CHAPTER 1

INTRODUCTION

FLUOROPYRIMIDINES

INTRODUCTION

Colorectal cancers account for about 15% of all deaths from malignant diseases in the Western world (1,2). The primary tumor can usually be adequately removed by surgery when diagnosed at an early stage. When the tumor has not infiltrated the muscular coats of the large bowel the 5 years survival rate is the same as that of the control group (3). However, when the tumor infiltrates the adipose tissue or spreads via lymphatics or the bloodstream to other sites (mainly the liver), the survival rate decreases sharply. At the time of diagnosis of the primary tumor, 25-30% of the patients already have hepatic tumor involvement, while in 40-50% of the patients the liver is a common site of recurrence (3-5). The survival is related to the extent of liver involvement and ranges from 3 months in case of wide spread disease, to 18 months with minimal disease in the liver (6,7).

Liver metastases are frequently incurable since resection is only possible in case of a solitary nodule or nodules confined to one lobe (5,6). Radiation therapy is only palliative and chemotherapy often fails because of the lack of sensitivity of these metastases to most antitumor agents (6,8,9). The highest tumor response rates are obtained with the fluoropyrimidines which therefore are an important group of antitumor drugs used in the treatment of disseminated colorectal cancer (5,6,8,10).

The fluoropyrimidines 5-fluorouracil (5FU) and 5-fluoro-2'-deoxyuridine (FUdR) were introduced more than 30 years ago by Heidelberger and co-workers as anticancer drugs (11,12). They are structural analogues of the naturally occurring pyrimidines uracil and 2'-deoxyuridine and are therefore classified as antineoplastic antimetabolites.

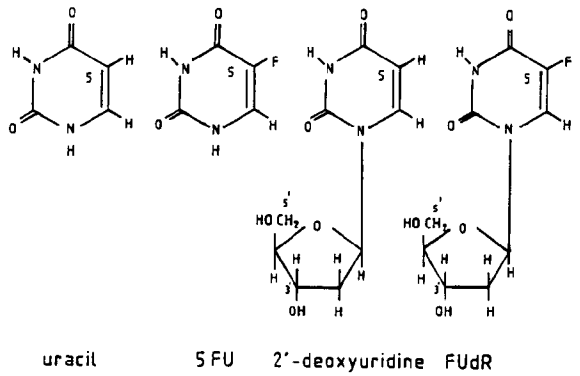


Fig. 1 *Structural formulas of fluoropyrimidines and their natural occurring counterparts*

The fluoropyrimidines were designed on a rational basis when it became known that certain tumor cells (rat hepatomas (13) and carcinomas (14)) used exogenous uracil more avidly for their pyrimidine synthesis than did healthy tissue. It was rationalized that replacement of the hydrogen at the C5 position in the pyrimidine ring by a fluorine atom (fig. 1) would yield a molecule which would be used as a substrate for biochemical reactions in virtually the same way as the naturally occurring pyrimidine (12). However, because of the structural modification it was expected to have an altered chemical reactivity, as the F-C bond is more stable than the H-C bond. This would lead to an interference with cellular processes required for RNA and DNA synthesis, and thus would lead to inhibition of cell growth.

The fate of 5FU and FUdR in vivo is complex and involves catabolic breakdown to inactive compounds and anabolic conversion to cytotoxic nucleotides which interfere with DNA and/or RNA synthesis (reviews 12, 15-20). The latter does not only takes place in tumor cells but also in normal, proliferating cells e.g. of gastro-intestinal tract and bone marrow. Thus, the balance between catabolism and anabolism on the one hand and the balance between anabolism in tumor and normal cells on the other hand is very important for the ultimate efficacy. Furthermore, there are several other factors which determine the extent of anabolism and the antitumor effect or toxicity, e.g., the presence and concentration of anabolic enzymes, of naturally occurring nucleosides and nucleotides, of various co-substrates and of the target enzymes.

ANABOLISM; MECHANISM OF ACTION

The fluoropyrimidines are active only after metabolic conversion to cytotoxic nucleotides (fig. 2) (15-20). 5FU can be metabolized to FUMP (5-fluorouridine-5'-monophosphate) via two different pathways depending on the cell type and on the concentration of co-substrates: it can be formed directly when a PRPP (5-phosphoribosyl-1-pyrophosphate) is coupled to N1 in the pyrimidine ring, or it can be formed after attachment of 5FU to ribose-1-phosphate, with the formation of 5-fluorouridine (FUR) and subsequent phosphorylation. FUMP, in turn, can be phosphorylated to FUDP (5-fluorouridine-5'-diphosphate) and finally to FUTP (5-fluorouridine-5'-triphosphate). The latter compound functions as a substrate for RNA polymerase and is incorporated into RNA.

5FU can also be enzymatically converted to 5-fluoro-2-'-deoxyuridine-5'-monophosphate (FdUMP), a powerful inhibitor of the enzyme thymidylate synthase (TS). Conversion to FdUMP proceeds either via FUdR or via reduction of FUDP and subsequent hydrolysis of the second phosphate bond. The first mechanism is important only when FUdR is given while the latter mechanism predominates for 5FU. Further, FdUMP can be converted to FdUTP which can then be incorporated into DNA. Also nucleotide sugars can be formed but the effects of these compounds on cellular functions are not fully understood.

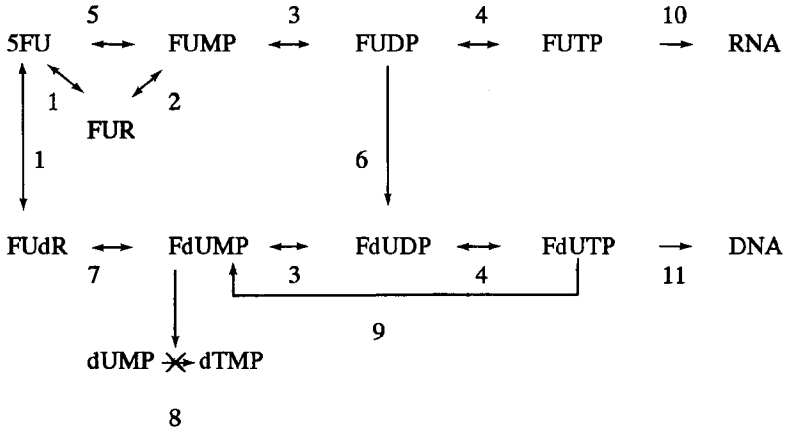


Fig. 2 *Mechanism of activation of fluoropyrimidines*
1 uridine (thymidine)phosphorylase; 2 uridine kinase; 3 (deoxy)uridine monophosphate kinase; 4 (deoxy)uridine diphosphate kinase; 5 orotidine monophosphate phosphoribosyltransferase; 6 ribonucleotide reductase; 7 thymidine kinase; 8 thymidylate synthase; 9 deoxyuridine triphosphate diphosphohydrolase; 10 RNA polymerase; 11 DNA polymerase

Inhibition of TS by FdUMP

The inhibition of TS by FdUMP is considered to be the most important mechanism in the development of cytotoxicity in several cell lines. The physiological function of TS is to catalyze the methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to thymidine-5'-monophosphate (dTMP) (17,21,22). dTMP is the precursor for dTTP which is incorporated in DNA. If the formation of this essential nucleotide is blocked, DNA synthesis is inhibited. FdUMP binds covalently to the active site of TS in the presence of reduced folates (5,10-methylene tetrahydrofolate) which function as the methyl group donor. The reaction proceeds in two steps:

1) transfer of the methylene group to the 5-position of the pyrimidine ring
2) reduction of the methylene group to a methyl group by removal of the proton from the 5-carbon. Because the FC-bond is stronger than HC-bond, fluorine is not removed and thus, in the presence of FdUMP, the reaction does not proceed past this point.

The inhibition of TS not only leads to an inhibiton of DNA synthesis but also disturbs the deoxynucleotide balance, which can have a major impact on cell functions (16). Firstly, dUMP levels increase and dTTP levels decrease (23). dTTP is an allosteric regulator of nucleoside-diphosphate reductase and when dTTP levels drop, the reduction of CDP increases

and reduction of GDP decreases (leading to elevated dCTP and lowered dGTP levels) (24,27). Thus, the inhibition of DNA synthesis is probably not solely due to depleted dTTP levels. A disturbed nucleotide balance after 5FU treatment is measured in a number of cell lines, e.g. in human melanoma IGR3 cells (increase in dATP and dCTP, decrease in and dTTP) (25), in mouse FM3A cells (increase in dATP, decrease in dGTP and dTTP) (26), and in HeLa-cells (increase in dATP, decrease in dTTP and dGTP) (27).

Incorporation of FdUTP in DNA

It has been recognized only a few years ago that FdUTP can be incorporated into DNA. This was thought to be unlikely because FdUTP was considered to be a substrate for dUTP-ase, an enzyme that normally hydrolyzes dUTP and prevents it from being incorporated into DNA. Furthermore, the enzyme uracil-(DNA)-glycosylase, which is a DNA repair enzyme, removes uracil-nucleotides from DNA (17). Later it was found that substantial levels of FdUTP were formed, that it functioned as a substrate for DNA-polymerase and that it could indeed be incorporated into DNA (28-31). Because of the incorporation of the faulty nucleotide and/or its subsequent removal, DNA is damaged. This leads to fragmentation (single strand breaks as well as double strand breaks). An other theory states that the DNA damage is caused by inefficient DNA repair due to a unbalanced nucleotide pool (dTTP and dGTP are decreased) (31). The altered nucleotide pool is a signal for endonuclease activity which cleaves DNA molecules at specific places (26).

Inhibition of RNA synthesis

5FU is incorporated as FUTP in all classes of RNA (17,18). It was shown to be incorporated in ribosomal RNA (32,33), messenger RNA (32,34), transfer RNA (35,36) as well as in small molecular weight, nuclear species (17). However, the damaging effect on RNA-level leading to cell death is thought to be the disruption of pre-rRNA processing in the nucleoli (32,33). The processing of nRNA into cytoplasmic rRNA is impaired either due to a block in processing of RNA or to a block in transport of nRNA to the cytoplasm. There is also a drop in RNA methylation, a step that is essential in RNA maturation. Due to the impaired maturation, the amount of cytoplasmic 18S and 28S rRNA is lowered and the amount of higher molecular RNA precursors in the nucleolus is raised. The latter can result in an increase in the size of nucleoli as found for example in human colonic carcinoma cells HT 29 (33), in liver parenchymal cells (37), enterocytes (38) and Ehrlich ascites cells (39). The effect on ribosomal RNA is strictly nuclear and there is no effect on mature cytoplasmic rRNA (32).

Other effects of fluoropyrimidines

Normally, the uridine metabolites are present in cells as nucleotide sugars (UDP-glucose and UDP-N-acetyl-hexosamine). These are substrates for glycosyltransferases which catalyze the glycosylation of proteins and lipids. It has been shown that FUDP-hexoses, hexosamines

and FdUDP-N-acetyl-hexosamines can be formed in liver cells after administration of 5FU (40,41). The contribution to cytotoxicity of these fluorinated nucleoside sugars is not yet clear but it is possible that they alter membrane structures by impairing biosynthesis of glycoprotein (42). Changes in transmembrane potential and in surface charge as well as an increase in cell volume have been measured. These effects make the cell more susceptible to lysis.

DNA VERSUS RNA TOXICITY

Due to the complicated metabolic activation and the several potential cellular targets, the question which mechanism is ultimately responsible for cytotoxicity is still the subject of many investigations. The mechanism depends on the intracellular as well as the extracellular environment:

1 the enzymatic machinery within the cell and the presence of cosubstrates (ribose, deoxyribose or ribose PP) determine the pathway of metabolic activation (25)
2 the turnover rate of the target enzyme and its affinity towards the fluorinated nucleotide determine the potency (17,18)
3 the concentration of reduced folates determines the potency and the duration of the TS-FdUMP block (43-46).
4 naturally occurring nucleotides can modulate the effect of fluoropyrimidines in different ways. The addition of thymidine to cells can either reverse (by circumventing the TS-FdUMP block) (17,24) or increase cell growth inhibition (elevated dTTP levels inhibit pyrimidine ribonucleotide reductase, which causes an increase in FUTP levels and RNA-directed toxicity). High concentrations of dUMP can replace or prevent TS-FdUMP binding (24,43,44), and high concentration of UTP can restore RNA synthesis (47-50).

It is thought that in vitro, TS-inhibition is the most important effect when FUdR is used (18,51). Very low doses of FUdR (sometimes even in the lower nM range) are sufficient to block TS and cell proliferation effectively (17,53). In vivo, FUdR is rapidly converted to 5FU (see below) and thus RNA effects might be considered to contribute to its antiproliferative action.
When 5FU is used, both in vitro and in vivo, TS inhibition and/or RNA-directed toxicity are important (depending on the cell type) (51,54). The cytotoxicity caused by 5FU is said to be due to TS inhibition when it can be reversed by the addition of thymidine. If the addition of thymidine leads to the same or even an increase in cell growth inhibition, RNA-directed toxicity is thought to be responsible.

For example, the cytotoxicity of FUdR in Sarcoma-180 tumor cells is caused by TS-inhibition, while part of the cytotoxicity of 5FU is due to the inhibition of (ribosomal) RNA synthesis (43,55). In a human colon adenocarcinoma cell line there is a linear relationship between TS inhibtion and the percentage cell survival when the 5FU dose is below 100 μM. At higher doses this linearity is absent, indicating that RNA-directed toxicity contributes to the overall cytotoxicity (56). In several cell lines TS inhibition becomes more important upon addition of reduced folates, which strengthen the TS-FdUMP bond (43-46).

Usually, higher doses of 5FU (more than 2 or 3 orders of magnitude) are necessary than of FUdR to inhibit cell proliferation in vitro (53). This is on the one hand due to a limited conversion of 5FU to FdUMP while FUdR is a more direct precursor (17,51). On the other hand, when reversing the TS inhibition caused by 5FU with thymidine, higher doses of 5FU were needed to inhibit RNA synthesis (55). Thus, higher doses of the drug are needed when the cytotoxicity shifts from inhibition of TS to inhibition of RNA synthesis. A survey of RNA- versus TS-directed toxicity of 5FU and FUdR in different tumor cell-lines is presented by Heidelberger (17) and Peters (25).

Several authors consider the incorporation of FdUTP in DNA and the resulting DNA damage to be the main effect leading to cytotoxicity in tumor cells (31,52), as well as in bone marrow cells (28-30).

CATABOLISM AND PHARMACOKINETICS

Fluoropyrimidines are subject to rapid intracellular catabolism in vivo via the same route as thymidine or uridine. After uptake into the cells by either facilitated diffusion or by active Na^+-dependent transport processes (57-59) FUdR is converted to 5FU. This first step in the catabolic breakdown of FUdR is mediated by phosphorylases. The process predominantly takes place in the liver but, because of the widespread presence of the enzyme, also in other organs (e.g. in intestine, kidney, and lungs) (17). Two types of phosphorylases have been identified, one of which specifically cleaves deoxyribose pyrimidines (thymidine phosphorylase) while the other cleaves ribose as well as deoxyribose pyrimidines (uridine phosphorylase) (60-63). It depends on the cell- or tissue-type which of the enzymes (or both) is present. Both enzymes are present in liver parenchymal cells (64), whereas in Kupffer cells only uridine phosphorylase is found (65).

The liver efficiently extracts FUdR from the circulation. After intraarterial hepatic injection in humans, 95% of the drug is removed during a single pass (66). The plasma half-life of FUdR after intravenous injection is therefore short (only a few minutes) and depends on the injected dose (67,68). Also, the elimination of FUdR by the isolated rat liver or hepatocytes is saturable and displays typical Michaelis-Menten kinetics (69,70). At concentrations higher than 250 μM the elimination rate becomes zero order (69-71). At low FUdR concentrations (20 μM), all FUdR is effectively converted within the cells to the end product α-fluoro-ß-alanine (FBAL) (71). At higher dose, part of the formed 5FU is released into the circulation (about 10-30% of the initial FUdR dose) (69-72) due to saturation of dihydrouracil dehydrogenase. This enzyme catalyzes the reduction of the double bond in the pyrimidine ring of 5FU to 5-fluoro-dihydrouracil (FUH2). This is the second and rate-limiting step in the catabolism of FUdR. After a bolus injection of 5FU ($t_{1/2}$ = 10 - 20 min) in humans, peak plasma concentrations of FUH2 were found 1 h after 5FU administration, slowly declining thereafter ($t_{1/2}$ = 62 min) (73). Dihydrouracil dehydrogenase is most abundant in liver but is also found in pancreas, lung, intestinal mucosa and lymphocytes (19). FUH2 has been shown to induce cytotoxicity in Ehrlich ascites cells (at concentration of 50 μM) through reverse conversion of the compound to 5FU by the same enzyme (74). The measurement of pharmacokinetic parameters of the fluoropyrimidines in vivo are hampered by the large intra-

and interindividual variations in the concentration-time product. This is due, amongst others, to the circadian rhythm displayed by uridine phosphorylase (75) and dihydrouracil-dehydrogenase (76,77).

FUH2 is catabolized further to fluoro-ureidopropionic acid (FUPA, a transient intracellular catabolite) and to FBAL (the major catabolite), NH_3 and CO_2 (19). FBAL can be detected in plasma for long periods of time (t½ = 32 h) after fluoropyrimidine administration (73). Elimination of this compound occurs mainly by urinary excretion, while part is conjugated with bile acids and excreted into the bile. In patients with an external biliary drainage, FBAL-*N*-cholic acid and FBAL-*N*-chenodeoxycholic acid have been detected (78-81). In humans with an intact entero hepatic circulation, FBAL-*N*-deoxycholic acid was also present (81). Furthermore, FBAL-*N*-muricholic acid was shown to be excreted in the bile of rats (82).

It has been suggested that FBAL can also be converted to the very toxic fluoroacetate (FA) but this compound has not yet been detected in vivo (83,84).

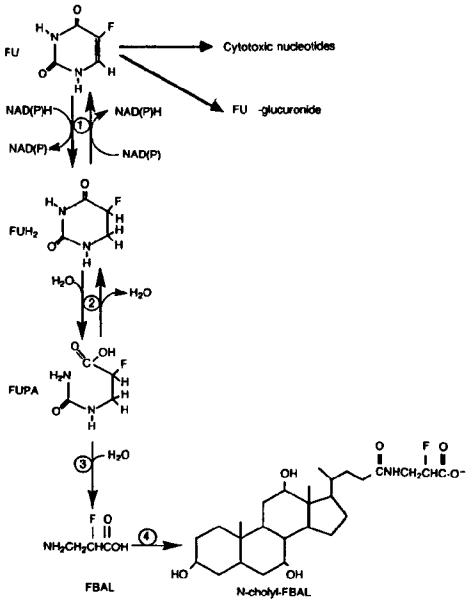


Fig. 3 *Catabolism of 5-fluorouracil*

1, dihydropyrimidine dehydrogenase; 2, dihydropyrimidinase; 3, ß-ureidopropionase; 4, *n*-acyl CoA transferase (taken from Diasio et al, 1989)

TOXICITY

The fluoropyrimidines are not only metabolized to cytotoxic nucleotides in tumor cells, but also in rapidly growing normal tissue. Cell proliferation is inhibited through the resulting repression of RNA and/or DNA synthesis (as described above). This leads to toxicity towards bone marrow (leukopenia, thrombocytopenia and anemia) and to the gastro-intestinal tract (stomatitis, mucositis, diarrhea, vomiting) (12,15-19). Also in normal rapidly dividing cells, the mechanism ultimately responsible for cell death is not fully elucidated. Bone marrow toxicity is assumed to be due to the incorporation of 5FU into RNA because high doses of uridine were shown to reverse the toxicity by replacement of FUTP from RNA (47-50). Others consider the contribution to toxicity of fluoropyrimidine incorporation into DNA to be important (28-30). Gastro-intestinal toxicity may be due to inhibition of RNA synthesis, because toxicity after administration of FUdR and 5FU correlated with FUTP- and not with FdUMP levels (85). However, in this study only free FdUMP levels were measured which do not necessarily reflect a true indication of TS inhibition. The latter depends on the level of unbound versus bound, inactivated, enzyme. Furthermore, the concentration of active nucleotides was measured only at one time point after drug administration .

The dose-limiting toxicity depends on the route and on the duration of the administration of 5FU and FUdR. Given as bolus injections, bone marrow toxicity predominates, while given as an intravenous infusion the toxic effects of the gastro-intestinal tract predominate (86-89). When FUdR is given by hepatic arterial infusion, neither bone marrow nor gastro-intestinal toxicity is dose-limiting. However, toxicity to the liver (biliary sclerosis) which develops after 1-2 months after the onset of therapy, necessitates a reduction or even cessation of the drug treatment (86,87,90,91). This toxicity must not be underestimated because it is held responsible for several drug-caused deaths (86). The mechanism of this particular toxicity is ascribed by some authors to FBAL-bile acids, which are thought to damage the canalicular membranes and induce cholestasis (92). Others consider the toxicity to be caused by an ischemic reaction to the infused drug (93).

Other toxicities (neurological and cardiological) are described but occur less frequently and are therefore less troublesome (94-97). Especially the neurological toxicity is sometimes ascribed to the catabolite fluoroacetic acid (FA) formed from FBAL. FA induces mitochondrial toxicity by impairing energy metabolism upon entering the citric acid cyclus as fluorocitrate (83). Although elevated citrate levels (caused by inhibition of the enzyme aconitase) have been measured in cats and humans, no one has yet been able to detect FA. It has been suggested that FBAL induces neurologic toxicity by a mechanism alternative to impairment of energy metabolism via conversion to FA, because FBAL was found to be more toxic than FA when injected directly into to left ventricle of cats (84). Therefore, the true mechanism of the neurological toxicity is not clarified.

The difference in dose that can be administered by either a bolus injection or by continuous infusion of 5FU and FUdR is noteworthy. 5FU is administered upto 500 mg/m^2 as a bolus injection in humans. Given as an infusion it is less toxic due to an increased catabolic breakdown (99). FUdR toxicity in contrast, increases considerably when the drug is given as an infusion: the tolerated dose is upto 1 g/m^2 when the drug is given as a bolus injection but only 0.075-0.2 mg/kg/day when administered as an intravenous infusion (86,87,89). The low toxicity of bolus injections is caused by the rapid degradation of the drug to 5FU and inactive catabolites.

POLICY OF 5FU AND FUdR ADMINISTRATION

As described above, the fluoropyrimidines are a very important class of drugs in the management of disseminated colorectal cancer because they display the highest tumor response rates of all chemotherapeutic agents tested. However, despite these objective responses, patient survival is usually not prolonged considerably. Therefore, many strategies have been tested in order to improve their therapeutic efficacy. These include variations in drug dose, administration route and time of administration (bolus injections versus infusion).

FUdR and 5FU are time-dependent drugs and therefore continuous exposure of tumor cells to the drug is favored. This will ensure the exposure of all tumor cells to the cytotoxic action throughout their entire cell cycle including their vulnerable mitotic phase. In vitro, the antiproliferative effect of FUdR in several cell-lines is reached at concentrations far lower (upto 2- 3 orders of magnitude) than of 5FU (53). In vivo, however, the effects are comparable due to the rapid conversion of FUdR to 5FU (100). If this conversion is reduced or prevented, prolonging the residence time of the drug in vivo, the higher activity observed in vitro could be expected to be found in vivo, as well.

There are several ways to prolong the residence time of the fluoropyrimidines in vivo.

1- Infusion: a continuous intravenous infusion of FUdR is relatively toxic and leads to low tumor response rates (10%). Therefore this treatment is not recommended. Regional delivery of the drug is favored e.g. in case of hepatic metastasis, ensuring high concentration to liver tumor cells while systemic concentrations will remain low because of the high extraction rate of the drug by the liver. Clinically, hepatic arterial infusion of FUdR leads to the highest tumor response rate (response rates of upto 80% are reported) (5). Hepatic arterial infusion is possible through the implantation of a pump device. This, however, necessitates hospitalization with surgical installment of the device. This can only be performed in patients who are in relatively good conditions and can tolerate this major surgery. A second disadvantage of this method is the growth of tumor foci outside the liver because systemic FUdR concentrations remain very low. Further, liver toxicity develops in a number of patients which necessitates dose reduction or cessation of the therapy (90,91).
2- Inhibitors of catabolic enzymes dihydrouracil-dehydrogenase (18) or phosphorylases. To date, several enzyme inhibitors have been tested in order to reduce the breakdown of the fluoropyrimidines. However, this approach has not yet been proven to be successful since the general toxicity increases even more than the antitumor activity.
3- Prodrugs. When FUdR is given as a prodrug it is no substrate for phosphorylases and it is thus protected from rapid degradation. A scala of different FUdR-prodrugs have been synthesized and partially tested (see below). The focus of the present research was on lipophilic FUdR-derivatives because they were expected to be excellent candidates for liposome incorporation.
4- Liposomes. By liposomal encapsulation, FUdR is protected against rapid degradation. Furthermore, liposomes can be directed to the liver, the target organ for FUdR therapy. A further advantage of liposomes is that their characteristics and thereby their behavior in vivo, and that of the encapsulated drug, can be readily manipulated (see below).

In this thesis the combination of lipophilic FUdR prodrug and liposome formulations has been tested; both are discussed in more detail below.

LIPOSOMES

INTRODUCTION

Liposomes are vesicles consisting of one or several concentric lipid bilayers, enclosing as many aqueous compartments (101, 102). They form spontaneously when suitable amphiphiles, like phospholipids, are allowed to hydrate in an aqueous medium (102). From the time Bangham first described the formation of vesicular structures from phospholipids in 1965 (103), research in this field has expanded enormously, especially when the use of liposomes as a drug carrier system was recognized (104). Liposomes are considered to be good candidates for drug carriers because they are relatively easy to prepare, exhibit little or no immunogenicity and toxicity by themselves, are biodegradable and can carry a diversity of compounds, either encapsulated in the water phase or incorporated in the lipid bilayer. Furthermore, liposome properties can be easily modified by changing the liposomal lipid composition, charge or particle size or chemical modification of the liposomal surface (reviews 105-110).

In many cases, therapeutic applicability was shown to improve, by using liposomes as a drug carrier system (111). As a matter of fact, liposomal formulations of several experimental as well as clinically applied drugs are currently being studied or, in some cases, already marketed e.g. as carrier for Doxorubicin in cancer chemotherapy (reviews 112-114) , for Amphotericin B in the treatment of fungal infections (115,116), and also as a carrier for contrast agents in tumor diagnosis (117,118).

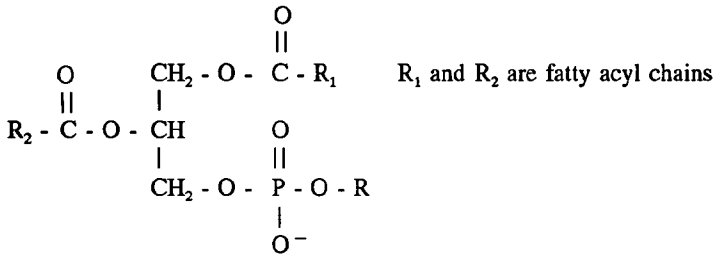
When a drug is incorporated in liposomes, its pharmacokinetic behavior in vivo usually differs considerably from that of the free drug. This may result in several advantages, one of which is a decreased toxicity, since the drug is prevented to reach organs which are otherwise affected adversely (e.g. Doxorubicin) (119). Also, it often leads to an increase in the in vivo residence time of the drug, in particular when this drug is normally subject to rapid catabolism (cytarabine) (111). In certain cases liposomal formulations of a drug may decrease its effective dose (cytarabine, methotrexate) (106, 111). Finally, liposomes are particularly good vehicles for lipophilic drugs, which would otherwise be difficult to administer.

PREPARATION, CHARACTERIZATION AND STABILITY OF LIPOSOMES

Phospholipids are amphiphiles that consist of a glycerol or a sphingosine backbone, conjugated with a hydrophobic moiety (one or two fatty acid chains) and a hydrophilic polar headgroup (a phosphorylated alcohol). They can be electrically neutral or bear a, usually, negative charge (101). Examples of some phospholipids are given in fig. 4.
Phospholipids can be recovered from biological material (e.g. PC from egg yolk or soy beans; PS from bovine brain). These natural phospholipids, usually are heterogeneous with respect to their fatty acid chains and usually have a high degree of polyunsaturation, which can affect

liposome characteristics. Synthetically prepared phospholipids have well-defined fatty acid compositions and can be obtained with different or identical saturated or unsaturated chains (101, 120).

Fig. 4 *Structure of phospholipids used in this thesis (120)*



Phospholipid	Abbreviation	source	R	acyl group	charge	T_t °C
Phosphatidylcholine	PC	egg-yolk	choline	mixed	0	- 15
Phosphatidylcholine (distearoyl)	DSPC	synthetic	choline	stearic acid	0	58
Phosphatidylglycerol (dipalmitoyl)	DPPG	synthetic	glycerol	palmitic acid	- 1	41
Phosphatidylserine	PS	bovine brain	L-serine	mixed	- 1	6 - 8

PC is the major component of most biological membranes and is often used as bulk lipid for the preparation of liposomes. The hydrated phospholipid bilayer is energetically very stable because the water exposed surface area of the lipophilic fatty acyl moieties is minimized, while Van der Waals forces allow strong interactions between the acyl chains (102).

Phospholipid bilayers can exist in different thermodynamic phases (fig. 5) whereby transition from one phase to another may occur upon a change in temperature. When the temperature is below this phase transition temperature (T_t) of the phospholipids they form a tightly ordered 'gel' or 'solid' phase. As the temperature increases the phospholipids pass into a fluid-crystal phase and finally into a fluid phase with increasing freedom of movement of the molecules. The T_t of phospholipids, which is defined within 0.5 °C for a pure

phospholipid species but may span a range of more than 10 °C for a mixture, depends on the length and the degree of saturation of the acyl chains (fig. 4). The thermodynamic phase condition of the liposomal bilayer is very important because it strongly influences the stability and behavior of the liposomes in biological systems (101,120).

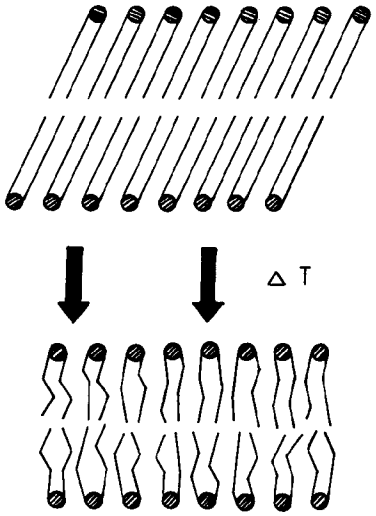


Fig. 5 *Thermodynamic phases of phospholipid bilayers (New,1990)*

Without further processing, a dispersion of phospholipids in aqueous media will consist of multilamellar vesicles (MLV) with particle sizes ranging from 0.4-3.5 μm. When, however, a liposomal preparation with vesicles of a well defined size and a narrow size distribution is desired, further processing of the initially obtained liposome suspension is necessary. This can be achieved amongst others by subjecting the crude liposome preparation to one of the following protocols:

- extrusion of the liposome suspension, at (high) pressure, through membranes with well-defined pore sizes. Thus, MLV with the desired vesicle diameter can be obtained (fig. 6) (102,121,122).

- ultrasonic irradiation of the liposome suspension, resulting in the foration of small unilamellar vesicles (SUV) (fig. 6) (102,122).

Depending on its physico-chemical properties, a drug can either be incorporated in the lipid bilayer (for lipophilic drugs) or encapsulated in the water phase of the liposome (for

hydrophilic drugs) (fig. 6). A lipophilic drug will be accommodated between the lipid molecules of the bilayer (102,123). The amount of drug that can be incorporated will depend on how well the drug fits in between the bilayer molecules and on the strength of the drug-lipid interaction. This will also determine the stability of the drug-liposome complex in vivo. Hydrophilic drugs, that have no interaction with the lipid bilayer, are encapsulated in the aqueous compartment of the liposomes. The amount of drug that thus can be encapsulated is proportional to the volume of the enclosed water phase (124). Upon liposome preparation, the drug will also be present in the water phase surrounding the liposomes and can be removed from the liposome-associated drug by gelfiltration chromatography, dialysis or centrifugation (102,122).

Upon storage, liposome-encapsulated drugs can cross the bilayer by passive diffusion. The rate at which a compound will 'leak' across the membrane, depends on its molecular weight, charge and hydrophilicity: leakage will be retarded when the molecular weight of the compound is high, the compound is charged and/or highly water-soluble (101). The composition of the bilayer also determines how efficiently a drug is retained within the liposomes. The permeability of the bilayer decreases with increasing saturation of the phospholipid acyl chains and with increasing acyl chain length. Both factors result in an increase in the transition temperature of the phospholipids and render the liposome-drug complex more stable (101,125).

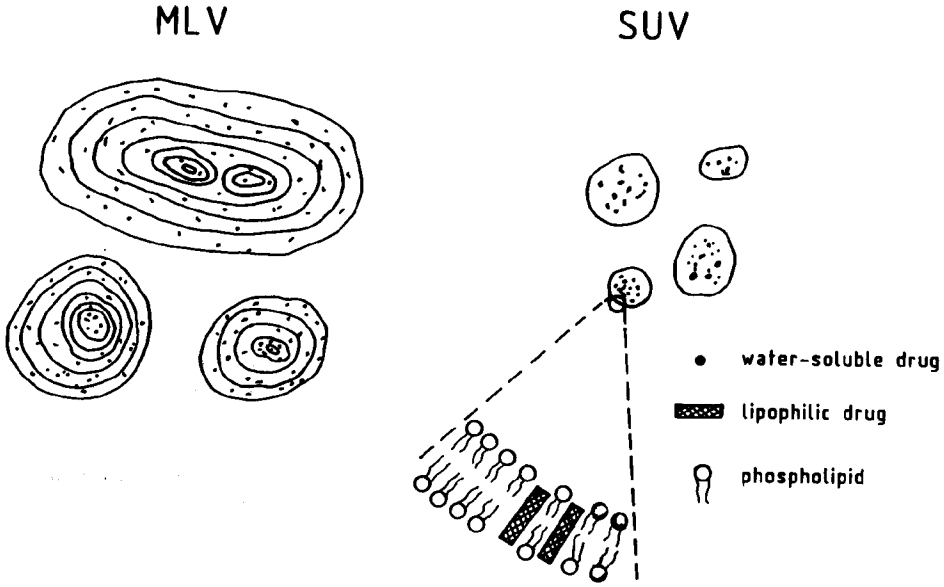


Fig. 6 *Schematic representation of multilamellar and small unilamellar vesicles and possible drug-liposome interactions*

Cholesterol influences the fluidity of the liposomal membrane and thus its permeability. It tends to rigidify bilayers of phospholipids below their Tt and thus to diminish their permeability towards solutes. Cholesterol can be incorporated into phospholipid bilayers upto 50 mol% (101).
The final preparation has to be characterized in order to obtain a well defined drug formulation. Characterization comprises the assessment of (102,124):

- particle size and size distribution
- particle charge
- concentration of drug and lipid, and thus the drug-to-lipid ratio
- stability of the liposome preparation upon storage (retention of contents, fusion, aggregation, chemical stability of contents and liposomal constituents)

INTERACTION OF LIPOSOMES WITH CELLS

Liposomes can interact with cells in several ways as is shown in fig. 7. These interactions depend on liposome characteristics (size, charge, lipid composition) as well as on cell characteristics. Interactions can be classified as (reviews 107-110,126):

- Adsorption:
  Liposome adsorption to a cell surface might take place as a result of physical attractive forces or as a result of binding by specific receptors to ligands on the vesicle membrane. Adsorption can be the first step to further interaction
- Lipid transfer:
  Phospholipid transfer between liposomes and biological membranes can take place when they are in close contact. It is assumed that a specific cell-surface exchange protein plays a prominent role because transfer is confined to certain phospholipids (PC and PE) and is reduced after trypsin treatment (126). Cholesterol, on the other hand, transfers readily, presumably through the aqueous phase. Lipid transfer does not necessarily lead to a disruption of the liposomal bilayer as the transfer is reversible. Lipid transfer between liposomes and lipoproteins is discussed below.
- Contact-release:
  Upon interaction of the liposome with the cell surface a partial and transient release of contents may take place.
- Fusion:
  Fusion of liposomes with the plasma membrane leads to the delivery of its aqueous phase content to the cytoplasm and mixing of the liposomal lipids with the cell membrane lipids. In vitro situations, fusion can be brought about by the presence of fusogens (detergents, surfactants or fusogenic proteins). In vivo, fusion is a rare event because liposomes are generally cleared rapidly from the circulation by phagocytic cells.
- Endocytosis:
  This is the main mechanism of interaction between liposomes and cells possessing endocytic capacity. Liposomes are taken up in endosomes which are formed by invagination of the plasma membrane. These endosomes then fuse with primary lysosomes to form secondary lysosomes where lysosomal enzymes digest the liposomes. During this process a liposome-associated drug may leak out of the lysosomes and gain access to the cytoplasm, alternatively it is degraded by lysosomal enzymes or it is stored in vacuoles

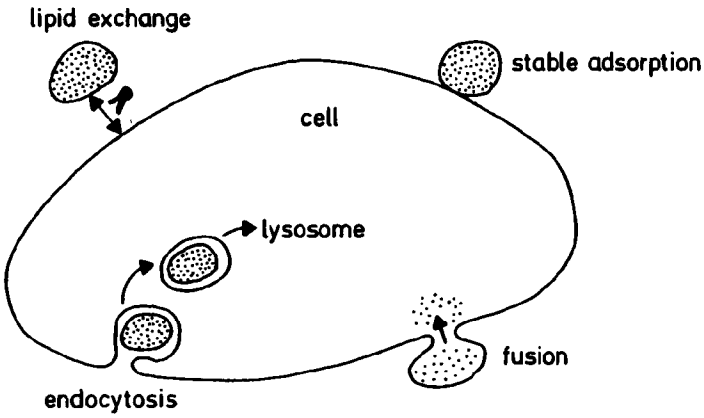


Fig. 7 *Possible liposome-cell interaction*

until exocytosis takes place. The rate of enzymatic degradation of the liposomes depends strongly on their lipid composition. Liposomes consisting of bilayers with high rigidity are far more resistant to intralysosomal digestion than more fluid-type liposomes. Cholesterol greatly contributes to liposomal resistance to lysosomal degradation.

STABILITY OF LIPOSOMES IN BLOOD

When liposomes are injected intravenously they first come into contact with blood. Of the various blood components especially HDL (high density lipoprotein) can destabilize liposomes due to transfer of (phospho)lipid components between liposome and HDL (127). This will result in an increase in liposome permeability and thus in release of an encapsulated solute. Whether an incorporated lipophilic drug itself can also transfer to blood components depends on the properties of the drug. Liposomes may also interact with opsonizing serum proteins that make liposomes attractive to cells of the mononuclear-phagocyte system (107-110, 126,128).

INTERACTION OF LIPOSOMES WITH THE MONONUCLEAR PHAGOCYTE SYSTEM (MPS) AND OTHER CELLS

Cells of the MPS are specialized in removing foreign particles from the blood stream. They are prominantly located in the liver (Kupffer cells), in the spleen and in the bone marrow.

When a compound is injected intravenously it reaches the liver via the portal vein or the hepatic artery and transverses the liver lobules via the sinusoids from the periportal to the centrilobular region and eventually may end up in the central vein. Kupffer cells are located within the sinusoids and thus are in direct contact with the bloodstream (129,130). The lining of the sinusoids, which is formed by endothelial cells, is fenestrated with pore sizes of about 100 nm (131). Particles (small liposomes) of 100 nm or smaller can cross the endothelial lining through these pores and end up in the space of Disse where they can interact with hepatocytes.

The liver anatomy is very important in the intrahepatic distribution of liposomes. Large liposomes (>200nm) end up in the Kupffer cells whereas smaller liposomes can transverse the endothelial lining and can be endocytosed by hepatocytes (132-134). Therefore, the size of the liposome is a major determinant for its intrahepatic distribution. Endothelial cells do not participate in liposome endocytosis (135).

Liposome elimination from the blood depends on the charge of the particles. Negatively charged liposomes (containing e.g. PS or PG), are cleared more rapidly than positively charged liposomes (containing e.g. stearylamine) (110). The rate of elimination also depends on the liposomal lipid composition and size. Liposomes composed of phospholipids with a high transition temperature usually exhibit a longer circulation half life than liposomes containing unsaturated, or short chain phospholipids (136,137). Also cholesterol influences liposome clearance. A high cholesterol content renders liposomes more resistant to opsonine proteins and therefore to endocytosis (138,139). Large liposomes are usually cleared more rapidly from the circulation than small liposomes (126,136). The clearence can be modulated by modifying the liposome surface with substances such as gangliosides (GM1) (140,141) or poly ethelyne glycol coupled to phospholipids (142,143). This leads to a reduced recognition of liposomes by macrophages. For this reason they have been dubbed "stealth" liposomes.

FUdR IN LIPOSOMES

Literature on liposomal FUdR formulations is scarce. Simmons (144) showed that the encapsulation efficiency of FUdR in liposomes is low, and depends on liposomal lipid composition. Incorporation efficiency of FUdR in liposomes consisting of SM/CH/DCP (sphingomyelin, cholesterol, dicetylphosphate) was higher than that of egg PC liposomes. Also, upon storage the drug was retained better within the SM-liposomes. 5FU, which is less water-soluble than FUdR, could not be adequately incorporated in liposomes and leaked out considerably faster than FUdR.

Another report on liposomal FUdR stems from Juliano and co-workers (145) who showed that the distribution of FUdR in rats could be changed after administration of FUdR in DPPC/DPPG/CHOL liposomes. It was found that liposomes can protect FUdR against rapid degradation to 5FU and that FUdR accumulated in liver and spleen (measured 90 min after a single injection). As far as we know, the therapeutic efficacy of liposomal FUdR in vivo has not been investigated.

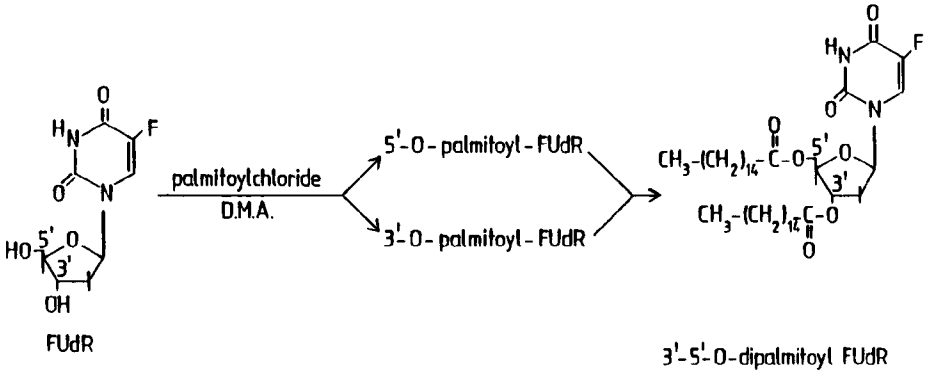


Fig. 8 *Synthesis of FUdR-dipalmitate*

LIPOPHILIC FUdR DERIVATIVES

The synthesis of lipophilic derivatives of FUdR is relatively simple. It can be accomplished, e.g., by incubating FUdR with a fatty acid anhydride or a fatty acid chloride in water-free solvent (pyrimidine or dimethyl acetamide) (fig. 8). In this procedure, the free hydroxyl groups in the deoxyribose moiety of FUdR are esterified. Depending on the molar ratio of fatty acid to FUdR, a 3'-O- and 5'-O-mono-ester or a 3'-5'-O-diester is obtained.

Nishizawa (146) was the first to synthesize lipophilic esters of FUdR, which appeared to be insensitive to nucleoside phosphorylases. This was shown for the methyl-esters (147) and for other esters (148). The lipophilic derivatives had antitumor activity after oral application in mice bearing adenocarcinoma 755 tumor cells (146). The esters were believed to be degraded by aspecific esterases which was later confirmed by (149-151). Esterase activity was demonstrated in several organs but was most abundant in liver, and intestine and was associated with a particulate fraction (147,148). Its activity appeared to be highest towards prodrugs of FUdR with aliphatic C8 or C10 chains and decreased steeply with increasing chain length (149). The prodrugs with longer aliphatic acyl chains, e.g. palmitic acid, were the FUdR-prodrugs with highest antitumor activity because FUdR plasma concentrations were elevated up to 48 hours after drug application (151).

The lipophilic prodrug FUdR-dioctanoate has been used in preclinical as well as in clinical studies for the treatment of liver tumor growth. In these cases the drug was dissolved in the lymphographic agent Lipiodol® which is selectively retained in the tumor area. This combination has been shown to display antitumor activity in a rabbit tumor model (150,152) as well as in humans (153), whereas its toxicity was low. FUdR was shown to be released

slowly after prodrug hydrolysis, and due to the selective retention of Lipiodol® in the tumor area only tumor cells were exposed continuously to the drug.

To our knowledge the only studies of the combination of FUdRdP and liposomes were reported by Schwendener and Supersaxo (154-156). These authors tested the antitumor activity of a liposomal formulation in different murine tumor models and found that FUdR applied in such a way retained its antitumor activity at dosages upto 75 times lower than those required for unmodified FUdR. In this study only one type of liposome was examined, small unilamellar vesicles consisting of egg PC/SA/CHOL/α-tocopherol prepared by means of a controlled dialysis technique.

SCOPE OF THIS THESIS

In this thesis attention is focussed on the use of liposomes to change the pharmacokinetics of FUdR in order to increase its efficacy. As stated above, FUdR is primarily used as an anticancer agent in the treatment of liver metastases in disseminated colorectal carcinoma. One of the reasons why the response rates to this drug are usually low is assumed to be the rapid degradation of the drug in vivo, which prevents an adequate exposure of tumor cells to the drug throughout their entire cell cycle including the vulnerable mitotic phase. To prolong the exposure time of liver tumor cells to the drug, liposomes were expected to be of value, for they are mainly taken up by Kupffer cells in the liver which can function as a drug-depot. In these cells, the liposomes are degraded slowly in the lysosomes whereupon the encapsulated drug is gradually released into the hepatic circulation. Thus, it is expected that higher concentrations of the drug in the surroundings of the target cells are sustained for longer periods of time than would be obtained upon administration of the free drug. (fig. 9)

The rate at which the drug is released from the Kupffer cells is believed to be of major importance to the antitumor activity and is controllable by changing the liposomal lipid composition. A rapid release of the drug will be attained by using readily degradable liposomes and a slower release by using liposomes with a higher resistance to lysosomal degradation. Therefore, two liposome types with quite different biodegradabilities were evaluated: egg PC/PS/CHOL and DSPC/DPPG/CHOL.

Most of the studies in this thesis were performed with lipophilic derivatives of FUdR because these were envisioned to display a higher incorporation efficiency and a better retention within the liposomes than the water-soluble FUdR. To investigate the contribution of the type of prodrug, two types of lipophilic FUdR-diesters were synthesized which are known to be catabolized at different rates in vivo: i.e. FUdR-dioctanoate and FUdR-dipalmitate.

A liposomal FUdR-prodrug can be considered as an entirely different administration form of FUdR and thus has to be regarded essentially as a new drug. Therefore, several questions had to be answered in order to assess the value of such liposomal lipophilic FUdR-prodrugs and to conclude which prodrug-liposome complex is to be used to obtain optimal efficacy. In this thesis, studies are described on different aspects of the application of FUdR-prodrugs in order to resolve some of the questions concerning the use of a this new drug formulation:

- In vitro characteristics of the liposome-drug complexes including stability in serum, and antiproliferative activity (chapter 2).
- Processing of FUdR-dipalmitate by Kupffer cells and the influence of liposome composition. Is the active compound FUdR released from the Kupffer cells? (chapters 3 and 4).
- Comparison of the in vivo distribution, catabolism and excretion of both liposomal FUdR-dipalmitate and free FUdR (chapter 5 and 6).
- Comparison of the antitumor activity of liposomal FUdR prodrugs and of free FUdR in different murine tumor models (chapter 7).
- Comparison of the toxicity of liposomal FUdR prodrugs and FUdR in vivo (chapter 8).

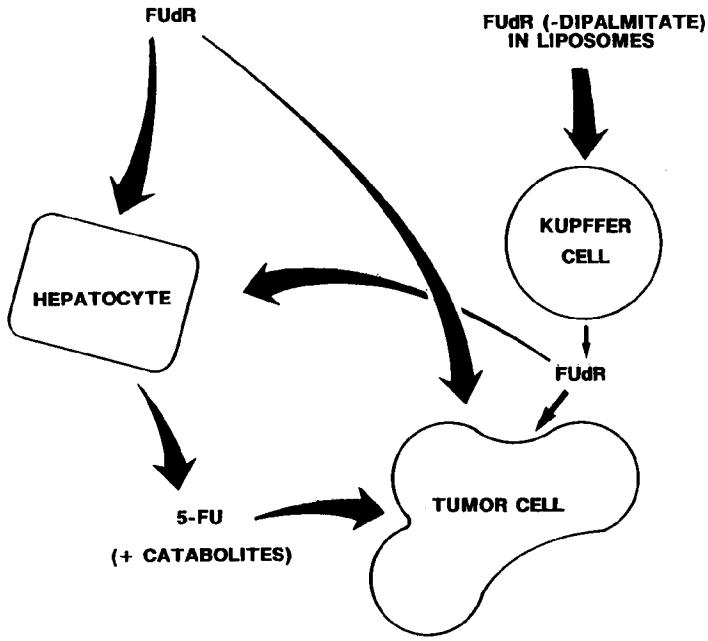


Fig 9. *Interaction of FUdR and liposomal FUdR-dipalmitate with liver cells*
Given as a bolus injection, FUdR is rapidly catabolized (in hepatocytes) to 5FU and inactive catabolites. This results in a short exposure time of liver tumor cells to the drug. In contrast, liposomal FUdR or FUdR-dipalmitate will accumulate in liver Kupffer cells, where the liposomes and prodrug are degraded intralysosomally. It is expected that this will result in a slow release of the active drug FUdR from the Kupffer cells, and thus in a prolonged exposure of tumor cells to this drug.

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