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MSc Dissertation

The toxicity and therapeutic index of a methotrexate polymeric compound, D-85.

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This dissertation is in partial fulfilment of MSc in Pharmacology.

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SUMMARY

Methotrexate (MTX) has been used for many years in the treatment of patients with cancer as a cytotoxic agent and as an anti-inflammatory drug for the treatment of inflammatory diseases, such as rheumatoid arthritis (RA). However, because of the side effects associated with MTX, there is a continuous search for drugs with less toxicity and hence a greater therapeutic index. In pursuit of a better and less toxic compound, researchers have coupled MTX to various polymeric drug carriers.

The objective of this study was to evaluate methotrexate and a methotrexate polymer (D-85) in *in vitro* and *in vivo* systems. It was hypothesized that D-85 would show improved anti-neoplastic and anti-inflammatory properties with decreased toxicity compared to MTX. The *in vitro* experiments included cell viability assays on cancerous and non-cancerous cell lines in order to compare the effects of the two drugs on normal and cancerous cells. Other *in vitro* assays were performed to assess the effect of the two compounds on cell cycle and mixed lymphocyte cultures. Finally, the toxic effects of both drugs were studied concentrating on two treatment regimens, namely that of an anti-inflammatory regimen and a chemotherapeutic regimen.

The results obtained during this study clearly illustrate the chemotherapeutic and anti-rheumatic activity of the two drugs, MTX and D-85. The methotrexate water-soluble drug D-85, however showed greater toxicity towards normal cells compared to the toxicity of methotrexate.

OPSOMMING

Metotreksaat word reeds vir baie jare gebruik by die behandeling van kanker pasiënte en as ‘n anti-inflammatoriese middel by die behandeling van inflammatoriese siektes soos rumatoïede arthritis (RA). As gevolg van metotreksaat se newe effekte is daar ‘n voortdurende soektog na minder toksiese middels is met dus ‘n groter terapeutiese indeks. Navorsers het in hierde soektog metotreksaat gebind aan verskeie polimeriese draers.

Die doel van hierdie studie was om metotreksaat en ‘n metotreksaat polimeer in *in vivo* en *in vitro* modelle te ondersoek. Die hipotese was dat D-85 beter anti-kanker en anti-inflammatoriese eienskappe sou toon met laer toksisiteit as gewone metotreksaat. Die *in vitro* toetse het sel lewensvatbaarheids analises op beide kanker- en nie-kanker sellyne ingesluit sodat die effek van die twee middels vergelyk kon word. Ander *in vitro* ontledings is gedoen om ‘n vergelyking te kon tref ten op sigte van die twee middels se effek op die sel siklus en gemengde limfosiet kulture. Laastens is die toksiese effekte van beide middels bestudeer deur op twee behandelings strategiëe te konsentreer, naamlik anti-inflammatoriese en anti-kanker.

Die resultate wat tydens hierdie projek bekom is, illustreer duidelik die anti-inflammatoriese en anti-kanker eienskappe van die twee middels. Die water-oplosbare middel, D-85 is egter meer toksies teenoor normale selle as metotreksaat.

LIST OF ABBREVIATIONS

A

AICAR	5-aminoimidazole-4-carboxamide-ribonucleotide
AMP	adenosine monophosphate
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
AST	aspartate aminotransferase
ATCC	American Type Culture Collection
AUCC	Animal Use and Care Committee
ALL	acute lymphoblastic leukemia

C

CF	citrivorum factor
CD	cluster of differentiation
CAM	chorioallantoic membrane
C_{max}	maximum concentration
$cc/min/m^2$	cubic cm per minute per square meter
CsA	cyclosporine A
CO_2	carbon dioxide
CHO	Chinese Hamster Ovarian

D

DHFR	dihydrofolate reductase
DAMPA	diaminio-2,4 N^{10} methylpteroïc acid
DNA	deoxyribonucleic acid

E

E-MEM	Eagle's Minimal Essential Medium
D-MEM	Dulbecco's Minimal Essential Medium

EDTA	ethylene diamine tetra-acetic acid disodium salt

F

4-amino PGA	4-amino pteroylglutamic acid
FH_2	dihydrofolate
FH_4	tetrahydrofolate
FBP	folate binding protein
4n	tetraploid

G

GVHD	graft versus host disease
GIT	gastrointestinal tract
Gy	gamma radiation

H

HPLC	high performance liquid chromatography
h	hour
HLA	human leukocyte antigen
HSA	human serum albumin
HAM	nutrient mixture F12 Ham Kaighn's modification

I

IL	interleukin
IFN	interferon
IV	intravenous
IC_{50}	50% of the inhibitory concentration

K

KDa	kilo Dalton

L

LPS	lipopolysaccharide
LD MTX	low dose methotrexate
LD_{25}	25% of the lethal dose

M

MTX	methotrexate
μM	micromolar
mg/m^2	milligram per square meter
μg/ml	micrograms per millilitre
mM	millimolar
M-phase	mitosis phase
MLR	mixed lymphocyte reaction
MLC	mixed lymphocyte cultures

N

NSAID's	non-steroidal anti-inflammatory drugs
NRBM	National Repository for Biological Material

O

OD_{nm}	optical density in nanometres

P

PMBC	peripheral mononuclear blood cells
PGE_2	prostaglandin 2
PHA	Phytohaemaglutinin
PBS	phosphate buffer solution
PCNA	proliferating cell nuclear antigen
PMA	poly-myristate acetate

PBL	peripheral blood lymphocytes

R

RA	rheumatoid arthritis
RNA	ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute
Rpm	revolutions per minute

S

7-OH MTX	7-hydroxymethotrexate
S-phase	synthesis phase
SEM	standard error from the median

T

TS	thymidylate synthase
TNF-α	tumor necrosis factor alpha
$t_{½}$	terminal half-life
2n	diploid
3n	triploid

V

Vd	volume of distribution
vs	versus

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Chapter 1:

Introduction

CHAPTER 1

1. Introduction

Methotrexate (MTX) was developed 50 years ago and was primarily used to treat malignancies such as breast cancer (Dollery, 1999). It can however also be used in the treatment of autoimmune diseases such as rheumatoid arthritis (RA), graft vs host disease (GVD), psoriasis and Chron's disease. This anti-inflammatory use started about 15 years ago in the treatment of RA (Majumdar & Aggarwal, 2001).

1.1 History of MTX

Folic acid is a vitamin in the B-complex family. After its identification and synthesis, researchers started to synthesize analogues of folic acid in which small changes were made to the molecule (Jukes, 1978). The first analogue researched was "crude x-methyl folic acid" which was prepared by using butyraldehyde in the synthetic reaction. In 1946 they found that this crude x-methyl folic acid inhibited the growth of two test organisms, *Streptococcus faecalis* and *Lactobacillus cassei*. This growth inhibition was readily reversed by folic acid. It was also found that this crude compound, x-methyl folic acid, slowed the growth of rats on a purified diet and the addition of folic acid restored the growth. The compound also affected the blood cell counts and bone marrow of the rats. These changes were completely prevented or reversed by the addition of folic acid as concomitant additives during the treatment period (Jukes, 1978).

The x-methyl folic acid did not have the same effects on humans as in the animal models (Jukes, 1978). Other analogues were synthesized that were not as readily reversible by the addition of folic acid. Even when folic acid was added in large quantities, it only resulted in a slight protective effect. The first of these new compounds were 4-amino-pteroylglutamic acid (4-amino PGA). In this compound the hydroxyl group on the pyrimidine configuration of the folic acid molecule was replaced by an amino group. This compound was

named aminopterin and it was the first compound used by Dr Sydney Farber to produce temporary remissions in children with leukaemia. However, its significant toxicity made it difficult to use as a chemotherapeutic agent on a sustained basis. Researchers then focussed on the search for a) a less toxic compound and b) to find a way to reverse the toxic effects of aminopterin. For the purpose of a), a new compound, namely 4-amino-10-methyl pteroylglutamic acid, was synthesized by a chemist from the Calco Chemical Company in 1948 (Jukes, 1978). This compound is known as methotrexate (MTX). The toxic effects of methotrexate in rats were reversible by folic acid over a narrow range. MTX is only slightly less toxic than aminopterin (Jukes, 1978).

High levels of folic acid were only weakly active when trying to reverse the toxic effects of these folic acid analogues. It was then suggested that folic acid was a precursor of a substance that was more active than folic acid. Säubelich & Bouman supported this theory in 1948 by using *Leuconostoc citrovorum* (Jukes, 1978). This organism, that usually required folic acid to grow, could also be made to grow when supplied with an unidentified factor present in the liver. This factor, later known as “citrivorum factor” (CF), could replace folic acid in promoting growth of other micro-organisms, such as *S. faecalis*. Concentrates of CF can also reverse the toxic effects of aminopterin on the growth of *Leuconostoc citrivorum*, as well as protect mice from the toxic effects of aminopterin (Jukes, 1978).

In 1950, Nicol & Welch demonstrated the conversion of folic acid to CF in rat liver slices (Goldin, 1978). Brockman and co-workers synthesized CF from folic acid in 1950. They reduced folic acid with zinc in the presence of formic acid. The active product was then prepared in a crystalline form through the catalytic reduction of folic acid over platinum. The crystal structure is 5-formyl-5,6,7,8-tetrahydropteroyl-glutamic acid. Folinic acid is currently on the market as Leucovorin® and is used to minimize the toxic effects of aminopterin in patients treated with methotrexate. Leucovorin® was registered in South Africa in January 2006

(www.doh.gov.zadocs/misc/approvejan6). The total dose of either MTX or aminopterin could be manipulated by the use of Leucovorin® (Goldin, 1978).

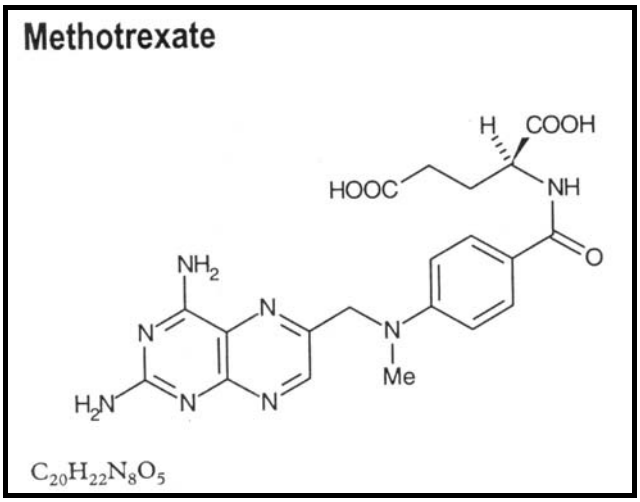

Figure 1.1: Structure of Methotrexate (British Pharmacopoeia, 1999)

Methotrexate ($C_{20}H_{22}N_8O_5$) is a mixture of 4-amino-10-methylfolic acid and is part of the anti-metabolite drug group which includes oncology medication and immune suppressants (Sommers, 2001; United States Pharmacopoeia, 1999; Dollery, 1999). It is a yellow to orange-brown crystalline powder and is presently used as an anti-metabolite in the treatment of cancer or in the treatment of auto-immune and other inflammatory disorders. Its structure can be seen in Figure 1.1.

1.2 Mechanism of action of methotrexate

The first target identified for MTX was the enzyme dihydrofolate reductase (DHFR). DHFR catalyzes the reduction of dihydrofolate (FH_2) to tetrahydrofolate (FH_4). MTX is similar in structure to dihydrofolate (FH_2) and is a competitive inhibitor of DHFR with the result that the tetrahydrofolate (FH_4), essential for DNA synthesis, is then not produced. This interferes with the mitosis of cancerous cells by inhibiting the *de novo* synthetic pathways for purines, pyrimidines, formation of polyamines and transmethylation of DNA, RNA, phospholipids and proteins (Lee & Weinblatt, 2001)(Figure 1.3 and Figure 1.4).

After entering the cell, MTX undergoes polyglutamation in the same way as the natural occurring folates (Figure 1.2) (Cutolo *et al*, 2001). When this happens, up to five additional glutamates can be added to the molecule (Dollery, 1999). The products of intracellular metabolism are MTX-polyglutamates, 7-hydroxymethotrexate and diamino-2,4 N^{10} methylpteroïc acid (DAMPA) (Genestier *et al*, 2000). MTX-polyglutamates also inhibit the enzymatic conversion of 5-aminoimidazole-4-carboxamide-ribonucleotide (AICAR) to formyl-AICAR by the enzyme AICAR-transformylase. This process plays a major role in the anti-inflammatory mechanism of action of MTX as discussed in paragraph (a) below (Majumdar & Aggarwal, 2001). The polyglutamation of MTX leads to its prolonged retention within cells. AICAR-transformylase, as thymidylate synthase (TS), requires folate co-factors. MTX has a fairly high affinity for these enzymes and cause the inhibition of these enzymes (Cutolo *et al*, 2001).

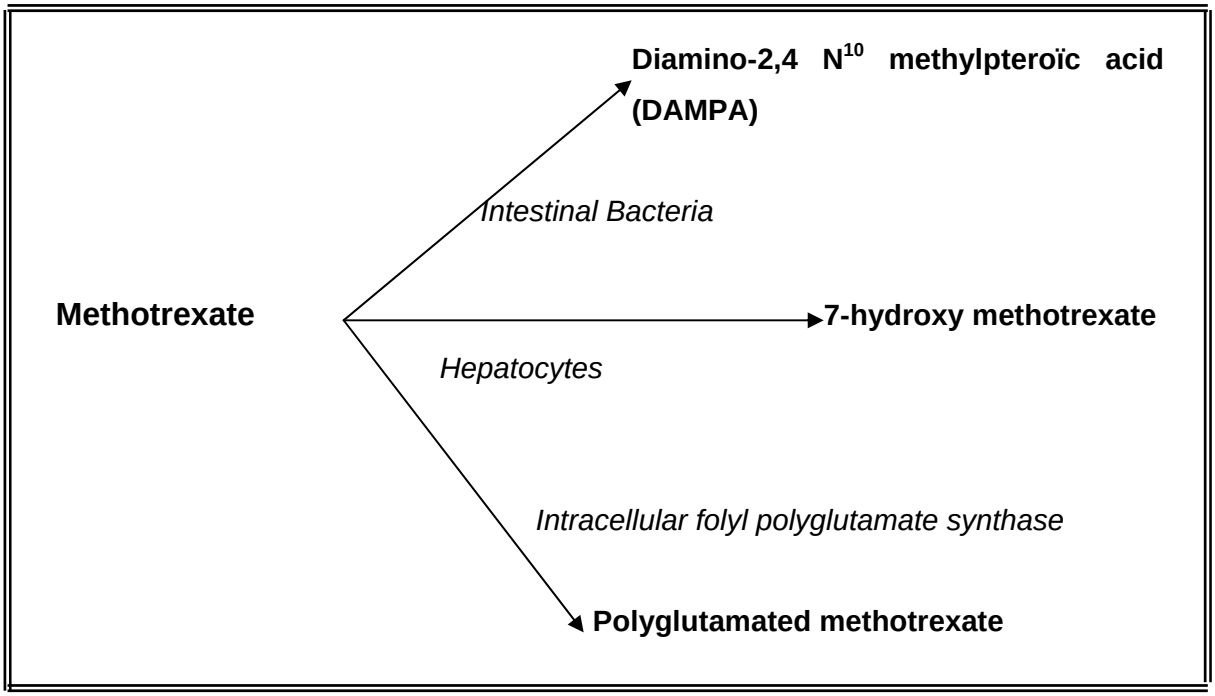

Figure 1.2: Intracellular metabolism of methotrexate in the intestine, liver and intracellular

a) Anti-inflammatory mechanism of action of methotrexate

MTX-polyglutamates inhibits the enzymatic conversion of 5-aminoimidazole-4-carboxamide-ribonucleotide (AICAR) to formyl-AICAR by the enzyme AICAR-transformylase. This leads to the intracellular accumulation of AICAR (Figure 1.3). This then in turn leads to the inhibition of adenosine production that interacts with specific receptors to diminish inflammation and tissue injury (Majumdar & Aggarwal, 2001). It also inhibits the production of inflammatory cytokines such as TNF-α, IL-6 and IL-8 in LPS-activated monocytes, macrophage cell lines and in an animal model of rheumatoid arthritis. It is believed that the suppression of inflammation during rheumatoid arthritis by low dose MTX is mediated by the release of adenosine.

Because AICAR inhibits the conversion of AMP to inosine mono-phosphate by AMP deaminase, the intracellular accumulation of AICAR will decrease inosine mono-phosphate production. AMP is converted to adenosine by 5'-ectonucleotidase (CD73). The expression of CD73 on lymphocytes depends

on their state of differentiation and function. CD73 expression on different cell types can be upregulated by IL-1, PGE_2, TNF-α and PMA (Majumdar & Aggarwal, 2001; Genestier *et al*, 2000). This surface enzyme can be down regulated by immune mediators, such as IL-4 and IFNγ, which decreases the activity of 5'-ectonucleotidase on peripheral blood mononuclear cells (PMBC). There have been reports that IL-1 and TNF- α increase the activity of this enzyme (Cutolo *et al*, 2001) (Figure 1.3).

b) Anti-cancer mechanism of action of methotrexate

It appears that methotrexate inhibits DNA synthesis more than RNA synthesis in humans (Bleyer, 1978). The drug is highly cell cycle dependent and acts predominantly in the S-phase (DNA synthesis). Low-dose MTX (< 20mg/m^2) inhibits leukemic myeloblasts in the S-phase for approximately 20 hours with little effect on the G_1, G_2 and M phases of the cell cycle. At higher doses, it inhibits the myeloblasts in the S-phase for more than 48 hours and slows the entry of cells from G_1 into the S-phase (Bleyer, 1978).

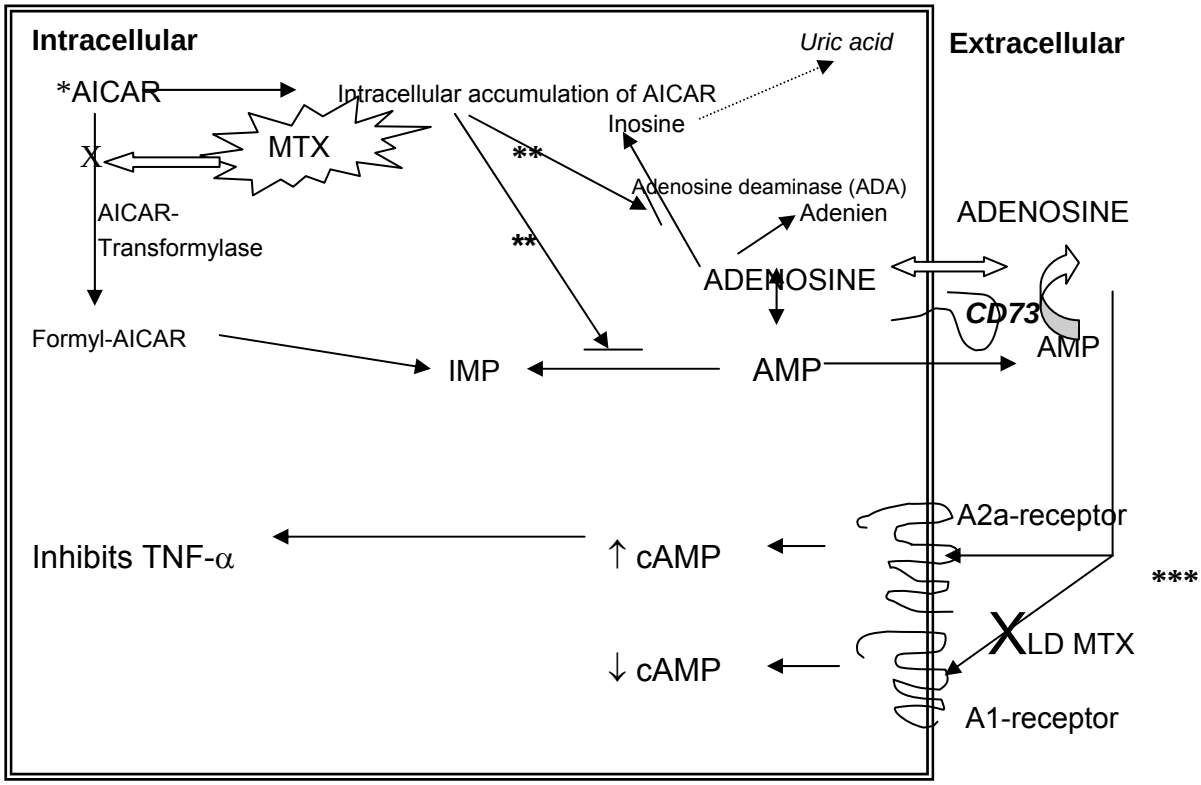

Figure 1.3: The intracellular and extracellular effects of methotrexate

** Inhibition of conversion of AICAR to formyl-AICAR by MTX that leads to the intracellular accumulation of AICAR*

*** The accumulation of AICAR leads to the inhibition of adenosine deaminas (ADA) and AMP deaminase – this lead to the increase of adenosine-5 monophosphate and adenosine intracellularly and extracellularly*

**** Extracellular adenosine bindes to A2a- and A1 receptors – the A2a receptor binding/pathway will be predominant causing an increase in cAMP intracellularly (increase in cAMP leads to immunosuppression)*

High dose methotrexate can also "arrest" cells in the G_2 phase (Bleyer, 1978). This can be ascribed to the inhibition of amino acid interconversions, homocysteine to methionine and glycine to serine, by methotrexate. Therefore, methotrexate interferes with protein synthesis that causes cell cycle arrest in the G_2 phase (Bleyer, 1978).

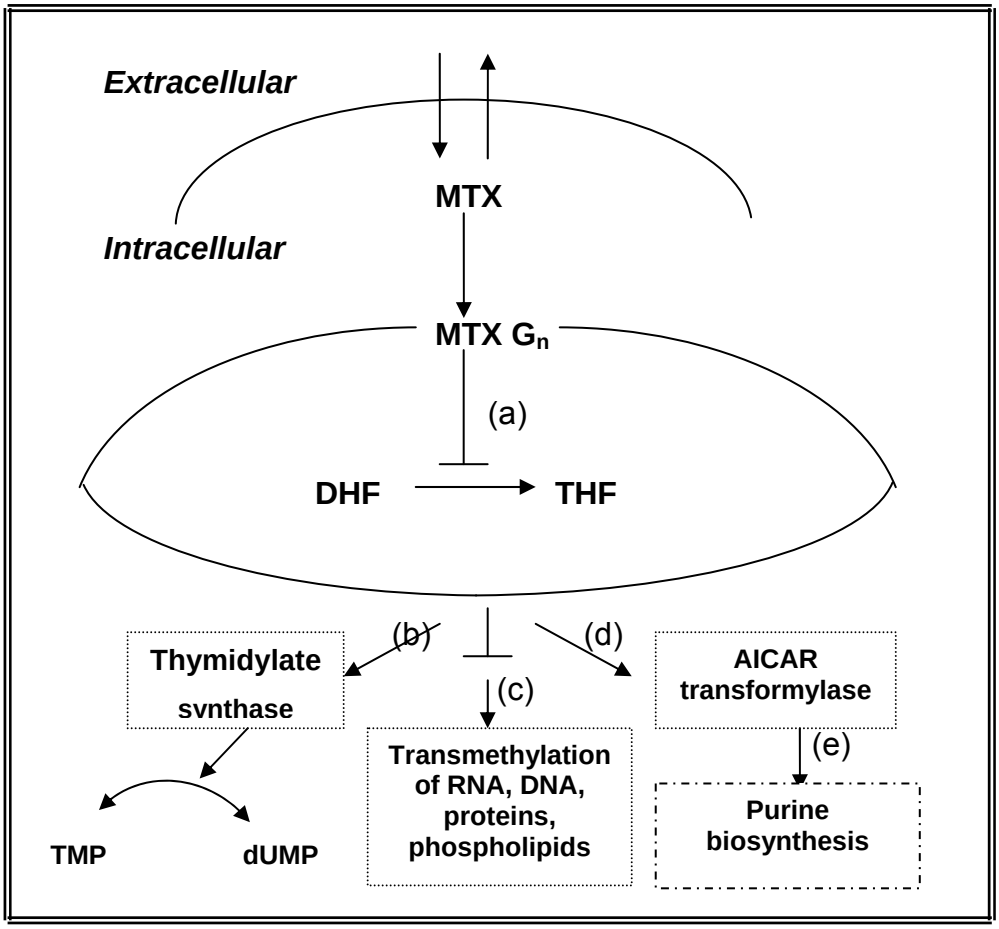

Figure 1.4: Possible sites of anti-cancer action of methotrexate (Seitz, 1999)

a) *Inhibition of dihydrofolate reductase (DHF) leads to deficit of tetrahydrofolaten (THF)*
b) *Inhibition of thymidylate synthase*
c) *Inhibition of transmethylation of RNA, DNA, Proteins and phospholipids*
d) *Inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase*
e) *Interference with **de novo** purine biosynthesis*

1.3 Pharmacokinetics of MTX

Methotrexate's volume of distribution (Vd) is 0.4-0.8L/kg. This refers to the ratio between the plasma concentration of a drug (as methotrexate in this

case) with the total amount of drug in the body (Reynolds, 1996). Peak concentrations in the plasma of 1 to 10μM are obtained after doses of 25-100mg/m². It penetrates ascetic fluid and effusions and this may act as a depot and thus enhance toxicity (Hardman et. al., 1996). Clearance from the plasma is reported to be triphasic. The first phase is the rapid distributive phase and is followed by the second phase, which reflects renal clearance (t½ of 2 to 3 hours). The terminal elimination t½ (third phase) is between 3-10 hours after low oral doses and 8-15 hours after high-dose parenteral treatment. The terminal half-life is prolonged by renal failure and may be responsible for the major toxic effects of the drug on the bone marrow and gastrointestinal tract (Hardman et. al., 1996; Reynolds, 1996).

Distribution of methotrexate into body spaces, such as the pleural or peritoneal cavity, occurs slowly (Hardman *et al*, 1996). If such spaces are expanded due to pathology, it may act as a site of storage and release of the drug, with the resultant prolonged elevation of plasma concentrations and more severe toxicity. MTX is also retained for several weeks in the kidneys and for months in the liver. Repeated doses of MTX can cause sustained serum concentrations and tissue accumulation (Hardman *et al*, 1996).MTX undergoes hepatic and cellular metabolism to form polyglutamates. These metabolites can be converted back to MTX through hydrolysis. Enterohepatic recirculation may occur. Small amounts are excreted in the faeces via bile excretion (Vucinic, 2002). Renal excretion of methotrexate occurs through glomerular filtration and active tubular secretion. Therefore, drugs that i) reduce renal blood flow (e.g. NSAID's), ii) that are nephrotoxic (e.g. cisplatin) or iii) that are weak organic acids (e.g. aspirin), can delay drug excretion and lead to myelosuppression (Vucinic, 2002).

Approximately 50% of methotrexate is bound to plasma proteins and may be displaced from plasma albumin by different drugs, such as sulphonamides, salicylates, tetracycline, chloramphenicol and phenytoin (Hardman *et al*, 1996). Caution should be used if these drugs are given concomitantly. Of the drug absorbed: 40-50% of a small dose to about 90% of a larger dose is excreted unchanged in the urine within 48 hours, mostly within the first 8-12

hours. A small amount is excreted in the faeces and bile. It has also been detected in small amounts in saliva and breast milk and has been shown to cross the placenta (Hardman *et al*, 1996).

When methotrexate is given in the treatment of resistant anti-inflammatory diseases, such as psoriasis and rheumatoid arthritis, low dosages are given orally once a week (7.5-20mg/week). These low dosages are rapidly absorbed from the gastro-intestinal tract, whereas higher dosages are less well absorbed (Hardman *et al*, 1996).

1.4 Drug resistance and drug-drug interactions

The acquired resistance of cells against drugs can be attributed to one of three mechanisms (Bleyer, 1978). Firstly, some cells develop resistance to MTX by synthesising more dihydrofolate reductase enzyme. Secondly, cells cease to transport methotrexate as readily over the cell membrane vs more sensitive cells. Thirdly, cells can produce an aberrant dihydrofolate reductase that induces resistance due to a bypass effect (Bleyer, 1978). A fourth mechanism can be due to the decreased ability of cells to convert MTX to polyglutamates intracellularly (Genestier *et al*, 2000).

Methotrexate also interacts with a variety of other drugs (Bleyer, 1978). Probenecid, salicylate and other weak organic acids can diminish its renal tubular transport. Salicylate and sulfisoxazole increases free MTX levels in plasma by displacing the drug from its binding sites on plasma proteins. Severe leucopenia (often fatal) has been shown in patients taking aspirin during MTX treatment. Cephalotin and hydrocortisone decreases the uptake of MTX by human leukaemia cells. A 40mg IV MTX dose slows down the biotransformation and presumably the activation of cyclophosphamide. Hydrocortisone and prednisone decreases the cytotoxic effects of MTX on bone marrow cells. Oral broad-spectrum, non-absorbable antibiotics decrease the intestinal absorption of MTX. Antibiotic may also interfere with the enterohepatic circulation of MTX by inhibiting bowel flora and suppressing the metabolism of the drug by these bacteria (Bleyer, 1978).

1.5 Toxicity

Toxicity studies on mice have shown that MTX interferes with embryogenesis and causes foetal growth retardation and embryo lethality (Dollery, 1999). However, the clinical significance of this in pregnant cancer patients receiving chemotherapy has not been defined yet. It can cause myelosuppression and one of MTX's metabolites, 7-hydroxymethotrexate, can lead to renal and hepatic toxicity following high dose therapy (Dollery, 1999). There are case studies that have shown that MTX treatment can lead to hepatotoxicity (Van Outryve *et al*, 2002). Some researchers measure liver transaminase enzyme levels, namely hepatic aspartate aminotransferase (AST), to determine whether MTX induces hepatotoxicity (Kremer, 2002). It has become clear that there is a significant correlation between the increase of hepatic aspartate aminotransferase (AST) and progression of histological deterioration in patients with RA receiving MTX weekly (Kremer, 2002). AST should be measured every 6-8 weeks when under MTX treatment. A mild increase can be noticed in 30% of patients receiving MTX. Increased values of AST can normalize without the discontinuation of treatment and only in some patients the doses should be reduced (Vucinic, 2002).

The possibility of MTX causing hepatotoxicity increases with pre-existing liver disease, obesity, diabetes mellitus, excessive alcohol use, renal failure and cumulative dose or 5mg (Vucinic, 2002).

1.6 Drug delivery systems

Methotrexate, which can be used for cancer treatment and as an anti-inflammatory drug have various adverse effects (Reynolds, 1996). To minimise the toxic effects of especially anti-neoplastic drugs, drug delivery systems are used to improve cancer cell specificity of a drug during chemotherapy (Swarts, 2002). The transporting device behaves as a shield or protective envelope into which the drug may be placed. While the drug is absorbed by or attached to the transporting device, the drug should be totally inert in a biological environment. Some of the properties that should be built into the polymeric drug carrier include biocompatibility and water-solubility (Swarts, 2002). Furthermore, it must have sufficiently large molecular mass to prevent quick excretion from the body and have a large amount of drug attachment sites that must allow easy drug-polymer coupling reactions without side reactions to generate biodegradable bonds between drug and polymer. It must also be biodegradable to allow elimination of the spent polymeric carrier from the body after the drug has been delivered. In the case of methotrexate, polymers are used to make the drug more water-soluble and therefore make the transport of MTX via the bloodstream, easier. Therefore, there is the assumption that a more water-soluble MTX-polymer has a greater therapeutic activity (Swarts, 2002).

There has been much research done on drug conjugates and their advantage in drug therapy (Garnett, 2001). Drugs may be linked to macromolecules and potent drugs, such as cisplatin and methotrexate. Drug-immuno-conjugates and antibody-carrier-drug conjugates has also been synthesized to try and increase the effectivity of the drug. These methods have been reviewed by Garnett (Garnett, 2001). A few MTX-conjugates have also been studied. A gelatine-methotrexate conjugate is such an example that showed moderate advantages to that of MTX use (Kosasih *et al*, 2000). A fibrinogen-methotrexate conjugate has also been studied. The fibrinogen macromolecule has a property of accumulation in tumors; therefore researchers tested it for selectivity to tumors. However, the conjugate did not

show improved properties compared to MTX in *in vitro* studies (Boratyński *et al*, 2000).

One such a drug that was engineered to attempt to overcome some of the problems is D-85, a methotrexate polymer. This is a molecule design where polyglutamic acid side chains were coupled onto a methotrexate molecule (Figure 1.5). The chain marked $[\ ]_x$ is coupled an average of 9.2 times onto the methotrexate molecule (x=9.2). The chain marked $[\ ]_y$ is coupled once to the methotrexate molecule (X=1). Researchers have also looked at other side chains, such as dextran, human albumin and soluble starch (Harding, 1971).

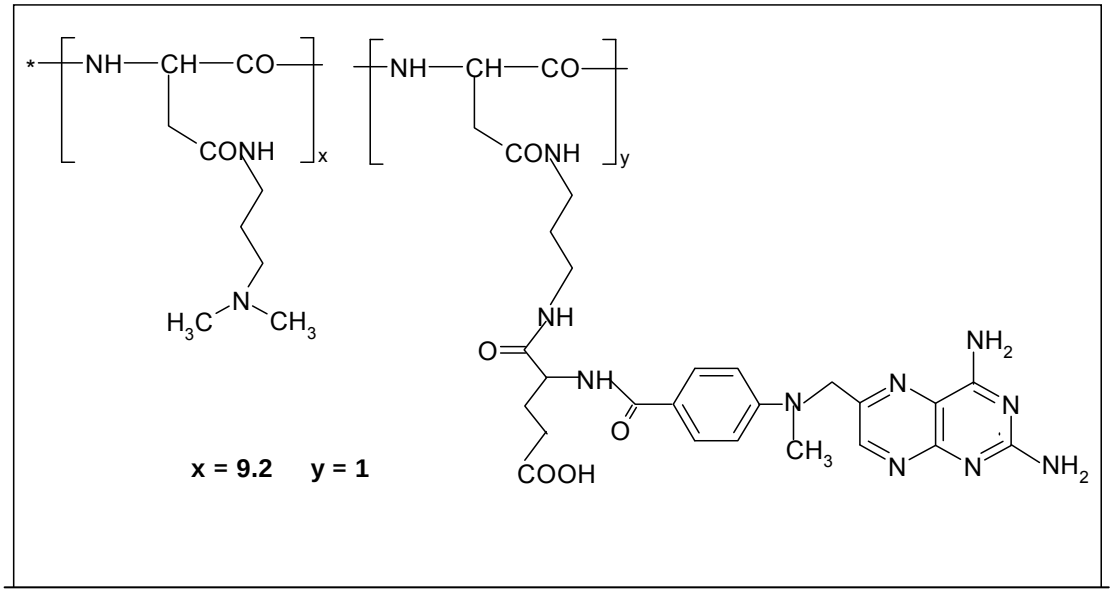

Figure 1.5: Structure of conjugate D-85

1.7 Hypothesis, aim and objective

In the dynamic field of drug development, it is fundamentally important to stay continuously on the forefront of drug development. This is a necessity enforced onto scientists and pharmaceuticals companies by the everlasting search for the "new penicillin" of our times. Therefore, it was decided to study polymers of methotrexate, moreover D-85, as methotrexate is still greatly used in the therapeutic fields of cancer therapy and the treatment of anti-inflammatory diseases, such as rheumatoid arthritis.

The hypothesis, aim and objective of this project was as follows:

Hypothesis:
The MTX polymer D-85 has superior anti-inflammatory and anti-cancer activity with less toxicity and better therapeutic index compared to MTX. The structure of D-85 can be seen in figure 1.5 (page 15).

Null hypothesis
The MTX polymer D-85 has equal anti-inflammatory and anti-cancer activity with the same therapeutic index when compared to MTX.

Aim:
The aim of this study was to test the above-mentioned hypothesis by evaluating and comparing MTX and D-85 in terms of the following criteria:

1. To determine if the MTX-polymer, D-85, has superior activity against cancer cells compared to MTX but with minimal cytotoxic activity against normal cells.

2. To screen D-85 in comparison with MTX as an immunosuppressive drug.

3. To screen D-85 in comparison with MTX as an anti-cancer drug.

4. To determine whether D-85 has lower toxicity than methotrexate.

Objectives:

1. ***Efficacy studies***
   a. *In vitro* studies to determine efficacy of D-85 and MTX on different cancer cell lines, namely cervix carcinoma cells (HeLa), breast carcinoma cells (MCF-7) and prostate cancer cells (DU145) ***(Chapter 2)***

2. ***Toxicity studies***
   a. *In vitro* studies to determine the toxicity of D-85 and MTX on different non-cancer cell lines, namely normal human lymphocytes, normal breast cancer cells (MCF 12A) and primary chick fibroblasts (harvested from 7-day old chick embryos) ***(Chapter 2)***
   b. *In vivo* studies to determine toxicity of D-85 and MTX:
      i) In Balb/C mice treated once a week for four weeks using an anti-inflammatory drug treatment regimen (15mg/kg per week, intraperitoneally) – *Experiment I* ***(Chapter 5)***
      ii) In Balb/C mice administered treatment for three weeks using an anti-cancer drug treatment regimen (3.5mg/kg every 2nd day for 21 days, intraperitoneally) – *Experiment II* ***(Chapter 5)***

3. ***Mechanism of action studies***
   a. *In vitro studies* to determine whether D-85 arrests MCF-7 breast carcinoma cells in the same phase of the cell cycle as methotrexate ***(Chapter 3)***
   b. *In vitro studies* to determine whether D-85 has superior immunosuppressive properties than MTX in mixed lymphocyte cultures, a graft-vs-host (GVHD) model ***(Chapter 4)***

1.8 Ethical considerations and funding

Funds for this project were made available from grants obtained from CANSA (Cancer Association of South Africa) and THRIP (Technology and Human Resources for Industry Programme).

Approval for the animal experiments has been obtained from the AUCC (Animal Use and Care Committee), Faculty of Veterinary Sciences, University of Pretoria. Blood from healthy, consenting donors were used for the *in vitro* experiments. A qualified nurse or doctor drew the blood from volunteers. All information regarding the donor's identity and laboratory data is kept confidential.

Chapter 2:

In vitro cytotoxicity

CHAPTER 2: *In vitro* cytotoxicity

AIM:

The aim of the *in vitro* studies that were performed were to determine the following:

a) The **efficacy** of D-85 and MTX on different **cancer cell lines**, namely cervix carcinoma cells (HeLa), breast carcinoma cells (MCF-7) and prostate cancer cells (DU145),

b) The **effects** of D-85 and MTX on different **non-cancer cell lines**, namely normal human lymphocytes, normal breast cancer cells (MCF 12A) and primary chick fibroblasts (harvested from 7-day old chick embryos)

2.1 Introduction

Methotrexate has a wide range of indications. It is used in the treatment of inflammatory diseases and in cancer chemotherapy. Its main use as an anti-inflammatory drug is in the treatment of rheumatoid arthritis (RA) (Dollery, 1999). Methorexate's primary mechanism of action as an anti-inflammatory agent is stimulation of adenosine mediated via CD73 ectonucleotidase cell receptors found on lymphocytes and other peripheral blood mononuclear cells (PBMC) (Majumdar & Aggarwal, 2001; Cutolo *et al*, 2001).

Methotrexate is commonly used in the treatment of acute lymphoblastic leukemia (ALL) (Hardman *et al*, 1996). It is also widely used in the treatment of other carcinoma's e.g. breast carcinoma, choriocarcinoma, gastric carcinoma, high-grade non-Hodgkin's lymphomas and head and neck carcinomas (Dollery, 1999). Its main mechanism of action as a chemotherapeutic drug is as an anti-folate. Methotrexate is a structural analogue of folic acid. Methotrexate inhibits the conversion of dihydrofolate (FH_2) to tetrahydrofolate (FH_4) by inhibiting the enzyme dihydrofolate reductase (DHFR) competitively. Therefore, it creates a shortage of

tetrahydrofolate that is needed for the *de novo* synthesis of purines and pyrimidines (nucleotides) that is required for cell division (Dollery, 1999).

Cancerous cell lines

In this study the cytotoxic effects of MTX and D-85 on cancerous and non-cancerous cell lines were investigated. The cancerous cell lines that were used are cervix carcinoma cells, breast cancer cells and prostate cancer cells. The non-cancerous cell lines that were used included normal human lymphocytes, non-malignant breast cancer cells and primary chicken fibroblasts (derived from 7-day old chick embryos). The cervix carcinoma cell line (HeLa) was used because of its general use in the screening of experimental drugs, as well as in the analyses of different pharmacodynamic aspects and characteristics of drugs, such as methotrexate (Zhao *et al*, 2004).

The breast cancer cell line, MCF 7 was initiated in 1973 from a malignant pleural effusion from a postmenopausal woman with metastatic infiltrating ductal carcinoma of the breast (Whang-Peng *et al*, 1983). These cells are commonly used in the study of methotrexate and related drugs because of its indication in breast cancer (Kennedy *et al*, 1985; Morrison & Allegra, 1989; Su & Cifti, 2002).

The prostate cancer cell line (DU 145) is not representative of the indications of methotrexate but is nevertheless used in MTX studies, such as drug-uptake in these cells (Wosikowski *et al*, 2003). DU 145 cells was derived from the brain of a Caucasian, male patient with metastatic prostate carcinoma. It is a hypotriploid human cell line with a single copy of the X chromosome. The Y chromosome is abnormal through its translocation to an unidentified chromosomal segment. These cells do not express prostate antigens (http://www.ATCC.org).

Non-cancerous cell lines

Normal human lymphocytes were obtained from healthy consenting volunteers. These cells were used because of their importance in inflammatory responses and to determine whether the drugs have negative effects on these cells when it is used for its anti-neoplastic indication. Methotrexate's mechanism of action as an anti-inflammatory drug can mainly be seen in its effects on immuno-competent cells such as lymphocytes, monocytes and neutrophils (Seitz, 1999; Cutolo *et al*, 2001; Majumdar & Aggarwal, 2001; Cronstein, 1996).

The non-malignant breast cancer cell line, MCF 12A, was used to see whether there is toxicity and selectivity differences of the drugs used compared to its effects on malignant breast cancer cells, MCF 7. This cell line was derived from non-tumorogenic epithelial tissue that was obtained during a reduction mammoplasty from a patient with fibrocystic breast disease (http://www.ATCC.org). These results are therefore indicative of whether the use of these drugs will be effective in the treatment of breast cancer.

Primary chick embryo cells are used to assess primary cell growth. Chicken embryo fibroblasts are widely used in the development of vaccines and studying of viruses (Balk *et al*, 1979). It is also used to determine the effects of drugs on primary cells. These cells maintain all their standard cell functions such as the cells' p53 regulation, whereas in cancerous cell lines, this gene regulation is not functioning optimally (Kim *et al*, 2001).

2.2 Materials and methods

2.2.1 Study drug

A novel methotrexate polymer, D-85, was supplied by Prof. EW Neuse (Department of Chemistry, University of the Witwatersrand). Methotrexate was purchased from Fluka BioChemika (Johannesburg, South Africa).

D-85 was dissolved in filtered distilled water. MTX was dissolved in filtered, distilled water, using 1M sodium hydroxide (NaOH) to aid the process of dissolving the MTX that is insoluble in water.

2.2.2 Cell cultures

The *in vitro* studies were divided into two subsections according to the cell lines used:

A) *In vitro* studies in cancerous cell lines:

- HeLa cervix carcinoma
- MCF 7 breast carcinoma cells
- DU 145 prostate carcinoma cells

B) *In vitro* studies in normal cells and non-cancerous cell lines.

- Normal human lymphocytes – normal cells
- MCF 12A non-malignant breast carcinoma cells – non-cancerous cell line
- Primary chicken fibroblasts – normal cells

The respective cells and cell lines were plated as described below. In addition refer to Table 2.1 and Table 2.2.

A. *In vitro* studies in cancer cell lines:

Cell suspensions were prepared and plated onto 96-well microtiter plates. Cells were then treated separately with MTX and D-85 at a concentration range (Table 2.1). Cells were incubated for 7 days after which a MTT assay (3-[4,5-dimethylthizol-2-yl]-2,5-difhenyltetrazolium bromide) was performed (Mosmann, 1983).

B. *In vitro* studies in normal cells and non-cancerous cell lines:

Cell suspensions were prepared and plated onto 96-well microtiter plates. Cells were then treated separately with MTX and D-85 at a concentration range (Table 2.2). Cells were incubated for 7 days after which a MTT assay (3-[4,5-dimethylthizol-2-yl]-2,5-difhenyltetrazolium bromide) was performed (Mosmann, 1983).

2.2.3 Cell culture preparation

A *In vitro* studies in cancer cell lines

The cells were cultured in a 37°C incubator in atmosphere of 5% CO_2 to 95% air. The mediums of the cell cultures were replaced in a 3-4 day cycle with fresh growth medium.

The HeLa, MCF 7 and DU 145 were removed from the cell culture flasks as follows:

1. The growth medium in the flasks were decanted and discarded.
2. The flasks were rinsed with a small amount of the respective growth medium for the respective cell line, to remove "old" medium.
3. *HeLa and DU 145 cells:*

   Trypsin, an enzyme used to loosen cells from the cell culture flask through its enzymatic activity on the cell surface, was added. Just enough trypsin was added to cover the cell culture flasks horizontal surface. Once the cells started lifting up from the surface, the cell suspension was poured into 15ml centrifuge tubes. The culture flasks were washed out with the respective growth medium. This was also poured into the 15ml centrifuge tubes – *step 5 followed next*
4. *MCF 7 cells:*

   Adhering MCF 7 cells were scraped loose from the cell culture flask with a cell scraper. The medium containing suspended MCF 7 cells were then decanted into a 50ml centrifuge tube. The flask was then washed with the respective growth medium and the growth medium was also decanted into the 50ml centrifuge tube - *step 5 followed next*
5. The tubes were centrifuged for 10 minutes at 1000 rpm. The supernatant was discarded and the cell pellet was resuspended in 1ml of the relevant growth medium. The cell suspension was made up to the concentrations noted in Table 2.1 and Table 2.2.

To obtain the above-mentioned cell concentration suspensions, the cells were counted with a haemacytometer using a Reichert Jung MicroStar110 microscope.

Cancer cell line	Culture media	Incubation period	Cells (per ml)	MTX drug concentration range	D-85 drug concentration range
1) HeLa (cervic carcinoma cells)	E-MEM + 10% fetal bovine calf serum (*)	7 days	$2.5x10^4$	0.0015-0.19 μg/ml	Equivalent of 0.0015-0.19 μg/ml MTX
2) MCF 7 (Breast carcinoma cells)	D-MEM + 10% fetal bovine calf serum (*)	7 days	$2x10^4$	0.003125-100 μg/ml	Equivalent of 0.003125-100 μg/ml MTX
3) DU 145 (Prostate carcinoma cells)	RPMI + 10% fetal bovine calf serum (*)	7 days	$2.5x10^4$	0.003125-100 μg/ml	Equivalent of 0.003125-100 μg/ml MTX

Table 2.1: Growth medium and criteria used for cancerous cells *in vitro* experiments

(* Penicillin/Streptomycin [Gray & Brenwald, 1999])

B *In vitro* studies in normal cells and non-cancerous cell lines

1) *Lymphocyte Transformation (Definition: cell activation and proliferation of lymphocytes induced by adding phytohaemaglutinin [PHA])* (Klein *et al*, 2004)
   a) Lymphocytes were prepared from whole blood collected from healthy, consenting donors.
   b) Blood was collected in flasks containing heparin to prevent the blood from clotting. This blood was separated into different layers using Histopaque (Sigma Aldrich, Johannesburg; density: 1.077±0.0001), a high viscosity fluid that separates the different cells and constituents of blood during centrifugation because of the different masses of the different cells and constituents.
   c) After centrifugation during which the blood separated into different layers, the lymphocytes formed a hazy, white layer in the centrifuge tube. This was drawn off with a pipette and transferred to a new centrifuge tube. These lymphocytes were then washed twice using RPMI growth medium to remove platelets.
   d) Erythrocytes that might have still been present after the above-mentioned separation method (as in *a*) were lysed by adding cold ammonium chloride to the cells for 10 minutes. The cell suspension was centrifuged to obtain a cell pellet.
   e) The lymphocyte cell pellet was then washed with RPMI medium. The suspension was centrifuged again to obtain a cell pellet – growth medium was discarded.
   f) Lymphocytes were then resuspended in RMPI medium supplemented with 10% fetal calf serum and antibiotics to obtain the cell concentration as referred to in Table 2.2.
   g) Lymphocytes were pipetted into 96-well microtiter plates at a concentration of $2x10^6$ cells per well and were treated separately with various concentrations of methotrexate and D-85.
   h) Lymphocytes were incubated for three days at 37°C in a 5% CO_2 atmosphere.

i) A metabolic assay was used based on the reactivity of MTT (3-[4,5-dimethylthizol-2-yl]-2,5-difhenyltetrazolium bromide) with viable cells (Mosmann, 1983) (Refer to paragraph ***2.2.4***).

2) Non-malignant breast carcinoma cells (MCF 12A)

The cells were maintained and prepared for experimental procedures as described in the above-mentioned paragraph 2.2.3 A, using the same method as for the preparation of MCF 7 cells. The cell suspensions for the cell viability assay (MTT assay) were prepared and standardized as in Table 2.2. The cells were transferred to a 96 well microtiter plate and were incubated for 7 days at 37°C.

3) *Primary chicken fibroblast cells*

The eggs were obtained from Boschkop hatcheries, Donkerhoek. Ethical approval was obtained from the Animal Use and Care Committee (AUCC), Faculty of Veterinary Sciences, University of Pretoria. Eggs were incubated at 37°C in a humid atmosphere for 7 days. The embryo was in stage 30-32 on day 7 according to the Hamburger & Hamilton staging, with only a few hours difference between these three stages (Hamburger & Hamilton, 1951). The chick embryos were harvested as follows:

1. Eggs were sprayed with 70% ethanol to sterilize the egg shell. The wide top of the egg was cracked open using a sterile forceps.
2. The forceps was resterilized after opening the egg.
3. The CAM (chorioallantoic membrane) was pierced with the sterile forceps and the embryo lifted up by grasping it under its neck. The umbilical cord of the embryo was cut and the embryo died within seconds. The embryo was then transferred to a 9cm Petri dish containing 20ml of EMEM containing 5% fetal calf serum.
4. The unwanted tissue (e.g. fat, necrotic material) was dissected off the embryo and discarded. The remaining embryo tissue was then cut into 3mm cubes with a sterile scalpel.
5. The tissue was transferred to a 50ml sterile centrifuge tube and the pieces were allowed to settle.

6. The tissue was washed with EMEM culture medium and time was allowed for the pieces to settle.
7. The supernatant, which is a suspension of the fibroblasts, was removed and put into sterile 50 ml tubes and were kept on ice.
8. Step 6 was repeated 2-3 more times. This was repeated until all the tissues were broken up into clusters (microscopic) or single fibroblasts during centrifugation.
9. The tissue sediment left after steps 6 was repeated 2-3 times was then transferred to empty sterile beaker and 180ml of PBS plus 20ml of 2.5% trypsin was added to promote cells to detach from each other. It was stirred with a magnetic stirrer at 100rpm for 30 minutes to obtain the last few fibroblasts from the tissue sediment.
10. The cell suspension was also decanted into a 50ml centrifuge tube.
11. This 50ml tubes with the fibroblast cell suspension, as well as the tubes from steps 5-7 (tubes that were kept on ice), were centrifuged at approximately 500g for 5 minutes to form a fibroblast cell pellet.
12. The pellet was resuspended in 10ml of RPMI medium with 10% fetal calf serum and the suspension was stored on ice until all the 50ml tubes have been centrifuged.
13. Each cell suspension was diluted to $1x10^6$ per ml in RPMI growth medium supplemented with HAM. This cell suspension was then put in 25 cm^2 cell culture flasks.
14. The medium of these cell culture flasks were changed every 2-4 days and the supernatant was checked daily for viable cells as some fibroblast cells can be slow to attach or may even prefer to proliferate in suspension (Freshney, 2000).
15. When fibroblasts attached to the cell culture flask's surface and formed a uniform monolayer, it was ready to be harvested.

Fibroblasts were harvested using cell scrapers and NOT trypsin as it is a primary cell line and is very sensitive. Trypsin might influence the fibroblasts cell viability negative.

Normal cells & Non-cancerous cell line	Culture media	Incubation period	Cells (per ml)	MTX drug concentration range	D-85 drug concentration range
1) Normal human lymphocytes	E-MEM + 10% fetal bovine calf serum (*)	3 days	2×10^6	0.025-200 μg/ml	Equivalent of 0.025-200 μg/ml MTX
2) MCF 12A (Non-malignant breast carcinoma cells)	D-MEM + 10% fetal bovine calf serum (*)	7 days	2×10^4	0.003125-100 μg/ml	Equivalent of 0.003125-100 μg/ml MTX
3) Normal Primary chick fibroblasts	RPMI + 10% fetal bovine calf serum (*)	3 days	1×10^5	0.003125-100 μg/ml	Equivalent of 0.003125-100 μg/ml MTX

Table 2.2: Growth medium and criteria used for normal cells and non cancerous cells in *in vitro* experiments

(* Penicillin/Streptomycin [Gray & Brenwald,999])

2.2.4 MTT cell viability assay

All the in vitro experiments were analysed using the MTT assay (Mosmann, 1983). This assay is used to determine the amount of viable cells that survived drug treatment, compared to the control. This MTT (3-[4,5-dimethylthizol-2-yl]-2,5-difhenyltetrazolium bromide) reacts with the mitochondria of viable cells to form violet coloured crystals. These crystals are dissolved with a solvent, namely DMSO (dimethyl sulfoxide). The plates were analysed spectrophotometrically after all these crystals have been dissolved. These results were analysed using GraphPad Prism Version 4, a statistical programme, to determine 50% of the inhibitory concentration value (IC50) of the respective drug. This was determined for each cell line used. A summary of the IC50 values are tabulated in Table 2.3.

2.2.5 *Growth mediums and other chemicals*

The growth mediums EMEM, DMEM and RPMI 1640 used in the cell culturing in the 96-well microtiter plates were purchased from Adcock (Johannesburg, South Africa). The heat-inactivated fetal calf serum used in these growth mediums to inactive possible compliment immunological effects, was purchased from Delta Bioproducts (Johannesburg, South Africa). The antibiotic mixture penicillin/streptomycin also used in these growth mediums was purchased from BioWhittaker^{TM}, CAMBREX Bio Science (10 000 U Pen/ml, 10 000 μg Strep/ml) (Walkersville, USA). The MTT that was used in the metabolic assays was purchased from Sigma Chemicals (Johannesburg South Africa).

The PBS (FTA Hemaglutination Buffer) that was used in the lymphocyte transformation cell separation procedure to wash the cell suspensions was purchased from BD-Becton, Dickenson & Company (Johannesburg, South Africa). The cold ammoniumchloride used to lyse the erythrocytes consisted of 8.3g NH_4Cl (Merck, Johannesburg, South Africa), 74mg EDTA (LabChem, Edenvale, South Africa) and 10g $NaHCO_3$ (Merck, Johannesburg, South

Africa) that was dissolved in 1L distilled water. The PHA that was used to stimulate the lymphocytes was purchased from Murex Biotech Ltd., Kent, England.

2.2.6 Equipment used in cell culture preparation

The cells were counted after resuspending it in growth media. It was counted with a haemacytometer using a Reichert Jung MicroStar110 microscope. Cell suspensions were vortexed using a Vortex-Genie 2 (LASEC [Laboratory & Scientific Equipment Co.], Johannesburg, South Africa). The centrifuges that were used are Allegra™ X-22 Centrifuge, S2096 rotor (Beckman Coulter, Johannesburg, South Africa) and Sigma 3K15 (Winsam Scientific & Precision Equipment, Johannesburg, South Africa). 96-well microtiter plates were analysed after the performing of the MTT assay with an ELx800UV spectrophotometer (Biotek Instruments, Analytical and Diagnostic Products, Weltevreden Park, South Africa).

2.3 Results

The results obtained from the experiments that were performed according to the procedures and methods discussed in **2.2** were analysed to obtain the respective cell lines' IC50 values in terms of both MTX and D-85 drug therapy. These results are tabled in Table 2.3 and Table 2.4. It refers to the concentration of the respective drug, MTX or D-85 that induced 50% of cell death in the respective cell lines.

In 2.3(a) and 2.3(b) here below the results of the cancerous cell lines, HeLa (cervix carcinoma), MCF 7 (breast carcinoma) and DU 145 (prostate carcinoma) are presented using line graphs (2.3(b), Figures 2.1, 2.2 and 2.3) that reflects the cell viability of the respective cells after treating the cells with a concentration range of respectively MTX and D-85.

In 2.3(c) and 2.3(d) here below the results of the non-cancerous cell lines, normal human lymphocytes, MCF 12A (non-malignant breast carcinoma) and primary chick fibroblasts are presented using line graphs (2.3(d), Figures 2.1, 2.2 and 2.3) that reflects the cell viability of the respective cells after treating the cells with a concentration range of respectively MTX and D-85.

a) Results obtained from cancerous cell lines (HeLa, MCF 7 and DU 145)

The results obtained from the *in vitro* assays performed on cancerous cell lines were analysed to determine the toxicity of D-85 and methotrexate. The results indicate that D-85 has a greater toxicity than methotrexate regarding the three cancerous cell lines used in this experiment. The IC50 values obtained for D-85 on both HeLa cervix carcinoma cells (0.015 µg/ml) and MCF-7 breast carcinoma cells (0.201 µg/ml) are lower than that of methotrexate (Table 2.3). An IC50 value for the treatment of DU 145 cells with the methotrexate polymer, D-85, were 22.174µg/ml whereas methotrexate possibly has a much higher IC50 value since no IC50 could be determined for MTX concentrations administered to DU 145 cells

(concentration range of 0.003125-100µg/ml MTX – refer to Table 2.1). Therefore one can postulate that methotrexate's IC50 value might be >100µg/ml as this was the upper concentration range administered to the cells.

In the treatment of HeLa cells with D-85 compared to methotrexate, significantly different results were obtained between 0.024-0.095 µg/ml. Cell growth was inhibited significantly at the concentrations 0.024µg/ml, 0.048µg/ml and 0.095µg/ml MTX or equivalent MTX in the polymer D-85 ($p<0.05$).

Significantly different results in the treatment of the MCF 7 breast carcinoma cells were obtained at the concentration range of 0.05-25µg/ml.

No significantly different results were obtained in terms of MTX and D-85 drug administration to the DU 145 cells, but further investigation at higher concentrations of MTX and D-85 (>100µg/ml MTX or D-85 equivalent) is needed to confirm or reject the possibility that the drugs do not have a cytotoxic effect of more than 50% on these cells.

IC50 (µg/ml)		
Cell Culture	**MTX**	**D-85**
HeLa	0.071 µg/ml	0.015 µg/ml
MCF-7	0.352 µg/ml	0.201 µg/ml
DU 145	no IC50	22.174 µg/ml

Table 2.3: IC50 values of D-85, compared to methotrexate, obtained after administration to three respective cancer cell lines

Cancerous cell lines	***Significant differences (p< 0.05) calculated at following concentrations****
HeLa	0.095 μg/ml, 0.048 μg/ml, 0.024 μg/ml
MCF 7	0.05 μg/ml, 6 μg/ml, 12 μg/ml, 25 μg/ml
DU 145	no p-values under 0.05

Table 2.4: Concentrations at which significant differences was calculated between MTX and D-85 treatment of non-cancerous cells

*** p-values determined using paired t-test and GraphPad Prism 4 statistical program**

b) Cancer cell lines: Results in line graph format (Figure 2.1, Figure 2.2 and Figure 2.3)

The *in vitro* toxicity experiments which were used to determine the 50% inhibitory concentration value of both MTX and D-85 in HeLa cervix carcinoma cells, MCF 7 breast carcinoma cells and DU 145 prostate carcinoma cells, were calculated using GraphPad Prism® Version 4 Statistical programme. These results can be seen in Figure 2.1, Figure 2.2 and Figure 2.3

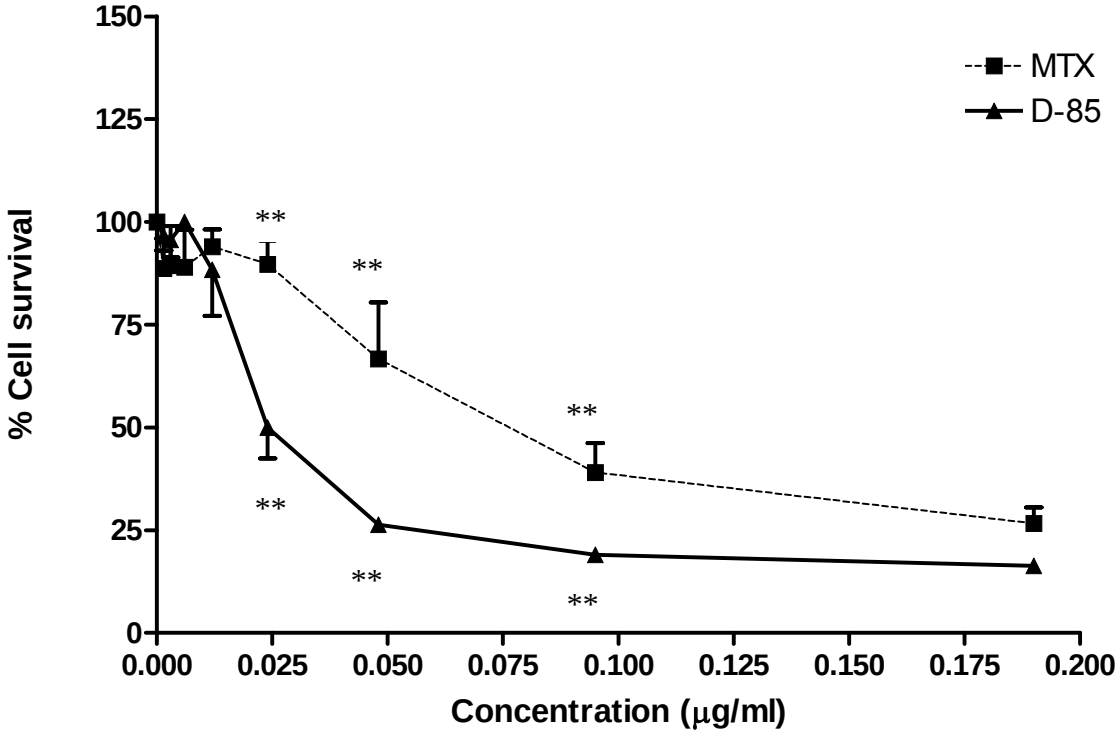

Figure 2.1: Cytotoxicity of D-85, compared to MTX, obtained after administration to HeLa (cervix carcinoma) cells

*** * p < 0.05 → significantly different from D-85**

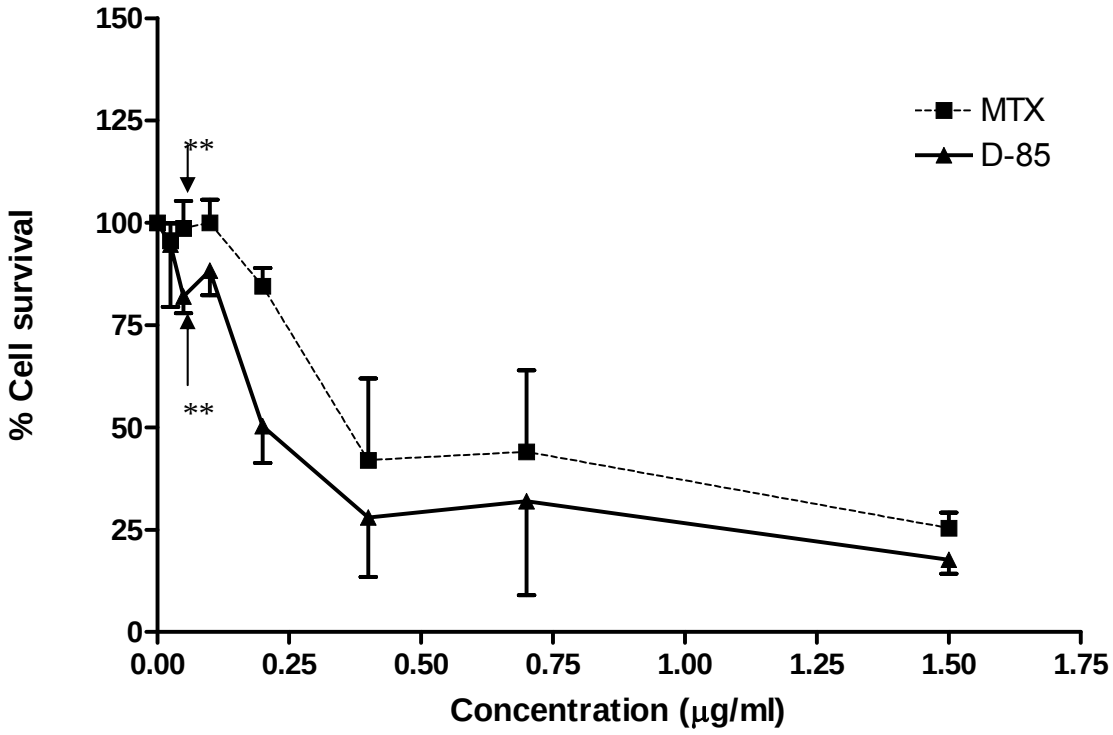

Figure 2.2: Cytotoxicity of D-85, compared to MTX, obtained after administration to MTX and D-85 to MCF 7 (breast carcinoma) cells

*** * $p < 0.05 \rightarrow$ significantly different from D-85**

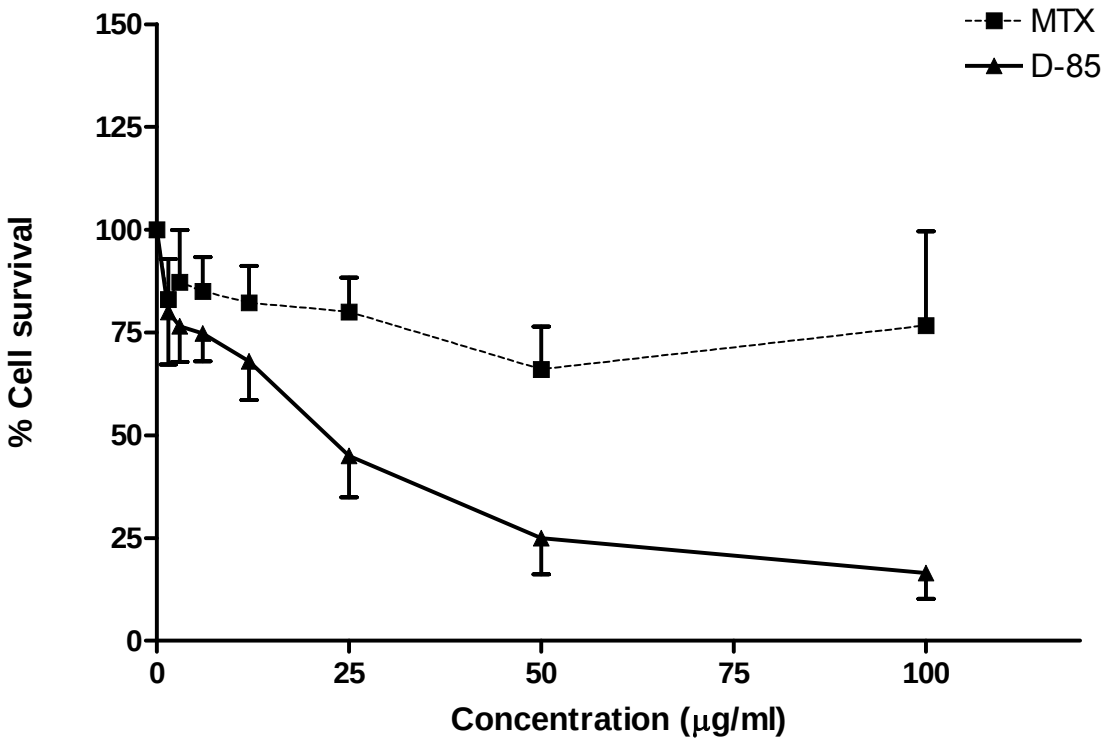

Figure 2.3: Cytotoxicity of D-85, compared to MTX, obtained after administration to DU145 cells

$0.05 < p < 0.5$

Although no significant differences between the cytotoxicity of D-85 and MTX on DU 145 were found, this might need further investigation. Concentrations of higher than 100μg/ml might obtain a significant difference. It could also possibly be attributed to a too large variation in the results obtained used to determine these results.

c) Results obtained from non-cancerous cell lines (normal human lymphocytes, MCF 12A non-malignant breast carcinoma cells and primary chicken fibroblasts)

The 50% inhibitory concentration that was determined for each of the non-cancerous cell lines are tabled in Table 2.4.

IC50 (µg/ml)		
Cell Culture	**MTX**	**D-85**
Lymphocytes: *Stimulated*	> 200 µg/ml	44.89 µg/ml
Lymphocytes: *Resting*	>200 µg/ml	24.12 µg/ml
MCF 12A	0.174 µg/ml	0.084 µg/ml
Primary chick fibroblasts	> 100 µg/ml	> 100 µg/ml

Table 2.5 (WAS 2.4): IC50 values of D-85, compared to methotrexate, obtained after administration to three respective non-cancerous cells and normal cells

Non-cancerous cells	***Significant differences (p< 0.05) calculated at following concentrations ****
Normal human lymphocytes:*Stimulated*	50 µg/ml, 100 µg/ml, 200 µg/ml
Resting	25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml
MCF 12 A	**p<0.05:** 25 µg/ml; **p<0.005:** 100 µg/ml
Primary chick fibroblasts	25 µg/ml, 100 µg/ml

Table 2.6: Concentrations at which significant differences was calculated between MTX and D-85 treatment of non-cancerous cells

*** p-values determined using paired t-test and GraphPad Prism 4 statistical program**

Although both MTX and D-85 are cytotoxic on normal cells, it is up to 100x less cytotoxic than when compared to the results obtained in the treatment of cancerous cells (Table 2.3). Significant differences in the treatment of these non-cancerous cells with MTX and D-85 are tabulated in Table 2.6.

The results in table 2.4 were obtained after repeating the experiments a minimum of 3 times and using the results obtained to determine the IC50 values with the statistical programme GraphPad Prism 4.0.

d) Non-cancerous cell lines: Results in graph format (Figure 2.4, Figure 2.5 and Figure 2.6

The *in vitro* toxicity experiments which were used to determine the 50% inhibitory concentration value of both MTX and D-85 in normal human lymphocytes, MCF 12A non-malignant breast carcinoma cells and primary chicken fibroblasts, were calculated using GraphPad Prism Version 4 Statistical programme. These results can be seen in Figure 2.1, Figure 2.2 and Figure 2.3.

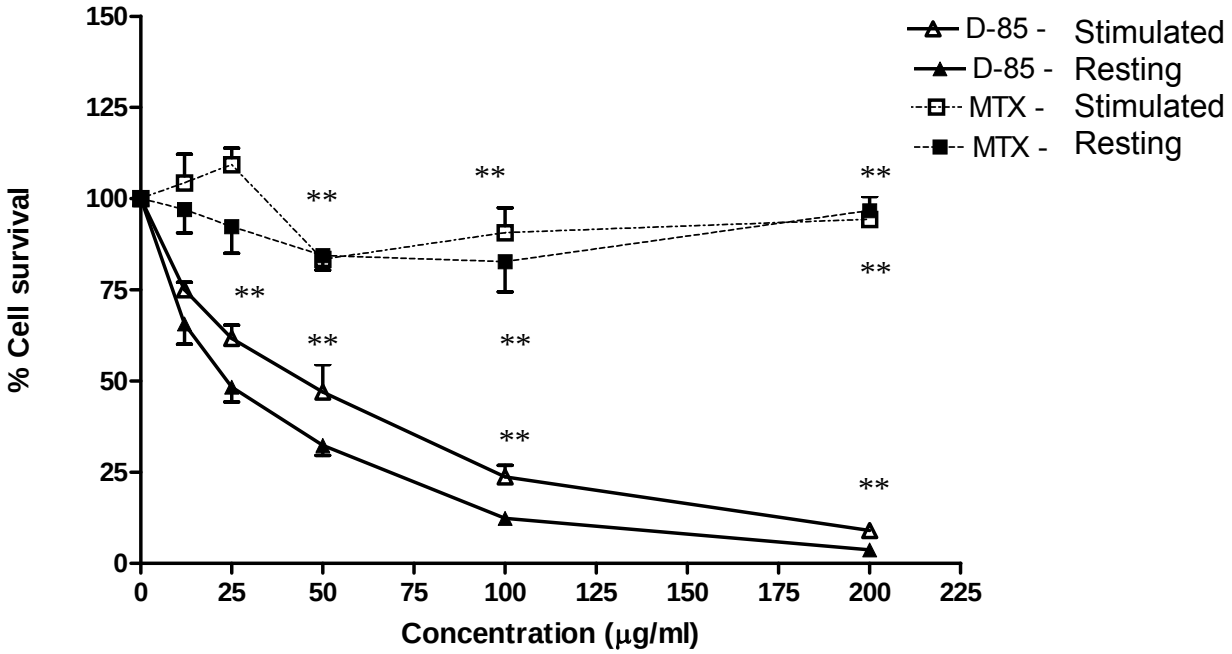

Figure 2.4: Cytotoxicity of D-85, compared to MTX, obtained after administration to stimulated and resting normal human lymphocytes

(Stimulated MTX & D-85: **p<0.05**: 50 µg/ml, 100 µg/ml, 200 µg/ml;
Resting MTX & D-85: **p<0.05**: 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml)

*** * p < 0.05 → MTX differs significantly from D-85**

D-85 shows a clear dose related toxicity not seen with methotrexate. This may be lymphocyte specific because other immune-competent cells were not tested.

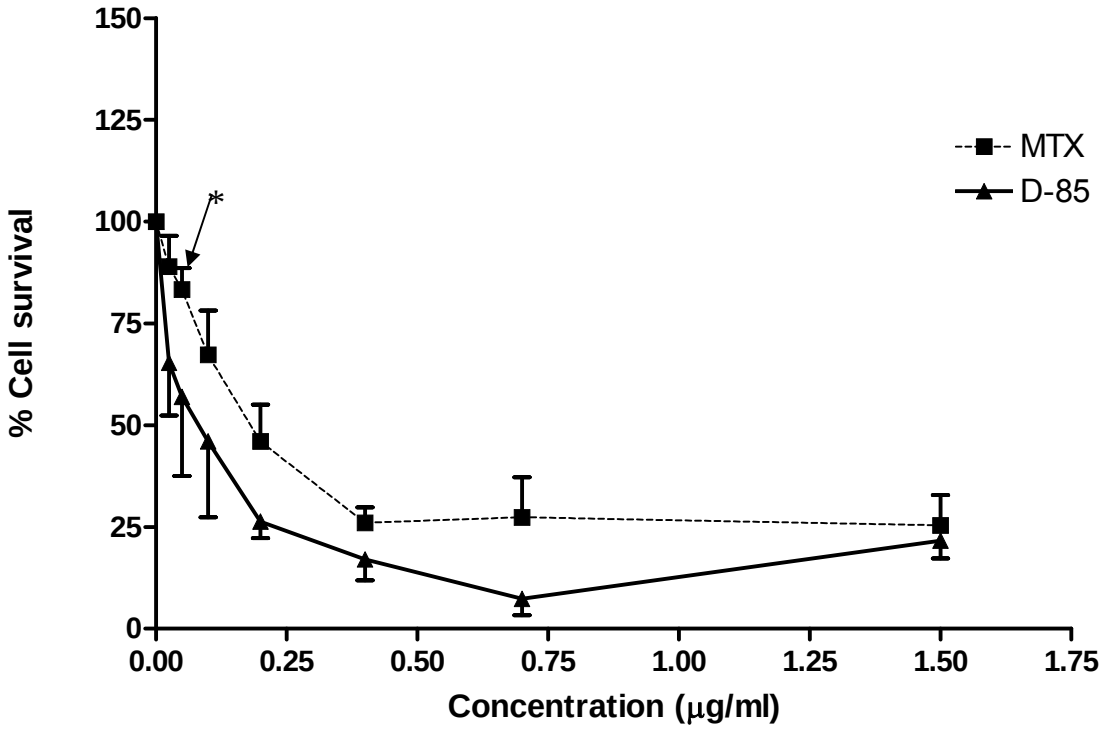

Figure 2.5: Cytotoxicity of D-85, compared to MTX, obtained after administration to MCF 12A (non-malignant breast carcinoma) cells

(**p<0.05**: 25μg/ml; **p<0.005**: 100 μg/ml –concentrations not shown on graph)

*** p < 0.1**

Significantly different results were obtained at 25μg/ml and 100μg/ml MTX and MTX equivalent concentration treatment of D-85. D-85 and MTX has approximately the same dose-related cytotoxic effects on MCF 12A cells.

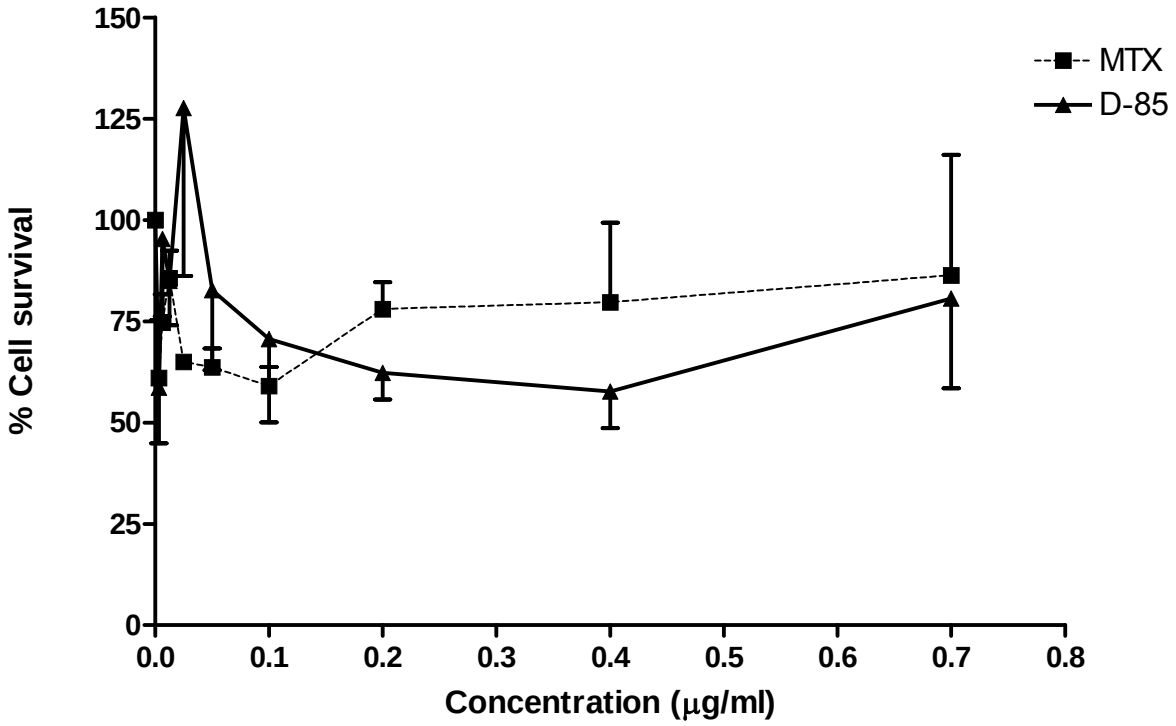

Figure 2.6: Cytotoxicity of D-85, compared to MTX, obtained after administration to primary chick fibroblasts

(**p<0.05**: 25 μg/ml, 100 μg/ml – concentrations not shown on graph)

No clear dose effect can be observed in both the MTX and D-85 treatment arm on primary chicken fibroblasts. The reason for the non-cytotoxic effects of MTX and D-85 needs further study.

e) Statistical results

The p-values were calculated for each experiment on the three cancerous and three non-cancerous cell lines. The p-values were determined for each of the MTX and D-85 concentrations that were administered to these cells. The significant p-values as determined per cell line are indicated on the respective figures.

2.4 Discussion

The significant differences ($p<0.05$) determined for the HeLa cells, are in the same range than the IC 50 values determined for methotrexate and MTX on this cell line. These values were determined by using a one-tailed, paired t-test with a 95% confidence interval. The significance of these results indicates that the MTX-polymer, D-85, have superior anti-cancer properties when comparing it with methotrexate in this cervix carcinoma cell line. It can therefore be said that it could be of greater value in the treatment of this cancer than the usage of methotrexate.

The IC50 values determined for MTX in the treatment of the MCF 7 cancer cells are similar to that of D-85. But D-85's IC50 value is more than 1.5 times less than that of methotrexate; thus having a slightly greater cytotoxicity and selectivity for this cell line than MTX.

No IC50 of methotrexate could be determined in the prostate cancer cell line, DU 145. It is not primarily used for the treatment of prostate cancer, but D-85 shows in a definite toxicity in the exposure of these cells. Therefore D-85 could also be indicated in the treatment of prostate cancer.

Normal human lymphocytes, MCF 12A cells and primary chick fibroblasts represent different areas of a healthy human. Lymphocytes, more specifically resting lymphocytes, refer to a healthy state and are found in blood which is the main carrier system for drugs in the body. These cells should only be affected by the drug when found in a *stimulated* phase. Stimulated lymphocytes are normally found when a human is ill, whether it is an acute or chronic disease.

In the treatment of normal human lymphocytes with methotrexate and D-85 it can be clearly seen that methotrexate is less toxic in both the stimulated and resting lymphocyte cultures than D-85. It can therefore be said that D-85 would be harmful to normal resting lymphocytes (dose-dependent), but would have positive cytostatic and cytotoxic effects on stimulated lymphocytes as in

the case of inflammatory diseases. On the other hand, D-85's effects on both stimulated and resting lymphocytes can be applied in the treatment of leukaemia. The significant p-values determined correlates with the IC50 values determined respectively for each cell line (Tables 2.6).

Although D-85 was cytotoxic to resting as well as stimulated lymphocytes (Table 2.4), this is fortunately not of such great concern, due to the fact that these IC50 are up to 100x higher than the IC50 calculated on cancerous cells (Table 2.3). On the other hand, the cytotoxicity of D-85 on lymphocytes (resting) can possibly be applied to the treatment of malignant lymphocyte diseases such as leukaemia.

The relative indiscriminate cytotoxic effects of both methotrexate and D-85 are perhaps a reason for concern in the treatment of the non-malignant breast cancer cell line, MCF 12A. Both these drugs have low IC50 values in the treatment of this cell line. These IC50 values are also more or less double than that of the cancerous breast cancer cell line, MCF7. *In vitro* cytotoxicity should guide initial *in vivo* experiments. It is not clear whether the higher toxicity of both compounds for MCF 12A cells *in vitro* is of any importance. MTX and D-85's IC 50 values are about twice as high in the treatment of MCF 7 cells than in that of MCF 12A. A possible explanation might be that MCF 7 cells are more resistant to MTX and D-85 than MCF 12A cells. This reiterates the use of folinic acid rescue during MTX, as well as D-85, therapy.

Better results were obtained in the treatment of the primary chick fibroblast cells. Both methotrexate and D-85 are much less toxic to these cells than in the other non-cancerous cells. There were no significant differences between the results obtained for D-85 and methotrexate treatment of chick fibroblasts..

No clear dose effect was observed in both the MTX and D-85 treatment of primary chicken fibroblasts (Figure 2.6). This may possibly be ascribed to a few different factors to be explored. One factor might be that these normal cells are not susceptible to the effects of both these drugs. Another hypothesis might be that the concentration range used during this experiment

needs to be explored further using a titration range to test the effects of MTX and D-85 on the cells at extreme drug concentrations. This refers to both extremely high and extremely low drug concentrations.

MCF 12A and primary chick fibroblasts represent normal tissue cells. If drugs are highly toxic on these “normal” cells, it indicates that the drug might be non-specific to cancerous cells and cells not involved in a disease etiology.

D-85 proved to be more toxic to cancerous cell lines than methotrexate; thus indicating superior anti-cancer activity compared to methotrexate. On the other hand, D-85 is also much more toxic towards non-cancerous cells it can probably cause greater toxicity in an *in vivo* model. This effect could be used in the treatment of diseases such as leukaemia where normal lymphocytes are affected.

Chapter 3:

Cell cycle analysis

CHAPTER 3: Cell cycle analysis

AIM:

The aim of the *In vitro studies* were to determine whether D-85 arrests MCF-7 breast carcinoma cells in the same phase of the cell cycle as methotrexate.

3.1 Introduction

The cell cycle is defined as an ordered set of events that results in cell growth and its division into two identical daughter cells (Satyanarayana *et al*, 2004). This complex process is initiated in normal cells only in the presence of mitogenic stimuli. Various proteins, e.g. cyclins, cyclin kinases and cyclin kinase inhibitors, regulate the process. In cancer cells this cell cycle regulation is lost and cells continue to divide irrespective of the presence or absence of a mitogenic stimulus (Satyanarayana *et al*, 2004).

The p53 tumor suppressor gene has been shown to affect cellular processes related to cell cycle control and gene amplification (Gudas *et al*, 1996). Cells that express wild-type p53 protein, e.g. MCF-7 cells, induce the downstream effector genes $p21^{CIP1/Waf1}$ and Gadd 45 and arrest cells in the G1 phase of the cell cycle after DNA damage or drug exposure. Earlier studies have shown that there is a loss in the G1 checkpoint and the expression of a mutant p53 gene that resulted in the increased survival of a methotrexate-resistant MCF-7 cell line. In experiments done on wild-type MCF cells, results show a 6-14 fold higher MTX concentration than the IC50 values determined by 7-day cytotoxicity assays. There was also an increase in the expression of the downstream target genes $p21^{CIP1/Waf1}$ and mdm2. The relative increase in the p53 levels did not always correlate with the relative increase in $p21^{CIP1/Waf1}$and mdm2 protein accumulation. Therefore, other factors may influence the expression of $p21^{CIP1/Waf1}$and mdm2 after its exposure to DNA damage and chemotherapeutic agents (Gudas *et al*, 1996).

A cytotoxic agent such as methotrexate, an anti-metabolite, causes DNA damage and leads to p53 enhancement of apoptosis (Kam & Ferch, 2000). Apoptosis is a process of cell suicide/death, which is a controlled and orchestrated event. Apoptotic mechanisms are encoded in the chromosomes of all nucleated cells. This process plays and important role in cell development, tissue homeostasis and defence against viral infections and mutations. These apoptotic cells undergo orderly, energy-dependent enzymatic breakdown into characteristic molecular fragments, deoxyribonucleic acid (DNA), lipids and other macromolecules, which are packaged into small vesicles that could be phagocytosed and re-used. Apoptosis results in cell death with minimal harm to adjacent cells. On the other hand, necrotic cell death is characterized by inflammation and wide-spread cell damage. Cancer can also be caused due to the cells' failure to initiate apoptosis following DNA damage (Kam & Ferch, 2000).

MTX inhibits the mitotic activity of cells (Egan & Sandborn, 1996). Low-dose MTX (< 20mg/m^2) arrests leukemic myeloblasts in the S phase for about 20h, with little or no effect on cells in G1, G2 or M-phases of the cell cycle; high doses of MTX (> 30mg/m^2) arrest human myeloblasts in S phase for more than 48 hours. It also slows the entry of cells from G1 into S phase (Bleyer, 1978). In a study done to analyze the cell-cycle-related staining patterns of PCNA (proliferating cell nuclear antigen), the treatment of MCF 7 cells with 10^{-6}mol/L MTX resulted in the rapid accumulation of cells with early S phase DNA content. This pattern of DNA content was observed for up to 48 hours after exposure to methotrexate (Van Dierendonck *et al*, 1991).

Methotrexate is often used in cell-cycle studies because of its G1/S border block. It inhibits the dihydrofolate reductase enzyme (DHFR), which in turn prevents the formation of thymidine monophosphate and blocks DNA synthesis. There are also conflicting reports as to exactly where in the cycle MTX blocks or even if there is a specific blocking site (Savage & Prasad, 1988). In an *in vivo* patient study, there was an even distribution of 2n and 4n cells in the S phase cell population; where 2n and 4n respectively indicate the DNA content of G1 and G2 cells. After 6 hours of MTX treatment, 76% of the

cells were between 2n and 3n, and 24% of the cells between 3n and 4n. All the cells were effectively arrested in the S phase. Cells that are arrested in the S phase can be lost due to cell death. The effects of MTX are rapid and cells are blocked effectively for 20 hours in the S phase. There is also evidence that after 24 hours, there are cells which proceeds through mitosis (Ernst & Killmann, 1971).

In this chapter the effects of MTX and D-85 (MTX-polymer) on MCF 7 cells were investigated. This cell line was selected because of the effectivity of MTX on these cells and because of the use of methotrexate in the treatment of breast cancer (Dollery, 1999).

3.2 Materials and methods

The cell cycle analysis was done by using MCF-7 breast carcinoma cells (ATCC HTB-22). Cells were washed with trypsin and were then scraped from the flasks' surface and resuspended in 1ml 10% D-MEM culture medium. Cells were then seeded in 25cm^2 cell culture flasks at a concentration of 2x10^5 cells.

3.2.1 MCF-7 cell preparation and drug treatment

Five culture flasks of MCF-7 viable cells were needed per one repetition of the experiment. The five flasks were used as follows:

1) flask one was a ***control*** (no drug added to this flask),
2) flask two was ***treated*** with MTX at a concentration of approximately 8 times the IC50 value of MCF-7 cells as determined earlier in the *in vitro* studies (IC50 = 0.352 μg/ml) – treatment: 2.779 μg/ml MTX,
3) flask three was ***treated*** with MTX at a concentration of approximately 16 times the IC50 value - treatment: 5.558 μg/ml),
4) flask four was ***treated*** with D-85 at a concentration of approximately 7 times IC50 value (IC50 = 1.419 μg/ml) – treatment: 1.419 μg/ml D-85
5) flask five was ***treated*** with D-85 at a concentration of approximately 12 times the IC50 value – treatment: 2.383 μg/ml D-85.

3.2.2 Incubation period of treated MCF-7 cells

The cells were then incubated for 29 hours before the drug was added to the cells. This incubation period was chosen because MCF-7 cells replicate within 29 hours; thus treating the cells as soon as it has replicated. Cells were then further incubated for another 29 hours before analysing cells with flow cytometry (Beckman Coulter Flow Cytometer).

3.3.3 Flow cytometric cell cycle analysis of treated MCF-7 cells

The treated MCF-7 cells were prepared as follows for its flow cytometric analysis:

I. Cells were scraped from the five culture flasks and were centrifuged at 1250rpm for 5 minutes in respective centrifuge tubes, marked according to the drug treatment administered to the cells.

II. The cell pellets were resuspended in 1ml of cold PBS (Phosphate buffer solution). The cells were then fixed by adding of 4ml of absolute ethanol which was at a temperature of -20°C and left to stand for 5-15 minutes.

III. The cell suspensions were centrifuged at 1250 rpm for 5 minutes.

IV. Each cell pellet was then resuspended in 1ml of PBS.

V. A 100μl of a 2mg/ml DNase-free, RNaseA solution (purchased from SIGMA) was added to the cells and incubated at 37°C for 30 minutes.

VI. After the incubation period was completed, 100μl of a 1mg/ml propidium iodide solution (purchased from SIGMA) was added to cells and incubated at room temperature for an added 5-15 minutes in darkness (propidium iodide is light sensitive).

VII. Cells were then analyzed with a Beckman Coulter Flow Cytometer. A specialized programme of this Flow Cytometer was used to determine the amount of cells which are in the different stages of the cell cycle, as described in paragraph 3.1, of the different treated MCF-7 cells with different MTX and D-85 drug concentrations. The control is used to indicate the normal cell cycle of the MCF-7 cells.

3.3 Results

Experimental results are presented in Table 3.1 and Figure 3.1. Figure 3.1 indicates the percentage of cells in the different cell stages according to the different treatment arms.

	Control	***A) MTX - 8 x IC50***	***B) MTX - 16 x IC50***	***C) D-85 - 7 x IC50***	***D) D-85 - 12 x IC50***
	Mean±SEM	***Mean± SEM***	***Mean± SEM***	***Mean± SEM***	***Mean± SEM***
G1-phase%	36.6 ± 2.8	50.96 ± 11.9	56.6± 7.4	36.3 ± 3.9	35.4 ± 4.5
S-phase %	29.3 ± 4.2.l	22.8 ± 6.9	12.0 ± 6.1	40.2 ± 2.4	40.6 ± 2.7
G2-phase %	34.1 ± 6.8	26.2 ± 6.9	31.4 ± 1.4	23.6 ± 2.6	24.02 ± 3.7

Table 3.1: Mean ± SEM values for each treatment in every cell cycle

The treatments that were administered were as follows: A) MTX at 8x its IC50 value, B) MTX at 8x its IC50* value C) D-85 at 7x its IC50** value, D) D-85 at 12x its IC50** value*

** MTX IC*

The mean (measured in %) and the standard error from the median is tabled in Table 3.1. This is a numeric reflection of the graphic presentation of the results in Figure 3.1. These results indicate the percentage of the amount of the cells that are in the different phases of the cell cycle during cell division.

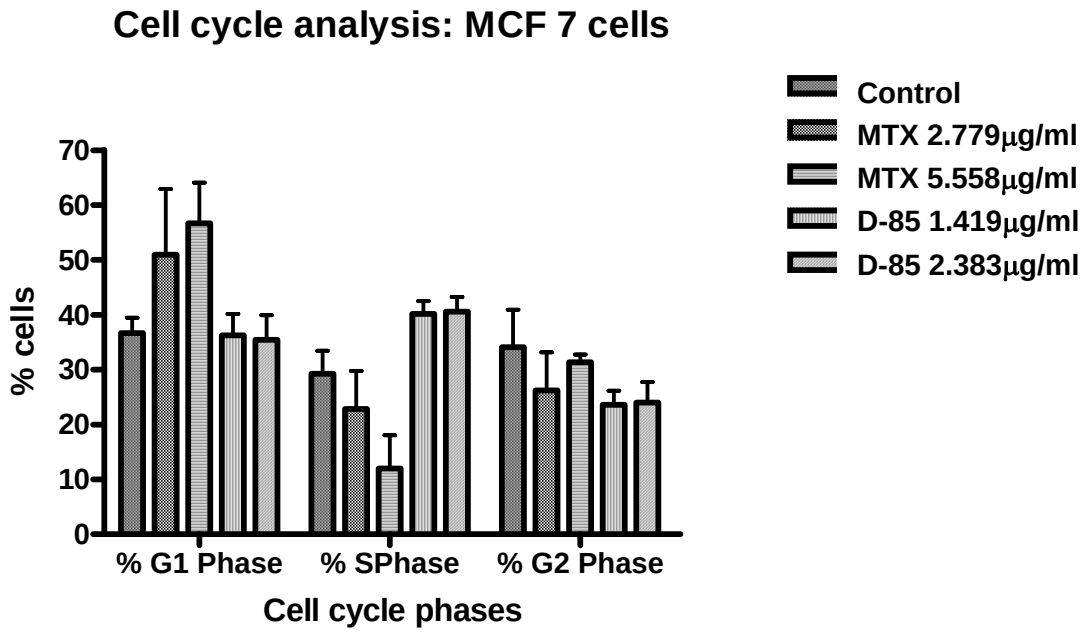

Figure 3.1: Percentage cells per cell cycle phase after administration of D-85 and MTX

Figure 3.1 shows that many cells were arrested in the G1 phase after MTX therapy with only few cells in the S-phase. Cells in the G2 phase after MTX exposure most probably proceed through the cell cycle after the 20 hour MTX sensitive period. D-85 on the other hand arrested cells through all the phases of the cell cycle with most of the cells arrested in S-phase.

□

3.4 Discussion

Methotrexate is a drug known to produce a G1/S border block during the normal cell process of mitosis. Due to the inhibition of dihydrofolate reductase, the formation of folate dependent enzymes such as thymidylate synthase is inhibited. This inhibits the formation of thymidine phosphate and causes the blocking of DNA synthesis (Savage & Prasad, 1988). It has also been proven with leukemic myeloblasts that MTX inhibits the cells' passage through S-phase and possibly produces extra recruitment of cells from G1- to S-phase. It was also hypothesized that this S-phase arresting effect of MTX seems restricted to the cells that are in S-phase during MTX exposure (Ernst & Killmann, 1971).

In a study done by Savage & Prasad, they have confirmed earlier findings that in human lymphocyte cultures and CHO cells there is no specific cell block for at least the first cell cycle (Carmargo & Cervenka, 1980; Ockey, 1972). The reason for this was most likely a sufficient precursor pool for the 1st cycle and that cells are in the different stages than the cells in the S-phase (Savage & Prasad, 1988). In another study done to determine the role of p53 protein in drug resistance in MCF-7 cells, it was shown that all the chemotherapeutic drugs that were used, including MTX, lead to increased levels of p53. The G1-checkpoint was analyzed after radiation of MTX-resistant MCF 7 cells had a minimal G1 arrest; thus suggesting that normal MCF-7 cells would probably experience a G1-checkpoint cell cycle arrest (Gudas et. al., 1996).

In figure 3.1 it can be seen that the two different treatments of MTX did cause a G1/S border block with most of the cells retained in G1. There are cells that did proceed through to the G2-phase. This could possibly be ascribed to the fact that at the moment of treatment, some cells were at a later stage in the S-phase and proceeded through to the G2-phase. It is also a rough estimate that cells are retained in the S-phase after MTX treatment for as long as the duration of the S-phase is for those specific cells. Therefore cells are retained for a certain period in the S-phase but would then pass through to the G2-phase.

A totally different trend than expected is seen in the results of D-85. No apparent G1/S block was induced and cells were spread out through all three phases of the cell cycle. Cells were most likely "recruited" into the S-phase. According to Swarts (2002) the polymer side chain, in this case MTX-polymer D-85, are supposed to be split off intracellularly by hydrolysis enzymes after fusion with lysosomes; thus releasing free MTX. Therefore it was postulated that the polymer would have the same cell cycle response as MTX. The results obtained do not indicate this. It seems that the period after treatment of the cells is not long enough for the hydrolytic enzymes to split off the side chain of D-85, releasing free MTX; thus there is a delay before seeing the effects of MTX. This can possibly explain why there is an increase of cells in the synthesis phase. The D-85 side chain comprises of glutamic acid side chains. The S-phase of cells has an increased demand for amino acids. When the glutamic acid side chains are split off, it is most probably free for any intracellular use. Therefore an increased supply of glutamic acid for protein synthesis may be available in the S-phase. Cells are then possibly "pulled" into the S-phase, decreasing the number of cells in the G1-phase. Whether the glutamic acid side chain is only split off after hydrolysis and no further metabolism occurs needs further investigation.

It is also possible that cell cycle arrest happens so fast that no progression is possible and therefore a more even spread of cells in various cycle are seen.

In a study done on a polymer of MTX and human serum albumin (HSA), it was demonstrated that there was a time difference in terms of when the effects of the respective drugs were observed. This resulted in different effects of MTX and the albumin MTX on cell cycle and thymidylate synthase (TS). It was also postulated that an extended cellular uptake period exists before the subsequent lysosomal degradation to the active form of MTX (Wosikowski *et al*, 2003). This supports the hypothesis for the differences in the results obtained for the effects of MTX and D-85 on cell cycle progression in MCF 7 breast cancer cells. Ultimately, this needs further investigation.

Chapter 4:

Mixed lymphocyte cultures

CHAPTER 4: Mixed lymphocyte reactions

AIM:

The aim of the *In vitro studies* was to determine whether D-85 has superior immunosuppressive properties than MTX in mixed lymphocyte cultures, a graft-vs-host (GVHD) model.

4.1 Introduction

Methotrexate was first developed for the treatment of malignancies, but was subsequently used in non-neoplastic diseases as an anti-inflammatory and immunosuppressive drug (Genestier *et al*, 1998; Genestier *et al*, 2000). MTX is often used in the treatment of rheumatoid arthritis (RA), chronic inflammatory disorders and as prophylaxis of acute-graft-vs-host disease (GVHD). MTX is used either alone in the treatment of acute GVHD or in association with cyclosporine A (CsA) and/or prednisone (Genestier *et al*, 1998; Genestier *et al*, 2000).

The anti-inflammatory properties of MTX are ascribed to an increased release of adenosine from cells such as fibroblasts and endothelial cells (Paillot *et al*, 1998). The extracellular adenosine may bind to any of the four adenosine receptors, namely A_1, A_{2a}, A_{2b} and A_3. These receptors are over expressed on inflammatory cells. The occupancy of these receptors on neutrophils inhibits the production of reactive oxygen species, synthesis and release of leucotrine β_4 and production of tumor necrosis factor α (TNFα). Adenosine also inhibits lymphocyte proliferation to mitogens. When adenosine acts on monocyte/macrophages, it inhibits TNFα, interleukin 6 (IL-6), interleukin 8 (IL-8), increases interleukin 10 (IL-10) secretion and promotes the transcription for IL-1 receptor antagonist (Cronstein, 1997). *In vitro* studies have also shown that MTX treatment reduces the chemotactic responses of PMN's, decreases synovial collegenase gene expression, prevents endothelial cell proliferation and neovascularization (Paillot *et al*, 1998).

The low-dose intermittent MTX exposure of human peripheral blood lymphocytes (PBL's) selectively induces apoptosis of activated but not resting lymphocytes. The selective susceptibility of activated T-cells in the S/G_2 phase of the cell cycle may result in the clonal deletion of these cells (Genestier *et al*, 1998). On the other hand, some studies have shown no evidence of inhibition of DNA synthesis or the inhibition of mitogen-stimulated lymphocyte proliferation in the presence of MTX. Therefore there have been no consistent observations in the inhibition of lymphocytes (Egan & Sandborn, 1996).

As already mentioned, MTX are used as monotherapy in the prophylaxis of GVHD or as adjunct therapy in allograft rejection (Paillot *et al*, 1998). Before the transplantation of tissues, the potential donors need to be evaluated. It has been demonstrated that when human peripheral blood leukocytes from different donors are co-cultured (mixed), then the thymidine incorporation into DNA is stimulated. In these mixed cell cultures, lymphocytes are stimulated to enlarge and divide. The maximum stimulation of these cell mixtures appears after approximately 7-8 days of incubation (Bach & Hirschlorn, 1964). This so-called mixed lymphocyte reaction (MLR) was one of the first *in vitro* analyses for quantifying alloreactivity. Unfortunately it is now accepted that the standard MLR assay has limited clinical value, for example in kidney transplantation (Cartwright *et al*, 2000). MLR results also do not correlate with the grade of GVHD (Lim *et al*, 1988).

In a study done to determine the importance of these mixed lymphocyte cultures (MLC) in the selection of a donor for bone marrow transplantation, the researchers concluded as follows:

- MLC does not give extra information in the cases of genotypically HLA-identical siblings;
- MLC reveals hidden differences in doubtful cases;
- MLC should be carried out in all cases of unrelated donor/recipient pairs.

- MLC and DNA typing methods are complimentary to each other and may contribute to the decision on the suitability of a potential donor (Pénzes *et al*, 1996).

The results of a MLC seem to be important in predicting a chronic GVH reaction. GVHD occurred more frequently and earlier in recipients who showed positive MLC results. MLC therefore has still a place in the screening for potential donors for organ transplantation. MLC is also used because it predicts the capacity of T-cells to produce cytokines through and allogeneic stimulus (Visetainer *et al*, 2002).

4.2 Materials and methods

Blood samples were obtained from two healthy consenting donors. The samples were labelled A and B respectively. Lymphocytes were then isolated according to the protocol as described in chapter 2, paragraph 2.2.3 B 1. Each of the lymphocyte cell suspensions were equally divided into two tubes, thus having two tubes with 500µl lymphocyte cell suspension of donor A and two tubes with 500µl lymphocyte cell suspension of donor B. This experiment was repeated four times using two *different* healthy consenting donors with each repeat.

4.2.1 The two different tubes of each donor were treated as follows:

1. Tubes A1 and B1 were resuspended in complete RPMI growth medium (medium containing 10% fetal calf serum and antibiotics);
2. Tubes A2 and B2 were resuspended in RPMI growth medium without fetal calf serum and the cells were inactivated by cobalt radiation at 20 Gy.

4.2.2 The experiment was done as follows:

1. 80µl complete RPMI growth medium was added to all the wells
2. 50µl viable cells at a concentration of $1x10^6$ from tubes A1 and B1 were added to the relevant wells and were incubated for one hour at 37°C with 5% CO_2 - (96 well microtiter plate was "divided" into 6x8 well columns, each column representing A1 and B1, with the top row of each column being the control)
3. 20µl of MTX and D-85 was added to the relevant cells according to a concentration range of 1.5-200 µg/ml MTX and D-85 with the equivalent of 1.5-200 µg/ml MTX.
4. Cells were incubated for another hour after which 50µl cell suspension from the irradiated tubes A2 and B2, were added to the wells (A2 cell suspension was added to column with B1

cells, B2 cell suspension was added to column with A1 cells). The irradiated cell suspensions were also standardized to contain $1x10^6$ cells per ml.

5. Cells were then incubated for 7 days at 37°C with 5% CO_2 concentration maintained in the incubator.
6. Cells were then analyzed by the MTT assay (refer to chapter 2, paragraph 2.2.4) which measures cell viability.

4.3 Results

The results obtained during the mixed lymphocyte reaction can be seen in Figure 4.1. The graph indicates the percentage of cell survival compared to the untreated control. The mixed lymphocyte cultures, both the stimulated and resting lymphocytes, were treated with the following MTX and D-85 concentration ranges:

1. MTX: 1.5 – 200 μg/ml
2. D-85: the equivalent of 1.5 – 200 μg/ml

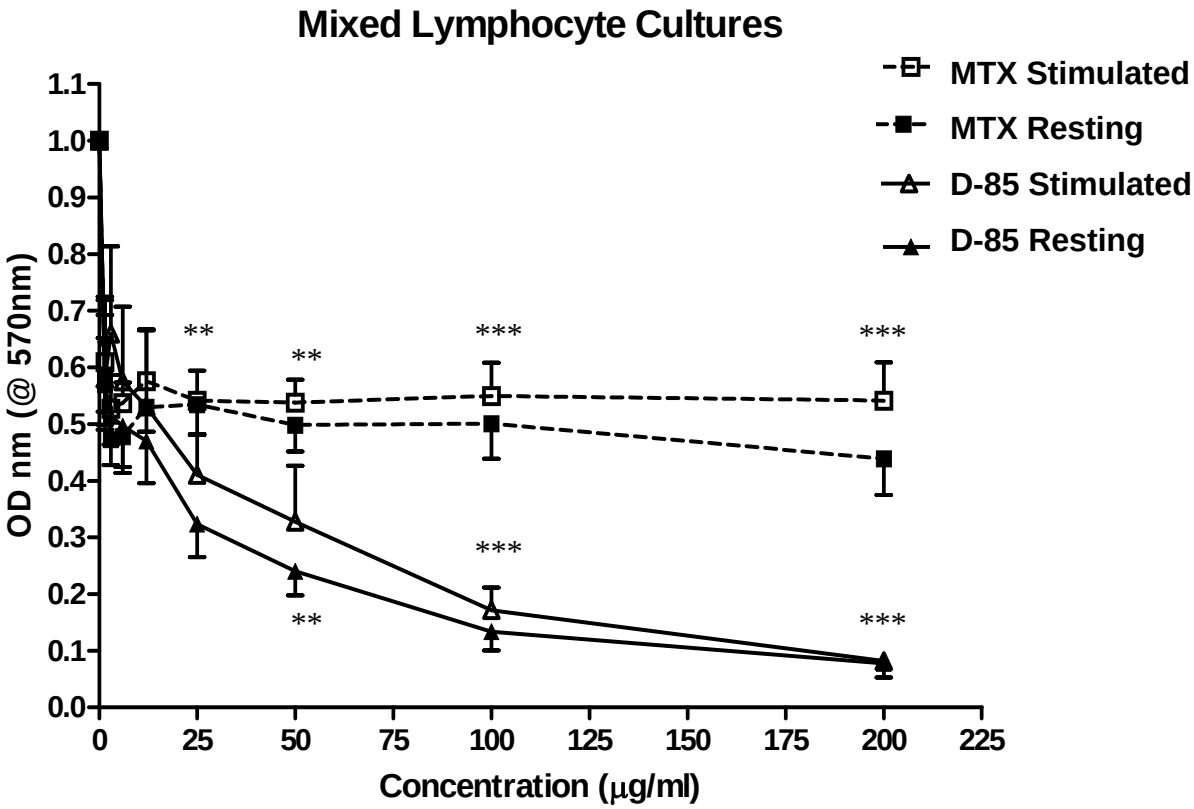

Figure 4.1: Cytotoxicity of D-85, compared to MTX, obtained after administration to stimulated and resting mixed lymphocyte cultures

[* p-value < 0.5 * * p-value < 0.05 * * * p-value< 0.005]

The results of the treatment of *stimulated* lymphocytes with D-85 and MTX were compared and the results of *resting* lymphocytes treated with D-85 and MTX. Significant differences were obtained at various concentrations of drug treatment.

4.4 Discussion

The importance of the mixed lymphocyte reaction in donor selection and prediction of graft-vs-host disease is a widely researched field. In the case of bone marrow transplantation, Pénzes *et al* (1996) concluded that mixed lymphocyte cultures are of importance in all cases of unrelated donor/recipient pairs. In doubtful cases it might reveal hidden genetic differences, especially regarding the "crossing over" and split differences for DRB1 and DQB1 alleles (Pénzes *et al*, 1996).

Mixed lymphocyte cultures also add value to DNA typing when considering the suitability of a potential donor (Pénzes *et al*, 1996). In studies performed by Visetainer *et al* (2002) in patients receiving bone marrow transplantation, mixed lymphocyte cultures was not useful in the prediction of GVHD. On the other hand results from mixed lymphocyte cultures seem to be important in the prediction of chronic GVHD, which is i) the decrease of graft monocytes and ii) the maintenance of T cells for a long period after transplantation (Visetainer *et al*, 2002).

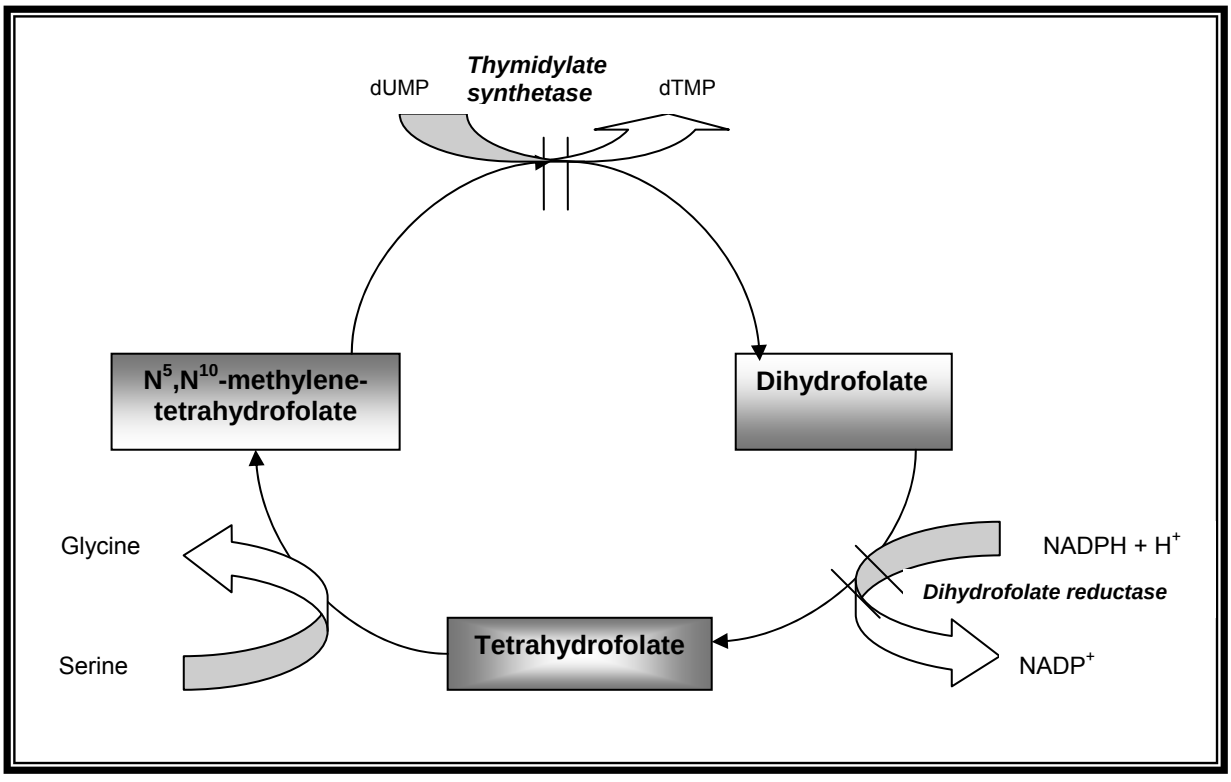

Figure 4.2: *De novo* synthesis of thymidylate

The production of the pyrimidine dTMP (di-thymidine-monophosphate) is a critical step in the *de novo* synthesis of DNA. It is of importance because it is the only *de novo* source of cellular thymidylate. The enzyme thymidylate synthase (TS) catalyzes the conversion of dUMP to dTMP by using 5,10-mehtylene tetrahydrofolate (Figure 4.2). Methotrexate inhibits this *de novo* thymidylate synthesis in a multifactorial process, consisting of direct and indirect inhibition (Genestier, 2000).

a) Direct inhibition of the *de novo* thymidylate synthesis

The direct inhibition of the enzyme TS is caused by the methotrexate polyglutamates formed during the intra-cellular metabolism of methotrexate (Genestier, 2000). It has also been demonstrated that MTX-Glu_1 has a much less inhibitory effect on the catalytic activity of TS than MTX-Glu_5 (Morrison & Allegra, 1989). This leads to a lack of intracellular thymine which is lethal to actively dividing cells. Uracil misincorporation and detrimental repair are also a factor in the cytotoxic events following TS inhibition. The inhibition of TS that is responsible for the reductive methylation of dUMP to yield TMP, leads to this misincorporation of dUMP in DNA (Figure 4.2 and Figure 4.3) (Ladner, 2001).

b) Indirect inhibition of the *de novo* thymidylate synthesis

The partial depletion of the substrate 5,10-methylene tetrahydrofolate also causes and inhibitory effect on dTMP production. This depletion of tetrahydrofolate can be attributed to the inhibition of the enzyme dihydrofolate reductase by methotrexate (as discussed in paragraph 1.2 of Chapter 1).

This thyminless intracellular state is toxic to actively dividing cells. This type of cell death due to thymine starvation is called “thymineless death” (TLD). Direct effects of thymine starvation involve both single- and double-strand DNA breaks. Single-strand DNA breaks may be repaired effectively, but double-strand DNA breaks leads to cell death (Ahmad *et al*, 1998). Studies done on thymine-deprived cultures demonstrated the loss of viability, formation of longer filaments and the decrease in thymidine nucleotides

(Ohkawa, 1975). This process of intra-cellular filamentation which is caused by thymine deprivation has also been demonstrated by Strauss *et al* (2004).

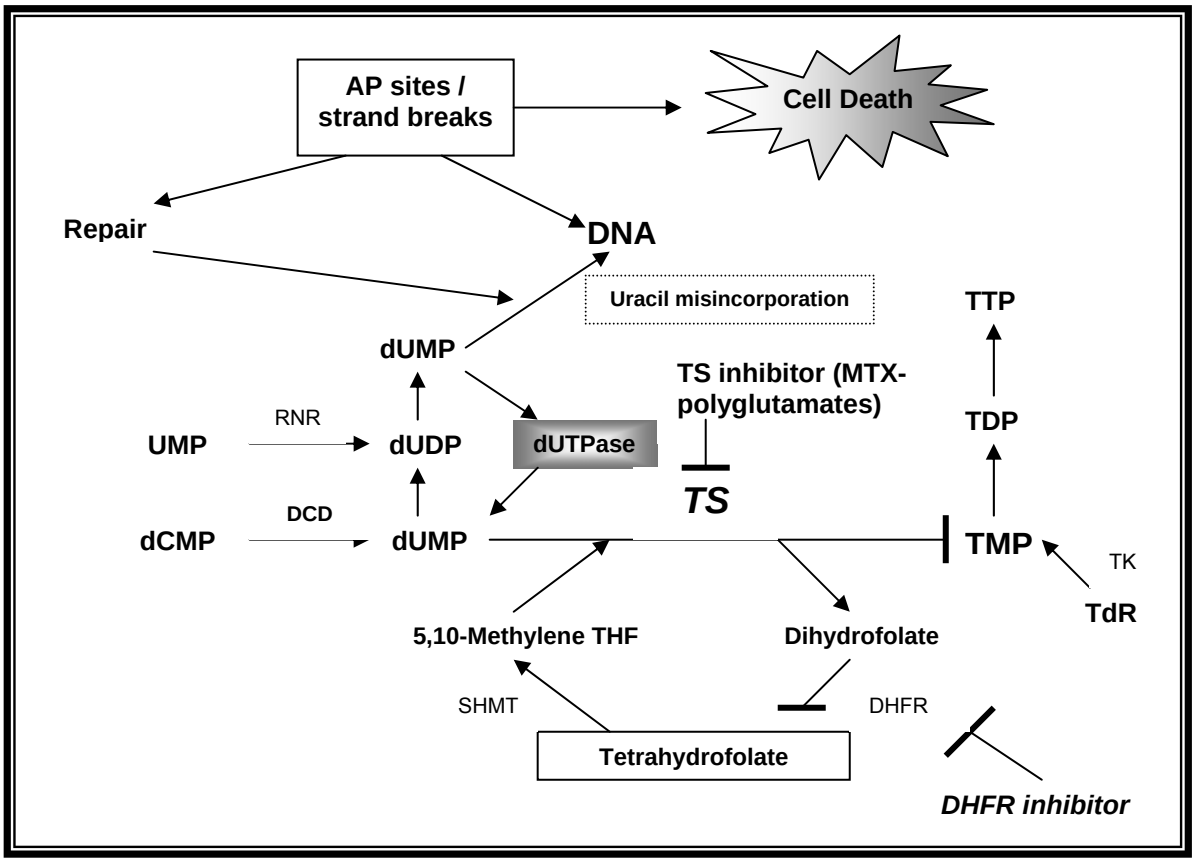

Figure 4.3: Inhibition of thymidylate synthase resulting in uracil misincorporation (Ladner, 2001)

There are also strong suggestions that cytokines are involved in the rejection process in transplantation (Cartwright *et al*, 2000). Theses cytokines may play and important role in the severity as well as the regulation of allograft rejection. T-helper lymphocyte cells play an important role in this immune process. These cells are sub-divided into two groups, namely Th1- and Th2-lymphocytes. This is a functional subdivision on the basis of cytokine secretion. Th1 lymphocytes secrete interleukin-2 (IL-2) and interferon-gamma (IFN-γ). These cytokines are associated with rejection. Th2 lymphocytes secrete interleukin-4 (IL-4) and interleukin-10 (IL-10). These latter cytokines

are thought to be associated with tolerance and low levels of secretion are associated with allograft rejection (Cartwright *et al*, 2000).

In MLC studies done to determine the roles of cytokines in allograft rejection, a significant association was found between a high IFN-γ secretion in MLC and rejection. On the other hand and though contradictory to literature, they have found associations between IL-4 and IL-10 secretion and rejection. These cytokines were previously found as down-regulators of the immune response in rejection (Cartwright *et al*, 2000). This finding is supported by other studies (El Gamal *et al*, 1998; Lang *et al*, 1995; Merville *et al*, 1995; Weimer *et al*, 1996). Researchers also demonstrated an association between renal allograft rejection and high IL-10 and TNF-α producer genotypes (Cartwright *et al*, 2000). After researchers performed a multivariate analysis on these results, the following variables were significantly associated with rejection:

- female sex,
- high degree of human leukocyte antigen (HLA) mismatching and
- high IL-10 secretion.

Therefore cytokine secretion measurement may be used as an additional tool for the prediction of HLA mismatching (Cartwright *et al*, 2000).

When looking at the results obtained in Figure 4.1, it is obvious that methotrexate inhibited both stimulated and resting lymphocytes up to a certain point. Although methotrexate had an inhibitory effect on the lymphocytes, an IC50 was never reached. Methotrexate's effect on lymphocytes and the drug's effect on graft-vs-host disease are well documented.

In contrast with MTX, D-85 had a more profound effect on stimulated and resting lymphocytes. This differed significantly from MTX. There is significant inhibition of both stimulated and resting lymphocytes which puts a question mark over the toxic effects of D-85 in the use of e.g. graft-vs-host disease. This does not mean that D-85 has no value as immunosuppressant or anti-

inflammatory drug, but has to be researched thoroughly to determine the polymer's therapeutic index and other relevant pharmacological parameters to conclude the polymer's possible use in humans.

D-85 showed superior effects over the conventional MTX used as immunosuppressant, which grants further investigations regarding the MTX polymer's future use in this therapeutic field.

Chapter 5:

In vivo toxicity

CHAPTER 5: In *vivo* toxicity

AIM:

The aim of the *In vivo* studies were to determine toxicity of D-85 and MTX:

a) In Balb/C mice treated once a week for four weeks using an anti-inflammatory drug treatment regimen (15mg/kg per week, intraperitoneally) – ***Experiment I***
b) In Balb/C mice administered treatment for three weeks using an anti-cancer drug treatment regimen (3.5mg/kg every 2nd day for 21 days, intraperitoneally) – ***Experiment II***

This study consisted of Experiment I and Experiment II as indicated in above mentioned **AIM**. Experiment I was performed to compare and determine whether MTX and D-85 caused toxicity in mice following a standard *anti-inflammatory dosing* regimen. Experiment II was performed to compare and determine whether MTX and D-85 caused toxicity, using a standard *anti-cancer dosing* regimen.

5.1 Introduction

After administration, methotrexate is retained for weeks in the kidney and for months in the liver (Vucinic, 2002). Repeated MTX doses can cause sustained serum concentrations and tissue accumulation of MTX and MTX-polyglutamates. After MTX is absorbed, it undergoes hepatic and intracellular metabolism forming MTX-polyglutamates. Small amounts of these metabolites may remain in tissues for extended periods. MTX is primarily excreted by the kidneys via glomerular filtration and is excreted by active transport. Enterohepatic recirculation of MTX may occur (Vucinic, 2002).

MTX toxicity is evident in rapidly dividing cells, mainly affecting bone marrow and the gastrointestinal mucosa (Vucinic, 2002). These side effects are listed in Table 5.1. Long-term, chronic MTX usage can cause hepatotoxicity that is sometimes irreversible. This risk increases with pre-existing diabetes mellitus,

liver disease, excessive alcohol use, obesity, cumulative dose over 5mg, and/or renal disease (Vucinic, 2002). MTX-polyglutamates accumulate in hepatocytes. The histological spectrum of MTX hepatic injury includes hepatocellular necrosis (death of hepatic cells; Thomas, 1993), macrovesicular steatosis (fatty degeneration of macrovessicle; Thomas, 1993) and portal inflammation, which can progress to cirrhosis. Low-dose MTX treatment can cause an increase in aspartate aminotransferase (AST) and alanine aminotransferase (Egan & Sandborn, 1996). The testing of liver enzymes was first thought to be of no value in predicting the development of clinically significant liver disease during MTX therapy. Later, researchers found a significant correlation between hepatic AST and histologic deterioration in patients receiving chronic weekly MTX treatment for rheumatoid arthritis. Liver toxicity is however not necessarily an inevitable outcome of long-term MTX treatment (Kremer, 2002).

	Toxicity	Pathophysiology
Major:	Hepatotoxicity	Direct drug effect
	Myelosuppression	Anti-proliferative effect on bone marrow
	Pulmonary toxicity	Idiopathic hypersensitivity
	Effects on fertility and fetus	Oligospermia, abortificient, teratogenic
	Enteritis-colitis	Anti-proliferative effect on intestinal mucosa
Minor:	Nausea or vomiting	
	Diarrhea	Anti-proliferative effect on intestinal mucosa
	Stomatitis	
	Alopecia	Anti-proliferative effect on hair follicle
	Rash	Allergy
	Neurologic	Unknown

Table 5.1: Side effects of methotrexate (Egan & Sandborn, 1996)

One of the most consistent renal effects associated with long-term, low-dose MTX therapy is a decrease in creatinine clearance. Direct creatinine clearance measurement in rheumatoid arthritis (RA) patients receiving 7.5mg MTX /week showed a significant 11% reduction in creatinine clearance after 6 months of treatment with MTX. At the same time, the renal clearance of MTX was also significantly reduced by 25%. Concomitant non-steroidal anti-inflammatory (NSAID's) therapy may also contribute to the renal toxicity of MTX. There have also been reports of reductions in renal function in patients receiving MTX alone. Data from 11 clinical trials of MTX involving 496 patients showed that patients with compromised renal function had the highest rate of methotrexate toxicity (Schiff & Whelton, 2000). Uraemia (a toxic condition associated with renal insufficiency produced by the retention in the blood of nitrogenous substances normally excreted by the kidney; Thomas, 1993) and haematuria (blood in urine; Thomas, 1993) has also been reported during MTX therapy; as well as acute renal failure following high dose MTX administration (Kintzel, 2001). MTX therapy should be stopped when patient has or develops renal failure, because MTX is excreted by glomerular filtration and active tubular secretion (Strang & Pullar, 2004).

MTX affects rapidly dividing cells; therefore cells of the gastrointestinal epithelium and the bone marrow are highly susceptible (Hardman *et al*, 1996). The most common gastrointestinal side effects associated with MTX therapy are: nausea, vomiting, diarrhea, stomatitis and anorexia (Belgi & Friedman, 2002; Egan & Sandborn, 1996). It also causes rapid morphological and functional abnormalities to the small intestine mucosa and thus occurs independently of the route of administration. These changes can cause a mal-absorption syndrome that is on the other hand dose-related (Egan & Sandborn, 1996). Nausea and vomiting can be reduced by administering MTX parenterally or by dose-splitting and administration of folic acid (Belgi & Friedman, 2002).

MTX therapy results in bone marrow suppression with putative cytopenias. Risk factors for myelosuppression are pre-existing cytopenias, concomitant NSAID administration, old age and renal insufficiency (Egan & Sandborn,

1996). Up to 10-20% of patients on long-term MTX therapy develop bone marrow suppression. Prolonged administration may also cause megaloblastic anaemia and aplastic anaemia (Belgi & Friedman. 2002). It is therefore important that a patients' full blood count should be monitored throughout the period of MTX treatment (Belgi & Friedman, 2002).

5.2 Materials and methods

5.2.1 *Experiment I and II*

Experiment I

Forty-five animals (BALB/c mice) were divided into three groups of 15 mice. The animals in the two experimental groups received a once weekly dosage of 15 mg/kg MTX and D-85 at a concentration equivalent to 15mg/kg MTX for 4 consecutive weeks, whereas the control group received the same volume of saline. Blood was drawn (after termination of the animals) on day 28. Blood samples were analysed at Ondestepoort at Clinical Pathology (Faculty of Veterinary Sciences, University of Pretoria) to determine serum thrombocyte, leucopenia, aspartate aminotransferase (AST) and creatinine levels [Thrombocytopoenia and renal damage are some of the toxic effects documented with MTX (Dollery, 1999)]. These levels were studied to see whether drug toxicity was induced (refer to paragraph 5.2.: Blood sample analysis 2).

Experiment II

Fifteen animals were divided into three groups for the extension toxicity study, each group consisting of 5 mice. The animals in the two experimental groups received one dose every other day for 21 days, the dose being 3.5 mg/kg MTX and D-85 at a concentration equivalent to 3.5mg/kg MTX. The control group received the same regimen but with saline. Blood was drawn intracardially on day 21 after anaesthetics. The animals were terminated after blood sampling was completed. Blood samples were analyzed at Ondestepoort to determine serum thrombocyte, aspartate, aminotransferase (AST) and creatinine levels [Thrombocytopoenia and renal damage are some of the toxic effects documented with MTX (Dollery, 1999)].

5.2.2 Method: Blood sample analysis

The blood sample preparation method that was used by Ondestepoort Clinical Pathology (Faculty of Veterinary Sciences, University of Pretoria) was as follow: The AST and creatinine heparin tubes were spun at 3 200 rpm for 10 minutes in a Jouan B3.10 bench top centrifuge (maximum RCF 1.730gt). Plasma was pipetted off with a pasteur pipette and was analysed with a VETEX NEXCT (Alfa Wasserman) chemistry analyser.

5.2.3 Pain and distress

Mammals display a range of more subtle stereotypical responses to stress or pain stimuli. Recognition for these changes in behaviour and the physical appearance of the animal was used for early identification of an animal experiencing pain or distress. None of the animals were terminated during the Experiment I and II studies.

5.3 Results

The results of both the Experiment I and Experiment II *in vivo* animal models are presented in sections 5.3*A* and 5.3*B*. In both sections there are two tables. Table 5.2 (Experiment I) and Table 5.5 (Experiment II) tabulates the changes in the mice' weight during the treatment period. These results were used to analyse if MTX and/or D-85 can lead to growth retardation. Table 5.3 (Experiment I) and Table 5.6 (Experiment II) tabulate the toxicology results, indicating the AST and creatinine values obtained in the two experiments of the study per respective treatment arm. Table 5.4 and Table 5.7 tabulate the mean±SD and p-values for the results obtained collectively in tables 5.2-5.3 and tables 5.5-5.6.

A) ***Experiment I*****:** Results

	Average weight gain per week (in *g*)*				**Week 1-4** (Weight gain over total 4 week period)
	Week 1	***Week 2***	***Week 3***	***Week 4***	
Control	0.71	0.66	0.39	0.56	2.32
MTX	0.89	0.78	0.5	0.69	2.86
D-85	0.53	1.01	0.24	0.58	2.36

Table 5.2: Average changes in weight of experimental mice over 4 week period

* Average of 15 replicates per group

	AST	**Creatinine**
Control	59.1 U/L	28.5 µmol/L
MTX	69.2 U/L	26.9 µmol/L
D-85	69.75 U/L	32.0 µmol/L

Table 5.3: Aspartate aminotransferase and creatinine serum levels

	AST U/L		**Creatinine µmol/L**		**Weight(g) (beginning)**		**Weight(g) (end)**	
	Mean ± SD	p-value	Mean ± SD	p-value	Mean ± SD	p-value	Mean ± SD	p-value
Control	55 ± 16		29 ± 3		23 ± 3		25 ± 3	
MTX	69 ± 24	0.0075	27 ± 4	0.0149	21 ± 3	0.2503	24 ± 2	0.0548
D-85	89 ± 31		24 ± 6		21 ± 3		23 ± 2	

Table 5.4: Calculated MEAN ± SD and p-values (calculated with One-way ANOVA) from results obtained in *Experiment I* (Tables 5.2 and 5.3)

During *Experiment I*, a single15mg/kg MTX dose was administered once per week, and a correlating D-85 polymer dose, was also administered once weekly. The drugs were administered to the mice over a period of 4 weeks. These regimens were selected as it simulated standard therapy in practice when treating patients with auto-immune diseases such as rheumatoid arthritis.

The first parameter that was observed was the average weight gain of the control and 2 treatment groups (Tables 5.2). The MTX-treatment group did gain weight at a more rapid rate than the control group. It is therefore apparent that low-dose methotrexate therapy does not influence normal growth significantly as it is expected according to literature that MTX will cause growth retardation according to literature.

The elevated aspartate aminotransferase (AST) levels in the *Experiment I* study are indicative of liver toxicity (Tables 5.3 and 5.6). In the MTX treatment group, there was an increase in the AST levels when compared to the control, but it is not a significant difference. In the D-85 treatment group, there was a significant increase in the AST levels when compared to the control.

There is a significant difference in these results when comparing MTX with D-85 with the control doing a one-way ANOVA analysis (p-value = 0.0075).

The creatinine levels were also influenced in the experiment I study. The creatinine levels in the MTX treatment group were lower than that of the control which is not the expected outcome. The p-value obtained when doing a one-way ANOVA analysis is 0.0149, which is a significant difference.

In the experiment I study, growth retardation was observed in the D-85 treatment group but not in the MTX treatment group as was expected.

On the other hand in the D-85, the growth retardation was much greater than in the MTX treatment group when compared to the control group and showed a significant difference when compared to the control. When analysing the results with a one-way ANOVA analysis, an insignificant p-value of 0.0548 is obtained.

B) ***Experiment II*****:** Results

Treatment Groups	**Weight gain (in *g*)**
Group 1: Control(Saline) *	0.66
Group 2: MTX *	0.43
Group 3: D-85 *	0.12

Table 5.5 : Average changes in weight of experimental mice over 21 days

* Average of 5 replicates per group

	AST	**Creatinine**
Control	168 U/L	27.4 µmol/L
MTX	100.4 U/L	32.2 µmol/L
D-85	129 U/L	32.7 µmol/L

Table 5.6 : Aspartate aminotransferase and creatinine serum levels Calculated MEAN ± SD and p-values (calculated with One-way ANOVA) from results obtained in *Experiment I*

	AST U/L		**Creatinine µmol/L**		**Weight(g) (beginning)**		**Weight(g) (end)**	
	Mean ± SD	p-value	Mean ± SD	p-value	Mean ± SD	p-value	Mean ± SD	p-value
Control	56 ± 25		30 ± 2		23 ± 2		23 ± 2	
MTX	56 ± 25	0.0363	30 ± 2	0.0169	23 ± 1	0.9270	23 ± 2	0.6966
D-85	56 ± 25		30 ± 2		23 ± 2		23 ± 2	

Table 5.7 : Calculated MEAN ± SD and p-values (calculated with One-way ANOVA) from results obtained in *Experiment II* (Tables 5.5 and 5.6)

As in experiment I, the first parameter that was observed was the average weight gain of the control and 2 treatment groups (Tables 5.5). In the experiment II study, growth retardation was not statistically significant. Although when referring to Table 5.5, it seems that if this treatment regimen is repeated in a bigger study population, significant values might be obtained. This needs further investigation to be substantiated.

The toxicology results of the experiment II study do not show the same clinical picture than in the experiment I study. In this study, the AST levels in the MTX treatment group decreased significantly. When reviewing the results of the D-85 group compared to that of the control, there is an insignificant decrease in the AST levels. When analysing the results with a one-way ANOVA analysis, a significant p-value of 0.0363 is obtained.

The creatinine levels in both treatment groups increased when compared to the control group. There is a significant increase in the creatinine levels in the MTX group. There is also a significant increase in the creatinine levels of the D-85 treatment group. When analysing the results with a one-way ANOVA analysis, a significant p-value of 0.0169 is obtained.

5.4 Discussion

Methotrexate therapy has substantial reported toxicity. Occasional deaths have been associated with patients treated with MTX for rheumatoid arthritis and psoriasis (Egan & Sandborn, 1996). Methotrexate has a low therapeutic index; therefore patients receiving MTX therapy should be closely monitored. Adverse reactions relative to the lack of efficacy are usually responsible for the discontinuation of MTX therapy (Egan & Sandborn, 1996).

Hepatotoxicity is one of the anticipated side effects of methotrexate therapy. MTX is absorbed in the small intestine and then enters the portal circulation. Hepatocellular uptake takes place where polyglutamation of MTX occurs. Storage of these MTX-polyglutamates in hepatocytes then takes place (Dollery, 1999). The accumulations of these MTX-polyglutamates have been detected in the liver months after the MTX was administered. It has been determined that the mean serum AST levels approximately doubles with long-term methotrexate therapy (Egan & Sandborn, 1996). In some instances this hepatotoxicity is irreversible (Vucinic, 2002). In paediatric acute lymphoblastic leukaemia patients studies showed that MTX chemotherapy decreased liver metabolic capacity (Wiela-Hojenska *et al*, 2001). In a study of patients receiving MTX for rheumatoid arthritis, the liver enzyme elevations did not exceed 2-3 times the normal value and was temporary with spontaneous regression during therapy or after temporary discontinuation of MTX therapy (Schnabel & Gross, 1994). Other studies done showed that liver toxicity could be substantial in RA patients receiving low-dose methotrexate therapy, with an increased risk the higher the cumulative dose (Whiting-O'Keefe *et al*, 1991).

Methotrexate is primarily excreted through active transport and glomerular filtration of the kidneys (Vucinic, 2002). Acute renal failure has also been reported after high dose methotrexate administration (Kintzel, 2001). The binding of the extracellular adenosine to A1-receptors diminishes renal blood flow. Researchers therefore suggest that excess adenosine release in the

kidneys and extra-articular sites may lead to this renal toxicity (Cronstein, 1996). The most consistent effect on the kidneys in RA patient treated with methotrexate is the decrease in the creatinine clearance. In such cases, folinic acid treatment should usually be considered (Schiff & Whelton, 2000).

Skeletal growth suppression has been demonstrated in mice. The major mechanism of action of methotrexate is its effect on dihydrofolate reductase. The inhibition of this enzyme has a direct effect on the pathway of the *de novo* synthesis of DNA. In a study done by Iqbal *et al* (2003), they have demonstrated this inhibitory effect of MTX on osteoblasts and chondrocytes. Abnormalities in the growth plates of the tibias and femurs of the MTX treated mice were observed. The addition of folinic acid after the MTX administration to the mice indicated the reversal of this side effect. Folinic acid diet supplementation is therefore advised for paediatric patients receiving methotrexate therapy (Iqbal *et al*, 2003).

The results in Experiment I regarding the effect of the anti-inflammatory dosing schedule of MTX and D-85 on the weight of the mice indicated that both treatment groups showed an increase in weight. When comparing the increase in weight of MTX to the control (p-value = 0.16) and D-85 with the control (p-value = 0.41), no significant differences in the mice weight gain was obtained. This is contradictory to the results by Iqbal (2003) and therefore these results need further investigation. But when comparing the weight changes between the MTX and D-85 treatment group, a p-value of 0.054 was obtained. This nearly significant value obtained is a possible indication of the difference in growth retardation between the two treatment groups. These results need further investigation to clarify the results obtained.

The toxicology results obtained in Experiment I is of concern as there was a significant increase in AST in both MTX (p-value = 0.05) and D-85 (p-value = 0.001) treatment groups when compared to the control. Also, when the AST levels of both treatment groups were compared, there was a significant difference (unpaired t-test: p-value = 0.05; one-way ANOVA: p-value =

0.0075). This is of some concern because it indicates a greater toxic effect on the liver with D-85. As methotrexate is biotransformed in the liver, this is an expected and known effect in literature. What is of concern is the significant increase of AST in the MTX-polymer, D-85, treatment group. The increased molecular weight of the drug and its glutamic acid side chains might be the reason for this result. As it is not precisely known whether the side chain of D-85 is split off the molecule before or after cellular entry, one can only postulate at this stage the cause of this toxicity. It is said that the side chain is only enzymatically cleaved off the molecule intracellularly (Swarts, 2002). It can be debated that this would hinder the hepatic intracellular biotransformation of the drug to its active polyglutamate form; therefore observing this increase in AST levels.

This indicates that the drug is not excreted according to normal MTX pharmacokinetics values. This could also indicate that this drug might cause renal failure in long-term, low-dose drug therapy.

This is of great concern as the drug regimen that was used, is comparable to the regimen that is commonly used in the treatment of inflammatory diseases such as RA. Therefore, great care should be taken when treating paediatric patients with rheumatoid arthritis.

This is opposite to what is described in literature, especially when treated with cumulative drug dosage, where liver toxicity is a well described side effect (Belgi & Friedman, 2002).

The creatinine level results in experiment II showed an increase when treated with MTX and D-85. There is a therefore an indication that both drugs might possibly induce renal failure if treatment is administered over a prolonged period. However, this experiment would have to be repeated in a bigger population group to determine whether this observation is correct.

Chapter 6:

Conclusion

CHAPTER 6

6.1 Conclusion

In vitro assays of drug toxicity and efficacy (mechanism of action / proof of concept) are done to guide researchers in the development process of the particular substance.

The *in vitro* cell viability analysis, MTT that was done (see chapter 4), indicates both that MTX and D-85 are cytotoxic towards cancerous cells. D-85 showed superiority in terms of the IC50 values obtained in these analyses. Significant differences between results of MTX and D-85 on both HeLa cervix carcinoma cells and MCF-7 breast carcinoma cells were obtained. No significant difference in the efficacy of MTX and D-85 was obtained on DU 145 prostate carcinoma cells.

The *in vitro* MTT assays were also performed on non-cancerous cells to test the effect of MTX and D-85 on normal cells. This was done to determine the effect of these drugs on "normal" cells to give an indication what these drugs may do in an *in vivo* model in terms of toxicity. MTX showed much less toxicity when treating normal stimulated and resting lymphocytes than in the case of D-85. D-85 however showed an inhibiting effect on stimulated lymphocytes that could not be seen with MTX. Therefore it may be said that D-85 could possibly be more effective as an anti-inflammatory drug, but with higher toxicity.

D-85 is a polymer consisting of a water-soluble side chain and MTX. According to Swarts (2002), these side chains are split off enzymatically intracellularly (Swarts, 2002). Therefore the hypothesis could be made that this drug should show the same mechanism of action as standard methotrexate. Thus, D-85 and methotrexate was compared relative to their effect on the cell cycle of MCF-7 cells, a breast carcinoma cell line. This cell line was selected because of the available literature on this cell line regarding methotrexate's effect on its cell cycle phases. These two compounds were

tested on MCF-7 cells to determine their effect on the cell cycle. The expected effect of MTX was obtained which was a G1/S-phase cell cycle block to a certain extent. D-85 did not present the same cellular effect. A more even cycle distribution was seen. As explained in section 3.4, this could possibly be attributed to the additive effect of the glutamic side chain of D-85 to the synthesis phase of the cells, thus causing a deviation in the normal distribution of the amount of cells in the different phases of the cell cycle. Another possible hypothesis could be that the cell cycle arrest happens so fast that no progression through the phases is possible. Previous research has shown that the effect of a human serum albumin MTX polymer is observed at a different time than that of MTX. This could also possible contribute to the different effects of the drugs on cell cycle. The different hypothesis has to be researched to obtain clarification of the actual cause for the different effects observed after the treatment of MCF 7 cells with MTX and D-85.

In a study done on a polymer of MTX and human serum albumin (HSA), it was demonstrated that there was a time difference in terms of when the effects of the respective drugs were seen. This resulted in different effects of MTX and the albumin MTX on cell cycle and thymidylate synthase (TS). It was also postulated that an extended cellular uptake period exists before the subsequent lysosomal degradation to the active form of MTX (Wosikowski et. al., 2003). This supports the hypothesis for the differences in the results obtained for the effects of MTX and D-85 on cell cycle progression in MCF 7 breast cancer cells. Ultimately, this needs further investigation.

Methotrexate is a well-known immunosuppressive drug. It is often used, as monotherapy or in combination with other drugs, in the prevention and treatment of graft-vs-host disease. To compare MTX and D-85 in terms of its mechanism of action in such an *in vitro* model, mixed lymphocyte cultures were used as described in chapter 4. Again, D-85 demonstrated a superior therapeutic value over MTX. However, in this model, as in the *in vitro* lymphocyte MTT analysis (Chapter 1), D-85 was also more toxic to the resting lymphocytes, indicating a possible toxicity in an *in vivo* model. It might be of

clinical value if treatment is done at a low concentration, such as 25 μg/ml, where toxicity towards resting lymphocytes, are not much different to that of MTX. But a significant difference is obtained in terms of its effect of stimulated lymphocytes. The toxicity that will be seen will possibly be treatable with folic acid supplementation and needs further exploration in future studies and the drug's possible use in diseases such as leukaemia.

When reviewing the results of the *in vitro* testing of these drugs in the above-mentioned experiments, its testing in an *in vivo* model is clearly justified and necessary. The *in vivo* experiments were done in two experiments. Experiment I mice were treated according to a generally used anti-inflammatory regimen. In experiment II, mice were treated according to a chemotherapeutic treatment regimen. Significant results were obtained when looking at both drugs' effects on the mice's AST and creatinine levels at the end of their treatment periods. In the experiment I model, mice treated with D-85 had a significant increase in their AST levels compared to that of methotrexate. The opposite is true for the experiment II results where methotrexate caused an increase in AST levels compared to that of D-85. Whether this increase was significant will need further research.

D-85 caused a significant change in its creatinine levels when compared to the effect of methotrexate in the experiment I study, but decreased the creatinine levels unexpectedly. Methotrexate did not cause any significant change in the mice creatinine levels. In the experiment II study, both MTX and D-85 caused a significant increase in the mice creatinine levels at the end of the treatment regimen.

Growth retardation in mice treated with MTX is well documented (Iqbal MP *et al*, 2003). However, this was not seen in both experiment I and II studies in terms of methotrexate's effect on the growth of the mice. On the other hand, D-85 had a nearly significant ($p=0.0548$) effect on growth in the experiment I study. However, this was not seen in the experiment II study. Results should possibly be tested on a bigger study population to increase the value and

power of the statistical analysis to draw a definite conclusion in terms of the above-mentioned parameters, namely AST-, creatinine levels and growth.

In review of all these experimental results, D-85 showed superiority in terms of selectivity and toxicity in comparison with methotrexate. It is not clear though what D-85' mechanism of action is. This would definitely need more research that would possibly clarify its greater toxicity towards cancerous, as well as non-cancerous cells. A concern is its toxicity in normal cells because this can be indicative of possibly serious toxicity in humans. Thus, D-85 illustrates a greater pharmacological effectivity and therapeutic value than methotrexate, but further research is needed to try and decrease is relative toxicity in normal cells and *in vivo* models and possible application in disease where the role of lymphocytes is significant.

Chapter 7:

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Title:

The toxicity and immunosuppresion of a methotrexate polymeric compound, D-85, compared to methotrexate.

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ABSTRACT

Methotrexate (MTX) has been used for many years in the treatment of patients with cancer as a cytotoxic agent and as an anti-inflammatory drug for the treatment of inflammatory diseases, such as rheumatoid arthritis (RA). However, because of the side effects associated with MTX, there is a continuous search for drugs with less toxicity and hence a greater therapeutic index. In pursuit of a better and less toxic compound, researchers have coupled MTX to various polymeric drug carriers.

In this article, we reviewed the in vitro toxicity of a novel MTX polymeric drug, D-85, compared to methotrexate. We also looked at its immunosuppressive properties using mixed lymphocyte cultures. The results obtained during this study clearly illustrate the chemotherapeutic effect of the D-85 polymer and its promising immunosuppressive properties.

1. INTRODUCTION

Methotrexate (MTX) was developed 50 years ago and was primarily used to treat malignancies such as breast cancer (Dollery, 1999). It can however also be used in the treatment of autoimmune diseases such as rheumatoid arthritis (RA), graft vs host disease (GVD), psoriasis and Chron's disease.

MTX unfortunately has various side effects such as growth retardation (Iqbal, 2003), myelosuppression, hepatotoxicity (Kremer, 2002), renal failure due to its effect on renal clearance (Vucinic, 2002). MTX has also a gastrointestinal side effects such as nausea, vomiting, diarrhea, stomatitis and anorexia (Belgi & Friedman, 2002; Egan & Sandborn, 1996).

MTX therapy results in bone marrow suppression with putative cytopenias. Risk factors for myelosuppression are pre-existing cytopenias, concomitant NSAID administration, old age and renal insufficiency (Egan & Sandborn, 1996). Up to 10-20% of patients on long-term MTX therapy develop bone marrow suppression. Prolonged administration may also cause

megaloblastic anaemia and aplastic anaemia (Belgi & Friedman. 2002). It is therefore important that a patients' full blood count should be monitored throughout the period of MTX treatment (Belgi & Friedman, 2002). Some of the main side effects of MTX are listed in Table I.

	Toxicity	Pathophysiology
Major:	Hepatotoxicity	Direct drug effect
	Myelosuppression	Anti-proliferative effect on bone marrow
	Pulmonary toxicity	Idiopathic hypersensitivity
	Effects on fertility and fetus	Oligospermia, abortificient, teratogenic
	Enteritis-colitis	Anti-proliferative effect on intestinal mucosa
Minor:	Nausea or vomiting	
	Diarrhea	Anti-proliferative effect on intestinal mucosa
	Stomatitis	
	Alopecia	Anti-proliferative effect on hair follicle
	Rash	Allergy
	Neurologic	Unknown

Table I: Side effects of methotrexate (Egan & Sandborn, 1996)

In the light of MTX low therapeutic index and various toxicities that researchers are continuously searching for drugs where these effects are minimized. There has been much research done on drug conjugates and their advantage in drug therapy (Garnett, 2001). Drugs may be linked to macromolecules and potent drugs, such as cisplatin and methotrexate. Drug-immuno-conjugates and antibody-carrier-drug conjugates has also been synthesized to try and increase the effectivity of the drug. These methods have been reviewed by Garnett (Garnett, 2001). A few MTX-conjugates have also been studied. A gelatine-methotrexate conjugate is such an example that showed moderate advantages to that of MTX use (Kosasih *et al*, 2000). A fibrinogen-methotrexate conjugate has also been studied. The fibrinogen macromolecule has a property of accumulation in tumors; therefore

researchers tested it for selectivity to tumors. However, the conjugate did not show improved properties compared to MTX in *in vitro* studies (Boratyński *et al*, 2000).

One such a drug that was engineered to attempt to overcome some of the problems is D-85, a methotrexate polymer. This is a molecule design where polyglutamic acid side chains were coupled onto a methotrexate molecule (Figure I). The chain marked $[\ ]_x$ is coupled an average of 9.2 times onto the methotrexate molecule (x=9.2). The chain marked $[\ ]_y$ is coupled once to the methotrexate molecule (X=1). Researchers have also looked at other side chains, such as dextran, human albumin and soluble starch (Harding, 1971).

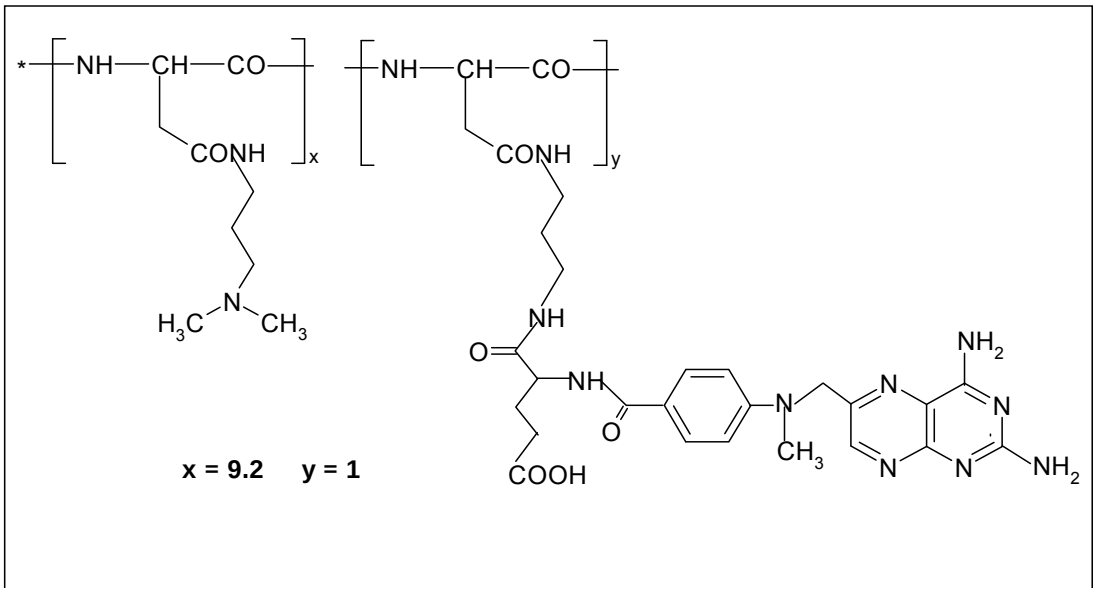

Figure I: Structure of conjugate D-85

In this article, we reviewed the in vitro toxicity of a novel MTX polymeric drug, D-85, compared to methotrexate. *In vitro* models (HeLa, MCF-7 and DU145 cell lines) were used to determine whether this drug conjugate, D-85, shows any superiority as an anti-cancer drug. We also looked at its effectivity and toxicity as an immunosuppressive drug compared to MTX by using mixed lymphocyte cultures. The results obtained during this study clearly illustrate the chemotherapeutic effect of the D-85 polymer and its promising immunosuppressive properties.

2. METHODS AND MATERIALS

MATERIALS:

2.1 *In vitro* toxicity studies in HeLa, MCF7 and DU145 cancer cells

The cells were cultured in a 37°C incubator in atmosphere of 5% CO_2 to 95% air. The mediums of the cell cultures were replaced in a 3-4 day cycle with fresh growth medium.

The HeLa, MCF 7 and DU 145 were removed from the cell culture flasks as follows:

a) The growth medium in the flasks were decanted and discarded.

b) The flasks were rinsed with a small amount of the respective growth medium for the respective cell line, to remove "old" medium.

c) *HeLa and DU 145 cells:*

d) Trypsin, an enzyme used to loosen cells from the cell culture flask through its enzymatic activity on the cell surface, was added. Just enough trypsin was added to cover the cell culture flasks horizontal surface. Once the cells started lifting up from the surface, the cell suspension was poured into 15ml centrifuge tubes. The culture flasks were washed out with the respective growth medium. This was also poured into the 15ml centrifuge tubes – *step 5 followed next*

e) *MCF 7 cells:*

f) Adhering MCF 7 cells were scraped loose from the cell culture flask with a cell scraper. The medium containing suspended MCF 7 cells were then decanted into a 50ml centrifuge tube. The flask was then washed with the respective growth medium and the growth medium was also decanted into the 50ml centrifuge tube - *step 5 followed next*

g) The tubes were centrifuged for 10 minutes at 1000 rpm. The supernatant was discarded and the cell pellet was resuspended in 1ml of the relevant growth medium. The cell suspension was made up to the concentrations noted in Table II.

To obtain the above-mentioned cell concentration suspensions, the cells were counted with a haemacytometer using a Reichert Jung MicroStar110 microscope.

Cancer cell line	Culture media	Incubation period	Cells (per ml)	MTX drug concentration range	D-85 drug concentration range
1) HeLa (cervic carcinoma cells)	E-MEM + 10% fetal bovine calf serum (*)	7 days	$2.5x10^4$	0.0015-0.19 μg/ml	Equivalent of 0.0015-0.19 μg/ml MTX
2) MCF 7 (Breast carcinoma cells)	D-MEM + 10% fetal bovine calf serum (*)	7 days	$2x10^4$	0.003125-100 μg/ml	Equivalent of 0.003125-100 μg/ml MTX
3) DU 145 (Prostate carcinoma cells)	RPMI + 10% fetal bovine calf serum (*)	7 days	$2.5x10^4$	0.003125-100 μg/ml	Equivalent of 0.003125-100 μg/ml MTX

Table II: Growth medium and criteria used for cancerous cells *in vitro* experiments

(* Penicillin/Streptomycin [Gray & Brenwald, 1999])

2.2 Mixed lymphocyte cultures

Blood samples were obtained from two healthy consenting donors. The samples were labelled A and B respectively. Lymphocytes were then isolated as follows:

a) Lymphocytes were prepared from whole blood collected from healthy, consenting donors.

b) Blood was collected in flasks containing heparin to prevent the blood from clotting. This blood was separated into different layers using Histopaque (Sigma Aldrich, Johannesburg; density: 1.077±0.0001), a high viscosity fluid that separates the

different cells and constituents of blood during centrifugation because of the different masses of the different cells and constituents.

c) After centrifugation during which the blood separated into different layers, the lymphocytes formed a hazy, white layer in the centrifuge tube. This was drawn off with a pipette and transferred to a new centrifuge tube. These lymphocytes were then washed twice using RPMI growth medium to remove platelets.

d) Erythrocytes that might have still been present after the above-mentioned separation method (as in *a*) were lysed by adding cold ammonium chloride to the cells for 10 minutes. The cell suspension was centrifuged to obtain a cell pellet.

e) The lymphocyte cell pellet was then washed with RPMI medium. The suspension was centrifuged again to obtain a cell pellet – growth medium was discarded.

f) Lymphocytes were then resuspended in RMPI medium supplemented with 10% fetal calf serum and antibiotics

Each of the lymphocyte cell suspensions were equally divided into two tubes, thus having two tubes with 500µl lymphocyte cell suspension of donor A and two tubes with 500µl lymphocyte cell suspension of donor B. This experiment was repeated four times using two *different* healthy consenting donors with each repeat.

a) *The two different tubes of each donor were treated as follows:*

1. Tubes A1 and B1 were resuspended in complete RPMI growth medium (medium containing 10% fetal calf serum and antibiotics);
2. Tubes A2 and B2 were resuspended in RPMI growth medium without fetal calf serum and the cells were inactivated by cobalt radiation at 20 Gy.

b) *The experiment was done as follows:*

1. 80µl complete RPMI growth medium was added to all the wells
2. 50µl viable cells at a concentration of $1x10^6$ from tubes A1 and B1 were added to the relevant wells and were incubated for one hour at 37°C with 5% CO_2 - (96 well microtiter plate was “divided” into 6x8 well columns, each column representing A1 and B1, with the top row of each column being the control)

3. 20μl of MTX and D-85 was added to the relevant cells according to a concentration range of 1.5-200 μg/ml MTX and D-85 with the equivalent of 1.5-200 μg/ml MTX.
4. Cells were incubated for another hour after which 50μl cell suspension from the irradiated tubes A2 and B2, were added to the wells (A2 cell suspension was added to column with B1 cells, B2 cell suspension was added to column with A1 cells). The irradiated cell suspensions were also standardized to contain $1x10^6$ cells per ml.
5. Cells were then incubated for 7 days at 37°C with 5% CO_2 concentration maintained in the incubator.
6. Cells were then analyzed by the MTT assay which measures cell viability.

2.3 Cell viability assay in *in vitro* toxicity studies and mixed lymphocyte cultures

In both the expirments the MTT assay was used to determine the cell viabilities of the respective cells. This assay is used to determine the amount of viable cells that survived drug treatment, compared to the control. This MTT (3-[4,5-dimethylthizol-2-yl]-2,5-difhenyltetrazolium bromide) reacts with the mitochondria of viable cells to form violet coloured crystals. These crystals are dissolved with a solvent, namely DMSO (dimethyl sulfoxide). The plates were analysed spectrophotometrically after all these crystals have been dissolved (Mosmann, 1983).

3. RESULTS

3.1 *In vitro* toxicity studies results in HeLa, MCF7 and DU145 cancer cells

The results obtained from the *in vitro* assays performed on cancerous cell lines were analysed to determine the toxicity of D-85 and methotrexate. The results indicate that D-85 has a greater toxicity than methotrexate regarding the three cancerous cell lines used in this experiment. The IC50 values obtained for D-85 on both HeLa cervix carcinoma cells (0.015 μg/ml) and MCF-7 breast carcinoma cells (0.201 μg/ml) are lower than that of methotrexate (Table III). An IC50 value for the treatment of DU 145 cells with the methotrexate polymer, D-

85, were 22.174µg/ml whereas methotrexate possibly has a much higher IC50 value since no IC50 could be determined for MTX concentrations administered to DU 145 cells (concentration range of 0.003125-100µg/ml MTX – refer to Table II). Therefore one can postulate that methotrexate's IC50 value might be >100µg/ml as this was the upper concentration range administered to the cells.

In the treatment of HeLa cells with D-85 compared to methotrexate, significantly different results were obtained between 0.024-0.095 µg/ml (Figure II). Cell growth was inhibited significantly at the concentrations 0.024µg/ml, 0.048µg/ml and 0.095µg/ml MTX or equivalent MTX in the polymer D-85 ($p<0.05$).

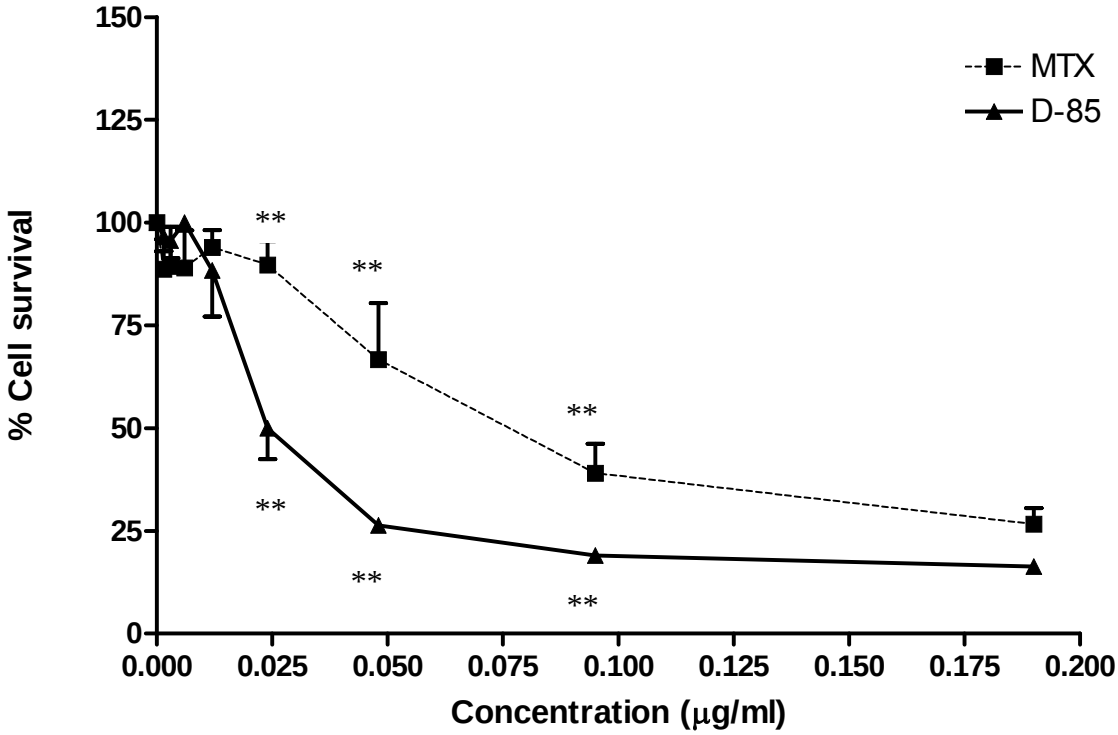

Figure II: Cytotoxicity of D-85, compared to MTX, obtained after administration to HeLa (cervix carcinoma) cells

[* * p value < 0.05 → significantly different from D-85]

Significantly different results in the treatment of the MCF 7 breast carcinoma cells were obtained at the concentration range of 0.05-25µg/ml (Figure III).

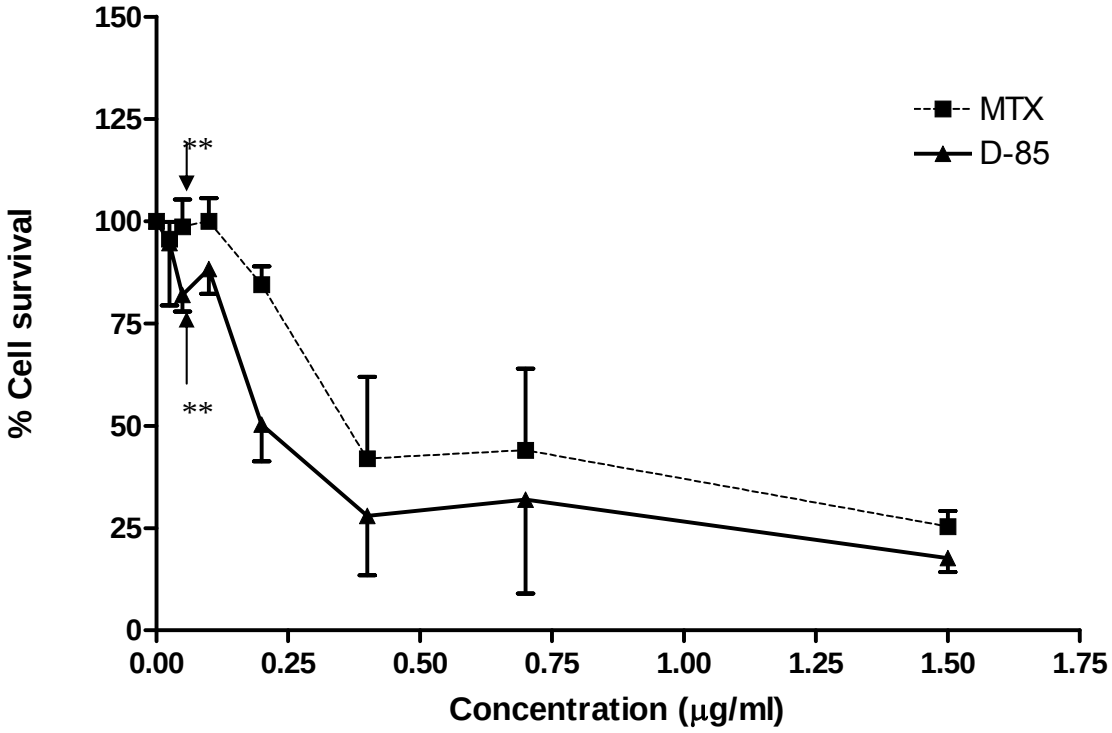

Figure III: Cytotoxicity of D-85, compared to MTX, obtained after administration to MTX and D-85 to MCF 7 (breast carcinoma) cells

[* * p value < 0.05 → significantly different from D-85]

No significantly different results were obtained in terms of MTX and D-85 drug administration to the DU 145 cells, but further investigation at higher concentrations of MTX and D-85 (>100µg/ml MTX or D-85 equivalent) is needed to confirm or reject the possibility that the drugs do not have a cytotoxic effect of more than 50% on these cells (Figure IV).

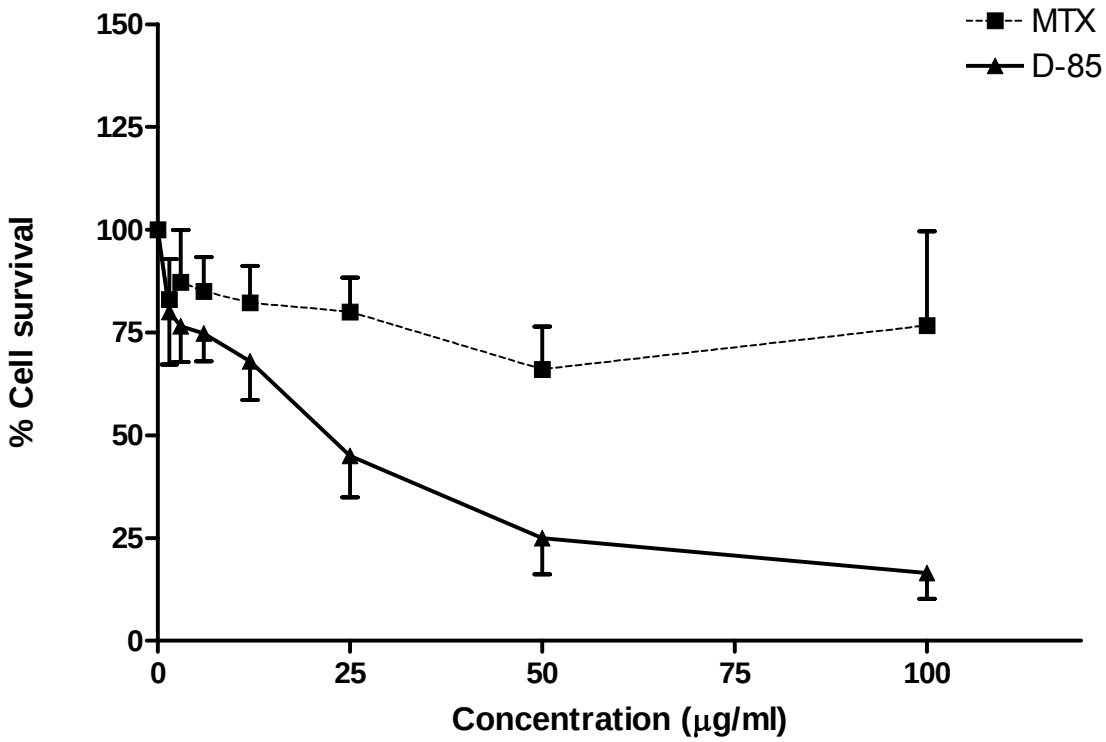

Figure IV: Cytotoxicity of D-85, compared to MTX, obtained after administration to DU145 cells

[0.05 < p value < 0.5]

The inhibitory concentration 50 (IC50) which indicates the drug concentration at which 50% of the cells survived, are tabled in Table 2. It clearly shows that D-85 shows superiority in the HeLa (servix carcinoma), MCF-7 (breast carcinoma) and DU145 (prostate carcinoma) assays. The significant values obtained during the in vitro experiments are tabled in Table IV.

IC50 (µg/ml)		
Cell Culture	MTX	D-85
HeLa	0.071 µg/ml	0.015 µg/ml
MCF-7	0.352 µg/ml	0.201 µg/ml
DU 145	no IC50	22.174 µg/ml

Table III: IC50 values of D-85, compared to methotrexate, obtained after administration to three respective cancer cell lines

Cancerous cell lines	*Significant differences (p< 0.05) calculated at following concentrations**
HeLa	0.095 μg/ml, 0.048 μg/ml, 0.024 μg/ml
MCF 7	0.05 μg/ml, 6 μg/ml, 12 μg/ml, 25 μg/ml
DU 145	no p-values under 0.05

Table IV: Concentrations at which significant differences was calculated between MTX and D-85 treatment of non-cancerous cells

[* p-values determined using paired t-test and GraphPad Prism 4 statistical program]

3.2 Mixed lymphocyte cultures results

The results obtained during the mixed lymphocyte reaction can be seen in Figure V. The graph indicates the percentage of cell survival compared to the untreated control. The mixed lymphocyte cultures, both the stimulated and resting lymphocytes, were treated with the following MTX and D-85 concentration ranges:

1. MTX: 1.5 – 200 μg/ml
2. D-85: the equivalent of 1.5 – 200 μg/ml

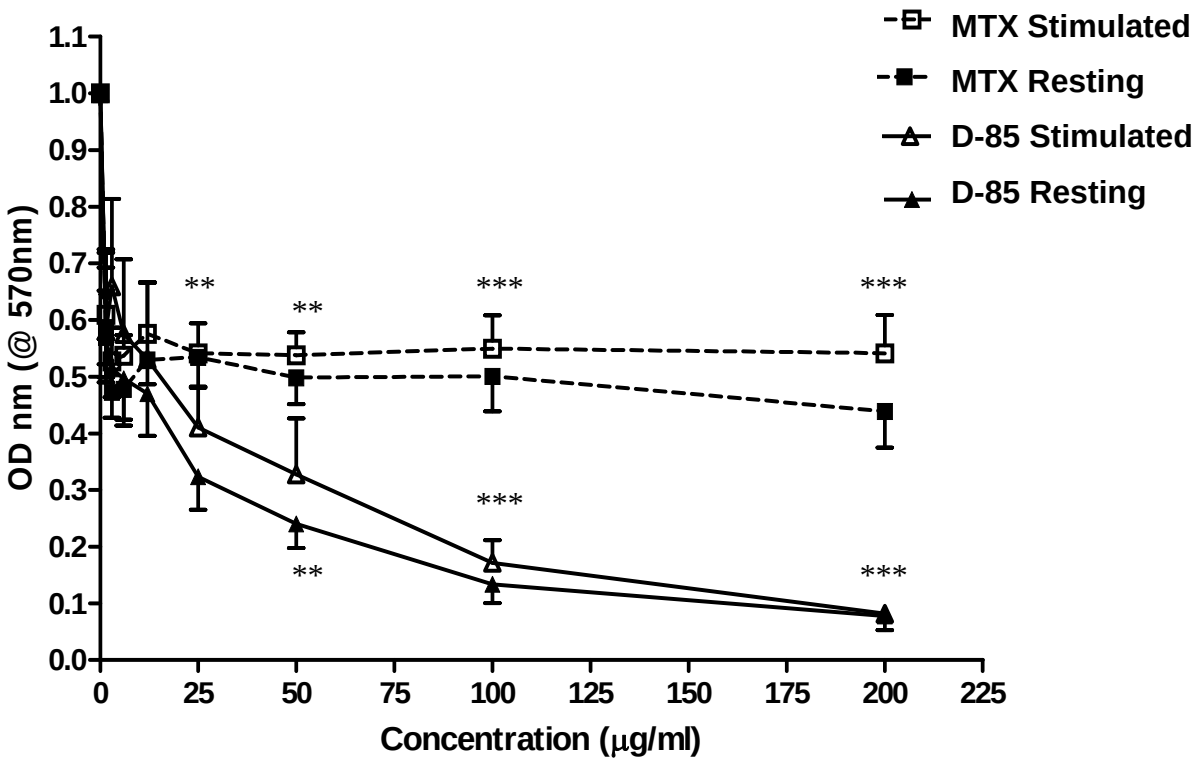

Figure V: Cytotoxicity of D-85, compared to MTX, obtained after administration to stimulated and resting mixed lymphocyte cultures

[* p-value < 0.5 * * p-value < 0.05 * * * p-value< 0.005]

The results of the treatment of *stimulated* lymphocytes with D-85 and MTX were compared and the results of *resting* lymphocytes treated with D-85 and MTX. Significant differences were obtained at various concentrations of drug treatment

3.3 Statistical methods

These results were analysed using GraphPad Prism Version 4, a statistical programme, to determine 50% of the inhibitory concentration value (IC50) of the respective drug. This was determined for each cell line used. The p-values are also determined by this programme with a 95% confidence interval.

4. DISCUSSION

The significant differences ($p<0.05$) determined for the HeLa cells, are in the same range than the IC 50 values determined for methotrexate and MTX on this cell line. These values were determined by using a one-tailed, paired t-test with a 95% confidence interval. The significance of these results indicates that the MTX-polymer, D-85, have superior anti-cancer properties when comparing it with methotrexate in this cervix carcinoma cell line. It can therefore be said that it could be of greater value in the treatment of this cancer than the usage of methotrexate.

The IC50 values determined for MTX in the treatment of the MCF 7 cancer cells are similar to that of D-85. But D-85's IC50 value is more than 1.5 times less than that of methotrexate; thus having a slightly greater cytotoxicity and selectivity for this cell line than MTX.

No IC50 of methotrexate could be determined in the prostate cancer cell line, DU 145. It is not primarily used for the treatment of prostate cancer, but D-85 shows in a definite toxicity in the exposure of these cells. Therefore D-85 could also be indicated in the treatment of prostate cancer.

D-85 proved to be more toxic to cancerous cell lines than methotrexate; thus indicating superior anti-cancer activity compared to methotrexate.

In the mixed lymphocyte cultures, (Figure 5), it is obvious that methotrexate inhibited both stimulated and resting lymphocytes up to a certain point. Although methotrexate had an inhibitory effect on the lymphocytes, an IC50 was never reached. Methotrexate's effect on lymphocytes and the drug's effect on graft-vs-host disease are well documented; however it was not clearly seen in these results.

In contrast with MTX, D-85 had a more profound effect on stimulated and resting lymphocytes. This differed significantly from MTX. There is significant inhibition of both

stimulated and resting lymphocytes which puts a question mark over the toxic effects of D-85 in the use of e.g. graft-vs-host disease. This does not mean that D-85 has no value as immunosuppressant or anti-inflammatory drug, but has to be researched thoroughly to determine the polymer's therapeutic index and other relevant pharmacological parameters to conclude the polymer's possible use in humans.

D-85 showed superior effects over the conventional MTX used as immunosuppressant, which grants further investigations regarding the MTX polymer's future use in this therapeutic field.

Conclusions:

In vitro assays of drug toxicity and efficacy (mechanism of action / proof of concept) are done to guide researchers in the development process of the particular substance.

The *in vitro* cell viability analysis, MTT that was done, indicates both that MTX and D-85 are cytotoxic towards cancerous cells. D-85 showed superiority in terms of the IC50 values obtained in these analyses. Significant differences between results of MTX and D-85 on both HeLa cervix carcinoma cells and MCF-7 breast carcinoma cells were obtained. No significant difference in the efficacy of MTX and D-85 was obtained on DU 145 prostate carcinoma cells.

Methotrexate is a well-known immunosuppressive drug. It is often used, as monotherapy or in combination with other drugs, in the prevention and treatment of graft-vs-host disease. To compare MTX and D-85 in terms of its mechanism of action in such an *in vitro* model, mixed lymphocyte cultures were used as described in chapter 4. Again, D-85 demonstrated a superior therapeutic value over MTX. However, in this model, D-85 was also more toxic to the resting lymphocytes, indicating a possible toxicity in an *in vivo* model. It might be of clinical value if treatment is done at a low concentration, such as 25 μg/ml, where toxicity towards resting lymphocytes, are not much different to that of MTX. But a significant difference is obtained in terms of its effect of stimulated lymphocytes. The toxicity that will be seen will possibly be treatable with folic acid supplementation and needs further exploration in future studies and the drug's possible use in diseases such as leukaemia.

Therefore, further investigations are required to determine whether D-85 shows promise as an anti-cancer and immunosuppressive drug.

Tables:

Table I: Side effects of methotrexate (Egan & Sandborn, 1996)
Table II: Growth medium and criteria used for cancerous cells *in vitro* experiments

Table III: IC50 values of D-85, compared to methotrexate, obtained after administration to three respective cancer cell lines

Table IV: Concentrations at which significant differences was calculated between MTX and D-85 treatment of non-cancerous cells

Figures:

Figure I: Structure of conjugate D-85

Figure II: Cytotoxicity of D-85, compared to MTX, obtained after administration to HeLa (cervix carcinoma) cells
Figure III: Cytotoxicity of D-85, compared to MTX, obtained after administration to MTX and D-85 to MCF 7 (breast carcinoma) cells

Figure IV: Cytotoxicity of D-85, compared to MTX, obtained after administration to DU145 cells

Legends:

Table II: * Penicillin/Streptomycin [Gray & Brenwald, 1999]

Table IV: * p-values determined using paired t-test and GraphPad Prism 4 statistical program

Figure II: * * p value < 0.05 $\rightarrow$ significantly different from D-85
Figure III: * * p value < 0.05 $\rightarrow$ significantly different from D-85

Figure IV: 0.05 < p value < 0.5

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