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The anticancer potential of CITme, a quinoline derivative

by

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Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae in Pharmacology

in the

FACULTY OF HEALTH SCIENCES
DEPARTMENT OF PHARMACOLOGY
UNIVERSITY OF PRETORIA

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Date of submission
17 November 2010

ACKNOWLEDGEMENTS

This master's thesis was written in partial fulfilment of the MSc degree in Pharmacology at the University of Pretoria. From an academic perspective, pharmacological and toxicological tests presented an interesting example of drug research. With support from the UPBRC and the Pharmaceutical Department of North West University, the research began in earnest during March 2007, and the final study was completed on 20 January 2010.

I would like to acknowledge the following people for their assistance, understanding and support throughout the process of completing this challenge venture:

- My study leader, PhD A.D Cromarty for his guidance, motivation, support and inspiration, for being a living example at all times and never limiting my ideas and questions.
- My co-supervisor, Professor C.E. Medlen, for giving me the opportunity to develop this project;
- Numerous executives/staff of the Pharmacology Department (University of Pretoria) for their cooperation, especially Dr Gisela Joone, Dr Irmgard von Teichman and Ms Maryna Steinmann for all the help, advice and collaboration; Grateful thanks go to Ms Margo Nell for great collaboration with the cell cultures;
- The Pharmaceutical Department at North-West University especially Professors Anne Grobler and Wilna Liebenberg, Dr Johan Lamprecht and Liezl-Marie for help and development of the formulation used in this study;
- Grateful thanks go to my family – Rui, Paula, Angela, Carlos, Mário and Victor – for their never-ending support and sacrifice in so many ways throughout the years. Sincere admiration to my husband, Mário, for his encouragement, inspiration and support throughout this project.

Finally, it should be recognised that this study and the research that underlies it, represents effort and cooperation of many more people than mentioned here but I wish to acknowledge their part and assistance in this work.

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ABSTRACT

3-[3-(7-chloro-quinolin-4-yl amino) phenyl]-1-(4-methoxy-phenyl) prop-2-enone citrate "CITme" is a substituted quinoline derivative, synthesized as a potential anti-tumour agent. The aim of this study was to investigate CITme with regard to anti-tumour activity, toxicity and pharmacokinetics.

In vitro screening for neoplastic cytotoxic effects using standard cell culture techniques revealed cytotoxic activity against HeLa, DU-145, MCF-7, Jurkat and CoLo 320 human derived cancer cell lines at low concentrations. Toxicity was significantly less in either normal resting and stimulated lymphocytes or primary chicken fibroblast cells. CITme showed highest cytotoxic specificity for DU-145 with an IC_{50} of 11 µM compared to 38.2 µM for lymphocytes after a three-day incubation period and 2.5 µM, after a seven-day incubation period.

CITme exhibited poor solubility in aqueous solutions and this had to be addressed prior to *in vivo* acute toxicity studies being performed. CITme was found to be insoluble in many biocompatible and acceptable formulating solvents. 2-*methyl* pyrrolidone and Cremophor EL showed promise as potential solvents but the solubility proved to be too low to enable therapeutic CITme dosing while avoiding solvent/carrier toxicity. An oral microcrystalline cellulose suspension, two combinations of 2-methyl pyrrolidone with plasma protein complexation and a propheroid formulation were also tested. These were tested in pilot acute toxicity studies using BALB/c mice or Sprague Dawley rats. No plasma concentrations were observed with the cellulose suspension and both the 2-*methyl* pyrrolidone formulations elicited toxic responses in vehicle control and experimental groups despite working well below the published LD_{50} of 2-*methyl* pyrrolidone.

A unique formulation developed by the Department of Pharmacy of NWU referred to as propheroid drug delivery system, provided sufficient solubility for acute and chronic toxicity studies of CITme. *In vivo* acute and chronic toxicity study of the propheroid delivery system formulation alone and the CITme propheroid at 30 mg/kg/day and 60 mg/kg body weight (acute toxicity study), and 10mg/kg/day (chronic toxicity study) demonstrated safety after seven and forty-five (six weeks) days administration by oral gavage.

The potential anti-tumour activity of CITme could not be determined in a nude mouse tumour model, using the most susceptible *in vitro* cell line, DU-145 due to the low success rate of tumour induction and the long lag period required for the development of subcutaneous prostate tumours after transplantation of either a DU-145 cell suspension or DU-145 tumour tissue blocks.

An LC-MS/MS method was developed to separate and quantify CITme from plasma and organ homogenates. The LC-MS/MS method was used to determine the pharmacokinetic parameters of I.V. administered CITme.

A pharmacokinetic study of CITme in mice indicated that CITme is absorbed after an oral administration and exhibits a very rapid distribution (metabolism) and/or elimination after IV administration. CITme could be found at relatively high concentrations 24 hours after oral dosing and to a smaller extent 10 minutes after I.V. administration in both the liver and kidney tissues.

Despite the fact that the efficacy and oral pharmacokinetics properties of CITme could not be determined, this study has proved that CITme is a selective and potent anti-tumour agent *in vitro* with low toxicity both *in vitro* and *in vivo* and therefore this compound warrants further scientific evaluation.

CHAPTER ONE

BACKGROUND AND SCOPE OF THE STUDY

1.1 INTRODUCTION

Cancer is generally defined as the uncontrolled and invasive growth of tissue, as a result of diverse genetic mutations that disrupts normal cell proliferation. This micro-evolutionary multi-step process is known as carcinogenesis that causes the cell to evolve into a highly aggressive neoplastic cell that possess the following traits: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless potential to replicate, the ability to initiate angiogenesis as well as the ability to invade other tissue or metastasise to other areas of the body (Hanahan and Weinberg, 2000). Carcinogenesis is a process that occurs over time and involves changes that result in acquisition of continuous growth and/or evasion of death stimuli through the inappropriate expression of certain mutant genes. Tumour suppressor genes that normally function to inhibit cellular proliferation, repair damaged genes and initiate apoptosis are inactivated during the changes that occur. Each new mutation/step (progression) confers a proliferative advantage to a particular cell compared to adjacent normal cells and consequent inappropriate growth (Hanahan and Weinberg, 2000; Lowe and Lin, 2000; Wyllie *et al.,* 1999).

1.2 INCIDENCE OF CANCER

Cancer remains a major public health issue and according to World Health Organization (www.who.int, 2005), it has become a leading cause of death worldwide. Cancer accounts for roughly 13% of all deaths worldwide (World Health Organization, 2003). From approximately 58 million deaths, cancer accounted for 7.6 million worldwide in 2004, which is more than the percentage of deaths caused by HIV/AIDS, tuberculosis and malaria combined (McIntyre, 2007). According to WHO estimates, there are currently 25 million people living with cancer. The main types of cancer leading to mortality are: Lung (1.3 million deaths/year); Stomach (803 000 deaths); Colorectal (639 000 deaths); Liver (610 000 deaths), and Breast (519 000 deaths).

In 2007 more than 70% of cancer, deaths occurred in low and middle income countries. Worldwide deaths from cancer are projected to continue rising, with an estimated 7.9 million people dying from cancer in 2015 and 11.5 million dying in 2030 (www.who.int/cancer/en/, 2009). However, new cases of cancer in the same period are estimated to jump from 11.3 million per year in 2007 to 15.5 million per year in 2030. As cancer is a disease mainly of old age, the worldwide cancer mortality rate is expected to rise from the present estimate of 7.9 million cancer deaths per year to 9 million by the year 2015. This estimate does take expected declines in death rates for certain cancers in high resource countries into account.

According to the World Health Organisation cancer report (World Health Organisation, 2003), this predicted sharp increase will be mainly due to steadily ageing populations in both developed and developing countries. Other factors include current trends in smoking prevalence and the growing adoption of unhealthy lifestyles. However, the report provides clear evidence that healthy lifestyles and public health action by governments and health practitioners could stem this trend, and prevent as many as one third of cancers worldwide. The report also reveals that cancer has emerged as a major public health problem in developing countries, matching the rates seen in industrialised nations.

Tobacco causes cancer at many sites including; lung, throat, mouth, pancreas, bladder, stomach, liver and kidney. Overweight and obesity are associated with colon, breast, uterus, oesophagus and kidney cancers. One fifth of cancers worldwide are reputedly due to infections (Fig 1.1), mainly from the hepatitis B and papilloma viruses, H. pylori bacterium, Schistosoma parasite, the liver fluke and human immunodeficiency virus (HIV) that affect the liver, cervix, stomach, bladder, bile duct, skin and blood cells respectively. The large contribution of tobacco, diet and infection to the development of various cancers is presented in Fig 1.1.

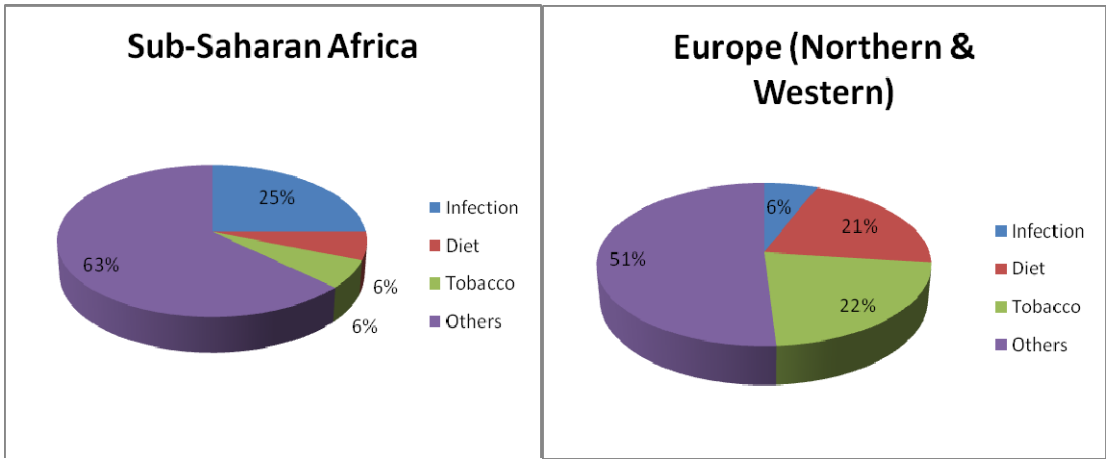

Figure 1.1: The major factors contributing to the development of cancer in Sub-Saharan Africa and Northern and Western Europe (Selikoff, 2005).

According to The National Cancer Registry of South Africa (2003), there were a total of 29,208 new cancer cases reported in females and 29,499 new cases reported in males. One in four males and one in five females (adjusted for under-reporting), aged 0 -74 years, were at risk of developing cancer. Table 1.1 summarises the most common cancers in South Africa.

Table 1.1: List of the most common cancers in South Africa (in descending order of occurrence) for males, females and children (National Cancer Registry, 2003).

MALES	FEMALES	CHILDREN (males & females, 0-14 years)
Prostate	Cervix	Leukaemia
Lung	Breast	Kidney
Oesophagus	Colorectal	Brain
Bladder	Oesophagus	Non-Hodgkin's lymphoma
Colorectal	Lung	Bone

In Southern Africa, the current cancer status is difficult to quantify and according to Albrecht (2006), The National Cancer Registry of South Africa is unreliable as a result of large scale under reporting and is furthermore a number of years behind. Mqoqi (Moqoqi *et al.,* 2004,) reported an incidence rate of 60343 new cancer cases in 1999 whereas Bradshaw (Bradshaw *et al.,* 2000) reported a mortality rate of 65 925 deaths in the year 2000. These two values (incidence and mortality rates) do

not instil faith in the cancer surveillance infrastructure of South Africa, as it suggests that there are more people dying of cancer than there are people diagnosed with cancer. This is not a realistic representation of the situation and a concerted effort to introduce new legislation related to the reporting of the incidence of cancer, to initiate better surveillance methods thereby making it possible for identification of new research areas especially in the field of cancer treatment (Albrecht, 2006).

1.3 CANCER THERAPY

In recent years, the body of knowledge concerning cancer has increased and it has been estimated that through several life style modifications, over 40% of all cancers could be prevented. Preventative strategies include a healthy diet and avoidance of certain physical and environmental factors such as carcinogens, radiation and oncogenic viruses such as the human papilloma and hepatitis B viruses (World Health Organization, 2003). Moreover, apart from preventative strategies, our treatment strategies include better surgical intervention, targeted radiation therapy and the development of new chemotherapeutic drugs (Albrecht, 2006; Grella *et al.,* 2003).

1.3.1 Surgery therapy

For centuries, surgery was the only cure for cancer (Shiu, 2003) and today is still the principal treatment for many of the common cancers. For example, treatment for hepatic colorectal metastases has greatly evolved over the two last decades, such that this rapidly fatal stage IV disease has become treatable by surgery and is potentially curable (Morris *et al.,* 2006). In recent years, there have been improvements in clinical and radiological staging resulting in more rational planning of surgical and adjuvant treatments.

1.3.2 Radiotherapy

Approximately 50% of all cancer patients are treated with radiation therapy, either as primary treatment with curative intent or for palliation of cancer related symptoms (Vink *et al.,* 2007). Likelihood of tumour response after radiotherapy is determined by the total radiation dose required for tumour cell kill and varies between tumours types, ranging from very sensitive low grades lymphomas (90%

tumour control at 4 Gy) to notoriously radio-resistant malignant gliomas (not responsive at clinically achievable doses). For patients treated with radiotherapy, fibrosis, necrosis and severe organ dysfunction may appear months to years after treatment (Koukourakis and Danielidis, 2005). Koukourakis and Danielidis (2005) define the therapeutic index (TI), as the ratio of the percentage of tumour control and the percentage of complications associated with a certain therapeutic regimen. In radiotherapy, radio-sensitizing agents aim to improve the TI by increasing the tumour control rate, while cyto-protective policies do the same by reducing the complication rate. Cisplatin and carboplatin have a substantial activity in sensitizing tumour cells to radiotherapy in head and neck, lung, oesophagus, cervix, bladder and rectal cancer as reported by Desoize and Madoulet (2002).

1.3.3 Chemotherapy

According to Espinosa (Espinosa *et al.,* 2003), classical anti-cancer drugs (Table 1.2) are grouped into chemotherapy, hormonal and immunotherapy. The subgroup “others” has expanded so much and it has been proposed that the group classification be based on the target cells. The target may be located in the tumour cell or in other elements that interact with the tumour cells (endothelium, extracellular matrix, immune system, host cells).

Table 1.2: Group classification of common anti-cancer drugs used in therapy

Chemotherapy	Alkylators
	Antibiotics
	Antimetabolites
	Topoisomerase inhibitors
	Mitosis inhibitors
	Others
Hormonal therapy	Steroids
	Anti-oestrogens
	Anti-androgens
	LH-RH analogues
	Anti-aromatase agents
Immunotherapy	Interferon
	Interleukin 2
	Vaccines

The chemical treatment of cancer has traditionally used agents that interfere with the cell division process (Don and Hogg, 2004). More recently, research and novel therapies have targeted the growth signals that drive the proliferation and survival of cancer cells (Herceptin for treatment of selected breast cancer) and the tumour vasculature (antibodies against vascular endothelial growth factor). DNA-interactive drugs in clinical use represent one of the most important drug classes in cancer therapy (Grella *et al.,* 2003). In general, there are three major types of the above mentioned clinically important drugs namely:

- The intercalators, which insert between the DNA base pairs of the double helix and cause a significant change of conformation being accompanied by unwinding and elongation of the duplex;
- The alkylators, which react covalently with DNA bases and;
- The DNA strand breakers, which generate reactive radicals that cleave of the polynucleotide strands.

Increased cure rates have been achieved in various cancer types mainly in: childhood cancer, testicular cancer, leukaemia and lymphoma, and survival

improvements have been obtained with adjuvant drug treatment of breast and ovarian cancer (Workman, 2001). However, the goal of routine cure or long-term management of cancer as a chronic disease remains elusive and the development of effective treatment through therapeutic agents is still very challenging. Early detection and the use of effective treatment modalities could prevent an estimated one-third of cancer deaths.

1.4 LIMITATION OF CURRENT CANCER TREATMENT

1.4.1 Lack of selectivity

According to Workman (2001), the majority of current cancer drugs are cytotoxic agents that exert their effects on all proliferating cells, which includes both normal healthy and cancerous cells. This is the case for very recently successful drugs such as irinotecan (colorectal cancer), taxanes (breast, ovarian and lung), and carboplatin (ovarian cancer). Since cytotoxic agents have a general anti-proliferative action rather than selective anti-cancer properties, the therapeutic efficacy for tumour versus normal tissue is still low, with toxic side effects being the norm. Exposure of normal tissues with a high cellular proliferation rate (such as bone marrow, gastrointestinal epithelial cells and cells of the hair follicles) to anti-proliferative drugs leads to tissue toxicity (Mollinedo and Gajate, 2006). Renal and gastrointestinal toxicity have been reduced considerably in the clinic during treatment with chemotherapeutic agents by intravenous hydration and use of anti-emetics drugs that antagonise the 5-hydroxytryptamine type 3 receptor (Screnci and Mckeage, 1999).

1.4.2 Resistance to drugs

Many tumours are resistant to current chemotherapy or become resistant during treatment (Broxterman *et al.,* 2003). During the past decades, drug resistance research has identified many mechanisms whereby cancer cells can elude chemotherapy (Nygren and Larsson, 2003). Resistance in the clinic manifests itself as an initial lack of significant response (refractiveness) to treatment or re-growth of the tumour after an initial response (Broxterman *et al.,* 2003). In the latter case, the recurrent tumour is almost invariably more resistant to treatment than it was initially. Another manifestation of clinical drug resistance is the frequent occurrence of partial responses. Different subpopulations of tumour cells will invariably be

present in tumours, where the predominant cell type will determine anti-tumour response, but the most resistant cells may determine the probability of complete cure. Another type of resistance, is caused by the multi-drug resistance (MDR) proteins, which are membrane-bound glycoprotein pumps in cells of humans, animals and several other organisms (Wang *et al.,* 2003). These pumps expel foreign substance such as xenobiotics, toxic agents and drugs directly after being absorbed, and they are commonly called multi-drug efflux pumps. The pumps are expressed and distributed in various organs and normal tissues, ranging from lung, liver, kidney, gastrointestinal tract, adrenals, uterus and even capillary endothelium of the brain. Based on impressive pre-clinical data, strategies for circumventing Pgp-mediated MDR using Pgp inhibiting agents like verapimil and cyclosporine A could improve chemotherapeutic outcomes (Nygren and Larsson, 2003).

1.5 DRUG DISCOVERY AND DEVELOPMENT

To successfully research and develop a new drug with sufficient efficacy and safety remains a time consuming and costly process. Despite considerable advances in pharmaceutical technology, a period of 10 to 15 years is still required at an estimated cost of several hundreds of millions US dollars before a drug reaches the market (Kidane and Bhatt, 2005; Lemma *et al.,* 2006). The success rate of compounds from first in human to registration was a meagre 11% for the pharmaceutical industry during the decade 1991–2000, whereas lack of efficacy was a leading cause of termination during the 1990s, toxicity and safety issues contributed together for approximately 30% of failures. Simultaneously, pharmacokinetic- and formulation-related losses decreased dramatically, largely because of changes in drug discovery practices (e.g. introduction of early predictive absorption, distribution, metabolism, elimination (ADME) assays and more-predictive pharmacokinetic (PK) models). The importance of this component of drug discovery was recognized a long time ago but was applied to few compounds usually just before clinical trials. This had a serious impact on the pipeline drug successes because at this advanced stage of the drug discovery process no chemical changes could be incorporated without having to repeat expensive pre-clinical assays. In many cases, projects were abandoned without establishing whether the undesirable side effect was associated with a particular active ingredient or just an accidental effect of the individual molecule. It is

important to emphasize that regulatory authorities require safety pharmacology investigations that integrate pharmacology, physiology and toxicology of new chemical entities to test and assure their safety for human (Whitebread *et al.,* 2005).

The development of new drugs involves the assessment of three major elements in drug design. First, the molecule needs to be active at the intended target (and preferably only the intended target). Second, the desired route of administration should provide bioavailability and therapeutic concentrations must reach the intended target in the patient (e.g. optimized for absorption, protein binding, membrane permeability and favourable metabolic clearance). Finally, the drug should be tolerated by the patient (Whitebread *et al.,* 2005).

The drug research process required to develop a new product is illustrated in Figure 1.2. The first phase in this process is initiated by the discovery of a new drug molecule via exploration of different sources followed by screening of this new molecule to determine its potential as a lead therapeutic agent. The second phase (pre-clinical) entails rigorous testing of the molecule to determine pharmacokinetics and target accumulation and the third phase (clinical studies) to prove safety and efficacy in order to obtain marketing approval from the relevant authorities.

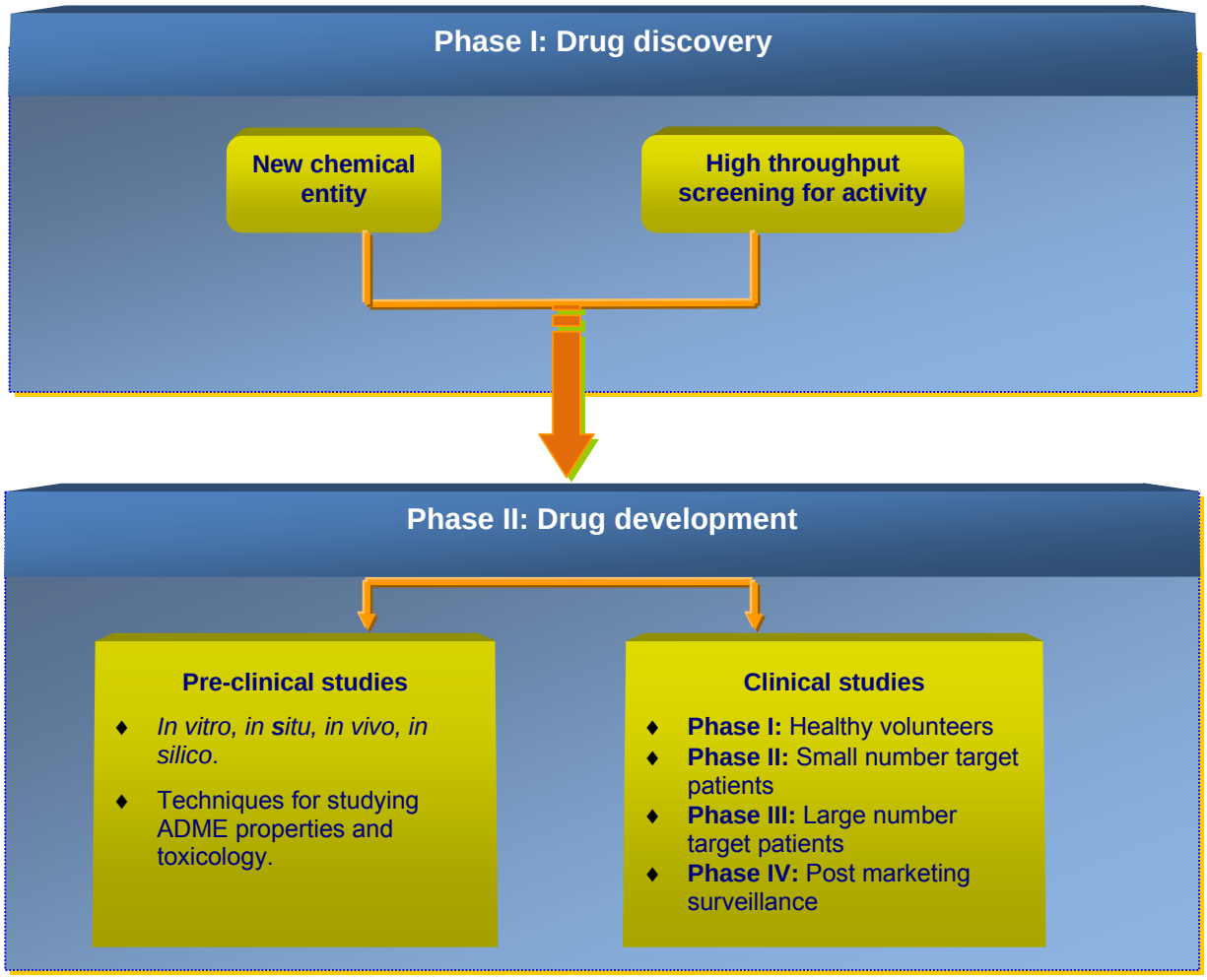

Figure 1.2: Illustration of the process required in the drug research process to develop new therapeutic agents (Adapted from Hamman, 2007).

In drug design and drug development, adequate model systems have to be used at as many early stages as possible to avoid rejection of promising compounds at an advanced stage due to insufficient absorption or distribution throughout the body. Besides toxicity, the pharmacokinetic characteristics were a major reason for failure of compounds in clinical studies in the past (Braun *et al.,* 2000). Simple screening systems and models are therefore very valuable in ranking lead molecules early in the drug development process (Rathbone and Martinez, 2002).

The pre-clinical and clinical stages of drug development are utilised to establish a scientific database on safety, pharmacokinetics and effectiveness of new lead

molecules. Accumulated data obtained from both these stages of drug development are required for registration of a product, especially if containing a new chemical entity, by the appropriate drug regulatory authorities before it may be successfully marketed and legally registered (Hamman, 2007).

1.6 PRE-CLINICAL STUDIES

According to Hamman (2007), the pre-clinical phase of the drug development process plays an important role in selecting candidates with the most appropriate drug-like properties for continuation into the clinical phase. This is an important stage of drug development where the risk of failure in the clinical phase could be avoided, thereby contributing to decreasing development costs when early candidate rejection occurs.

Wide-scale *in vitro* pharmacology profiling of new chemical entities during early phases of drug discovery has become essential to predict possible adverse clinical effects. Modern, relatively inexpensive assay technologies and rapidly expanding knowledge with respect to targets such as G-protein coupled receptors, nuclear receptors, ion channels and enzymes, have made it possible to implement a large number of *in vitro* assays predicting possible adverse clinical effects. Together with appropriate *in vitro* assays focusing on toxicology and bioavailability, this knowledge is a powerful tool to aid drug development. Preclinical safety pharmacology integrates *in vitro* and *in vivo* data to assess potential undesirable pharmacodynamic effects in humans (Whitebread *et al.,* 2005).

Drug discovery and toxicological testing share the need for dependable *in vitro* (cellular) and *in vivo* (animal) toxicity tests. Paillard *et al* (2002), reported that *in vitro* methods for toxicity assessment are the experimental models of choice because of their simplicity in drug development. Animals have conventionally been and still are used in pre-clinical studies to determine the absorption, distribution, metabolism, elimination and toxicity (ADMET) parameters, pharmacological efficacy and the dose ranging of the new drug entities (Streubel *et al.,* 2006).

1.7 DRUG DESIGN AND TARGETS

Advances in science have greatly improved the understanding of drug action, diseases and biological processes, which has resulted in an increased number of potential mechanisms for disease management (Peppas, 2004). Davvis (2005) reported that potential targets that may serve as a starting point for the production of a new drug molecule include receptors, ion channels, proteins and enzymes. These targets can be identified by means of published data in the literature or by research in the fields of cell chemistry and molecular biology and from the human genome. After identification of the drug target, the process then continues with the synthesis of new lead molecules and the screening of their *in vitro* biological activities as well as characterisation of their physicochemical properties and biopharmaceutical characteristics (Kidane and Bhatt, 2005).

According to Landis-Piwowar, advances in anti-cancer therapies have increasingly involved combinations of current cytotoxic agents with new molecular targeting agents (Landis-Piwowar *et al.,* 2006). The literature emphasis on combinational chemistry and the screening of millions of compounds tends to obscure the fact that most desirable drug-like properties are restricted to a relatively small number of compounds (Lipinski, 2000). When evaluating molecular targets for potential combinational therapies, significant drug-development challenges arise. For example, whether to use a single molecular targeting agent, a molecular targeting agent combined with a standard chemotherapeutic drug, or a combination of various molecular targeting agents.

New agents discovered for possible use as anti-cancer therapy include genes, proteins, growth factors and receptors, and compounds involved in specific pathways, such as angiogenesis, signal transduction, the cell cycle, cell apoptosis, cell invasion, metastasis, drug resistance and blood flow (Gupta, 2002) Many current drugs were discovered by trial and error (Landis-Piwowar *et al.,* 2006). Therefore, the brute force methods such as high-throughput synthesis and screening can be an effective approach when the target information is unknown. If the structure of the target is known, a process called ligand-based drug design can be applied in which analysis of known, active ligands are used to find similarity among other, novel ligands that could alter the activity of a target.

1.8 QUINOLINE DERIVATIVES

Medicinal chemistry is paying increasing attention to the development of methods for the synthesis of condensed heterocyclic structures containing indole and pyridine fragments (pyrrole and/or quinoline). The interest in such compounds is due to the prospect of seeking new biologically active substances, since these molecules contain two widely known pharmacophoric moieties (Ferlin *et al.,* 2005).

Sissi and Palumbo (2003) have described quinoline derivatives as topoisomerase II toxins. Antibacterial agents, such as ciprofloxicin, inactivate the bacterial equivalent DNA gyrase (Gyr) and topoisomerase IV (Top4) enzymes, both of which belong to the type II topoisomerase family. These enzymes are essential in all bacteria and are expressed in prokaryotic organisms only. In eukaryote organisms DNA gyrase and topoisomerase IV are functionally replaced by two isoenzymes: topoisomerase II α and β. Eukaryotic topoisomerase II (Top2) is a homodimer. The quinolines and drugs such as etoposide and teniposide do not intercalate with DNA, but increase the DNA cleavage rate. It has been shown that quinolines and etoposide share overlapping but not identical binding sites on topoisomerase II but both need a metal cofactor to stabilize the drug-cleavage complex interactions. (Sissi and Palumbo, 2003).

The quinoline ring is one of the most commonly encountered heterocycles in medicinal chemistry and plays an important role in many biological systems (Metwally *et al.,* 2006). Their chemical synthesis is flexible, can be easily adapted to prepare new derivatives and rationally devised structures, and hopefully can be a source of useful drugs in future. Quinolines possess diverse chemotherapeutic activities including: antibacterial (Metwally *et al.,* 2006; Kayirere *et al.,* 1998), antifungal (Metwally *et al.,* 2006; Musiol *et al.,* 2006), anti-amoebic (Metwally *et al.,* 2006), antimicrobial (Jain *et al.,* 2005), anti-leishmanial (Jain *et al.,* 2005; Metwally *et al.,* 2006), antimalarial (Fujita *et al.,* 2000), augment myocardial contractility (Fujita *et al.,* 2000), inhibit HIV replication (Koga *et al.,* 1998), anti-arteriostenotic (Koga *et al*,1998), and anticonvulsant (Jin *et al.,* 2006; Yanai *et al.,* 2007).

Quinoline derivatives have been shown to be effective against various types of auto-immune diseases such as multiple sclerosis, rheumatoid arthritis, systemic

lupus erythematosis, lupus nephritis (Li *et al.*,2007), and appear to be very effective against select cancer cells (Hiroshi *et al.,* 1999; Chen *et al.,* 2004; Chen *et al.,* 2005; Ferlin *et al.,* 2005; He *et al.,* 2005; Chen *et al.,* 2005; Li *et al.,* 2006; Musiol *et al.,* 2007;Singh *et al.,* 2007).

Vesnarinone (as illustrated in Fig 1.3) is a well-known quinoline derivative that has been reported to have anti-tumour effects. In mice at an administered dose of 30 mg/kg body weight, vesnarinone showed no toxic effects and no significant weight loss. After intraperitoneal injection of MH134 tumour cells, vesnarinone at same concentration of 30 mg/kg body weight increased the mice survival rate up to 21 days compared to 15.7 days in the untreated control group (Hiroshi *et al.,* 1999).

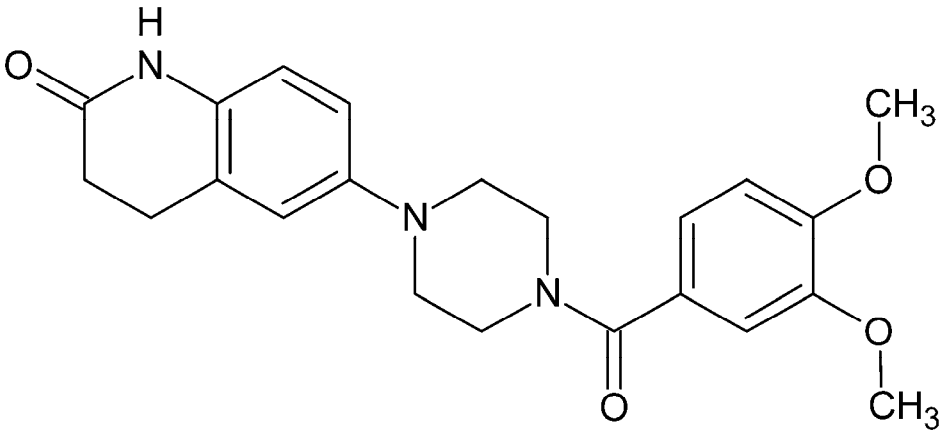

Figure 1.3: Chemical structure of Vesnarinone (3,4-dihydro-6-[4-(3,4-dimethoxybenzoyl)-1-piperazinyl]-2(1H)-quinolinone.

Linomide (Fig 1.4) is a quinine-3-carboxamide, which has no anti-proliferative activity against prostate cancer cells, but was shown to be effective in treatment for prostate cancer due to its anti-angiogenic effects (Vukamovic *et al.,* 1993; Vukanovic and Isaacs, 1995).

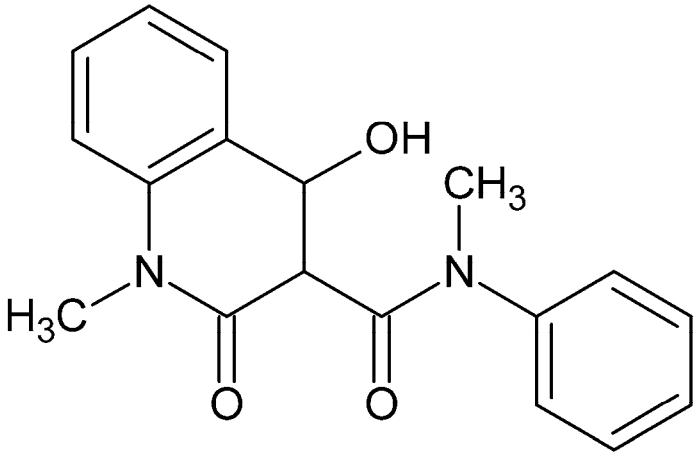

Figure 1.4: Chemical structure of Linomide (N-phenylmethyl-1,2-dihydro-4-hydroxyl-l-methyl-2-oxoquinolone-3-carboxamide)

Various quinoline derivatives, based on the quinoline anti-malarial drug chloroquine (illustrated in Fig 1.5), were designed and successfully synthesised for exploratory anticancer studies by Professor Chibale's medicinal chemistry team at University of Cape Town.

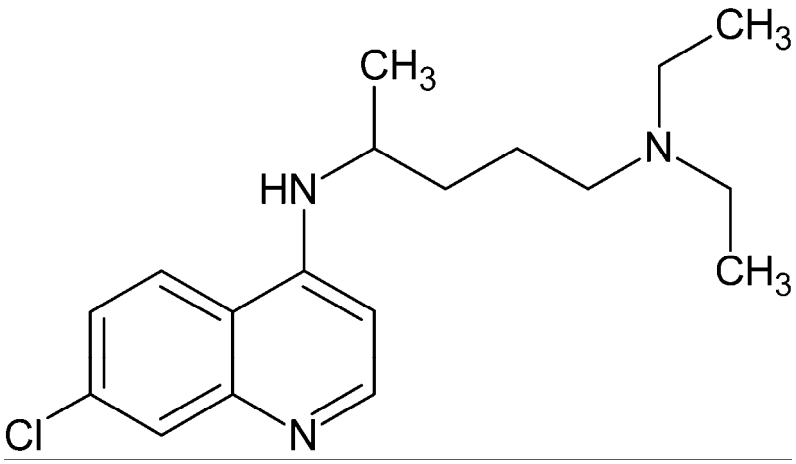

Figure 1.5: Chemical structure of Chloroquine (7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline

Several of these quinoline derivates have been shown to possess potent *in vitro* anti-tumour and multidrug resistant (MDR) reversal properties. Potential compounds were developed and synthesised on a continuous basis and screened for anticancer activity. After *in vitro* screening, a promising lead compound was

identified. 3-[3-(7-chloro-quinolin-4-yl amino) phenyl]-1-(4-methoxy-phenyl) prop-2-enone citrate, “CITme” a quinoline derivative, was identified with cytotoxic anti-tumour activity and subjected to further testing. Although the mechanism of action of CITme is not well understood, it appears to act as a topoisomerase II inhibitor, similar to other quinoline compounds.

In a previous study done in this Department (unpublished results), CITme was compared to linomide to assess anti-angiogenic effects. CITme induced apoptosis in bovine microvascular endothelial cell (BME) cultures similar to that of linomide, thus showing potential antiangiogenic activity.

1.9 PROBLEM STATEMENT

Cancer is one of the leading causes of death all over the world. Although improvements in diagnostic and surgical procedures have made this disease curable if detected and treated appropriately in the early clinical stages, advanced cancer is difficult to remove completely by surgery and is often resistant to available chemotherapeutic agents (Hiroshi *et al.,* 1999).

The difficulty to diagnose the disease at an early stage, the narrow therapeutic indices of anti-cancer compounds, and the problem of multi-drug resistance are some of the major hurdles in the successful application of chemotherapy for cancer (Li *et al.,* 2007; Singh *et al.,* 2007). Therefore, efforts into the development of effective anti-cancer drugs with minimal toxic side effects both *in vitro* and *in vivo* must be continued.

This project continued the growing experience that the Pharmacology Department has with respect to assessing potential anticancer agents and especially CITme, as a quinoline derivative and the toxicological testing experience of the University of Pretoria Biomedical Research Centre (UPBRC).

A thorough search of the PubMed database revealed no such compound has been tested either *in vitro*, using cell culture systems or *in vivo* animal studies.

This study assessed the *in vitro* cytotoxic activity, *in vivo* safety and the pharmacokinetic parameters of CITme, before embarking on a tumour efficacy study on the nude mouse model.

1.10 RESEARCH HYPOTHESIS

3-[3-(7-chloro-quinolin-4-yl amino) phenyl]-1-(4-methoxy-phenyl) prop-2-enone citrate "CITme", a novel quinoline derivative is a highly selective and potent anti-tumour agent with low systemic toxicity *in vivo*.

1.11 STUDY AIM

The aim of this study is to investigate the novel quinoline derivative CITme with regards to cytotoxicity, systemic toxicity, anti-tumour activity and pharmacokinetics.

1.12 OBJECTIVES OF THE STUDY

The specific objectives of this study are:

- Assess the cytotoxic effects and cell line specificity of CITme using primary cell cultures and various selected cancer cell lines;
- Find a suitable formulation of the water insoluble CITme for oral and/or intravenous administration;
- Assess the *in vivo* toxicity and the safe dose of CITme in the BALB/c mouse model using an acute and chronic toxicity study;
- The development and validation of a LC/MS/MS method to determine plasma levels of CITme;
- Determine the basic pharmacokinetic parameters of CITme in BALB/c mice;
- Determine the mouse gender and the cell number required of the prostate cancer cell line (DU-145) to induce tumour growth in nude mice.

1.13 STRUCTURE OF THE STUDY

This research study is divided into seven chapters. The information that will be included in each chapter is as follows:

- ***Chapter One***: The first chapter reviews relevant literature, the research objectives and the purpose of the research.

- ***Chapter Two***: The second chapter discusses the *in vitro* cytotoxicity assays in detail, in order to establish the sensitivity of cancer cell lines and primary cell cultures as well as the tumour selectivity of the experimental compound.
- ***Chapter Three***: The third chapter extensively investigates the solubility problems of the experimental compound and the advantages of using a non-toxic formulation in animal studies. Relationship between solubility and formulation are discussed in detail. The emphasis is on the benefits of using a nano-emulsion formulation that is non-toxic and capable of retaining the drug in solution.
- ***Chapter Four***: This chapter describes and discusses the *in vivo* acute and chronic toxicity studies performed on BALB/c mice to determine the changes in toxicity markers for a week and the safe oral dose to be administered in BALB/c mice for a period of six weeks. All relevant processes will be defined and discussed.
- ***Chapter Five***: This chapter describes a new LC-MS/MS method developed to analyse CITme in plasma and organs, and the pharmacokinetic properties of CITme. Solubility problems will be discussed in this chapter.
- ***Chapter Six***: This chapter will explain the various studies done to induce prostate tumours in nude mice and the problems with this model are discussed in detail.
- ***Chapter Seven***: The research findings are interpreted and discussed and the chapter will contain conclusions and recommendations for further studies.

CHAPTER TWO

IN VITRO CYTOTOXICITY AND TUMOUR SPECIFICITY

2.1 INTRODUCTION

Choosing a good screening system to assess the anti-tumour activity of potential lead chemicals is not a trivial problem. It is even more complicated if, in addition to screening for anti-tumour activity the objective is also to predict real tumour responses in the clinical setting (Hanauske, 1993).

Since the discovery in the 1950's that some human tumours cells could be propagated indefinitely in culture in the presence of the appropriate nutrients and growth factors, human cancer cell lines have played an important part in the discovery, testing and characterization of new chemotherapeutic drugs. A potential weakness of such cell lines is that they may have lost important properties originally possessed *in vivo*, including potential chemotherapeutic targets (Baguley, 2004). Early toxicity prediction has become a very important time and cost concern in drug development. *In vitro* methods for toxicity assessment are the experimental models of choice because of their speed, simplicity and their reduced requirement of test material, and have therefore become an absolute necessity in early drug development (Paillard *et al.,* 2002). Ideally, these tests should be objective, quantitative and reproducible (Cree and Andreotti, 1997) but if toxicity assays use only the accepted standard *in vivo* testing methods, laboratory animal requirements and test material needed per test would increase. By using *in vitro* cell culture based cytotoxicity tests, these requirements could be significantly reduced (Ekwall *et al.,* 2000).

According to Tuschl and Schwab (2004), cytotoxicity can be defined as the adverse effects resulting from interference with structures and processes essential for cell proliferation, function and survival after exposure to potential therapeutic agents. In antineoplastic drugs this cytotoxic effect is sought, but must show a higher selectivity for the cancer cells than the healthy cells of the same person.

In the field of cancer research, a number of *in vitro* tests to assess the growth inhibiting effects of chemotherapeutic agents can be used (Ulukaya *et al.,* 2008).

Many biological assays require the measurement of surviving and/or proliferating mammalian cells in culture and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as originally described by Mossman (1983) is the most commonly used assay at present for the evaluation of cytotoxicity (Ulukaya *et al.,* 2008). According to several authors the MTT assay is widely used for the determination of *in vitro* resistance and sensitivity studies (Nakamura *et al.,* 2006; Kurbacher and Cree, 2005; Furukawa, 2004) where new-drug treated cell numbers are compared to the cell number of untreated controls or to that of cells treated with a known cytotoxic drug.

In this study, a series of *in vitro* cytotoxicity assays were undertaken to establish the sensitivity of several different cancer cell lines and normal primary cell cultures to the experimental drug compound, CITme.

2.2 STUDY AIM

The aim of this study was to investigate the cytotoxicity and tumour specificity of CITme using various cancer cell lines and primary cell cultures.

2.3 MATERIALS

All miscellaneous consumables and chemicals unless otherwise indicated were of highest grade available purchased from Sigma Chemicals and Reagents (St Louis, Missouri). Centrifuge tubes (15 and 50ml), 96 well plates and 75cm^2 culture flasks were purchased from AEC- Amersham (PTY) Ltd (JHB, SA). Glacial acetic acid, ammonium chloride (NH_4Cl), crystal violet solution (0.1%) and sodium bicarbonate ($NaHCO_3$) were purchased from Merck (JHB, SA). Foetal calf serum (FCS) and penicillin/streptomycin were purchased from Adcock Ingram, JHB, SA). FTA hemagglutinine buffer was purchased from scientific group (JHB, SA). Trypsin/EDTA solution (0.25%) was purchased as a sterile solution from Highveld Biological (JHB, SA). Phytohemagglutinine (PHA) was purchased from Bioweb (PTY) Ltd., (JHB, SA).

2.3.1 Experimental drug, CITme

CITme (Figure 2.1) was synthesized for testing at PET Labs facilities in Pretoria following the procedures established by Professor K. Chibale of the Department of Chemistry, University of Cape Town.

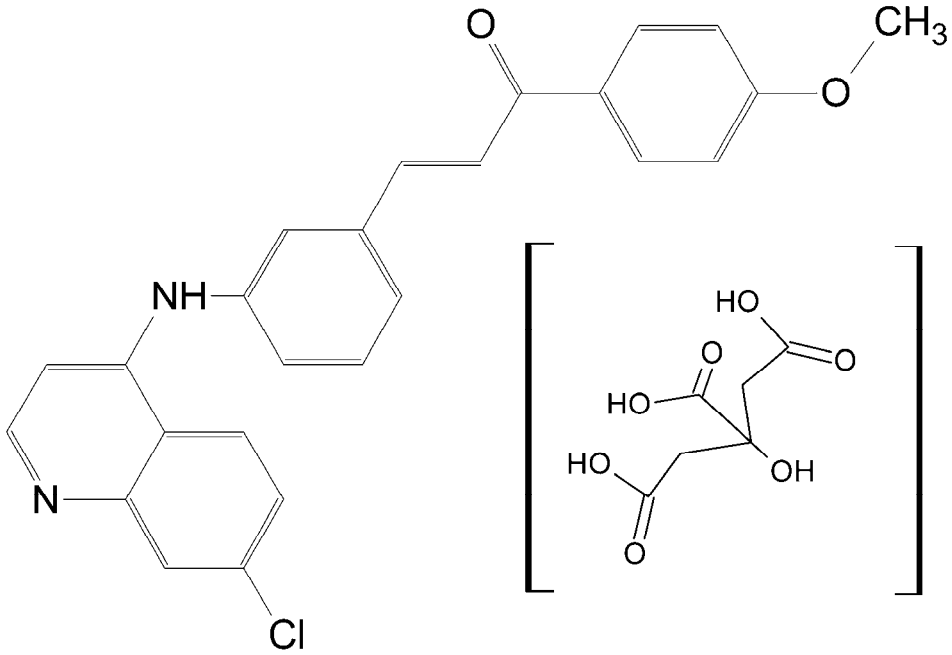

Figure 2.1: Chemical structure of CITme (3-[3-(7 chloro-quinolin-4-yl amino) phenyl]-1-(4 methoxy-phenyl) prop-2-enone) citrate

CITme has a molecular weight of 607.03 g/mol. A 20 mM stock solution was prepared by dissolving 12.14 mg of the compound in 1 ml of DMSO. The CITme was completely soluble in DMSO at this concentration. The stock solution was then frozen in 50 µl aliquots at -70°C. Subsequent dilutions were done in the appropriate tissue culture medium which was supplemented with 10% bovine FCS just before use on the day of the experiment.

2.3.2 Preparation of reagents

The following reagents were used and made up as following:

- **Trypsin/EDTA solution**

A Trypsin/EDTA solution containing the following: 0.25% Trypsin, 0.1% EDTA in Ca^{++} and Mg^{++} free phosphate buffered saline, was supplied by Highveld Biologicals (Sandringham, RSA).

- **White blood cell counting fluid**

One millilitre of 0.1% crystal violet solution was added to 2 ml acetic acid and diluted to 100 ml with de-ionised water.

- **Phosphate buffered saline (PBS)**

Phosphate buffered saline was made by dissolving 9.23 g FTA powder in 1,0 litre de-ionised water as per manufacturer directions.

- **MTT solution**

Two hundred and fifty milligrams of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide),was dissolved in 50 ml PBS solution (5 mg/ml) and filter sterilized through a 0.22 µm syringe filter and stored refrigerated at 4°C in a dark container until used.

- **Penicillin/streptomycin**

Five millilitres of purchased penicillin/streptomycin solution was added to 500 ml tissue culture media.

- **Phytohemagglutinin (PHA)**

The content of the vial as purchased was dissolved in 5 ml of sterile saline and dispensed into 0.4 ml aliquots. Aliquots were stored at -20°C. When required an aliquot was added to1.6 ml RPMI supplemented with 10% FCS for use in the cell cultures requiring the presence of a mitogen.

- **Foetal calf serum (FCS)**

Purchased FCS was heat inactivated in the original container under sterile conditions for 45 minutes at 56°C. This solution was centrifuged under sterile conditions at 2000*g* and the supernatant added to the relevant tissue culture media at a concentration of 10%.

- **Ammonium chloride solution**

8,3g NH_4Cl, 1g $NaHCO_3$ and 74mg EDTA were dissolved in distilled water, diluted to 1 litre and filter sterilised using a 0.22µm syringe filter.

- **Tissue culture media**

The pH was adjusted further, if necessary, by adding small quantities of 1N HCl or 1N NaOH until an end pH of 7.4 was obtained. Medium was filtered under vacuum using a Sartorius 0.22 µm cellulose acetate filter. The medium was filtered a second time using two Sartorius glass-fibre pre-filters which preceded the sterile Sartolab P pressure filtration unit (0.2 µm). A Heidolph peristaltic pump was used to force the medium through the filters. Medium was dispensed into sterile 500 ml flasks containing 1% penicillin/streptomycin. Medium was stored at 4°C until needed. Just before use the medium was supplemented with 5 - 10% bovine FCS as and if required.

- **RPMI (RPMI-1640)**

RPMI was purchased Sigma (St Louis, USA). Fifty-two grams was dissolved in 5 L of autoclaved ultra pure, pyrogen-free, de-ionised water (produce by an in house Elga PureLab Ultra water unit). Ten grams of $NaHCO_3$ (Sigma S5761) was added just before filtration to adjust the pH.

- **EMEM (Earl's Minimum Essential Medium)**

EMEM was purchased from Sigma (St Louis, USA). Forty-eight grams of EMEM was dissolved in 5 L of autoclaved ultra pure, pyrogen-free, de-ionized water (produce by an in house Elga PureLab Ultra water unit). Eleven grams of $NaHCO_3$ was added just before filtration to adjust the pH.

- **DMEM (Dulbecco's Minimum Essential Medium)**

DMEM was purchased from Sigma (St Louis, USA). Sixty-seven point three five grams of DMEM was dissolved in 5 L of autoclaved ultra pure, pyrogen-free, de-ionised water (produce by an in house Elga PureLab Ultra water unit). Eighteen grams of $NaHCO_3$ was added just before filtration to adjust the pH.

2.3.3 Cell line and media required

For *in vitro* toxicity study, cancer cell lines and primary cell cultures were used in order to determine CITme cytotoxic activity. Table 2.1 summarises the cells used

in this study with their main characteristics. The sections following describe the growth conditions for each of the cell lines.

Table 2.1: Cancer cells and primary cell cultures used in this study to determine tumour specificity and selectivity (tumour vs. normal)

Tumour cell line	Species	Tissue type	Parameter evaluated with MTT assay
HeLa	Human	Cervical	Concentration range, tumour specificity
CoLo	Human	Colon	Tumour specificity
MCF-7	Human	Breast	Tumour specificity
DU-145	Human	Prostate	Tumour specificity
Jurkat T	Human	T-Lymphocyte	Tumour specificity
Primary cell cultures	**Species**	**Tissue type**	**Parameter evaluated with MTT assay**
Embryo fibroblasts	Chicken	Fibroblasts	Selective toxicity
Resting lymphocytes	Human	Lymphocytes	Selective toxicity
PHA stimulated lymphocytes	Human	Lymphocytes	Selective toxicity

2.3.3.1 Cancer cell lines

- HeLa cells (ATCC no CCL-2) are adherent cervix derived adenocarcinoma cells, maintained in Eagles minimum essential medium (EMEM), containing 2mM L-glutamine, 0.1mM non-essential amino acids, 1.0mM sodium pyruvate and 5% FCS (EMEM/5%FCS).

- MCF-7 cells (ATCC no HTB-22) are adherent breast derived adenocarcinoma cells, maintained in Dulbecco's minimum essential medium (DMEM), containing 2mM L-glutamine, 0.1mM non-essential amino acids, 1mM sodium pyruvate and 5% FCS (DMEM/5%FCS).

- DU-145 cells (ATCC no HTB 81) are adherent prostrate derived carcinoma cells, maintained in RPMI 1640 medium with 10% FCS (RPMI/10%FCS).

- CoLo 320 DM cells (ATCC no CCL-220) are non-adherent colon derived adenocarcinoma cells that grow loosely attached and in suspension which are rounded and refractile. These cells were maintained in RPMI/10%FCS.

- Jurkat cells (NRBM no 0062) are non-adherent immortal T-lymphocyte cells that grow loosely attached and in suspension, are rounded and refractile and were maintained in RPMI/10% FCS.

2.3.3.2 Primary cell cultures

- Chicken embryo fibroblasts are primary cell culture, which are isolated from chicken embryos as described below and were maintained in RPMI/10% FCS.

- Human lymphocytes are primary cell culture, which are isolated from blood of healthy donors. Lymphocytes were prepared as previously described by Anderson and co-workers (Anderson, Smit and van Rensburg, 1993) as described below and were cultured in RPMI/10% FCS.

2.4 METHODS

2.4.1 Cultivation of cancer cell lines

Cell cultures were prepared in the prescribed ATCC tissue culture medium (specified for each cell line) supplemented with 10% bovine FCS in 75 cm^2 culture flasks and sub-cultured three times a week. Tissue culture medium was discarded from the tissue culture flask containing the cells at approximately 80% confluence that were to be used for the cytotoxicity assays. Approximately 5 ml trypsin/EDTA solution was added, and care was taken to gently coat all interior surfaces with the solution, before it was decanted. Another 2 to 5 ml was added to cover the adherent cells on the bottom of the flask. The culture flask with trypsin/EDTA solution was then incubated at 37˚C for a few minutes until the cells detached from the flask. The trypsin/EDTA cell suspension was transferred to a sterile 15 ml centrifuge tube. Tissue culture media supplemented with 10% bovine FCS was used to rinse the culture flask and this was used to top up the cell suspension in the centrifuge tube, thus neutralizing the action of trypsin/EDTA.

Cell cultures that grow in suspension were decanted directly into 15 ml centrifuge tubes without trypsin/EDTA solution addition. The cell suspension was centrifuged for 6 minutes at 200*g*. The supernatant was then discarded and the cell pellet was re-suspended in 1 ml of the tissue culture medium specified for each cell line, supplemented with bovine FCS. The cell suspension was mixed by gently pipetting repeatedly to make sure that the cells were separated from each other and that a uniform suspension was formed.

2.4.2 Establishment of primary cell cultures

- **Chicken embryo fibroblasts**

Fertilised chicken eggs were incubated at 38.5°C in a 65% relative humidity atmosphere for six days. They were kept in an egg incubator that rolled them over once in 24 hours. After that, all procedures were carried out under sterile conditions.

Eggs were swabbed with 70% alcohol and placed with its blunt end facing up in a small beaker. The top of the shell was gently cracked and the shell peeled off to the edge of the air sac, by using forceps. The forceps were re-sterilized and used to peel off the white shell membrane to reveal the chorioallantoic membrane (CAM) below, with its blood vessels. The CAM was pierced with sterile curved forceps and the embryo lifted out by grasping it gently under the head. Embryos were transferred to a sterile Petri dish and decapitated to kill them instantly. Fat and any loose or necrotic material was removed from the embryos, and then the embryos were transferred to a second sterile Petri dish.

Embryos were cut with crossed scalpels into very small pieces and transferred to a sterile 50 ml centrifuge tube and washed three times with RPMI/10% FCS. Tissue was allowed to settle each time before the medium was discarded. Tissue was transferred to a sterile 100 ml glass bottle with a screw cap (Schott bottle), where most of the residual fluid was removed with a Pasteur pipette. Forty five millilitres of PBS (filter sterilised) with 5 ml of 2.5% trypsin/EDTA were added to the tissue mixture together with a magnetic stirrer and the flask closed and stirred at 100 rpm for 30 min at 37°C. Pieces of tissue were allowed to settle, and the supernatant was poured (with disaggregated cells in suspension) into a sterile 50ml centrifuge

tube and placed on ice. These steps were repeated until the disaggregation was complete (3-4 hours). The disaggregated cell suspension that was stored on ice was decanted into new sterile tubes (leaving the debris behind) and then centrifuged at 200*g* for 5 minutes. The supernatant was discarded and cell pellet re-suspended in RPMI/10%FCS. The cell suspension was transferred to 75 cm^2 cell culture flasks and incubated at 37°C in an atmosphere containing 5% CO_2. Cultures were maintained in RPMI/10%FCS and the medium was changed at regular intervals (2-4 days) as pH decreased (growth medium turns yellow).

- **Human lymphocytes**

The lymphocytes were prepared under sterile conditions from whole blood, as follows: Thirty millilitres of heparinised blood was loaded carefully onto 15 ml Histopaque 1077 in a sterile 50 ml Falcon centrifuge tube with a Pasteur pipette and centrifuged for 25 minutes at 650*g* at room temperature. The top plasma layer was removed with a Pasteur pipette and discarded. The lymphocyte/monocyte layer (±12 ml the distinct band of cells that had just penetrated the Histopaque layer) was transferred to a sterile 50 ml centrifuge tube with a Pasteur pipette. The tube was filled with sterile RPMI/10% FCS and centrifuged for 15 minutes at 200*g* at 25°C (to remove contaminating platelets). The supernatant fluid was discarded, the lymphocyte pellet re-suspended in sterile RPMI-1640 without bovine FCS. This cell suspension was once again centrifuged for 10 min at 200g at room temperature of 25°C.The supernatant was discarded and the cell pellet was re-suspended in 50 ml sterile, ice cold ammonium chloride. The cell suspension was left on ice for ±10 minutes (to lyse contaminating red cells). The cell suspension was centrifuged for 10 minutes at 200g at 25°C. The supernatant was discarded and the pellet was re-suspended in 50 ml sterile RPMI medium without bovine FCS. The cell suspension was centrifuged for 10 minutes at 200*g* at 25°C and the supernatant discarded. The cell pellet was re-suspended in 1 ml of sterile RPMI medium supplemented with 10% bovine FCS and a 50 µL aliquot counted in a haemocytometer. Cells were diluted in sterile RPMI/10%FCS to $2x10^6$ cells per ml, the concentration needed for the proliferation experiments. Resting and PHA stimulated lymphocytes were used in cytotoxicity tests.

- **Cells counting**

Fifty microlitres of the cell suspension that was prepared as described above was added to 450 µl of cell counting fluid and mixed well. A small amount of this (± 20 µl) was placed onto a haemocytometer and the cells were counted using a Reichert Jung Micro Star microscope at 400 x magnification. Dilutions of the cell suspensions were then made with the specific tissue culture medium for each cell line used, supplemented with 10% bovine FCS to achieve the required cell concentration for the cytotoxicity assay (or cell proliferation assay).

2.4.3 Cytotoxicity assay

Tissue culture plates (96 well plates) were divided in two sections for controls (untreated cells) and experimental compound (treated cells). Eighty microlitres of the specific tissue culture medium for each cell line supplemented with 10% bovine FCS (60 µl in the case of lymphocytes earmarked to be PHA stimulated) was dispensed into each of the wells of a 96 well tissue culture plate. One hundred microlitres of homogenous cell suspension was added to each well. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 for one hour. Twenty microlitres of the test compound (at different concentrations) was added to the "treated" cells wells. Untreated control wells received 20 µl of culture medium only. A control set with DMSO at the same concentration as in the test compound were also made to assess any DMSO toxicity effect. Lymphocytes earmarked to be stimulated, received 20 µl of PHA five minutes after the addition of the drug. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 for three and seven days (with the exception of the lymphocytes) respectively before performing the MTT assay.

HeLa and DU-145 cells were used at a final concentration of 500 cells per well. CoLo, MCF-7, were used with a final concentration of 400 cells per well, whereas Jurkat cells had a concentration of 600 cells per well. Primary chicken fibroblasts were used with a final concentration of 50 000 cells per well, while the human lymphocytes had a final concentration of 200 000 cells per well.

2.4.4 MTT assay (Mossman, 1983)

After the incubation period (3 or 7 days) at 5% CO_2 and 37°C, 20 µl of sterile MTT stock solution in PBS was added to each well. All cultures were then incubated for

a further four hours (except the chicken fibroblasts that had to be incubated for 24 hours at 37°C in an atmosphere of 5% CO_2) and then centrifuged for 10 minutes at 800*g*. Supernatant was carefully removed from each well without disturbing the pellet with a Pasteur pipette and cells were washed by addition of 150 µl of PBS and centrifuged for 10 minutes at 800*g*. Supernatant was again removed with a Pasteur pipette from each well without disturbing the pellet and allowed to dry for approximately an hour before adding 100ul DMSO. The plates were put on a shaker for about 1 to 2 hours to solubilise the coloured formazan crystals and absorbance was measured on a Universal Microplate Reader (ELx800 UV, Bio-Tek Instruments) using a wavelength of 570 nm and a reference wavelength of 630 nm.

2.5 DATA AND STATISTACAL ANALYSIS

Percentage of control of cell growth in drug treated cells was calculated by dividing the mean absorbance of the different concentrations of drug treated cell replicates by the mean absorbance of all the untreated controls x100, and these values were used to determine the IC_{50} (the concentration of the experimental compound inducing a 50% decrease in cell growth). IC_{50} value was calculated with the GraphPad software program Prism version 5 using the Lowess curve fit algorithm. "Relative tumour specificity" was calculated by dividing the mean IC_{50} values of the primary cell cultures by the mean of the cancer cells. Tumour specificity is a very broad indicator of the selectivity of the drug.

Results are expressed as mean IC_{50} ± SEM for treated cells. Each value represents the mean of three independent experiments using at least three replicates per experiment. The Student's t-test for unpaired values was used for the statistical analysis of the data. *P* values of less than 0.05 ($p<0.05$) were considered to be statistically significant.

2.6 RESULTS

Cytotoxicity assays were conducted to determine the level of sensitivity as well as the selectivity (relative tumour specificity) of cancer and normal cells to the experimental compound. In the present study, the cell growth/survival rate was assessed by the MTT assay.

Figures 2.2-2.8 and Figures 2.9-2.14 represents the percentage of control ± SEM of cell growth of the cell lines treated with CITme for a period of three and seven days respectively, using different CITme concentrations on primary cell cultures (resting and stimulated (PHA) lymphocytes and chicken embryo fibroblasts) and various cancer cell lines (DU-145, HeLa, CoLo, MCF-7 and Jurkat cells). CITme was more cytotoxic against cancer cell lines than in normal cells after both three and seven days in culture. After three days incubation period, resting lymphocytes appeared to be more sensitive than stimulated (PHA) lymphocytes. However, the degree of sensitivity of resting lymphocytes was still less than the cancer cell lines used in this study, such as DU-145, CoLo and Jurkat cancer cells. Of all the cell lines (both normal and cancer cells) treated with CITme for three days, DU-145 cells appeared to be the most sensitive; whereas, HeLa and PHA stimulated lymphocytes were the least sensitive.

On the other hand, when cells were treated with CITme for seven days, which is the normal period of incubation for cancer cell lines and the primary chicken embryo fibroblast cells, HeLa cells were the most sensitive followed by DU-145 cells, while chicken embryo fibroblasts did not show any sensitivity to CITme, even when treated at higher concentrations.

Table 2.2 represents the mean IC_{50} ± SEM for cancer and normal cell lines treated with different concentrations of CITme for three and seven days incubation. After three days incubation period, CITme was more cytotoxic towards DU-145 cells, with an IC_{50} of 11.030 µM, while on resting lymphocytes, it had an IC_{50} of 26.488 µM. No sign of cytotoxicity was observed on stimulated lymphocytes, even at concentrations of 50 µM. In the seven days incubation time experiments, CITme was more cytotoxic against HeLa cells, with an IC_{50} of 0.872 µM, followed by DU-145 with an IC_{50} of 2.543 µM, with no observed toxic effect against chicken embryo fibroblasts, at any of the concentrations used for this test.

Tumour specificity was calculated as the average of the IC_{50} values of the primary lymphocytes cell cultures divided by the average of the IC_{50} values of the cancer cells. Table 2.3 represents the tumour specificity of CITme, which was calculated by dividing the mean IC_{50} values of the primary lymphocytes cell cultures by the

mean IC_{50} values of the cancer cells. CITme appeared to be more selectively cytotoxic towards cancer cells, with more specificity to HeLa and DU-145 than to normal cell lines.

According to Mossman (1983), MTT is a convenient and rapid colorimetric assay for cell viability. This substance has a pale yellow colour and is reduced by mitochondrial dehydrogenases enzymes, normally responsible for the production and maintenance of ATP levels for the cellular function. A dark purple insoluble formazan results from the reduction of the tetrazolium ring of MTT and is an indicator of mitochondrial function. As this conversion takes place only in living cells, the amount of formazan produced correlates with the number of viable cells present.

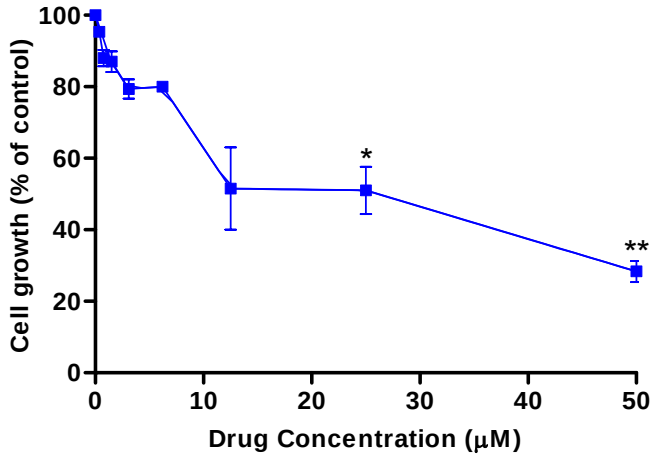

Figure 2.2: Mean growth inhibition of CITme on resting lymphocytes cells after three days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean; *p < 0.05, **p < 0.01

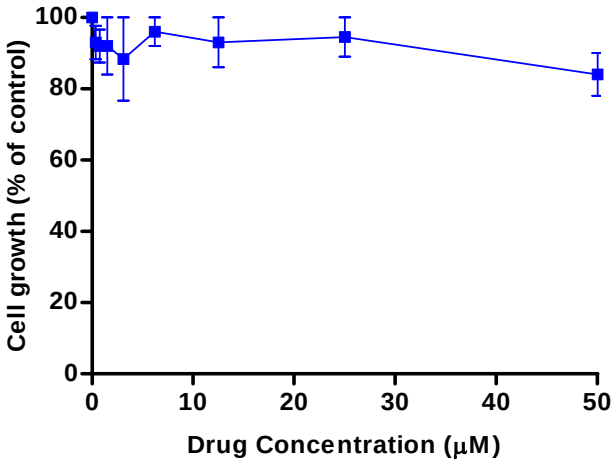

Figure 2.3: Mean growth inhibition of CITme on PHA lymphocytes cells after three days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean

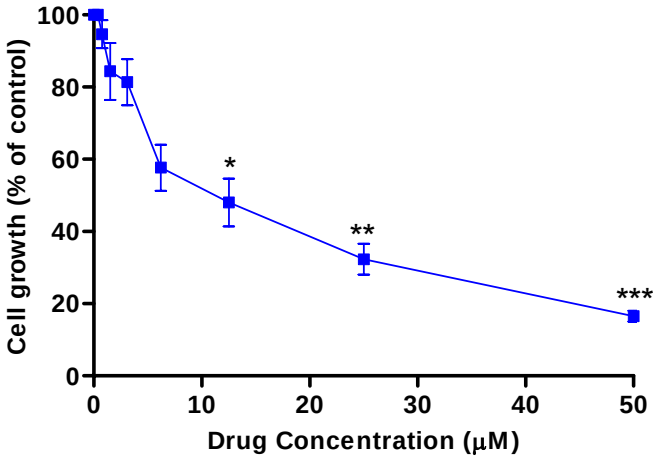

Figure 2.4: Mean growth inhibition of CITme on DU-145 cells after three days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean; *p < 0.05, **p < 0.01, ***p < 0.005

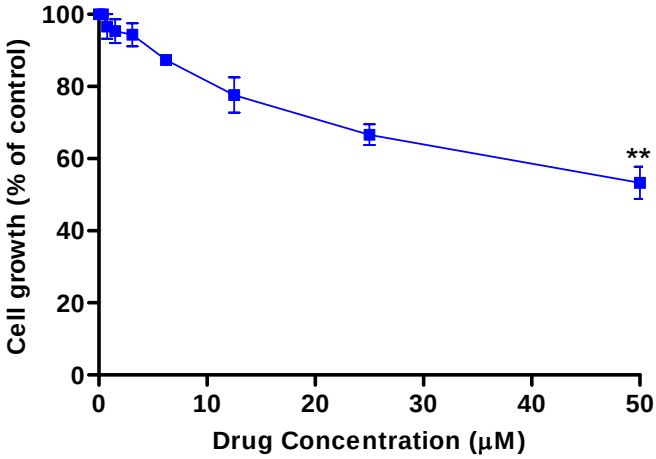

Figure 2.5: Mean growth inhibition of CITme on HeLa cells after three days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean; *p < 0.05, **p < 0.01

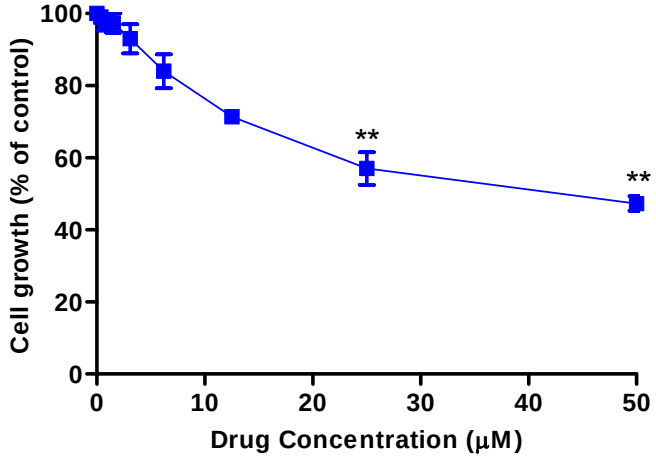

Figure 2.6: Mean growth inhibition of CITme on MCF-7 cells after three days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean; *p < 0.05, **p < 0.01

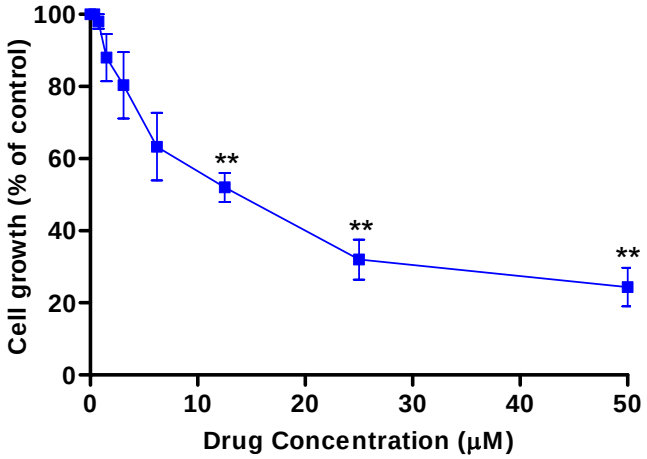

Figure 2.7: Mean growth inhibition of CITme on CoLo cells after three days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean; *p < 0.05, **p < 0.01

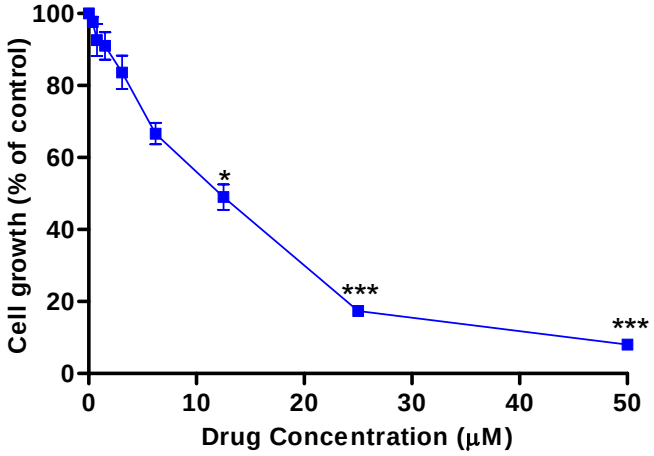

Figure 2.8: Mean growth inhibition of CITme on Jurkat cells after three days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean; *p < 0.05, **p < 0.01, ***p < 0.005

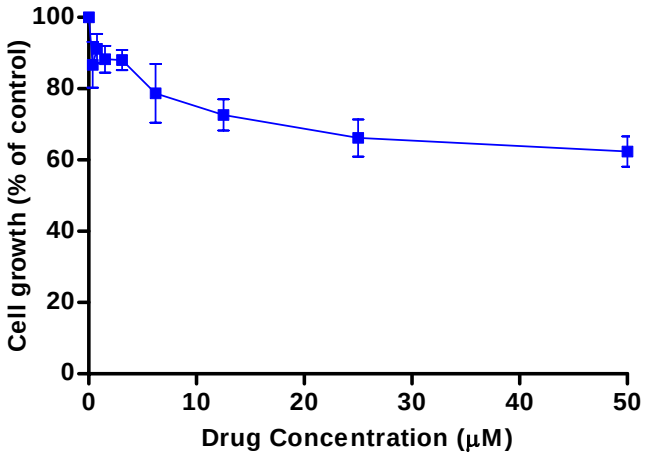

Figure 2.9: Mean growth inhibition of CITme on chicken embryo fibroblasts cells after seven days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean

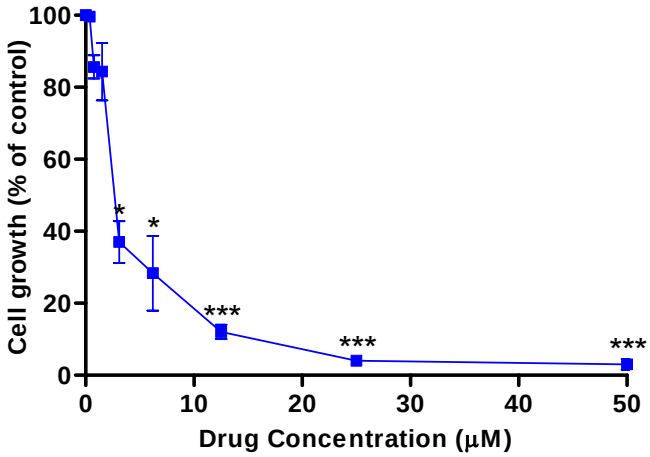

Figure 2.10: Mean growth inhibition of CITme on DU-145 cells after seven days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean; *p < 0.05, **p < 0.01, ***p < 0.005

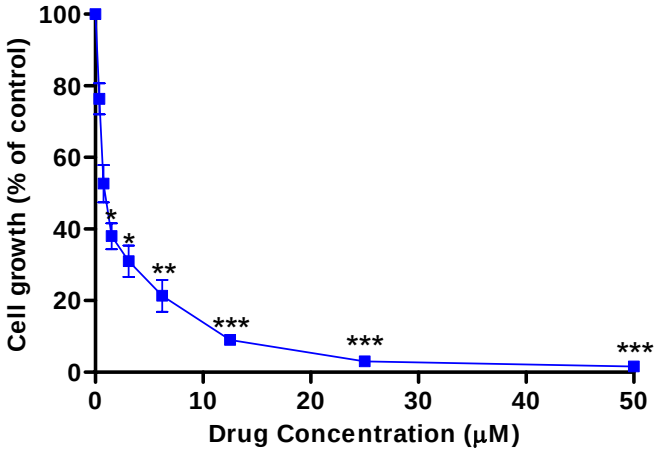

Figure 2.11: Mean growth inhibition of CITme on HeLa cells after seven days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean; *p < 0.05, **p < 0.01, ***p < 0.005

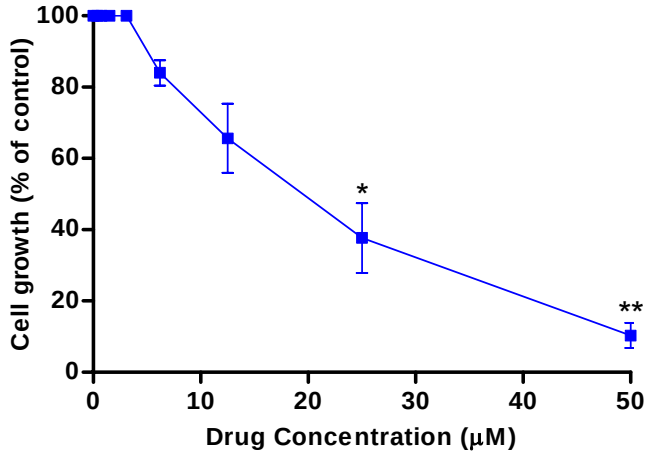

Figure 2.12: Mean growth inhibition of CITme on MCF-7 cells after seven days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean; *p < 0.05, **p < 0.01

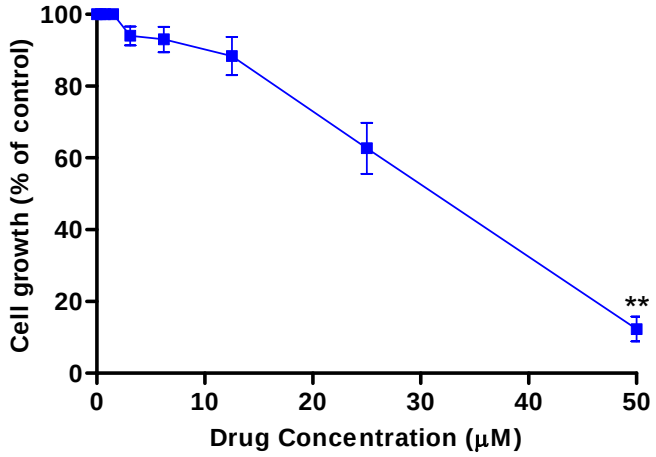

Figure 2.13: Mean growth inhibition of CITme on CoLo cells after seven days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean; *p < 0.05, **p < 0.01

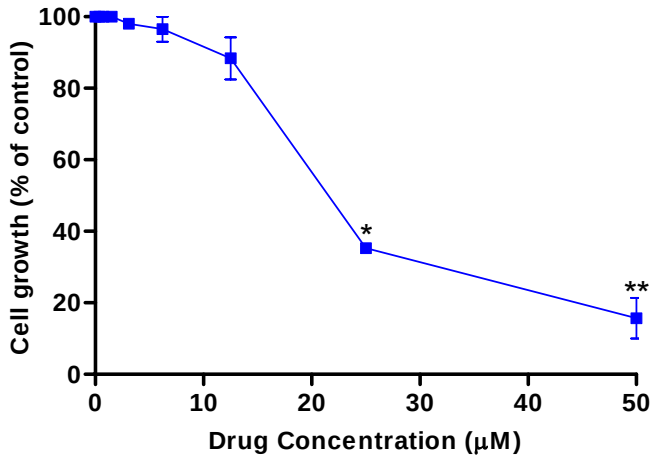

Figure 2.14: Mean growth inhibition of CITme on Jurkat cells after seven days incubation period. Data presented as percentage of control (n=9) ±SEM; SEM: standard error mean; *p < 0.05, **p < 0.01

Table 2.2: Represents the mean IC50 ± SEM of CITme for cancer and normal cells lines after three and seven day's incubation periods

CITme IC_{50} (µM) values and SEM (±) of CITme on various cancer and normal cell lines after three days incubation period		
Cell line	**CITme IC_{50} (µM)**	**SEM (±)**
HeLa	46.063	± 1.31
DU-145	**11.030**	± 4.403
MCF-7	29.777	± 6.090
CoLo	12.459	± 3.742
Jurkat	12.479	± 1.237
Resting Lymphocytes	**26.488**	± 7.904
PHA Lymphocytes	**>50**	-
CITme IC_{50} (µM) values and SEM (±) of CITme on various cancer and normal cell lines after seven days incubation period		
Cell line	**CITme IC50 (µM)**	**SEM (±)**
HeLa	**0.872**	± 0.157
DU-145	**2.543**	± 0.372
MCF-7	19.283	± 4.33
CoLo	30.813	± 2.269
Jurkat	21.385	± 0.476
Chicken embryo fibroblasts	**>50**	-

Table 2.3: Represents the tumour specificity of CITme, which was calculated by dividing the mean IC50 values of primary cultures by the mean IC50 values of the cancer cells

Calculated tumour specificity of CITme tested on cancer and normal cell cultures	
CITme	**43.86**

2.7 DISCUSSION

As been reported by Spielmann and Goldberg (1999), *in vitro* experiments involving cell and tissue cultures have become a crucial approach to elucidate the consequences of exposure and to assign risk since the early 1990s. *In vitro* experiments are routinely used to screen compounds for their biological effects, to determine the presence or absence of a specific biological effect within a chemical class and to attempt to identify the mechanism of action of a chemical or group of chemicals. Furthermore, all industries and regulatory authorities for toxicity testing, safety assessment and risk evaluation routinely use *in vitro* techniques.

The current use of *in vitro* techniques in drug development in order to assess the cytotoxicity properties of novel compounds, offers several advantages such as:

- controlled testing conditions,
- reduction of systemic effects,
- reduction of variability between experiments,
- same dose range can be tested in a variety of test systems,
- time-dependent studies can be performed and
- time-dependent samples taken,
- testing is fast and
- relatively cheap,
- small amounts of test material are required,
- limited amount of toxic waste is produced,
- human cells and tissues can be used,
- transgenic cells carrying human genes can be used and
- a reduction of testing animals

All these factors taken together make *in vitro* culture techniques an optimal screening method to be used in the early pre-clinical drug development phase (Li, 2004).

In this study, selected cancer cells were treated with CITme for three and seven consecutive days. The reason for this was to have a good comparison between the cancer cell lines and the normal primary cells, since normal lymphocytes can only

be successfully incubated for three days without addition of a cytokine cocktail. The results suggest that CITme starts exerting its cytotoxic effects immediately after exposure to treatment, especially against DU-145. However, its full cytotoxic effects were better observed after seven days treatment probably due to a larger percentage of cells entering the cell cycle during this longer incubation time.

It is well known that the utility of cancer chemotherapy is considerably restricted by the toxic side effects of anticancer drugs to healthy tissue. This restriction results from the fact that the anticancer drugs used in the present chemotherapy lack high selectivity for malignant cells (Masayuki *et al.,* 1990).

CITme proved to be cytotoxic towards cancer cells at very low concentrations, more specifically to HeLa (human adenocarcinoma of the cervix) and to a lesser extent to DU-145 (prostate cancer) cancer cells with no or minimal effect against normal cells (resting and stimulated lymphocytes and chicken embryo fibroblasts). The potential cytotoxic activity of CITme against cancer cells are in line with other quinoline derivatives (Ferlin *et al.,* 2005; He *et al.,* 2005; Chen *et al.,* 2005; Li *et al.,* 2006; Musiol *et al.,* 2007; Singh *et al.,* 2007). Furthermore, this efficient cytotoxicity against cancer cells and the potential selectivity property makes CITme a potential drug of choice for cervix adenocarcinoma and/or prostate cancer chemotherapy. However, further pre-clinical data would still need to be obtained before a first in human trial can be planned and developed.

In vitro methods that use mammalian cell cultures and various cytotoxic endpoints have been proposed as alternatives to *in vivo* acute oral toxicity tests that use rodents (http://iccvam.niehs.nih.gov/). Spielmann *et al.* (1999) also proposed that cytotoxicity data obtained *in vitro* could be used to predict the *in vivo* acute toxicity of chemicals. The number of animals used in acute toxicity testing could therefore be reduced by applying this approach. However, a major drawback of *in vitro* system is that each cell type is studied in isolation, whereas in the human body, there might be multiple organ interactions that are critical to drug toxicity (Li, 2005; Li *et al.,* 2004). Besides that, there are several limitations to the use of *in vitro* studies such as:

- general side effects cannot be assessed,

- systemic effects cannot be evaluated,
- interaction between tissues and organs cannot be evaluated,
- specific organ sensitivity cannot be assessed,
- chronic effects cannot be tested (Li, 2004).

Furthermore, no drug can enter the clinical phase based only on data from *in vitro* assays. A series of *in vivo* examinations in animal species are essential for clearance, before entering clinical trials. Nevertheless, general pharmacological, toxicological and ADME evaluations *in vivo* are prerequisites for any clinical trial. *In vitro* studies cannot predict cumulative effects during chronic treatment and dose related pharmacokinetic-pharmacodynamic (PK–PD) relationship, neither can these assays address the complexity of the *in vivo* performance of the compounds at promiscuous targets (Whitebread *et al.,* 2005).

CHAPTER THREE

FORMULATION OF THE NON SOLUBLE QUINOLINE DERIVATIVE WITH POTENTIAL ANTICANCER ACTIVITY

3.1 INTRODUCTION

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. In recent years, research and development in the field of site-specific drug therapy has progressed significantly due to an understanding of the effect at various target sites or receptors (Katzung, 2004). However, poor aqueous solubility has limited the efficacy of many potential drugs and increases their observed side effects (Monzer, 2007).

According to Parijat and Yalkowsky (2007), poor drug solubility is a widespread concern in the pharmaceutical sciences, especially when screening new chemical compounds and in formulation research. Development of new drug delivery systems to enhance the effectiveness of existing drugs is an important research area within anticancer research. One useful new pharmaceutical dosage form for overcoming solubility problems is solubilisation of cancer drugs using surfactant systems in the form of micro- or nano- emulsions (Monzer, 2007).

The design of effective formulations for drugs has long been a major challenge, because drug efficacy can be severely limited by instability or poor solubility within the vehicle excipients. One of the most promising new technologies is the nanoemulsion drug delivery system, which is being applied to enhance the solubility and oral bioavailability of the poorly water-soluble drugs. In addition, nanoemulsions have been reported to improve the plasma concentration profiles and bioavailability of drugs and show better reproducibility of these plasma concentration profiles (Sheikh *et al.,* 2007).

The ability of nanoparticles to improve drug diffusion through biological barriers is another typical benefit for the delivery of anticancer agents. Some studies have indicated that administration of nanoparticle-bound anti-tumour agents showed

longer drug retention in tumours, improved reduction in tumour growth and prolonged survival of tumour-bearing animals (Mu and Feng, 2003). Therefore, there is strong argument to develop drug delivery systems by which cytotoxic drugs can very selectively be targeted at tumour tissue and effectively act on mainly cancer cells within functional living systems (Hamaguchi *et al.,* 2005).

Previous studies done in our department (unpublished results) showed that CITme is not sufficiently soluble for *in vivo* dosing in water (or aqueous buffers), ethanol, ethyl acetate, chloroform, dilute sodium hydroxide, ammonium hydroxide, glycerine, sesame oil or olive oil. CITme showed limited solubility in pure methanol, acetonitrile and the lipophilic emulsion stabiliser, Cremophor EI after solubilising in DMSO. However, on addition of distilled water even at low percentages CITme precipitated from these emulsions, in some cases within minutes. Of all the solvents tested, CITme was only soluble in pure DMSO (dimethyl sulphoxide), DMF (dimethyl formamide), and formic acid. Unfortunately, none of these solvents are suitable for *in vivo* studies due to high toxicity. The highest acceptable dose of DMSO for use in animals is 0.5%. A suspension, using DMSO (0.5%) and Cremophor EL was tested, but unsuccessful. At a low concentration, CITme was partially soluble in Cremophor EI, but as soon as the drug concentration was increased to dose relevant concentrations, the CITme precipitated from the solution.

Due to the known water solubility problems and the requirement for a non-toxic formulation for either oral or intravenous *in vivo* administration of CITme, various approaches were used to develop a suitable formulation that would be stable and non-toxic for use in acute and chronic toxicity studies.

3.2 STUDY AIM

The purpose of this investigation was to formulate the poorly soluble drug into a non-toxic formulation and to determine the *in vivo* acute and chronic toxicity of CITme in BALB/c mice. Several similar quinoline derivatives have been tested *in vivo* for toxicity and found to have no significant toxic effects at concentrations of up to 30 mg/Kg in healthy mice (Hiroshi *et al.,* 1999), CITme was therefore initially tested at an equivalent concentration in an acute toxicity mouse model.

3.3 MATERIALS AND METHODS

Ethical approvals by the AUCC (Animal Use and Care Committee) of the University of Pretoria were obtained (Protocol No. H009/07 and H030/07). The study was conducted at the University of Pretoria Biomedical Research Centre (UPBRC) from August 2007 to February 2008.

- **Animals**

The animals (female BALB/c mice and Sprague Dawley rats) used for these studies were housed at the UPBRC for at least one week before each study was initiated. The animals were housed in SPF mouse/rat cages, in groups of five with 12-hour light/dark cycles, in rooms with controlled environmental conditions. All animals had free access to both water and a normal commercial pelleted mouse/rat chow diet. The animals had additional shredded paper with no ink provided as nesting materials after the paper had been sterilised. The animals were monitored daily for pain or signs of distress (behavioural changes) and weighed every second day. Daily distress and morbidity was determined on a regular basis by the trained personnel of UPBRC using a welfare score sheet.

Evaluation of reduced food and water intake, weight loss (humane endpoint at 20% weight loss relative to untreated controls), observation of excessive grooming, disinterest in environment, piloerection, isolation abnormal movement (particularly as it pertains to the ability of the animal to obtain food and water) and ease of breathing were used as observable indicators of toxicity. Animals would be terminated if 25% or more showed signs of distress or adverse reactions, as described above. The final decision to terminate before the end of the study was made by the veterinarian in charge, Dr R. Auer, and was to be done if any signs of discomfort were observed.

At the end of the study, all animals were euthanased by anaesthetic overdose and CO_2 asphyxiation after collection of blood for haematological and selected blood toxicity markers. The animals were dissected to determine any changes in gross anatomy and visual signs of organ toxicity.

All collected blood samples were taken immediately for analysis and analysed at the Clinical Pathology Laboratories, Faculty of Veterinary Sciences, University of Pretoria on the same day of collection.

- **Chemicals and reagents**

The quinoline derivative, CITme, was synthesized and purified by qualified personnel from our Department at the PET Labs facilities following the same procedures used by Professor K. Chibale (Department of Chemistry, University of Cape Town).

2-*methyl*-pyrrolidone (BASF-Johannesburg) and microcrystalline cellulose (Warrenchem) were used to formulate the in the initial experiments.

Due to poor formulation stability or toxicity of the first formulations attempted, a propheroid formulation (a triphasic drug delivery system) and CITme propheroid formulation were prepared by qualified personnel from the Pharmacy Department of North-West University.

This formulation phase of the study was divided in three phases:

- ***Phase one***: Pilot study using a formulation of CITme using 2-*methyl*-pyrrolidone;
- ***Phase two***: Pilot study using a formulation of CITme by using an oral microcrystalline cellulose suspension; and
- ***Phase three***: Pilot study using a formulation of CITme in a patented sterile non-toxic drug delivery system using the propheroid drug delivery system.

3.3.1 Phase one: Pilot study using a formulation of CITme using 2-*methyl*-pyrrolidone

A stock solution of 8 mg/ml CITme was prepared in 2-methyl-pyrrolidone pH adjusted with formic acid and complexed using plasma protein in the following ratios: 8:1:1 and 5:0.5:4.5 to form two different stable soluble formulations of the drug for a pilot acute toxicity tests.

Two pilot acute toxicity studies were done in ten BALB/c mice (five for control, plus five for experimental) for each formulation for seven days using 0.1 ml oral gavage, to test the safety of the formulation vehicle, 2-*methyl*-pyrrolidone that was pH adjusted with formic acid and complexed using plasma protein formulation, as well as 30 mg/kg/day CITme using the same formulation loaded with CITme.

A total of 20 BALB/c mice (10 mice per formulation) were used in this experiment.

The mice were monitored for signs of adverse effects as well as behavioural changes. The weights were recorded every second day. At the end of the study, kidney function markers (urea, creatinine) and liver toxicity marker enzyme levels (ALT, AST, and GGT) were analysed for indications of toxicity. Post-mortem of the euthanased mice was undertaken to establish any signs of toxicity for the different organs.

An intravenous formulation of CITme at 5mg/kg was prepared with 2-methyl-pyrrolidone pH adjusted with formic acid and plasma protein, (5:0.5:4.5) and then injected into one Sprague Dawley rat in an attempt to get an indication about the optimal route of administration as well as the distribution rate of CITme after I.V. injection. While the rat was at the surgical plane of anaesthesia, 1.5ml of uncoagulated blood sample (Li-heparin anticoagulant) was collected via cardiac puncture by trained personnel from the UPBRC. Four timed blood samples were collected from rats at baseline (0), 1, 3, and 5 minutes after CITme injection. The rats were terminated after blood collection according to standard UPBRC procedures using isoflurane overdose followed by CO_2 asphyxiation.

The blood was immediately processed by centrifuging for 8 minutes at 4°C and 800*g* to remove the blood cells, the supernatant transferred into vial inserts, snap frozen and taken for analysis of plasma concentrations of CITme by LC-MS/MS at the Department of Pharmacology on the Medical campus of University of Pretoria.

3.3.2 Phase two: Pilot study using a formulation of CITme by using an insoluble oral microcrystalline cellulose suspension

An oral uptake study was done using eight Sprague Dawley rats to ascertain whether CITme could be absorbed from the gut when given in an insoluble complexed formulation as opposed to a soluble formulation. This was done to confirm whether there was a need to develop a suitable soluble formulation to be used for future studies. The rats were divided into 4 groups according to different time intervals (0.5; 1; 1.5 and 2 hours). There were 2 rats per time interval. A total of eight rats (4 groups of 2 rats) were used for this pilot study.

On the day of the experiment, animals received an oral dose (by gavage) of 30mg/kg body weight of CITme suspended in 400µl of microcrystalline cellulose gel to prevent rapid separation of the insoluble CITme (CITme powder was mixed and vortex in cellulose gel).

At each of the following time intervals: 0.5; 1; 1.5 and 2 hours, two rats were anaesthetised via isoflurane inhalation. While the rat was at the surgical plane of anaesthesia, approximately 3 ml of uncoagulated blood sample (3 ml/rat – Li-heparin anticoagulant) was collected via cardiac puncture by trained personnel from the UPBRC. The rats were then terminated according to standard UPBRC procedures using isoflurane overdose followed by CO_2 asphyxiation.

The blood was immediately processed by centrifuging for 8 minutes at 4°C and 800*g* to remove the blood cells, the supernatant transferred into vial inserts, snap frozen and taken for analysis of plasma concentrations of CITme by LC-MS/MS at the Department of Pharmacology on the Medical campus of University of Pretoria.

3.3.3 Phase three: Pilot study using a formulation of CITme in a patented sterile non-toxic drug delivery system using propheroid.

A pilot acute toxicity study was carried out in ten BALB/c mice (five for control, plus five for experimental) for seven days after daily oral doses (by gavage) of 0.1 ml, to test the safety of the propheroid formulation as well as propheroid formulation

containing CITme at 30 mg/kg/day by oral gavage. The mice were monitored for signs of adverse effects as well as behavioural changes. The weights were recorded every second day. At the end of the study, blood was drawn (Li-heparin anticoagulant) by cardiac puncture while the animals were at surgical plane of anaesthesia after which the animals were euthanased by Isoflurane overdose followed by CO_2 asphyxiation.

Kidney function markers (urea, creatinine) and liver marker enzyme levels (ALT, AST, and GGT) were analysed for indications of toxicity. Post-mortem of the euthanased mice was undertaken to establish any signs of toxicity for the different major organs.

3.4 STATISTICAL ANALYSIS

All the available data were analysed using the unpaired Mann-Whitney test. *P* values of less than 0.05 were considered statistically significant.

3.5 RESULTS AND DISCUSSION

Formulation screening must be initiated early in development of the pre-clinical tests, even before knowing the viable drug dose. Challenges including the purity of excipients used in the formulation of the poorly soluble drugs can compromise the consistency of the drug product and can ultimately lead to wrong or false conclusions.

In this study, different solvents were used in an attempt to obtain a suitable formulation of the poorly soluble drug without exerting harmful effects. However, biologically compatible solvents that are commonly used to solubilise lipophilic drugs showed poor solvation of CITme. CITme was found to be soluble in DMSO, DMF and formic acid, which are all toxic *in vivo* at relatively low concentrations. By using 2-*methyl*-pyrrolidone, a unique and uncommon drug delivery solvent, or Cremophor EL emulsions some promise as potential solvent for CITme was observed at low concentrations (<5 mg/ml) but the solubility was still too low to allow stable formulations and to avoid solvent/carrier toxicity at the required CITme doses.

Solubility and formulation problems of the poorly soluble CITme were addressed by using a combination of solvent (2-*methyl*-pyrrolidone, pH adjusted with formic acid) and then complexed to plasma protein. A stock solution of CITme at a final concentration of 10 mg/kg was prepared in 2-*methyl*-pyrrolidone which was then pH adjusted to 7.6 with-formic acid and complexed using mouse plasma protein in the following ratios: 8:1:1, and 5:0.5:4.5 to form two separate soluble and stable formulations of the drug for a pilot acute toxicity tests.

A parallel acute pilot toxicity study was performed using a total of 40 BALB/c mice (ten mice per formulation plus ten per vehicle control group). All mice were dosed orally on a daily basis. However, both these pyrrolidone formulations elicited acute toxic responses. The first signs of toxicity, including death were observed between the second and third dose of a planned seven day dosing period in both the vehicle controls as well as the two experimental groups. All the mice were terminated before the end of the study according to the approved ethics protocol) This toxic effect was not expected as the administered doses were far below published LD_{50} concentrations of 2-*methyl*-pyrrolidone (Payan *et al.,* 2003; Dudeck *et al.,* 2006).

In the intravenous formulation, of CITme at 5 mg/kg body weight, four blood samples were collected [at baseline (0), 1, 3, and 5 minutes] after CITme injection. However, very low concentrations, at the limit of detection for CITme were detected in only the 5 minute post administration plasma sample. This is not an expected result as the plasma concentration should be at its highest at the first collection time point after the bolus IV injection of the compound. One explanation may be the possibility that the 2-*methyl*-pyrrolidone formulation causes vasoconstriction at the site of injection resulting in a reduced blood circulation through the injected vein. An alternative explanation would be that the protein complexation may be altered due to the 2-*methyl*-pyrrolidone causing partial protein denaturation which alters the ability to effectively recover the CITme from this protein or that the protein may be insoluble and rapidly filtered from the blood in the liver, kidney or lungs prior to blood collection. As the protein then resolubilises as the 2-*methyl*-pyrrolidone is diluted by the circulating plasma or is hydrolysed by plasma enzymes, the CITme may be released into the circulation. A further possibility is that the injections for the administration were not into the vein but into the tissue

next to the vein where it responded as a subcutaneous injection. All three of these possibilities could result in the observed results and it cannot be determined, which is the most probable reason from the observed data.

In the oral uptake study using the insoluble microcrystalline CITme suspension in 8 Sprague Dawley rats, no quantifiable CITme was detected in any of the blood samples collected at any of the time points (0.5, 1, 1.5 and 2 hours) post gavage. In these samples, there was not even the slightest increase at the expected retention time for the CITme even after increasing the injected sample volumes five fold during the LCMSMS analysis. A low concentration spike of CITme into the sample did however show the analyte when reanalysing the sample proving that the analytical method was working and was in fact capable of detecting low concentrations of CITme. This was initially interpreted that the CITme formulation was not stable and that the CITme was not solubilised preventing absorption from the GIT.

It is well known that cancer chemotherapy is considerably restricted by toxic side effects of many of the anticancer drugs. This restriction results from the fact that the anticancer drugs used for chemotherapy lack solubility in common drug administration solvents and a lack of efficient target selectivity for the malignant cells. A promising approach to suppress the toxic side effects of the anticancer drugs towards normal dividing cells and to improve their efficiency against malignant cells is to conjugate the anticancer drugs to targeting delivery systems (Yokoyama *et al.,* 1990).

According to various studies, drug formulations using nano particle delivery systems were successful for systemic, oral, pulmonary and transdermal administration for different aims including drug targeting, enhancement of drug bioavailability and protection of drug bioactivity and stability (Maincent *et al.,* 1986; Cappel and Krewuter, 1991; Zeltner *et al.,* 1991; Mu and Feng, 2003). Moreover, the ability of nano systems to improve drug diffusion through biological barriers is a typical benefit for the delivery of anticancer agents, especially for those drugs with poor aqueous solubility properties (Mu and Feng, 2003).

In this study, it was found that CITme was a suitable candidate for formulation with a patented non-toxic “propheroid drug delivery system” prepared by the Pharmacy Department at North-West University (NWU). The propheroid drug delivery system could be loaded with relatively high concentrations up to 20 mg/ml of CITme and was found to be stable for at least three weeks. CITme stock solution in the propheroid delivery system at a concentration of 15.0 mg/ml was enough to achieve the required *in vivo* acute toxicity dose in a 100 µl aliquot which was the maximum allowed oral gavage volume of the formulation. This propheroid formulation could therefore be used to circumvent the solubility and toxicity problems experienced in the early phases of the study where 2-*methyl*-pyrrolidone was used as the main drug solvent.

The pilot acute toxicity study using the propheroid delivery system was carried out for seven consecutive days on ten BALB/c mice (five for propheroid vehicle control, plus five experimental dosed with CITme loaded propheroid system), to test the acute toxicity profiles and safety of the propheroid formulation (control) as well as a CITme loaded propheroid formulation at 30 mg/kg/day (experimental) by oral gavage. All the animals in both control and experimental group survived the seven-day duration of the study without any observed unusual or adverse effects. None of the animals showed any signs of excessive stress or abnormal behaviour during this time. There was no need to add a third group for a double negative control (animals feed with water only), because the Department of Pharmacy from NWU, claimed that the propheroid drug delivery system has been tested before both in animals (mice) and humans, in order to determine its toxicity and efficacy properties, and was found to be non-toxic while enhancing drug activity in both species. Furthermore this formulation is currently being commercialised for delivery of other classes of drugs and cosmetic products in humans.

The body mass (Figure 3.1) from both propheroid formulation groups showed a slight decrease over the seven days but no significant differences were observed between the control group (propheroid formulation) and the experimental drug (CITme propheroid formulation), given at an orally administered dose of 30 mg CITme/kg/day. The organ masses (Figure 3.2), kidney function markers (creatinine, urea) (Figure 3.3) and liver toxicity marker enzymes (ALT, AST, GGT) (Figure 3.4)

showed no signs of toxicity with no significant difference between the control group (propheroid formulation) and the experimental drug group (CITme propheroid formulation).

Blood cell counts were not analysed due to insufficient blood volumes being collected and the priority was to assess the biological markers. Macroscopic and histopathology analysis during the post mortem examinations concluded that there were no significant pathological lesions compatible with organ toxicity that could be associated with the administration of the experimental drug (CITme propheroid formulation), when given at an orally administered dose of 30 mg/kg/day.

Below is a summary of the results shown graphically (Figures 3.1-3.5) in related groups of information obtained from the experimental group and compared to the control mice that were sham dosed with propheroid formulation. The data was analysed using GraphPad Prism software applying the unpaired Mann-Whitney test. *P* values of less than 0.05 were considered statistically significant. As can be seen from the reported data no significance could be demonstrated for any of the measured parameters between the CITme treated and the drug free propheroid formulations.

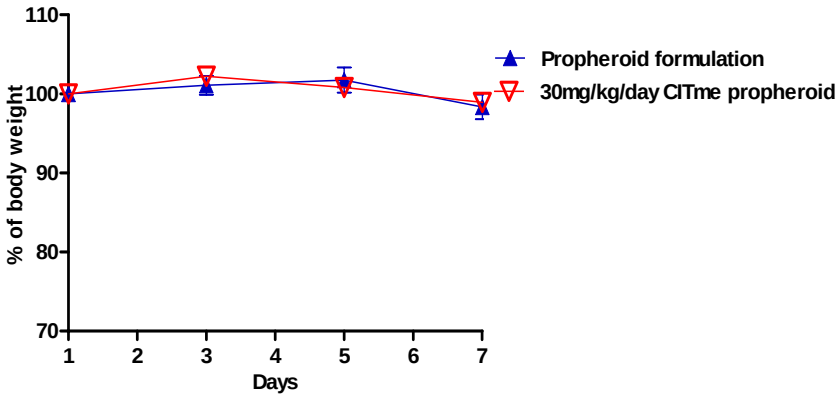

Figure 3.1: Changes in body mass of ten mice undergoing daily gavage for seven days with either propheroid (control) or CITme propheroid (experimental) formulations. A slight but insignificant decline in body mass was evident at the end of the study. n=5 mice per group

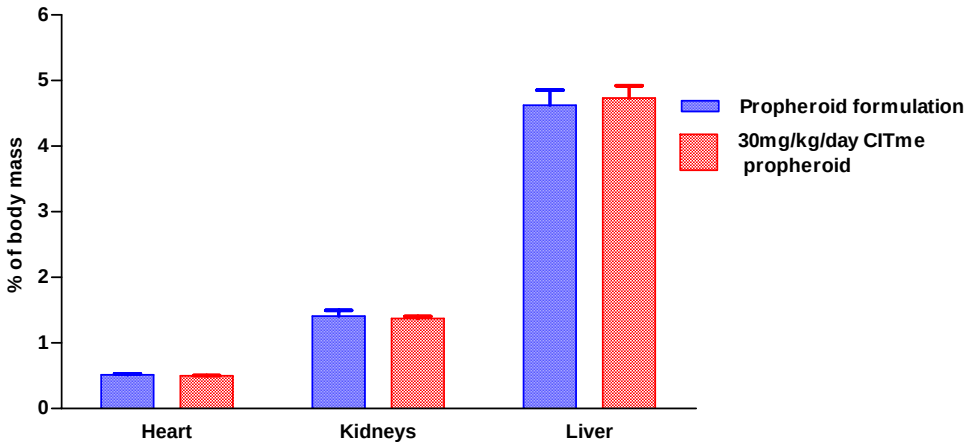

Figure 3.2: Comparison of the mass of the heart, kidneys and liver, as a percentage of the total body mass of ten mice for both the propheroid (control) and CITme propheroid (experimental) formulations. No significant differences were evident between groups after the seven daily dosing period. n=5 mice per group

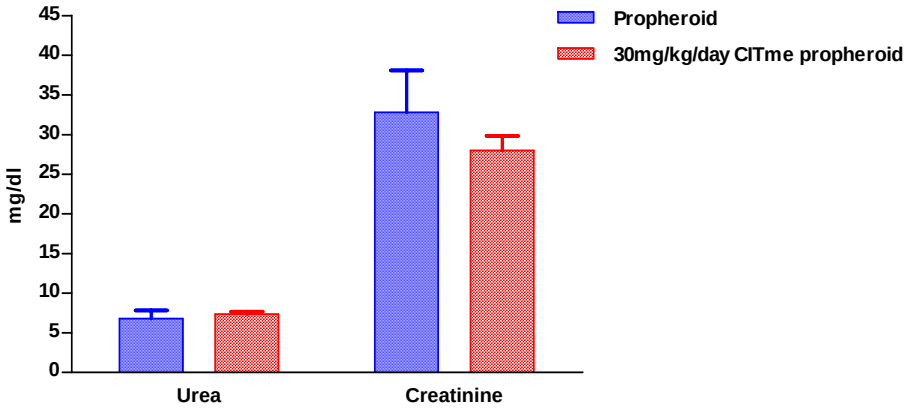

Figure 3.3: Comparison of Urea and Creatinine concentrations in the plasma as markers of kidney function of mice for both the propheroid (control) and CITme propheroid (experimental) treated mice. No significant difference was evident between groups for the seven daily dosing period. n=5 mice per group

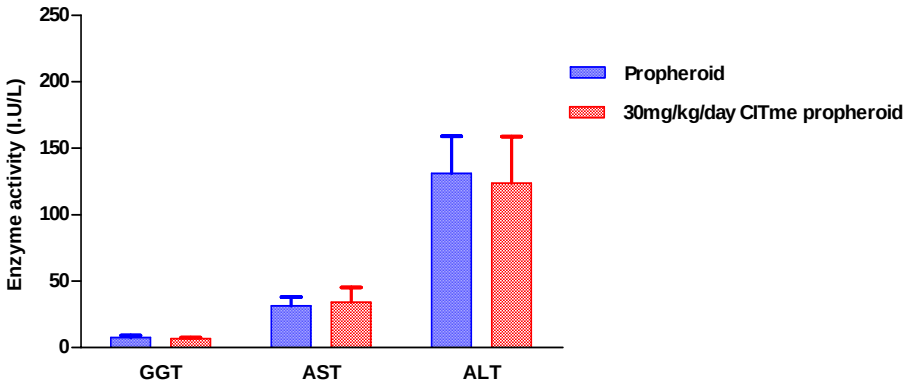

Figure 3.4: Comparison of GGT, AST and ALT concentrations in the plasma as markers of liver toxicity of mice for both the propheroid (control) and CITme propheroid (experimental) treated mice. No significant difference was evident between groups for the seven daily dosing period. n=5 mice per group

Due to the solubility characteristics of CITme several problems were encountered in finding a suitable formulation that was both stable and suitable for *in vivo* administration by either oral or parental routes. The use of solvent based formulations that could be used during *in vitro* studies was unsuccessful due to toxic side effects, with protein complexed formulations also showing toxicity and not being suitable for injection based administration.

During these early phases of the formulation assessment, it was also found that the use of microcrystalline cellulose in the oral formulation gave debatable uptake results. This almost resulted in the project being suspended due firstly to insufficient proof that absorption of CITme was in fact taking place via the oral route and secondly, the severe toxicity of the solvent based formulations that did indeed give a stable CITme solutions.

The use of a unique formulation was then assessed with the help of the available expertise of the North West University's Pharmacy Department to determine whether a stable lipid based CITme formulation could be produced. The initial formulation results using the patented "propheroid drug delivery system" resulted in a stable formulation with a high enough CITme concentration to allow the assessment of the acute toxicity of the CITme at high concentrations in the mouse model.

CHAPTER FOUR

IN VIVO TOXICITY PROFILE OF CITME, A QUINOLINE DERIVATIVE

4.1 INTRODUCTION

Toxicity profiling is an essential component in all drug development. A drug that is highly effective yet extremely toxic is worthless as a therapeutic agent. An agency such as the Medicines Control Council of South Africa (MCC), the U.S. Food and Drug Administration (FDA) and the World Health Organisation (WHO) requires extensive pre-clinical studies to be conducted before a drug is allowed to be tested in humans.

To protect human health, the risks of poisoning from new chemical entities are assessed by various research industries and agencies. Risk assessment is most often based on the results of animal toxicity tests, each test measuring one or more of many aspects of toxicity like acute and chronic toxicity, local irritancy to the skin and eye, and teratogenicity (Ekwall *et al.,* 1999).

Early screening for drug toxicity, especially human-specific toxicity, has become a routine practice in drug discovery and development. It can be argued that because drug candidates will be vigorously tested in laboratory animals, assays to identify human-specific toxicity (which, by definition, could probably not be detected in non-human animals) are critical to drug development (Li, 2005). In the process of drug discovery, selected active substances are further differentiated according to their toxicity (Popiolkiewicz *et al.,* 2005). It is desirable to find not only the most active, but simultaneously the substances with the least systemic toxic effects (Ecobichon, 1997).

Pre-clinical animal safety studies are conducted to provide evidence that the drug in question, in the words of the regulations, “reasonably safe for the proposed clinical trials” and should be designed to test the margin of safety, the type of toxicity (to assess the benefit/risk ratio) and to test the reversibility of any toxic effects (Gumer, 1989; Tanojo *et al.,* 1997).

Toxicity studies on animals, which must be conducted before planning any clinical trial, constitute an important part of the complete drug discovery process. Acute and chronic toxicity studies are conducted in animals to ascertain the total adverse biological effects caused during a finite period of time following administration of a single, frequently a large single dose of an agent and a extended daily dose (which is intended to be the therapeutic dose) respectively for a long term period (Ecobichon, 1997). Besides that, acute toxicity testing in animals is typically the initial step in the evaluation of the health effects of a chemical, and its primary purpose is to provide information on potential health hazards that may result from short-term or high dose exposure (Zhang *et al.,* 2007). The target tissue or organs are also investigated to determine whether any organ accumulation of either the parent drug or the drug metabolites takes place which could further predict potential toxicity or physiological processes that are at risk after exposure to the new chemical entity. These are parameters that cannot be evaluated *in vitro* as they rely on various organ contributions and the circulation between these organs.

Overall, animal toxicity studies are intended to inform the clinical investigators about the potential toxicities associated with the investigational drugs so that those effects may be monitored closely during the clinical investigations. Baseline evaluation of safety includes monitoring for signs and symptoms of adverse events by observing the test animals behaviour and post mortem gross anatomy, histology and further laboratory screening for known toxicity markers (e.g., chemistry, haematology, and urinalysis).

CITme was found to have no obvious toxicity in BALB/c mice after seven daily doses of 30 mg CITme/kg body weight by oral gavage when using the stable propheroid drug delivery system. Furthermore, several similar quinoline derivatives have been tested *in vivo* and found not to have significant toxic effects at a concentration of up to 60 mg/kg in healthy mice (Fujita *et al.,* 2000). The structurally similar CITme could be expected to elicit low toxicity at equivalent concentrations.

4.2 STUDY AIM

The aim of this study was to determine the maximum tolerated and the safety dose of CITme in the BALB/c mouse model using an acute toxicity dose limited ranging study followed by a chronic toxicity study before embarking on the tumour efficacy study on the nude mice.

4.3 MATERIAL AND METHODS

Ethical approval was obtained by the AUCC (Animal Use and Care Committee) (Protocol No. H09/07). The study was conducted at the University of Pretoria Biomedical Research Centre (UPBRC) from June to August 2008.

- **Chemical and reagents**

All chemicals used, unless otherwise indicated, were of highest grade available purchased from Sigma Chemicals and Reagents (St Louis, Missouri). The quinoline derivative, CITme was synthesized by qualified personnel from our department at PET Labs facilities following the same procedures used by Professor K. Chibale (Department of Chemistry, University of Cape Town), and then formulated with a patented sterile drug delivery system using the propheroid delivery system by the Pharmacy Department of the North-West University (UNW) for all the *in vivo* toxicity studies.

- **Animals**

Due to the expense and current unavailability of nu/nu mice, inbred female BALB/c mice (6-8 weeks old) from which the nude mice were derived, were used for the toxicity studies before embarking on the tumour efficacy study on the nude mice. Mice were used and housed in IVC mouse cages, in groups of five with 12-hour light/dark cycles, in rooms with controlled environmental conditions. The mice had free access to both sterilised water and a sterilised normal mouse chow diet. The animals had additional nesting materials provided as long as there was no ink and that the paper had been sterilised. Mice were weighed every second and fifth consecutive day for acute and chronic toxicity study respectively, and monitored daily for pain and distress (behavioural changes). Morbidity was determined on a regular basis by the trained personnel of UPBRC using welfare monitoring sheets.

- **Evaluation of pain and distress**

Toxicity was implied by evaluation of reduced food and water intake, weight loss, observation of abnormal movement (particularly as it pertains to the ability of the animal to obtain food and water) and ease of breathing. All animals would be terminated if 25% or more showed signs of severe distress or adverse reactions, as described above. Any adverse effects or morbidity were determined by trained UPBRC personnel members on a daily basis by evaluation of these factors for a period of 1 week for acute toxicity study and 6 weeks for the chronic toxicity study.

Animal weights were measured every second day or fifth day for the acute and chronic toxicity studies respectively at approximately the same time of day. If there was a body mass reduction of 20% or more from initiation of the study, the animals would be deemed to be showing signs of toxicity and would be terminated by isoflurane overdose according to standard UPBRC procedures. LD_{25} was used as a cut-off norm for toxicity. In the event that five mice per group (25%) show signs of toxicity the experiment would be terminated and the remaining mice euthanased as would have taken place at the end of the study. All mice that died before the end of the 1 and 6-weeks study were dissected and the organs checked to determine a reason of death and which organ was targeted.

The final decision to terminate any mice prematurely was made by the allocated veterinarian, Dr Auer, and was to be done if any signs of toxicity (as described above) or discomfort were observed. Animals were euthanased by anaesthetic overdose if deemed necessary.

4.3.1 The toxicity study in BALB/c mice

This study involved an acute and chronic toxicity study on the BALB/c mouse model using CITme. The acute and chronic toxicity study were carried out in two groups of 40 mice each (20 for control, plus 20 for experimental). The number of 20 mice per group was based on the statistical analysis of the expected differences in measured blood parameters between control and experimental animals and the possibility of losing any animals during handling and gavage. A total of eighty mice were used for the complete *in vi*vo toxicity study.

If NOAEL (no observed adverse effect level) concentration could be determined from the concentration used for the acute toxicity study, this would form the basis for the concentration of CITme that would be used for the chronic toxicity study. The dose to be used for the chronic study would be either 1/3 NOAEL concentration of CITme or 60 mg CITme/kg body weight. The weight of the mice was determined to adapt the dosages according to their body weight. The chronic toxicity study testing the long-term dose safety of CITme was only initiated after been proved that there was no signs of toxicity during the acute toxicity test.

Uncoagulated blood samples (±0.8 ml/mice Li-heparin anticoagulant) were drawn by trained personnel from the UPBRC (on day 7 and day 45 for the acute and chronic toxicity study respectively) from all the mice in each study. The mice were anaesthetised via isoflurane inhalation, and while at the surgical plane of anaesthesia, blood sample was collected from each mouse via cardiac puncture. The mice were terminated by isoflurane overdose according to standard UPBRC procedures and dissected for necropsy examinations and recording of the organ weights of the heart, liver, kidneys and spleen.

The blood was analysed for haematological (haemoglobin, red and white cells, and platelet counts) and clinical chemistry analysis (kidney function markers and liver enzyme levels: creatinine, urea, ALT, AST and GGT) by the Clinical Pathology Laboratories, Faculty of Veterinary Sciences, University of Pretoria. All blood samples were taken immediately for analysis. The mice were checked for gross internal anatomical changes, histology and the organ weights of the heart, spleen, kidneys and liver were determined.

Blood samples (if possible), organ weights and body mass data were collected in the event of an early termination or death prior to the end of the study.

4.3.2 Acute toxicity study

A one week toxicity study was performed on 40 female BALB/c mice (±20 grams). The mice were divided into 2 groups, one experimental plus one vehicle only treated control group.

Mice in the experimental group received a single oral dose (by gavage) of 0.1 ml of 60 mg CITme/kg body weight in a propheroid drug delivery system. Control animals received a single oral dose (by gavage) of 0.1 ml of propheroid nanoemulsion delivery system alone, to control for the procedure used in the experimental group.

4.3.3 Chronic toxicity study

A chronic toxicity study was completed on 40 female BALB/c mice (±20 grams). The mice were divided into 2 groups (of twenty mice each), one experimental plus one vehicle only treated control group.

The mice in the experimental group received an oral dose (by gavage) of 10 mg CITme /kg/day in approximately 0.1 ml of propheroid drug delivery system per day for 6 weeks. The control group received a sham dose of 0.1 ml of propheroid drug delivery system without CITme per day at the same time.

4.4 STATISTICAL ANALYSIS

All data from the acute and chronic toxicity study were analysed using the unpaired Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

4.5 RESULTS

4.5.1 Acute toxicity study

The acute toxicity study was carried out on forty BALB/c mice (twenty for control, plus twenty for experimental), to test the acute toxicity profile and safety of CITme propheroid formulation at 60 mg/kg body weight (experimental) by oral gavage.

From the forty mice in total, three mice from the control group died, while all the rest of the animals from both the control as well as from the experimental group survived the entire duration of the study without any observed unusual or adverse effects. None of the surviving animals showed any signs of excessive stress or abnormal behaviour during this time. All three animals that died were in the control group and died within 45 minutes of being gavaged. These animals all appeared to

have lung problems and it is thought that these animals may have aspirated the relatively oily propheroid emulsion either during or shortly after oral gavage. Post mortem studies showed no organ abnormalities other than congested lungs.

No significant body weight loss was observed in either groups (Figure 4.1). The organ mass (heart, liver, spleen and kidneys) as percent of body mass (Figure 4.2), liver toxicity marker enzymes (ALT, AST, GGT) (Figure 4.3), kidney function markers (creatinine, urea) (Figure 4.4) and haematology parameters (WCC, RCC, Hb) (Figure 4.5), indicated that no signs of toxicity were evident in either the control group (propheroid formulation) nor the CITme propheroid formulation at a single orally administered dose of 60 mg CITme/kg body weight.

Macroscopic and histopathology analysis from the three mice that died early in the study concluded that there was severe diffuse pulmonary congestion and multifocal moderate oedema. The alveolar capillaries revealed mild leukostasis, and in focal areas showed well-demarcated small foci of fibrin deposition associated with small numbers of neutrophils and even scattered single eosinophils. Foreign material was not clearly visible in these areas. Multifocal mild haemorrhage was also present in the areas of pneumonia, but also in the surrounding parenchyma. No further notable lesions were visible in the other organs examined (brain, heart, spleen, liver, kidneys, stomach, small and large intestine) from these animals.

From the rest of animals that survived for the entire study there were no significant pathological lesions compatible with organ toxicity that could be associated with the administration of the delivery system (propheroid formulation) or with the experimental drug (CITme propheroid formulation), after a single orally administered dose of 60 mg CITme/kg body weight.

Incidental findings such as haemopericardium, haemothorax, lung haemorrhages and myocardial haemorrhages are signs associated with the blood collections, while idiopathic hereditary cataracts were observed in many of these mice.

Idiopathic mineralization of the epicardium was also found as well as multiple other non-specific incidental observations.

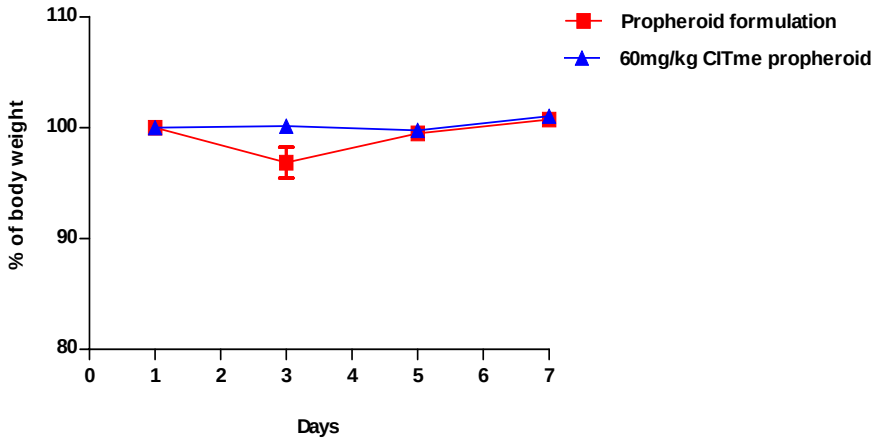

Figure 4.1: Changes in body mass as a percentage of the total body mass of 37 mice undergoing a once off oral gavage dose with either propheroid delivery system (control) or CITme propheroid (experimental) formulation at 60 mg/kg body weight. No significant difference was evident in either group for the seven-day follow up period. n=20 mice per group

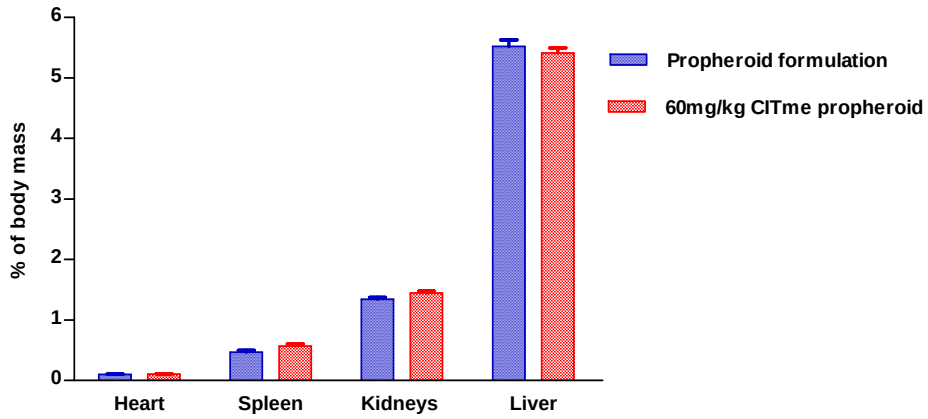

Figure 4.2: Comparison of the mass of the heart, spleen, kidneys and liver, as a percentage of the total body mass of 37 mice undergoing a once off oral gavage dose with either propheroid delivery system (control) or CITme propheroid (experimental) formulation at 60 mg/kg body weight. No significant difference was evident in both groups for the seven-day follow up period. n=20 mice per group

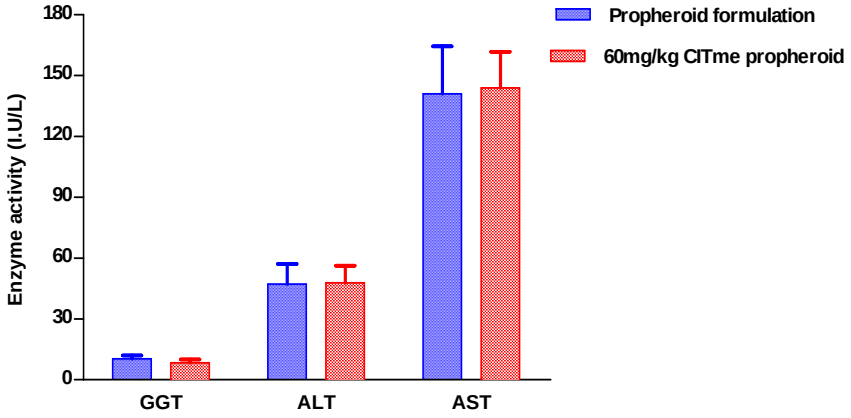

Figure 4.3: Comparison of GGT, ALT and AST concentrations in the plasma as markers of liver damage of 37 mice undergoing a once off oral gavage dose with either propheroid delivery system (control) or CITme propheroid (experimental) formulation at 60 mg/kg body weight. No significant difference was evident in both groups for the seven days follow up period. n=20 mice per group

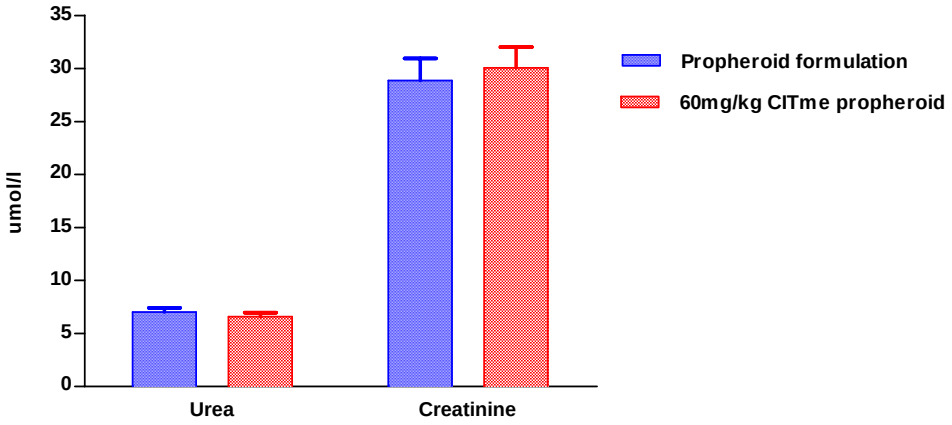

Figure 4.4: Comparison of Creatinine and Urea concentrations in the plasma as markers of kidney function of 37 mice undergoing a once off oral gavage dose with either propheroid delivery system (control) or CITme propheroid (experimental) formulation at 60 mg/kg body weight. No significant difference was evident in both groups for the seven days follow up period. n=20 mice per group

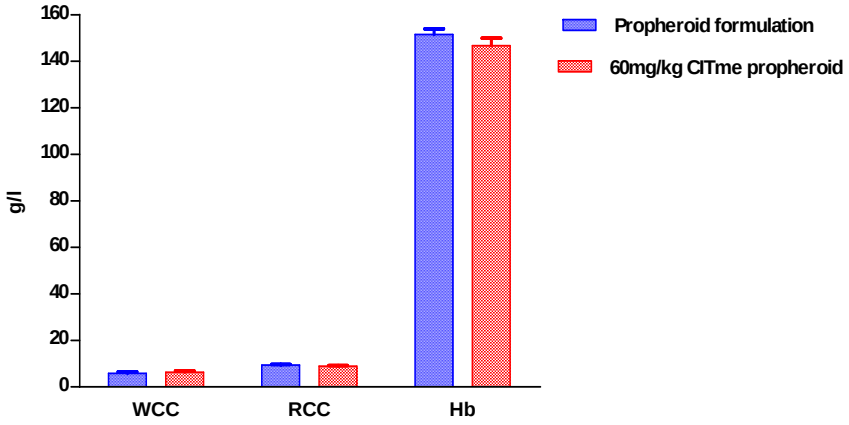

Figure 4.5: Comparison of white blood cell count (WCC), red blood cell count (RCC) and haemoglobin (Hb) concentrations in the blood of 37 mice undergoing a once off oral gavage dose with either propheroid delivery system (control) or CITme propheroid (experimental) formulation at 60 mg/kg body weight. No significant difference was evident in both groups for the seven days follow up period. n=20 mice per group.

4.5.2 Chronic toxicity study

The *in vivo* chronic toxicity study was carried out on forty BALB/c mice (twenty for control, plus twenty for experimental), to test the safety of CITme propheroid formulation after a long-term chronic dose of 10 mg CITme/kg/day for six weeks.

From the forty mice in total, one mouse from the control group and three mice from the experimental group died (three days after initiating the study), while all the rest of the animals from both the control as well as from the experimental groups survived the entire duration of the study without any observed unusual or adverse effects. None of the surviving animals showed any signs of severe stress or abnormal behaviour during this time.

An increase in body weight was observed in both groups, which was greater in experimental group. However, the difference in weight gain was not statistically significant (Figure 4.6).

The organ masses (heart, liver, spleen and kidneys) (Figure 4.7), liver toxicity marker enzymes (ALT, AST, GGT) (Figure 4.8), kidney function markers

(creatinine, urea) (Figure 4.9) and haematology parameters (WCC, RCC, Hb) (Figure 4.10), showed no signs of toxicity, in either the control group (propheroid formulation) or the experimental group CITme administered propheroid formulation at an orally dose of 10 mg CITme/kg/day for six weeks.

Macroscopic and histopathology analysis from the four mice that died early shortly after initiation of the study concluded that there was an acute shock condition with especially shock lung where protein-rich oedema and severe pulmonary congestion was observed. The alveolar capillaries revealed mild leukostasis, and in focal areas also showed well-demarcated small foci of fibrin deposition associated with small numbers of neutrophils and even scattered single eosinophils. Foreign material was again not clearly visible in these areas. Multifocal mild haemorrhage was also present in the areas of pneumonia, and in the surrounding parenchyma. No further notable lesions were visible in the other organs examined (brain, heart, spleen, liver, kidneys, stomach, small and large intestine) from these animals. The shock reaction could possibly be associated with the oral gavage or the effect of the propheroid formulation dose administered the previous day to these four mice.

The remaining 36 animals survived for the entire study and there were no significant pathological lesions compatible with organ toxicity that could be associated with the administration of the delivery system (propheroid formulation) or with the experimental drug (CITme propheroid formulation), given at an orally administered dose of 10 mg CITme/kg/day for an extended chronic exposure period of six weeks.

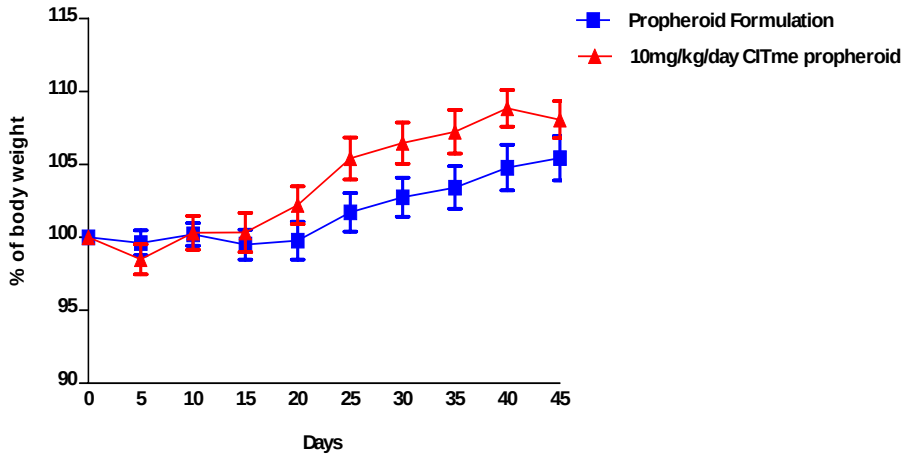

Figure 4.6: Changes in body mass of 36 mice undergoing daily oral gavage for six weeks with either propheroid delivery system (control) or CITme propheroid (experimental) formulation at 10 mg/kg/day body weight. No significant difference was evident between groups for the six weeks follow up period. n= 20 mice per group

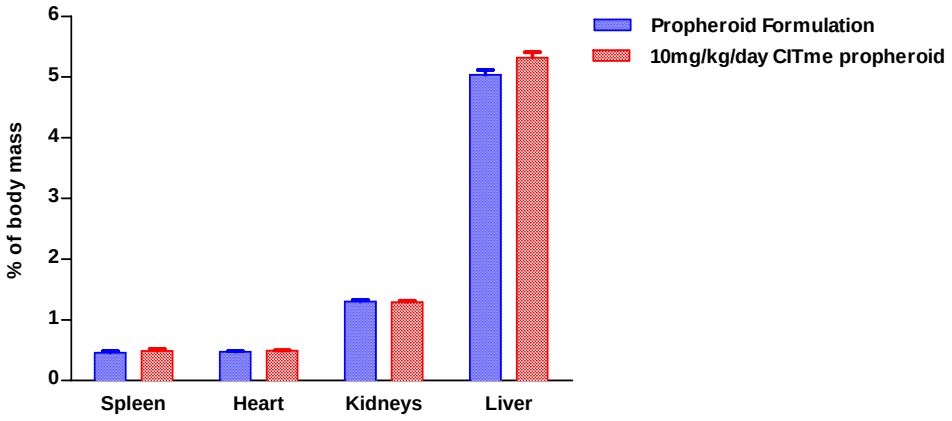

Figure 4.7: Comparison of the mass of the heart, spleen, kidneys and liver, as a percentage of the total body mass of 36 mice undergoing daily oral gavage for six weeks with either propheroid delivery system (control) or CITme propheroid (experimental) formulation at 10 mg/kg/day body weight. No significant difference was evident between groups for the six weeks follow up period. n=20 mice per group

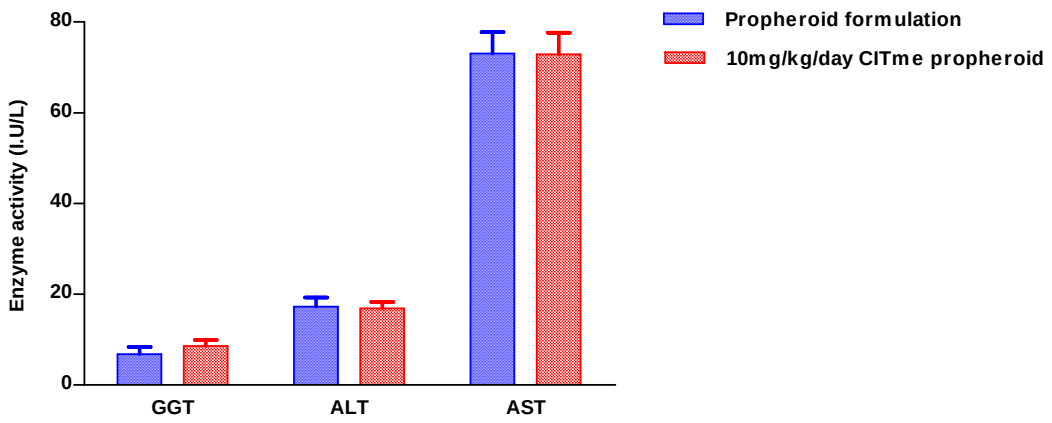

Figure 4.8: Comparison of ALT, AST and GGT concentrations in the plasma as markers of liver damage of 36 mice undergoing daily oral gavage for six weeks with either propheroid delivery system (control) or CITme propheroid (experimental) formulation at 10 mg/kg/day body weight. No significant difference was evident in both groups for the six weeks follow up period. n=20 mice per group

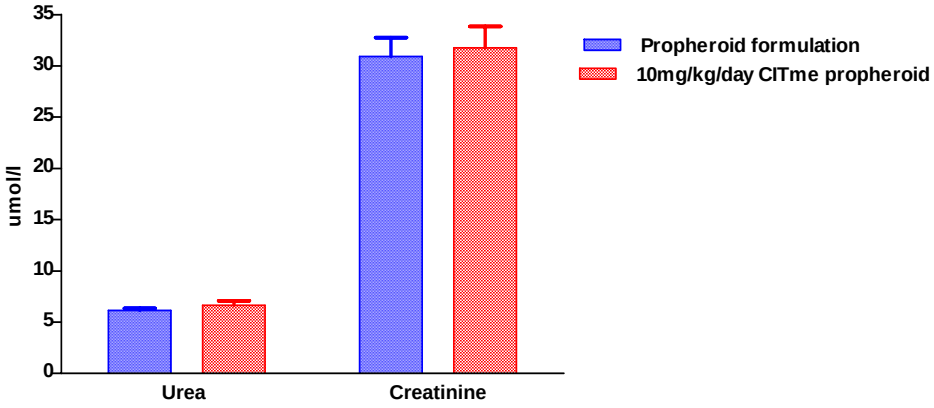

Figure 4.9: Comparison of Creatinine and Urea concentrations in the plasma as markers of kidney function of 36 mice undergoing daily oral gavage for six weeks with either propheroid delivery system (control) or CITme propheroid (experimental) formulation at 10 mg/kg/day body weight. No significant difference was evident in both groups for the six weeks follow up period. n=20 mice per group

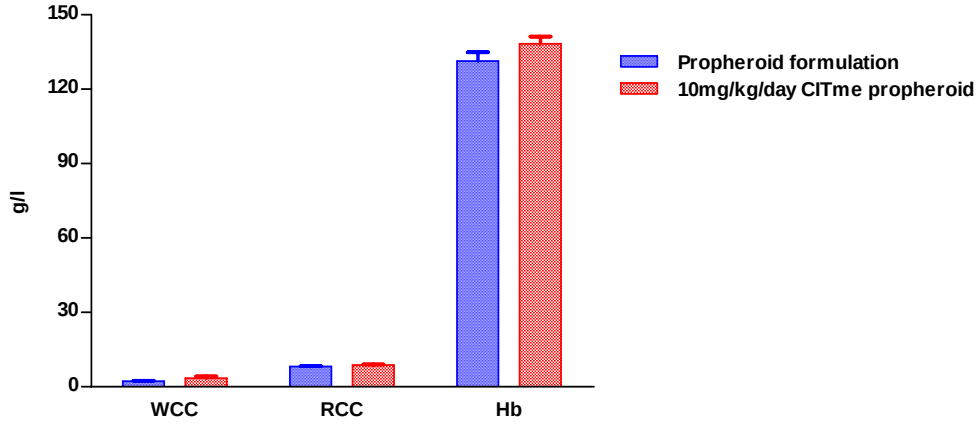

Figure 4.10: Comparison of white blood cell count (WCC), red blood cell count (RCC) and haemoglobin (Hb) concentrations in the blood of 36 mice undergoing a daily oral gavage dose for six weeks with either propheroid delivery system (control) or CITme propheroid (experimental) formulation at 10 mg/kg/day body weight. No significant difference was evident in both groups for the six weeks follow up period. n=20 mice per group

4.6 DISCUSSION

In society as a whole as well as in the scientific world, criticism on the use of laboratory animals for the safety testing of chemicals is increasing. This criticism is not only limited to ethical concerns, but scientific considerations also play a significant role. However, it should be realised that the animal bioassays presently used in toxicity testing are model systems for the prediction of toxicity in humans or the environment. An advantage of these models is the very long experience with their use with a relatively low number of serious pitfalls (Vinardell, 2005; Gubbels-van Hal *et al.,* 2005).

Accurate prediction of drug safety remains the major challenge for the pharmaceutical industry. The routine practice of preclinical and clinical drug safety evaluation is apparently inadequate, as evidenced by the yearly withdrawal or severe limitations of marketed drugs due to unexpected adverse effects. Early screening for drug toxicity, especially human-specific toxicity, has become a routine practice in drug discovery and drug development (Li, 2005).

Studies required by the U.S.A. FDA include acute toxicity studies in two rodent species, one month toxicity studies in a rodent and non-rodent species, reproductive toxicity studies and long term toxicity studies (Gumer, 1989; Tanojo *et al.,* 1997). The duration of treatment in animal studies should always be at least as long as the duration of treatment in the intended clinical study and at the later stages of development, longer than the therapeutic indication. This provides an extra element of safety (Gumer, 1989).

In this study, acute and chronic toxicity studies were carried out in order to determine the safety parameters of CITme after one and six week's exposure respectively using appropriate dosing levels.

Of the total of eighty mice used (forty for acute toxicity study plus forty for chronic toxicity study), three mice (all from the control group) in the acute toxicity study died, while four (one from control plus three from experimental group) mice from the chronic toxicity study died, despite the fact that propheroid drug delivery system has been proved to be safe on mice in previous studies, while the rest of the animals from the control group as well as from the experimental group survived for the entire duration of the study without any observed unusual or adverse effects.

The early death of these animals do not match with the findings observed in previous studies (studies from NWU in animals and humans, and the toxicity study with a daily dose of 30 mg CITme/kg/day for seven consecutive days), leading to the conclusion that the cause of death of those mice was most probably due to handling problems, especially during gavage and therefore not clinically relevant to this study.

Changes in body and internal organ weights can indicate toxicity or serious adverse side effects. Generally, weight loss is a simple and relatively sensitive index of toxicity after exposure to toxic substances (Valenta *et al.,* 2005; Degim, 2006). In the acute toxicity study, animals maintained more or less the same body weight with no significant difference between both groups. On the other hand, an increase in body weight was observed in both control and experimental animal

groups during the chronic toxicity study. This weight gain is an expected outcome due to the extended period of the study where the animals doubled their age. However, the increase in body weight showed no significant difference in the weight gain patterns of the CITme treatment group compared to that of the control group. Organ weights (liver, spleen and kidney) recorded from both the acute and chronic toxicity study also did not show any significant difference compared to that of the relevant controls nor was any pathological tissue damage seen that could be associated with either the administered drug or formulation.

Blood is an important index of physiological and pathological changes in an organism and can really only be considered valuable if the normal values are known (Swarbrick and Boylan, 2002). In the absence of known normal values, the treatment groups were compared to the sham dosed controls. In these studies, there were no significant differences on blood parameters, between the control and experimental groups from either the acute or chronic toxicity studies.

Hepatotoxicity is measured by biological parameters such as elevations of liver enzymes (ALT, AST and GGT) in the circulating blood or clinical abnormalities (hepatitis, jaundice, cholestasis) (Ozcanli *et al.,* 2006).

According to Evans (1996), enzymes are proteins that have catalytic properties that include specific activation of their respective substrates. Much emphasis is been placed on the application of plasma enzyme concentrations as markers of organ damage, with many enzymes used in toxicological studies to measure cellular injury, enzyme induction, activation or inhibition of enzymes. The distribution of enzymes in different tissues varies between species, and therefore influences their diagnostic value in a particular species.

The degree of damage to the tissue or whole body respectively, can be assessed by specific tests for enzymes that do not normally occur in the plasma (Conkova *et al.,* 2005). In clinical diagnostics, determination of transferases (ALT, AST and GGT), creatinine and urea are of great importance as they are excellent indicators of liver and kidney toxicity (Schacter, 2006). AST is an intracellular enzyme that is released into the blood stream in proportion to the number of damaged cells and is

found in the cytosol and/or mitochondria of different tissues such as heart, skeletal muscle, liver, kidneys, pancreas and erythrocytes. ALT is primarily located in the cytosol of hepatocytes and therefore it is an important parameter in identifying specific liver damage or disorders. GGT is specific to the hepato-biliary system and is therefore used to identify the exact location of hepatic damage (Degim, 2006; Schacter, 2006; Elovaara *et al.,* 2007). Furthermore, increased plasma levels of ALT are attributed to liver damage but those of AST may imply damage to organs other than the liver. The absence of any notable increase in plasma GGT indicates that there are no marked changes involving the biliary system. Several chemicals and medications can induce clinical or biological hepatic toxicity and they need to be carefully supervised (Ozcanli *et al.,* 2006).

In this study no significant induction, activation or inhibition in assayed enzyme levels or kidney markers were observed, nor were there any macroscopically or histologically relevant signs that could be assigned to hepatotoxicity or nephrotoxicity in either the control or experimental groups from both the acute and chronic toxicity studies.

The results from these two toxicity studies indicate that the CITme formulated into the propheroid delivery system was shown to be non-toxic at concentrations several fold higher than those that would be expected to be used in human treatment regimens. This would imply that the formulation is safe for oral administration and therefore warrants evaluation using a larger group of animals and possibly a different species e.g. rabbit or canine

CHAPTER FIVE

A PILOT TUMOUR INDUCTION STUDY IN NUDE MICE

5.1 INTRODUCTION

One of the greatest challenges faced in drug development for cancer, is the obvious need to test new drugs and new treatment strategies in *in vivo* models (pre-clinical stage) that have a high probability of being predictive of similar activity in humans.

The laboratory mouse has been the primary species in which experimental cancer treatments have been tested. The use of a syngeneic transplantable tumour model, a mouse tumour growing in mice of the strain in which the tumour originated, was pioneered by the National Cancer Institute (NCI) in the United States in the 1950's for preclinical therapy studies (Troiani *et al*., 2008). Since then, human tumour cell lines and even primary biopsy tumour specimens have been used to induce progressively growing and potentially lethal cancers in immune deficient mice (Shimosato *et al*., 1976; Kozlowski et al., 1984; Troiani *et al*., 2008). These murine cancer models are used to investigate the factors involved in malignant transformation, invasion and metastasis, as well as to examine response to new therapies (Morton and Houghton 2007). The availability of genetically athymic mice (nude) and immuno-deficient mouse strains with other genetic lesions (severe combined immunodeficiency, SCID) allowed the widespread possibility of testing for response to cancer drugs by human tumour explants and cell lines grown as xeno-transplants (Fig. 5.1). These models raised the hope that they would reveal agents more likely to have activity in solid human cancers. One of the strengths of these models is the broad spectrum of available tumour types (patient explant and many cell line–derived models have been described) that grow in the nude mouse model and the possibility of *ex vivo* genetic or therapeutic manipulation before xeno-transplantation (Burger and Fiebig, 2001; Fiebig and Burger, 2001; Alley *et al*., 2004; Sausville and Burger, 2006).

One of the most widely used pre-clinical model systems for efficacy testing of potential anticancer compounds is the human tumour xenograft growing in nude or

SCID mice (Morton and Houghton, 2007). The development of these models was a big step towards more clinically relevant tumour models (Kelland, 2004; Peterson and Houghton, 2004). In the human tumour xenograft model, human tumour cells are transplanted, either under the skin or into the same organ in which the tumour originated, into immuno-compromised mice that cannot reject the human cells (Morton and Houghton, 2007; Troiani *et al*., 2008). The better option is to transplant the cancer cells into nude or SCID mice. Because nude mice have the nu gene "knocked out", these animals are genetically athymic and therefore unable to generate mature T lymphocytes, whereas SCID mice lack functional T and B lymphocytes, thus showing a limited rejection of xenografts, (Plowman, 1997; Kelland, 2004; Bernard *et al*., 2008). Depending upon the number of cells injected, tumour type, or the size of the tumour transplanted, the tumour will develop over one to eight weeks, or in some instances one to four months (or even longer), and the response to appropriate therapeutic regimes can be studied *in vivo* (Morton and Houghton, 2007).

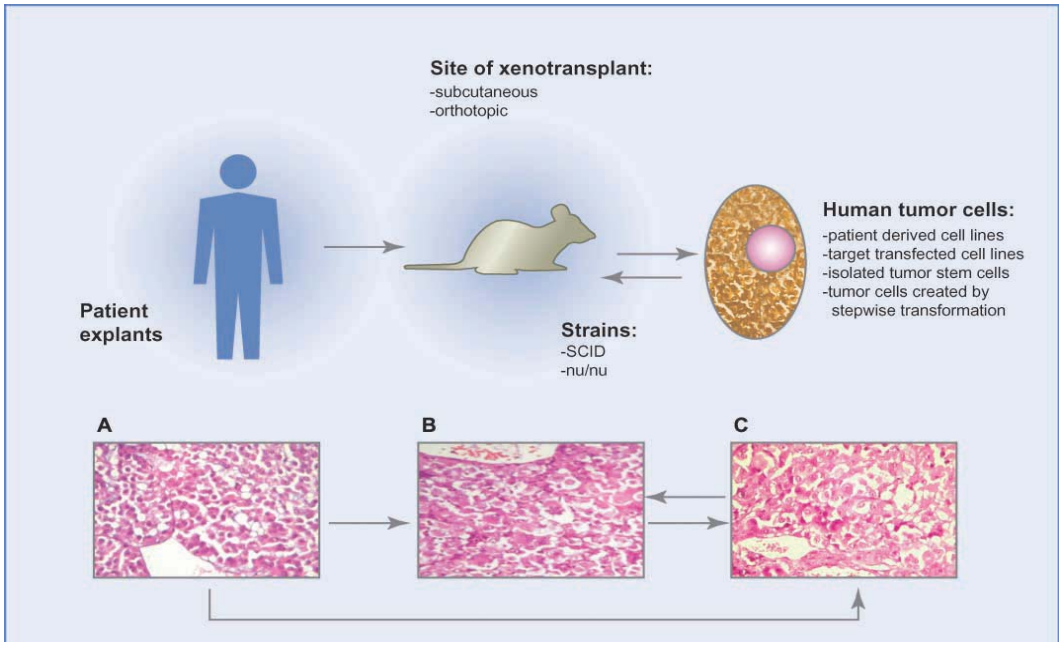

Figure 5.1: Overview of the variety of available xenograft models according to Sausville and Burger (2006). Most frequently used is s.c. growing xenografts from cell lines and patient explants. The histology of a large cell lung cancer (LXFL 529) is shown for the original patient tumour (A), the xenograft from this patient specimen after passage 5 in the mouse (B), and for the xenograft derived from a permanent cell line of LXFL 529 after

s.c. injection (C). The similarity of (A) to (C) indicates that human tumour xenografts can closely resemble clinical disease

In this study, it was found in initial *in vitro* cell culture experiments that the prostrate cancer cell line DU-145 was the most sensitive to CITme after three or seven days of treatment with impressively low IC_{50} of 2.54 µM. Based on these results it was decided to establish a nude mouse model for these cell to use as the mouse model to assess the efficacy of CITme as an anti-cancer agent *in vivo*.

5.2 STUDY AIM

The aim of this part of the study was to establish a mouse model for the DU-145 prostrate cancer cell line. This required the determination of which mouse gender to use as well as the number of cultured DU-145 cancer cells required for transplant into nude mice to induce a high incidence of tumour growth.

5.3 MATERIALS AND METHODS

Ethical approval by the AUCC (Animal Use and Care Committee) was obtained prior to the start of the study (Protocol No. H020-07). The study was conducted at the University of Pretoria Biomedical Research Centre (UPBRC).

- **Animals**

Inbred (from the UPBRC) nude mice (female and male) of four to six weeks old were housed at approximately 22ºC and 40-60% relative humidity in Isolated Ventilated Cages (IVCs) (Type II long Techniplast) under sterile conditions. Mice were housed in groups of four to reduce the stress of solitary confinement except in cases where signs of aggression occurred. Sterilised food and water were provided *ad libitum* with 12 hour day/night light cycles. All cages, feed, bedding, and drinking water were sterilized prior to use. Their care was in accordance to the institutional guidelines.

- **Cell culture**

The human androgen-independent prostatic carcinoma cell line, DU-145, was obtained from ATCC (catalogue number HTB 81). DU-145 cells were grown as a

monolayer in RPMI 1640 medium (GIBCO) supplemented with 10% FCS with penicillin /streptomycin (20 units/ml and 20 µg/ml, respectively) in 75 cm^2 culture flasks at 37°C in a humidified 95% air 5% CO_2 atmosphere and sub-cultured three times a week .

- **Cell line preparation**

After incubation, the tissue culture flasks containing cells were examined under an inverted microscope to check for satisfactory growth, percentage confluence and an aliquot was examined under high magnification after staining to confirm that the culture was not contaminated with any microorganism or mycoplasma.

Tumour cells growing exponentially were harvested by discarding the tissue culture medium from the tissue culture flask containing the cells that were to be used and ± 5 ml trypsin/EDTA was added. Care was taken to coat all interior surfaces with the solution, before it was discarded. Another 2 to 5 ml was added to cover the adherent cells on the bottom of the flask. The culture flask with trypsin/EDTA cell mixture was incubated at 37ºC for a few minutes until the cells showed signs of detaching from the flask. The trypsin/EDTA cell suspension was transferred to a 15 ml centrifuge tube. Tissue culture media supplemented with 10% bovine FCS was used to rinse the culture flask and this was used to fill the centrifuge tube, thus neutralizing the action of the trypsin/EDTA. The cell suspension was centrifuged for 6 minutes at 200*g*. The supernatant was then discarded and the cell pellet was re-suspended in 1 ml of RPMI 1640, supplemented with 5% bovine FCS with penicillin /streptomycin (20 units/ml and 20 µg/ml, respectively) the cell concentration determined by counting a 50 µl aliquot using a haemocytometer. Various cell concentration suspensions were made. The cell suspension was mixed by pipetting repeatedly to make sure that the cells were separated from each other and that a uniform suspension was formed.

Two cell concentrations were tested: $1x10^7$ and $5x10^7$ cells/ml. These were the total cell numbers used to determine which cell concentrations would most effectively induce a solid tumour in nude mice. Two full 75 cm^2 flasks were sufficient to make 400 µl of each concentration.

- **Tumour tissue transplant**

Solid DU-145 tumours of approximately 5 mm^3 resulting after nine weeks of growth after a subcutaneous cell suspension transplant in nude mice (both female and male) were aseptically dissected and mechanically minced; 3mm^3 pieces of each tumour tissue were transplanted subcutaneously by microsurgery into both male and female nude mice under isoflurane anaesthesia. After transplantation, when tumours had grown to a volume between 120 and 150 mm^3, tumours were to be aseptically dissected and transplanted again to another group of nude mice to validate the method to be used for future efficacy study on CITme compound.

This study was divided in two phases:

- ***Phase one***: Tumour induction by injecting in vitro cultured DU-145 tumour cells in suspension under the skin on the flank of nude mice; and
- ***Phase two***: Tumour induction in nude mice by tumour tissue transplantation from solid tumours dissected from mice carrying subcutaneous DU-145 tumours

5.3.1 Phase one: Tumour induction in nude mice by injecting tumour cells in suspension

All the tumour induction studies were performed at University of Pretoria Biomedical Research Centre (UPBRC) at Onderstepoort.

Two different concentrations as a total of cell number of DU-145 cells ($1x10^7$and $5x10^7$cells/ml) where suspended in 1ml each, of RPMI 1640, supplemented with 5% bovine FCS with penicillin /streptomycin (20 units/ml and 20 µg/ml, respectively) for a pilot tumour induction test.

Two pilot studies were completed in four nude mice of four to six weeks old (2 female plus 2 male) for each of the cell concentrations to determine the optimum number of cells necessary to induce a solid tumour in nude mice in a short period of time. A total of eight nude mice (4 mice per cell concentration) were used in these experiments.

For the first pilot study, four mice were injected with 75 µl of DU-145 cancer cells with either $1x10^{7,}$ or $5x10^{7}$ cells/ml suspended in RPMI 1640 supplemented with 5% FCS via a 25-gauge needle into the subcutaneous space of the left flank region.

The second pilot study used another group of four mice that were injected with 200 µl of DU-145 cancer cells at $1x10^{7}$ or $5x10^{7}$ cells/ml. In both studies, palpable tumours were measured through the skin twice a week using a digital calliper at the same time that the mice were weighed. Tumours volumes were measured in mm^3 and were calculated according to the method reported by Tang *et al.,* (2004) by the following formula:

Tumour Volume = $(width)^2$ x length/2.

Mice were terminated according to standard procedures (anaesthetic overdose) used at UPBRC twelve weeks after inoculation. The solid tumours that had formed were aseptically dissected out and weighed to determine their weight.

The mouse cadavers were sent for autopsy and histopathology to ascertain whether any metastasis had occurred.

5.3.2 Phase two: Tumour induction in nude mice by tumour tissue transplantation

In this phase of the experiment, a tumour tissue transplantation technique using microsurgery was used. Solid prostate tumour tissue (±5 mm^3) was inserted subcutaneously into the flank region instead of a prostate tumour cell suspension (DU-145 cells). The solid tumour tissue was the tumour tissue that had developed in nude mice after cell suspension implants.

A pilot study was completed in eight nude mice of four weeks old (four female plus four male) in an attempt to develop solid tumours in a shorter period of time, and also to validate this technique for future efficacy study on CITme compound.

Solid prostate tumours (size of 135 and 151 mm^3) resulting after 9 weeks of growth in nude mice (both female and male) were aseptically dissected and mechanically minced using opposing scalpels; ±5 mm^3 pieces of each tumour tissue were transplanted subcutaneously by microsurgery into four female and four male nude mice under isoflurane anaesthesia. After transplantation, tumours were measured twice a week using a digital calliper at the same time that the mice were weighed. Tumour volumes were measured in mm^3 and were calculated according to Tang *et al*. (2004). When tumours had grown to a volume between 40 and 50 mm^3, tumours would be aseptically dissected and transplanted again to a second larger group of nude mice to validate the method to be used for future efficacy study on CITme compound.

Mice were terminated according to standard procedures (anaesthetic overdose) used at UPBRC twelve weeks after inoculation. The solid tumours that had formed were aseptically dissected out and weighed to determine its weight. Tumour tissue samples were sent for histological analysis to confirm that the growths were tumour tissue and not fibrosis.

The mouse cadavers were sent for autopsy and histopathology to ascertain whether any metastasis had occurred.

5.4 RESULTS AND DISCUSSIONS

The outcome of long-term treatment of prostate cancer (PCa) is poor and this is probably the main reason that PCa is still the leading cause of death from malignant disease among men in the Western world despite curatively intended therapies such as radical surgery and radiotherapy (Rydh, 1999). Because of the poor results in the treatment of prostate cancer, significant efforts have been made to promote research in order to find new therapeutic alternatives.

In the cancer research field, human tumour xenograft model growing in nude mice is widely used as a pre-clinical model system for efficacy testing of new therapeutical drugs. Moreover, the use of human tumour xenografts to examine therapeutic responses to drugs offers several advantages, such as:

- the use of human tumour cells or tissues featuring the complexity of genetic and epigenetic abnormalities that exist in the human tumour population;
- the fact that many of these models are fairly reproducible;
- the hosts are readily available;
- statistically valid numbers of mice can be used in studies;
- the wide variety of tumour lines available, which many of these lines have a long history and therefore a strong baseline of drug response data;
- human tumour xenografts can be used to aid in the development of individualized molecular therapeutic approaches;
- results can be obtained in a matter of a few weeks from a human tumour biopsy regarding response to therapy;
- multiple therapies can be tested from a single tumour biopsy;
- data from tissue microarrays and genetic microarrays can be readily obtained from the human biopsy and xenograft tissue before and after drug therapy, for extensive analysis before the patient is subjected to therapy that may not work and stroma from the human tumour microenvironment can be included in the xenograft to more completely mimic the human tumour microenvironment (Kerbel, 2003; Troiani et al., 2008).

Prostate cancer xenograft models are powerful tools for cancer drug discovery. The recent expansion in the number of cancer cell xenografts has already lead to important new discoveries relevant to the molecular biology of prostate cancer progression.

The DU-145 cancer cell line, derived from a human adenocarcinoma metastatic to the brain, is androgen-independent (Lamharzi *et al*., 1998). This cell line has been reported to have been xenografted into nude mice and represents a suitable model for investigating the effects of various classes of antitumour peptides on androgen-independent prostate cancers both *in vitro* and *in vivo (*Jungwirth *et al*., 1997; Jungwirth *et al*., 1997; Lamharzi *et al*., 1998).

In vitro studies demonstrated the high cytotoxic efficacy of CITme, against the DU-145 prostate cancer cells. Furthermore, the steady increase in the rates of new

cases (or deaths) of prostate cancer made this prostate tumour cell line the cancer cell line of choice to be used for development and validation of a protocol to induce tumours in nude mice that could be used in future studies to test the *in vivo* efficacy of CITme and potentially other drugs.

However, it was observed that the cell volume, the number of cells injected to induce tumour, as well as the gender of the nude mice (since this type of tumour is hormone independent) is not clearly defined in the literature (Steiner *et al.,* 2000; Wells *et al.,* 2002). In an attempt to determine the volume of cell suspension, the optimal cell number as well as the best mouse gender to induce prostate tumour in nude mice, two pilot studies using 75µl and 200µl respectively of $1x10^7$and $5x10^7$ cells /ml of DU-145 cell suspensions were done in 8 mice (4 mice for each volume of cell suspension) of each gender (1 female plus one male mouse for each cell concentration). Moreover, similar studies (using the same and even less cell concentration) applying the same technique in a large animal sample size was proven to develop tumours successfully (in more them 90% of the animals) in a very short period of time (one to three weeks after inoculation) (Jungwirth *et al.,* 1997; Dondi *et al.,* 1998; Steiner *et al.,* 2000; Mikata *et al.,* 2002). However, in this study, none of the mice that received 75µl of either $1x10^7$ or $5x10^7$ cells/ml developed tumours. In previous studies using different cancer cell lines for tumour induction (Protocol No H011-07 using HeLa or CoLo 320 cells), this volume and the total number of cancer cells proved to successfully to induce tumours in nude mice in less than three weeks. Of the four nude mice that received 200 µL of either $1x10^7$ or $5x10^7$ cells/ml suspension, only two mice (one female and one male) injected with the higher concentration of $5x10^7$ of DU-145 cell suspensions developed palpable solid prostate tumours. However, these tumours only started developing seven weeks after inoculation. No signs of tumour growth were observed in any of the other mice even in the fourteenth week after inoculation.

Histological reports on all the animals used in this study, indicated no significant anatomical or histopathological changes in any of the organs. No metastases were found in any of the tissues from any of the inoculated mice. No metastases were found in the two mice that did develop palpable tumours in the area of the cell

transplant. No visible tumour metastasis could be observed in any organs or draining lymph nodes of the left flank of the mice.

The results obtained in this study do not correlate with results obtained by other researchers using a similar tumour induction protocol with the same cell volume and cell concentration (Jungwirth *et al.,* 1997; Dondi *et al.,* 1998; Steiner *et al.,* 2000; Wells *et al.,* 2002; Mikata *et al.,* 2002).

According to Morton and Houghton (2007), primary human tumour models are established by transplanting fresh human tumour fragments onto immune-deficient mice. Compared with traditional cell line based xenograft models, primary models better mimic original patient histopathology, as well as the gene expression profile, and can therefore provide better predictive value for cancer prioritisation and clinical efficacy estimation.

Due to the low success rate of tumour induction and the long lag period that the nude mice required to develop solid subcutaneous prostate tumours after transplant of DU-145 cell suspensions in this study, an alternative experimental technique described by Jungwirth *et al.* (1997); Lamharzi *et al.* (1998); and Mikata *et al.* (2002) of inducing prostrate tumours by using solid tumour segments that had been grown in other nude mice instead of cells suspension were used.

A second pilot study was done in a further eight four-week old nude mice (four female, plus four male). In this study, solid tumours were dissected from the initial two mice that developed solid tumours (from the nude mice transplanted with DU-145 cell suspensions) and diced using opposing scalpels (±5 mm^3) and transplanted subcutaneously by microsurgery into the subcutaneous space of the flank region of new healthy nude mice while under anaesthesia. Because tissue blocks of newly formed solid tumours were used, it was expected that solid tumour would grow in all the animals in a shorter period.

However, none of the nude mice used in this pilot experiment developed any palpable solid tumours within fourteen weeks, despite using this technique of solid tumour block implantation. Macroscopic and histological analysis of the mice cadavers as well as their organs confirmed the absence of any sign of solid tumour formation.

Although our results contradict the results published by other researchers (Jungwirth *et al*., 1997; Lamharzi *et al*., 1998; Mikata *et al*., 2002), our results are in accordance with Charles River Laboratory findings (personal email communication, 2008), where DU-145 cells are no longer used for tumour induction in nude mice, either due to the long lag time required for tumour growth or the highly inconsistent induction of tumours. In addition, our findings are supported by recently published literature, where Morton and Houghton (2007) report that not all tumour cell lines are suitable for *in vivo* tumour induction in nude mice, due to either inconsistent tumour induction rate or unacceptably large tumour size variation within the same mice species.

Taking into consideration the failure in developing prostate tumours derived from DU-145 cell line, an alternative tumour cell line that has proven to be significantly sensitive towards CITme should be used to develop significantly solid tumours in a short period of time.

Although nude mice are a very expensive model, a larger sample size should be used due to the difficulty in establishing growth in this model.

CHAPTER SIX

PHARMACOKINETIC PARAMETERS OF CITME

6.1 INTRODUCTION

Typically, new drug approval is a protracted, expensive and complex process during drug development. It is estimated that approximately 90% of clinical drug candidates fail before registration with estimated costs of 50 to 70 million US dollars. These failures are often due to poor absorption, distribution, metabolism, elimination and/or toxicity properties (http://www.scientistlive.com/European-Science-News/Drug_Discovery/Early_screening_and_innovative_solutions_key_to_drug_development/14641/). Extensive efforts have been invested to develop scientifically relevant approaches to evaluate these properties. The application of *in vivo* assays with laboratory animal species relevant to humans represents one of the best approaches to assess these important drug-like properties and should therefore allow reasonably accurate prediction of *in vivo* human drug properties, giving guidance for the design and selection of drug candidates with high probability of clinical success (Li, 2005).

The treatment of cancer has for many years focused on systemic, low-specificity, high-dose chemotherapy, but there is a growing trend to find a drug that balances minimal adverse events with maximal anti-tumour activity (Abou-Jawde *et al.,* 2003). Initial steps in the development of drugs for cancer treatments using this approach have lead to the creation of several new anti-cancer agents. Improved systemic drug therapy is particularly important for the treatment of patients with advanced metastatic cancer, for whom surgery and radiation therapy can no longer be curative (Workman, 2001).

The clinical application of new anti-neoplastic drugs has been hindered by their narrow therapeutic index and lack of efficacy (Sanchez *et al.,* 2001). Thus, an approach to improve old and new anti-cancer drugs has been to manipulate their pharmacokinetic properties. Pharmacokinetics is the study of the way drug molecules behave in the body after administration (Sanchez *et al.,* 2001). Four

interrelated processes occur between the administration and the elimination of a drug from the body: after oral administration, drug molecules are absorbed via the enterocytes from the gastrointestinal lumen into the portal vein, pass through the liver and the lungs, reach the systemic circulation, and then further distribute into various tissues and organs via blood vessels, some of which may have metabolic or excretory activity for eliminating the drug. These sequential events namely, Absorption, Distribution, Metabolism, and Excretion are referred to as the ADME processes of the drug., The purpose of PK studies, is to define and characterise the ADME processes of drugs in the body by examining the time course of drug concentration profiles in readily accessible body fluids such as blood, plasma, urine, and/or bile (Kwon, 2001).

Previously, pharmacokinetics and toxicity tests were performed in the later stages of drug development. However, with the number of drug targets as well as the volume of assays performed in high-throughput screening expanding, it has become critical to rapidly and efficiently identify potential drugs with advantageous ADME and toxicity (ADME/Tox) profiles at an early stage of development. To this end, the introduction of improved ADME/Tox screens as early as possible in the drug discovery process is gaining appeal. This will help yield compounds with good target affinity, reasonable drug properties and ensure greater likelihood of acceptable ADME/Tox properties. Moreover, it is likely to accelerate the selection process, reduce the overall cost of preclinical and clinical studies and boost the prospects of success.

3-[3-(7 chloro-quinolin- 4-yl amino) phenyl]-1-(4 methoxy-phenyl) prop-2-enone citrate, “CITme” was synthesized as a potential anti-tumour agent. As no data on the absorption parameters of CITme was available and no toxicity was demonstrated during the acute and chronic *in vivo* toxicity studies using Sprague Dawley rats, there was concern whether the experimental compound was significantly absorbed after oral administration. A pilot study comparing oral versus intravenous administration was performed on Sprague Dawley rats in order to confirm that oral uptake does occur and to determine the basic pharmacokinetic parameters of CITme. The best route of administration was also to be determined. The study made use of a limited number of animals, using volumes of <50 µl blood

drawn into a capillary haematocrit tubes after a tail vein prick or other suitable bleed site. The pilot study was carried out using 10 males (five oral plus five I.V. dosed) Sprague Dawley rats (±325 grams) at University of Pretoria Biomedical Research Centre (UPBRC), to determine the suitability of a method that would require small amounts of blood (<50 µl) collected into heparinised haematocrit tubes, instead of catheterisation of a major vein. If this method proved successful and mastered it would dramatically reduce the number of rats required for pharmacokinetics studies in the future. If this same pharmacokinetic study were to be done using the "standard" pharmacokinetic assay method, 101 mice (three mice per time interval) would be required, whereas only 20 rats (ten rats per route of administration) in total would be required for this new method. Therefore development of this method could significantly reduce the time required, number of animals used and the cost required for a pharmacokinetic study.

6.2 STUDY AIM

The aim of this study was to develop a suitable liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to separate and quantitate CITme from biological samples (mainly plasma) when given as a single dose at concentration of 5 or 30 mg/kg body weight by either I.V. or oral administration respectively, and to perform a pharmacokinetic parameter study of oral and I.V. administered doses of CITme.

6.3 MATERIALS AND METHODS

Ethical approval by the AUCC (Animal Use and Care Committee) was obtained for this study (Protocol No H021-07). The study was conducted at the University of Pretoria Biomedical Research Centre (UPBRC).

- **Animals**

Twenty male Sprague Dawley rats of eight to twelve weeks old (±325 grams) were used for this study and were housed at the UPBRC for at least one week before the study was initiated to acclimatise. They were housed in standard plastic rat cages, in pairs with 12-hour light/dark cycles, in rooms with controlled environmental conditions. They had free access to both water and a normal rat

chow diet. The animals had additional nesting materials provided. Their care was in accordance with the institutional guidelines.

- **Chemicals and reagents**

CITme (mol wt. 607.03), (±90% purity) was synthesized and provided by PetLab, Pretoria. Formic acid and triethylamine (TEA) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and 2-methyl pyrrolidone was a gift from BASF (Johannesburg, SA). HPLC-grade acetonitrile was purchased from Fisher Chemicals (Fair Lawn, NJ, USA). In-house purified water (Elga "Genetic" deionisation system) was used for all aqueous solutions. Drug-free pooled mouse and rat plasma was obtained from UPBRC.

6.3.1 Pharmacokinetic pilot study

The rats were weight matched and randomly divided into two groups of five each (one-group rats for oral plus one group for intravenous administration dose).

On the day of the experiment, the animals were treated either with a dose of 30 mg/kg body weight CITme orally by gavage or with 5 mg/kg by intravenous injection into the tail vein. Blood samples (±50 µl/sample) were collected at baseline (0) then at the following times after administration of the CITme: 1, 3, 5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 180, 240, 360, 480 minutes for the I.V. administration and at baseline (0), 10, 15, 30, 60, 120, 180, 240, 360, 480, 720, 1440 minutes for the oral administration. Trained personnel from the UPBRC performed the blood collection. The rats were lightly anaesthetised with isoflurane and a suitable bleed point (saphenous vein) sterilized and punctured with a finger prick lance. Blood droplets were drawn directly into a heparinised haematocrit tube and processed. On completion of the sampling phase of the protocol (i.e. at the 24 hr blood collection) all animals were terminated by CO_2 asphyxiation while under anaesthesia. The kidneys and liver were dissected out and assayed for CITme to determine the extent of organ accumulation.

6.3.2 Preparation of stock solution and calibration standards

Standard stock solution of CITme was made at concentration of 2 mg/ml in 2-*methyl*-pyrrolidone, aliquoted into 100 µL samples and stored at -20°C. Stock solution of the internal standard, chloroquine diphosphate, was accurately prepared at approximately 1 mg/ml in distilled water, aliquoted into 100 µl samples and stored in amber vials at -20°C. The working solution of internal standard was prepared fresh daily from aliquots of the stock solution. For the standards calibration curve, aliquots of stock solution of CITme were added to either distilled water or drug free rat plasma respectively, and then diluted serially with either water or plasma to achieve concentration ranges from 0.5 to 100 µg/ml.

CITme was extracted from the plasma standards as follows: 100 µl of plasma in a 1.5 ml micro-reaction tube was deproteinised with 300 µl of acetonitrile (MeCN) and vortex mixed for approximate 1 minute. After centrifugation at 14000*g* for 5 minutes, the clear upper layer was recovered and 5 µl aliquot of the resulting solution was injected by auto sampler onto the LC-MS/MS system for analysis.

6.3.3 Sample preparation

For analysis of plasma samples, the end of the heparinised capillary tube used to collect the blood samples was plugged with clay, centrifuged at 500*g* for 5 minutes in a haematocrit centrifuge and the plasma harvested using a 10 µl GC syringe, transferred to 50 µl autosampler vial inserts. The samples were diluted with an equal volume of acetonitrile, mixed, sealed and stored at -70°C.

From each organ (kidneys and liver) collected per animal, approximately 540 mg of each organ was weighing off and homogenised in a total of 5 ml of acetonitrile containing chloroquine (IS) and 2-*methyl*-pyrrolidone (3:2) first by cutting with opposing scalpels then by 6 strokes using an Ultraturax homogeniser at 20000 rpm. After that, organ homogenates were centrifuged at 1000*g* for five minutes and the supernatant was collected and stored at -70°C until analysis. Five microlitres of the resultant extract was injected onto the LCMS/MS system by auto sampler.

6.3.4 LC-MS/MS instrumentation and conditions

The LC 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, 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 AB Sciex 4000 QTrap MS/MS system (Applied Biosystems MDS/Sciex, Concord, Canada) with a "Turbo V" electrospray ionisation source operated in positive ion mode.

Diluted plasma and organ homogenate samples (5µl) were injected directly into the LC/MS/MS and slowly transferred onto the C18 capture column (SecurityGuard cartridge of 4.0mm x 3.0mm, Phenomenex, Torrance, CA) with 0.1% formic acid in water before washing at 300 µl/min for 1.75 minutes. At 2.5 minutes the switching valve was actuated and the capture column back flushed with 0.1% formic acid and 0.05% triethylamine (TEA) in 30% MeCN onto a C18 SecurityGuard cartridge (4.0mm x 3.0mm, Phenomenex, Torrance, CA) protected analytical column (Phenomenex, C18 Hydro 100A, 4µm particle size, 75mm x 2.0mm, Torrance, CA) maintained at 45°C. Chromatographic separation was achieved by using a mobile phase of 0.1% formic acid and 0.05% TEA in 100% MeCN and delivered at a flow rate of 300 µl/min. The column effluent was monitored using the following multiple reaction monitoring (MRM) transitions: CITme m/z 415.4→279.1 and 415.4→371.2 m/z, with a dwell time of 80 ms 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 5200V and the drying gas temperature was set 450°C. The collision energy for collision induced dissociation for the aforementioned transitions were 75V. All instrument controls and data collection was performed by the Analyst 1.4.2 (Applied Biosystems/ MDS SCIEX) software program.

A single sample analysis cycle (Figure 6.1) consisted of the following phases:

- ***Phase 1 (A):*** Capture and wash phase
  Firstly an injection volume of 5 µl was transferred to the capture column by the Agilent 1200 pump at 100 µl/min using a mobile phase of 0.1% formic acid where the analytes are captured allowing the proteins to pass to waste. The cartridge was then washed at 1 ml/min for 1 minute.

- ***Phase 2 (B):*** Elution and analysis phase
  After 1.75 minutes the switching valve switches allowing the Agilent 1100 pump to elute the analytes from the capture column using a mobile phase of 30% MeCN with 0.1% formic acid and 0.05%TEA. The analytes were transferred to the analytical column where separation took place with an increasing gradient of acetonitrile to 98%. The column eluent was transferred to the 4000QTrap MS/MS where identification and quantitation took place using positive mode MRM and SRM.

6.4 METHOD VALIDATION

The precision, accuracy and recovery of the assay were based on analyses of samples made up in both water and plasma containing known concentrations of CITme. Plasma and water quality control samples were included within the ranges of all calibration curves and processed in triplicate. The specificity of the assay for the experimental compound in plasma was evaluated using different batches of pooled mouse or rat plasma from healthy animals that had not been exposed to the analytes.

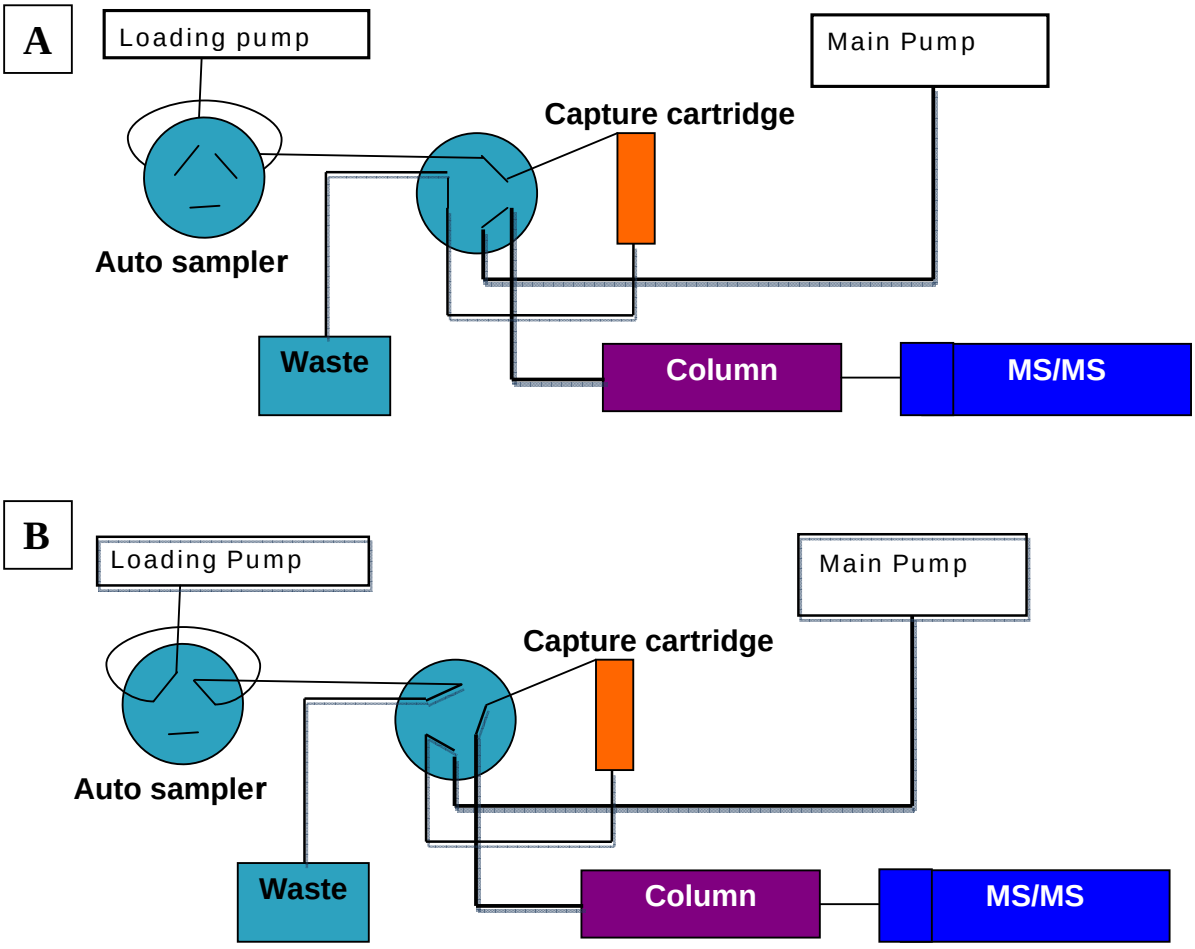

Figure 6.1: Diagrammatic representation of the semi-automated online solid phase extraction (SPE) LC-MS/MS method used for the detection of the parental drug CITme. [A]: Capture and wash phase. [B]: Elute and analysis phase

6.5 RESULTS

6.5.1 Mass spectrometry and chromatography

The positive ion scans of standard solutions indicated that CITme had a protonated molecular ion (M+H)+ of m/z 415.5. Fragmentation of this ion using collision induced dissociation at collision energies ramped from 25 to 75V resulted in two strong product ions for CITme. Under the chromatographic conditions the transition of 415.3→371.2 and 415.3→279.1 were selected for optimal monitoring and quantitation of CITme in rat plasma and organ homogenate samples. The ion spray voltage at 5200V 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.

The mobile phase had a significant effect on the retention time of CITme with retention times shorter than five minutes obtained due to the addition of 0.1% formic acid and 0.05% TEA. A representative chromatogram of CITme from rat plasma after an I.V. injection of CITme at 5mg/kg body weight with a retention time of 4.50 minutes is presented in Figure. 6.2.

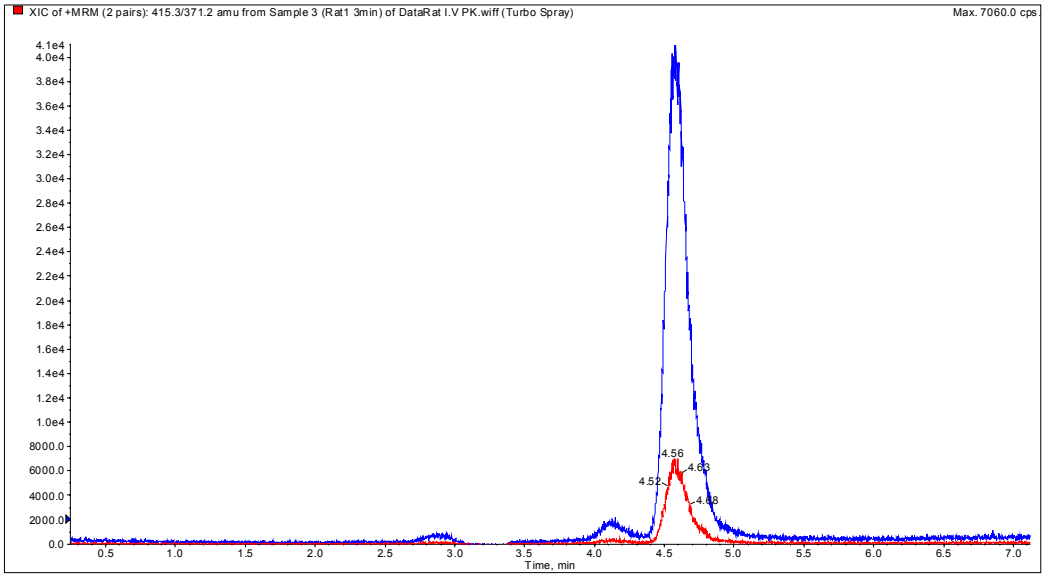

Figure 6.2: A typical chromatogram showing the monitored MRM chromatograms of CITme as seen in a rat plasma sample drawn three minutes after administering an I.V dose

6.5.2 Method validation

The method was validated using both water and plasma for CITme at concentration ranges from 0.5 to 100 µg/ml. Calibration curves prepared over these concentration ranges were linear with average correlation coefficients greater than 0.99 for CITme, in both water and plasma (Figure. 6.3 and 6.4).

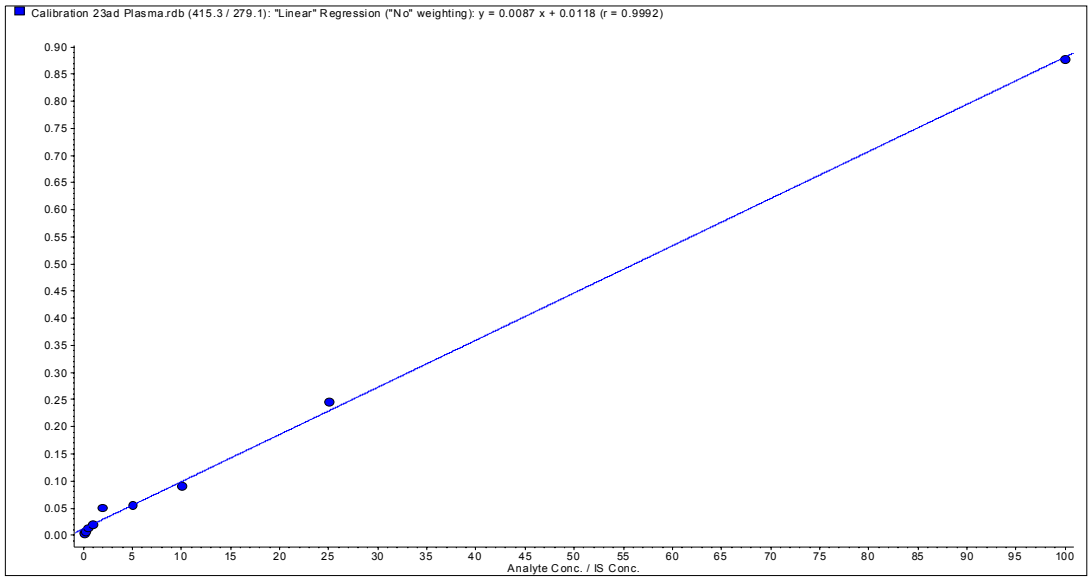

Figure 6.3: Diagrammatic representation of the calibration curve of CITme in distilled water

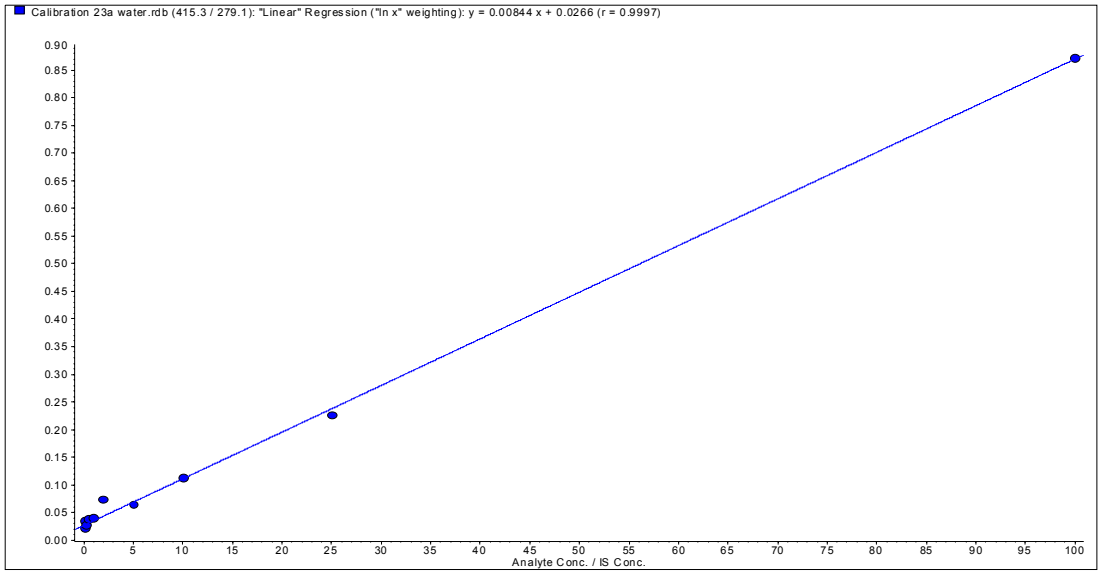

Figure 6.4: Diagrammatic representation of the calibration curve of CITme in rat plasma

6.5.3 Pharmacokinetic pilot study and method application

The method of blood sampling proved to be efficient and could provide enough plasma for the analysis, therefore this technique of blood sampling was implemented in the pharmacokinetic studies.

CITme was found in liver and kidney tissue (Figure 6.5) of all five rats receiving an oral administration of 30 mg/kg body weight. However, the plasma concentrations were very low and appear below the quantitation limit of the method and significantly lower than the concentration found in both liver and kidney tissue at 24 hours after dosing.

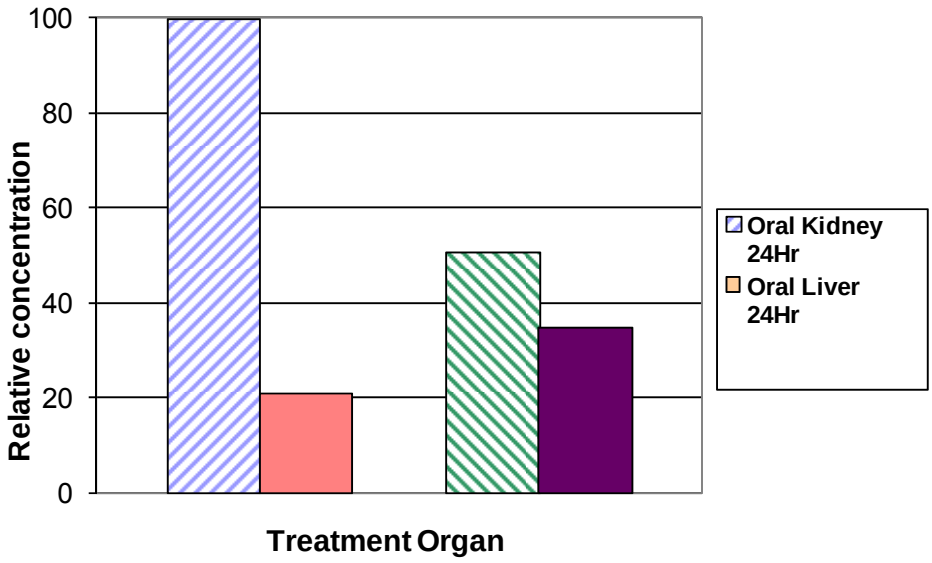

Figure 6.5: Relative concentration of organ distribution of CITme in Sprague Dawley rats after Oral & I.V dosing

The orally dosed rats (30 mg/kg body weight) plasma concentrations were also significantly lower than those found after an I.V. administration (5 mg/kg body weight) despite the lower dose of CITme used for IV administration (Figure 6.6).

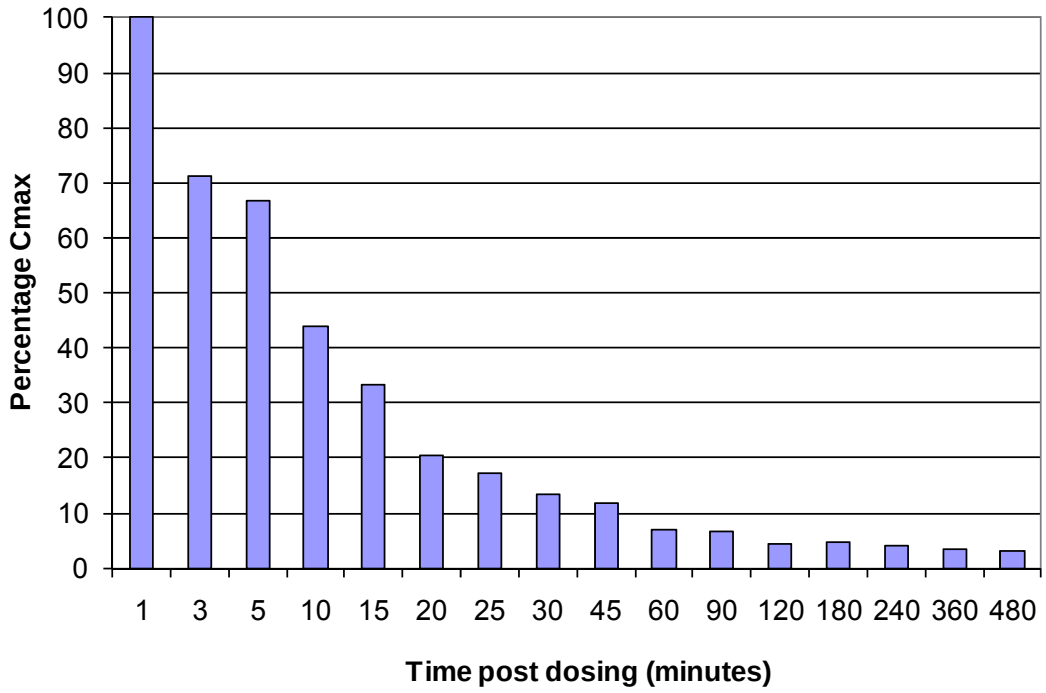

Figure 6.6: Average CITme plasma concentrations as a percent of Cmax after a single I.V. administration of 5 mg/kg body weight in 5 Sprague Dawley rats. Note that the time axis on the histogram is not linear

The maximum concentration (C_{max}) in the I.V. treated rat plasma was seen at the first sampling time at one minute, and the initial elimination half life ($t_{1/2}$) was found to be less than ten minutes (Figure 6.6 and 6.7). CITme was detected in liver and kidney tissues at relatively high concentrations within ten minutes of I.V. administration (Figure 6.5). However, these organ concentrations after I.V. administration were lower than what was found in the same organs 24 hours after the oral administration after the higher administered dose of 30 mg/kg body weight. This finding confirmed that the orally administered CITme was absorbed and that the lack of detectable plasma levels was due to the rate of distribution/elimination exceeding that of the rate of absorption. Although the plasma elimination half-life of CITme is rapid, CITme was still present in plasma at very low concentration eight hours after I.V. administration of relatively small doses (Figure 6.5).

The best route for CITme administration would be the I.V. route as the fast elimination from the plasma would prevent the drug from reaching therapeutically relevant concentrations after oral administration. The rapid plasma elimination rate

could be a negative characteristic of the drug even after an intravenous administered dose due to a limited area under the curve (AUC) and the fact that the organs involved in metabolism and excretion showed high accumulation.

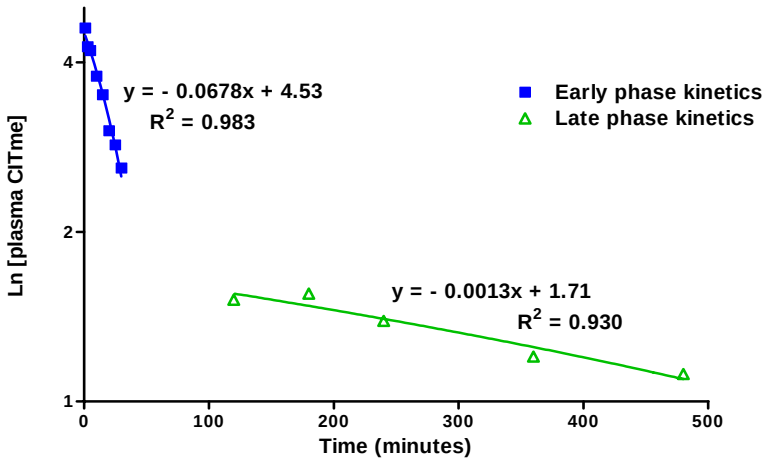

Figure 6.7: Semi-ln plot of plasma concentration of CITme at early and late phase distribution in Sprague Dawley rats after I.V. dosing. There is an obvious two compartment model effect with a very rapid decrease in plasma concentration during the first 30 minutes. From 120 minutes the plasma concentration decreases at an almost constant rate of less than 1/40th of the initial rate

6.6 DISCUSSION

The high cost of drug development is partly due to the high failure rate of drug candidates in clinical trials. A successful drug candidate needs to be efficacious without unacceptable adverse effects (Li, 2005). According to Kwon (2001), pharmacokinetics (PK) and pharmacodynamics (PD), are the most important fields of pharmaceutical sciences for investigating disposition profiles and the pharmacological efficacy of drugs in the body under various experimental and clinical conditions.

During drug development, one needs to select drug candidates with the most appropriate drug characteristics and properties. The critical drug properties can be best assessed by examining the fate of an orally administered drug. Upon ingestion of a drug, the following events would occur:

I. Dissolution: dissociation of the active ingredients of a drug from its matrix and dissolving in the body fluid at the site of absorption.
II. Absorption: passage of the drug through the intestinal epithelium into the systemic circulation.
III. Disposition: entrance of the drug into the systemic circulation and distribution to various organs and tissues.
IV. Metabolism: biotransformation of the drug by the drug-metabolizing enzymes.
V. Biological interaction: interaction of the drug and its metabolites with intended and unintended targets.
VI. Elimination: removal of the drug from the body.

The desirable drug properties in addition to efficacy are high solubility and absorption, rapid distribution to intended target tissues, appropriate metabolic stability with minimal formation of toxic metabolites, minimal toxicity and an appropriate rate of elimination. A drug candidate with these desirable drug properties will be probable to be successful in clinical trials. (Li, 2005).

Furthermore, most pharmaceuticals currently in use are not based on their ability to accumulate in or selectively target the pathological organs, tissues or cells. Usually, the pharmaceutical agent is almost evenly distributed throughout the body. To reach the site of action, the drug has to cross several biological barriers, such as vascular endothelium, organ compartments, cell membranes and intracellular compartments, where it can be blocked, inactivated or express undesirable influence on organs and tissues that are not involved in the pathological process. As a result, to achieve a required therapeutic concentration of a drug at the target site, one has to administer the drug in large quantities, of which a fairly high proportion is “wasted” in normal healthy tissues. In addition, under these circumstances, cytotoxic and/or antigenic drugs can cause many negative side effects in non-target tissue (Torchilin, 2000).

Poor bioavailability can be due to poor solubility, degradation in GI lumen, poor membrane permeation and pre-systemic elimination. By many estimates up to 40 percent of new chemical entities discovered by the pharmaceutical industry today

and many existing drugs are poorly soluble or hydrophilic compounds, which lead to poor oral bioavailability, high intra- and inter-subject variability and lack of dose proportionality (Sheikh *et al.,* 2007).

CITme, was synthesized as a potential anti-tumour agent but has poor aqueous solubility. Because no data on the absorption parameters of CITme existed and the *in vivo* toxicity studies using oral administration showed no signs of toxicity there was doubt that the compound was being absorbed at all. In an attempt to determine the pharmacokinetic parameters including bioavailability of oral doses a pilot pharmacokinetic study comparing oral *vs* intravenous administration was performed on a small number of rats.

An analytical method for CITme in rat plasma that required only small amounts of blood (<50 µl) to give quantitative results was developed and assessed. The blood was collected into heparinized heamatocrit tubes after a tail vein or other suitable bleed site prick, instead of catheterization of a major vein. If the same type of pharmacokinetic study were to be done using the standard methods in use today, 77 mice (three mice per time interval) would be required, whereas only 20 rats (ten rats per route of administration) was required for this new method.

This pharmacokinetic assay method offered several advantages such as:

- reduction of the number of animals required for the study,
- sequential sampling from each animal due to small sample volume required,
- reduction on the period of time needed for the whole assay,
- fewer qualified personnel required,
- reduced cost of the study.

However, this method also presented some disadvantages that should be taken in consideration such as:

- the requirement of personnel skilled and qualified to perform intravenous animal injections,
- the stress and alteration of animal behaviour due to handling,

- repeated bleeding, which can cause blockage of the vessels (arteries and veins) during the bleeding process,
- very small blood samples collection
- delay of bleeding time points that are relatively rapidly spaced.

Although CITme was found in plasma, liver and kidneys of all the rats after an oral administration of 30 mg/kg body weight, the pharmacokinetic parameters could not be determined due to the very low levels of the drug found in plasma. Even though the relative plasma concentrations of CITme found were irregular and very low, CITme's presence at significantly higher levels in kidneys and liver 24 hours after dosing proves that CITme is substantially absorbed after oral administration. With *in vivo* oral uptake being confirmed the problems with apparent poor bioavailability cannot be related with poor absorption, but with other pharmacokinetic factors that are not well defined yet.

With regards to the pharmacokinetics parameters of CITme after an I.V. dose, CITme showed maximal plasma concentrations at the first bleed time (one minute after administration), confirming that the CITme was correctly administered (I.V. route). With regards to its pharmacokinetics, CITme followed a distinct two compartment model, with an initial (0 – 30 minutes) elimination rate constant of 0.068 min^{-1} with an elimination half life ($t_{1/2}$) of 10.2 minutes, and a final elimination (2 – 8 hours) of 0.0013 min^{-1} with an elimination half life ($t_{1/2}$) of 8.9 hours. There is thus an initial fast distribution out of the plasma and/or elimination. CITme was however found in liver and kidneys at significant concentrations within ten minutes after I.V. administration showing rapid organ accumulation. Although it is initially very rapidly eliminated from the plasma, it does remain in plasma at very low concentration for at least eight hours after administration. These results are in agreement with other investigations of similar lipophilic quinolines that appear to be highly protein bound and several drugs of this class (chloroquine) have been shown to accumulate mainly in red blood cells (erythrocytes) and remain in body for very long periods. Future studies would need to assess the red blood cell and other lipid rich organ (such as the brain, lungs and adipose tissue) accumulation of

CITme to better understand the distribution of the drug (Aderounmu and Fleckenstein, 1983; Virendra *et al.,* 1999).

The LC-MS/MS method was developed to separate and quantitate CITme from rat plasma and organ homogenate samples. The doses that were required to be analysed were a single dose of 30 and 5 mg/kg body weight after an oral and I.V. administration respectively. The assay is based on injecting the equivalent of 5 µl of pre-treated (protein crash) rat plasma directly onto an capture column mounted in a column switching system, washing off the remaining contaminating protein then back eluting the captured analytes onto a 75x2.0 mm with 4 µm particle size column for analysis using optimized mass spectral conditions and analyte specific MRM transitions. This concept allows for minimal sample preparation as the automatic switching valve has a capture column in the “sample loop” position, which is used advantageously to eliminate the contaminating protein fraction that remains in solution after an acetonitrile “protein crash” sample-preparation step. The utility of the method was demonstrated by determination of the pharmacokinetic parameters for CITme in Sprague Dawley rats. This LC-MS/MS method has several advantages such as: low sample volume requirement, simple sample manipulations and preparation steps, rapid clean up and extraction of the experimental compound from the sample, high sensitivity and quick quantification of CITme in plasma samples. The ability to measure CITme in small plasma volumes permits serial sampling protocols in individual rats that will support comprehensive drug pharmacokinetics studies designed to understand the factors that determine CITme pharmacokinetics.

However, CITme concentrations could not be quantitated in the orally dosed rats, due to the very low concentrations found in the plasma after dosing by this route and a confounding continuous low analyte bleed or carry over seen in all samples, including the control (blank) samples after several assays. This analyte bleed appears to be related to the very low solubility of the analyte in the protic solvents that are part of the mobile phases.

CHAPTER SEVEN

FINAL CONCLUSIONS AND RECOMMENDATIONS

7.1 DISCUSSION AND CONLUSIONS

This study served to provide pre-clinical information of the potential anticancer quinoline derivative compound, CITme, regarding its efficacy, pharmacokinetic and toxicity both *in vitro* and *in vivo* respectively.

The aim of this study was to investigate CITme with regards to its cytotoxicity effect and tumour specificity against primary cell cultures and various cancer cell lines, to formulate the poorly soluble drug into a non-toxic formulation, to determine the *in vivo* toxicity of CITme in BALB/c mice, to determine the tolerated and the safety dose of CITme in the BALB/c mouse model using an acute toxicity study followed by a chronic toxicity study before embarking on the tumour efficacy study in the nude mouse model. Several objectives were defined like the determination of which gender of mouse to use as well as the number of DU-145 cancer cells to induce tumour growth in nude mice, to determine the anti-tumour effect of CITme in BALB/c mice, and to determine CITme pharmacokinetic parameters, after oral and I.V. administration and to develop a suitable LC-MS/MS method to separate and quantitate CITme from biological samples when given as a single bolus dose at concentration of 5 and 30 mg/kg body weight for I.V. and oral administration respectively.

To determine CITme anticancer cytotoxicity various *in vitro* studies were performed using various cancer cell lines as well as normal primary cultured cells. The toxicity study, efficacy study and pharmacokinetic parameters were to be determined using either BALB/c mice or Sprague Dawley rats, which is drug related data that is required before embarking on human clinical trials.

Cytotoxicity assays were conducted to determine the level of sensitivity of different cancer cells lines as well as the selectivity (tumour specificity) of cancerous verses normal untransformed cells to the experimental compound. In the present study, the cell survival rate was assessed using the MTT cell enumeration assay method

after exposure to CITme and culturing for seven days. The results obtained in this study indicated that CITme is very cytotoxic to cancer cells, specifically to HeLa (0.872 µM) and DU-145 (2.543 µM) cells with no or minimal effect against normal cells such as resting and stimulated lymphocytes from healthy donors (26.488 µM, >50 µM) and chicken embryo fibroblasts (>50µM).

The potent cytotoxic activity of CITme against cancer cells were not surprisingly because various quinoline derivatives have been demonstrated to have anticancer effects (Chen *et al.,* 2005; Ferlin *et al.,* 2005; He *et al.,* 2005; Li *et al.,* 2006; Musiol *et al.,* 2007; Singh *et al.,* 2007). Based on these impressive *in vitro* results, it could be concluded that CITme exhibited selectivity for the tumour cells and was far less toxic to the normal cells, which is a desirable combination of characteristics for a successful anticancer drug.

Due to the lack of solubility of CITme in aqueous matrices, which would be a requirement for effective drug absorption, a non-toxic formulation for CITme was required. Various formulations that would be stable for use in *in vivo* toxicity studies were attempted. Different low toxicity solvents were used in an attempt to obtain a stable solvent based formulation of the low solubility drug without exerting harmful effects. Biologically compatible solvents that are commonly used to solubilise lipophilic drugs showed poor solvent ability for CITme, yet it was found to be soluble in DMSO, DMF and formic acid, which all have toxic or adverse *in vivo* effects at relatively low concentrations at which the CITme would start precipitating out of the solution.

However, CITme was found to be a suitable candidate for formulation with the patented non-toxic propheroid drug delivery system prepared by the Pharmacy Department at North-West University (NWU). The propheroid drug delivery system could circumvent the solubility problems experienced with the solvent-based formulations. A pilot acute toxicity study was carried out on ten BALB/c mice (five for control, plus five for experimental), to test the acute toxicity profile and safety of the propheroid formulation without CITme (control) as well as a propheroid formulation of CITme at 30 mg/kg/day (experimental) by oral gavage. The pilot acute toxicity study showed that neither the propheroid drug delivery system nor

propheroid/CITme formulation at 30 mg/kg/day was toxic in mice after oral gavage for seven consecutive days. The use of the propheroid drug delivery system could circumvent the initially experienced solubility problems and could be used for the chronic toxicity study as well as an anticancer efficacy study. A large-scale acute toxicity study with a large sample size (40 mice) still needed to be performed using this delivery system. The pilot study also proved that the toxicity levels of CITme, was acceptable and was comparable to other quinoline derivatives.

Acute and chronic toxicity studies were carried out to determine the *in vivo* toxicity of CITme. During the acute toxicity study using forty mice divided into equal groups of propheroid CITme formulation treated and non drug containing propheroid formulation treated control animals, three mice from the control group died shortly after dosing by gavage. Post mortem on these animals showed lung lesions. All other animals in the study survived without showing any observed adverse effects.

During the chronic study, forty mice divided into equal groups of CITme treated and propheroid treated control animals, were treated daily by gavage for 45 days. Four mice (one from the control group and three from the experimental group) died during this phase of the study, all shortly after gavage. The propheroid delivery system has previously been proven to be safe for oral administration in mice. It was concluded that the cause of death of the mice was most probably due to handling problems during gavage and therefore not clinical relevant.

In the acute toxicity study, animals maintained their body weight with no significant mass differences between the treatment and control groups. On the other hand, an increase in body weight was observed in both treatment groups (control and experimental) during the extended period of the chronic toxicity study. When comparing the increase in body mass between the two groups no significant differences in the growth rates could be seen. Organ weights (liver, spleen and kidney) recorded from both the acute and chronic toxicity study post mortems did not show any significant difference when comparing the controls and the treated animals. No significant differences in haematological parameters could be observed between the control and experimental groups in these two toxicity studies.

Taking all the *in vivo* toxicity assay data collected into consideration, it could be conclude that CITme formulated into the propheroid drug delivery system was safe for oral use and could be administered safely at up to 30 mg CITme /kg body mass representing the doses used for the periods tested.

In vitro studies demonstrated a high cytotoxic efficacy of CITme against a prostate cancer cell line (DU-145). The increase in the reported rate of new cases (or deaths) of prostate cancer made this prostate tumour cell line the choice for development and validation of a protocol to induce human tumours in nude mice that could be used in studies to test the *in vivo* efficacy of CITme and potentially other drugs.

Tumour induction was attempted by inoculating various concentrations (75 or 200 µL of $1x10^7$and $5x10^7$as total of cell number) of *in vitro* cultured DU-145 cancer cells in suspension subcutaneously into the left flank region of eight nude (immune compromised) mice. Only two mice (one female and one male) injected with $5x10^7$ as total number of cells of DU-145 cell suspensions developed any tumours at the inoculation site and this was only palpable seven weeks after inoculation.

Due to the low success rate of tumour induction and the long lag period that the nude mice required to develop solid subcutaneous prostate tumours after transplant of DU-145 cells suspension in this study, a second experimental technique described by Jungwirth *et al* (1997); Lamharzi *et al* (1998); and Mikata *et al* (2002) of inducing solid prostrate tumours by using solid tumour segments grown in nude mice instead of *in vitro* cultured cells suspension was used. In this study solid prostate tumour tissue of ±5 mm^3 from the previous pilot study were transplanted into a further eight nude mice. However, none of the animals developed any palpable tumour and histological reports from all the animals used in this study, indicated no significant anatomical or histopathological changes in any of the organs, nor could any metastases be found in any of the tissues from these eight animals.

In conclusion, the hormone independent tumour cells, DU-145 proved to be an unreliable cell line for human xenografts studies into nude mice, due to the poor rate of successful tumour development and secondly because of the long time period required for solid palpable tumours to develop using either the subcutaneous injection of cultured cell suspensions or by transplanting segments of solid tumour tissue. For these reasons, the potential *in vivo* anti-tumour activity of CITme could not be determined as yet, and an alternative suitable cancer cell line would be required to develop a successful human xenograft model in nude mice for testing the anticancer efficacy of CITme. The cell line used would need to be significantly sensitive to CITme as was seen with the DU-145 cell line.

Because no data on the pharmacokinetic parameters of CITme existed and there was concern whether CITme was significantly absorbed after oral administration, an oral versus I.V. pharmacokinetic study was performed. Due to the total lack of signs of toxicity during either the acute or chronic studies, it was proposed that CITme may not be absorbed in significant amounts from the GIT after oral administration. The fraction absorbed needed to be determined to confirm that sufficient drug was being taken up to provide therapeutic doses and that the lack of any toxicity was in fact due to the drug being non-toxic and not due to poor systemic absorption.

An LC-MS/MS method was developed to separate and quantitate CITme in plasma and organ homogenate samples. The LC-MS/MS method could quantitate CITme from blood volumes of less than 50 µL. The application of this method that requires only small amounts of blood sample per time interval allows for easy sampling from the same animal at multiple time intervals, thus reducing the number of animals to be used in a study and giving better quality data due to the collection of sequential repeated measures or single sample multipoint data. Such a method was successful implemented, and can therefore be used for future animal pharmacokinetic studies using CITme or closely related compounds.

Results from this pharmacokinetic study indicate that CITme's bioavailability in plasma is very erratic after oral dosing and presents a very fast initial elimination (metabolism and/or distribution) after an I.V. dose.

Despite the fact that the efficacy and oral pharmacokinetics properties of CITme could not be determined, this study has (partially) proven the research hypothesis that CITme, a novel quinoline derivative is a selective and potent *in vitro* anti-tumour agent with low toxicity both *in vitro* and *in vivo* for healthy tissue.

In conclusion, CITme has showed the potential to be developed as an anti-cancer drug based on *in vitro* cytotoxicity assay as well as it lacks of *in vivo* toxicity.

7.2 RECOMENDATIONS

The results obtained in this study clearly indicate the potential anti-cancer effect of CITme against cancer cells with minimal toxicity effect against normal cells *in vitro* cultures, and *in vivo* in mice. However, several problems were encountered during this investigation that could have been avoided. Firstly, not all the information with regards to the chemical synthesis, purity, solubility, and stability of the experimental compound was provided before the initiation of this investigation. Moreover, the synthetic process of CITme should include an effective cleanup procedure as well as solubility tests in order to describe the physico-chemical properties that are important in describing drug lipophilicity of this compound. This data should be available together with sufficient pure material from a single batch synthesis before embarking on any preclinical tests. A potential stable formulation should also be available before embarking on studies that will be used in *in vivo* testing of either toxicity or efficacy.

The failure to develop a human xenograft model using the DU-145 cell line for prostate tumours in nude mice has been found to be a common problem and an alternative prostate cancer cell line should be used. Personal communication with researchers at Charles River advised against the use of DU-145 for reasons of poor reproducibility of the tumour model in mice. An alternative prostate cancer cell line would need to be used, but before developing the model it would be advisable to test for the *in vitro* sensitivity of the alternative cell line to CITme. Although nude mice are a very expensive model, a larger sample size should be use due to the difficulty of establishing high enough numbers of animals with solid tumour growth to obtain statistically meaningful results.

Although this novel compound presented some serious problems to work with, the data obtained during the study gives a clear indication of its anti-cancer potential, and this project should be continued to establish the anti-tumour properties of CITme, rather than abandoning it due to solubility problems or the absence of a reliable and functional tumour xenograft model at present.

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APPENDIX

- ETHICS PROTOCOL APPROVALS -

Gisela

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Animal Use and Care Committee

Dr Roland Auer
Acting AUCC Co-ordinator: Faculty of Health Sciences

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University of Pretoria

Prof C Medlen
Department of Pharmacology
Faculty of Health Sciences
BMW Building, Prinshof Campus
PO Box 2034
Pretoria
0001

June 27, 2006

Dear Prof Medlen,

Protocol H22/06 - The use of chicken embryo fibroblast cultures in toxicity assays

The Animal Use and Care Committee approved the above-mentioned request at a meeting held on Monday, June 26, 2006.

The AUCC however requested that you report the number of embryonated eggs used at the end of the year.

Please contact this office should you have any questions.

Yours sincerely,

Dr Daan Verwoerd
Chair: AUCC

Cc Prof Medlen

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H009/07

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27 February 2007

Prof CE Medlen
(connie.medlen@up.ac.za)

Dear Prof Medlen

H009/07– An *in vivo* investigation of the toxicity of CITme, a quinoline derivative compound in Balb/c mince (SCC Martins)

I am pleased to inform you that protocol H009/07 was approved by the Animal Use and Care Committee meeting held on 26 February 2007 and that you may commence with the project.

Please contact this office should you have any questions.

Kind regards

Elmarie Mostert
Contact Person: AUCC

Copy SCC Martins, Dept of Pharmacology, Faculty of Health Science, Room 7-25, BMW-Building, Prinshof Campus
Lita Pauw (lita.pauw@up.ac.za)

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27 June 2007

Dr A D Cromarty
Department of Pharmacology
Faculty of Health Science
University of Pretoria
(duncan.cromarty@up.ac.za)

Dear Dr Cromarty

H020-07 - An *in vivo* investigation of the anti-tumor properties of ClTme, a quinoline derivative compound using human cancer xenografts in nude mice *SCC Martins)*

I am pleased to inform you that the abovementioned protocol was approved and signed by the Animal Use and Care Committee meeting held on 25 June 2007.

Please contact this office should you have any questions.

Kind regards

E Mostert

Elmarie Mostert
Contact Person: AUCC

Copy: SCC Martins (Researcher) scc_martins@yahoo.com.br
Dr R Auer (UPBRC) roland.auer@up.ac.za

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H021-07

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27 June 2007

Dr A D Cromarty
Department of Pharmacology
Faculty of Health Science
University of Pretoria
(duncan.cromarty@up.ac.za)

Dear Dr Cromarty

H021-07 - The pharmacokinetic profile of CITme, a quinoline derivative compound using Sprague Dawley rats or BALB/c mice (*SCC Martins)*

I am pleased to inform you that the abovementioned protocol was approved and signed by the Animal Use and Care Committee meeting held on 25 June 2007.

Please contact this office should you have any questions.

Kind regards

Elmostert

Elmarie Mostert
Contact Person: AUCC

Copy: SCC Martins (Researcher) scc_martins@yahoo.com.br
Dr R Auer (UPBRC) roland.auer@up.ac.za

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H030-07

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30 October 2007

Dr AD Cromarty
Department of Pharmacology
Faculty of Health Science
(Duncan.cromarty@up.ac.za)

Dear Dr Cromarty

H030-07 – A pilot uptake study of a CITmc suspension using Sprague Dawley rats *(SCC Martins)*

I am pleased to inform you that the abovementioned protocol was approved by the Animal Use and Care Committee at its meeting held on 1 October 2007.

Please contact this office should you have any questions.

Kind regards

EMostert

Elmarie Mostert
Contact Person: AUCC

Copy: Dr R Auer (roland.auer@up.ac.za)
Mrs. M Watson (marie.watson@up.ac.za)