

*The toxicity, pharmacokinetics, anti-inflammatory
and anti -tumour properties of a methotrexate
polymer*

by

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Submitted in fulfilment of the requirements for the degree in the Faculty of
Health Sciences, University of Pretoria

Year: 2009

Date of submission: 25 June 2009

Abstract

A major effort to develop anticancer drugs through both empiric screening and rational design of new compounds has been under way for over 30 years (Katzung, 2004). In recent years, research and development in the field of site-specific drug therapy has progressed significantly. Safe and non-toxic formulations of cytotoxic drugs based on polymers with their improved site-specific delivery and effective activation to biologically active cytotoxic compounds at the targeted tumours have become a promising approach to cancer therapy. Drug delivery systems based on polymer micelles, coated micro- and nanoparticles, liposomes and various pro-drug systems including water-soluble polymer–drug conjugates and immunoconjugates have been prepared and extensively studied as novel drug delivery systems designed for cancer chemotherapy. Amongst these drug delivery systems that enable specific drug delivery and release, water-soluble polymer–drug conjugates rank among the most promising, versatile and efficient systems.

This dissertation reviews the preclinical testing and pharmacokinetic study of D85, a novel water-soluble macromolecular pro-drug that is a polymer with pH-controlled methotrexate (MTX) release with potential for treatment of cancer in humans (Ulbrich & Subr, 2004). As MTX is also indicated in low doses for the treatment of chronic inflammatory conditions, the polymer was further tested in an acute inflammatory model to determine whether the polymer would be more effective than MTX in controlling inflammation.

The objective of this study was to compare the potency and efficacy of D85 to MTX. D85, a MTX conjugated polymeric lead compound, was designed and synthesised as a potential anti-neoplastic and anti-inflammatory agent. It was initially tested *in vitro* on three different cancer cell lines where selective toxicity towards the cancer cell cultures compared to primary cell cultures and greater toxicity than MTX was observed. The initial *in vitro* tests showed very promising

results with D85 demonstrating approximately 300 times greater cytotoxicity than MTX against a colon cancer cell line (COLO 320 DM). This high cytotoxic effect warranted further investigation in an *in vivo* colon cancer tumour model.

An induced murine tumour model of COLO 320 DM was successfully developed in nude mice, and the anti-tumour efficacy of D85 tested in this model. The maximum tolerated dose of D85 was established by carrying out an *in vivo* dose ranging toxicity test in BALB/c mice.

The anti-inflammatory effects of D85 were also determined using the carrageenan-induced paw oedema model in rats where carrageenan was injected into a footpad of a rat causing acute oedema, which was measured using a water displacement plethysmometer. D85 was found to exacerbate the inflammatory response.

Finally, the pharmacokinetic parameters of MTX and D85 were assessed using a LC/MS/MS method specifically developed and validated to determine low concentrations of MTX in small volumes of plasma. This new method made use of online solid phase extraction and sample cleanup on 2µl injections of diluted plasma allowing an entire pharmacokinetics study to be completed on an individual rat. Fairly similar pharmacokinetics were determined from both compounds.

Key words: Methotrexate, D85, anti-inflammatory, anti-cancer, carrageenan inflammation model, pharmacokinetics, nude mice



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Glossary of abbreviations

AICAR	5-amino-imidazole-4-carboxamide ribonucleotide
ALL	Acute lymphoblastic leukaemia
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BFM	Berlin-Frankfurt-Munster
CANSA	Cancer Association of South Africa
CHCl ₃	Chloroform
CO ₂	Carbon dioxide
D85	The methotrexate conjugated polymer used in this study
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's Minimal Essential Medium
DMSO	Dimethyl Sulfoxide
EPR	Enhanced Permeability and Retention
EtOH	Ethanol / Ethyl alcohol
ESI	Electrospray ionisation
GGT	γ -glutamyl transferase
HCl	Hydrochloric acid
HCO ₂ H	Formic acid
HI FCS	Heat inactivated foetal calf serum
HNO ₃	Nitric acid
HPLC	High pressure liquid chromatography
i.v.	Intravenous
LC/MS/MS	Liquid chromatography tandem mass spectrometry
LLOD	Lower limit of detection
LLOQ	Lower limit of quantitation
NaOH	Sodium hydroxide
nm	Nanometre



MeCN	Acetonitrile
MeOH	Methanol
MS	Mass spectrometry
MTT	(3-{4, 5-dimethylthiazol-2-yl}-2, 5-diphenyltetrazolium bromide)
MTX	Methotrexate
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinine
PK	Pharmacokinetic
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
s.c.	Subcutaneous
SCID	Severe combined immune deficient mice
SD	Sprague Dawley
SFs	Synovial fibroblasts
μl	Microlitre
UPBRC	University of Pretoria Biomedical Research Centre



Declaration by candidate

The experimental work discussed in this dissertation was carried out by the author (S. S. Sayed) in the Department of Pharmacology, Faculty of Health Sciences, University of Pretoria under the supervision of Dr. A.D. Cromarty and Prof. C.E. Medlen.

I declare that the dissertation, which I hereby submit for the degree MSc: Pharmacology at the University of Pretoria, is my own work and has not previously been submitted for a degree at this or any other tertiary institution.

.....
Sharfuddin Sakil Sayed

.....
Date

Acknowledgements

Several people have in some way contributed to the completion of this study and I wish to express my sincere gratitude to them.

My grandmother, mom, uncle, brother, Fatima, family and friends for their continued support and encouragement.

My supervisor, Dr. A.D. Cromarty has served as an educator, a mentor and as my best friend and has always been there when I needed him, at any time of the day.

Prof. C.E. Medlen was instrumental in several aspects of this study from start to finish.

Dr Auer played an important role with all the animal related work, my sincerest gratitude to him.

Prof. E. W. Neuse kindly provided the methotrexate-conjugated polymer.

Animal ethics, use and care

The following protocols were approved by the Animal Use and Care Committee (AUCC) of the University of Pretoria and carried out at the University of Pretoria Biomedical Research Centre (UPBRC).

1. An *in vivo* investigation of the anti-tumour properties of a novel methotrexate polymer using human cancer xenografts in nude mice (Protocol no: H014-06).
2. An *in vivo* investigation of the toxicity profile of a novel methotrexate polymer in BALB/c mice (Protocol no: H029-06).
3. The pharmacokinetic profile of a methotrexate polymer, compared to methotrexate in Sprague Dawley rats (Protocol no: H016-06).
4. The anti-inflammatory property of a novel methotrexate polymer (D85) administered intravenously; using the carrageenan rat model of inflammation (Protocol no: H019-07).

Trained personnel at the UPBRC cared for all animals used during the various studies. Nude mice were housed in pairs to reduce the stress of solitary living. Mice were housed in Isolated Ventilated Cages (IVCs) (Type II long; Techniplast) under sterile conditions at approximately 22°C and 40-60% relative humidity. Food and water were provided *ad libitum*. All cages, feed, bedding and drinking water were sterilized prior to use.

The rats used for the study were purchased from a certified animal breeder and acclimatized at the UPBRC for at least one week before the study was initiated.

Food and water were provided *ad libitum* with a 12-hour day/night light cycle.

Condition and morbidity were determined at relevant times by the trained personnel of UPBRC. Evaluation of reduced food and water intake, weight loss [more than 10% original weight], observation of abnormal behaviour or movement (particularly as it pertained to the ability of the animal to obtain food and water) and ease of breathing were determined.

1. Introduction

1.1. Background

Methotrexate (MTX) is the common name for 4-amino-N-10-methylpteroylglutamic acid (Figure. 1) (Bleyer, 1978). It differs from the natural vitamin folic acid by two substitutions: (1) a hydroxyl is replaced by an amino group at C4 position of the pterine ring; (2) a methyl group replaces a hydrogen at N-10 position (Santos *et al.*, 2006). MTX, also known as amethopterin, differs from aminopterin (another folic acid antagonist) in that the latter is not methylated at the N-10 position (Bleyer, 1978).

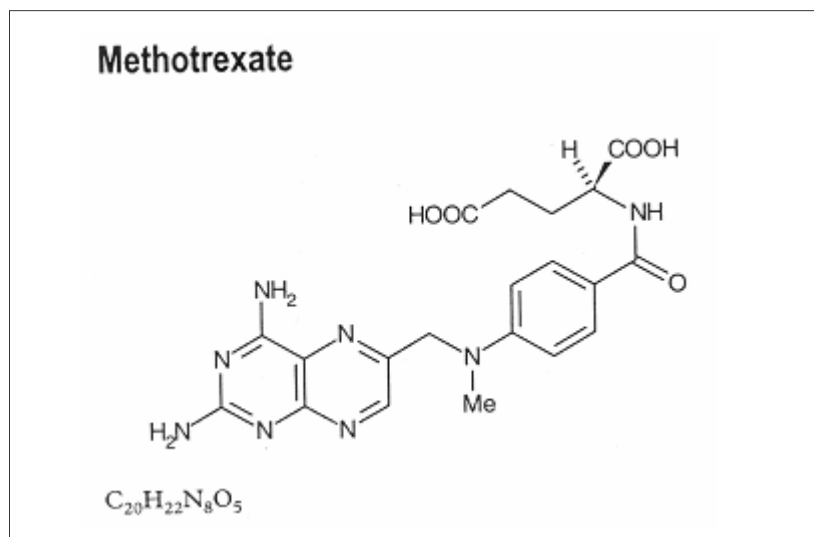


Figure 1: Structure of Methotrexate.

The molecular weight of MTX is 454.46 Dalton. The organic:aqueous partition coefficients at pH 7.5 are between 1:50 - 1:10,000 depending on the organic solvent used. MTX is a weak dicarboxylic acid with two acid groups with pK_a's of 4.8 and 5.5 respectively. As predicted from these physicochemical characteristics, MTX is essentially ionized and lipid insoluble at physiological pH.

MTX represents one of the most potent classical anti-tumour drugs in clinical administration, and is used alone or in combination regimens (Moscow *et al.*, 1998). It has a broad range of cytostatic activity, especially when given in high doses with a follow-up folinic acid rescue (Ofner *et al.*, 2006). MTX is an important drug in the treatment of Acute Lymphoblastic Leukaemia (ALL), choriocarcinoma, related trophoblastic tumours and more recently has been used in low doses for conditions such as rheumatoid arthritis and psoriasis (Ofner *et al.*, 2006).

The biological activity of the molecule is thought to involve the pterine ring portion (Bleyer, 1978). The cytotoxic effects of MTX are due to the inhibition of the dihydrofolate reductase enzyme resulting in downstream blockade of DNA and RNA synthesis followed by cell death of the most rapidly dividing cells (Tjaden & De Bruinn, 1990). In humans, MTX appears to inhibit DNA synthesis to a greater extent than RNA synthesis, suggesting that inhibition of thymidylate synthesis is the more important mechanism of MTX cytotoxicity (Bleyer, 1978). The drug efficacy is highly dependent on cell cycle phase, acting primarily during rapid DNA synthesis (S-phase) (Bleyer, 1978). As a result, those tissues undergoing rapid cellular division with a high fraction of the cells in DNA synthesis are the most susceptible to the drug's cytotoxic effects (Bleyer, 1978). The mechanisms by which MTX enters normal and neoplastic cells has not been fully characterized but current evidence suggests that reduced folates necessary for normal cellular metabolism gain entry to cells via an energy-coupled, carrier-mediated mechanism in the cell membrane with MTX and other folate analogues appearing to be transported by the same mechanism (Bleyer, 1978). The Martindale monograph (Sweetman, 2004) on MTX states that the drug enters the cells in part by an active transport mechanism and is then trapped as a polyglutamate conjugate. This intracellular formation of polyglutamate derivatives appears to be important in the therapeutic mechanism of MTX (Katzung, 2004). MTX polyglutamates are selectively retained within cancer cells and have increased inhibitory effects on enzymes involved in folate metabolism, making

them important metabolites extending the duration of action of MTX (Katzung, 2004).

MTX doses of less than $20\text{mg}/\text{m}^2$ arrest leukaemia myeloblasts in S-phase for about 20 hours, with little effect on cells in the G1, G2, or M-phases of the cell cycle (Bleyer, 1978). At higher doses ($>30\text{mg}/\text{m}^2$), MTX arrests human myeloblasts in S-phase for more than 48 hours but also slows the transition of cells from G1 to S-phase (Bleyer, 1978).

Reduced folates are also required as co-factors in the methylation of glycine to serine and of homocysteine to methionine (Bleyer, 1978) and by preventing these amino acid conversions, MTX may interfere with normal protein synthesis. This effect may be the mechanism by which high dose MTX “arrests” cells in G1-phase (Bleyer, 1978). Rapidly dividing tissue that survive MTX exposure may be protected from fatal injury by a temporary arrest of cells in the G0/G1 phase of the cell cycle, preventing their progression into the drug-sensitive S-phase (Bleyer, 1978). According to this hypothesis, high-dose therapy would have a “self-limiting” effect. On the other hand, cells deprived of essential protein synthesis for prolonged intervals may also be irreversibly damaged and would succumb (Bleyer, 1978).

High-dose MTX was reported to be effective in central nervous system (CNS) malignancies where high or even massive dosages of the drug were given in order to achieve high enough serum MTX concentrations. This enabled sufficient blood-brain barrier migration to reach tumouricidal levels in the cerebrospinal fluid (Reggev & Djerassi, 1988).

The mechanism of action of high dose MTX has still not been entirely elucidated despite studies starting almost 30 years ago (Ambinder *et al.*, 1979). One rationale for treating with high dose MTX is based on the assumption that drug resistance is due to inadequate intracellular drug concentration, a restriction that can be corrected by either a higher MTX dose or by extended drug exposure time

(Ambinder *et al.*, 1979). In mixed cell populations with overlapping cell division cycles, prolonged high dose infusions of drug may be required to ensure sufficiently high intracellular MTX concentrations during the susceptible phase of the cell cycle for all the dividing cell populations (Ambinder *et al.*, 1979).

MTX has a relatively short biological half-life and therefore its tumour exposure time is considered to be short (Ofner *et al.*, 2006). Consequently, its therapeutic efficacy is impaired by its short *in vivo* half-life and exacerbated by its low tumour accumulation which then requires higher doses or extended treatment regimens that increases the adverse effects in susceptible healthy tissue (Ofner *et al.*, 2006). These problems contribute substantially to the potentially life threatening adverse effects of bone marrow depression, ulcerative colitis, hepatotoxicity and nephrotoxicity (Ofner *et al.*, 2006).

In addition to the non-selective toxicities mentioned above, MTX has a high incidence of drug resistance which further limits its effectiveness (Ofner *et al.*, 2006). MTX resistance develops through several mechanisms including decreased folate carrier-mediated membrane transport, dihydrofolate reductase gene amplification, specific transcription–translational modifications, and down regulation of intracellular MTX polyglutamation (Lindgren *et al.*, 2006). It has been reported that 30% of remission failures are due to MTX resistance in the treatment of ALL (Ofner *et al.*, 2006).

Water soluble conjugates of low molecular mass drugs to high molecular mass proteins, peptides or polymers are being investigated in several laboratories due to the potential therapeutic advantages compared to the free drug (Ofner *et al.*, 2006). One potential advantage is the passive accumulation of conjugates in solid tumours due to the reputed enhanced permeability and retention (EPR) effect caused by “leaky” tumour vasculature combined with a poorly developed lymphatic drainage (Ofner *et al.*, 2006). Due to permeability enhancing factors, such as vascular endothelial growth factor (VEGF) and bradykinin, the

endothelium of the tumour vasculature becomes discontinuous. This leads to the extravasation of macromolecules from the bloodstream in the tumour tissue (Hoste *et al.*, 2004). Additionally, the lack of effective lymphatic drainage in the tumours prevents the macromolecules or macromolecular pro-drugs that accumulate as a result of the vascular leakage from being removed rapidly and subsequently this results in an extravascular retention of the macromolecules or macromolecular drug conjugates (Hoste *et al.*, 2004). This so-called 'enhanced permeability and retention effect' (EPR effect) has also been observed in inflamed tissue (Hoste *et al.*, 2004). The EPR effect is now generally accepted and considered as a major rationale for using polymeric pro-drugs (Figure. 2).

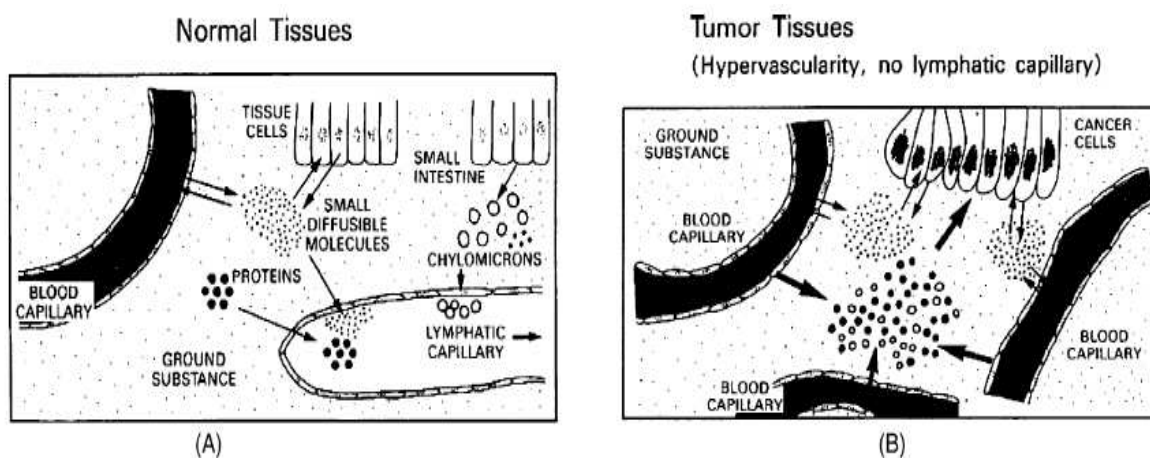


Figure 2: A diagram illustrating the Enhanced Permeability and Retention effect (Hoste *et al.*, 2004).

This tumour specific accumulation of polymeric conjugated drug reduces systemic toxicity by reducing toxicity to healthy tissues (Ofner *et al.*, 2006). The EPR enhanced accumulation may account for a several fold higher maximum tolerated dose of a polymer conjugated drug compared to free drug (Ofner *et al.*, 2006). In addition, the EPR effect is amplified by the cytotoxicity of the conjugate metabolic products, which increases the average drug concentration within the tumour, further enhancing the efficacy of the conjugate (Ofner *et al.*, 2006). It has been reported that the EPR effect is most effective for macromolecules of greater than 40 kDa, but less effective for smaller molecules that can be cleared more rapidly from tumour interstitium (Ofner *et al.*, 2006).

A promising strategy to overcome the pharmacokinetic deficiencies and premature MTX release, leading to an altered bio-distribution, involves the bio-reversible conjugation of MTX to a water-soluble macromolecular polymer. The physical and chemical properties of macromolecular drug-conjugates substantially influences conjugate behaviour under various biological conditions to which it is exposed during drug delivery (Ofner *et al.*, 2006). After intravenous administration, the conjugate is exposed to plasma (~ pH 7.4 and proteolytic enzymes), then tumour interstitium (~pH 6.5 and proteolytic enzymes) and after uptake into the cell, it is ultimately degraded under lysosomal conditions (~ pH 4.8 and proteolytic enzymes) with drug release (Ofner *et al.*, 2006). Therefore, after receptor-mediated cellular entry, the active drug could be released in early or secondary endosomes by pH-controlled hydrolysis (pH drop from physiological 7.4 - 5~6 in endosomes or 4~5 in lysosomes) or specifically by enzymatic lysis within lysosomes (Ulbrich & Subr, 2004).

The carrier polymer used in this study, D85, was synthesised from aspartic acid, a natural amino acid, and then conjugated with MTX. This conjugate was designed and synthesized in the Department of Chemistry, University of the Witwatersrand, Johannesburg. The final concentration of MTX in the D85 was 17% based on mass. This product was submitted for evaluation to the “Anti-Cancer Drug Development Consortium” supported by the Cancer Association of South Africa (CANSA).

MTX was selected as the anti-neoplastic drug for conjugation to the polymer for the following reasons:

- It is a cell-cycle phase specific drug where extended exposure of cancer cells to the drug improves the drug’s cytotoxic efficacy and
- The release of the conjugated drug from the macromolecular polymers is expected to occur intracellularly over a prolonged period (Singh & Udupa, 1997).

As the carrier is based on a substituted asparagine, it is biodegradable, biocompatible, reputedly shows low immunogenic activity and is completely soluble in water (Figure. 3). The polymer conjugate should accumulate in solid tumours because of enhanced intra-tumour vascular permeability, allowing a substantial leakage/uptake of the polymeric molecules into the tumour. The main advantage of localized drug delivery is a high loco-regional concentration of the therapeutic agent with prolonged retention and consequently a lower total systemic dose can be administered, reducing the chance of concentration dependant adverse effects by avoiding the “normal” high doses of the free drug required to achieve therapeutic concentrations at diseased sites (Modi *et al.*, 2006). The subsequent accumulation of the released drug within tumour cells due to the increased bioavailability of the conjugated drug in the immediate vicinity of the rapidly dividing cells should reduce the required systemic dose and therefore the adverse side effects, making this conjugate safer for the treatment of cancer.

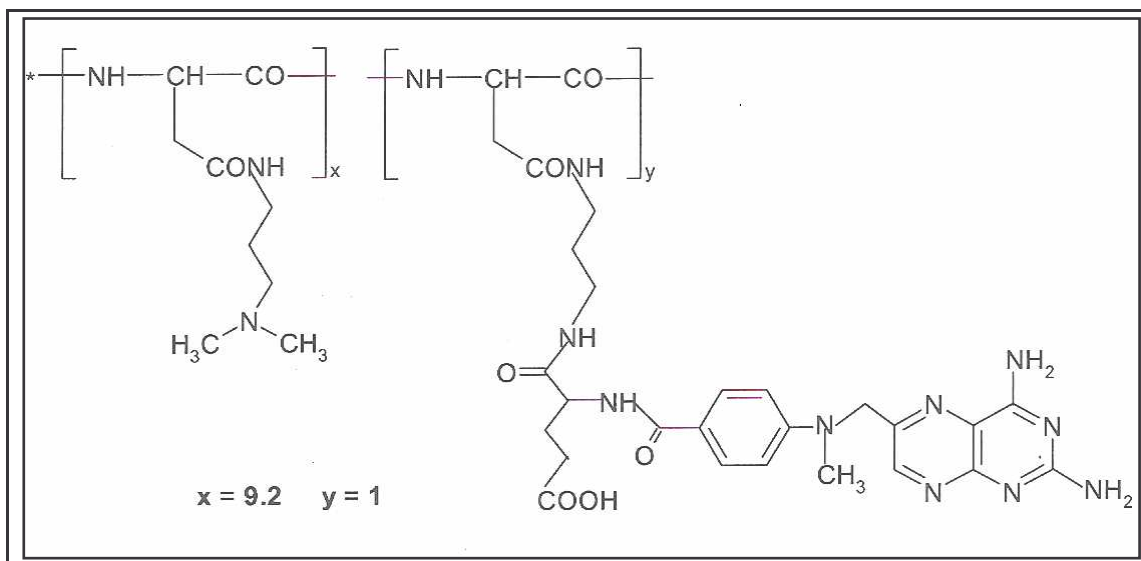


Figure 3: Structure of D85.

1.2. Pharmacokinetics of MTX

MTX is distributed in tissues and extra cellular fluid with an apparent steady-state volume of distribution of 0.4-0.8 L/kg (Sweetman, 2004). After intravenous administration, MTX follows a typical two-compartment model, rapidly distributing into a volume of approximately 18% of body weight and then equilibrating into a larger volume of approximately 76% of body weight. These apparent volumes of distribution approximate those of the extracellular space and total body water, respectively (Bleyer, 1978). After intravenous administration, the drug is cleared from the plasma in a triphasic manner; the first phase has an elimination half life of 45min, the second phase 2.5h and the final phase 7h (Singh & Udupa, 1997).

Toxic side effects in patients receiving high dose therapy, results partially from formation of an active metabolite (7-OH-Methotrexate) and delayed excretion, both of which continue to restrict its widespread therapeutic use. The principal toxicities caused by MTX are myelosuppression, gastrointestinal mucositis and hepatitis. With high-dose systemic therapy, renal dysfunction, vomiting, acute desquamative dermatitis and B-lymphocyte dysfunction are also encountered (Bleyer, 1978). Seizures, reactivation of solar dermatitis, vasculitis of hands and feet, conjunctivitis and radiation toxicity recall have been reported (Bleyer, 1978). Nephrotoxicity during high-dose systemic therapy is particularly problematic because the drug and the active metabolite are retained even longer as a result of impaired renal function (Bleyer, 1978).

Conjugation of MTX to a polymeric carrier is expected to change the physicochemical and pharmacokinetic properties of the drug. As a result of these changes, biocompatibility, water solubility, improved efficacy and increased mean retention time due to the large molecular mass slowing kidney excretion, contribute to less toxicity and greater efficacy (Swarts, 2002).

1.3. Anti-Inflammatory effects of MTX

MTX exerts a variety of anti-inflammatory actions and is a potent anti-inflammatory agent (Dalmarco *et al.*, 2002). The mechanism by which MTX inhibits both acute and chronic inflammation remains controversial (Dalmarco *et al.*, 2002). Evidence indicates that MTX treatment decreases neutrophil-endothelial adhesion *in vitro* by modulating the expression of integrins (Dalmarco *et al.*, 2002). Others believe that it is mediated via the increased release of adenosine because of 5-amino-imidazole-4-carboxamide ribonucleotide transformylase (AICAR) inhibition by MTX polyglutamates (Wunder *et al.*, 2003). Inhibition of this enzyme results in the accumulation of AICAR, which in turn inhibits adenosine deaminase, thereby increasing the intracellular adenosine concentration and the subsequent release of adenosine into the extracellular fluid (Wunder *et al.*, 2003). Adenosine exhibits a range of anti-inflammatory activities, including the inhibition of leukocyte accumulation and neutrophil-mediated endothelial injury at sites of inflammation; the reduction in tumour necrosis factor alpha synthesis, and inhibition of a variety of natural killer cell, monocyte/macrophage, and T-cell activities (Wunder *et al.*, 2003).

In rheumatoid arthritis (RA), the synovial layer lining affected joints is transformed into a highly proliferative so-called pannus-like tissue consisting of synovial fibroblasts (SFs), synovial macrophages, and various infiltrating inflammatory cells (Wunder *et al.*, 2003). This hypertrophic and oedematous tissue increasingly invades adjacent cartilage and bone (Wunder *et al.*, 2003). Subsequently, joint remodeling is mediated by matrix-degrading enzymes released by apparently transformed and activated SFs (Wunder *et al.*, 2003). Similar transformation and activation phenomena are also observed in neoplastic diseases (Wunder *et al.*, 2003). Because of the similarity of amplified rates of cell division in RA and cancer, both diseases can be treated with anti-proliferative drugs like MTX, which is one of the most frequent drugs in refractory RA treatment and is also part of numerous treatment protocols in oncology (Wunder *et al.*, 2003). MTX was first used for the treatment of RA in 1951 and has proved

to be effective in the treatment of RA in several randomized clinical trials since then (Alarcon, 2000).

The progression of joint destruction cannot be totally inhibited by MTX treatment in most RA patients (Fiehn *et al.*, 2004). One reason might be that low drug accumulation in the poorly vascularized joints (Fiehn *et al.*, 2004) and the rapid elimination of the circulating MTX by the kidneys.

Although increasing the dose of MTX does result in higher therapeutic efficacy, the risk of adverse side effects also increases (Fiehn *et al.*, 2004). Thus, the disadvantageous pharmacokinetic properties of MTX are probably the basis of inadequate treatment outcomes (Wunder *et al.*, 2003). To overcome the lack of specificity with regard to the target tissue, drugs can be covalently conjugated to suitable drug carriers (Wunder *et al.*, 2003).

The main features of an appropriate drug carrier are accumulation in the target tissue, low uptake rates in normal tissue and low toxicity towards healthy tissue, especially liver and kidneys. The biochemical potential to be linked to drugs, the release of the drug in the target tissue, and the general availability and affordability (Wunder *et al.*, 2003) are also important. Polymers of the type used to synthesize D85 have however never been tested *in vivo* for bioavailability or efficacy as anti-inflammatory agents.

1.4. Hypothesis

1. The methotrexate-conjugated polymer, D85, is a more effective anti-tumour agent with less adverse effects and an improved pharmacokinetic profile compared to free MTX.
2. The MTX-conjugated polymer, D85, is a more effective anti-inflammatory agent with less adverse effects than free MTX.

1.5. Aims

The aim of the study was to test whether the novel MTX polymer (D85) is superior to MTX with regards to:

- anti-tumour activity
- anti-inflammatory activity
- pharmacokinetic profile
- systemic toxicity

1.6. Objectives

The objectives of this study were:

1. The *in vitro* evaluation of the cytotoxic activity of D85 compared to MTX against various cancer cell lines and primary lymphocyte cultures.
2. To evaluate the systemic toxicity of D85 in BALB/c mice.
3. To establish a xenografted human tumour model in *nu/nu* knockout nude mice.
4. To evaluate the anti-tumour properties of D85 in the established xenografted human tumour nude mouse model.
5. To evaluate the anti-inflammatory activity of D85 compared to MTX in the carrageenan inflammation model in Sprague Dawley rats.
6. To evaluate the pharmacokinetic properties of D85 and MTX using a LC/MS/MS method developed and validated for determining free MTX levels (derived from D85) in plasma of Sprague Dawley rats.

2. In vitro cytotoxicity

2.1. Introduction

MTX continues to play an important role in the treatment of a variety of malignancies and its mechanism of action is well understood at a molecular level (Wosikowski *et al.*, 2003). As a folate antagonist MTX inhibits synthesis of tetrahydrofolate, pyrimidines and purines, and induces differentiation in several cell types. MTX enters the cell via an active transport system for reduced folate and is then converted into a polyglutamated form by folylpolyglutamyl synthetase inside the cell. The polyglutamated form is retained in the cell and not only inhibits dihydrofolate reductase (DHFR) but has an increased affinity for certain folate dependent enzymes such as thymidylate synthase and enzymes involved in purine synthesis (Singh *et al.*, 2006). The development of several new MTX conjugates with distinctive chemical features has provided new opportunities to expand the role of antifolates in cancer chemotherapy. In an attempt to improve selectivity and decrease adverse systemic toxicity, numerous derivatives of MTX have been synthesized (Santos *et al.*, 2007). The advantage of this approach is the altered specificity of the MTX conjugate to kill cancerous cells more selectively. MTX was selected as a model drug since it has numerous proven anti-cancer properties and is one of the most widely used drugs in medical oncology for the treatment of cancers (Santos *et al.*, 2007).

In this phase of the study, the cytotoxic effects of MTX and D85 on cancerous and non-cancerous cell lines were investigated. *In vitro* cytotoxicity assays were performed to establish sensitivity of cancer cell lines and of normal primary cell cultures to MTX and D85 using standard cell culture techniques. A selected concentration of cells was exposed to different concentrations of each of the experimental drugs in 96 well tissue culture plates and thereafter incubated for an appropriate period of time depending on the cells in culture. After the incubation period the relative percentage of viable cells in each treated well compared to

equivalent untreated controls was determined using the 3-[4, 5 dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) cell enumeration method (Mossman, 1983). The results obtained in this phase of the study allowed the calculation of the concentrations of MTX and D85 that inhibited treated cell proliferation by 50% (IC_{50}).

2.2. Materials and Methods

Prof. EW Neuse (Department of Chemistry, University of the Witwatersrand) kindly provided the MTX-conjugated polymer (D85). MTX was purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO).

A stock solution of 4.4 μ M MTX was made up in dimethyl sulfoxide (DMSO). The stock solution of D85 was made up in water at a weight equivalent to 4.4 μ M MTX (11.69mg of D85 is equivalent to 2mg of MTX).

The following cell cultures were used in this study: HeLa (Human adenocarcinoma of the cervix, ATCC no. CCL-2), COLO 320 DM (Human colon cancer, ATCC no. CCL-220), MCF-7 (Human breast cancer, ATCC no. HTB 22) and primary mixed lymphocytes from healthy volunteers.

The HeLa cell line is used for initial screening of all new experimental drugs that are tested by the “Anti-Cancer Drug Development Consortium” and gives some comparative data for all the drugs tested. MCF-7 and COLO 320 DM cell lines were chosen as models because MTX is used in the treatment of selected breast and colon cancers and represent adhesive and free floating type cancer cells.

Lymphocyte proliferation with exposure to the test drugs was also measured in this phase of the study as a model of normal healthy cells. These cells were used to indicate whether the drugs had adverse effects on these healthy cells at concentrations used for anti-neoplastic indications.

Heparinized blood was collected from healthy, consenting volunteers and the mixed lymphocyte population isolated under sterile conditions and exposed to different concentrations of either MTX or D85 for 3 days in culture using both resting and phytohemagglutinin (PHA) stimulated cells. The effect of both MTX and D85 on lymphocyte proliferation in resting and stimulated states were compared.

2.2A. Method for cancer cell line preparations

1. Eighty microlitres medium (HeLa: EMEM medium, COLO 320 DM: RPMI+ medium, MCF-7: DMEM medium) was placed into each well of a round-bottomed 96-well tissue culture plate.
2. One hundred microlitres cell suspension in the appropriate medium ([HeLa] = 2.5×10^4 , [COLO 320 DM] = 2×10^4 , [MCF-7] = 2.5×10^4) was added to each well.
3. Cultures were then incubated at 37°C in an atmosphere of 5% CO₂ and 95% relative humidity for an hour.
4. A stock solution of 4.4µM was made up for both MTX and D85 and serially diluted one in two down to a concentration of 3.4µM. Thereafter twenty microlitres of each test compound at each concentration was added to three triplicate wells. Control wells received 20µl of growth medium only.
5. The culture was incubated for 7 days at 37°C in an atmosphere of 5% CO₂ and 95% relative humidity.
6. On day 7 of the incubation period the viable cells were enumerated by using the MTT method as explained below.
7. Percentage survival relative to the controls was calculated and this value then used to determine the IC₅₀ value.

IC₅₀ = the concentration (µM) of the experimental compound inducing a 50% decrease in cell growth relative to the controls.

IC₅₀ value was determined using the GraphPad Prism 4 statistical analysis program.

2.2B. Method for lymphocyte isolation and preparation

Complete RPMI (RPMI+) consisting of RPMI-1640 supplemented with 1% glutamine, a penicillin and streptomycin cocktail (BioWhittaker™, CAMBREX Bio Science, Walkersville, USA) at 100µg/ml each and 10% heat inactivated foetal calf serum was used for culturing freshly isolated mixed lymphocyte populations.

The lymphocytes were prepared under sterile conditions from whole blood, as follows:

- Thirty millilitres heparinised blood was carefully layered onto 15ml Histopaque 1077 (Sigma Aldrich, St. Louis, MO) in a sterile 50ml Falcon centrifuge tube.
- This was centrifuged (Allegra™ X-22 Centrifuge, S2096 rotor, Beckman Coulter, Johannesburg, South Africa) for 25 min at 800g at room temperature.
- The top plasma layer was removed.
- The mixed lymphocyte/monocyte layer near the top of the Histopaque layer was transferred into new sterile 50ml tubes.
- The tubes were filled with sterile RPMI-1640 medium (Sterilab, Johannesburg, South Africa).
- The tubes were centrifuged for 15 min at 500g to wash the cells and to remove any contaminating platelets.
- The supernatant fluid was discarded, the lymphocyte pellet resuspended in sterile RPMI-1640 and centrifuged for 10 min at 500g.
- The supernatant was discarded and the pellet resuspended in sterile, cold NH₄Cl lysing solution. It stood for approximately 10 minutes on ice to lyse any contaminating erythrocytes.
- Thereafter it was centrifuged for 10 minutes at 500g.
- Supernatant was discarded and the pellet resuspended in RPMI-1640.
- It was centrifuged for 10 min at 500g and the supernatant was discarded.

- The pellet was resuspended in 1ml RPMI+, thereafter a 50 μ l aliquot was diluted into counting fluid and the concentration determined using a haemocytometer.
- The cells were diluted with an appropriate volume of RPMI+ to a final concentration of 2×10^6 cells/ml for the experiment.

Method for *in vitro* toxicity study

The toxicity of D85 (the polymer conjugated with MTX), MTX as the comparison and the carrier polymer as control were tested on 96 well microtiter plates as follows:

1. The entire top row of each plate were used as untreated control wells.
2. First 6 columns were used to test the effect of the drugs on PHA (Murex, Biotec Ltd, Kent, England) stimulated lymphocytes.
3. The second set of 6 columns were used to test the drugs on resting lymphocytes.
4. A stock solution of 4.4 μ M was made up for both, MTX or D85 and serially diluted one in two until a concentration of 3.4 μ M. Thereafter twenty microlitres of each test compound at each concentration was added to three different replicate wells. Control wells received 20 μ l of growth medium only.
5. The culture plate was incubated for 3 days at 37 $^{\circ}$ C in an atmosphere of 5% CO₂ and 95% relative humidity.
6. Viable cells were enumerated using the MTT assay.
7. Percentage cell survival compared to the average of the untreated controls was calculated and these values then used to determine the IC₅₀ value, which is the inhibitory concentration where 50% of the cells survive.

IC₅₀ = the concentration (μ M) of the experimental compound inducing a 50% decrease in cell growth compared to the untreated controls.

IC₅₀ was determined using GraphPad Prism 4 statistical analysis programme that determines the best-fit curve for the data.

2.3. MTT staining principle

The reduction of the tetrazolium salt 3-[4, 5 dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT), a pale yellow substance, has several desirable properties for assaying cell survival and proliferation. The tetrazolium salt is water-soluble yet easily crosses the plasma membrane as well as the mitochondrial membranes by passive diffusion. The tetrazolium ring is rapidly reduced in active mitochondria by the mitochondrial dehydrogenase enzymes to form dark purple insoluble formazan crystals when incubated with live cells, but not by dead cells, erythrocytes or tissue culture medium. The amount of formazan generated is directly proportional to the number of viable, metabolically active cells and can be measured colorimetrically after solubilisation in a suitable organic solvent.

2.3A. Method

- Twenty microlitres of a filter sterilised MTT (Sigma Chemicals, St Louis, MO) solution (5 mg/ml PBS) was added to each well of the 96 well plate after incubation period.
- The plate was then incubated for a further 3½ - 4 h at 37°C in a CO₂ incubator.
- The plate was then centrifuged at 1000g/10min using an appropriate rotor.
- The supernatant was carefully removed without disturbing the cell pellet.
- The pellets were washed with 150 µl PBS and recentrifuged.
- The PBS (BD, Johannesburg, South Africa) was again carefully removed and the plate allowed to dry for at least 1 hour.
- One hundred microlitres DMSO was added and the plate was shaken gently for 1 hour on a plate shaker until the purple formazan had dissolved.
- Finally, the plate was read using a plate reader spectrophotometer (ELx800UV spectrophotometer, Biotek Instruments, Analytical and Diagnostic Products, Weltevreden Park, South Africa) at a wavelength of 570 nm (reference 630 nm).

2.4. Results

The experiments that were performed according to the methods described above were analysed to obtain the respective IC₅₀ values for each tested cell line in terms of both MTX and D85 drug therapy. These results are presented in Table 1. It summarises the cytotoxicity of MTX compared to D85 as measured by cell survival using the MTT assay for the different cell lines and expressed as the IC₅₀ with the values expressed as concentration of MTX (in µM) therefore the IC₅₀ values for the D85 are adjusted for the MTX content.

Table 1: IC₅₀ values of D85 and MTX, obtained after administration to various primary and neoplastic cells in culture.

IC ₅₀ Values (in µM MTX equivalents)		
Cell culture	MTX	D85
Stimulated lymphocytes	> 440	> 440
Resting lymphocytes	> 440	25.2
HeLa	29.7	3.2
COLO 320 DM	28.8	0.09
MCF-7	26.3	2.5

The *in vitro* results indicated that the selected cancer cell lines tested were more sensitive than normal primary cell cultures to both D85 and MTX. Furthermore, D85 was approximately ten times more cytotoxic against the three tested cancer cell lines than MTX. D85 showed the greatest cytotoxic activity against the COLO 320 DM, being over 300 times more potent than MTX to the same cells. D85 showed very selective toxicity against the cancer cell lines compared to the stimulated lymphocytes. MTX was less toxic to lymphocyte cultures, in both stimulated and resting states compared to D85. This would imply that there is a possibility that D85 would be cytotoxic to normal resting lymphocytes at therapeutic concentrations, which required further confirmation in an *in vivo* model of toxicity. This would add value to the *in vitro* results since it's a holistic test in a living organism and would confirm or reject the data observed above.

3. In vivo toxicity

3.1. Introduction

In vivo toxicology testing is an integral part of pre-clinical testing. Animals have been used as models to study the causes, pathogenesis, progression, and treatment of diseases or as sentinels for early detection of potential risk to humans. This gave rise to the field of investigative toxicology, wherein animals are used as first line surrogates to predict possible adverse health effects to humans arising from exposure to various chemicals or potential therapeutic agents. Toxicity testing in animals is conducted in various phases to identify possible adverse effects resulting from acute exposure and to evaluate dose-response relationships to lower chronic doses. Toxicity tests need to be designed to minimize variance, bias, and reduce the potential for false-positive and false-negative results.

In vivo toxicological studies should enhance the value of *in vitro* toxicological data, both in terms of understanding the results of the toxicity tests and in comparison to clinical data as part of the assessment of the potential risk and safety in humans.

The need for toxicology data and the assessment of exposure in individual toxicity studies should be based on a flexible approach and a process that provides sufficient quality data to identify potential risks and establish safety with confidence. However, the primary objective of toxicology studies is to determine whether the product is safe to use.

In this study, the *in vivo* toxicity of intravenously administered experimental compound (D85) was determined in BALB/c mice. The toxicity of MTX in nude mice was previously evaluated where 50mg MTX/kg administered i.v. was deemed to be safe in nude mice (Wosikowski *et al* 2003). This dosage is in line

with the Berlin-Frankfurt-Munster (BFM) protocol used in the treatment of ALL patients (Pees *et al.*, 1992). MTX has been in use for more than 40 years and numerous studies have been performed to establish the safety and efficacy of the free drug or combination therapy in humans and animals with cancer and more recently chronic inflammatory conditions. Over the last decade, MTX has also become one of the most frequently prescribed disease modifying anti-rheumatic drug (DMARD) for the treatment of severe refractory rheumatoid arthritis (Yazici *et al.*, 2005). According to Yazici *et al.*, 2005, MTX treatments are continued longer than any other DMARD, indicating long term effectiveness, including long-term absence of adverse effects. It has also been reported that the development of liver function abnormalities is relatively unusual (Yazici *et al.*, 2002), perhaps even more so since the introduction of folic acid supplementation (Yazici *et al.*, 2005).

3.2. Materials and methods

This study consisted of 3 groups of 10 mice per group.

Two different doses of D85 were administered and compared to a control group dosed with an equivalent volume of sterile saline. All administrations were 50µl-bolus i.v. (via the tail vein) once a week for 3 weeks according to Table 2.

Table 2: Experimental design of *in vivo* toxicity study in BALB/c mice.

Number of mice	Experimental compound	Dosage/week for 3weeks
10	Saline	0mg/kg
10	D85	5.84mg D85/kg (equivalent to 1mg MTX/kg)
10	D85	8.77mg D85/kg (equivalent to 1.5mg MTX/kg)

After initiation of the study by administration of the first dose, the mice were monitored for signs of adverse effects and behavioural changes on a regular basis. The weight of the mice was recorded every second day. At the end of the study trained personnel from the UPBRC drew heparinised blood samples

(500µl/mouse) via cardiac puncture for haematological analysis (haematocrit, haemoglobin, red blood cell, white blood cell), kidney function markers (urea, creatinine) and liver marker-enzyme levels (ALT, AST, and GGT) while the animals were under isofluorane anaesthesia. The animals were then terminated via anaesthetic overdose.

The blood analyses were carried out immediately after collection at the Clinical Pathology Laboratories, Faculty of Veterinary Sciences, University of Pretoria.

The mouse cadavers were sent for post mortem and histopathology analysis with Dr J. Steyl, Section Pathology, Department of Paraclinical Sciences, University of Pretoria.

3.3. Results

The animals receiving both the lower and higher D85 dosages survived for the duration of the study. After the blood samples were drawn at the end of the study for haematological and marker levels analysis, the gross anatomy was evaluated before the heart, kidneys and liver removed and weighed. Thereafter histopathology was performed on the relevant organs.

The haematology results showed no significant differences between the control and the treated groups for either dosage of D85.

ALT and AST serum levels showed no significant changes but a significant difference in the serum level of GGT was observed at the higher D85 dose when compared to the control (Figure. 4).

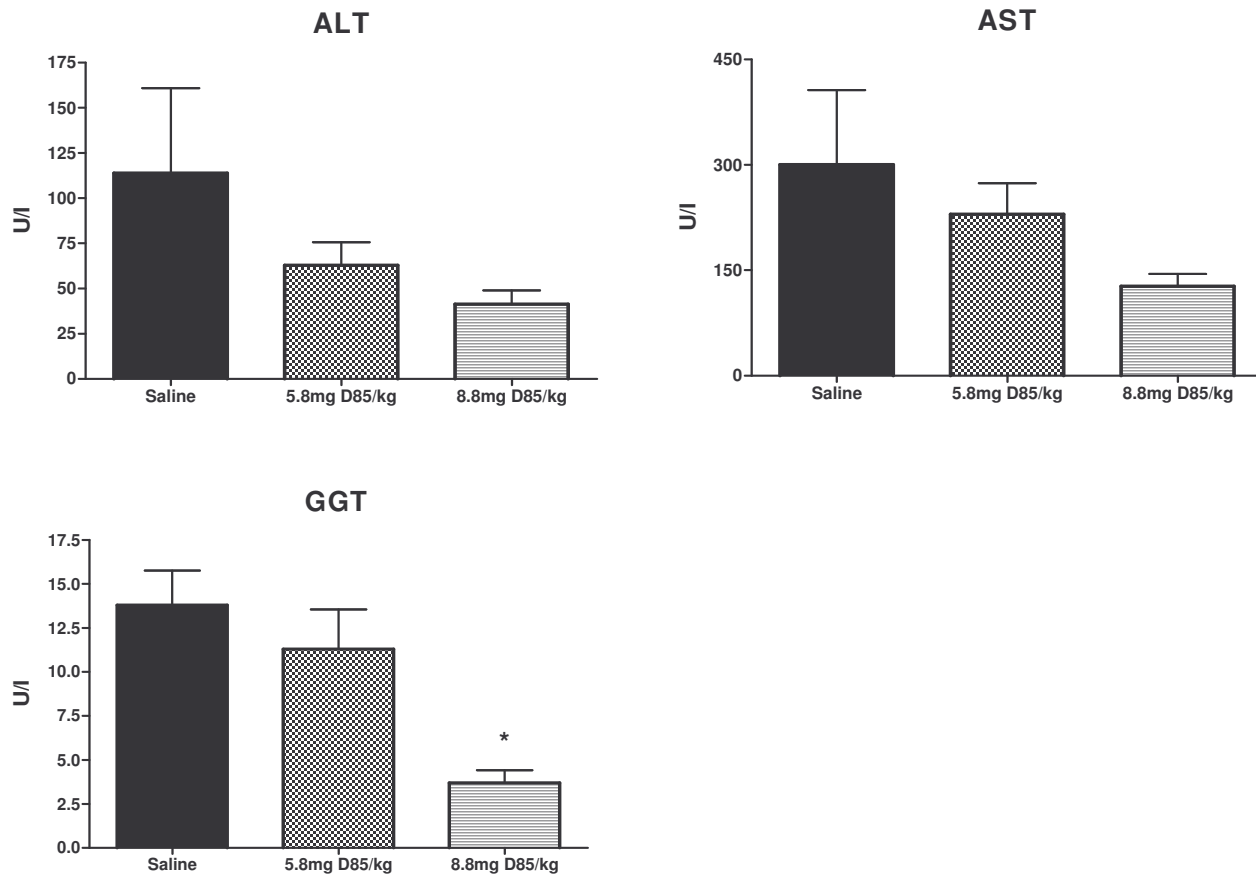


Figure 4: Plasma concentration levels of the toxicity marker liver enzymes ALT, AST, and GGT at termination of D85 toxicity study. A significant decrease in the GGT concentration of the 8.8mg D85/kg group compared to both the saline and 5.8mg D85/kg group ($P < 0.01$). No significant difference in the AST and ALT levels was observed between the groups despite a definite trend of decreased concentrations of all these markers.

Analysis of the organs weights (Heart, Liver, and Kidney) indicated that a significant decrease in the kidney weights existed between the control and the treated groups (Figure. 5, 6, 7).

Histopathology results concluded that there were no specific pathological lesions compatible with organ toxicity that could be associated with the intravenous administration of D85 between the groups.

The results obtained indicated two points of concern. Firstly the kidney weight of both D85 treated groups was lower compared to the control and secondly the GGT enzyme of the higher dose group was also significantly depressed. Kidney mass changes could be due to renal toxicity but the decrease in the GGT level is an anomaly since GGT levels increases in liver damage and not decreases as observed in the study. However, if renal toxicity occurred the most common effect would be a decreased glomerular filtration, which would have been seen in an increase in the kidney toxicity marker levels which showed a small but insignificant decrease for both urea and creatinine indicating a small improvement in kidney function or a change (decrease) in the normal protein metabolism in the animals. Considering all the toxicity results together, especially the preliminary *in vitro* cytotoxicity results and the expected ability of the polymer to increase the bioavailability of a drug into targeted tumour regions, the lower dose of 5.84mg D85/kg, i.e. equivalent to 1-mg MTX/kg was used in all further *in vivo* investigations.

Kidney weight

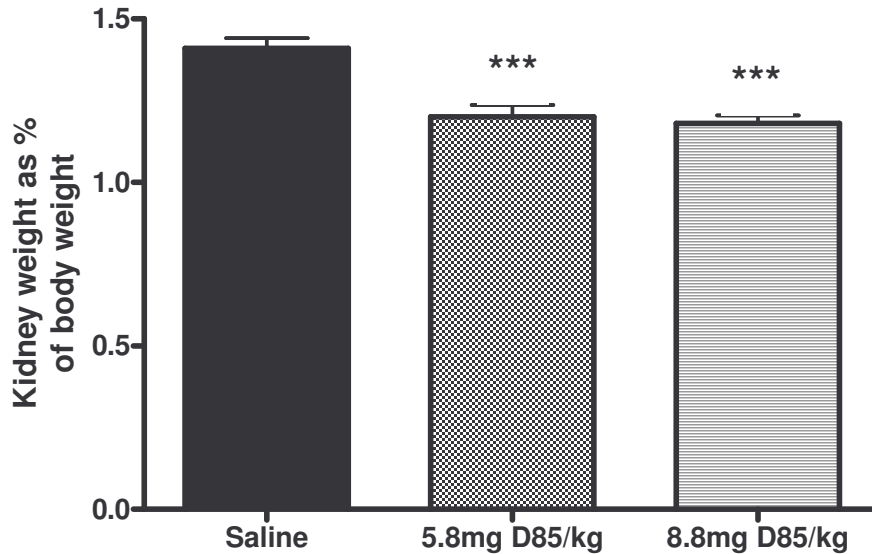


Figure 5: Kidney weight as a percentage of body weight of mice at termination of study. The D85 at 5.8mg/kg equivalent to 1mg MTX/kg and 8.8mg D85/kg equivalent to 1.5mg MTX/kg groups were significantly lower when compared to the saline treated group (***) = $P < 0.0001$).

Heart weight

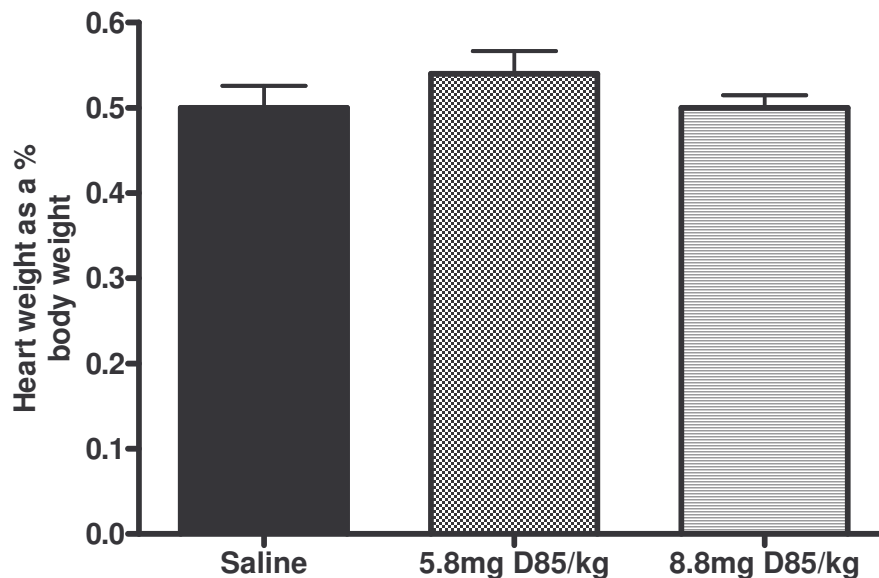


Figure 6: Heart weight as a percentage of body weight of mice at termination of study. There was no significant difference between the groups.

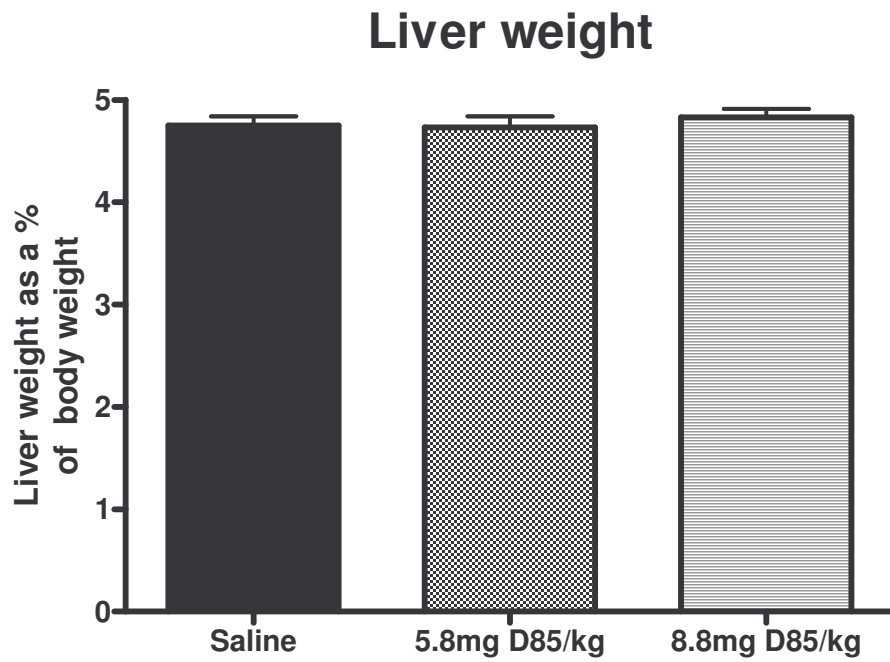


Figure 7: Liver weight as a percentage of body weight of mice at termination of study. There was no significant difference between the groups.

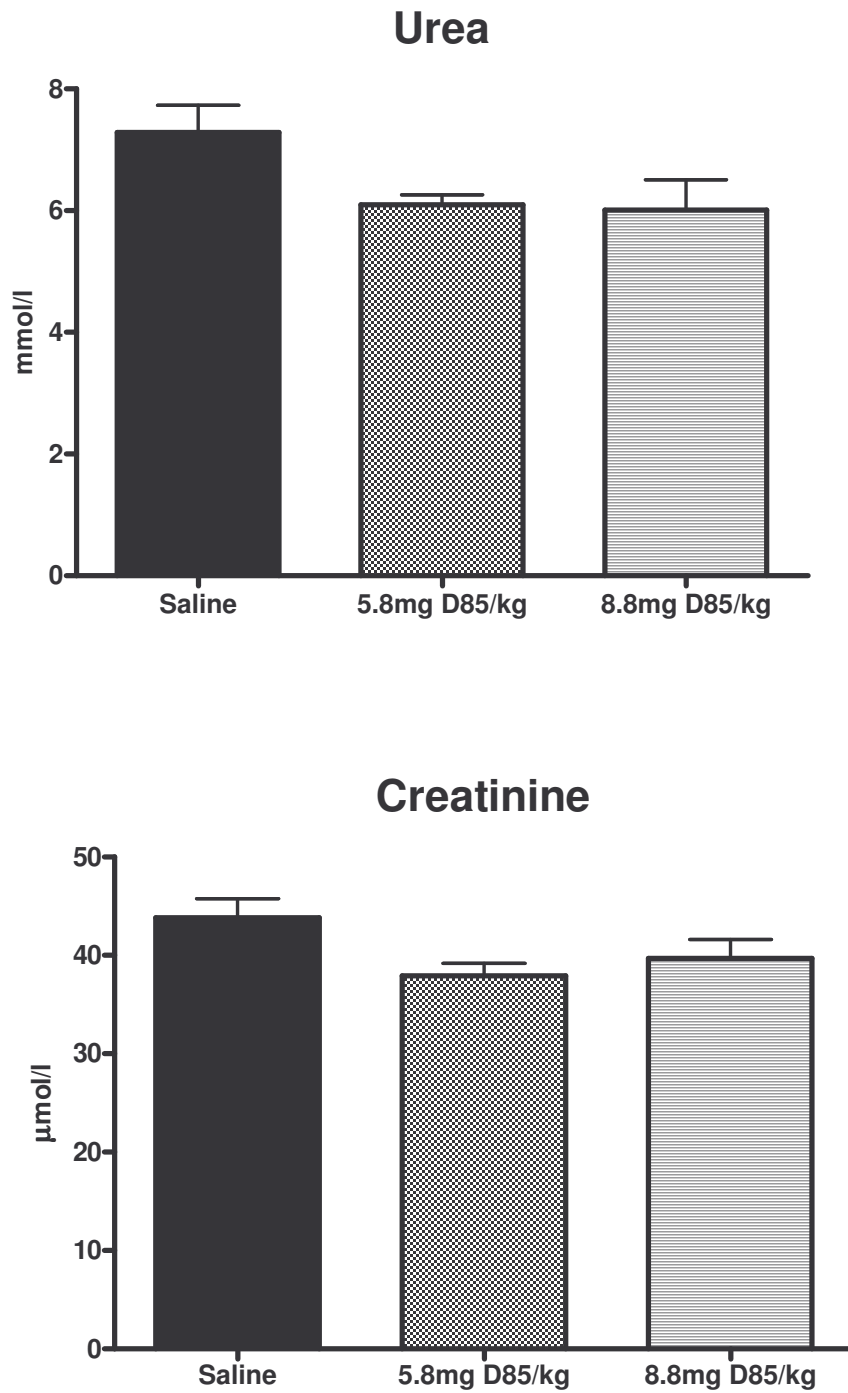


Figure 8: Plasma concentrations of the kidney function markers, urea and creatinine at study termination. There was no significant difference in the levels of urea and creatinine between the groups.

4. Tumour induction in nude mice

4.1. Introduction

Most anti-cancer research is performed *in vitro* in cell culture systems, but these systems have limitations when compared to *in vivo* conditions. Test tube experiments are often effective in early phases of research. However, to be able to mimic the same conditions as would be expected in the human, the drug requires a “complete organism” for evaluation. Drugs must at some point be tested on living systems in order to determine pharmacokinetics, enable dose ranging and to evaluate possible side effects in human bodies. Furthermore, animal research is crucial for understanding the causes of cancer, development and testing of new drugs, studying new forms of treatment, and identifying possible ways to prevent cancer.

Mouse models of cancer have consistently been used to qualify new anti-cancer drugs for development of human clinical trials (Troiani *et al.*, 2008). The most commonly used models are xenografts of human tumour cells grown subcutaneously in immunodeficient mice such as athymic (nude) or severe combined immune deficient (SCID) mice (Troiani *et al.*, 2008). Nude mice have the Nu gene knocked out, resulting in athymic (and hairless) animals, which are unable to generate T lymphocytes, whereas SCID mice lack functional T and B cells (Troiani *et al.*, 2008). Both mouse models show limited rejection of xenograft tissue, enabling human derived tumours to be grown *in vivo* in these mice (Troiani *et al.*, 2008). The model tumours are grown from cultured cancer cells that are injected subcutaneously, intraperitoneally, intravascularly or directly into an organ, such as the spleen, liver and pancreas (Jacob *et al.*, 2004). Subcutaneous models are frequently used because of the simplicity of the method of implanting tumour cells and the ease of monitoring the progress of solid tumour growth (Jacob *et al.*, 2004). The inoculation procedure is

uncomplicated and tumour growth is generally consistent and reproducible (Jacob *et al.*, 2004).

The success of an *in vivo* tumour model begins with the initial cell culture (Jacob *et al.*, 2004). According to Jacob *et al.*, before cancer cells are harvested from *in vitro* culture they must be verified to be free of any contaminants, such as yeast or mycoplasma. The medium must be supplemented with 10% heat inactivated foetal bovine serum, 1% glutamine and 100µg/ml penicillin and streptomycin to achieve viable cells for implantation. The cells are cultured at 37°C in a humidified incubator containing 5% CO₂. Cells should be 70%-80% confluent before being harvested. They should be actively growing to ensure that they possess the viability, vitality, and morphological properties crucial for implantation (Jacob *et al.*, 2004).

During this study, an initial *in vivo* pilot study was performed using 10 (male and female) *nu/nu* mice to determine:

1. The optimum number of cancer cells to inject subcutaneously to induce a palpable tumour within 6 weeks.
2. Which of two cancer cell lines (COLO 320 DM or MCF-7) would be the most effective to induce a palpable tumour within 6 weeks. These cell lines were selected due to MTX being used as potential therapy for these cancers (Wosikowski *et al.*, 2003; Sweetman, 2004).

4.2. Preparation of tumour cells

4.2.1. Materials and Methods

4.2.1.1. Specimen Required

- COLO 320 DM suspension in complete RPMI. Complete RPMI (RPMI+) consists of RPMI-1640 supplemented with 1% glutamine, penicillin and streptomycin cocktail (BioWhittaker™, CAMBREX BioScience, Walkersville, USA) of 100µg/ml each and 10% heat inactivated foetal calf serum.
- MCF-7 suspension in complete DMEM. Complete DMEM (DMEM+) consists of DMEM supplemented with 1% penicillin and streptomycin (BioWhittaker™, CAMBREX BioScience, Walkersville, USA) at 100µg/ml each and 5% heat inactivated foetal calf serum.

All procedures had to be carried out under strictly sterile conditions. Plastic tips and tubes for freezing cells had to be autoclaved and kept sterile. Only sterile, disposable plasticware was used. Bottles containing culture media had to be kept sterile (opened only in laminar flow cabinet) and stored at 4°C. The laminar flow cabinet had to be kept clean and resterilized on a regular basis by wiping with 70% alcohol.

4.2.1.2. Maintenance of COLO 320 DM culture

Preparation of cell culture medium (RPMI+)

This medium contains components such as essential amino acids, vitamins, inorganic salts, hormones and nutrients. (All these procedures were performed sterile):

- Fifty-five millilitres was removed from the 500 ml bottle RPMI-1640 medium (Sigma Aldrich, St. Louis, MO).
- Five millilitres of a Penicillin/streptomycin mixture BioWhittaker™, CAMBREX Bio Science, Walkersville, USA) was added.
- Thereafter 50 ml of heat inactivated foetal calf serum (HI FCS) (Delta Bioproducts, Johannesburg, South Africa) was added. Foetal calf serum

was added because it contains growth factors, hormones as well as proteins necessary for attaching to the culture flask (fibronectin and albumin). Foetal serum contains high levels of the required growth factors and hormones.

- Lastly, this was mixed gently and stored at 4°C until used.

Subculture procedure:

Viable cells in the above culture medium were kept in culture flasks at 37°C in an atmosphere of 5% carbon dioxide – 95% relative humidity. They multiplied 8-fold in 7 days, provided fresh medium was added after 3-4 days. Healthy cells were rounded and refractile. The subcultures were prepared by gently shaking the culture flask and dividing the supernatant into new culture flasks and re-feeding both flasks. The total volume RPMI+ in a 75cm² flask was approximately 15ml. The lids of the flasks were not tightened completely since the inflow of CO₂ (in the incubator) regulates the pH of the medium in the flask.

4.2.1.2A. Harvesting of cells

After incubation, the flasks were examined under an inverted microscope to check for satisfactory growth and an aliquot was examined under high magnification after staining to confirm that the culture is not contaminated.

The floating COLO 320 DM cells were poured into 15ml tapered centrifuge tubes and spun down for 5 min/800g.

The supernatant was discarded and the pellet re-suspended in 1ml RPMI+, the cell concentration determined by manually counting a stained aliquot using a haemocytometer and dilutions made to achieve the required cell concentrations. Two full 75cm² flasks were sufficient to make 200µl of each of the five concentrations tested *in vivo*.

The following cell concentrations were prepared: 1x10⁶, 5x10⁶, 1x10⁷, 5x10⁷ and 1x10⁸/ml. These different cell concentrations were injected subcutaneously into the flank of different nude mice to determine which cell concentrations would effectively induce a solid palpable tumour within 6 weeks.

4.2.1.3. Maintenance of MCF-7 cultures

Preparation of cell culture medium (DMEM+)

This medium contains added components such as essential amino acids, vitamins, inorganic salts, hormones and nutrients. (All these procedures were performed sterile.):

- Thirty millilitres was removed from a 500ml bottle Dulbecco's Modified Eagle's Medium (Sigma Aldrich, St. Louis, MO).
- Five millilitres of a Penicillin/streptomycin mixture (BioWhittaker™, CAMBREX Bio Science, Walkersville, USA) was added.
- Thereafter 25ml of heat inactivated foetal calf serum (to a final concentration of 5% HI FCS) (Delta Bioproducts, Johannesburg, South Africa) was added. Foetal calf serum is added because it contains growth factors and hormones as well as proteins necessary for attaching to the culture flask (fibronectin and albumin). Foetal serum contains high levels of growth factors and hormones.
- Lastly, this was mixed gently and stored at 4°C until used.

Subculture procedure:

Viable MCF-7 cells were kept in culture flasks at 37°C in an atmosphere of 5% carbon dioxide – 95% relative humidity. They have epithelial-like morphology and they grow as a monolayer on the bottom of the flask and form domes in confluent cultures. Adherent cells produce a layer of adhesion proteins on the flask surface. The total volume DMEM+ in a 75cm² flask is approximately 15ml. Fresh medium was renewed 2-3 times per week. The lids of the flasks were not tightened completely since the inflow of CO₂ (in the incubator) regulates the pH of the medium in the flask.

4.2.1.3A. Harvesting of cells

After incubation, the cells were examined using an invert microscope to confirm satisfactory growth.

The medium was decanted, \pm 5ml Trypsin/Versene solution (0.25% Trypsin + 0.05% EDTA solution in Ca^{2+} and Mg^{2+} free Dulbecco Buffer) (Adcock, Johannesburg, South Africa) was added, rinsed and removed (to remove all traces of serum which contains trypsin inhibitor). Just enough trypsin/versene solution was then added to cover the cells in the flask and incubated at 37°C until the cells detached (10-20 minutes). Fresh culture medium (\pm 15ml) was added, aspirated into 15ml tubes and spun down for 5 min/800g. The calf serum in the culture medium contained proteins that prevent cell damage caused by the trypsin.

The supernatant was decanted and the pellet was re-suspended in 1ml DMEM+. Cell concentration was determined manually and the suspension diluted to the various dilutions required for injection. Two full 75cm^2 flasks were sufficient to make 200 μl of each of the five concentrations tested *in vivo*.

The following cell concentrations were made: 1×10^6 , 5×10^6 , 1×10^7 , 5×10^7 and $1 \times 10^8/\text{ml}$. These cell concentrations were used to determine the cell concentration that would most effectively induce a solid palpable tumour in nude mice within 6 weeks.

Trypsin/Versene solution is described as follows:

A) Trypsin: a proteolytic enzyme that disrupts the adhesion protein/cell binding. Serum proteins, Ca^{2+} , Mg^{2+} must be removed from the medium to promote enzyme access to the lateral and basal surfaces of the cells as these ions promote cell/cell contact.

B) EDTA: a complexing agent that removes Ca^{2+} and Mg^{2+} ions from solution resulting in disruption of cell adhesion.

4.3. Preparation of the cancer cell injection

Tumour implantation and/or metastasis is proportional to the number of viable cells injected (Jacob *et al.*, 2004). However, an increase of viable cell number does not automatically lead to more metastasis or larger local tumour volume (Jacob *et al.*, 2004). To establish tumours *in vivo*, it is more important to give a consistent, homogenous mixture of viable cells, rather than an injection consisting of clumped, unevenly mixed and/or unviable cells (Jacob *et al.*, 2004). For the preparation of the tumour cells for injection, it is important to draw the cells into a syringe without a needle. Using a needle causes a strong, negative pressure to form, which can cause considerable cell membrane damage, lysis and even cell death. The most practical injection method is to use a 1-ml syringe with a 27-gauge needle that can penetrate the skin efficiently.

It is an absolute requirement that the cells be resuspended frequently, if not continuously, in the syringe prior to the injection. Cells suspended in medium will quickly sediment and adhere to each other, forming clumps of cells that hinder distribution therefore the metastatic potential (Jacob *et al.*, 2004).

Ten nude mice were sequentially anaesthetized using isofluorane, and the inoculation area cleaned and sterilised with ethanol. Each mouse was subcutaneously injected with a 75µl suspension containing either of 1×10^6 , 5×10^6 , 1×10^7 , 5×10^7 or 1×10^8 /ml of COLO 320 DM or MCF-7 cells. The cells were injected into the subcutaneous space of the left flank region.

The mice were returned to their cages immediately after introduction of the cancer cell suspensions and monitored for any abnormal behaviour for the following few hours. The tumour growth was monitored twice weekly. Any palpable tumours were measured using digital callipers, and the tumour volume in mm³ was calculated by the following formula (Tang *et al.*, 2004):

$$\text{Tumour Volume} = (\text{width})^2 \times \text{length}/2$$

Mice were terminated according to standard procedures (anaesthetic overdose) used at UPBRC six weeks after inoculation. Any solid tumours that had formed were carefully dissected out and measured and weighed to determine its size. The mouse cadavers were sent for autopsy and histopathology to ascertain whether any metastasis had occurred.

4.4. Results

Both the cell types used, namely COLO 320 DM (colon cancer) and MCF-7 (breast cancer) induced solid subcutaneous tumours. Palpable growth could be detected one week after administering the cell suspension and the rate of tumour growth depended on the cancer cell concentration injected. The growth (measured as tumour size) of the COLO 320 DM at a concentration of 5×10^7 /ml (Figure. 9) and MCF-7 at a concentration of 1×10^8 /ml (Figure. 10) showed the most rapid growth. After 6 weeks, the mice were euthanased and the tumours dissected out and weighed. All the excised tumours weighed more than 150mg (Figure. 11). This implied that it was possible to grow tumours at any of the cell concentrations used during this phase of the study. Histopathological reports on all the animals indicated no significant anatomical or histopathological changes in any of the major organs. No macroscopically visible tumour metastasis could be recognized in any organs or draining lymph nodes of the left flank. This verified previous literature that subcutaneous tumours rarely develop metastases (Jacob *et al.*, 2004).

The age of mice played an important role regarding the success of tumour induction. It appeared that older mice had taken longer to form a solid tumour or in some instances did not form a tumour at all. This was however not a parameter that was tested but just an observation that confirmed reports suggesting that animals should be as young as possible, because they have lower natural killer (NK) cell activity and there is an inverse correlation between NK cell activity and the formation of metastases (Jacob *et al.*, 2004). In addition,

age dependent haemodynamic parameters such as vessel diameter and endothelium thickness can affect tumourigenesis and the likelihood of metastasis (Jacob *et al.*, 2004).

Tumour size (CoLo)

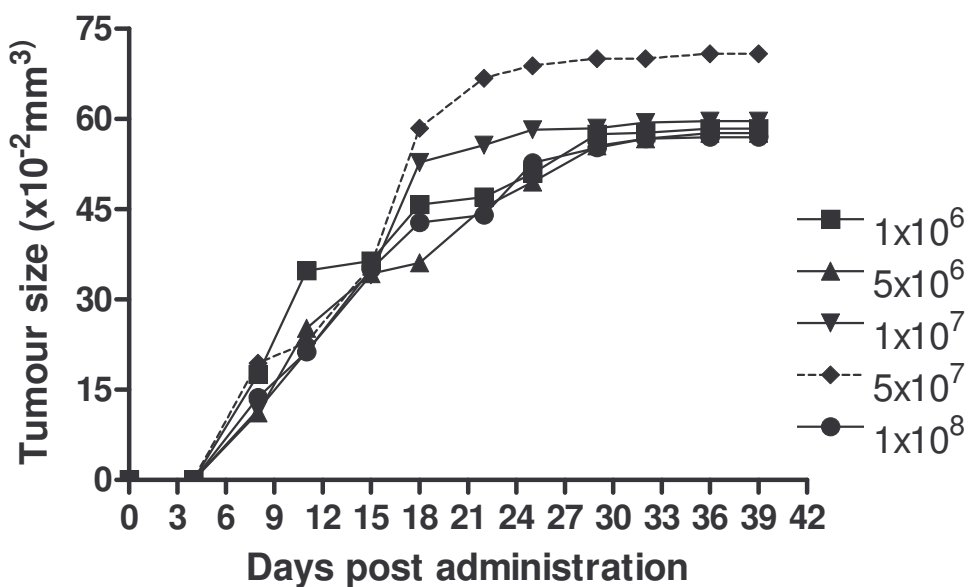


Figure 9: A graph indicating tumour sizes induced by injecting colon cancer cells subcutaneously in nude mice at different cell concentrations over a period of time. One animal per concentration was used.

Tumour size (MCF-7)

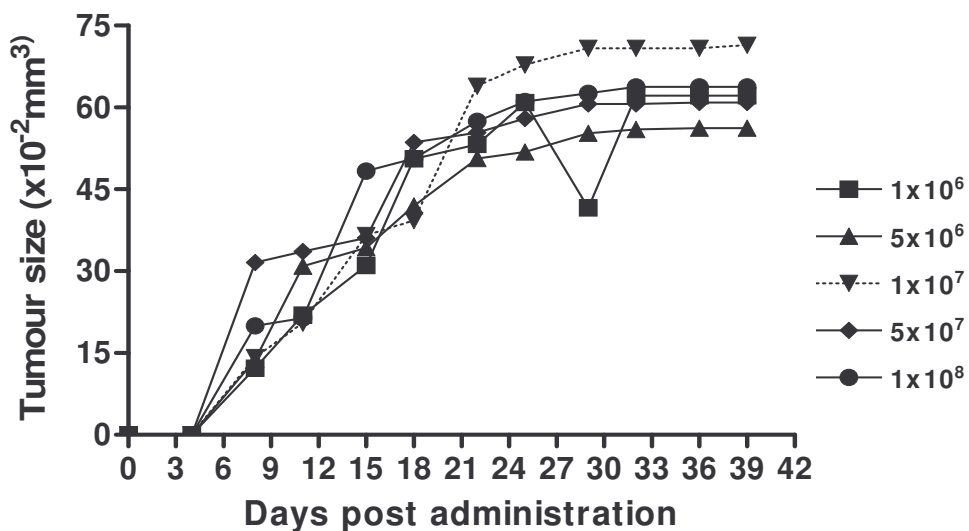


Figure 10: A graph indicating MCF-7 tumour sizes in nude mice after inoculating with different concentrations of cultured MCF-7 cells over a period of time. One animal per concentration was used.

Tumour Weight

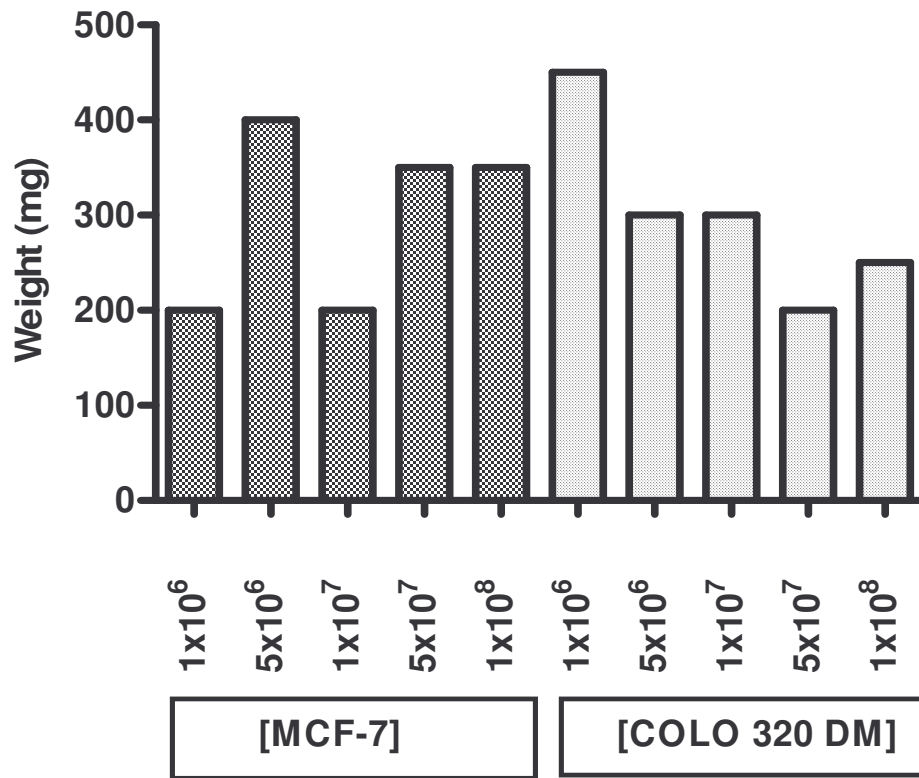


Figure 11: Histogram indicating the relative tumour weight (in mg) of MCF-7 and COLO 320 DM cell induced tumours in nude mice at various injected cell concentrations six weeks after introduction by subcutaneous injection. One animal per concentration was used.

5. Study of anti cancer efficacy in nude mice

5.1. Introduction

Biologically and clinically relevant animal studies are essential in the investigation of disease progression and therapeutic protocols using appropriate models (Mognetti *et al.*, 2006). These models enable the development and testing of new approaches to disease prevention and treatment. Furthermore, they can provide an understanding of the *in vivo* biology and genetic events involved in tumour initiation, promotion, progression and metastasis. However, no animal model is perfectly applicable to human cancers but it is generally accepted that use of an animal model is a requirement to understanding how neoplasms develop and assessing the efficacy of new therapeutic approaches. Experimental animal models that accurately represent the cellular and molecular changes associated with the initiation and progression of human cancer are thus of crucial importance, though some are better suited for particular applications. Therefore, their optimization and application in future research may improve the early detection and treatment of cancer (Mognetti *et al.*, 2006).

Subcutaneous models are regularly used because of the simple inoculation procedure, no requirement for a general anaesthetic, the consistency and reproducibility of tumour growth, and the accessibility of the tumour for measurement during treatment (Jacob *et al.*, 2004).

In this study the xenograft model of a human colon cancer (COLO 320 DM) was used to test the efficacy of both MTX and D85 based on the *in vitro* sensitivity of this cancer cell line to the D85 as demonstrated in the *in vitro* phase of this study and the ability to initiate *in vivo* growth of tumours from this cell line in the nude mouse model.

5.2. Materials and methods

The cancer cell xenograft model of human colon cancer in nude mice as developed and described in the previous chapter was used to test the efficacy of the experimental compound D85 against an induced subcutaneous colon cancer tumour at a dose equivalent to 1mg MTX/kg, calculated according to the MTX content of the polymer, and compared the efficacy to two different MTX concentrations (1mg/kg and 50mg/kg).

COLO 320 DM cells were grown to approximately 80% confluence as described in the previous chapter, checked for any contamination by yeasts, fungi or mycoplasma before being harvested for the implantation into nude mice used in this study.

Forty nude mice of both genders were used and were randomly divided into 4 weight matched groups of 10 mice each.

The nude mice were anaesthetized using isofluorane, and the inoculation area cleaned and sterilised with ethanol swabs. Mice were injected with 75 μ l of a suspension of COLO 320 DM cells containing a total of 5×10^7 /ml cells in RPMI supplemented with 10% heat inactivated FCS via a 27-gauge needle into the subcutaneous space of the left flank region. This concentration of cells was used as it showed the best results during the pilot study.

Tumour growth was monitored by measuring any palpable nodules at the injection site twice weekly using a digital engineering calliper. This was measured at the same time that the mice were routinely weighed. Skin thickness was negligible since it remained relatively constant in all the mice and did not influence the results.

Tumours were all induced on the same day but the treatment of the animals was started on the day that the measured tumour size reached 1mm^3 as calculated from the formula

$$\text{Tumour Volume} = \frac{1}{2}(\text{width})^2 \times \text{length}$$

Treatment was continued on a once a week basis according to the following protocol:

- 1) Group 1 was injected i.v. with $50\mu\text{l}$ saline (control group).
- 2) Group 2 was injected i.v. with $50\mu\text{l}$ MTX dissolved in saline at a concentration of 25mg MTX/ml to deliver 50mg MTX/kg .
- 3) Group 3 was injected i.v. with $50\mu\text{l}$ MTX dissolved in saline at a concentration of 0.5mg MTX/ml to deliver 1mg MTX/kg .
- 4) Group 4 was injected i.v. with $50\mu\text{l}$ of D85 dissolved in saline at a D85 concentration equivalent to 0.5mg MTX/ml to deliver 1mg MTX/kg . (5.84mg of D85/kg is equivalent to 1mg MTX/kg).

Treatment was continued for 3 weeks and the mice were then terminated at the end of the fourth week after treatment was initiated.

The mice were euthanased by isofluorane overdose, weighed and the tumours were dissected out and weighed and checked microscopically to assess the tumour characteristics.

The mice cadavers were sent for histopathology to determine whether any metastasis had occurred and whether there were any signs of macroscopic anatomical changes.

5.3. Results

The MTX dose used for the treatment of human breast cancer is 50mg/kg body mass. The therapeutic effect of the classical drug MTX (1mg/kg and 50mg/kg) and D85, dose which is the polymeric conjugate of MTX, were investigated *in vivo* against a colon cancer xenograft growing subcutaneously in nude mice. Not all the mice developed palpable solid tumours within the study time, which resulted in different numbers of animals in each group receiving treatment. The control group that was treated with saline had 5 animals while both the MTX treated groups had 6 animals each with the D85 treated group having 9 animals. As expected the saline treated control group showed a continuous fairly rapid growth in tumour size during the four weeks of treatment which was started once the tumour size was determined to be 1mm³. The trend of increase in tumour size was seen in all the treatment groups, but the rate of increase in tumour size was slower in all the treatment groups than in the saline treated control group. The tumour size increase is summarized in Figure 12, where it is clearly seen that all the treatments applied induced anti-tumour activity *in vivo* with an obvious dose dependent effect for free MTX. The greatest anti-tumour effect was observed in the MTX (50mg/kg) group, which is the recommended dose for MTX in the treatment of ALL (Pees *et al.*, 1992). When comparing the lower doses administered as either the free MTX or as a polymer conjugated MTX, no significant superiority of D85 was observed. It should be remembered that the polymer was expected to have an increased accumulation in the tumour due to the enhanced permeability and retention effect that should have shown a greater anti tumour effect than the free MTX. These results would suggest that cytotoxic concentrations of MTX for the COLO 320 DM type tumours are only reached in the tumour tissue at the higher dosage of free MTX, which confirms that MTX at high concentrations is an effective anti-tumour agent against COLO 320 DM tumours in the nude mouse model as used in this study.

The expected and important difference between MTX and D85 with respect to pharmacodynamic behaviour, tumour uptake mechanism and accumulation of D85 within tumour tissue cells resulting from the enhanced microvascular permeability could unfortunately not be demonstrated in these experiments.

Effect of various treatments on tumour growth

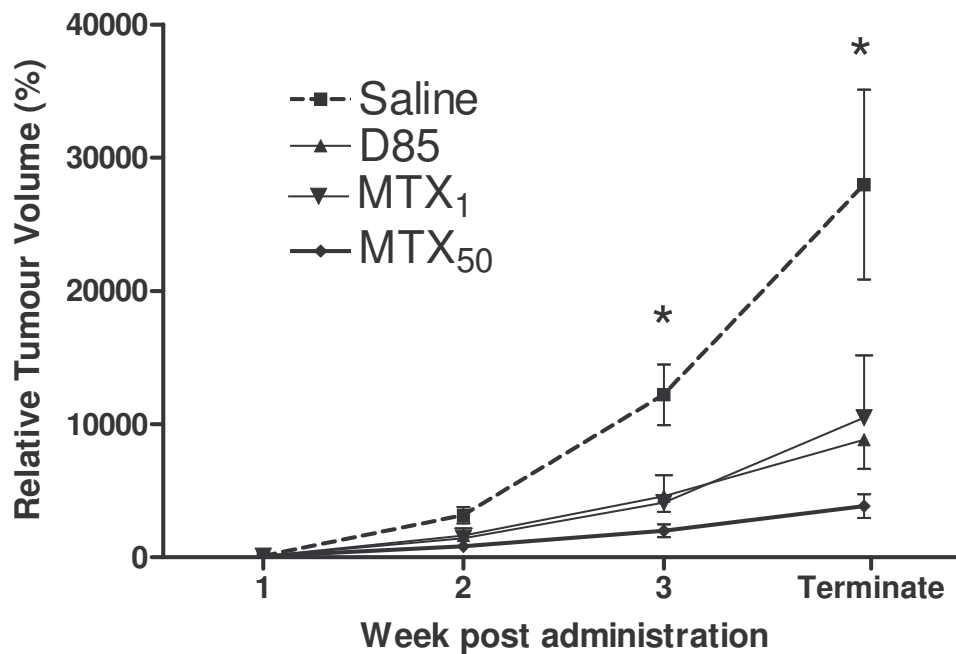


Figure 12: The effect of MTX (at two different doses) and D85 treatment on COLO 320 DM xenografts. Control mice were treated with 0.9% NaCl solution (n=5), D85 (equivalent to 1mg/kg MTX, n=9), MTX₁ (1mg/kg, n=6) or MTX₅₀ (50mg/kg, n=6) as indicated in the figure. Data represented as mean ± SEM. *Indicates a significant difference between the saline and MTX₅₀ at week 3 and at termination (P<0.0001). A number of animals in each group did not grow tumours and data presented are of those that did develop palpable tumours.

6. In vivo inflammation model

6.1. Introduction

Chronic inflammation is an important pathological problem and various therapeutic procedures aimed at its treatment have been proposed (Bertani *et al.*, 1999). Inflammation is part of the body's natural defence mechanism involving both chemical and cellular processes that are aimed at potential invasive organisms, foreign material and for normal turnover of dead or senescent tissue or cells. In most inflammatory processes there are distinct phases that involve various cytokines that initially play a role in activation of the process and later a shift in the cytokines and other mediators to inhibit the process when the threat to the body has been eliminated. Two classical symptoms of inflammation are swelling (oedema) and redness, which is accompanied by warmth (pyretic effect) and pain. Oedema is one of the major initial responses during inflammation and although it is a defensive reaction, it sometimes represents the main pathology (Bertani *et al.*, 1999). Among the several models of acute inflammation, carrageenan-induced inflammation has been well established as a valid model due to the cellular and molecular mechanism of this carrageenan-induced inflammation being well characterised (Bilici *et al.*, 2002) and is therefore a widely used model to investigate the pathophysiology of an acute localised inflammation (Cicala *et al.*, 2007). It is a classical laboratory model usually used to screen for anti-inflammatory activity of various potential therapeutic compounds (Bertani *et al.*, 1999) and has been in use since 1962 (Winter *et al.*, 1962). Carrageenans are a heterogeneous mixture of high-molecular-weight linear sulphonated polysaccharides extracted from the cell walls of certain algae of the Rhodophyta phylum. The main source of carrageenan is *Chondrus crispus*. It is categorised into kappa-, lambda-, or iota-carrageenan, depending on the degree of sulphonation and the nature of the intra-galactan bonding. Carrageenans are widely used in the food industry as a thickening, gelling and protein-suspending agent.

Oedema develops when carrageenan is injected sub-plantar in the paw of the rat and is the result of a biphasic event (Vinegar *et al.*, 1987). Initially the oedema is attributed to the release of histamine and serotonin, whereas the second phase is attributed to the release of prostaglandins (Di Rosa & Willoughby, 1971).

In the rat model, acute inflammation is induced by injecting a small volume of a solution of carrageenan (the mucopolysaccharide from sea moss) into the plantar region of one of the hind paws (Bertani *et al.*, 1999). Carrageenan-induced rat paw oedema remains localized in the injection site, thus advantageous for local acute inflammation experiments (Cicala *et al.*, 2007).

In this study, a carrageenan-induced paw oedema model of acute inflammation was used according to previously described methods (Recio *et al.*, 2000; Smit *et al.*, 2000; Petersson *et al.*, 2001) to determine the difference in the effects of D85 and MTX on acute inflammation.

MTX was introduced for the treatment of inflammatory diseases, with very little understanding of its mechanism of action (Montesinos *et al.*, 2000). Although the original rationale for the use of MTX in the treatment of rheumatoid arthritis was "immunosuppression", the molecular mechanism by which MTX suppresses inflammation is not well understood. Proposed mechanisms included a decrease in neutrophil (but not macrophage) leukotriene synthesis and inhibition of transmethylation reactions by inhibiting the formation of S-adenosyl-methionine (Cronstein *et al.*, 1993). Other studies demonstrated that adenosine, acting at one or more of its receptors, mediate the anti-inflammatory effects of MTX in animal models of both acute and chronic inflammation (Montesinos *et al.*, 2006). However, this biochemical mechanism by which MTX promotes adenosine release is not fully established. Montesinos *et al.* suggests that MTX is taken up by cells and polyglutamated; the polyglutamates of MTX remaining metabolically active (Montesinos *et al.*, 2000). These metabolites, in addition to inhibiting folate metabolism, directly inhibit 5-aminoimidazole-4-carboxamide ribonucleotide

transformylase, resulting in an intracellular accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide, which is an intermediate metabolite in the *de novo* pathway of purine synthesis, and has been associated with increases in extracellular adenosine (Montesinos *et al.*, 2006). Adenosine, whether released from injured cells or tissues or from exogenous sources, regulates inflammation via interaction with one or more of the 4 known receptors for adenosine (A_1 , A_{2A} , A_{2B} , and A_3), as confirmed by many *in vitro* and *in vivo* pharmacologic studies (Montesinos *et al.*, 2003). The demonstration that adenosine mediates the anti-inflammatory effects of MTX in *in vivo* models of acute inflammation rests upon reversal of the anti-inflammatory effects of MTX, either by enzymatic hydrolysis of adenosine by adenosine deaminase or by administration of adenosine receptor antagonists that reverse the anti-inflammatory effects of MTX treatment (Montesinos *et al.*, 2003). Investigators in Montesinos group have previously demonstrated that adenosine, acting at A_2 receptors, mediates the anti-inflammatory effects of MTX in a murine model of acute inflammation (Montesinos *et al.*, 2003). In contrast, a review by Montesino *et al.* mentioned that adenosine A_1 receptor agonists have been reported to be the most potent anti-inflammatory adenosine receptor agonists in other *in vivo* models of acute inflammation (Montesinos *et al.*, 2000). Therefore, no matter which receptor is responsible for the anti-inflammatory effect, there is evidence that MTX can be used to treat acute inflammation.

6.2. Materials and methods

In this study, a 50 μ l injection of a 2% λ -carrageenan solution (Sigma–Aldrich, Inc. St. Louis, MO, USA) in saline was injected subplantar into the right hind paw of anaesthetized Sprague-Dawley rats. The resulting paw oedema was measured hourly, from the time of injection, for 7 hours using a water displacement plethysmometer (LE 7500 Digital Plethysmometer, Panlab, Barcelona, Spain) that measures the total paw volume (Table 3).

Thirty female Sprague Dawley (SD) rats of 12 weeks old and weighing between 200g-300g were used. The rats were randomly allocated into one of the following three groups:

1. Untreated control group (250µl saline administered i.v. (tail vein)).
2. Experimental group 2 (250µl MTX administered i.v. (tail vein) at 20mg/kg).
3. Experimental group 3 (250µl D85 administered i.v. (tail vein) at 5.84mg D85/kg that is a dose equivalent to 1mg MTX/kg).

6.2.1. Experimental design

The control group received 250µl saline intravenously into the lateral tail vein. The two experimental groups (groups 2-3) received 250µl of either MTX (at a concentration of 20mg MTX/ml) or D85 (equivalent to 1mg MTX/ml) to deliver 20mg MTX/kg or D85 equivalent to 1mg MTX/kg respectively. All the solutions were filter sterilized through 0.22µm filters before use.

Half an hour after the intravenous drug administration, the following procedures were followed:

- The right hind paw of each rat was marked at a point above the expected swelling region with a permanent marker. The paw was accurately submerged in a water displacement plethysmometer to this mark for each measurement of the paw volume at each of the time intervals. This was done before the inflammatory oedema was initiated to obtain a starting value.
- Fifty microlitres of a 2% λ-carrageenan solution in saline was then injected subplantar into the right hind paw to induce inflammation. The inflammation reaction started almost immediately after the carrageenan injection.
- Extent of paw oedema was measured in each rat at hourly intervals for 7 hours from the time of injection by placing the paw up to the previously made mark into the plethysmometer solution.
- At the end of the study, the rats were euthanased by isofluorane overdose.

Table 3: Summary of experimental procedures for the determination of the degree of rat paw oedema in carrageenan induced inflammation and the effect of different treatments on the oedema.

	Time (0)	Time (0.5hrs)	Time (1.5-7.5hrs)	Time (7.5hrs)
Control Group	Intravenous saline	Carrageenan induced inflammation	Hourly paw volume measurement for seven hours	Termination
Experimental Group 1	Intravenous MTX	Carrageenan induced inflammation	Hourly paw volume measurement for seven hours	Termination
Experimental Group 2	Intravenous D85	Carrageenan induced inflammation	Hourly paw volume measurement for seven hours	Termination

6.3. Results

The drug doses selected for this experiment were for the following reasons: Dalmarco *et al.*, (2002) tested several doses of MTX to determine the most effective anti-inflammatory dose. They concluded that an anti-inflammatory effect of MTX would only be seen if the dose is high enough. Therefore, 20mg MTX/kg was selected since an inhibitory effect by Dalmarco *et al.*, (2002) was observed in a carrageenan model only after administering 20mg/kg MTX. The D85 experimental group was dosed with 5.84mg D85/kg, as this was the maximum tested dose of D85 during the acute toxicity study in BALB/c mice and due to the expected drug delivery profile of polymers, its biodegradability, biocompatibility and its ability to release the active compound in the target tissue as found by other investigators (Wunder *et al.*, 2003) justified the use of this dose for further investigations of D85.

While preparing animals for experimental procedures, three rats from the control group died due to an overdose of anaesthetic. These deaths were not related to the experimental drug.

The injection of carrageenan into the hind paw produced an inflammatory response for all animals in all three experimental groups and was quantified by measuring the changes in footpad volume using a water displacement plethysmometer on an hourly basis for 7 hours. The saline control, MTX and D85 treated groups all showed significant increases in paw volume after injection of the carrageenan which lasted for the seven hour period ($P < 0.01$) of the study indicating oedema due to an acute inflammatory response (Figure. 13). A general pattern seen in all three groups was that there was a rapid increase in the footpad volume for the first two hours followed by a slight recovery for the next 2 hours after which there was a slow increase in volume again which continued until the end of the study at 7.5 hours. The general curve for all the groups followed a different swelling effect compared to other studies (Tsukada *et al.*, 1974; Kim *et al.*, 2008), where once the carrageenan had been injected a steady increase in oedema was observed over the time of the observation. The oedema observed in this study was unique in that a decrease in oedema was observed between 2 and five hours after which the oedema increased again. This effect was observed in all the treatment groups of rats.

The footpad oedema of the MTX treated group was significantly lower than that of both the control and D85 treated groups ($P < 0.05$) despite following a similar swelling profile. This finding confirmed previous research by Montesinos *et al* that MTX can be used as a treatment acute inflammation.

An unexpected result was that the footpad oedema of the D85 treated group was significantly greater than both the saline control and MTX-treated groups ($P < 0.05$) at all measurement times up to the end of the study where the D85 group appeared to show a further increase in swelling whereas the other two groups had reached a plateau by 6 hours. This would imply that the D85 was perhaps leaking through the vasculature in the region of the inflammation in the same way that enhanced permeation and retention takes place in the cancer tissue and that the polymer caused an increase in the inflammatory effect due to

an increased activation of infiltrating leucocytes or the release of higher concentrations of pro-inflammatory cytokines. This effect would be an advantage in the cancer tissue where localised inflammation would probably lead to greater effect of the immune system in the region of the tumour but is an undesirable adverse effect in areas of inflammatory oedema.

The D85 treated group showed a mean maximum increases in footpad volume of approximately 17.6% two hours after injection of the carrageenan, whereas the MTX group showed a significantly slower and overall smaller mean increase in footpad volume, reaching a maximum of only approximately 7.9% seven hours after the carrageenan injection. This was 1.12% higher than the extent of swelling seen at 2 hours. The saline control already showed a mean increase in footpad swelling of 13.7% at two hours.

The evidence presented here indicates that MTX at a I.V. dose of 20mg/kg reduces acute inflammation significantly compared to the saline control. However, D85 at 5.84mg/kg, which is a dose equivalent to 1mg MTX/kg, exacerbated the swelling at both the 2 hour high and again from 4 to 7 hours after carrageenan injection indicating that the D85 was in fact pro-inflammatory from the outset.

Effect of test drugs on carrageenan induced paw oedema

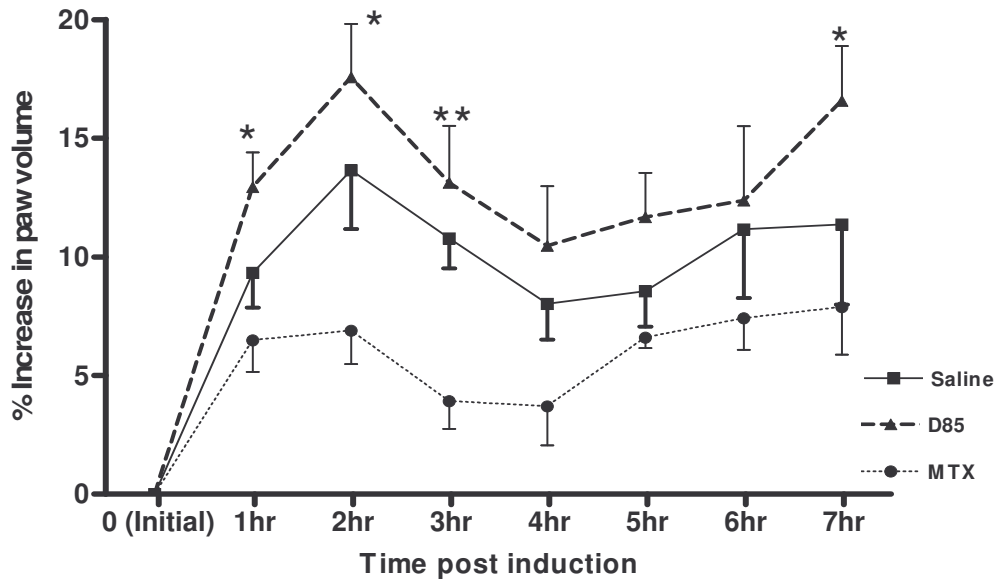


Figure 13: The anti-inflammatory properties of a MTX conjugated polymer (D85) compared to MTX and saline in female Sprague Dawley rats. Rats were treated with 0.9% NaCl solution (saline controls), D85 (equivalent to 1mg/kg MTX), and MTX (20mg/kg) as indicated in the figure. Results are the mean of seven to ten rats. Data is expressed as means \pm SEM. * indicates a significant difference between the D85 and MTX at 1hr, 2hr, 3hr and 7hrs after induction (* $P < 0.05$ and ** $P < 0.005$, One way ANOVA). There were no significant differences between Saline and MTX or between Saline and D85.

7. Pharmacokinetics of methotrexate compared to D85

7.1. Introduction

MTX is an anticancer drug widely used to treat a variety of tumour types. However, potentially toxic side effects in patients receiving high-dose therapy and those with MTX elimination dysfunction continue to restrict its therapeutic use as there are no convenient, quick, sensitive methods to determine the plasma concentrations. Monitoring of plasma MTX levels for efficacy studies, dosing schedule, different individual responses related to genetic polymorphism and adverse drug reactions requires a rapid, selective and sensitive analytical method (Li *et al.*, 2006). A quick, selective and sensitive method for MTX analysis in small volumes of biological samples would enable plasma concentration monitoring for MTX to allow dose adjustments to achieve optimal efficacy without causing adverse effects. Procedures presently available for monitoring MTX include: fluorometry, RIA, enzyme inhibition assay, HPLC, MS, isotachopheresis and capillary electrophoresis but all these methods suffer from long analysis times, complex sample pre-treatment, accuracy, selectivity or sensitivity concerns (Tjaden & De Bruinn, 1990). A high-performance liquid chromatography tandem mass spectrometric (LC/MS/MS) method has recently been developed for quantitation of MTX in environmental samples (Sabatini *et al.*, 2005). This method however cannot detect sub ng/ml concentrations and an improved method needed to be developed to follow low dose pharmacokinetics of MTX in biological fluids.

A new LC/MS/MS method has been developed for the determination of MTX in little samples of plasma or serum. The assay is based on directly injecting the equivalent of 1µl of plasma directly onto a small ODS capture column mounted on a column switching system, washing off the contaminating protein then back eluting the captured MTX onto a 20x2mm ODS 3µm particle size column for analysis using optimized conditions and specific MRM transitions. This method

was validated with respect to reproducibility, specificity, recovery, linearity, lower limit of quantitation (12.5ng/ml) and maximum limit of quantitation (125µg/ml).

The utility of the method was demonstrated by determination of pharmacokinetics (PK) for MTX in Sprague Dawley Rats. This method has the advantage of low sample volume requirement, rapid and simple sample clean up, measurement of MTX and an internal standard allowing a complete detailed PK study to be completed in an individual rat as opposed to 3 rats per time interval using existing methods.

7.2. Experimental

7.2.1. Chemicals and reagents

MTX (>98.0%), aminopterin, dimethyl sulfoxide (DMSO) and formic acid of highest purity were purchased (Sigma–Aldrich, Inc. St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were purchased from Rathburn Chemicals (Peebles, UK). In-house 18MΩ, pyrogen free water (Elga Genetic water purification system) was used for all aqueous solutions. Drug-free pooled rat plasma for spiking was obtained from University of Pretoria Biomedical Research Centre (UPBRC).

7.2.2. Preparation of stock solutions, calibration standards and quality control samples

Standard solutions of MTX were prepared separately at a concentration of 2.50 mg/ml using different batches of pooled rat plasma from healthy animals that had not been exposed to any of the analytes. Thereafter, the standard solutions were aliquoted into 100µl samples and stored in amber vials at -20°C. Stock solutions of the internal standard, folinic acid, were accurately prepared at approximately 4.7mg/ml in distilled water, aliquoted into 1ml samples and stored in amber vials at -20°C. The working solution of internal standard were prepared fresh daily from aliquots of the stock solution.

To prepare calibration standards, stock solution of MTX was diluted 1/100 with plasma, and then diluted serially with plasma to achieve concentration ranges from 0.025 to 1000 μ g/ml for MTX. Quality control samples were prepared from separately weighed samples in an analogous manner to the standards at a concentration range of 0.25 μ g/ml, and were assayed on each day that samples were analyzed.

7.2.3. Sample preparation

Blood was collected from the rats via the saphenous vein into a 33 μ l heparinized capillary tube after venipuncture with a finger-prick lance. The end of the capillary tube was plugged with haematocrit clay, centrifuged at 500g for 10 minutes and the plasma harvested using a 10 μ l GC syringe. Internal standard solution (47 μ g/ml) of exactly the same volume as the harvested plasma was added and the combined mixture added to a tapered microvial insert and vortex mixed for 20 seconds. Two microlitres of the resultant mixture was injected onto the LC/MS/MS system by the autosampler.

7.2.4. LC/MS/MS system and conditions

The LC system was an Agilent 1100 HPLC system consisting of a well-plate autosampler, a mobile phase degasser, a binary gradient pump, column oven with an automated six port switching valve and a diode array detector and an additional 1200 series binary pump with a reduced flow path volume, (all from Agilent Technologies, Waldbronn, Germany). The mass spectrometer was an API 4000 QTrap MS/MS system (Applied Biosystems MDS/SCIEX, Concord, Canada) with a “Turbo V” electrospray ionization source operated in positive ion mode.

Diluted plasma samples (2 μ l) were injected directly onto the system and transferred at 50 μ l/min onto the capture column (C18 SecurityGuard cartridge of 4.0mm x 2.0mm, Phenomenex, Torrance, CA) with 0.1% formic acid and 1g/l ascorbic acid in water at pH 2.8 before washing at 1000 μ l/min for 1.5 minutes. At 2.5 minutes the switching valve was actuated and the capture column

backflushed with 0.1% formic acid in methanol at pH 8.8 (using NH_4OH) onto a second C18 SecurityGuard cartridge (4.0mm x 2.0mm, Phenomenex, Torrance, CA) coupled to an analytical column (Alltech, C18 100Å, 3µm particle size, 20mm x 2.0mm) combination maintained at 35°C. Chromatographic separation was achieved by using a mobile phase of 0.1% formic acid and 1g/l ascorbic acid in 20% acetonitrile pH 6.0 adjusted with NH_4OH and delivered at a flow rate of 200µl/min. The column effluent was monitored using the following multiple reaction monitoring (MRM) transitions: MTX m/z 455.4 > 308.0 and folinic acid 474 > 327 with a dwell time of 80ms for each channel. The nebuliser gas, drying gas, curtain gas and collision gas were all nitrogen set at 30, 40, 23 and “medium” respectively. The ion spray voltage was set to 3500V and the drying gas temperature was set 550°C. The collision energies for collision induced dissociation for the aforementioned transitions were 27.4, and 28V, for MTX and folinic acid, respectively. All instrument controls and data collection was performed by Analyst 1.4.2 (Applied Biosystems/ MDS SCIEX) software program.

A single sample analysis cycle consisted of the following phases:

Phase 1: Sample injected and transferred onto the capture column at a slow flowrate using the 1200 series pump followed by a 1.5 minutes wash period using 0.1% formic acid solution (eluent to waste) at a flow rate of 1ml/min. This deproteinised the sample and extracted the MTX and the added folinic acid internal standard from the plasma.

Phase 2: After actuating the switching valve, the capture column was back-flushed with a 25µl slug of 0.1% formic acid in methanol at pH 8.8 onto a second C18 SecurityGuard cartridge coupled to a 20 x 2mm and 3µm C18 analytical column to desorb the analytes from the capture column, transferring the analytes onto the analytical column where it was separated using an isocratic mobile phase of 0.1% formic acid, 0.1% ascorbic acid and 20% CH_3CN in water that was eluted directly into the ESI source of the MS/MS for analysis.

7.2.5. Method validation

The precision and accuracy of the assay was based on analyses of both spiked plasma and water samples containing known concentrations of MTX. Plasma and water quality control samples were included within the ranges of all calibration curves and processed in triplicate. The intra-day and inter-day mean concentrations, standard deviations, percentage biases and percentage coefficients of variations (% CVs) were calculated. The LLOQ in plasma and water samples was defined as the lowest concentration at which the signal-to-noise peak height ratio was greater than 10:1 and both intra-day and inter-day percentage CVs and percentage biases had to be less than 15%. The specificity of the assay for MTX and folinic acid (internal standard) in plasma was evaluated using different batches of pooled rat plasma from healthy animals that had not been exposed to any of the analytes.

7.2.6. Method application

The analytical method was subsequently used to analyze samples from a pharmacokinetic study of MTX and D85 in rats. For the pharmacokinetic studies, each rat received 1 mg/kg of MTX as an intravenous bolus and sequential timed blood samples were collected from the saphenous vein following drug administration. Blood samples (20-30µl/time interval/rat) were drawn into heparinized 33µl glass capillary haematocrit tubes by trained personnel from the UPBRC at the following time intervals: 0, 1, 10, 15, 30, 60, 120, 240, 360, 720, 1440 min for LC/MS/MS analysis (to assay the free MTX levels in plasma). Blood was collected via the saphenous vein after venipuncture with a finger-prick lance. Plasma, harvested by centrifugation after plugging the bottom of the capillaries was processed and stored on ice. These samples were analyzed as soon as possible after collection. MTX levels were determined at the Department of Pharmacology, Faculty of Health Sciences, University of Pretoria.

7.3. Results

7.3.1. Mass spectrometry and chromatography

The positive ion scans of standard solutions of MTX indicated that MTX, and folic acid had protonated molecular ions $[M+H]^+$ of m/z 455.4 and 474, respectively. Under the chromatographic conditions the transition of $455.4>308.0$ and $474.0>327$ were selected for quantitation of MTX and folic acid, respectively. An ion spray voltage of 3500V provided a sufficient response under the selected chromatographic conditions, and no further increase in the response was found when the ion spray voltage was increased further. Baseline noise was found to be fairly significant at a source drying gas temperature of 450°C , but by increasing the temperature to 550°C a much reduced background noise was evident and yielded smoother peaks (Guo *et al.*, 2007).

The pH of the mobile phase had a significant effect on the retention time of folic acid, with longer retention times obtained at lower pH. The retention time of MTX was not similarly affected, and thus, suitable chromatographic resolution of both compounds could be achieved by controlling the mobile phase pH. The mobile phase yielded retention times of less than 4 min for both analytes allowing high sample throughput. Representative chromatograms of MTX and folic acid in water are shown in (Figure. 15) respectively. Retention times were 2.4 min for folic acid and 2.75 min for MTX (Figure. 15).

7.3.2. Method validation

The method was validated in plasma for MTX and folic acid at a concentration range of 2.5 to 2500 ng/ml. Calibration curves prepared over these concentration ranges were linear with average correlation coefficients greater than 0.99 in water for both drug and internal standard (Figure. 16). The method yielded mean intra-day and inter-day values of less than $\pm 15\%$. The recoveries were routinely greater than 85%. The recoveries were usually the lowest at lower concentrations.

7.3.3. Method application

Representative concentration–time profiles of MTX in rat plasma after intravenous administration of 1mg/kg MTX and D85 equivalent to 1mg/kg in rats are shown in Figure. 14. The results shown clearly indicate the subtle pharmacokinetic differences between the two compounds. Hence, these parameters provide the necessary foundation to pursue additional investigations on the mechanisms controlling MTX distribution and further demonstrate the suitability of the assay method to explore the disposition of MTX using new delivery systems in preclinical pharmacokinetics.

Rat pharmacokinetics of MTX and D85

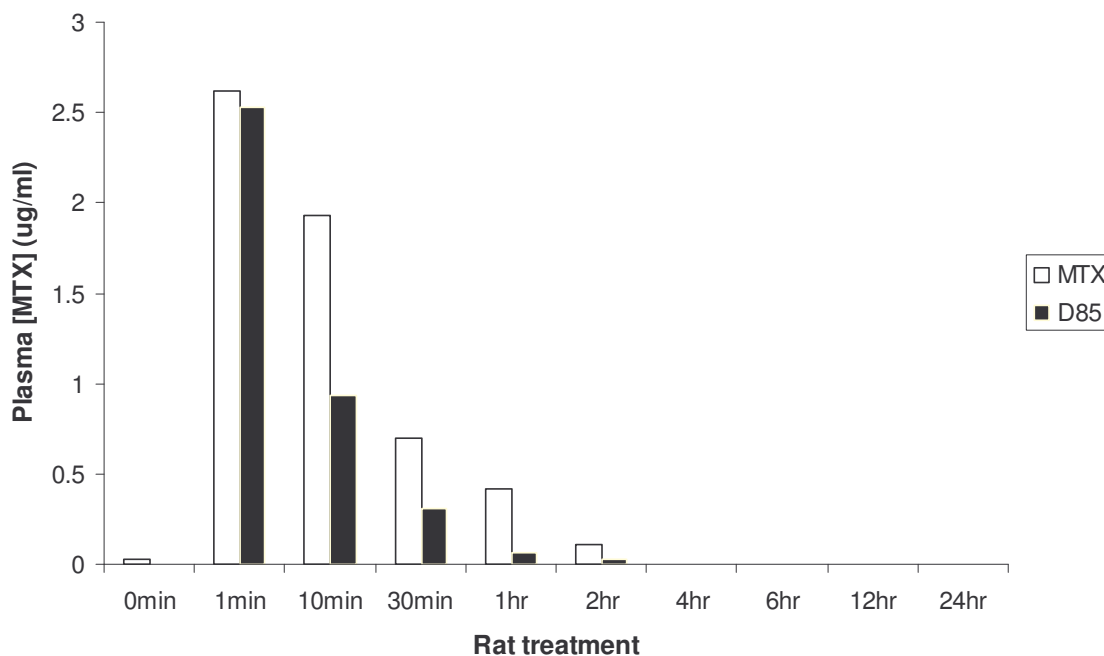


Figure 14: An example showing the pharmacokinetics of D85 compared to MTX using Sprague Dawley rats. Rats were injected i.v. with D85 (equivalent to 1mg/kg MTX), or MTX (1mg/kg) as indicated. The average T_{max} and C_{max} for both compounds were very similar. T_{max} and C_{max} were approximately 1min and 2.5 μ g/ml respectively.

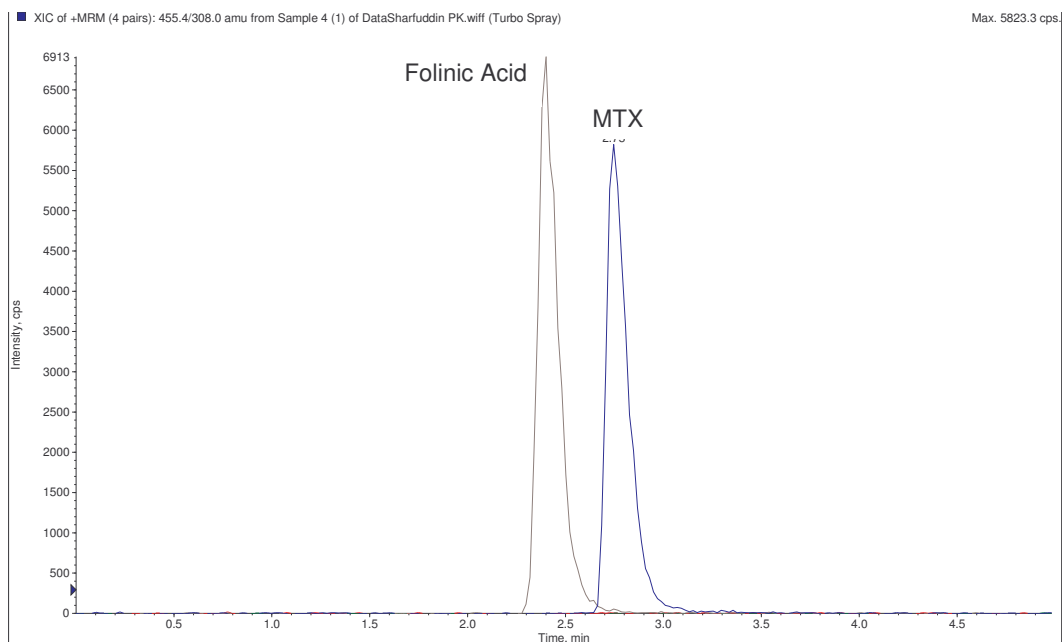


Figure 15: An example of a sample chromatogram showing MTX and folinic acid (internal standard). Retention times were 2.4 min for folinic acid and 2.75 min for MTX. The column effluent was monitored using the following multiple reaction monitoring (MRM) transitions: MTX m/z 455.4 > 308.0 and folinic acid 474 > 327 with a dwell time of 80ms for each channel.

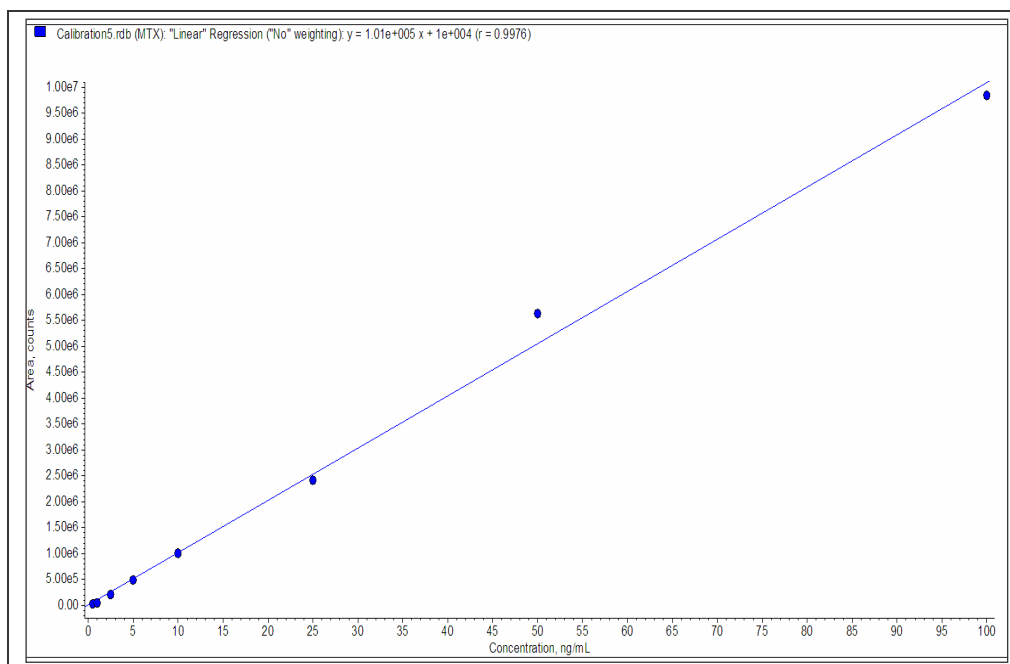


Figure 16: Results of a single calibration curve of MTX in plasma. The method was validated in plasma for MTX and folinic acid at a concentration range of 2.5 to 2500 ng/ml. Calibration curves prepared over these concentration ranges were linear with average correlation coefficients greater than 0.99 in plasma for both drug and internal standard.

7.4. Conclusion

A rapid and sensitive LC/MS/MS method for the quantification of MTX in rat plasma was presented. The unique features of the new assay are the requirement of only small sample volumes (<10 µl blood), the use of an effective online SPE extraction procedure for plasma and the use of a small guard cartridge as a capture column. The ability to measure MTX in small plasma volumes permits serial sampling protocols in individual rats that will support comprehensive drug distribution studies to understand the factors that determine MTX's distribution.

Given the attributes of this LC/MS/MS technique, it should be possible to use a finger prick technique for blood sampling during therapeutic drug monitoring, especially in young children on MTX treatment.

8. Discussion

MTX continues to play an important role in the treatment of a variety of malignancies and is well understood with respect to its mechanism of action at the molecular level during cancer treatment. However, it has limitations due to the lack of selectivity for cancerous tissue and severe dose related vital organ toxicity. In an attempt to avoid systemic toxicity of this useful cytotoxic drug and to improve tumour selectivity, MTX was bound to an amino acid polymer which was expected to improve target selectivity and improve pharmacokinetic properties. In this study, a new MTX conjugated polymer, D85, was assessed for anti-cancer and anti-inflammatory characteristics using different experimental techniques.

The first step was the screening of the experimental compound for cytotoxicity, done by *in vitro* cell culture assays to determine the selectivity and effectivity of the experimental drug. *In vitro* tests were done on selected adherent and free floating cancer cell lines and on normal primary cell cultures to determine whether there was any selectivity for the more rapidly dividing cancer cells compared to non-transformed but dividing cells. D85 was approximately ten times more cytotoxic against the three tested cancer cell lines than was MTX. The most potent cytotoxic effect was seen against the free floating COLO 320 DM cell line, being over 300 times more effective than MTX. This was an interesting finding because MTX is active against most cancers but not reported to be specifically more potent for any one particular cancer type.

D85 showed very selective toxicity against the cancer cell lines compared to the mitogen stimulated lymphocytes; however the resting lymphocytes showed cytotoxic effects similar to that of the tested adherent cancer cell line. This difference compared to the stimulated lymphocytes was unexpected and is an anomaly, since the resting lymphocytes are expected to be less vulnerable to cytotoxic drugs than the stimulated lymphocytes. However, the results obtained

were confirmed by repeating the *in vitro* experiments three times using the same batch of D85 throughout all the *in vitro* experiments.

The EPR effect does not play a role in the *in vitro* assays, as the drug is in direct contact with the cells during the experiment and uptake would not be influenced by a vascular system showing an EPR effect. Furthermore, it is unlikely that pH played a role since no change in pH was observed in the culture medium. A possible reason for the difference in toxicity of the resting versus stimulated lymphocytes could have been that the stimulated lymphocyte uptake or accumulation of the polymer was influenced by the membrane polarity state. In any case MTX was far less toxic to lymphocytes, in both stimulated and resting states compared to D85.

There are however a few shortcomings with the *in vitro* work done. Cancer cell lines are usually incubated for 7 days but lymphocytes are only viable for 3 days without the addition of specialised growth factors or conditioned media. It would be interesting to incubate the different cancer cells for only 3 days and do a cytotoxicity comparison without having time as a variable in the experiment. This would be ideal, but from most reports, cancer cell lines need more than 3 days for all cells to divide in order to get useful results while lymphocytes cannot easily be cultured for longer than 3 days.

The positive anti-cancer results obtained from the *in vitro* experiments justified further investigation of D85 *in vivo*. The proof of concept lay in the fact that there were cytotoxic effects resulting from the D85 which implied that the compound was in fact being taken up by the cancer cells tested and that there appeared to be a fairly high selectivity for the polymeric drug. Although toxicity was noted in resting lymphocytes, this effect could be different in living organisms, since the pharmacokinetics and pharmacodynamics can change due to metabolism, redistribution and specific target site accumulation *in vivo*. Furthermore, *in vivo*, an important difference between D85 and MTX is their expected different

pharmacokinetic behaviour, with the accumulation of D85 in tumour tissue due to the enhanced microvascular permeability, as well as different cellular uptake mechanisms, which cannot be imitated by *in vitro* assays. Thus, the main advantages of D85 over MTX cannot be demonstrated fully in the *in vitro* cell culture experiments.

Before an efficacy study could be carried out, an *in vivo* toxicity study was done at the concentrations that were to be used for an efficacy study to ascertain potential systemic toxicity risks and safety. There were no significant differences in the haematology results between the groups especially with respect to the lymphocyte populations which had shown sensitivity to the drug in the *in vitro* assays. This confirmed the haematological safety profile of D85 at the dose equivalent of 1.5mg MTX per kg body mass. The liver toxicity markers, ALT and AST serum levels showed an unusual trend in that their concentrations appeared to be decreasing although this was not significant. However a significant decrease in the serum level of a third hepatotoxicity marker, GGT was observed at the higher D85 dose when compared to the control. This decrease was not what was expected if liver toxicity was evident; therefore the decrease in the GGT level is an anomaly.

Analysis of the organs weights (heart, liver, and kidney) indicated that a small but significant decrease in the kidney weights existed between the control and the treated groups. Despite this the histopathology results concluded that there were no specific pathological lesions and no signs compatible with organ toxicity that could be associated with the intravenous administration of the test substance between the groups.

The results obtained indicated two points of concern. Firstly the kidney size of both D85 treated groups was lower compared to the control and secondly the GGT enzyme of the higher dose group was significantly lower. Kidney mass changes could be the result of renal toxicity but this was not reflected in the

markers of kidney function, urea and creatinine. If renal toxicity occurred the most common effect would be a decreased glomerular filtration, which would have been seen as an increase in the kidney toxicity marker levels which in contrast showed a small insignificant decrease for both urea and creatinine indicating if anything, a small improvement in kidney function or a change (decrease) in the normal protein metabolism in the animals.

The toxicity data would have been more useful if larger sample sizes were used. This would eliminate any statistical bias and would have added more power to the statistical test.

Considering all the toxicity results that were obtained with the current numbers, the *in vitro* cytotoxicity results and the expected ability of the polymer to selectively increase the bioavailability of the drug into targeted tumour regions, the lower dose of 5.84mg D85/kg body mass which was equivalent to 1mg MTX/kg was used in further efficacy investigations.

Before the efficacy study could be performed, a tumour model had to be established and to this end the most suitable cancer cell line and number of relevant cancer cells to introduce subcutaneously had to be determined in a pilot study. The COLO 320 DM and MCF-7 cancer cell lines both developed tumours at all the cell concentrations tested. The reason that 5×10^7 cells were used in the final efficacy experiment was based on the results obtained from the pilot study. The COLO 320 DM tumours grew well and faster compared to the other concentrations tested. Also this concentration of cells was easy to produce within a short time. The colon cancer cell line was used since it was most sensitive cell line to D85 during the *in vitro* testing stages. The greatest anti tumour effect was expected using this cell line once a tumour was formed.

The dose recommended for the treatment of human breast cancer was 50mg MTX/kg body mass. The therapeutic effect of the classical drug MTX (1mg/kg

and 50mg/kg) and D85, which is the studied polymeric conjugate of MTX, were investigated *in vivo* against a colon cancer xenograft growing subcutaneously in nude mice. Unfortunately not all the mice developed solid tumours within the time constraint of the study, which resulted in different numbers of animals in each group receiving treatment. The control group that was treated with saline only had 5 animals develop any palpable tumours while both the MTX treated groups had 6 animals each with the D85 treated group having 9 animals with palpable tumours. The possible reasons that tumours did not grow in all the animals are numerous. The fact that they were done in batches could have played a role, due to the growth state of the tumour cells at the time of harvesting. Other plausible reasons could have been the age differences in the mice (which were mass matched randomly selected), which was reported to have played a role in other studies. As the mice grew older, the chances of developing a tumour are slimmer since increased numbers of NK cells are formed (Jacob *et al.*, 2004).

As far as the results of the anti-cancer efficacy study were concerned all the mice that developed palpable tumours survived the 4 weeks during which treatment was administered, including the untreated control group. The saline treated group showed a continuous fairly rapid growth in tumour size during the four weeks of “treatment” that was started once the tumour size was determined to be 1mm³. This increase in tumour size was a trend seen in all the treatment groups as well, but the rate of increase of tumour size was slower than in the saline treated control group.

The measured tumour size showed that the three treatments tested all induced anti-tumour activity *in vivo* with a distinct dose dependent effect for free MTX. The greatest anti-tumour effect was observed in the MTX (50mg/kg) group, which is the recommended dose for MTX in the treatment of breast cancer and ALL (Pees *et al.*, 1992). When comparing the lower dose of MTX to the comparable MTX dose delivered as the polymeric D85 when both were administered intravenously, no significant superiority of D85 was observed. It should be

remembered that the polymer was expected to have an increased accumulation in the tumour due to the enhanced permeability and retention effect and was expected to have shown a greater anti tumour effect than the free MTX at the equivalent dose of active drug (Ofner *et al.*, 2006). These results would suggest that effective cytotoxic concentrations of MTX against the COLO 320 DM type tumours are only reached in the tumour tissue after administration of high concentrations of free MTX, which only confirms that MTX at high concentrations is an effective anti-tumour agent against COLO 320 DM tumours in the nude mouse model, as tested in this study.

The important and expected advantage of D85 above MTX with respect to pharmacokinetic behaviour, tumour uptake mechanism and accumulation of D85 within the “leaky” tumour tissue cells resulting from the enhanced microvascular permeability could unfortunately not be demonstrated in these experiments.

MTX is reported to be a potent anti-inflammatory agent (Dalmarco., 2002). Low-dose MTX is efficacious in the treatment of rheumatoid arthritis (Baggott *et al.*, 2007). MTX treatment produces increased levels of adenosine and/or its metabolites *in vivo*, and this leads to the suppression of the immune system (Baggott *et al.*, 2007). As far as the acute inflammatory model experiments were concerned, some interesting observations were made. The saline control, MTX and D85 treated groups all showed significant increases in paw volume after injection of the carrageenan which lasted for the seven hour period of the study indicating oedema due to an acute inflammatory response. This was expected and confirmed the inflammatory reaction as described by Bilici *et al.*, (2002). A general pattern was seen in all three groups, in that there was a rapid increase in the footpad volume for the first two hours followed by a slight decrease in the total oedema over the next 2 hours after which there was a slow increase in volume again which continued until the end of the study period at 7.5 hours post carrageenan injection. The general foot volume curve for all the groups followed an abnormal swelling effect (i.e. an abnormal increase in swelling once the

swelling had begun to decrease) compared to other studies (Tsukada *et al.*, 1974; Kim *et al.*, 2008) where this second phase increase was not observed. This was a unique difference observed in all the treatment groups of rats in this study and no explanation can be found for this unusual effect.

The footpad oedema of the MTX treated group was significantly lower than that of both the control and D85 treated groups ($P < 0.05$) despite all groups following a similar time profile of swelling. This finding confirmed previous research by Montesinos *et al.* that MTX can be used to treat acute inflammation.

An unexpected result was that the footpad oedema which implies the inflammatory reaction of the D85 treated group was significantly greater than both the saline control and MTX-treated groups ($P < 0.05$) at all measurement times through to the end of the study where this treatment group appeared to show an even greater increase in swelling whereas the other two treatment groups had reached a maximal plateau by 6 hours.

This could be due to the D85 leaking through the vasculature in the region of the inflammation/oedema in the same way that enhanced permeation and retention takes place in the cancer tissue and that the polymer caused an increase in the inflammatory effect due to an increased accumulation or activation of infiltrating leukocytes or the release of higher concentrations of pro-inflammatory cytokines by the affected tissue. This effect could be regarded as an advantage in the cancer tissue where localised inflammation would probably lead to greater anticancer effects by the immune system in the region of the tumour, but is an adverse effect in areas of inflammatory oedema. The D85 treated group showed a maximum increase in mean footpad volume of approximately 17.6% two hours after injection of the carrageenan, whereas the MTX group showed a significantly slower and an overall smaller increase in footpad volume, reaching a maximum of only approximately 7.9% larger volume than the initial pre-carrageenan injection measurement at seven hours after the carrageenan injection. This was only 1.12% more than the extent of swelling seen at 2 hours. The saline control

showed a maximum mean increase in footpad swelling of 13.7% at two hours with the 7 hour oedema less severe. However, D85 at 5.84mg/kg, which is a dose equivalent to 1mg MTX/kg, exacerbated the swelling at all measurement times of the study indicating that the D85 was in fact pro-inflammatory. The evidence presented indicated that MTX at a dose of 20mg/kg reduces acute inflammation significantly compared to the saline control and the polymeric MTX drug, D85.

To prove that the pharmacokinetics differ between MTX and D85, pharmacokinetics assays were carried out after administration of each of the drugs. The pharmacokinetics of D85 had never been done before and it was of interest to see the difference in the two pharmacokinetic profiles which was used to correlate to its efficacy. There was no *in vitro* pharmacokinetic method that mimicked i.v. drug delivery, thus this needed to be done *in vivo*. Furthermore, no artificial membrane could imitate capillaries accurately to justify the study *in vitro*. Therefore, a rapid and sensitive LC/MS/MS method for the quantification of MTX in rat plasma was developed and validated. There were no interfering peaks co-eluting with the analytes of interest (Figure. 15). Separation of the MTX and the internal standard in rat serum were successfully achieved. An excellent linear relationship ($r^2=0.99$) was demonstrated between MTX and folinic acid over a concentration range of 2.5 to 2500ng/ml. This method was then applied to the determination of MTX in pharmacokinetics studies in rats. There were no previously published studies or information of the pharmacokinetics of plasma concentrations of MTX following D85 administration in any species. Following administration of D85 and MTX, a very minor subtle difference in the pharmacokinetic profile was noticed. This was unexpected because the pharmacokinetics profile should have been very different from each other due to the molecular mass, solubility differences, charge differences and percentage of the molecule that was in the form of MTX. MTX concentrations peaked immediately which was the expected outcome after an intravenous bolus administration of the drug, but finding the same kinetic parameters for the D85

group was not at all what was expected. The polymer MTX concentration was not envisaged to have peaked immediately since it is supposed to have been bound to the polymer until enzymatically cleaved and this was expected to occur intracellularly not in the plasma. The plasma MTX concentrations resulting from D85 administration should have remained low but relatively constant for a relatively long time. There is a possibility that the covalent MTX-polymer bond was broken/cleaved during the first pass effect in the liver or perhaps due to the plasma pH. Plasma MTX concentrations were below the detection limit two hours after administration either by elimination or distribution into tissue after administration of both free MTX and D85 compounds. The elimination period half-life of MTX after administering the polymer was expected to have been longer than the free MTX, but this was however not the case. Further investigations would be required to determine the exact reasons for the very rapid release of MTX from the polymer carrier.

The unique features of the new assay are the requirement of only small sample volumes (<10 µl blood), the high sensitivity and selectivity, reproducibility, accuracy and specificity. The developed method was successfully applied to the longitudinal pharmacokinetic study of MTX in single rats which allows each rat to act as its own initial control and to give better statistics due to the same animal being used for a complete study for the first time. During this study, the number of animals required for the complete assay was reduced to only 10 rats per treatment despite collecting data from 12 different time points. The ability to measure MTX in small plasma volumes permits longitudinal/serial sampling protocols in individual rats that would support comprehensive drug distribution studies to understand the factors that determine MTX's distribution. Given the attributes of this LC/MS/MS technique, it should be possible to use a finger prick technique for blood sampling during therapeutic drug monitoring of MTX, especially in young children on MTX treatment. Further studies are ongoing in our laboratory to further characterize the MTX and D85 metabolites and their pharmacological and toxicological activity.

8.1. Final Conclusion

In conclusion, D85 is a potential new delivery system for the anti-cancer compound MTX that shows promise as a useful drug for many different tumour types, but especially colon cancer. It appears to enhance the effective anti-tumour agent MTX and warrants further investigation as an anti-cancer agent for other cancers normally treated with MTX. Studies using larger sample sizes should be performed to determine both the efficacy for different cancer types and to assay any long term toxicity effects that may not be evident in short term acute toxicity assays. Furthermore, intensive research needs to be carried out to improve acute toxicity challenges currently faced, and this will bestow fruitful rewards long term. Difficulties in trans-membrane movement can not be achieved with standard diffusion cell techniques due to the large molecular size requiring poorly formed pervious vasculature. Chemical stability studies (pH, chelating agents, transport enhancers) could however indicate what condition would be conducive to the breakdown of D85.

The properties of D85 with respect to inflammation proved to be pro-inflammatory. The D85 appeared to be a potent pro-inflammatory agent compared to both free MTX and the saline treated control. This pro-inflammatory effect is probably is due to the polymer size that allows leakage from the vasculature into the region of inflammation where the inflammatory response is exacerbated rather than suppressed by the polymer without the release of the MTX which would result in an anti-inflammatory effect. Although this effect is a negative result for the drug it does confirm that the D85 would in fact leak from the vascular system where the endothelium is compromised or not fully developed as is reputed to occur in tumour tissue (Ofner *et al.*, 2006). Taking all the data collected during this study into consideration, D85 does show promise as an anti-cancer agent but would require several further confirmatory experiments and efficacy dose ranging studies. Once intensive research has been completed, D85 shows promising commercial viability.

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