formulations. The receptor phase was extracted every hour for six hours, giving extraction times of 1.0; 2.0; 3.0; 4.0; 5.0 and 6.0 h. Twelve hour skin diffusion studies were done and the amount of 5-fluorouracil that diffused through the skin *in vitro* was determined. It was observed during a pilot study that very low amounts of 5-fluorouracil permeated through the skin for the greater duration of the experiments, leading to problems with accurate detection and quantification. So, it was decided to do a single extraction after 12 h, thus analyzing only the total amount of 5-fluorouracil that diffused through the skin. Tape-stripping was performed after the 12 h diffusion.

Tape-stripping procedure

The tape-stripping technique is useful in dermatopharmacological research for selectively removing the skin's outermost layer, the stratum corneum.¹⁷ This study focused on topical drug delivery so tape-stripping was performed in order to determine the amount of drug that was retained in the layers of the skin. The tape-stripping technique as described by Pellet *et al.*¹⁷ was followed but phosphate buffer solution, pH 7.4, was used as the solvent and the samples in buffer were stored overnight at 4 °C. This procedure was repeated for each Franz cell. On the following day the tape-strip samples (represented the stratum corneum-epidermis) and skin samples (represented the epidermis-dermis) in buffer were filtered then analyzed by HPLC. Hereinafter the stratum corneum-epidermis is referred to as the epidermis and the epidermis-dermis is referred to as the dermis.

Statistical analysis

The Shapiro-Wilk test and quantile-quantile (q-q) plots were used to determine the normality of the data. Statistical analysis was done on the results of the drug efficacy test but the results may be unreliable because there were only three observations per group. Descriptive statistics for each group were obtained; these include the mean for normal data, median for non-normal data and standard deviation. It was decided that the data had a sufficiently normal distribution for the performance of robust tests so omnibus tests were performed which included the one-way analysis of variance (ANOVA), the Welch test and the Brown-Forsythe test. To uncover the individual differences the Games-Howell for unequal variances and the Tukey's B post-hoc tests were conducted. In order to determine the practical significance of the differences between groups the effect size (Cohen's d-value) between each treatment was calculated. An absolute

value, $|d|$, is calculated to represent practical significance. If d is bigger than 0.2 but less than 0.5 the difference is not practically significant ($0.2 < |d| < 0.5$), if d is bigger than 0.5 but less than 0.8 there is a practically visible effect or difference in the mean ($0.5 < |d| < 0.8$) and if d is bigger than 0.8 ($|d| > 0.8$) there is a practical significance in the difference of the mean. The same statistical analysis methods were used to analyze the skin diffusion and tape-stripping data with the exception of the Tukey's B post hoc test.

RESULTS AND DISCUSSION

Optimization of lotion concentration

The concentrated dilutions did not mix wells with the cell suspensions and gave either erratic results or percentage viability values within the range of the ethanol positive control. It was found that the 0.1% dilutions of the placebo lotion and placebo Pheroid™ lotion treatments resulted in 94.3% and 95.0% cell viability, respectively. This was acceptable because they were in close range of the cell viability due to treatment with the positive controls (PBS and complete culture media) which meant that they were essentially harmless. Therefore the 0.1% dilutions together with different API concentrations were utilized in the apoptosis assay. The results are tabulated in Table 1.

Table 1: Viability of cells after treatment with dilutions of the placebo Pheroid™ and non-Pheroid™ lotions for the purpose of optimization

Drug efficacy testing

The geometric mean fluorescence intensity (MFI) was utilized for the data analysis. The MFI for each treatment was averaged and expressed as a percentage of the positive control (ethanol) MFI. Line graphs of 5-fluororacil concentration versus %MFI were drawn for each of the drug delivery vehicles (lotion, Pheroid™ lotion and API solution) and they are shown in Figure 1.

Early signs of apoptosis were evident after the 24 h incubation period with the treatments. The highest %MFI was detected in the cells treated with the 13.30 $\mu\text{g/ml}$ Pheroid™ lotion (5.36%), followed by 6.67 $\mu\text{g/ml}$ Pheroid™ lotion (2.77%), 13.30 $\mu\text{g/ml}$ lotion (1.91%), 13.30 $\mu\text{g/ml}$ solution (1.66%), 6.67 $\mu\text{g/ml}$ lotion (1.33%), 3.33 $\mu\text{g/ml}$ lotion (1.04%), 1.67 $\mu\text{g/ml}$ Pheroid™ lotion (0.91%), 6.67 $\mu\text{g/ml}$ solution (0.74%), 1.67 $\mu\text{g/ml}$ lotion (0.71%), 3.33 $\mu\text{g/ml}$ Pheroid™ lotion (0.70%), 1.67 $\mu\text{g/ml}$ solution (0.65%) and 3.33 $\mu\text{g/ml}$ solution (0.63%) in that order.

All the Pheroid™ lotion treatments except the 3.33 $\mu\text{g/ml}$ treatment resulted in higher %MFI values than the corresponding lotion and solution treatments. This may be due to the fact that Pheroid™ are composed of fatty acids and therefore an affinity exists between Pheroid™ and cell membranes, resulting in more effective and fast uptake of Pheroid™ by cells.¹⁹ The

solution treatments produced the lowest %MFI in the cells in comparison to the lotion and Pheroid™ lotion treatments at the same concentrations. The %MFI produced by the lotion treated cells increased with increase in concentration in a relatively linear manner. The Pheroid™ lotion treatments resulted in a lower %MFI in the 3.33 µg/ml treatment than in the 1.67 µg/ml. From the 3.33 µg/ml treatment to the 13.30 µg/ml treatment of the Pheroid™ lotion the %MFI increased with concentration. A trend similar to that of the Pheroid™ lotions was observed with the API solutions. The effects of Pheroid™ and of increasing the concentration became clearer at the higher concentrations than at the lower concentrations.

Figure 1: Line graph showing comparisons between the %MFI induced in the A375 cells treated with 1) the lotion; 2) the API solution and 3) Pheroid™ lotion

According to the ANOVA and Welch tests there was a significant difference between the formulations with both tests giving p-values < 0.05. The Brown-Forsythe test, however, gave a p-value of 0.16 which meant that there were no significant differences. The Games-Howell post-hoc test was used to compare the effects of the drug delivery vehicles (lotion, Pheroid™ lotion and solution) at the same concentration. No statistical significance was determined for all the relevant comparisons. Tukeys B test identified two groups. The ethanol treatment was in one group and the rest of the treatments in another group. Since the assumptions for ANOVA were not 100% met this may have contributed to the tests giving varied conclusions.

Effect sizes were calculated to determine the practical significance of differences between treatments. Comparisons were made among treatments of the same concentrations. The effect size test identified either a practically visible difference in the mean or a practical significance in all the comparisons except the comparisons between 1) 1.67 µg/ml lotion and 1.67 µg/ml solution; 2) 3.33 µg/ml solution and 3.33 µg/ml Pheroid™ lotion and 3) 13.3 µg/ml solution and 13.3 µg/ml lotion. Generally these results show that a change in formulation but with a constant 5-fluorouracil concentration produces effects that are practically visible or significant.

The results obtained in this study confirmed that 5-fluorouracil does result in apoptotic cell death and is effective against human melanoma (A375) cells. The apoptotic effect of 5-fluorouracil seems to differ according to the vehicle that the API has been incorporated into and this needs to be researched further.

Aqueous solubility

The aqueous solubility of 5-fluorouracil was determined to be 17.6 mg/ml which is acceptable although slightly higher than the values obtained in literature.²⁰ An aqueous solubility above 1 mg/ml is ideal for optimum skin permeability⁹ so the aqueous solubility of 5-fluorouracil was ideal for skin permeation.

Octanol-buffer distribution coefficient (log D)

A log D value of -1.15 was obtained for 5-fluorouracil in this study, which is close to the values in literature.¹⁰ A log P value between 1 and 3 is required for optimum skin permeability⁹ so from these results it was tentatively predicted that 5-fluorouracil would not easily permeate through the skin.

The release of 5-fluorouracil from formulations

For ease of writing each formulation had a number designated to it for identification. The designations are as follows: **(1)** 0.5% lotion; **(2)** 0.5% Pheroid™ lotion; **(3)** 1.0% lotion; **(4)** 1.0% Pheroid™ lotion; **(5)** 2.0% lotion; **(6)** 2.0% Pheroid™ lotion; **(7)** 4.0% lotion and **(8)** 4.0% Pheroid™ lotion. These number references have been used for the drug release and skin diffusion data only.

The results from the drug release studies indicated that **(6)** released the highest % of 5-fluorouracil (13.9%), followed by **(8)** (10.2%), **(2)** (8.16%), **(4)** (7.37%), **(5)** (6.19%), **(1)** (5.66%), **(3)** (4.96%) and lastly **(7)** (4.20%) in that order. The percentage of 5-fluorouracil that was released from the formulations did not remain constant with a change in formulation and/or initial concentration. When comparisons were made between a lotion and a Pheroid™ lotion of the same concentration level it was revealed that the presence of Pheroid™ resulted in an increased average % of API release. The penetration enhancing effect of Pheroid™ vesicles has been documented by Grobler *et al.*¹² and these results concur with the results expected with use of Pheroid™. An enhanced release from formulations results in a higher amount of 5-fluorouracil available on the skin surface for skin penetration.

The average cumulative amount released per unit area from the lotions generally increased with an increase in concentration and the same is true of the Pheroid™ lotions. The overall conclusions were that 5-fluorouracil was released from all the formulations and Pheroid™ resulted in an increased release of 5-fluorouracil from the formulations.

Skin diffusion studies

The highest total amount diffused was detected from **(8)** ($57.6 \mu\text{g}/\text{cm}^2$) followed by **(2)** ($31.6 \mu\text{g}/\text{cm}^2$), **(6)** ($28.5 \mu\text{g}/\text{cm}^2$), **(4)** ($16.2 \mu\text{g}/\text{cm}^2$), **(7)** ($15.9 \mu\text{g}/\text{cm}^2$), **(3)** ($11.9 \mu\text{g}/\text{cm}^2$) **(5)** ($9.71 \mu\text{g}/\text{cm}^2$) and lastly **(1)** ($4.63 \mu\text{g}/\text{cm}^2$) in that order. The Pheroid™ lotions resulted in higher 5-fluorouracil amounts diffused than their lotion counter-parts at the same concentration. From an overall perspective all the Pheroid™ lotions had higher total amounts diffused than all the lotions. Figure 2 shows the average amounts of 5-fluorouracil that diffused through the skin after 12 h from the different formulations.

A comparison of the total amount diffused per unit area from the lotions was made. The 4.0% lotion **(7)** had the highest total amount diffused ($15.9 \mu\text{g}/\text{cm}^2$) followed by **(3)** ($11.9 \mu\text{g}/\text{cm}^2$), **(5)** ($9.71 \mu\text{g}/\text{cm}^2$) and lastly **(1)** ($4.63 \mu\text{g}/\text{cm}^2$).

A comparison of the total amount diffused per unit area from the Pheroid™ lotions was made. The 4.0% Pheroid™ lotion **(8)** resulted in the highest amount of 5-fluorouracil diffusing through the skin per unit area with $57.6 \mu\text{g}/\text{cm}^2$. This was followed by **(2)** ($31.6 \mu\text{g}/\text{cm}^2$), **(6)** ($28.5 \mu\text{g}/\text{cm}^2$) and lastly **(4)** ($16.2 \mu\text{g}/\text{cm}^2$). These results revealed that a low concentration of 5-fluorouracil in the formulation (as shown by the 0.5% Pheroid™ lotion) can result in a relatively high concentration of 5-fluorouracil diffusing through the skin.

The total amount diffused per unit area from each lotion was compared with the total amount diffused per unit area from the Pheroid™ lotion of the same concentration. This was done in order to observe the effect of Pheroid™ on the transdermal delivery of 5-fluorouracil. Figure 2 shows the total amount diffused per unit area from the lotions and Pheroid™ lotions, with the same concentrations adjacent to each other. All the Pheroid™ lotions resulted in a higher total amount diffused than the lotions without Pheroid™. This illustrates the enhancing effect of Pheroid™ on skin penetration as noted in previous studies.^{21, 22} It is possible that due to the lipophilic nature of Pheroid™ vesicles,¹² 5-fluorouracil was easily carried into the skin while encapsulated in Pheroid™. However, 5-fluorouracil is hydrophilic in nature which means that it is “water-loving”, so after reaching the dermis of the skin the API could easily diffuse into the aqueous receptor phase. Without the aid of enhancers, hydrophilic drugs such as 5-fluorouracil are less likely to diffuse into or remain in the skin because the skin is a lipophilic membrane.¹² Therefore a low percentage of 5-fluorouracil was likely to diffuse into the skin alone and in turn into the aqueous receptor phase. This may explain why the amounts diffused from the non-Pheroid™ lotions were lower than those from the corresponding Pheroid™ lotions. Vermaas¹³ performed skin diffusion studies with 0.5% 5-fluorouracil lotions (with and without Pheroid™) and found that the Pheroid™ lotion ($10.01 \mu\text{g}/\text{cm}^2$) resulted in a slightly higher amount diffused than the non-Pheroid™ lotion ($9.06 \mu\text{g}/\text{cm}^2$), which is consistent with the results found in this study. The lotions that were formulated by Vermaas had a composition similar to the lotions

that were utilized in this study with the exception of 15.0% ethanol which was used as a preservative in this study instead of the parabens. When taking into consideration the rest of the results found by Vermaas we see that the penetration enhancing effect of Pheroid™ may depend on the nature of the formulation into which Pheroid™ are incorporated.

Figure 2: Comparisons of the average amount of 5-fluorouracil (in $\mu\text{g}/\text{cm}^2$) that diffused through human skin after 12 h from each lotion with the amounts diffused from the respective Pheroid™ lotion of the same concentration (n=10)

According to the one-way ANOVA, the Welch test and the Brown-Forsythe test there was a statistical significance between the formulations for the total amount diffused per unit area, all with p values below 0.000001. In essence there were significant differences in the means of the eight formulations for the total amount diffused. The Games-Howell post-hoc test was conducted to determine where the differences between the groups lie. A statistically significant difference in the total amount diffused per unit area existed between the lotions and their Pheroid™ lotion counter-parts at three of the four concentrations (0.5%; 2.0% and 4.0%). The comparison between 1.0% lotion and 1.0% Pheroid™ lotion was not statistically significant (Figure 2).

Effect sizes were calculated in order to determine the practical significance of results obtained by the lotions versus those obtained by the Pheroid™ lotions. A practically significant difference in the total amount diffused per unit area existed between the lotions and their Pheroid™ lotion counter-parts at the four concentrations (Figure 2).

Results of the tape-stripping procedure

The results from the tape-stripping experiment have been put into Table 2. The Pheroid™ lotions all resulted in a higher average concentration of 5-fluorouracil in the epidermis and dermis than their corresponding lotions of the same concentration. The only exception was the dermis comparison at the 1.0% concentration. The overall observed effect of Pheroid™ could have been due to the lipophilicity rendered to the formulations by the presence of Pheroid™ which resulted in the vesicle entrapped 5-fluorouracil being retained in the lipophilic epidermis. Another possibility is that a higher concentration of 5-fluorouracil was able to penetrate into the skin layers from the Pheroid™ lotion than the lotion so consequentially a higher concentration of 5-fluorouracil was available to remain in the skin. The concentration of 5-fluorouracil in the lotions had an effect on the epidermal concentration of 5-fluorouracil, this is implied by the increase in epidermal concentrations that occurred with an increase in the percentage of 5-fluorouracil used in the lotions.

The one-way ANOVA, Welch and Brown Forsythe tests detected statistically significant differences between the formulations for the epidermis and dermis 5-fluorouracil concentrations with p values below 0.001. The influence of Pheroid™ on the 5-fluorouracil concentration in the epidermis was not statistically significant at all four concentrations but it was practically visible at the 0.5% and 2.0% concentrations. For the 5-fluorouracil concentrations in the dermis a statistically significant result was only obtained with the comparison of the 4.0% lotion with the 4.0% Pheroid™ lotion. Pheroid™ had a practically significant influence on the dermis values resulting from the 0.5% and 4.0% lotions (Pheroid™ vs. non-Pheroid™).

Table 2: Average concentrations of 5-fluorouracil that remained in the epidermis and dermis after the 12 hour diffusion studies (n=10)

CONCLUSION AND FUTURE RECOMMENDATIONS

The results obtained showed that 5-fluorouracil (1.67 – 13.33 µg/ml) can induce apoptosis in human melanoma (A375) cells *in vitro*. The optimum concentration for apoptosis induction, however, has not been determined. The results obtained suggest the enhancement of the *in vitro* effect of 5-fluorouracil by Pheroid™. Further research shall need to be done with a wider variation of 5-fluorouracil concentrations to confirm and characterize the effect of Pheroid™ on *in vitro* apoptosis induction by 5-fluorouracil. A combination of different measurements and techniques such as, light microscopy, fluorescence microscopy, enzyme-linked immunosorbent assays (ELISA) and colorimetric assays, must be used in future studies because no single parameter defines apoptosis in all systems. The drug delivery vehicle also seems to have an effect on the efficacy of 5-fluorouracil.

An ideal topical anticancer formulation would result in release of the API from the formulation, penetration of the API into the skin, diffusion of the API within the skin and its retainment in the skin layers. In this study the formulation which showed such optimal characteristics was the 2.0% lotion because a relatively low amount of 5-fluorouracil diffused through the skin per unit area (9.71 µg/cm²), but relatively high concentrations of 5-fluorouracil were detected in the epidermis (1.56 µg/ml) and dermis (1.58 µg/ml). More formulations with such characteristics must be made for topical use.

The results obtained in this study show that 5-fluorouracil was able to diffuse through the skin regardless of the presence or absence of Pheroid™, although Pheroid™ enhanced the effect. The use of lower concentrations may result in an improved side-effect profile after application of 5-fluorouracil to the skin. Non-cutaneous cancers can be targeted with 5-fluorouracil via the transdermal route using these formulations instead of the intravenous route. Pheroid™ resulted in increased epidermal and dermal 5-fluorouracil concentrations with the exception of the dermal concentrations resulting from the 1.0% 5-fluorouracil formulations. However, Pheroid™

did not seem to have any effect on the distribution of the API between the epidermis and dermis.

The lotions prepared and used in this study all had 5-fluorouracil concentrations that are below the 5.0% 5-fluorouracil market product concentration. According to Vermaas,¹³ the total API diffused after 12 h from the commercial product was 2.52 $\mu\text{g}/\text{cm}^2$, the concentration in the epidermis was 0.12 $\mu\text{g}/\text{ml}$ and the concentration in the dermis was 0.05 $\mu\text{g}/\text{ml}$. The results that were obtained from the commercial product are lower than all the results that were obtained in this study at the different concentrations. It can be tentatively predicted that clinical use of any one of the formulations from this study will result in a clinical effect similar to or better than the clinical effect of the commercial product.

The penetration enhancing effect of Pheroid™ on 5-fluorouracil and the effect of concentration on topical and transdermal delivery need to be investigated further to confirm and build on the findings from studies done thus far. The combination of these results could result in a Pheroid™ formulation that delivers the optimum amount of drug to the site of action with reduced risk of side effects due to the convenient enhancing effects of Pheroid™.

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Figure legend

Figure 1: Line graph showing comparisons between the %MFI induced in the A375 cells treated with 1) the lotion; 2) the API solution and 3) Pheroid™ lotion (n=3)

Figure 2: Comparisons of the average amount of 5-fluorouracil (in $\mu\text{g}/\text{cm}^2$) that diffused through human skin after 12 h from each lotion with the amounts diffused from the respective Pheroid™ lotion of the same concentration (n=10)

Tables

Table 1: Viability of cells after treatment with dilutions of the placebo Pheroid™ and non-Pheroid™ lotions for the purpose of optimization (n=3)

TREATMENT TYPE	MEAN %VIABILITY ±SD*
PBS	93.3 ± 5.5
Complete culture media	93.7 ± 2.5
0.5% 5-fluorouracil solution	19.3 ± 4.0
Ethanol control	30.7 ± 5.5
1.0% dilution of placebo lotion	89.3 ± 4.0
1.0% dilution of placebo Pheroid™ lotion	53.3 ± 17.7
0.1% dilution of placebo lotion	94.3 ± 1.5
0.1% dilution of placebo Pheroid™ lotion	95.0 ± 1.0

*SD – Standard deviation

Table 2: Average concentrations of 5-fluorouracil that remained in the epidermis and dermis 12 h after application of the selected formulations (n=10)

Formulation	Average concentration in epidermis (µg/ml)	Average concentration in dermis (µg/ml)
0.5% lotion (1)	0.49 ± 0.10 ^{•#}	0.99 ± 0.36 ^{•#}
0.5% Pheroid™ lotion (2)	0.56 ± 0.10	1.58 ± 0.70
1.0% lotion (3)	1.11 ± 0.3 ^{•°}	0.71 ± 0.37 ^{•°}
1.0% Pheroid™ lotion (4)	1.26 ± 0.20	0.69 ± 0.26
2.0% lotion (5)	1.56 ± 0.37 ^{•#}	1.58 ± 0.94 ^{•#}
2.0% Pheroid™ lotion (6)	2.63 ± 1.42	2.27 ± 1.23
4.0% lotion (7)	1.95 ± 0.77 ^{•°}	0.78 ± 0.29 ^{•#}
4.0% Pheroid™ lotion (8)	2.31 ± 0.67	6.69 ± 4.74

* - (p<0.05) statistically significant difference present between lotion and Pheroid™ lotion results at given concentration

• - (p>0.05) no statistically significant difference present between lotion and Pheroid™ lotion results at given concentration

- (d>0.5) a practically visible or practically significant difference present between lotion and Pheroid™ lotion results at given concentration

° - (d<0.5) no practical significance present between lotion and Pheroid™ lotion results at given concentration

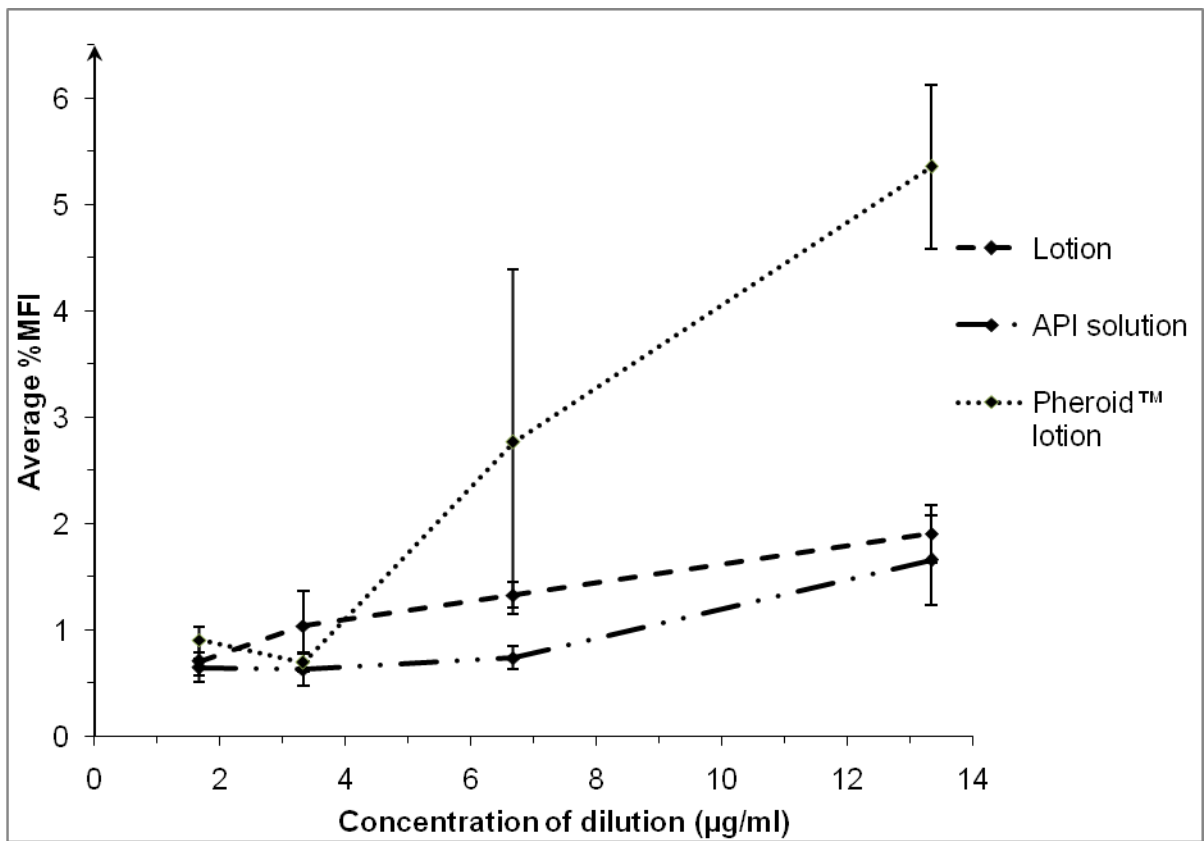


Figure 1: Line graph showing comparisons between the %MFI induced in the A375 cells treated with 1) the lotion; 2) the API solution and 3) Pheroid™ lotion (n=3)

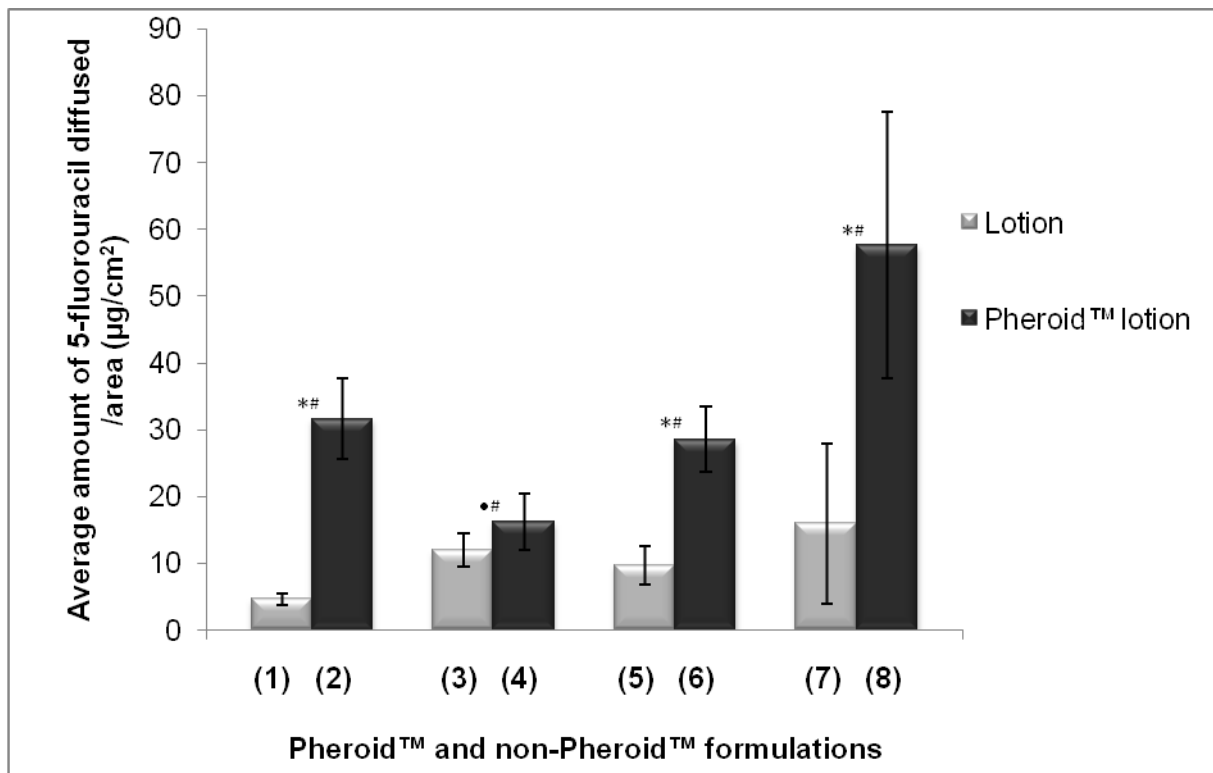


Figure 2: Comparisons of the average amount of 5-fluorouracil (in $\mu\text{g}/\text{cm}^2$) that diffused through human skin after 12 h from each lotion with the amounts diffused from the respective Pheroid™ lotion of the same concentration (n=10). **($p < 0.05$ and $|d| > 0.8$), statistical and practical significance between Pheroid™ and non-Pheroid™; •#($p > 0.05$ and $|d| > 0.8$), practical significance but no statistical significance between Pheroid™ and non-Pheroid™.

CHAPTER FOUR

FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

Skin cancer is characterised by a dysregulation of apoptosis which results in either too little apoptosis or too much cell survival. Topical chemotherapy of skin cancer requires treatment modalities that can reduce the imbalance either by increasing apoptosis or reducing the survival of cells (Lippens *et al.*, 2011:329). 5-Fluorouracil is an active pharmaceutical ingredient (API) which acts by inhibiting thymidylate synthase, an enzyme necessary in DNA replication. This results in intracellular cell damage which in turn results in induction of apoptosis (Longley *et al.*, 2003:330). The main aims of this project were to (i) determine the efficacy of 5-fluorouracil in formulation as an anti-cancer agent and to investigate the influence of drug delivery vehicles on the efficacy and (ii) determine the influence of concentration and Pheroid™ on the topical delivery of 5-fluorouracil. In order to achieve these aims, the following objectives were set:

- ❖ development and validation of a high performance liquid chromatography (HPLC) method for analysis of samples from the diffusion studies;
- ❖ development of lotions (with and without Pheroid™) that contain varying concentrations of 5-fluorouracil;
- ❖ performing an apoptotic assay to determine the *in vitro* efficacy of 5-fluorouracil and the influence of Pheroid™ on the efficacy of 5-fluorouracil;
- ❖ determining the aqueous solubility and the *n*-octanol-buffer distribution coefficient of 5-fluorouracil;
- ❖ performing drug release studies to determine whether 5-fluorouracil is released from the formulations;
- ❖ performing *in vitro* skin diffusion studies to investigate the diffusion of 5-fluorouracil into and through the skin;
- ❖ using the tape-stripping technique to determine the concentration of 5-fluorouracil within the stratum corneum-epidermis and the epidermis-dermis after 12 h skin diffusion and
- ❖ determining the influence of Pheroid™ on the transdermal and topical delivery of 5-fluorouracil.

The HPLC analytical method that was utilised in this study was validated and found to be reliable, reproducible and suitable for its intended use. Therefore, the method was used to determine the concentration of analyte that was present in the receptor phase of the Franz diffusion cells.

White, smooth, homogenous, odourless and non-tacky lotions (with and without Pheroid™) containing 0.5, 1.0, 2.0 or 4.0% 5-fluorouracil were formulated in this study. The Pheroid™ containing lotions however had a Pheroid™ characteristic “fish-oil” odour. These lotions were utilised in the Franz cell diffusion studies and in the drug efficacy tests.

The effect of 5-fluorouracil on human melanoma cells (A375) was investigated during this study. The A375 cells were exposed to different concentrations (1.67; 3.33; 6.67 and 13.33 µg/ml) of 5-fluorouracil incorporated in solutions, lotions and Pheroid™ lotions. After exposure the cells were analysed to observe whether or not 5-fluorouracil induced apoptosis. The drug efficacy tests reflected that 5-fluorouracil (1.67 – 13.33 µg/ml) can induce apoptosis in human melanoma cells *in vitro*. Results obtained *in vitro* differ from what could be found *in vivo* because of the presence of various physiological factors found *in vivo*. Therefore the *in vitro* drug efficacy tests need to be corroborated with some *in vivo* testing. This study served as a pilot study with respect to the drug efficacy tests and further investigations, with a wider variation of 5-fluorouracil concentrations, need to be done in order to get a clearer picture. The results obtained suggest that the use of a 5-fluorouracil Pheroid™ formulation may lead to an enhanced *in vitro* apoptotic effect. Pheroid™ are composed of fatty acids and an affinity exists between Pheroid™ and cell membranes which may result in a more effective and faster uptake of Pheroid™ by cells (Grobler, 2004:6). The effect of Pheroid™ on apoptosis induction by 5-fluorouracil must be determined and characterised. A combination of different measurements and techniques, such as light microscopy, fluorescence microscopy, enzyme-linked immunosorbent assay (ELISA) and colorimetric assay should be used when studying the apoptotic effect of 5-fluorouracil in future because no single parameter defines apoptosis in all systems.

In this study an aqueous solubility of 17.6 mg/ml was determined for 5-fluorouracil. This value is close to the value of 12.5 mg/ml as determined by Troy (2005:1573), and according to Naik *et al.* (2000:319) it is ideal for skin permeation. The log D that was obtained in this study (-1.15) is close to the value of -0.83 that was determined by Buur *et al.* (1985:55) and it does not lie within the optimal range as stated by Naik *et al.* (2000:319). The log D value was indicative of a low potential for 5-fluorouracil skin diffusion. However, a single physico-chemical parameter cannot be relied on to predict skin diffusion because it is not known to what extent each parameter influences skin diffusion.

5-Fluorouracil was released from all the formulations. The Pheroid™ lotions however, resulted in a higher release of 5-fluorouracil than their lotion counter-parts of the same API concentration. This may have been due to the documented penetration enhancing effects of Pheroid™.

The results from the skin diffusion studies revealed that, regardless of the unfavourable log D value 5-fluorouracil was able to diffuse from all the formulations and through the skin. Even in the absence of the Pheroid™ permeation enhancer, 5-fluorouracil permeated through the skin. The skin permeation of 5-fluorouracil did not increase with an increase in concentration but rather no trend could be observed. Differences in the skin samples that were used may have been responsible for this. The tape-stripping revealed that 5-fluorouracil was retained within the stratum corneum-epidermis. Concentrations of 5-fluorouracil were also detected in the epidermis-dermis after the tape-stripping. Therefore, 5-fluorouracil managed to reach the relevant site of action. From an overall perspective 5-fluorouracil diffused into the skin from all the formulations that were prepared in this study.

In this study, the Pheroid™ lotions generally resulted in higher amounts of 5-fluorouracil diffusing through the skin and higher concentrations of 5-fluorouracil within the skin as compared to their respective lotions. The penetration enhancing effect of Pheroid™ was therefore observed. This could allow for the use of lower doses of API with resulting effects that are comparable with those of the high dose formulations.

The topical delivery of 5-fluorouracil is required in order to treat cutaneous cancer so the diffusion of 5-fluorouracil across the skin was not required in this study. Nonetheless 5-fluorouracil managed to diffuse across the layer of skin and into the aqueous receptor phase. These results suggest that 5-fluorouracil will diffuse into the bloodstream *in vivo*. The systemic side effects of 5-fluorouracil are relatively harmful and should preferably be avoided (McEvoy, 2011). An ideal result would have been the penetration of 5-fluorouracil into the skin and it remaining within the skin. A perfect anticancer topical formulation would result in the API being released from the formulation, penetrating the skin hydrophobic layer and remaining within the skin. The 2.0% Pheroid™ lotion managed to provide a balance between relatively high concentrations within the skin and relatively low amounts in the receptor phase. Therefore, it may be possible to reduce the 5-fluorouracil dose while maintaining (or increasing) efficacy. A reduced dose means that a lower concentration of 5-fluorouracil will get in contact with skin thus lowering the superficial adverse effects of 5-fluorouracil. As with most things the ideal is hard to attain so a balance may be necessary in order to be able to treat cutaneous cancers topically.

Future recommendations include:

- ❖ using an increased number of concentrations in order to enable deduction of concentration dependent trends in the efficacy and topical delivery of 5-fluorouracil;
- ❖ conduction of *in vivo* tests (animal) to determine the effect of 5-fluorouracil in the presence of physiological factors;

- ❖ further investigations into the effect of the drug delivery vehicle (e.g. Pheroid™ formulation, cream, lotion) on the apoptosis inducing effect of 5-fluorouracil and
- ❖ optimised formulation of semi-solid products that result in a depot of 5-fluorouracil in the epidermis and dermis, thereby reducing systemic uptake.

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APPENDIX A

VALIDATION OF THE HPLC ANALYTICAL METHOD FOR 5-FLUOROURACIL ANALYSIS

A.1 Introduction

An analytical method must be validated so as to ensure that it is suitable for its intended purpose (ICH, 2005:1). During validation it must be determined that the method is sensitive and reliable in the determination of the analyte under investigation. In this study high performance liquid chromatography (HPLC) was used to determine the concentration of 5-fluorouracil in the receptor phase of the Franz cells during the skin diffusion studies.

A.2 Chromatographic conditions

The analytical method was developed with the assistance and knowledge of Professor Jan du Preez of the North-West University (NWU), Potchefstroom. All analyses were done in the Analytical Technology Laboratory (ATL) of the NWU. There are various factors that must be considered when developing an analytical method and these include: detection wavelength; run time; type of column; type of mobile phase; flow rate and injection volume. The parameters that were used in this method are:

Analytical instrument:	An Agilent® 1100 series (Agilent Technologies, Palo Alto, CA) was used for the analyses. The machine consists of a quaternary pump, a degasser, an auto-sampler injection mechanism and a diode array detector. Chemstation Rev. A.10.02 software was used for the acquisition of data and analysis.
Column:	A Phenomenex® Synergi Fusion Reversed Phase column (4.6 mm x 250.0 mm), with a 4 µm particle size was used (Phenomenex®, Torrance, CA).
Mobile phase:	4% acetonitrile solution was prepared by mixing 40 ml acetonitrile with 1 ml ortho-phosphoric acid (H ₃ PO ₄) and adding sufficient Milli-Q water to 1000 ml.
Flow rate:	1 ml/min
Injection volume:	50 µl
Detection wavelength:	266 nm
Retention time:	4.7 min
Stop time:	6 min

Solvent: The standard solutions were prepared in phosphate buffered solution at pH 7.4. Phosphate buffer solution at pH 7.4 was also the solvent (receptor phase) for the extracted samples during the diffusion studies.

A.3 Preparation of standard

The standard prepared consisted of 5-fluorouracil (DBFine, South Africa) in phosphate buffered solution (pH 7.4). 5-fluorouracil (25 mg) was weighed and dissolved in phosphate buffer solution (pH 7.4) to make 100 ml of a 250 µg/ml solution. The 250 µg/ml solution was then diluted by 10 to give a standard with a final concentration of 25 µg/ml.

A.4 Validation parameters

The parameters that were investigated during the method validation process are: linearity; limit of detection (LOD); lower limit of quantification (LLOQ); accuracy; specificity; precision; ruggedness, system repeatability and system suitability.

A.4.1 Limit of detection and lower limit of quantification

Limit of detection is the lowest concentration of an analyte that can be reliably differentiated from background noise by the analytical method but not necessarily quantified. The LLOQ, however, is the lowest amount of an analyte in a sample that can be determined quantitatively with suitable accuracy and precision (FDA, 2001:20).

The LOD and LLOQ are tested by preparing various standards of very low concentrations and injecting each standard six consecutive times. The acceptance criteria for LOD and LLOQ are that the % relative standard deviation (%RSD) should not exceed 20% and 15%, respectively. The LOD for this method was observed to be 0.005 µg/ml with a %RSD of 15.474%. The LLOQ was 0.012 µg/ml with a %RSD of 4.297%. If a concentration below the LLOQ was detected during any of the analyses the results were deemed as unreliable and therefore rejected.

Table A.1: Results for limit of detection and lower limit of quantification

STD ¹ µg/ml	Peak area (1)	Peak area (2)	Peak area (3)	Peak area (4)	Peak area (5)	Peak area (6)	Mean	SD ²	%RSD
0.005	1.617	1.209	1.596	1.652	1.103	1.668	1.474	0.228	15.474
0.012	3.307	3.288	3.477	3.660	3.269	3.269	3.378	0.145	4.297
0.025	6.636	6.757	6.845	6.828	6.694	6.804	6.761	0.075	1.104
0.049	13.505	13.771	13.302	13.599	14.031	13.471	13.613	0.234	1.721

1 - Standard (STD)

2 - Standard deviation (SD)

A.4.2 Linearity

The linearity of an analytical method refers to a methods ability (within a given range) to present with test results that are directly proportional to the concentration of analyte in the sample (ICH, 2005:5). The method must have an acceptable level of accuracy, precision and linearity within the given range. A regression coefficient (R^2) value close to 1 is reflective of high linearity and therefore indicates the stability and reliability of the analysis system. Standards of eight differing concentrations were prepared and injected in duplicate. Linear regression analysis was performed on the plot of the peak area versus the average concentration ($\mu\text{g/ml}$).

The data obtained are best described by the general equation of a line.

$$y = mx + c$$

Equation A.1

Where:

y = peak area ratio of the analyte

m = slope of the line

x = concentration of the analyte in $\mu\text{g/ml}$

c = y-intercept

Table A.2: Results for linearity of 5-fluorouracil

Standard ($\mu\text{g/ml}$)	Peak area ratio
0.25	39.5
0.50	78.8
1.25	194.2
2.50	390.9
4.99	804.5
12.48	1965.8
24.95	3936.2
49.90	7944.2
Slope (m)	159.01
y-intercept (c)	-4.819
R^2	1

The acceptance criteria for linearity is that the R^2 value should be ≥ 0.99 (Du Preez, 2010a:5). Such a value indicates that the method is highly linear and thus should give accurate and reliable results.

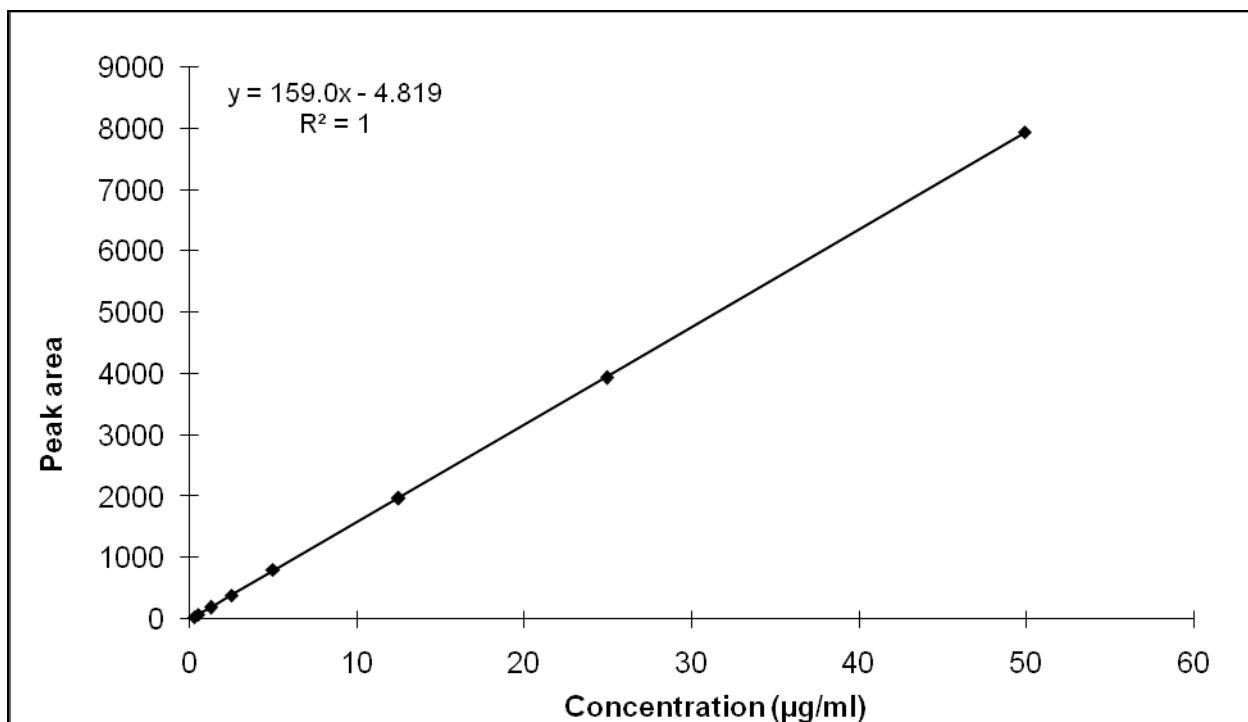


Figure A.1: Linear regression curve of 5-fluorouracil

An R^2 value of 1.0 was obtained for the linearity of this analytical method. This indicates that the method is stable, highly reliable and accurate within the range 0.25 – 50.00 µg/ml.

A.4.3 Accuracy

Table A.3: Results for accuracy of 5-fluorouracil

Concentration spiked µg/ml	Peak area (1)	Peak area (2)	Mean	Recovery	
				µg/ml	%
1.3 (1)	192.5	193.8	193	1.3	103.1
1.3 (2)	191.9	193.3	193	1.3	102.8
1.3 (3)	189.4	188.9	189	1.3	101.1
12.6 (1)	1940.5	1973.5	1957	12.6	99.7
12.6 (2)	1958.5	2065.2	2012	12.9	102.5
12.6 (3)	1921.2	1898.8	1910	12.3	97.3
25.2 (1)	4073.8	4027.1	4050	26.0	102.9
25.2 (2)	4033.5	3934.5	3984	25.6	101.3
25.2 (3)	3931.7	3916.7	3924	25.2	99.7
				Mean	101.2
				SD	1.8
				%RSD	1.8

Accuracy of an analytical method expresses the closeness of the value obtained to the actual or true concentration of the analyte under the prescribed conditions (Du Preez, 2010b:5). Percentage recovery by the assay of the known amount of analyte in the sample gives a reflection of the accuracy. A minimum of nine determinations over a minimum of three concentration levels are utilised to obtain the accuracy of a particular method (ICH, 2005:10). In this instance three concentrations of samples were prepared in triplicate and injected into the HPLC in duplicate.

The acceptance criterion for accuracy is that the recovery must be between 98 – 102% (Du Preez, 2010a:6). The mean recovery was 101.2% which led to the conclusion that the analytical method was accurate.

A.4.4 Precision

Precision of an analytical method is the closeness of agreement between a series of measurements obtained after repeatedly applying the method to the same homogenous sample under the set conditions (ICH, 2005:4). It is recommended to use a minimum of three concentrations within the range of expected concentrations (FDA, 2001:5). This parameter can be further sub-categorised into intra-day and inter-day precision.

A.4.3.1. Intra-day precision

Table A.4: Results for intra-day precision of 5-fluorouracil

Concentration spiked µg/ml	Peak area (1)	Peak area (2)	Mean	Recovery	
				µg/ml	%
1.26 (1)	192.5	193.8	193.15	1.30	103.12
1.26 (1)	191.9	193.3	192.60	1.30	102.84
1.26 (1)	189.4	188.9	189.15	1.27	101.09
12.62 (2)	1940.5	1973.5	1957.00	12.59	99.73
12.62 (2)	1958.5	2065.2	2012.85	12.94	102.51
12.62 (2)	1921.2	1898.8	1910.00	12.28	97.34
25.24 (3)	4073.8	4027.1	4050.45	25.98	102.94
25.24 (3)	4033.5	3934.5	3984.00	25.56	101.25
25.24 (3)	3931.7	3916.7	3924.20	25.17	99.73
				Mean	101.2
				SD	1.8
				%RSD	1.8

Intraday precision is conducted to observe the within-run precision. This can also be described as the reproducibility of the method on the same day. Three samples of analyte in phosphate

buffer solution (pH 7.4) were prepared according to the method for preparing the standard in Section A.3. The concentrations utilised were concentrations in the range of expected results. The range was from 1.26 – 25.24 µg/ml. Each of these samples were prepared in triplicate then injected in duplicate into the HPLC.

If there are nine samples, as it is in this case, the percentage relative standard deviation (%RSD) should be better than 2% (Du Preez, 2010a:7). The intra-day precision of 5-fluorouracil had a %RSD of 1.8, leading to the conclusion that the method was precise.

A.4.3.2. Inter-day precision

Inter-day precision expresses variations that may occur within the same laboratory on two or more different days. It is also known as intermediate precision. Inter-day precision was conducted on three consecutive days. Three samples of 12.5 µg/ml were prepared on each day and injected in duplicate. The % recovery of these samples was then obtained using a standard as a reference.

Table A.5: Results for inter-day precision of 5-fluorouracil

	Day 1	Day 2	Day 3	Between days
	99.7	99.6	95.7	
	102.5	97.4	96.6	
	97.3	95.3	97.5	
Mean	99.86	97.41	96.60	97.96
SD	2.11	1.73	0.73	1.39
%RSD	2.11	1.78	0.76	1.42

The %RSD for inter-day precision should be less than 5% (Du Preez, 2010a:7). A %RSD of 1.42% was obtained for the inter-day precision which was acceptable.

A.4.5 Stability

It is necessary for samples to be stable for the duration of the period during which they shall stand before analytical analysis. A sample standard was prepared according to the method in Section A.3 and injected every hour for 24 h. The peak areas were compared with the peak area obtained at time point 0.0 h.

Table A.6: Results for stability of 5-fluorouracil

Time (h)	Peak area	%
0	3995.5	100.00
1	4154.3	103.97
2	4047.3	101.30
3	3883.2	97.19
4	3978.8	99.58
5	4030.3	100.87
6	3894.7	97.48
7	3972.8	99.43
8	3918.2	98.07
9	3917.6	98.05
10	3929.7	98.35
11	3941.9	98.66
12	4164.3	104.23
13	3902.4	97.67
14	3919.4	98.10
15	4094.4	102.48
16	3925.9	98.26
17	3830.4	95.87
18	3982.6	99.68
19	3952.9	98.93
20	3871.4	96.89
21	4072.4	101.92
22	4070.9	101.89
23	3951.7	98.90
24	3893.9	97.46
Mean	3970.4	99.4
SD	84.46	2.15
%RSD	2.13	2.16

Samples should not be used for a period longer than it takes for the analyte to degrade by 2% (Du Preez, 2010a:7). According to the results obtained there wasn't a clear degradation pattern for 5-fluorouracil and this may have been due to environmental disturbances or human error. Despite this the method was utilised as it is and no stability problems were encountered.

A.4.6 System repeatability

System repeatability expresses the precision of the analytical method over a short interval of time (Du Preez, 2010b:6). It was tested by preparing a 100% standard solution of the analyte as described in Section A.3 and injecting it six consecutive times.

Table A.7: Results for system repeatability of 5-fluorouracil

Injection number	Peak area	Retention time (min)
1	3847.74	4.734
2	3845.04	4.732
3	3846.96	4.731
4	3845.94	4.724
5	3846.29	4.726
6	3846.45	4.721
Mean	3846.40	4.728
SD	0.84	0.005
%RSD	0.02	0.098

The peak area and the retention times should have %RSD values that do not exceed 2% (Du Preez, 2010a:8). The %RSD of 5-fluorouracil was 0.098% for retention time and 0.02% for peak area, which was very good as this reflects that there was minimal relative standard deviation.

A.4.7 Specificity

Specificity is the ability to explicitly assess the analyte in the presence of other components (degraded analytes, matrix components or impurities) that may be expected to be present. The analytical method should be able to distinguish between compounds that have closely related structures. To determine the specificity of the analyte a 100% standard solution was prepared as described in Section A.3. The standard solution was placed in four test-tubes with 1 ml in each test-tube. Water (1 ml), sodium hydroxide (1 ml), hydrochloric acid (1 ml) and 10% hydrogen peroxide (1 ml) were added to a test-tube each. The stressed samples were analysed immediately then left to stand overnight and analysed again the following day.

The placebo and degraded samples must not contain peaks that may interfere with the determination of the analyte (Du Preez, 2010a:4). The peaks of 5-fluorouracil were clearly visible although other peaks due to the degradation were also now present, but did not interfere with the determination of the 5-fluorouracil.

A.4.8 System suitability

System suitability tests are used to verify that the resolution and reproducibility of the chromatographic system are suitable for the analysis to be done. Suitability testing ascertains the effectiveness of the final operating system (USP, 2002:1991). A standard was prepared according to the method in Section A.3 and injected six consecutive times. System suitability parameters were determined from the analyte peak. The number of theoretical plates and the %RSD were then calculated. Below are the system suitability parameters that were tested and their respective results.

Retention time (minutes): 4.705

Number of theoretical plates (N) plates/column (5 sigma method): 10 056

USP tailing factor: 0.873

Capacity factor (k'): 0.665

A chromatographic system is said to be suitable if the %RSD of six injections is not more than 2% and if the column has more than 6000 theoretical plates. The system performed well with the given method and was considered suitable for analysing 5-fluorouracil in diffusion samples.

A.5 Conclusion

The HPLC analytical method was found to be reliable, reproducible and suitable for its intended use of determining the concentration of analyte present in the receptor phase of the Franz diffusion cells. All the validation parameters complied with the relevant acceptance criteria except for one parameter. The 24 h stability did not comply with the requirements but the outcome was close enough to the acceptance criterion so the method was used as it is for the analysis of 5-fluorouracil during the skin diffusion studies. The skin diffusion studies will be discussed further in Appendix D.

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FDA **see** Food and Drug Administration

ICH **see** International Conference on Harmonisation

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APPENDIX B

FORMULATION OF TOPICAL SEMI-SOLID PRODUCTS WITH 5-FLUOROURACIL AS THE ACTIVE PHARMACEUTICAL INGREDIENT

B.1 Introduction

An active pharmaceutical ingredient (API) is incorporated into a dosage form so as to provide safe, effective and convenient delivery of accurate dosages (Fishburn, 1965:1). If it was left to the patient to measure and administer the required API concentration there would be a lot of inconsistencies and pandemonium, especially with low dose and highly potent APIs. It is thus the role of the pharmaceutical scientist to ensure that the API gets to the patient in forms that can easily be administered and handled. Dosage forms may differ with respect to physical and pharmaceutical characteristics. Differences in dosage forms provide the physician with options on choice of API and type of delivery systems that are applicable to each patient.

The successful formulation of dosage forms entails the consideration of physical, chemical and biological characteristics of all the APIs and excipients that will be used in producing the end product. The API must be physically and chemically compatible with the excipients in order to produce a stable, efficacious, safe, convenient (easy to administer) and attractive product. It is also possible for excipients to influence release of the API in an undesirable way and this effect must be monitored and controlled as much as possible (Fishburn, 1965:8). Quality control measures are also put in place during manufacture to maintain product stability and the packaging should be such that the stability is maintained during the products shelf-life (Gibson, 2004:301).

Prior to commencement of formulation, toxicity data of all excipients and APIs to be used must be collected and the formulating scientist must ensure that the chosen excipients are not toxic via the chosen route of administration. Another consideration that may be often overlooked is whether the substance is non-toxic upon acute use but toxic upon chronic use. Toxicity, however, does not only refer to toxicity to the patient but also to the formulator especially when it comes to large-scale formulating. In the case of 5-fluorouracil it is a highly toxic drug as it is an anti-metabolite used to treat cancer and cancer related ailments (Chu & Sartorelli, 2009:935). To avoid any harm to the researcher gloves and other relevant protective clothing were worn at all times when handling the API. Notice was also given to fellow researchers not to let their skin get in contact with the API in question.

A convenient formulation must be easy to administer precisely and it must have a reasonable shelf-life. Preferably a formulation should be “ready-for-use” by the patient (Fishburn, 1965:9).

B.2 Formulation of semi-solid topical products

Topical products are usually liquid or semi-solid preparations that may contain dissolved and suspended APIs. Semi-solid dosage forms have a unique rheological character in that they can retain their shape until acted upon by an outside force, like solids. However, semi-solid dosage forms are easily deformed unlike solids, hence the name semi-solid (Flynn, 2002:214). On application, semi-solid dosage forms cling to the surface of the skin generally, until being washed off or worn off (Flynn, 2002:213). In this study, topical application refers to semi-solid applications for skin application, although, topical application generally includes application to skin, eye or mucous membranes (Mahato, 2007:183). During formulation of topical products there are five aspects that must be taken into consideration. The first three were listed by Polderman (1977:6), namely:

- ❖ the API must be released from the semi-solid topical dosage form;
- ❖ the product must have good physical, chemical and microbiological qualities inclusive of stability;
- ❖ it must be possible to manufacture the product on any scale without significant changes to the formulation
- ❖ the API must reach the site of action and
- ❖ the API should exert efficacy against the ailment intended to treat.

These five factors were considered in the progression of this research project. The final two aspects will be described in Appendix C and Appendix D.

Membrane release studies were conducted on the formulations before the skin diffusion studies so as to determine whether the API was released from the formulation. Simple over-night and 7-day stability determinations were done during early formulation on small batches. The lotions were visually observed for any signs of separation, creaming or colour changes. The formula and method that were utilised are relatively simple and it should be possible to scale up production without any major complications.

A wide assortment of topical products is available for the treatment of skin conditions. These include, but are not limited to:

- ❖ solutions;
- ❖ lotions;
- ❖ creams;
- ❖ hydrogels;

- ❖ emulgels;
- ❖ ointments;
- ❖ pastes and
- ❖ aerosols.

The semi-solid dosage form formulated at any given time must be compatible with the API, the sickness that is to be treated and with the probable areas of application. After considering all these factors, a lotion was the chosen semi-solid dosage form. The aim was to formulate an aesthetically acceptable lotion that results in optimum 5-fluorouracil delivery to the skin.

B.2.1 Preformulation

According to Steele (2004: 21), preformulation is the physico-chemical characterisation of the solid and solution properties of an API. The goals are to establish the relevant physico-chemical parameters, determine the kinetic rate profile, to establish the physical characteristics and to find out the API's compatibility with frequently used excipients. The parameters that are usually investigated are pKa or pKb, melting point and polymorphism, vapour pressure, solubility, hygroscopicity and log P/log D to mention a few. If the API was already discovered and has been in use, preformulation will entail doing a thorough literature study on the API so as to obtain details on the physico-chemical properties of the API. In this study, a literature study on 5-fluorouracil was done in addition to determining the log D (*n*-octanol-phosphate buffer solution at pH 7.4) and aqueous solubility (phosphate buffer solution at pH 7.4) of 5-fluorouracil. The values obtained for log D and aqueous solubility were then compared to the values found in literature (see Appendix D).

B.2.2 Early formulation

Early formulation was done on a relatively trial and error basis. A literature study regarding preservative efficacy, excipient compatibilities, skin diffusivity and formulation stability was done. This information was reconciled with the information obtained on 5-fluorouracil and in turn used to come up with the formulations of choice. It is during this stage that it was decided that 5-fluorouracil lotions will be formulated.

Formulas were taken from literature and tweaked to fit the properties of the API and excipients. The hydrophilic-lipophilic balance (HLB) was used as an aid in tweaking the formulas so as to prepare relatively stable emulsion-based formulations.

While stability and diffusivity are important, the cosmetic elegance is also important, thus the feel (non-grainy, non-greasy, non-sticky and non-watery), colour, scent, spreadability, absorbability and general appearance of the lotion were taken into consideration throughout

formulation. The exact masses of excipients required for the lotions were determined during this stage.

B.2.3 Final formulation

The final formulations were prepared for the Franz cell diffusion studies and the drug efficacy studies. Four different concentrations of 5-fluorouracil were utilised in the research as an attempt to determine which dose results in the minimal effective concentration at the site of action. The concentrations of 5-fluorouracil that were used are 0.5%, 1.0%, 2.0% and 4.0% (m/m). This range of concentrations was selected because it lies within the 5-fluorouracil concentration range of topical semi-solid products that are found on the market worldwide (0.5% - 5.0%). At each concentration a lotion was formulated with and without Pheroid™ technology making it a total of eight lotions. The water used in all the non-Pheroid™ lotions was HPLC-grade water.

B.3 Formulation of a lotion

A lotion is either a fluid (low to medium viscosity) emulsion or suspension that is designed for external application. The key components of a lotion are the aqueous and oily phases, an emulsifier to prevent separation of the two phases and the active substance, if used (Mahato, 2007:187). Most emulsions that are prepared for commercial use in the pharmaceuticals industry are dermatological products for topical and transdermal application. The emulsions are usually either lotions or creams. Emulsions are commonly used in the pharmaceuticals and cosmetic industry due to their application acceptability and their ability to excellently solubilise hydrophilic and lipophilic APIs (Otto *et al.*, 2009:5).

Emulsions are heterogeneous systems that are composed of at least two liquids that are immiscible. One liquid becomes dispersed as fine droplets throughout the other liquid by mechanical agitation. The liquid existing as the fine droplets is termed the dispersed phase while the other is the continuous phase. Emulsions generally consist of an oil phase and an aqueous phase and are thus either referred to as oil-in-water (o/w) or water-in-oil (w/o) emulsions. More complex emulsions include multiple emulsions such as the oil-in-water-in-oil (o/w/o) variety, water-in-oil-in-water (w/o/w), microemulsions and nanoemulsions (Walters & Brain, 2004:556).

Emulsions are thermodynamically unstable because phase inversion and separation to the dispersed and continuous phases can occur with time. Therefore surfactants are utilised to increase the stability because they have emulsification properties. The phase in which the surfactant is most soluble usually becomes the continuous phase. , however, a combination of surfactants is usually used to achieve better stability. While surfactants are necessary to

formulate an acceptable emulsion the downside is that impurities found in surfactants may have a negative effect on the API and preservatives included in some formulations. Stability can thus become a problematic issue with emulsion-based formulations (Wei Lu & Gao, 2010:84).

The type of lotion formulated in this study was of the o/w variety. The advantages of o/w lotions are that they produce a more stable emulsion, they have non-greasy application characteristics and they have a “dry” end feel for light bodied cosmetic effects (Brooks, 2000:22). These properties are usually more appealing to the consumer, hence being advantageous.

B.3.1 Applications of a lotion

Due to their fluidity lotions are usually applied on areas where the skin rubs together e.g. under the arms, between the fingers or thighs. They are also dosage forms of choice for products to be applied in hairy and “hard to reach” areas. Lotions are advantageous because they can be spread thinly and can therefore economically cover a larger area of skin than more viscous preparations (Mahato, 2007:187). The cutaneous conditions for which 5-fluorouracil is used mainly present on sun-exposed areas such as balding heads and the neck which can be hairy and have skin folds respectively. A lotion would thus allow for easy application of the medicament.

B.3.2 Stability and storage of a lotion

A requirement for any dosage form in the pharmaceutical industry is that it should be stable. Stability criteria that should be monitored include the appearance (creaming, coalescence or colour change), particle size uniformity, weight changes, viscosity, clarity, pH, microbial contamination and smell of the lotion. The package material has a large role to play in the long-term stability of a manufactured product. Semi-solid dosage forms must be stored in air-tight containers in a refrigerator or at room temperature.

There are certain criteria and objectives that must be met by the packaging. Points to be considered when selecting appropriate packaging are, the package must:

- ❖ not affect the product negatively;
- ❖ not be affected negatively by the product;
- ❖ have a tight seal to avoid excessive loss of constituents e.g. water evaporation;
- ❖ be suitable for the physical form of the product, e.g. a thick lotion or cream must either be in a squeezable container or a container with a large orifice to make it accessible;
- ❖ be able to protect the product from the effects of its environment;
- ❖ be easy to handle and

- ❖ be aesthetically appealing to the consumer.

In this study, amber glass jars with screw-on lids were used for storage. Glass jars were chosen because glass is inert and has excellent barrier properties (Romanowski & Schueller, 2000:798). The jars had to be amber in order to block out light for two reasons. Firstly, 5-fluorouracil is light-sensitive and secondly, light can cause product discolouration or change in fragrance. If amber jars were unavailable clear jars were used and covered with foil paper to avoid the penetration of light. For short-term storage (overnight) the formulations were placed in clean opaque plastic containers. Stability and the choice of packaging were, however, not part of the primary focus in this study.

B.3.3 Preservation of a lotion

Contamination of lotions should be avoided as much as possible during preparation or during use because emulsions support microbiological growth. The work area and equipment should be kept clean to minimise contamination, but if the product is going to be kept for any length of time a preservative must be added (Allen, 1998:180).

A preservative must be non-toxic, stable, inexpensive and compatible with the rest of the excipients in the formulation. In addition, a preservative must be compatible with the type of formulation and it must have an acceptable colour, taste (if applicable) and odour. Bacterial growth will normally occur in the aqueous phase so the preservative must be concentrated in the aqueous phase. The parabens (methylparaben, propylparaben and butylparaben) are very popular preservatives in semi-solid formulations and are considered to be some of the most satisfactory (Allen, 1998:180).

Vermaas (2010:117), used parabens as the preservatives in the formulations with 5-fluorouracil as the API. After the 6 month stability testing period, Vermaas (2010:80) found that 5-fluorouracil was unstable in the different formulations. This may have been due to the fact that parabens have reduced activity in the presence of non-ionic surfactants (such as tween 80) and tween 80 is one of the emulsifiers that were used in the formulations. The formulations in this study were based on the same basic formula as Vermaas' formulations but it was decided to use a different preservative and note any changes in stability. Ethanol (15% w/w), instead of parabens was used as the preservative in all the formulations. Choice of preservative must always be considered carefully during formulation, preservative studies, however, were not a primary focus of this study.

B.3.4 General method for the formulation of a lotion

While manufacturing lotions, heating processes are usually employed to prevent microbial contamination and to facilitate emulsification. The formulation ingredients are separated into two separate phases, namely the oil and water phase. The phases are heated separately to about 60-70 °C, if necessary. The dispersed phase is then stirred into the dispersion medium (Allen, 1998:175). After carrying out the initial emulsification, the emulsion is homogenised by using a mixer. The viscosity and other physical properties of the end-product are largely influenced by the manufacturing conditions. Therefore, the proper conditions for the emulsification (order of addition of excipients), stirring and cooling processes must be selected and maintained during manufacturing. Lastly, the emulsion obtained is de-aired and cooled to produce the finalised product (Mitsui, 1997).

B.4 Formulation of 5-fluorouracil lotions in this study

The general method for formulating a lotion (Section B.3.4) was followed in preparing the lotions in this study but the conditions for emulsification, stirring and cooling were specific to the method and ingredients used. The same basic formula was utilised for the four non-Pheroid™ lotions, with the distinguishing factor being the API concentration. The concentrations that were used are 0.5%, 1.0%, 2.0% and 4.0% (w/w) 5-fluorouracil. In order to observe the effect of Pheroid™ technology on topical drug delivery and drug efficacy of 5-fluorouracil an additional four lotions were prepared utilising Pheroid™ technology. Each of the Pheroid™ lotions differed in API concentration just as the non-Pheroid™ lotions. The same concentrations of 0.5%, 1.0%, 2.0% and 4.0% (m/m) 5-fluorouracil were utilised in the Pheroid™ lotions.

B.4.1 Ingredients used in the formulations

A lotion is an emulsion, therefore, it requires all the ingredients necessary for formulating an emulsion plus thickening agents. These ingredients include an emollient (oil phase), aqueous phase, emulsifier, emulsion stabilisers, preservatives and the relevant API. It must be ascertained beforehand that the excipients used in a lotion are not toxic when applied topically. Table B.1 lists the ingredients that were used in this study, their purpose, suppliers and batch numbers.

Table B.1: Ingredients used during formulation of the lotions

INGREDIENT	PURPOSE	SUPPLIER	BATCH NUMBER
Cetyl alcohol	Thickening agent; Emollient; Emulsion stabiliser	Merck Chemicals	S5183304904
<i>d</i> - α -tocopherol	Anti-oxidant	Chempure	UTI0020016
Ethanol (96%)	Preservative	Rochelle Chemicals	230811EX
Liquid paraffin	Emollient	Merck Chemicals	1035428
Span 60	Emulsifier	Merck Chemicals	S5361721034
Tween 80	Emulsifier	Merck Chemicals	1035460
5-fluorouracil	API	DBFine Chemicals	IF-FL-111226

B.4.1.1 Cetyl alcohol

Cetyl alcohol is a mixture of solid aliphatic alcohols and it comprises mainly of 1-hexadecanol. It functions as a coating agent, emulsifying agent and thickening agent in cosmetic and pharmaceutical formulations such as suppositories, modified release dosage forms, creams, lotions and ointments. Cetyl alcohol is an emollient because it is absorbed and retained in the epidermis, where it lubricates and softens the skin while imparting a velvety texture. These properties of cetyl alcohol give it importance in topical formulations. The stability enhancing effect of cetyl alcohol is due to it combining with the water-soluble emulsifier in o/w emulsions and forming a close-packed monomolecular barrier at the oil-water interface. The monomolecular barrier acts as a mechanical barrier against droplet coalescence. In the lotions formulated in this study cetyl alcohol acted as a thickening agent and an emulsion stabiliser (Unvala, 2005:155).

B.4.1.2 *Dl*- α -tocopherol

The naturally occurring form of α -tocopherol is known as *d*- α -tocopherol but a racemic mixture of tocopherol can be synthesised and this is known as the *dl*- α -tocopherol. In pharmaceutical formulations *dl*- α -tocopherol is used for its anti-oxidant activity and for its use as a source of vitamin E. Vitamin E contains essential fatty acids that are found in skin consequently the *dl*- α -tocopherol had therapeutic and anti-oxidant actions in the lotions (Owen, 2005a:32).

B.4.1.3 Ethanol

Ethanol is widely used in pharmaceutical formulations because it may act as a preservative, topical penetration enhancer or a solvent. Ethanol has antimicrobial preservative properties when used at concentrations above 10% (v/v). It is a clear, colourless, highly volatile liquid, with

a characteristic odour. Due to its volatility, ethanol was added to the lotions after the heating and cooling stage of the formulation method (Owen, 2005b:18).

B.4.1.4 Liquid paraffin

Paraffin liquid is a mixture of refined liquid saturated aliphatic and cyclic hydrocarbons obtained from petroleum. It is a transparent, colourless, viscous oily liquid which is often used as an emollient and solvent in topical pharmaceutical formulations. Paraffin liquid has widespread use and has rarely been associated with instances of allergic reactions making it a relatively safe emollient for topical products. The recommended use level of paraffin liquid in topical pharmaceutical formulations varies from 0.1-95.0% depending on whether it is being incorporated into a lotion, cream or ointment, to name a few (Owen, 2005c:471).

B.4.1.5 Span 60

Span 60 is a monostearate ester of sorbitol that exists as a cream solid with a characteristic odour. It is also known as sorbitan monostearate. Sorbitan esters are used as lipophilic non-ionic surfactants in cosmetics, pharmaceuticals and in food products. They are also used in conjunction with other surfactants, usually polysorbates, in the emulsification of creams, ointments and emulsions for topical application. The emulsification system of o/w emulsions is generally hydrophilic and the opposite is true for w/o emulsions. The lotions formulated were of the o/w variant so the span 60 which is a lipophilic surfactant was acting as an aid to tween 80 which is a hydrophilic surfactant (Lawrence, 2005a:713).

B.4.1.6 Tween 80

Tween 80 is a polyoxyethylene sorbitan fatty acid ester also known as polysorbate 80. Tween 80 functions in pharmaceutical formulations as an emulsifying agent, non-ionic surfactant, solubilising agent and a wetting agent. It is a hydrophilic surfactant so its main use in emulsions is in o/w emulsions. Polysorbates are generally regarded as nontoxic and non-irritant materials but the potential for hypersensitivity is always present (Lawrence, 2005b:581).

B.4.2 Formulation of 5-fluorouracil lotions (non-Pheroid™)

The general formula for the non-Pheroid™ 5-fluorouracil lotions is outlined in Table B.2.

Table B.2: Formula for 5-fluorouracil lotions formulated in this study

Ingredient	Amount m/m	Function
A: Liquid paraffin	20.0%	Emollient (oil phase)
Cetyl alcohol	4.0%	Emulsifier; thickening agent; emulsion stabiliser
Span 60	2.0%	Emulsifier
Tween 80	7.0%	Emulsifier
B: Ethanol	15.0%	Preservative
C: 5-fluorouracil	0.5%*	Active pharmaceutical ingredient
Distilled water	to 100%	Solvent (aqueous phase)

* - variations of 5-fluorouracil concentrations were used (0.5%, 1.0%, 2.0% and 4.0%).

B.4.2.1 Method used to formulate 5-fluorouracil non-Pheroid™ lotions

The oil phase consisted of all the ingredients labelled under A while the aqueous phase consisted of all ingredients under the label C. The ethanol (B) was not classified under the aqueous or oil phase.

- ❖ All the excipients on **A** were weighed and heated to 80 °C.
- ❖ Simultaneously the respective amount of 5-fluorouracil was weighed and dissolved in the respective amount of distilled water.
- ❖ The API dissolved in distilled water was then heated to 80 °C taking care not to overheat.
- ❖ **A** was added to **C** and the mixture homogenised at 13 500 rpm for at least 4 min until a temperature of 40 °C was reached.
- ❖ **B** was added to the emulsified mixture of **A** and **C**. Homogenisation continued for 1 min.
- ❖ The mixture above was mixed at a speed of 200 rpm, to remove air bubbles, until room temperature was reached.

B.4.2.2 Outcome

White, smooth, homogenous, odourless, non-tacky lotions with short “rub-out” times were formulated. The lotions were not too oily or too hydrous. Viscosity of the lotions increased with increase in API concentration, probably due to the reduced water content.

B.4.3 Formulation of 5-fluorouracil Pheroid™ lotions

The Pheroid™ lotions were formulated using the same method as the non-Pheroid™ lotions except that: 1) *dl*- α -tocopherol was added as an anti-oxidant and 2) two other Pheroid™ ingredients were added to the formulation. The two Pheroid™ ingredients shall not be mentioned by name as Pheroid™ technology is patented technology and is protected by the laws of intellectual property with patent number SA93/3895, 1994 (Pitmeij International NV Patent Portfolio, 1994).

B.4.3.1 Outcome

White, smooth, homogenous, non-tacky lotions with short “rub-out” times were formulated. The lotions were not too oily or too hydrous. Viscosity of the lotions increased with increase in API concentration, probably due to the reduced water content.

The outcome was similar to that of the non-Pheroid™ lotions except that the Pheroid™ lotions had a Pheroid™ characteristic “fish oil” odour.

B.5 Conclusion

Eight topical semi-solid products were formulated with 5-fluorouracil as the API. These are the products that were formulated:

- ❖ 0.5% 5-fluorouracil lotion;
- ❖ 1.0% 5-fluorouracil lotion;
- ❖ 2.0% 5-fluorouracil lotion;
- ❖ 4.0% 5-fluorouracil lotion;
- ❖ 0.5% 5-fluorouracil Pheroid™ lotion;
- ❖ 1.0% 5-fluorouracil Pheroid™ lotion;
- ❖ 2.0% 5-fluorouracil Pheroid™ lotion and
- ❖ 4.0% 5-fluorouracil Pheroid™ lotion.

The lotions were checked for appearance and homogeneity after every formulation process and were found to be acceptable for use in the skin diffusion studies and the drug efficacy tests. Freshly prepared lotions were used for each experiment.

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APPENDIX C

THE *IN VITRO* DETERMINATION OF 5-FLUOROURACIL CYTOTOXIC EFFICACY AGAINST HUMAN MELANOMA CELLS USING FLOW CYTOMETRY

C.1 Introduction

Drug efficacy and drug toxicity tests are often conducted in animals but with the advancement of technology it is possible to minimise the use of animals. The use of animals as surrogates for humans has been criticised because of the assumptions and extrapolations involved. Test animals are at times exposed to higher active pharmaceutical ingredient (API) doses than those typically expected for human exposure in turn resulting in assumptions being made about effects at the low doses (Holmes *et al.*, 2010:15). Russell and Burch (cited by Doyle & Griffiths, 2000:402) developed the concept of the three R's and it became the root of the development of *in vitro* alternatives to animal experimentation. The three R's are replacement, reduction and refinement. Replacement refers to using methods that can replace the use of conscious living vertebrates, reduction refers to lowering the number of animals required to obtain certain information with a particular level of precision and refinement is the elimination of any inhumane conduct to animals that have to be used (Doyle & Griffiths, 2000:402; Holmes *et al.*, 2010:15). The limitations involved with animal testing in combination with the economic and ethical concerns are the driving force for the exploration of potential non-animal alternative approaches to drug toxicity and drug efficacy testing (Holmes *et al.*, 2010:15). With this in mind *in vitro* and *in silico* drug efficacy and drug toxicity testing have gained popularity in the bio-sciences field because they do not include the use of animals for testing. There are various other methods that have been implemented over the years in order adhere to the concept of the three R's. Tissue engineering (Holmes *et al.*, 2010:15) and early microdose drug studies in humans (Combes *et al.*, 2003:4) have been utilised as viable alternatives to animal testing. The alternative methods still need to be perfected therefore a combination of animal *in vivo* testing and alternative methods is still used in most drug efficacy and drug toxicity studies (Combes *et al.*, 2003:9).

There are various limitations to *in vitro* drug testing because the *in vitro* tests generally account for what happens at a cellular level and do not account for the various relevant conditions and events (e.g. metabolism) taking place outside of the cell. It is also important to take note of the differences that may exist between the *in vitro* measurement taken and the *in vivo* response. If a tissue response (e.g. an inflammatory reaction, fibrosis, kidney transport) or a systemic response (e.g. pyrexia, vascular dilatation) is required *in vivo* the researcher must have an accurate understanding of the cell-cell interaction and the interplay of endocrine hormones with local paracrine and autocrine factors (Freshney, 2000:330). Subsequent to gaining this

understanding it becomes possible to simulate the complex tissue and systemic reactions *in vitro*. However, a novel method of working with microfluidic cell culture systems (MCCS) is a promising alternative to the conventional methods of cell culture (Ziolkowska *et al.*, 2010:542), because a flow of medium and test solutions through the microfluidic systems can simulate *in vivo* conditions (Jedrych *et al.*, 2011:1544). Jedrych *et al.* (2011:1550) tested the toxicity of 5-fluorouracil on cancer cells using the conventional method and the MCCS. A comparison of the results showed that cells were more sensitive to 5-fluorouracil in the conventional method than the MCCS but there was a correlation between the results of the two methods. This suggests that the method of cultivation and analysis affects the efficacy of the API. The results obtained in a single *in vitro* cell culture study are therefore not conclusive and some form of *in vivo* or further *in vitro* testing must follow.

Little research has been conducted on the *in vitro* effects of 5-fluorouracil on human melanoma cells so *in vitro* drug efficacy tests were conducted in this study to determine the cytotoxic potential of 5-fluorouracil to human melanoma cells that originate from the skin. 5-Fluorouracil is an anti-metabolite that is used in the treatment of cancer (Chu & Sartorelli, 2009:935), so the desired outcome was toxicity to the melanoma cells. Cancer, auto-immune diseases, reperfusion injury and immunodeficiency diseases are mainly characterised by dysregulation of apoptosis. Apoptosis is an organised and genetically directed process that leads to cell death. It is sometimes referred to as programmed cell death. If apoptosis is not regulated the organism is unable to protect itself from rogue cells that threaten homeostasis and it is also unable to control cell number and tissue size (Barisic *et al.*, 2003:151). Apoptotic cells are characterised by a very specific pattern of metabolic, biochemical and molecular features which can be probed by flow cytometry.

The API used in this study, 5-fluorouracil, interferes with deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis. This in turn results in apoptotic death of cells which curbs the cell excess that would have been caused by apoptosis dysregulation. Currently 5-fluorouracil is topically used in the treatment of actinic keratoses, superficial basal cell carcinoma and Bowen's disease. 5-Fluorouracil is useful in the overall treatment of some cancers and cancer-related ailments so it was decided to investigate the effect of various concentrations of 5-fluorouracil on cutaneous human melanoma cells. The cell line for this study was procured from American Type Culture Collection (ATCC), USA.

The aim of this part of the study was to establish whether:

- ❖ 5-fluorouracil in a topical formulation can be a viable alternative form of therapy for cutaneous cancer and
- ❖ the Pheroid™ drug delivery system has an impact on the efficacy of 5-fluorouracil against human melanoma cells.

The outcome of this study could form a basis for further research on this topic.

C.1.1 Flow cytometry analytical method for *in vitro* drug efficacy testing

The constitution of a cell population can be analysed using flow cytometry. This technology concurrently measures and analyses multiple physical characteristics of single cells as they flow in a fluid stream through a beam of light (Shapiro, 2003:1). In the flow cytometer particles are carried in a fluid stream to the laser intercept where they scatter light, which is detected as forward scatter (FSC) or side scatter (SSC). If there are fluorescent molecules present on the particles they fluoresce and also scatter light. The scattered and fluorescent light is collected by appropriately positioned lenses then steered to the appropriate detectors by beam splitters and filters. Electronic signals that are proportional to the optical signals striking the detectors are produced and processed by the computer (BD Biosciences, 2000:5).

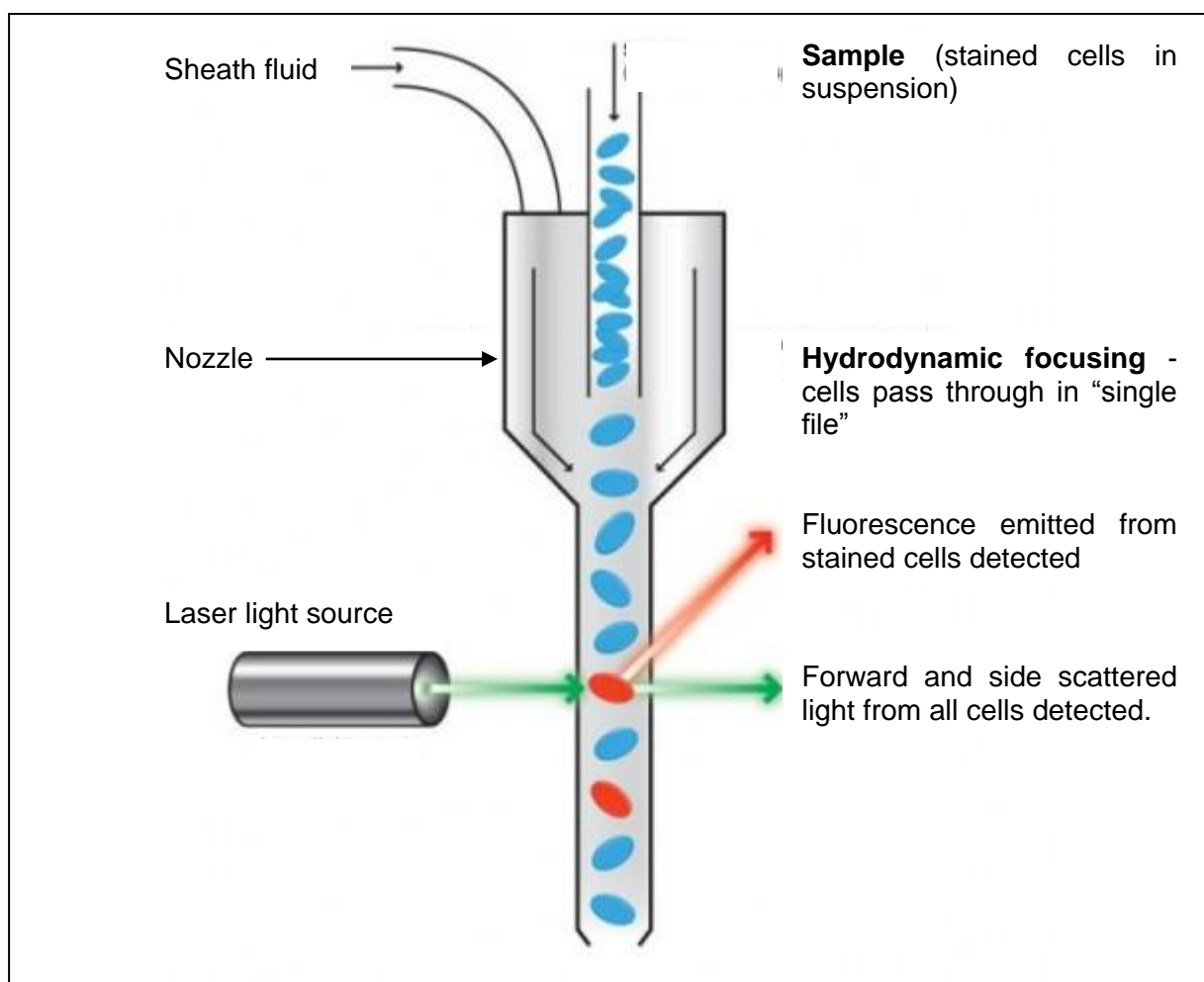


Figure C.1: Basics of flow cytometry (Adapted from Sony Insider, 2010)

Each detector analyses the fluctuating brightness and in turn deduces various facts about the physical and chemical structure of individual particles. Forward scatter correlates with the cell size/volume while SSC depends on the inner complexity (nucleus shape, amount and type of

cytoplasmic granules or the membrane roughness) of the cell. Some commonly measured parameters are volume and morphological complexity of cells, DNA and RNA, chromosome analysis and sorting, proteins and cell surface antigens to name a few (Shapiro, 2003:3).

Fluorescence activated cell sorting (FACS) is a special type of flow cytometry in which the desired population of cells is labelled with a fluorescent marker and suspended in solution. Fluorescent cells are detected by the laser beam and the information displayed on a screen as a graph. The cells are then sorted by passing them between two oppositely charged plates and similarly charged cells are collected together (Doyle & Griffiths, 2000:35). However, in this study only the fluorescence emission by the cells needed to be detected so simple flow cytometry was used.

C.2 Selection of an appropriate cell line

There are parameters apart from specific functional requirements that must be taken into consideration when selecting an appropriate cell line for a particular study. These include, but are not limited to finite vs. continuous cell line; normal vs. transformed cell line; human vs. non-human; growth characteristics and availability (Freshney, 2000:181). In order to meet up with the scope of the study the cell line had to be 1) from human tissue, 2) tumorigenic and 3) originating from the skin. The cell line had to be human-derived because the *in vitro* effect of various concentrations of 5-fluorouracil on cutaneous cancer and related illnesses in humans was under investigation. A continuous human melanoma cell line of skin origin was selected for the study. The cell line is designated as A375 and its ATCC number is CRL1619.

C.3 Selection of appropriate drug concentrations for use

Tsuji & Karasek (1986:474) conducted a study to determine the differential effects of 5-fluorouracil on human skin melanocytes and malignant melanoma *in vitro*. They exposed the cells to 1.92×10^{-5} - 3.84×10^{-4} M 5-fluorouracil for 7 or 14 days and observed that all the melanoma cells died by the end of 5 weeks. This concentration of 5-fluorouracil translates to 2.50 - 49.95 µg/ml. This concentration range was used as a guideline. It was decided to utilise three concentrations within the lower end of the range and one concentration below the minimum concentration but the API exposure time was 24 h instead of 7 or 14 days. This was done in order to determine the concentrations of 5-fluorouracil that result in the required response within 24 h *in vitro*. Concentrations of 1.67; 3.33; 6.67 and 13.33 µg/ml 5-fluorouracil were deemed as appropriate for this study and utilised for the *in vitro* drug efficacy tests. The drug efficacy tests were conducted utilising the different API concentrations in solution, non-Pheroid™ and Pheroid™ lotions in order to also determine variations due to the differing drug delivery systems.

C.4 Non-assay experimental procedures

Cell cultivation, feeding, freezing, seeding and treating the cells are the experimental procedures that were done prior to the analytical assay. All experiments were carried out under aseptic conditions in the laboratory for applied molecular biology (LAMB) of the North-West University (NWU). Extra care was taken to avoid contamination of the cells in culture.

C.4.1 Materials

The plastic consumables were purchased from Separations (Johannesburg) in sterile packaging and only handled in the laminar air flow cabinet to maintain sterility. The reagents that were used in the study have been illustrated in Table C.1.

Table C.1: Materials that were used during the *in vitro* drug efficacy study

Material	Storage	Supplier	Batch #
Human melanoma cell line (skin origin): A375	Liquid nitrogen vapour phase	ATCC, USA	-
Dulbecco's Modified Eagle's Medium (DMEM)	2 - 8 °C	Sigma-Aldrich, Germany	LKNBC1537
Foetal bovine serum (FBS)	-20 °C	Sigma-Aldrich, Germany	L019K3396
Pen-Strep (PS)	-20 °C	Sigma-Aldrich, Germany	L08M0850
Trypsin 25% EDTA	Frozen	Gibco, USA	L645445
Phosphate buffered saline 0.0067 M (PBS)	15 - 30 °C	Thermo-Scientific, USA	AWG16132
Dimethylsulphoxide (DMSO)	Room temperature	Merck, South Africa	1025926
Ethanol 96%	Room temperature	Merck, Germany	K42193571
Vybrant® apoptosis assay kit #1	2-6 °C	Invitrogen, USA	487373

C.4.2 Cultivation of cells

The A375 cells were cultivated in 75 cm² flasks and DMEM, 10% FBS and 1% Pen-Strep. The cells were viewed under an inverted microscope everyday to observe their growth and check for any irregularities. Fresh culture media were added to the cells every day to ensure that the cells had a constant and sufficient supply of nutrients and the cells were split every second day when ~80% confluency was reached.

Cell scrapers or trypsin (0.25%) were used to detach the cells from the flask wall. The detached cells from each flask were split into two new flasks which were labelled with the passage number, date and designation of cells. The cells were maintained at 37 °C in an atmosphere of

5% CO₂. Every two weeks cells were frozen in DMEM-DMSO (7.5%) in order to have a backup supply of cells.

C.4.3 Determination of cell viability

Experiments were conducted on cells that were at least 95% viable. The trypan blue dye exclusion test was used to determine cell viability. The concept of the test is that viable cells do not take up trypan blue, whereas non-viable cells are permeable to trypan blue and get stained on exposure to it (Griffiths, 2000:20). Cells are loaded into a chamber on a haemocytometer slide and five squares (four corners and one centre) are counted in each chamber (Figure C.2). Only cells touching the middle line of the triple line on the top and left of the squares are counted (Phelan, 1998:7).

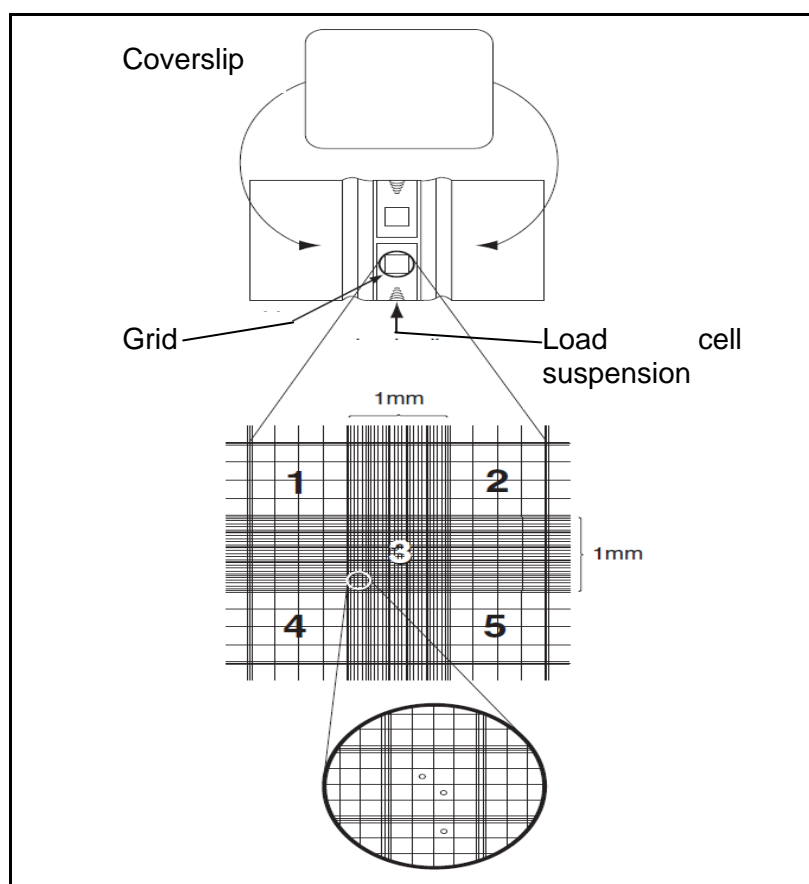


Figure C.2: Generalised haemocytometer slide (Adapted from Phelan, 1998:6)

Cell viability was determined with the Countess automated cell counter (haemocytometer). Firstly, a uniform cell suspension was obtained by repeatedly pipetting and aspirating the cells to break cell clumps and disperse the cells subsequent to detachment. To determine cell density and viability 10 µl of 0.4% trypan blue was mixed with 10 µl of the cell suspension in an eppendorf[®] microtube. The cells were left for ~3 min for the cells to stain and loaded onto a

chamber of a Countess slide for examination. The instrument then gave a reading for the total number of cells, viable cells, dead cells and the percentage viability.

The percentage viable cells are calculated as follows:

$$\% \text{viable cells} = \frac{\text{number of unstained cells}}{\text{total number of cells}} \times 100 \quad \text{Equation C.1}$$

C.4.4 Procedure for preparing and treating the cells

A cell suspension containing 1×10^5 cells/ml was prepared for the experiments and seeded into 6 or 24-well multiwell culture plates. The cell suspension was seeded at volumes of 2 ml or 500 μ l per well, respectively and placed in the 37 °C incubator for 24 h. Preparation of the cell suspension and seeding of the wells was done three days prior to the day of the experiment. This counted as day one. On the second day the cells were treated with 1 ml or 250 μ l of the test substances and left to incubate for another 24 h. On day three the cells were either counted using a haemocytometer or analysed by flow cytometry.

C.4.5 Optimisation of the formulation concentration

The topical formulation (lotion) was maintained for the *in vitro* drug efficacy tests because an API's diffusive properties (through the cell membrane) may vary with formulation variables. However, prior to starting with the assays the optimum lotion concentration had to be determined. The lotion and Pheroid™ lotion both had oil components which could have been potentially harmful to the cells and in turn resulted in cell death (the terms lotion and non-Pheroid™ lotion have been used interchangeably in the text). It was decided to dilute the placebo lotion and the placebo Pheroid™ lotion and note the effects of the different placebo dilutions on cell growth. An appropriately selected dilution of the lotions together with API was utilised in the assays to rule out cell death due to the effects of the lotion components and not the API. Any other potentially problematic issues with the experiment were detected during the optimisation.

C.4.5.1 Day one

On day one, 2 ml of 1×10^5 cells/ml were seeded into 6-well multiwell culture plates and incubated at 37 °C for 24 h for the cells to adhere.

C.4.5.2 Day two

Placebo lotions (Pheroid™ and non-Pheroid™) were formulated for the optimisation and dilutions prepared. The lotions were appropriately diluted with PBS and sonicated in order to end up with 100.0%; 50.0%; 25.0%; 12.5%; 1.0% and 0.1% of the original lotion formulations.

Ethanol, PBS, a 0.5% 5-fluorouracil solution and complete culture media were used as control test solutions. The treatment solutions (1 ml) were added to a well each and the culture plates placed back in the incubator. Experiments were conducted in triplicate. The 12.5 - 100.0% lotion dilutions were relatively viscous and did not mix well with the culture media already present in the wells thus giving erratic and unreliable results. The wells with 1.0% and 0.1% placebo dilutions; ethanol; PBS and complete culture media gave reproducible results. Phosphate buffered saline was added as a treatment solution so as to rule out the possibility of PBS in the dilutions having a negative effect on cell growth *in vitro*. Treatment with a solution of 5-fluorouracil was also necessary in order to validate the cytotoxic potential of 5-fluorouracil *in vitro*.

C.4.5.3 Day three

The culture media-treatment solution was aspirated from the wells. Fresh complete culture media (2 ml) was added to each well and the cells detached from the well surface using cell scrapers. The cells were pipetted and aspirated a few times to break cell clumps. The method as described in Section C.4.3 was used to determine the cell viability subsequent to the different treatments.

C.4.5.4 Results and discussion of the optimisation

The viability readings that were recorded by the haemocytometer are shown in Table C.2.

Table C.2: Results for optimisation of the lotion concentrations

TREATMENT TYPE	PERCENTAGE VIABILITY (%)			MEAN	SD*
	Repeat 1	Repeat 2	Repeat 3		
1.0% dilution of placebo lotion	93	85	90	89.3	4.0
1.0% dilution of placebo Pheroid™ lotion	65	62	33	53.3	17.7
0.1% dilution of placebo lotion	93	96	94	94.3	1.5
0.1% dilution of placebo Pheroid™ lotion	94	96	95	95.0	1.0
PBS	96	87	97	93.3	5.5
Complete culture media	91	94	96	93.7	2.5
0.5% 5-fluorouracil solution	17	24	17	19.3	4.0
Ethanol control	37	27	28	30.7	5.5

* SD - Standard deviation

It is evident from Table C.2 that the 0.1% dilutions of both the placebo lotion and placebo Pheroid™ lotion resulted in a percentage viability that was within proximity of the media and PBS controls, leading to the conclusion that they did not negatively affect cell growth *in vitro*.

The 1.0% dilution of the placebo lotion did not negatively affect cell growth either but treatment with the 1.0% dilution of the placebo Pheroid™ lotion negatively affected cell growth. The difference may have been due to the extra ingredients incorporated in Pheroid™ which increase its oiliness and lower the accessibility of oxygen to the cells. However, for the sake of uniformity the 0.1% lotion dilution was decided upon for the assays. The PBS and complete culture media controls had high percentage viability values demonstrating that the PBS and the media did not have a negative effect on the A375 cells after the 24 h of treatment. Ethanol and 5-fluorouracil solution (0.5%) treatments consequentially resulted in reduced viability. The optimisation process established reliable controls for the experimental procedure.

C.5 Apoptosis analytical method

The Single Channel Annexin V/ Dead Cell Apoptosis kit with Alexa Fluor® 488 annexin V and SYTOX® Green for Flow Cytometry (Invitrogen) was used to separate live, apoptotic and dead cells. Normal live cells have a negatively charged phospholipid, phosphatidylserine (PS), on the cytoplasmic surface of the cell membrane. In the early phases of apoptosis, PS is translocated to the external surface of the cell membrane thus exposing it to the external cellular environment. Annexin V is a Ca²⁺ dependent phospholipid binding protein which was originally discovered as a vascular anticoagulant. Annexin V has a high affinity for PS and is labelled with a fluorophore or biotin for use in apoptosis assays for flow cytometry (Vermes, 1995:40).

C.5.1 Apoptosis assay method

C.5.1.1 Day one

A375 cells (1×10^5 /ml) were seeded into 24-well multiwell culture plates. Each well received 500 µl of the cell suspension and the culture plates were placed into the 37 °C incubator for 24 h.

C.5.1.2 Day two

Lotions and Pheroid™ lotions with original concentrations of 0.5; 1.0; 2.0 and 4.0% m/m were formulated and diluted by 1 000 (to 0.1%) to give 5; 10; 20 and 40 µg/ml dilutions. The pre-diluted lotions were of the same concentrations as the lotions used in the diffusion studies and were formulated according to the method described in Appendix B. Twenty-four hours after seeding the multiwell culture plates with A375 cells, three wells each were treated with 250 µl of one of the following:

- ❖ 5 µg/ml lotion
- ❖ 10 µg/ml lotion
- ❖ 5 µg/ml solution
- ❖ 10 µg/ml solution

- ❖ 20 µg/ml lotion
- ❖ 40 µg/ml lotion
- ❖ 5 µg/ml Pheroid™ lotion
- ❖ 10 µg/ml Pheroid™ lotion
- ❖ 20 µg/ml Pheroid™ lotion
- ❖ 40 µg/ml Pheroid™ lotion
- ❖ 20 µg/ml solution
- ❖ 40 µg/ml solution
- ❖ PBS
- ❖ Non-Pheroid™ placebo lotion
- ❖ Pheroid™ placebo lotion
- ❖ Ethanol (96%)

The final concentration of the treatments became 1.67; 3.33; 6.67 and 13.3 µg/ml instead of 5; 10; 20 and 40 µg/ml, respectively because of the dilution rendered by the 500 µl of culture media already present in the wells. The effect of 16 different treatments was investigated in triplicate. This gave a total of 48 wells.

The annexin binding buffer and the SYTOX® working solution from the assay the kit were prepared a day before the assay was conducted. Annexin binding buffer (5 ml) was mixed with 20 ml of milli-Q water in a 50 ml cell culture tube to make 1 x annexin binding buffer which was stored between 2 - 6 °C. A working solution of 5 µM SYTOX® Green dye was prepared by diluting 10 µl of 50 µM SYTOX® Green stock solution with 90 µl of the prepared 1 x annexin binding buffer. The 5 µM SYTOX® Green dye was stored in an eppendorf® microtube at -20 °C.

C.5.1.3 Day three

The multiwell cell culture plates were removed from the incubator and the cells centrifuged for 5 min at 800 rpm. The supernatant was discarded by pipetting after removing the cells from the centrifuge. Cells in each well were rinsed with 250 µl trypsin-PBS (0.25%) and it was immediately discarded from the wells. Trypsin-PBS (500 µl) was added to each well and the cells incubated for 10 min in order to allow the cells to detach from the walls of the cell culture plates. The cells were centrifuged again at 800 rpm for 5 min and the trypsin-PBS supernatant discarded. The following steps were performed in the absence of artificial light sources because the Alexa Fluor® 488 annexin V and SYTOX® Green components are photosensitive.

The cells were resuspended in 100 µl of 1 x annexin binding buffer (prepared on day 2) and transferred from the wells to respectively labelled round-bottom tubes.

- 1) Alexa Fluor® 488 annexin V (5 µl) and 5 µM SYTOX® Green working solution (1 µl) were added to each tube.
- 2) The cells were incubated at room temperature for 15 min.
- 3) After the incubation period 400 µl of 1 x annexin binding buffer was added to each tube and the contents mixed gently.

The samples were placed on ice and analysed with a BD FACSCalibur (BD Biosciences) by measuring the fluorescence emission at 530 nm. The cells were analysed with FCSExpress Software version 3 (DeNovo Software).

Table C.3: Concise summary of methods followed during the formulation optimisation experiments and apoptosis assay

	Day 1	Day 2	Day 3
Optimisation	<ol style="list-style-type: none"> 1. Seeded 2 ml of 1×10^5 cells/ml in 6-well multiwell culture plates. Left for 24 h for adherence to occur. 	<ol style="list-style-type: none"> 1. Formulated placebo lotion and placebo Pheroid™ lotion as described in Appendix B. 2. Diluted formulated lotions with PBS to give 100.0%; 50.0%; 25.0%; 12.5%; 1.0% and 0.1% dilutions of original formulations. 3. Treated cells with 1 ml of treatment solutions – see Section C.4.5.2 for the treatments that were used. 	<ol style="list-style-type: none"> 1. Detached cells using cell scrapers and counted the cells on the haemocytometer using the trypan blue dye exclusion test see Section C.4.3.
Assay	<ol style="list-style-type: none"> 1. Seeded 500 µl of 1×10^5 cells/ml in 24-well multiwell culture plates. 2. Left for 24 h for adherence to occur. 	<ol style="list-style-type: none"> 1. Formulated 0.5%, 1.0%, 2.0% and 4.0% (5; 10; 20 and 40 mg/ml) 5-fluorouracil lotions and Pheroid™ lotions. Placebo lotions were also formulated. Method is described in Appendix B. 2. Diluted all the formulations by 1 000 resulting in 5; 10; 20 and 40 µg/ml solutions of 5-fluorouracil lotions and Pheroid™ lotions. 3. Treated cells with 250 µl of treatment solutions – see Section C.5.1.2 for full list of treatments used. (Due to the 500 µl of media that was already present the concentration of API that the cells were subjected to was 1.67; 3.33; 6.67 and 13.33 µg/ml). 4. Prepared 1 x annexin binding buffer and working solution of 5 µM SYTOX® Green dye. 	<ol style="list-style-type: none"> 1. Detached cells using 0.25% trypsin-PBS. 2. Performed the apoptosis assay and analysed the cells using flow cytometry – see Section C.5.1.3.

C.5.2 Assay results and discussion

The first step in the data analysis is the identification of the region of interest and to remove debris and possible other interferences that may negatively influence the data or misrepresent the data and present inaccuracies. The cells are plotted on a FCS/SSC dot plot (Figure C.3). The dot plot differentiates cell populations based on size (FCS) and complexity or granularity (SSC). In Figure C.3 a representative plot of the A375 cells with a random treatment (PBS) is shown and a gate is drawn (gate 1) to select the region of interest. A gate is a numerical or graphical boundary that is selected based on cell populations that can be discriminated; it can be used to define the characteristics of cells for further analysis (BD Biosciences, 2000:29). However, this region is based on visual inspection of the plot only and needs confirmation. The data are further analysed by using a histogram (Figure C.4). The representative histogram of fluorescence intensity (FL1-H) vs. number of events in Figure C.4a illustrates the ungated data, whereas Figure C.4b shows the gated data. After gating, the histogram of the gated events allows us to view the data more clearly for the cell population, and the selected parameter (FL1-H) can be analysed (Figure C.4b). By using the marker of the histogram the amount of cells can exactly be matched to the gate set in the dot plot and the region of interest is confirmed and used for further analysis.

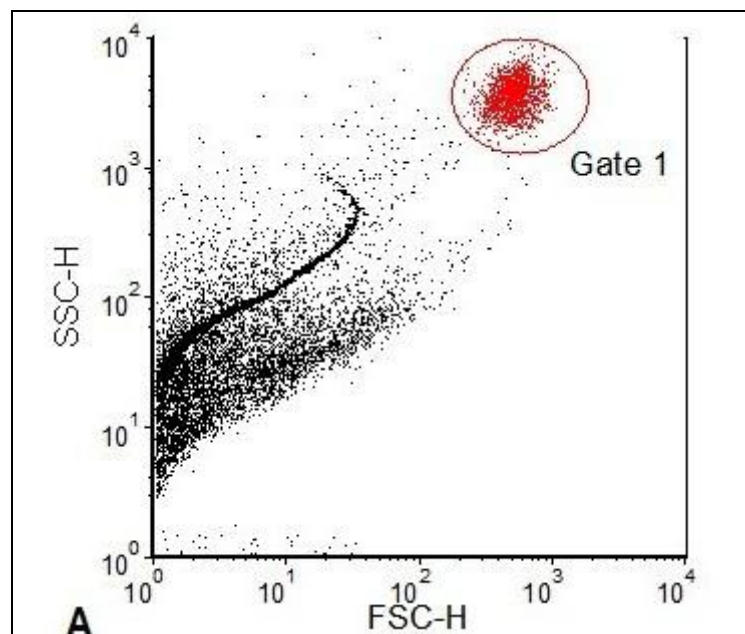


Figure C.3: Representative dot plot of the A375 cells in PBS illustrating the gated cell population (Gate 1; circle) of interest that was further used in all analyses

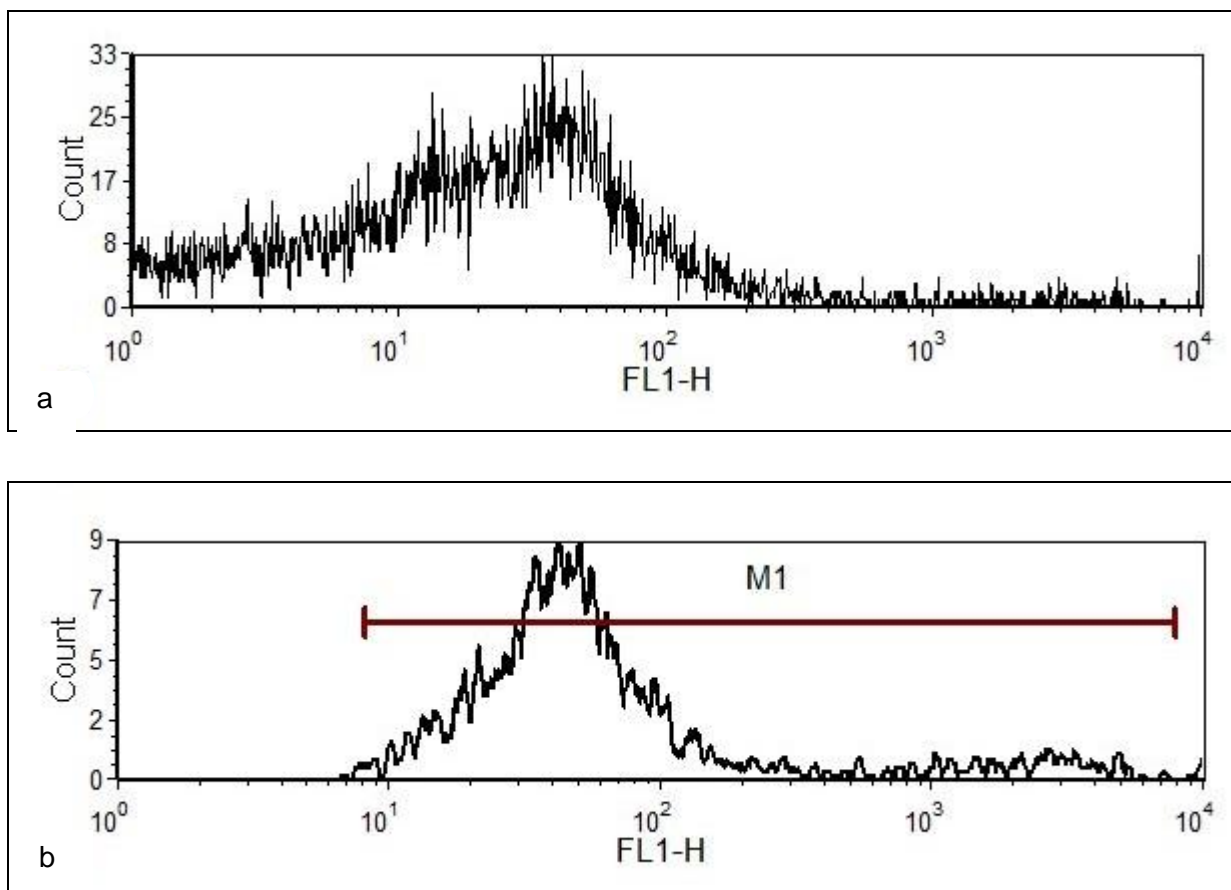


Figure C.4: Representative histograms (FL1-H) of the A375 cells in PBS: a) the cell population in Figure C.3 prior to gating and b) the gated cell population in Figure C.3. The marker (M1) used for validation of the region of interest is also shown.

Figure C.5 gives a representation of a histogram with the lotion, Pheroid™ lotion, PBS and ethanol controls. On analysis of the controls, the cells separated into three groups: live cells with low levels of fluorescence ($FL1-H < 10^1$), apoptotic cells with medium fluorescence ($10^1 < FL1-H < 10^4$) and dead cells with high intensity green fluorescence due to staining by SYTOX® green ($FL1-H > 10^4$). The placebo lotions showed low levels of fluorescence ($FL1-H < 10^1$) reflecting that there was a high proportion of live cells present after treatment when compared with the PBS (Figure C.5). Of these, the placebo Pheroid™ lotion showed the largest peak, followed by the PBS and lastly the placebo lotion. The ethanol control, however, resulted in medium density fluorescence ($10^3 < FL1-H$) which was characteristic of apoptosis. There was no fluorescence in the live range after treatment with ethanol and this showed that ethanol resulted in apoptosis and death of most of the treated cells.

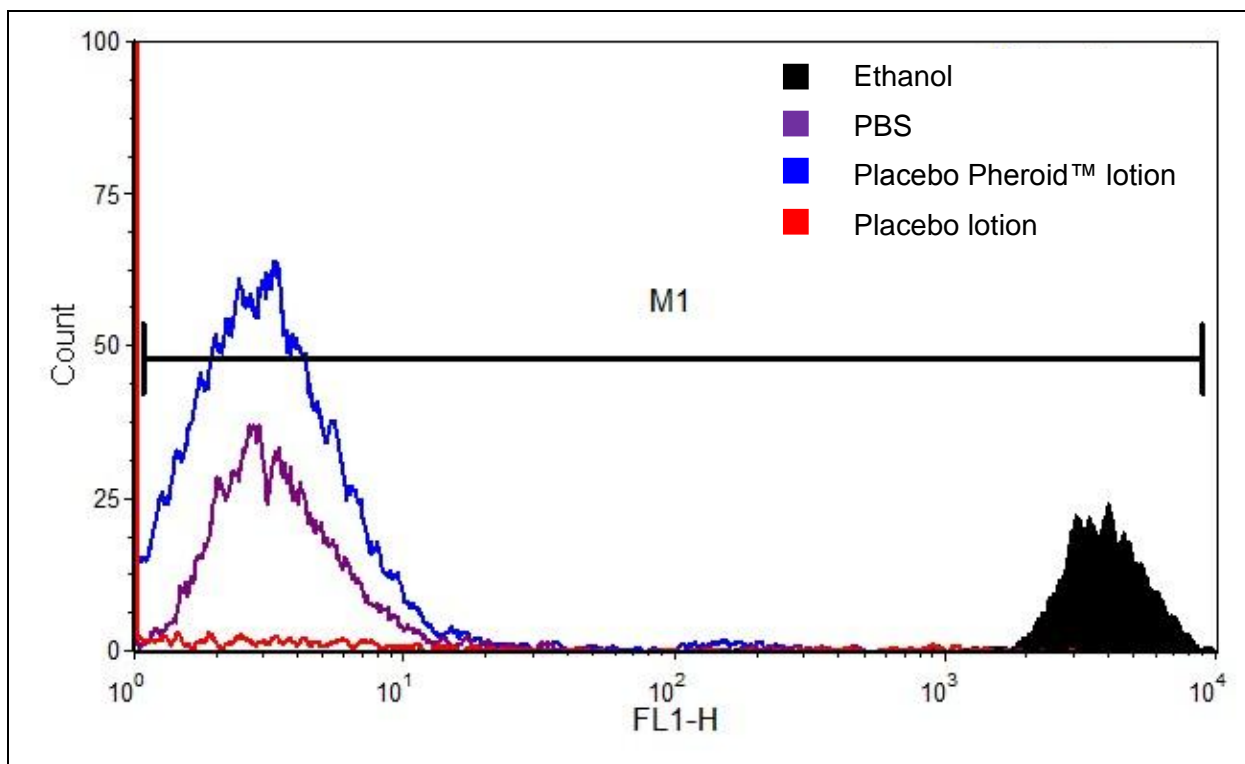


Figure C.5: A representative overlay histogram (FL1-H) of the A375 cells with the control treatments. Ethanol (positive control) is the shaded histogram in the 10^3 to 10^4 region, whereas the PBS, placebo Pheroid lotion and placebo lotion are in the unshaded 10^0 to 10^1 region.

The treatment samples all produced some fluorescence within the spectrum of interest. The API solution had a high event count at low fluorescence levels which indicated the presence of live cells within this cell population. Lotion and Pheroid™ lotion samples mainly resulted in medium intensity fluorescence which showed that apoptosis had occurred in the cell population. Due to formulation effects among other things, the rate and extent of penetration of 5-fluorouracil into the cells differed and may have resulted in varying rates of apoptotic cell death. This translated to the differences in fluorescence intensity shown in Figure C.6. Figure C.6 is a histogram representation of the fluorescence emitted by the different treatments at a constant concentration ($13.33 \mu\text{g/ml}$). The $13.33 \mu\text{g/ml}$ 5-fluorouracil solution resulted in fluorescence mainly in the live range ($\text{FL1-H} < 10^1$), while the $13.33 \mu\text{g/ml}$ lotion and Pheroid™ lotion mainly resulted in fluorescence in the apoptotic range ($10^1 < \text{FL1-H} < 10^4$) with the Pheroid™ lotion giving a smaller peak. Figure C.6 is a representative histogram of the fluorescence emitted after 24 h treatment with 5-fluorouracil in the diluted dosage form.

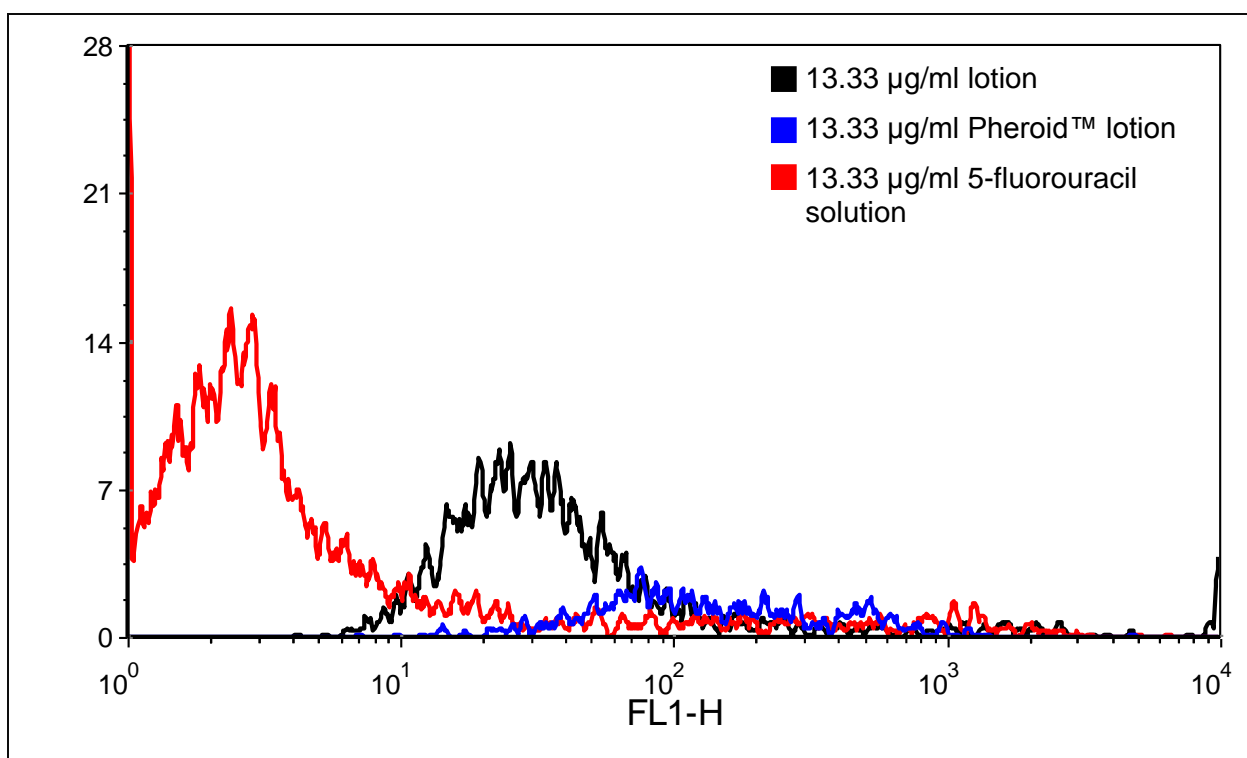


Figure C.6: Overlay histogram (FL1-H) with representations of the A375 cells treated with the 13.33 µg/ml lotion, Pheroid™ lotion and 5-fluorouracil solution

The flow cytometry analysis produces typical statistical values after the analysis and the analyst then decides what is relevant to their study. In this study it was decided to use the geometric mean fluorescence intensity (MFI). The MFI for each treatment was averaged and expressed as a percentage of the positive control (ethanol) MFI.

Table C.4: The average MFI expressed as a percentage of the positive control and the standard deviation (SD)

Treatment sample	Average %MFI	SD
13.33 µg/ml Lotion	1.91	0.27
6.67 µg/ml Lotion	1.33	0.12
3.33 µg/ml Lotion	1.04	0.33
1.67 µg/ml Lotion	0.71	0.20
13.33 µg/ml API solution	1.66	0.42
6.67 µg/ml API solution	0.74	0.11
3.33 µg/ml API solution	0.63	0.15
1.67 µg/ml API solution	0.65	0.08
13.33 µg/ml Pheroid™ lotion	5.36	0.77
6.67 µg/ml Pheroid™ lotion	2.77	1.62
3.33 µg/ml Pheroid™ lotion	0.70	0.09
1.67 µg/ml Pheroid™ lotion	0.91	0.12

The results in Table C.4 reflect that the early signs of apoptosis were evident after the 24 h incubation period with treatment. The highest %MFI was detected in the cells treated with the 13.33 µg/ml Pheroid™ lotion (5.36%), followed by 6.67 µg/ml Pheroid™ lotion (2.77%), 13.33 µg/ml lotion (1.91%), 13.33 µg/ml solution (1.66%), 6.67 µg/ml lotion (1.33%), 3.33 µg/ml lotion (1.04%), 1.67 µg/ml Pheroid™ lotion (0.91%), 6.67 µg/ml solution (0.74%), 1.67 µg/ml lotion (0.71%), 3.33 µg/ml Pheroid™ lotion (0.70%), 1.67 µg/ml solution (0.65%) and 3.33 µg/ml solution (0.63%) in that order.

All the Pheroid™ lotion treatments except the 3.33 µg/ml treatment resulted in higher %MFI values than the corresponding lotion and solution treatments of the same concentration. The solution treatments produced the lowest %MFI in the cells in comparison to the lotion and Pheroid™ lotion treatments at the same concentrations. This concurs with what was observed on the histogram representation in Figure C.6.

The %MFI produced by the lotion treated cells increased with increase in concentration in an almost linear manner. The Pheroid™ lotion treatments resulted in a lower %MFI in the 3.33 µg/ml treatment than in the 1.67 µg/ml. From the 3.33 µg/ml treatment to the 13.33 µg/ml treatment of the Pheroid™ lotion the %MFI increased with concentration. A trend similar to that of the Pheroid™ lotions was observed with the API solutions.

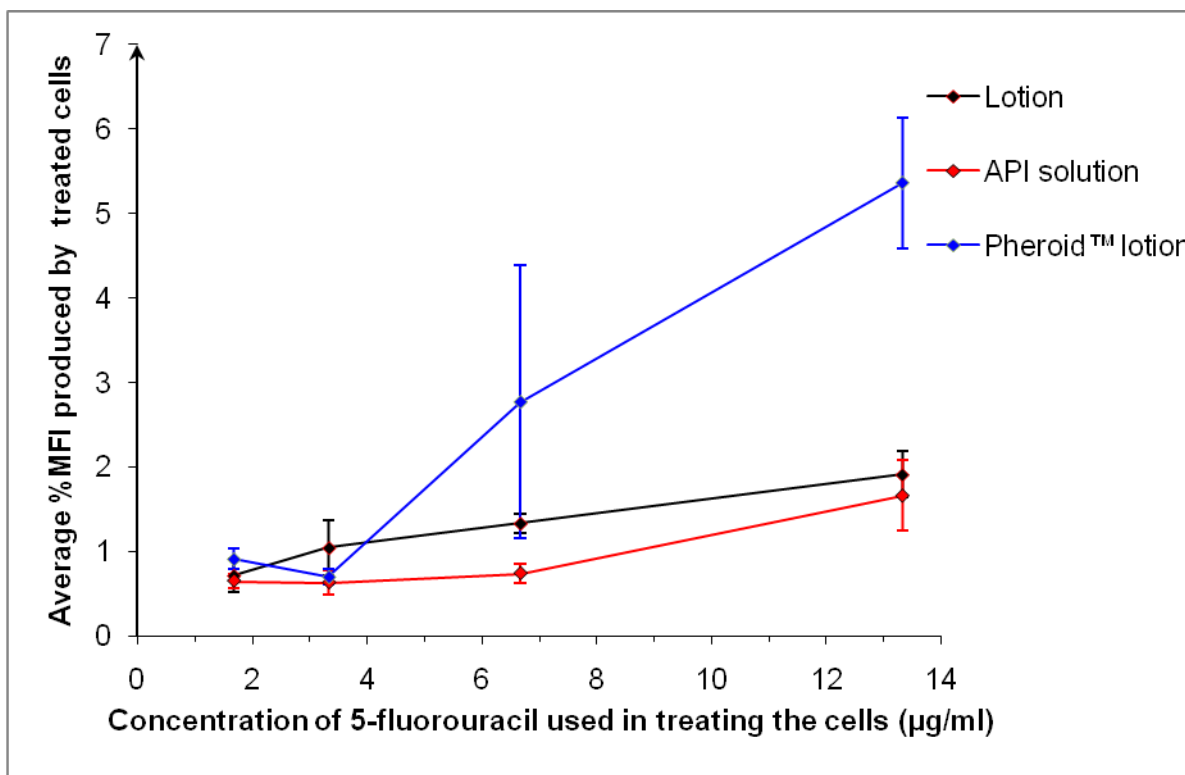


Figure C.7: Line graph showing comparisons between the %MFI induced in the A375 cells treated with 1) the non-Pheroid™ lotion; 2) the API solution and 3) Pheroid™ lotion

On Figure C.7 we see that as the API concentration doubled the %MFI did not in turn necessarily double. The Pheroid™ lotion treatments, however, showed a relatively linear increase in response (%MFI) as the concentration increased from 3.33 - 13.33 µg/ml. With Pheroid™ the effect of increasing concentration became clearer at the higher concentrations than at the lower concentrations. Treatment with the lotions seemed to give a relatively linear relationship between the API concentration and the response observed (%MFI) while no clear relationship could be deduced for the solution treatments. These results confirmed that 5-fluorouracil does result in apoptotic cell death and is effective against human melanoma (A375) cells. At 6.67 µg/ml and 13.33 µg/ml the Pheroid™ treatments resulted in a much higher response than the lotion and solution treatments. This may be due to the fact that Pheroid™ are composed of fatty acids and therefore an affinity exists between Pheroid™ and cell membranes, resulting in more effective and fast uptake of Pheroid™ by cells Grobler (2004:6). The apoptotic effect of 5-fluorouracil clearly differs according to the vehicle that the API has been incorporated into and this needs to be researched further.

C.6 Statistical analysis

Any comparisons made in scientific research are bound to have differences but the differences can be due to real effects, random variation or both. It is the job of the researcher to investigate and decide how much variation should be ascribed to chance so that the remaining variation can be assumed to be due to a real effect (Machin *et al.*, 2007:3). Statistical analysis is a tool used to test the significance of variations obtained. A statistically significant result addresses the question of whether or not the treatments truly vary but it does not necessarily apply to the practical magnitude of the treatment effects (Bolton, 1990:130). In this study the statistical and practical significance was measured but attention was mainly on the practical significance of the treatment effects.

The wide varieties of statistical techniques are grouped into parametric and non-parametric techniques. Parametric statistics are more powerful but they make assumptions about the data that are more stringent. The key assumptions of parametric tests are: 1) the data should be normally distributed and 2) the variances of the groups under analysis should be equal. Non-parametric statistics on the other hand have less stringent assumptions and therefore tend to be not as powerful as parametric statistics. The weakness of non-parametric statistics results in less sensitivity in detecting a relationship or difference among groups (Pallant, 2005:102). However, both non-parametric and parametric statistical techniques were applied in this study.

C.6.1 Statistical methods

Statistical analysis was done on the results of the drug efficacy test but the results may be unreliable because there were only three observations per group. Firstly, the descriptive statistics for each group were obtained; these include the mean for normal data, median for non-normal and standard deviation. Several tests were applied to test the normality of the data. The Shapiro-Wilk test was used to determine the normality of the data. Normality of the data were described as normally distributed if $p > 0.05$. Quantile-quantile (q-q) plots were also drawn to determine the normality of the data. If the data are normally distributed a relatively straight line should be obtained.

After exploration of the data it was decided that the data were sufficiently normal to perform a robust test. An omnibus test was performed which included the one-way analysis of variance (ANOVA) and the robust tests (the Welch test and the Brown-Forsythe test). These three tests tell us if there are differences between groups but they do not indicate between which specific groups the differences lie. To uncover the individual differences the Games-Howell and the Tukey's B post-hoc tests were conducted. In order to determine the practical significance of the differences between groups the effect size (Cohen's d-value) between each treatment was calculated. It was advantageous to calculate the effect size because these measurements are

independent of sample size and this study had only three observations per group. Effect size refers to a family of indices that measure the magnitude of a treatment effect (Becker, 2000).

C.6.2 Statistical analysis of results

Table C.5: Descriptive statistics of the measured MFI

Treatment	Mean MFI	Median MFI	SD
1.67 µg/ml API solution	13.17	13.05	2.01
1.67 µg/ml lotion	14.41	15.53	4.99
1.67 µg/ml Pheroid™ lotion	18.47	19.65	2.96
3.33 µg/ml API solution	12.93	11.02	3.70
3.33 µg/ml lotion	21.24	22.33	8.21
3.33 µg/ml Pheroid™ lotion	14.30	15.17	2.21
6.67 µg/ml API solution	15.08	15.96	2.65
6.67 µg/ml lotion	27.14	27.27	3.05
6.67 µg/ml Pheroid™ lotion	56.47	59.91	40.50
13.33 µg/ml API solution	33.92	36.65	10.43
13.33 µg/ml lotion	55.61	38.57	6.74
13.33 µg/ml Pheroid™ lotion	109.20	102.45	19.14
Ethanol (+control)	2038.00	1602.30	1460.36

Mean fluorescence intensity was the primary outcome that was analysed statistically while the results discussion utilised MFI as a percentage of the positive control (%MFI). Table C.5 shows the calculated mean, median and SD of the MFI values. However, the mean MFI was used for the statistical analysis because the data followed a relatively normal distribution.

Omnibus tests consisting of the ANOVA, Welch and Brown-Forsythe tests were conducted to determine if the mean MFI levels differ across the treatment groups for the human melanoma cells. A significant difference between groups was reflected by a p-value that is less than 0.05 ($p < 0.05$). According to the ANOVA and Welch tests there was a significant difference between groups with both tests giving p-values < 0.05 . The Brown-Forsythe test, however, gave a p-value of 0.16 which meant that there were no significant differences. The Games-Howell post-hoc test showed that there were significant differences between 1) the 6.67 µg/ml lotion and the PBS control ($p=0.04$); 2) the 6.67 µg/ml lotion and the placebo lotion control ($p=0.021$) and 3) the 6.67 µg/ml lotion and the placebo Pheroid™ lotion control ($p=0.049$). The main focus was however on comparisons between drug delivery vehicles at the same concentrations of 5-fluorouracil (See Table C.6). Tukeys B test identified two groups. The ethanol treatment was in one group and the rest of the treatments in another group. Since the assumptions for ANOVA were not 100% this may have contributed to the tests giving varied conclusions.

Effect sizes were calculated to determine the practical significance of differences between treatments. An absolute value, *d*, is calculated to represent practical significance. If *d* is bigger than 0.2 but less than 0.5 the difference is not practically significant ($0.2 < |d| < 0.5$), if *d* is bigger than 0.5 but less than 0.8 there is a practically visible effect or difference in the mean ($0.5 < |d| < 0.8$) and if *d* is bigger than 0.8 ($|d| > 0.8$) there is a practical significance in the difference of the mean. Comparisons were made among treatments of the same concentrations (Table C.6) and among treatments of the same type of formulation. The effect size test identified either a practically visible difference in the mean or a practical significance in all the comparisons except the comparisons between 1) the 1.67 µg/ml lotion and 1.67 µg/ml solution; 2) the 3.33 µg/ml solution and 3.33 µg/ml Pheroid™ lotion and 3) the 13.33 µg/ml solution and 13.33 µg/ml lotion. Generally these results show that a difference in concentration between formulations of the same type produces effects that are practically significant. The same is true of the effects of a change in formulation with a constant API concentration.

Table C.6: Results of the Games-Howell test and effect size determinations for the drug efficacy tests

Comparisons	p-value*	d-value
1.67 µg/ml API solution vs. 1.67 µg/ml lotion	1.00	0.25 [#]
1.67 µg/ml API solution vs. 1.67 µg/ml Pheroid™ lotion	0.56	1.79
1.67 µg/ml lotion vs. 1.67 µg/ml Pheroid™ lotion	0.98	0.81
3.33 µg/ml API solution vs. 3.33 µg/ml lotion	0.89	1.01
3.33 µg/ml API solution vs. 3.33 µg/ml Pheroid™ lotion	1.00	0.37 [#]
3.33 µg/ml lotion vs. 3.33 µg/ml Pheroid™ lotion	0.93	0.85
6.67 µg/ml API solution vs. 6.67 µg/ml lotion	0.10	3.95
6.67 µg/ml API solution vs. 6.67 µg/ml Pheroid™ lotion	0.83	1.02
6.67 µg/ml lotion vs. 6.67 µg/ml Pheroid™ lotion	0.96	0.72
13.3 µg/ml API solution vs. 13.3 µg/ml lotion	1.00	0.47 [#]
13.3 µg/ml API solution vs. 13.3 µg/ml Pheroid™ lotion	0.09	3.94
13.3 µg/ml lotion vs. 13.3 µg/ml Pheroid™ lotion	0.12	3.68

- $|d| < 0.5$ (no practical significance in the difference of means)

* - p is significant at 0.05 level

C.7 Conclusion

The results obtained clearly show that 5-fluorouracil (1.67 - 13.33 µg/ml) can induce apoptosis in human melanoma (A375) cells *in vitro*. The optimum concentration for apoptosis induction, however, has not been determined. The results obtained suggest the enhancement of the *in vitro* effect of 5-fluorouracil by Pheroid™. Further research shall need to be done with a wider variation of 5-fluorouracil concentrations to confirm and characterise the effect of Pheroid™ on *in vitro* apoptosis induction by 5-fluorouracil. A combination of different measurements and

techniques must also be used in future studies because no single parameter defines apoptosis in all systems. In this study it was shown in the skin diffusion studies that Pheroid™ enhances the delivery of 5-fluorouracil through the skin, to the stratum corneum-epidermis and to the epidermis-dermis (see Appendix D). The combination of these results could result in a Pheroid™ formulation that delivers the optimum amount of API to the site of action with reduced risk of side effects due to the convenient enhancing effects of Pheroid™.

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APPENDIX D

FRANZ CELL DIFFUSION STUDIES OF FORMULATIONS CONTAINING 5-FLUOROURACIL

D.1 Introduction

Knowledge of percutaneous permeation and absorption of an active pharmaceutical ingredient (API) is necessary in the development of topical and transdermal pharmaceutical formulations (Wester & Maibach, 1990:213). *In vivo* (animal and human) and *in vitro* (animal and human) techniques are employed to determine the transdermal permeation and delivery of APIs to the skin. Since the ultimate goal is to clinically deliver the API into or through the skin in humans the relevant technique is human *in vivo* testing (Behl, 2003:231). However, human *in vivo* diffusion studies are not recommended because of the impracticality, ethical implications and dangers involved, especially in studies pertaining to relatively 'unsafe' drugs or enhanced drug delivery (Williams, 2003:51).

In this study, *in vitro* skin diffusion studies of formulations containing 5-fluorouracil were performed with excised human skin. Lotions with four different concentrations of 5-fluorouracil (0.5%; 1.0%; 2.0% and 4.0%) were formulated according to the method in Section B.4 for the diffusion studies. The lotions were formulated with and without Pheroid™ technology at the different concentration levels. The aim of the skin diffusion studies was to,

- ❖ investigate the effect of Pheroid™ technology on topical drug delivery;
- ❖ investigate the effect of 5-fluorouracil concentration on drug delivery and
- ❖ investigate the tendency of 5-fluorouracil to remain in the skin layers or diffuse through the skin during a 12 h skin diffusion.

The focus of this study was the possible application of 5-fluorouracil in the topical treatment of cutaneous cancer. Squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) originate from cutaneous keratinocytes in the epidermis and cutaneous malignant melanoma (CMM) originates from cutaneous melanocytes in the base of the epidermis. Therefore, topical delivery of 5-fluorouracil was sought after in this study and the desired outcome was 5-fluorouracil concentrating within the skin layers.

D.2 Methods

D.2.1 High performance liquid chromatography analytical method

The samples extracted during the diffusion studies were analysed with high performance liquid chromatography (HPLC) in the Analytical Technology Laboratory (ATL) of the North-West University, Potchefstroom Campus, South Africa and the HPLC analytical method was

developed with the assistance and expertise of Prof Professor Jan Du Preez. Appendix A shows the results of the analytical method validation, which showed that the method was suitable for analysis of 5-fluorouracil.

An Agilent® 1100 series (Agilent Technologies, Palo Alto, CA) which consists of a quaternary pump, a degasser, an auto-sampler injection mechanism and a diode array detector was used for the analyses. Data acquisition and analysis was done with Chemstation Rev. A.10.02 software. A Phenomenex® Synergi Fusion Reversed Phase column (4.6 mm x 250.0 mm), with a 4 µm particle size was used (Phenomenex®, Torrance, CA). Table D.1 shows the HPLC analytical conditions.

Table D.1: HPLC analytical conditions for the determination of 5-fluorouracil concentrations in the skin layers and receptor phase

Mobile phase	4% acetonitrile solution (40 ml acetonitrile, 1 ml H ₃ PO ₄ and HPLC-grade water to 1 000 ml)
Flow rate	1 ml/min
Injection volume	50 µl
Detection wavelength	266 nm
Run time	4.6 min
Stop time	6 min
Solvent/receptor phase	Phosphate buffer solution, pH 7.4

D.2.2 Preparation of phosphate buffer solution

An isotonic solution buffered to pH 7.4 is a preferred and suitable receptor fluid because it mimics the blood conditions. To allow the API to diffuse from the donor phase to the receptor phase of the Franz cell the thermodynamic activity of the API in the receptor fluid should not exceed 10% of its thermodynamic activity in the donor phase. Phosphate buffer solution at pH 7.4 was used as the receptor phase for the skin diffusion studies. The phosphate buffer solution was prepared by weighing 3.147 g and 13.620 g of sodium hydroxide (NaOH) and potassium orthophosphate dihydrogen (KH₂PO₄) respectively. The NaOH (3.147 g) was diluted to 786.8 ml with HPLC-grade water and the KH₂PO₄ was diluted to 500 ml with HPLC-grade water. The NaOH solution and the KH₂PO₄ solution were mixed together and stirred with a magnetic stirrer. The pH of the phosphate buffer solution prepared was measured with a Mettler Toledo pH meter (Switzerland) and adjusted to 7.4 with either 10% NaOH or 10% orthophosphoric acid.

D.2.3 Aqueous solubility determination

The aqueous solubility of 5-fluorouracil was determined by adding an excess of 5-fluorouracil in 5 ml phosphate buffer solution (pH 7.4) in three glass polytops. A magnetic stirrer was placed in each polytop to speed up equilibration. Unlike the normal procedure of determining solubility at 25 °C, a temperature of 32 °C was maintained using a Grant® constant temperature water-bath, as this is the temperature at the surface of the skin (Williams, 2003:63). The solutions were constantly checked up on to ensure that there was still an excess of 5-fluorouracil present. After 24 h the supersaturated samples were filtered, diluted 500 times and analysed in duplicate by HPLC to determine drug concentrations. Prior to analysis of the samples a standard of 50 µg/ml 5-fluorouracil in phosphate buffer solution (pH 7.4) was prepared and injected.

D.2.4 *n*-Octanol-buffer distribution coefficient (log D)

Pre-saturated *n*-octanol-buffer was prepared by mixing 50 ml of phosphate buffer solution (pH 7.4) with 50 ml of *n*-octanol. The mixture was stirred vigorously and left to equilibrate over 24 h. The two layers were separated into different containers after the 24 h period and the containers labelled respectively. If the compound is hydrophilic it is firstly dissolved in pre-saturated *n*-octanol and then in buffer (pH 7.4) because it is less likely to dissolve in the *n*-octanol. Due to the hydrophilic nature of 5-fluorouracil, a 1 mg/ml solution of 5-fluorouracil in pre-saturated *n*-octanol was prepared by weighing 5 mg of 5-fluorouracil and dissolving it in 5 ml of pre-saturated *n*-octanol in a round-bottomed tube. This was done in triplicate. An equal volume of pre-saturated buffer (5 ml) was added to the *n*-octanol 5-fluorouracil solution in each tube. The tubes were then closed and placed in a temperature controlled (32 °C) auto-rotator for 24 h. After 24 h the solutions was centrifuged for 15 min at 5 000 rpm with an Eppendorf Centrifuge 5804 R. The buffer phase (bottom) was then extracted and analysed in duplicate using HPLC. Prior to analysing the samples a standard of 5-fluorouracil was injected in order to obtain a standard curve. The log D value was calculated as the logarithmic ratio of the concentration of 5-fluorouracil in the *n*-octanol phase relative to the concentration in the buffer phase, as shown in Equation D.1.

$$\log D = \frac{\text{pre-saturated } n\text{-octanol}}{\text{pre-saturated buffer}}$$

Equation D.1

D.2.5 Preparation of skin

Abdominal human skin obtained from female Caucasian cosmetic abdominoplasty surgery patients was used for the skin diffusion studies. Ethical approval for obtaining, preparing and using human skin in the research was obtained from the Research Ethics Committee of the

NWU (Ethical approval number - NWU-00114-11-A5). Informed consent was obtained from the participating anonymous patients, allowing the use of their excised skin in this study. After surgery the skin was transported in a cooler box with ice packs, prepared and frozen within 24 h of the surgery. The freshly excised skin was visually inspected to ensure that it had no imperfections (e.g. holes or stretch marks). The skin samples were prepared using an electric Zimmer[®] dermatome (USA) at a thickness of 400 μm . This thickness included the epidermis and part of the dermis. The dermatomed skin was placed on top of Whatman[®] filter paper and at least 12 circles with a diameter of approximately 15 mm were punched into the skin. The skin circles were wrapped in foil, placed in a Ziploc[®] bag and then frozen at -20 °C until needed. The frozen skin was used as soon as possible.

In a skin diffusion study, it is advised to use multiple human skin sources due to individual variations that occur in skin and may affect the outcome of the experiment. However, due to the limited availability of human skin, it is permissible to use only one skin source per study in order to conserve supply (Wester & Maibach, 1993:9). Care must be taken when interpreting and using such results. In this study, one skin source was used per experiment because there was a limited and unpredictable supply of excised human skin.

D.2.6 Franz cell diffusion studies

Vertical Franz cells were used for the diffusion studies. A typical Franz cell is made with inert glass and consists of two compartments, with a membrane clamped between the donor (top) and receptor (bottom) sections. The Franz cells used had a receptor volume capacity of approximately 2 ml and a diffusion area of 1.075 cm². In order to maintain a mimic of *in vivo* conditions the receptor phase of a cell was immersed in a water bath set at 37 °C (the temperature of blood in the body). An internal temperature of 37 °C in turn results in a skin surface temperature of 32 °C (Williams, 2003:62). Twelve Franz cells were used for each experiment, ten with the test formulation and two control cells. The method that was followed throughout the drug release and skin diffusion studies is outlined below.

- 1) To reach experimental temperature the buffer (pH 7.4) was placed in a water bath at 37 °C for an hour.
- 2) Prior to the experiment the donor phase formulation was placed in a 32 °C water bath, where the temperature was maintained, for at least an hour to mimic the temperature at the surface of the skin.
- 3) Dow Corning[®] vacuum grease was applied to the donor and receptor compartments of the cells and a magnetic stirring rod placed into the receptor compartment.

- 4) The circles of skin (stratum corneum facing up towards the donor compartment) or polytetrafluoroethylene (PTFE) membranes were placed on to the lower half (receptor) of the Franz cell.
- 5) The two compartments of the Franz cell were placed together and greased to ensure no leakage would occur. The compartments were then securely fastened together with a horseshoe clamp.
- 6) The receptor phase was filled with 2.0 ml of buffer (pH 7.4) ensuring that no air bubbles were present and 1.0 ml (an infinite dose) of the formulation under investigation was placed into the donor phase. The donor compartment was covered with Parafilm[®] and a plastic cap to avoid the loss of constituents by evaporation. An infinite amount of donor phase was used to rule out the effects of concentration change or donor depletion (Williams, 2003:65).
- 7) The assembled Franz cells were placed on a Franz cell stand and placed in a water bath with a Variomag[®] magnetic stirrer ensuring that only the receptor compartment of the Franz cell was immersed. The Variomag[®] magnetic stirrer ensured that the contents of the receptor phase were constantly mixed keeping them homogenous. This was recorded as time 0.0 h.
- 8) The entire receptor buffer phase was removed at predetermined time intervals and replaced with fresh buffer to maintain sink conditions.
- 9) Respectively labelled HPLC vials were filled with the extracted receptor solutions and immediately analysed.
- 10) The HPLC method in Section D.2.1 was used to analyse the samples and determine the drug concentrations in the receptor phase.

D.2.6.1 *In vitro* drug release studies

In order to assess the ability of a formulation to deliver an API to the skin, it is important to determine the release rate of the API from the vehicle (Ademola, 1997:527). Therefore, *in vitro* drug release studies (also known as membrane diffusion studies) were performed before the skin diffusion studies to determine the release of 5-fluorouracil from the formulations. PTFE membrane filters with a thickness of 0.45 µm were used instead of skin. The method described in Section D.2.6 was followed for the drug release studies. The entire receptor phase was extracted and replaced with fresh buffer (pH 7.4) every hour for six hours (step 7) resulting in extraction times of 1; 2; 3; 4; 5 and 6 h.

D.2.6.2 *In vitro* skin diffusion studies

The method described in Section D.2.6 was followed for the skin diffusion studies. The number of extractions made in any *in vitro* skin diffusion experiment usually allow for a trend in the diffusion to be observed. In this study, it was observed during a pilot study that very low amounts of 5-fluorouracil permeated through the skin for the greater duration of the experiments leading to problems with accurate detection and quantification. So, it was decided to do a single extraction after 12 h, thus analysing only the total amount of 5-fluorouracil diffused. Tape-stripping as described in Section D.2.7 was performed after the 12 h diffusion study.

D.2.7 Tape-stripping technique

The tape-stripping technique is useful in dermatopharmacological research for selectively removing the skin's outermost layer, the stratum corneum (Surber *et al.*, 1999:395). This study focused on topical drug delivery so it was necessary to perform tape-stripping in order to determine the amount of drug that was retained in the layers of the skin. After the skin diffusion study, the pieces of skin were removed from the Franz diffusion cell and pinned onto Whatmann® filter paper which was stapled to a hardboard. The diffusion area was clearly visible due to the imprint left by the formulation and the Franz cell. The skin was gently cleaned by wiping it with tissue paper. Strips of 3M Scotch® Magic™ tape that were big enough to cover the diffusion area were cut. As part of the cleaning procedure, one strip of 3M Scotch® Magic™ tape was applied with uniform pressure over the clearly marked diffusion area and discarded. This represents unabsorbed drug on the skin surface. The stripping procedure over the diffusion area was repeated with 15 more tape strips (until the skin glistened) but these were placed in a labelled polytop containing 5 ml buffer (pH 7.4). The non-diffusion area of the skin was cut off and disposed of. The diffusion area was then cut into small pieces to increase surface area and soaked in a labelled polytop containing 5 ml buffer (pH 7.4). The skin and tape-strip samples in buffer were then stored overnight at 4 °C. On the following day the tape-strip samples (representing the stratum corneum-epidermis) and skin samples (representing the epidermis-dermis) in buffer were filtered then analysed by HPLC according to the method in Section D.2.1. This method was repeated for each Franz cell.

D.2.8 Transdermal and statistical data analysis

The data obtained for the skin diffusion experiments were processed to calculate the average percentage diffused and the total amount diffused per unit area after 12 h. The drug release data were processed to calculate the average percentage released and the average cumulative amount per unit area after 6 h.

Statistical analysis was done on the skin diffusion data and some of the tests that were conducted for the *in vitro* drug efficacy data (Section C.7.1) were used to analyse the skin diffusion data. The Shapiro-Wilk and Kolmogorov-Smirnov tests were conducted to determine the normality of the data. The data were said to have a normal distribution if $p > 0.05$. Quantile-quantile (q-q) plots were drawn as an aid in determining the normality of the data. Normal data must produce a relatively straight line. The data were found to have a sufficient normal distribution to perform parametric tests. Descriptive statistics were obtained and in this study we focused on the mean and standard deviation.

A one-way analysis of variance (ANOVA) test was performed together with the Welch and the Brown-Forsythe tests to determine the statistical significance of the results. The data were said to be statistically significant if p was less than 0.05 ($p < 0.05$). These tests inform us about the presence of statistically significant differences between groups but they give no indication of where the variations lie. To determine where differences between groups lay post-hoc tests had to be conducted. The Games-Howell post-hoc test was conducted in this study.

Statistical significance concentrates on the significance of the variation of the treatments but it does not necessarily address the practical magnitude of the treatment effects (Bolton, 1990:130). Effect sizes ($|d|$) were calculated to determine the practical significance of the different formulations. A difference is said to be practically significant if d is bigger than 0.8 ($|d| > 0.8$). If d is bigger than 0.2 but less than 0.5 the difference is not practically significant ($0.2 < |d| < 0.5$) and if d is bigger than 0.5 but less than 0.8 there is a practically visible effect or difference in the mean ($0.5 < |d| < 0.8$). Effect size refers to a relevant interpretation of an estimated magnitude of a treatment effect from the effect statistics. It is sometimes referred to as the biological, practical or clinical importance of the effect (Nakagawa & Cuthill, 2007:593).

D.3 Results and discussion

D.3.1 Aqueous solubility

According to Troy (2005:1573) the aqueous solubility of 5-fluorouracil is 1 g in 80 ml water which translates to 12.5 mg/ml. This study determined the aqueous solubility of 5-fluorouracil to be 17.6 mg/ml which is acceptable although slightly higher than the values obtained in literature. The discrepancy may have been due to differences in temperature (25 °C vs. 32 °C), solvent (water vs. phosphate buffered solution) or pH. An aqueous solubility above 1 mg/ml is ideal for optimum skin permeability (Naik *et al.*, 2000:319) so the aqueous solubility of 5-fluorouracil (17.6 mg/ml) was ideal for skin permeation.

D.3.2 *n*-Octanol-buffer distribution coefficient (log D)

For a molecule to permeate through the skin it must have both lipophilic and hydrophilic properties hence a log P value between 1 and 3 (Naik *et al.*, 2000:319) promotes the permeation of an API through the skin. A log D value of -1.15 was determined for 5-fluorouracil in this study. Buur *et al.* (1985:55) determined a log D value of -0.83. The conditions that were used by Buur *et al.* (1985:55) are 0.02 M acetate buffer, pH 4.0, at a temperature between 20 – 25 °C. The value that was obtained during this study (-1.15) is within close range of the value obtained in literature (-0.83). The difference observed may be due to differing experimental conditions. From these results it could be predicted that 5-fluorouracil would not easily permeate through the skin.

D.3.3 *In vitro* drug release studies

The results that were obtained from the drug release studies have been tabulated in Table D.2. After statistical exploration of the data, the data were found to be relatively normal so the arithmetic mean could be used for the transdermal and statistical data analysis. Table D.2 shows the average % of 5-fluorouracil that was released and the average cumulative amount per unit area ($\mu\text{g}/\text{cm}^2$).

Table D.2: Total amount of 5-fluorouracil diffused as a percentage of initial amount in donor formulation and average cumulative amount of 5-fluorouracil that diffused from the formulations per unit area after the 6 h drug release studies

Formulation	Average % diffused after 6 h	Average cumulative amount/area ($\mu\text{g}/\text{cm}^2$)
0.5% lotion (1)	5.66	526.65 \pm 82.52
0.5% Pheroid™ lotion (2)	8.16	769.68 \pm 133.64
1.0% lotion (3)	4.96	931.66 \pm 183.07
1.0% Pheroid™ lotion (4)	7.37	1454.45 \pm 96.13
2.0% lotion (5)	6.19	2302.57 \pm 222.67
2.0% Pheroid™ lotion (6)	13.94	5220.28 \pm 871.63
4.0% lotion (7)	4.20	3154.35 \pm 355.05
4.0% Pheroid™ lotion (8)	10.25	7629.42 \pm 927.92

In the 6 h-period, (6) released the highest % of 5-fluorouracil (13.94%), followed by (8) (10.25%), (2) (8.16%), (4) (7.37%), (5) (6.19%), (1) (5.66%), (3) (4.96%) and (7) (4.20%) in that order. The percentage of 5-fluorouracil that was released did not increase with an increase in the concentration of 5-fluorouracil in the formulations. These results show that the average percentage of 5-fluorouracil diffused after 6 h did not increase with an increase in the concentration of 5-fluorouracil in the formulation but it varied. When comparisons were made

between a lotion and a Pheroid™ lotion of the same concentration level it was revealed that the presence of Pheroid™ resulted in an increased average %diffused. The penetration enhancing effect of Pheroid™ vesicles has been documented by Grobler *et al.* (2008:284) and these results concur with the results expected with use of Pheroid™.

The average cumulative amount per unit area of the lotions increased with an increase in concentration and the same is true of the Pheroid™ lotions. The overall conclusions from the drug release studies are that 5-fluorouracil was released from all the formulations and Pheroid™ resulted in an increase in the release of 5-fluorouracil from the formulations. 5-Fluorouracil would thus be available on the skin surface for diffusion.

D.3.4 *In vitro* skin diffusion studies

After HPLC analysis the data obtained were processed to obtain the average percentage diffused and the total amount diffused per unit area ($\mu\text{g}/\text{cm}^2$). The total amount diffused is the arithmetic mean of the cumulative amounts diffused from the ten Franz cells in each experiment. Table D.3 shows the results that were obtained for the different formulations.

Table D.3: Total amount of 5-fluorouracil diffused as a percentage of initial amount in donor formulation and average cumulative amount of 5-fluorouracil that diffused from the lotion per unit area after the 12 h skin diffusion studies (n = 10)

Formulation	Average %diffused after 12 h	Average amount diffused/area ($\mu\text{g}/\text{cm}^2$)
0.5% lotion (1)	0.05	4.63 ± 0.84
0.5% Pheroid™ lotion (2)	0.34	31.56 ± 6.10
1.0% lotion (3)	0.06	11.88 ± 2.50
1.0% Pheroid™ lotion (4)	0.08	16.15 ± 4.25
2.0% lotion (5)	0.03	9.71 ± 2.92
2.0% Pheroid™ lotion (6)	0.08	28.48 ± 4.89
4.0% lotion (7)	0.02	15.92 ± 12.01
4.0% Pheroid™ lotion (8)	0.08	57.59 ± 20.00

D.3.4.1 Discussion of *in vitro* skin diffusion results

The average %diffused after 12 h was highest from **(2)** (0.34%) followed by **(4)** (0.08%), **(8)** (0.08%), **(6)** (0.08%), **(3)** (0.06%), **(1)** (0.05%), **(5)** (0.03%) and lastly **(7)** (0.02%). Most of the average %diffused values were within the range 0.02 – 0.08% except for the average %diffused from **(2)** which was much higher with a value of 0.34%. In this study the average %diffused was mainly important when comparing a Pheroid™ lotion with a non-Pheroid™ lotion of the same concentration.

The highest total amount diffused was detected from the 4.0% Pheroid™ lotion **(8)** ($57.59 \mu\text{g}/\text{cm}^2$) followed by **(2)** ($31.56 \mu\text{g}/\text{cm}^2$), **(6)** ($28.48 \mu\text{g}/\text{cm}^2$), **(4)** ($16.15 \mu\text{g}/\text{cm}^2$), **(7)** ($15.92 \mu\text{g}/\text{cm}^2$), **(3)** ($11.88 \mu\text{g}/\text{cm}^2$), **(5)** ($9.71 \mu\text{g}/\text{cm}^2$) and lastly **(1)** ($4.63 \mu\text{g}/\text{cm}^2$). The Pheroid™ lotions resulted in higher 5-fluorouracil amounts diffused than their lotion counterparts at the same concentration. From an overall perspective all the Pheroid™ lotions had higher total amounts diffused than all the lotions.

Figures D.1 – D.8 illustrate the Franz cells and the average cumulative amount diffused from the formulations. In the figures FC is an abbreviation for Franz Cell.

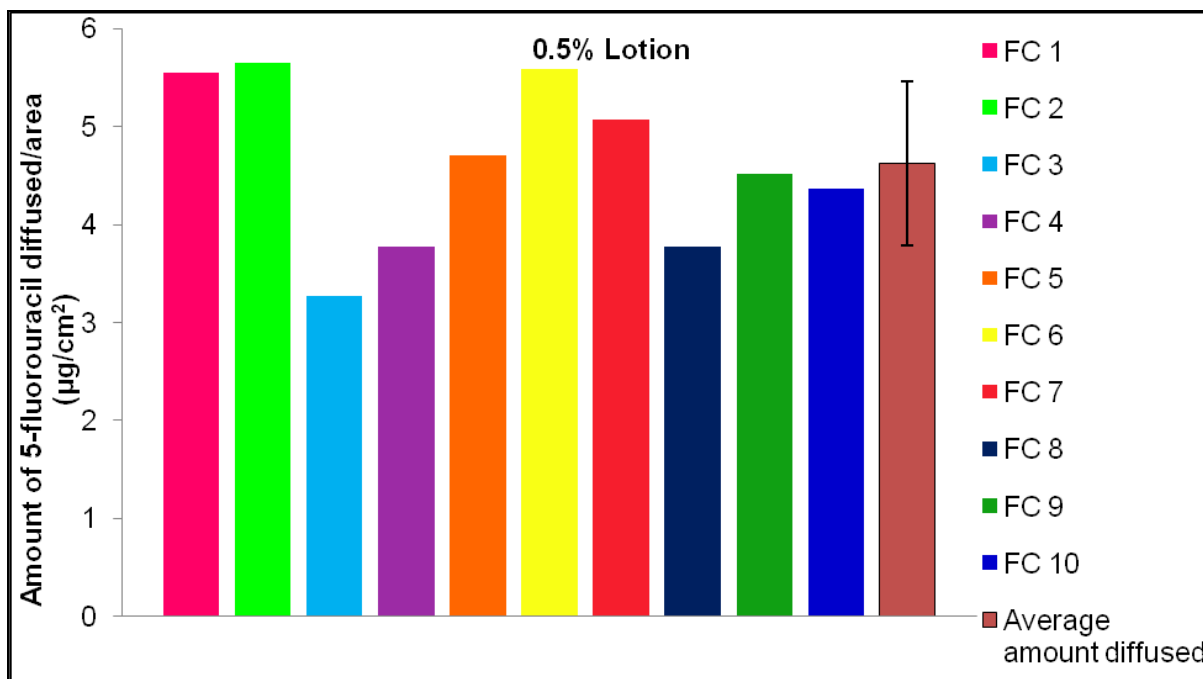


Figure D.1: Amount of 5-fluorouracil that diffused through human skin from the 0.5% lotion (1) after 12 h (n = 10)

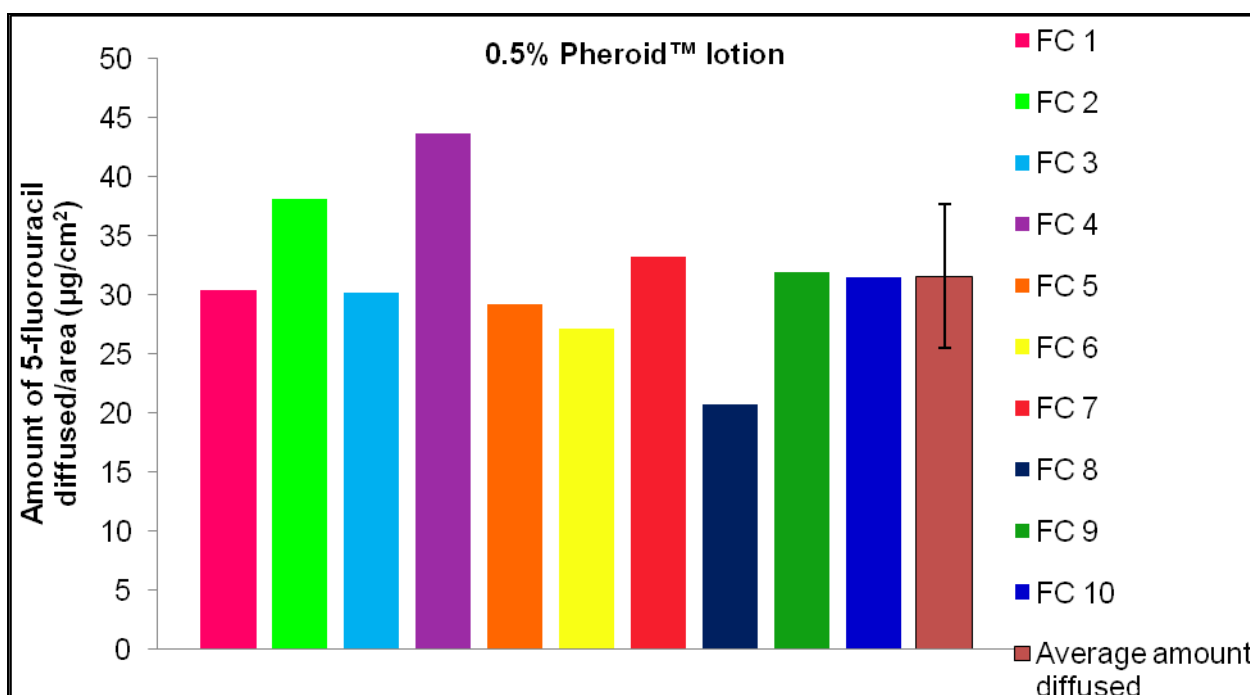


Figure D.2: Amount of 5-fluorouracil that diffused through human skin from the 0.5% Pheroid™ lotion (2) after 12 h (n = 10)

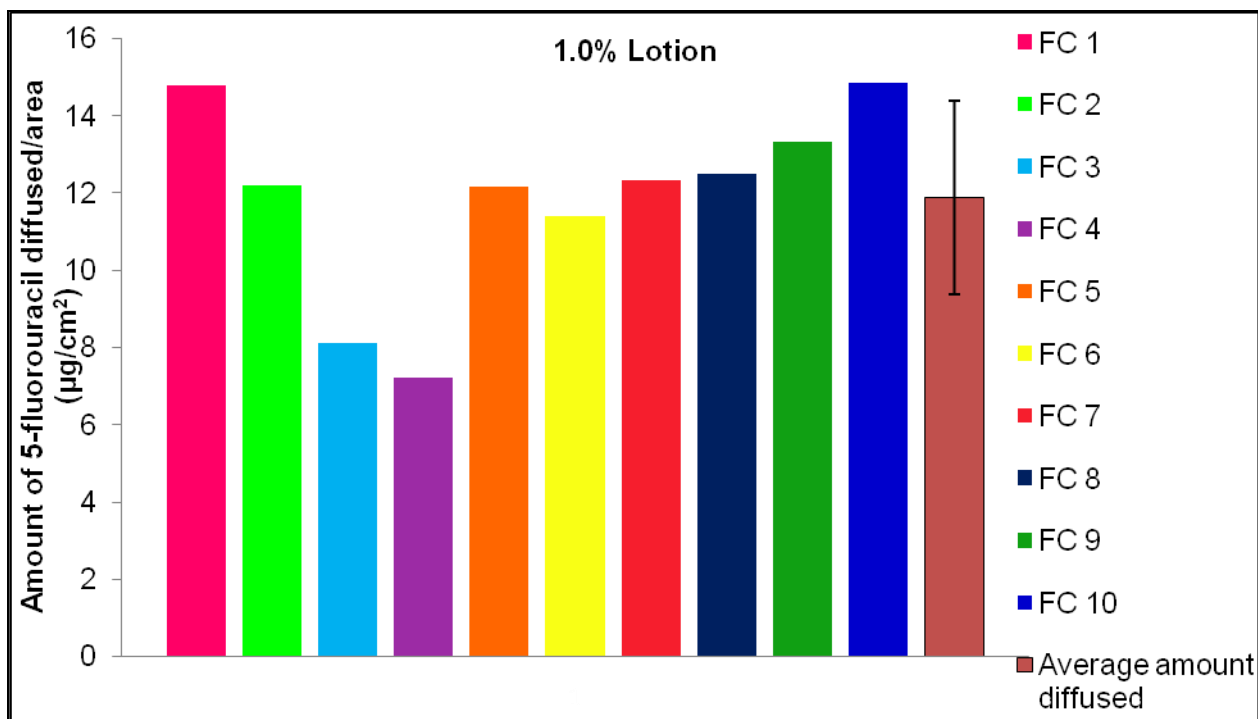


Figure D.3: Amount of 5-fluorouracil that diffused through human skin from the 1.0% lotion (3) after 12 h (n = 10)

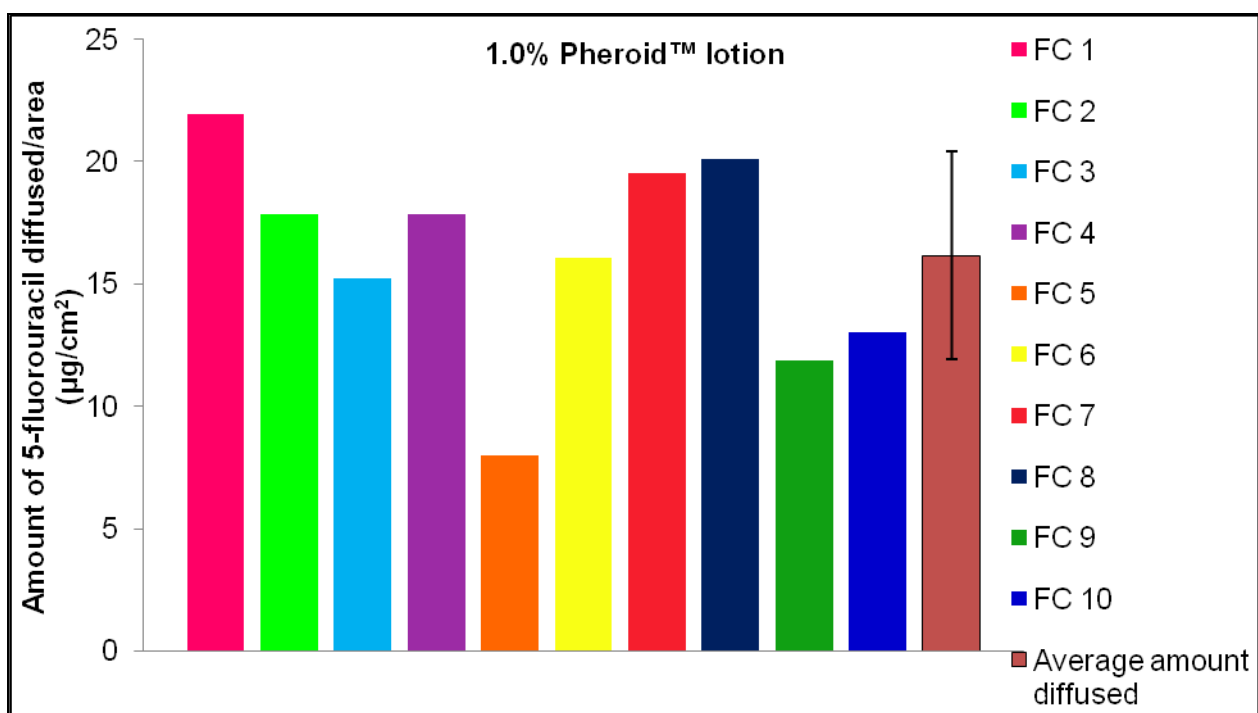


Figure D.4: Amount of 5-fluorouracil that diffused through human skin from the 1.0% Pheroid™ lotion (4) after 12 h (n = 10)

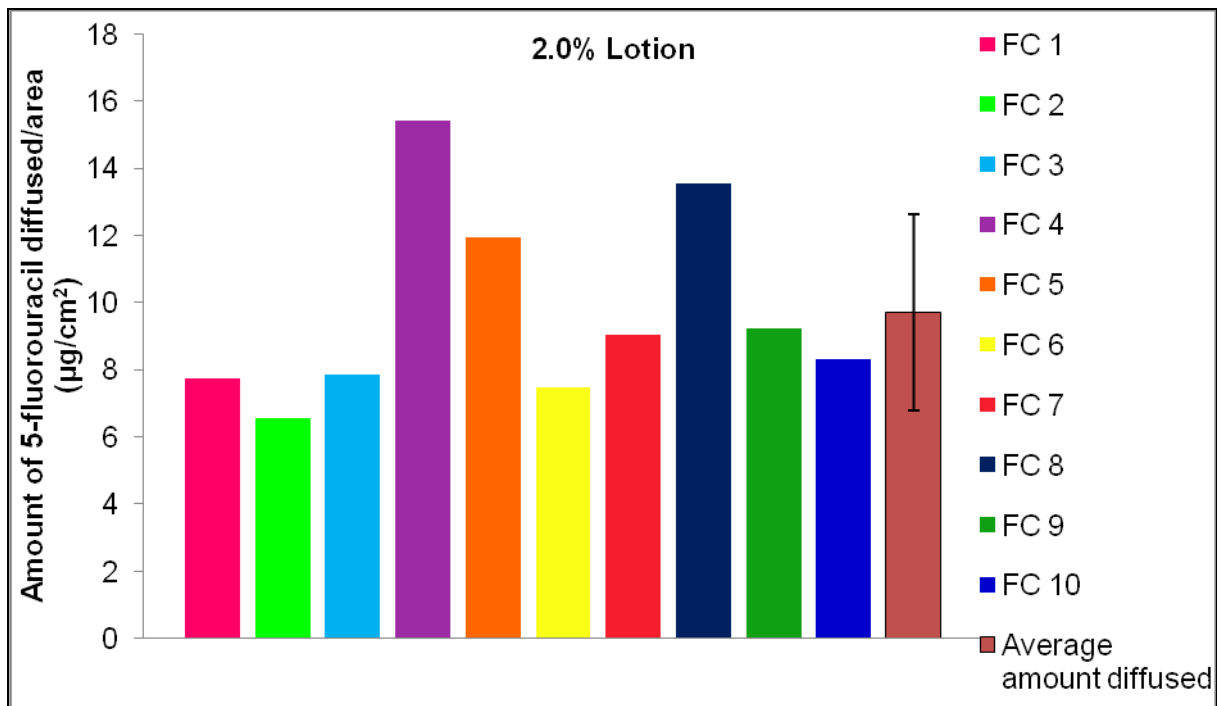


Figure D.5: Amount of 5-fluorouracil that diffused through human skin from the 2.0% lotion (5) after 12 h (n = 10)

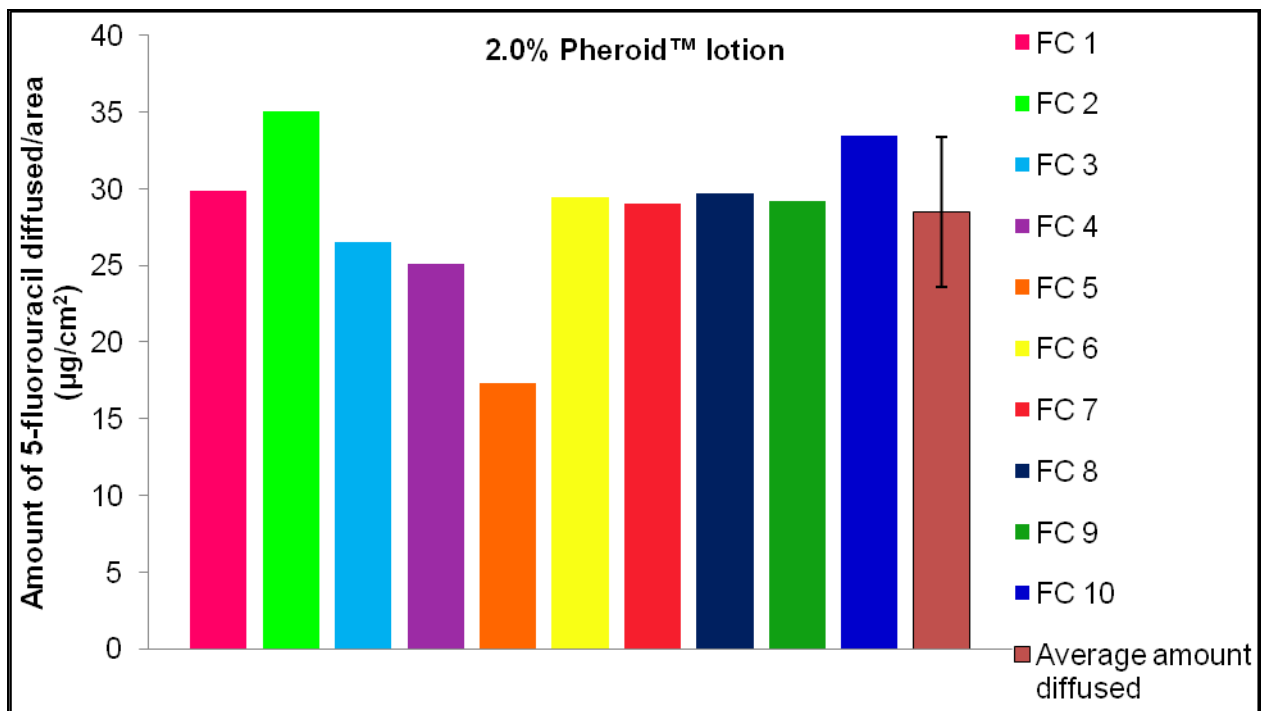


Figure D.6: Amount of 5-fluorouracil that diffused through human skin from the 2.0% Pheroid™ lotion after (6) 12 h (n = 10)

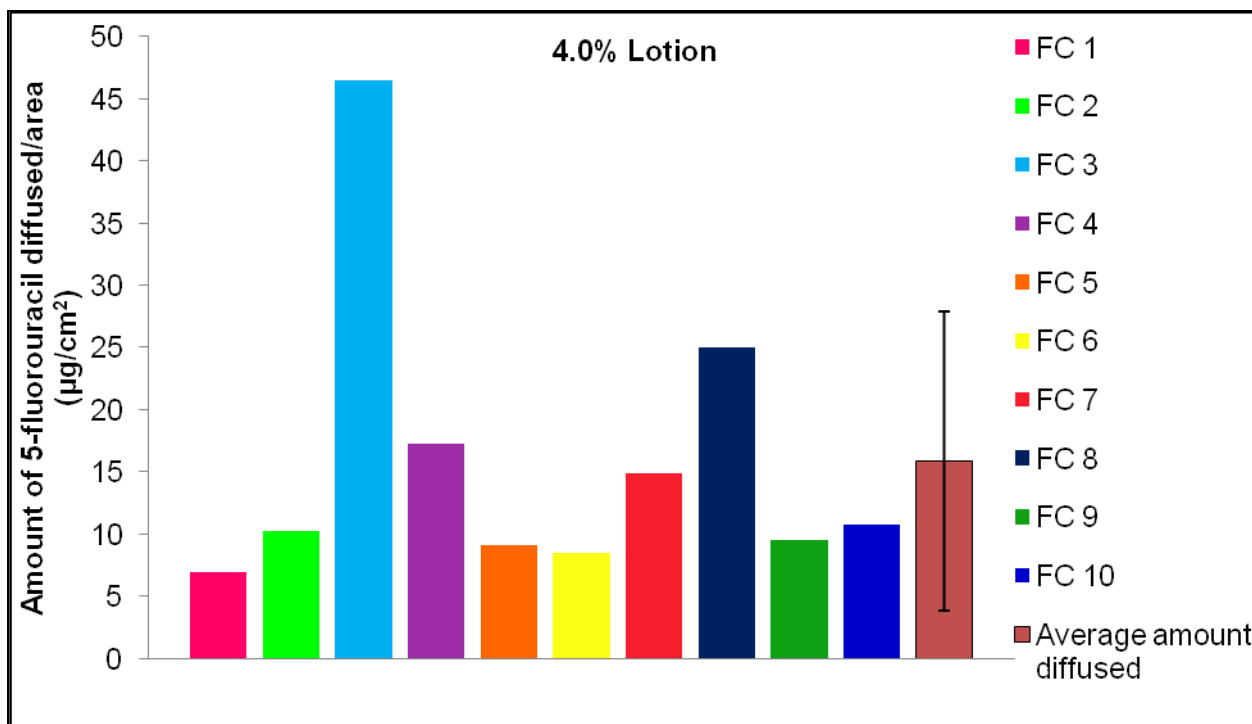


Figure D.7: Amount of 5-fluorouracil that diffused through human skin from the 4.0% lotion (7) after 12 h (n = 10)

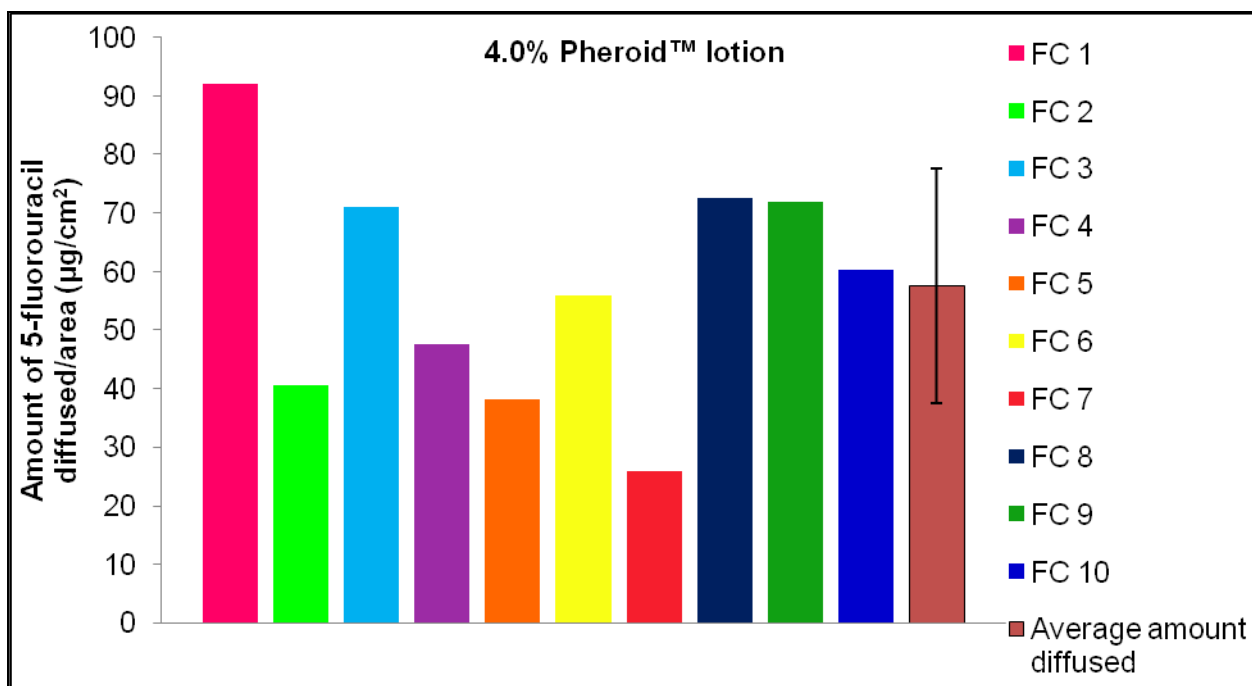


Figure D.8: Amount of 5-fluorouracil that diffused through human skin from the 4.0% Pheroid™ lotion (8) after 12 h (n = 10)

The depictions in Figures D.1 – D.8 show that the Franz cells in some of the experiments had amounts diffused per unit area that were either relatively small or large in comparison to the other Franz cells. This may have been due to biological variation and the unpredictable nature of skin.

A comparison of the total amounts diffused per unit area from the lotions was made (Figure D.9). The 4.0% lotion **(7)** had the highest total amount diffused ($15.92 \mu\text{g}/\text{cm}^2$) followed by **(3)** ($11.88 \mu\text{g}/\text{cm}^2$), **(5)** ($9.71 \mu\text{g}/\text{cm}^2$) and lastly **(1)** ($4.63 \mu\text{g}/\text{cm}^2$). This order corresponded with the ranking that was obtained with the drug release studies. It is not known if this was a coincidence or if there was a particular trend that was present in the release and permeation of 5-fluorouracil. The trend can be investigated in depth in the future using additional 5-fluorouracil concentrations within the same range and various skin samples.

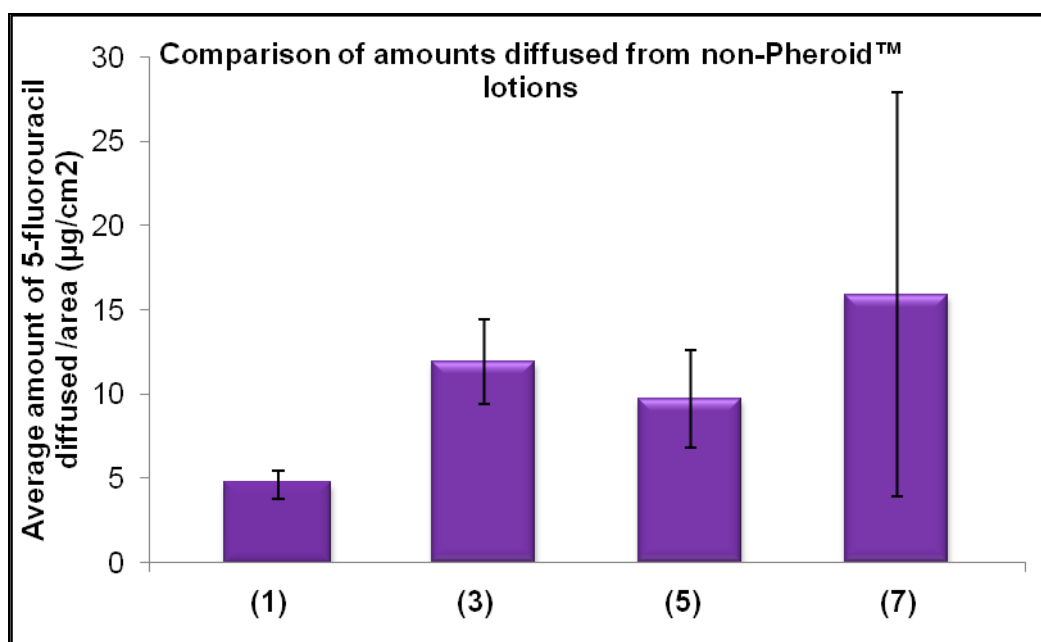


Figure D.9: Comparison of the total amounts of 5-fluorouracil that diffused through human skin per unit area from the lotions of different 5-fluorouracil concentrations, **(1)**; **(3)**; **(5)** and **(7)**, ($n = 10$)

A comparison of the total amount diffused per unit area from the Pheroid™ lotions was made. **(8)** resulted in the highest amount of 5-fluorouracil diffusing through the skin per unit area with $57.59 \mu\text{g}/\text{cm}^2$. This was followed by **(2)** ($31.56 \mu\text{g}/\text{cm}^2$), **(6)** ($28.48 \mu\text{g}/\text{cm}^2$) and lastly **(4)** ($16.15 \mu\text{g}/\text{cm}^2$). The comparison is depicted in Figure D.10. These results revealed that a low concentration of 5-fluorouracil in the formulation can result in a relatively high concentration of 5-fluorouracil diffusing through the skin, as shown by the 0.5% Pheroid™ lotion. The impact of changes in initial 5-fluorouracil concentration on diffusion through the skin has not been fully

investigated. In order to get a clearer picture of this effect, skin diffusion studies with additional API concentrations within the same range may need to be done.

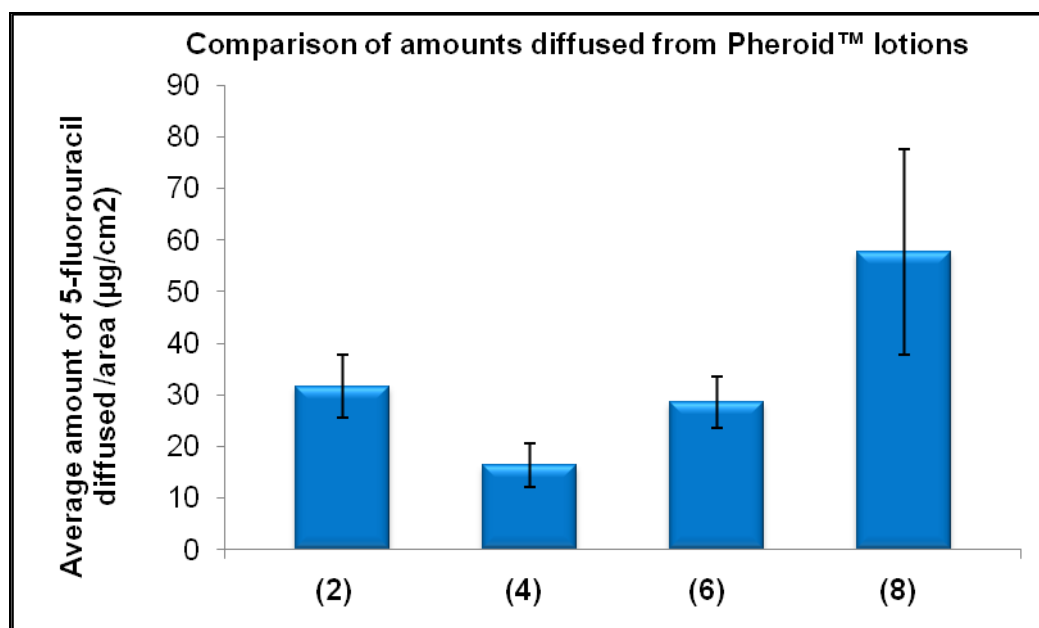


Figure D.10: Comparisons of the total amount of 5-fluorouracil diffused through human skin per unit area from the Pheroid™ lotions of different 5-fluorouracil concentrations, **(2); (4); (6)** and **(8)**, (n = 10)

The total amount diffused per unit area from each lotion was compared with the total amount diffused per unit area from the Pheroid™ lotion of the same concentration. This was done in order to observe the effect of Pheroid™ on the transdermal delivery of 5-fluorouracil. Figure D.11 shows the total amount diffused per unit area of lotions and Pheroid™ lotions, with the same concentrations adjacent to each other. All the Pheroid™ lotions resulted in a higher total amount diffused than the lotions without Pheroid™. This clearly illustrates the skin permeation enhancing effect of Pheroid™ as noted in previous studies (Kilian, 2004:78; Van Dyk, 2008:61). It is possible that due to the lipophilic nature of Pheroid™ vesicles, 5-fluorouracil was easily carried into the skin while encapsulated in Pheroid™. However, 5-fluorouracil is hydrophilic in nature which means that it is “water-loving”, so after reaching the dermis of the skin the API could have easily diffused into the aqueous receptor phase. Hydrophilic drugs such as 5-fluorouracil are less likely to diffuse into or remain in the skin because the skin is a hydrophobic membrane. Therefore a low percentage of 5-fluorouracil was likely to diffuse into the skin and in turn into the aqueous receptor phase. This may explain the results obtained with the skin diffusion studies.

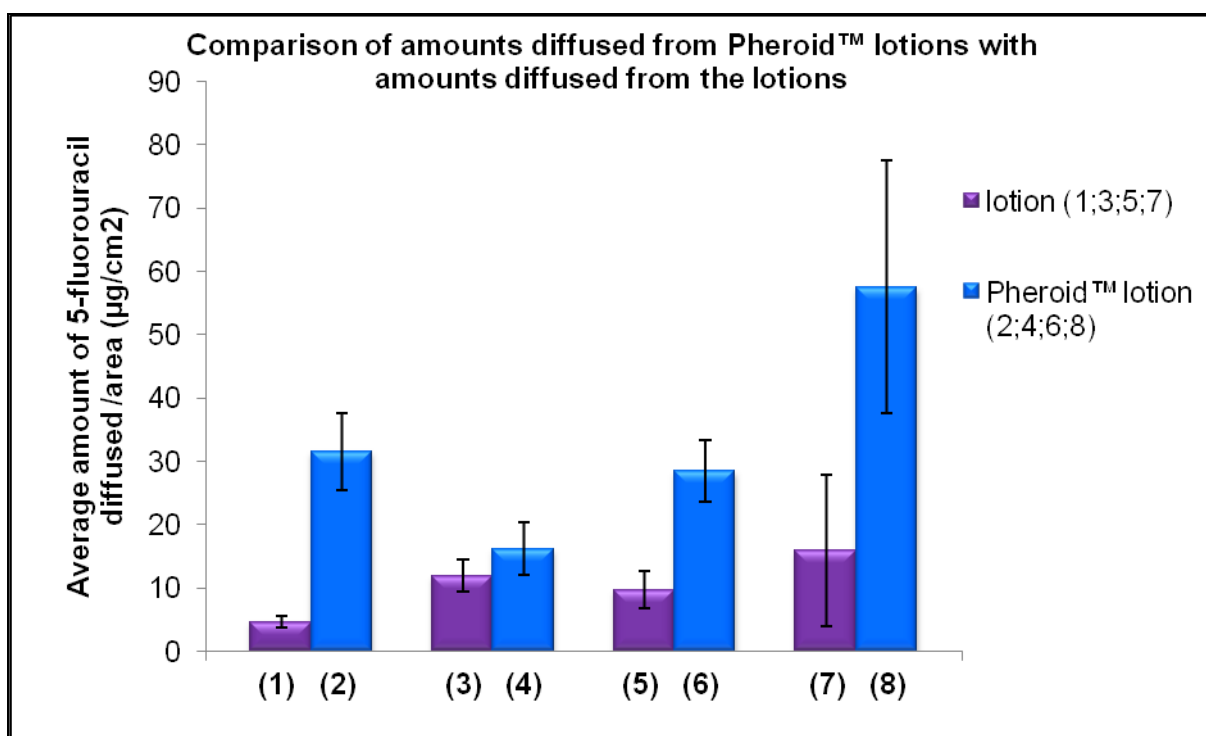


Figure D.11: Comparisons of the amount of 5-fluorouracil that diffused through human skin from each lotion with the amounts diffused from the respective Pheroid™ lotion of the same concentration (n = 10)

Vermaas (2010:183) performed skin diffusion studies with 0.5% 5-fluorouracil lotions (with and without Pheroid™) and found that the Pheroid™ lotion ($10.01 \mu\text{g}/\text{cm}^2$) resulted in a slightly higher amount diffused than the non-Pheroid™ lotion ($9.06 \mu\text{g}/\text{cm}^2$), which is consistent with the results found in this study. When taking into consideration the rest of the results found by Vermaas (2010:183) we see that the penetration enhancing effect of Pheroid™ may depend on the nature of the formulation into which Pheroid™ are incorporated. A 24 h skin diffusion study was conducted by Alvi *et al.*, (2011:774) to compare the topical delivery of 5-fluorouracil in niosomes, transfersomes and liposomes using rat abdominal skin. Alvi *et al.* (2011:779) found that the transfersome formulation resulted in the highest percentage diffusion of 5-fluorouracil (68.25%), followed by the liposome formulation (49.85%), niosome formulation (41.14%), aqueous API solution (11.77%) and lastly the gel formulation (7.0%). The %diffused values that were obtained in the current study are much lower (below 0.5%) than those that were obtained by Alvi *et al.* (2011:779). The differences may have been exacerbated by species differences (rat vs. human skin), pH differences (pH 6.5 vs. pH 7.4), differences in extraction times (hourly extractions vs. single extraction after 12 h) or viability of the membrane during the experiment (24 h vs. 12 h). The results obtained by Alvi *et al.* (2011:779) showed that like Pheroid™ these drug delivery vesicles (niosomes, transfersomes and liposomes) enhanced the topical delivery of 5-fluorouracil.

D.3.5 Tape-stripping

The results obtained from the tape-stripping procedure have been tabulated in Table D.4. The average concentration of 5-fluorouracil in the stratum corneum-epidermis (hereinafter referred to as the epidermis) and in the epidermis-dermis (hereinafter referred to as the dermis) is shown. Skin cancer mainly originates from cells that are found in the basal layer of the epidermis (stratum basale); these being the keratinocytes and melanocytes. Therefore a topical 5-fluorouracil formulation of choice should deliver the API to the epidermis and dermis (to act on possibly spreading cancerous cells) with minimal diffusion to the blood. In the case of 5-fluorouracil this is difficult because once in the skin its hydrophilic nature results in a tendency to diffuse into the surrounding aqueous environment

Table D.4: Average concentrations of 5-fluorouracil that remained in the epidermis and dermis after the 12 h skin diffusion studies (n = 10)

Formulation	Average concentration in epidermis ($\mu\text{g/ml}$)	Average concentration in dermis ($\mu\text{g/ml}$)
0.5% lotion (1)	0.49 \pm 0.10	0.99 \pm 0.36
0.5% Pheroid™ lotion (2)	0.56 \pm 0.10	1.58 \pm 0.70
1.0% lotion (3)	1.11 \pm 0.36	0.71 \pm 0.37
1.0% Pheroid™ lotion (4)	1.26 \pm 0.20	0.69 \pm 0.26
2.0% lotion (5)	1.56 \pm 0.37	1.58 \pm 0.94
2.0% Pheroid™ lotion (6)	2.63 \pm 1.42	2.27 \pm 1.23
4.0% lotion (7)	1.95 \pm 0.77	0.78 \pm 0.29
4.0% Pheroid™ lotion (8)	2.31 \pm 0.67	6.69 \pm 4.74

D.3.5.1 Concentration in the epidermis

The amount of 5-fluorouracil that concentrated in the epidermis was highest after the 12 h skin diffusion with **(6)** (2.63 $\mu\text{g/ml}$), followed by **(8)** (2.31 $\mu\text{g/ml}$), **(7)** (1.95 $\mu\text{g/ml}$), **(5)** (1.56 $\mu\text{g/ml}$), **(4)** (1.26 $\mu\text{g/ml}$), **(3)** (1.11 $\mu\text{g/ml}$), **(2)** (0.56 $\mu\text{g/ml}$) and lastly **(1)** (0.49 $\mu\text{g/ml}$) in that order. The Pheroid™ lotions all resulted in a higher average concentration of 5-fluorouracil in the epidermis than their corresponding lotions of the same concentration. This could have been due to the lipophilicity rendered to the formulations by the presence of Pheroid™ which resulted in the vesicle entrapped 5-fluorouracil being retained in the lipophilic epidermis. Another possibility is that a higher concentration of 5-fluorouracil was able to penetrate into the skin layers from the Pheroid™ lotion than the lotion as mentioned in Section D.3.4 so consequentially a higher concentration of 5-fluorouracil remained in the skin. The concentration of 5-fluorouracil in the lotions had an effect on the epidermal concentration of 5-fluorouracil, this is seen by the

increase in epidermal concentrations that occurred with an increase in the percentage of 5-fluorouracil used in the lotions.

In a previous study on 5-fluorouracil topical formulations, 0.5% Pheroid™ and non-Pheroid™ lotions resulted in 5-fluorouracil concentrations of 0.18 µg/ml and 0.14 µg/ml respectively (Vermaas, 2010:193). The current study obtained epidermal results of 0.49 µg/ml from the 0.5% lotion and 0.56 µg/ml from the 0.5% Pheroid™ lotion. These results were more than double the concentrations that were obtained by Vermaas (2010:193). This was possibly due to the presence of ethanol in the lotions, which may have altered the solubility properties of the skin, resulting in a consequential improvement in the partitioning of the API into the skin (Williams, 2003:94).

D.3.5.2 Concentration in the dermis

The dermal concentration of 5-fluorouracil determined after tape-stripping was highest due to **(8)** (6.69 µg/ml), followed by **(6)** (2.27 µg/ml), **(5)** (1.58 µg/ml), **(2)** (1.58 µg/ml), **(1)** (0.99 µg/ml), **(7)** (0.78 µg/ml), **(3)** (0.71 µg/ml) and **(4)** (0.69 µg/ml) in that order. All the Pheroid™ lotions resulted in a higher 5-fluorouracil concentration in the dermis than the non-Pheroid™ lotions with the exception of the 1.0% lotion. The dermis is the relatively hydrophilic layer of the skin so the expectation was that a higher concentration of 5-fluorouracil would be found in the dermis in comparison to the epidermis. To the contrary, the results did not reflect any deducible trend in the distribution of 5-fluorouracil between the dermis and the epidermis. Vermaas (2010:193) found that a 0.5% 5-fluorouracil lotion resulted in an API dermal concentration of 0.42 µg/ml without Pheroid™ and 0.31 µg/ml with Pheroid™. The results that were obtained in the current study are higher (0.99 µg/ml and 1.58 µg/ml respectively). This may have been due to the permeation enhancing effects of ethanol which was present in the formula of the lotions that were utilised in the current study.

D.3.6 Statistical analysis of results

The skin used in this study was not randomly selected but rather the availability of the skin determined the samples used in the experiments. Usually you cannot generalise with an availability sample, as is the case here, but statistical significance is reported for the sake of being complete. The main focus should however be on the practical significance. In this study, the skin samples in each experiment originated from one piece of skin but the ideal situation is whereby there is a source variation within the samples in an experiment. To enable comparison between different treatments a variation within samples is desired because this enables the differentiation between effects due to the skin and the effect of treatment. The lack of variation is referred to as a confounding factor. To enable analysis the confounding impact of skin was ignored.

Omnibus tests comprising of the one-way ANOVA, Welch test and the Brown-Forsythe test were conducted to determine if there were any significant differences between the effects (total amount diffused, epidermal concentrations and dermal concentrations) of the different formulations. A statistically significant result gave a p value below 0.05 ($p < 0.05$). According to the one-way ANOVA, the Welch test and the Brown-Forsythe test there was a statistical significance between the formulations for the total amount diffused per unit area, all with p values below 0.000001. A statistically significant difference was also detected between the formulations for the epidermis and dermis 5-fluorouracil concentrations with p values below 0.001. In essence, there were significant differences in the means of the eight formulations for the total amount diffused, epidermis data and dermis data. The Games-Howell post-hoc test was conducted in order to determine where the differences between the groups lie.

The influence of Pheroid™ on the total amount diffused was statistically significant at the 0.5% ($p = 0.000002$), 2.0% ($p = 0.000007$) and 4.0% ($p = 0.001$) concentrations. There were no significant differences between the lotion and Pheroid™ lotion 5-fluorouracil concentrations in the epidermis ($p > 0.05$). For the 5-fluorouracil concentrations in the dermis a statistically significant result was only obtained with the comparison of the 4.0% lotion with the 4.0% Pheroid™ lotion ($p = 0.043$).

To determine the effect of a change in concentration comparisons were made between the different concentrations of lotions. Similar comparisons were then repeated with Pheroid™ lotions. A statistically significant difference in the total amounts diffused was present between the 0.5% and 1.0% lotions ($p = 0.00005$) and between the 0.5% and 2.0% lotions ($p = 0.005$). The average 5-fluorouracil concentration in the epidermis due to the 0.5% lotion had a statistically significant difference from the concentrations resulting from the 1.0% ($p = 0.006$), 2.0% ($p = 0.00008$) and the 4.0% ($p = 0.003$) lotions. There were no statistically significant differences between the effects of any of the lotions in the dermis ($p > 0.05$). There were statistically significant differences in the total amount diffused from the Pheroid™ lotions between: the 0.5% and 1.0% ($p = 0.0001$); 0.5% and 4.0% ($p = 0.035$); 1.0% and 2.0% ($p = 0.0002$); 1.0% and 4.0% ($p = 0.001$); and lastly the 2.0% and 4.0% ($p = 0.017$) concentrations. The 0.5% and 1.0% ($p = 0.000006$); 0.5% and 2.0% ($p = 0.017$); 0.5% and 4.0% ($p = 0.0002$); and lastly the 1.0% and 4.0% ($p = 0.011$) Pheroid™ lotions had statistically significant differences between their average 5-fluorouracil concentrations in the epidermis. The dermis average concentrations of 5-fluorouracil differed significantly between the 0.5% and 1.0% ($p = 0.04$); 1.0% and 2.0% ($p = 0.037$); and lastly the 1.0% and 4.0% ($p = 0.04$) Pheroid™ lotions.

Effect sizes were calculated in order to determine the practical significance of results obtained by the lotions versus those obtained by the Pheroid™ lotions. The practical significance of a change in concentration within the same formulation type (lotion or Pheroid™ lotion) was also

determined. A practically significant difference in the total amount diffused per unit area existed between the lotions and their Pheroid™ lotion counter-parts at the four concentrations. The influence of Pheroid™ on the 5-fluorouracil concentration in the epidermis was practically visible at the 0.5% and 2.0% concentrations. Pheroid™ had a practically significant effect on the dermis values resulting from the 0.5% and 4.0% lotions (Pheroid™ vs. non-Pheroid™).

Comparisons were made between the different 5-fluorouracil concentrations within the lotions and Pheroid™ lotions used for the experiments. Majority of the comparisons made reflected either a practically visible effect or a practical significance in the difference of the results. The change from the 2.0% Pheroid™ lotion to the 4.0% Pheroid™ lotion had no practical significance on the epidermis concentrations. A comparison between the dermis concentrations due to the 1.0% and 4.0% lotion showed that there was no practical significance in the difference of the results.

Table D.5 shows the calculated p-values and d-values for all the comparisons between formulations and between concentrations.

Table D.5: Results of the Games-Howell test and effect size determinations for the skin diffusion studies

Comparison	p-value			d-value		
	Total amount diffused	Epidermis	Dermis	Total amount diffused	Epidermis	Dermis
(1) vs. (2)	2.74x10 ^{-6*}	8.57x10 ⁻¹	3.18x10 ⁻¹	4.41*	0.61 [#]	0.85*
(3) vs. (4)	1.85x10 ⁻¹	9.34x10 ⁻¹	1.00x10 ⁻⁰	1.01*	0.42	0.06
(5) vs. (6)	7.72 x10 ^{-7*}	3.71x10 ⁻¹	8.68x10 ⁻¹	3.84*	0.76 [#]	0.53 [#]
(7) vs. (8)	9.74 x10 ^{-4*}	9.49x10 ⁻¹	4.30x10 ^{-2*}	2.08*	0.46	1.25*
(1) vs. (3)	5.52 x10 ^{-5*}	6.27x10 ^{-3*}	6.87x10 ⁻¹	2.90*	1.68*	0.74 [#]
(1) vs. (5)	4.90 x10 ^{-3*}	8.38x10 ^{-5*}	5.38x10 ⁻¹	1.74*	2.84*	0.67 [#]
(1) vs. (7)	1.65 x10 ⁻¹	2.98x10 ^{-3*}	8.14x10 ⁻¹	0.94*	1.89*	0.60 [#]
(3) vs. (5)	6.40 x10 ⁻¹	1.76x10 ⁻¹	1.76x10 ⁻¹	0.74 [#]	1.20*	0.96*
(3) vs. (7)	9.56 x10 ⁻¹	1.04x10 ⁻¹	1.00x10 ⁻⁰	0.34	1.10*	0.17
(5) vs. (7)	7.48 x10 ⁻¹	8.16x10 ⁻¹	2.20x10 ⁻¹	0.52 [#]	0.51 [#]	0.89*
(2) vs. (4)	1.40 x10 ^{-4*}	4.39x10 ^{-6*}	4.01x10 ^{-2*}	2.52*	3.46*	1.28*
(2) vs. (6)	9.06 x10 ⁻¹	1.73x10 ^{-2*}	7.77x10 ⁻¹	0.50 [#]	1.46*	0.56 [#]
(2) vs. (8)	3.49 x10 ^{-2*}	2.45x10 ⁻¹	9.21x10 ⁻¹	1.30*	2.61*	1.08*
(4) vs. (6)	2.53 x10 ^{-4*}	1.50x10 ⁻¹	3.69x10 ^{-2*}	2.52*	0.97*	1.29*
(4) vs. (8)	1.38 x10 ^{-3*}	1.06x10 ^{-2*}	4.00x10 ^{-2*}	2.07*	1.56*	1.27*
(6) vs. (8)	1.72 x10 ^{-2*}	9.97x10 ⁻¹	1.81x10 ⁻¹	1.46*	0.23 ^o	0.93*

* - Statistically or practically significant difference

- Practically visible difference

D.4 Conclusion

The 5-fluorouracil aqueous solubility of 17.6 mg/ml that was obtained in this study led to the prediction that the API would easily diffuse into and through the skin. To the contrary the log D value of -1.15 that was obtained led to an opposing conclusion; there was a low potential for 5-fluorouracil skin diffusion. The results from the skin diffusion studies and the tape-stripping revealed that regardless of the unfavourable log D value 5-fluorouracil was able to diffuse into and through the skin. This confirmed that one parameter cannot be singled out to predict skin diffusion because many physiological and physico-chemical factors influence skin diffusion. Seeing as 5-fluorouracil is a hydrophilic molecule its diffusion into and through the skin may have been through the transappendageal route.

The topical delivery of 5-fluorouracil was required in this study but the API managed to traverse the layer of skin and into the aqueous receptor phase. The desired effect was the penetration of 5-fluorouracil into the skin and its retainment within the skin. Systemic absorption of 5-fluorouracil is not required in the treatment of the various skin cancers because it may result in unwanted cytotoxic side effects. An ideal anticancer topical formulation would result in release of the API, penetration of the API into the skin, diffusion of the API within the skin and its retainment in the skin layers.

The results obtained in this study show that 5-fluorouracil was able to diffuse through the skin regardless of the presence or absence of Pheroid™. The transdermal delivery of 5-fluorouracil was, however, increased to a high extent in the presence of Pheroid™. The use of lower concentrations may result in an improved side-effect profile after transdermal application of 5-fluorouracil. Non-cutaneous cancers can be targeted with 5-fluorouracil via the transdermal route using these formulations instead of the intravenous route.

The use of Pheroid™ also resulted in increased epidermal and dermal 5-fluorouracil concentrations with the exception of the dermal concentrations resulting from the 1.0% 5-fluorouracil formulations. However, Pheroid™ did not seem to have any effect on the distribution of the API between the epidermis and dermis.

The lotions prepared and used in this study all had 5-fluorouracil concentrations that are below the 5-fluorouracil market product concentration (5.0%). According to Vermaas (2010:183), the total API diffused after 12 h from the commercial product was 2.516 $\mu\text{g}/\text{cm}^2$, the concentration in the epidermis was 0.120 $\mu\text{g}/\text{ml}$ (Vermaas, 2010:193) and the concentration in the dermis was 0.052 $\mu\text{g}/\text{ml}$ (Vermaas, 2010:193). The results that were obtained from the commercial product are below all the results that were obtained in this study at the different concentrations (compare with Tables D.3 and D.4). It can thus be assumed that clinical use of any one of the

formulations from this study will result in a clinical effect similar to or better than the clinical effect of the commercial product.

The optimal formulation should result in maximal penetration of the skin but with minimal absorption through the skin to the systemic circulation. In this study the formulation which showed such optimal characteristics was the 2.0% lotion because a relatively low amount of 5-fluorouracil diffused through the skin per unit area ($9.709 \mu\text{g}/\text{cm}^2$), but relatively high concentrations of 5-fluorouracil were detected in the epidermis ($1.56 \mu\text{g}/\text{ml}$) and dermis ($1.58 \mu\text{g}/\text{ml}$). More formulations with such characteristics must be made for topical use. The penetration enhancing effect of Pheroid™ on 5-fluorouracil and the effect of concentration need to be investigated further to confirm the findings from studies done thus far.

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APPENDIX E

JOURNAL OF PHARMACEUTICAL SCIENCES: GUIDELINES FOR AUTHORS

E.1 General guidelines

All submitted manuscripts should contain previously unpublished original research. Submitted manuscripts should not be under consideration for publication elsewhere.

E.1.1 Online Submission and Peer Review

Authors should ensure that papers conform to the scientific and style instructions given below. In order to expedite the publication process the Journal requires that manuscripts be submitted online at <http://mc.manuscriptcentral.com/jpharmsci>.

Journal of Pharmaceutical Sciences has a completely digital submission, review, and production process. We therefore ask for production-quality files at the time of submission of your manuscript. This will speed the production and distribution of your work across a variety of print and electronic platforms. If you don't follow the simple guidelines given below, your submission will be returned to you for additional revision. This will of course delay review and, in the event that your work is accepted, would delay publication. Therefore we ask that you pay careful attention at this time and we thank you for your cooperation.

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Online help is available to you at all times during the process. You are also able to exit/re-enter at any stage before finally "submitting" your work. All submissions are kept strictly confidential. If you have any questions, do not hesitate to contact us at support@scholarone.com. You may also contact the Journal's Editorial Assistant, Tammy Dunning, at tdunning@ku.edu, or at tel.785-864-5919, fax 785-864-5875.

E.1.2 Text

Submit your text in DOC or RTF format. Do not embed figures or tables in this document. These should be submitted as separate files.

E.1.3 Tables

Tables should be created with a word processor and saved in either DOC or RTF format. Do not embed tables in your text. Tables should be on separate pages and saved as one file in DOC format.

E.1.4 Figures

To ensure the highest print quality, your figures must be submitted in either TIF or EPS format according to the following minimum resolutions:

1200 dpi (dots per inch) for black and white line art (simple bar graphs, charts, etc.)

300 dpi for halftones (black and white photographs)

600 dpi for combination halftones (photographs that also contain line art such as labeling or thin lines), figures should be saved as individual files. Vector-based figures (e.g. figures created in Adobe Illustrator) should be submitted in EPS format.

E.1.4.1 Color figures

In addition to the above resolution guidelines, color figures must be submitted in a CMYK color. Do not submit color figures as RGB.

E.1.4.2 Unacceptable figure formats

Do not submit figures in any of the following formats: JPG, GIF, PSD, CRD, PCT, PPT, PDF, XLS, DOC, BMP, 123 (or other Lotus formats).

E.2 Scope of the Journal of Pharmaceutical Sciences.

The *Journal* focuses on two major questions of importance to pharmaceutical scientists: (1) "What are the physical and biological barriers that limit the access of drugs to their therapeutic targets?", and (2) "How can drugs, dosage forms and delivery systems be designed to maximize therapeutic efficacy?" Rapid Communications, Research Articles, Notes, Lessons Learned, Perspectives, Reviews, Minireviews and Commentaries that address these questions quantitatively and mechanistically will be considered for publication in the *Journal*. It is expected that these contributions will span a variety of disciplines, including physical pharmacy,

pharmaceutical engineering, materials science, pharmacokinetics/ pharmacodynamics, pharmacogenomics, animal and human biopharmaceutics, cellular and molecular biopharmaceutics, drug delivery, drug metabolism, pharmaceutical biotechnology, analytical chemistry, computer modeling and medicinal chemistry. Specific scientific topics of interest to the Journal include, but are not limited to, preformulation and formulation studies, crystallization, polymorphism, drug stability, selection of salt forms and excipients, pharmaceutical process design and manufacturing, preclinical and clinical pharmacokinetics, cell and tissue culture models, transport in biological membranes and associated transport proteins, controlled release, drug targeting, oral and parenteral drug delivery, prodrugs, formulation and delivery of biomacromolecules (peptides, proteins, antibodies, DNA, RNAi), vaccines and stem cells, high throughput screening, novel analytical methods for drug characterization, and computational and modeling approaches to drug design, formulation and delivery.

Most manuscripts are evaluated for their scientific content and significance by the Editor-in-Chief, an Editor, a member of the Editorial Advisory Board and at least two independent reviewers. , however, Editors reserve the right to reject a manuscript without conducting an in-depth review if they feel that the manuscript does not meet the minimal acceptance criteria for publication in the Journal of Pharmaceutical Sciences.

E.3 Types of Manuscripts.

Rapid Communications are preliminary accounts of significant and original experimental and/or theoretical results that fit within the scope of *JPharmSci*TM. The results must be of sufficient significance, originality and general interest to justify accelerated publication in hard copy form. Authors are asked to write their manuscripts in a clear and concise manner and to include only data crucial to arriving at their final conclusions. Preferably manuscripts should not exceed 2,000 words of text and a total of 4 figures and/or tables. Extra experimental and/or theoretical data in the form of figures and tables should be deposited under Supporting Information.

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Notes differ from Rapid Communications in that they are final reports and from Research Articles in that they are limited in scope. Authors are asked to write their manuscripts in a clear and concise manner and to include only data crucial to arriving at their final conclusions. Preferably manuscripts should not exceed 2,000 words of text and a total of 4 figures and/or

tables. Extra experimental and/or theoretical data in the form of figures and tables should be deposited under Supporting Information.

Commentaries present the author's considered opinion on a scientific or technical subject within the scope of the *Journal*. If the Commentary criticizes an article published in the *Journal*, the authors of the original article will be given an opportunity to reply in the same issue in which the Commentary is published.

Perspectives articles summarize the viewpoints of distinguished pharmaceutical scientists with regard to the current status and future direction of the field. Perspectives are similar in length to Commentaries and Reviews, and may be submitted only by invitation.

Reviews provide a comprehensive summary of broadly-based topics of general interest to pharmaceutical scientists. Reviews are not limited as to the number of words, tables, figures and references that may be included.

Minireviews are well-focused, well-documented examinations of timely issues in the pharmaceutical sciences. The issues may be of a controversial nature, or may address a more narrowly focused area than those typically covered in a Review. Minireviews are limited to approximately 3,000-4,000 words, including tables, figures and references.

Lessons Learned are short articles (600 words) which provide authors with a means of informing other scientists about critical issues, experiences and observations, the descriptions of which would not be appropriate for a typical Research Article, Communication, Note, Commentary or Review. Examples include, but are not limited to, key insights into an unanticipated manufacturing problem, knowledge accumulated over a career of "tricks of the trade" for a given analytical or formulation method, how to avoid a mistake that is repeated over and over again by scientists in industry and academia. Each article will be reviewed directly by an Editor who has expertise in the relevant scientific area. Because each of these articles represents the personal opinion, experience and/or insights of the author(s), data are not required (but could be described) nor does the identity of a given drug need to be divulged. Articles may contain up to three key references.

Reviews, Minireviews, and Commentaries are by invitation only. Authors interested in preparing a Commentary, Review or Minireview should provide a brief outline to Editor John Carpenter, requesting an invitation to submit a manuscript in one of these categories.

E.4 Preparation of Manuscripts.

E.4.1 General Considerations

In order to expedite peer review, authors are required to submit their manuscripts online at <http://mc.manuscriptcentral.com/jpharmsci>. (See Section 1.1 above for details about the on-line submission process.)

Authors should write manuscripts in clear, concise English. The responsibility for all aspects of manuscript preparation rests with the authors. Extensive changes or rewriting of the manuscript will not be undertaken by the editors.

There are no page charges for publication in the Journal of Pharmaceutical Sciences.

E.4.2 Suggested Reviewers

The Journal requires that corresponding authors suggest at least four reviewers, up to a maximum of six reviewers, two of which must be an Editorial Advisory Board (EAB) member. Please include contact information for suggested reviewers. Please note that upon electronic submission of a manuscript, the corresponding author will be given the opportunity to identify "preferred" and "non-preferred" reviewers. Non-preferred reviewers would be individuals who, for whatever reason, the corresponding author prefers not be invited to review their manuscript. Preferred reviewers would be individuals who have expertise directly related to the science described in their manuscript.

E.4.3 Title

Titles are of great importance for current awareness and for information retrieval. The wording of titles should be chosen carefully to provide information on the contents and to function as "points of entry" for information retrieval. Symbols, formulas, or arbitrary abbreviations should not be included in the title, except chemical symbols to indicate the structure of isotopically labeled compounds.

E.4.4 Abstract

The abstract should briefly (80-200 words) present, in one paragraph, the problem and experimental approach and state the major findings and conclusions. It should be self-explanatory and suitable for reproduction without rewriting. Footnotes or undefined abbreviations may not be used. If a reference must be cited, complete publication data must be given.

E.4.5 Keywords

Please provide up to 10 keywords that reflect the scientific content of your manuscript. At least 5 of your selected keywords must come from the Journal's official keyword list. In addition to facilitating indexing of articles, our keyword system assists in the assignment of qualified reviewers for your manuscript. In addition, each member of our Editorial Advisory Board has selected keywords that are applicable to their own work.

E.4.6 Abbreviations

Abbreviations are used without periods. Standard abbreviations should be used throughout the manuscript. All nonstandard abbreviations should be kept to a minimum and must be defined in the text following their first use and in a footnote at the beginning of the manuscript.

E.4.7 QSAR/QSPR

All manuscripts dealing with quantitative structure activity relationships (QSAR) and quantitative structure property relationships (QSPR) must identify individual chemical structures using Chemical Abstracts Service (CAS) SciFinder. To aid authors in the use of CAS SciFinder for structure searching, please go to <http://onlinelibrary.wiley.com/doi/10.1002/jps.10260/pdf> for a commentary written by Dr. Christopher Lipinski describing the procedure. This Commentary appears in *Journal of Pharmaceutical Sciences* 91(12): 2470-2472.

E.4.8 Experimental Section

The experimental procedures should be described in sufficient detail to enable others to repeat the experiments. Names of products and manufacturers [with city, state, and country (if other than the U.S.)] should be included only if alternate sources are deemed unsatisfactory. Brand names may be used only once in the manuscript. For subsequent designation, use "formulation A", product B", etc. Novel experimental procedures should be described in detail, but published procedures should merely be referred to by literature citation of both the original and any published modifications. The purity of key compounds and descriptions(s) of the method(s) used to determine purity should be included in this section. For buffers, use terminology such as "20 mM potassium phosphate buffer (pH 7.7) containing...". Also, state w/v or v/v when appropriate.

Identification of and precautions for handling hazardous chemicals and dangerous procedures should be placed at the beginning of the section. An example would be " **Caution:** *The following chemicals are hazardous and should be handled carefully; (list of chemicals and handling procedures or references)* ".

Manuscripts containing data generated from animal and/or human studies must specify the committee and the institution that approved the experimental protocols used to generate these data.

E.4.9 Results

The results should be presented concisely. Tables and figures should be designed to maximize the presentation and comprehension of the experimental data. Attention should be paid to the matter of significant figures (usually, no more than three). The same data should not be presented in more than one figure or in both a figure and a table. As a rule, interpretation of the results should be reserved for the discussion section of a Research Article, but under some circumstances it may be desirable to combine results and discussion in a single section.

E.4.10 Discussion

The purpose of the discussion is to interpret the results and to relate them to existing knowledge in the field in as clear and brief a fashion as possible. Information given elsewhere in the manuscript should not be repeated in the discussion. Extensive reviews of the literature should be avoided.

E.4.11 References and Notes

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Macmillan Publishing Co. p 446-472. List submitted manuscripts as "in press" only if formally accepted for publication; otherwise, use "unpublished results" after the names of authors. Any footnotes to the text should be incorporated in the correct numerical sequence with the references.

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The Supporting Information format of this journal can accommodate and make readily available almost any type of supplementary figures or data (e.g., reproductions of spectra, experimental procedures, tabulated data, expanded discussion of peripheral findings, etc.). The author should include a Supporting Information Available statement that describes the material at the end of the printed manuscript text. Consult a current issue of the Journal for the proper wording of this statement. Supporting Information should be clear and of high contrast (suitable for direct photoreproduction) and submitted in quadruplicate on 8.5- × 11-in. paper. All pages of Supporting Information must be consecutively numbered. Captions or legends for figures, spectra, etc., must appear directly on the figure. Supporting Information is available free of charge via the Internet (www.wileyonlinelibrary.com).

E.4.13 Acknowledgments

This section should acknowledge financial support, technical assistance, advice from colleagues, gifts, etc. Permission must be received from persons whose contribution to the work is acknowledged in the manuscript.

E.4.14 Spectral Data

It may be desirable to include such data for representative compounds in a series, for novel classes of compounds, and in structural determinations. Usually, it is not desirable to include routine spectral data for every compound in the manuscript. Papers where interpretations of spectra are critical to structural elucidation and those in which band shape or fine structure needs to be illustrated may be published with spectra included. When such presentations are deemed essential, only pertinent sections should be reproduced.

E.4.15 Experimental Data

Experimental methods must be referenced or described in sufficient detail to permit the experiments to be repeated by others. Detailed descriptions of experimental methods should be placed in the experimental procedures section. Data may be presented as numerical expressions in tables or in graphical form with no duplication of information in the text. If tables or figures include a minimal number of experimental values (< four), the data should be presented in the text. Units should be abbreviated without punctuation and with no distinction

between singular and plural forms (e.g., 1 mg, 25 mg). If possible, statistical significance of the experimental data should be provided. Statistical probability (p) in tables, figures, figure legends and text should be expressed as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. For multiple comparisons within a table, footnotes italicized in lower case, superscript letters should be used and defined in the table legend. References to statistical methods of calculation should be provided. If statistical limits cannot be provided, the number of determinations and some indication of the variability and reliability of the results should be provided. For animal experimental data, doses and concentrations should be expressed as molar quantities (e.g., mmol/kg, mM) when comparisons are made between compounds having large differences in molecular weights. The routes of administration of test compounds and vehicles used should be indicated. For animal and human studies, authors must specify the committee and the institution that approved the experimental protocols used to generate these data.

If experimental data on proprietary compounds (i.e. compounds whose chemical structures are not available in the public domain) and/or using proprietary procedures (i.e. experimental procedures and/or components of procedures that are not described in the public domain) are provided in a manuscript, authors should carefully read the next two paragraphs.

Traditionally, scientific papers must reveal sufficient information for the work to be repeated by others. That tradition led to the policy that *JPharmSci*TM has applied to manuscripts that contain information on proprietary small molecules. This policy essentially states that information pertaining to proprietary (small molecule) compounds can be published providing that, in the opinion of the reviewers and editors, the paper would be publishable based solely on the information derived from studies of known compounds. Thus, information on proprietary compounds has been considered to be supplemental while the decision to publish or not has relied on compounds for which structures were disclosed.

Studies of proprietary proteins and other biologicals pose a difficult situation for *JPharmSci*TM. In some cases, complete structures may not have been determined or, even if the structure is available, compounds having identical structures may be difficult for others to generate (e.g., the amino acid sequences of immunoglobulin hypervariable regions). Yet, interesting studies of proprietary biologicals can nevertheless be envisioned that may be deemed to have sufficient value that the failure to reveal detailed structural information should not be a deterrent to publication. Therefore, the journal will determine the acceptability of such papers on an individual basis. Decisions of acceptability will be made using the following criteria: (a) the structural information provided is adequate for the purpose of evaluating the paper using rigorous scientific standards; (b) the structural information provided is sufficient to enable others to verify the results by conducting essentially the same experiments; and (c) the work is judged

to be of sufficient importance that a lack of complete structural information does not significantly detract from its scientific contributions.

E.4.16 Tables

Tabulation of experimental results is encouraged when this leads to more effective presentation or to more economical use of space. Tables should be numbered consecutively with Arabic numerals. Provide a brief title with each table and a brief heading for each column. Clearly indicate the units of measure (preferably SI). Data should be rounded to the nearest significant figure. Explanatory material referring to the whole table is to be included as a footnote to the title (a). Footnotes in tables should be given lower case letter designations and cited in the tables as italicized superscripts. Tables that require special treatment, such as insertion of arrows or other special symbols under or over alphanumeric characters, or contain many structures should be submitted as camera-ready copy. All tables should be cited in the text.

E.4.17 Illustrations

The quality of the illustrations printed in your paper depends on the quality of the originals you provide. Electronic submission of illustrations is required. Preferred formats for graphics and artwork are TIFF (tagged image file format) and EPS (Encapsulated PostScript). John Wiley & Sons, Inc. journal pages are now produced completely electronically.

In preparing illustrations, contrast is important. Use dark black ink on high quality, smooth, opaque white paper. Ordinary white bond paper works well. Avoid tracing paper or textured "artist" papers.

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The chemical names for drugs should be used. If the terminology is unwieldy, nonproprietary names of drugs may be used throughout the manuscript after the first mention and identification. Formally adopted nonproprietary names listed in *United States Adopted Names (USAN)* should be used. In cases in which a name has not been assigned by *USAN*, the *International Nonproprietary Names (INN)*, approved by the World Health Organization, should be used. Trade names and laboratory codes should not be used except as additional information.

E.4.19 Analyses

Adequate evidence to establish identity and purity should be provided for new compounds. When possible, this should include elemental analysis. The purity of compounds used for biological testing should be stated with a description of the method used to evaluate it.

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All hazardous chemicals should be clearly identified as such. Precautions for handling dangerous materials or for performing hazardous procedures should be explicitly stated and referenced.

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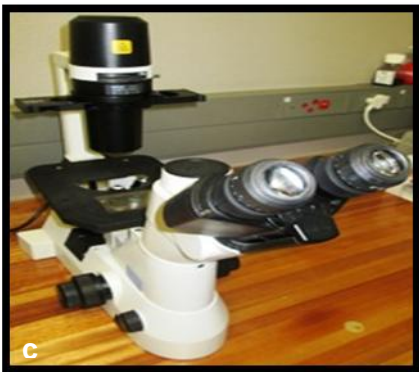
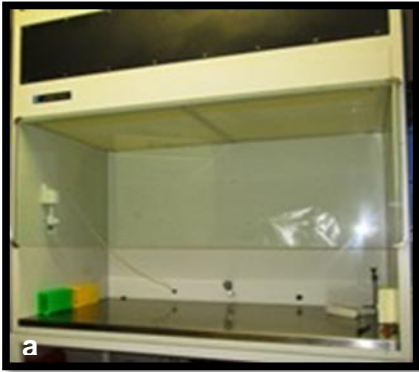
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APPENDIX F

PHOTOGRAPHS OF EQUIPMENT USED IN THE DRUG DELIVERY AND DRUG EFFICACY STUDIES



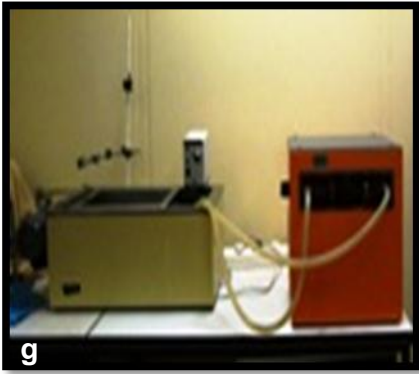


Figure F1: The apparatus used during the drug efficacy and drug delivery testing were: **(a)** a laminar air flow cabinet (Labotec) in which aseptic handling of cells was conducted; **(b)** an incubator (Forma Scientific); **(c)** an inverted microscope (Nikon); **(d)** a Countess™ automated cell counter (Invitrogen™); **(e)** a centrifuge 3K15 (Sigma Zentrifugen); **(f)** a BD FACSCalibur (BD Biosciences); **(g)** a temperature controlled auto-rotator (Labotec); **(h)** an electric dermatome (Zimmer®); **(i)** Dow Corning® high vacuum grease; **(j)** a Variomag® magnetic stirrer plate; **(k)** fully assembled Franz diffusion cells on a tray and **(l)** a Grant JB Series water bath.