SYNTHESIS AND BIOLOGICAL EVALUATION OF RETINOYL AND DOCOSAHEXAENOYL DERIVATIVES OF 5-FLUORO-2'-DEOXYURIDINE AS ANTICANCER PRODRUGS

LIPING FENG

Synthesis and Biological Evaluation of Retinoyl and Docosahexaenoyl Derivatives of 5-Fluoro-2'-deoxyuridine as Anticancer Prodrugs

by

Liping Feng

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Master of Science

School of Pharmacy
Memorial University of Newfoundland

September 2003

St. John's Newfoundland and Labrador Canada

Your file Votre référence
ISBN: 0-612-99073-7
Our file Notre référence
ISBN: 0-612-99073-7

Abstract

According to the survey (Gaudett *et al.* 1998, 1996), cancer is the number one disease that causes death in Canada and U.S. Many approaches have been used to treat cancer. Chemotherapy has played and will continue to play an important role in caner treatment. Although many anticancer drugs are available, there are serious problems associated with cancer chemotherapy including toxicity and development of drug resistance. Retinoids such as all-*trans* retinoic acid, omega-3 polyunsaturated fatty acids such as *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) and fluoropyrimidines such as 5-fluoro-2'-deoxyuridine (FUdR) have potent distinct anticancer mechanisms. Since many cancer cells are known to overexpress low density lipoprotein (LDL) receptors, LDL has been proposed as a cancer specific carrier. In this study, LDL was investigated as a drug carrier to enhance the drug delivery to cancer cells (Hela, MCF7, MB231 and HepG2 cell lines). Four derivatives of FUdR (3'-*O*-retinoyl-FUdR, 3'-*O*-docosahexaenoyl-FUdR, 5'-*O*-retinoyl-FUdR and 3', 5'-di-*O*-retinoyl-FUdR) were synthesized as prodrugs of FUdR. The prodrugs were incorporated into LDL. The results showed that the cytotoxicity of the respective prodrugs was increased compared with parent drug FUdR. The prodrug/LDL complex was more effective than the prodrug without LDL as a carrier in Hela cells.

Acknowledgments

I am greatly indebted to my supervising professor Dr. Hu Liu for his continuous support, guidance and understanding to me during these years and friendship beyond his duty. I particularly thank Dr. Lili Wang for her tolerance, encouragement to me and critical assessment of this thesis. Their trust and faith in my abilities have helped me re-establish the confidence in myself. I have learned so much from them during these years.

I enjoyed many conversations with Dr. Joseph Banoub from Northwest Atlantic Fisheries Centre, St. John's, Newfoundland. He shared his advice with me on NMR and MS spectra. I thank Dr Graham Bodwell from the Chemistry Department, Memorial University of Newfoundland for his help on this thesis. I would like to thank Dr. Mohamedtaki Kara and Dr. Alan Pater for their helps. I have benefited from the friendship and expertise of many colleagues during my graduate study. Ms. Margaret Connors, Yu Shu and Jiahui Hu helped me laugh during my first year. I am glad to have worked with them.

Most of all, I thank my parents for their love and encouragement through these years. I am especially grateful to my husband Shiju Wang for his patience, understanding and constant support of my goals.

Table of Contents

List of Tables

List of Figures

List of Schemes

List of Abbreviations

3'-*O*-retinoyl-FUdR	3'-*O*-Retinoyl-5-fluoro-2'-deoxyuridine
3'-*O*-DHA-FUdR	3'-*O*-Docosahexaenoyl-5-fluoro-2'-deoxyuridine
5'-*O*-retinoyl-FUdR	5'-*O*-Retinoyl-5-fluoro-2'-deoxyuridine
3',5'-di-*O*-retinoyl-FUdR	3',5'-Di-*O*-retinoyl-5-fluoro-2'-deoxyuridine
APL	Acute promyelocytic leukemia
BSA	Bovine serum albumin
CH_2Cl_2	Dichloromethane
$CHCl_3$	Chloroform
CM	Chylomicron
CRBP-I	Cellular retinol-binding protein I
CRBP-II	Cellular retinol-binding protein II
dTDP	2'-Deoxythymidine-5'-diphosphate
dTMP	2'-Deoxythymidine-5'-monophosphate
dTTP	2'-Deoxythmidine-5'-triphospate
dUMP	2'-Deoxyuridine monophosphate
DCC	4-Dicyclohexylcarbodiimide
DHA	*Cis*-4,7,10,13,16,19-Docosahexaenoic acid
DMAP	4-(Dimethylamino)pyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide

DPPE	*DL*-α-Phosphatidylethanolamine dipalmitoyl
DPPC	*L*-α-Phosphatidylcholine dipalmitoyl
FBS	Fetal bovine serum
FUdR	5-Fluoro-2'-deoxyuridine
FdUMP	5-Fluoro-2'-deoxyuridine-5'-monophosphate
FdUDP	5-Fluoro-2'-deoxyuridine-5'-diphosphate
FdUTP	5-Fluoro-2'-deoxyuridine-5'-triphosphate
HDL	High density lipoprotein
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
IC_{50}	Inhibitory concentration 50%
LDL	Low density lipoprotein
MTT	3-(4,5-Dimethyliazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT	Nitro blue tetrazolium
Na_2SO_4	Sodium sulfate
PBS	Phosphate-buffered saline
PMA	Phorbol 12-myristate 13-acetate
RBP	Retinol-binding protein
Retinoic acid	All-*trans* retinoic acid
RXRs	Retinoid X receptors
RARs	Retinoic acid receptors
TLC	Thin layer chromatography

TBAF	Tetrabutylammonium fluoride
TEA	Triethylamine
THF	Tetrahydrofuran
TS	Thymidylate synthetase
μM	Micro molar concentration
VLDL	Very low density lipoprotein

Chapter 1 Introduction

1.1 Cancer and Chemotherapy

Cancers are diseases in which unremitting clonal expansion of somatic cells kill normal cells by invading, subverting and eroding (Evan *et al.* 2001, 1998). According to Gaudette's survey (Gaudette *et al.* 1996, 1998), cancer is one of the leading causes of death in Canada and the U.S. It is estimated that one in every three Americans will develop cancer in their lifetime (Leszczniecka *et al.* 2001). Data (Parkin *et al.* 2001) indicate that lung cancer and breast cancer are the most common cancers in the world.

It is recognized that cancer progression is a complex, multi-step process. Numerous factors may induce cancer including environmental factors and genetic factors (Haldane *et al.* 1985). Environmental factors include chemicals, biological and physical agents (Tomatis *et al.* 1997, Strickland *et al.* 1995, Weichselbaum *et al.* 1989, Mann *et al.* 1987). Evidence indicates that smoking, alcohol, certain medications, electromagnetic radiation and infection play crucial roles in neoplasm (Montesano *et al.* 2001, Doll, 1999). For example, tobacco is known to be associated with various cancers, especially lung cancer (Parkin *et al.* 1994, Doll *et al.* 1994, Liu *et al.* 1998, Niu *et al.* 1998). While many cancers are believed to be closely linked to environmental factors, some cancers are due to inherited mutations in certain genes and genetic susceptibility causes a large proportion of cancers (Peto *et al.* 2001). Many factors are known to enhance genetic susceptibility and the most important ones are immune system, aging and living

environment. The host immune system, especially a weakened immune system, is often not sensitive enough to respond to the self-cellular malignancy process (Wooldridge et al, 2003). Inside mammalian cells, there are two components: a nucleus and its surrounding cytoplasm. Division of nucleus into two daughter nuclei occurs at mitosis. It is believed that one complete cell cycle comprises the following stages: G_1- an initial resting phase; S- the synthetic phase, during which the doubling of the DNA occurs; G_2- a second resting phase or premitotic phase; and M- the actual process of mitosis (Campbell *et al.* 1990).

Cells die by two primary processes: A) necrosis, in which the release of intracellular proteases and lysozymes results in an inflammatory response, or B) apoptosis, a programmed cell death process, which is an active, genetically regulated process of cell suicide. Apoptosis is characterized by specific morphological changes including cell shrinkage, loss of contacts, condensation, and segregation of chromatin around the nuclear membrane. In contrast to necrosis, apoptosis does not cause inflammation (Farkas *et al.* 2001, Israels *et al.* 1999, Hale *et al.* 1996). The major mechanism of cell removal is believed to be apoptosis. Normal apoptotic cell removal and cell replacement in tissue are estimated at 1×10^{11} cells per day – equivalent to the turnover of an adult's total body weight every 18 to 24 months (Israels *et al.* 1999).

Although the underlying mechanism(s) of apoptosis are not fully understood, it is believed that apoptosis occurs in a multi-step process and is regulated by genes such as p53 gene, bcl-2/bax gene family, protease, c-myc, c-fun and/or endonuclease depending on cellular development context or cell type (Parton *et al.* 2001, Ellis *et al.* 1998, Ferreira

et al. 1999, Bergh 1999, Hamilton *et al.* 2000, Kumar 1997, Raff 1998, Jehn et al. 1997). For instance, p53 regulatory gene can induce cell cycle arrest in G_1 or it can promote apoptosis. If cell damage is considered repairable, p53-induced cell cycle will allow time for DNA repair. With more extensive damage, p53 will signal cell to undergo apoptosis pathway. Although apoptosis is regulated by gene expression, it can be triggered by genotoxic damage, such as chemotherapy and radiation, or deprivation of cytokines such as erythropoietin (Ehlert *et al.* 2001, Kaufmann *et al.* 2000, Mendenhall *et al.* 2002).

It was found that specific neoplastic cells exhibit abnormal patterns of differentiation (Waxman *et al.* 1991, 1996). The therapy that can induce cancer cell differentiation is referred to as differentiation therapy. Differentiation therapy has drawn much attention recently since it is less toxic and more selective compared with conventional chemotherapy. Differentiation therapy is based on that the abnormality of cancer cells can be killed by differentiation inducers which can result in tumor reprogramming, in a loss in proliferating capacity and induction of terminal differentiation (Ebert *et al.* 1994, Jiang *et al.* 1994). Over the last decade, differentiation agents have been successfully used in the treatment of leukemia. Among the drugs that can induce the differentiation in cancer cells, all-*trans* retinoic acid is the first and most effective one used for the treatment of acute promyelocytic leukemia (APL) (Huang *et al.* 1988, Warrell *et al.* 1993). Studies show that treatment by differentiation drugs also resulted in the apoptosis of leukemic cells (Gianni *et al.* 2000).

Many approaches have been used to treat cancer, such as surgery (De la Habra Rodriguez *et al.* 2002), radiotherapy (Mendenhall *et al.* 2002, Weichselbaum *et al.* 2001),

chemotherapy (Goodman 2000), immunotherapy (Haviv *et al.* 2001, Jaffar *et al.* 2001, Scollay 2001), gene therapy (Idowu 2001), photodynamic therapy (Bown *et al.* 2002, Dima *et al.* 1998) and hormonal therapy (Krauss *et al.* 2002). The most commonly used treatment involves the removal of a tumor by surgery or radiation therapy, combined with chemotherapy that may eradicate micro-tumor. Although biological treatments such as gene therapy and immunotherapy have recently attracted attention, they still cannot replace other treatments, especially chemotherapy. Chemotherapy has played and will continue to play an important role in cancer treatment.

There are many chemotherapeutic anticancer drugs available, which act at specific phases of the cell cycle and some of them are summarized in Figure 1.1.

The currently available anticancer drugs can be classified into several major groups according to their structures and/or mechanisms of action, such as alkylating agents, antimetabolites and natural products, etc. Within each category, drugs may be further classified into various sub-groups. For example, antimetabolites can be divided as cytidine analogues, purine analogues, pyrimidine analogues and others. Some of the anti-cancer drugs are shown in Figure 1.2.

Alkylating agents were among the first anticancer drugs and examples include nitrogen mustards, mechlorethamine and tris(β-chloroethyl)amine. This class of drugs acts through the covalent bonding of alkyl groups to a number of nucleophilic groups such as amino, carboxyl, sulfhydryl or imidazoles moieties in proteins and nucleic acids and form the alkylated products.

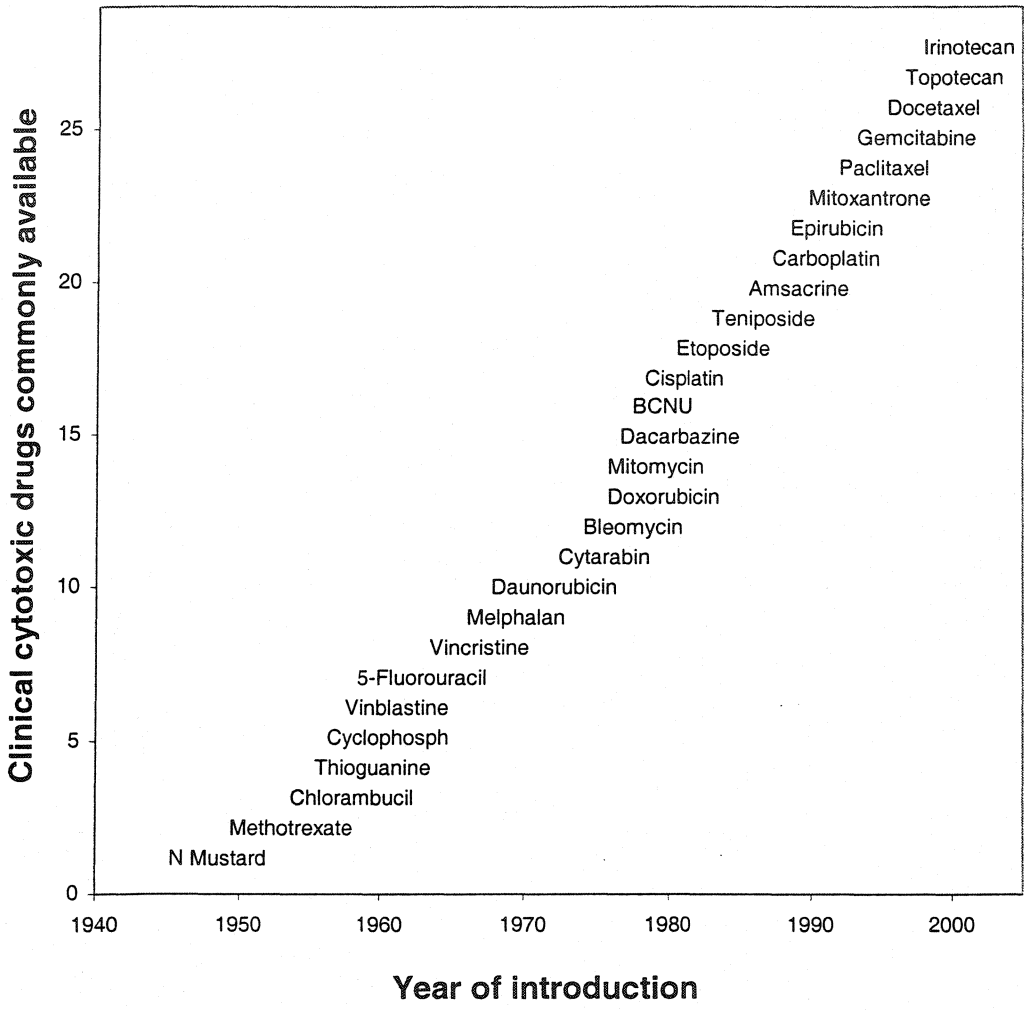
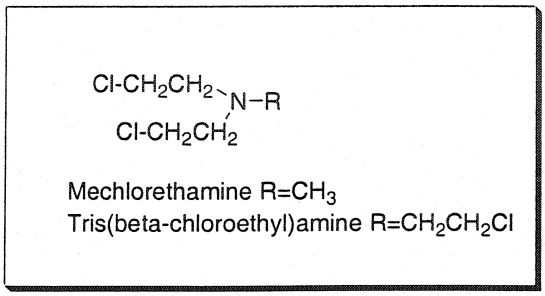
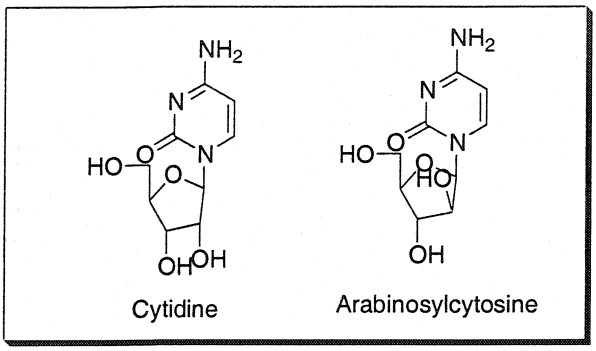


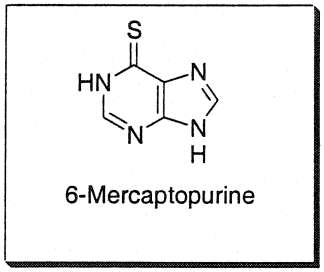
Figure 1.1. Chronology of the development of some of the anticancer drugs, taken from Baguley 2002. N Mustard stands for nitrogen mustard, and Cytarabin and BCNU represent cytosine arabinoside and bischloroethylnitrosourea, respectively.



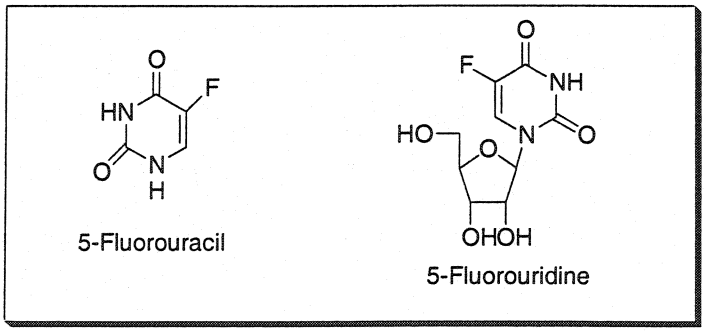
Alkylating Agents



Cytidine Analogues



Purine/Purine Nucleoside Antimetabolites



Fluorinated Pyrimidines

Figure 1.2. Structures of some anticancer drugs

Most antimetabolites destroy cancer cells by interfering with nucleic acid synthesis or nucleotide synthesis. Many antimetabolites are nucleoside analogues (Baguley 2002, Manfredini *et al.* 1997). Nucleosides are fundamental components in nucleic acid synthesis. Nucleoside analogues upon being taken up by cancer cells interfere with nucleoside and nucleotide synthesis and metabolism of cancer cells, thus resulting in growth inhibition of cancer cells (Daher *et al.* 1990). Cytidine analogues were originally isolated from the sponge *Cryptothethya crypta*. There are three major mechanisms of action believed to be responsible for cytidine analogues: inhibition of DNA polymerase alpha, incorporation into DNA, and termination of DNA chain elongation. The most active agent of this class is arabinosylcytosine (ara-C) which is used mainly in combination with doxorubicin or daunomycin for the treatment of acute myelocytic leukemia (Ellison *et al.* 1968). Purine analogues, on the other hand, inhibit *de novo* purine synthesis/purine interconversion and incorporate into DNA or RNA. Pyrimidines are another class of antimetabolite anticancer drugs. 5-Flurouracil (5-FU) and 5-fluoro-2'-deoxyuridine (FUdR) are examples of fluorinated pyrimidine analogues. Both of them act as anticancer drugs by inhibiting thymidylate synthetase (TS) and by incorporation into DNA leading to cell death.

1.2 5-Fluoro-2'-deoxyuridine (FUdR)

FUdR, one of the pyrimidine nucleoside analogues, is used extensively for the treatment of many cancers, such as colon cancer, colorectal cancer, pancreatic cancer,

liver cancer and breast cancer (Heidelberger *et al.* 1957, Nicum *et al.* 2000). The chemical structure of FUdR is shown in Figure 1.3.

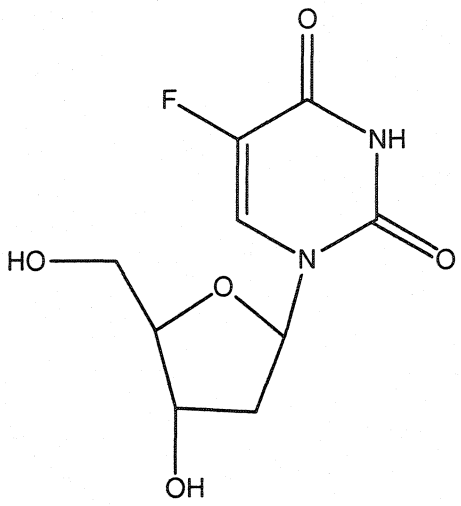


Figure 1.3. The structure of FUdR

FUdR first undergoes phosphorylation forming 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) followed by 5-fluoro-2'-deoxyuridine-5'-diphosphate (FdUDP) and 5-fluoro-2'-deoxyuridine-5'-triphosphate (FdUTP). Then it interferes with DNA synthesis by at least two mechanisms (Mader *et al.* 1998, Pratt *et al.* 1994, Schuetz *et al.* 1984, 1986) as shown in Figure 1.4.

The first possible mechanism is that FdUMP, a product of the phosphorylation of FUdR, inhibits of thymidylate synthetase (TS) (Santi *et al.* 1974, Pratt *et al.* 1994, Mader *et al.* 1998). TS is an essential enzyme for the synthesis of 2'-deoxythymidine-5'-monophosphate (dTMP) as shown in Figure 1.4 and inhibition of TS leads to impaired synthesis of thymidylate and cell death as a result of a "thymidineless state". It is believed that FdUMP competes with deoxyuridine monophosphate (dUMP) for TS and its co-factor, *N*-5,10-methylenetetrahydrofolate, thus inhibiting conversion of dUMP to dTMP and subsequently synthesis of 2'-deoxythymidine-5'-diphosphate (dTDP) and 2'-deoxythymidine-5'-triphosphate (dTTP). Ultimately it inhibits DNA synthesis. Figure 1.5 further illustrates the interaction of FdUMP with TS and *N*-5,10-methylenetetrahydrofolate.

The second possible mechanism is that FdUTP, a product of the phosphorylation of FUdR, acts as a substrate for DNA polymerase (Tanaka *et al.* 1981, Ingraham *et al.* 1980), and is incorporated into DNA (Schuetz *et al.* 1984, 1986, Mader *et al.* 1998), particularly in the presence of decreased dTTP resulted from the above process (inhibition of TS). This mis-incorporation of FdUTP into DNA can change the stability of DNA due to the production of DNA strands which have small fragments, leading ultimately to cell death (Cheng and Nakayama 1983).

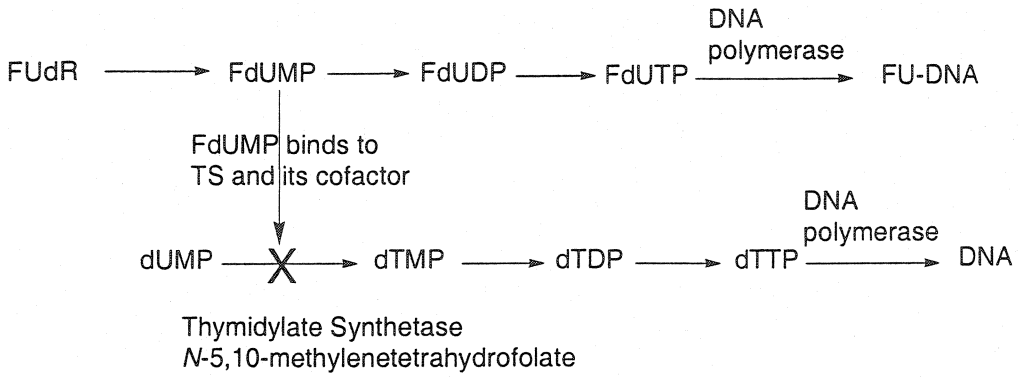


Figure 1.4. Activation and mechanism of action of FUdR. This diagram was taken from Mader *et al.* 1998.

Due to its poor bioavailability, FUdR is usually given by intra-arterial infusion rather than oral administration. FUdR is known to suffer from a number of problems including toxicity to normal tissues especially rapidly dividing tissues, such as gastrointerstinal mucosa and bone marrow (Hohn *et al.* 1985), rapid blood clearance and drug resistance. These problems limit FUdR's success in the clinic. Many strategies have been devised to enhance FUdR's clinical activity and/or minimize its toxicity. Some of these approaches involve a combination of FUdR with other cytotoxic agents such as methotrexate (Hull *et al.* 1988).

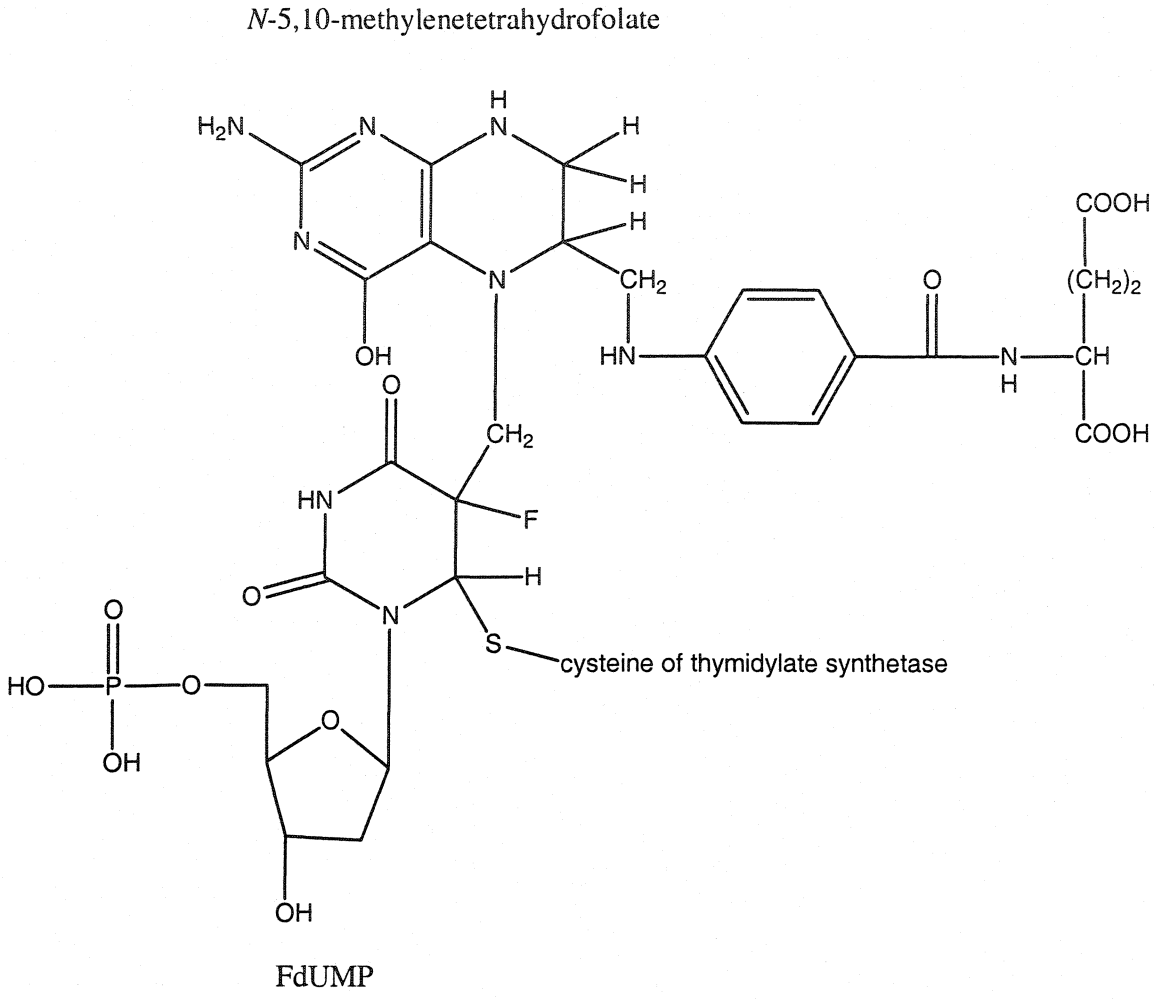
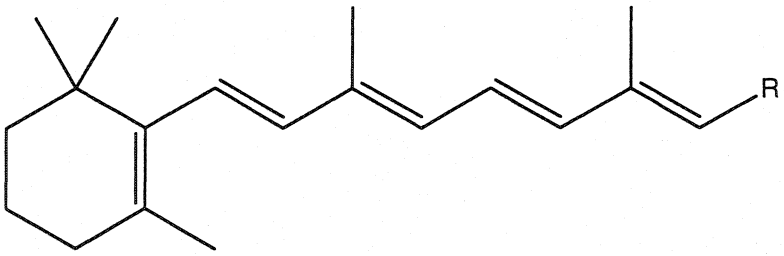


Figure 1.5. Interaction of FdUMP with TS and *N*-5,10-methylenetetrahydrofolate (Santi *et al.* 1974, Sommer and Santi, 1974).

1.3 All-*trans* retinoic acid (retinoic acid)

All-*trans* retinoic acid (3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid) is a polyene carboxylic acid and a derivative of retinol, also known as Vitamin A. Its chemical structure and related retinoid compounds are shown in Figure 1.6. The resemblance of this compound to a long-chain polyunsaturated fatty acid explains its limited aqueous solubility. Because of the existence of the conjugated polyene group, retinoic acid is relatively susceptible to chemical reactions, which is believed to attribute to its biological activity and site of action.



All-*trans* retinoic acid R=COOH
Retinal R=CHO
Retinol (vitamin A) R=CH_2OH

Figure 1.6. The structures of all-*trans* retinoic acid and related retinoid compounds.

In the body, retinoic acid is generated through irreversible oxidation of retinal which is biosynthesized by reversible oxidation of Vitamin A (Blaner and Olson, 1994). The latter mainly originates from the diet and derives from provitamin A carotenoids from vegetables or retinyl esters from animal sources. Retinoic acid is present at low concentration in the blood and the rate of retinoic acid formation varies in tissues depending on specific need of the cell. It is believed that retinoic acid is the most active metabolite of Vitamin A, which plays important roles in human visual function as well as in controlling normal cell growth, differentiation and metabolism.

The mechanism of entry of retinol into normal cells is not fully understood. It may enter cells via specific receptors for retinol-binding protein (RBP) on cell surfaces (Rask and Peterson 1976) as shown in Figure 1.7 or by simple diffusion of retinoic acid (Noy and Xu 1990). The entry of retinol into cells has at least two purposes: storage of retinol as retinyl esters, or undergoing metabolism to produce active metabolites, mainly retinoic acid, which regulates nuclear retinoid receptors after entering into the cell nucleus. Once retinol gets into cells, it may bind to cellular retinol-binding proteins, CRBP-I and CRBP-II (Napoli *et al.* 1991, Fiorella *et al.* 1991). After retinoic acid is generated through enzymatic oxidation of retinol, one of the following or a combination would occur: binding to cellular retinoic acid binding proteins (CRABP-I and CRABP-II), which would facilitate retinoic acid passing through the nuclear membrane, interacting with proteins such as P_{450} enzymes leading to inactive metabolites, or undergoing isomerization to 9-*cis* retinoic acid and/or 13-*cis* retinoic acid by the catalysis of isomerases. Although the functions of CRABPs are still not clear, it has been suggested

that CRABPs may regulate intracellular concentration of retinoic acid (Boylan and Gudas, 1992), may facilitate the transport of retinoic acid to the nucleus, and may deliver retinoic acid to specific chromatin sites in order to regulate gene expression (Takase *et al.* 1986, Fiorella *et al.* 1991). In the nucleus, retinoic acid and its isomers control gene expression upon binding to nuclear retinoid X receptors (RXRs) and retinoic acid receptors (RARs), which bind to specific DNA sequences, or response elements RARE and RXRE (Mangelsdorf *et al.* 1994).

To date, three subtypes of retinoic acid receptors: RAR-α, -β, and –γ, and three subtypes of retinoid X receptors: RXR-α, -β, and –γ, have been characterized (Benbrook *et al.* 1988, Brand *et al.* 1988, Zelent *et al.* 1988, Chambon 1996, Mangelsdorf *et al.* 1992). It has been found that retinoic acid exclusively binds to RAR receptors and 9-*cis* retinoic acid would bind to both RAR and RXR receptors. In conclusion, retinoids modulate cell phenotypes by binding to a number of retinoid receptors (RARs and RXRs). It has been suggested that RARs may regulate the cell proliferation and differentiation, while RXRs may modulate cell apoptosis (Mehta *et al.* 1996, Boehm *et al.* 1997).

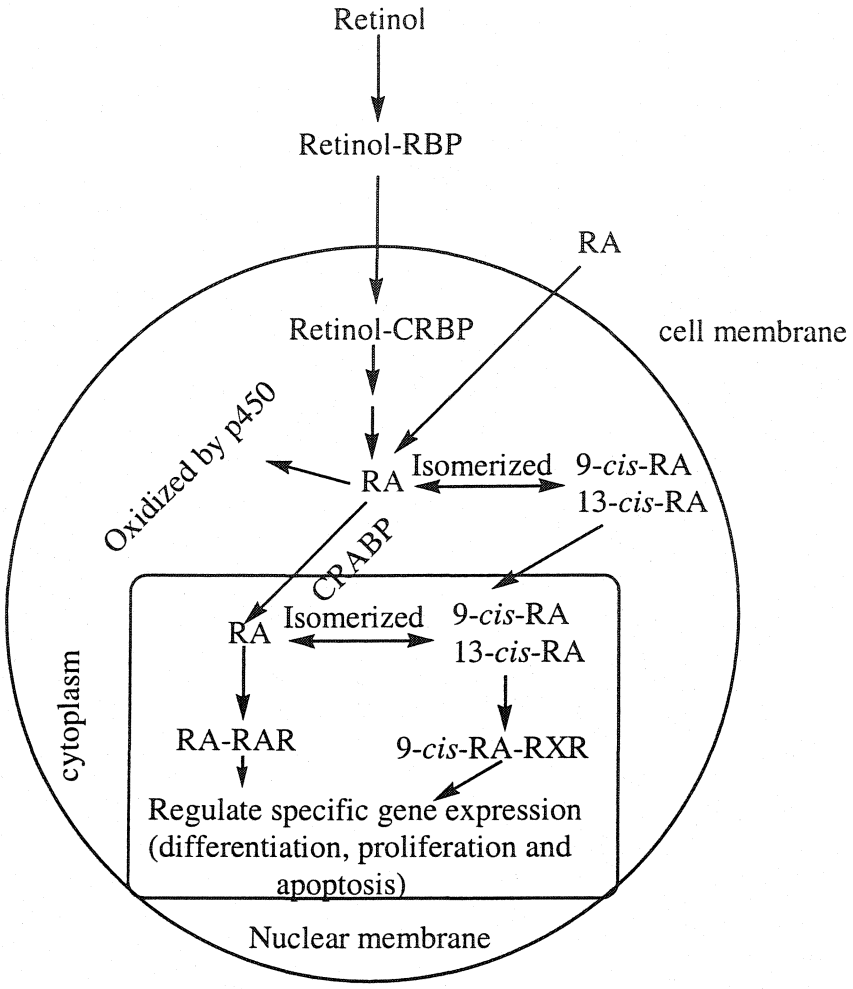


Figure 1.7. Retinoic acid signaling pathway in liver cells. Retinoic acid enters the cells by free diffusion or by conversion from retinol that has been absorbed from the gastrointestinal tract, bound in circulation to retinol-binding proteins (RBPs), and rebound intracellularly to cellular retinol-binding proteins (CRBPs) after entering into the cell. Retinoic acid can be immediately metabolized upon binding to cellular RA-binding proteins (CRABPs), oxidized by cytochrome P_{450} enzymes and/or isomerized to 9-*cis* retinoic acid and 13-*cis* retinoic acid. Retinoic acid nuclear receptor RAR and retinoid X receptor RXR upon binding to retinoic acid, 9/13-*cis* retinoic acid interact with specific DNA response elements to regulate gene expression (Warrell 1994, Mangelsdorf *et al.* 1994, Agadir *et al.* 1999).

Most of the metabolites of retinoic acid have been proven to be inactive (Blaner *et al.* 1994, Curley *et al.* 1997) (Figure 1.8). However, recent studies showed that 9-*cis* retinoic acid and 13-*cis* retinoic acid may have activity for regulating retinoic acid receptors (RAR) or retinoid X receptors (RXR) (Giguere 1994, Mangelsdorf *et al.* 1994). The pathways for their biosynthesis *in vivo* are unknown. The isomerization of retinoic acid to 9-*cis* retinoic acid and/or 13-*cis* retinoic acid could be one of *in vivo* pathways for biosynthesis of these species. In addition, isomerization of retinoic acid and 9-*cis* retinoic acid within the cell may provide a way to regulate the activity of retinoic acid.

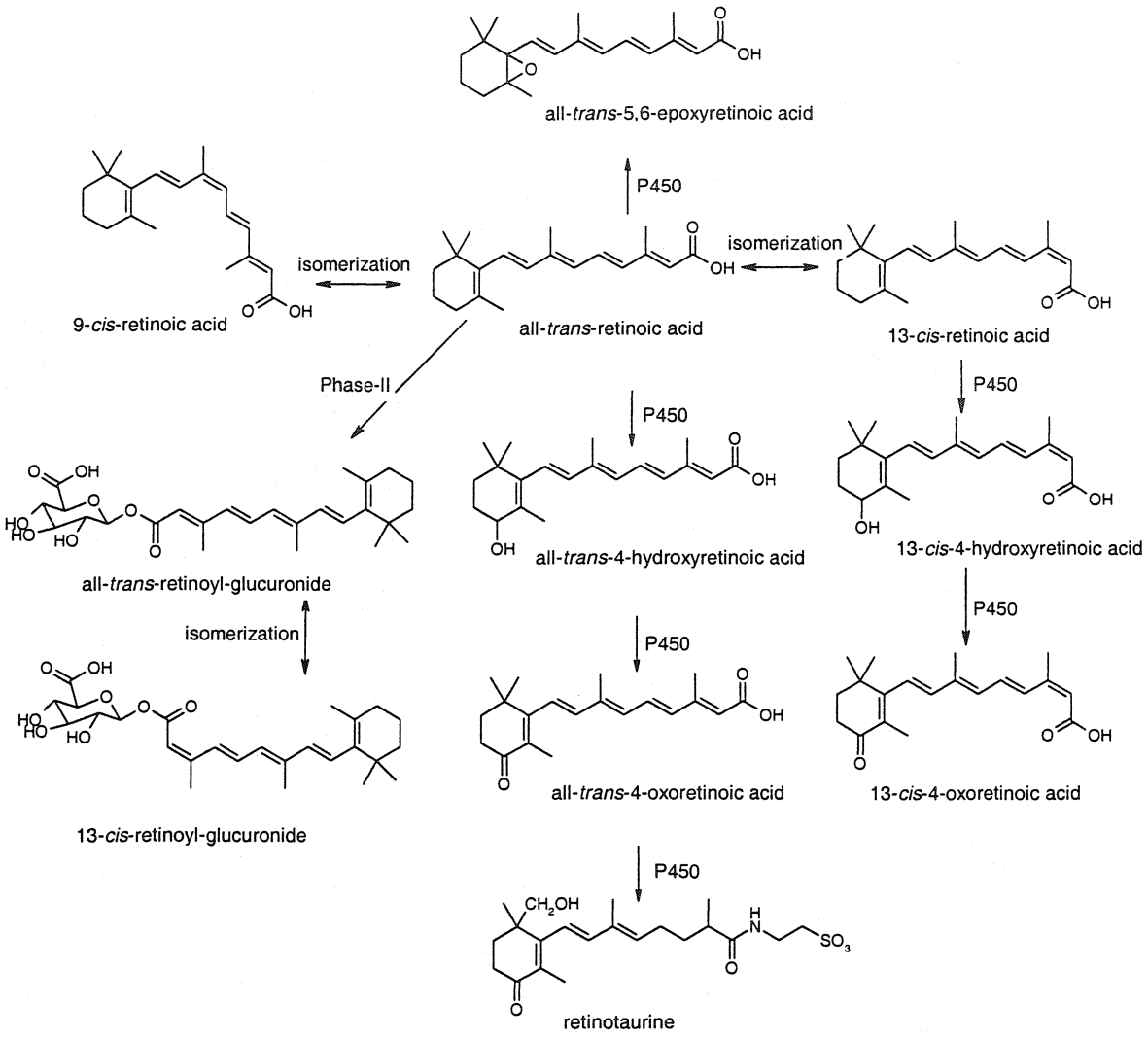


Figure 1.8. The major metabolites of retinoic acid (Blaner *et al.* 1994, Curley *et al.* 1997)

Retinoic acid has been reported to induce *in vitro* malignant cell differentiation or to suppress cell proliferation in numerous cancer cell lines (Kizaki *et al.* 1994, Bollag *et al.* 1994, Muccio *et al.* 1998, Nayl *et al.* 1998). In addition, retinoic acid has been used clinically in cancer therapy and prevention. These include treatment of the premalignant lesions in oral leukoplakia (Hong *et al.* 1986), acute promyelocytic leukemia (APL) (Huang *et al.* 1988), and other cancers such as skin, head, breast and liver cancers (Hansen *et al.* 2000, Bollag *et al.* 1994, Muccio *et al.* 1998), with the greatest success treating the patients with APL.

There is a close relationship between the expression of specific retinoid receptors and clinical retinoid responses, and some of them are listed below (after Nason-Burchenal and Dmitrovsky, 1999):

- Acute promyelocytic leukemia : PML/RARα
- Myeloid leukemia (HL-60): RARα
- Oral leukoplakia: RARβ
- Breast cancer: RARα + RARβ

For example, the rearranged receptors PML/RARα in APL cells have been credited with the successful treatment of APL by retinoic acid.

The predominant side effects of retinoic acid therapy include dryness of skin and mucous membranes, headache, hypertriglyceridemia, bone pain, and pseudotumor cerebri, particularly in pediatric patients (Castaigne *et al.* 1990, Toh *et al.* 1992, Hakimian *et al.* 1993). The other major side effect is the retinoic acid syndrome characterized by fever, respiratory distress, pulmonary infiltrates, pleuropericardial effusions, and edema after 2-

28 days of treatment (Frankel *et al.* 1992). In addition, it has been observed that prolonged use of retinoic acid alone may result in development of drug resistance (Muindi *et al.* 1992).

1.4 *Cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA)

DHA is one of the essential omega-3 unsaturated fatty acids (Conner 2000). It is found in marine oils in high amounts (5-15%) and also in human tissues such as brain gray matter, retina, nerve, heart, sperm, etc. In addition, it is found in human milk (Kuroda *et al.* 2001). The structure of DHA is shown in Figure 1.9.

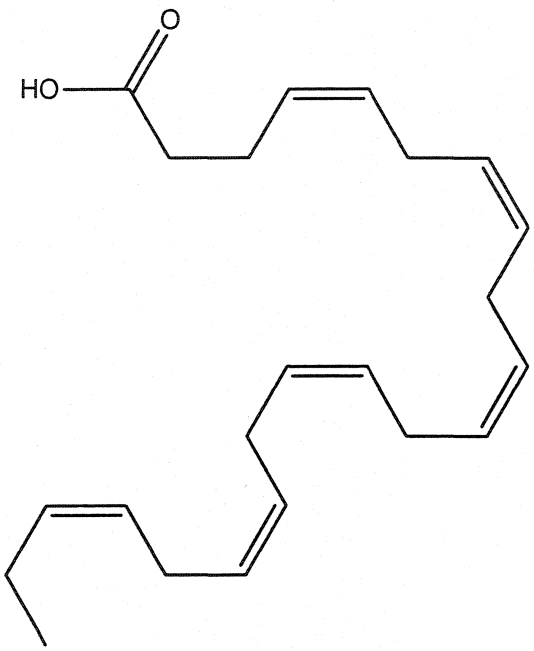


Figure 1.9. The structure of DHA

Unsaturated fatty acids including DHA have been shown to have anti-proliferation and cancer growth inhibitory properties (Plumb *et al.* 1993, Das 1991, Das and Ramesh 1998, Guffy *et al.* 1984, Horrobin 1990, Jiang *et al.* 1998a, Connolly *et al.* 1999, Pascale *et al.* 1993). Statistics also showed that the cancer incidence is generally low in the Eskimos in Greenland and Alaska where people consume large amounts of oils derived from marine animals (Blot *et al.* 1975, Bang *et al.* 1976). Epidemiological studies in Swedish found that those who consumed large quantity of fish have significantly lower incidences of prostate cancer than those who consumed little fish in their diet (Terry *et al.* 2001).

It has been reported that exogenous unsaturated fatty acids resulted in increased accumulation of anticancer drugs such as doxorubicin in cancer cells such as L1210 murine leukemia cell line or small-cell lung cancer cell line, which is believed to be responsible for the observed elevated cytotoxicity either *in vitro* and/or *in vivo* (Tan *et al.* 2000, Menendez *et al.* 2001, Rudra *et al.* 2001, Germain *et al.* 1998, Shao *et al.* 1995). Precise mechanisms for the increased intracellular accumulation of anticancer drugs resulted from the use of unsaturated fatty acids have not been determined. However, it has been proposed that the increased accumulation of anticancer drugs inside cells could result from changes in biophysical properties and functions of tumor cell membranes brought about by fatty acid supplementation (Timmer-Bosscha *et al.* 1989, Zijlstra *et al.* 1987, Burns and North, 1986, Jiang *et al.* 1998b). Membrane fluidity and drug transport are influenced by the compositional ratio of saturated fatty acids to unsaturated fatty acids: the greater the percentage of unsaturated fatty acids, the greater membrane fluidity,

and the greater proportion of anticancer drugs enter the cells, since many anticancer drugs are considered to enter the cell by passive diffusion (Avdeef 2001).

The increased cytotoxicity observed with the use of unsaturated fatty acids has also been suggested to be due to the formation of peroxidation products and free radicals involved in the modulation of drug efficacy by fatty acids, especially by unsaturated fatty acids (Das 1991, Igarashi *et al.* 2000, Menendez *et al.* 2001, Sagar *et al.* 1992). Some anticancer drugs yield oxygen-reactive species that could react with the unsaturated fatty acids and induce the peroxidation process (Berchekroum *et al.* 1993). In addition, it has been speculated that unsaturated fatty acids may affect the protein expression related to cell adhesion and motility (Jiang *et al.* 1995a, 1998c, 1995b). It has been proven that DHA-induced apoptosis can be inhibited by phosphatidic acid (PA), a specific protein phosphatase-1 (PP1) inhibitor and hence this process is considered to be mediated through activation of PP1 (Siddiqui *et al.* 2001). In addition, others suggested that unsaturated fatty acids modulate the expression of some early genes, such as c-*jun* and c-*fos*, which are associated with cell apoptosis (Sellmayer *et al.* 1997).

1.5 Targeted drug delivery

Although there are many drugs available for cancer chemotherapy, there are problems associated including drug toxicity to normal cells and development of drug resistance. Targeted delivery of anticancer drugs to cancer cells would reduce the toxicity and increase the efficacy of anticancer drugs.

The concept of drug targeting was first suggested by Paul Ehrlich almost a century ago and an idea of "magic bullets" was proposed (Ehrlich 1906). Since then, drug targeting or targeted delivery has drawn a lot of attention. Theoretically, a good anticancer drug carrier for *in vivo* use should meet the following criteria (Kader 1997):

- The drug-carrier conjugate must be stable, both during storage and *in vivo*.
- The carrier should be biocompatible and biodegradable, and the drug conjugate must not produce unacceptable levels of toxicity or immunological reactions.
- The carrier should be suitable for targeting.
- The drug-carrier conjugate must allow release of the drug at the target site.
- The carrier must not cause unspecific uptake by non-target cells.

To date, a number of particulate systems have been proposed as drug carriers such as emulsions, microspheres, lipoproteins, nanoparticles and liposomes (Davis *et al.* 1986, Schilsky *et al.* 1996, Schmidt-Erfurth *et al.* 1994, Lundberg 1994, Filipowska *et al.* 1992, Nishioka *et al.* 2001, Kreuter 2001). Monoclonal antibodies have also attracted a lot of attention (Dubowchik and Walker 1999, Garnett 2001). In addition, cells, cell ghosts, and micelles have been studied as targeted drug carriers (Torchilin 2000). Recently, magnetic drug targeting, which is based on binding anticancer drug with ferrofluids that concentrate the drug in the tumor site under magnetic fields, has also been investigated (Lubbe *et al.* 2001).

Although many drug carriers have been investigated, there are many obstacles to the successful delivery of drugs using these carriers, such as rapid clearance by the

reticulo-endothelial system (RES) resulting in low plasma half-life and instability of the carrier-drug conjugates *in vivo* (Kader *et al.* 1998). Low density lipoprotein (LDL) has been proposed and studied as a carrier for cytotoxic agents recently (De Smidt *et al.* 1990, Shaw *et al.* 1987, Counsell and Pohland 1982). LDL is a quasispherical endogenous nanopartical with a long serum half life of 2-4 days. Over the past several years our group has investigated the potential of using LDL as a drug carrier for anti-cancer drugs.

1.6 LDL as a carrier for cytotoxic agents

Lipoproteins are complexes that serve to transport cholesterol and triglycerides in the blood stream. According to their buoyant density, plasma lipoproteins are classified into four major groups called chylomicrons (CM), very low density lipoprotein (VLDL), LDL and high density lipoprotein (HDL) (Bradley *et al.* 1978). CM and VLDL are the largest and lightest of lipoproteins and are the major carriers for triglycerides. LDL and HDL contain cholesterol esters as the predominant lipid. In man, LDL cholesterol contributes about two-thirds of the circulating plasma cholesterol, which is a major constituent of the mammalian cell membrane.

Figure 1.10 shows the schematic structure of LDL. LDL is a spherical particle with a diameter of approximately 22 nm and a mass of over 1 x 10^6 daltons. LDL contains a hydrophobic core of approximately 1500 molecules of cholesteryl ester. It is shielded from the aqueous blood by an amphiphilic shell that consists of monolayer of phospholipids, free cholesterol as well as proteins known as apoproteins. The cholesterol

on the surface can exchange rapidly with cholesterol in other structures such as cellular membranes, whereas the cholesteryl ester molecules remain more firmly in the oily core (Firestone 1994, Van Berkel 1993, Kader *et al.* 1998).

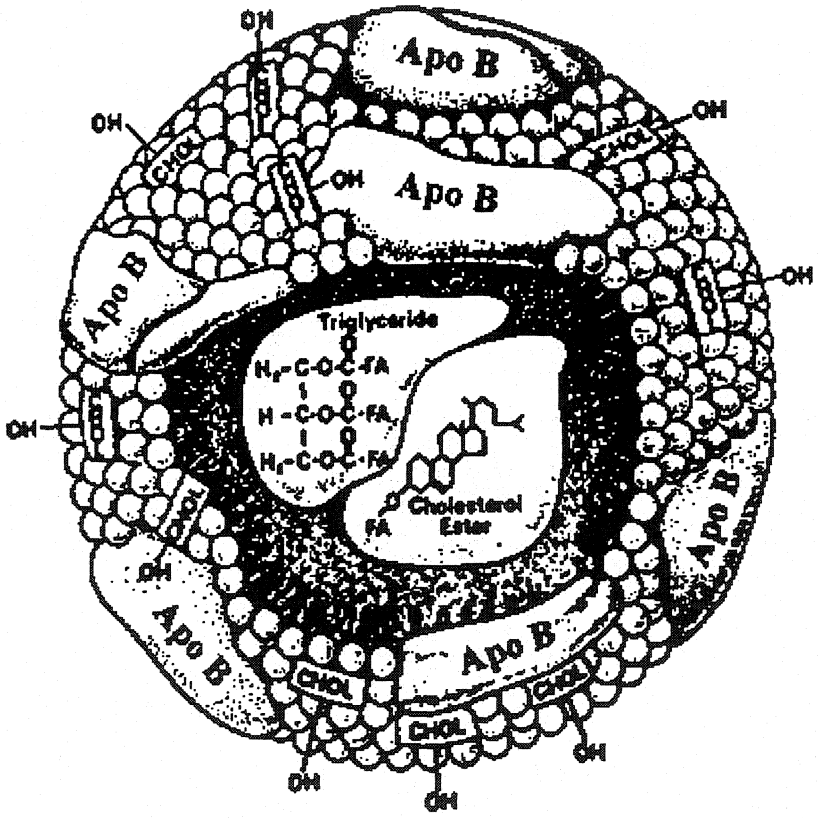


Figure 1.10. The schematic structure of LDL

Apoprotein on the surface of the LDL phospholipid monolayer is a single 514,000-dalton protein called apoprotein B (Apo-B). Apo-B is recognized by specific cell surface receptors, i.e., LDL receptors, which carry LDL into the cell by receptor-mediated endocytosis. Figure 1.11 shows the fate of LDL particles and LDL receptors after endocytosis.

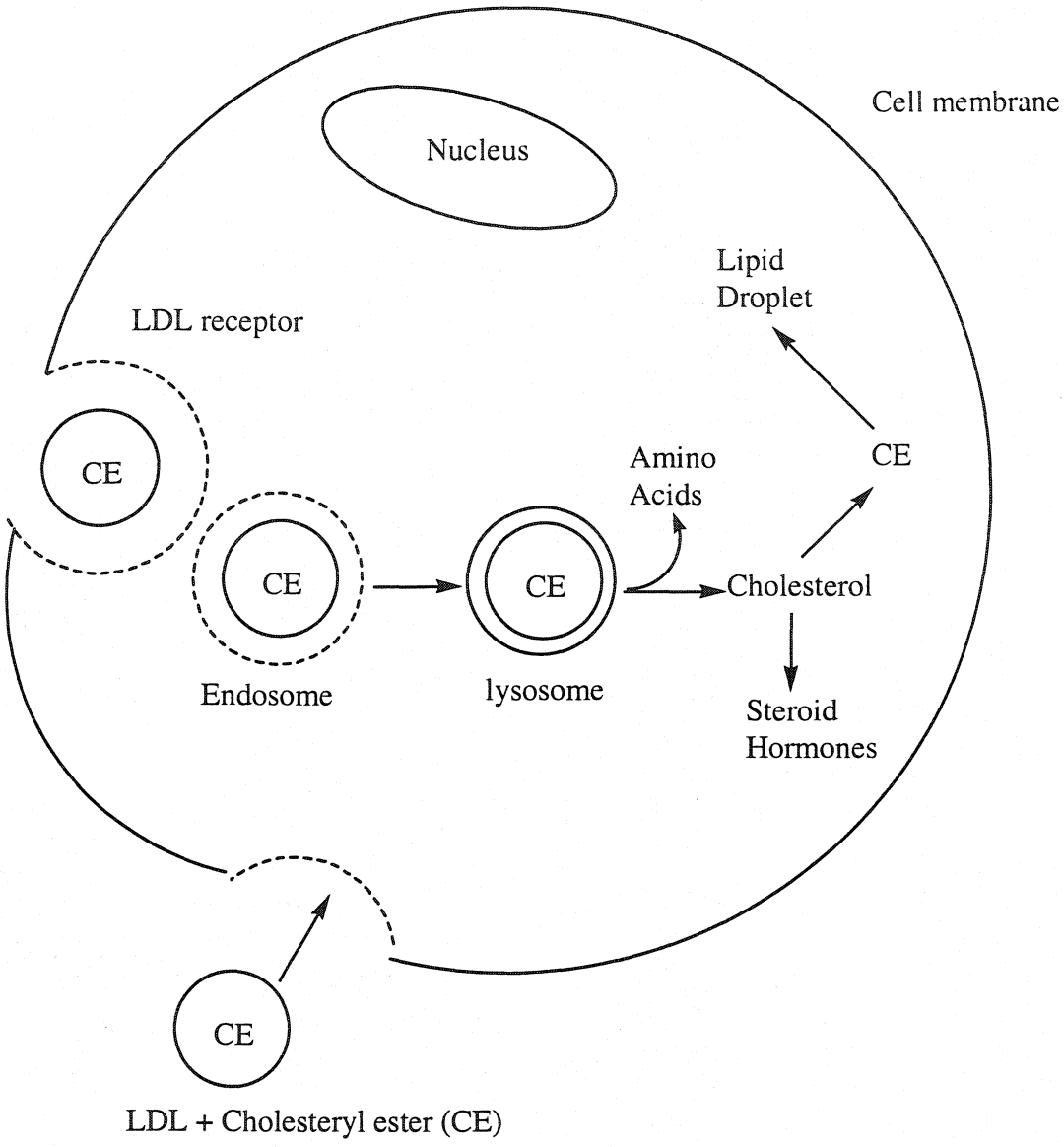


Figure 1.11. Fate of LDL particles and LDL receptors after endocytosis. After binding of LDL to the LDL receptor, the lipoprotein is internalized and delivered to the lysosomes, where the cholesteryl esters can be hydrolyzed. Liberated cholesterol can pass the lysosomal membrane and is available for cellular metabolism, such as formation of membranes or steroids (De Smidt *et al.* 1990, Counsell *et al.* 1982).

After a LDL particle binds to the LDL receptor, the receptor-ligand complex is internalized in a coated pit by endocytosis, where the lower pH causes the dissolution of LDL from its receptor. In a subsequent step, the LDL dissociates from the receptor and the receptor is transported back to the plasma membrane. The endosome containing LDL now fuses with lysosomes, forming a large secondary lysosome. In this lysosome the apoprotein B is degraded into amino acids by proteases, and the cholesteryl esters are hydrolyzed to fatty acids and cholesterol by lipases, and cholesterol is available for the cellular metabolism such as formation of membranes or as the precursor for the biosynthesis of steroids (Figure 1.11). Excess amounts of cholesterol can be re-esterified into CE and stored as oil droplets for future use.

Interest in LDL as a drug carrier has been triggered by the discovery that many cancer cells show a high receptor-mediated uptake of LDL (Ho, *et al.* 1978, Tokui *et al.* 1995, Maletinska *et al.* 2000). The high levels of LDL receptors present on the surface of cancer cells may be explained by the fact that cancer cells presumably need more cholesterol because of the fast dividing nature of cancer cells. The increased need for the formation of new membrane material is met by the increased LDL uptake from the blood circulation. Thus, if LDL can be an anticancer drug carrier, it might serve as a targeting carrier. It has been shown that cancer cells are associated with elevated levels of LDL receptors (Vitol *et al.* 1985, Rudling *et al.* 1986, 1990). LDL could deliver anticancer drugs more specifically to cancer cells. The concern of using LDL as a drug carrier for anticancer drugs is the significant dumping of anticancer drugs to normal tissues where high levels of LDL receptors exist such as liver. However, it has been demonstrated that

the level of LDL receptors in those tissues can be effectively down-regulated. For example, the LDL receptor in the liver can be successfully lowered by means of dietary fats, cholesterol and bile salts (Dietschy *et al.* 1986).

Furthermore, LDL lacks immunogenicity (Filipowska *et al.* 1992, Van Berkel 1993), which is an advantage over many foreign particles. Its native structure has a lipid droplet that maybe used to store lipid soluble drugs (Firestone 1994).

1.7 Design of prodrugs

LDL appears to be a good drug carrier for delivering anticancer drugs since cancer cells have higher LDL receptor levels than normal cells. However, due to the high hydrophilicity of FUdR, it is expected to be difficult to incorporate it into LDL which is rather hydrophobic. Therefore, chemical modification or preparation of prodrugs is necessary to increase the hydrophobicity of FUdR. To increase hydrophobicity, esterification is usually the first choice, since esters are easily hydrolyzed by esterases which are rich in the human body (Bundgaard 1991). Given the chemical structure of FUdR (with two hydroxyl groups), it is possible to design an ester which is more hydrophobic. With the increased lipophilicity, the prodrug is expected to be easily incorporated into the LDL carrier.

Although many esters of FUdR have been reported in literature (Schwendener *et al.* 1985, Kawaguchi *et al.* 1988, Yamashita *et al.* 1988, Halmos *et al.* 1992), we decided to prepare retinoyl and DHA derivatives of FUdR. There is evidence of interesting

synergistic or additive antitumor effects by simple physical combination of retinoic acid with other anticancer drugs such as cytosine arabinoside (Hassan *et al.* 1986, Chomience *et al.* 1986, Ponzoni *et al.* 1991, Li *et al.* 1997, Nudelman and Rephaeli 2000). Therefore, the retinoyl derivative of FUdR or DHA derivative of FUdR have the potential to display increased lipophilicity and also improved efficacy, since retinoic acid or DHA will be released in addition to FUdR upon hydrolysis of the respective prodrugs. In addition, retinoic acid and DHA function via different mechanisms which may result in synergistic effect. It has been proved that some retinoic acid prodrugs are more effective inducers of cell differentiation and/or apoptosis in a number of neoplasias (Formelli *et al.* 1996, Manfredini *et al.* 1997).

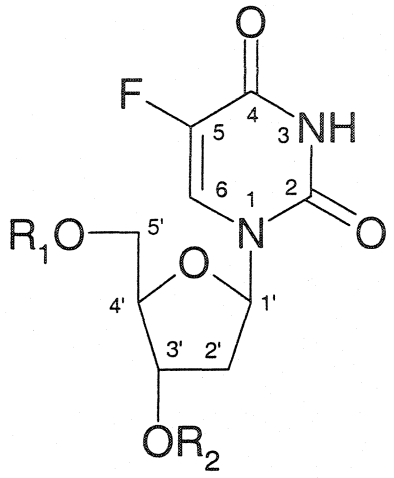
Chapter 2 Objectives

The objectives of this research project are as follows:

1. to design and synthesize novel prodrugs of FUdR,
2. to incorporate the proposed prodrugs into LDL, a drug carrier,
3. to evaluate and compare the cytotoxicity of the proposed prodrugs, prodrugs/LDL with that of FUdR.

The novel prodrugs designed for this project were the retinoic acid and DHA conjugates of FUdR as shown in Figure 2.1 based on the following reasons:

- With retinoic acid or DHA conjugated to FUdR, it is anticipated that the resultant compounds will possess an enhanced lipophilicity which is essential for the incorporation into the proposed drug carrier, LDL.
- As discussed earlier in the Introduction, both retinoic acid and DHA have been proven to be beneficial against cancer. The two species (retinoic acid and FUdR or DHA and FUdR) released upon hydrolysis of the derivatives will be active via different mechanisms.



Compounds	R_1	R_2
3',5'-di-*O*-retinoyl-FUdR (Compound 4)	$-C_{20}H_{27}O$	$-C_{20}H_{27}O$
5'-*O*-retinoyl-FUdR (Compound 11)	$-C_{20}H_{27}O$	H
3'-*O*-retinoyl-FUdR (Compound 7)	H	$-C_{20}H_{27}O$
3'-*O*-DHA-FUdR (Compound 13)	H	$-C_{22}H_{31}O$

Figure 2.1. The chemical structures of the proposed FUdR prodrugs

The prodrugs synthesized were incorporated into LDL, a tumor seeking carrier, forming prodrug/LDL complexes.

The following studies were carried out to evaluate the prodrugs and prodrugs/LDL in comparison with FUdR.

- Cytotoxicity using MTT assay in two human breast cancer cell lines: MB231 and MCF7, one cervical cancer cell line: Hela, and one hepatic cancer cell line: HepG2.
- Differentiation effect of these prodrugs against leukemia cell line: CEM/T4.

Chapter 3 Materials and Methods

3.1 Materials

FUdR, retinoic acid, DHA, 4-(dimethylamino)pyridine (DMAP), and all other chemicals used in synthesis were purchased from Sigma-Aldrich Chemical Co and were of chemical grade unless otherwise specified. All chemicals were used without further purification unless otherwise noted. Benzene, dichloromethane (CH_2Cl_2), and triethylamine (TEA) were freshly distilled from calcium hydride prior to use. Tetrahydrofuran (THF) was distilled from sodium in the presence of benzophenone prior to use.

All reactions were conducted under argon or nitrogen atmosphere protected from light. Unless otherwise noted, organic extracts were dried with sodium sulfate (Na_2SO_4), filtered through a fritted glass funnel and concentrated to dryness under reduced pressure (20-30 mmHg) with a rotary evaporator below 30 °C. Thin layer chromatography (TLC) was performed using Silica Gel 60 F_{254} (Fisher chemical Company). Silica Gel 60 (230-400 mesh) was used for flash chromatography (Still *et al.* 1978). Nucleoside derivatives were examined under UV light and unsaturated fatty acid was located by exposure to iodine vapor. Proton nuclear magnetic resonance spectra (^{1}H NMR) were determined at 300 MHz or 500 MHz and $CDCl_3$ or DMSO-d_6 was used as solvent. FAB Mass spectra (MS) were obtained at the Department of Fisheries and Oceans, Newfoundland.

All solvents used for HPLC analyses were of HPLC grade. All reagents used for *in vitro* tests were of cell culture grade. 3-(4,5-Dimethyliazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Company. Nitro blue tetrazolium (NBT) and phorbol 12-myristate 13-acetate (PMA) were kindly provided by Dr. Alan Pater (Faculty of Medicine, Memorial University of Newfoundland). Fetal bovine serum (FBS) was purchased from GIBCO BRL Co. RPMI-1640 culture medium, Dulbecco's modified Eagle's minimal essential medium (DMEM), non-essential amino acids and penicillin/streptomycin were purchased from Sigma biochem Co. HPLC analyses were conducted using an *HP* 1100 chromatographic system, with a C18 reverse-phase column (150 × 3.9 mm, Phenomenex).

Fresh human plasma was obtained from the Canadian Blood Services, St. John's, Newfoundland, Canada.

Human cancer cell lines, MCF7 and MB231, and cervical cell line, Hela, were obtained from the American Type Culture Collection (Manassas, VA, USA). Leukemia cell line, CEM/T4, and Hepatic Cancer cell line, HepG2, were kindly provided by Dr. Alan Pater (Faculty of Medicine, Memorial University of Newfoundland).

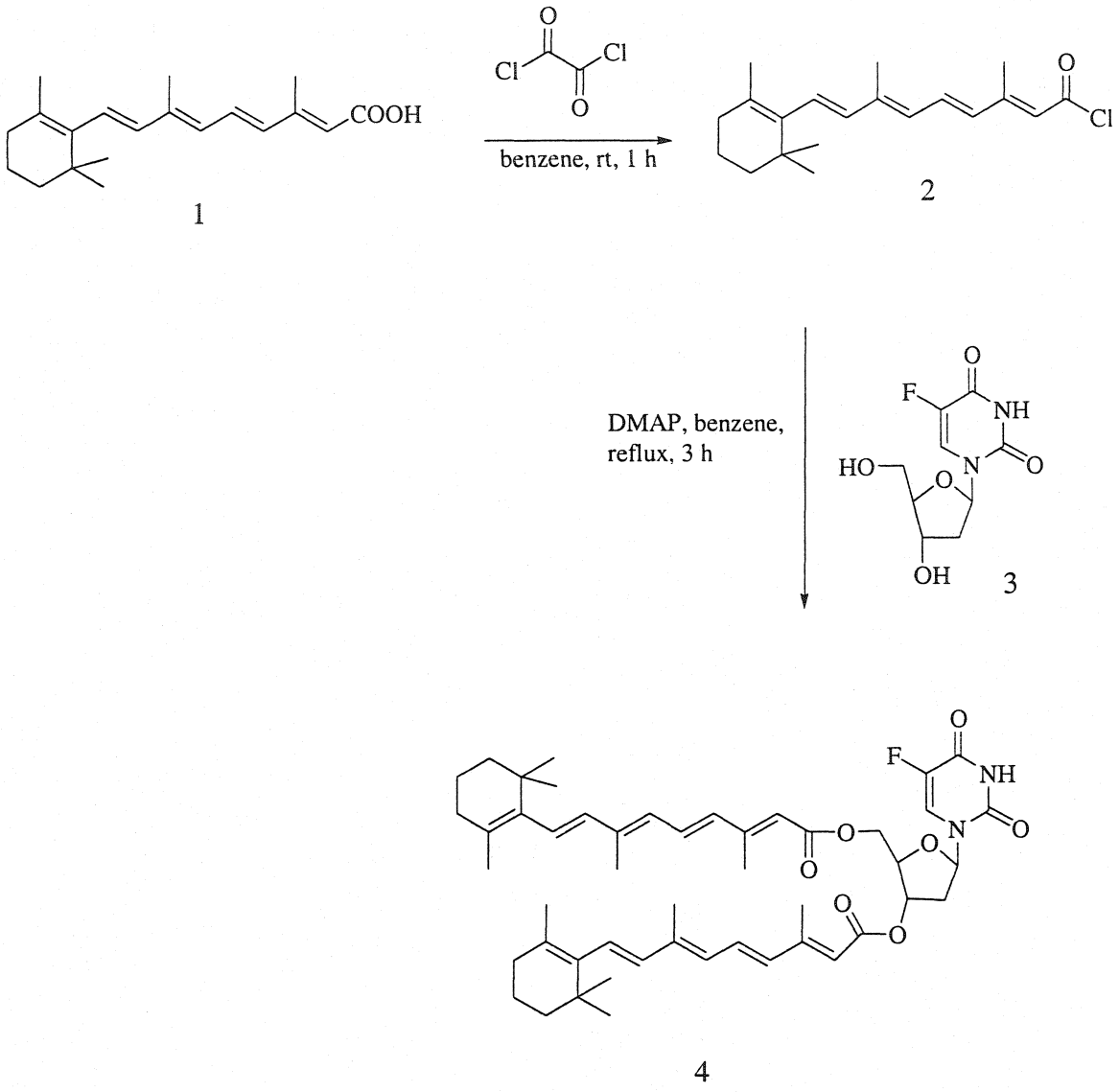
3.2 Chemical synthesis

3.2.1 Synthesis of retinoyl chloride (Compound 2)

The reaction of retinoic acid with oxalyl chloride at ambient temperature resulted in retinoyl chloride. Due to its instability, retinoyl chloride produced as such was used for the subsequent reaction without being separated from the reaction mixture.

3.2.2 Synthesis of 3', 5'-di-*O*-retinoyl-FUdR (Compound 4)

Retinoyl chloride (2) generated above reacted with FUdR at a molar ratio of 1:2 using DMAP as a catalyst to obtain 3', 5'-di-*O*-retinoyl-FUdR, which appeared as a yellow solid as shown in Scheme 3.1.



Scheme 3.1. Synthesis of 3', 5'-di-*O*-retinoyl-FUdR

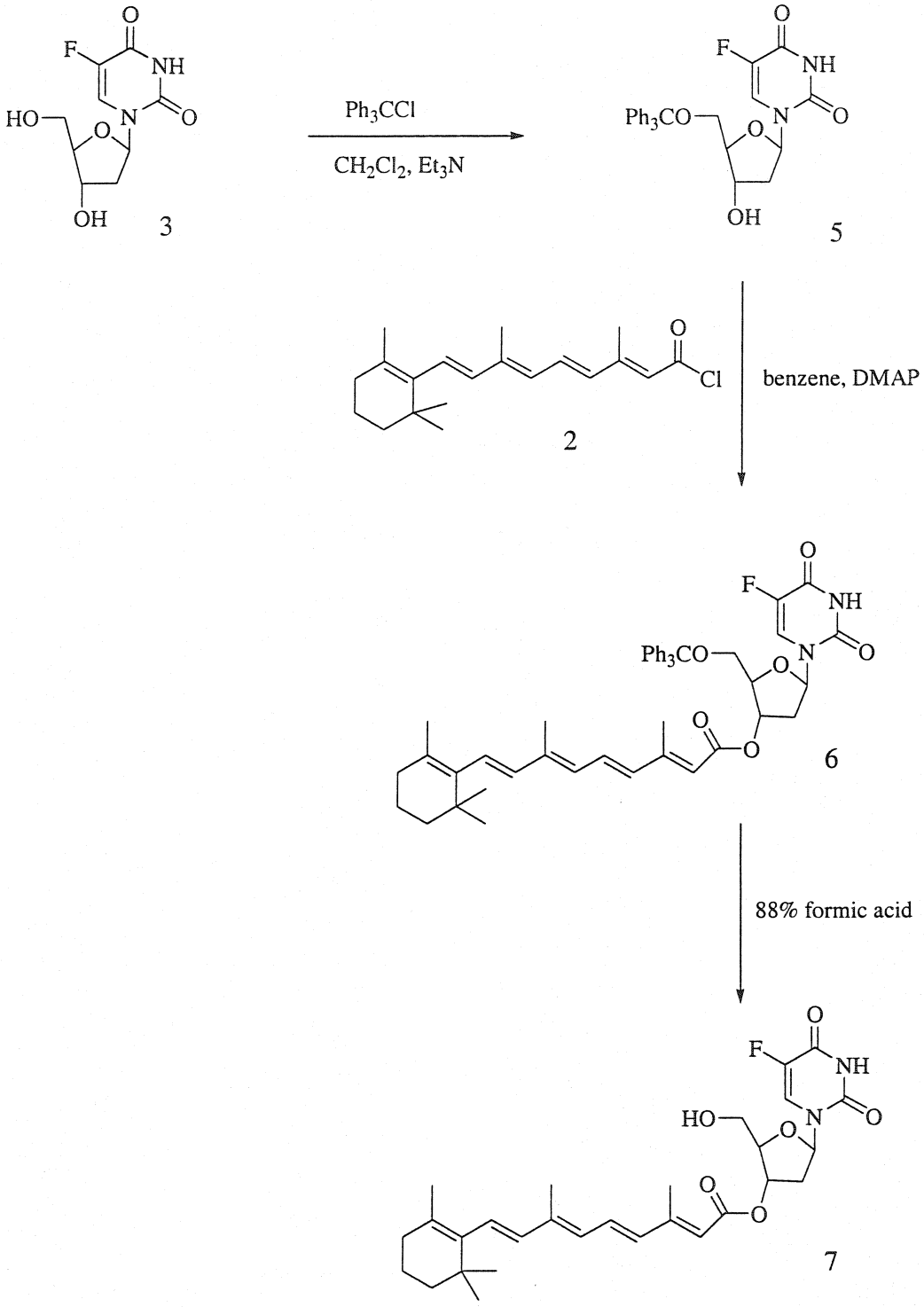
3.2.3 Synthesis of 5'-*O*-triphenylmethyl-FUdR (Compound 5), 3'-*O*-retinoyl-5'-*O*-triphenylmethyl-FUdR (Compound 6), and 3'-O-retinoyl-FUdR (Compound 7)

FUdR possesses two hydroxyl groups on its sugar moiety with 5'-OH (primary alcohol) being more reactive than 3'-OH (secondary alcohol). In order to prepare 5'-*O*-retinoyl-FUdR, we first tried to condense FUdR with retinoyl chloride as with the preparation of 3', 5'-di-*O*-retinoyl-FUdR as shown in Scheme 3.1 with exception of a molar ratio of 1:1 (retinoyl chloride:FUdR). However, the chemical yield of 5'-*O*-retinoyl-FUdR was less than 10%. The major product was still 3', 5'-di-*O*-retinoyl-FUdR and the amount of 3'-*O*-retinoyl-FUdR was undetectable. It was then concluded that protection of 5'-OH or 3'-OH was necessary in order to obtain 3'-*O*-retinoyl-FUdR or 5'-*O*-retinoyl-FUdR.

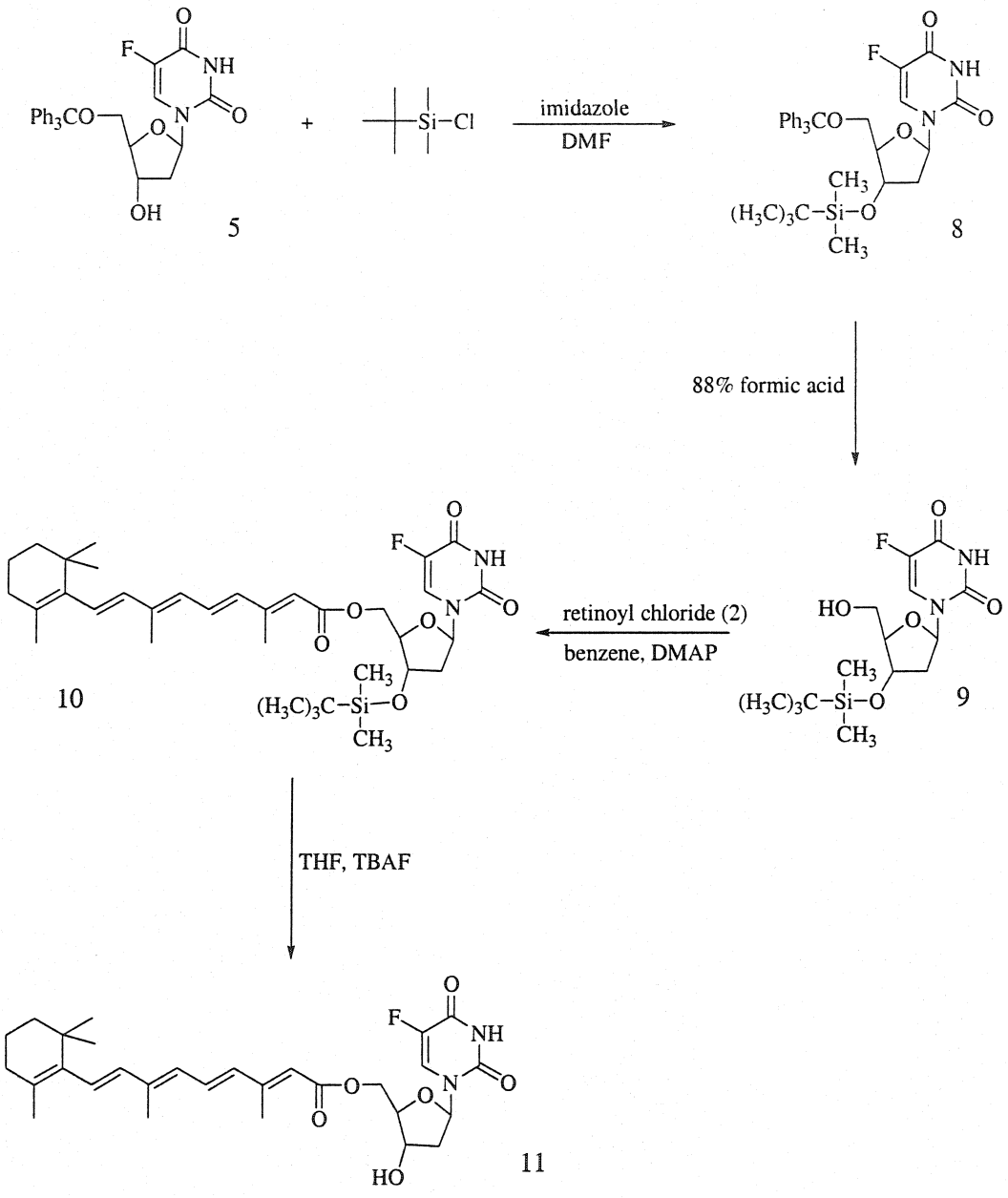
To prepare 3'-*O*-retinoyl-FUdR, FUdR was treated with chlorotriphenylmethane in the presence of triethylamine and DMAP (Chaudary *et al.* 1979) to obtain mainly 5'-*O*-triphenylmethyl-FUdR (5) which was then allowed to react with retinoyl chloride to afford 3'-*O*-retinoyl-5'-*O*-triphenylmethyl-FUdR (Compound 6). The latter was hydrolyzed with 88 % formic acid (Bessodes *et al.* 1986) to yield 3'-*O*-retinoyl-FUdR.

3.2.4 Synthesis of 3'-*O*-*tert*-butyldimethylsilyl-5'-*O*-triphenylmethyl-FUdR (Compound 8), 3'-*O*-*tert*-butyldimethylsilyl-FUdR (Compound 9), 3'-*O*-*tert*-butyldimethylsilyl-5'-*O*-retinoyl-FUdR (Compound 10), and 5'-*O*-retinoyl-FUdR (Compound 11)

Since 5'-OH is more reactive than 3'-OH, introducing the second protective group was deemed necessary for the preparation of 5'-*O*-retinoyl-FUdR and a two-step protection as shown in Scheme 3.3 was employed. 5'-*O*-Triphenylmethyl-FUdR prepared as above was first treated with *tert*-butyldimethylsilyl chloride in the presence of imidazole to obtain 5'-*O*-triphenylmethyl-3'-*O*-silylated FUdR (Compound 8). The triphenylmethyl group at the 5'-position was removed with 88 % formic acid resulting in 3'-*O*-*tert*-butyldimethylsilyl-FUdR (Compound 9). Next, reaction of retinoyl chloride with compound 9 resulted in 5'-*O*-retinoyl-3'-*O*-butyldimethylsilyl-FUdR (Compound 10). Removal of the silyl group with anhydrous tetrabutylammonium fluoride (TBAF) yielded 5'-*O*-retinoyl-FUdR.



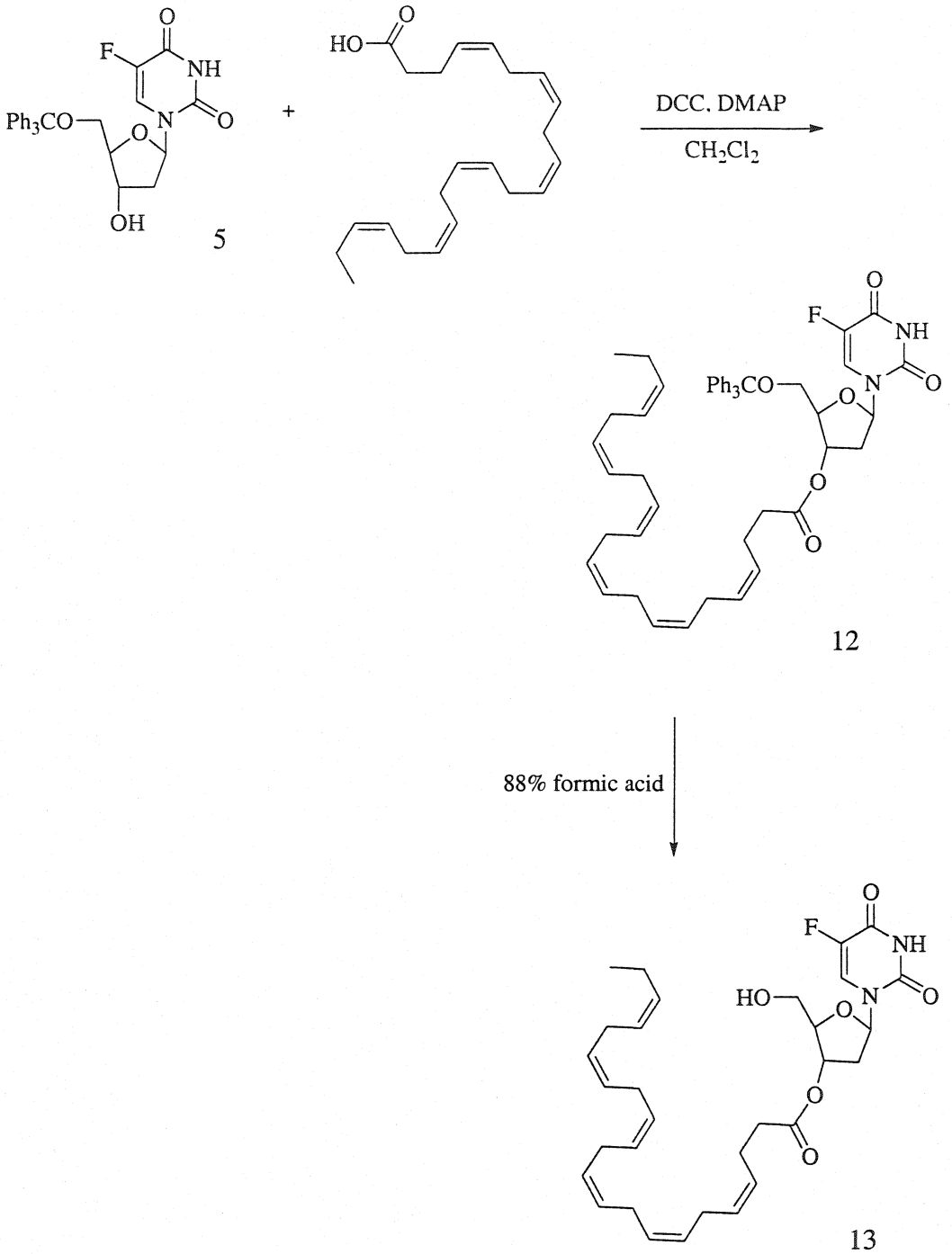
Scheme 3.2. Synthesis of 3'-*O*-retinoyl-FUdR



Scheme 3.3. Synthesis of 5'-*O*-retinoyl-FUdR

3.2.5 Synthesis of 3'-*O*-DHA-5'-*O*-triphenylmethyl-FUdR (Compound 12) and 3'-*O*-DHA-FUdR (Compound 13)

To prepare 3'-*O*-DHA-FUdR, FUdR was treated with chlorotriphenylmethane in the presence of triethylamine and DMAP (Chaudary *et al.* 1979) to obtain mainly 5'-*O*-triphenylmethyl-FUdR (5) which was then allowed to react with DHA in the presence of DMAP to afford 3'-*O*-DHA-5'-*O*-triphenylmethyl-FUdR (Compound 12). The latter was hydrolyzed with 88 % formic acid (Bessodes *et al.* 1986) to yield 3'-*O*-DHA-FUdR as shown in Scheme 3.4.



Scheme 3.4. Synthesis of 3'-*O*-DHA-FUdR

3.3 Isolation of LDL from human plasma

Human LDL (density 1.020-1.063 g/mL) was isolated by ultracentrifugation of fresh human plasma according to a published method (Schumaker *et al.* 1986). Briefly, phenylmethylsulfonylfluoride (PMSF) was added to the fresh human plasma obtained from the Canadian Blood Services (St. John's, Newfoundland) up to 0.015 % in a final concentration as a protease inhibitor to prevent the degradation of plasma proteins. Potassium bromide (KBr) was used to adjust the density of plasma to 1.020 g/mL. The plasma was then centrifuged at 4 °C at 40,000 rpm for 24 h using a Beckman L8-M ultracentrifuge and a 60 Ti or 75 Ti rotor. After centrifugation, VLDL that floated to the top of the centrifuge tubes as a white layer was removed carefully using a disposable plastic pipette. The rest of the plasma was re-suspended and the density was adjusted to 1.063 g/mL again using KBr. The mixture was then subjected to centrifugation at 40,000 rpm at 4 °C for 24 h. LDL floated to the top of the centrifuge tubes as a yellow layer was collected carefully using a disposable plastic pipette. The collected LDL was dialyzed overnight at 4 °C against a dialysis buffer containing 0.3 mM EDTA, 150 mM NaCl, and 50 mM Tris (pH 7.4) to remove KBr. LDL after dialysis was sterilized by filtration through a sterile 0.20 µm Corning® syringe filter and was stored at -20 °C in autoclaved 1.5 mL Eppendorf tubes.

3.4.1 Determination of the protein concentration in LDL preparations

The protein concentration of the LDL preparations was determined using the method of Bradford (Bradford 1976) with bovine serum albumin (BSA) as standard. Bio-Rad Protein Assay Dye Reagent Concentrate was diluted 5 times in saline prior to use. Briefly, four standard BSA solutions in saline, 0.05, 0.1, 0.2 and 0.4 mg/mL, were prepared and stored in 1.5 mL autoclaved Eppendorf tubes. The LDL isolated above was diluted 10 times in saline prior to the assay. The assay was preformed in a 96-well microtitre plate. Ten μL of the standard BSA solutions and the diluted LDL solution were pipetted in quadruplicate into a 96-well microtitre plate. To these protein solutions was added 200 μL of the diluted dye reagent. The contents were mixed by vortexing for 5 min. Immediately after vortexing, the absorbance at 595 nm was measured on a Bio-Rad Model 550 microplate reader interfaced with an EM PaC 386 computer. The standard curve was constructed by plotting the absorbance versus the concentration of the standard BSA solutions. The protein concentration of the LDL preparation was then determined using the standard curve.

3.5 Incorporation of the prodrugs into LDL

3.5.1 Preparation of 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and 3',5'-di-*O*-retinoyl-FUdR microemulsions

The 3'-*O*-retinoyl-FUdR microemulsion, 5'-*O*-retinoyl-FUdR microemulsion, 3'-*O*-DHA-FUdR microemulsion and 3',5'-di-*O*-retinoyl-FUdR microemulsion were prepared using the method previously developed in our lab (Xiao *et al.* 1999) with minor

modification. Briefly, *L*-α-phosphatidylcholine dipalmitoyl (DPPC) (12 mg), *DL*-α-phosphatidylethanolamine dipalmitoyl (DPPE) (10 mg), seal oil (20 mg), and 10 mg of the respective compound (3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR or 3',5'-di-*O*-retinoyl-FUdR) were dissolved in 10 mL of chloroform. The chloroform solution was dried with a gentle stream of nitrogen. The residue was re-suspended in 10 mL of saline. The suspension was sonicated for 2 h with a Virosonic Cell Disrupter Model 16-850 at 40-50 watts while being kept in salt-ice bath (-10 °C) under a nitrogen stream. The mixture was then subjected to ultracentrifugation at 40,000 rpm for 7-8 h at 4 °C using a Beckman L8-M ultracentrifuge with a 60 Ti or 75 Ti rotor. The microemulsion floated to the top of centrifuge tubes was collected carefully by a disposable plastic pipette and was stored at -20 °C in 1.5 mL Eppendorf tubes.

3.5.2 Incorporation of the prodrug 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and 3',5'-di-*O*-retinoyl-FUdR into LDL

The respective microemulsions (3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and 3',5'-di-*O*-retinoyl-FUdR) obtained above were incubated with the LDL preparation at 37 °C for 7-8 h at a molar ratio of 1000:1 (prodrug to LDL). The LDL loaded with 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR or 3',5'-di-*O*-retinoyl-FUdR was then separated by ultracentrifugation at 40,000 rpm for 24 h at 4 °C using a Beckman L8-M ultracentrifuge with a Beckman 60 Ti or 75 Ti rotor. The LDL layer spun down to the bottom of centrifuge tubes was collected carefully and sterilized

using a sterile 0.20 μm Corning® syringe filter. The LDL/prodrug preparation was stored in autoclaved 1.5 mL Eppendorf tubes at -20 °C.

3.6 Determination of the prodrug concentration in the prodrug/LDL complex

The respective prodrugs (3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3', 5'-di-*O*-retinoyl-FUdR and 3'-*O*-DHA-FUdR) in the prodrug/LDL complexes were first extracted with chloroform. Briefly, 1 mL of $CHCl_3$ was added in a 1.5 mL Eppendorf tube containing 50 μL of prodrug/LDL complex. The contents were mixed by vortexing for 2-3 min. The chloroform layer was then spun down by centrifugation using an Eppendorf® tabletop centrifuge at 5,000 rpm for 3 min. The LDL layer floated to the top was pipetted and transferred to an Eppendorf tube and then extracted with chloroform two more times in order to extract as much prodrug as possible. The chloroform extracts were combined and dried under a gentle stream of nitrogen. The residue was re-dissolved in 50 μL of chloroform. HPLC analysis was performed by injecting 10 μL of the sample. The concentration of 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3', 5'-di-*O*-retinoyl-FUdR and 3'-*O*-DHA-FUdR was then determined using an *HP*® 1100 HPLC with a Phenomenex® C18 reverse phase column (15 cm x 3.9 mm, particle size 5 μm). Methanol/H_2O (9/1, v/v) at 1.0 mL/min was used as the mobile phase with UV detection at 267 nm.

The standard prodrug solutions (0.2 mg/mL, 0.4 mg/mL, 0.8 mg/mL, and 1.6 mg/mL) were prepared in chloroform. HPLC analysis was performed by injecting 10 μL

of each sample with methanol/H_2O (9/1, v/v) at 1.0 mL/min as the mobile phase and UV detection at 267 nm. The area under the curve of the respective peak was calculated. The analysis for each sample was repeated three times. A standard curve was obtained by plotting the concentrations of the standard samples versus the corresponding peak area. The analysis was repeated three times for each sample and the results were expressed as the average of three analysis. The standard curve was used to determine the concentration of prodrug in the prodrug/LDL complexes.

3.7 Preparation of prodrugs in ethanol and FUdR in PBS stock solutions

Twenty mM of 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and 3',5'-di-*O*-retinoyl-FUdR were prepared in 99.9% ethanol as stock solution. FUdR (20 mM) was prepared in phosphate-buffered saline (PBS) (2.67 mM KCl, 1.47 mM KH_2PO_4, 138 mM NaCl and 8.10 mM $NaHPO_4 \cdot 7H_2O$). PBS was freshly prepared and autoclaved prior to use. The stock solutions were then sterilized using a sterile 0.20 μm Corning® syringe filter and stored at -20°C in 1.5 mL autoclaved Eppendorf tubes. The working solutions were obtained by diluting the stock solutions in DMEM or RPMI-1640 medium to the appropriate concentration prior to use. In no case throughout the experimental assays did the final ethanol concentration of the prodrugs/ethanol working solution exceed 3 % or did the final LDL protein concentration of the prodrugs/LDL working solution exceed 0.35 mg/mL.

3.8 Cell culture

Five cancer cell lines which contain different levels of LDL receptors were used in this study: two human breast cancer cell lines, MCF7 and MB231, one human cervical cancer cell line, Hela, one human hepatic cancer cell line, HepG2, and one leukemia cancer cell line, CEM/T4. These cancer cell lines were grown in a 25 cm^2 Corning® flask at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. MCF-7, MB231, HepG2 and Hela cells were maintained as monolayer in DMEM medium supplemented with 10% FBS, 1% non-essential amino acids, 2 mM *L*-glutamin, 50 IU penicillin/mL and 50 μg/mL streptomycin. CEM/T4 cells were cultured in suspension in RPMI-1640 medium supplemented with 10% FBS.

These cancer cell lines were cultured according to the routine culture procedure. During subculturing, the suspension of CEM/T4 cells in a 4 mL medium in a 25 cm^2 Corning® flask were transferred into a 15 mL conical centrifuge tube. CEM/T4 cells were harvested by centrifugation using a Thermo IEC tabletop centrifuge at 1200 rpm for 5 min. The medium layer was then carefully removed by a sterile pipette. The cell pellet was re-suspended in 12 mL DMEM medium and transferred 3 or 4 mL into 25 cm^2 Corning® flask for subculturing. The doubling time for CEM/T4 cell was 40 h under these conditions. The medium was changed every two days to maintain cell growth.

MCF-7, MB231, HepG2 and Hela cells were attached on the bottom of a 25 cm^2 Corning® flask during culture. Trypsin-EDTA solution was used to detach the cells from a flask. Briefly, the medium was removed upon the cells reached subconfluence, and the

cell monolayer was washed with 10 mL PBS. After removal of PBS, the 3 mL trypsin-EDTA solution was added to the flask. The flask was then placed back into a humidified incubator with an atmosphere of 5% CO_2 at 37 °C for 5 min in order to detach the cells from the flask. The cells suspension in the trypsin-EDTA solution was transferred into a 15 mL centrifuge tube and the trypsin-EDTA solution was removed by centrifugation at 1,200 rpm for 5 min as described previously. The cells pellet was re-suspended with 10 mL PBS in order to remove as much trypsin-EDTA solution as possible. Once PBS solution was removed by centrifugation, the cell pellet was re-suspended in 12 mL medium. The cell suspension was then reseeded into 3-4 flasks. Under these conditions, the doubling times for MB231, MCF-7, Hela and HepG2 cells were 35, 30, 48 and 17 h, respectively. During normal culture, the medium in a flask was changed every two days.

3.9 Assessment of cytotoxicity using Tetrazolium (MTT) assay

Cytotoxicity test was performed using a MTT assay as described by Carimichael (Carmichael *et al.* 1987, Mosmann 1983, Li *et al.* 1999) with minor modification. This assay is based on the reduction of MTT to a colored formazen product by mitochondrial dehydrogenase present only in living, metabolically active cells. A 10 mg/mL stock MTT solution was prepared in PBS. The stock MTT solution was filtered using a sterile 0.20 μm Corning® syringe filter to remove any insoluble residue and stored at 4 °C in a autoclaved 15 mL centrifuge tube. The MTT working solution (1.0 mg/mL) was prepared by diluting the stock solution in PBS prior to use.

Since the prodrug solution was prepared in ethanol, the cytotoxicity of ethanol was assessed in Hela, MCF7, MB231 and HepG2 cell lines, respectively, to ensure that the amount of ethanol used was not toxic and all cell death observed with the prodrug/ethanol solutions was attributed to prodrug alone. In addition, the cytotoxicity of LDL alone was also determined. Briefly, 10^4 cells/100 μL/well of cell suspension in DMEM were plated in quadruplicate into a 96-well microtitre plate and were kept at 37 °C in a 5% CO_2 humidified incubator for 24 h. The medium was then removed and replaced with 100 μL medium containing 1.5%, 3% and 6% of ethanol or 100 μL medium containing 0.125 mg/mL, 0.25 mg/mL and 0.50 mg/mL of LDL (protein concentration of LDL). The plate was returned to the incubator for 72 h. Cells in DMEM were also cultured under the same conditions as control. At the end of 72 h, the culture medium was removed, and 100 μL of a 1.0 mg/mL MTT solution in PBS was added to each well and the plate was returned to the incubator for another 4 h. The supernatant was then removed by aspiration and 100 μL dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The plate was placed on a plate shaker for 10 min at room temperature to ensure that all the crystals had been dissolved in DMSO. The absorbance was recorded on a Bio-Rad Model 550 microplate reader interfaced with an EM PaC 386 computer at 570 nm using DMSO as a blank measured at 630 nm. Data analysis was carried out using the Bio-Rad Microplate Manager®/ PC version 4 software program. Tests were repeated at least twice at different times for each cell line.

The cytotoxicity of 3',5'-di-*O*-retinoyl-FUdR/LDL, 3'-*O*-retinoyl-FUdR/LDL and 5'-*O*-retinoyl-FUdR/LDL in Hela cell line and the cytotoxicity of 3'-*O*-retinoyl-

FUdR/LDL and 3'-*O*-DHA-FUdR/LDL in MCF7 and MB231 cell lines were also performed using the MTT assay. The respective prodrug/LDL working solutions were prepared by diluting the prodrug/LDL prepared as described in Section 3.5 in DMEM to appropriate concentrations (8-10 concentrations of the prodrugs were tested, with the highest concentration being between 380 μM and 190 μM, depending on the individual prodrug/LDL, from which 7-9 serial 1:1 dilutions were made). Briefly, 10^4 cells/100 μL/well of cell suspension in DMEM was seeded in quadruplicate into 96-well microtitre plates and incubated at 37 °C in a 5% CO_2 humidified incubator for 24 h. The medium was then removed and replaced with 100 μL medium containing appropriate concentrations of the prodrug/LDL (8-10 concentrations as described earlier). The plates were returned to the incubator for 24 h, 48 h or 72 h, respectively. Cells in the presence of cultural medium only (without the prodrug/LDL treatment) and medium containing no cells were cultured under the same conditions. At the end of each time interval (24, 48 or 72 h), the culture medium was removed, and 100 μL of a 1.0 mg/mL MTT solution in PBS was added to each well. After additional 4 h incubation at 37 °C, the supernatant was removed by aspiration and 100 μL of DMSO was added to dissolve the formazan crystals. The plates were then placed on a plate shaker for 10 min at room temperature to ensure that all the crystals have been dissolved in DMSO. The absorbance was recorded on a Bio-Rad Model 550 microplate reader interfaced with an EM PaC 386 computer at 570 nm using DMSO as blank measured at 630 nm. Data analysis was carried out using the Bio-Rad Microplate Manager®/ PC version 4 software program. Cell survival (%) was calculated as the fraction of drug treated samples relative to control (cells containing

no drug) or mean absorbance of the treated wells divided by the mean absorbance of the control wells x 100. The IC_{50} was defined as the drug concentration causing 50% inhibition of cell growth as compared to the untreated cells. Tests were repeated at least twice at different times for each cell line and each compound.

The cytotoxicity of respective prodrugs (3',5'-di-*O*-retinoyl-, 3'-*O*-retinoyl and 5'-*O*-retinoyl-FUdR, and 3'-*O*-DHA-FUdR) in ethanol was also performed in Hela, MCF7, MB231 and HepG2 cell lines using the MTT assay. The prodrugs/ethanol working solutions were prepared by diluting the 20 mM prodrugs/ethanol stock solution prepared according to Section 3.7 in DMEM medium to approximately similar concentration to the prodrugs/LDL working concentration. Briefly, 10^4 cells/100 μL/well of cell suspension was plated in quadruplicate into 96-well microtitre plates. After 24 h seeding at 37 °C in a 5% CO_2 humidified incubator, the media were removed and replaced with 100 μL-medium containing appropriate concentrations of prodrug/ethanol (8-10 concentrations). The plates were incubated for 24 h, 48 h or 72 h at 37 °C in a 5% CO_2 humidified incubator. Control cells without prodrug/ethanol treatment and blank wells containing medium with no cells were cultured under the same conditions. At the end of each time point, the culture medium was removed, and 100 μL of a 1.0 mg/mL MTT solution in PBS was added to each well. After additional 4 h incubation at 37 °C, the supernatant was removed by aspiration and 100 μL DMSO was added to dissolve the formazan crystals. Plate was then placed on a plate shaker for 10 min at room temperature to ensure that all the crystals have been dissolved in DMSO. The absorbance was recorded on a Bio-Rad Model 550 microplate reader interfaced with an EM PaC 386

computer at 570 nm using DMSO as a blank measured at 630 nm. Data analysis was carried out using the Bio-Rad Microplate Manager®/ PC version 4 software program. Cell survival (%) was calculated as the fraction of cell alive relative to control (mean absorbance in the treated wells/mean absorbance in the control wells x 100). The IC_{50} (Inhibitory Concentration 50%) was defined as the drug concentration causing 50% inhibition of cell growth as compared to the untreated cells. Tests were repeated at least twice at different times for each cell line and each compound.

3.10 *In vitro* differentiation test in CEM/T4 cell line

The differentiating activity (Balzarini *et al.* 1995; Manfredini *et al.* 1997) of retinoic acid, FUdR, 3'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and 5'-*O*-retinoyl-FUdR was examined in leukemia cell line, CEM/T4. Briefly, the differentiation was characterized by superoxide production after stimulation with phorbol 12-myristate 13-acetate (PMA). Nitro-blue tetrazolium (NBT) is a pale yellow dye that is reduced by superoxide to a dark blue water-insoluble fromazan. In the NBT reduction assay, 10^6 CEM/T4 cells per 1.0 mL of cell suspension in RPMI-1640 culture medium were seeded in triplicate in a 24-well plate. One hundred μL of the respective prodrugs in ethanol in various concentrations (0.01 μM, 0.1 μM and 1 μM) was added to each well. The plates were then incubated for 72 h at 37 °C in a 5% CO_2 humidified incubator, following which 0.5 mL of a freshly prepared NBT solution (2.0 mL/mg, containing PMA at 200 ng/mL in PBS, pH 7.2) was added to each well. The mixture was further incubated for 40

min in a shaker kept in a water bath at 37 °C. The plate was cooled on ice for 5 min to stop the reaction. The cell suspension was transferred into 1.5 mL Eppendorf tubes. The cells were spun down at 1,000 rpm for 10 min at 4 °C, following which the supernatant was carefully removed. The cell pellet was re-suspended in 0.5 mL of RPMI-1640 medium, kept on ice and protected from light until the percentage of cells containing dark blue formazan precipitates was determined in a hemocytometer under an inverted light microscopy. For each sample, at least a total of 200 cells were scored, and the percentage of formazan-positive (NBT-positive) cells was calculated. Cells alone (in the absence of any prodrugs) were examined and used as control. It was found that the control contained not more than 10% NBT-positive cells. Experiments were repeated at least twice at different times for each compound.

Chapter 4 Results and Discussion

4.1 Retinoyl chloride (Compound 2)

To a solution of retinoic acid 1 (106 mg, 0.36 mmol) in freshly dried benzene (10 mL) was added oxalyl chloride (46 μL, 0.54 mmol). The reaction mixture was stirred at ambient temperature and protected from light for 2 h under positive argon pressure. The resultant mixture (dark yellow) was then subjected to evaporation on a rotary evaporator, and the residue was re-dissolved in freshly dried benzene (5 mL). Retinoyl chloride was used immediately for the next reaction.

4.2 3', 5'-Di-*O*-retinoyl-FUdR (Compound 4)

To a solution of FUdR (85.4 mg, 0.333 mmol), DMAP (85.52 mg, 0.70 mmol) in 7 ml dry benzene, kept in an ice-bath, was added retinoyl chloride (0.667 mmol) in 5 ml dry benzene slowly. The reaction mixture was stirred in ice-bath for 1 h, then refluxed for 3 h. After cooling down to the room temperature, the mixture was diluted with 30 ml ethyl acetate, then washed by saturated $NaHCO_3$ (2 x 20 ml), water (2 x 15 ml), and dried with Na_2SO_4. After removal of the solvent *in vacuo*, the residue was chromatographed on a silica gel column using gradient elution (from hexanes to hexanes-ethyl acetate, 1.5:1, v/v) to afford 3',5'-di-*O*-retinoyl-FUdR as a yellow solid (200 mg). The R_f value was 0.59 in ethyl acetate:hexanes 1:1.5 (v/v). The chemical yield was determined to be 72%.

The melting point of 3', 5'-di-*O*-retinoyl-FUdR was in the range of 97.5-99.5 °C. ^{1}H NMR ($CDCl_3$) data: δ 1.04 (s, 12H, 4 x cyclohexene-Me), 1.30-1.70 (m, 8H, cyclohexene), 1.73 (s, 6H, 2 x cyclohexene-Me), 2.02-2.23 (m, 11H, H-2', retinoyl-Me, cyclohexene), 2.39 and 2.42 (two s, 6H total, 2 x retinoyl-Me), 2.53-2.61 (m, 1H, H-2'), 4.32-4.50 (m, 3H, H-4' and H-5'), 5.29-5.36 (m, 1H, H-3'), 5.76-5.79 (m, 2H, retinoyl), 6.14-6.24 (m, 9H, H-1', retinoyl), 7.02-7.13 (m, 2H, retinoyl), 7.80 (d, J_{HF}= 6.5 Hz, 1H, H-6). Referring to the structure on page 33 for numbering.

4.3 5'-*O*-Triphenylmethyl-FUdR (Compound 5)

A mixture of FUdR (123 mg, 0.5 mmol), DMAP (10 mg, 0.08 mmol), TEA (153 mg, 1.1 mmol), and chlorotriphenylmethane (153 mg, 0.55 mmol) in dry dichloromethane (5 mL) was stirred overnight at room temperature. The resultant mixture was concentrated and the residue was purified by flash chromatography (continuous gradient from dichloromethane to ethyl acetate) to yield 5'-*O*-triphenylmethyl-FUdR (213 mg). The product was then crystallized from dichloromethane-hexane. After purification, the chemical yield was found to be 87%. The melting point of the product was 116.5-118.5 °C which is comparable with reported melting point of 116 °C (Thomas and Montgomery 1962). The R_f value in ethyl acetate was 0.81. ^{1}H NMR ($CDCl_3$) data: δ 2.20-2.24 and 2.41-2.60 (two m, 2H total, H-2'), 3.42-3.47 (m, 2H, H-5'), 4.02-4.07 (m, 1H, H-4'), 4.45-4.55 (m, 1H, H-3'), 6.22-6.24 (m, 1H, H-1'), 7.20-7.41 (m, 15H, phenyl

group), 7.82 (d, J_{HF} = 6.5 Hz, 1H, H-6). Referring to the structure on page 33 for numbering.

4.4 3'-*O*-Retinoyl-5'-*O*-triphenylmethyl-FUdR (Compound 6)

To a solution of 5'-*O*-triphenylmethyl-FUdR (146 mg, 0.30 mmol) and DMAP (61 mg, 0.50 mmol) in 8 mL freshly dried benzene, kept at 10 °C, was added retinoyl chloride (0.33 mmol) in 5 mL freshly dried benzene gradually. The mixture was stirred for one hour at 10 °C, then heated to reflux for 2-3 h. The mixture was cooled to room temperature and washed with water (3×15 mL), then dried with Na_2SO_4. Flash chromatography (ethyl acetate : chloroform = 1:9) of the residue resulted in 3'-*O*-retinoyl-5'-*O*-triphenylmethyl-FUdR (220 mg). After purification, it was found that the chemical yield of 3'-*O*-retinoyl-5'-*O*-triphenylmethyl-FUdR was 94%. The R_f value in ethyl acetate:hexanes 1:1 (v/v) was 0.68. ^{1}H NMR ($CDCl_3$) data: δ 0.98 (s, 6H, 2 X cyclohexene-Me), 1.08-1.34 (m, 4H, cyclohexene), 1.71 (s, 3H, cyclohexene-Me), 2.02-2.12 (m, 5H, retinoyl-Me and cyclohexene), 2.33-2.60 (m, 5H, retinoyl-Me and H-2'), 3.38-3.65 (m, 2H, H-5'), 4.23-4.30 (m, 1H, H-4'), 5.49-5.56 (m, 1H, H-3'), 5.79 (s, 1H, retinoyl), 6.12-6.39 (m, 5H, H-1' and retinoyl), 7.02-7.10 (m, 1H, retinoyl), 7.23-7.50 (m, 15H, phenyl group), 7.88 (d, J_{HF} = 6.5 Hz, 1H, H-6), 8.25 (s, 1H, NH). Referring to the structure on page 33 for numbering.

4.5 3'-*O*-Retinoyl-FUdR (Compound 7)

To a solution of Compound 6 (200 mg) in ethyl acetate (1.5 ml) was added 1.5 ml aqueous 88 % formic acid. The reaction mixture was stirred at room temperature for 1 hour, then diluted with ethyl acetate (25 ml). After organic layer was washed with half-saturated NaCl (2 x 10 ml), saturated $NaHCO_3$ (3 x 8 ml) and water (2 x 10 ml), it was then dried with Na_2SO_4 overnight. After removal of the solvent *in vacuo*, the residue was chromatographed on a silica gel column using gradient elution (0-50 % ethyl acetate in hexanes) to afford the product 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine (7) (120 mg) as a yellow solid. The chemical yield was 90%. The melting point was determined to be 80.5-82.0 °C. The R_f value in ethyl acetate:hexanes 1:1 (v/v) was 0.34. ^{1}H NMR ($CDCl_3$) data: δ 1.06 (s, 6H, cyclohexene-Me), 1.09-1.54 (m, 4H, cyclohexene), 1.77 (s, 3H, cyclohexene-Me), 1.98-2.11 (m, 5H, retinoyl-Me and cyclohexene), 2.30-2.54 (m, 5H, retinoyl-Me and H-2'), 3.95-4.04 (m, 2H, H-5'), 4.15-4.20 (m, 1H, H-4'), 5.40-5.45 (m, 1H, H-3'), 5.80 (m, 1H, retinoyl), 6.14-6.40 (m, 5H, retinoyl and H-1'), 7.04-7.09 (m, 1H, retinoyl), 8.10 (d, J_{HF} = 7.0 Hz, 1H, H-6), 8.64 (brs, 1H, NH). Referring to the structure on page 33 for numbering.

4.6 3'-*O*-*tert*-Butyldimethylsilyl-5'-*O*-triphenylmethyl-FUdR (Compound 8)

To a solution of Compound 5 (400 mg, 0.819 mmol) in dry dimethylformamide (DMF) (1.5 mL) was added imidazole (134 mg, 1.98 mmol) and *tert*-butyldimethylsilyl

chloride (147 mg, 0.98 mmol). The reaction mixture was stirred for 5 h at room temperature, then diluted with 80 mL dichloromethane. The resultant mixture was washed with water (3 × 40 mL), and was then dried with Na_2SO_4. After filtration and removal the solvent, flash chromatography (continuous gradient from hexanes to hexanes/ethyl acetate, 1:1, v/v) was carried out to obtain 3'-*O-tert*-butyldimethylsilyl-5'-*O*-triphenylmethyl-FUdR (410 mg). It was found that the chemical yield was 83%. The melting point was found to be 144.5-146.5 °C and the R_f value of the product was 0.75 in ethyl acetate:hexane 1:1 (v/v). ^1H NMR ($CDCl_3$) data: δ 0.22 (s, 6H, CH_3), 0.86 (s, 9H, CH_3), 2.12-2.20 and 2.36-2.41 (two m, 2H total, H-2'), 3.37-3.42 (m, 2H, H-5'), 3.97-4.02 (m, 1H, H-4'), 4.44-4.47 (m, 1H, H-3'), 6.22-6.25 (m, 1H, H-1'), 7.22-7.32 (m, 9H, phenyl), 7.33-7.38 (m, 6H, phenyl), 7.83 (d, J_{HF} = 6.5 Hz, 1H, H-6), 8.24 (brs, 1H, NH). Referring to the structure on page 33 for numbering.

4.7 3'-*O-tert*-Butyldimethylsilyl-FUdR (Compound 9)

To a solution of Compound 8 (400 mg, 0.67 mmol) in ethyl acetate (2 mL) was added 88% formic acid (3 mL). The reaction mixture was stirred at room temperature for 1 h, then diluted with ethyl acetate (40 mL). The mixture was washed with half-saturated NaCl solution (2×10 mL), saturated $NaHSO_4$ (2×10 mL) and water (2×10 mL). It was then dried with Na_2SO_4 and concentrated. The residue was subjected to flash chromatography eluted with continuous gradient from hexane to ethyl acetate/hexane 1:1, v/v) to obtain 3'-*O-tert*-butyldimethylsilyl-FUdR as a white solid (114 mg). The chemical

yield of 3'-*O-tert*-butyldimethylsilyl-FUdR prepared was 48%. The melting point of 3'-*O-tert*-butyldimethylsilyl-FUdR was 173.5-175.0 °C and the R_f value in ethyl acetate:hexane 1:1 (v/v) was 0.44. ^{1}H NMR ($CDCl_3$) data: δ 0.21 (s, 6H), 0.87 (s, 9H), 1.89 (brs, 1H, -OH), 2.18-2.22 and 2.24-2.32 (two m, 2H total, H-2'), 3.79-3.82 (m, 1H, H-4'), 3.96-4.00 (m, 2H, H-5'), 4.48-4.52 (m, 1H, H-3'), 6.21-6.25 (m, 1H, H-1'), 8.95 (d, J_{HF} = 6.2 Hz, 1H, H-6), 8.22 (s, 1H, NH). Referring to the structure on page 33 for numbering.

4.8 3'-*O-tert*-Butyldimethylsilyl-5'-*O*-retinoyl-FUdR (Compound 10)

To a stirred solution of Compound 9 (100 mg, 0.28 mmol), DMAP (61 mg, 0.5 mmol) in dry benzene (7 mL), kept at 10 °C, was added retinoyl chloride (0.30 mmol) in 5 mL benzene dropwise. After the reaction mixture was stirred at 10 °C for 1 h, it was heated to reflux for 2 h. After cooling down to room temperature, 20 mL ethyl acetate was added. The mixture was washed with water (2 × 20 mL), then dried with Na_2SO_4. Flash chromatography eluted with continuous gradient from hexanes to ethyl acetate/hexanes (1:1, v/v) of the residue resulted in 3'-*O-tert*-butyldimethylsilyl-5'-*O*-retinoyl-FUdR (110 mg), which appeared as a yellow solid. The chemical yield was 60%. The R_f value of the product in ethyl acetate:hexanes 1:1 (v/v) was 0.80. ^{1}H NMR ($CDCl_3$) data: δ 0.25 (s, 6H), 0.94 (s, 9H), 1.04 (s, 6H, 2 x cyclohexene-Me), 1.44-1.68 (m, 4H, cyclohexene), 1.72 (s, 3H, cyclohexene-Me), 1.94-2.08 (m, 5H, retinoyl-Me and cyclohexene), 2.35-2.47 (m, 5H, H-2' and retinoyl-Me), 4.06-4.24 (m, 2H, H-5'), 4.28-

4.48 (m, 2H, H-4', H-3'), 5.82-5.84 (m, 1H, retinoyl), 6.15-6.40 (m, 5H, H-1' and retinoyl), 7.03-7.08 (m, 1H, retinoyl), 7.89 (d, J_{HF} = 6.1 Hz, 1H, H-6), 8.12 (s, brs, 1H, NH). Referring to the structure on page 33 for numbering.

4.9 5'-*O*-retinoyl-FUdR (Compound 11)

Compound 10 (58 mg, 0.088 mmol) in dry tetrahydrofuran (2.2 ml) was treated with anhydrous tetrabutylammonium fluoride (76 mg, 0.29 mmol) and acetic acid (9.37 µl, 0.164 mmol) for 3.5 h at room temperature. The solution was then concentrated to dryness *in vacuo*. Flash chromatography (gradient, 0-3% v/v methanol in chloroform) of the residue yielded a yellow solid compound 9 (41 mg). The chemical yield obtained was 88%. The R_f value in ethyl acetate:hexanes, 1:1 (v/v) was 0.89. ^{1}H NMR ($CDCl_3$) data: δ δ1.03 (s, 6H, cyclohexene-Me), 1.04-1.43 (m, 4H, cyclohexene), 1.72 (s, 3H, cyclohexene-Me), 1.80-2.21 (m, 5H, retinoyl-Me and cyclohexene), 2.22-2.45 (m, 5H, retinoyl-Me and H-2'), 3.27 (s, 1H, -OH), 4.03-4.15 (m, 2H, H-5'), 4.25-4.32 (m, 1H, H-4'), 4.45-4.54 (m, 1H, H-3'), 5.56-5.62 (m, 1H, retinoyl), 6.20-6.40 (m, 5H, retinoyl and H-1'), 7.20-7.28 (m, 1H, retinoyl), 7.94 (d, J_{HF} = 5.6 Hz, 1H, H-6), 8.46 (brs, 1H, NH). Referring to the structure on page 33 for numbering.

4.10 3'-*O*-DHA-5'-*O*-triphenylmethyl-FUdR (Compound 12)

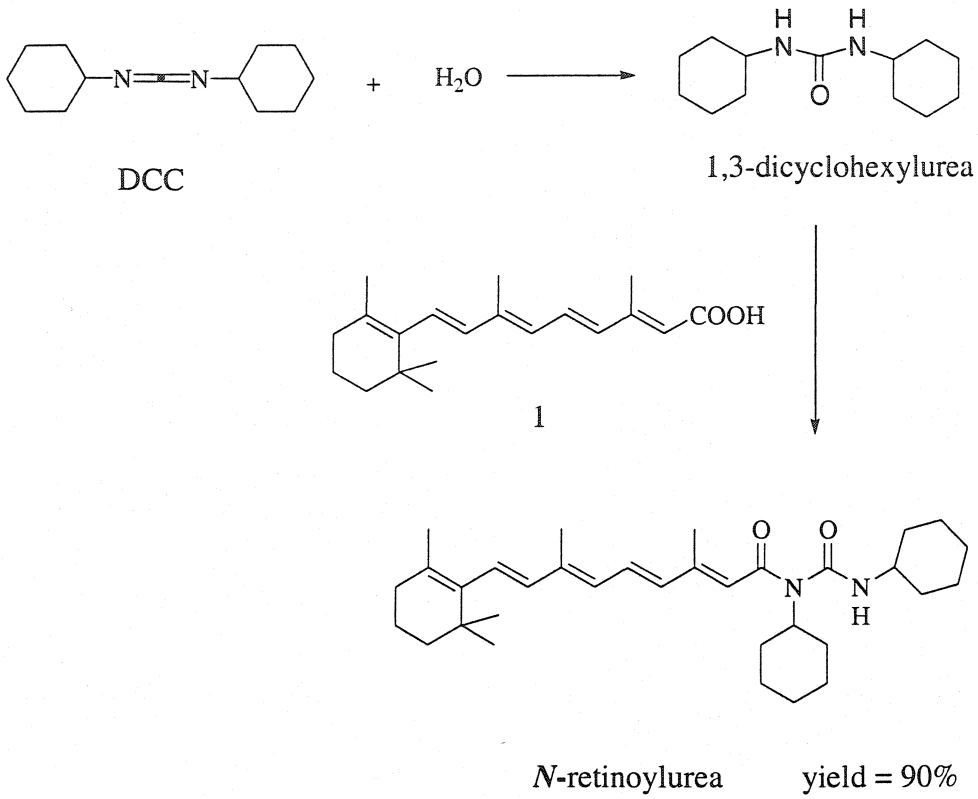
To a stirred solution of 5'-*O*-triphenylmethyl-FUdR (79 mg, 0.16 mmol) was added dicyclohexylcarbodiimide (DCC) (45.5 mg, 0.22 mmol), DMAP (1-1.5 mg) in 0.5

mL dichloromethane, and DHA (56 mg, 0.165 mmol) in 1.0 mL dichloromethane. The mixture was stirred for 1.5 h at room temperature, then diluted with dichloromethane, washed with water (2×10 mL) and dried with Na_2SO_4. Flash chromatography (gradient from hexane to ethyl acetate/hexane 1/1) of the residue yielded an amorphous product of 3'-*O*-DHA-5'-*O*-triphenylmethyl-FUdR (100 mg). The chemical yield was found to be 79%. The R_f in ethyl acetate:hexane 1:1 (v/v) was 0.80.

4.11 3'-*O*-DHA-FUdR (Compound 13)

To a solution of Compound 12 (100 mg, 0.13 mmol) in ethyl acetate (1.5 mL) was added 88% formic acid (1.5 mL). The reaction mixture was stirred at room temperature for 1 h, then diluted with ethyl acetate (25 mL). After organic layer was washed with half-saturated NaCl (2×10 mL), saturated $NaHCO_3$ (3×8 mL) and water (2×10 mL), it was then dried with Na_2SO_4. After removal of the solvent *in vacuo*, the residue was subjected to silica gel column chromatography with a gradient elution system (from hexane to hexane/ethyl acetate 1:1, v/v) to afford 3'-*O*-DHA-FUdR (44 mg) as a waxy product. The chemical yield was found to be 65%. The R_f value in ethyl acetate:hexane 1:1 (v/v) was 0.40. ^{1}H NMR ($CDCl_3$) data: δ 0.95-1.05 (t, J = 7.0 Hz, 3H, -CH_3), 2.01-2.07 (q, J = 7.0 Hz, 2H, -CH_2-), 2.20-2.30 (m, 2H, H-2') 2.38-2.49 (m, 5H, 5'-OH and -CH_2-CH_2-), 2.80-2.94 (m, 10H, $(CH_2)_5$), 3.95-4.00 (m, 2H, H-5'), 4.05-4.10 (m, 1H, H-4'), 5.40-5.53 (m, 13H, $(CH{=}CH)_6$ and H-3'), 6.32-6.40 (m, 1H, H-1'), 8.05 (d, J_{HF} = 6.3 Hz, 1H, H-6), 8.67 (brs, 1H, NH). Referring to the structure on page 33 for numbering.

DCC is known as a carboxyl-group activating reagent for the synthesis of peptides (Jones 1994). However, in an attempt to synthesize retinoyl derivatives of FUdR using DCC as a coupling agent with FUdR and retinoic acid as starting materials, no retinoyl derivatives of FUdR were found. Instead, the major product isolated was found to be the corresponding *N*-retinoylurea as a yellow solid (Scheme 4.1). The product was purified by flash chromatography and the chemical yield was found to be 90%. The structure of the product was confirmed by NMR and FAB MS. As shown in Scheme 4.1, DCC hydrolyzed partially to 1,3-dicyclohexylurea, which was coupled with the retinoic acid to form the observed product *N*-retinoylurea.



Scheme 4.1. Formation of *N*-retinoylurea

4.12 Isolation of LDL from human plasma

According to the method described by Schumaker *et al.* in 1986, human plasma was first mixed with PMSF (up to 0.015%) which was used as a protease inhibitor. LDL was then obtained based the density gradient which was achieved using KBr. The density of plasma was first adjusted to 1.020 g/mL to remove VLDL and then adjusted to 1.063 g/mL to obtain LDL that had a light yellow color.

4.13 Determination of the protein concentration in LDL preparations

The protein concentration of the LDL preparations was determined using the method of Bradford (Bradford 1976) with BSA as standard. The standard curve was prepared using BSA in saline (0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, and 0.4 mg/mL) solutions and is as shown in Figure 4.2. The LDL isolated above was then analyzed and the protein concentration was determined using the standard curve. The protein concentration in the LDL preparations was found to be 1.2-2.0 mg/mL.

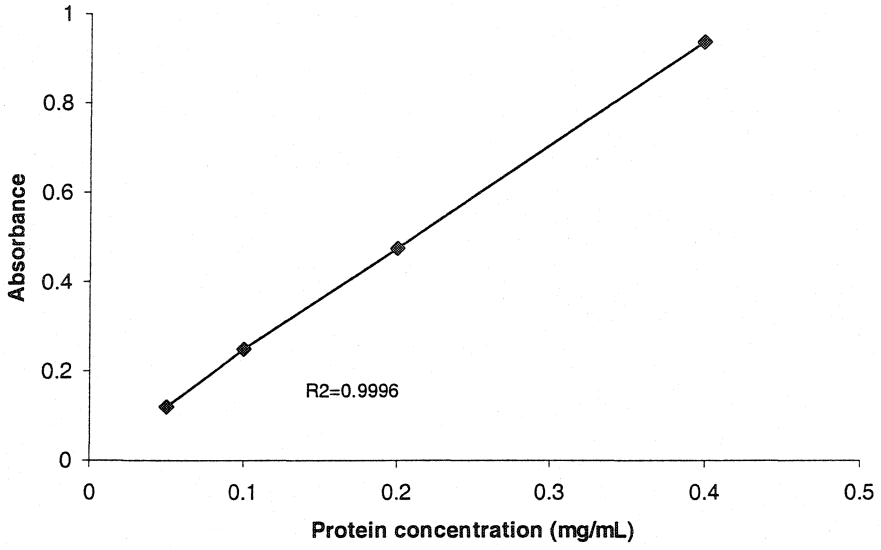


Figure 4.1. The standard curve prepared using BSA. Each data point represents the average of 4-5 measurements.

4.14 Incorporation of prodrugs into LDL

4.14.1 Preparation of 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and 3', 5'-di-*O*-retinoyl-FUdR microemulsions

The microemulsion of the respective compounds was prepared using the method described in Section 3.5.1. They were used for subsequent incorporation of the prodrugs into LDL or preparation of complexes of LDL with the respective prodrugs.

4.14.2 Incorporation of 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and 3', 5'-di-*O*-retinoyl-FUdR into LDL

It is known that the lipophilicity of the compound to be incorporated affects the incorporation efficiency. The retinoic acid and DHA derivatives of FUdR possess increased lipophilicity in comparison to FUdR. Therefore, 3', 5'-di-*O*-retinoyl-FUdR, 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR and 3'-*O*-DHA-FUdR were expected to be more easily incorporated into LDL than FUdR. The incorporation efficiency expressed as the number of molecules incorporated per LDL particle for 3', 5'-di-*O*-retinoyl-FUdR, 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl and 3'-*O*-DHA-FUdR was found to be 120, 1000, 870 and 900, respectively. The lower incorporation efficiency for 3', 5'-di-*O*-retinoyl-FUdR is likely due to the larger molecular size of 3', 5'-di-*O*-retinoyl-FUdR.

4.15 Determination of the prodrug concentration in the prodrug/LDL complex

The concentration of 3', 5'-di-*O*-retinoyl-FUdR, 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR and 3'-*O*-DHA-FUdR contained in the prodrug/LDL complex was determined using an *HP*® 1100 HPLC with a Phenomenex® C18 reverse phase column (15 cm x 3.9 mm, particle size 5 μm). The respective prodrugs in the prodrug/LDL complexes were first extracted with chloroform as described in Section 3.6.

The standard prodrug solutions (0.2 mg/mL, 0.4 mg/mL, 0.8 mg/mL, and 1.6 mg/mL) were prepared in chloroform. HPLC analysis was performed by injecting 10 μL of each sample with methanol/H_2O (9/1, v/v) at 1.0 mL/min as the mobile phase and UV detection at 267 nm. Under these conditions, the retention times for 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and 3', 5'-di-*O*-retinoyl-FUdR were 9, 6, 7 and 5 min, respectively. The area under the curve of the respective peak was calculated. The analysis for each sample was repeated three times. A standard curve was obtained by plotting the concentrations of the standard samples versus the corresponding peak area. The analysis was repeated three times for each sample and the results were expressed as the average of three analysis. The standard curve was used to determine the concentration of prodrug in the prodrug/LDL complexes.

4.16 Cytotoxicity of 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3',5'-di-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and their LDL complexes determined in Hela cells

The cytotoxicity of 3', 5'-di-*O*-retinoyl-FUdR/LDL, 3'-*O*-retinoyl-FUdR/LDL, 5'-*O*-retinoyl-FUdR/LDL, 3', 5'-di-*O*-retinoyl-FUdR in ethanol, 3'-*O*-retinoyl-FUdR in ethanol, 5'-*O*-retinoyl-FUdR in ethanol, 3'-*O*-DHA-FUdR in ethanol and FUdR/PBS were determined using the MTT assay in Hela cells (cervical cancer). Tests were repeated at least twice at different times for each compound.

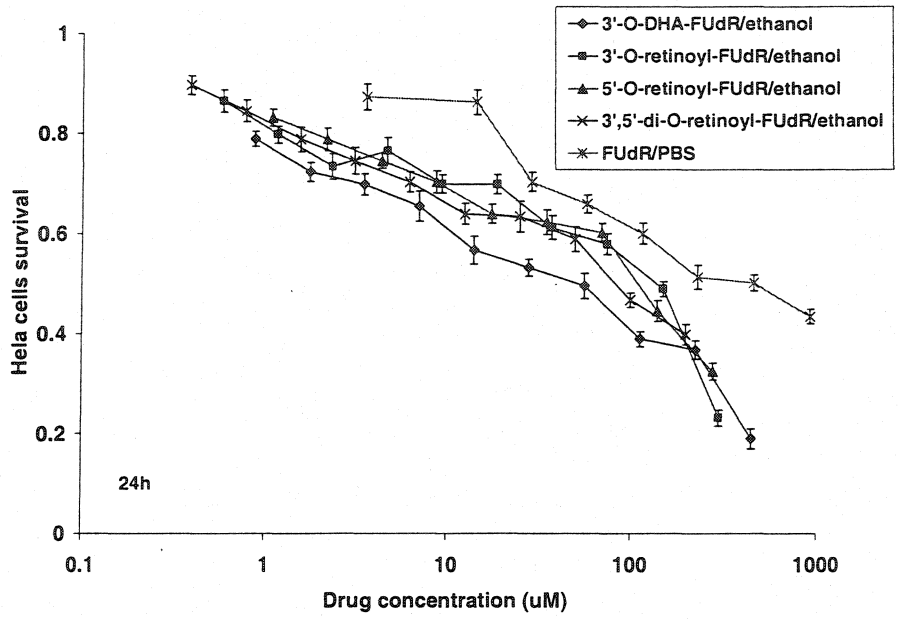
Since the prodrugs were incorporated into LDL, it was essential to ensure that the amount of LDL used in this study was not toxic and did not have any effect on the cells. The cytotoxicity of LDL (0.125 mg/mL, 0.25 mg/mL and 0.50 mg/mL, protein concentration) in Hela cell line was determined using MTT assay. Our results showed that LDL (0.125 mg/mL, 0.25 mg/mL and 0.50 mg/mL) did not cause any cell death. Since the concentration of LDL in each of the prodrug/LDL complex was less than 0.35 mg/mL, the cytotoxicity of prodrug/LDL was attributed to the prodrug alone.

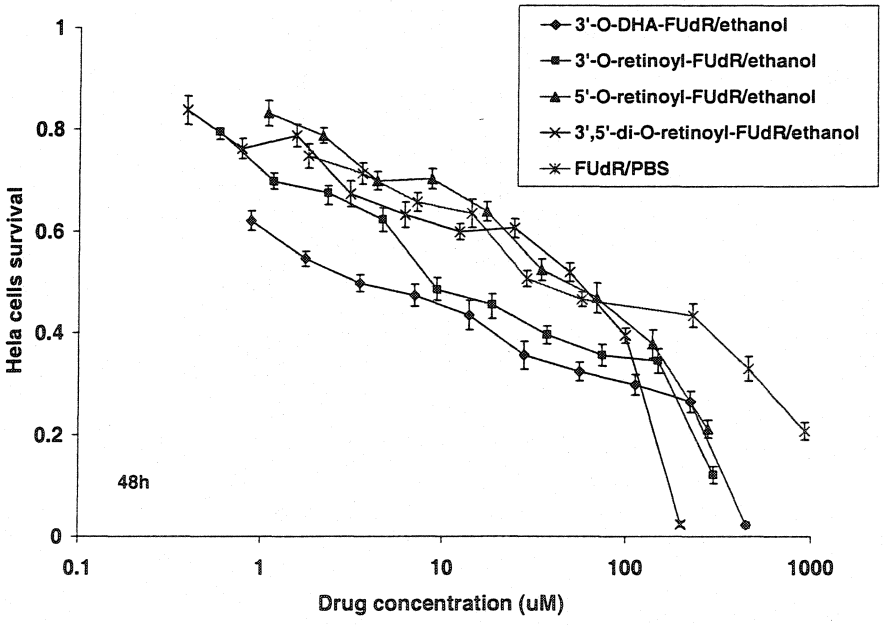
Since ethanol was used to dissolve the prodrugs, 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoy-FUdR 3'-*O*-DHA-FUdR and 3', 5'-di-*O*-retinoyl-FUdR, the cytotoxicity of ethanol was determined using the MTT assay. It was found that ethanol at the concentrations tested (1.5%, 3% and 6%) was not toxic to Hela cells. The concentration of ethanol used in preparation of prodrugs was thus kept to be 3% or lower.

The results of cytotoxicity determined in Hela cells are shown in Figures 4.2 and 4.3, and Table 4.1. As shown in Figure 4.2, the results demonstrated that all prodrugs, 3'-*O*-DHA-FUdR, 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR and 3', 5'-di-*O*-retinoyl-FUdR, were more cytotoxic than FUdR while 3'-*O*-DHA-FUdR and 3'-*O*-retinoyl-FUdR were more potent than 5'-*O*-retinoyl-FUdR and 3', 5'-di-*O*-retinoyl-FUdR, especially

upon incubation of 48 h. We believe that this is because 3'-esters are more easily hydrolyzed resulting in two active drugs released with different anticancer mechanisms. The hydrolysis process for the 5'- and 3', 5'- derivatives is relatively slow. Our data also showed that 3'-*O*-DHA-FUdR was superior to 3'-*O*-retinoyl-FUdR and the IC_{50} values upon incubation of 24 h are 48 μM and 92 μM, respectively. One explanation could be that the hydrolysis of the DHA derivative may be faster than the retinoyl derivative, or DHA may alter the pharmacokinetics of DHA conjugated FUdR. It has been reported that the DHA-paclitaxel conjugation showed increased cytotoxicity in certain tumors such as CD2F1 tumor compared with paclitaxel due to the alteration of pharmacokinetics of DHA-paclitaxel by DHA (Bradley *et al.* 2001). The other explanation could be that the Hela cells are more sensitive to DHA than retinoic acid. DHA has shown to enhance anticancer drugs' cytotoxicity, such as paclitaxel and doxorubicin, in many human breast cancer cell lines and other cancer cell lines (Menendez *et al.* 2001, Liu and Tan 2000). Although FUdR did not show any cytotoxicity upon incubation with Hela cells for 24 h, it was quite effective after 72 h with an IC_{50} of 10 μM. As shown in Figure 4.3, all the prodrug/LDL were more effective than FUdR upon incubation of 24 and 48 h. However, the cytotoxicity demonstrated by the prodrug/LDL was found to be similar to that of FUdR upon incubation of 72 h. The IC_{50} values are summarized in Table 4.1. It has been reported that Hela cells internalize LDL by a receptor-mediated process (Johnson *et al.* 1983, Lestavel-Delattra *et al.* 1992). Previously, it has been demonstrated that the loading procedure we used did not alter the recognition of LDL by the LDL receptors on the Hela cell surface (Kader 1998). Our results suggested that the respective prodrug/LDL

complex is recognized by the LDL receptors. Upon internalization, hydrolysis of the prodrug would result in two active anticancer compounds, FUdR and DHA or FUdR and retinoic acid.


3'-O-DHA-FUdR/ethanol
3'-O-retinoyl-FUdR/ethanol
5'-O-retinoyl-FUdR/ethanol
3',5'-di-O-retinoyl-FUdR/ethanol
FUdR/PBS
Hela cells survival
1
0.8
0.6
0.4
0.2
0
24h
0.1
1
10
100
1000
Drug concentration (uM)


3'-O-DHA-FUdR/ethanol
3'-O-retinoyl-FUdR/ethanol
5'-O-retinoyl-FUdR/ethanol
3',5'-di-O-retinoyl-FUdR/ethanol
FUdR/PBS
Hela cells survival
1
0.8
0.6
0.4
0.2
0
48h
0.1
1
10
100
1000
Drug concentration (uM)

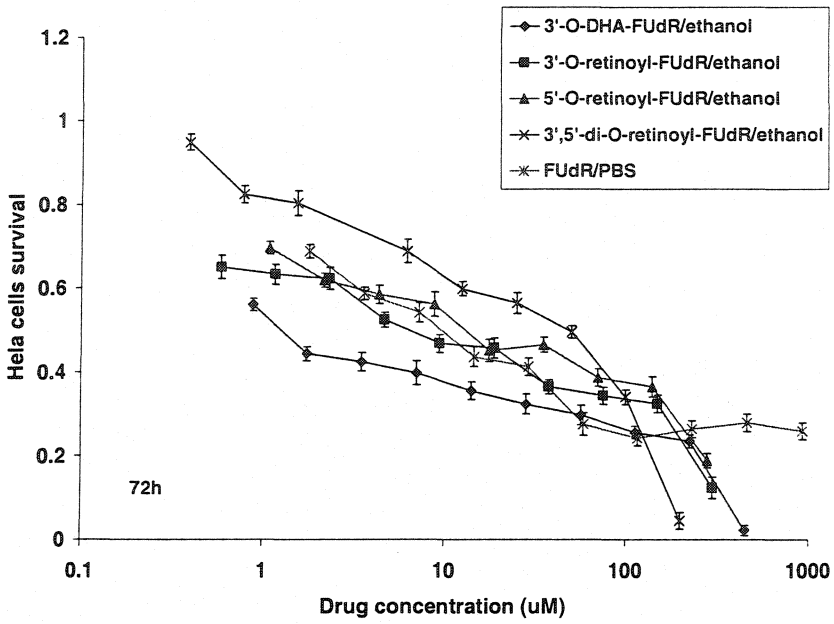
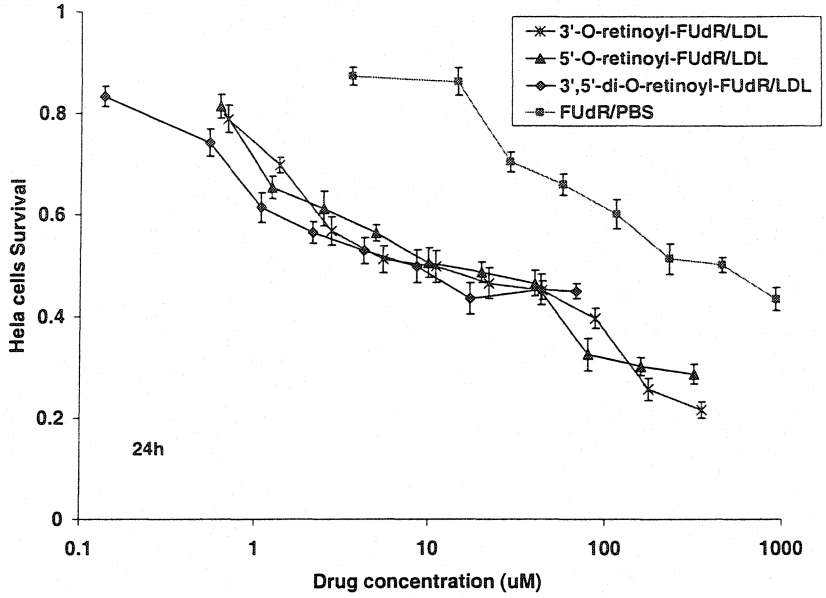
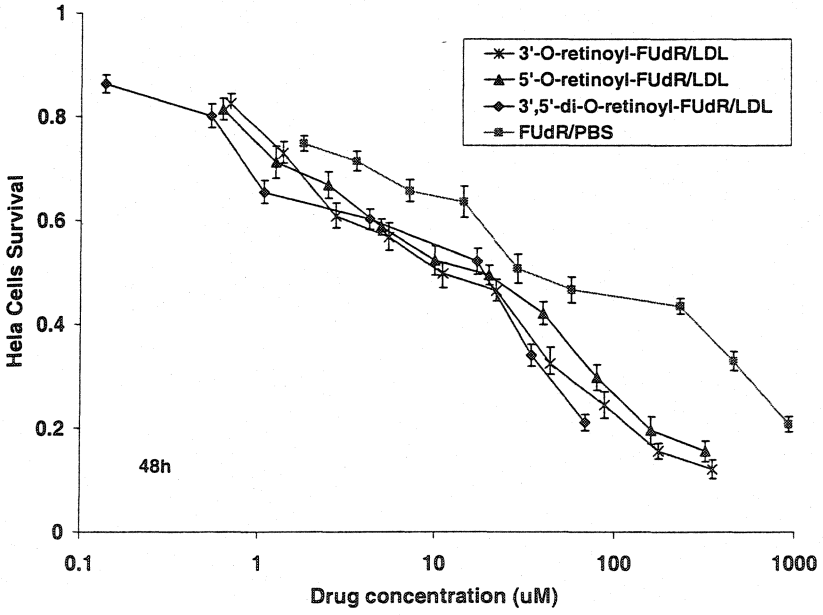


Figure 4.2. Cytotoxicity of 3'-*O*-DHA-FUdR, 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, and 3', 5'-di-*O*-retinoyl-FUdR dissolved in ethanol, and FUdR dissolved in PBS in Hela cells upon incubation of 24 h, 48 h and 72 h, respectively. Student T-test was carried out for the statistical analysis.


3'-O-retinoyl-FUdR/LDL
5'-O-retinoyl-FUdR/LDL
3',5'-di-O-retinoyl-FUdR/LDL
FUdR/PBS
Hela cells Survival
24h
Drug concentration (uM)
0
0.2
0.4
0.6
0.8
1
0.1
1
10
100
1000


3'-O-retinoyl-FUdR/LDL
5'-O-retinoyl-FUdR/LDL
3',5'-di-O-retinoyl-FUdR/LDL
FUdR/PBS
Hela Cells Survival
48h
Drug concentration (uM)
0
0.2
0.4
0.6
0.8
1
0.1
1
10
100
1000

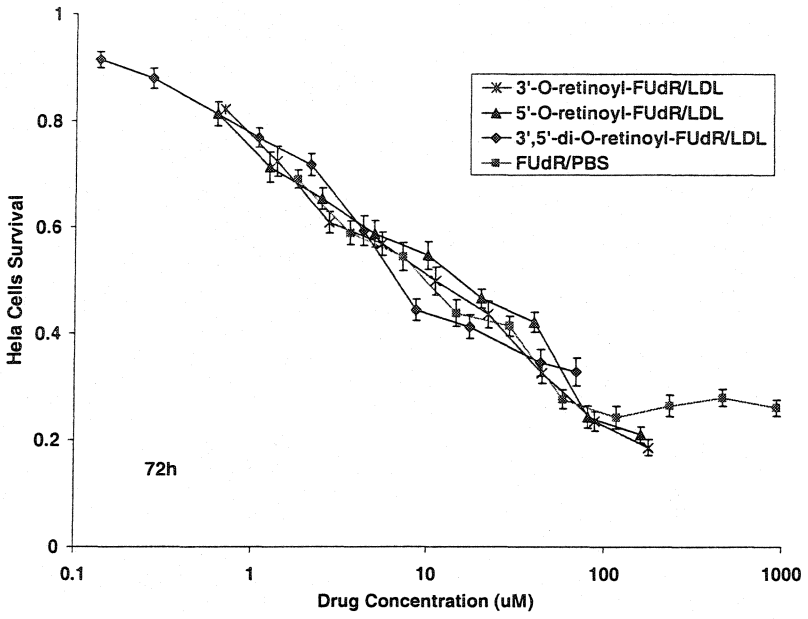


Figure 4.3. Cytotoxicity of 3'-*O*-retinoyl-FUdR/LDL, 5'-*O*-retinoyl-FUdR/LDL, 3', 5'-di-*O*-retinoyl-FUdR/LDL, and FUdR dissolved in PBS in Hela cells upon incubation of 24 h, 48 h and 72 h, respectively. Student T-test was carried out for the statistical analysis.

Table 4.1 Summary of IC_{50} (μM) values determined in Hela cells under different conditions

Compounds tested	IC_{50} values (μM)		
	24h	48h	72h
3'-*O*-retinoyl-FUdR/LDL	10	7.9	6.5
5'-*O*-retinoyl-FUdR/LDL	11	8.4	6.5
3', 5'-*O*-diretinoyl-FUdR/LDL	12	8.8	6.8
3'-*O*-retinoyl-FUdR[a]	92	13	7.3
5'-*O*-retinoyl-FUdR[a]	100	39	11
3'-*O*-DHA-FUdR[a]	48	4.4	1.6
3', 5'-di-*O*-retinoyl-FUdR[a]	102	43	20
FUdR[b]	765	52	10

Notes:

a. The compounds were dissolved in ethanol and in no case did the final concentration of ethanol exceed 3 %.

b. FUdR was dissolved in PBS (pH 7.4) and in no case did the final concentration of PBS exceed 2 %.

4.17 Cytotoxicity of 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3',5'-di-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and their LDL complexs determined in MB231 and MCF7 cells

The cytotoxicity of 3'-*O*-retinoyl-FUdR/LDL, and 3'-*O*-DHA-FUdR/LDL together with 3', 5'-di-*O*-retinoyl-FUdR, 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR in ethanol and FUdR in PBS were determined using the MTT assay in MB231 and MCF7 (breast cancer). Tests were repeated at least twice at different times for each cell line and each compound.

Since ethanol was used to dissolve the prodrugs, the cytotoxicity of ethanol (1.5%, 3% and 6%) was determined in MB231 and MCF7. The cytotoxicity of LDL (0.125 mg/mL, 0.25 mg/mL and 0.50 mg/mL) in MB231 and MCF7 cells was also evaluated. Our results showed that both ethanol and LDL at the concentrations tested were not toxic to either MB231 or MCF7 cells. The concentration of ethanol used in preparation of prodrugs was kept to be 3% or lower and the concentration of LDL in each prodrug/LDL complex was less than 0.35 mg/mL.

The results of cytotoxicity of the four respective prodrugs (3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3', 5'-di-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR) dissolved in ethanol in MB231 upon incubation of 24 and 72 h are shown in Figure 4.4. The results in MCF7 upon incubation of 24 and 48 h are shown in Figure 4.5. It is demonstrated that the prodrugs were more potent than their parent drug, FUdR, upon incubation of 24 h.

However, upon incubation of 72 h with MB231 cells, the difference of IC_{50} values between the respective prodrugs and FUdR was not significant. FUdR did not exert cytotoxicity in MCF7 upon incubation of 24 or 48 h. In addition, 3'-*O*-DHA-FUdR was superior to the retinoyl esters of FUdR, suggesting that the hydrolysis of the DHA derivative may be faster than the retinoyl derivatives or the cells may be more sensitive to DHA than to retinoic acid.

The cytotoxicity of 3'-*O*-DHA-FUdR/LDL and 3'-*O*-retinoyl-FUdR/LDL was evaluated in MB231 and MCF7 together with FUdR in PBS. The results are shown in Figures 4.6 and 4.7, and Table 4.2. It is interesting to note that the respective prodrug/LDL complexes did not demonstrate improved cytotoxicity.

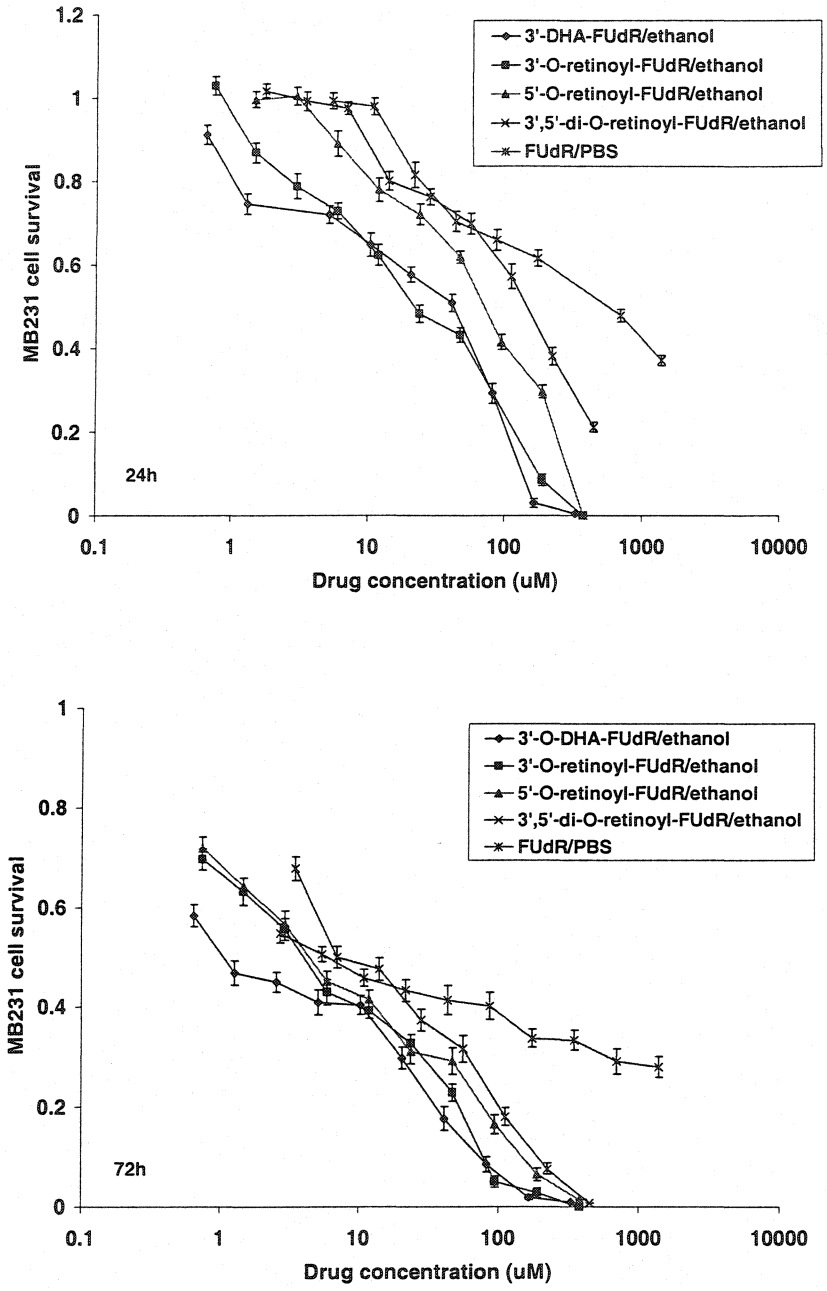


Figure 4.4. Cytotoxicity of 3'-*O*-DHA-FUdR, 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR and 3', 5'-di-*O*-retinoyl-FUdR dissolved in ethanol and FUdR dissolved in PBS in MB231 cells upon incubation of 24 h and 72 h, respectively. Student T-test was carried out for the statistical analysis.

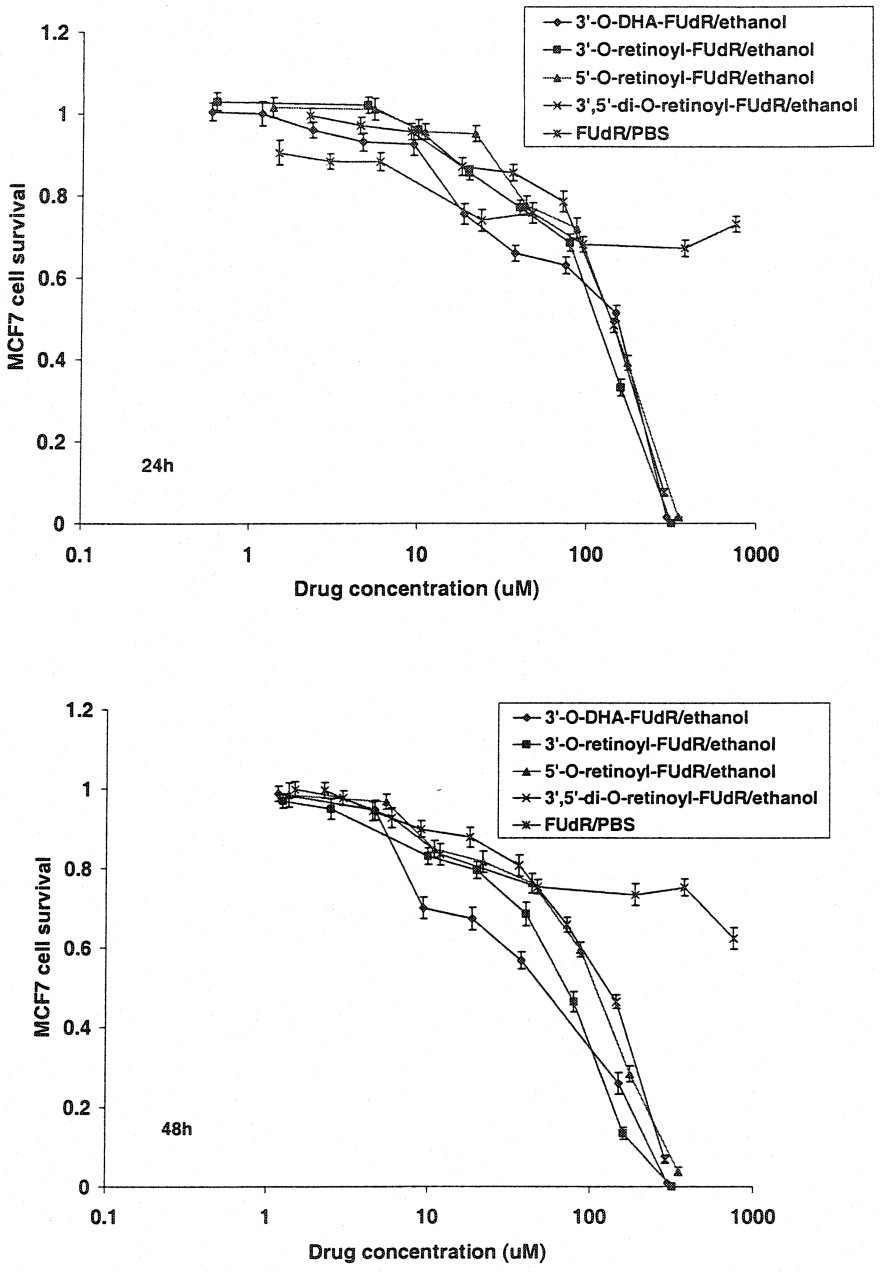


Figure 4.5. Cytotoxicity of 3'-*O*-DHA-FUdR, 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3', 5'-di-*O*-retinoyl-FUdR dissolved in ethanol and FUdR dissolved in PBS in MCF7 cells upon incubation of 24 h and 48 h, respectively. Student T-test was carried out for the statistical analysis.

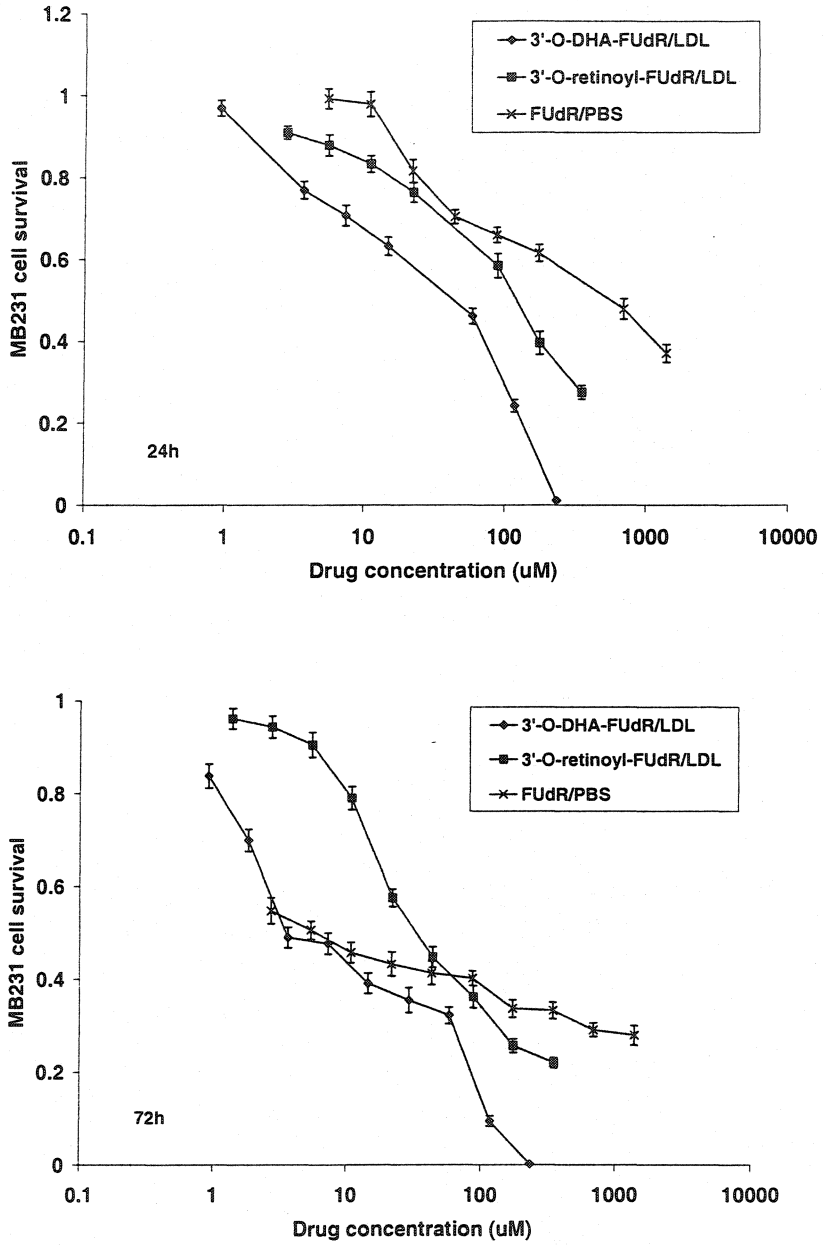


Figure 4.6. Cytotoxicity of 3'-*O*-DHA-FUdR/LDL, 3'-*O*-retinoyl-FUdR/LDL and FUdR dissolved in PBS in MB231 cells upon incubation of 24 h and 72 h, respectively. Student T-test was carried out for the statistical analysis.

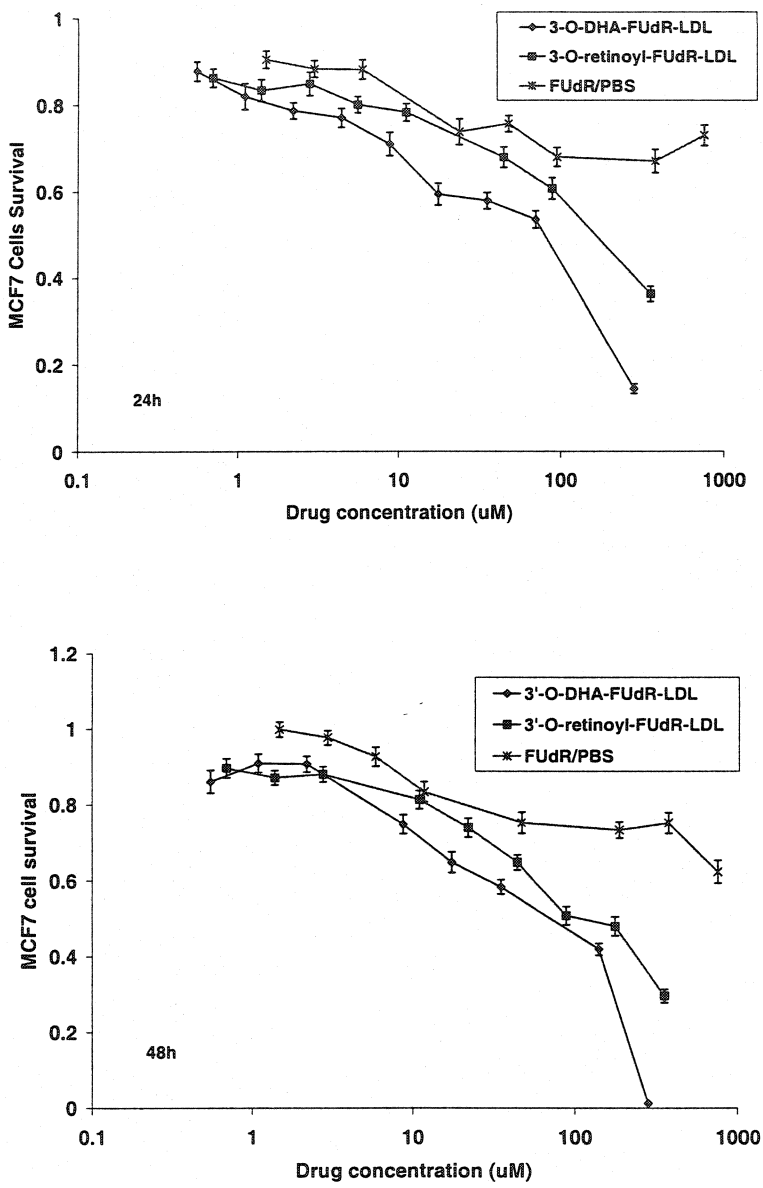


Figure 4.7. Cytotoxicity of 3'-*O*-DHA-FUdR/LDL, 3'-*O*-retinoyl-FUdR/LDL, and FUdR dissolved in PBS in MCF7 cells upon incubation of 24 h and 48 h, respectively. Student T-test was carried out for the statistical analysis.

Table 4.2 Summary of IC_{50} (μM) values determined in MB231 and MCF7 cells under different conditions

Cell line	Compounds tested	IC $_{50}$ values (μM)		
		24h	48h	72h
MB231	3'-*O*-retinoyl-FUdR/LDL	98	/[c]	40
	3'-*O*-DHA-FudR/LDL	22	/	5.6
	3'-*O*-retinoyl-FUdR[a]	87	/	34
	5'-*O*-retinoyl-FUdR[a]	79	/	44
	3'-*O*-DHA-FudR[a]	45	/	4.9
	3', 5'-di-*O*-retinoyl-FUdR[a]	126	/	32
	FUdR[b]	195	/	5.8
MCF7	3'-*O*-retinoyl-FUdR/LDL	231	88	/
	3'-*O*-DHA-FUdR/LDL	54	34	/
	3'-*O*-retinoyl-FUdR[a]	243	102	/
	5'-*O*-retinoyl-FUdR[a]	357	223	/
	3'-*O*-DHA-FUdR[a]	92	41	/
	3', 5'-di-*O*-retinoyl-FUdR[a]	387	274	/
	FUdR[b]	∞[d]	∞	/

Notes:

a. Prodrugs were dissolved in ethanol, and in no case did the final concentration of ethanol in the medium exceed 3%.

b. FUdR was dissolved in PBS (pH 7.4), and in no case did the final concentration of PBS in the medium exceed 2%.

c. Not determined.

d. IC_{50} value was greater than 5,000 (μM).

4.18 Cytotoxicity of 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3',5'-di-*O*-retinoyl-FUdR, and 3'-*O*-DHA-FUdR in ethanol determined in HepG2 cells

The cytotoxicity of 3', 5'-di-*O*-retinoyl-FUdR in ethanol, 3'-*O*-retinoyl-FUdR in ethanol, 5'-*O*-retinoyl-FUdR in ethanol, 3'-*O*-DHA-FUdR in ethanol and FUdR dissolved in PBS were determined using the MTT assay in HepG2 (hepatic) cells. Tests were repeated at least twice at different times for each cell line and each compound.

Again the cytotoxicity of ethanol was determined using the MTT assay. Ethanol at the concentrations tested (1.5%, 3% and 6%) was not found to be toxic to HepG2 cells. The concentration of ethanol used in the preparation of prodrug solutions was kept to be 3% or lower.

The results of cytotoxicity of the respective prodrugs are illustrated in Figure 4.8 and Table 4.3. It was found that the four prodrugs were more potent than their parent drug, FUdR, while 3'-*O*-retinoyl-FUdR and 3'-*O*-DHA-FUdR were superior to 5'-*O*-retinoyl-FUdR and 3', 5'-di-*O*-retinoyl-FUdR.

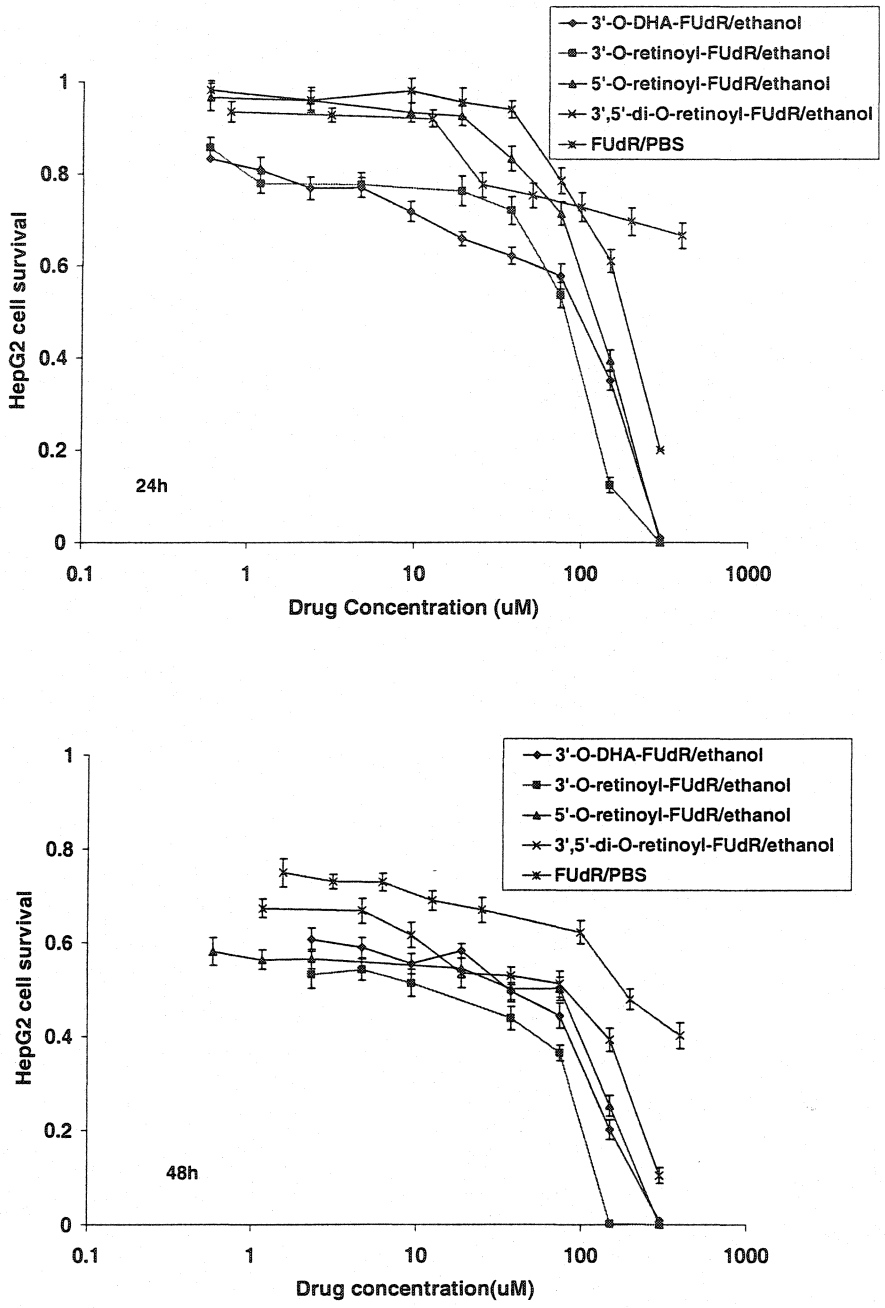


Figure 4.8. Cytotoxicity of 3'-*O*-DHA-FUdR, 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, and 3', 5'-di-*O*-retinoyl-FUdR dissolved in ethanol, and FUdR dissolved in PBS in HepG2 cells upon incubation of 24 h and 48 h, respectively. Student T-test was carried out for the statistical analysis.

Table 4.3 Summary of IC_{50} (μM) values determined in HepG2 cells under different conditions

Compounds tested	IC_{50} values (μM)	
	24h	48h
3'-*O*-retinoyl-FUdR[a]	33	7.8
5'-*O*-retinoyl-FUdR[a]	85	5.2
3'-*O*-DHA-FUdR[a]	35	14
3', 5'-di-*O*-retinoyl-FUdR[a]	321	19
FUdR[b]	∞[c]	214

Notes:

a. Prodrugs were dissolved in ethanol, and in no case did the final concentration of ethanol in the medium exceed 3%.

b. FUdR was dissolved in PBS (pH 7.4), and in no case did the final concentration of PBS in the medium exceed 2%.

c. IC_{50} value was greater than 5,000 (μM).

4.19 *In vitro* differentiation test in CEM/T4 cells

Retinoic acid and DHA released from the prodrugs were expected to be able to induce malignant cell differentiation and then to lead to cell apoptosis. Therefore, differentiation effect of prodrugs in ethanol was examined in leukemia cell line, CEM/T4 cells. The results are shown in Table 4.4. 3'-*O*-DHA-FUdR, 3'-*O*-retinoyl-FUdR and 5'-*O*-retinoyl-FUdR were shown to be slightly superior to FUdR.

Table 4.4 Differentiation activity of retinoic acid, FUdR, 3'-*O*-DHA-FUdR, 3'-*O*-retinoyl-FUdR and 5'-*O*-retinoyl-FUdR determined in CEM/T4 cells

	Concentration (μM) / Differentiation (% NBT-positive cells)		
Compounds tested	1 μM	0.1 μM	0.01 μM
Retinoic acid	43%	40%	32%
FUdR	18%	14%	14%
3'-*O*-retinoyl-FUdR	29%	25%	18%
3'-*O*-DHA-FUdR	20%	20%	14%
5'-*O*-retinoyl-FUdR	27%	21%	16%
Control	< 10%		

Chapter 5 Conclusions

In this study, four FUdR derivatives (3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and 3', 5'-di-*O*-retinoyl-FUdR) were designed and synthesized as prodrugs of FUdR. It is believed that the respective prodrugs upon being taken into cells would be hydrolyzed to generate two compounds (FUdR and retinoic acid or FUdR and DHA) which would act as anticancer drugs via different mechanisms. To facilitate the specific uptake of these prodrugs by cancer drugs, LDL was used as a drug carrier. It is known that most cancer cells express elevated levels of LDL receptors on their surface compared to normal cells. The prodrugs synthesized were thus incorporated into LDL resulting in the formation of prodrug/LDL complexes. It was found that the incorporation efficiencies of the prodrugs were > 120 molecules per LDL particle.

The cytotoxicity of 3'-*O*-retinoyl-FUdR/LDL, 5'-*O*-retinoyl-FUdR/LDL, and 3', 5'-di-*O*-retinoyl-FUdR/LDL complexes together with 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3', 5'-di-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR, dissolved in ethanol and FUdR dissolved in PBS, were tested in cervical cancer cell line, Hela cells. In all cases, the prodrugs showed increased cytotoxicity compared with the parent drug, FUdR. Our results also showed the cytotoxicity of prodrugs/LDL complexes was increased compared with the corresponding prodrug dissolved in ethanol in Hela cells, especially upon incubation of 24 h (Table 4.1). It has been reported that LDL receptors are expressed on the surface of the Hela cells which internalize LDL particles via a receptor-mediated

process (Johnson *et al.* 1983; Lestavel-Delattra *et al.* 1992). The increased cytotoxicity demonstrated by the prodrug/LDL complexes is consistent with the increased cell surface LDL receptor levels on Hela cells which resulted in the enhanced cellular uptake of 3',5'-di-*O*-retinoyl-FUdR/LDL, 3'-*O*-retinoyl-FUdR and 5'-*O*-retinoyl-FUdR. The results showed that LDL could be a useful drug carrier for targeting anticancer drugs to Hela cells.

The cytotoxicity of 3'-*O*-retinoyl-FUdR/LDL, 5'-*O*-DHA-FUdR/LDL complex together with 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3', 5'-di-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR, dissolved in ethanol and FUdR dissolved in PBS, were also tested in breast cancer cell lines, MB231 and MCF7 cells. Our results indicated that all prodrugs were more potent than the parent drug, FUdR. However, the prodrug/LDL complexes (3'-*O*-retinoyl-FUdR/LDL and 3'-*O*-DHA-FUdR/LDL) did not demonstrate much improved cytotoxicity in MCF7 and MB231 over the corresponding prodrugs dissolved in ethanol (Table 4.2).

In addition, the cytotoxicity of 3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3', 5'-di-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR dissolved in ethanol, and FUdR dissolved in PBS, were tested in hepatic cancer cell line-HepG2 cells. Our results showed that all prodrugs showed the increased cytotoxicity compared with the parent drug, FUdR.

Among the four prodrugs (3'-*O*-retinoyl-FUdR, 5'-*O*-retinoyl-FUdR, 3'-*O*-DHA-FUdR and 3',5'-di-*O*-retinoyl-FUdR), 3'-*O*-DHA-FUdR and 3'-*O*-retinoyl-FUdR were found to be more potent than the other two prodrugs. This may indicate that the different derivatives have different hydrolysis rates and the 3'-esters are more susceptible to

hydrolysis than the 5'- or 3',5'-esters. More studies are needed in this regard. A comparison of 3'-*O*-DHA-FUdR and 3'-*O*-retinoyl-FUdR showed that 3'-*O*-DHA-FUdR was superior to 3'-*O*-retinoyl-FUdR when incubated with Hela and MCF7 cells, which may suggest that the cells tested are more sensitive to DHA than retinoic acid or the 3'-*O*-DHA derivative undergoes a faster hydrolysis. However, more studies are needed.

Differentiation effect of the prodrugs dissolved in ethanol (3'-*O*-DHA-FUdR, 3'-*O*-retinoyl-FUdR and 5'-*O*-retinoyl-FUdR) was examined in leukemia cell line, CEM/T4 cells. The results showed that 3'-*O*-DHA-FUdR, 3'-*O*-retinoyl-FUdR and 5'-*O*-retinoyl-FUdR were slightly superior to FUdR.

Reference:

Agadir A. and Chomienne C., Retinoids and differentiation of normal and malignant hematopoietic cells. in *Retinoids: the biochemical and molecular basis of vitamin A and retinoid action*. Nau H. and Blaner W.S. Eds., Springer, **1999**, 277-291.

Avdeef A. Physicochemical profiling (solubility, permeability and charge state). *Curr Top Med Chem.* **2001**, 1: 277-351.

Baguley B.C., A brief history of cancer chemotherapy in "Anticancer drug development". Baguley B.C. and Kerr D.J., Eds., Academic Press, **2002**, 1-9.

Balzarini J., Verstuyf A., Hatse S., Goebels J., Sobis H., Vandeputte M., and De Clerco E., The human immunodeficiency virus (HIV) inhibitor 9-(2-phosphonylmethoxyethyl) adenine (PMEA) is a strong inducer of differentiation of several tumor cell lines. *Int. J. Cancer,* **1995,** 61: 130-137.

Bang H.O., Dyerberg J., and Hjorne N., The composition of food consumed by Greenland Eskimos. *Acta Med. Scand.*, **1976**, 200: 67-73.

Benbrook D., Lernhardt E., and Pfahl M., A new retinoic acid receptor identified from a hepatocellular carcinoma. *Nature,* **1988**, 333: 669-672.

Berchekroun N.M., Pourquier P., Schott B., and Robert J., Doxorubicin-induced lipid peroxidation and gluthathione peroxidase activity in tumor cell lines selected for resistance to doxorubicin. *Eur. J. Biochem.*, **1993**, 211: 141-146.

Bergh J., Clinical studies of p53 in treatment and benefit of breast cancer patients. *Endocr. Relat. Cancer*, **1999**, 6: 51-59.

Bessodes M., Komiotis D., and Antonakis K., Rapid and selective detritylation of primary alcohols using formic acid. *Tetrahedron Lett.* **1986,** 27: 579-580.

Blaner W.S. and Olson J.A., Retinoid and retinoic acid metabolism in *The Retinoids Biology, Chemistry and Medicine.* 2nd Ed, Edited by Sporn M.B., Roberts A.B., and Goodman D.S., New York, Raven Press. **1994**, 229-255

Blot W.J., Lanier A., Fraumeni Tr J.F., Bender T.R., Cancer mortality among Alaskan natives, 1960-1969, *J. Natl. Cancer Inst.*, **1975**, 55: 546-554.

Boehm M.F., Heyman R.A., and Nagpal S., A new generation of retinoid drugs for the treatment of dermatological diseases. *Emerging Drugs,* **1997,** 2: 287-303.

Bollag W., Majewski B., and Jabloska S., Cancer combination chemotherapy with retinoids: experimental rationale. *Leukemia,* **1994,** 8: 1453-1457.

Bosch L., Harbers E. and Heidelberger C., Studies of fluorinated pyrimidines and their effects on nuleic acid metabolism. *Cancer Res.*, **1958**, 18: 335-343.

Bown S.G., Rogowaka A.Z., Whitelaw D.E., Lees W.R., Lovat L.B., Ripley P., Jones L., Wyld P., Gillams A., and Hatfield A.W., Photodynamic therapy for cancer of the pancreas. *Gut*, **2002**, 50: 549-556.

Boylan J.F. and Gudas L.J., The level of CRABP-I expression influences the amounts and types of all-*trans* retinoic acid metabolites in F9 teratocarcinoma stem cells. *J. Biol. Chem.* **1992,** 267: 21486-21491.

Bradford M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, 72: 248-251.

Bradley W.A. and Goto A.M., in Disturbances in lipid and lipoprotein metabolism. Distschy J.M., Goto A.M., and Ontko J.A. Eds., American physiological society, Bethesda, MD, **1978**, pp 111-137.

Bradley M.O., Swindell C.S., Anthony F.H., Witman P.A., Devanesan P., Webb N.L., Baker S.D., Wolff A.C., and Donehower R.C., Tumor targeting by conjugation of DHA to paclitaxel. *J Controlled Release*, **2001**, 74: 233-236.

Brand N., Petkovich M., Krust A., Chambon P, de The H, Marchio A, Tiollais P, Dejean A, Identification of a second human retinoic acid receptor. *Nature,* **1988,** 332: 850-853.

Bundgaard H., Novel chemical approaches in prodrug design. *Drugs of the Future,* **1991,** 16: 443-458.

Burns C.P. and North J.A., Adriamycin transport and sensitivity in fatty acid-modified leukemia cells. *Biochim. Biophys. Acta*, **1986**, 29: 10-17.

Campbell N.A., Lawrence G.M., Jane B.R. Biology: Concepts and Connections, 3rd Ed, **1999**, Menlo Park, CA.

Carmichael J, Degraff W.G., Gazdar A.F., Minna J.D. and Mitchell J.B., Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.*, **1987**, 47: 936-942.

Castaigne S., Chomienne C., and Fenaux P., Hyperleukocytosis during all-trans retinoic acid for acute promyelocytic leukemia. *Blood,* **1990,** 76: 260a (suppl 1, abstr).

Chambon P., A decade of molecular biology of retinoic acid receptors. *FASEB J.,* **1996,** 10: 940-954.

Chaudary S.K. and Hernandez O., A simplified procedure for the preparation of triphenylesters. *Tetrahedron Lett.* **1979,** 20: 95-98.

Cheng Y-C. and Nakayama K., Effects of 5-fluoro-2'-deoxyuridine on DNA metabolism in HeLa cells. *Mol. Pharmocol.*, **1983**, 23: 171-174.

Chomienne C., Balitrand N., Degos L., and Abita J.P., 1-β-*D*-arabinofuranosyl cytosine and retinoic acid in combination accelerates and increases monocyte differentiation of myeloid leukemic cells. *Leuk. Res.* **1986,** 10: 631-636.

Connolly J.M., Gilhooly E.M., and Rose D.P., Effects of reduced dietary linoleic acid intake, alone or combined with an algal source of docosahexaenoic acid, on MDA-MB-231 Breast cancer cell growth and apoptosis in nude mice. *Nutrition and Cancer*, **1999**, 35: 44-49.

Connor W.E., Importance of omega-3 fatty acids in health and disease. *Am. J. Clin. Nutr.*, **2000**, 71(suppl): 171S-175S.

Counsell R.E., and Pohland R.C. Lipoproteins as potential site specific delivery systems for diagnostic and therapeutic agents. *J. Med. Chem.*, **1982**, 25: 1115-1122.

Curley R.W. and Robarge M.J., Retinoid structure, chemistry, and biologically active derivatives. *Advances in Organ Biology*, **1997**, 3: 1-34.

Daher G.C., Harris B.E. and Diasio R.B., Metabolism of pyrimidine analogues and their nucleosides. *Pharmacol. Ther.* **1990,** 48: 189-222.

Das U.N., Tumoricidal action of *cis*-unsaturated fatty acids and their relationship to free radicals and lipid peroxidation. *Cancer Lett.*, **1991**, 56: 235-243.

Das U.N. and Ramesh G., Effect of *cis*-unsaturated fatty acids on Meth-A ascetic tumor cells in vitro and in vivo. *Cancer Lett.*, **1998**, 123: 207-214.

Davis S.S. and Illum L., in *Site-specific drug delivery,* eds. TomLinson E. and Davis S.S., John Wiley, Chichester, New York, Brisbane, Toronto, and Singapore, **1986**, p 93-122.

De la Haba Rodrigues J.R., Algar J., Mendez Vidal M.J., Aranda I.B., Alvarez Kindelan A., Lopez Pujol J., and Aguilar E.A., Surgical treatment of pulmonary metastases: experience with 40 patients. *Eur. J. Surg. Oncol.*, **2002**, 28: 49-54.

De Smidt P.C. and Van Berkel T.J.C., Prolonged serum half-life of antineoplastic drugs by incorporation into the low density lipoprotein. *Cancer Res.*, **1990**, 50: 7476-7482.

Dietschy J.M. and Spady D.K., in *Receptor-Mediated uptake in the liver* (Greten H., Windler E., and Beisiegel U., eds), Springer-Verlag, Berlin, Heidelberg, **1986**, p56.

Dima V.F., Vasiliu V., and Dima S.V., Photodynamic therapy: an update. *Roum. Arch. Microbiol. Immunol.*, **1998**, 57: 207-230.

Doll R., The Pierre Denoix Memorial Lecture: Nature and nurture in the control of cancer. *European J. Cancer*, **1999**, 35: 16-23.

Doll R., Peto R., Wheatley K., Gray R., and Sutherland I., Mortality in relation to smoking: 40 years' observations on male British doctors. *Br. Med. J.*, **1994**, 309: 901-11.

Dubowchik G.M. and Walker M.A., Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer drugs. *Pharmacology & Therapeutics*, **1999**, 83: 76-123.

Ebert P.S. and Salcman M., Differentiation therapy is potentiated by chemotherapy and hyperthermia in human and canine brain tumor cell *in vitro*. *Neurosurgery*, **1994**, 34: 657-664.

Ehlert J.E. and Kubbutat M.H., Apoptosis and its relevance in cancer therapy. *Onkologie,* **2001**, 24: 433-440.

Ehrlich P., Studies in immunity, Plenum Press, New York, **1906**.

Ellis P.A., Smith I.E., Detre S., Burton S.A., Salter J., and A'Hern R. et al., Reduced apoptosis and proliferations and increased bcl-2 in residual breast cancer following preoperative chemotherapy. *Breast Cancer Res. Treat*, **1998**, 48: 107-16.

Ellison R.R., Holland J.F. and Weil M., Arabinosyl cytosine: a useful agent in the treatment of acute leukemia in adults. *Blood*, **1968**, 32: 507-523.

Evan G.I., and Vousden K.H., Proliferation, Cell Cycle and Apoptosis in Cancer. *Nature,* **2001**, 411: 342-348.

Evan G.I., and Littlewood T., A matter of life and cell death, *Science*, **1998**, 281: 1317-1322.

Farkas R.H, and Grosskreutz C.L., Apoptosis, Neuroprotection, and Retinal Ganglion Cell Death: A review. *Int. Ophthalmol. Clin.*, **2001**, 41: 111-130.

Ferreira C.G., Tolis C., and Giaccone G., P53 and chemosensitivity. *Ann. Oncol.*, **1999**, 10: 1011-1021.

Filipowska D., Filipowska T., Morelowska B., Kazanowska W., Laudanski T., Lapinjoki S., Akerlund M., and Breeze A., Treatment of cancer patients with a low

density lipoprotein delivery vehicle containing a cytotoxic drug. *Cancer Chemotherapy & Pharmacology*. **1992**, 29: 396-400.

Fiorella P.D. and Napoli J.L., Expression of cellular retinoic acid binding protein (CRABP) in Escherichia coli: Characterization and evidence that holo-CRABP is a substrate in retinoic acid metabolism. *J.biol. Chem.* **1991,** 266: 16570-16579.

Firestone R.A., Low density lipoprotein as a vehicle for targeting antitumor compounds to cancer cells. *Bioconjugate Chem.* **1994,** 5: 105-113.

Formelli F., Barua A.B., and Olson J.A., Bioactivities of *N*-(4-hydroxyphenyl) retinamide and retinoyl β-glucuronide. *EASEB J.* **1996,** 10: 1014-1024.

Frankel S.R., Eardley A., Lauwers G., and et al., The "retinoic acid syndrome" in acute promyelocytic leukemia. *Ann. Intern. Med.* **1992,** 117: 292-296.

Garnett M.C., Targeted drug conjugates: principles and progress. *Advanced Drug Delivery Rev.*, **2001**, 53: 171-216.

Gaudette L.A., and Lee J., Eds. *Canadian Cancer Statistics, National Cancer Institute of Canada, the Canadian Cancer Society, Statistics Canada, Health Canada, and the Canadian Council of Cancer Registries,* **1996**, 1-84.

Gaudette L.A., Altmayer C.A., Wysocki M., and Gao R.N., *Cancer incidence and mortality across Canada. Health Rep.* **1998** Autumn, 10(2): 66.

Germain E., Chajes V., Cognault S., Lhuiller C., and Bougnoux P., Enhancement of Doxorubcin cytotoxicity by polyunsaturated fatty acids in the human breast tumor cell line MDA-MB-231: Relationship to lipid peroxidation. *Int. J. Cancer*, **1998**, 75: 578-583.

Gianni M., Ponzanelli I., Mologni L., Reichert U., Rambaldi A., Terao M., and Garattini E., Retinoid-dependent growth inhibition, differentiation and apoptosis in acute promyelocytic leukemia cells: expression and activation of caspases. *Cell Death Differ.*, **2000**, 7: 447-460.

Giguere V., Retinoic acid receptors and cellular retinoid binding proteins: Complex interplay in retinoid signaling. *Endocrine Rev.* **1994,** 15: 61-79.

Goodman G.E., Prevention of lung cancer. *Crit. Rev. Oncol/Hematol.*, **2000**, 33: 187-197.

Guffy M.M., North J.A., and Burns C.P., Effect of cellular fatty acid alteration on adriamycin sensitivity in cultured L1210 murine leukemia cells. *Cancer Res.*, **1984**, 84: 1863-1866.

Hakimian D., Tallman M.S., Zugerman C., and Caro WA., Erythema nodosum associated with all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. *Leukemia,* **1993,** 7: 758-759.

Haldane J.B.S., On being the right size and other essays, Maynard Smith J., Ed. Oxford, Oxford University Press, **1985**, 113-134.

Hale A.J., Smith C.A., Sutherland L.C., Stoneman V.E. et al., Apoptosis: molecular regulation of cell death. *Eur. J. Biochem.*, **1996**, 236: 1-26.

Halmos T., Moroni P., Antonakis K., and Uriel J., Fatty acid conjugates of 2'-deoxy-5-fluorouridine as prodrugs for the selective delivery of 5-fluorouracil to tumor cells. *Biochem. Pharm.* **1992,** 44: 149-155.

Hamilton A., and Piccart M., The contribution of molecular markers to the prediction of response in the treatment of breast cancer: a review of the literature on HER-2, p53 and *bcl-2*. *Ann. Oncol.*, **2000**, 11: 647-663.

Hansen L.A., Sigman C.C., Andreola F., Ross S.A., Kelloff G.J., and De Luca L.M., Retinoids in chemoprevention and differentiation therapy. *Carcinogensis,* **2000,** 21: 1271-1279,

Hassan H.T. and Rees J.K.H., Triple combination of retinoic acid, low concentration of cytarabine and dimethylformamide induces differentiation of human acute myeloid leukemic blasts. *Chemotherapy* **1986,** 10: 631-636.

Haviv Y.S. and Curiel D.T., Conditional gene targeting for cancer gene therapy. *Advanced Drug Delivery Rev.*, **2001**, 53: 135-154.

Heidelberger C., Chaudhuary N.K., and Danenberg P., Fluorinated pyrimidines: a new class of tumor inhibitory compounds. *Nature*, **1957**, 179: 663-666.

Hohn D.C., Stagg R.J. and Price D.C., Avoidance of gastroduodenal toxicity in patients receiving hepatic arterial 5-fluoro-2'-deoxyuridine. *J. Clin. Oncol.,* **1985**, 3: 1257-1260.

Hong W.M., Endicott J., Itri L.M., Doos W., Batsakis J.G., Bell R., Fofonoff S., Strong S., et al., 13-*cis* retinoic acid in the treatment of oral leukoplakia. *N. Engl. J. Med.*, **1986**, 315: 1501-1505.

Horrobin D.F. (Eds), Omega-6 *cis*-unsaturated fatty acids, **1990,** 351-378, Wiley-Liss, New York.

Ho Y.K., Smith R.G., Brown M.S., and Golstein J.L., Low-density lipoprotein (LDL) receptor activity in human acute myelogenous leukemia cells, *Blood* **1978,** 52: 1099-1114.

Hull W.E., Port R.E., Herrmann R., Britsch B., and Kunz W., Metabolism of 5-fluorouracil in plasma and urine, as monitored by ^{19}F nuclear magnetic resonance spectroscopy, for patients receiving chemotherapy with or without methotrexate pretreatment. *Cancer Res.*, **1988,** 48: 1680-1688.

Huang M.E., Ye Y.C., Chen S.R., Chair J. R., Lu X., Zhao L., Gu L.J., and Wang Z.Y., Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. *Blood*, **1988**, 72: 567-572.

Idowu O.E., Biological therapy in cancer. *Niger. J. Med.*, **2001**, 10: 102-105.

Ingraham H.A., Tseng B.Y. and Goulian M., Mechanism for exclusion of 5-fluorouracil from DNA. *Cancer Res.,* **1980**, 40: 998-1001.

Igarashi M.,and Miyazawa T., Do conjugated eicosapentaenoic acid and conjugated docosahexaenoic acid induce apoptosis via lipid peroxidation in cultured human tumor cells? *Biochem. Biophys. Res. Commun.*, **2000**, 270: 649-656.

Israels L.G., and Israels E.D., Apoptosis. *Stem. Cells*, **1999**, 17: 306-313.

Jaffar M., Williams K.J., and Stratford I.J., Bioreductive and gene therapy approaches to hypoxic diseases. *Advanced Drug Delivery Rev.*, **2001**, 53: 217-228.

Jehn B.M., and Osborne B.A., Gene regulation associated with apoptosis. *Critical Reviews in Eukaryotic Gene Expression*, **1997**, 7: 179-193.

Jiang H., Lin J., and Fisher P.B., A molecular definition of terminal cell differentiation in human melanoma cells. *Mol. Cell Differ.*, **1994**, 2: 221-239.

Jiang W.G., Bryce R.P., and Horrobin D.F., Essential fatty acids: molecular and cellular basis of their anti-cancer action and clinical implications. *Crit. Rev. Oncol. Hematol.*, **1998a**, 27: 179-209.

Jiang W.G., Bryce R.P., and Horrobin D.F., Regulation of tight junction permeability and occludin expression by polyunsaturated fatty acid. *Biochem. Biophys. Res. Commun.*, **1998b**, 244: 414-420.

Jiang W.G., Hiscox S., Bryce R.P., Horrobin D.F., and Mansel R.E., The effects of n-6 polyunsaturated fatty acids on the expression of nm-23 in human cancer cells. *Br. J. Cancer*, **1998c**, 77: 731-738.

Jiang W.G., Hiscox S., Hallett M.B., Horrobin D.F., Mansel R.E., and Puntis M.C.A., Regulation of the expression of *E*-cadherin on human cancer cells by gamma-linolenic acid (GLA). *Cancer Res*, **1995a**, 55: 5043-5048.

Jiang W.G., Hiscox S., Hallett M.B., Scott C., Horrobin D.F., and Puntis M.C.A., Inhibition of hepatocyte growth factor-induced motility and in vivo invasion of human colon cancer cells by gamma linolenic acid. *Br. J. Cancer*, **1995b**, 77: 744-752.

Johnson F.L., St Clair R.W. and Rudel L.L., Studies on the production of low density lipoproteins by perfused livers from nonhuman primates. Effect of dietary cholesterol. *J. Clin. Invest.*, **1983**, 72: 221-236.

Jones J., *The chemical synthesis of peptides, international series of monographs on chemistry.* Oxford University, Oxford, **1994,** Vol 23 (Z25,92-99).

Kader A., Davis P.J., Kara M., and Liu H., Drug targeting using low density lipoprotein (LDL): physicochemical factors affecting drug loading into LDL partical. *J. Controlled Release*, **1998**, 55: 231-243.

Kader A. Master Thesis from Memorial University of Newfoundland. **1997**.

Kaufmann S.H., and Earnshaw W.C., Induction of apoptosis by cancer chemotherapy. *Experimental Cell Research*, **2000**, 256: 42-49.

Kawaguchi T., Fukushima S., Hayashi Y., and Nakano M., Nonenzymatic and enzymatic hydrolysis of 5-fluoro-2'-deoxyuridine (FUdR) esters. *Pharm. Res.* **1988,** 5: 741-744.

Kizaki M., Nakajima H., Mori S., Koike T., Morikawa M., Ohta M., Saito M., Koeffler H.P., and Ikeda Y., Novel retinoic acid, 9-*cis*-retinoic acid, in combination with all-*trans*-retinoic acid –resistant HL-60 cells. *Blood,* **1994,** 83: 3289-3297.

Krauss T., Huschmand H., Hinney B., Viereck V., and Emons G., Hormonal therapy and chemotherapy of endometrial cancer. *Zentralbl. Gynakol.*, **2002**, 124: 45-50.

Kreuter J., Nanoparticulate systems for brain delivery of drugs. *Advanced Drug Delivery Rev.*, **2001**, 47: 65-81.

Kumar S., The *Bcl-2* family of proteins and activation of ICE-CED-3 family of proteases: a balancing act in apoptosis? *Cell Death Differ.*, **1997**, 4: 2-3.

Kuroda Y., Shima N., Yazawa K., and Kaji K., Desmutagenic and bio-antimutagenic activity of docosahexaenoic acid and eicosapentaenoic acid in cultured Chinese hamster V79 cells. *Mutation Res.*, **2001**, 497: 123-130.

Lestavel-Delattre S., Martin-Nizard F., Clavey V., Testard P., Favre G., Doualin G., Hossaini H.S., Bard J.M., Duriez P., Delbart C., Soula G., Lesieur D., Lesieur I., Cazin J.C., Cazin M., and Fruchart J.C., Low-density lipoprotein for delivery of an acrylophenone antineoplastic molecule into malignant cells. *Cancer Res.* **1992,** 52: 3629-3635.

Leszczyniecka M., Roberts T., Dent P., Grant S., and Fisher P.B., Differentiation therapy of human cancer: basic science and clinical applications. *Pharmacology & Therapeutics*, **2001**, 90: 105-156.

Li Y.X., Coucke P.A., Paschoud N., and Mirimanoh R. O., Cytotoxic interactions of 5-fluorouracil and nucleoside analogues *in vitro*. *Anticancer Res.* **1997**, 17: 21-28.

Li Y.X., Sun L.Q., Weber-Johnson K., Paschoud N., and Coucke P.A., Potentiation of cytotoxicity and radiosensitization of (*E*)-2-deoxy-2'-(fluoromethylene) cytidine by pentoxifyline *in vitro*. *Int. J. Cancer,* **1999,** 80: 155-160.

Liu B.Q., Peto R., and Chen Z.M., Emerging tobacco hazards in China: 1. Retrospective proportional mortality study of one million deaths. *Br. Med. J.*, **1998**, 317: 1411-1422.

Liu Q.Y. and Tan B.K.H., Effects of *cis*-unsaturated fatty acids on doxorubicin sensitivity in P388/DOX resistant and P388 parental cell lines. *Life Sciences* **2000**, 67: 1207-1218.

Lubbe A.S., Alexiou C., and Bergemann C., Clinical application of magnetic drug targeting. *J. Surg. Res.*, **2001**, 95: 200-206.

Lundberg B., Cytotoxic activity of two new lipophilic steroid nitrogen carbamates incorporated into low-density lipoprotein. *Anticancer Drug Des.*, **1994**, 9: 471-476.

Mader R.M., Muller M., and Steger G.G., Resistance to 5-fluorouracil. *Gen. Pharmac.*, **1998**, 31: 661-666.

Maletinska L., Blakely E.A., Bjornstad K.A., Deen D.F., Knoff L.J., and Forte T.M., Human glioblastoma cell lines: levels of low-density lipoprotein receptor and low-density lipoprotein receptor-related protein. *Cancer Res.* **2000,** 60: 2300-2303.

Manfredini S, Simoni D., Ferroni R., Bazzanini R., Vertuani S., Hatse S., Balzarini J., and Clercq E.D., Retinoic acid conjugates as potential antitumor agents: sythesis and biological activity of conjugates with Ara-A, Ara-C, 3(2H)-Furanone, and Aniline mustard moieties. *J. Med. Chem.* **1997,** 40: 3851-3857.

Mangelsdorf D.J., Umesono K., and Evans R.M., The retinoid receptors in *The retinoids: Biology, Chemistry, and Medicine, 2nd* , Edits: Sporn, M.B., Roberts A.B., and Goodman D.S., Wiley, New York, **1994,** p319-349.

Mangelsdorf D.J., Borgmeyer U., Heyman R.A., Zhou J.Y., Ong E.S., Oro A.E., Kakizuka A. and Evans R.M., Characterization of three RXR genes that mediate the action of 9-*cis*-retinoic acid. *Genes and Development*, **1992**, 6: 329-344.

Mann D.L., DeSantis P., Mark G., Pfeifer A., Newman M., Gibbs N., Popovic M., Sarngadharan M.G., Gallo R.C., Clark J., and Blattner W., HTLV-1 associated B-cell CLL: indirect role for retrovirus in leukemogenesis. *Science,* **1987**, 236: 1103-1106.

Mehta K., McQueen T., Neamati N., Collins S., and Andreeff M., Activation of retinoid receptors RARα and RXRα induces differentiation and apoptosis, respectively, in HL-60 cells. *Cell Growth Differ.* **1996,** 7: 179-186.

Mendenhall W.M., Rout W.R., Lind D.S., Zlotecki R.A., Hochwald S.N., Schell S.R., and Copeland III E.M., Role of radiation therapy in the treatment of respectable rectal adenocarcinoma. *J. Surg. Oncol.*, **2002**, 79: 107-117.

Menendezetal J.A., Barbacid M. der M., Montero S., Sevilla E., Escrich E., SolanasM., Cortes-Funes H., and Colomer R., Effects of gamma-linolenic acid and oleic acid on paclitaxel cytotoxicity in human breast cancer cells. *European J. Cancer*, **2001**, 37: 402-413.

Montesano R., and Hall J., Environmental causes of human cancers. *European J. Cancer*, **2001**, 37: S67-S87.

Mosmann, T., Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays *J. Immunol. Methodes*, **1983**, 65: 55-63.

Muccio D.D., Brouillette W.J., Breitman T.R., Taimi M., Emanuel P.D., Zhang X.K., Chen G.Q., Sani B.P., Venepally P., Reddy L., Alam M., Simpson-Herren L., Hill D.L., Conformationally defined retinoic acid analogs: 4. Potential new agents for acute promyelocytic and juvenile myelomonocytic leukemias. *J. Med. Chem.* **1998,** 41: 1679-1687.

Muindi J.R.F., Frankel S.R., Miller W.H.Jr., Jakubowski A., Scheinberg D.A., Young C.W., Dmitrovsky E., and Warrell R.P.Jr., Continuous treatment with all-*trans* retinoic acid caused a progressive reduction in plasma drug concentration: implication for

relapse and retinoid "resistance" in patients with acute promyelocytic leukemia. *Blood,* **1992,** 79: 299-303.

Napoli J.L., Posch K.P., Fiorella P.D., and Boerman M., Physiological occurrence, biosynthesis and metabolism of retinoic acid – evidence for roles of cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) in the pathway of retinoic acid homeostasis. *Biomed. Pharmacoth.,* **1991**, 45, 131-143.

Nason-Burchenal K. and Dmitrovsky E., The retinoids: cancer therapy and prevention mechanisms in "Retinoids: the biochemical and molecular basis of vitamin A and retinoid action". (Nau H. and Blaner W.S., eds), **1999**, p301-322.

Nayl L., Thomazy V.A., Heyman R.A. and Davies P.J.A., Retinoid-induced apotosis in normal and neoplastic tissues. *Cell Death & Differ.* **1998**, 5: 11-19.

Nicum S., midgley R., and Kerr D.J., Chemotherapy for colorectal cancer. *J. The Royal Society of Med.* **2000,** 93: 416-419.

Nishioka Y. and Yoshino H., Lymphatic targeting with nanoparticulate system. *Advanced Drug Delivery Rev.*, **2001**, 47: 55-64.

Niu S.R., Yang G.H., and Chen Z.M., Emerging tobacco hazards in China: 2. Early mortality results from a prospective study. *Br. Med. J.*, **1998**, 317: 1423-1424.

Noy N. and Xu Z-J., Interactions of retinol with binding proteins: implications for the mechanism of uptake by cells. *Biochemistry*, **1990**, 29: 3878-3883.

Nudelman A. and Rephaeli A., Novel mutual prodrug of retinoic and butyric acids with enhanced anticancer activity. *J. Med. Chem.* **2000,** 43: 2962-2966.

Pascale A.W., Ehringer W.D., Stilwell W., Sturdevant L.K., and Jenski L.J., Omega-3 fatty acid modification of human membrane structure and function. II. Alteration by docosahexaenoic acid of tumor cell sensitivity to immune cytolysis. *Nutr. Cancer*, **1993**, 19: 147-157.

Parkin D.M., Bray F.I. and Devesa S.S., Cancer Burden in the year 2000, the Global Picture. *European J. Cancer*, **2001**, 37: S4-S66.

Parkin D.M., Pisani P., Lopez A.D., and Masuyer E., At least one in seven cases of cancer is caused by smoking. Global estimated for 1985. *Int. J. Cancer*, **1994**, 59: 494-504.

Parton M., Dowsett M., and Smith I., Studies of apoptosis in breast cancer. *Br. Med. J.*, **2001**, 322: 1528-1532.

Peto J., and Houlston R.S., Genetics and the common cancers. *European J. Cancer*, **2001**, 37: S88-S96.

Plumb J.A., Lou W., and Kerr D.J., Effect of polyunsaturated fatty acids on the drug sensitivity of human tumor cell lines resistant to either cisplatin or doxorubicin. *Br. J. Cancer*, **1993**, 67: 728-733.

Ponzoni M., Lanciotti M., Montaldo P.G., and Cornaglia-Ferraris P., Gamma interferon, retinoic acid and cytosine arabinoside induce neuroblastoma differentiation mechanism, *Cell Mol. Neurobiol.* **1991,** 11: 397-413.

Pratt W.B., Ruddon R.W., Ensminger W.D., and Maybaum J., The anticancer drugs. New York, Oxford University Press, Inc. **1994,** 69-107.

Priestman T.J. Eds, Cancer chemotherapy: an introduction. Third edition. **1989**, 2-26, Springer-Verlag.

Raff M., Cell suicide for beginners. *Nature*, **1998**, 396: 119-122.

Rask L. and Peterson P.A., In vitro uptake of vitamin A from the retinol-binding plasma protein to mucosal epithelial cells from the monkey's small intestine. *J. Biol. Chem.*, **1976**, 251: 6360-6366.

Rudra P.K. and Krokan H.E., Cell-specific enhancement of doxorubicin toxicity in human tumor cells by docosahexaenoic acid. *Anticancer Res.*, **2001**, 21: 29-38.

Rudling M.J., Stihle L., Peterson C.O., and Skoog L., Content of low density lipoprotein receptors in breast cancer tissues related to survival of patients. *Brit. Med.J.* **1986,** 292: 580-582.

Rudling M.J., Angelin B., Peterson C.O., and Collins V.P., Low density lipoprotein receptor activity in human intracranial tumors and its relation to the cholesterol requirment. *Cancer Res.* **1990,** 50: 483-487.

Sagar P.S., Das U.N., Koratkar R., Ramesh G., Padma M., and Kumar G.S., Cytotoxoc action of *cis*-unsaturated fatty acids on human cervical carcinoma (HeLa) cells: relationship to free radicals and lipid peroxidation and its modulation by calmodulin antagonists. *Cancer Lett.*, **1992**, 63: 189-198.

Santi D.V., McHenry C.S., and Sommer H., Mechanisms of interactions of thymidylate synthetase with 5-fluorodeoxyuridylate. *Biochemistry,* **1974,** 13: 471-481.

Schilsky R.T., Milano G.A., and Ratain M.J., Principles of antineoplastic drug development and pharmacology. New York: Dekker, **1996**.

Schmidt-Erfurth U., Bauman W., Gragoudas E., Flotte T.J., Michaud N.A., Birngruber R., and Hasan T., Photodynamic therapy of experimental choroidal melanoma using lipoprotein-delivered benzoporphyrin. *Ophthalmology*, **1994**, 101: 89-99.

Schuetz J.D., Wallace H.J., and Diasio R.B., 5-Fluorouracil incorporation into DNA of CF-1 mouse bone marrow cells as a possible mechanism of toxicity. *Cancer Res.* **1984,** 44: 1358-1363.

Schuetz J.D., Collins J.M., Wallance H.J., and Diasio R.B., Alteration of the secondary structure of newly synthesized DNA from murine marrow cells by 5-fluorouracil. *Cancer Res.* **1986,** 46: 119-123.

Schumaker V.N. and Puppione D.L., Sequential flotation ultracentrifugation. *Methods in Enzymology* **1986**, 128: 155-170.

Schwendener R.A., Supersaxo A., Rubas W., Weder H.G., Hartmann H.R., Schott H., Ziegler A., and Hengartner H., 5'-*O*-palmitoyl- and 3',5'-*O*-dipalmitoyl-5-fluoro-2'-deoxyuridine-novel lipophilic analogues of 5'-fluoro-2'-deoxyuridine: synthesis, incorporation into liposomes and preliminary biological results. *Biochem. Biophy. Res. Comm.* **1985,** 126: 660-666.

Scollay R., Gene therapy: a brief overview of the past, present, and future. *Ann N.Y. Acad. Sci.* **2001**, 953: 26-30.

Sellmayer A., Danesch U., and Weber P.C., Prostaglandins leukot. *cis*-unsaturated fatty acids, **1997**, 57: 353-357.

Shao Y., Pardini L., and Pardini R.S., Dietary menhaden oil enhances mitomycin C anticancer activity toward human mammary carcinoma MX-1. *Lipids*, **1995**, 30: 1035-1045.

Shaw J.M., Shaw K.V., Yanovich S., Iwanik M., Futch W.S., Rosowsky A., and Schook L.B., Delivery of lipophilic drugs using lipoproteins. *Ann. NY Acid Sci.*, **1987**, 507: 252-271.

Siddiqui R.A., Jenski L.J., Weisehan J.D., Hunter M.V., Kovacs R.J., and Stilwell W., Prevention of docosahexaenoic acid-induced cytotoxicity by phosphatidic acid in Jurkat leukemic cells: the role of protein phosphatase-1. *Biochim. Biophys. Acta*, **2001**, 1541: 188-200.

Sommer A. and Santi D.V., Purification and amino acid analysis of an active site peptide from thymidylate synthetase containing covalently bound 5-fluoro-2'-deoxyuridine and methylene tetrachloride. *Biochem. Biophys. Res. Commun.*, **1974**, 57: 689-696.

Strickland P.T. and Kensler T.W., Chemical and physical agents in our environment in *clinical Oncology.* Edited by Abeloff M.D., Armitage J.O., Lichter A.S., and Niederhuber J.E. NewYork, Churchill Livingstone Inc., **1995,** 151-166.

Takase S., Ong D.E. and Chytil F., Transfer of retinoic acid from its complex with cellular retinoic acid-binding protein to the nucleus. *Arch. Biochem. Biophys.* **1986**, 247: 328-334.

Tanaka M., Yoshida S., Saneyoshi M., Utilization of 5-fluoro-2'-deoxyuridine triphosphate and 5-fluoro-2'-deoxycytidine triphosphate in DNA synthesis by DNA polymerases alpha and beta from calf thymus. *Cancer Res.,* **1981**, 41: 4132-4135.

Tan B.K.H., and Liu Q.Y., Effects of *cis*-unsaturated fatty acids on doxorubicin sensitivity in P388/DOX resistant and P388 parental cell lines. *Life Sci.*, **2000**, 76: 1207-1218.

Terry P., Lichtenstein P., Feychting M., Ahlbom A., and Wolk A., Fatty fish consumption and risk of prostate cancer. *Lancet.*, **2001**, 357: 1764-1766.

Thomas J.C. and Montgomery J.A., Complex esters of 5'-thioinosinic acid. *J. Med. Pharm. Chem.* **1962,** 5: 24-32.

Timmer-Bosscha H., Hospers G.A.P., and Meijer C., Influence of docosahexaenoic acid on cisplatin resistance in a human small cell lung carcinoma cell line. *J. Natl. Cancer Inst.*, **1989**, 81: 1069-1075.

Tokui T., Kuroiwa C., Muramatsu S., Tokui Y., Sasagawa K., Ikeda T., and Komai T., Plasma lipoproteins as targeting carrier to tumor tissues after administration of a lipophilic agent to mice. *Biopharmaceutics & Drug Disposition,* **1995,** 16: 91-103.

Toh C.H. and Winfield D.A., All-*trans* retinoic acid and side effects. *Lancet,* **1992,** 339: 1239-1240.

Tomatis L., Huff J., and Hertz-Picciotto I., Avoided and avoidable risks of cancer. *Carcinogenesis*, **1997**, 18: 97-105.

Torchilin V.P., Drug targeting. *Eur. J. Pharmaceu. Sci.*, **2000**, 11 (supply): S81-S91.

Uchida M., Brown N., and Ho D.H.W., Enzymatic conversion of 5-fluoro-2'-deoxyuridine to 5-fluorouracil or 5-fluoro-2'-deoxyuridine-5'-monophosphate in human tissues. *Anticancer Res.* **1990,** 10: 779-783.

Van Berkel T.J.C., Drug targeting: application of endogenous carriers for site-specific delivery of drugs. *J. Controlled Release.* **1993**, 24: 145-155.

Vitol S., Gahrton G., Bjorkholm M., and Peterson C., Hypocholesterolemia in malignancy due to elevated LDL receptor activity in tumor cells: evidence from studies in leukemia patients. *Lancet. II.* **1985,** 1150-1154.

Warrell R.P.Jr., de The H., Wang Z.Y., and Degos L., Acute promyelocytic leukemia. *N. Engl. J. Med.*, **1993**, 329: 177-189.

Warrell R.P.Jr, Application for retinoids in cancer therapy. *Semin. Hematol.* **1994,** 31: 1-13,

Waxman S. (Eds), *Differentiation therapy, challenges of modern medicine*, Vol 10. **1996**, Rome: Ares-Serono symposia publishers.

Waxman S., Rossi G.B. and Takaku F. (Eds), *The status of differentiation therapy*, Vol 2, **1991**, New York: Raven Press.

Weichselbaum R.R., Beckett M.A., and Diamond A.A., An important step in radiation carcinogenesis may be inactivation of cellular genes. *Int. J. Radiat. Oncol. Biol. Phys.*, **1989,** 16: 272-282.

Weichselbaum R.R., Kufe D.W., Advani S.J., and Roizman B., Molecular targeting of gene therapy and radiotherapy. *Acta Oncol.*, **2001**, 40: 735-738.

Xiao W., Wang L., Davis P.J. and Liu H., Seal oil markedly enhanced the transfer of a hydrophobic radiopharmaceutical into acetylated low density lipoprotein. *Lipids.* **1999,** 34: 503-509.

Wooldridge JE and Weiner GJ., CpG DNA and cancer immunotherapy: orchestrating the antitumor immune response. *Curr Opin Oncol.* **2003**,15:440-445.

Yamashita J., Takeda S., Matsumoto H., Unemi N., and Yasumoto M., Studies on antitumor agents. 8. Antitumor activities of *O*-alkyl derivatives of 2'-deoxy-5-(trifluoromethyl)uridine and 2'-deoxy-5-fluorouridine. *J. Med. Chem.* 32: 136-139, **1988.**

Zelent A., Krust A., Petkovich M., Kastner P, and Chambon P, cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin. *Nature,* **1988,** 339: 714-717.

Zijlstra J.G., de Vries E.G.E., Muskiet F.A.J., Martini L.A., Timmer-Bosscha H., and Mulder N.H., Influence of docosahexaenoic acid *in vitro* on intracellular adriamycin concentration in lymphocytes and human adriamycin-sensitive and –resistant small cell lung cancer cell line and on cytotoxicity in the tumor cell lines. *Int. J. Cancer*, **1987**, 40: 850-856.