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New Amphiphilic Heterodinucleoside Phosphate Dimers of 5-Fluorodeoxyuridine: Anticancer Activity and Cellular Pharmacology in Human Prostate Tumour Cells

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Table of Contents

Summary 3

Zusammenfassung 6

Abbreviations 9

1 Introduction 13

1.1 Cancer of the Prostate 13
1.1.1 Introduction 14
1.1.2 Anatomy, Physiology and Pathology 14
1.1.3 Epidemiology 15
1.1.4 Carcinogenesis 16
1.1.5 Treatment 16
1.2 Apoptosis and Necrosis 18
1.2.1 Introduction 18
1.2.2 Characteristics of Apoptosis and Necrosis 18
1.2.3 Importance of Apoptosis in Oncology 20
1.2.4 P53 and its Role in Oncology 21
1.3 Anticancer Drugs 22
1.3.1 A new Class of anticancer Drugs: Amphiphilic heterodinucleoside phosphate Dimers 22
1.3.2 Fluoropyrimidines 24
1.3.2.1 Introduction 24
1.3.2.2 Mechanism of Action 24
1.3.2.3 Mechanisms of 5-FU Resistance 26
1.3.2.4 Pharmacokinetics and Toxicity 26
1.3.3 Cytosine arabinoside (ara-C) 28
1.3.3.1 Introduction 28
1.3.3.2 Mechanism of Action 28
1.3.3.3 Pharmacokinetics and Toxicity 29
1.3.3.4 Hydrophilic Ara-C-Derivatives 29
1.3.4 Lipophilic Ara-C-Derivatives 30
1.3.4.1 Introduction 30
1.3.4.2 Antitumour Activity and Toxicity 32
1.3.4.3 Mechanism of Action 32
1.3.4.4 Metabolism in Mice 33
1.3.4.5 Distribution in Human Blood 33

1.4 Liposomes 33
1.4.1 Introduction 33
1.4.2 Composition, Characterisation and Preparation of Liposomes 35
1.4.3 Interactions of Liposomes with Cells 36
1.4.4 Interactions of Liposomes with the Mononuclear Phagocyte System (MPS) 37
1.4.5 Active and passive Targeting of Liposomes 38

2 Materials and Methods 41

2.1 Reagents, Drugs and Antibodies 42
2.2 Cells 42
2.3 Liposome Preparation 42
2.4 Drug Uptake 43
2.5 Haemolytic Activity in Vitro 43
2.6 Cytotoxicity Assay 43
2.7 Cell Cycle Distribution Analysis 44
2.8 Quantification of the apoptotic Cell Fraction 44
2.9 Caspase-3 Activity 45
2.10 DNA Fragmentation 45
2.11 Immunofluorescence Labelling and Confocal Microscopy 45
2.12 Electron Microscopy 46
2.13 Thymidylate Synthase Activity 47

3 Results 49

3.1 Inhibition of Cell Growth 50
3.2 Cellular Drug Uptake 54
3.3 Haemolytic Activity in Vitro 55
3.4 Cell Cycle Arrest 57
3.5 Induction of Apoptosis 61
3.6 DNA Fragmentation 63
3.7 Increase of Caspase-3 Activity 64
3.8 Disruption of Cytoskeleton and Formation of Apoptotic Nuclei . 66
3.9 Inhibition of Thymidylate Synthase Activity 68

4 Discussion 71

5 References 79

Appendix..... 101

List of Publications and Presentations 102

Publications 102
Poster Presentations..... 102
Annual Reports 103
Oral Presentations 103

Curriculum vitae 104

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Summary

Prostate cancer is a leading cause of morbidity and mortality among men. Current therapies, including treatment with 5-fluorodeoxyuridine (5-FdU), a chemotherapeutic agent frequently used against solid tumours, have limited impact on the progression of metastatic hormone-refractory prostate cancer. The usefulness of 5-FdU is impaired by the frequent development of resistance in tumour cells, which can develop by deletion of one of the key enzymes required for its activation or by mutations in the p53 gene. To overcome the resistance and enhance the effectiveness of this drug new amphiphilic heterodinucleoside phosphate dimers of 5-FdU were synthesised. As second molecules substances with antitumour activity were chosen and linked to 5-FdU trough a 3'→5' or 5'→5' phosphate bond. The best studied molecule linked to 5-FdU was N^4-octadecyl-1-β-D-arabinofuranosylcytosine (NOAC), a new anticancer drug effective in the L1210 leukaemia mouse model and in different solid human tumour xenograpfts, including PC-3 human prostate tumour xenografts. In the present study the usefulness of the dimers as new drugs against p53 mutated, androgen-independent DU-145 and PC-3 human prostate tumour cells was examined, comparing them to 5-FdU and NOAC for their cytotoxic effect and the cell cycle dependence of cytotoxicity, as well as for their capacity to induce apoptosis and inhibit thymidylate synthase (TS).

Treatment of the cells with the new dimers N^4-palmitoyl-2'-deoxycytidylyl-(3'→5')-5-fluoro-2'-deoxyuridine (dCpam-5-FdU), 2'-deoxy-5-fluorouridylyl-(3'→5')-2'-deoxy-5-fluoro-N^4-octadecylcytidine (5-FdU-5-FdC18) and 2'-deoxy-5-fluorouridylyl-(5'→5')-N^4-octadecyl-1-β-D-arabinofuranosylcytosine (5-FdU-NOAC) resulted in a marked cytotoxicity with IC_{50} values of 3-5 μM, as determined with the WST-1 cytotoxicity assay. 5-FdU-5-FdC18 and 5-FdU-NOAC at 100-200 μM were able to overcome 5-FdU resistance in both cell lines, eradicating 100% of the tumour cells.

Cytotoxicity was caused by S-phase arrest. Flow cytometric analysis revealed a dramatic increase of the cell population in early S-phase after treatment with 5-FdU, 5-FdU-5-FdC18 and dCpam-5-FdU. The latter was the most potent agent arresting the cell cycle, resulting in an increase of this cell population from 35 to 84 % in DU-145 and from 36 to 78% in PC-3 cells after 24 h incubation at 50 μM. Significant S-phase arrest was indicated by a decreased proportion of cells in G1- and G2/M-phases. 5-

FdU-NOAC and NOAC did not alter cell cycle drastically causing only a slight increase in S-phase cells.

Cell cycle arrest and inhibition of cell proliferation were followed by apoptosis. Quantification of apoptotic cell death was determined with the monoclonal antibody Apo 2.7 using flow cytometry for detection. 5-FdU, dCpam-5-FdU, 5-FdU-5-FdC18 and 5-FdU-NOAC caused a significant induction of apoptosis resulting in 67-87% of DU-145 and 22-54% of PC-3 cells being apoptotic after 96 h incubation with 50 µM. 5-FdU-5-FdC18 increased the number of apoptotic PC-3 cells up to a factor of 1.6 compared to 5-FdU. Contemporaneous, a 6-8 -fold increase in caspase-3 activity in DU-145 and a 8-11 -fold increase in PC-3 cells was found in cells treated with 5-FdU, dCpam-5-FdU and 5-FdU-5-FdC18. NOAC induced only 18% of DU-145 and 6% of PC-3 cells to undergo apoptotic cell death. 5-FdU-NOAC and NOAC did not induce caspase-3. DNA fragmentation further confirmed the induction of apoptosis in both cell lines. Confocal laser scanning and electron microscopy revealed the disruption of the cells into apoptotic bodies after treatment with 5-FdU-5-FdC18.

As 5-FdU the dimers also specifically inhibited TS in a time- and concentration-dependent manner. The enzyme activity was inhibited by 50% after 90 min at 5-6 nM 5-FdU and 0.6-0.7 µM dimer concentration in both cell lines, whereas NOAC did not alter TS activity.

In conclusion, the results of this study demonstrate that the new amphiphilic dimers are able to overcome 5-FdU resistance in androgen-independent DU-145 and PC-3 cells. It can be assumed that the dimers are cleaved into the monophosphorylated form 5-FdUMP, resulting in sustained intracellular drug concentration over an extended period and consequently increasing the duration and magnitude of the cytotoxic effect. This hypothesis is supported by the fact that the new dimers exert a cell cycle phase-dependent cytotoxicity and specifically inhibit TS activity, two mechanisms characteristic for 5-FdU. Furthermore, the dimers are able to induce apoptosis, a process often hindered or suppressed in cancer cells. In summary, findings of the present study suggest the great potential value of the dimers as new therapeutic agents against p53 mutated, hormone-independent prostate carcinoma.

Zusammenfassung

Prostatakrebs ist eine der häufigsten Ursachen für Krankheit und Sterblichkeit bei Männern. Gegenwärtige Therapien, inklusive Behandlungen mit 5-Fluorodeoxyuridin (5-FdU), ein häufig verwendetes Chemotherapeutikum gegen solide Tumoren, haben nur beschränkt Einfluss auf die Weiterentwicklung von metastatischen hormon-resistenten Prostatatumoren. Die Nützlichkeit von 5-FdU wird durch die häufige Resistenzentwicklung in Tumorzellen eingeschränkt. Die Resistenz gegen 5-FdU kann durch die Deletion eines Schlüsselenzymes, welches für die Aktivierung benötigt wird oder durch Mutationen im p53 Gen verursacht werden. Um die Resistenzentwicklung gegen 5-FdU zu überwinden und dessen Wirksamkeit zu erhöhen, wurden neue amphiphile heterodinucleosidphosphate Dimere von 5-FdU synthetisiert. Als zweite Moleküle wurden Substanzen mit Antitumoraktivität gewählt und durch eine 3'→5' oder eine 5'→5' Phosphatbindung an 5-FdU gekoppelt. Das am besten untersuchte Molekül, welches an 5-FdU gebunden wurde, ist N^4-octadecyl-1-β-D-arabinofuranosylcytosin (NOAC). NOAC ist eine neues Chemotherapeutikum, welches im L1210 Mausleukämie-Model und in verschiedenen soliden humanen Tumorxenograften, inklusive im PC-3 humanen Prostatatumorxenograft, ausgezeichnete Wirkung aufweist. In der vorliegenden Arbeit wurde die Bedeutung der Dimere als neue Zytostatika gegen humane, p53 mutierte, androgen-unabhängige DU-145 und PC-3 Prostatatumorzellen untersucht. Zytotoxische Effekte, die Zellzyklus-Abhängigkeit der Toxizität der Dimere, Apoptose-Induktion und die Inhibition der Thymidylat Synthase (TS), wurden mit 5-FdU und NOAC verglichen.

Behandlung der Zellen mit den neuen Dimeren N^4-palmitoyl-2'-deoxycytidylyl-(3'→5')-5-fluoro-2'-deoxyuridin (dCpam-5-FdU), 2'-deoxy-5-fluorouridylyl-(3'→5')-2'-deoxy-5-fluoro-N^4-octadecylcytidin (5-FdU-5-FdC18) und 2'-deoxy-5-fluorouridylyl-(5'→5')-N^4-octadecyl-1-β-D-arabinofuranosylcytosin (5-FdU-NOAC) ergab eine ausgeprägte Zytotoxizität mit IC_{50}-Werten zwischen 3-5 μM. Die Zytotoxizität wurde mit dem WST-1 Test bestimmt. Bei einer Konzentration von 100-200 μM konnten 5-FdU-5-FdC18 und 5-FdU-NOAC die 5-FdU-Resistenz in beiden Zelllinien überwinden und 100% der Tumorzellen eliminieren.

Zellzyklus-Untersuchungen mittels Durchflusszytometer zeigten, dass die Zytotoxizität durch einen Stillstand der Zellen in der S-Phase verursacht wurde. Nach der Behandlung mit 5-FdU, dCpam-5-FdU und 5-FdU-

5-FdC18 nahm die S-Phase Population dramatisch zu. Der Zellzyklus wurde am wirksamsten von dCpam-5-FdU arretiert mit einer Erhöhung der S-Phase Population von 35 auf 84% in DU-145 Zellen, respektive von 36 auf 78% in PC-3 Zellen nach 24 h Inkubation mit 50 µM. Der S-Phase Stillstand wurde von einer Abnahme in den G1- und G2/M-Phasen begleitet. 5-FdU-NOAC und NOAC verursachten keine starke Veränderung des Zellzyklus, sondern nur eine leichte Zunahme der S-Phase Population.

Auf den Zellzyklus-Stillstand und die Inhibition der Zellproliferation folgte Apoptose. Das Ausmass des apoptotischen Zelltodes wurde mit dem monoklonalen Antikörper Apo 2.7 und mittels Durchflusszytometrie bestimmt. Eine deutliche Induktion von Apoptose wurde durch 5-FdU, dCpam-5-FdU, 5-FdU-5-FdC18 und 5-FdU-NOAC bewirkt. Nach 96 h Inkubation mit 50 µM Wirkstoffkonzentration wurde eine apoptotische Zellfraktion von 67-87% in DU-145 und von 22-54% in PC-3 Zellen nachgewiesen. 5-FdU-5-FdC18 erhöht im Vergleich zu 5-FdU die apoptotische PC-3 Zellfraktion um den Faktor 1.6. Gleichzeitig wurde eine 6-8-fache Erhöhung der Caspase-3 Aktivität in DU-145 und eine 8-11-fache Erhöhung in PC-3 Zellen gefunden, die mit 5-FdU, dCpam-5-FdU und 5-FdU-5-FdC18 behandelt worden waren. NOAC verursachte nur in 18% der DU-145 und in 6% der PC-3 Zellen Apoptose. 5-FdU-NOAC und NOAC erhöhten die Caspase-3 Aktivität nicht. DNA Fragmentierung bestätigte ferner Apoptose-Induktion in beiden Zelllinien. Untersuchungen mit dem Konfokalen Laser Scanning- und mit dem Elektronen-Mikroskop zeigten den Zerfall der Zellen in apoptotische Körper nach Behandlung mit 5-FdU-5-FdC18.

Die Dimere inhibierten spezifisch die TS wie 5-FdU zeit- und konzentrationsabhängig. Eine 50%ige Inhibition des Enzyms wurde nach 90 min bei 5-6 nM 5-FdU und bei 0.6-0.7 µM Dimerkonzentration in beiden Zelllinien erreicht. NOAC hingegen bewirkte keine Inhibition der Thymidylat Synthase.

Mit den vorliegenden Untersuchungen wurde gezeigt, dass die neuen amphiphilen Dimere die 5-FdU Resistenz in androgen-unabhängigen DU-145 und PC-3 Zellen überwinden können. Es kann angenommen werden, dass die Dimere zum monophosphorylierten Molekül 5-FdUMP gespalten werden. Dies bewirkt erhöhte intrazelluläre Zytostatikakonzentration über längere Zeit, was wiederum die Dauer und das Ausmass des zytotoxischen Effektes erhöht. Diese Hypothese wird durch die Tatsache unterstützt, dass die neuen Dimere eine zellzyklusabhänginge Zytotoxizität aufweisen und spezifisch TS inhibieren, zwei Mechanismen, die für 5-FdU charakteristisch sind. Zusätzlich sind die Dimere in der Lage Apoptose, ein Vor-

gang, der in Krebszellen häufig erschwert oder verhindert ist, zu induzieren. Die durchgeführten Untersuchungen weisen auf die grosse Bedeutung dieser Dimere in der Behandlung von p53 mutierten, hormonunabhängigen Prostatatumoren hin.

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Abbreviations

AAH	atypical adenomatous hyperplasia
ara-C	1-β-D-arabinofuranosylcytosine
ara-CMP	1-β-D-arabinofuranosylcytosine monophosphate
ara-CTP	1-β-D-arabinofuranosylcytosine triphosphate
ara-U	1-β-D-arabinofuranosyluracil
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CLSM	confocal laser scanning microscopy
CTP	cytidine triphosphate
DAPI	4',6-diamidino-2-phenylindole
dCpam-5-FdU	N^4-palmitoyl-2'-deoxycytidylyl-(3'→5')-5-fluoro-2'-deoxyuridine
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
dUMP	2'-deoxyuridine monophosphate
[5-^{3}H]dUMP	tritium-labelled dUMP in position 5
FCS	foetal calf serum
5-FdU	5-fluoro-2'-deoxyuridine
5-FdU-5-FdC18	2'-deoxy-5-fluorouridylyl-(3'→5')-2'-deoxy-5-fluoro-N^4-octadecylcytidine
5-FdU-NOAC	2'-deoxy-5-fluorouridylyl-(5'→5')-N^4-octadecyl-1-β-D-arabinofuranosylcytosine
5-FdUMP	5-fluoro-2'-deoxyuridine monophosphate
5-FU	fluorouracil
G1-phase	first gap phase in cell cycle (before DNA synthesis)
G2-phase	second gap phase in cell cycle (after DNA synthesis)
HBSS	Hanks' balanced salt solution
IC	inhibitory concentration
LUV	large unilamellar vesicle
M-phase	mitotic phase in cell cycle
MLV	multilamellar vesicle
MPS	mononuclear phagocyte system
MT	microtubule protective buffer
NHAC	N^4-hexadecyl-1-β-D-arabinofuranosylcytosine
NOAC	N^4-octadecyl-1-β-D-arabinofuranosylcytosine
[5-^{3}H]NOAC	tritium-labelled NOAC in position 5
PARP	poly(ADP-ribose) polymerase

PB	phosphate buffer
PBS	phosphate buffered saline
PEG	poly(ethylene glycol)
PI	propidium iodide
PIN	prostatic intraepithelial neoplasia
PSA	prostate-specific antigen
RNA	ribonucleic acid
S-phase	synthetic phase in cell cycle
SPC	soy phosphatidylcholine
SUV	small unilamellar vesicles
T_C	phase transition temperature
TS	thymidylate synthase
WST-1	(4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate)

1 Introduction

1.1 Cancer of the Prostate

1.1.1 Introduction

Since 1970 incidence and mortality from prostate cancer has been increasing in most developed countries representing today 36% of all newly diagnosed cancers in males (1). In the United States it has become the most common cancer, second only to lung cancer as a cause of cancer deaths (2). Because the incidence of prostate cancer increases rapidly with age and the average life expectancy of men is rising, the number of patients with this disease is expected to increase steadily over the next decade (3). More efficient diagnostic tests, including measurement of prostate-specific antigen (PSA) levels and transrectal ultrasonography, will further increase the number of cases detected (4-6).

More than half of the patients diagnosed with prostate cancer die from the disease within 10 years. Despite therapy, more than 65% suffer local or systemic progression of the disease (7-9) and the prognosis for those with advanced prostate cancer remains dismal. In 30% of men older than 50 years foci of cancer are found in the prostate (10, 11). This remarkably high prevalence of cancer makes prostate cancer the most common malignancy in humans. There is an enormous discrepancy between the high prevalence of the disease at autopsy and the low incidence of the disease. In fact, only 1% of men over 50 years will be diagnosed with prostate cancer each year, and only 0.3% will die from the disease. This discrepancy has confounded the understanding of its clinical significance (3, 8, 11).

1.1.2 Anatomy, Physiology and Pathology

The prostate is a small accessory sex gland located at the base of the bladder. This organ is composed of branching tubuloalveolar glands, that eventually enter the prostatic urethra. They are arranged in lobules and surrounded by a stroma that is rich in nerve fibers, smooth muscle cells, collagen, and lymphatics. The epithelial component includes prevalently secretory epithelial cells. They contain androgen receptors on their surface, are androgen dependent for growth, and synthesise and secrete PSA and prostate-specific acid phosphatase. Both are mixed with the prostatic fluid of ejaculate and are used as markers for prostate cancer. When an-

drogen is withdrawn, these cells die through the activation of a cell death program called apoptosis. Basal cells contain the stem cell population of the epithelial compartment. They lack androgen receptors and do not die when androgens are withdrawn. In contrast to epithelial cells, stromal fibroblasts and smooth muscle cells do not undergo apoptosis and are not androgen dependent (12-14).

The main function of prostatic fluid is to aid sperm in traversing the female genital tract by adjusting the pH of semen and liquefying cervical mucus (13). The development and maintenance of the prostate is largely controlled by the male sex hormone testosterone (15).

Over 99% of the cancers that develop in the prostate are adenocarcinomas derived from epithelial cells (16). Cancer of the prostate regularly spreads by direct extension and lymphatic and vascular routes. Local extension tends to grow into and through the prostatic capsule, the bladder base, and seminal vesicles, whereas extension into the urethra and rectum is uncommon. As a result of direct invasion of the venous system and systemic dissemination, metastases to bone have been observed in up to 80% of patients (17).

1.1.3 Epidemiology

Incidence of prostate cancer depends on age, geography, race, ethnicity, diet, occupation, and genetic predisposition. Age is the most important risk factor (18). It is estimated that 70% of men over 80 years have some histological evidence of cancer in their prostate. Men in their 30s and 40s have a high incidence of small foci of cancer, whereas older men have larger lesions (19). However, the finding of histological cancer does not necessarily imply the clinical manifestation of disease (20).

The frequency of histological cancers in men of equivalent age is similar around the world (21), but the clinically evident carcinoma of the prostate shows a more than 10-fold difference in incidence according to geography, race, or ethnicity. In parts of the United States and Europe, there are more than 200 cases per 100 000 people, whereas in Thailand it is less than 50 people per 100 000 (22). One reason for the geographic differences in incidence of this disease is diet. Fat is the most important factor (23), but also selenium (24) and a lack in vitamin A (25) are associated with prostate cancer incidence.

The disease affects ethnic groups differentially. Prostate cancer is more common in African American than in American Caucasians (26,

27). A possible explanation is the higher testosterone level found in this group (28).

Occupational studies of prostate cancer have found statistically significant excess risk for people who report frequent occupational exposure to cadmium (29, 30).

Genetic predisposition also influences the development of prostate cancer (31). Familial clusters have been documented (32, 33). One common genetic mechanism found in cancer families is the loss of tumour suppressor genes such as p53. Alterations in this gene have been reported in up to 38% of metastatic lesions, compared to less than 5% of early-stage primary tumours, suggesting that loss of p53 function is a late event and may be associated with metastasis and androgen independence (34). Other molecular factors that influence prostate cancer progression include growth factor expression (transforming growth factor-β, epithelial growth factors, fibroblast growth factors), oncogenes (ras, myc, bcl-2), and tumour suppressor genes (retinoblastoma, nm 23) (35).

1.1.4 Carcinogenesis

Carcinogenesis is a multistep accumulation of genetic lesions that may result in uncontrolled cellular proliferation, a decrease in cell death or apoptosis, invasion, metastatic spread, and blockage of differentiation. The clonal growth of these partially transformed cells results in morphologically identifiable premalignant lesions termed atypical adenomatous hyperplasia (AAH) or prostatic intraepithelial neoplasia (PIN) (36). AAH is defined as a proliferation of microglandular structures that are insufficient for the diagnosis of well-differentiated adenocarcinoma. PIN is subdivided into low and high grade (19, 37). Alterations during prostatic carcinogenesis include loss of glandular formation with development of a more anaplastic morphology (38), nuclear pleomorphism (39), invasion of the basement membrane, an increase in cell motility (40), loss of contact inhibition, and angiogenesis (41, 42).

1.1.5 Treatment

The natural history of a prostate cancer is to progress and to become more malignant over time (43, 44). The frequently late diagnosis contributes to the poor prognosis. The median survival is generally less than two years due to metastasic disease (45), which is generally resistant to systemic therapies. The first decision to be made is whether treatment is re-

quired or careful observation is sufficient. This decision is greatly influenced by tumour volume, histologic differentiation, patient age, and patient comorbidities (46). The data from the literature do not provide clear-cut evidence for the superiority of any one treatment (47, 48).

Radical prostatectomy is indicated for cancers that are clinically confined to the prostate without evidence of regional lymph node invasion or distant metastasis (49). Two radiation modalities are currently used in the treatment of early prostate cancer. The first is external beam irradiation, which uses high-energy photon beams (50). The second and less commonly used modality is interstitial, isotopic radioactive seed implantation using iodine 125 or palladium 103 radioactive sources (51).

Despite initial surgery or radiation therapy, the disease recurs in many patients. Standard androgen-ablation therapy with orchiectomy or luteinizing hormone-releasing hormone agonists can produce significant responses, but they tend to be of short duration. Androgen-responsive tumours contain an androgen-independent population before the initiation of hormone therapy and this population emerges as a result of selection when androgen is withdrawn (52, 53). Resistance to androgen ablation develops eventually in nearly all cases (54).

Despite the testing of numerous drugs and drug combinations, chemotherapy has only limited success when given after the failure of androgen ablation (55). To date, the major benefit of these therapies have been palliative in nature, resulting in an improvement in quality of life. None of these agents or regimens have been shown to affect survival significantly, and none can be considered to be standard therapy for this disease (56). Application of these regimens earlier in the course of the disease may have a more significant impact on the morbidity and mortality of prostate cancer (57).

Most prostate cancers eventually develop resistance to hormonal therapy and chemotherapy regimens. Resistance can develop by mutations in the p53 gene (58, 59). In patients with metastatic prostate cancer mutations of this gene are seen more commonly than in those with primary tumours (60). Loss of p53 function facilitates tumour cell progression through the cell cycle and renders induction of apoptosis difficult. Isaacs and co-workers (61) demonstrated that growth of prostate cells with p53 mutations can be inhibited by introduction of the wild-type gene. However, in tumour cells that are p53 null or have a mutated p53 gene apoptosis can occur in a p53-independent manner (59, 62, 63). Thus, the identification of new agents able to trigger p53-independent apoptosis may be

of clinical relevance seen the commonly occurring loss of p53 function in many tumours (64).

1.2 Apoptosis and Necrosis

1.2.1 Introduction

The two major processes that contribute to the progression of tumour cell growth are increased proliferation and decreased cell death. It has been generally accepted that apoptosis and necrosis are two distinct, mutually exclusive, modes of cell death (65-68). Apoptosis or programmed cell death is an active physiological mode of cell death. A multistep mechanism regulates the cell's propensity to respond to various stimuli by apoptosis, the complexity of which has only become apparent recently (69). The regulation system is controlled by at least two distinct checkpoints. One is the bcl-2/bax family of proteins (70, 71), another the cysteine- (72, 73) and possibly the serine-proteases (74, 75). These checkpoints interact through several oncogenes and tumour suppressor genes such as p53 with the system regulating cell proliferation and DNA repair.

While apoptosis is characterised as active cell death, necrosis is a passive, catabolic and degenerative process. Necrosis generally represents a cell's response to gross injury and can be induced by an overdose of cytotoxic agents. If apoptosis can be compared to 'cell suicide', necrosis represents an accidental death and is often referred to as 'cell murder' (66).

1.2.2 Characteristics of Apoptosis and Necrosis

A cell triggered to undergo apoptosis activates a cascade of molecular events which lead to its total disintegration. Many of these changes are characteristic and appear to be unique to apoptosis (Figure 1). One of the early events in apoptotic cells is dehydration. Loss of intracellular water leads to condensation of the cytoplasm followed by a change in cell shape and size. The originally spherical cells may become elongated and smaller.

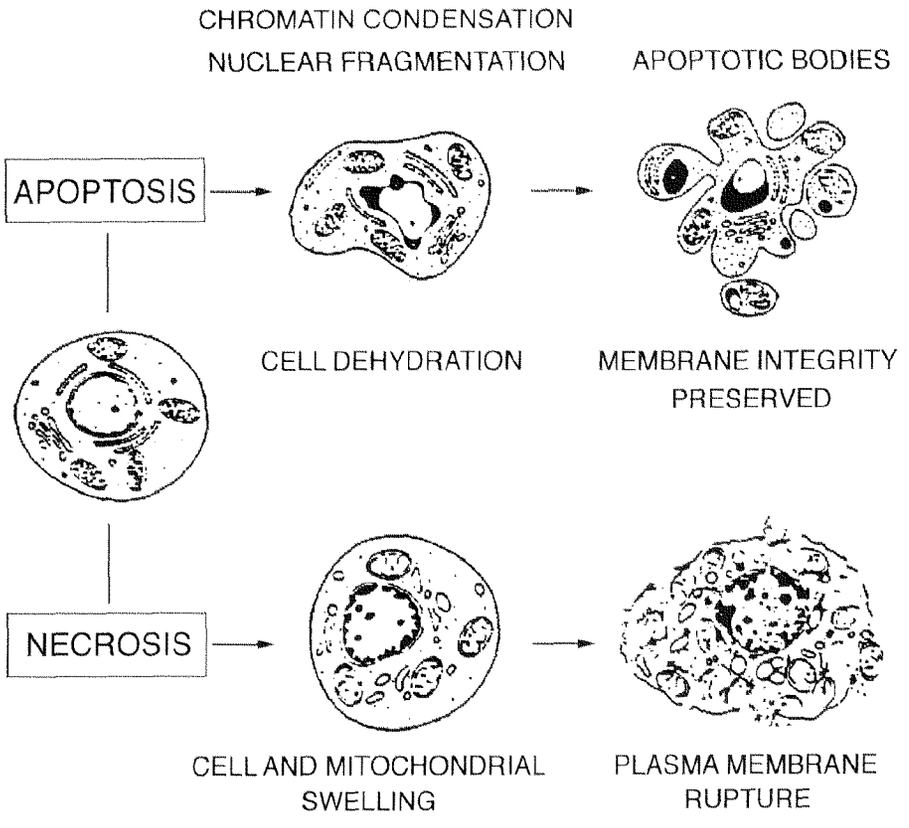


Figure 1
Schematic representation of morphological and biochemical changes during apoptosis and necrosis. Figure adapted from reference (76).

The most characteristic feature of apoptosis is the condensation of nuclear chromatin. The condensation starts at the nuclear periphery, and results in condensed chromatin often acquiring a concave shape resembling a half-moon or sickle. The condensed chromatin has an uniform, smooth appearance, with no evidence of any texture normally seen in the nucleus. Condensed DNA stains strongly with fluorescent light absorbing dyes. The nuclear envelope disintegrates, with laminin proteins undergoing proteolytic degradation, followed by nuclear fragmentation. The nuclear fragments, together with constituents of the cytoplasm, including intact organelles, are then packaged and enveloped by fragments of the plasma membrane. These structures, called apoptotic bodies, are then shed from the dying cell. When apoptosis occurs in vivo apoptotic bodies are phagocytosed by neighbouring cells (macrophages, fibroblasts or epithelial cells), without triggering an inflammatory reaction in the tissue (68, 77).

Activation of endonuclease(s) which preferentially cleave DNA at the internucleosomal section is another characteristic apoptotic event (78-80). The products of DNA degradation generate a characteristic ladder pattern when analysed by agarose gel electrophoresis. In many cell types DNA degradation does not proceed to nucleosome sized fragments but rather results in small 50-300-kb DNA fragments (81).

In apoptosis the structural integrity and most of the plasma membrane function is preserved, at least during the initial phase of cell death. However, while cellular organelles including mitochondria and lysosomes remain preserved during this process, the mitochondrial transmembrane potential is markedly decreased (82, 83).

Other features of apoptosis include mobilisation of intracellular ionised calcium (84), activation of transglutaminase which crosslinks cytoplasmic proteins (85), loss of microtubules (86) and loss of asymmetry of the phospholipids on the plasma membrane leading to exposure of phosphatidylserine on the outer surface (87). The latter results in the recognition of apoptotic cells by phagocytosing cells. The time span for complete apoptosis varies, but is generally short: apoptotic bodies may form and disappear within 24 h (68, 77).

The early event of necrosis is manifested by mitochondrial swelling followed by rupture of plasma membrane and release of cytoplasmic constituents which include proteolytic enzymes (66, 68) (Figure 1). The nucleus undergoes slow dissolution. Necrosis triggers an inflammatory reaction in the tissue. DNA degradation is not as extensive during necrosis as in the case of apoptosis, and the products of degradation are heterogeneous in size, failing to form discrete bands on electrophoretic gels.

1.2.3 Importance of Apoptosis in Oncology

The discovery of oncogenes like bcl-2 (88), which protect cells from apoptosis indicated that not only increased cell proliferation but also the loss of their ability to die may be a cause of cancer. It also became apparent that tumour progression and the increase in malignancy may be associated with the change in propensity of tumour cells to undergo spontaneous apoptosis (89-91). Therefore, new antitumour strategies based on modulation of the cancer cell propensity to undergo apoptosis, are subject of great interest (65, 92-95).

The regulation of the apoptotic machinery consists of several checkpoints at which interacting molecules either promote or prevent apoptosis (67, 69). Consequently, possibilities for interactions with the regulatory

machinery, thereby influencing modulation of the cell propensity to respond to intrinsic or exogenous signals by death are existing. Such possibilities are of great interest in oncology. Strategies involving the regulatory mechanisms of apoptosis to modulate the sensitivity of tumour and/or normal cells to antitumour agents, to increase treatment efficiency, and to lower toxicity are currently being explored (92-95).

1.2.4 P53 and its Role in Oncology

P53 is a protein consisting of 393 amino acids, which resides in the nucleus of the cell. The nonmutated wild-type p53 protein is often found within cells in a latent state and is activated by various intracellular (e.g. DNA damage) and extracellular signals. Activation involves an increase in overall p53 protein levels, as well as qualitative changes of the protein such as phosphorylation state. Through the activation of specific target genes wild-type p53 can induce a variety of cellular responses, the most notable being cell cycle arrest and apoptosis. The physiological role of p53 is to prevent the formation of tumours (96).

Loss of p53 function can be a predisposition for tumour formation due to tumour cell progression through the cell cycle and hampered induction of apoptosis. Isaacs and co-workers (61) demonstrated that growth of prostate cells with p53 mutations can be inhibited by introduction of the wild-type gene. The p53 gene plays a central role in determining the response of tumour cells to chemotherapy (97). Although it represents only one of several important genetic regulators, the fact that p53 is probably the most frequently mutated gene in human cancer indicates its importance.

This finding has led to the examination of a number of means for exploiting this lack of function as part of therapeutic attempts for the reversal of resistance. These include the development of new cytotoxic agents which are capable of initiating apoptosis in cells with dysfunctional p53. In tumour cells that are p53 null or have a mutated p53 gene apoptosis can occur in a p53-independent manner. A number of experimental studies have shown that drug-resistant cells remain sensitive to agents that are capable of inducing p53-independent cell death (59, 62, 63, 98). Thus, the identification of new agents able to trigger p53-independent apoptosis may be of clinical relevance seen the commonly occurring loss of p53 function in many tumours (64).

1.3 Anticancer Drugs

A large number of cytostatic drugs have been studied in patients in whom prostate cancer has progressed despite hormonal therapy (99). None of these agents (cisplatin, doxorubicin, mitoxantrone, cyclophosphamide, 5-fluorouracil, or combined therapies) have been shown to affect survival significantly (56, 57) (see chapter 1.1.5).

1.3.1 A new Class of anticancer Drugs: Amphiphilic heterodinucleoside Phosphate Dimers

The deoxyribonucleoside derivative of 5-fluorouracil (5-FU), 5-fluoro-2'-deoxyuridine (5-FdU) has been limited in its clinical use due to rapid degradation and the formation of resistance in normal and tumour tissues (see chapter 1.3.2.3). To enhance the cytotoxic activity of 5-FdU a new strategy of masking nucleoside phosphates by the synthesis of amphiphilic dinucleoside phosphates was developed (100). These dimers (Figure 7) contain the active metabolite 5-fluoro-2'-deoxyuridine monophosphate (5-FdUMP), which is the primary metabolite in the phosphorylation chain of 5-FdU. As second molecule structures with antitumour activity were chosen. The two nucleosides are linked through a 3'→5' or a 5'→5' phosphate bond. The best studied single molecule linked to 5-FdU is N^4-octacecyl-1-β-D-arabinofuranosylcytosine (NOAC), a new lipophilic cytosine arabinoside (ara-C) derivative effective in the L1210 leukaemia mouse model and in different solid tumour xenografts. The amphiphilic/lipophilic nature provides the dimers with new pharmacokinetic properties. It is expected that after cellular uptake the 5'-monophosphate is released by enzymatic cleavage. Consequently, low activities of nucleoside-5'-monophosphate kinases could be circumvented by these dimers, resulting in increased anti-tumour activities.

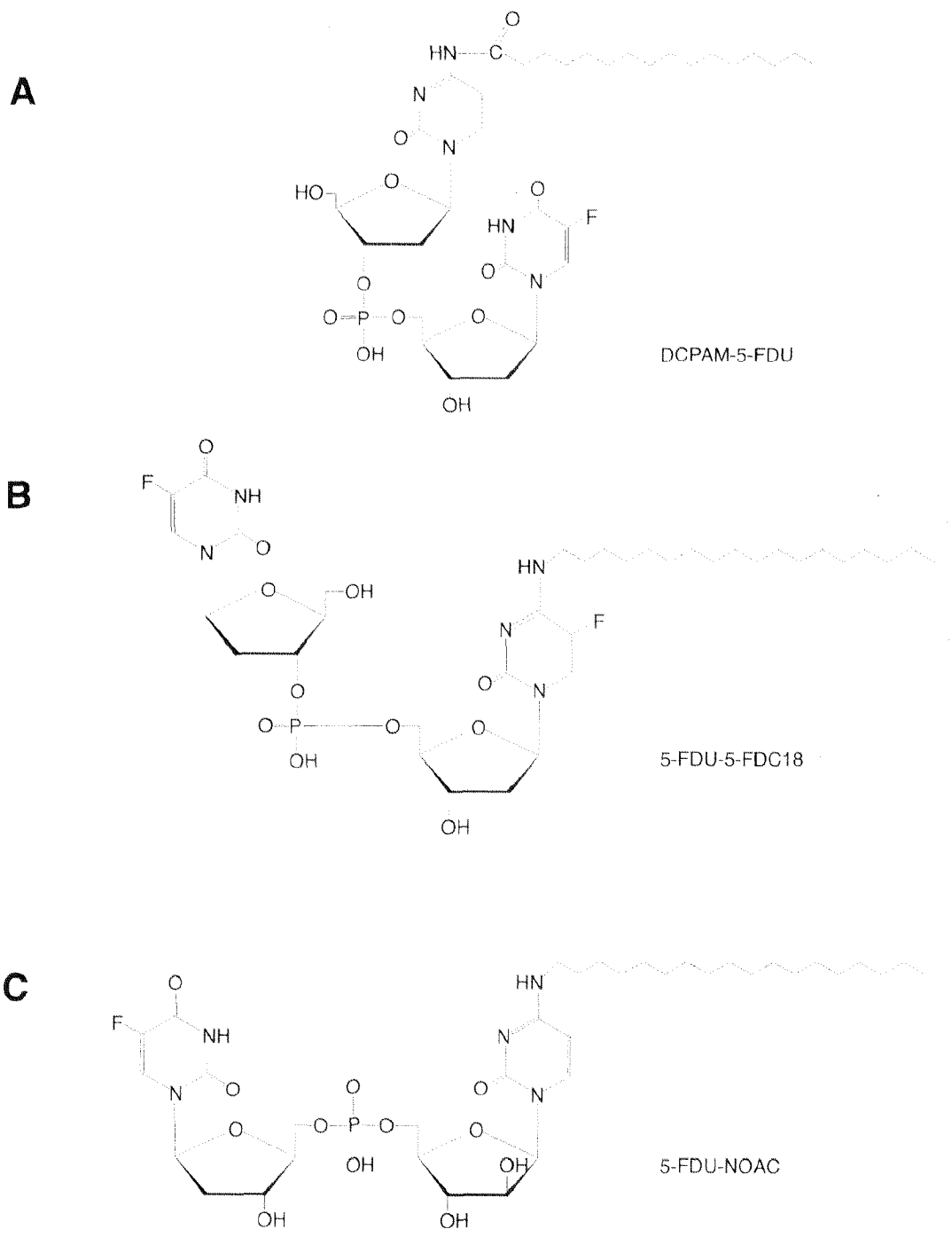


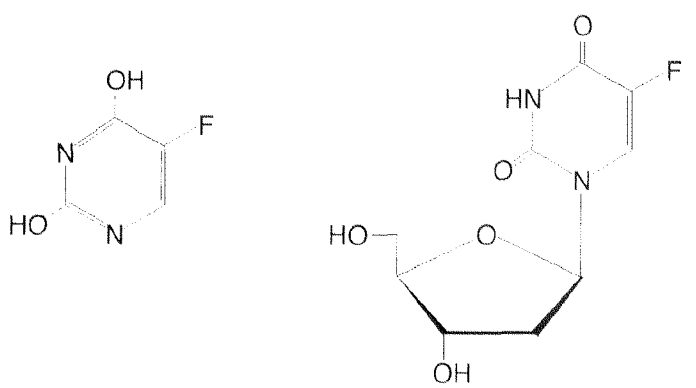
Figure 7
Chemical structures of the amphiphilic heterodinucleoside phosphate dimers N^4-palmitoyl-2'-deoxycytidylyl-(3'→5')-5-fluoro-2'-deoxyuridine (A: dCpam-5-FdU), 2'-deoxy-5-fluorouridylyl-(3'→5')-2'-deoxy-5-fluoro-N^4-octadecylcytidine (B: 5-FdU-5-FdC18) and 2'-deoxy-5-fluorouridylyl-(5'→5')-N^4-octadecyl-1-β-D-arabinofuranosyl-cytosine (C: 5-FdU-NOAC)

1.3.2 Fluoropyrimidines

1.3.2.1 Introduction

Most of the active antitumour agents presently in clinical use have been discovered by serendipitous observation or screening. 5-Fluorouracil (5-FU), synthesised by Dr. Charles Heidelberger and colleagues, represents a notable exeption (101). The rationale for the synthesis of fluorinated pyrimidines originated from the observation that rat hepatoma cells use uracil more efficiently than normal rat intestinal mucosa. This finding suggested that uracil might represent an exploitable target for cancer chemotherapy.

5-FU has a fluorine atom substituted for hydrogen at the 5-carbon position of the pyrimidine ring (Figure 2). 5-FU has antitumor activity against many solid tumours, including breast, gastrointestinal, head and neck, and ovarian carcinomas (10-40% overall response rate). Because of its synergistic interactions with other antineoplastic agents, with irradiation, with physiologic nucleosides such as thymidine and uridine, and with the interferons, 5-FU is currently most often administered in the context of combination therapy.



5-FU 5-FDU

Figure 2
Chemical structures of 5-fluoropyrimidines

1.3.2.2 Mechanism of Action

To exert their cytotoxic effects the fluoropyrimidines require intracellular activation. Three mechanisms of action are responsible for the effect of 5-FU (Figure 3). First, 5-FU is converted to 5-FdU (Figure 2) by thymidine phosphorylase. Subsequent phosphorylation of 5-FdU by

thymidine kinase results in formation of the active metabolite 5-fluoro-2'-deoxyuridine monophosphate (5-FdUMP). In the presence of the reduced folate 5,10-methylenetetrahydrofolate, 5-FdU forms a stable covalent complex with thymidylate synthase (TS), inhibiting TS enzyme activity and leading to depletion of deoxythymidine triphosphate, a necessary precursor for DNA synthesis (102). Secondly, 5-FU may be anabolised to 5-fluorouridine monophosphate which is further metabolised to 5-fluorouridine triphosphate. The latter can be incorporated into RNA or converted to the deoxyribonucleotide 5-FdUMP (103). Thirdly, 5-FdUMP may subsequently be phosphorylated to 5-fluoro-2'-deoxyuridne-5'-triphosphate, which is incorporated into DNA (104).

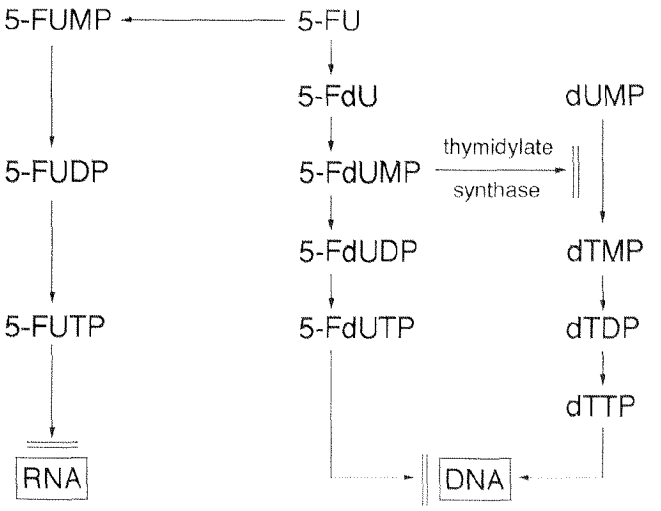


Figure 3
Mechanisms of action of 5-FU

The central mechanism of 5-FU action is the inhibition of TS by 5-FdU (102). The TS-5-FdU-folate complex is slowly dissociable, with a half-life of 6 hours in intact cells. The presence of the reduced folate co-factor is critical for complex formation as well as for sustaining enzyme inhibition.

5-FU is extensively incorporated into both nuclear and cytoplasmic RNA, and this incorporation alters RNA processing and function. Incorporation of 5-FU into RNA inhibits the conversion of high-molecular-weight nuclear RNA species to lower-molecular-weight ribosomal RNA (105). Polyadenylation of mRNA is inhibited by relatively low concentrations of 5-FU, thereby affecting the stability of this RNA species (106). Quantitative as well as qualitative aspects of protein synthesis are affected

by incorporation of 5-FU into RNA. In the presence of RNA-containing 5-FU moieties, translational miscoding can occur (107).

Another mechanism of cytotoxicity of 5-FU is its incorporation into DNA resulting in inhibition of DNA elongation and alteration of DNA stability, production of DNA single-strand breaks and DNA fragmentation (108). The fluoropyrimidines may also induce DNA strand breaks without being directly incorporated into DNA, possibly through inhibition of DNA repair caused by dTTP depletion (109).

1.3.2.3 Mechanisms of 5-FU Resistance

A number of different mechanisms of resistance have been identified. The reason are the various sites of cytotoxic action of 5-FU and the multiple steps required for its activation. The relative frequency with which each of these mechanisms is responsible for resistance in humans, however, is unknown.

Resistance to 5-FU in human and murine tumour cells can develop through deletion of one of the key enzymes required for its activation. Increased activity of catabolic enzymes such as acid and alkaline phosphatases leading to decreased accumulation of 5-FU nucleotides has been implicated (110). A relative deficiency of the reduced folate substrate 5,10-methylenetetrahydrofolate may also compromise the cytotoxic action of 5-FdUMP on TS. Some resistant mutant cell lines have elevated intracellular cytidine triphosphate (CTP) (111). This increase in CTP pools results in feedback inhibition of uridine kinase and conversion of 5-FU to the active nucleotide forms. Decreased incorporation of 5-FU into both RNA and DNA has also been found in various cell lines resistant to fluoropyrimidines (112). Finally, alterations in the target enzyme TS, such as decreased binding of 5-FdU to TS and increased TS expression, can lead to resistance to 5-FU.

1.3.2.4 Pharmacokinetics and Toxicity

There are different schedules, doses and various routes of administration of 5-FU such as oral, intravenous, intraarterial, or intraperitoneal. Each of it has unique advantages and disadvantages that determine its usefulness in cancer chemotherapy.

5-FU should not be given by the oral route because less than 75% reach the systemic circulation (113). After intravenous bolus infusion, 5-FU penetrates well into the cerebrospinal fluid and extracellular third-space fluids, such as ascites and pleural effusions. After intravenous administration of conventional single doses of 400 to 600 mg/m^2, peak

plasma concentrations reach 0.2 to 1 mM. The plasma concentration declines rapidly, with a primary distribution half-life of 6 to 20 minutes. Intrahepatic arterial infusion for the treatment of hepatic metastases at a rate of 30 mg/kg/day results in plasma levels in the range of 0.13 to 0.35 μM. This explains the relative lack of myelosuppression resulting from this form of therapy (113).

More than 80% of 5-FU administered by an intravenous or intraarterial route are inactivated by metabolic conversion by dihydropyrimidine dehydrogenase to dihydrofluorouracil, and 20% are excreted intact in the urine. Unlike the parent compound, the active 5-FU nucleotides, 5-FdUMP and 5-fluorouridinetriphosphate have prolonged intracellular half-lives. Their decay rates vary among individual tissues, and their continued presence is a critical determinant of duration and magnitude of drug effect.

5-FU may also be administered by the intraperitoneal route, particularly for the treatment of ovarian cancer to take advantage of the high intraperitoneal drug concentration (4 mM), the slow absorption of the drug into the portal circulation, its rapid metabolism in liver, and the relatively small amounts of drug that reach the systemic circulation (114).

The spectrum of toxicities associated with 5-FU varies considerably according to the dose, schedule, and route of administration. Prolonged exposures to low concentrations of 5-FU cause gastrointestinal toxicity and mucositis, and higher intermittent doses result in myelosupression.

The dose-limiting toxicity after bolus intravenous therapy, using a 5-day course or single, weekly doses is myelosuppression, the nadir of leukopenia and thrombocytopenia generally occurring between day 9 and 14 after the first injection of the drug. The most frequent symptoms of gastrointestinal toxicity are stomatitis and diarrhoea. Continuous intravenous infusion of 5-FU at doses of 30 mg/kg/day for 5 days also causes gastrointestinal symptoms such as stomatitis and diarrhoea but myelosuppression is less intense (115). 5-FU is also associated with significant ocular toxicity that includes blepharitis, epiphora, tear-duct stenosis, and acute and chronic conjunctivitis. The acute inflammatory response is reversible when the drug is discontinued early in the treatment course.

1.3.3 Cytosine arabinoside (ara-C)

1.3.3.1 Introduction

Originally, 1-β-D-arabinofuranosylcytosine (Cytarabine, ara-C) was isolated from the sponge Cryptothethya crypta (116). It differs from its physiologic counterpart 2'-deoxycytidine by the presence of a β-OH group in the 2'-position of the sugar (Figure 4). Other arabinose nucleosides with useful antitumour and antiviral effects have been synthesised or isolated from bacterial broth. Ara-C is the most cytotoxic agent of this class and has important clinical activity against human acute myelogenous leukaemia. As single agent, ara-C induces remission in 50% of patients with this disease. Combination therapy with anthracyclines like doxorubicine or daunomycin induce complete remission in 60% to 80%. Ara-C is also used in combination therapy for the blast crisis of chronic granulocytic leukaemia (117), for non-Hodgkins's lymphoma (118), and for childhood acute lymphocytic leukaemia (119). As single agent however, ara-C has only minimal activity against solid tumours, presumably because of its lack of metabolic activation in solid tumours.

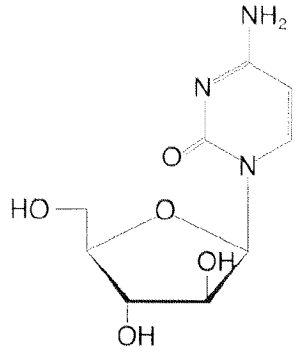


Figure 4
Chemical structure of ara-C

1.3.3.2 Mechanism of Action

After entering the cells by an active nucleoside transport system (120, 121), ara-C is converted to its active form, ara-C-5'-triphosphate (ara-CTP). Ara-C is recognised as an analogue of the physiologic nucleoside 2'-deoxycytidine in human cells. The first phosphorylation step to ara-C-5'-monophosphate catalysed by deoxycytidine kinase is believed to be the rate-limiting step (122). Ara-CTP accumulates inside leukaemic

cells because the natural feedback mechanism of deoxycytidine 5'-triphosphate (dCTP) regulating deoxycytidine kinase activity is lacking for ara-CTP (122). This nucleotide inhibits DNA polymerase α by competing with the normal substrate dCTP (123). However, more important than the effects of ara-C on DNA synthesis is its incorporation into DNA (124) which correlates strongly with cytotoxic effects (125). Once incorporated into DNA the tumour cells are not able to excise the nucleotide. Inhibition of template function and slowing down of chain elongation are the consequences (125, 126). Strongest cytotoxic effects are reached during S-phase of the cell cycle by inhibiting DNA synthesis (127) and during maximal rates of cell proliferation in tissue cultures (128).

1.3.3.3 Pharmacokinetics and Toxicity

Orally administered ara-C is not effective because of the presence of high concentrations of cytidine deaminase in the gastrointestinal epithelium and the liver (129). Nevertheless, upon parental administration 70% to 80% of ara-C are deaminated and excreted as inactive ara-U (130). Consequently, it is given intravenously at doses ranging from 3 mg/m^2 twice weekly to 3 g/m^2 every 12 h for 6 days (131). Constant intravenous infusion of 2 g/m^2/day results in a plasma concentration of 5 μM. The plasma concentration declines rapidly, with a distribution half-life of 7 to 20 min, followed by an elimination half-life of 30 to 150 min (132, 133).

The toxic side effects of ara-C are myelosuppression and gastrointestinal injury (134). With standard doses of 200 mg/m^2/day leukopenia and thrombocytopenia reach their maximum after 7 to 14 days, whereby myelosuppression usually lasts 14 to 21 days (135). High-dose ara-C treatment produce cerebral and cerebellar dysfunction, leading to ataxia, confusion and coma in 20% of patients (136). In most cases the central nervous system toxicity is reversible.

1.3.3.4 Hydrophilic Ara-C-Derivatives

With the goal to overcome the rapid deamination of ara-C to the inactive metabolite ara-U, to increase cytotoxic activity, to overcome ara-C resistance and to alter the pharmacokinetic properties a large number of cytidine derivatives have been developed. The derivatives can be subdivided into a hydrophilic and a lipophilic class (see chapter 1.3.4).

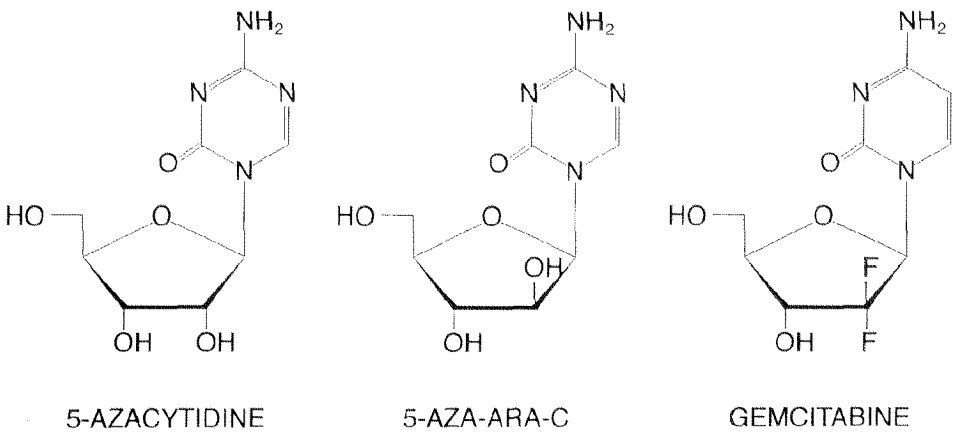


Figure 5
Chemical structure of hydrophilic ara-C derivatives

Three hydrophilic cytidine analogues have entered clinical trials (Figure 5). 5-Azacytidine has significant activity in the clinical treatment of leukaemia. In plasma, liver and tumour cells however, it is deaminated like ara-C. In contrast to ara-C, it is phosphorylated by uridinecytidine kinase to its monophosphate form, which is then metabolised to its active form 5-azacytidinetriphosphate (137). The side effects are more severe than those of ara-C. Furthermore, the drug was found to be mutagenic and teratogenic. A synthetic cytidine analogue containing the structural features of ara-C and 5-azacytidine is 5-azacytosine arabinoside. It is phosphorylated and incorporated into DNA as ara-C, but it is a poor substrate for cytidine deaminase. In addition it is active against a broad spectrum of solid tumours (138). 2',2'-difluorodeoxycytidine (gemcitabine) is also phosphorylated by deoxycytidine kinase (139). It shows a much stronger effect on DNA termination and DNA repair inhibition and exerts promising antitumour activity against solid tumours in humans (140).

1.3.4 Lipophilic Ara-C-Derivatives

1.3.4.1 Introduction

Many lipophilic 5'- and N^4-derivatives of ara-C have been synthesised. Often long chain fatty acids (141-144), phospholipids (145, 146) or steroids (147) were chosen as modifications. All 5'- and N^4- substituted ara-C derivatives with the exception of the new class of N^4-alkyl-ara-C

derivatives are prodrugs of ara-C, acting exclusively by ara-CTP and following the ara-C pathway. These lipophilic prodrugs have the ability to form spontaneously micelles (148). Because of the abolition of water solubility it is necessary to use other ways of drug administration, e.g. incorporation into liposomes (149, 150) (see chapter 1.4), emulsions (151) or addition of potentially toxic solubilising agents (152). The major disadvantage of these substances is their haemolytic activity. Consequently, an intravenous application is impossible (153) and some of these drugs are administered orally.

A new class of lipophilic cytosine derivatives are the N^4-alkyl-ara-C derivatives. In contrast to the other 5'- and N^4-derivatives, these substances are not prodrugs of ara-C. These new compounds were developed by Professor Schott of the University of Tübingen (154). The long alkyl chain protects the amino group susceptible to deamination, and renders these drugs highly resistant to deaminase. No hydrolysis of the alkylamino group occurs after incubation with human plasma or mouse liver microsomes (155). Therefore, these new compounds are significantly more stable than the acyl compounds. There is a structure-activity relationship between the alkyl chain length and the cytostatic drug effect. In the L1210 leukaemia mouse model derivatives with a chain length of 16-22 carbon atoms showed highest antitumour activity, whereas short chain compounds (C_6-C_8 atoms) were inactive (155). Preclinical studies were performed with N^4-hexadecyl-1-β-D-arabinofuranosylcytosine (NHAC) and N^4- octacecyl-1-β-D-arabinofuranosylcytosine (NOAC; Figure 6). After incorporation into the lipid membranes of small unilamellar liposomes or solution in dimethyl sulfoxide or ethanol the lipophilic substances can be administered by parenteral routes.

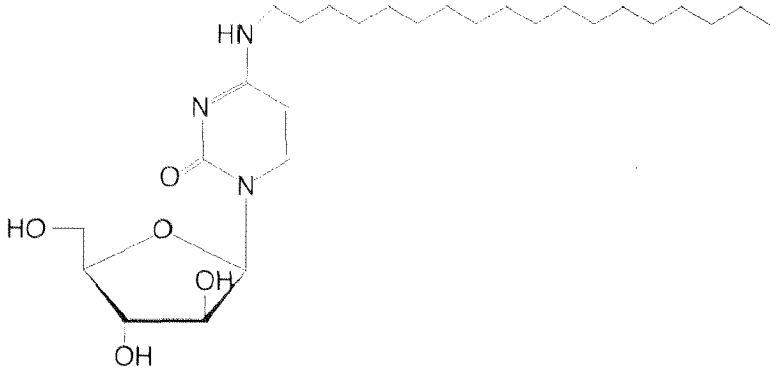


Figure 6
Chemical structure of NOAC

1.3.4.2 Antitumour Activity and Toxicity

Ara-C, NHAC and NOAC were compared in the L1210 leukaemia mouse model. The best treatment effects were obtained with NOAC at 50 μmol/kg given on days 2 and 6. NHAC showed the same efficiency only with 2-fold higher doses and ara-C did not reach the same effects as NOAC even at 2 to 8 times higher doses. In contrast to ara-C, NHAC and NOAC exert excellent antitumour effects after oral therapy at a 10 to 20-fold higher dose than needed for intravenous therapy (156, 157). NOAC was able to overcome ara-C resistance and had high cytotoxicity in ara-C resistant HL-60 cells (158).

Tested in human tumour xenografts in nude mice, NOAC was significantly more effective than ara-C in various leukaemias. An impressive antitumour activity against breast, prostate, small and large cell lung carcinoma was found with NOAC in solid tumour xenografts. In the PC-3 prostate cancer model NOAC had higher cytostatic activity than several standard antitumour agents (159).

The biodistribution of liposomal NOAC in ICR mice after intravenous application revealed a biphasic blood concentration versus time curve with a distribution half-life of 23 min and an elimination half-life of 7 h. The drug was distributed mainly into the liver with an elimination half-life of 8 h (160). The LD_{50} in ICR mice after a single intraperitoneal application was 524 mg/kg for NOAC, whereas NHAC was not toxic. The haemolytic toxicity remained moderate for both drugs with a mild leucopenia and a drop in platelet counts, which recovered 4 to 6 days after treatment. The erythrocytes were not affected. A pronounced atrophy of the rapidly dividing epithelial cells of the small intestine and the white pulp of the spleen were observed. The damage was reversible because intestinal structures recovered 48 h after treatment (156).

1.3.4.3 Mechanism of Action

Horber et co-workers suggest that NHAC and NOAC have cytotoxic mechanisms which are significantly different from ara-C and that these lipophilic derivatives are able to overcome ara-C resistance (158). After nucleoside transporter independent cell uptake only low amounts of ara-CTP are formed. Considering the 2.5-150 fold lower ara-CTP formation as compared to ara-C, the cytotoxicity of NHAC seems not to follow the ara-C pathway in HL-60, K-562 and U-937 cells (158, 161). Furthermore, NHAC induced apoptosis in HL-60 cells only at concentrations 20 times higher than those observed for ara-C and cytotoxicity was less S-phase specific (162).

1.3.4.4 Metabolism in Mice

Metabolism and excretion of NOAC was investigated in mice. Forty-eight hours after the injection of tritium-labelled NOAC, 39% of the radioactivity was excreted in urine and 16% in faeces, whereas ara-C radioactivity was only found in urine with 48% of the injected dose. The radioactivity of faeces extracts of NOAC treated mice was composed of unmetabolised NOAC (2% of injected dose), hydroxylated NOAC (NOAC+OH), its sulphated derivative (NOAC+OSO_3H) and unidentified metabolites. In urine the hydrophilic molecules ara-C (25% of injected dose) and ara-U were found. Consequently, NOAC is metabolised by two major pathways, one leading to the hydrophilic metabolites ara-C and ara-U and the other to hydroxylated and sulphated NOAC. Urine collected during 48 h of ara-C treated mice contained 33% of the injected dose as unmetabolised drug and 13% as the main metabolite ara-U (163).

1.3.4.5 Distribution in Human Blood

In human blood, liposomal NOAC was distributed in vitro to low-density proteins at 36%, to high density lipoproteins at 21%, to albumin and other proteins at 12% and to very-low density lipoproteins at 5% (160).

1.4 Liposomes

1.4.1 Introduction

Liposomes were first described in 1965 (164) and have ever since attracted attention because of their potential as drug delivery system for both hydrophilic and lipophilic drugs (165) (Figure 8). Liposomes are vesicles consisting of one (unilamellar) or several (multilamellar) concentric lipid bilayers, enclosing as many aqueous compartments. The unilamellar liposomes are classified into small (20-200 nm; SUV) and large (> 200 nm; MLV) unilamellar vesicles, whereas the multilamellar vesicles are of heterogeneous size (0.1-5 μM) (166). Liposomes exhibit little or no immunogenicity and intrinsic toxicities, and are biodegradable.

Perhaps the most compelling property of liposomes is their ability to significantly alter pharmacokinetics and biodistribution of many of their associated drugs (168, 169). Because liposomes and their associated drug are confined largely to the central compartment, uptake of the drug into normal tissue is decreased, leading to decreased toxicities in sensitive

normal tissues (170). Liposomes can be excellent solubising agents for lipophilic drugs and prevent toxicities associated with some of the more traditional excipients such as propylene glycol, cremophor or dimethyl sulfoxide (171-173). Substances associated with liposomes receive substantial protection from interaction with degrading enzymes, resulting in an increased half-time. This is particularly useful for rapidly degrading drugs (ara-C) (174). Liposomes function as sustained release system, continually releasing their entrapped drugs over several hours to several days (174-177). There is evidence that liposome association of drugs may help to overcome multidrug resistance mediated by the multidrug resistance-associated protein or p-glycoprotein (178-181).

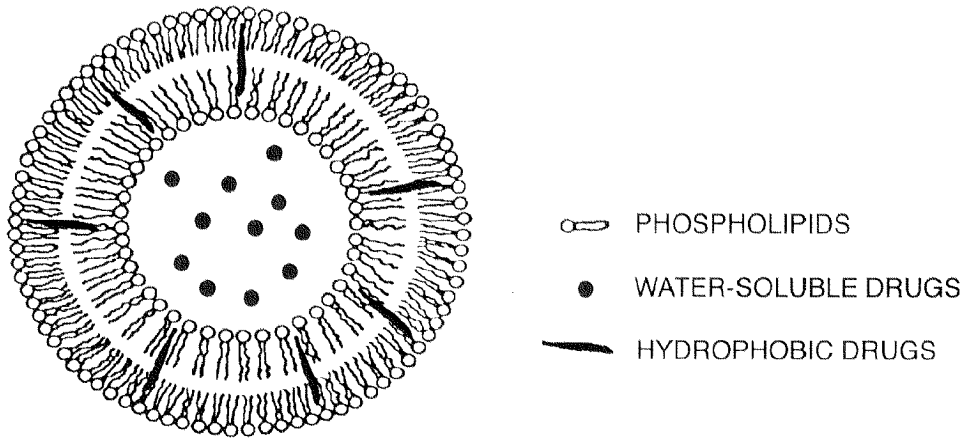


Figure 8
Schematic representation of an unilamellar liposome containing water-soluble and hydrophobic drugs. Figure adapted from reference (167).

Presently, three liposomal drug delivery systems have been approved for clinical use. These include a liposomal formulation of the antifungal drug amphotericin B, and two liposomal formulations of the anticancer drugs daunorubicin and doxorubicin. Additional antitumour drugs which are in clinical development include liposomal vincristine, cisplatin, paclitaxel, annamycin and a lipophilic cisplatin derivative. Further applications under development are carriers for contrast agents in tumour diagnosis (182) and vaccines (183, 184). A new field represents the use of liposomes in gene therapy approaches, e.g. for cystic fibrosis (185-187) and for AIDS (188).

1.4.2 Composition, Characterisation and Preparation of Liposomes

Liposomes form spontaneously when suitable amphiphilic compounds, such as phospholipids are hydrated in aqueous media (189). Phospholipids are amphiphilic compounds which consist of glycerol or a sphingosine backbone, conjugated with a hydrophobic moiety and a hydrophilic polar head. The hydrophilic parts of the phospholipids (e.g. phosphocholine) are always oriented towards the aqueous solution, forming a bimolecular layer, whereas the hydrophobic parts (one or two fatty acid chains) form a continuous hydrocarbon bilayer (Figure 8).

Phosphatidylcholine is the major component of most biological membranes and is frequently used as a standard lipid for liposomes. In this work phosphatidylcholine isolated from soy bean lecithin (SPC) was used to prepare liposomes. Phospholipid bilayers composed of saturated phospholipids (e.g. dipalmitoyl phosphatidylcholine, distearoyl phosphatidylcholine) can exist in different thermodynamic phases, such as 'gel', 'solid', 'fluid-crystal' or fluid phases, depending on their liquid crystalline phase transition temperature (T_C). Due to the parallel arrangement of the fatty acids, the hydrated phospholipid bilayers are tightly packed. Strong Van der Waals forces are exerted, stabilising the bilayer and raising the T_C (189). The phase condition of the liposomal bilayer strongly influences stability and behaviour of the liposomes in biological systems.

Eucaryotic plasma membranes contain large amounts of cholesterol, reaching an amount of up to one molecule for every phospholipid molecule. Liposomal preparations normally also contain cholesterol. Incorporation of cholesterol decreases the fluidity and permeability of liposomal membranes. Cholesterol stabilises and rigidifies the lipid bilayer and renders it more resistant to in vivo degradation (190).

After the phospholipids have been dried and hydrated, they spontaneously form MLV with particle sizes ranging from 0.4-5 µm. The preferred size for clinical applications, are SUV with 50 to 200 nm in diameter. Liposomes of this size are small enough to avoid or reduce uptake by the mononuclear phagocyte system (MPS) better than larger liposomes and to permit localisation in diseased tissue, yet large enough to trap useful drug loads (191). To obtain SUV different preparation methods are available. For this work the extrusion method was chosen. The MLV suspension was filtered at high pressure through polycarbonate filters with well defined pore size using a Lipex™ extruder (192), resulting in SUV with the desired vesicle diameter (see chapter 2.3).

1.4.3 Interactions of Liposomes with Cells

Liposomes can interact with cells in different ways (Figure 9). These interactions depend on liposome characteristics (lipid composition, size, charge, surface modification) and cell type (193-195). Phagocytosis is the main mechanism of interaction between liposomes and cells possessing endocytotic capacity such as macrophages and cells of the mononuclear phagocyte system (MPS) (196-199). Invagination of the plasma membrane leads to phagocytic uptake of liposomes into endosomes. Subsequently, the endosomes fuse with lysosomes to form endolysosomes, where lysosomal enzymes digest the liposomes. During the process of breakdown of the liposome membranes, the contents of the aqueous compartment are released. They may leak out of the endolysosomes, be degraded by lysosomal enzymes or be stored in vacuoles until exocytosis. The rate of phagocytosis strongly depends on the lipid composition of the liposomes. Liposomes consisting of bilayers of high rigidity are far more resistant to intralysosomal digestion than more fluid-type liposomes (200).

Liposomes can adsorb to a cell surface, resulting in an increased permeability of the liposome membrane. This leads to release of water-soluble solutes at high concentrations in the close vicinity of the cell membrane, some of which may enter the cell by crossing the cell membrane.

After close interaction of the liposome with the cell surface, intermembrane transfer of lipid components can take place between the two phospholipid bilayers without need for disruption of the liposome or damage of the membrane integrity. Indeed, it is possible for such transfer to occur (often in both directions) with complete retention of the contents of the liposome's aqueous compartment. Depending on the composition of the lipids, they can remain there over long periods of time or can be redistributed into a variety of intracellular membranes after incorporation into the cellular bilayer. Cholesterol transfers very rapidly between bilayers reaching an equimolar concentration throughout all membranes.

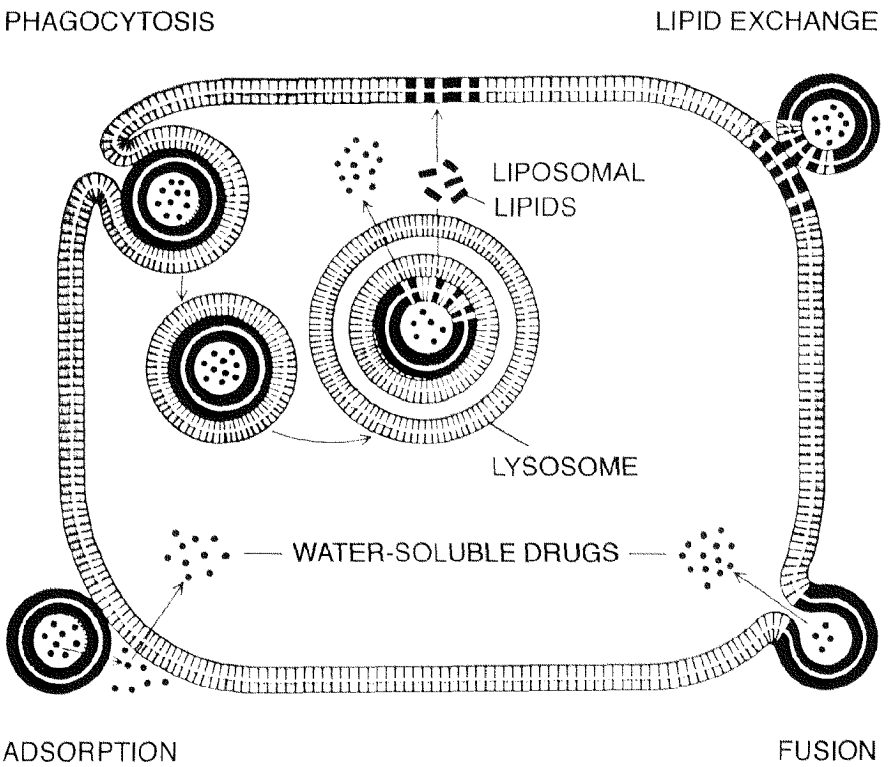


Figure 9
Schematic representation of possible interactions of liposomes with cells. Figure adapted from reference (201).

Close interaction of liposomes with cell membranes can lead to fusion, resulting in the release of aqueous contents into the cytoplasm and in the mixing of liposomal lipids with those of the plasma membrane. However, in vivo fusion is a rare event because liposomes are cleared far too rapidly from the bloodstream by phagocytic cells (197).

1.4.4 Interactions of Liposomes with the Mononuclear Phagocyte System (MPS)

The main site of clearance of liposomes from the blood is the MPS. These cells are specialised in removing foreign particles from the blood stream. After parenteral administration, plasma proteins (opsonins) are absorbed onto the surface of liposomes, triggering recognition and liposome uptake by MPS cells through receptors such as the complement C3b receptor and others (167, 168, 202, 203). Mononuclear cells in blood and in the bone marrow, macrophages in tissues, and dendritic cells in

skin belong to this system. These cells are predominantly located in the liver (Kupffer cells), in the spleen and in the bone marrow. Consequently, liposomes accumulate mainly in these organs.

After intravenous administration, the liposomes circulate in the blood with half-lives determined by liposome size, composition, surface properties and charge. Smaller liposomes have slower clearance rates than larger (168). Generally, liposomes are too large to pass the barrier represented by the capillary endothelium. Therefore liposomes can only enter organs with fenestrated endothelia such as liver or spleen. Large liposomes rapidly end up in the Kupffer cells of the liver and are digested, whereas smaller liposomes can traverse the endothelial lining and can be endocytosed by hepatocytes (204, 205). Generally, liposomes composed of lipids with high transition temperature exhibit longer circulation half-lives than liposomes containing unsaturated, or short chain phospholipids (203, 206). Cholesterol also influences the liposome clearance. A high cholesterol content renders liposomes more resistant to opsonising proteins and consequently to endocytosis (207, 208). Negatively charged liposomes (containing e.g. phosphatidylserine) are cleared more rapidly than neutral or positively charged liposomes (containing e.g. stearylamine) (209-211).

In recent years new liposome formulations were developed by coating of the liposome surface with polyethylene glycols. This leads to reduced recognition of liposomes by macrophages and to a significantly prolonged circulation half-life. These formulations, known as sterically stabilised liposomes, long-circulating liposomes or 'stealth' liposomes result in increased circulation times and thus in an alteration of the biodistribution of their associated drugs (212-215).

1.4.5 Active and passive Targeting of Liposomes

A number of investigators are exploring ligand-targeted liposomes where antibodies, proteins or peptides attached to the surface of the liposome increase the binding of liposomes to specific epitopes or receptors at the target cell surface (216-218). Use of antibodies against internalising epitopes are thought to be of particular advantage, as binding of the liposome to its target will trigger the entry of the entire drug package into the interior of the target cell (217).

The time of residence of liposomes in the vasculature can affect their biodistribution, and prolonged circulation times of liposome-associated drugs appear to increase their localisation into diseased tissues (219-223). Regions of solid tumour growth, as well as regions of infection and in-

flammation, have capillaries with increased permeability because of the disease process (224). Therefore, drug-containing liposomes (SUV) with long circulating times are able to localise in greater quantities in these regions than in normal tissue, which have intact capillaries that are impermeable to liposomes (225, 226).

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2 Materials and Methods

2.1 Reagents, Drugs and Antibodies

Bovine serum albumin (BSA), 5-bromo-2'-deoxyuridine (BrdU), propidium iodide (PI), Triton X-100, acid washed activated charcoal, dUMP, 4',6-diamidino-2-phenylindole (DAPI), the mouse anti-α-tubulin and the goat anti-mouse IgG-Cy3 antibodies were purchased from Fluka Chemie (Buchs, Switzerland). Phalloidin-oregon green, SYTOX green and SYBR green II were from Molecular Probes (Eugene, OR, USA) and the 123-bp marker from Gibco (Paisley, UK). RPMI-1640 medium, foetal calf serum (FCS), penicillin-streptomycin, L-glutamine, Hank's balanced salt solution (HBSS) and agarose were from Life Technologies (Basel, Switzerland). Trypsin-EDTA was obtained from Biochrom KG (Berlin, Germany). The WST-1 assay kit, RNAse A and proteinase K were from Boehringer Mannheim (Rotkreuz, Switzerland), Tween 20 from Merck (Darmstadt, Germany) and T-70 dextran from Pharmacia (Dübendorf, Switzerland). Digitonin and Mowiol were purchased from Calbiochem (Juro Supply AG, Lucerne, Switzerland). The monoclonal antibody Apo 2.7-PE was from IL Instrumentation Laboratory AG (Zurich, Switzerland). The FITC-labelled anti-BrdU antibody and the Ac-DEVD-AMC fluorogenic substrate were from Becton-Dickinson (Basel, Switzerland). The lipophilic heteronucleoside dimers were synthesised according to the methods described previously (100). 5-FdU was obtained from Hoffman La-Roche, Basel, Switzerland. The drugs were dissolved in 0.9% NaCl.

2.2 Cells

The human epithelial prostate tumour cell lines PC-3 and DU-145 were obtained from the German Collection of Microorganisms and Cell Cultures, DSMZ, Braunschweig, Germany. The cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine in a humidified 5% CO_2 atmosphere at 37°C.

2.3 Liposome Preparation

SUV of 100 ± 30 nm mean diameter were prepared by filter extrusion as described by Hope et al. (192). Briefly, lipid mixtures composed

of SPC, cholesterol, D, L-α-tocopherol and NOAC or drugs at a molar ratio of 1:0.2:0.01:0.2 were hydrated with phosphate buffer (PB; 13 mM KH_2PO_4, 54 mM Na_2HPO_4 pH 7.4) and sequentially filtered through Nucleopore (Costar, Sterico, Dietikon, Switzerland) filters of decreasing pore size (400 nm, 100 nm). Liposomes without NOAC or drugs were used as control. Liposomes were sterile filtered through 0.2 µm filters (Acrodisc, Gelman Sciences, Ann Arbor, MI, USA), stored at 4°C and used within 48 h. Trace amounts of [5-^{3}H]NOAC were added for detection and quantification.

2.4 Drug Uptake

Cells (10^5 cells per well) were seeded and incubated in 24-well plates for 24 h. Cells were exposed to various concentrations (0-400 µM) of [5-^{3}H]NOAC for 90 min or for various time periods (0-48 h) with 40 µM of [5-^{3}H]NOAC at 37°C (5% CO_2). After washing twice with cold PBS (8 mM $NaPO_4$, 1.5 mM KH_2PO_4, 0.14 M NaCl, 2.6 mM KCl) total drug uptake was determined by scintillation counting. For each concentration and time period the cell numbers were determined and drug uptake/10^6 cells was calculated.

2.5 Haemolytic Activity in Vitro

Liposomal preparations of dCpam-5-FdU, 5-FdU-5-FdC18 and 5-FdU-NOAC were incubated at different concentrations (0.6-5 mM) with freshly collected human blood from healthy donors for 60 min at 37°C. Aliquots of the supernatants obtained after centrifugation (200 g, 10 min) were diluted 1:100 in 0.9% NaCl and the concentration of haemoglobin was determined by calculating the difference between the absorption at 577 nm and 561 nm. Total haemolysis (100%) was obtained by incubation of blood in water containing 0.5% Triton X-100 at a 1:1 (v/v) ratio.

2.6 Cytotoxicity Assay

To evaluate cell proliferation the WST-1 kit was used. Exponentially growing cells were seeded in sterile 96-well plates and incubated for 24 h. Drugs were added to a final concentration of 12-200 µM. The supernatant was removed after 96 h and 100 µl of freshly diluted WST solution were

added. The plates were incubated for 30-60 min at 37°C (5% CO_2). Cell viability was evaluated by measurement of the absorption at 450 nm using a Dynatech MR5000 plate reader (Microtec Produkte, Embrach, Switzerland). Fifty percent growth-inhibitory concentrations (IC_{50}) were calculated from interpolations of the graphical data.

2.7 Cell Cycle Distribution Analysis

Cells were seeded in 100-mm culture dishes, incubated for 48 h and exposed to various concentrations (0-200 µM) of dimers and 5-FdU for 24 h at 37°C (5% CO_2) or for various time periods (0-48 h) with 50 µM of the drugs. After the specified period the cells were incubated with 10 µM BrdU for 30 min at 37°C (5% CO_2). The supernatant with dead cells and the harvested living cells were fixed in pre-cooled (-20°C) ethanol (80%) and stored at -20°C for up to 3 days. BrdU/PI staining was carried out as described previously (227). Briefly, after centrifugation, the cells were treated with 2 M HCl for 30 min at 20°C and re-suspended in 50 µl PBS, 0.5% Tween-20, 1% BSA and incubated with FITC-labelled anti-BrdU antibody for 30 min at 20°C followed by addition of 1 ml PBS/PI (10 µg/ml). Stained cells were analysed with an Epics Elite Analyser (Coulter, Florida, USA). Single fluorescent samples (FITC or PI) were used to optimise instrument settings and ensure proper electronic compensation.

2.8 Quantification of the apoptotic Cell Fraction

Cells were treated as described for cell cycle analysis. After incubation the supernatant with dead cells and the harvested living cells were pooled and permeabilised by incubation on ice for 20 min with 100 µg/ml digitonin in PBS supplied with 2.5% FCS (v/v) and 0.01% NaN_3. After permeabilisation the cells were labelled with Apo 2.7-PE for 15 min at room temperature in the dark. For flow cytometric analysis cells were re-suspended in PBS supplied with 2.5% FCS (v/v) and 0.01% NaN_3 and stored on ice in the dark until analysis.

2.9 Caspase-3 Activity

Cells were treated as described for cell cycle analysis. After incubation with the drugs dead cells in supernatant and the harvested living cells were counted and lysed with 10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaH_2PO_4, 10 mM $Na_4P_2O_7$ (2 x 10^6 cells/ml). After centrifugation (5 min, 1400 g) 100 µl of the cell lysate were reacted with 20 µM Ac-DEVD-AMC fluorogenic substrate in 20 mM HEPES, pH 7.5, 10% glycerol, 2 mM dithiothreitol for 2 h at 37°C. Released AMC from Ac-DEVD-AMC was measured using a spectrofluorometer (Kontron SFM 23/23 LC) with excitation and emission wavelengths of 380 nm and 440 nm, respectively.

2.10 DNA Fragmentation

Cells were exposed for various time periods (0-96 h) with 50 µM of 5-FdU, dCpam-5-FdU, 5-FdU-5-FdC18, 5-FdU-NOAC and NOAC at a 37°C (5% CO_2). As positive control colcemide was included (1 µg/ml; 24-96 h). DNA extraction was performed with modifications as described by Kaufmann (228). Briefly, supernatants and harvested cells were pooled, washed once with PBS and lysed in 300 µl lysis buffer (0.5 M Tris-HCl pH 9.0, 2 mM EDTA, 10 mM NaCl, 1% SDS, 0.33 mg/ml proteinase K). The samples were incubated at 55°C for 24 h, extracted twice with phenol/chloroform (1:1, v/v) and once with chloroform. The probes were then incubated with 300 µg/ml DNAse free RNAse A and loaded onto 1.2 % (w/v) agarose gels. Staining of DNA was performed using SYBR green II dye. Gels were scanned at 488 nm on a FluorImager 595 (Molecular Dynamics, CA, USA) using a SYBR green filter (530DF30).

2.11 Immunofluorescence Labelling and Confocal Microscopy

Cells were incorporated in a 4 : 1 (v/v) mixture of rat tail collagen type I (50'000 cells/100 µl) and RPMI 10x supplemented with 292 mM $NaHCO_3$ and 75 mM NaOH. The collagen was isolated with minor modifications as described by Elsdale and Bard (229). The collagen-cell suspensions (20 µl) were seeded on Permanox chamber slides (Life Technologies, Basel, Switzerland). After solidification of the collagen at 37°C, the cells were incubated for 1 week in medium at 37°C (5% CO_2). Consecutively, they were treated with 50 µM 5-FdU-5-FdC18 or 0.5 µg/ml col-

cemide for 5 days. To preserve the structural organisation of microtubules cells were washed in a microtubule protective (MT) buffer (230), permeabilised for 15 min with 1% Triton X-100 in MT buffer, and fixed for 30 min with 3% para-formaldehyde in MT buffer at room temperature, followed by treatment with 0.1 M glycine in PBS at 4°C for 15 min. For triple staining cells were incubated twice over night with the first and second antibody at 4°C. Antibodies were diluted in PBS containing 3% BSA: mouse anti-α-tubulin 1:500 (v/v) goat anti-mouse IgG Cy 3 1:50 (v/v). For phalloidin-oregon green the dilution was 1:10 (v/v) and for the DAPI stain 1:100 (v/v). Alternatively cell nuclei were stained with SYTOX green overnight at 4°C after permeabilisation. The dilution of the dye was 1:5000 (v/v) in Tris-EDTA buffer (10 mM Tris pH 7.4, 1 mM EDTA). Cells were embedded in Mowiol and coverslipped. The samples were analysed on a Zeiss LSM 410 inverted microscope (lasers: HeNe 543 nm, Ar 488/514 nm and Ar UV 364 nm). IMARIS, a 3D multi-channel image processing software for confocal microscopic images (Bitplane AG, Zurich, Switzerland) was used for image processing on a Silicon Graphics workstation.

2.12 Electron Microscopy

PC-3 cells were seeded in 100-mm culture dishes and incubated for 48 h. Medium was exchanged and cells were exposed to 50 µM 5-FdU-5FdC18 for 48 h at 37°C (5% CO_2). Control cells were not treated. The supernatant with dead cells and the trypsinised living cells were pooled, washed once with medium and resuspended in 1 ml RPMI-1640. Cells were incubated at 37°C (5% CO_2) for 30 min on a shaker, fixed with an equal volume of 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (76 mM Na_2HPO_4, 23 mM NaH_2PO_4, 1 mM $MgSO_4$, pH 7.4) at room temperature for 30 min. The cell pellets were postfixed overnight with 1% OsO_4 in 0.1 M sodium phosphate buffer at 4°C. Pellets were dehydrated in serial ethanol solutions (10-90%, v/v). The samples were embedded in Epon and polymerised at 60°C for 2 days. Thin sections were cut on a ultracut E ultrotome (Reichert-Jung). The sections were stained with 0.5% uranyl acetate and lead citrate (8.8 mM Pb2(NO_3), 13.1 mM $C_6H_5Na_3O_7$, pH 12) and examined on a Philips CM 10 electron microscope.

2.13 Thymidylate Synthase Activity

Activity of TS was measured by the release of tritium from [5-^{3}H]dUMP. Cells were seeded in 6-well plates and incubated for 48 h. After exposition to the drugs for 90 min at different concentrations (0.01-100 µM) or for various time periods (0-8 h) with 0.1 µM at 37°C (5% CO_2) the cells were treated with deoxyuridine-5'-monophosphate (10 µM) trace labelled with 0.5 µCi/ml [5-^{3}H]deoxyuridine-5'-monophosphate (Amersham Pharmacia Biotech, Dübendorf, Switzerland) (231). After incubation at 37°C for 60 min, 0.2 ml medium were removed and added to 1 ml of a mixture of ice-cold T-70-dextran and BSA-treated charcoal to terminate the reaction. After 30 min at room temperature the probes were centrifuged (30 min, 4400 g) and the radioactivity of the supernatant determined in a liquid scintillation instrument (1900 TR Packard). Fifty percent inhibitory concentrations were calculated from interpolations of the graphical data.

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3 Results

3.1 Inhibition of Cell Growth

Various newly synthesised 5-FdU, ara-C and NOAC derivatives were tested for their efficacy in the two human prostate tumour cell lines DU-145 and PC-3. The 50% inhibitory concentrations (IC_{50}) of the tested compounds after 96 h of drug exposure are summarised in Table 1. The derivatives were tested in both aqueous and liposomal formulations. Untreated cells or cells treated with empty liposomes were used as controls for 100% viability. Empty liposomes were not toxic for the cells at a lipid concentration up to 0.8 mg/ml SPC (corresponding to liposomes with 200 µM drug). Dimers with 5-FdU had strong cytotoxic effects in both cell lines, resulting in IC_{50} values of 3-8 µM. One exeption was the liposomal formulation of 5-FdU-(3'→5')-NOAC, which does not reach an IC_{50} in PC-3 cells in the concentration range tested. At low concentrations of 12 µM DU-145 are more sensitive than PC-3 cells, whereas at 200 µM there are no significant differences (data not shown). Compared to the liposomal formulation the drugs dissolved in 0.9% NaCl act at lower concentrations as shown in the example of 5-FdU-5-FdC18 (Figure 10). In contrast to many new derivatives (5-FdU-5-FdC16, 5-FdU-5-FdC18, 5-FdU-(5'→5')-NOAC, Ara-C-(5'→1')-L(ocd)-(3'→5')-5-FdU), 5-FdU and Ara-C were not able to reach a 100% cytotoxicity neither in DU-145 nor in PC-3 cells. Both substances reach the highest toxicity at 12 µM in both cell lines and an increase in concentration did not increase the cytotoxicity further (Figure 11).

Based on these results the following derivatives with excellent cytotoxic effects on both cell lines were chosen for further analysis: dCpam-5-FdU, 5-FdU-(3'→5')-5-FdC18 and 5-FdU-(5'→5')-NOAC. 5-FdU and NOAC were included as controls. The cytotoxic effects of 5-FdU, dCpam-5-FdU, 5-FdU-5-FdC18, 5-FdU-NOAC and NOAC are shown in Figure 12. The IC_{50} values of the drugs (Table 1) show that dCpam-5-FdU, 5-FdU-5-FdC18 and 5-FdU-NOAC have a cytotoxicity comparable to 5-FdU. Concentration dependent studies demonstrated that 5-FdU reached maximal growth inhibition at a concentration of 12 µM in both cell lines (Figure 12A-B). Higher concentrations did not produce additional cytotoxicity. In contrast, the cytotoxicity of 5-FdU-5-FdC18 and 5-FdU-NOAC increased continuously with higher concentrations resulting in complete cell death at 200 µM in both DU-145 and PC-3 cells.

Table 1
IC_{50} values in DU-145 and PC-3 cells after incubation with the various drugs for 96 h.[a]

Drug	IC_{50} (μM)			
	DU-145 cells		PC-3 cells	
	liposomal[b]	aqueous[c]	liposomal[b]	aqueous[c]
5-FdU	na[d]	3.35[e]	na	3.39
5-FdU-(3'→5')-NOAC	4.20	3.68	nr[f]	6.00
5-FdU-(5'→5')-NOAC	4.20	3.90	8.22	5.00
dCpam-(3'→5')-5-FdU	3.68	3.59	3.55	4.00
MOPA-5-FdU	4.29	na	5.00	na
5-FdU-(3'→5')-5-FdC16	3.97	3.44	4.81	3.83
5-FdU-(3'→5')-5-FdC18	4.30	3.48	8.16	4.26
NOAC	133.65	na	109.86	na
NOAC-(5'→5')-ddI	nr	na	nr	na
NOAC-(5'→5')-5-FdC18	nr	na	nr	na
NOAC-(2',3'→1)-Lpam-(3→5')-5-FdC18	nr	na	nr	na
Ara C-(5'→5')-NOAC	11.70	4.11	123.00	54.45
Ara C	na	5.39	na	7.33
Ara C-(5'→1')-L(ocd)-(3→5')-5-FdU	na	3.67	na	9.40
Ara C-(5'→5')-5-FdC18	15.48	na	nr	na
5-FdU-Ara C-N^4-oleate	4.37	3.52	4.66	43.10
5-FdC18	4.87	na	5.41	na
5-FdC18-PO_4	na	6.88	na	33.80

[a] Cytotoxicity determined using the WST-1 cell proliferation assay.
[b] Drugs in liposomes.
[c] Drugs in 0.9% NaCl.
[d] na = not available
[e] Mean of three separate experiments performed in triplicates. SD was < 10% of mean values.
[f] nr = not reached within a drug concentration range of 1-200 μM

The time-dependent cytotoxic activity of the various drugs at 50 μM on DU-145 and PC-3 cells is shown in Figure 12C-D. 5-FdU, dCpam-5-FdU and 5-FdU-5-FdC18 behaved in a similar way, reaching 50% growth inhibition in DU-145 cells after approximately 30 h incubation. In PC-3 cells 90% cytotoxicity was reached after the same time period. At 200 μM and 24 h incubation with 5-FdU-5-FdC18 and 5-FdU-NOAC a cytotoxic

effect of 77% and 82% was obtained in DU-145 cells, and of 93% and 84% in PC-3 cells, respectively, whereas the effects of 200 µM 5-FdU, dCpam-5-FdU or NOAC were not notably different from those with 50 µM (data not shown).

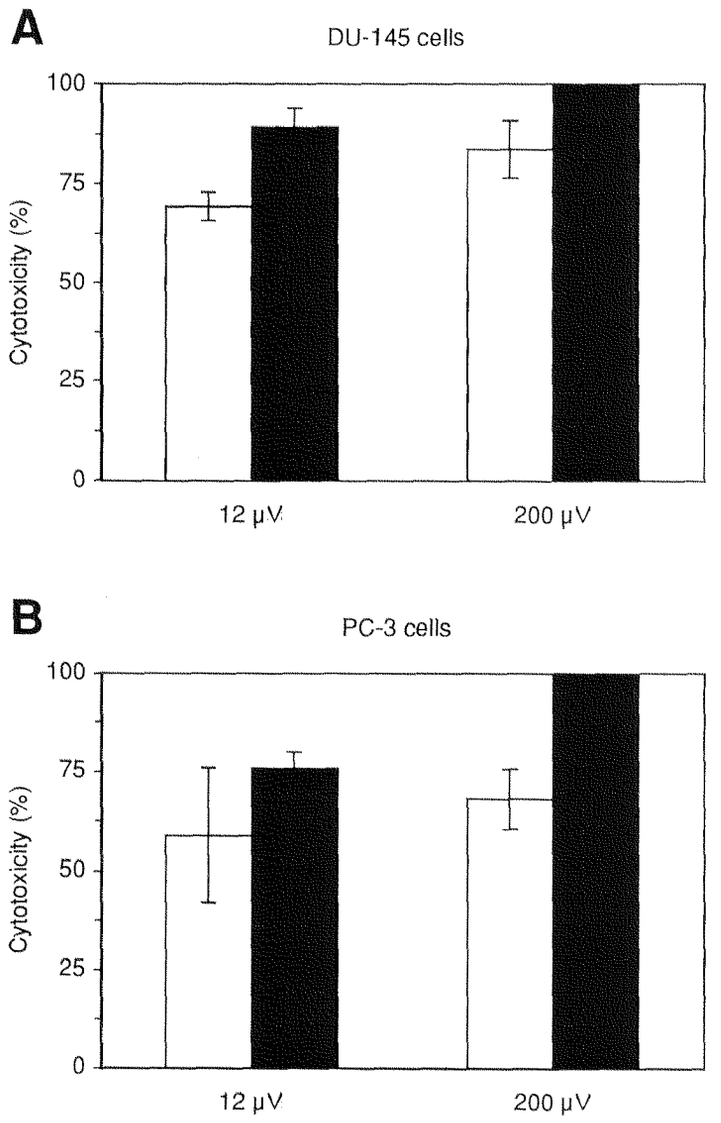

Figure 10
Cytotoxicity determined by WST-1 dye reduction in DU-145 (A) and PC-3 cells (B). Cells were treated for 96 h with 5-FdU-5-FdC18 administered in liposomal formulation (❑) or dissolved in 0.9% NaCl (■). Results are shown as means ± standard deviation (SD) of at least three independent experiments performed in triplicates.

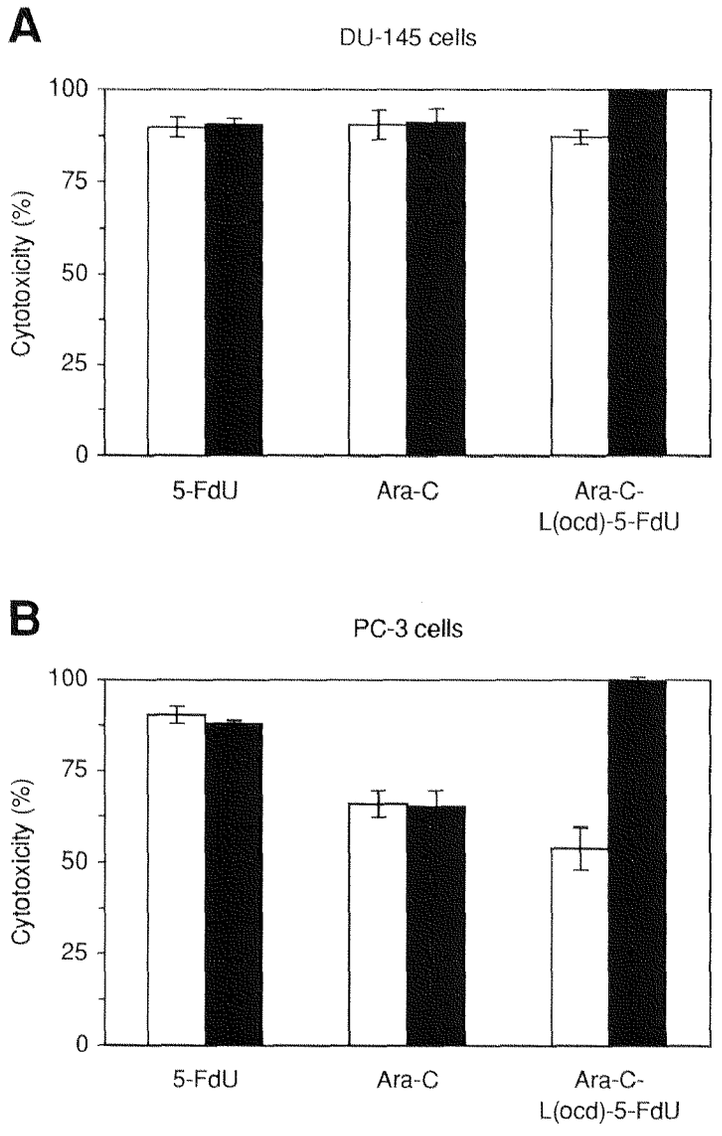

Figure 11
Cytotoxicity determined by WST-1 dye reduction in DU-145 (A) and PC-3 cells (B). Cells were treated with 5-FdU, Ara-C and Ara-C-L(ocd)-5-FdU at concentrations of 12 µM (❑) and 200 µM (■). Results are shown as means ± SD of at least three independent experiments performed in triplicates.

Comparison of the cytotoxicity of the two cell lines shows that DU-145 are more sensitive at low drug concentrations of 12 µM and long incubation times, but PC-3 cells respond faster at higher concentrations and 10% cell viability is reached after 30 h with 50 µM in contrast to a viability of 50% in DU-145. As described before, NOAC had strong antitumour activity in a PC-3 prostate cancer xenograft model (159). Surprisingly, NOAC did not show strong efficacy against these human prostate tumour cell lines in vitro.

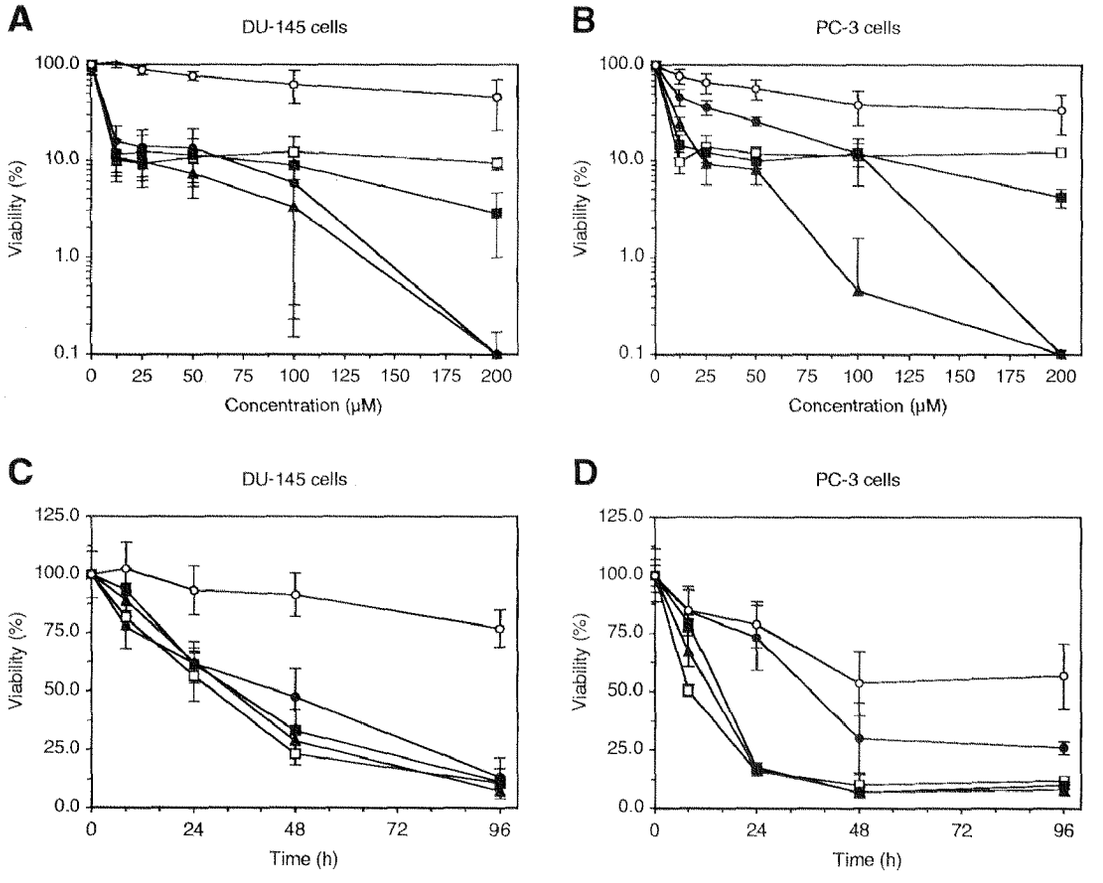

Figure 12
Cytotoxicity determined by WST-1 dye reduction in DU-145 and PC-3 cells. DU-145 (A) and PC-3 cells (B) were treated with 5-FdU (❑), dCpam-5-FdU (■), 5-FdU-5-FdC18 (▲), 5-FdU-NOAC (●) or NOAC (○) for 96 h at a concentration range of 12 - 200 µM. Alternatively, DU-145 (C) and PC-3 cells (D) were treated with 50 µM for various time periods. Results are shown as means ± SD of at least three independent experiments performed in triplicates.

3.2 Cellular Drug Uptake

The cellular uptake of NOAC in DU-145 and PC-3 cells was concentration and time dependent (Figure 13). The uptake of NOAC revealed typical Michaelis-Menten kinetics. Saturation was reached at 100 µM and after 12 h in DU-145 cells, and at 200 µM and after 24 h in PC-3 cells, respectively. The highest uptake occurred at 9.3 ± 0.4 nmol/10^6 DU-145 cells and 22.8 ± 1.3 nmol/10^6 PC-3 cells after 24 h. Accordingly, the IC_{50} for NOAC in PC-3 cells was found to be lower than in DU-145 cells. The uptake of the new dinucleoside derivatives could not been determined, because no tritium-marked dimers were available.

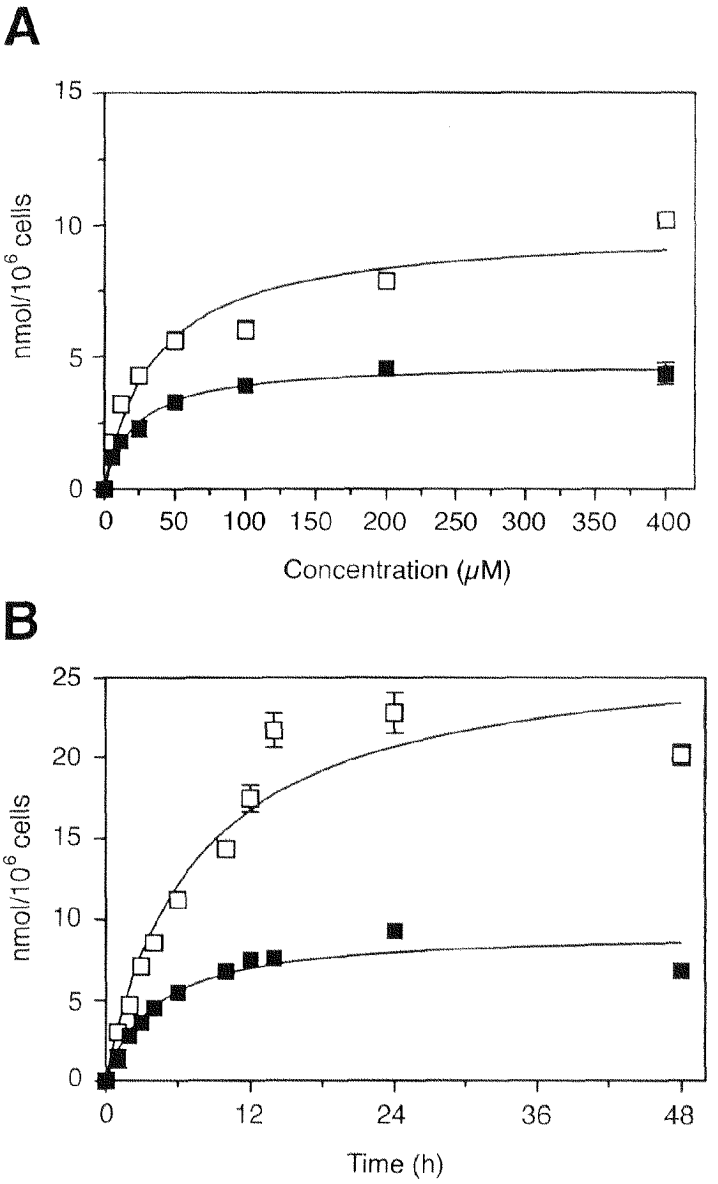

Figure 13
Uptake of [5-^{3}H]NOAC in DU-145 (■) and PC-3 cells (❑). Cells were exposed to various concentrations of NOAC for 90 min (A) or to 40 μM for increasing time periods (B). Data are means ± SD from three separate experiments performed in triplicates.

3.3 Haemolytic Activity in Vitro

The various drugs were tested for their haemolytic effect. For this purpose drug containing liposomes or drugs dissolved in 0.9% NaCl were incubated with fresh human blood. Comparison of haemolytic effects of drugs administered in liposomal formulations of the dimers and aqueous solutions at 2 mM are summarised in Table 2. Liposomal formulations did

not induce haemolysis in contrast to drugs dissolved in aqueous solution. The haemolytic activity of dimeric drugs tested in liposomes was 0% up to the highest possible concentration of 5 mM (data not shown), whereas with liposomal NOAC 3-9 % of the erythrocytes were lysed above 2 mM (156). Figure 14 depicts the concentration dependent haemolytic activity of drugs dissolved in 0.9% NaCl. 5-FdU-5-FdC18 had the strongest haemolytic effects on human blood, resulting in complete haemolysis at a concentration of 6 mM. As expected for hydrophilic substances, 5-FdU did not lyse erythrocytes. All drugs were tested up to the highest possible concentration.

Table 2

Haemolytic effects in human blood of 2 mM 5-FdU, dCpam-5-FdU, 5-FdU-5-FdC18 or 5-FdU-NOAC incorporated in liposomes or dissolved in 0.9% NaCl after 60 min incubation.

Drug	Haemolytic activity (%)	
	liposomal [a]	aqueous [b]
5-FdU	na [c]	0 ± 0
dCpam-5-FdU	0 ± 0.29 [d]	15.3 ± 3.8
5-FdU-5-FdC18	0 ± 0.33	79.7 ± 8.4
5-FdU-NOAC	0 ± 0.55	40.7 ± 11.2

[a] Drugs in liposomes.
[b] Drugs in 0.9% NaCl.
[c] na = not available
[d] Mean ± SD of two separate experiments performed in duplicates.

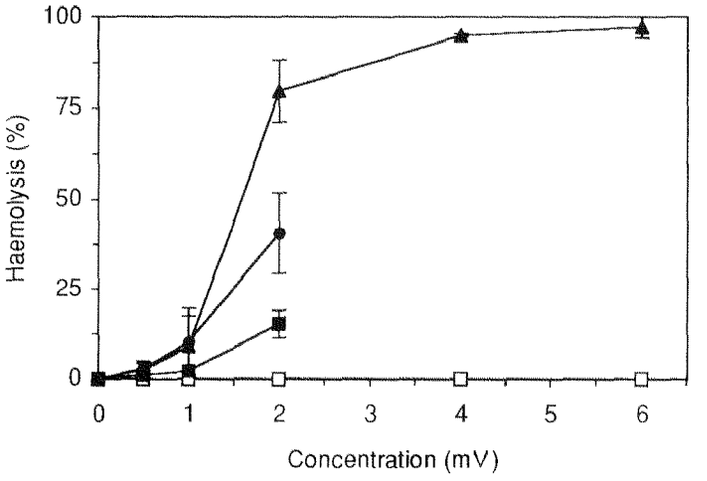

Figure 14
Haemolytic effects of 5-FdU (❑), dCpam-5-FdU (■), 5-FdU-5-FdC18 (▲) and 5-FdU-NOAC (●) dissolved in 0.9% NaCl in human blood after 60 min incubation. Higher drug concentrations in 0.9% NaCl could not be reached due to insolubility. Data are means ± SD of two separate experiments performed in duplicates.

3.4 Cell Cycle Arrest

The influence of the drugs on the cell cycle was analysed by flow cytometry (Figure 15; see chapter 2.7). In Table 3 time-dependent changes in cell cycle distribution of DU-145 and PC-3 cells at increasing incubation times with 50 µM 5-FdU, dCpam-5-FdU, 5-FdU-5-FdC18, 5-FdU-NOAC and NOAC are summarised. In DU-145 as well as in PC-3 cells 5-FdU, dCpam-5-FdU and 5-FdU-5-FdC18 caused a pronounced growth arrest in S-phase. After initiation of drug exposure first effects were observed within 8 h and after 24 h 84% of DU-145 and 78% of PC-3 cells treated with dCpam-5-FdU were in S-phase compared to only 35-36% of untreated cells (Figure 16). Correspondingly, 5-FdU and 5-FdU-5-FdC18 increased the S-phase population to 83-88% in DU-145 and to 54-64% in PC-3 cells after 24 h exposure (Figure 16). The dramatic S-phase arrest always correlated with an increase in early S-phase cells which was accompanied by a decreased proportion of cells in G1- and G2/M-phases (Figure 16). Figure 15 shows the marked cell cycle arrest of PC-3 cells in the early S-phase caused by treatment with dCpam-5-FdU (50 µM, 24 h). After a prolonged drug exposure of 48 h the S-phase cell numbers decreased with all three drugs, probably due to induction of

apoptosis and subsequent cell fragmentation of cells arrested in the S-phase after 24 h (see below). 5-FdU-NOAC had different effects depending on the cell type. In DU-145 it caused an increase in S-phase, whereas in PC-3 no marked cell cycle arrest was caused after 24 h (Figure 16). Accordingly, cytotoxicity after 24 h exposure at 50 µM was higher in DU-145 than in PC-3 cells. S-arrested cells are defined as cells, which, due to their DNA content belong to the middle S-phase fraction, but do not incorporate BrdU into DNA. This cell fraction increased after longer incubation times reaching 8.8% in PC-3 cells after treatment with 5-FdU-NOAC. NOAC alone caused only a slight increase in S-phase cells, suggesting that NOAC is not S-phase limited in its cytotoxicity.

Exposure of DU-145 cells to 100 µM 5-FdU, dCpam-5-FdU and 5-FdU-5-FdC18 for 24 h increased the S-phase population to 91-94%. Further increase in drug concentration up to 200 µM did not alter the cell cycle distribution (Table 4).

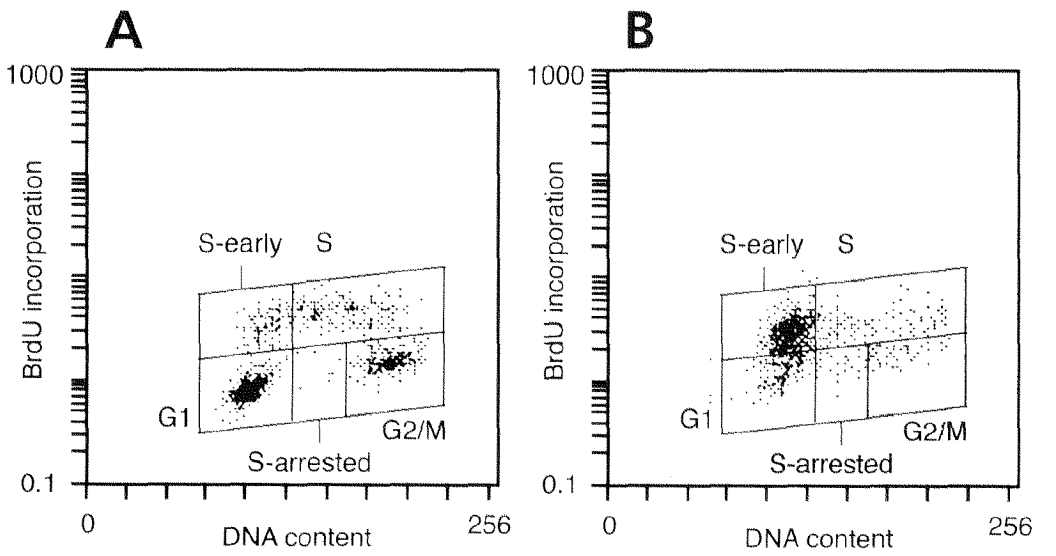

Figure 15

Alteration in cell cycle distribution of PC-3 cells. Cell cycle distribution of untreated cells (A) and cells treated with 50 µM dCpam-5-FdU for 24 h (B). Data are shown as contour plots with DNA content on the x-axis (PI staining) and BrdU content on the y-axis (BrdU-FITC antibody). G1-, S-, S-early, S-arrested and G2/M-phase distribution was quantified by gating the respective cell populations.

Table 3
Cell cycle distribution in DU-145 and PC-3 cells after incubation with 50 µM 5-FdU, dCpam-5-FdU, 5-FdU-5-FdC18, 5-FdU-NOAC or NOAC for increasing incubation times.[a]

Drug	Cell cycle distribution (%)									
Time	DU-145 cells					PC-3 cells				
(h)	G1	S	S-e[b]	S-a[b]	G2/M	G1	S	S-e[b]	S-a[b]	G2/M
5-FdU										
0	51.2[c]	34.7	13.8	0.4	14.1	51.0	36.3	15.9	0.3	12.7
8	43.4	50.9	27.2	0.4	5.7	47.4	47.3	35.3	1.1	5.4
24	13.4	82.9	62.3	2.2	3.7	30.8	64.3	50.5	1.8	4.8
48	39.5	56.7	33.8	5.8	3.8	25.2	70.1	47.9	2.6	4.7
dCpam-5-FdU										
0	51.2[c]	34.7	13.8	0.4	14.1	51.0	36.3	15.9	0.3	12.7
8	45.2	50.8	28.3	0.4	4.0	50.5	43.5	29.4	0.6	6.0
24	12.6	84.2	67.0	3.2	3.2	17.9	78.4	66.4	1.8	3.7
48	36.3	57.6	41.8	6.0	6.1	36.4	56.5	41.6	2.7	7.1
5-FdU-5-FdC18										
0	51.2[c]	34.7	13.8	0.4	14.1	51.0	36.3	15.9	0.3	12.7
8	46.5	48.8	29.1	0.4	4.6	53.1	38.8	25.3	1.3	8.0
24	10.7	87.5	72.5	2.1	1.8	39.6	54.1	42.5	3.8	6.3
48	38.7	57.7	44.9	5.8	3.6	37.3	55.7	36.2	6.1	7.0
5-FdU-NOAC										
0	51.2[c]	34.7	13.8	0.4	14.1	51.0	36.3	15.9	0.3	12.7
8	50.8	42.9	22.1	0.4	6.3	60.1	34.9	18.9	0.4	5.1
24	23.5	73.2	55.3	1.5	3.3	57.4	31.7	16.3	7.2	10.9
48	39.1	55.2	39.4	5.4	5.7	63.0	23.6	10.0	8.8	13.4
NOAC										
0	49.2[c]	35.6	14.8	0.5	15.1	49.3	37.4	16.6	0.3	13.3
8	49.9	41.9	18.5	0.4	8.2	49.3	46.2	24.0	0.2	4.5
24	41.2	49.5	17.7	0.6	9.3	46.2	45.3	20.3	0.4	8.5
48	52.7	39.0	11.3	0.7	8.3	58.8	31.6	14.0	0.8	9.6

[a] Cell cycle distribution determined using the BrdU-PI method.
[b] S-e (S-early); S-a (S-arrested) as described in the text.
[c] Mean of cell cycle fractions (%) of two separate experiments performed in duplicates. SD was < 10% of mean values.

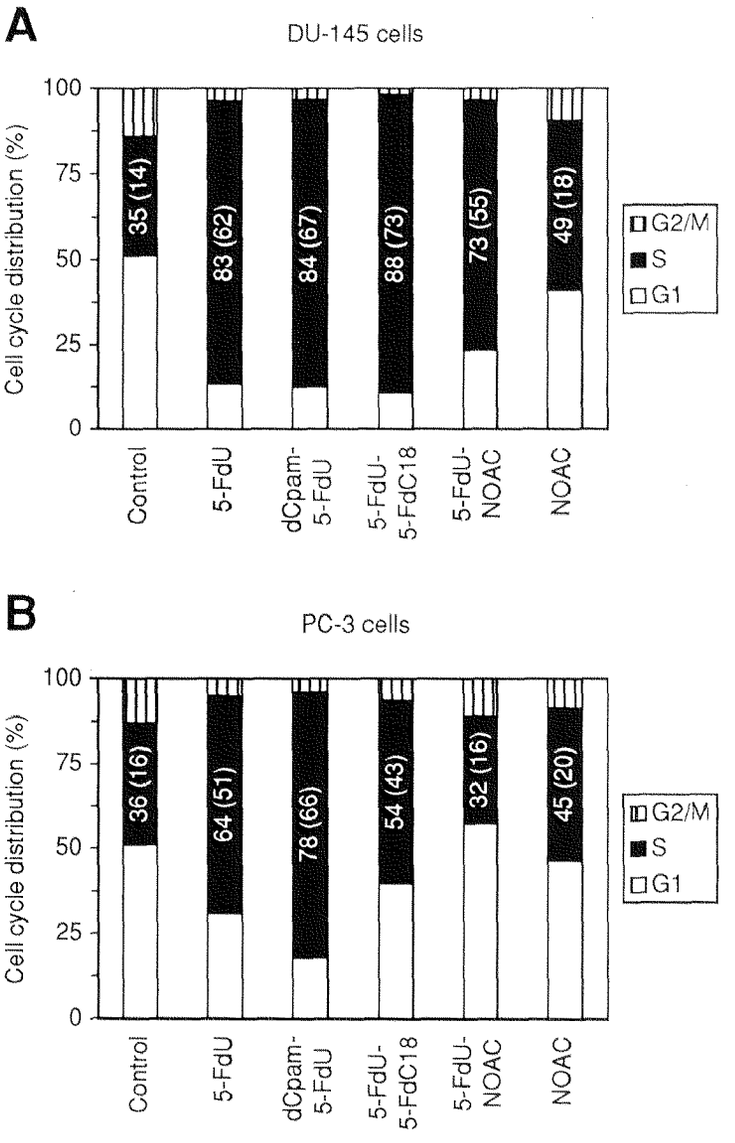

Figure 16

Cell cycle distribution after 24 h incubation with 50 µM 5-FdU, dCpam-5-FdU, 5-FdU-5-FdC18, 5-FdU-NOAC or NOAC in DU-145 (A) and PC-3 cells (B). The first values correspond to the total S-phase cells, whereas the numbers in brackets represent the early S-phase cell fraction in percent of the total cell population. Data are means of at least two independent experiments performed in duplicates.

Table 4
Cell cycle distribution in DU-145 and PC-3 cells after incubation with 5-FdU, dCpam-5-FdU, 5-FdU-5-FdC18, 5-FdU-NOAC or NOAC for 24 h at increasing concentrations.[a]

Drug	Cell cycle distribution (%)									
Conc	DU-145 cells					PC-3 cells				
(μM)	G1	S	S-e[b]	S-a[b]	G2/M	G1	S	S-e[b]	S-a[b]	G2/M
5-FdU										
0	52.0[c]	33.5	12.3	0.4	14.5	49.7	34.6	13.2	0.3	15.7
75	13.3	84.3	66.2	1.3	2.3	24.9	69.6	55.2	1.3	5.5
100	7.3	91.7	69.9	0.6	1.1	28.9	66.3	42.6	2.4	4.7
200	5.1	94.3	67.8	0.2	0.5	21.9	73.9	52.9	1.5	4.2
dCpam-5-FdU										
0	52.0[c]	33.5	12.3	0.4	14.5	49.7	34.6	13.2	0.3	15.7
75	7.3	91.2	73.6	1.1	1.4	25.6	68.7	56.7	2.5	5.7
100	5.4	93.7	76.1	0.8	0.9	22.6	72.4	58.2	2.5	5.0
200	11.6	87.4	69.5	0.2	1.0	33.2	60.8	46.8	2.8	6.0
5-FdU-5-FdC18										
0	52.0[c]	33.5	12.3	0.4	14.5	49.7	34.6	13.2	0.3	15.7
75	8.1	90.3	75.4	0.9	1.5	35.6	56.8	42.0	4.7	7.5
100	8.3	90.6	75.7	0.7	1.1	40.4	52.0	34.8	5.7	7.5
200	37.2	60.1	37.8	1.5	2.7	62.4	27.2	11.1	6.6	10.4
5-FdU-NOAC										
0	52.0[c]	33.5	12.3	0.4	14.5	49.7	34.6	13.2	0.3	15.7
75	32.1	65.7	44.3	0.6	2.2	59.1	28.6	12.4	6.1	12.2
100	44.7	50.2	30.4	1.1	5.2	65.6	20.9	6.9	4.4	13.5
200	33.4	63.0	41.2	3.4	3.6	51.1	30.2	8.4	10.5	18.8
NOAC										
0	50.3[c]	35.0	13.0	0.5	14.7	46.8	37.1	14.5	0.4	16.0
75	44.5	45.8	14.7	0.5	9.7	43.4	49.5	15.4	0.4	7.1
100	45.2	46.4	14.5	0.4	8.4	36.8	51.4	19.9	0.3	11.8
200	41.1	50.9	13.9	0.5	8.1	41.1	53.0	14.6	0.6	5.9

[a] Cell cycle distribution determined using the BrdU-PI method.
[b] S-e (S-early); S-a (S-arrested) as described in the text.
[c] Mean of cell cycle fractions (%) of two separate experiments performed in duplicates. SD was < 10% of mean values.

3.5 Induction of Apoptosis

The quantitative determination of apoptotic cell fractions was carried out with the Apo 2.7 monoclonal antibody that reacts preferentially with cells undergoing apoptosis (232). The histograms in Figure 17 depict the time-dependent increase of apoptotic cells after treatment with dCpam-5-FdU at 50 µM in DU-145 cells. The induction of apoptosis started after 24 h and the strongest effect was found between 24 and 72 h of drug exposure (Figure 18). After this time point there was a only slight further increase. 5-FdU-5-FdC18 and 5-FdU acted in a similar way on DU-145 cells. The quantification of the apoptotic cell fractions in the two cell lines after incubation with 5-FdU, dCpam-5-FdU, 5-FdU-5-FdC18, 5-FdU-NOAC and NOAC were quantified by gating the cell populations and they are summarised in Figure 18. Induction of apoptosis recurred after drug exposures of 24 h in DU-145 and of 48 h in PC-3 cells. 5-FdU-5-FdC18 was the most potent inducer of apoptosis, resulting in an apoptotic cell fraction of 84 ± 1% in DU-145 and 55 ± 10% in PC-3 cells after 96 h. 5-FdU and dCpam-5-FdU resulted in only 34 ± 7% and 22 ± 2% apoptotic cells after 96 h in PC-3 cells, which corresponds to nearly half of the inducing capacity of 5-FdU-5-FdC18. 5-FdU-NOAC was less active, and induced after 96 h 67 ± 5% apoptotic cells in DU-145 and 31 ± 3% in PC-3 cells. NOAC was not able to substantially increase the apoptotic cell fraction in either cell line. Control cells and cells treated with control liposomes did not show any changes. These results correspond exactly to the cell cycle distribution findings (Table 3), where S-phase arrested cell fractions decreased rapidly in DU-145 after 24 h, whereas in PC-3 cells no or only a weak regression of this cell population was found.

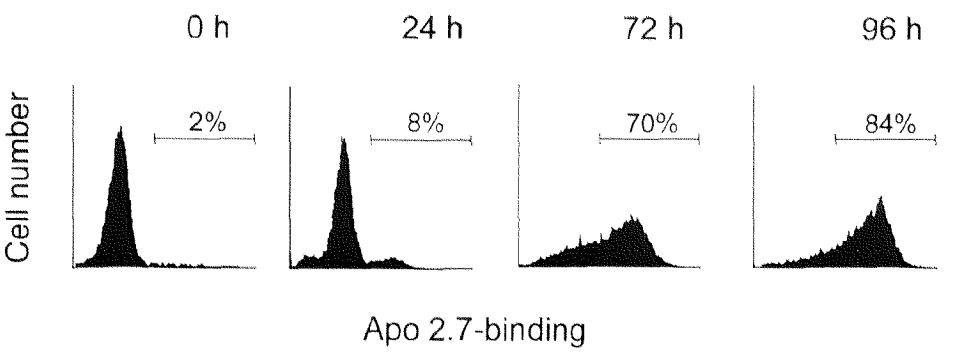

Figure 17
Flow cytometric detection of apoptotic DU-145 cells by Apo 2.7-PE staining in untreated cells (0 h) and in cells treated for 24-96 h with 50 µM dCpam-5-FdU. Apoptotic cell death was quantified by gating the Apo 2.7-PE positive cell population.

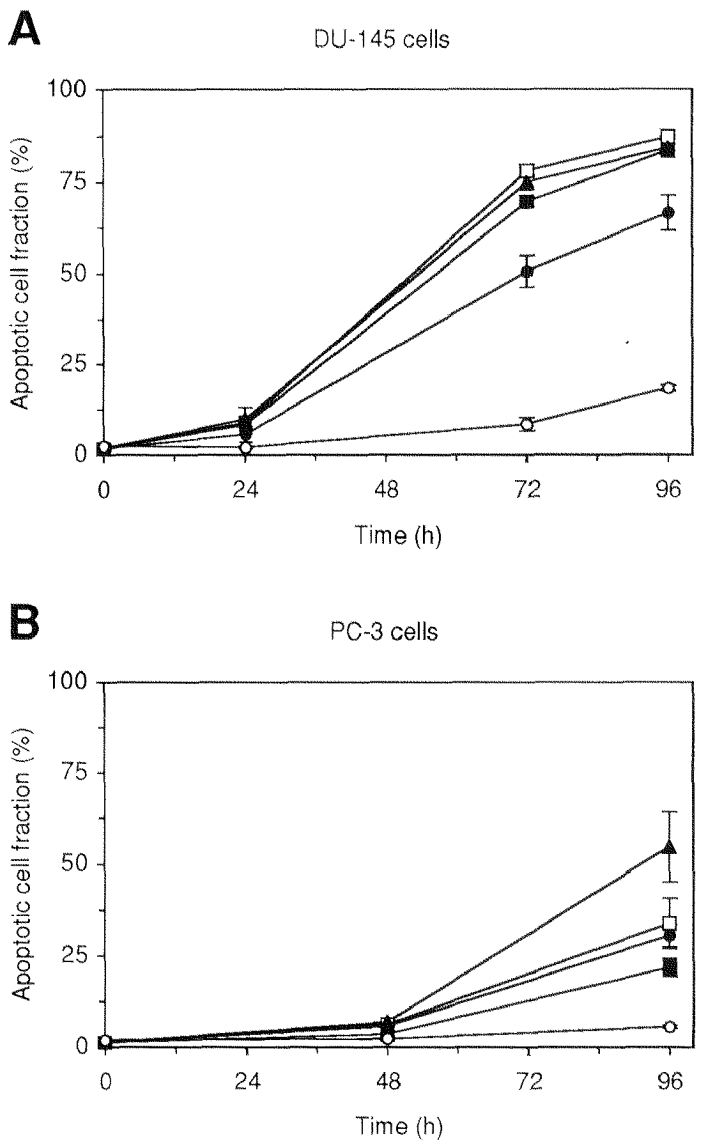

Figure 18
Apoptotic cell fraction after incubation with 50 µM 5-FdU (❑), dCpam-5-FdU (■), 5-FdU-5-FdC18 (▲), 5-FdU-NOAC (●) or NOAC (○) for various incubation times in DU-145 (A) and PC-3 cells (B). Results are means ± SD of at least two separate experiments performed in duplicates.

3.6 DNA Fragmentation

Apoptosis was confirmed by agarose gel electrophoresis as shown in Figure 19 demonstrating the characteristic DNA-ladders after drug treatment. DNA fragmentation was initiated 24 h after exposure with 5-FdU-5-FdC18 at 50 µM in DU-145 cells and was more pronounced after longer incubation times (Figure 19A; lane 3-6). 5-FdU, dCpam-5-FdU

and 5-FdU-NOAC had similar effects on DNA (Figure 19A; lanes 7-9), whereas NOAC caused no DNA fragmentation (Figure 19A; lane 10). Untreated cells (Figure 19A; lane 2) and cells treated with control liposomes (not shown) did not show an apoptotic DNA pattern. Colcemide-treated cells (Figure 19A; lane 11) were included as a positive control for DNA fragmentation. As expected, the same results in PC-3 cells were found with the difference, that DNA fragmentation was delayed and only visible after 48 h (Figure 19B). NOAC induced weak DNA fragmentation in PC-3 cells, while in DU-145 cells no fragmentation was observed (Figure 19A-B; lane 10).

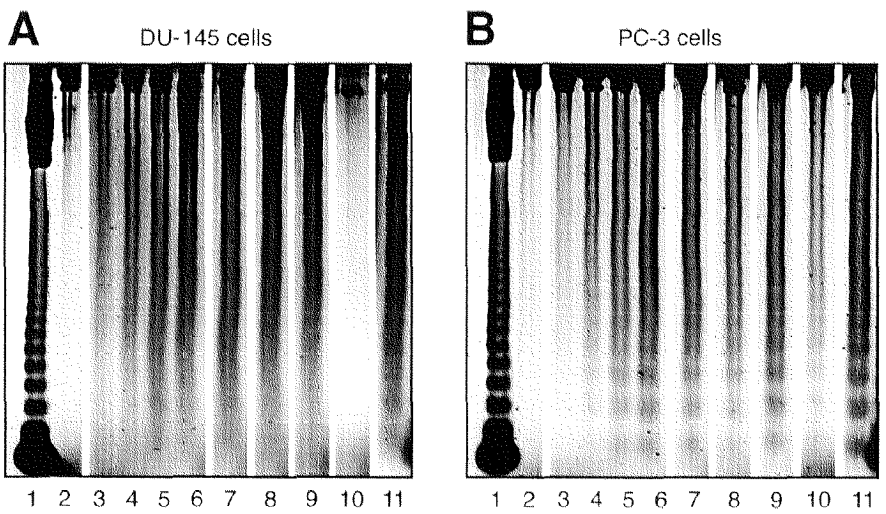

Figure 19
Endonucleolytic DNA fragmentation in DU-145 (A) and PC-3 cells (B) induced by incubation with 50 µM 5-FdU-5-FdC18 (lanes 3-6) for various time periods (24-96 h) and after induction with 5-FdU (lane 7), dCpam-5-FdU (lane 8), 5-FdU-NOAC (lane 9) and NOAC (lane 10) for 96 h. Agarose gel electrophoresis was used for the detection of DNA fragmentation. Untreated cells are shown in lane 2. Colcemide (1 µg/ml, 96 h; lane 11) was used as positive control for apoptosis. A 123-bp ladder was used as marker (lane 1).

3.7 Increase of Caspase-3 Activity

The results of the qualitative caspase-3 apoptosis assay after increasing incubation periods of the different drugs with the cell lines are summarised in Figure 20. The results are given as relative activities of caspase-3 compared to untreated control cells or to cells treated with control liposomes. 5-FdU, dCpam-5-FdU and 5-FdU-5-FdC18 induced a time dependent activation of the enzyme in both DU-145 and PC-3 cells. These drugs were able to increase the enzyme activity up to a factor of 8

in DU-145 (Figure 20A) and 11 in PC-3 cells (Figure 20B). Interestingly, in DU-145 cells 5-FdU reached its highest activity after 72 h, whereas with 5-FdU-5-FdC18 and dCpam-5-FdU a further increase was observed after 96 h (Figure 20A). According to the results with Apo 2.7 antibody and DNA fragmentation the increase in caspase-3 enzyme activity was initiated after 24 h in DU-145 and after 48 h in PC-3 cells. The dimer 5-FdU-NOAC and NOAC had no inducing activities.

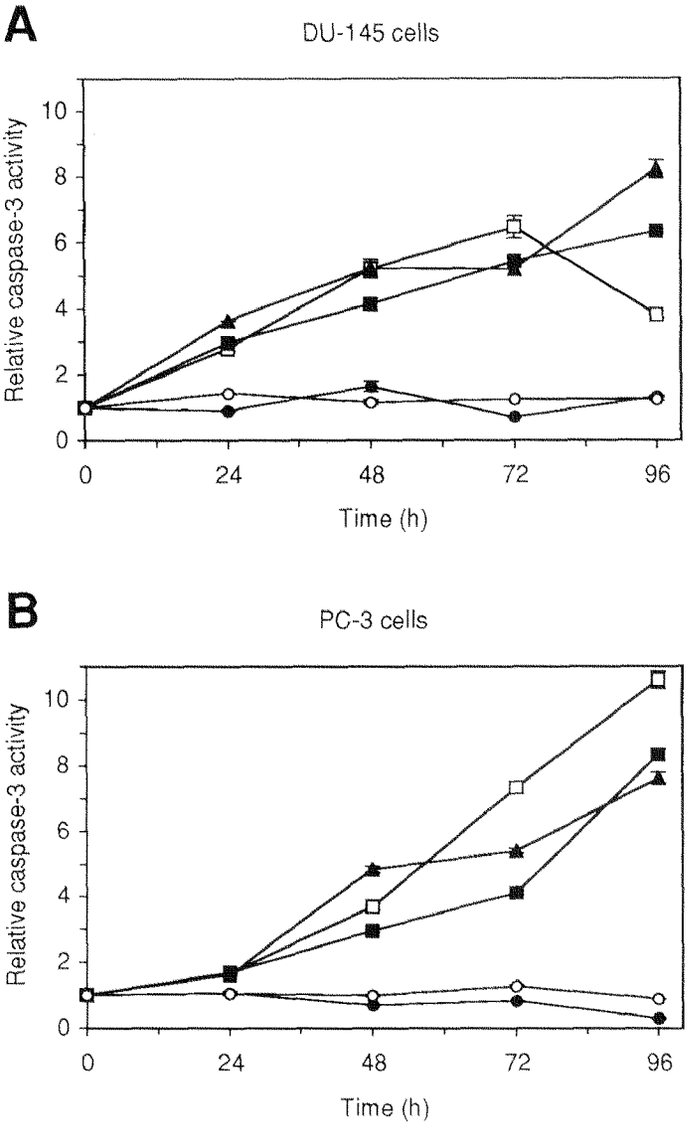

Figure 20

Relative caspase-3 activity after incubation with 50 µM 5-FdU (❑), dCpam-5-FdU (■), 5-FdU-5-FdC18 (▲), 5-FdU-NOAC (●) or NOAC (○) for various incubation times in DU-145 (A) and PC-3 cells (B). Results are given as increase of activity compared to untreated control cells or cells treated with control liposomes. Results are shown as means ± SD of one representative experiment performed in duplicates.

3.8 Disruption of Cytoskeleton and Formation of Apoptotic Nuclei

To illustrate the morphological changes after drug treatment, cells were embedded in a 3-dimensional collagen I matrix and analysed by confocal laser scanning microscopy. As shown in Figure 21 we examined the structure of the microtubules, actin filaments and nuclei of untreated control cells (Figure 21A-D) compared to cells treated with 5-FdU-5-FdC18 (50 µM, 120 h; Figure 21E-H) and colcemide (0.5 µg/ml, 120 h; Figure 21I-M) in DU-145. Compared with the controls (Figure 21A), treatment with 5-FdU-5-FdC18 resulted in a complete disruption of actin filaments (Figure 21E). Likewise, the structure of the microtubules was disrupted (Figure 21F). Fluorescence microscopy of cells stained with the

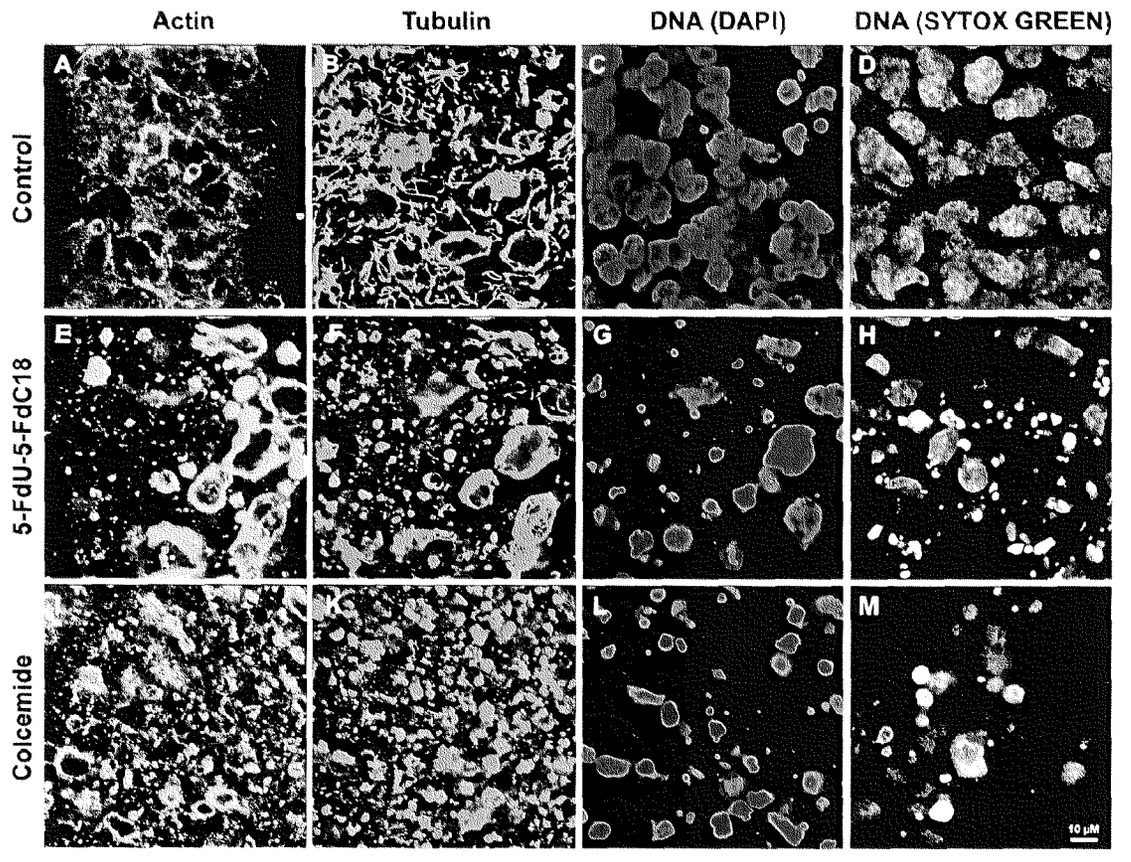

Figure 21
Confocal laser scanning microscopy of collagen embedded DU-145 cells after treatment for 120 h with 50 µM 5-FdU-5-FdC18 (E-H) and 0.5 µg/ml colcemide (I-M). Untreated cells are shown in panels A-D. The cells were triple-stained for F-actin (green; A, E, I), α-tubulin (red; B, F, K) and DAPI for cell nuclei (blue; C, G, L). Alternatively, cell nuclei were stained with SYTOX green (D, H, M). All pictures represent single optical sections.

DNA fluorochrome DAPI (Figure 21G) or with SYTOX green (Figure 21H) revealed the presence of apoptotic nuclei with condensed and fragmented DNA in drug treated DU-145 cells. Apoptotic nuclei were observed only sporadically in control cells (Figure 21C-D). Colcemide-treated cells were included as positive control for apoptosis showing complete cytoskeleton disruption (Figure 21I-K) and DNA fragmentation (Figure 21L-M).

5-FdU-5-FdC18 had similar effects on PC-3 cells (Figure 22). Analysis of these cells showed that in contrast to untreated cells (Figure 22A-C), drug treated tumour cells (Figure 22E-G) did not proliferate. Compared to the well developed cytoskeleton of actin in controls, PC-3 cells treated for 120 h with 50 µM 5-FdU-5-FdC18 had completely disrupted actin filaments (Figure 22B,F), as visualised by phalloidin-oregon green staining. DAPI-stained PC-3 cells also revealed the presence of apoptotic nuclei with condensed and fragmented DNA in cells treated with 5-FdU-5-FdC18 (Figure 22G).

The morphological changes of PC-3 cells after a 48 h exposure to 5-FdU-5-FdC18 at 50 µM were also assessed by electron microscopy

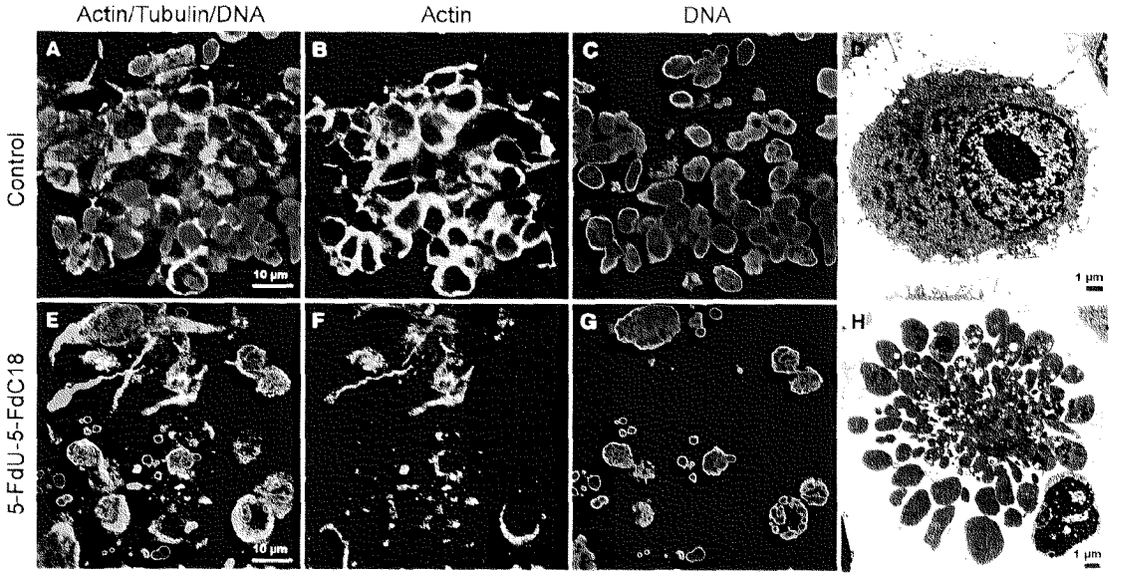

Figure 22
Confocal laser scanning microscopy of collagen embedded PC-3 cells treated with 50 µM 5-FdU-5-FdC18 for 120 h and untreated control cells. The cells were triple-stained for F-actin (green; A-B,E-F), α-tubulin (red; A,E) and cell nuclei (blue; A,C,E,G). Untreated cells are shown in panels A-C and cells treated with 5-FdU-5-FdC18 in E-G. All pictures represent single optical sections. Electron micrographs of an untreated PC-3 cell and a cell treated with 50 µM of 5-FdU-5-FdC18 for 48 h are shown in panels D and H.

(Figure 22D,H). About 10% of the analysed cells displayed the characteristic formation of apoptotic bodies, containing condensed chromatin and intact organelles such as mitochondria and endoplasmatic reticulum. In the cell depicted in Figure 5H the cytoplasm was almost completely fractionated into vesicles. Control cells (Figure 22D) did not display abnormal chromatin condensation or apoptotic bodies. Less than five percent of the control cells had disrupted membranes and organelles, the characteristic features of late apoptosis or necrosis.

3.9 Inhibition of Thymidylate Synthase Activity

The chemotherapeutic effect of 5-FdU is primarily due to the inhibition of TS by 5-FdUMP. Measurement of TS activity in DU-145 and PC-3 cells in situ indicated that all tested dimeric compounds inhibited TS activity in a concentration- and time-dependent manner (Figure 23). DCpam-5-FdU, 5-FdU-5-FdC18 and 5-FdU-NOAC were effective as inhibitors of TS and complete enzyme inhibition was reached after 90 min incubation with 10 µM of the dimers. 5-FdU had the same effect at a concentration of 0.1 µM, whereas NOAC did not alter TS activity. The concentrations at which TS activity was inhibited by 50% are shown in Table 5. The time-dependent analysis showed that 5-FdU induced complete TS inhibition after 30 min. DCpam-5-FdU reached 50% TS inhibition after 3 h, whereas 5-FdU-5-FdC18 and 5-FdU-NOAC required 5, respectively 7 h for the same effect in PC-3 cells (Figure 23D). In DU-145 cells, inhibitory activity of 5-FdU decreased after 5 h, probably due to the dissociation of 5-FdUMP from TS (Figure 23C).

Table 5
50% TS inhibition values in DU-145 and PC-3 cells after incubation for 90 min with 5-FdU, dCpam-5-FdU, 5-FdU-5-FdC18, 5-FdU-NOAC or NOAC.

Drug	50% TS inhibition values (µM)	
	DU-145 cells	PC-3 cells
5-FdU	0.005 ± 0.003 [a]	0.006 ± 0.000
dCpam-5-FdU	0.710 ± 0.085	0.610 ± 0.042
5-FdU-5-FdC18	0.615 ± 0.049	0.670 ± 0.042
5-FdU-NOAC	0.660 ± 0.028	0.640 ± 0.156
NOAC	nr [b]	nr

[a] Mean ± SD of two separate experiments performed in duplicates.
[b] nr = not reached within a drug concentration of 0.01 nM -100 µM

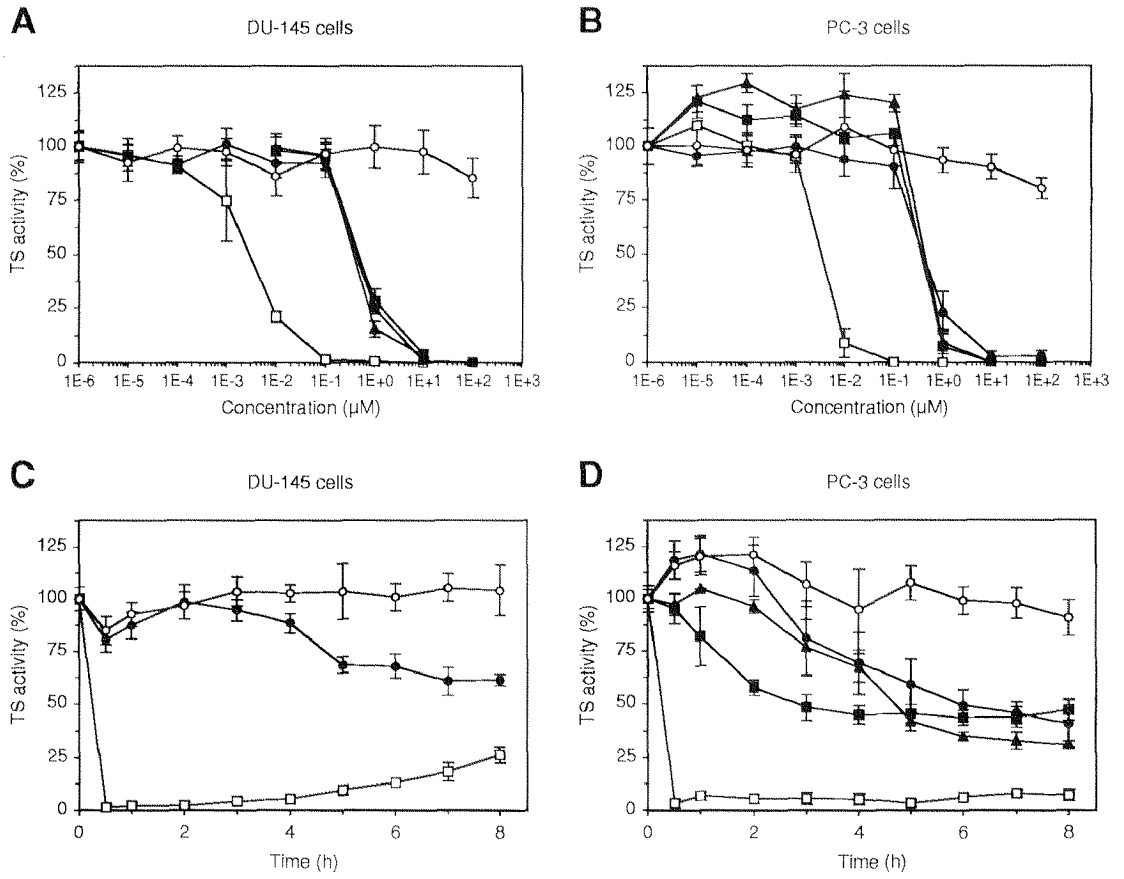

Figure 23

TS enzyme activity of DU-145 and PC-3 cells *in situ*. DU-145 (A) and PC-3 cells (B) were treated with 5-FdU (❑), dCpam-5-FdU (■), 5-FdU-5-FdC18 (▲), 5-FdU-NOAC (●) or NOAC (○) for 90 min at increasing concentrations. Alternatively, DU-145 (C) and PC-3 cells (D) were incubated at 0.1 µM drug for various time periods. TS activity values are shown as percent of untreated control. Data are means ± SD of at least two independent experiments performed in duplicates.

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4 Discussion

Increased proliferation and decreased cell death (apoptosis) are two major processes that contribute to the progression of tumour cell growth. Consequently, agents which can inhibit cell proliferation or induce apoptosis are of important therapeutic value in preventing tumour cell growth (233). In the present study the effect of new phosphate dinucleosides of 5-FdU and NOAC on cell growth and apoptosis in human DU-145 and PC-3 prostate tumour cells was evaluated. The results show that compared to the clinically used drug 5-FdU these novel antitumour agents exert stronger cytotoxicity by drastically arresting the cell cycle and inducing apoptosis.

The antiproliferative effect of 5-FU has previously been associated with therapeutic benefit against different cancers including breast, gastrointestinal, head and neck, and ovarian carcinomas. However, the clinical utility of 5-FU is limited by rapid degradation and the formation of resistance. More than 80% of 5-FU administered by an intravenous or intraarterial route are inactivated by metabolic conversion by dihydropyrimidine dehydrogenase to dihydrofluorouracil. Resistance to 5-FU can develop by deletion of one of the key enzymes required for its activation or by mutations in the p53 gene (58, 59). The new amphiphilic heterodinucleoside phosphate dimers of 5-FdU, the primary metabolite of 5-FU, strongly inhibited cell proliferation in both DU-145 and PC-3 cells in a time- and dose-dependent manner (Figure 12). At a concentration of 12 µM the dimers had cytotoxic effects comparable to the parent drug 5-FdU. While higher concentrations of 5-FdU did not produce additional cytotoxicity, 5-FdU-5-FdC18 and 5-FdU-NOAC at a concentration of 200 µM incubated for 96 h, were capable to induce 100% toxicity, overcoming 5-FdU resistance (Figure 12).

This is in contrast to other new compounds recently found to be cytotoxic for human prostate tumour cells. The nucleoside drug gemcitabine (Figure 5) inhibited cell growth and colony formation of the androgen sensitive prostate tumour cell line LNCaP as well as the androgen insensitive cell lines PC-3 and DU-145 (234). Although colony formation could be suppressed, cell viability was never less than 10%. The synthetic retinoids fenretinide and CD437 induced S-phase arrest and apoptosis in human prostate tumour cells (235, 236). While fenretinide completely inhibited PC-3 cell growth as the dimers did, the synthetic retinoid CD437 did not reach this cytotoxicity even after 6 days of continuous incubation.

Previous findings had revealed impressive efficacy of NOAC in vivo in solid tumour xenografts with PC-3 cancer cells (159). Surprisingly, NOAC did not exert strong cytotoxicity on this prostate tumour cell line

in vitro eradicating only 23-66% of cells in the concentration range tested. This may be explained by a reduced uptake of NOAC containing liposomes in tumour cells in vitro compared to solid tumours in vivo.

5-FdU inhibits cell proliferation by S-phase arrest. Cell cycle arrest is due to TS inhibition (102), single-strand breaks and DNA fragmentation (108). DCpam-5-FdU and 5-FdU-5-FdC18 caused a S-phase arrest similar to 5-FdU in DU-145 and PC-3 cells. The effect of the two dimers was seen within 8 h incubation and it increased after 24 h as demonstrated by flow cytometric analysis (Table 3). After this time point a decrease of cells arrested in S-phase was observed in DU-145 cells. These findings correlate exactly with the results of the Apo 2.7 binding and DNA fragmentation analysis, where an increase in the apoptotic cell population was observed after 24 h (Figures 18A and 19A). Consequently, the decrease in S-phase cells after 24 h was due to induction of apoptosis and subsequent cell fragmentation. In PC-3 cells, where apoptosis was induced after 48 h in parallel with Apo 2.7 and DNA fragmentation studies (Figures 18B and 19B) there was no decrease in S-phase cells after treatment with 5-FdU and 5-FdU-5-FdC18 and 24 h incubation. In DU-145 cells 5-FdU-NOAC had a similar effect as 5-FdU, dCpam-5-FdU and 5-FdU-5-FdC18. However, in PC-3 it caused an increase in G1-phase cells and in S-arrested cells. NOAC treatment resulted in a slight S-phase arrest after 24 h at 50 µM, probably caused by ara-C formed from metabolised NOAC as previously described for NHAC (162).

Apoptosis has emerged as a significant therapeutic target for the effective elimination of cancer cells (94, 237). Many cancer malignancies have a mutated p53 gene which is associated with decreased induction of apoptosis, resulting in chemotherapeutic resistance. In patients with metastatic prostate cancer mutations of this gene are seen more commonly than in those with primary tumours (60). Therefore, the development of new pharmacological agents able to trigger p53-independent apoptosis may be of clinical relevance (64). Endonucleolytic DNA fragmentation and Apo 2.7 studies showed that the dimeric drugs were able to induce apoptosis after incubations of 24 h in DU-145 and after 48 h in PC-3 cells (Figure 18) which are both p53 negative. Simultaneously, the activity of caspase-3 was 3-5 times higher than in untreated control cells or in cells treated with control liposomes (Figure 20). Prolonged exposure of the cells to dCpam-5-FdU and 5-FdU-5-FdC18 resulted in a linear increase of caspase-3 activity up to a 96 h incubation time (6-11-fold increase) in both cell lines, whereas in DU-145 cells 5-FdU induced maximal caspase-3 activity after 72 h (6.5-fold increase), followed by a decline at 96 h (Figure

20A). The apoptotic cell fraction slightly increased after prolonged treatment with 5-FdU in these cells (Figure 18). This was not surprising considering that enhanced caspase-3 activity preceded the increase of the apoptotic cell fraction. Likewise, a decline in enzyme activity was expected to precede a decline of the apoptotic cell fraction. Thus, it can be hypothesised that dCpam-5-FdU and 5-FdU-5FdC18 continued to activate caspase-3, while 5-FdU induced caspase-3 activity was not sustained.

5-FdU-NOAC and NOAC had no inducing activity on caspase-3. Nevertheless, 5-FdU-NOAC had the capability to induce apoptosis in DU-145 and PC-3 cells as shown by DNA laddering and Apo 2.7 antibody binding (Figures 18 and 19). Therefore, apoptosis induced via 5-FdU-NOAC was found to be caspase-3 independent.

In PC-3 cells 5-FdU-5-FdC18 was the most potent apoptosis inducing agent at 50 µM with an 1.6-fold increase of the apoptotic cell population as compared to 5-FdU. Interestingly, 5-FdU, dCpam-5-FdU and 5-FdU-5-FdC18 exhibited comparable toxicities on PC-3 cells after 48 and 96 h incubation (Figure 12D), but only 5-FdU-5-FdC18 induced more than 50% of the cells to undergo apoptosis after 96 h continuous incubation (Figure 18). This finding can be explained with the other drugs possibly inducing more non-apoptotic cell deaths.

Confocal microscopy confirmed the induction of apoptosis after treatment with 5-FdU-5-FdC18 on the morphological level, revealing cells with characteristic apoptotic bodies (Figure 21 and 22). To avoid floating of detached apoptotic cells, cultivated cells were embedded in collagen and cultivated in a three-dimensional culture. Three-dimensional cultures of prostatic and other human cells are increasingly used in preclinical research. These tissue-like structures more realistically model the structural architecture and differentiated function of the human prostate than a cellular monolayer. Thereby, three-dimensional cultures produce an in vivo-like response to therapeutic agents (238). Not only DAPI but also the novel fluorescent dye SYTOX green was used for the detection of apoptotic bodies in collagen-embedded DU-145 cells by confocal microscopy. The use of SYTOX green resulted in superior staining of condensed and fragmented DNA which is present in apoptotic cells. 5-FdU-5-FdC18 induced the formation of apoptotic bodies. Probably as late effect of apoptosis the actin-filaments and microtubules were disrupted. For 5-FdU-5-FdC18 apoptosis was confirmed by electron microscopy in PC-3 cells, where after 48 h exposure about 10% of all cells had condensed chromatin and apoptotic bodies, whereas organelles such as mitochondria and endoplasmatic reticulum were intact.

The overcoming of 5-FdU resistance can possibly be explained by the prodrug nature of the dimers, resulting in persisting intracellular drug concentrations of the monophosphorylated cleavage product over longer time periods compared to 5-FdU. Unlike the parent compound, the active 5-FU nucleotide 5-FdUMP has a prolonged intracellular half life. Although the decay rate varies among different tissues, the continued presence is an important determinant for duration and magnitude of drug effects. In addition, a conceivable reason for the improved potency of 5-FdU-5-FdC18 and 5-FdU-NOAC over 5-FdU and NOAC could be that the molecule contains not only the masked 5-FdU or its monophosphate (5-FdUMP) but also an additional molecule with a cytotoxic activity, namely the 5-FdC18 or the NOAC moiety of the dimer (Table 1). With NOAC, which is similar to 5-FdC18 the hydrophilic metabolites ara-C and ara-U were identified which were formed by metabolic cleavage of the alkyl chain from the parent molecule (163). Thus, it might be possible that in 5-FdU-5-FdC18 the alkyl chain can also be cleaved and, after an additional oxidation reaction, 5-FdU and 5-FdUMP are formed.

The hypothesis, that the dimers are effective prodrugs of 5-FdU is supported by three facts.

Firstly, the dimers exert their cytotoxicity by inhibiting DNA synthesis like 5-FdU, arresting cells in early S-phase.

Secondly, the derivatives specifically inhibited TS activity. Inhibition of cell proliferation through 5-FdU is predominantly due to TS inhibition, followed by thymidine depletion and S-phase arrest (102). 5-FdUMP forms a stable covalent complex with TS, which is slowly dissociable with a half-life of 6 h in intact cells (102). The delayed inhibition of TS through the dimers (Figure 23C-D) further sustains the hypothesis that cleavage of the dimeric drugs into the monophosphorylated molecule 5-FdUMP took place.

Thirdly, the dimers are hydrolysed by phosphodiesterase I and human serum (Cattaneo-Pangrazzi 1999, submitted).

It remains to be investigated whether the dimers are taken up by cells in their unchanged form and whether they are degraded intracellularly to yield active metabolites such as 5-FdU or their corresponding monophosphates. Further studies with the dimers are needed to verify these assumptions including cytotoxicity in *in vivo* experiments.

The comparison of apoptosis induction (Figure 18) with cell cycle distribution (Table 3) and proliferation rates in the time-dependent studies (Figure 12) showed that the inhibition of DNA synthesis and proliferation in DU-145 and PC-3 cells was evident 8 h after exposure to 5-FdU,

dCpam-5-FdU and 5-FdU-5-FdC18. Apoptotic cells, however, appeared only after longer lasting drug exposure of 24 h in DU-145 and of 48 h in PC-3 cells (Figure 18). These findings were in agreement with others who also found a continuous drug exposure to be required to induce apoptosis in DU-145 and PC-3 cells (235, 239). The delayed induction of apoptotic cell death can possibly be explained by these cell lines having low propensity to undergo apoptosis. The p53 tumour suppressor gene has been shown to be an essential component of the apoptotic pathway induced by genotoxic insults (240). Therefore, it can be assumed that the absence of wild-type p53 in DU-145 and PC-3 cells was a contributing factor for the long induction times. Experiments on lymphocytes of p53 deficient mice showed that p53-null thymocytes were resistant to DNA damage-induced apoptosis by etoposide and irradiation, but that their response to glucocorticoids was unaltered by the absence of p53 (241-243). These data imply the existence of p53-dependent and -independent pathways for the induction of apoptosis. In DU-145 and PC-3 cell lines wild-type p53 appears to be ineffective as inducer of apoptosis. It seems rather involved in setting the threshold for apoptosis induction directly or by affecting the transcription of other regulatory apoptosis genes (94, 244).

According to these findings the existence of an 'apostat', a conceptual organelle-like complex in which cellular life or death decisions are made was proposed in a recent review (245). Signals received by the apostat from sensors of cellular damage, such as p53 from DNA damage, as well as signals promoting survival, such as those coming from insulin-like growth factor-1, would be weighed one against the other, ultimately determining the cell's fate. Integral to such an apostat would also be the molecules of the bcl-2 family. Some of them promote survival (bcl-2, bcl-w, bcl-x_L), others promote death by apoptosis (bax, bak, bad) (246). Essentially, the stoichiometry of pro- and anti-apoptotic molecules, perhaps integrated with survival signals together set the threshold for survival of a particular cell type.

In conclusion, the results of this study demonstrate that the new amphiphilic dimers containing 5-FdU are able to overcome 5-FdU resistance in p53 mutated and androgen-independent DU-145 and PC-3 cells. This is an important finding taking into consideration that mutations of p53 and changes in the expression of this gene frequently lead to drug resistance in advanced prostate cancer (60) while resistance to androgen ablation develops eventually in nearly all cases (54). It can be assumed that the dimers are cleaved into 5-FdUMP, resulting in sustained intracellular drug concentration over an extended period and consequently increasing the

duration and magnitude of the cytotoxic effect. This hypothesis is supported by the fact that the new dimers exert a cell cycle phase-dependent cytotoxicity and specifically inhibit TS activity, two mechanisms characteristic for 5-FdU. Furthermore, the dimers are able to induce apoptosis, a process often hindered or suppressed in cancer cells. In summary, findings of the present study suggest the great potential value of the dimers as new therapeutic agents against hormone-refractory prostate carcinomas.

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Appendix

List of Publications and Presentations

Publications

Cattaneo-Pangrazzi RMC, Schott H, Wunderli-Allenspach H, Rothen-Rutishauser B, Günthert M, Schwendener RA (2000) Cell cycle arrest and p53-independent induction of apoptosis by the new anticancer drugs 5-FdU-5-FdC18 and dCpam-5-FdU in DU-145 human prostate cancer cells. Journal of Cancer Research and Clinical Oncology. In press

Cattaneo-Pangrazzi RMC, Schott H, Wunderli-Allenspach H, Derighetti MI, Schwendener RA (2000) New heterodinucleoside phosphate dimers of 5-Fluorodeoxyuridine induce cell cycle dependent cytotoxicity and apoptosis in human prostate PC-3 cells. Submitted to Biochemical Pharmacology

Cattaneo-Pangrazzi RMC, Schott H, Schwendener RA (2000) Cellular pharmacology of the novel antitumor drug 5-FdU-NOAC in human prostate cancer cells. Submitted to The Prostate

Horber DH, Cattaneo-Pangrazzi RMC, von Ballmoos P, Schott H, Ludwig PS, Eriksson S, Schwendener RA (2000) Cytotoxicity, cell cycle perturbations and apoptosis in human tumour cells by lipophilic N^4-alkyl-1-ß-D-arabinofuranosylcytosine derivatives and the new heteronucleoside phosphate dimer arabinocytidylyl-(5'→5')-N^4-octadecyl-1-ß-D-ara-C. Journal of Cancer Research and Clinical Oncology. In press

Schlosser V, Koechli OR, Cattaneo RMC, Jentsch B, Haller U, Walt H (1999) Photodynamic effects in vitro in fresh gynecologic tumors analyzed with a bioluminescence method. *Clin Chem Lab Med* **37**: 115-120

Poster Presentations

Cattaneo-Pangrazzi RMC, Schott H, Wunderli-Allenspach H, Schwendener RA (1999) New amphiphilic heterodinucleoside phosphates of 5-FdU: cytotoxicity, induction of apoptosis and cell cycle arrest. AEK-Symposium. Heidelberg (BRD). *J Cancer Res Clin Oncology* **5125**: 580

Cattaneo-Pangrazzi RMC, Schott H, Wunderli-Allenspach H, Schwendener RA (1998) New dinucleoside Dimers of 5-FdU and NOAC: Cell cycle dependent cytotoxicity and induction of apoptosis. GPEN98. Zurich

Pangrazzi RMC, Schwendener RA, Schenk V, Haller U, Köchli OR (1996) Untersuchung der Wirkung von N^4-octadecyl-1-ß-D-Arabinofuranosylcytosin (NOAC) auf Ovarialkarzinome mit dem ATP-Zytotoxizitätstest. Jahreskongress der Schweizerischen Gesellschaft für Gynäkologie und Geburtshilfe. Interlaken

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Cattaneo-Pangrazzi RMC (1996) Pharmakologische Untersuchung des neuen Zytostatikums N^4-octadecyl-1-ß-D-Arabinofuranosylcytosin (NOAC) an humanen Tumorzellen. Abteilung für Biopharmazie, Departement Pharmazie, ETH Zürich

Pangrazzi RMC (1993) Untersuchung über die kausalen Beziehungen zwischen dem Kationengehalt der Nematocysten einerseits und deren Funktionalität andererseits. Diplomarbeit. Zoologisches Institut, Universität Zürich

Oral Presentations

Zelluläre Pharmakologie von neuen Dimerverbindungen von N^4-octadecyl-1-ß-D-Arabinofuranosylcytosin (NOAC) und 5-Fluorodeoxyuridine (5-FdU) an humanen Prostata-Tumorzellen (1998). Doktorandentag des Departements Pharmazie, ETH Zürich

Curriculum vitae

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Citizen of Zurich and Italy

1975-1981	Primary School, Zurich
1981-1988	Gymnasium, Zurich
1988	Graduation diploma (Matura Typ B)
1988-1993	Studies in Zoology and Molecular Biology at the University of Zurich
1993	Diploma in Zoology (dipl. zool.)
1994	Round-the-world journey
1995-1996	Scientific assistant in the Chemosensitivity Laboratory at the University Hospital of Zurich
1996-1999	Ph. D. student at the Division of Cancer Research, Department of Pathology, University Hospital Zurich under the guidance of Prof. Dr. R.A. Schwendener
1999	Examination to obtain the degree of Doctor of Natural Sciences, Swiss Federal Institute of Technology, ETH Zurich, Switzerland