Studies of Cytotoxicity and Anticancer Mechanism of Cytotoxic Agents: Cyclopentenones, Cyclohexenones, Oxysterols and LR6M

by

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The biological activity of a series of natural products containing the group O=C-C=CH_2 was reported. Dr. Wang's Medicinal Chemistry group synthesized a number of possible drug candidates bearing this group or not to observe whether it is essential to biological activity with the aim of finding effective clinical drugs. In the project MAC13 and MAC16 cell lines were used to evaluate the cytotoxicity. From the values of IC_{50} (drug concentrations 50% inhibition of growth in culture) shows that ZK3 and ZK6 have the highest cytotoxicity for both MAC13 and MAC16 cell lines. ZK5 and ZK1 have low cytotoxicity for both cell lines. Of the other series of compounds most have high cytotoxicity for the MAC13 and moderate cytotoxicity for the MAC16 cell line. The aryl group plays an important role in cytotoxicity as seen by comparing the ZK1 and ZK3 series. For each series the substituent has a different effect on the cytotoxicity, but no regular pattern was obtained. Incorporation of radiolabelled thymidine, uridine and leucine was significantly reduced suggesting that they inhibit DNA, RNA and protein synthesis. Antioxidants can not reverse the cytotoxicity effect of ZK compounds and the results of interaction of 6,6-dithiodinicotinic acid with tumour cells indicated that free radicals and alkylating species are not the main mechanism of action and the action position is not on the cell membrane. For selectivity evaluation, comparing the IC_{50} values between the tumour cell lines (MAC16, MAC13) and the normal cell lines C_2C_{12} (mouse myoblast cell lines), 3T3 (mouse fibroblast cell lines) suggested there was no selective toxicity towards tumour cells.

Dr Wang's chemistry group synthesized another series of compounds named oxysterols, derivatives of cholesterol that contain additional oxygen atoms either on the steroid nucleus or on the side chains. The cytotoxicity of the 24 compounds was evaluated against the MAC16 cell line. Most of these compounds are inactive, and some of them were even stimulating the growth of the cell lines. Some of them were synthesized as isomers to evaluate the effect of stereo structure on biological activity. Comparing the cytotoxicity of the isomers, there was no significant difference between them. So the stereo difference might not affect the cytotoxicity against the MAC16 cell line. They are not stereo specific. The mechanism of the effect on cell growth of oxysterols still needs to be investigated.

Dr. Ayuko's group discovered a novel anticancer agent from a reaction between N-methylformamide and dichloroacetylchloride. LR6M (reaction product), OXOFORM-CF (intermediate) and LR6H (prodrug) were evaluated for cytotoxicity in the MAC13 and MAC16 cell lines. LR6H has high cytotoxicity in the MAC13 and low cytotoxicity in the MAC16 cell lines. LR6M and OXOFORM-CF have no cytotoxicity in both cell lines. It is suggested that LR6M might act as a prodrug. Isolation experiments suggested that HPLC is not suitable to purify these compounds.

Keywords: cytotoxicity, oxysterol, α , β -unsaturated ketone, NMF derivatives

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Abbreviations

ATP	Adenosine 5'-triphosphate
BHT	Butylated hydroxytoluene
CPDS	6,6-Dithiodinicotinic acid
CT	Cholestanetriol
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
GSH	Glutathione
HMG-CoA reductase	3-Hydroxy-3-methylglutaryl coenzyme A reductase
^{3}H-thymidine	Methyl labelled tritiated thymidine
HPLC	High performance liquid chromatography
LR6M	3,4-Dichloro-6-hydroxy-1-methyl-1, 6-dihydro-pyridine-2, 5-dione
MAC	Murine colon adenocarcinoma cell
NMF	N-Methylformamide
OS	Oxysterol
PBS	Phosphate-buffered saline
RPMI1640	Roswell Park Memorial Institute medium
TCA	Trichloro-acetic acid
7-KC	7-Ketocholesterol
7-HC	7-Hydroxycholesterol

Chapter

1.0 Introduction

Cancer is a disease whose existence was first recognized thousands of years ago. The term cancer, which means "crab" in Latin, was coined by Hippocrates in the fifth century B.C. to describe diseases, in which particular tissues grow and spread unrestrained throughout the body, eventually choking off life.

Cancer, the uncontrolled proliferation and invasion of neoplastic tissue into the surrounding organs, with consequent metastasis to other sites, is the second most common cause of death in the developed world after cardiovascular disease (Murray et al 1990). A defining characteristic of cancer cells is their ability to divide under conditions where their normal counterparts do not. The gain or loss of function in proteins that constitute the cell cycle machinery itself can largely explain this unconstrained proliferation (Clurmun and Robert 1995). Cancer cells are inherently disordered, genetically unstable and extremely heterogeneous.

1.1 Chemotherapy

Chemotherapy is the term given to drugs that influence many tumour cell types and whose actions are largely based on blocking cell proliferation. It is most effective against rapidly dividing cells and its efficacy depends on the concentration of drugs reaching the tumour and the duration of this exposure.

While surgery for cancer was already reported centuries ago, practical limitations restricted its use. With the advent of anaesthesia its use become more wide spread and for more than 100 years surgically removing tumours had now become a standard of care. After the discovery of the principles of radiotherapy this technique was rapidly developed and also became a standard treatment quite some time ago. In contrast to those two treatment modalities, chemotherapy is a relatively young strategy. The first steps in its development were made in the late 1930's, and followed-up at the end of the Second World War. Since the late 1950's in particular, there has been steadily increasing interest in the development, which should not be underestimated. While

surgery and radiotherapy beyond any doubt have their merits and result in a cure for some 40% of all cancer patients, the remaining 60% die as a result of metastatic disease. For those patients cancer has to be considered as a systemic therapy.

In the 50 years of its development, chemotherapy has yielded important achievements. While the most important effect is an improvement in survival (McVie 1999), it is obvious that in this respect gains have to be balanced against toxicity in indications where efficacy is limited. However, palliative effects such as symptom control and an increase in quality of life cannot be ignored (Verweij and de Jonge 2000). As chemotherapeutic drugs also affect normal cells, their side affects can be considerable. The gap between the minimum effective dose of drug and the maximum dose tolerated by the patient can be small.

Over the last few decades chemotherapy has evolved to become an important integral part of the multidisciplinary approach to the treatment of cancer. In a limited number of cases chemotherapy has become the backbone of curative treatment. Now chemotherapy is the most widely used type of treatment and can be used at different stages of cancer progression.

1.1.1 **Cure by chemotherapy**

Certainly among non-oncologists there is limited awareness of the curative potential of chemotherapy, which can occur frequently in spite of the extensive spread of disease. In several diseases, particularly a few that were extremely lethal before the era of chemotherapy, chemotherapy can now be considered the main curative treatment. The treatment of metastatic germ cell tumours with chemotherapy is one example of the success of modern medical oncology. The first advantage in the treatment of germ cell tumours was achieved in 1960 with the introduction of chlorambucil, dactinomycin and methotrexate resulting in tumour regression in 30% of patients (Mccaffrey and Bajorin 1998). The next major improvement came with the recognition of the efficacy of cisplatin. Currently, approximately 70%-80% of patients presenting with metastatic disease can be cured with cisplatin–based chemotherapy. Chemotherapy also had a major impact on the cure rate of female patients with choriocarcinoma. Before the

introduction of methotrexate, choriocarcinoma resulted in the death of 60% of patients with disease confined to the uterus and 90% of those with metastatic disease (Li et al 1956). As for testicular cancer, the treatment of these patients is currently also based upon prognostic factors. Good prognosis patients are treated with single-agent methotrexate resulting in a 100% cure rate. In intermediate and high-risk patients combination chemotherapy is the treatment of choice. And also in the management of Hodgkin's and non-Hodgkin's disease, the development of chemotherapy resulted in a major improvement in survival.

1.1.2 **Adjuvant chemotherapy and chemoradiation therapy**

For many solid tumours, a certain level of incurability is still a reality. The incurability mostly relates to residual cancer in an occult and microscopically stage, eventually leading to the outgrowth of metastases. At this stage, local therapy apparently fails to be sufficient and systemic therapy may be attractive. It is actually at this stage where over the years some important achievements have been made. One of the diseases where adjuvant (postsurgery) chemotherapy has had a major impact is breast cancer. It led to a significant reduction in recurrence as well as an increase in survival in patients under the age of 70 years. In view of reported response rates to chemotherapy, breast cancer, even when metastatic, has always been considered as a relatively chemosensitive disease. In contrast, colon cancer has always been considered as relatively chemotherapy-resistant once it has metastasised, with much lower response rates being reported (Verweij and de Jonge 2000).

The primary goal of concomitant chemoradiotherapy has largely been to use cytotoxic agents to sensitise tumour cells to the effects of the radiotherapy. Concurrent chemotherapy inhibits the repair of the sublethal damage from irradiation, synchronises cells to a particularly radio sensitive phase of the cell cycle, reduces the fraction of hypoxic cells that are resistant to radiation and initiates proliferation in nonproliferating cells. The chemotherapy has been combined with radiotherapy in the treatment of locally advanced cervical cancer and squamous cell cancer of the head and neck region with nodal involvement, in an attempt to reduce the rate of local failure and distant

metastases, and thereby improve overall survival and organ preservation.

1.1.3 Drug progress and targets

Most of the currently available drugs are designed to be selectively toxic against rapidly dividing cells. This gives a low therapeutic index, which causes unacceptable damage to normal organs, limiting the drug dose that can be administered (Deonarain and Epenetos 1994).

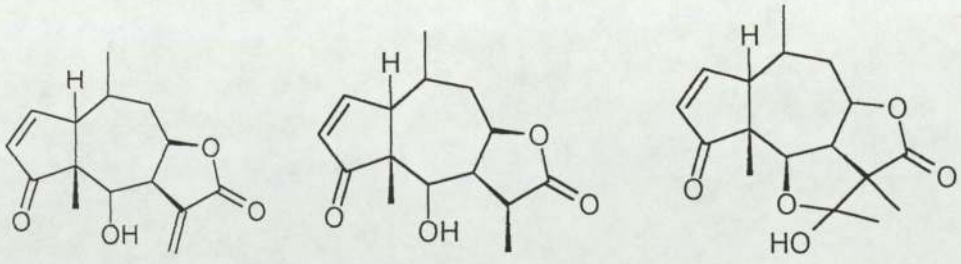
Over the decades the search for new agents against cancer has become increasingly systematic and increasingly focused. In the 1980s special attention was given to the development of analogues with the hope of developing agents that were either more efficacious or at least as efficacious as the parent drug, but with less toxicity. The search for analogues has unfortunately not been very rewarding and only a few agents have been added to the chemotherapeutic armamentarium. In the 1990s, there has again been a shift towards the development of new classes of agents although the term "new" might not always be fully appropriate. The two most successful class developed in the last decade have been the taxanes and the topoisomerase I inhibitors. Although the search for analogues is not very successful, it is still a common method to find active compounds for drug candidates.

Recent achievements in the field of molecular biology have unraveled many of the processes in the development and growth of cancer cells, as well as factors in the microenvironment that are a prerequisite to this growth and to the metastatic potential of cancer cells. The Human Genome Project has resulted in a true plethora of potential targets for anticancer drugs. Actually, since the number of potential targets has now become more and more it is likely that the art of drug development will become choosing the right target, rather than anything else. Such as the blood vessels (angiogenesis) is an absolute necessity for tumour growth and survival (Guardiola et al 1996).

1.2. The progress of α , β −unsaturated ketone research

Many natural and synthetic α , β -unsaturated ketones have significant anticancer activity (Hall et al 1977). The presence of a methylene functional group to the carbonyl group of γ -lactone or cyclopentanone has given rise to promising anti-cancer and cytotoxic activities. The cyclopentanone framework containing an α -methylene group, which is isosteric with an α -methylene- γ -lactone, has elicited substances with anti-cancer effects. The α -methylene cyclopentanone nucleus appears in natural products such as Helenalin, Tenulin, Plenolin **(Figure 1)** as well as in many synthetic analogues.

Helenalin (isolated from the extraction of *Texas Helenium microcephalum*) caused 99% inhibition of Ehrlich ascites cell growth and produced a T/C =316 for the survival in Walker 256 ascites carcinosarcoma (Lee et al 1975). Tenulin (isolated from the extraction of *Helenium amarum*) and Plenolin (isolated from the extraction of *Florida Helenium autumnale*) were also found to have potent cytotoxic activity (Hall et al 1977) These compounds contain α -methylene- γ -lactone or α -methylene cyclopentenone ring system.



Helenalin Tenulin Plenolin

Figure 1. Structures of Helenalin, Tenulin and Plenolin

Studies have demonstrated that these ring structures interact with reduced glutathione and L-cysteine. And the O=CC=C system of the cyclopentenone ring represents a major alkylating centre of antitumour agents for Tenulin and Plenolin, which is particularly reactive with thiol groups of proteins (Hall et al 1977). Both Helenalin and Tenulin reduced DNA synthesis, DNA levels. This is not due to alkylation of nucleophilic sites of purine bases but rather appears to be reaction with thiol groups of enzymes necessary for DNA replication. Furthermore, these agents

cause an increase in cAMP levels of tumour cells, which might be correlated, with the suppression of chromatin protein phosphorylation necessary for cell replication and differentiation (Lee et al 1977). The inhibition of cellular enzyme activities and metabolism with these drugs might be explained by a Michael-type addition reaction. (Lee et al 1977).

As a characteristic structural component of these natural compounds, the natural α -methylene- γ -butyrolactone isolated from some natural products also has biological activity (Chen and Lai-Yuan 1998). Due to the unique structural features, as well as the interesting biological activities, a series of α -methylene- γ -butyrolactone **(Figure 2)** was synthesized and evaluated for their cytotoxicities. These compounds demonstrated a strong growth inhibitory activity against leukemia cell lines, but were relatively inactive against non-small cell lung cancers and CNS cancers. The α -methylene- γ -butyrolactone moiety may be considered as the determinant pharmacophore for their activities, and also proved that the enone component is essential for their activities. The substituents which included both aryl group and γ -substituents play a modulatory role in which the aryl group is preferred to be quinolin-2 (1H)-one and the γ -substituent preferred to be a biphenyl (Cheng-Chyi 2000).

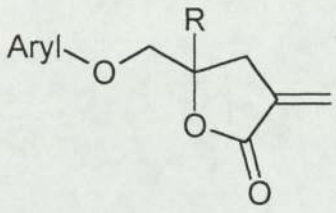
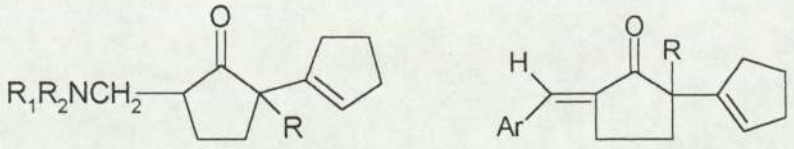


Figure 2. Structure of γ -Substituent – γ –Aryloxymethyl- α -methylene- γ -butyrolactones

The α , β -unsaturated cyclopentanone might be the alkylating centre of antitumour agents. (Powell et al 1972) More work was done to enhance the biological activity – cyclopentenyl was introduced at the α '–position in an α , β -unsaturated (or α -aminomethyl) cyclopentanone. The reason is that natural and synthetic compounds containing the cyclopentenyl group afforded significant inhibitive activity toward some tumour cells (Powell et al 1972). The cytotoxicity of α -aminomethyl-

α '-(1-cyclopentyl) cyclopentanone **(Figure 3)** was evaluated. The test *in vitro* showed that the compounds 1 and 2 possessed significant activity against P_{388}, Hela, HepA, and EAC. Their inhibitions reached or approached 100% against HepA and EAC when their concentration is 1.5uM for 4hours. Compounds 1 exhibited higher activity than 2. The conjugated enone (or β -amino methyl ketone) and cyclopentenyl were required for the anticancer activity. When these two conditions were satisfied, changes at Ar and R modulated the bioactivity. *In vivo* they also showed the potent activity against EAC. (Chuan-Min and Zhi-Zhong 1995).



1 2

Figure 3. Structure of α -aminomethyl (or α , β -unsaturated)- α '-(1-cyclopentyl) cyclopentanone

Although lots of natural or synthetic compounds containing the enone group were found that have the cytotoxicity, the therapeutic value of both the natural products and synthetic analogs has been limited due to their undiscriminating toxicity, and to some extent, epidemic allergic reactivity. As the methylene function may play a significant role for both anti-cancer action and related effects, many attempts have been made to mask the activity of the double bond in order to increase selectivity and reduce toxicity (Stang and Treptow 1981).

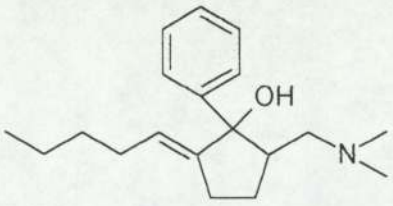
Mannich bases of cyclopentanones can produce the methylene substances upon 1,2-elimination of the amine. This masking function may have significant ramification as the mechanism of the cytotoxic and anticancer activities. When the Mannich base part is -N $(CH_3)_2$ higher activity appears compared to other substituents (Chen and Zhi-zhong 1996). The alkylidene moiety can increase the cytotoxicity of the cyclopentanone Mannich base, but the disubstituted alkylidene has no longer potent activity.

1.2.1. Aims of the investigation of ZK compounds

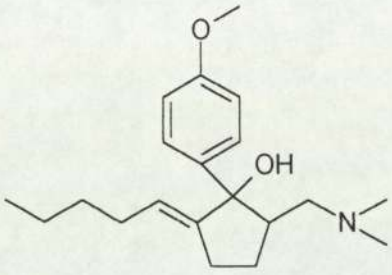
The principle aim of research is discovery of novel cytotoxic and anticancer agents. From the finding that α , β -unsaturated ketones have cytotoxicity, a lot of work has been done about these kinds of compounds in the field of biological activity evaluation, structure-activity relationship research and structure optimum. On the basis of these results, Dr. Wang's Medicinal Chemistry group synthesized a number of possible drug candidates bearing this functional enone-group or not to observe whether it is essential to biological activity (**Figure 4**). Although the enone component is important for their biological activity, the substituent also played an important role for their pharmacological properties. To explore the effect of substitution with respect to cytotoxicities of these compounds, we also synthesized and evaluated a series of compounds containing different substituents and containing the same substituent, but in a different place. In the past research several cell lines have been used to evaluate the biological activity of this kind of compound including human leukemia, small cell lung cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer cell lines. In this project MAC13 and MAC16 (murine colon adenocarcinoma cell lines) were used to evaluate the cytotoxicity. MAC16 is resistant to most chemicals and MAC13 is sensitive to most chemicals. Primary tumours of the colon were induced by weekly injection of 1,2-dimethylhydrazine in NMRI mice. All viable polyps were removed from the colon and transplanted in recipient NMRI mice. Tumours developed from subcutaneous transplantation of induced tumour fragments no larger than 1-2mm^3 by trocar (Double et al 1975). These tumours produced a series of transplantable murine adenocarcinomas (MACs) or MAC cell lines with varying degrees of differentiation and histology similar to human colorectal cancer. MAC tumours and cell lines have been shown to have a range of responses to cytotoxic agents.

The second part of this study was to investigate the mechanism of action of these agents. Some possible mechanisms of action of this kind of compound were investigated. Is it acting as an alkylating agent on the nucleophiles and how does it work? In addition the action on DNA, RNA, protein synthesis and cellular ATP levels were explored.

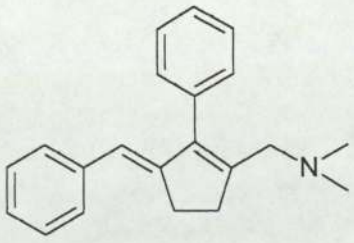
FIGURE 4. Structure List of ZK compounds



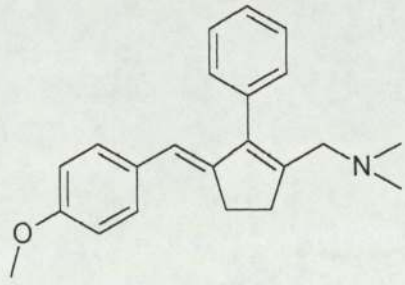
1-Phenyl-2(E)-phentylidene-5-dimethylaminomethyl cyclopentanol (ZK1-1)



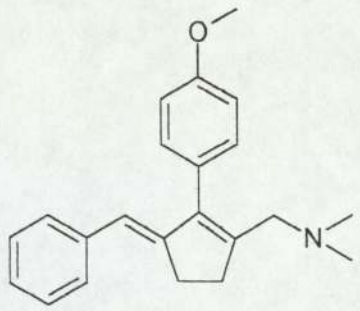
1-(4-Methoxy)phenyl-2(E)-phentylidene-5-dimethylaminomethyl cyclopentanol (ZK1-2)



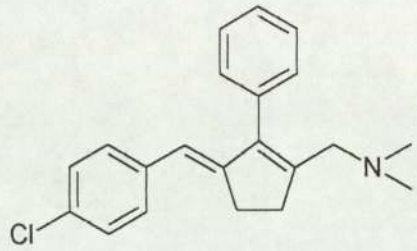
1-Phenyl-2-dimethylaminomethyl-5-benzylidenecyclopentene (ZK2-1)



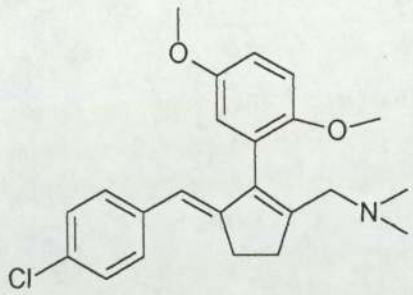
1-Phenyl-2-dimethylaminomethyl-5-(4-methoxy)benzylidenecyclopentene (ZK2-2)



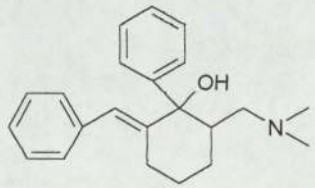
1-(4-Methoxy)phenyl-2-dimethylaminomethyl-5-benzylidenecyclopentene (ZK2-4)



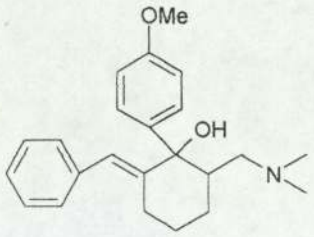
1-(4-Methoxy)phenyl-2-dimethylaminomethyl-5-(4-chloro)benzylidenecyclopentene (ZK2-6)



1-(2,5-Dimethoxy)phenyl-2-dimethylaminomethyl-5-(4-chloro)benzylidenecyclopentene (ZK2-7)



1-Phenyl-2(E)-benzylidene-6-dimethylaminomethylcyclohexanol (ZK3-1)



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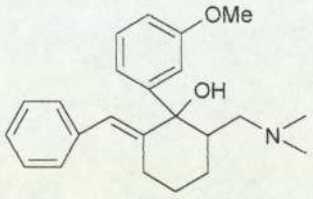
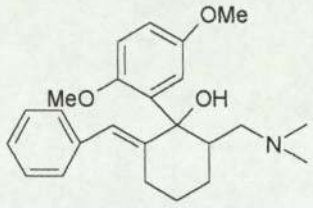


1-(3-Methoxy)phenyl-2(E)-benzylidene-6-dimethylaminomethylcyclohexanol (ZK3-3)



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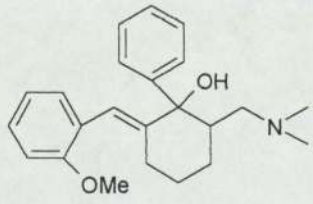
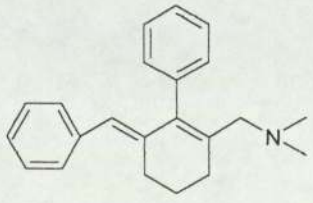
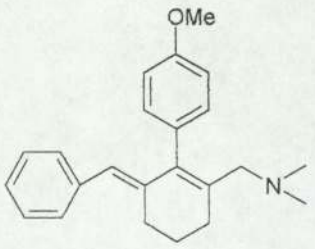


1-Phenyl-2(E)-(2-methoxy)benzylidene-6-dimethylaminomethylcyclohexanol (ZK3-5)



1-Dimethylaminomethyl-2-phenyl-3-benzylidenecyclohexene (ZK4-1)



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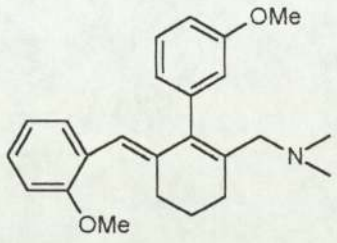

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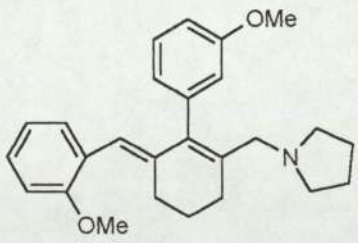

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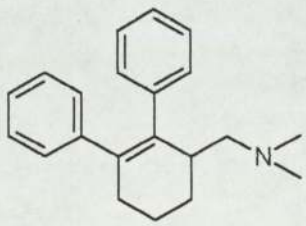
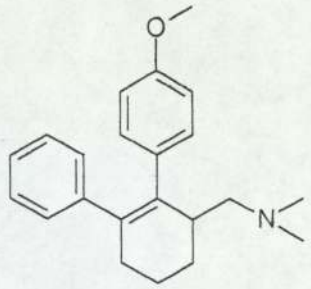


1,2-Diphenyl-3-dimethylaminomethylcyclohexene (ZK5-1)



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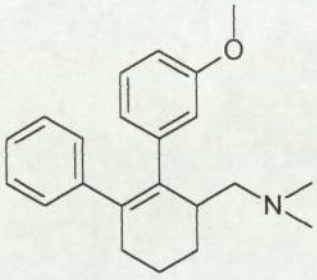

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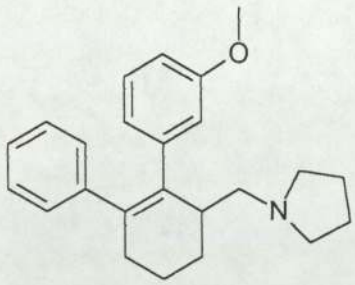

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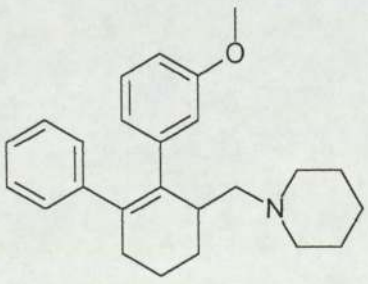

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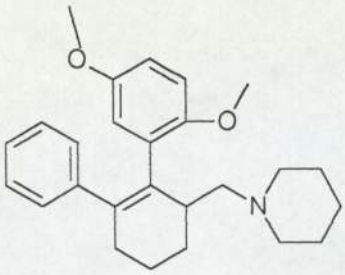

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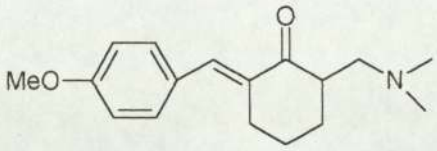

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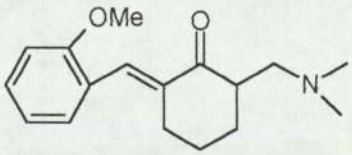

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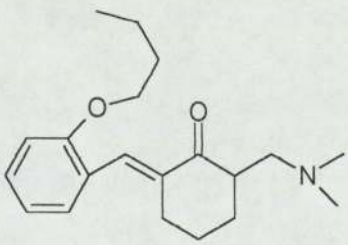

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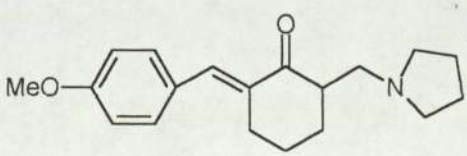

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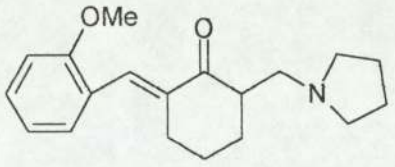
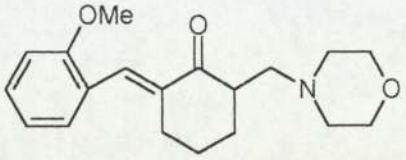
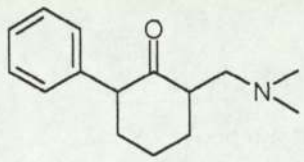


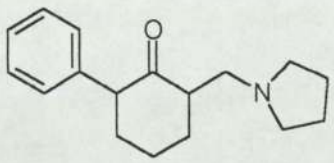
2(E)-(2-Methoxy)benzylidene-6-cyclopentylaminomethylcyclohexanone (ZK6-5)



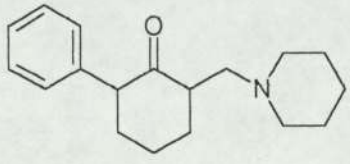
2(E)-(2-Methoxy)benzylidene-6-(4-oxo)cyclopentylaminomethylcyclohexanone (ZK6-6)



2-Phenyl-5-dimethylaminomethylcyclohexanone (ZK6-7)



3-Phenyl-5-cyclopentylaminomethylcyclohexanone (ZK6-8)



2-Phenyl-5-cyclohexylaminomethylcyclohexanone (ZK6-9)

All the compounds were tested as their hydrochloride salts

Discriminating toxicity between tumour cells and normal cells is an important evaluation factor for a drug candidate. C_2C_{12} (mouse myoblast cell lines) and 3T3 (mouse fibroblast cell lines) were chosen to evaluate the selectivity. All these investigations are worthwhile to drug design and future research.

1.3 Biological effects of oxysterol compounds

Oxysterols are derivatives of cholesterol **(Figure 5)** that contain additional oxygen atoms either on the steroid nucleus or the side chains. This family comprises a large number of compounds formed from cholesterol through enzymatic and non-enzymatic oxidation. These oxides form in the human body by endogenous oxidation and may also be derived from food, since absorption of OS has been demonstrated (Bascoul et al 1985). Initially, it was thought that OS from endogenous origin were formed solely by enzymatic processes, mainly in biosynthesis of bile acids and steroid hormones. However, it is now known that non-enzymatic processes can also occur in the body. In foods, OSs are formed by non-enzymatic oxidation processes, especially by autoxidation (Smith et al 1990). These compounds have been isolated from some fresh foods and many processed foods: dairy, egg, meat and fish products, fried foods and frying media (Guardiola et al 1996).

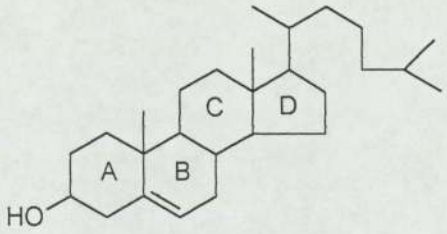


Figure 5. Cholesterol

OSs have several *in vitro* and *in vivo* biological effects at the cellular level, illustrating potentially significant biological effects that demand further investigation. The effects that have been reported are: gene regulation, cytotoxicity, atherogenesis, changes in cellular membrane properties and inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) activity. Furthermore, OSs are mutagenic and carcinogenic, although some have been studied as antitumour agents

based on their cytotoxic properties. The cytotoxicity has been the central focus of these bioactivities, and two mechanisms have been postulated: the inhibition of endogenous cholesterol synthesis and alteration of membrane properties. However, direct evidence of the cytotoxic mechanisms remains obscure, while plenty of indirect evidence is reported as well as some studies intended to develop anti-tumour agents from OS (Guardiola et al. 1996).

1.3.1 OS cytotoxicity and antitumour activity

Attempts have been made to capitalize on the cytotoxicity of OS and to use these compounds as antitumour agents. This activity has been demonstrated in some in vitro studies. Cholestanetriol (CT), 7-ketocholesterol (7-KC), 7-hydroxycholesterol (7-HC) and other commonly appearing OS are reported to be cytotoxic to many normal and malignant cells. In the middle of the 1970's, Cheng et al extracted 7 β -HC from *Bombyx cum Botryte*, a traditional Chinese medicine curing tumour, and found this compound is cytotoxic to hepatoma HTC and ZHC *in vitro* at 33 μ g/ml, but is not toxic to normal murine fibroblast cell 3T3 at 80 μ g/ml (Cheng et al. 1971). Hietter (1986) and Luu (1986) reported a toxic effect of 7 β -hydroxycholesterol (7 β -HC) and its sodium dihemisuccinate on rat Morris hepatoma cells (tissue culture). OS showed higher cytotoxicity in highly proliferative cells. 7 β -HC is more toxic on hepatoma cells than on normal hepatocytes (Nordman et al. 1989). Hietter (1986) also noted that 7 β -HC was more toxic on cultured mouse lymphoma cells than on normal lymphocytes. CT was found to be cytotoxic to murine L-cells (Higley et al 1984), macrophages and pig arterial smooth muscle cells (Baranowski 1982). 7-KC, 22-HC, 25-HC with some other analogues are also reported to be cytotoxic (Smith et al 1989). In the last 15 years a lot of naturally occurring oxysterols have been found from plant or marine sources that show cytotoxicity to tumour cell lines.

In recent years, some groups have started to design OS derivatives, which can be applied as antitumour drugs. Christ et al. (1991) administered intraperitoneally two water-soluble 7 β -HC phosphodiesters to mice in which P815 mastocytoma cells had been subcutaneously implanted. Optimal doses were those found between 20 and

40mg/kg/day. Further studies on OS phosphodiesters (Allemand et al., 1993; Moog et al., 1993) show that these compounds display anticancer activity in mice bearing experimental tumours. In addition, Moog et al. (1993) showed that phosphodiesters act as prodrugs, slowly releasing free OS in the organism. Werthle et al. (1994) showed antitumour activity of 7 β -HC oleate in experimental rat brain C6 glioblastoma and that clearance of this compound reached 99% after 48hours.

1.3.2 Mechanisms of OS cytotoxicity

The cytotoxicity properties of these OSs are complicated and far from clear as more than one mechanism exists and each individual OS acts differently. Their cytotoxic mechanisms are usually derived from the biological effects mentioned above. As mentioned before, two actions of OSs have been postulated as the main mechanisms: the inhibition of HMG-CoA reductase activity, leading to reduced endogenous cholesterol synthesis and replacement of cholesterol by OS molecules in membranes, perturbing permeability, stability and other membrane properties. Some other mechanisms have been reported as relevant. HMG-CoA reductase, as well as being the key enzyme in cholesterol synthesis, is also the key enzyme in the synthesis of prenyl alcohols, activators of some oncogenes (Glomset et al 1990; Schafer et al 1989). Some studies suggest a possible mechanism related to immune system alteration induced by OS, since attraction of macrophages and neutrophils by OS has been observed in vivo (Moog et al., 1990). Further, an inhibition effect on the first steps of lymphocyte division, induced by mitogens or alloantigens, was observed (Christ et al., 1991; Luu and Moog, 1991). Other authors suggest that OS affect genesis and functionality of cytotoxic lymphocytes by inhibiting proliferation and transformation of lymphocytes (blastogenesis) mixed lymphocyte response and activity of NK cells (Smith and Johnson, 1989). This mechanism has been demonstrated for 25-HC, 7-KC. Hwang (1992) observed that 7-KC and 25-HC showed the ability to decrease viability of murine cells (EL4 lymphoma and K36 leukaemia cell lines). However, the presence of inhibitors of protein or RNA synthesis increases this viability. This finding was confirmed later by Christ et al. (1993), who showed that cycloheximide and

actinomycin D increase viability of murine lymphoma cells (RDM4) in the presence of 25-HC and 7β, 25-dihydroxycholesterol, which have antiproliferative activity. This suggests that OS toxicity could be mediated by mechanisms involving protein or RNA synthesis. It would be interesting to find what proteins are key factors in cellular death induced by these OS. Furthermore, Bakos et al. (1993) identified an OS binding protein and showed that 25-HC induced cell death of different human leukemic T-lymphocyte clones which correlated with OS affinity for this cytosolic-binding protein, which has been related to the inhibition mechanism of HMG-CoA reductase. A recent study suggested a role for a DNA-binding protein in OS-induced regulation of lymphoid cell viability and growth (Ayala-Torres et al., 1994).

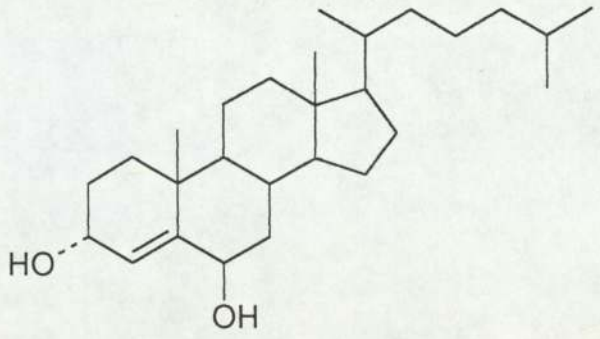
1.3.3 Aim of investigation of oxysterol compounds

Interests in many biological effects, the research about oxysterol has persisted for a long time. From the view of medicinal chemistry, more stereo-specific derivatives are needed for studies of their bioactivity. Dr Wang's chemistry group synthesized a series of stereo oxysterols **(Figure 6).** The mechanisms of cytotoxicity are relevant to several biological effects. Further research can be done according to the cytotoxicity of these compounds, and the results of cytotoxicity can be used for QAR analysis to direct future chemistry synthesis. In my project MAC16 cell lines were chosen to preliminarily evaluate the cytotoxicity.

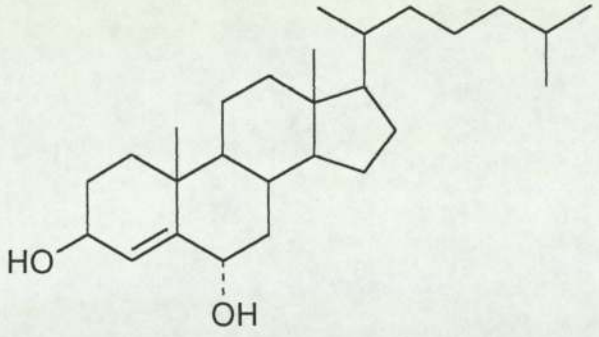
1.4 Introduction of LR6M and related compounds

The antitumour activity of N-methylformamide (NMF, $HCONH.CH_3$), a derivative of methacrylic acid, against murine tumours was first described in the early 1950s (Clarke et al., 1953; Furst et al., 1955). Dr. Washington Ayuko in our group has used NMF together with dichloroacetylchloride, in a reaction mixture which yielded several products, some of which have been shown to exhibit *in vivo* antitumour activity, without toxicity. From this discovery, they prepared a number of analogues, screened the active ones and developed the chemical approach of selected agents. The final selected synthetic example is 3,4-dichloro-6-hydroxy-1- methyl-1,6-dihydro- pyridine

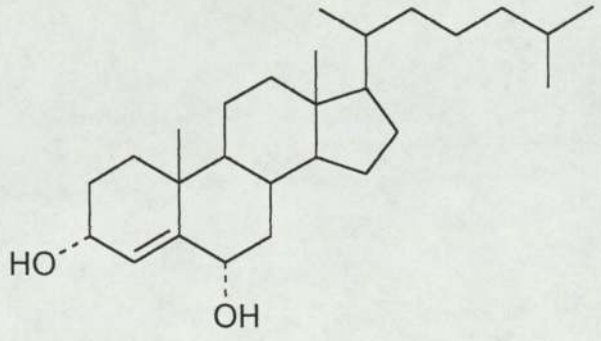
FIGURE 6. THE STRUCTURE LIST OF OXYSTEROLS (OSTL)



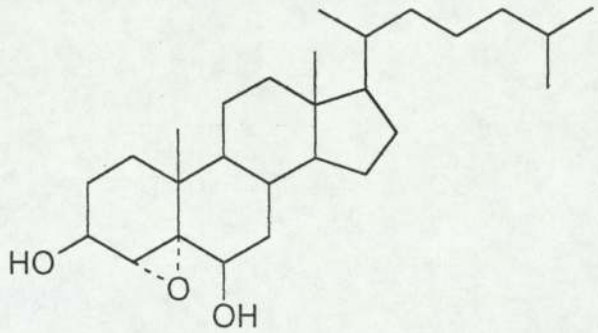
Cholest-4-en-3α,6β-diol (OSTL-2)



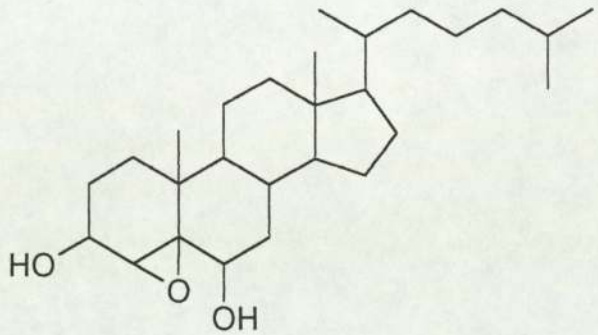
Cholest-4-en-3β,6α-diol (OSTL-3)



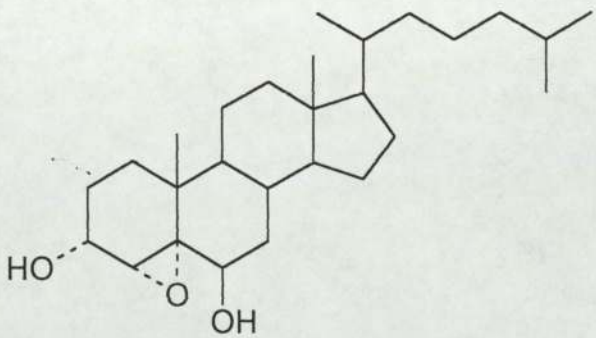
Cholest-4-en-3α,6α-diol (OSTL-4)



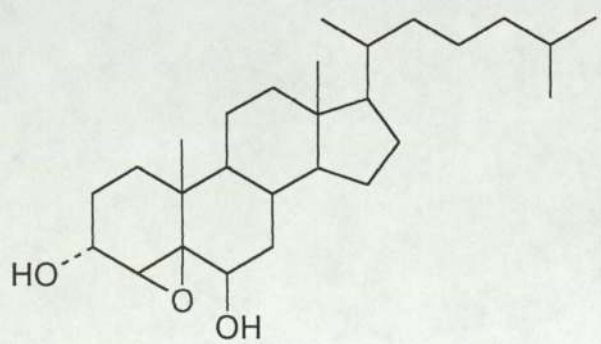
4α,5-Epoxy-5α-cholestan-3β,6β-diol (OSTL-5)



4β,5-Epoxy-5β-cholestan-3β,6β-diol (OSTL-6)



4α,5-Epoxy-5α-cholestan-3α,6β-diol (OSTL-7)



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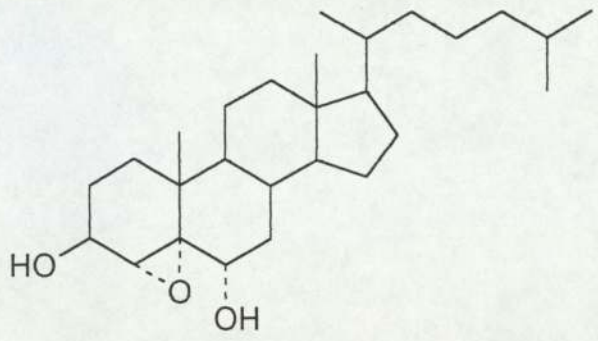

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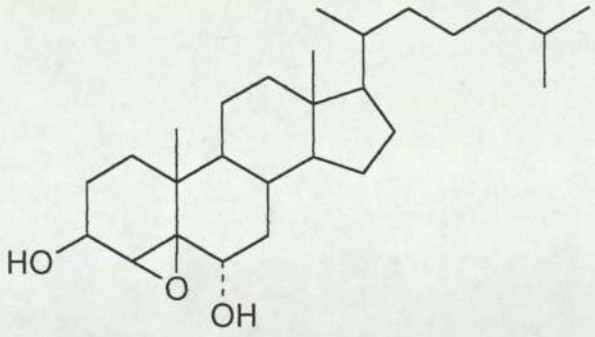
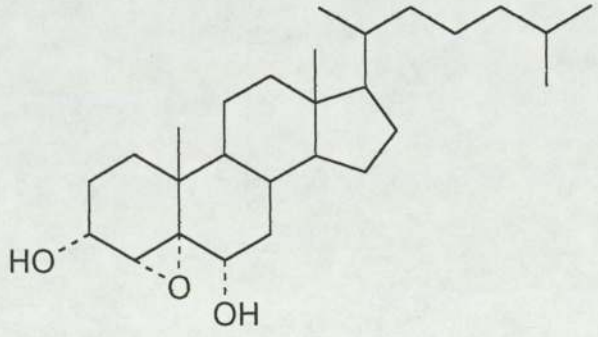
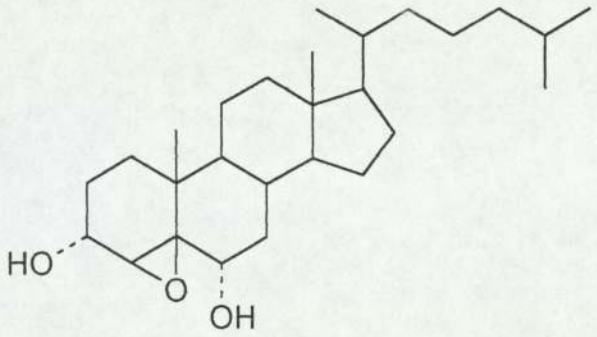


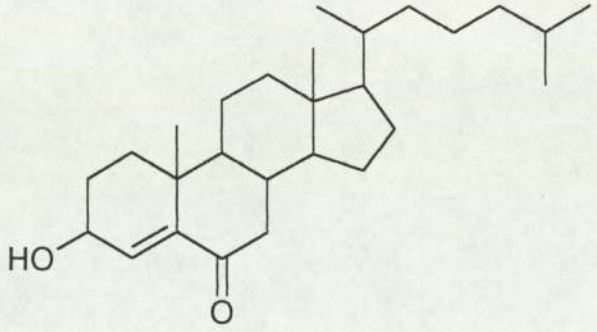
4β,5-Epoxy-5β-cholestan-3β,6α-diol (OSTL-10)



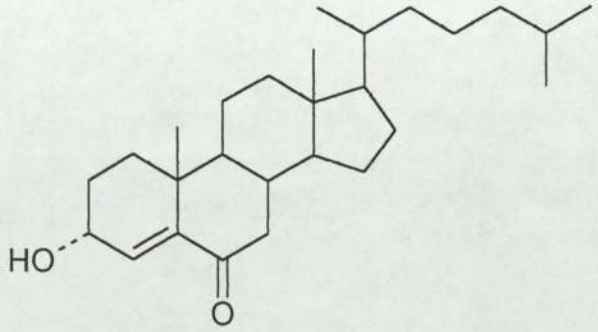
4α,5-Epoxy-5α-cholestan-3α,6α-diol (OSTL-11)



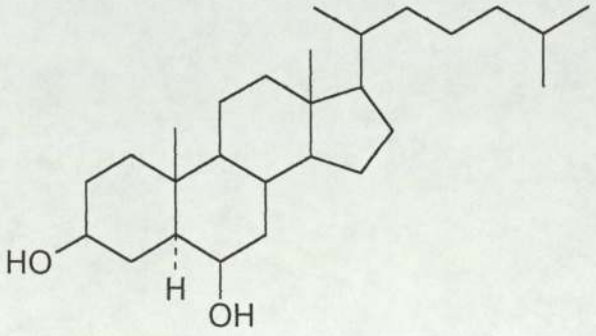
4β,5-Epoxy-5β-cholestan-3α,6α-diol (OSTL-12)



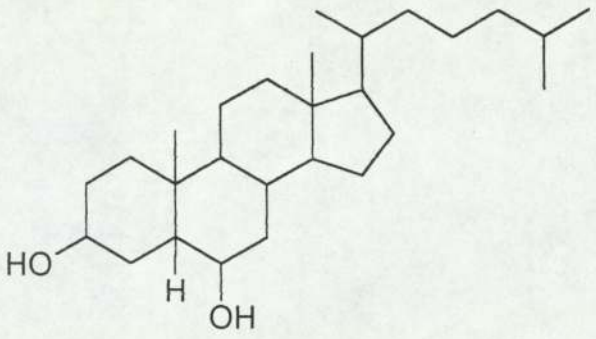
3β-Hydroxycholest-4-en-6-one (OSTL-13)



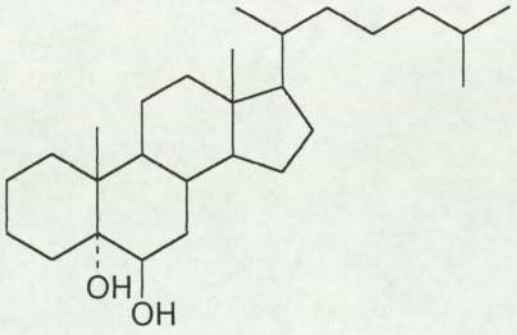
3α-Hydroxycholest-4-en-6-one (OSTL-14)



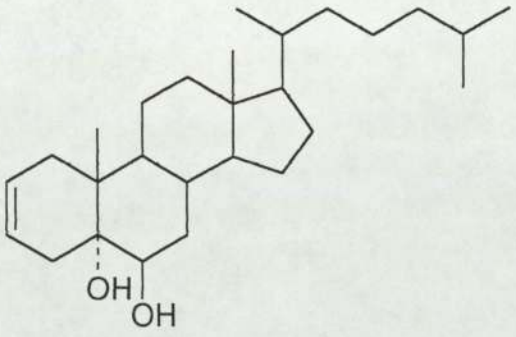
5α-Cholestan-3β,6β-diol (OSTL-18)



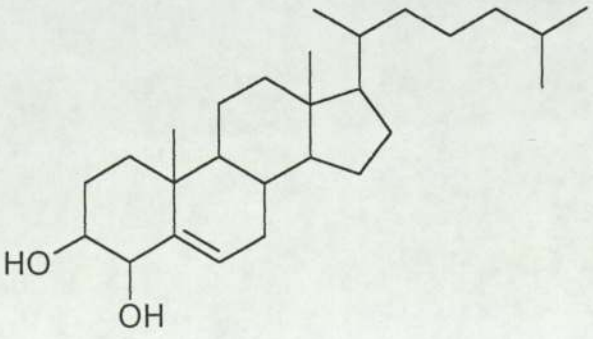
5β-Cholestan-3β,6β-diol (OSTL-22)



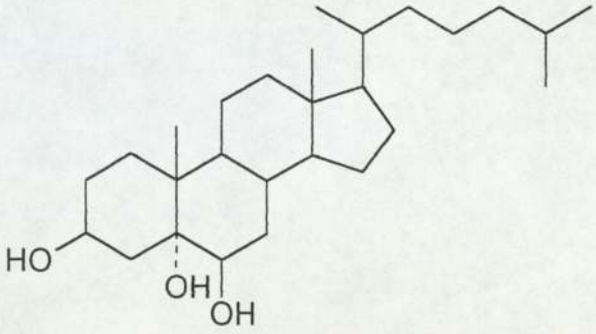
5α-Cholestan-5α,6β-diol (OSTL-26)



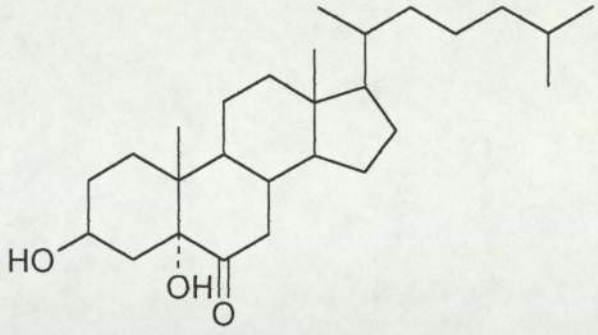
5α-Cholestan-2-en-5α,6β-diol (OSTL-30)



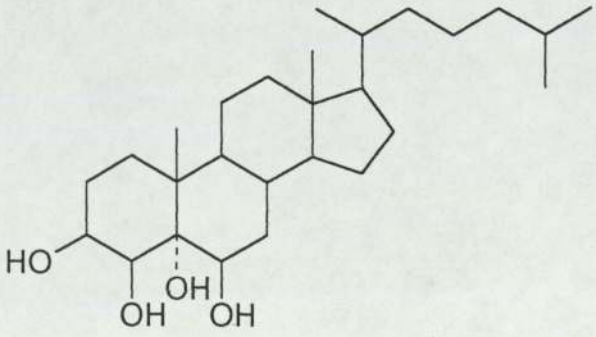
Cholest-5-en-3β,4β-diol (OSTL-34)



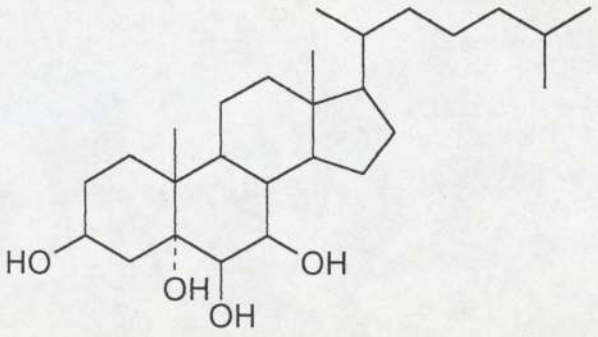
5α-Cholestan-3β,5α,6β-triol (OSTL-48)



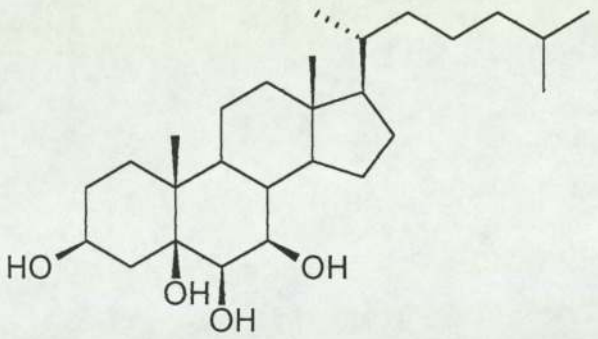
3β,5α-Dihydroxy-5α-cholestan-6-one (OSTL-56)



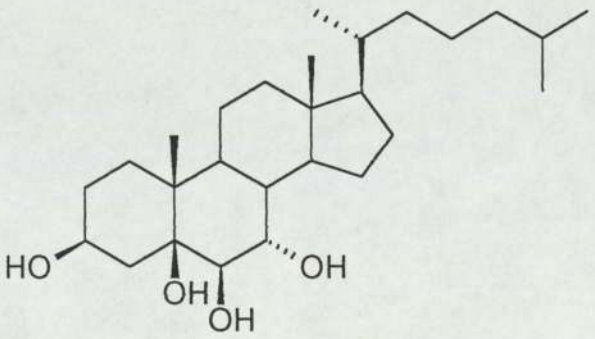
5α-Cholestan-3β,4β,5α,6β-tetrol (OSTL-188)



5α-Cholestan-3β,5α,6β,7β-tetrol (OSTL-204)



5β-Cholestane-3β,5,6β,7β-tetrol (OSTL-214)



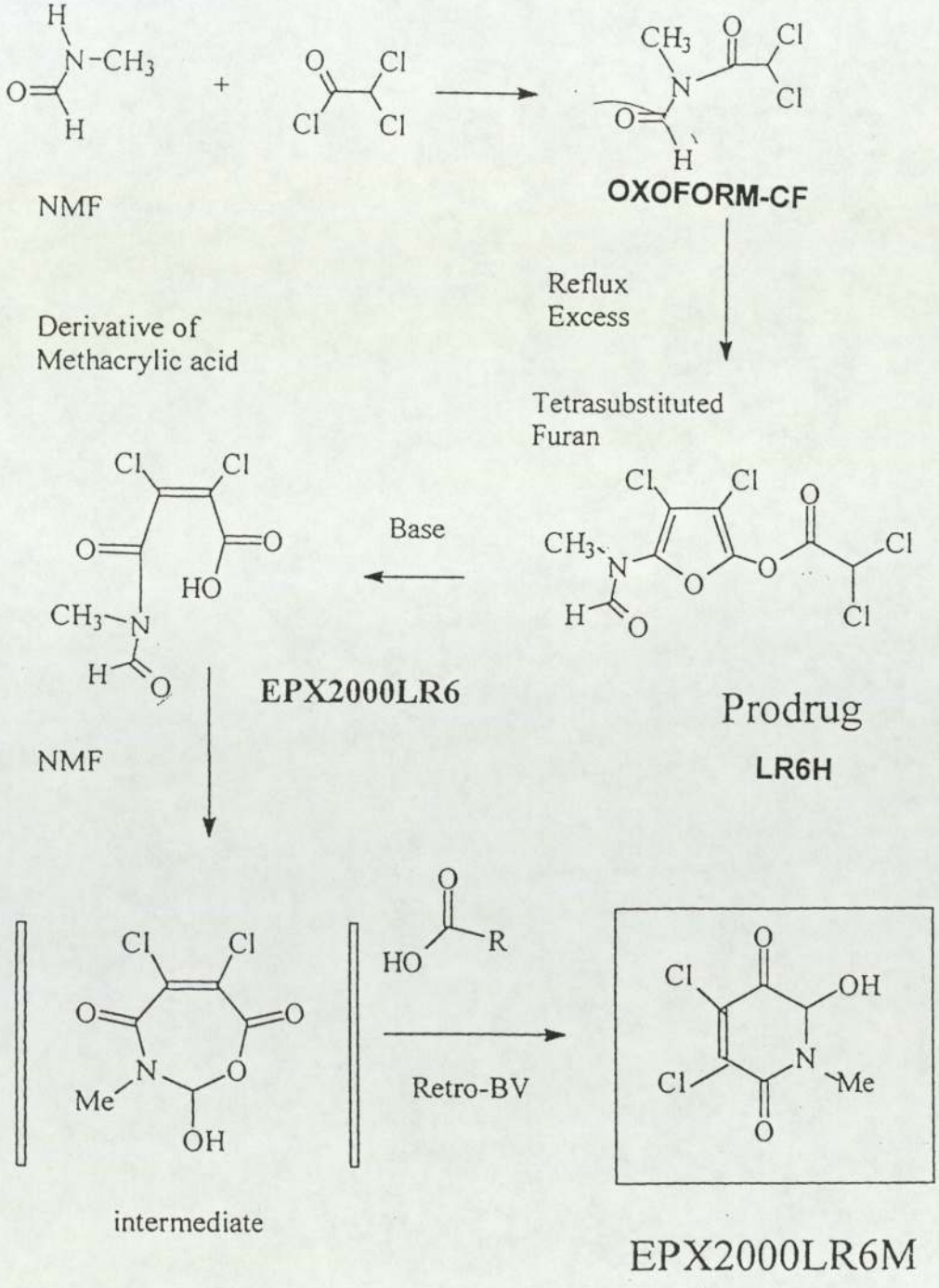
5β-Cholestane-3β,5,6β,7α-tetrol (OSTL-215)

-2,5-dione (code name LR6M). The preferable method is shown in **Figure 7**.

In this synthetic procedure OXOFORM-CF (intermediate), LR6H (prodrug) and LR6M have been evaluated for antitumour activity *in vivo*. OXOFORM-CF and LR6H as intermediary reaction products showed no toxicity and were inactive *in vivo*. The reaction product-LR6M exhibited high antitumour activity *in vivo*. This project aims to evaluate the cytotoxicity of these compounds *in vitro*. In the procedure of synthesis the reaction product is not pure, which may affect the biological activity. So isolation and purification is another part of the project. Dr. Ayuko used different methods to try to purify the products. That is why LR6M was tested several times. These LR6M came from different procedures and were dissolved in different solvents. For the final reaction products different HPLC conditions were improved for further purification.

Figure 7. The synthetic procedure of LR6M

Selected synthetic example



Example : Synthesis of **3,4-dichloro-6-hydroxy-1-methyl-1,6-dihydro-pyridine-2,5-dione**

2.0 Materials

All chemicals used for tissue culture were tissue culture grade. Chemicals used for analytical techniques were ANALAR grade, whilst solvents (unless otherwise stated e.g. for HPLC) were GPR.

2.1 Tissue culture

Bovine Serum Albumin, fatty acid free (BSA)	Sigma
Foetal calf serum (FCS)	Gibco
Dulbecco's Modified Eagle Medium without sodium pyruvate, with 4500mg/glucose, with nonessential amino acids.	Gibco
EDTA (disodium salt)	Sigma
PBS without calcium and magnesium x10 strength	Gibco
Nunc 250ml flasks	Gibco
Nunc 24-well multi-dishes	Gibco
Nunc 6-well multi-dishes	Gibco
RPMI 1640 with 1 ml glutamine and 25 mM Hepes Buffer	Gibco
0.5% Trypsin: 0.25%EDTA x 10 strength	Gibco
L-Glutamine	Gibco

2.2 Chemicals

DMSO	Sigma
BHT	Sigma
Catalase	Sigma
Cysteine	Sigma
Glutathione	Sigma
Indomethacin	Sigma
Trypan blue(B.D.H. Standard stain)	The British drug house LTD.
Trichloro-acetic acid (TCA)	Sigma

Sodium hydroxide	Fisons
Hydrochloric acid	Fisons
ATP mix solution	Sigma
ATP assay mix dilution buffer	Sigma
6,6-Dithiodinicotinic acid	Sigma

2.3 Gases

Carbon Dioxide	BOC. Ltd.
Oxygen	Medical Gases BOC. Ltd.

2.4 HPLC Analysis

Column: C18 (3.9mmx300mm)

C2 (5 µ mx250mm)

Methanol (HPLC grade)	Fisons
Acetonitrile (HPLC grade)	Fisons
Water (HPLC grade)	Fisons

2.5 Radioactive Chemicals

[Methyl-^{3}H]-thymidine (activity 5 Ci/mmol)	Amersham International Ltd
[^{3}H]-uridine (activity 31 Ci/mmol)	Amersham International Ltd
[^{3}H]-leucine (activity 34 Ci/mmol)	Amersham International Ltd
[^{3}H]-phenylalanine (activity 5Ci/mmol)	Amersham International Ltd

2.5 Name and Address of Suppliers

Amersham International Ltd.

Amersham Place, Little Chalfort, Buckinghamshire, UK

BOC.Ltd.(British Oxygen Company)

Lower Walsall Street, Wolverhampton, West Midlands.

Gibco

Life Technologies Limited, Unit 4, Coweley Mill Trading Eastate, Longbridge Way, Uxbridge, Middlesex UK.

Fisons

Bishop Meadow Road, Loughborough, Leicestershire, UK

Waters

Waters Division of Millipore, Millipore Corporation, 34 Maple Street, Milford, UK.

3.0 Methods

3.1 Methods of ZK compounds tested

3.1.1 *Anti-cancer biological screening*

3.1.1.1 Maintenance of cell lines and preparation for use

Cell origins and storage

The cell lines MAC13, MAC16 were originally derived from colon tumours induced by 1,2-dimethylhydrazine (DMH) in NMRI mice. Cells were stored in RPMI 1640 culture medium with a supplement of 20%foetal calf serum (FCS) and 10% DMSO at $2x10^6$cells/ml and kept under liquid nitrogen.

Freezing the cells in liquid nitrogen (-185 ℃)

Use the cells that are rapidly dividing. On the day before freezing, split the cells 1:10 into fresh medium. On the day of freezing, transfer the cells to a sterile chilled centrifuge tube. Spin the tubes at 400xg for 5min. Remove the supernatant, resuspend the cells in 1ml medium (10%DMSO, 20%FCS, 70%RPMI). The final cell concentration should be approximately $5x10^6$cells/ml. Transfer to a chilled freezing vial and place the vials on a freezing rack at -20℃ about 2 or 3 hours. Then transfer the vials to liquid nitrogen.

Preparation of cell lines for use

MAC13 cells have a doubling time of 18 hours and grow as a monolayer. The cells were cultured in the growth media RPMI1640, supplemented with 10%FCS, 1%glutamine. The cells need to be subcultured every 3 or 4 days. Due to the nature of the cell lines they adhere to the bottom of the flask and have to be removed by proteolytic enzymes, namely trypsin, which digests the protein to help cells to be removed from the bottom. The subculture of MAC13 was taken from the incubator, which was maintained at 37℃, and the medium decanted to remove the dead cells. Five millilitres of PBS was added to rinse the cells and then removed. One millilitre trypsin (0.025%EDTA) diluted in PBS (1:9) was added and the flask was incubated at

37°C for ten minutes. On removal from the incubator they were inspected under the microscope to ensure the majority of the cells had been removed, and then transferred to a sterile universal tube. The remaining subculture flask was refilled with 20 millilitre RPMI1640, and then the flasks were gassed at 5%CO_2 for 20 seconds.

MAC16 cells have a doubling time of 32 hours and grow in suspension. The cells were cultured in the growth medium RPMI 1640, supplemented with 5%FCS, 1%glutamine. Every 3 or 4 days the cells were subcultured. Any cells adhering to the flask surface were removed with a firm tap. When the cells were confluent, took out 3ml cell lines into a flask, added thirty millilitre RPMI medium and then the flask was gassed at 5%CO_2.

3.1.1.2 Method

MAC13, MAC16 total cell number count

MAC13, MAC16 cells were maintained in RPMI 1640 medium supplemented with 10%FCS at 37°C under an atmosphere of 5% CO_2. MAC13 cells were seeded at $5x10^3$cells/ml. MAC16 cells were seeded at $2x10^4$cells/ml. Both cell lines were seeded in 24 well plates and incubated for 24 hours before the test compound addition. Compounds were dissolved in PBS to make a 1ml stock solution and added to wells in 10ul aliquots to give the final compound concentrations ranging from 0.1uM to 100uM. Each concentration had two replicates and a control well. MAC13 cell counts were made 72 hr after drug addition. MAC16 cell counts were made 48hr and 72hr after drug addition using a **ZM** electronic particle Coulter Counter. Coulter Counter settings are upperthreshold T_U (15um), lowerthreshold T_L (8um), attenuation (A) 16 and current I (200).

3.1.1.3 Presentation data

Data were represented in tabular form for the number of cells counted at each concentration tested and for each compound the graph of the percent inhibition against concentration was plotted. From the graph the IC_{50} value can be obtained to enable

comparison.

3.1.2 Further experiments with ZK-1, ZK-2 on mechanism of action

3.1.2.1. Determine whether free radicals or alkylating species are involved.

3.1.2.1.1. Introduction

We think the enone group ($O=C-C=CH_2$) is essential for their biological activities, by acting as an alkylating agent through a Michael-type reaction with bionucleophiles. In this process free radicals or alkylating species might be produced. Thus different antioxidants of free radicals were used to investigate, including BHT, catalase, cysteine, glutathione and indomethacin.

3.1.2.1.2 *Total cell number count*

Prepare the inhibitors solution

BHT solution: BHT was dissolved in DMSO and take 10 ul of the DMSO solution to each well to make the final concentration of 5uM.

Catalase solution: Catalase is 1600units/mg solid. Take 3.125 mg catalase solid, dissolve in 1 ml PBS and add 10ul of this catalase PBS solution in each well to get the final concentration 50units/well.

Cysteine-HCl solution: Cysteine was dissolved in PBS and according to the molecular weight to make solution concentration 100uM, add 10ul in each well to make the final concentration of 1uM. Before added in the well, it was neutralized by 0.1mol/l HCl.

Glutathione solution: Glutathione was dissolved in PBS and according to the molecular weight, the solution concentration was made 100mM. Add 10 ul to each well to make the final concentration of 1mM.

Indomethacin solution: The same as glutathione solution but dissolved in DMSO.

Methods:

MAC13 and MAC16 cell lines were seeded in 24 well plates. MAC13 cell lines were seeded at $5x10^3$/ml and MAC16 were seeded at $2x10^4$/ml. After incubation for 24 hours

the test compounds and inhibitors were added at the same time. For MAC13 after incubation for 72 hr the total cell number was counted by using a Coulter Counter, for MAC16 the cell number was counted after incubation for 48hr.

3.1.2.1.3 ***Cell viability assay***

Use trypan blue stain and the hemacytometer to determine the cell viability. This is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do.

Methods:

MAC16 cell lines were seeded in 6-well dishes at a concentration of $7x10^4$/ml and incubated at 37℃ for 24 hours. ZK6-1 and ZK6-2 were dissolved in PBS, 10u l of the PBS solution was added to each of 6 wells, to give the final concentration of 50 μM. After incubation transfer the medium to a centrifuge tube, centrifuge at 1500g for 5min. Wash cells with 1ml PBS twice. Resuspend cells in 0.5ml PBS, transfer 0.5ml of 0.4% Trypan Blue (w/v) to the tube and mix thoroughly. Allow standing for 5 to 15min, with the cover-slip in place, use a Pasteur pipette to transfer a small amount of Trypan Blue-cell suspension mixture to the chamber of the hemacytometer. Carefully touch the edge of the cover-slip with the pipette tip and allow each chamber to fill by capillary action. Do not overfill or underfill the chambers. Count all the cells in the 1mm center square and four 1mm corner squares.

CELL VIABILITY (%)= total viable cells (unstained) / total cells (unstained and stained) x 100

3.1.2.1.4. ***Structure change evaluation by NMR***

GSH and ZK6-1 mixture solution (GSH: ZK6-1=2:1mole) were dissolved in 5ml D_2O (deuterated water), keep in 37°C overnight. The NMR (XL-100) was used to test the sample.

3.1.2.2 Measurement of [^{3}H]-thymidine, [^{3}H]-uridine and [^{3}H]-leucine (or [^{3}H]-phenylalanine) uptake

3.1.2.2.1. Methods:

Cells were seeded at the following densities in 6-well dishes (2ml/well): MAC13 $4x10^4$cells/ml; MAC16: $4x10^4$cells/ml

MAC13 cells: After the cells had been seeded and incubated for 24 hours in a humid atmosphere at 37℃, 5%CO_2, the 50uM final concentration of ZK-1, ZK-2 were added to the medium in triplicate wells. After incubation for another 24 hours, the isotope [^{3}H]-thymidine (1 μ Ci/well), [^{3}H]-uridine (1 μ Ci/well) and [^{3}H]-leucine (or phenylalanine) (2 μ Ci/well) were added to the wells. The reaction was terminated by aspirating the radioactive medium after incubation for 1hr, 2hr or 48hr. The cells were washed with 1ml ice-cold PBS three times. Cellular protein was precipitated with 1ml/well of 5% trichloro-acetic acid (TCA) and incubated for 1hr on ice. The TCA containing the cellular protein was transferred to tubes. Tubes were centrifuged at 1500xg for 15 min and the supernatant was removed to another tube for scintillation analyses. The precipitate was dissolve in 0.3M NaOH at 37°C for 30 min. An aliquot of the dissolved material was taken and mixed with scintillation fluid and the amount of the radioactivity incorporated measured in a 2000CA Tri-Carb liquid scintillation counter. For protein synthesis measurement another aliquot was used to determine protein concentration using Bio-Rad solution.

MAC16 cells: Because of the nature of suspension, the cells were mechanically dislodged in the wells and transferred to centrifuge tubes, and subsequent steps carried in these tubes the same as MAC13 cells.

3.1.2.3 ATP assay

MAC16 cell lines were seeded at $7x10^4$cells/ml in 6 well multidishes. After incubation for 24 hours ZK6-1, ZK6-2 were added at the concentration of 50 μ M. After1, 2, 4, 6 and 24 hours triplicate wells were used to test intracellular ATP concentrations.

ATP mix solution was diluted in ATP assay mix dilution buffer to give the appropriate concentration range for the sample.

At the end of an experimental incubation period, gently take out the medium; add 1ml PBS to rinse the cells twice. The third time put 1ml PBS and add an equal volume of 2% TCA to the culture well.

Incubate for 15 minutes at room temperature to extract ATP.

Remove a 100 μ l exact from each well and neutralize with 100 μ l 0.1M Tris- HCl buffer (ρ H=9.0), mix by gentle inversion.

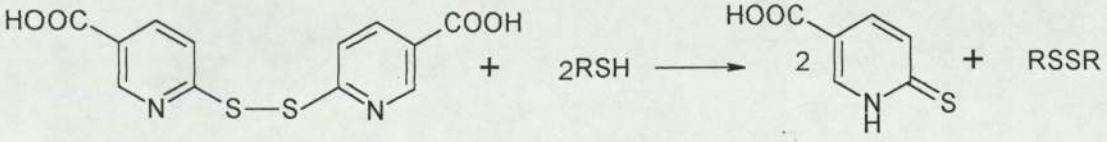
Add 0.1ml of ATP assay mix solution to a 1ml centrifuge tube, allow standing at room temperature for approximately 3 minutes. During this period any endogenous ATP will be hydrolyzed, decreasing the background.

Begin the assay by rapidly adding 0.1ml of neutralized sample. Swirl briskly to mix and immediately measure the amount of light produced with a 2000CA Tri-Carb liquid scintillation counter.

Add 0.1ml sterile water to 0.1ml of ATP assay mix solution as a blank.

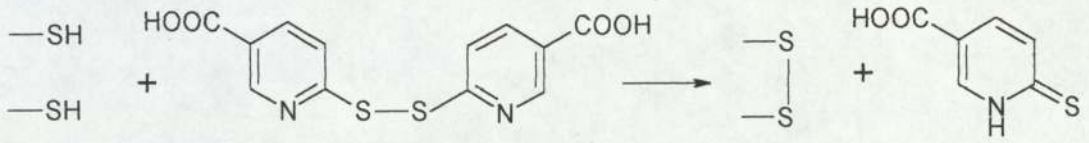
3.1.2.4. The interaction of 6,6- dithiodinicotinic acid with tumour cells treated by compounds

6,6-Dithiodinicotinic acid (CPDS) reacts with thiols and forms a disulphide and 6-mercaptonicotinic acid (6MNA). The reaction can be followed spectrophotometrically by monitoring absorption at 344nm.



The fact that 6-dithiodinicotinic acid is virtually completely in the thione form prevents further interaction of this compound with disulphides. This reaction can be used for the estimation of cellular thiol groups present in glutathione and cysteine. In addition during a short incubation 6,6-dithiodinicotinic acid does not penetrate cells

and can thus be used for measurement of the cell surface bound sulphydryl groups.



Methods:

MAC16 cell lines were seeded at 10^6cells/well in 6 well plates (2ml/well). After incubation for 24 hours ZK6-1 and ZK6-2 were added at the concentration of 50 μ M. Triplicate wells were used to test each compound. After 2 hours the cells were mechanically dislodged in the wells and transferred to centrifuge tubes. Centrifuge at 1000xg for 10min. Remove the medium and resuspend cells in 2ml PBS. 10u l CPDS (1mm) dissolved in DMSO solution was added, to give the final concentration of 5 μ M. Determine the absorption at 344nm after incubation for 5min at 37℃. 10ul CPDS (1mm) solution was dissolved in 2ml PBS as a blank. For the estimation of cellular thiol groups before adding the CPDS sonicate the cell lines, subsequent steps carried out in these tubes were the same as cell membrane estimation.

3.1.3. Selectivity evaluation

These compounds showed high cell –growth inhibitory activity in the MAC13 cells and some of them showed high cell-growth inhibitory activity in the MAC16 cells. The cytotoxicity in normal cells is an important factor to evaluate drug candidates. C_2C_{12} (mouse myoblast cell lines) and 3T3 (mouse fibroblast cell lines) were chosen to evaluate the selectivity of these compounds in normal cells.

3.1.3.1. Preparation of cell lines for use

The C_2C_{12} cells grow as a monolayer. The cells were cultured in the growth media DMEM, supplemented with 10%FCS, 1%penicillin-streptomycin in a humid

atmosphere of O_2: CO_2 (95: 5) at 37℃. The cells need to be subcultured every 3 or 4 days. Due to the nature of the cell lines they adhere to the bottom of the flask and have to be removed by trypsin.

The 3T3 cells were cultured the same as C_2C_{12} cells, expect the atmosphere of O_2: CO_2 (90: 10).

3.1.3.2. Method:

C_2C_{12} cells were seeded at $5x10^3$cells/ml. 3T3 cells were seeded at $1.5x10^4$cells/ml. Both cell lines were seeded in 24 well plates and incubated for 24 hours before the tested compound addition. ZK6-1, ZK6-2 were dissolved in PBS and serially diluted, 10ul of the final dilutions was added to each of two wells, to give final concentrations of 100, 50, 10, 1, 0.1uM. Counts on both cell lines were made after incubation for 72hr using a **ZM** electronic particle Coulter Counter. For C_2C_{12} cells the settings are upperthreshold T_U (16um), lowerthreshold T_L (15um). For 3T3 cells the settings are upperthreshold T_U (10um), lowerthreshold T_L (5um).

3.2 Methods of evaluation of oxysterol compounds

3.2.1. Cytotoxicity assay

Prepare the cell lines for use (see 3.1.1.1)

Prepare the compound solution

Each of the compounds was weighed and dissolved in DMSO. The concentrations range from 10mM, 5mM, 1mM to 0.01mM to find the highest soluble concentration. Then add 10ul compound DMSO solution to each well, the concentrations range from the highest soluble concentration to 0.01mM.

Total cell number count (see 3.1.1.2)

Present the data (see 3.1.1.3)

3.3 Methods of evaluation of LR6M and related compounds

3.3.1. Cytotoxicity assay

Prepare MAC16 and MAC13 cell lines for use (see 3.1.1.1.1)

Prepare the compound solution

LR6M sample was a rose red, sticky liquid, dissolved in PBS to make the serial concentrations from 200uM to 0.1uM. LR6H sample was a yellow liquid; diluted in PBS to make the serial concentrations, ranging from 150ug/ml to 0.5ug/ml. OXOFORM-CF was a white solid, dissolved in DMSO to make the final concentration from 100uM to 0.1uM according to the molecular weight (received from Mass Spectrum).

Total cell number count (see 3.1.1.2)

Present the data (see 3.1.1.3)

3.3.2 Stability test

LR6M sample was dissolved in PBS to make a 10^{-4}M solution. LR6H sample was diluted to 500ug/ml. Four condition factors were tested for the compound stability– room temperature (dark), room temperature (in light), 4℃(dark) and -20℃ (dark). For each compound two samples were tested for each factor. After 0,1,2 and 4 weeks the samples were tested by HPLC.

A μ Bondapak C2 column (5 μ mx250mm) was attached to a Shimazdu System with the UV detector set at 200nm (LR6M), 201nm (LR6H) and absorbance at 0.32. There was a constant flow rate of 1ml/min. The liquid phase was 10%Methanol and 90% water. 50 μ l sample was injected by using an auto-analyser.

3.3.3 Isolation and characterization by HPLC

HPLC condition:

Solid phase: μ Bondapak C2 column (5 μ mx250mm)

μ Bondapak C18 column (3.9mmx300mm)

Liquid phase: Use different liquid phase according to different compounds

Flow rate: 1ml/min

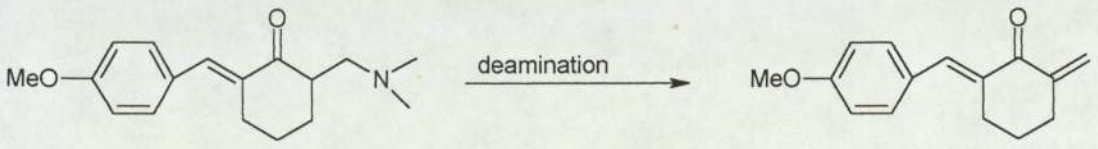
4. 0 Results and discussion:

4.1 Results and discussion about ZK compounds

The curves of percent inhibition to compound concentrations of 31 compounds are shown in **Figure 8**. According to these graphs, the IC_{50} values (the concentration required to inhibit cell growth by 50%) obtained in two cell lines (MAC16, MAC13) are summarized in **Table 1**. Comparing the data in **Table 1** shows that ZK3 and ZK6 have the highest cytotoxicity for both MAC13 and MAC16 cell lines. ZK5 and ZK1 have low cytotoxicity for both cell lines. Of the other series of compounds most have high cytotoxicity for the MAC13 cell line and moderate cytotoxicity for the MAC16 cell line. The MAC16 is resistant to most antiproliferative agents, so ZK6 and ZK3 express strong cytotoxicity. Analysing the structures shows that the basic skeleton of ZK6 contains the O=C-C=C group (enone group) and ZK3 contains C=C-C-OH group which might transform to the enone group in solution. The enone group plays an important role in cytotoxicity (see introduction). The aryl group plays an important role in toxicity as seen by comparing ZK1 and ZK3. For each series the substituent has a different effect on the cytotoxicity, but no regular pattern was obtained.

What kind of mechanisms of action makes these compounds have so strong cytotoxicity? From the sixth series we choose ZK6-1 and ZK6-2, which have the highest cytotoxicity for further investigation of the mechanism of action. The structure of the compounds is not complex, the compounds undergo deamination to form the α -methylene function in solution and the O=C-C=C system (enone group) is formed (Hai-tao Chen and Zhi-zhong 1996). One hypothesis which has been put forward to explain the mode of cytotoxic action is that the enone group alkylates the SH biological nucleophiles of key regulatory enzymes of nucleic acid synthesis by rapid Michael addition (Lee et al 1971). So the mechanism of action is enzyme-alkylating agents (Cavallito and Haskell 1945). The compounds might attack a number of enzymes taking part in RNA, protein synthesis and also the linking enzyme which are needed for the construction of new DNA stands on their parent templates. Alkylation prevents these enzymes from carrying out their biological role

within the cell and so stops the formation of new DNA, which in turn inhibits mitosis. The enone group represents a major alkylating center. The second hypothesis assumes that activated species of oxygen, mostly superoxide ion O_2^- are generated in *situ* by the reductive activation of molecular oxygen. The compounds served as electron carriers during the electron transport process, the compound would be transiently reduced to the corresponding unstable free radicals. The cellular damage is generally considered as the consequence of the formation of hydroxyl radicals. It has also been claimed that free radicals were able to covalently bind DNA and proteins. To determine whether free radicals or alkylating species were produced, five kinds of antioxidants were used– BHT, catalase, GSH, cysteine and indomethacin. Originally the ZK compounds and antioxidants were added at the same time, no increase of cell number can be observed. (**Figure 9**) Then the antioxidants were added in advance for two hours but little change could be observed. The mixture of ZK compounds and GSH was put in the incubator at 37°C overnight to give enough time for reaction, and then added to MAC16 cell lines. The cell number was counted after 72 hours. The results were shown in **Figure 10.** The total cell number had increased. To get the precise difference the cell viability was measured **(Figure 11).** An increase can be seen, but there was not complete reverse of the effect of ZK compounds on cell lines. Also by comparing the NMR graph of blank and GSH-ZK mixture the change of chemical shift in δ -3.5 (methylene hydrogen) can be observed. Although we cannot explain each change of the graph, the structure of the compounds shows the active group only can be the enone group. Also after deamination two enone groups were produced (**See below**), so the chemical shift change means one of the two groups or the two groups were involved in the reaction. The reaction of GSH and ZK proceeds.



If the two enone groups are all active groups in the action, another hypothesis is the formation of cross-linkages between DNA chains, damages DNA, which requires two alkylating functional groups for this mechanism of action. In the ZK6-1 and

ZK6-2 structure if this requirement can be satisfied then the C=C group is another active centre. This is only a hypothesis and still needs to be investigated.

Measurement of radiolabeled thymidine, uridine and leucine (or phenylalanine) uptake was done to determine the effect on DNA, RNA and protein synthesis. The effect of ZK6-1 and ZK6-2 on radiolabeled thymidine, uridine, leucine (or phenylalanine) uptake in the MAC16 cell lines was shown in **Figure 12.** The isotope uptake in the pool was measured to erase the effect of dead cells on the data. After two hours the incorporation of labeled ^{3}H-thymidine, uridine and leucine (or phenylalanine) uptake decreased. ZK6-1 and ZK6-2 both have the obvious effects on DNA, RNA, protein synthesis and the action is so rapid, especial for RNA and protein synthesis. From these results we can know that the compounds might have the mechanism of action as alkylators, but if they only act as alkylators acting on the enzyme it cannot have the action on DNA, RNA and protein synthesis so quick. So it cannot be the main mechanism of action. There must be other mechanisms of action.

For reducing the DNA, RNA and protein levels so quickly, a possible action position is on the cell membrane which broke quickly after treatment by ZK compounds. If these compounds act as alkylating agents the enone group alkylates the SH biological nucleophiles of key regulatory enzymes. So thiol groups of the cell membrane were measured to determine whether the action position is on the cell membrane. At the same time the cellular thiol groups were measured, to evaluate whether soluble thiol groups were involved in the enone group action. The 6,6-dithiodinicotinic acid (CPDS) was used to estimate the thiol group remaining on the cell membrane and in the cells after treatment by ZK6-1 or ZK6-2 compounds. The results were shown in **Figure 13.** The reaction products of CPDS and thiol groups both on the cell membrane and in the cells did not decrease. It is thus suggested that the thiol groups are not involved in the reaction with ZK compounds, and the action position is not on the cell membrane through reaction with the thiol groups. These compounds do not alkylate the SH groups of enzymes. It confirmed there must be some other mechanisms of action.

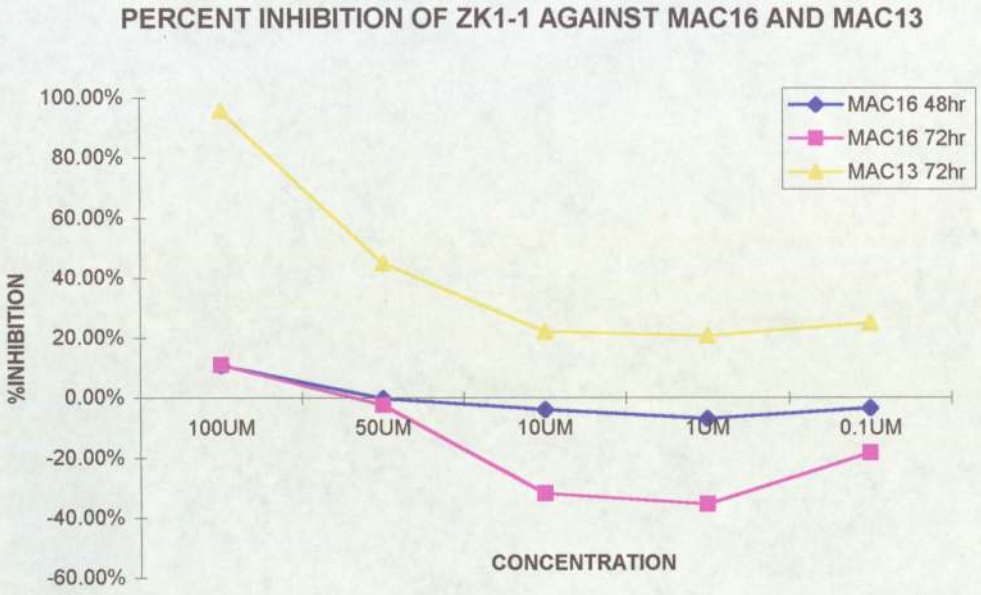
Measurement of intracellular ATP is also an important part of understanding the

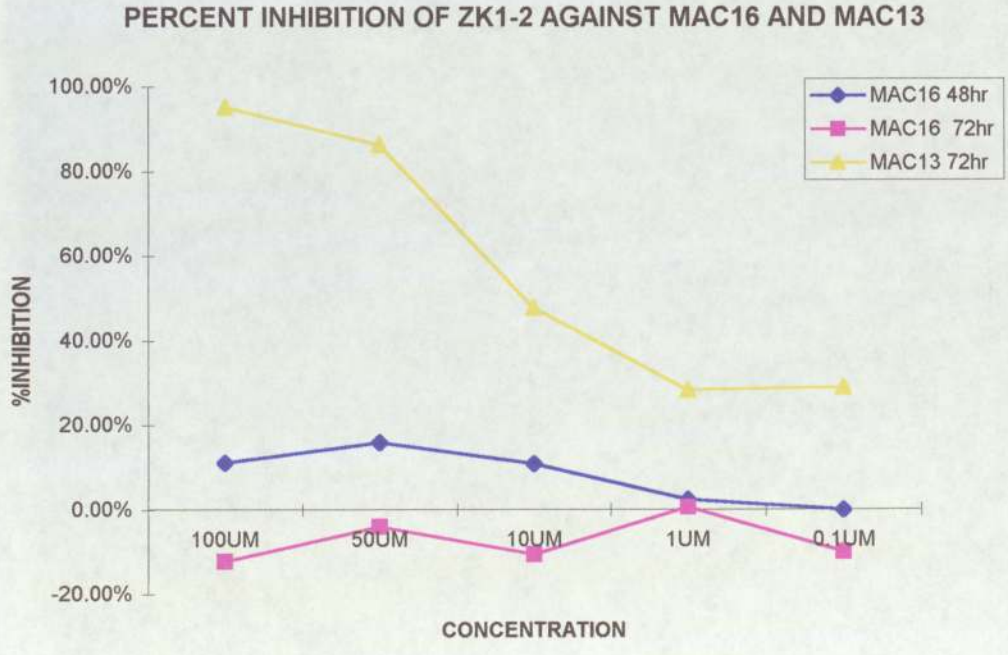
mechanism of action. The ATP-driven luciferin-luciferase system that produces light is the basis for an exquisitely sensitive assay that can detect as little as 10^{-12} moles of ATP. ATP is the primary donor of chemical high energy in living cells. It is present in all metabolically active cells, drives a wide range of biochemical reactions, and is rapidly degraded by ATPases in dead and dying cells. These properties make ATP levels a good index of viable biomass in a wide range of biological systems. According to this property the ATP graphs **(Figure 15)** also give the information of the high cytotoxicity- after 4 hours the ATP decreased to below 10% of control values. Combining the rapid reduction of DNA, RNA and protein synthesis these compounds might have other actions. When comparing the cellular ATP **(Figure 15)** with the cell viability **(Figure 14)** we can see that the ATP decreases a little faster than cell viability. There is not an obvious action on energy pathway.

For selectivity evaluation, from each series one compound was chosen for general evaluation. A comparison was made of the IC_{50} values between the tumour cell lines (MAC16, MAC13) and the normal cell lines (C_2C_{12}, 3T3) **(Table 2)**. This suggested there was no selective toxicity towards tumour cells. Some of the compounds even had more cytotoxicity for normal cells than for tumour cells. This is a long- term problem for this kind of compound.

The ZK compounds which contain the enone group do have high cytotoxicity and they have the effects on DNA, RNA and protein synthesis, reducing the levels very quickly. But there is no free radicals or alkylating species involved in the action. They do not act as alkylators. Also the thiol group on the cell membrane isn't the action target. The mechanism of action of these agents is complex and only series six was evaluated. For each series the structure is different, and they might act differently. The mechanism of action of these agents still needs to be investigated. The undiscriminating cytotoxicity is an obvious drawback of these agents. To increase selectivity and reduce toxicity, ZK3-2 and ZK3-3 might be the important point, which have high cytotoxicity but contain the C=C-C-OH group which might relieve the cytotoxicity to normal cells. The research about the two compounds is under active investigation.

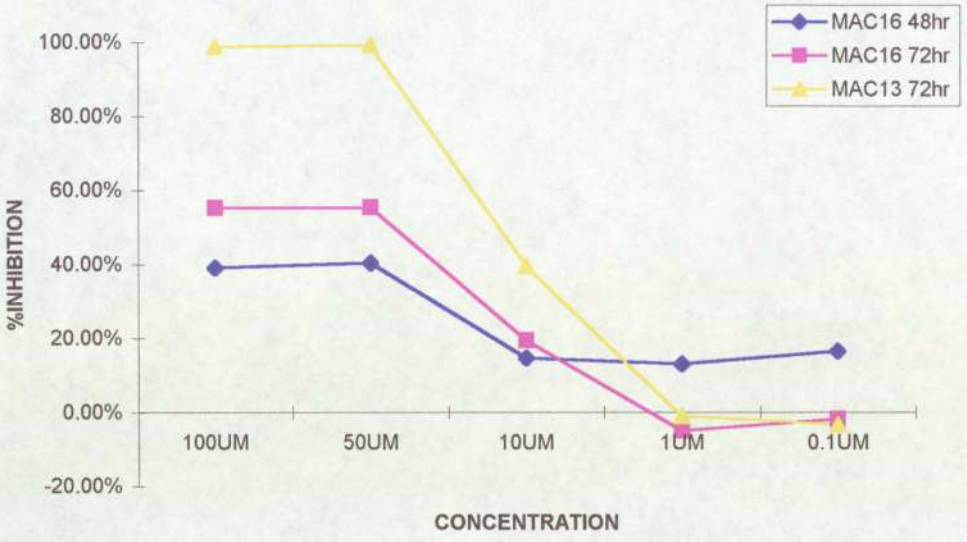
FIGURE 8. The cytotoxicity evaluation of ZK compounds against MAC16 and MAC13



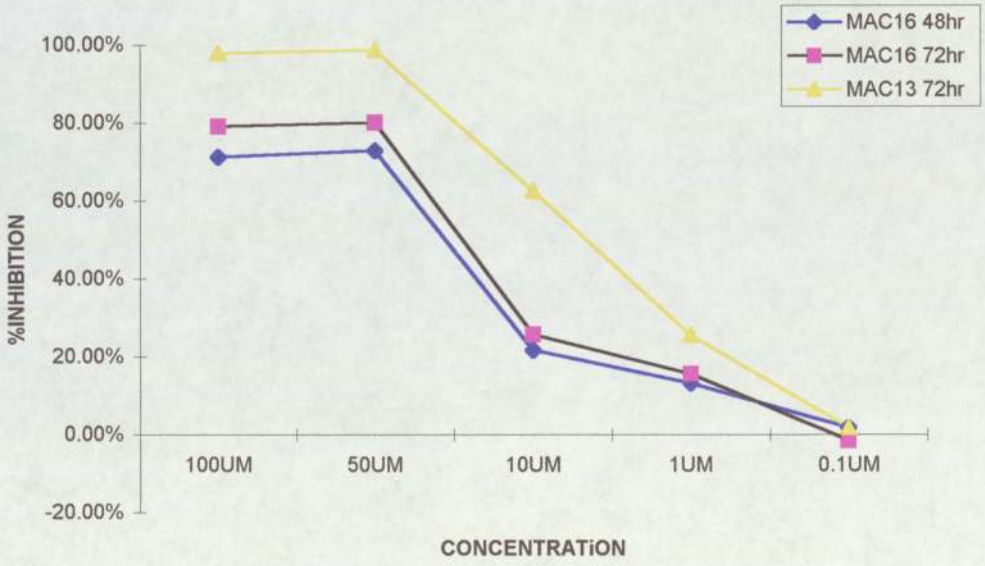


For each concentration had two replicates and presented as mean value.

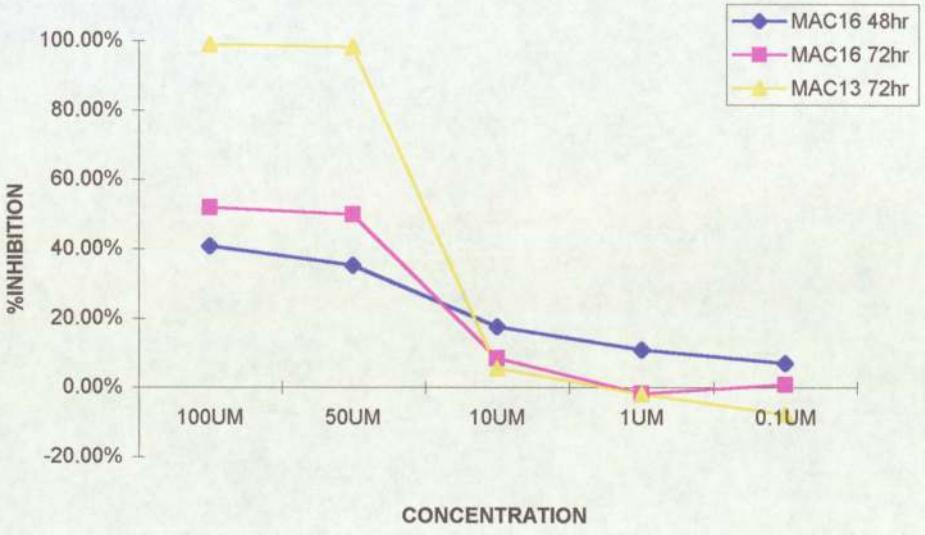
PERCENT INHIBITION OF ZK2-1 AGAINST MAC16 AND MAC13



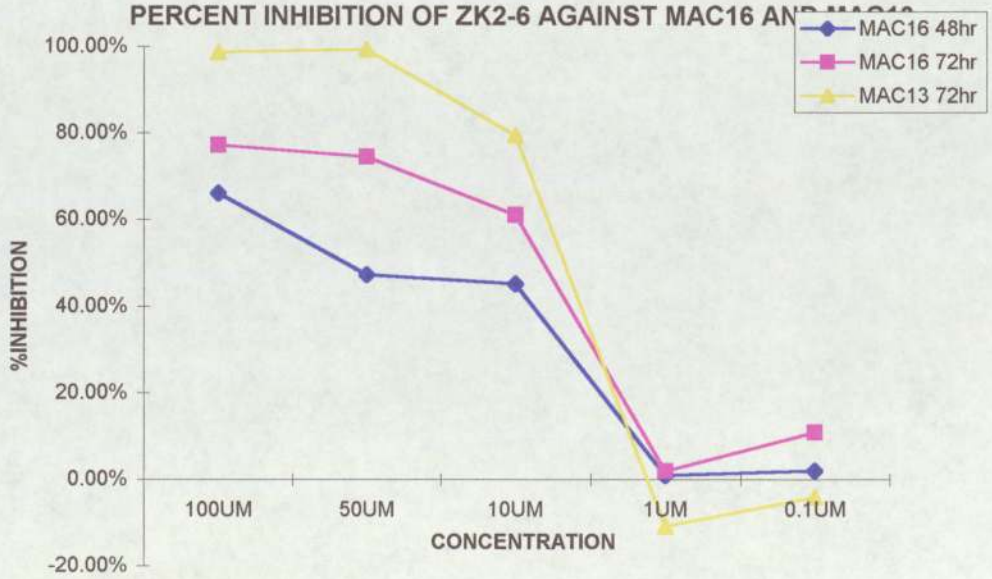
PERCENT INHIBITION OF ZK2-2 AGAINST MAC16 AND MAC13



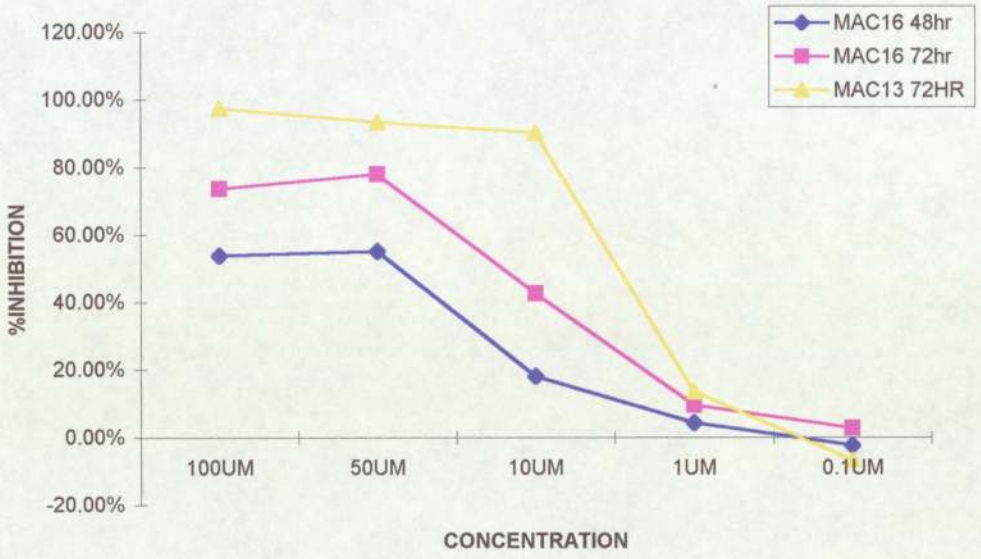
PERCENT INHIBITION OF ZK2-4 AGAINST MAC16 AND MAC13



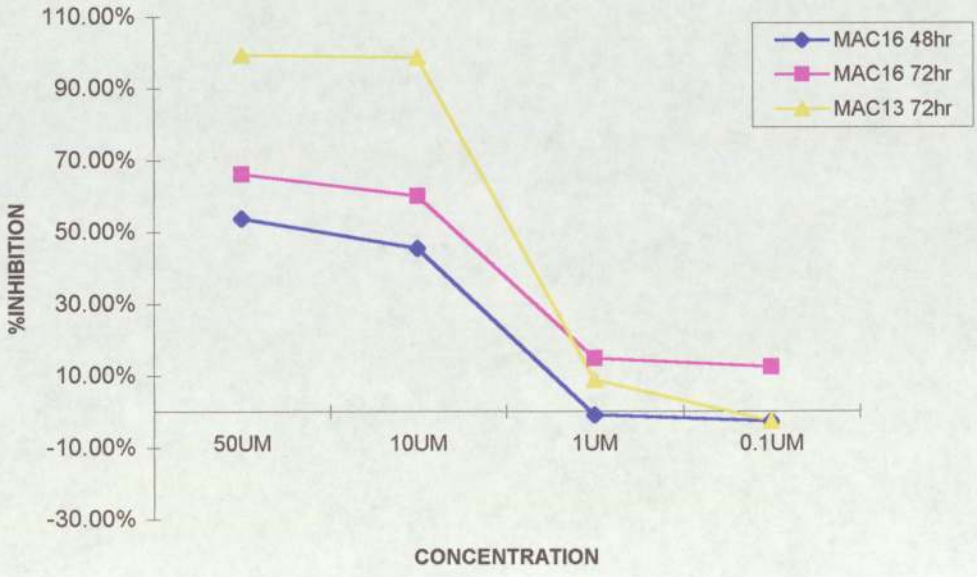
PERCENT INHIBITION OF ZK2-6 AGAINST MAC16 AN



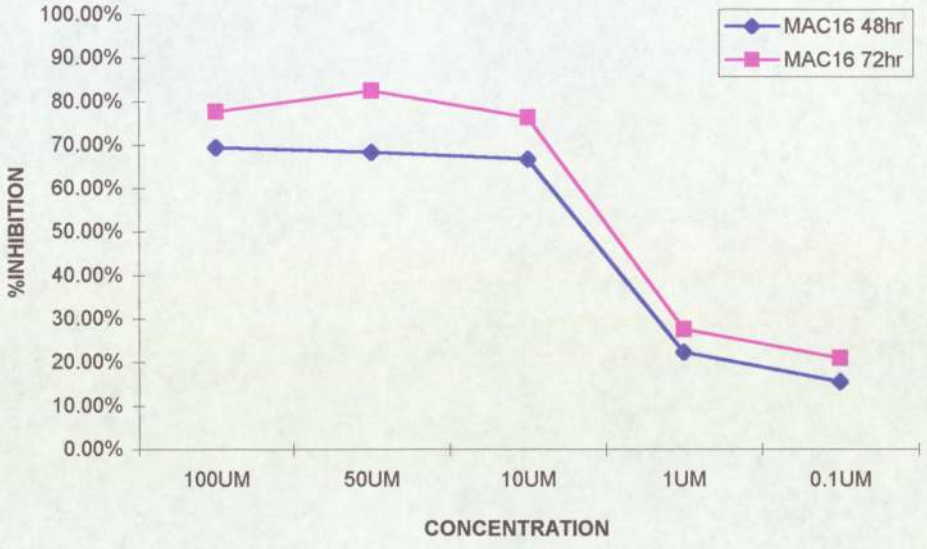
PERCENT INHIBITION OF ZK2-7 AGAINST MAC16 AND MAC13



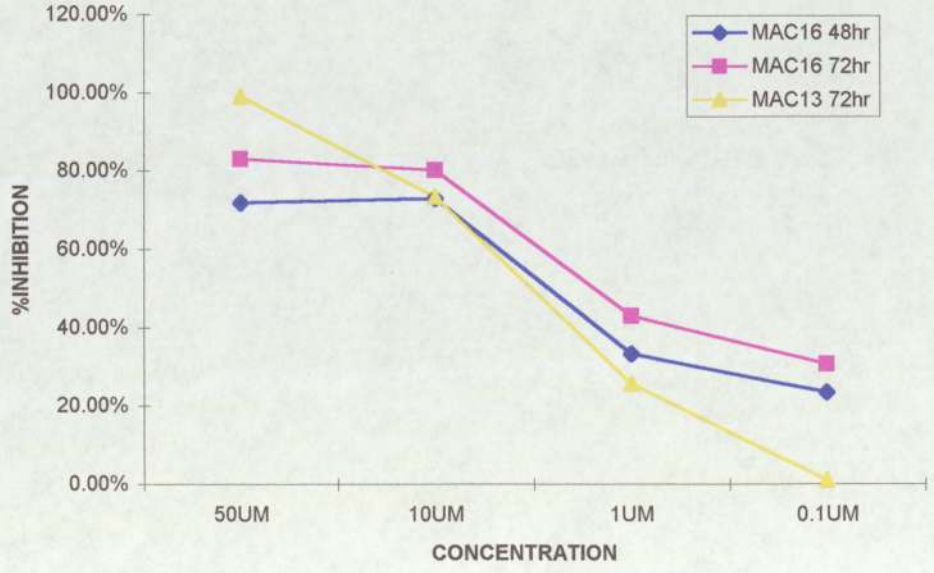
PERCENT INHIBITION OF ZK3-1 AGAINST MAC16 AND MAC13



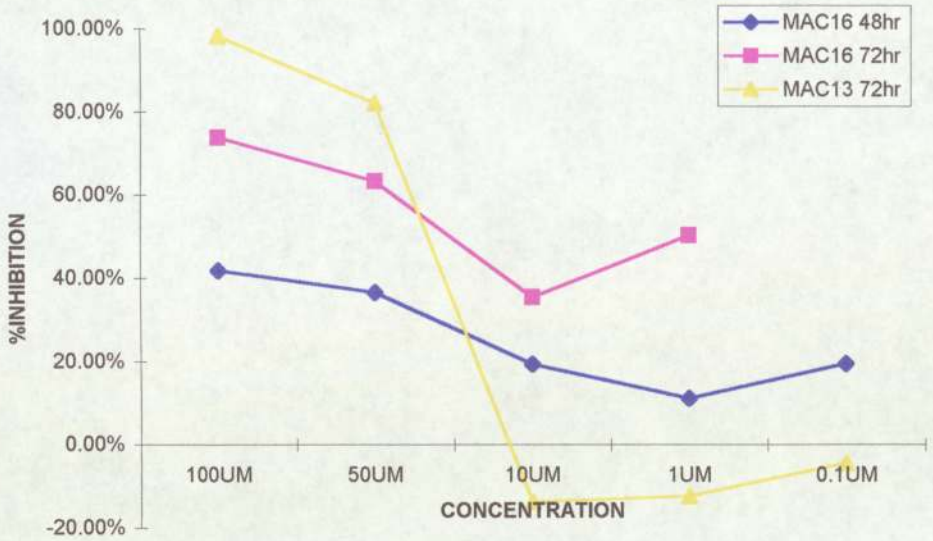
PERCENT INHIBITION OF ZK3-2 AGAINST MAC16 AND MAC13



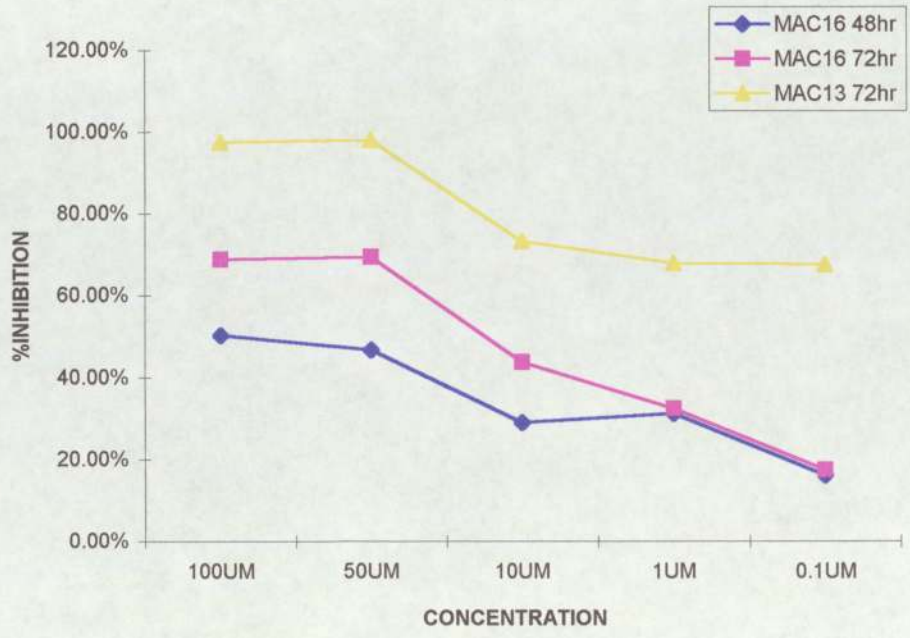
PERCENT INHIBITION OF ZK3-3 AGAINST MAC16 AND MAC13

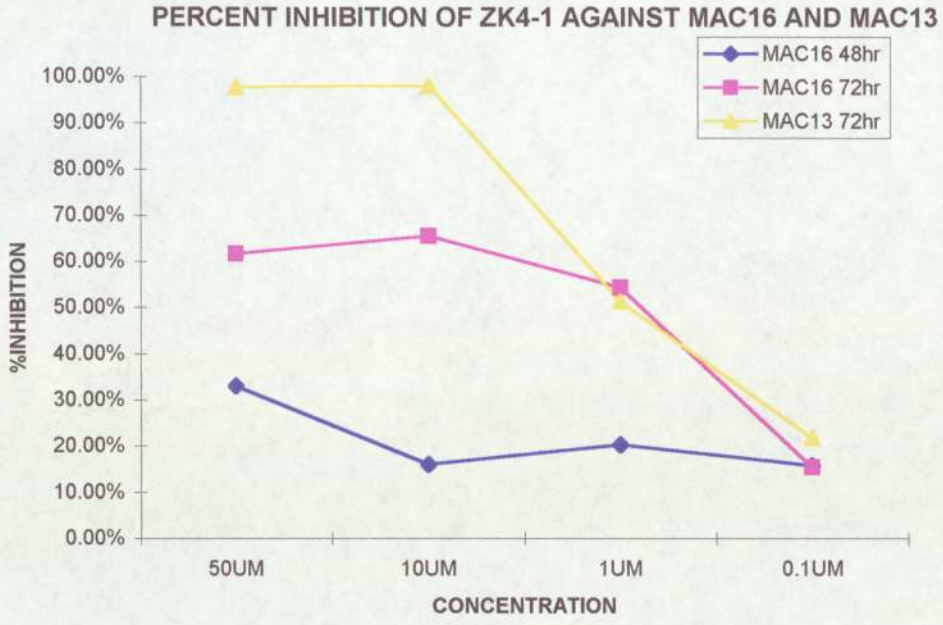


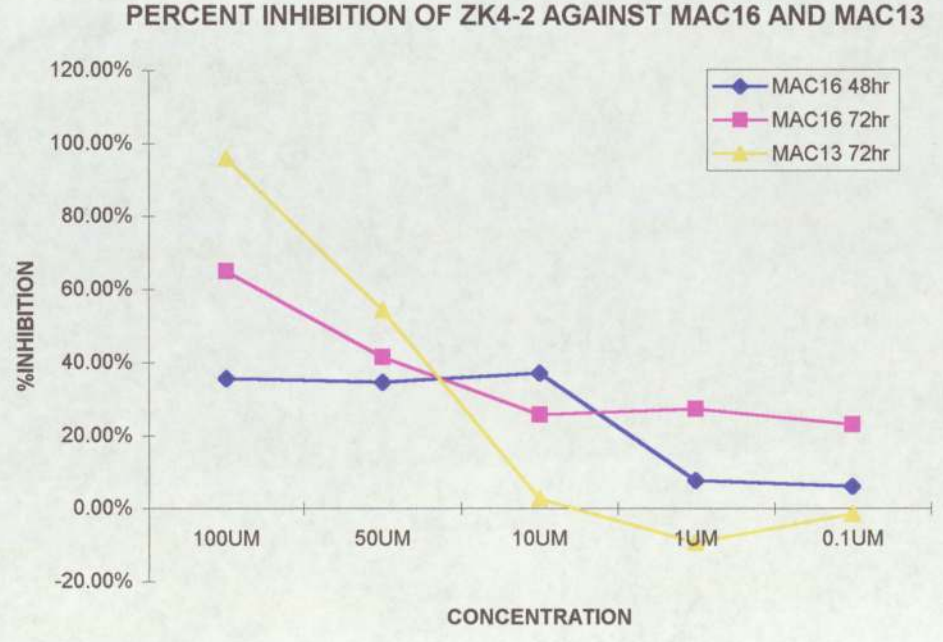
PERCENT INHIBITION OF ZK3-4 AGAINST MAC16 AND MAC13



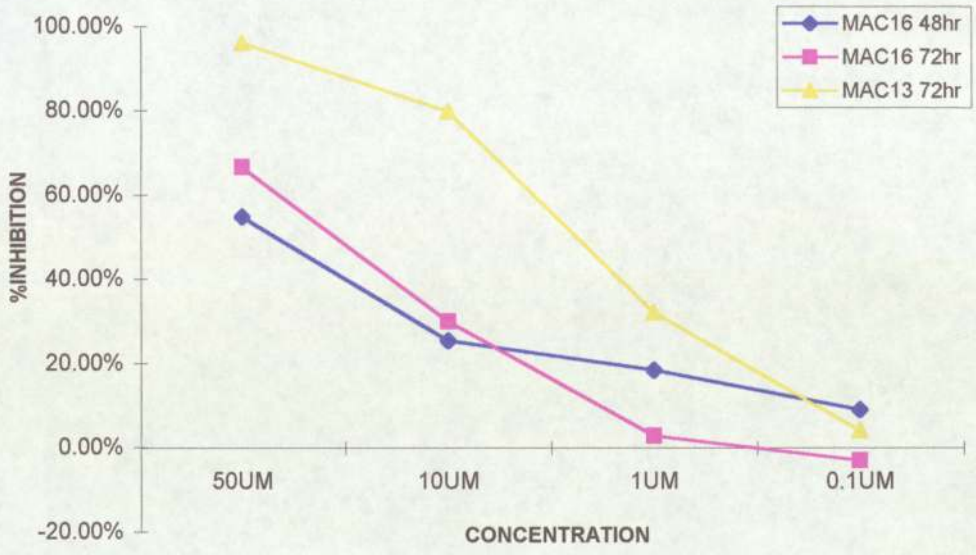
PERCENT INHIBITION OF ZK3-5 AGAINST MAC16 AND MAC13



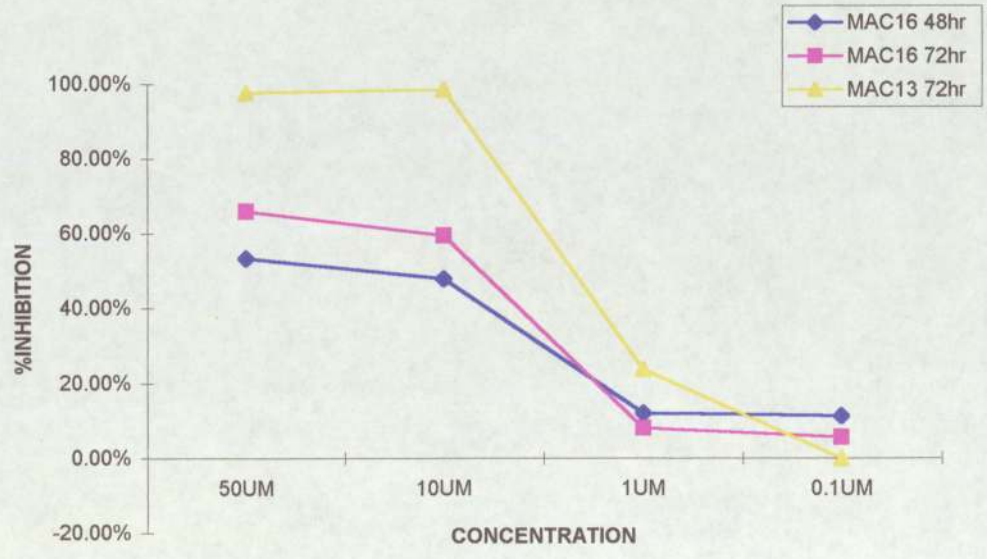

PERCENT INHIBITION OF ZK4-1 AGAINST MAC16 AND MAC13
MAC16 48hr
MAC16 72hr
MAC13 72hr
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
%INHIBITION
50UM
10UM
1UM
0.1UM
CONCENTRATION


PERCENT INHIBITION OF ZK4-2 AGAINST MAC16 AND MAC13
MAC16 48hr
MAC16 72hr
MAC13 72hr
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
-20.00%
%INHIBITION
100UM
50UM
10UM
1UM
0.1UM
CONCENTRATION

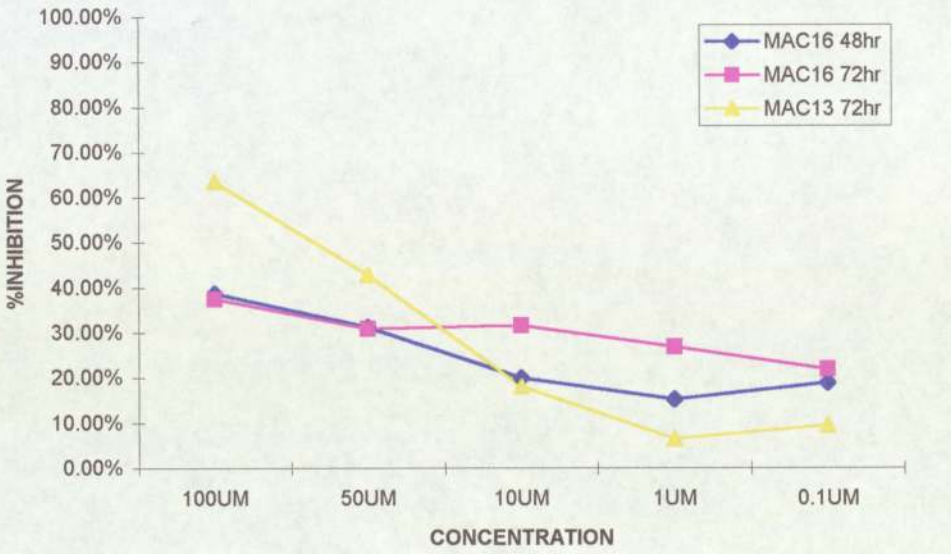
PERCENT INHIBITION OF ZK4-4 AGAINST MAC16 AND MAC13



PERCENT INHIBITION OF ZK4-5 AGAINST MAC16 AND MAC13



PERCENT INHIBITION OF ZK5-1 AGAINST MAC16 AND MAC13



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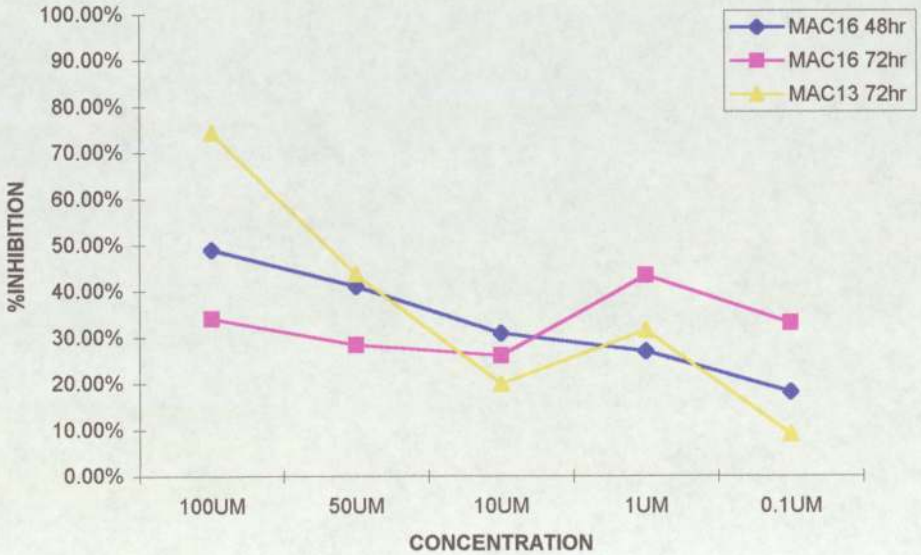
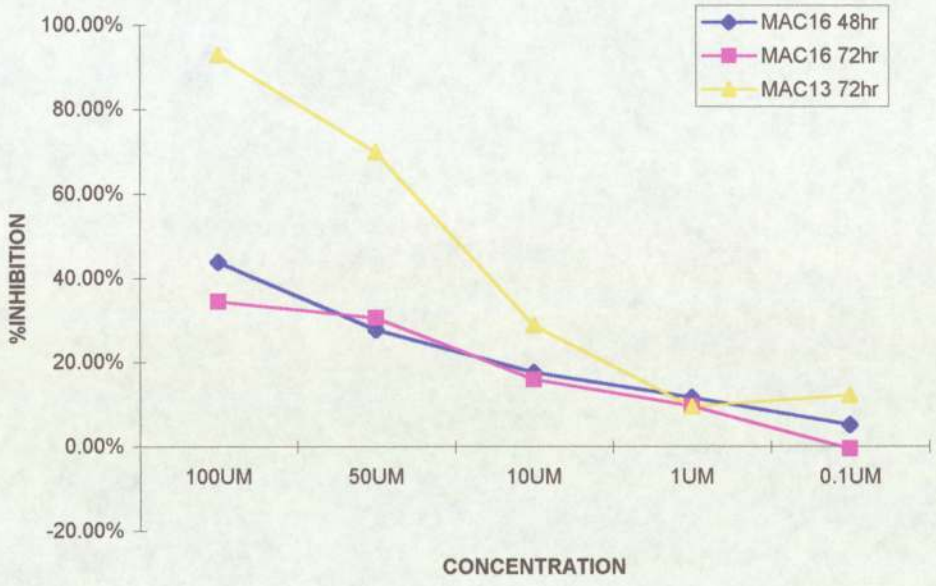
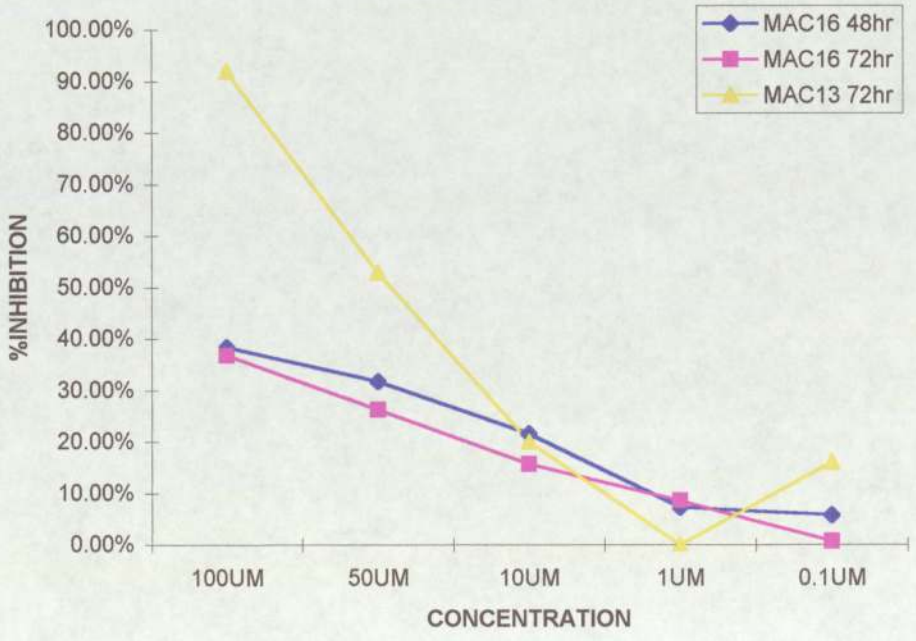


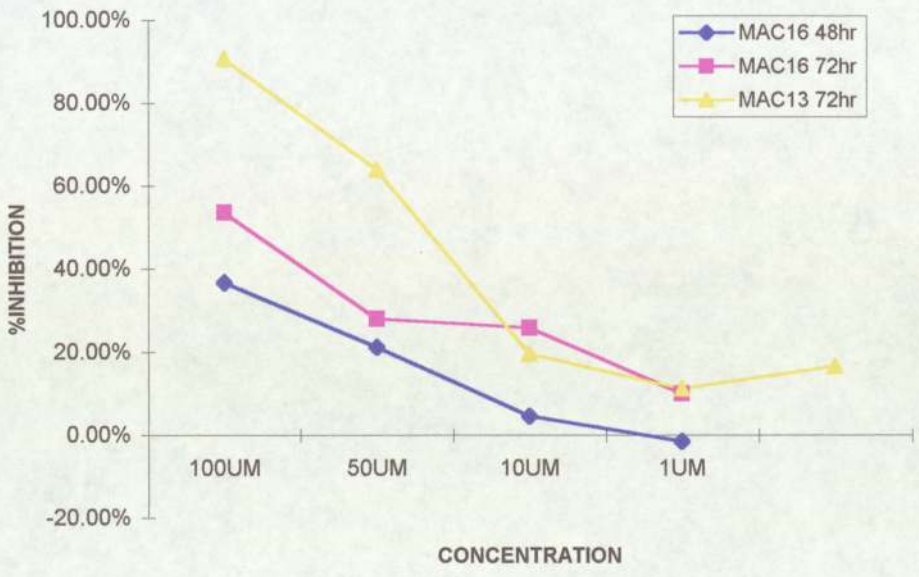
PERCENT INHIBITION OF ZK5-3 AGAINST MAC16 AND MAC13



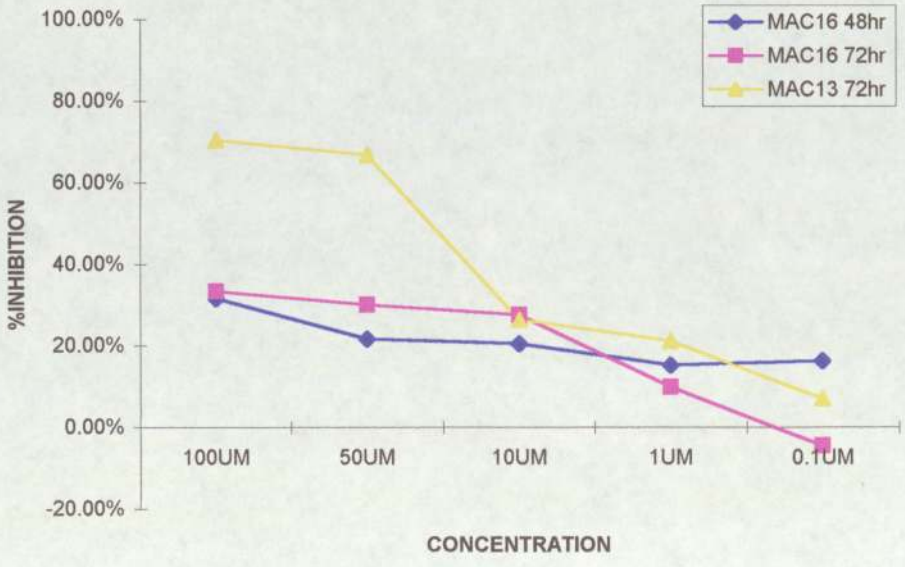
PERCENT INHIBITION OF ZK5-4 AGAINST MAC16 AND MAC13



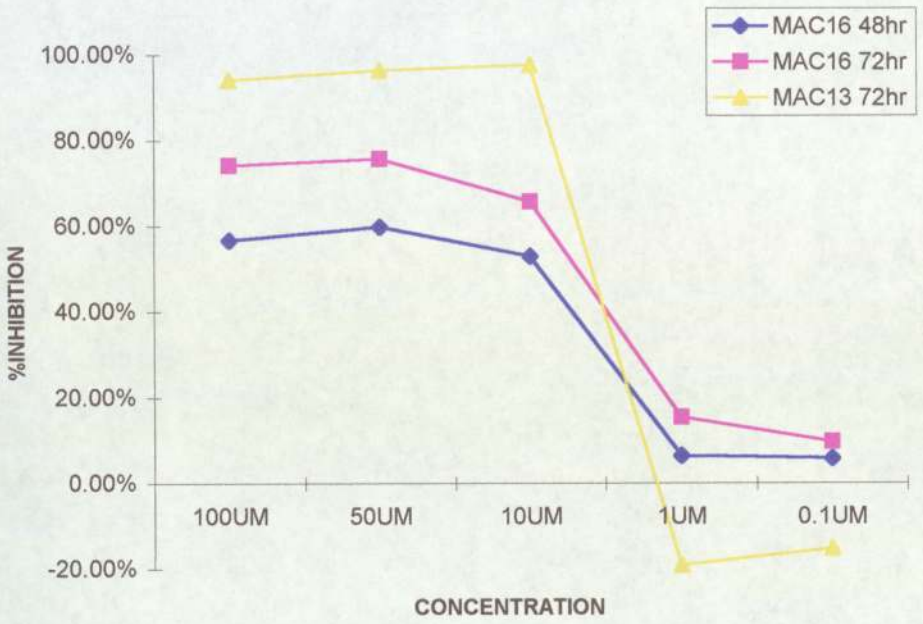
PERCENT INHIBITION OF ZK5-5 AGAINST MAC16 AND MAC13



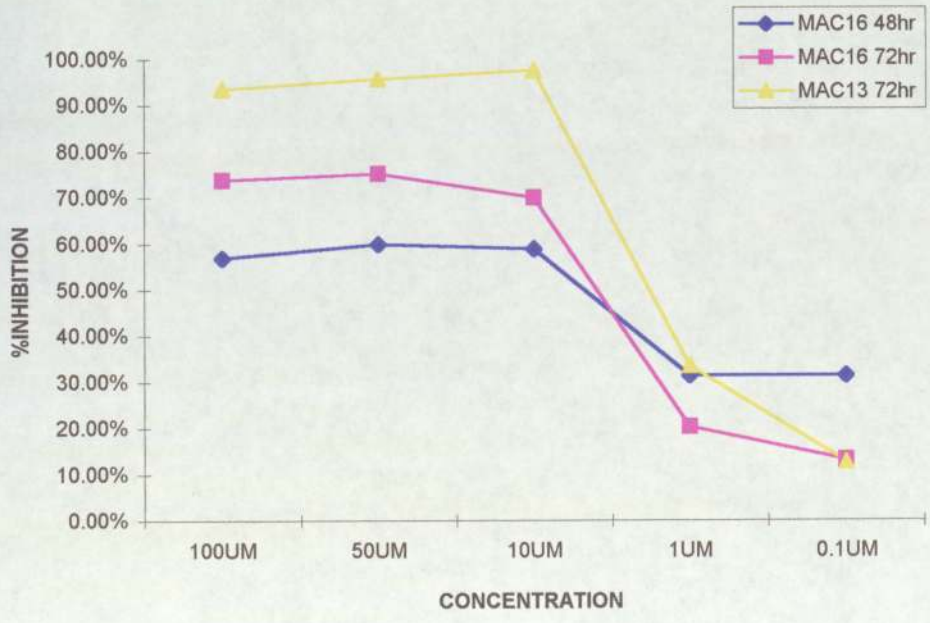
PERCENT INHIBITION OF ZK5-6 AGAINST MAC16 AND MAC13



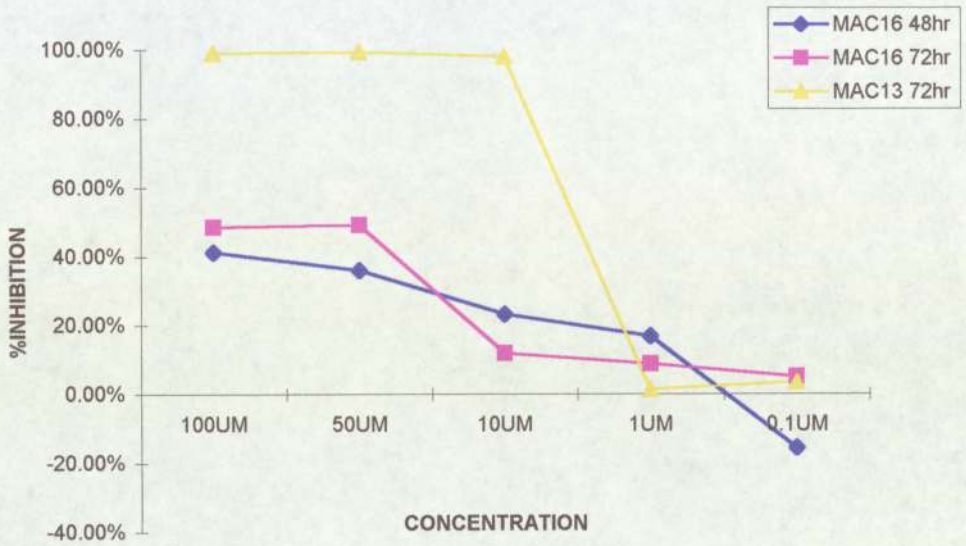
PERCENT INHIBITION OF ZK6-1 AGAINST MAC16 AND MAC13



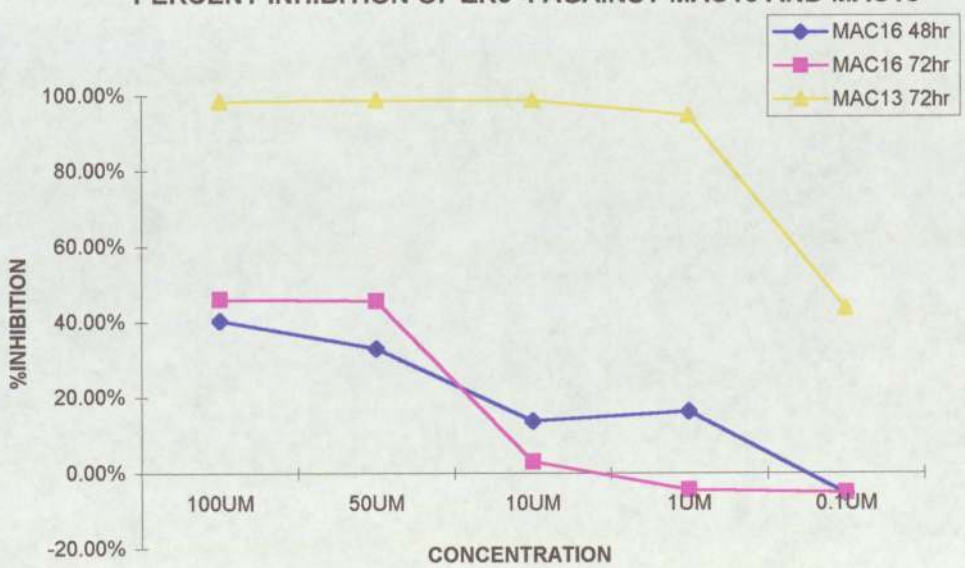
PERCENT INHIBITION OF ZK6-2 AGAINST MAC16 AND MAC13



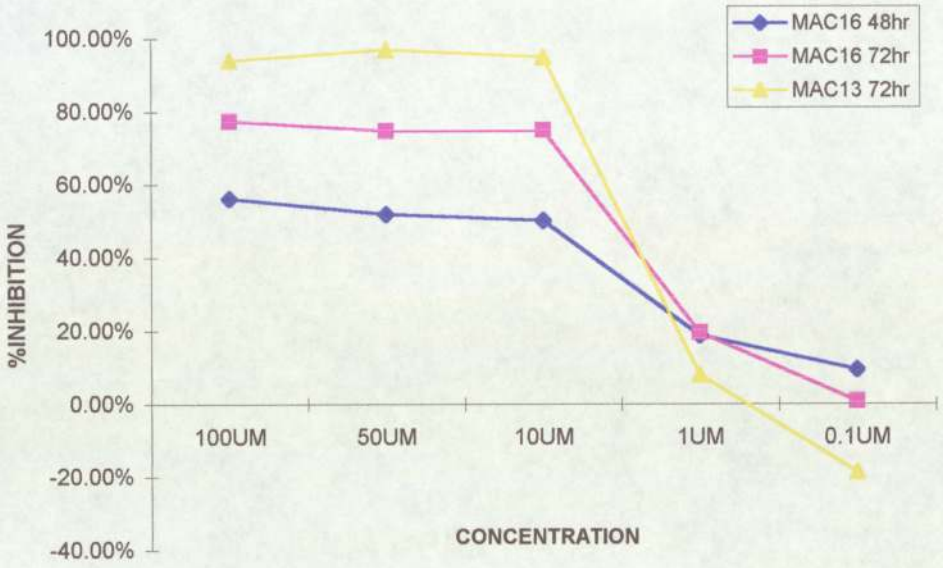
PERCENT INHIBITION OF ZK6-3 AGAINST MAC16 AND MAC13



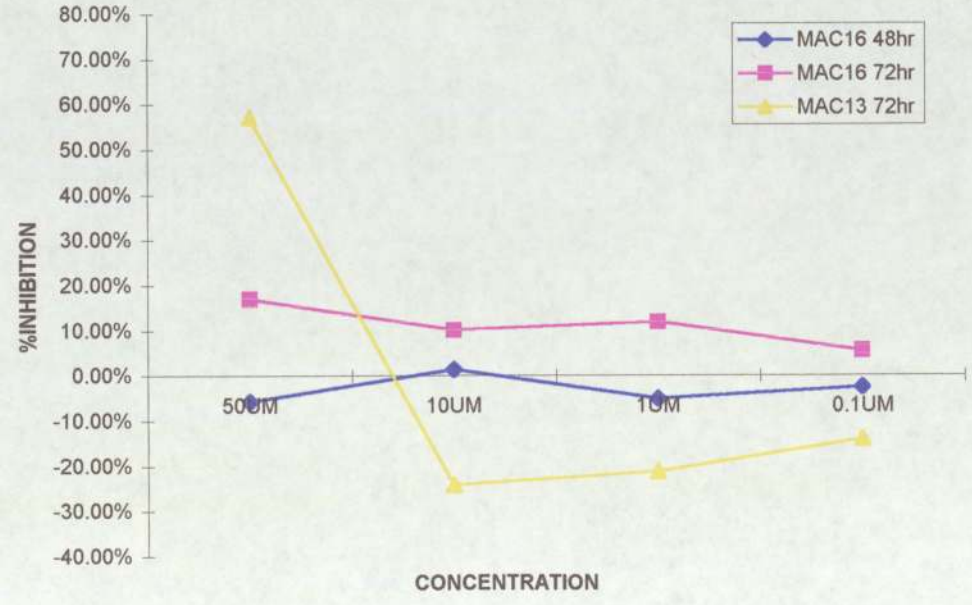
PERCENT INHIBITION OF ZK6-4 AGAINST MAC16 AND MAC13

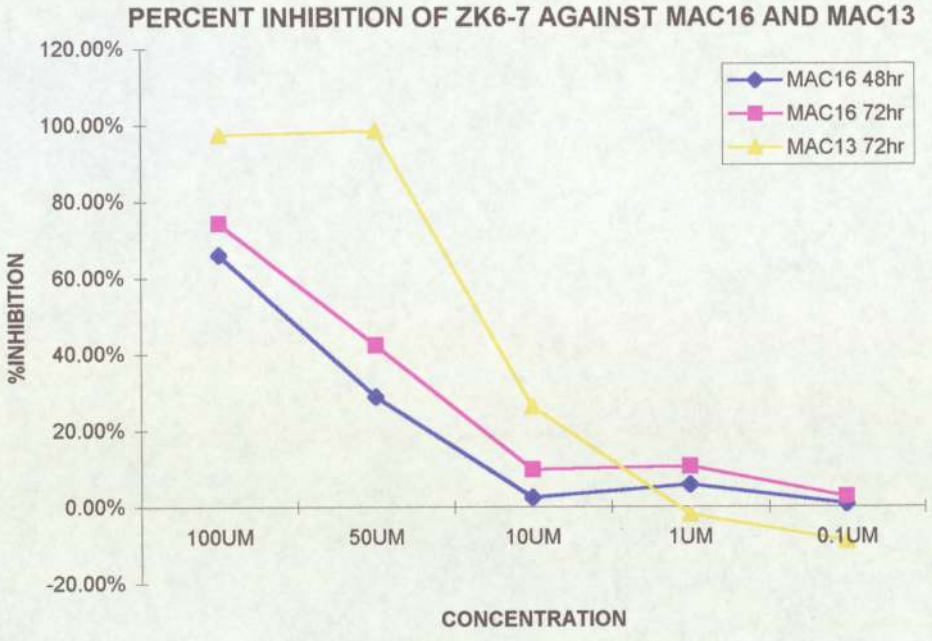


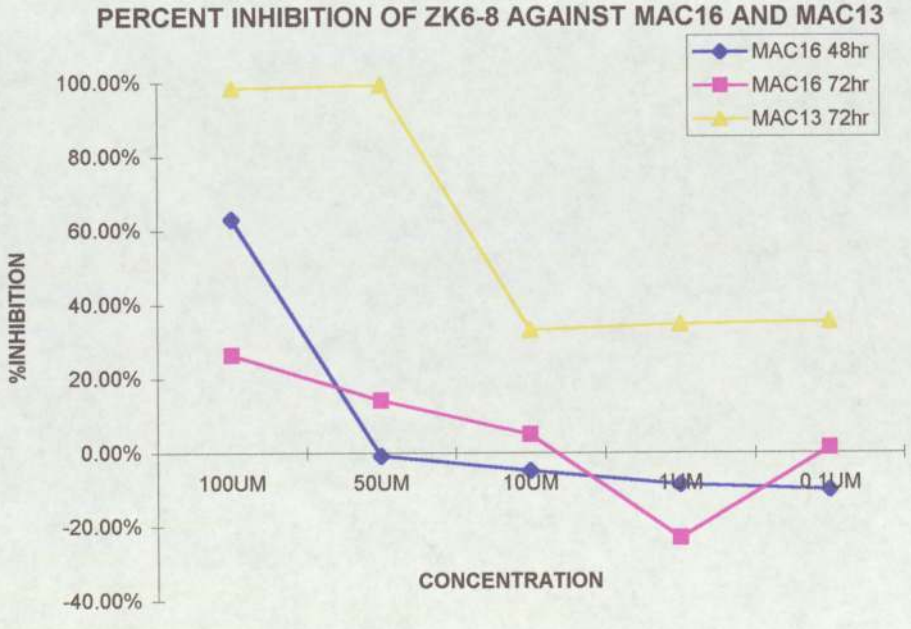
PERCENT INHIBITION OF ZK6-5 AGAINST MAC16 AND MAC13



PERCENT INHIBITION OF ZK6-6 AGAINST MAC16 AND MAC13




PERCENT INHIBITION OF ZK6-7 AGAINST MAC16 AND MAC13
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
-20.00%
%INHIBITION
MAC16 48hr
MAC16 72hr
MAC13 72hr
100UM
50UM
10UM
1UM
0.1UM
CONCENTRATION


PERCENT INHIBITION OF ZK6-8 AGAINST MAC16 AND MAC13
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
-20.00%
-40.00%
%INHIBITION
MAC16 48hr
MAC16 72hr
MAC13 72hr
100UM
50UM
10UM
1UM
0.1UM
CONCENTRATION

PERCENT INHIBITION OF ZK6-9 AGAINST MAC16 AND MAC13

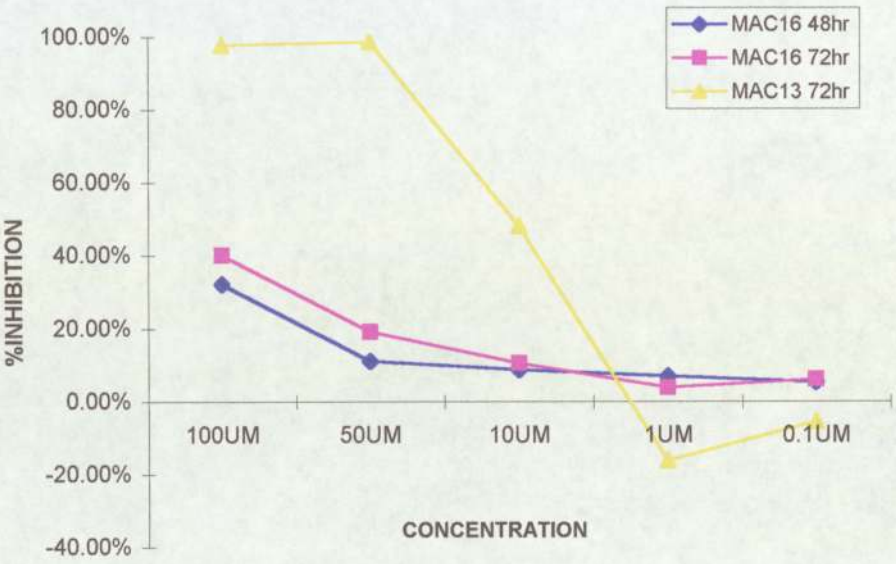
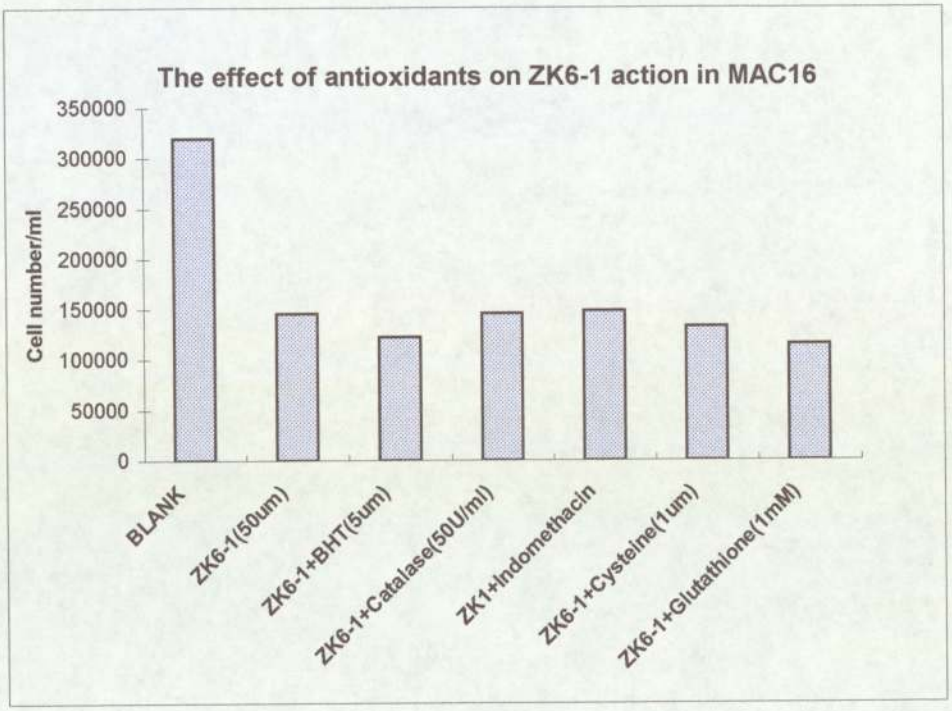
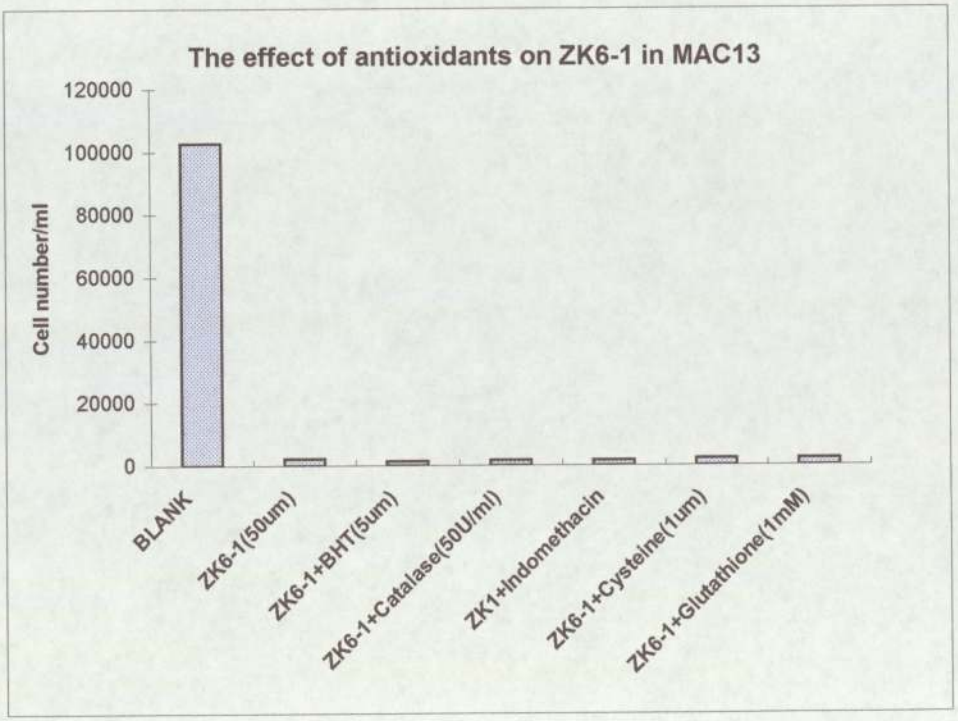


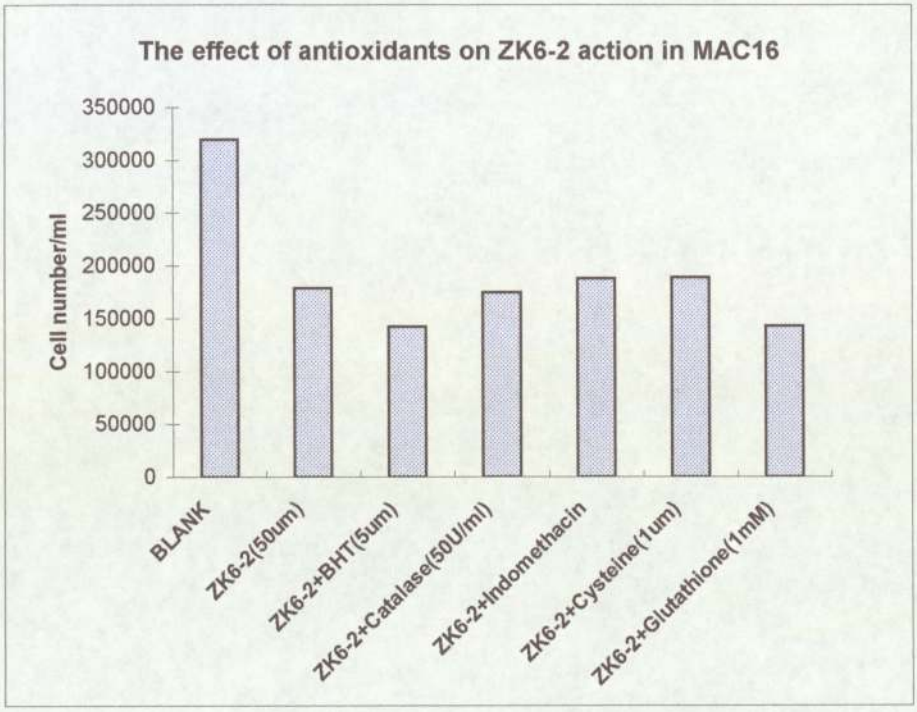
Figure 9.The effect of antioxidants on ZK6-1 action in MAC13 and MAC16

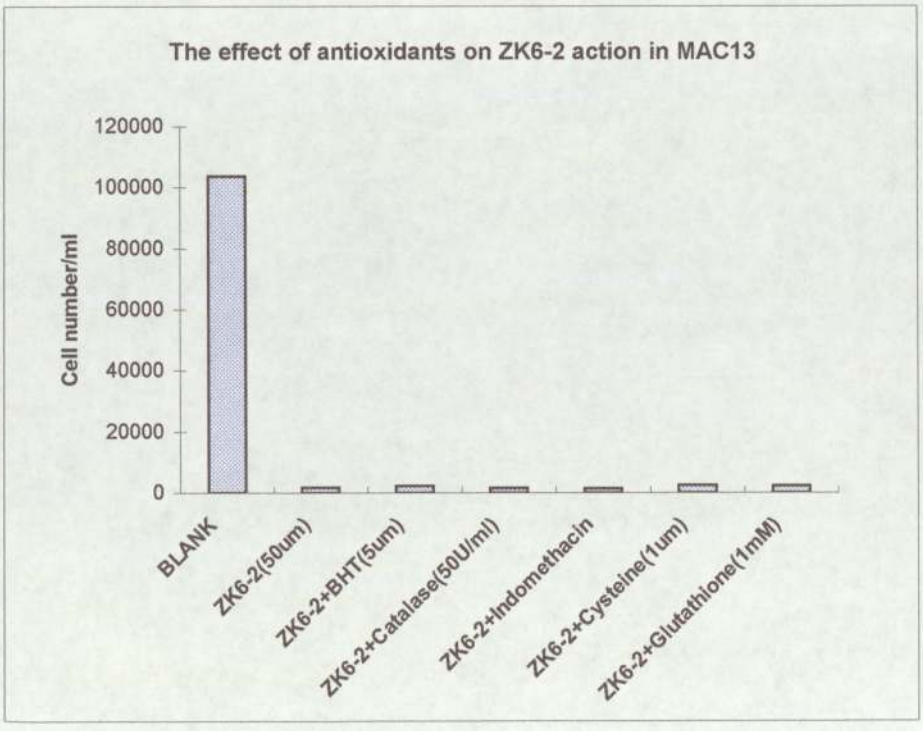




(After incubation for 24 hr the antioxidants and ZK6-1 compounds were added at the same time and for MAC16 after 48 hr cell number was counted, for MAC13 after 72 hr)
For each antioxidant had three replicates and presented as mean value

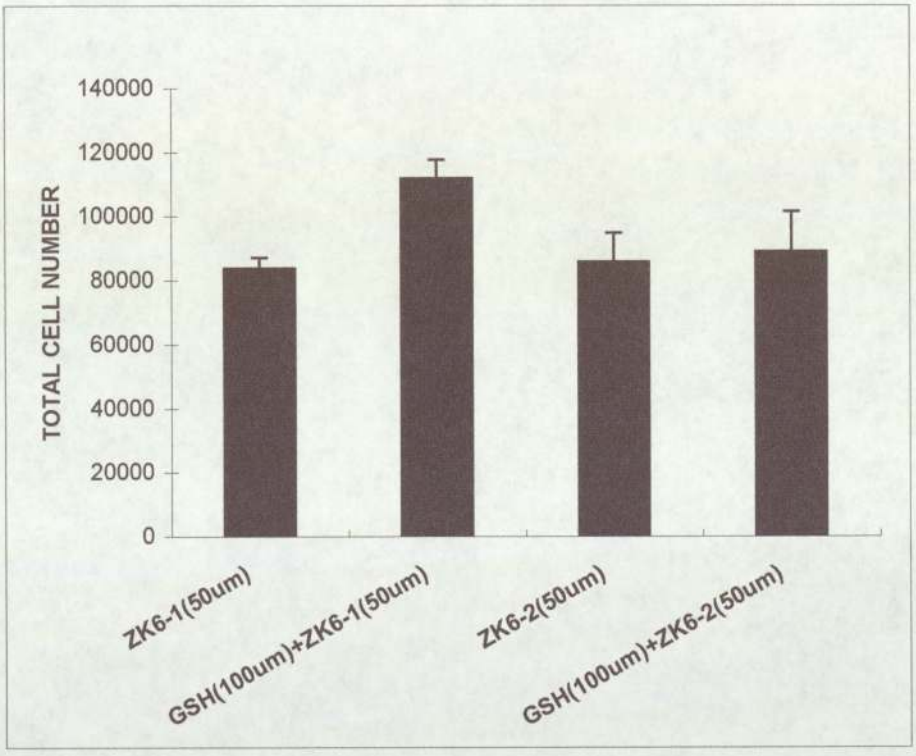
Figure 9.The effect of antioxidants on ZK6-2 action in MAC13, MAC16





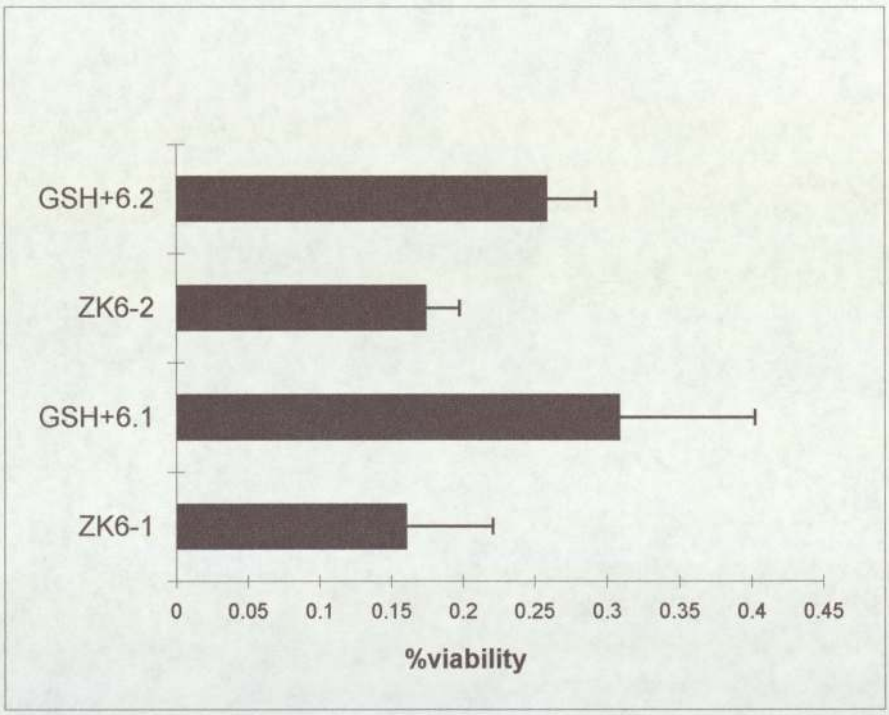
(After incubation for 24 hr the antioxidants and ZK6-2 compounds were added at the same time. For MAC16 after 48 hr cell number was counted, for MAC13 after 72 hr) For each antioxidant had three replicates and presented as mean value

FIGURE 10. The effect of GSH on ZK6-1 and ZK6-2 action in MAC16



(After incubation for 24 hours the mixture of GSH and ZK and ZK compounds were added at the save time and after another 24hrs cell number was counted)
The mixture of GSH and ZK compounds was incubated at 37C overnight before adding to the cells
Results are given as means±s.e.m.(n=6)
Differences are expressed as $P<0.005$(ZK6-1), $P>0.01$(ZK6-2)determined by t-test.

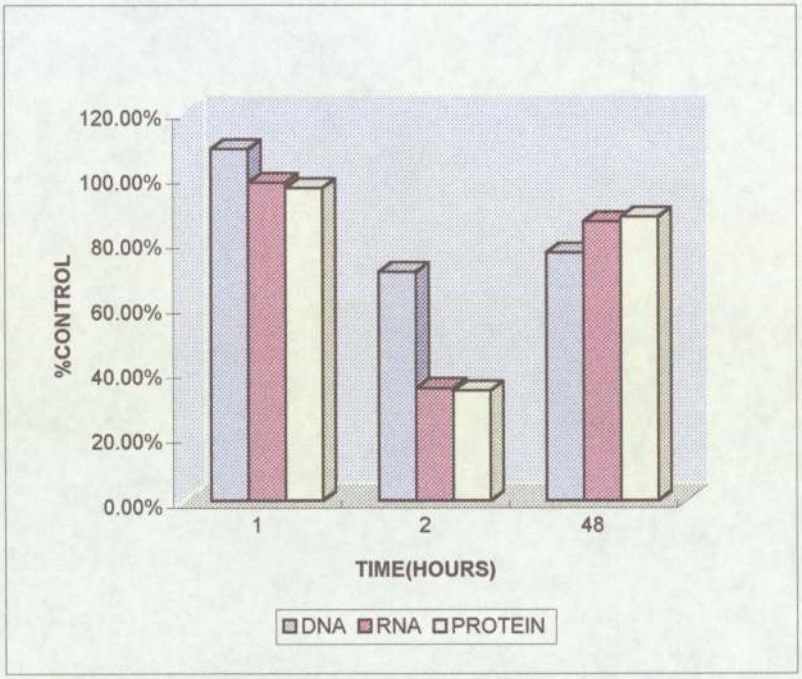
FIGURE 11. The effect of GSH on ZK6-1 and ZK6-2 action in cell viability of MAC16 cell lines



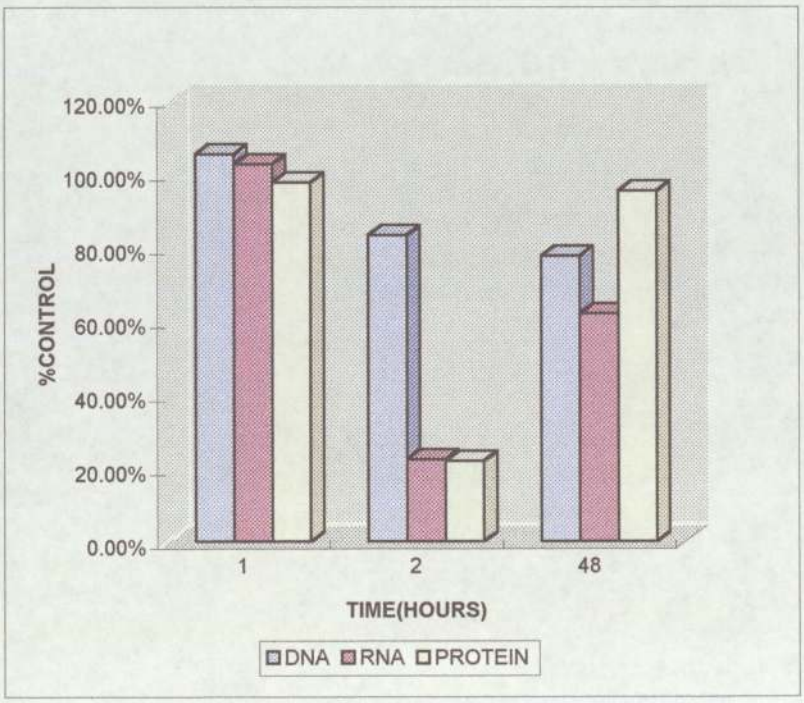
(After incubation for 24 hours the mixture of GSH and ZK and ZK compounds were added and after another 24 hours cell viability was evaluated)
The mixture of GSH and ZK compounds was incubated at 37C overnight before adding to the cells
The concentration of GSH is 100uM and the concentration of ZK compounds is 50uM
Results are given as means±s.e.m.(n=3)
Differences are expressed as $P>0.01$(ZK6-1), $P<0.05$(ZK6-2)determined by t-test.

FIGURE 12. The effect of ZK6-1,ZK6-2 on DNA RNA protein synthesis

THE EFFECT OF ZK6-1 ON DNA RNA PROTEIN SYNTHESIS IN MAC16

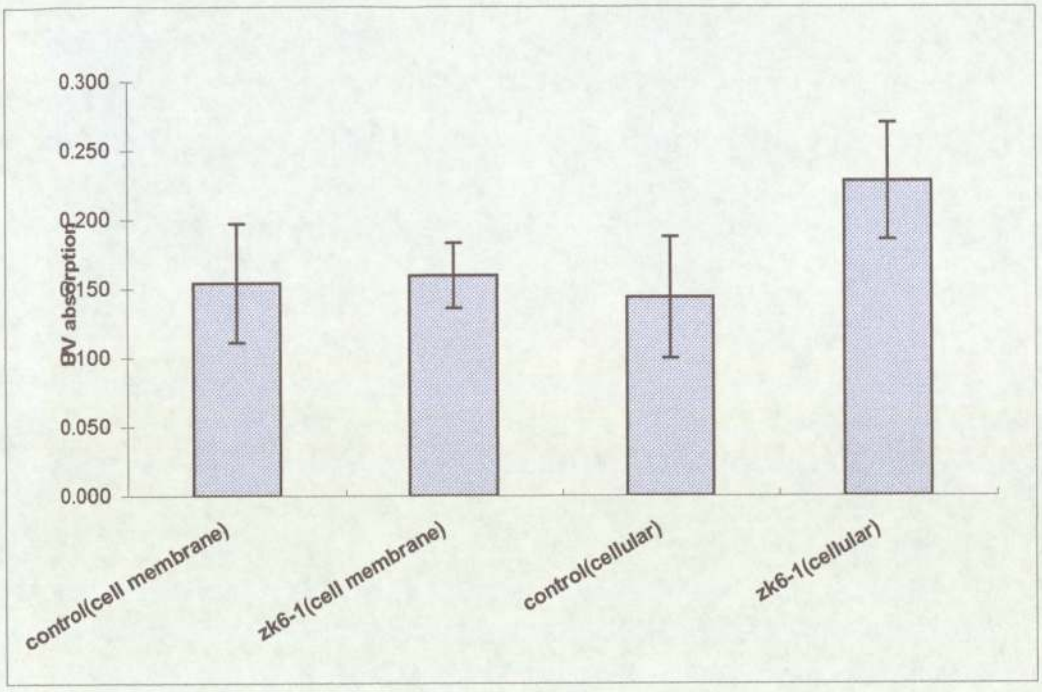


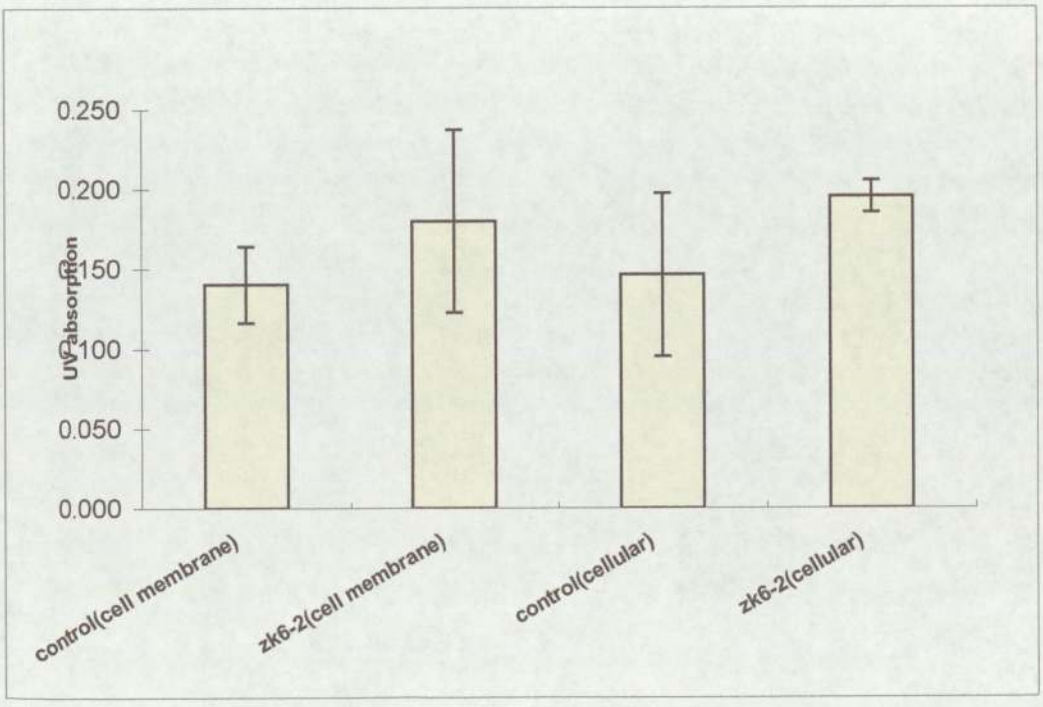
THE EFFECT OF ZK6-2 ON DNA RNA PROTEIN SYNTHESIS IN MAC16



%control=(the incorporation of labelled [^{3}H]-thymidine, [^{3}H]-uridine, [^{3}H]-leucine into acid insoluble material to the ratio of these isotopes in the cytoplasmic pool)/ control
For each column had three replicates and presented as mean value

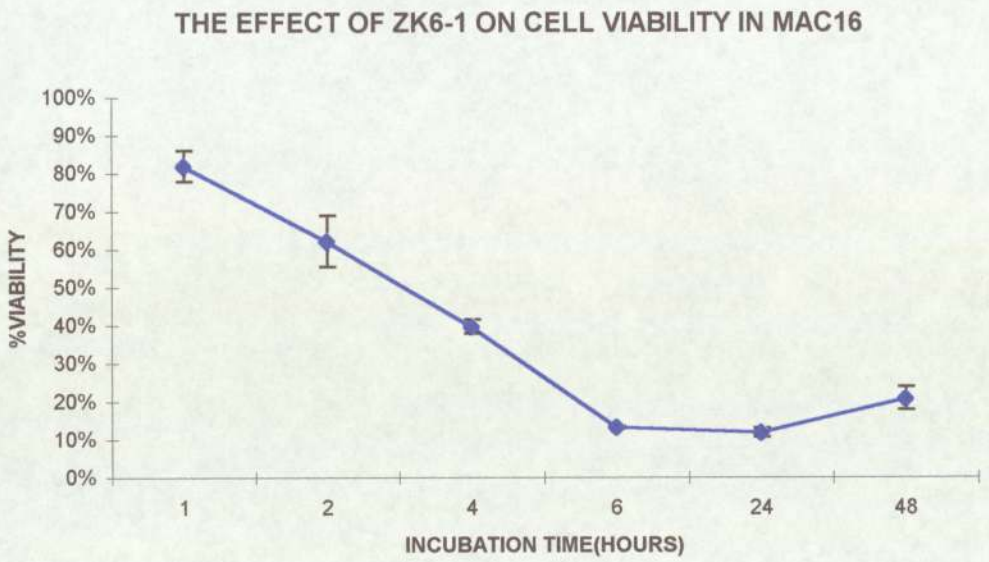
Figure 13. The evaluation of effect of ZK compounds on the cell membrane and cellular thiol groups

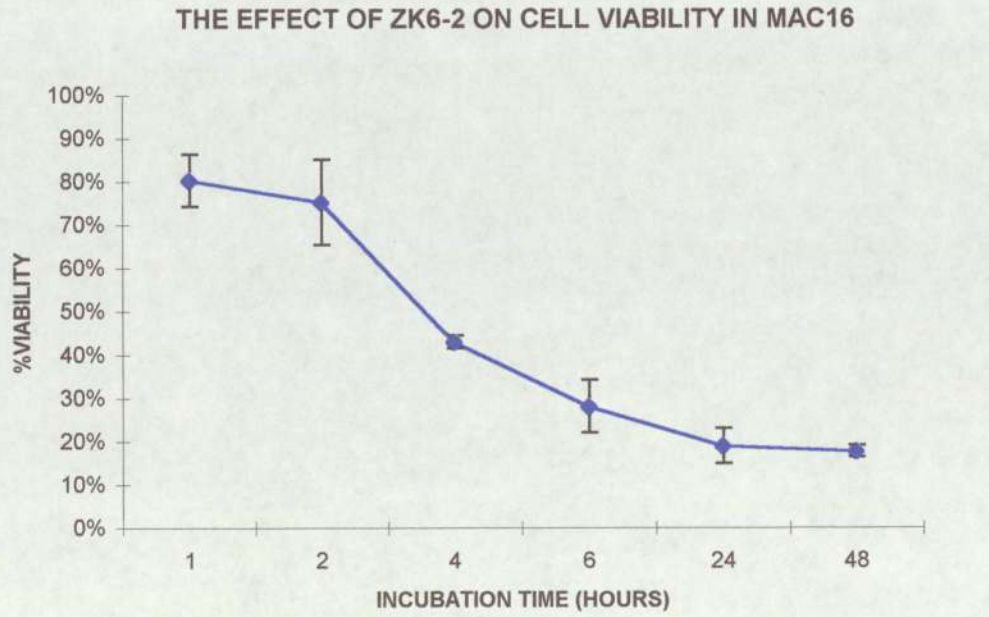




After incubation for 24 hours compounds were added.
Then after 2 hours the cells were resuspended in PBS.CPDS solution was added.
Incubation for 5min at 37□ the absorption was determined at 344nm .
10ul CPDS(1mm) solution dissolved in 2ml PBS as a blank.
For the estimation of cellular thiol groups sonicate the cell lines before adding the CPDS.
Results are given as means±s.e.m (n=3)

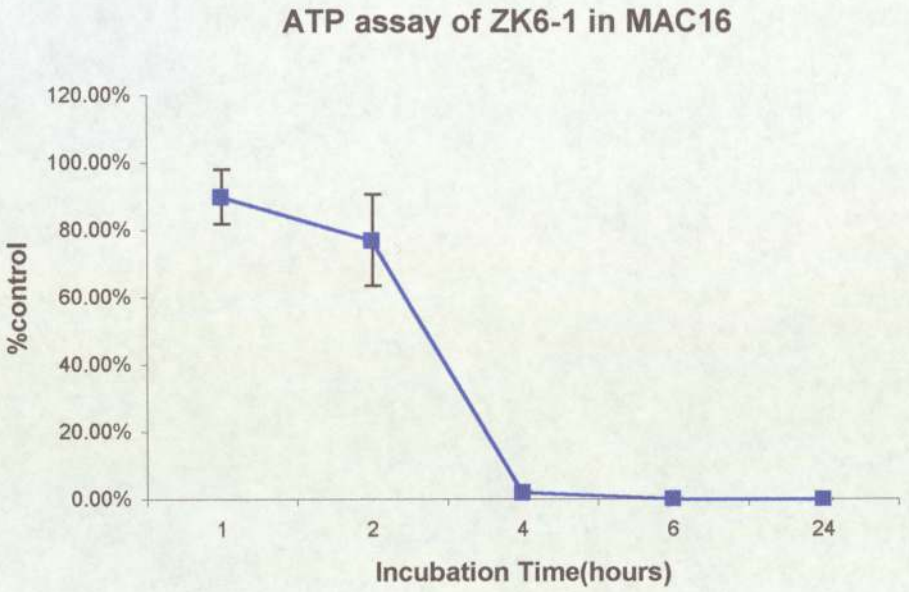
FIGURE 14. The effect of ZK6-1,ZK6-2 on cell viability in MAC16

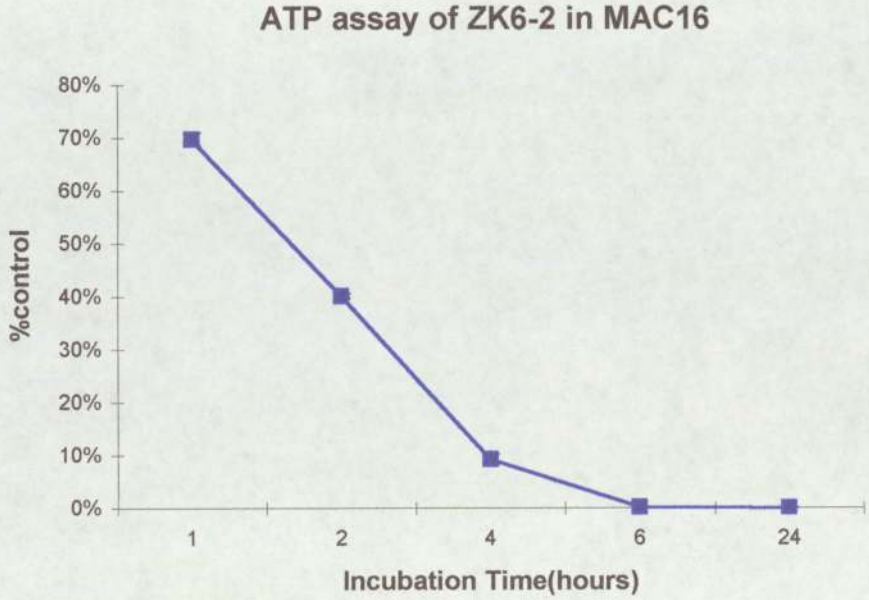




Results are given as means±s.e.m (n=4)

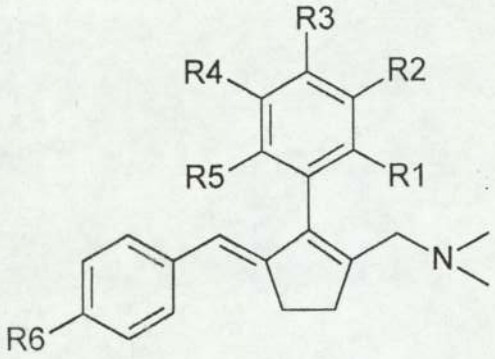
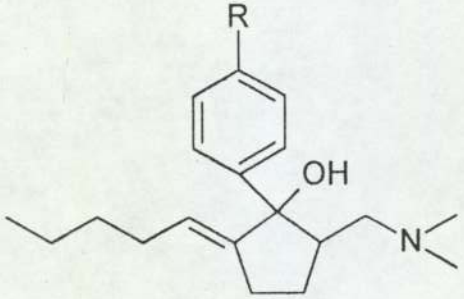
FIGURE 15. ATP assay graph of ZK6-1 and ZK6-2 in MAC16

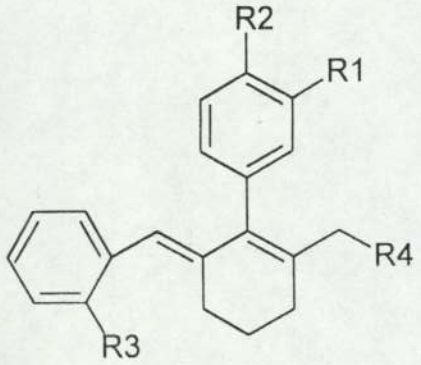
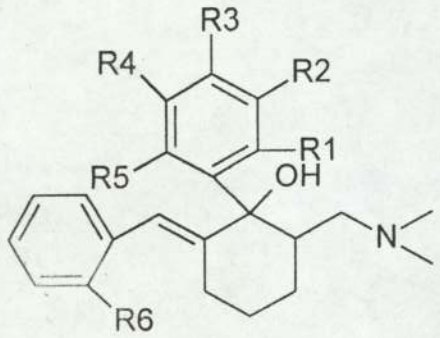


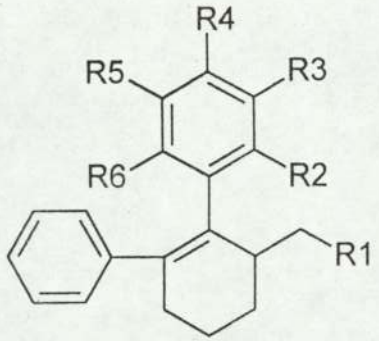


Results are presented as mean ±s.e.m (n=4)

Table 1. The IC50 values of ZK compounds in MAC13 and MAC16

STRUCTURE	SUBSTITUENT	MAC16(48hr) IC_{50}(um)	MAC16(72hr) IC_{50}(um)	MAC13(48hr) IC_{50}(um
	$R_1R_2R_3R_4R_5R_6$=H	>100	43.88	18.33
 ZK-2	$R_1R_2R_3R_4R_5$=H R_6=— OMe	32.03	27.86	6.93
	$R_1R_2R_4R_5R_6$=H R_3=— OMe	>100	51.17	29.17
	$R_1R_2R_3R_4R_5$=H R_6=—Cl	57.04	8.31	7.31
	$R_1R_3R_4$=H R_2R_5=— OMe R_6=—Cl	44.08	18.23	18.23
 ZK-1	R=H	>100	>100	54.98
	R=— OMe	>100	>100	12.33

STRUCTURE	SUBSTITUENT			MAC16(48hr) IC_{50}(um)	MAC16(72hr) IC_{50}(um)	MAC13(48hr) IC_{50}(um
 ZK-4	$R_1R_2R_3$=H		R_4=—N<	>50	0.9	0.96
	R_1R_3=H	R_2=— OMe	R_4=—N<	>100	67.88	46.71
	R_2=H	R_1R_3=— OMe	R_4=—N<	43.26	31.75	4.35
	R_2=H	R_1R_3=— OMe	R_4=—N⟨ ⟩	23.81	8.32	4.15
 ZK-3	$R_1R_2R_3R_4R_5R_6$=H			31.17	7.97	4.71
	$R_1R_2R_4R_5R_6$=H		R_3=— OMe	6.61	5.13	
	$R_1R_3R_4R_5R_6$=H		R_2=— OMe	4.76	2.67	5.56
	$R_1R_3R_4R_6$=H		R_2R_5=— OMe	>100	30.78	36.57
	$R_1R_2R_3R_4R_5$=H		R_6=— OMe	57.20	19.55	<0.1

STRUCTURE	SUBSTITUENT			MAC16(48hr) IC_{50}(um)	MAC16(72hr) IC_{50}(um)	MAC13(48hr) IC_{50}(um
 ZK-5	R_1=—N<	$R_2R_3R_4R_5R_6$=H		>100	>100	67.18
	R_1=—N<	$R_2R_3R_5R_6$=H	R_4=— OMe	>100	>100	60.27
	R_1=—N<	$R_2R_4R_5R_6$=H	R_3=— OMe	>100	>100	30.69
	R_1=—N	$R_2R_4R_5R_6$=H	R_3=— OMe	>100	>100	46.37
	R_1=—N	$R_2R_4R_5R_6$=H	R_3=— OMe	>100	92.69	37.34
	R_1=—N	R_3R_4=H	R_2R_5=— OMe	>100	>100	33.36

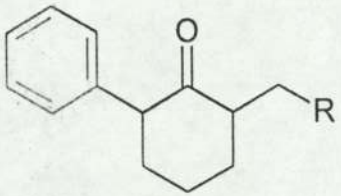
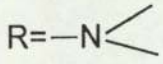
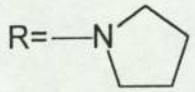
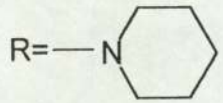
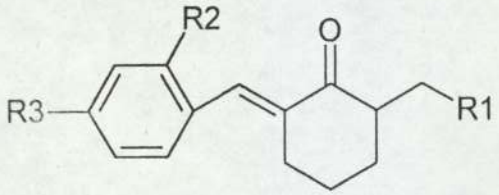
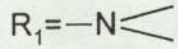
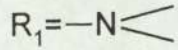
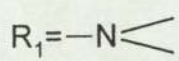
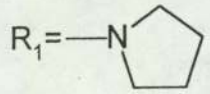
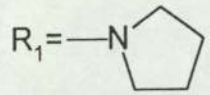
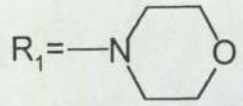
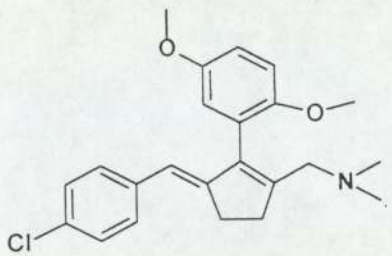
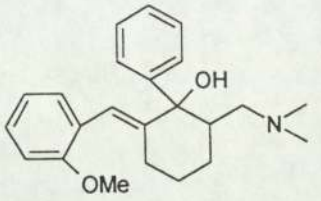
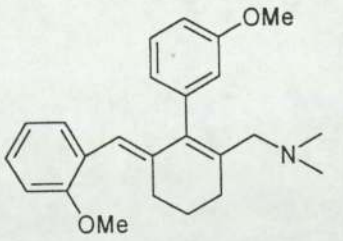
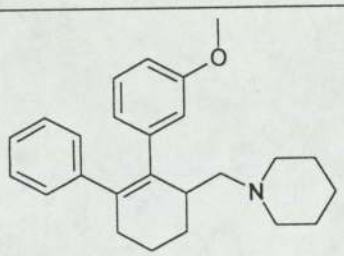
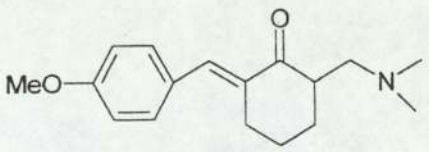
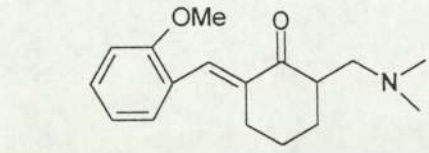
STRUCTURE	SUBSTITUENT			MAC16(48hr) IC_{50}(um)	MAC16(72hr) IC_{50}(um)	MAC13(48hr) IC_{50}(um)
 ZK-6				78.31	61.79	23.13
				89.68	>100	20.16
				>100	>100	11.46
 ZK-6		R_2=H	R_3=—OMe	9.41	7.18	6.84
		R_2=— OMe	R_3=H	7.07	6.38	3.32
		R_2=—O 	R_3=H	>100	>100	25.31
		R_2=H	R_3=—OMe	>100	>100	0.21
		R_2=— OMe	R_3=H	9.93	5.95	5.34
		R_2=— OMe	R_3=H	>100	>100	46.46

Table 2. Selectivity evaluation of ZK compounds

STRUCTURE	THE CYTOTOXICITY TO CANCER AND NORMAL CELL LINES	
 ZK2-7	MAC16 IC_{50}(μ M)	18.23
	MAC13 IC_{50}(μ M)	18.00
	C2C12 IC_{50}(μ M)	1.82
	3T3 IC_{50}(μ M)	3.74
 ZK3-5	MAC16 IC_{50}(μ M)	19.55
	MAC13 IC_{50}(μ M)	0.1
	C2C12 IC_{50}(μ M)	25.85
	3T3 IC_{50}(μ M)	23.97
 ZK4-4	MAC16 IC_{50}(μ M)	31.75
	MAC13 IC_{50}(μ M)	4.35
	C2C12 IC_{50}(μ M)	26.54
	3T3 IC_{50}(μ M)	4.22
 ZK5-5	MAC16 IC_{50}(μ M)	92.69
	MAC13 IC_{50}(μ M)	37.34
	C2C12 IC_{50}(μ M)	>50
	3T3 IC_{50}(μ M)	9.66

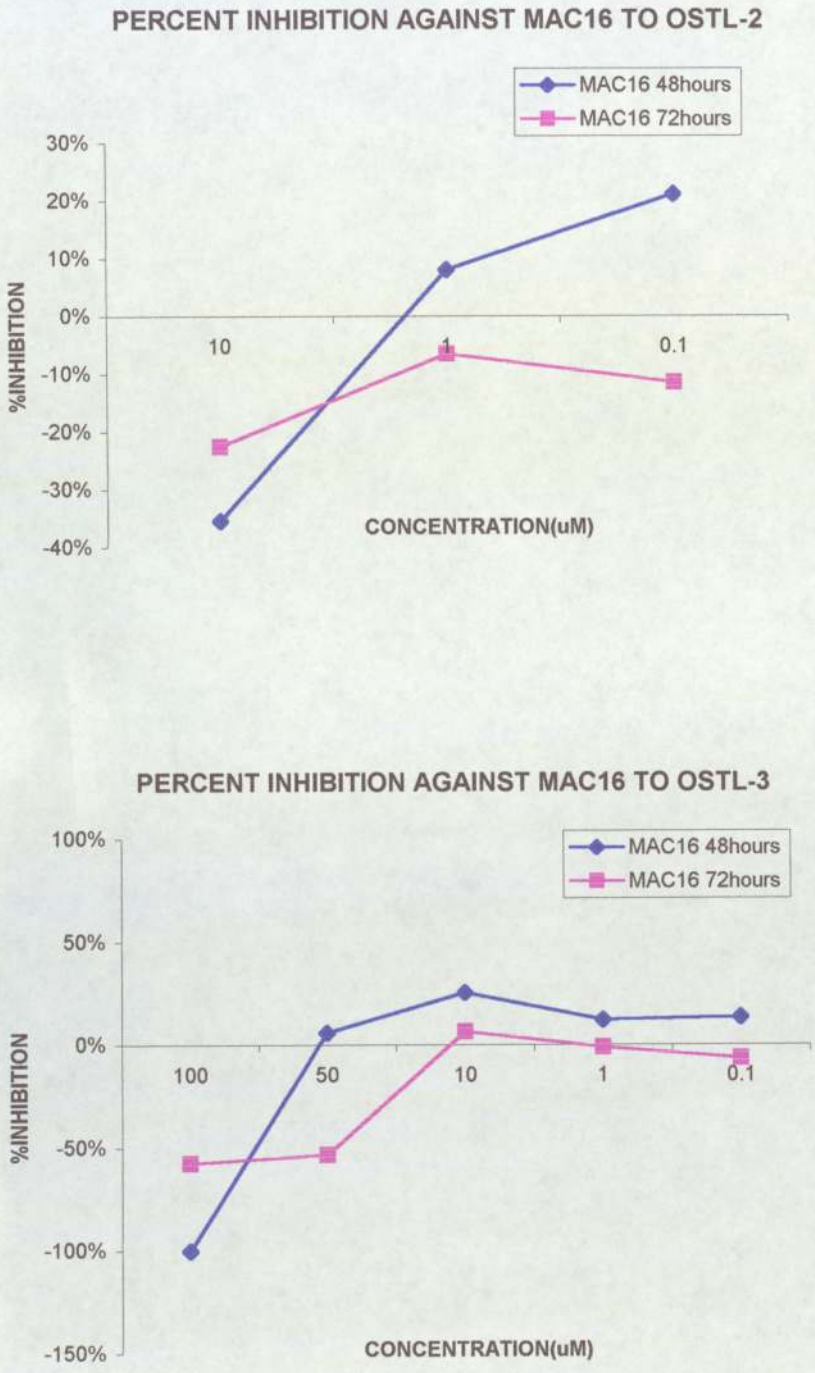
 ZK6-1	MAC16 IC_{50}(μ M)	7.18
	MAC13 IC_{50}(μ M)	6.84
	C2C12 IC_{50}(μ M)	4.28
	3T3 IC_{50}(μ M)	1.36
 ZK6-2	MAC16 IC_{50}(μ M)	6.38
	MAC13 IC_{50}(μ M)	3.32
	C2C12 IC_{50}(μ M)	2.97
	3T3 IC_{50}(μ M)	0.47

4.2 Results and discussion about oxysterol compounds

The cytotoxicity of 24 compounds was evaluated against the MAC16 cell lines after treatment for 48 and 72hours. The graph of percent inhibition to compound concentration is shown in **Figure 16**. As shown in the Figure, most of these compounds are inactive, such as ostl-11, ostl-12, ostl-14, ostl-18, ostl-26, ostl-30, ostl-204, ostl-215 and some of them were even stimulating the growth of the cell lines. Some of these compounds were synthesized as isomers to evaluate the effect of stereo structure on biological activity, such as 2, 3 and 4; 5, 6, 7, 8, 9, 10, 11 and 12; 13.and 14; 18 and 22; 214 and 215. Comparing the cytotoxicity of these isomers, there is no significant difference between them. The stereo difference does not affect the cytotoxicity against the MAC16 cell lines. They are not stereo specific.

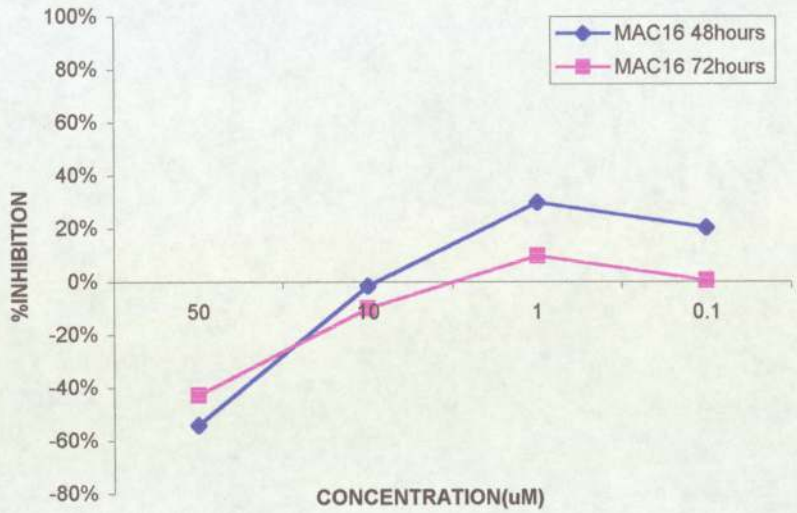
It is interesting that some of these agents stimulate cell growth. As their structures are quite similar to cholesterol. They might act as cholesterol agonists or stimulate the synthesis of cholesterol to stimulate cell growth. No detailed study was made here. The mechanism of action is unclear. In the previous test conducted in the NCI screen, Ostl-48 and ostl-56 have demonstrated a potent cytotoxicity against certain number of cancer cell lines. But the results of cytotoxicity testing against the MAC16 cells showed they have no activity suggesting that the MAC16 cells might be not sensitive to these compounds. For more comprehensive information, the compounds need to be tested on different cell lines. The mechanism effect on cell growth of oxysterols still needs to be investigated.

FIGURE 16. The cytotoxicity evaluation of oxysterols in MAC16

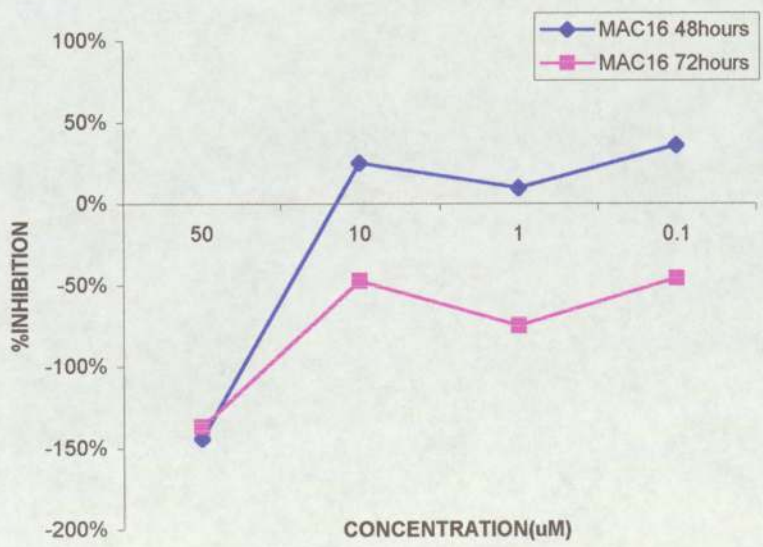


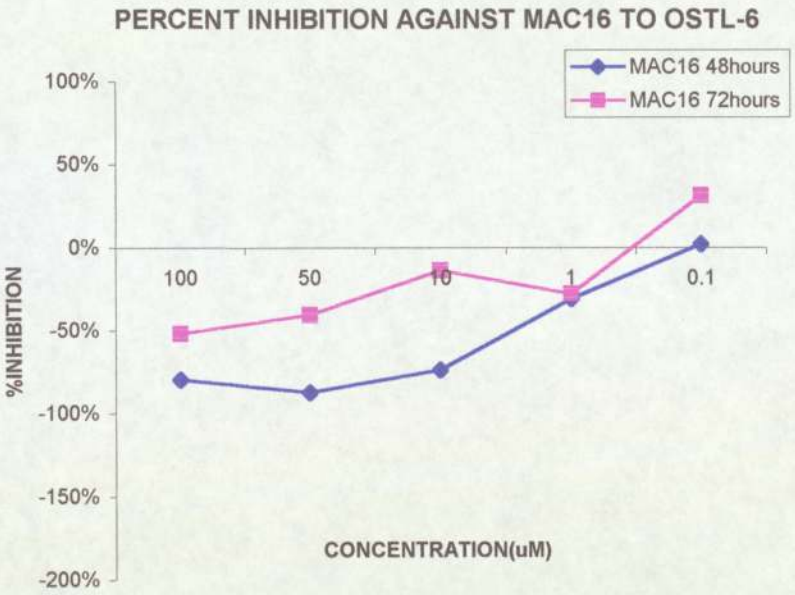
For each concentration had two replicates and presented as mean value.

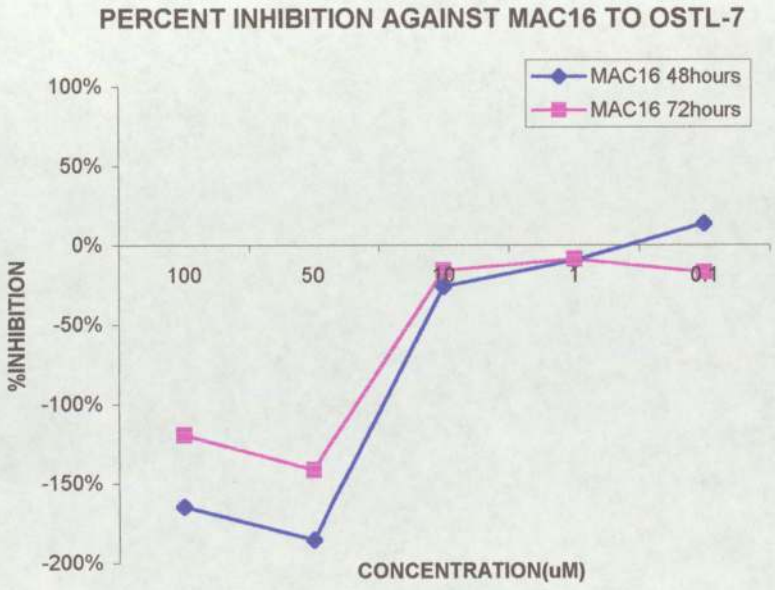
PERCENT INHIBITION AGAINST MAC16 TO OSTL-4



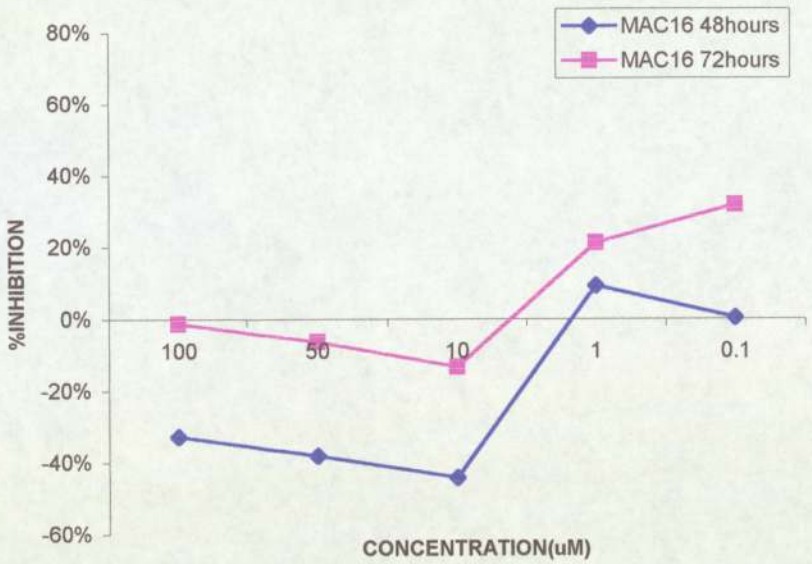
PERCENT INHIBITION AGAINST MAC16 TO OSTL-5



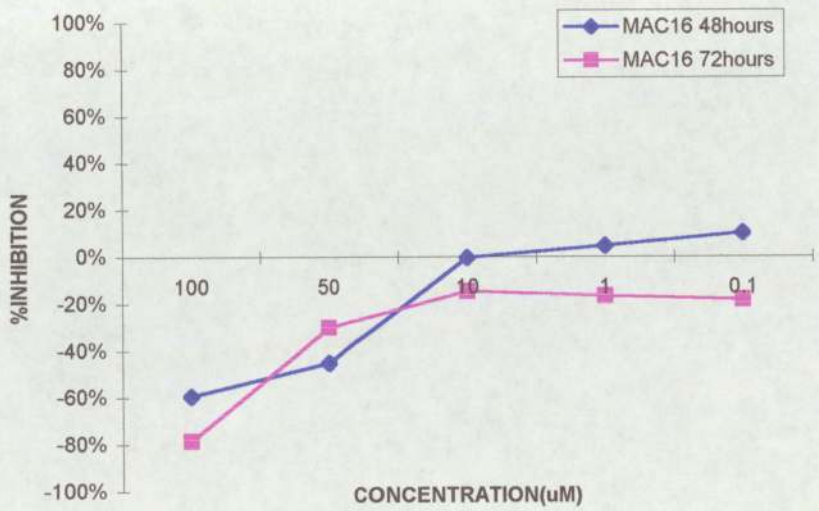

PERCENT INHIBITION AGAINST MAC16 TO OSTL-6
MAC16 48hours
MAC16 72hours
100%
50%
0%
-50%
-100%
-150%
-200%
%INHIBITION
100
50
10
1
0.1
CONCENTRATION(uM)


PERCENT INHIBITION AGAINST MAC16 TO OSTL-7
MAC16 48hours
MAC16 72hours
100%
50%
0%
-50%
-100%
-150%
-200%
%INHIBITION
100
50
10
1
0.1
CONCENTRATION(uM)

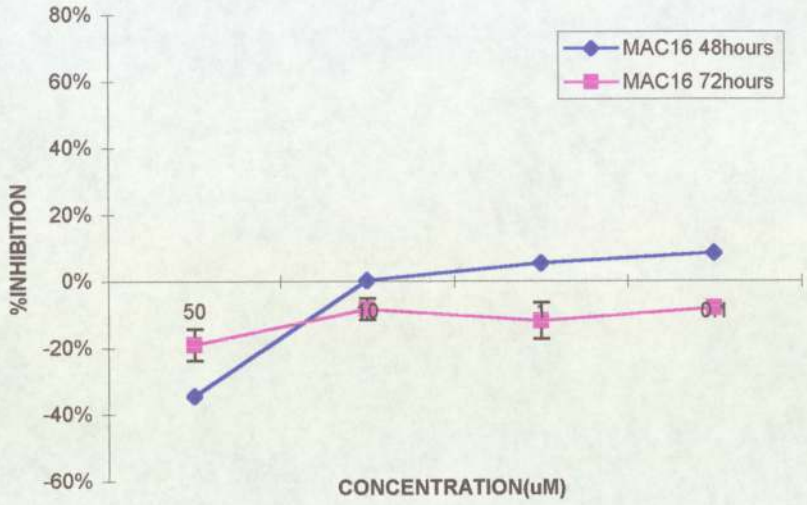
PERCENT INHIBITION AGAINST MAC16 TO OSTL-8



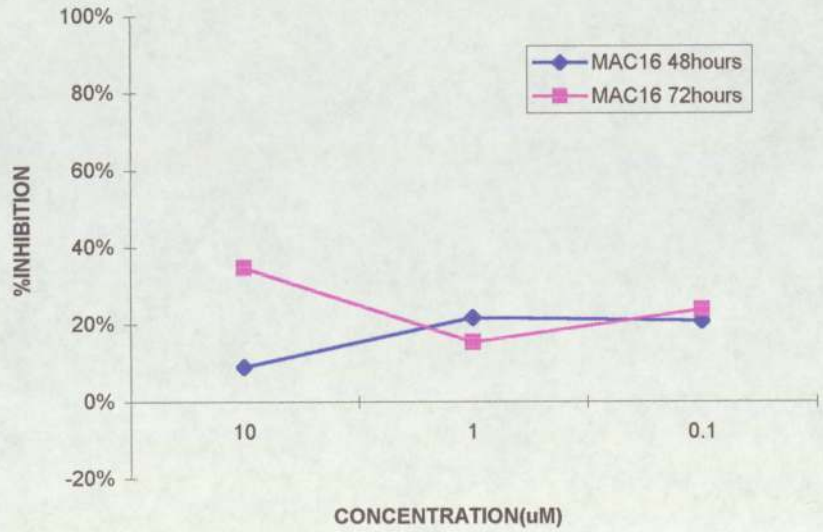
PERCENT INHIBTION AGAINST MAC16 TO OSTL-9



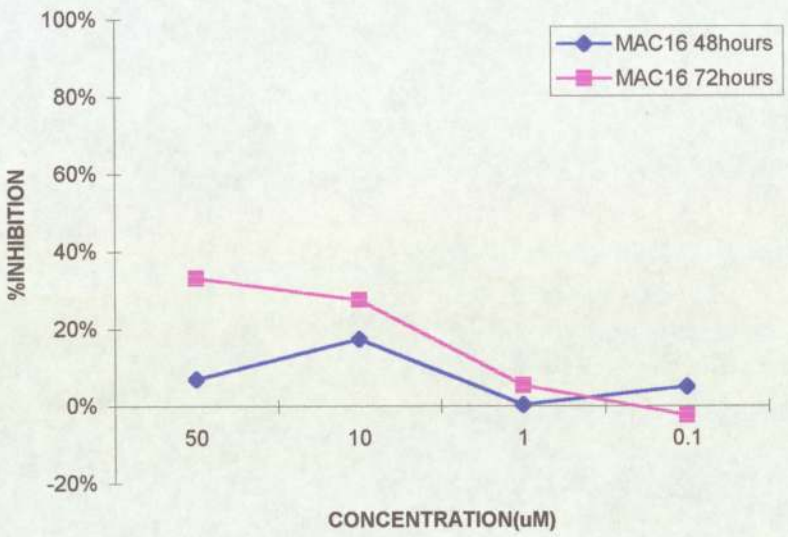
PERCENT INHIBITION AGAINST MAC16 TO OSTL-10



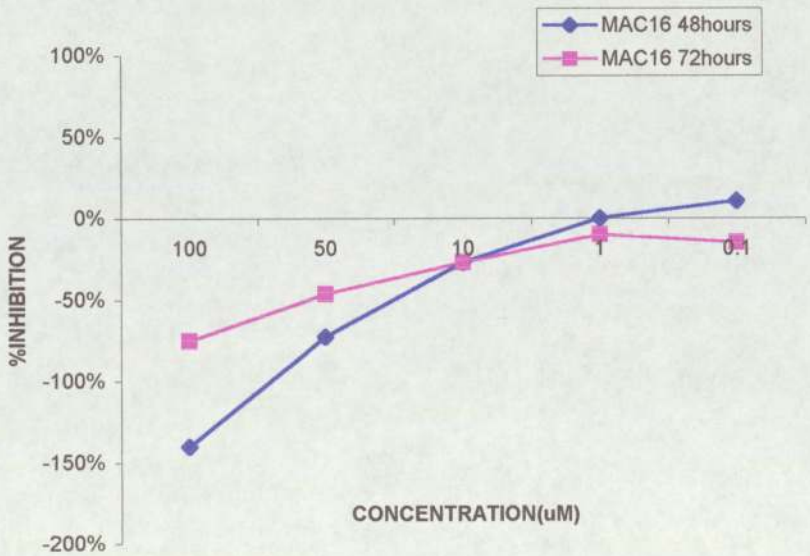
PERCENT INHIBITION AGAINST MAC16 TO OSTL-11



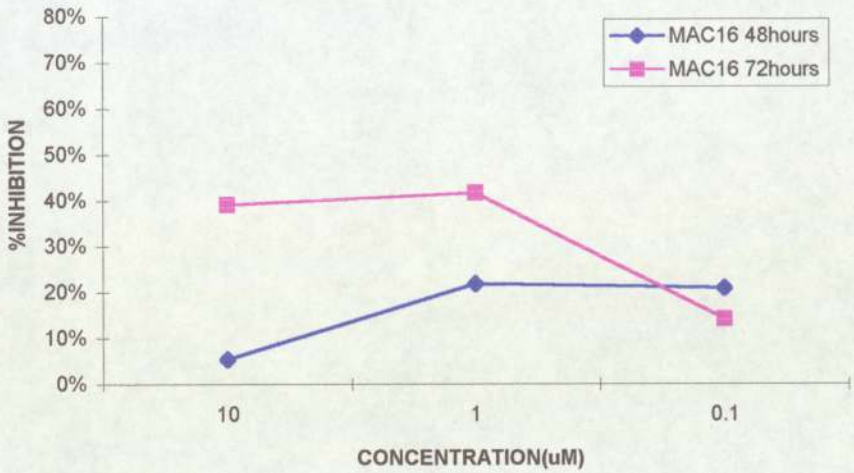
PERCENT INHIBITION AGAINST MAC16 TO OSTL-12



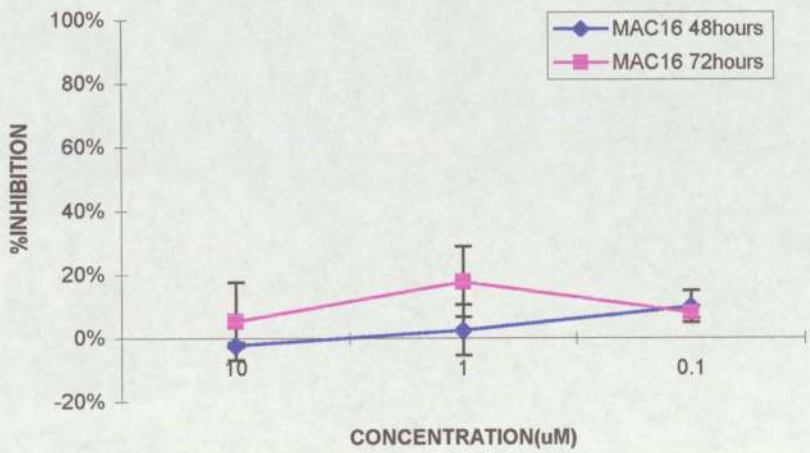
PERCENT INHIBITION AGAINST MAC16 TO OSTL-13



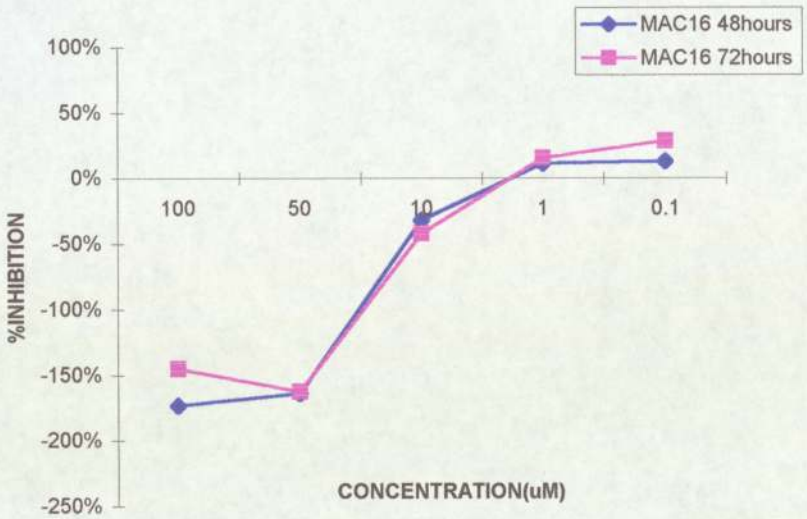
PERCENT INHIBITION AGAINST MAC16 TO OSTL-14



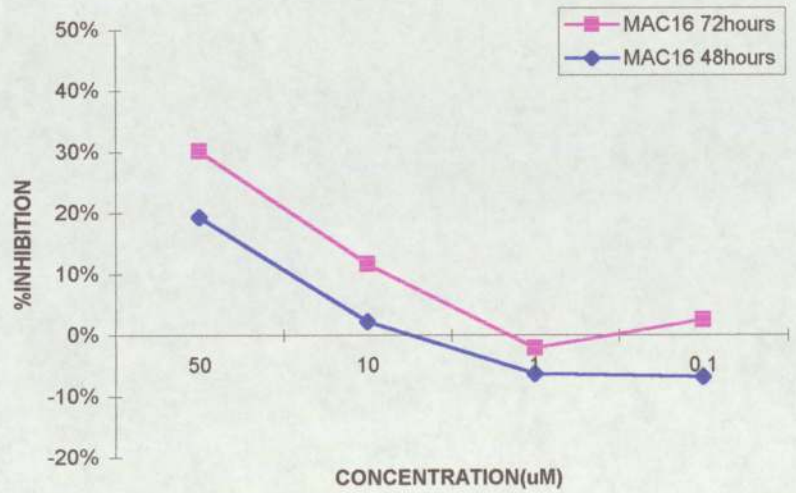
PERCENT INHIBITION AGAINST MAC16 TO OSTL-18



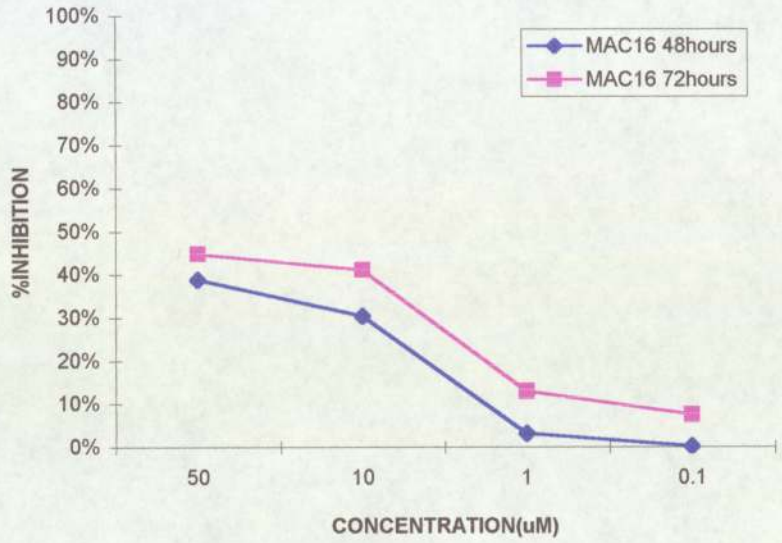
PERCENT INHIBITION AGAINST MAC16 TO OSTL-22



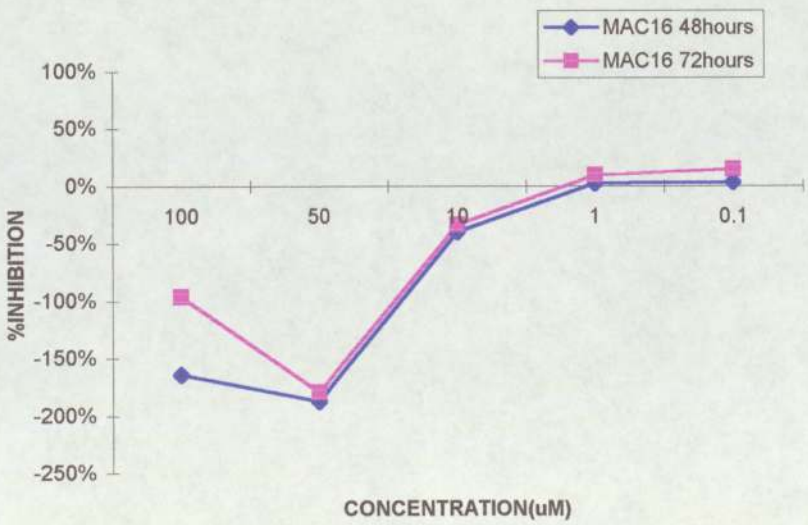
PERCENT INHIBITION AGAINST MAC16 TO OSTL-26



PERCENT INHIBITION AGAINST MAC16 TO OSTL-30



PERCENT INHIBITION AGAINST MAC16 TO OSTL-34



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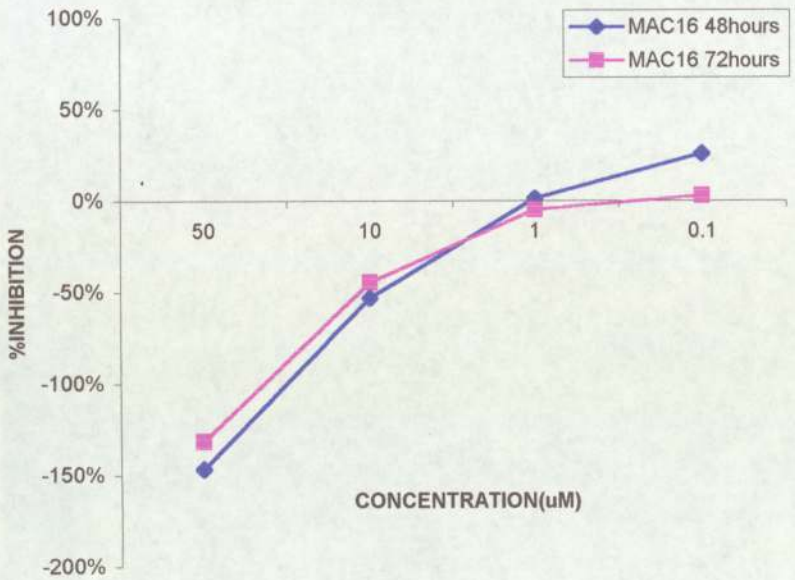
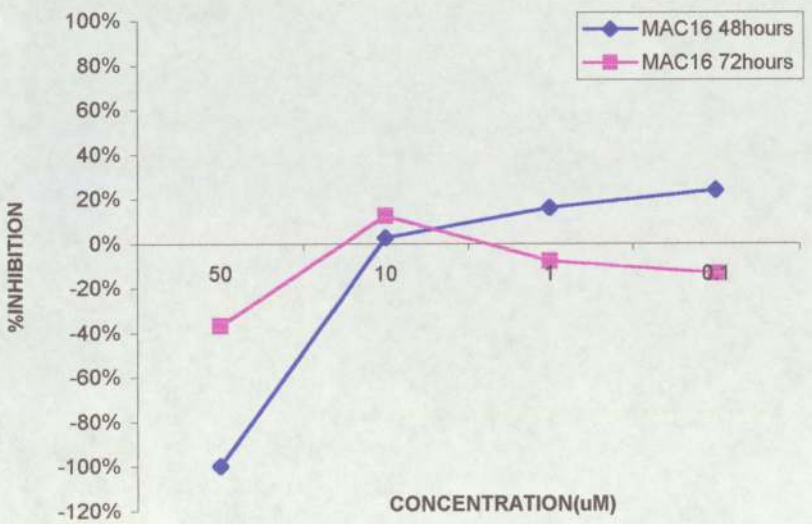


PERCENT INHIBITION AGAINST MAC16 TO OSTL-56



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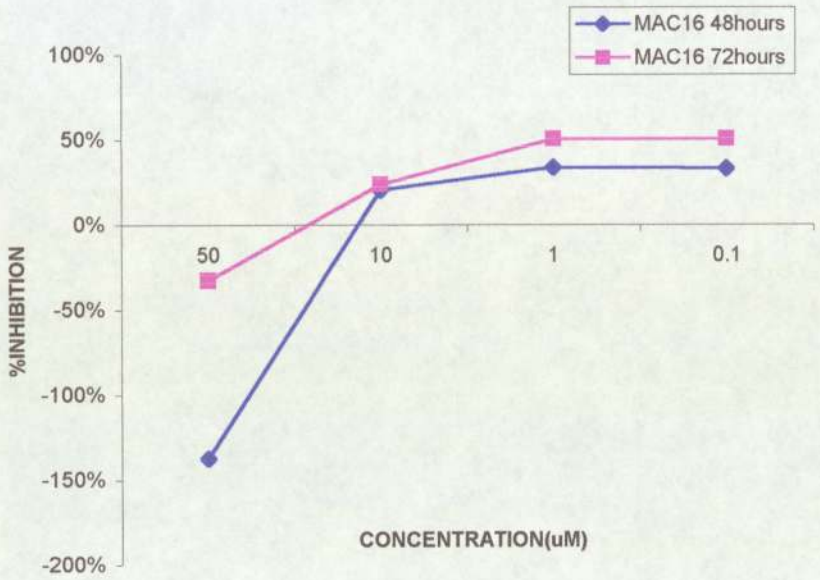
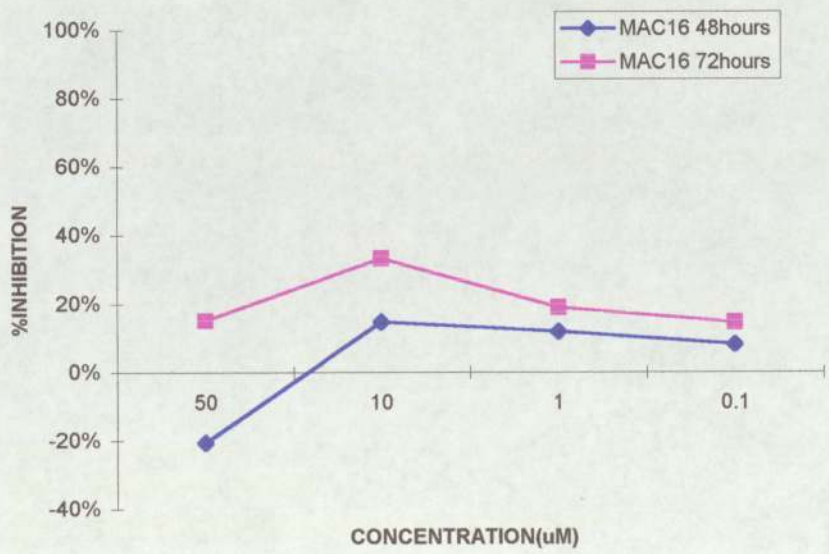


PERCENT INHIBITION AGAINST MAC16 TO OSTL-204



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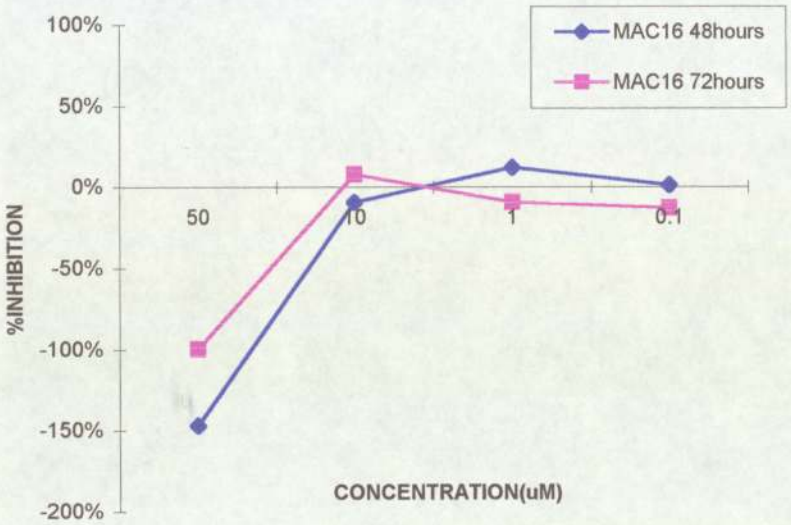
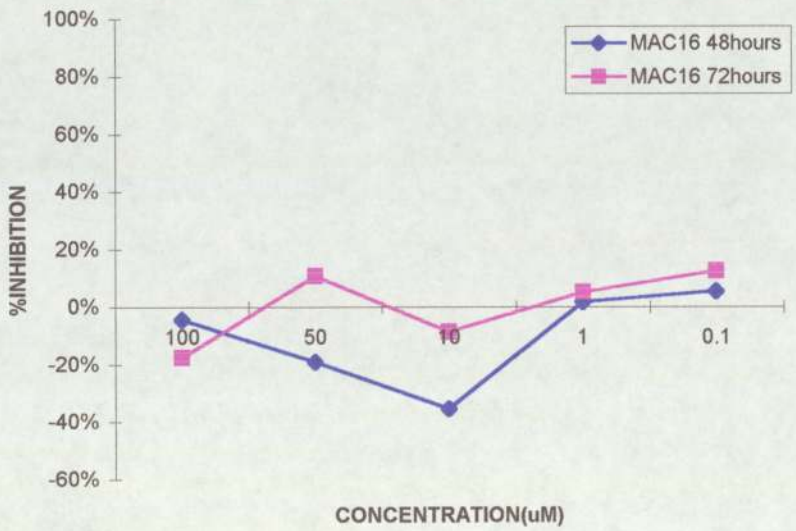


PERCENT INHIBITION AGAINST MAC16 TO OSTL-215



4.3 Results and discussion of LR6M and related compounds

The stability test results are shown in **Figure 17**. The peak that the retention time is about 3.8min is PBS solvent peak. The peak that the retention time is about 4.3min is LR6M peak, and the peak that the retention time is about 5.7min is LR6H peak. After one, two and four weeks the retention time and the ratio of intensity of the peaks of LR6M and LR6H did not change compared with zero day samples. So LR6M and LR6H were stable at the four conditions- room temperature (dark), room temperature (in light), 4℃(dark) and -20℃ (dark).

LR6M, LR6H and OXOFORM-CF were evaluated for cytotoxicity in the MAC13 and MAC16 cell lines. As shown in **Figure 18**, LR6H has high cytotoxicity in the MAC13 cell lines and low cytotoxicity in the MAC16 cell lines. LR6M and OXOFORM-CF have no cytotoxicity in both MAC13 and MAC16 cell lines. LR6M has been evaluated and has been found to exhibit antitumour activity *in vivo*. Comparing the different activity *in vitro* and *in vivo* it is suggested that LR6M might act as a prodrug when it comes into the body. It is metabolised to the compound, which has activity.

As shown in **Figure 7**, LR6M was synthesized as a target compound and LR6H was a prodrug. Further isolation and purification of LR6M and LR6H was done by using HPLC. Different liquid phases and solid phases were combined to get the premier isolation conditions. NMF was the main reactant and it might exist as the main impurity in the reaction products. First of all, the task was to identify whether NMF existed in these products. Two different HPLC conditions were used:

1. Solid phase: C2 column (5μmx250mm)
   Liquid phase: Methanol: H2O = 1: 9
   Compound solvent: distilled water
2. Solid phase: C2 column (5μmx250mm)
   Liquid phase: 50% acetonitrile
   Compound solvent: 50% acetonitrile

For condition 1, comparing the HPLC graph of NMF, LR6M and LR6H, the time of the strongest peak is the same as the peak of NMF (about 4min) in LR6M and LR6H graph. (**Figure 19**) Except this strongest peak and solvent peak in LR6M and LR6H graph they still have a small peak for each one. In LR6H the small peak time is

5.408min and in LR6M it is in 4.803min. For condition 2, it was the same as condition 1—the time of the strongest peak and the peak of NMF were the same (**Figure 20**). Further comparison was done, comparing the elution profile of a mixture of NMF and LR6M (shown in **Figure 21**) with LR6M. There was no significant difference between them. In case the reason is due to incomplete isolation we also tried several conditions and repeated them several times, as following:

C18, Methanol: H_2O=10:9;
C18, Methanol: H_2O=25:75;
C18, CH_3CN: H_2O=75:25
C18, Methanol: CH_3CN: H_2O=1:1:1
C18, Methanol: CH_3CN: H_2O=2:1:7
C18, Methanol: H_2O=30:70;
C18, CH_3CN H_2O=10:90;
C18, 100% CH_3CN;
C18, Methanol: CH_3CN: H_2O=3:2:5

There was no new peak coming out. So we cannot get a new component from these compounds. These results confirmed that both LR6M and LR6H do have some component the peak time of which is the same as for NMF. According to these results the following hypotheses were formulated:

1. The component is LR6M or LR6H. For NMF, LR6M and LR6H the diffusion rate is the same, so they come out at the same time. The peaks of these compounds are overlapped.

2. LR6M and LR6H are not stable in silica gel. They broke up to NMF, so actually there were no LR6M and LR6H, the peak coming out being NMF. In each elution profile there still remained a small peak, which might be LR6M and LR6H undecomposed. For further identification of the small peaks, collected the peak2 of LR6H. Concentrate it by freeze-drying or vacuum pump. Test the cytotoxicity of the collected liquid in MAC13 and MAC16 cell lines. The result of cytotoxicity test was shown in **Figure 22.** That it has no cytotoxicity suggest that it is not LR6H but impurity. All this suggests that they might break up before they come out.

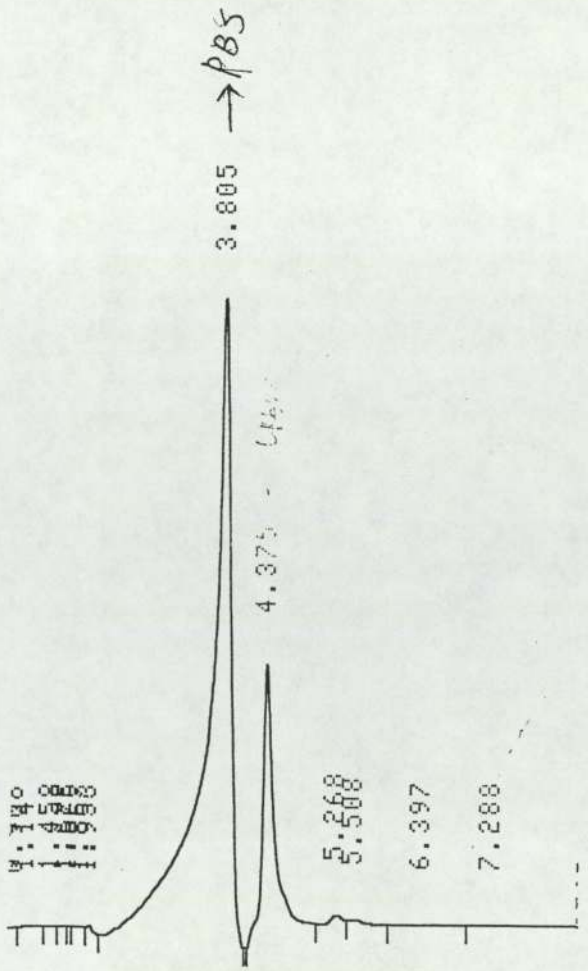
3. These compounds remained in the column, they did not come out. But it has a drawback that the peak of NMF cannot be so strong as an impurity.

For all these possibilities, the HPLC is not suitable to purify these compounds. We should try other methods from chemistry routes and others. And if hypotheses 2 is true, they do decompose in column that the results of stability test is unreliable.

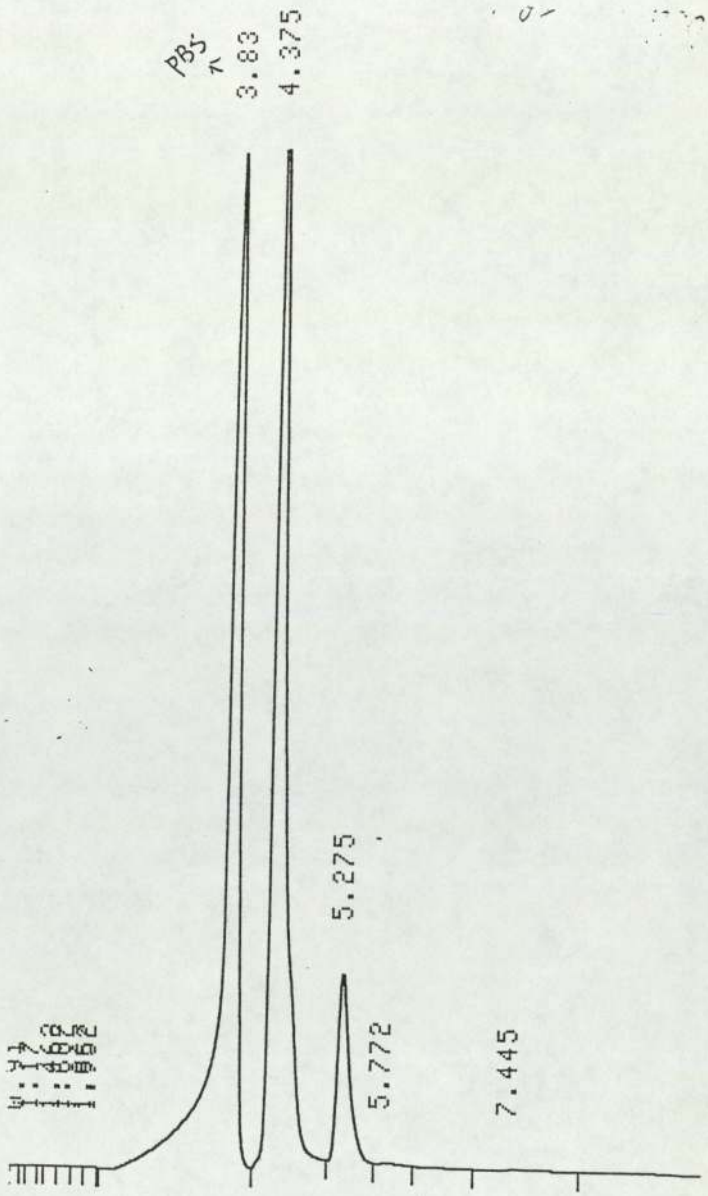
Figure 17. Stability test of LR6M and LR6H

(zero day)

Solid Phase:	**C2 column (5µmx250mm)**
Liquid Phase:	**Methanol : Water = 10:90**
Flow Rate:	**1ml/min**
Solution:	**PBS**
Concentration:	**LR6M- 10^{-4}mol/ml**
	LR6H 500ug/ml



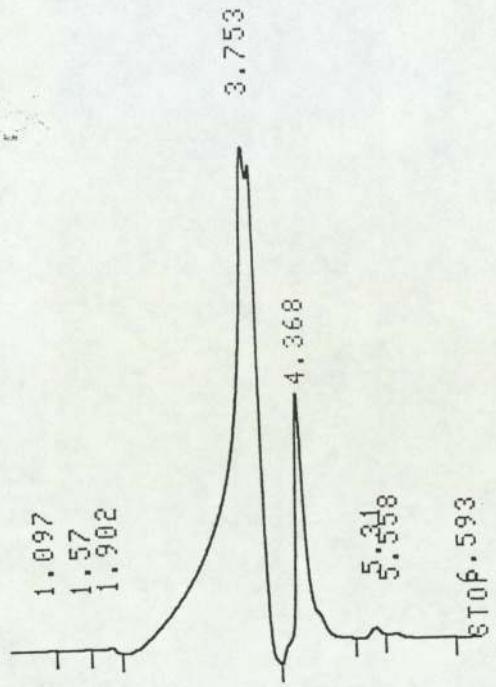
LR6M



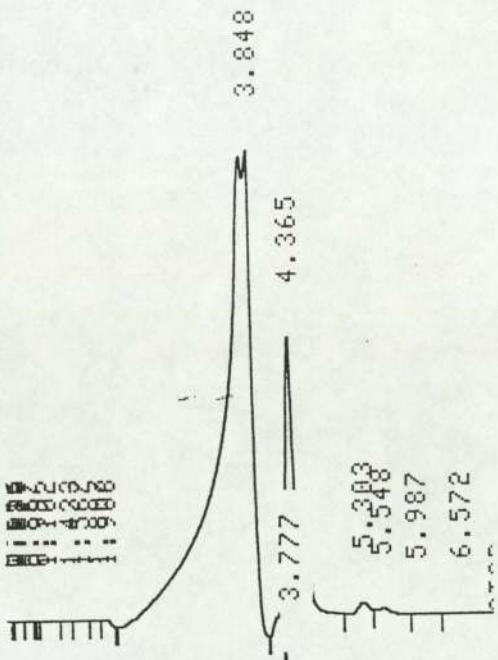
LR6H

Stability test of LR6M

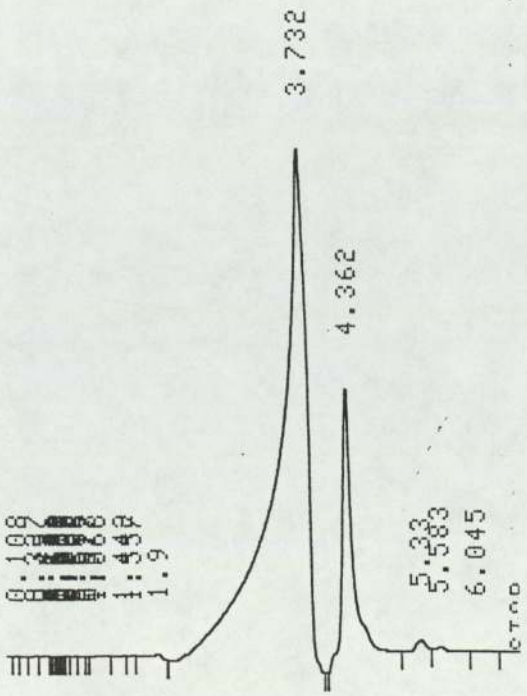
(after one week)



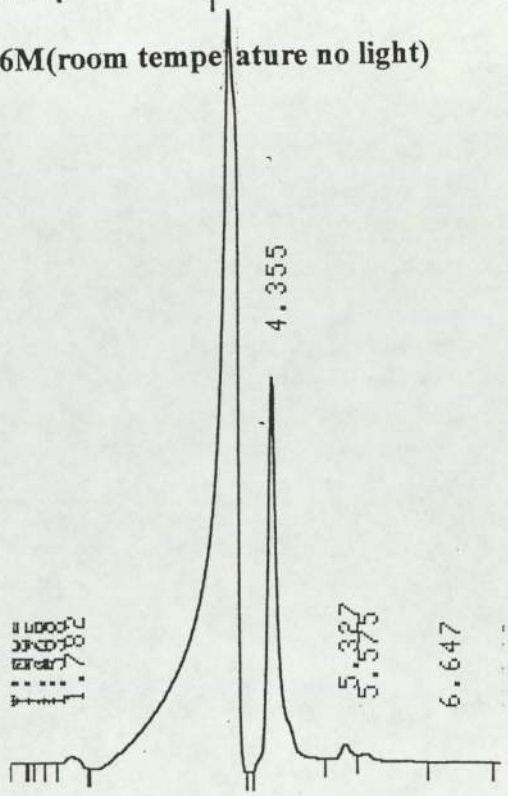
LR6M(room temperature + light)



LR6M(room temperature no light)



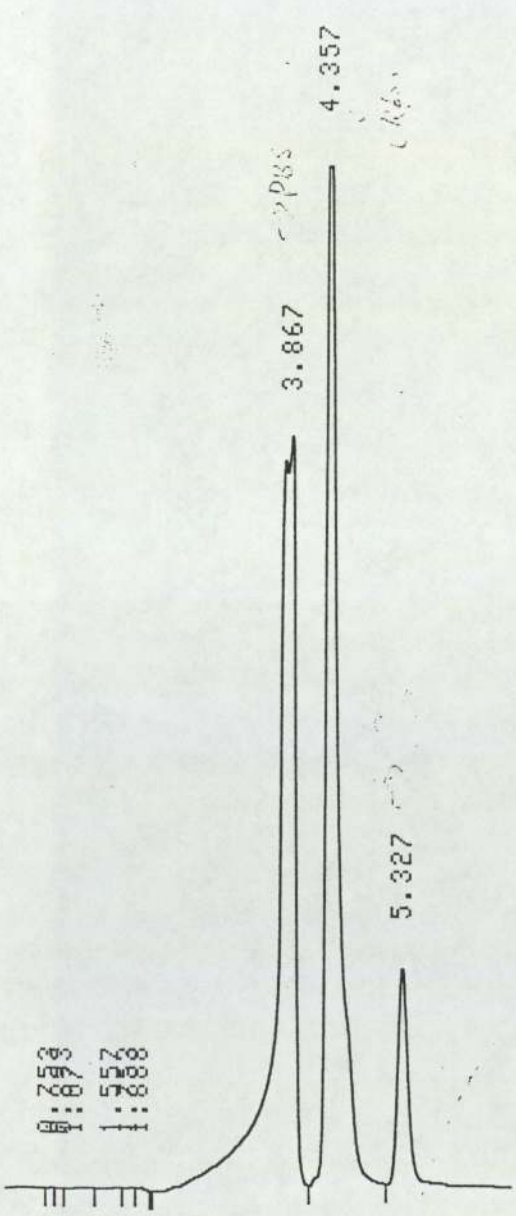
LR6M (4℃ no light)



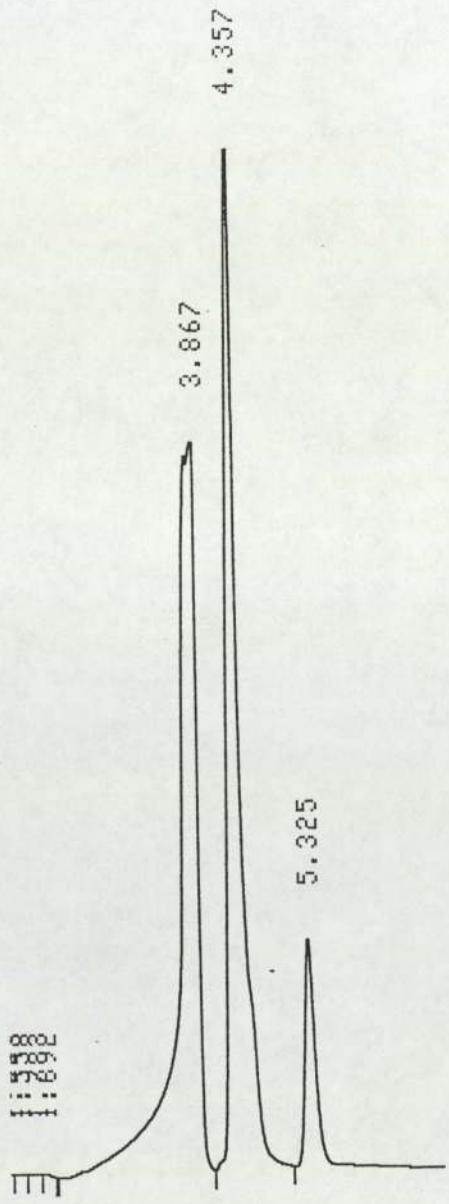
LR6M (-20℃ no light)

Stability test of LR6H

(after one week)



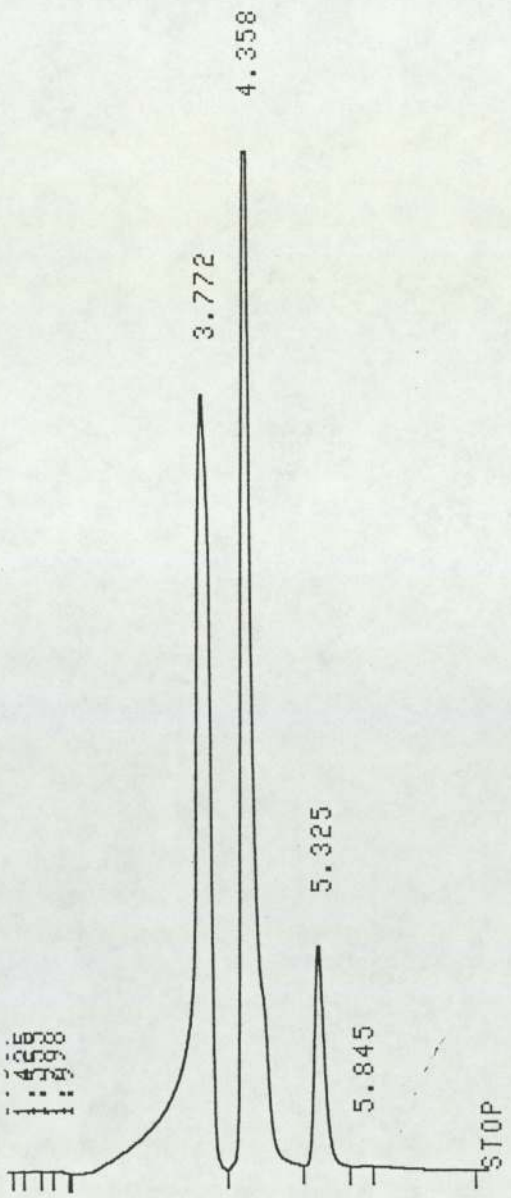
LR6H (room temperature + light)



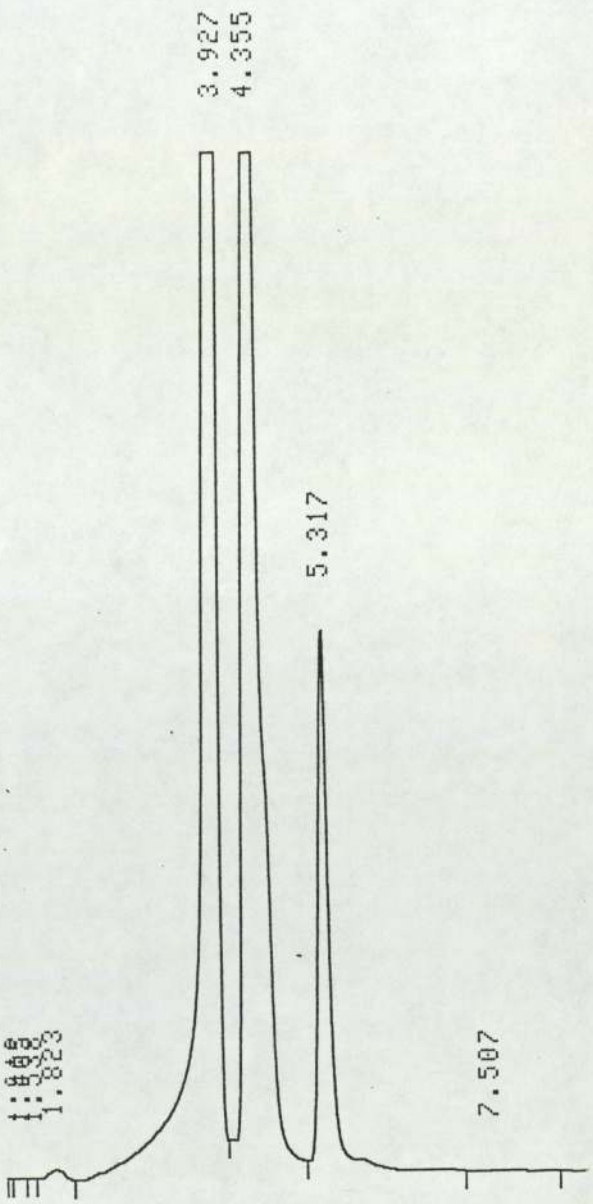
LR6H (room temperature no light)

Stability test of LR6H

(after one week)



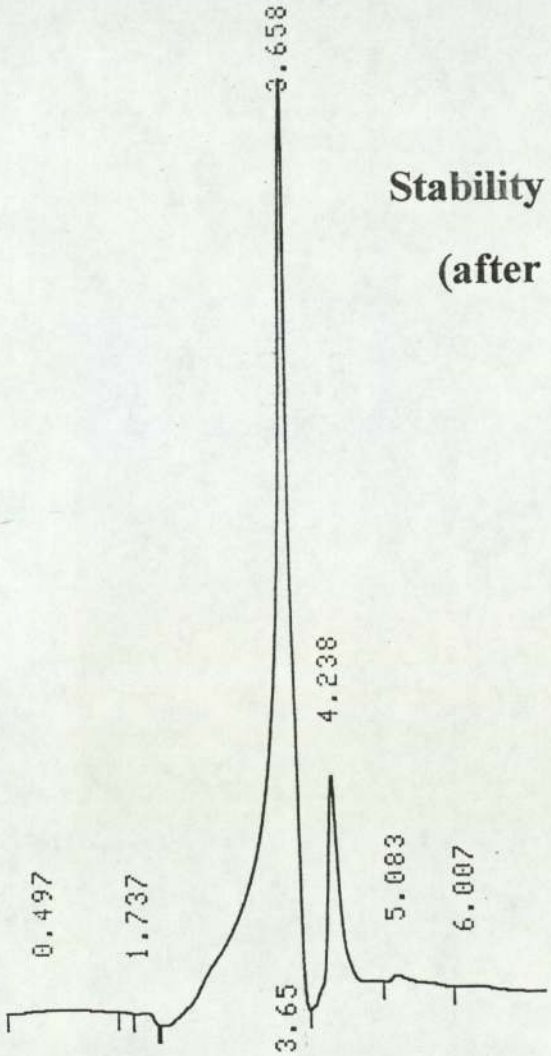
LR6H (4℃ no light)



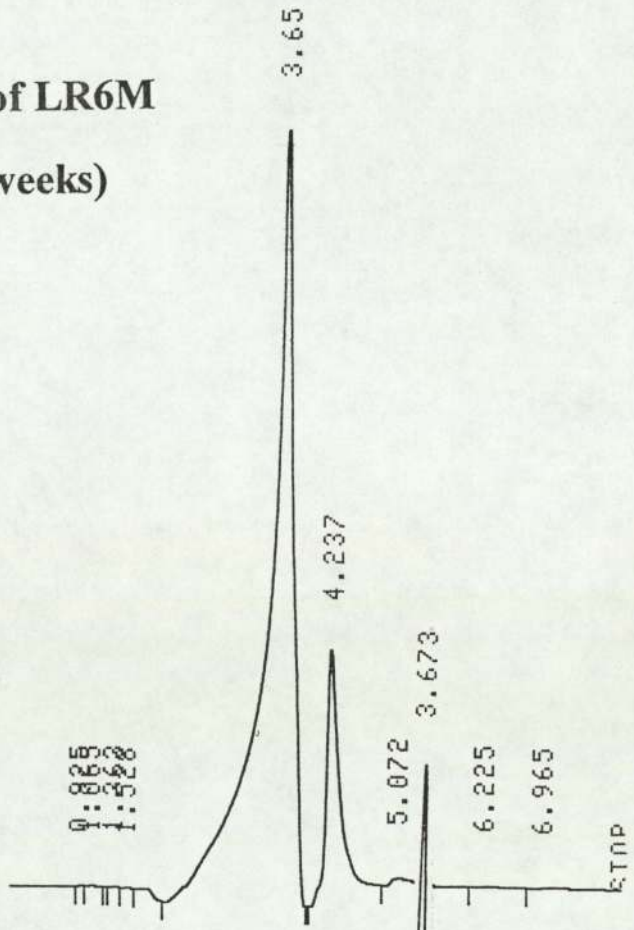
LR6H (-20℃ no light)

Stability test of LR6M

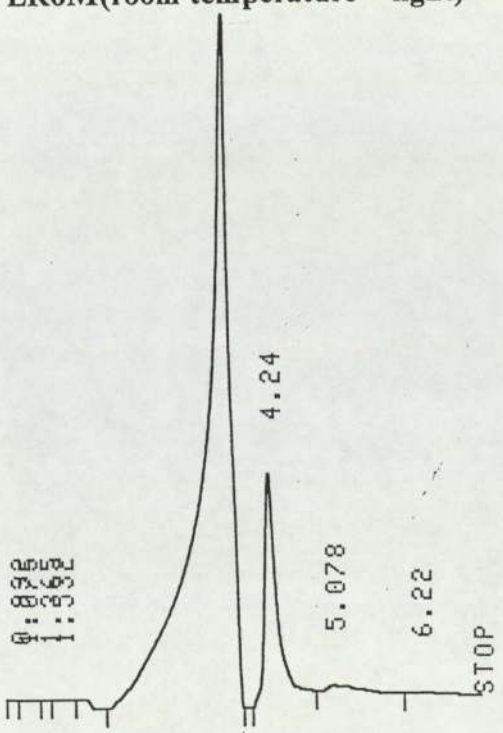
(after two weeks)



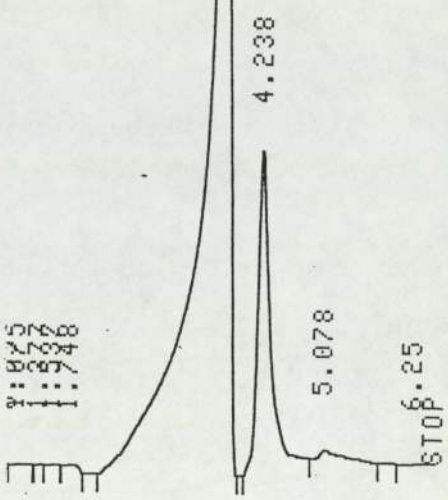
LR6M(room temperature + light)



LR6M(room temperature n light)



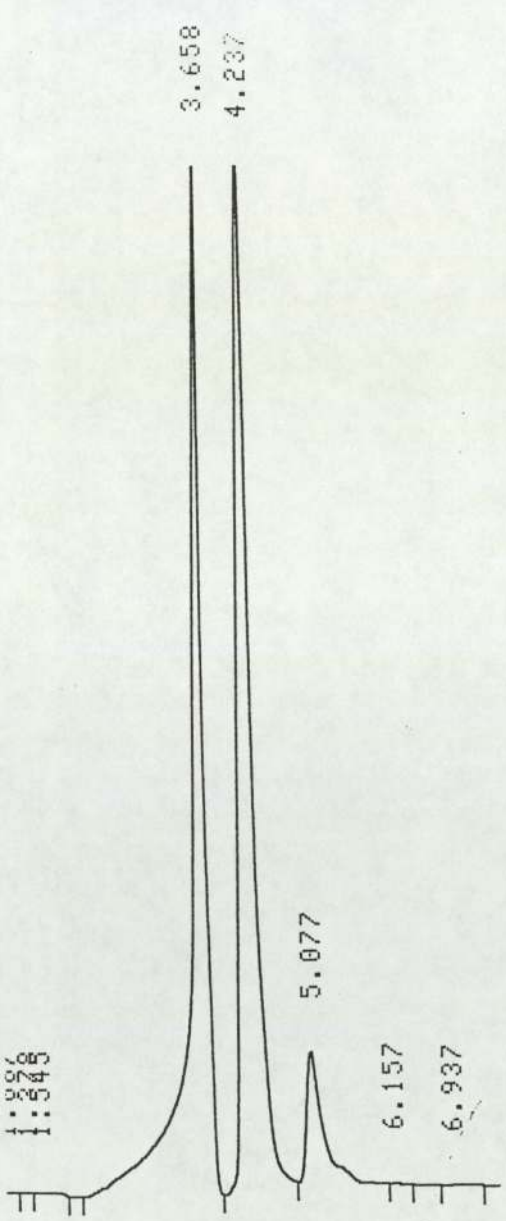
LR6M (4℃ no light)



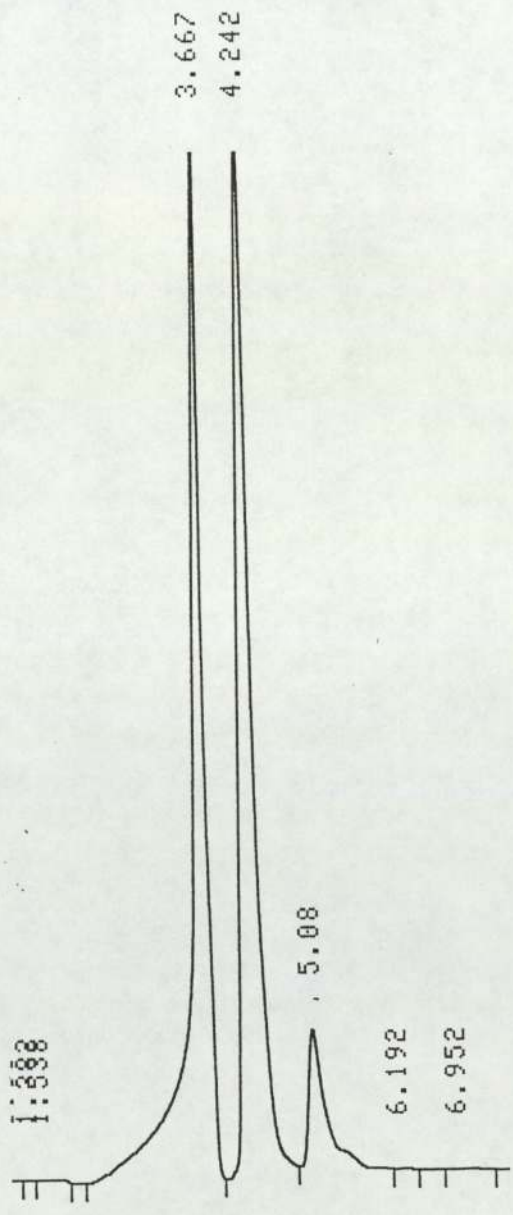
LR6M (-20℃ no light)

Stability test of LR6H

(after two weeks)



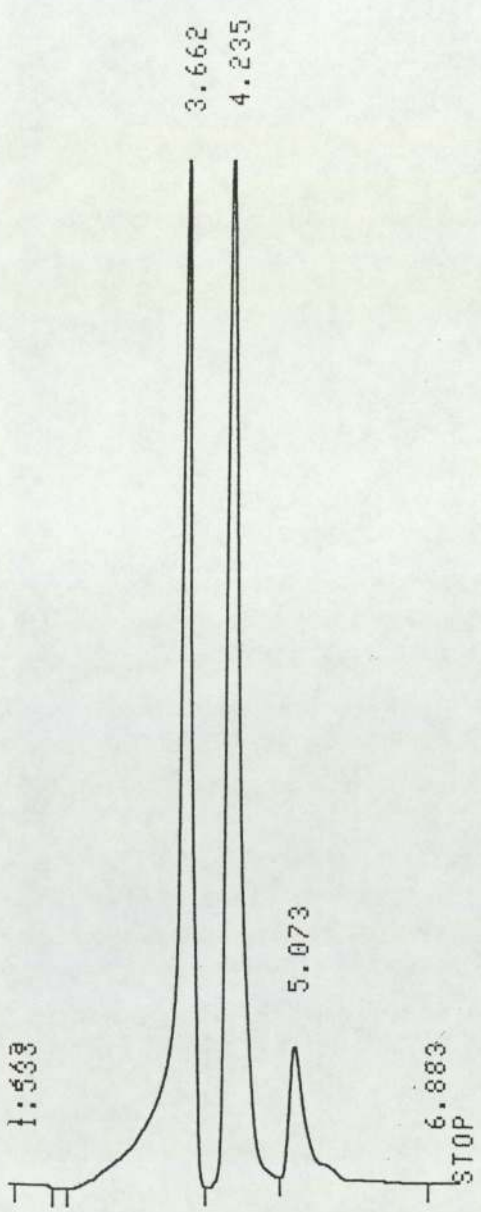
LR6H (room temperature + light)



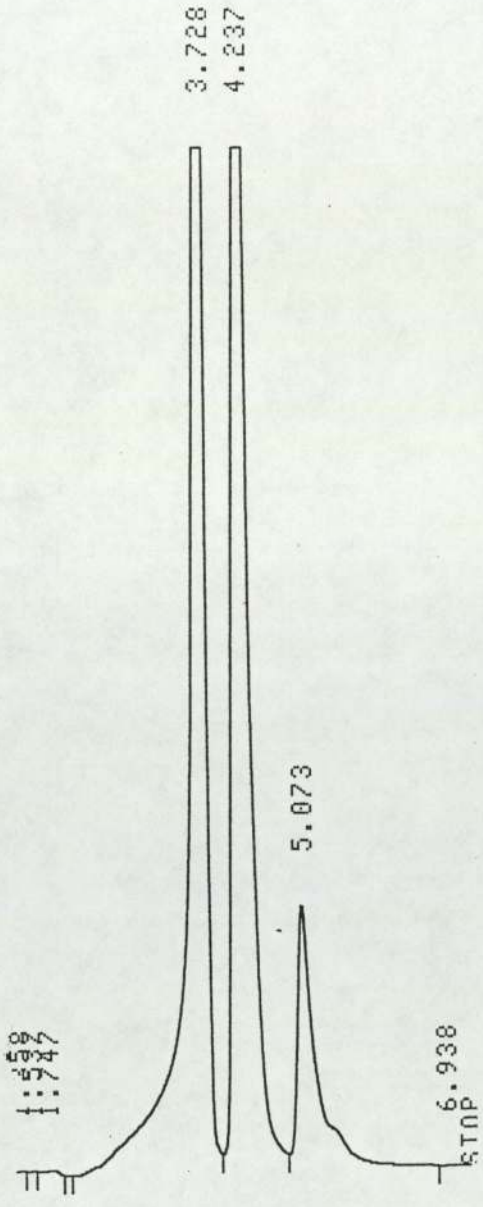
LR6H (room temperature no light)

Stability test of LR6H

(after two weeks)



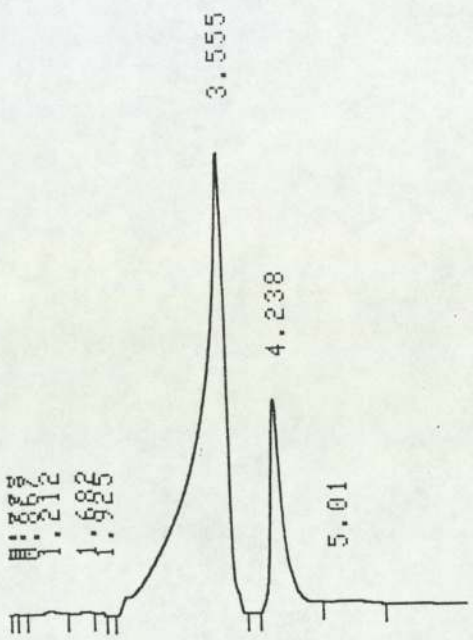
LR6H (4℃ no light)



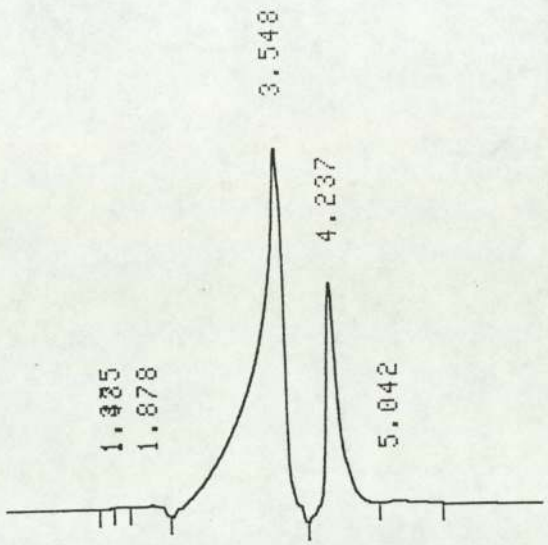
LR6H (-20℃ no light)

Stability test of LR6M

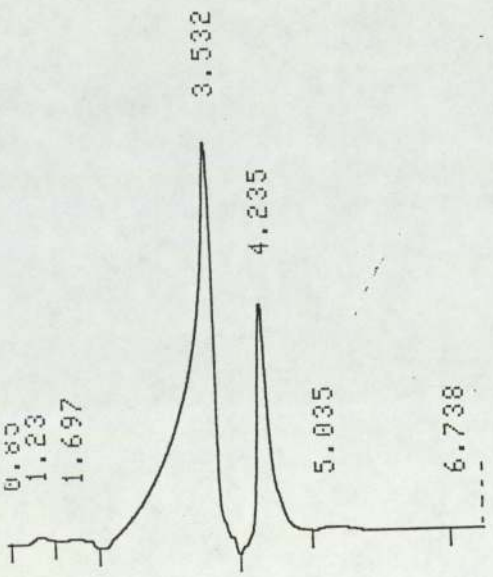
(after four weeks)



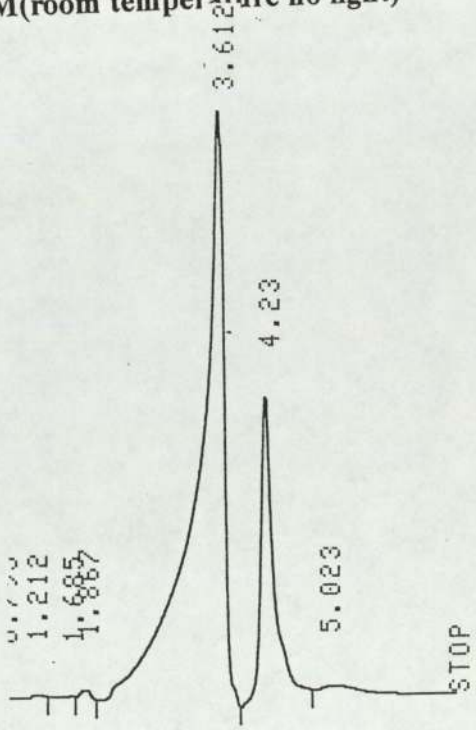
LR6M(room temperature + light)



LR6M(room temperature no light)



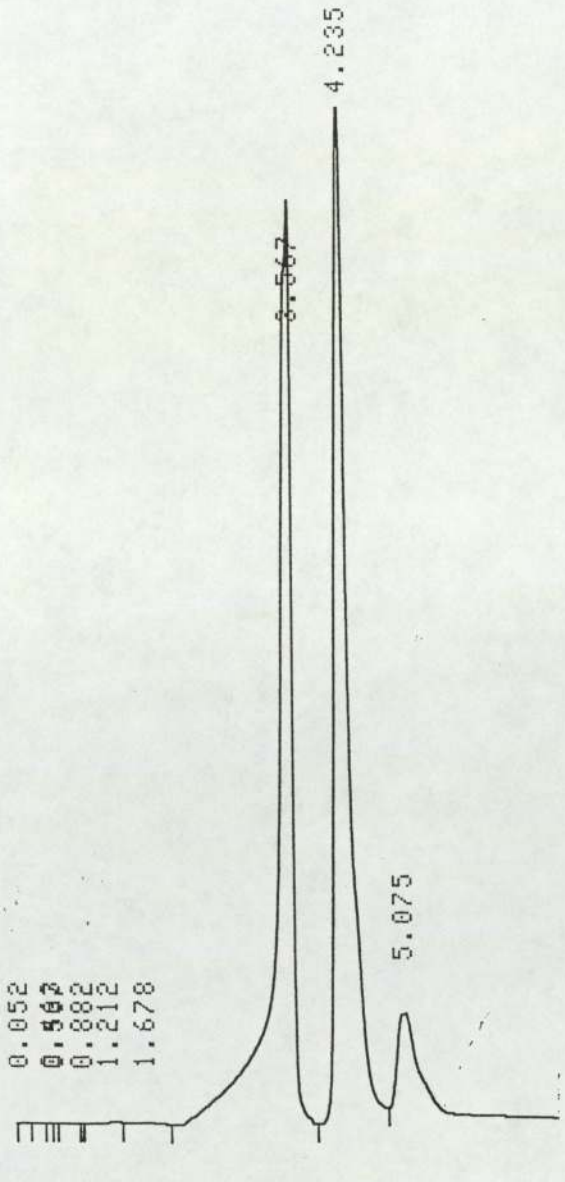
LR6M (4℃ no light)



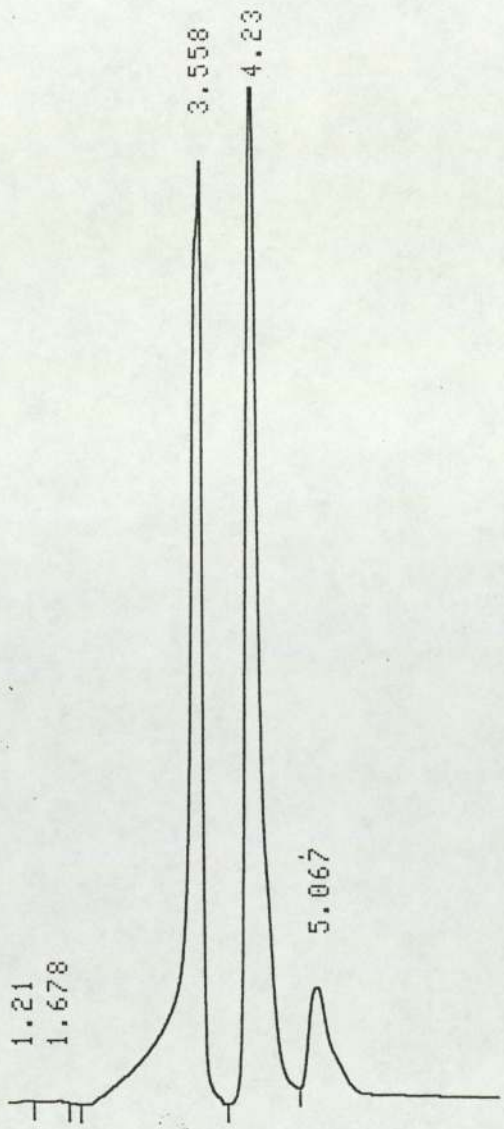
LR6M (-20℃ no light)

Stability test of LR6H

(after four weeks)



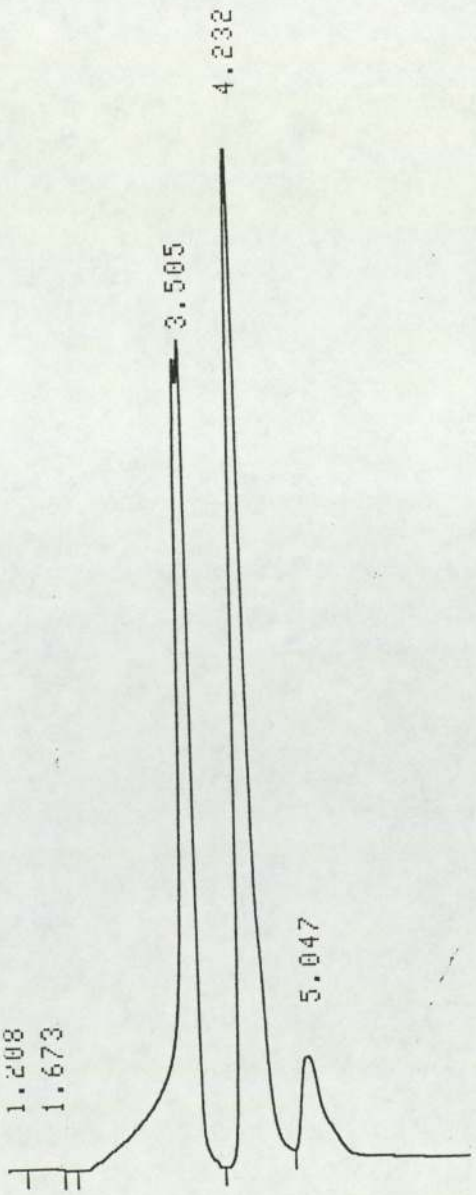
LR6H (room temperature + light)



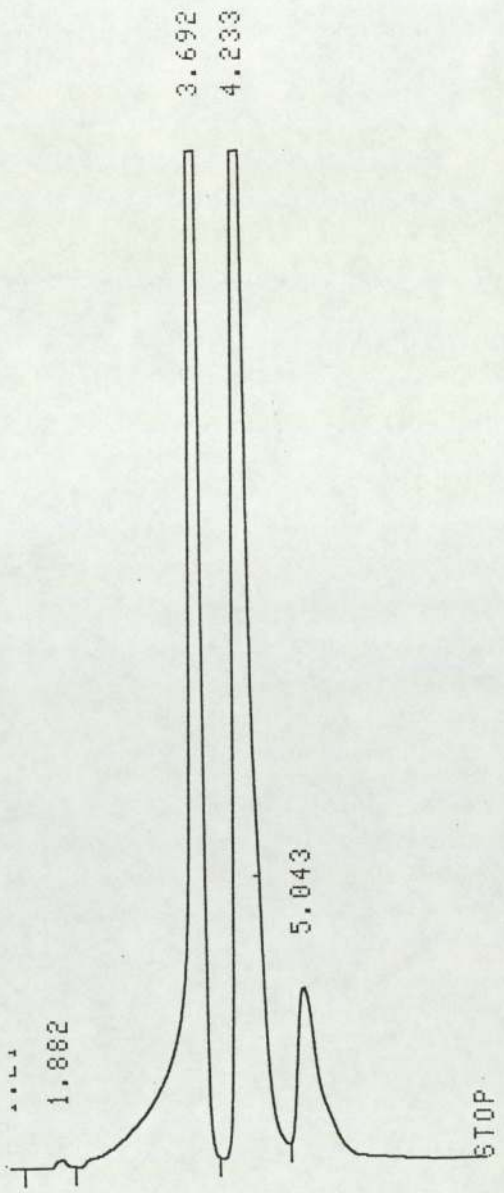
LR6H (room temperature no light)

Stability test of LR6H

(after four weeks)

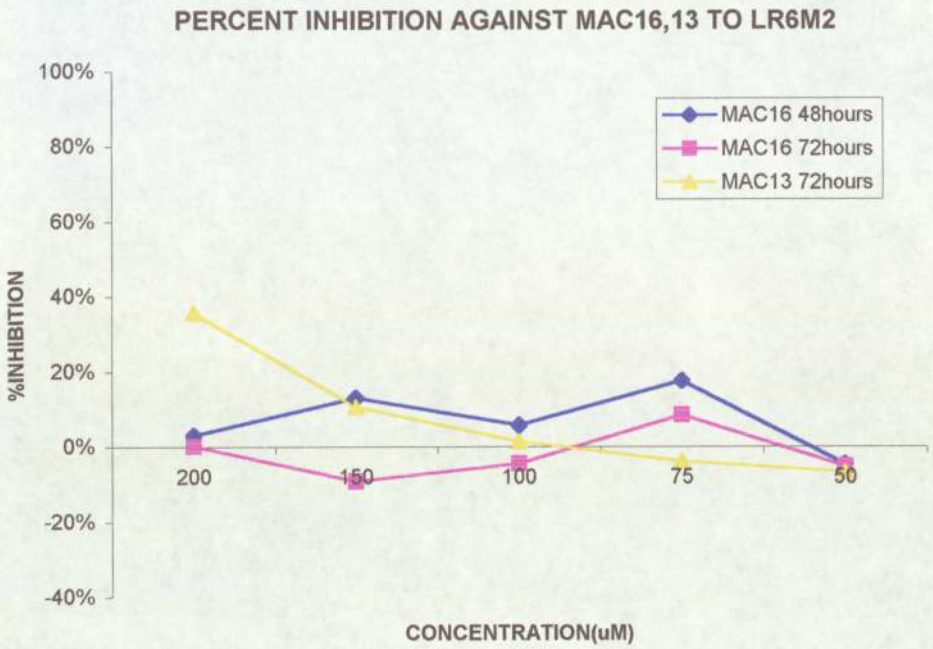


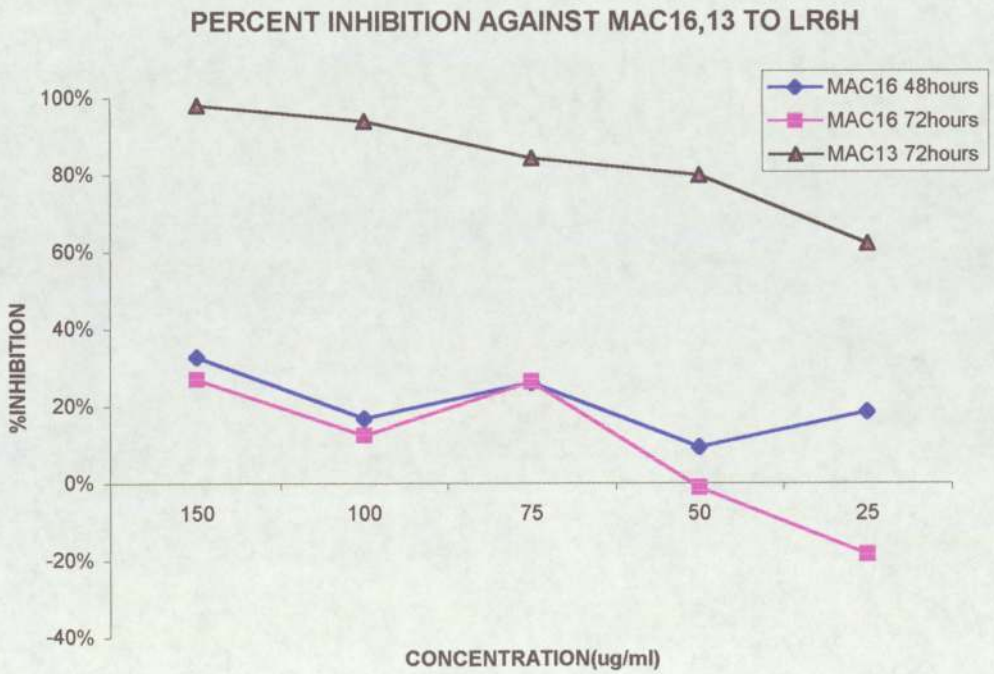
LR6H (4℃ no light)



LR6H (-20℃ no light)

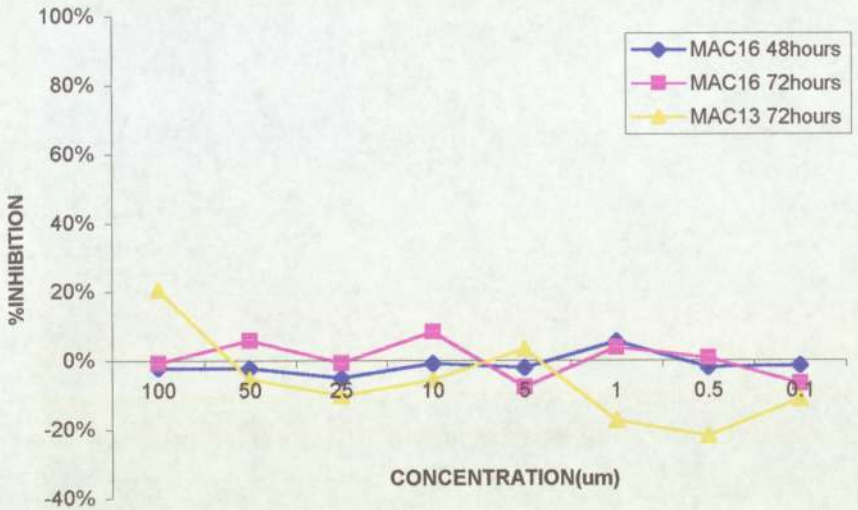
Figure 18. The curves of cytotoxicity evaluation of LR6H, LR6M and OXOFORM-CF in MAC13 and MAC16



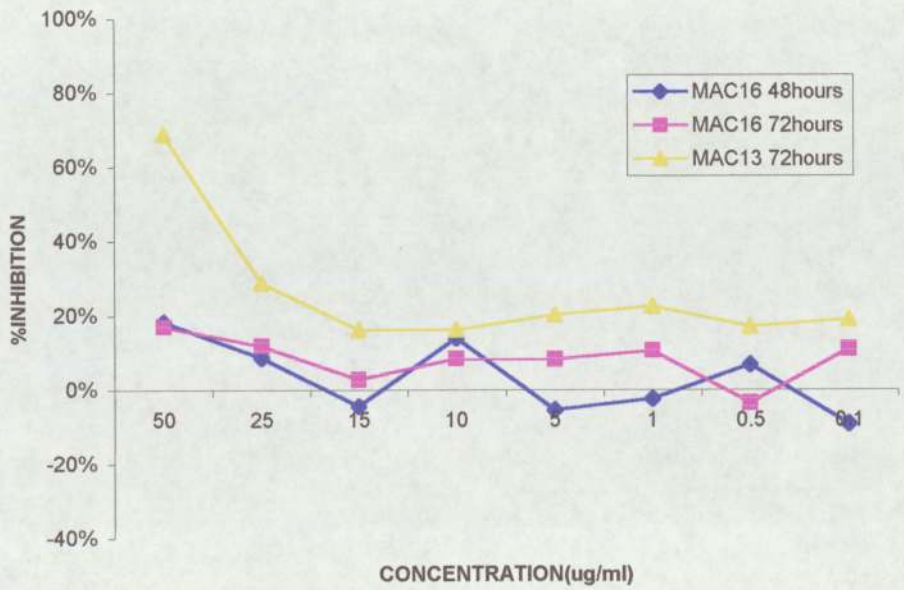


For each concentration had two replicates and presented as mean value.

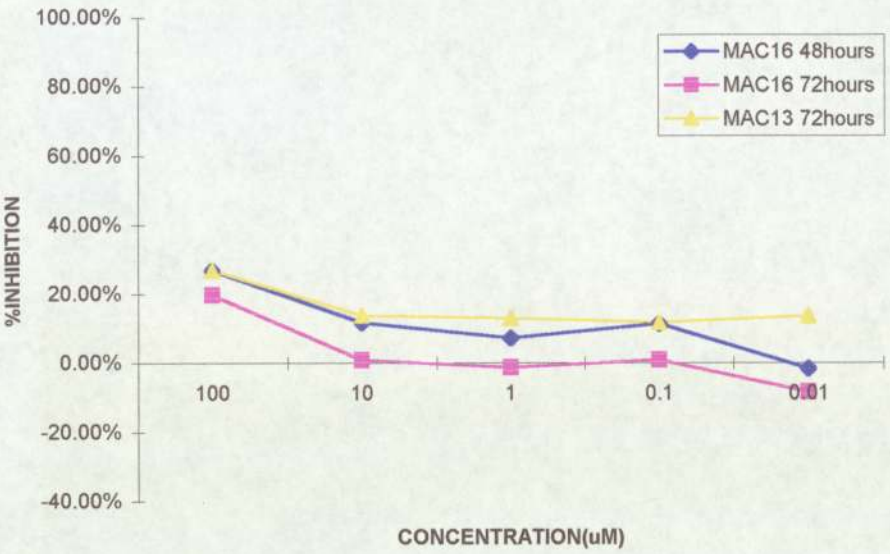
PERCENT INHIBITION AGAINST MAC16,13 TO LR6M2



PERCENT INHIBITION AGAINST MAC16,13 TO LR6H



PERCENT INHIBITION AGAINST MAC16,13 TO LR6M



PERCENT INHIBITION AGAINST MAC16,13 TO OXOFORM-CF

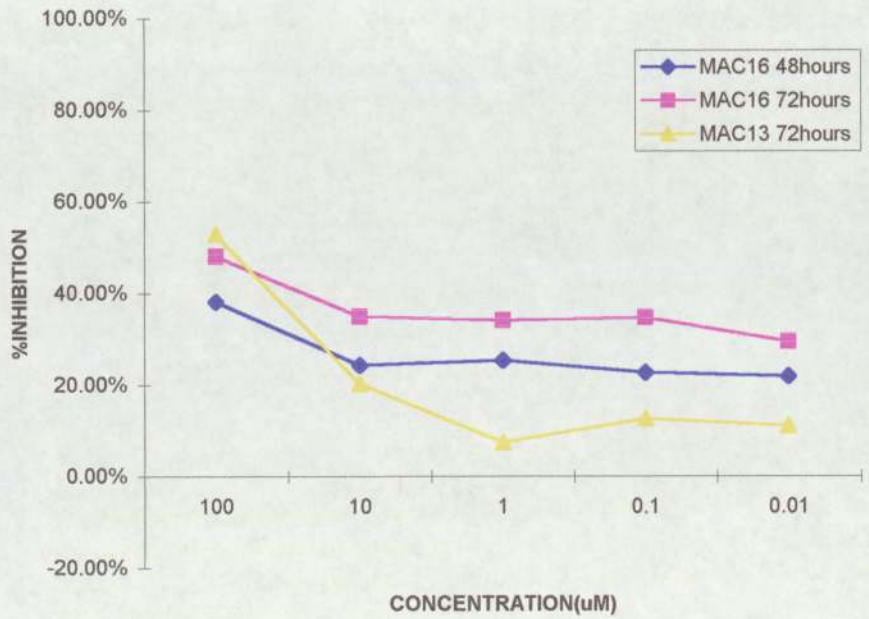


Figure 19. HPLC graph of NMF, LR6M and LR6H in condition 1(see 4.3)

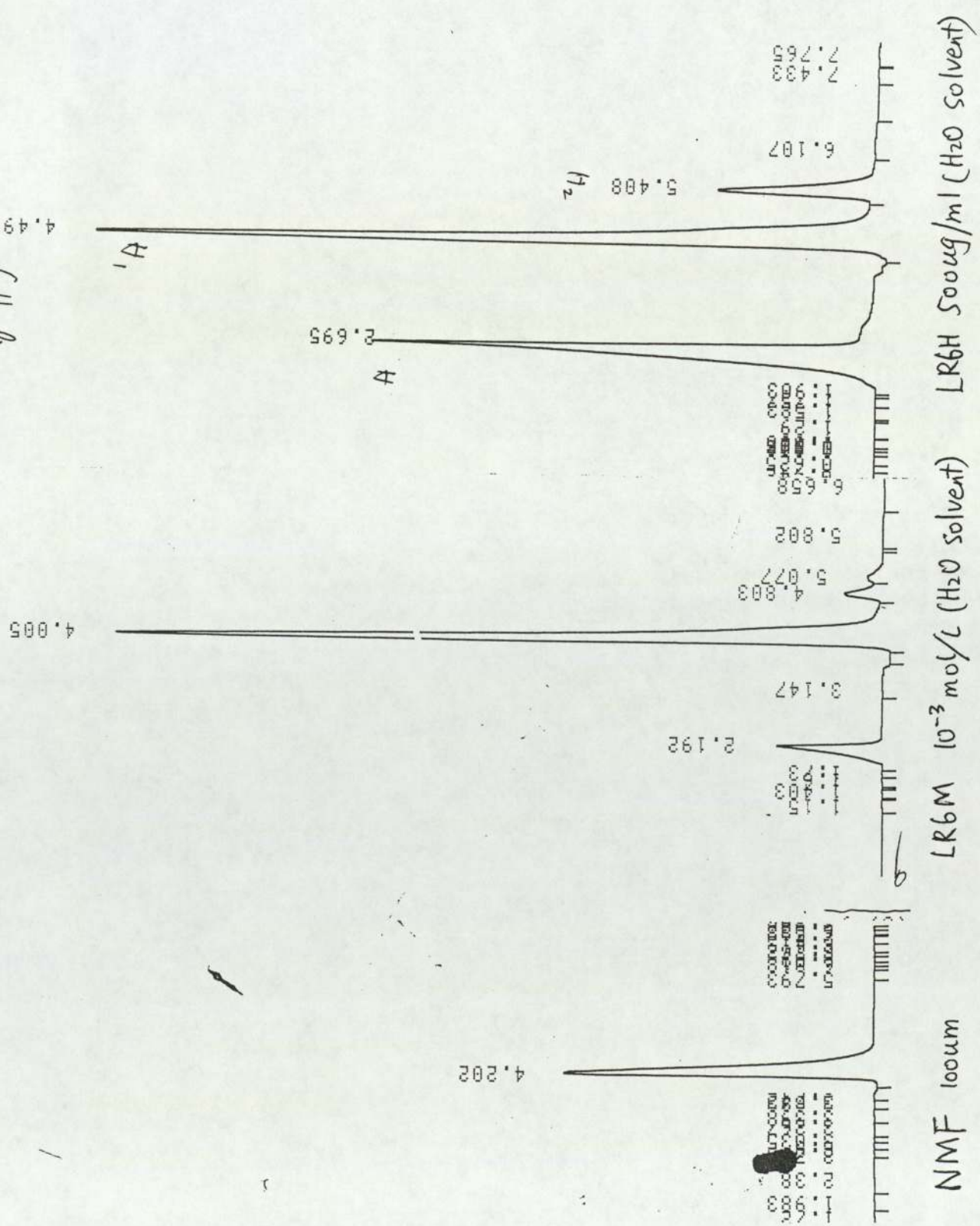


Figure 20. HPLC graph of NMF, LR6M and LR6H in condition 2 (see 4.3)

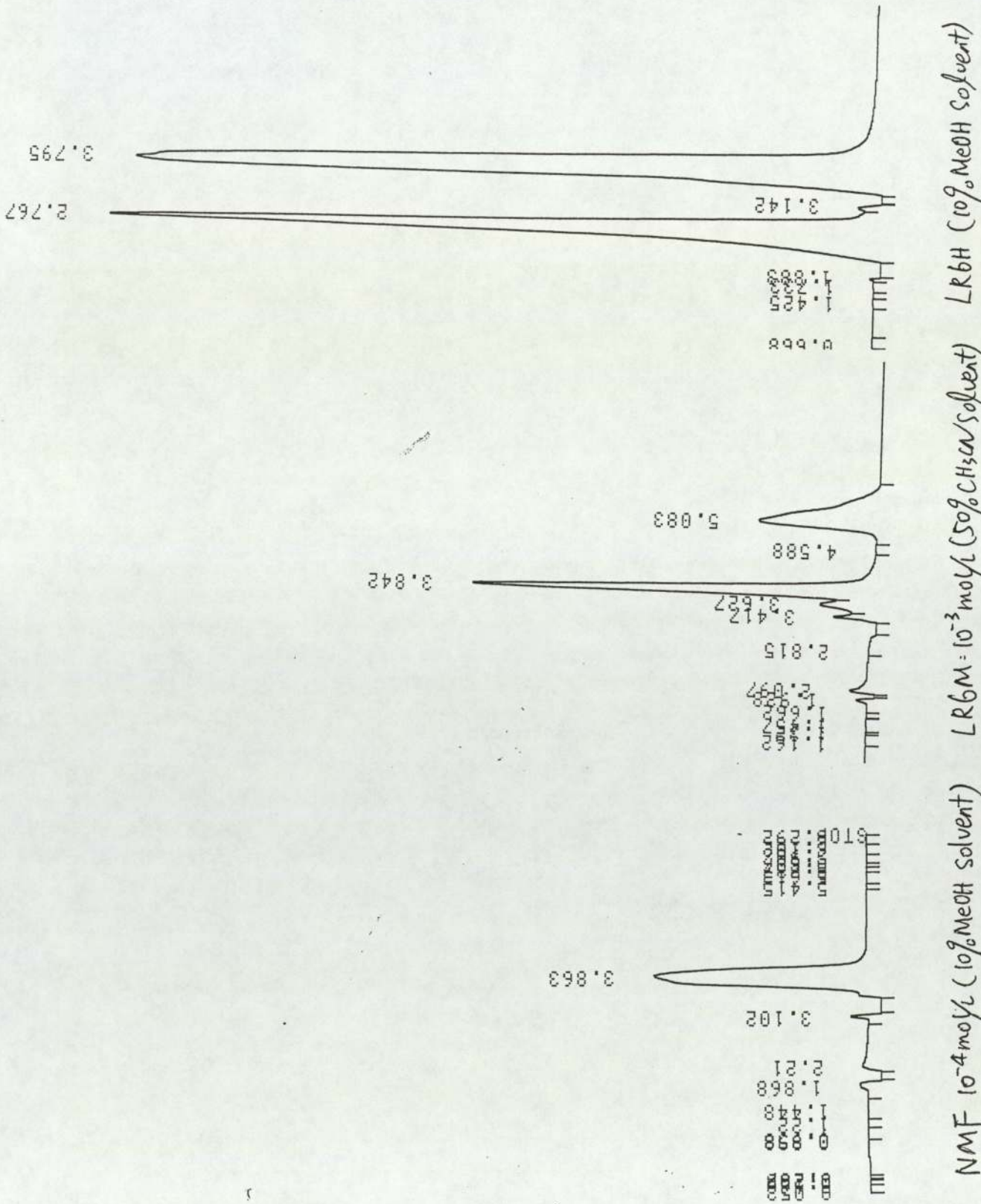
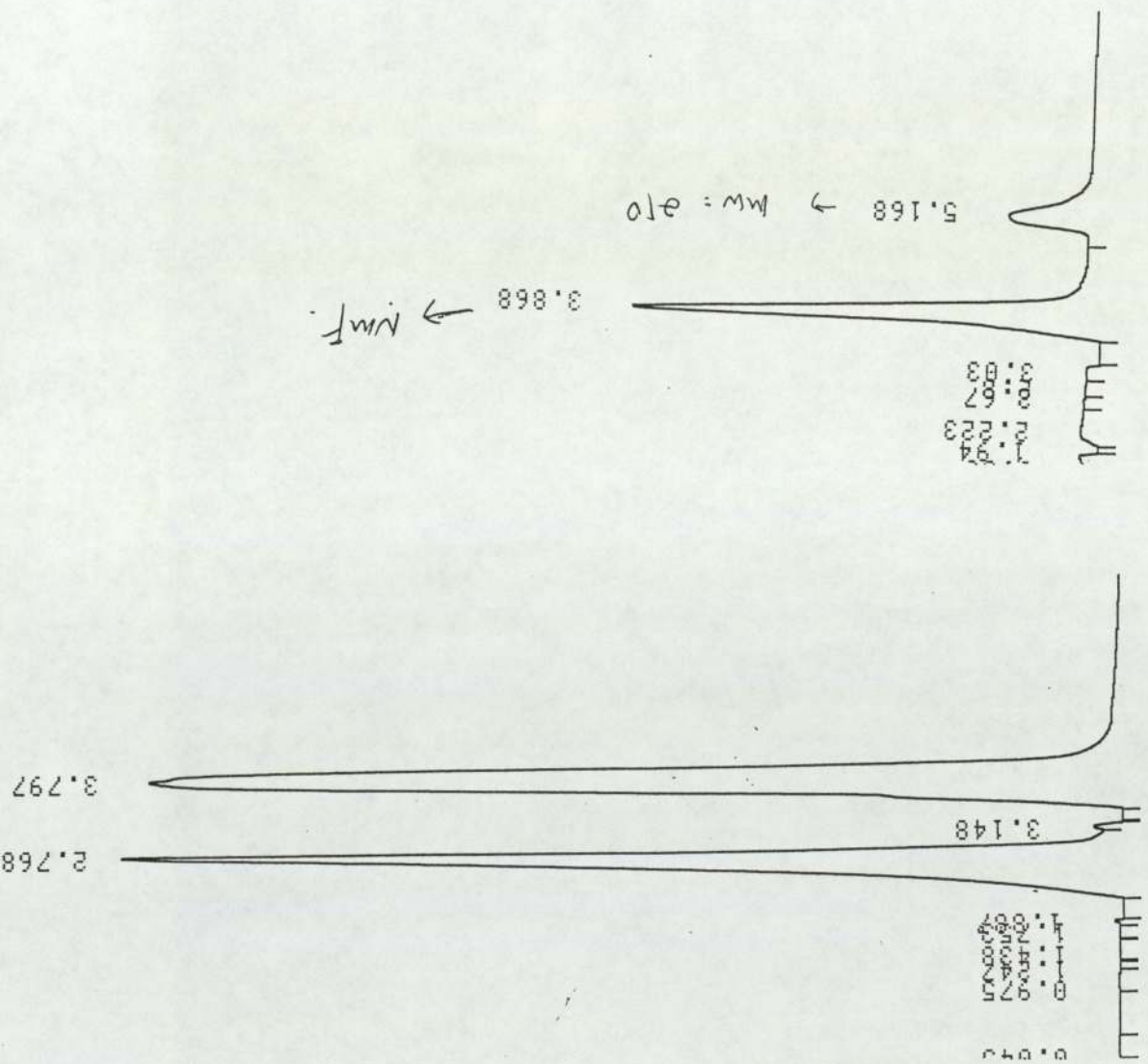


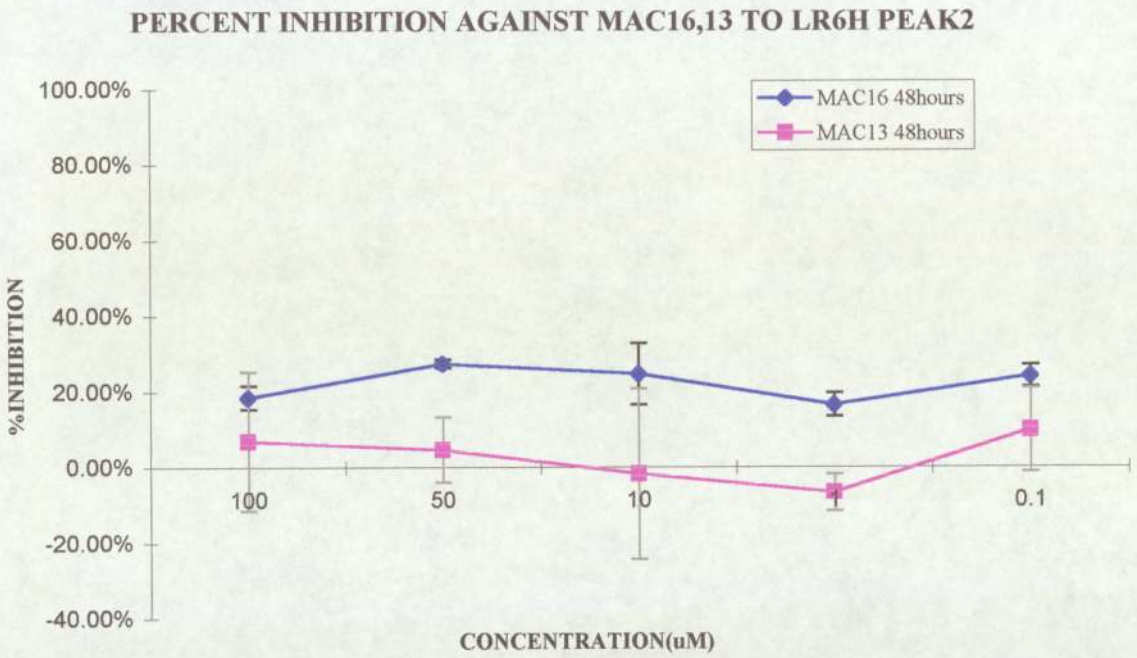
Figure 21. HPLC graph of the mixture of NMF and LR6M, NMF and LR6H in condition 2 (see 4.3)



LR6H (50 mg/ml) + NMF (10^{-3} mol/l)

LR6M (10^{-2} mol/l) + NMF (10^{-3} mol/l)
50% DMSO solvent

Figure 22. Cytotoxicity evaluation of LR6H peak2



Collect the peak2 of LR6H from HPLC and use the ratio of peak area to know the quality then calculate mole concentrations (suppose the molecular weight is 210)

5. References

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