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ESTRAMUSTINE AND ITS DERIVATIVES IN POTENTIATING RADIOTHERAPY OF PROSTATE CANCER

by

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Academic Dissertation

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals I–IV:

I Ståhlberg K, Kairemo K, Karonen S-L, Jekunen A, Taari K and Rannikko S. Radio iodinated estramustine phosphate and estramustine binding protein antibody accumulate in the prostate of a mouse. *Prostate* 1997; 32(1): 1–8.

II Ståhlberg K, Kairemo K, Karonen S-L, Taari K and Rannikko S. Distribution of radio iodinated estramustine binding protein antibody in mice with DU-145 prostate cancer xenograft. *Anticancer Res* 2007; 27: 2275–2278.

III Ståhlberg K, Kairemo K, Erkkilä K, Pentikäinen V, Sorvari P, Taari K, Dunkel, L and Rannikko S. Radiation sensitizing effect of estramustine is not dependent on apoptosis. *Anticancer Res* 2005; 25(4): 2873–2878.

IV Ståhlberg K, Kämäräinen E-L, Keyriläinen J, Virtanen I, Taari K and Kairemo K. Hypoxia in DU-145 prostate cancer xenografts after estramustine phosphate and radiotherapy. *Current Radiopharmaceuticals* 2010; 3: 297–303.

Article IV has also appeared in the following academic dissertation by Eeva-Liisa Kämäräinen:

F-18 Labelling Synthesis, Radioanalysis and Evaluation of A Dopamine Transporter and A Hypoxia Tracer. University of Helsinki, Faculty of Science, Department of Chemistry, 2006.

2. ABBREVIATIONS

ANOVA	analysis of variance
BT4C	rat glioma model
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DU 145	a human prostate cancer cell line
EM	estramustine
EMBP	estramustine binding protein
EMBP-AB	antibody against estramustine binding protein
EMP	estramustine phosphate
FITC	fluorescein isothiocyanate
FMISO	fluoromisonidazole
G2/M	a phase of cell cycle
HSF	hypoxia-specific factor
MAP	microtubulus-associated protein
NOR	Nitrogen ohne Radikal
p53	nuclear phosphoprotein p 53
PET	positron emission tomography
PMMA	polymethylmethacrylate
RBE	relative biological efficiency
R3327	a rat prostate cancer model
RI	radioiodine
RI-EMBP-AB	Radioiodinated estramustine binding protein antibody
RI-EMP	Radioiodinated estramustine phosphate
VEGF	vascular endothelial growth factor

3. ABSTRACT

Estramustine, a cytostatic drug used for treating advanced cancer of the prostate, has been shown to inhibit prostate cancer progression and also to increase the sensitivity of cancer cells to radiotherapy. The goals of this study were, first, to find out whether it is possible to use either estramustine or an antibody against estramustine binding protein as carrier molecules for bringing therapeutic radioisotopes into prostate cancer cells, and, secondly, to gain more understanding of the mechanisms behind the known radiosensitising effect of estramustine.

Estramustine and estramustine binding protein antibody were labelled with iodine-125 to study the biodistribution of these substances in mice. In the first experiment, both of the substances accumulated in the prostate, but radioiodinated estramustine also showed affinity to the liver and the lungs. Since the radiolabelled antibody was found out to accumulate more selectively to the prostate, we studied its biodistribution in nude mice with DU-145 human prostate cancer implants. In this experiment, the prostate and the tumour accumulated more radioactivity than other organs, but we concluded that the difference in the dose of radiation compared to other organs was not sufficient for the radioiodinated antibody to be advocated as a carrier molecule for treating prostate cancer.

Mice with similar DU-145 prostate cancer implants were then treated with estramustine and external beam irradiation, with and without neoadjuvant estramustine treatment. The tumours responded to the treatment as expected, showing the radiation potentiating effect of estramustine. In the third experiment, this effect was found without an increase in the amount of apoptosis in the tumour cells, despite previous suggestions to the contrary. In the fourth

experiment, we gave a similar treatment to the mice with DU-145 tumours. A reduction in proliferation was found in the groups treated with radiotherapy, and an increased amount of tumour hypoxia and tumour necrosis in the group treated with both neoadjuvant estramustine and radiation. This finding is contradictory to the suggestion that the radiation sensitising effect of estramustine could be attributed to its angiogenic activity.

4. INTRODUCTION

The purpose of this study was to deepen our knowledge of the combined use of estramustine (EM) and radiotherapy for the treatment of prostate cancer. Prostate cancer is a common disease, with a high variability between subjects in its malignant potential. In many cases, the disease is an incidental finding with little or no clinical significance, because the slow progression of the tumour might not have caused any symptoms, nor mortality. In other cases, however, prostate cancer may be an aggressive malignant disease, which, if the initial treatment fails, lacks an effective cure and may lead to severe symptoms, metastasis, and death despite all treatment. In many cases, the methods of treatment available at the moment provide cure or significant regression of symptoms, but often at the cost of considerable side effects.

Research on the treatment of prostate cancer may be considered to have three somewhat differing goals, corresponding to the different types or stages of the disease:

1. To develop diagnostic tools for risk assessment of newly diagnosed prostate cancer in order to differentiate between high risk patients that benefit from active treatment in spite of its side effects, and low risk patients with whom refraining from treatment may be a better option, despite the risk of progressive disease.
2. To improve or supplement existing treatment modalities for the more aggressive prostate cancer, in order to improve disease control and survival and diminish side effects.
3. To develop treatment modalities for the advanced cases, where no effective treatment is available at the moment.

This is a study of one specific medical substance used in the treatment of prostate cancer, estramustine. This study was initiated primarily to look into the possibility of developing a targeting system for bringing a molecular radiation source into the substance of prostate cancer, and secondarily to give more insight into the mechanism of the known radiation sensitising effect of estramustine. The targeting system as well as radiosensitization could potentially be utilized in all types and stages of the disease mentioned above.

The effectiveness and tolerability of molecular radioisotope treatment relies on targeting: the isotope must be retained in the organ to be treated, or, preferably, incorporated into the tumour cells, as close to nuclear DNA as possible. Thus, the development of suitable carrier molecules to take the isotopes selectively into the cancer cells is crucial. Both antibodies and other substances that have specific affinity to cancer cell targets have shown promising results *in vitro* and even in clinical trials.

The radiosensitising effect of estramustine has been shown in many experimental studies and has been the subject of clinical trials as well. A considerable amount of research data is available on the mechanisms of the effect. This study adds to our knowledge on the subject, focusing on some specific, somewhat controversial issues.

5. REVIEW OF THE LITERATURE

The following review will first briefly describe the prostate gland and the concept and treatment of cancer in general, then provide some more specific information on prostate cancer and its treatment options. Thereafter, the focus will be on EM and radiotherapy, and the radiosensitising effect of EM.

5.1 The prostate gland – anatomy and function

The prostate is an exocrine gland found only in the male. About four centimetres in diameter, it is the largest accessory gland of the male reproductive tract. The prostate is partly glandular and partly fibromuscular and is surrounded by a dense fascial sheath. The prostate is somewhat conical, with the base upwards and four surfaces (anterior, posterior, and two inferolateral surfaces) and the apex downwards. The lower part of the prostate faces the urogenital diaphragm, the urethral sphincter and levator ani muscles. The base is related to the neck of the urinary bladder, and the urethra enters the prostate in the base, near the anterior surface, and exits in the apex. The two ejaculatory ducts pass through the substance of the prostate to open into the prostatic urethra. The ejaculatory duct is formed by the union of the ductus deferens and the duct of the seminal vesicle on each side. The prostatic secretion is discharged by smooth muscle contraction into the urethra through 20 to 30 prostatic ducts that open into sinuses on each side of the posterior wall of prostatic urethra. The prostatic fluid constitutes up to one third of the semen. [1]

The prostate is small at birth but enlarges at puberty. In most males it starts to enlarge in middle age, often leading to benign prostatic hyperplasia, which is a common cause of urinary obstruction. Cancer of the prostate gland is one of the

most common malignant tumours in men, microscopically detectable in about 60% of men over 80 years of age. [1]

5.2 Cancer

Cancer is a generic term for a group of more than 100 diseases that can affect any part of the body. The defining feature of cancer is the rapid creation of abnormal cells which grow beyond their usual boundaries, and which can invade adjoining parts of the body and spread to other organs. The direct infiltration of cancer cells into adjacent tissue is referred to as invasion, and distinct tumours that arise from seeding of cancer cells via blood or lymphatic vessels are known as metastases. [2]

Development of cancer

The transformation from a normal cell into a malignant cell (cancer cell) is a multistage process, typically a progression from a pre-cancerous lesion to malignant tumours. The development of cancer may be initiated by external and genetic factors. The external factors include physical carcinogens such as radiation, chemical carcinogens such as tobacco smoke and biological carcinogens such as certain viral infections or microbial toxins. Ageing is also a factor in the development of cancer. The incidence of cancer rises dramatically with age, most likely due to risk accumulation over the life course combined with the tendency for cellular repair mechanisms to be less effective as a person grows older. Another distinct risk factor is lack of physical activity. [2]

Cancer mortality

Metastases are the major cause of death from cancer; the primary tumour, often causing local and systemic symptoms, is a less common cause of mortality. From a total of 58 million deaths worldwide in 2005, cancer accounts for 7.6 million (13%). The main types of cancer leading to overall cancer mortality among women are (in order of number of global deaths): breast, lung, stomach, colorectal and cervical cancer, and among men: lung, stomach, liver, colorectal, oesophagus and prostate cancer. [2]

Treatment of cancer

The four major types of treatment for cancer are surgery, radiotherapy, chemotherapy, and biologic therapies (according to [3]):

Surgery may aim to radical or marginal excision of malignant tissue, but sometimes it is only possible to reduce tumour mass. Another important indication for surgery is to obtain tissue samples to establish the diagnosis and characterize the tumour, and to define the stage of the disease. A third indication is the palliation of symptoms caused by incurable cancer.

Radiotherapy means treating disorders by submitting the affected tissue to ionising radiation. Most often it is used in combination with surgery to improve local control of the tumour, especially when the excision margins are not secure or when it has not been possible or desirable to remove the entire organ or compartment containing the tumour. In some cancers, radiotherapy alone may achieve cure or long-term remission and in others it is used in combination with other treatments.

Chemotherapy refers to treatment by medication. In some cancers, total cure or long remission can be achieved by chemotherapy alone. In others, it is used to

decrease the likelihood of recurrence or spreading after surgery or radiation (adjuvant chemotherapy), to make radiotherapy more effective (concurrent chemotherapy) or to shrink large tumours to operable size (neoadjuvant chemotherapy). In incurable or recurrent cancers chemotherapy may slow the growth and alleviate symptoms (palliative chemotherapy).

Biologic therapy is sometimes called immunotherapy, biotherapy, or biological response modifier therapy. Biologic therapies use the body's immune system to fight cancer or to lessen the side effects of some cancer treatments. [3] Treatments utilizing antibodies may also be included in this category.

5.3 Carcinoma of the prostate

Risk factors

About 80% of cases of prostate cancer occur in men over the age of 65. The prevalence of subclinical prostate cancer is high at all ages, but the lifetime risk of dying of prostate cancer is only 3%.

Prostate cancer is more common in black men and those with a first-degree relative who has had prostate cancer. Obesity may increase mortality, and high intake of dairy products and calcium as well as red meat may slightly increase the risk of prostate cancer. Testosterone replacement therapy is considered a potential risk. [4, 5]

Diagnosis and prediction of progression

Patients with urinary symptoms or abnormal blood prostate specific antigen (PSA) levels are referred to examinations, which can lead to diagnosis of prostate cancer. Clinical findings may include a prostatic mass in rectal

examination or in transrectal ultrasonography. Multiple tissue samples are obtained under transrectal ultrasound guidance. The aggressiveness of prostate cancer varies from indolent, not requiring treatment, to aggressive, which may progress and metastasise despite treatment. The risk of progressive disease is estimated from tumour volume, aggressiveness, and extent of cancer. The primary measure of aggressiveness is the Gleason histological score: tumours scored 8-10 are considered the most aggressive, while those with scores under 6 are potentially indolent. [3, 6, 7]

The risk of progression, or recurrence after treatment, may be predicted in many ways, for example:

Low risk:	PSA <10 ng/ml, Gleason score <6, and clinical stage T1c or T2a
Intermediate risk:	PSA > 10–20 ng/ml, Gleason score 7, or clinical stage T2b
High risk:	PSA > 20 ng/ml, Gleason score 8–10, or clinical stage T2c

The percentage of cancer positive core biopsies is considered an important risk factor, and various biochemical markers are being advocated as important tools in the assessment of the malignant potential of prostate cancer [8].

Current treatment options of prostate cancer

The choice of treatment is based on the risk estimate, as well as other factors, such as patient preference, age and comorbidity. Radical prostatectomy is the most common curative treatment [5, 9]. The options include:

Watchful waiting / active surveillance, an active plan to postpone intervention of localized prostate cancer. The disease is monitored with digital rectal examination, ultrasonography, prostate biopsies and/or PSA blood tests. Active treatment is begun based on patient preference, symptoms, and clinical findings. The regimen causes no immediate side effects and has a low initial cost. Most patients, especially of low to intermediate risk, do not need other treatment and survive at least 10 years. The potential risks include the advancement of cancer, progression into an incurable disease, and patient anxiety. [3, 4, 6]

Radical prostatectomy, the complete surgical removal of prostate gland with seminal vesicles, ampulla of vas, and sometimes pelvic lymph nodes. The operation may be done retropubically, perineally or laparoscopically. Efforts are made to preserve the nerves for erectile function. Radical surgery may eliminate cancer in some but not all cases. According to a randomised controlled trial, mortality from prostate cancer and metastasis is reduced compared with watchful waiting [10]. The operation is generally well tolerated. However, it requires hospitalisation and bears the risks of major surgery: perioperative death, cardiovascular complications, bleeding, urinary incontinence, urethral stricture, bladder neck contracture, and bowel and erectile dysfunction. In recent years, technological advances such as laparoscopy and robotic assistance have gained more popularity and may potentially improve the surgical outcome. [3, 11-13]

External beam radiation therapy, ionising radiation from an external source applied in multiple doses over several weeks. Conformal radiotherapy uses three-dimensional planning systems to maximise dose to prostate cancer and to minimise it on adjacent tissue. In modern practice, external beam radiation is delivered by intensity-modulated radiotherapy (IMRT) or image guided radiotherapy (IGRT) [14].

In some cases, external radiation may eliminate cancer. It is generally well tolerated. However, it does not remove the prostate gland and may not eradicate the cancer. The risk profile is somewhat more benign than with surgical treatment: incontinence, proctitis, diarrhoea, cystitis, erectile dysfunction, urethral stricture, bladder neck contracture, and rectal bleeding are the most common adverse effects, but their incidence has diminished with the new methods of delivery of radiation. Five to eight weeks of outpatient therapy is needed. Radiotherapy is contraindicated in the presence of an inflammatory bowel disease because of risk of bowel injury. [3, 15, 16]

Brachytherapy is administered by temporarily placing radioactive implants into or in the proximity of the target tissue under anaesthesia using radiological guidance. External beam “boost” radiotherapy or androgen deprivation is sometimes recommended in combination. This treatment may also eliminate cancer and is generally well tolerated. It only requires a single outpatient session. As in external beam radiotherapy, it does not remove prostate gland and may not eradicate cancer. It may not be effective for larger prostate glands or more aggressive tumours and it is contraindicated in patients with prior transurethral resection of the prostate. The possible side effects include urinary retention, incontinence, impotence, cystitis or urethritis, and proctitis. [3, 8, 17]

Androgen deprivation can be achieved either by oral or injected drugs or surgical removal of testicles to lower or block circulating androgens. The operation is smaller and avoids most of the risks of prostatectomy and radiotherapy. It typically lowers PSA levels and may slow cancer progression but does not remove the prostate and may not eradicate cancer. Side effects include gynaecomastia, impotence, diarrhoea, osteoporosis, lost libido, hot flushes, and “androgen deprivation syndrome” (depression, memory difficulties and fatigue). [3, 19, 19]

Chemotherapy, the use of cytostatic drugs in the treatment of prostate cancer, is mainly useful in the advanced stages of the disease [20]. Clinical trials have shown that docetaxel alone or in combination with estramustine improves the survival of patients with metastatic androgen independent prostate cancer [21, 22]. Docetaxel is a taxane group cytostat. Estramustine alone has not been shown to improve survival. Other chemotherapeutic agents that are being investigated include vinca alkaloids (such as vinblastine and vinorelbine) and epothilones (such as ixabepilone and patupilone), but they have not improved survival. The effect of all of the above medications is based on anti-microtubule activity. [20]

Cryoablation means destruction of cells through rapid freezing and thawing using transrectal guided placement of probes and injection of freezing and thawing gases into the prostate. In some cases, this may eliminate the cancer. It is generally well tolerated, avoids some of the operative risks and only requires a single outpatient session. However, it does not remove prostate gland and may not eradicate cancer, and has the risk of impotence, incontinence, scrotal oedema, pelvic pain, sloughed urethral tissue, prostatic abscess, urethrorectal fistula. No large long-term outcome reports are available on this treatment. [3, 23, 24]

5.4 Estramustine

Estramustine phosphate (EMP), *Estra-1,3,5(10)-triene-3,17-diol(17 β)-3[bis(2-chloroethyl)carbamate]17-(dihydrogen-phosphate), disodium salt, hydrate*, is a cytostatic pharmaceutical that has long been used for treatment of advanced, hormone refractory prostate cancer. Until recently, it was used as a second line

treatment against metastatic or advanced prostate cancer that did not respond to hormonal treatment or that had developed resistance to hormonal treatment after an initial response. [25-29] During the last decade EM has largely been replaced by docetaxel [30]. EM has also been used in clinical trials against malignant brain tumours [31-34]. In clinical experiments it is often used in combination with other chemotherapeutic agents, such as vinblastine and docetaxel [35-44], or with radiotherapy [31, 45]. In recent studies, treatment of hormone refractory prostate cancer with the combination of docetaxel and estramustine resulted in statistically significantly prolonged survival [21, 46]. The side effects of EM treatment include thromboembolic events, nausea, oedema, impotence and gynaecomastia [20, 47].

Structure

Estramustine is a NOR-nitrogen mustard derivative of estradiol-17 β [48]. Estradiol-17-beta was earlier considered a potential hormonal treatment option for prostate cancer but it was later replaced by more complex oestrogen derivatives [49-51]. NOR-nitrogen mustard is used as an alkylating agent in chemical industry. It has been found to cause genomic damage, more effectively than nitrogen mustard does. [52, 53] NOR means *Nitrogen ohne Radikal*, nitrogen without methyl radical $-\text{CH}_3$ [54].

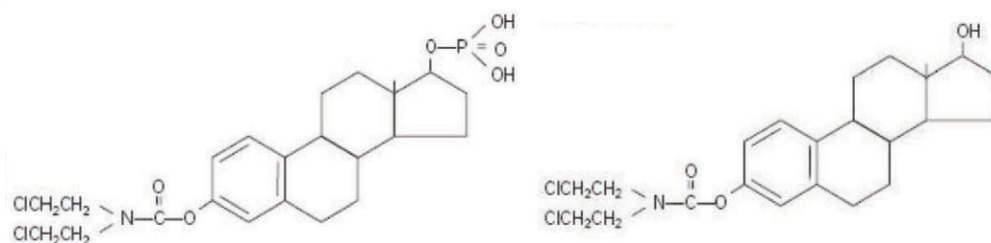


Figure 1. Structure of estramustine phosphate (left) and estramustine (right).

Mechanism of anti-tumour effect

Despite the presence of an alkylating agent and an oestrogen in EM, the anti tumour effect of EM is not based on alkylating or steroid (hormonal) activity [55]. It is believed to be due to direct and specific binding to tubulin, causing depolymerization of microtubules at high concentrations and, perhaps more importantly, stabilization of microtubule dynamics at much lower concentrations. Microtubules are responsible for the separation of the divided chromosomes in mitosis, and the impaired function of microtubules causes mitotic arrest at the transition from metaphase to anaphase (G2/M). The antimitotic effect of EM is reversible and it is not associated with genomic damage. Mitotic arrest may lead to cell death by apoptosis. Apoptosis may be an important mediator in the anti tumour action of EM. EM has been shown to cause low molecular weight (<1000 bp) DNA fragmentation and the morphological changes typical to apoptosis in glioma cells, in human gliomas and in prostate cancer cells. [34, 56-60]

Other modes of action have been suggested, among them interaction with microtubulus-associated protein (MAP) [61-64] and inhibition of invasion by suppression of matrix metalloproteinase-2 and collagenase activity [65-67]. Both these actions and the induction of apoptosis may be consequences of the action on microtubules or MAP.

Pharmacological properties

Estramustine is administered perorally or intravenously as the water-soluble compound estramustine phosphate (EMP). EM is phosphorylated in the 17-beta position. EMP has no effect on cultured tumour cells *in vitro*, but *in vivo* it is rapidly dephosphorylated and hydroxylated into estramustine (EM) and further oxidized into estromustine [68-70], which has a dose-dependent antimitotic and antiproliferative effect on malignant cells. Such an effect has been described *in*

vitro and *in vivo* on many different cancer cell cultures, including prostate cancer [48, 71-76], glioblastoma [77-79], renal cell carcinoma [80], colon cancer [81-83] and breast cancer [84]. Estromustine, the predominant metabolite found in the circulation, is then metabolised into nitrogen mustard, estrone and estradiol and they are degraded and excreted so that prolonged use of EM does not lead to long-term accumulation of any of the substances [65, 66].

5.5 Radiosensitising effect of estramustine

EM has been found to enhance the effect of irradiation *in vitro* on a variety of malignant cells, including prostate cancer (DU 145), breast cancer (MCF7), glioma (U-251, BT4C) and renal cell cancer (A498, CAKI 2) [80, 85-89]. *In vivo* experiments with DU-145 human prostate cancer cell xenografts implanted in nude mice, as well as those with Dunning R3327 prostate cancer and BT4C glioma cell xenografts in rats, have shown an increased sensitivity to irradiation when subjected to EM as compared to irradiation or EM therapy only [86, 89-91]. In the 1994 experiment by Solveig Eklöv *et al.*, DU 145 xenografts in nude mice responded with decreased tumour volume and increased necrotic content to combined treatment with estramustine and irradiation. Tumour growth was unaffected by estramustine alone and the radiation effect was statistically significantly higher than after radiation alone in tumour growth curves. The results were similar in the other studies with the above mentioned cell lines. Certain other malignant cells, including colon cancer (HT 29) and cervical cancer (HeLa S3) did not show significant radiosensitizing effect [88].

The radiosensitizing effect of estramustine, often combined with other chemotherapeutic agents, has been used in clinical trials in different settings for

the treatment of hormone refractory prostate cancer and glioma, often with promising results. [31, 32, 45, 92, 93]

5.6 Estramustine binding protein

One reason for the suitability of EM for treating prostate cancer is its tendency to accumulate in the prostate and prostate cancer. The reason for this is that EM binds not only to microtubules, but also to a protein called estramustine binding protein (EMBP). EMBP is found in high concentrations in cytoplasmic vesicles of epithelial cells of the prostate, constituting 18% of total protein in the cytosol of the rat ventral prostate epithelium. [94-96] It has also been detected in several other organs, including cerebral cortex, salivary glands, the thyroid gland, adrenal glands, seminal vesicles, epididymis, pancreas and kidney [94, 97] and in some, but not all, malignant cells including prostate cancer, breast cancer, melanoma, colon cancer, glioma, non-small cell lung cancer and renal cell cancer [82, 98-103].

The biological function of EMBP is not fully understood, but it is a secretory protein of the prostate and may have an immunosuppressive function [97, 98]. EMBP has a proteolytic effect on MAP, but not on tubulin [98]. The NOR-nitrogen mustard component of EM is a necessity in binding to EMBP; estradiol and other steroids do not bind to the protein [95].

In the prostate, the concentration of EMBP is higher in the epithelium than in the stroma, and it is higher in benign prostatic hyperplasia than in prostate cancer [104, 105]. The appearance of EMBP in a tumour is not dependent on androgen or estrogen effect, and is not related to morphology or growth rate of

the tumour, but may be associated with androgen responsivity, androgen receptor content, anaplasia and metastatic potential [61, 106].

Estramustine binding protein antibody

Antibodies against EMBP (EMBP-AB) accumulate in tissue that contains EMBP, and have been used to detect EMBP *in vitro* and *in vivo* [98, 103, 107].

5.7 Radiotherapy

Radiotherapy means treating disorders by submitting the affected tissue to ionising radiation.

Ionising radiation

Radiation may be classified as directly or indirectly ionising.

Directly ionising radiation includes neutrons and charged particles, such as electrons, protons, alpha particles (helium nucleus: two protons and two neutrons), mesons and heavy ions (nuclei of nitrogen, carbon, neon, argon *etc.*). Provided that the above-mentioned particles have sufficient kinetic energy, they can directly disrupt the atomic structure of the substance, which they traverse, and produce chemical and biological changes. Electromagnetic radiation (x-rays, gamma rays) is ***indirectly ionising***. When absorbed to the substance they traverse, they give up energy to produce fast-moving charged particles, which in turn ionise other atoms of the absorbing substance and break chemical bonds. [108]

Biological effects of radiation

Directly and indirectly ionising radiation is not to be confused with direct and indirect actions of radiation in the cell.

Direct action is radiation damage caused directly to DNA, or another vital structure such as the cell membrane, by any form of directly or indirectly ionising radiation. It is the dominant process when using neutrons or alpha particles. *Indirect action* of radiation means that radiation interacts with other atoms or molecules in the cell, usually water, to produce free radicals, which are able to diffuse to critical targets. A free radical is an atom or a molecule carrying an unpaired orbital electron. An unpaired, or odd, orbital electron makes the atom highly reactive, enabling it to damage DNA by interacting with it. [108]

5.8 Radiotherapy of prostate cancer with radioactive isotopes

Iodine-125 is used as brachytherapy against prostate cancer. The long half-life of the isotope and the slow progression rate of the disease may be a cause for its clinical efficacy. Other advantages of a local iodine-125 radiation source are localized dose distribution, reduced development of radioresistance and reduced repopulation. [109-111] The Auger-electron radiation of I-125 has a short range. It is most effective when incorporated into DNA; this increases the biological effect of radiation by a factor of about ten as compared with extracellular radiation. The medium-energy beta-emitter I-131 has a longer range and more capacity to penetrate tissue, and the effect is little enhanced by proximity to DNA. [112, 113] Iodine-131 has been used in interesting experiments using, in a clinical trial, an antibody [114] or, in a xenograft model, a replication-defective adenovirus expressing the rat NIS gene (Ad-rNIS) [115, 116] as a vector to get therapeutic doses of I-131 into prostate cancer cells. In clinical use, systemically

administered radiolabelled antibodies have so far only proved useful in the treatment of haematological malignancies but not solid tumours [117-120].

Study of radioisotope treatment of prostate cancer is mainly focused on palliative treatment of painful bone metastases. Current treatment options include Re-186-HEDP (Rhenium 153 hydroxyethylidene diphosphonate) [121-123], Sm-153-EDTMP (Samarium 153 ethylenediaminetetramethylene phosphonic acid), Ho-166 DOTMP (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethylenephosphonate) [124-126], and Sr-89 (Strontium 89) [127-129]. These isotopes with the relevant carrier molecules (or, in the case of Strontium, Strontium chloride) have given good results in terms of pain palliation and tolerable side effects.

5.9 Radiosensitization by estramustine

There are a number of chemical agents that have been shown to increase the effectiveness of radiotherapy. The following paragraphs describe some of the key concepts of radiosensitization in relation to the mechanisms of radiosensitization by EM.

Radiosensitivity and cell cycle

The cell cycle of mammalian cells can be divided into phases:

G₀, cells that are out of the cell cycle, not dividing

G₁ (gap 1), the first phase after mitosis, when DNA is not being synthesized. The duration of this phase varies and causes the variation between the length of the cell cycles of different types of cell.

S (synthesis), the phase during which DNA is synthesized to reduplicate it.

G2 (gap 2), in which reduplicated DNA is segregated and condensed.

M (mitosis), the separation of the reduplicated DNA and other cell structures and division of the cell into two identical cells, which enter the G1 phase.

The sensitivity of cells to radiation varies during the cell cycle as follows:

G0: low sensitivity.

G1: sensitivity at the lowest in the beginning, then increases towards the end if duration of G1 is long enough.

S: fairly sensitive in the beginning, then decreases towards the end.

G2: low sensitivity at the beginning, increases towards the end, reaches maximum at transition to M.

M: sensitivity at the highest in transition from G2 to M, decreases towards the end of M.

The steepness of the radiation dose-response curve in G2/M is about 2,5 times that in late S. The reason for this difference is not entirely known. [108]

The principal mechanism of the radiosensitizing effect of EM is the anti-microtubule action of EM, causing the cell cycle to arrest in the G2/M phase, while cells in this phase are most sensitive to irradiation [130]. Treatment of prostate cancer cells with estramustine resulted in 95% of cells being in G2/M state (arrested metaphase with contracted chromosomes not aligned in the metaphase plane), and with no cells in anaphase [71]. The importance of this mechanism is underlined by the finding that cell lines that do not react to EM by arresting in metaphase are not sensitised to irradiation [88].

Hypoxia in malignant tumours

Solid malignant tumours are often hypoxic. Especially the core of the tumour may be too far from normally functioning blood vessels to receive adequate amounts of oxygen. Radiotherapy tends to further increase hypoxia by causing damage to microvasculature, although this effect may only be transitory due to the process of reoxygenation, which means improved oxygenation in the remaining hypoxic cancer cells after the successful deletion of well oxygenated cells by radiotherapy. Tumour hypoxia is a consequence of the way malignant tumours arise, whether primary or metastatic: they grow from a single, mutated normal cell into a solid tumour and, once their size exceeds the distance that oxygen is able to diffuse through their substance (about 100 μm), have to develop their own blood supply. They do this by stimulating the growth of cells from surrounding vessels into the tumour, a process known as angiogenesis. However, these newly formed blood vessels are irregular and tortuous, have arteriovenous shunts and blind ends, lack smooth muscle or nerves and have incomplete endothelium and basement membrane. As a result, blood flow is slow and irregular and the level of oxygen in most tumour cells is lower than in normal tissue. Hypoxic cells are less sensitive to irradiation because oxygen molecules, if present, react rapidly with free-radical damage produced by ionising radiation in the DNA, thereby making the damage irreversible and leading to cell death. [131]

Some investigators suggest the induction of release of free oxygen radicals by EM as a mechanism of radiosensitisation [83, 89]. Another possible mechanism is increased tumour blood flow, shown in Dunning R 3327 rat prostate carcinoma model [91] and in BT4C rat glioma model [57]. EMP did not affect blood flow of normal brain tissue but increased the flow in the glioma. Increased tumour blood flow may relieve tumour hypoxia and thus potentiate the effect of irradiation.

Role of apoptosis

Some of the DNA damage caused by irradiation may be repaired during the cell cycle, but if genomic damage persists, the cell cycle is arrested by suppressor genes. This, by mechanisms not known in full detail, leads to programmed cell death, apoptosis. The p53 suppressor gene is the most commonly mutated gene in human malignancies, leading to a defect in cell cycle control and decreased radiosensitivity of tumour cells. [132-134] However, other factors are involved, and radiation-induced apoptosis in prostate cancer cells is not dependent on over expression of mutant p53 [131].

Apoptosis might also have a role in radiosensitisation by EM, either by EM modulating the mechanisms that lead to apoptosis after irradiation, or by it having a direct additive effect. EM has been shown to cause apoptosis in cancer cells [34, 56-60]. It induced apoptosis by causing early DNA damage in glioma cells but not in normal brain tissue [34]. DNA damage has not been observed in all studies. However, mitotic arrest leads to apoptotic cell death even in the absence of direct damage to DNA [135-137].

6. AIMS OF THE STUDY

This study was based on certain special features of EMP: its ability to accumulate in the prostate and in cancer cells, the radiation sensitising effect, and the ability to cause apoptosis. Initially, the aim was to use EMP as a vehicle for transporting a radioactive isotope into cancer cells, where the isotope would function as an intracellular source of radiation, and EMP as a carrier molecule and as a radiation sensitiser. Secondly, we investigated the mechanisms of radiosensitization by EM. The aims of the study were as follows:

I - To find out whether the known ability of EMP and EMBP-AB to accumulate in the prostate is present after radioiodination of the substances, and whether their biodistribution in terms of tissue uptake and clearance makes them suitable for use as carrier molecules for radioiodine into prostate cancer.

II - To assess the uptake of a therapeutic dose of RI-EMBP-AB in prostate cancer in order to determine whether the dose of radioactivity absorbed by the tumour is sufficient to achieve therapeutic effects, and to compare the dose of radioactivity in the tumour with that in other organs to evaluate the safety of the treatment.

III - To assess the role of apoptosis in the radiosensitizing effect of EM; to find out whether the EM-enhanced effect of radiation is mediated by an increased amount of apoptosis.

IV - To determine the effect of EMP-treatment to the status of oxygenation in prostate cancer, when used either alone or as a radiosensitizing neoadjuvant treatment before and during radiotherapy.

7. MATERIALS AND METHODS

Four experiments were designed, corresponding to the aims of the study:

Experiment I - The biodistribution of radioiodinated EMP and EMBP-AB and pure radioiodine was determined by injecting mice with the substances and measuring the radioactivity of different organs at time points.

Experiment II – The first experiment was repeated with EMBP-AB, using nude mice with implanted tumours of DU-145 human prostate cancer.

Experiment III – Similar nude mice with DU-145 tumours were irradiated with or without neoadjuvant EMP-treatment. Samples of the tumours and of the testes were analysed to determine the amount of apoptosis.

Experiment IV – Nude mice with DU-145 tumours were irradiated with or without neoadjuvant EMP-treatment. The amount of hypoxia after the treatment was determined by measuring the accumulation of 18-fluoromisonidazole, injected at the end of the treatment, into the tumours and the testes. In addition, histological and immunohistochemical analyses were performed.

The following chapter will describe the materials and methods used in the experiments in detail.

7.1 Estramustine phosphate and estramustine binding protein antibody

Estramustine phosphate, *Estra-1,3,5(10)-triene-3,17-diol(17 β)-3[bis(2-chloroethyl)carbamate]17-(dihydrogen-phosphate), disodium salt, hydrate (Estracyt ®) was obtained from Pharmacia-Upjohn, Lund, Sweden and from Pharmacia & Upjohn GmbH, Erlangen, Germany.*

Clone A8-G11-C10-F9-B2 antibody against EMBP was obtained from Pharmacia-Upjohn, Lund, Sweden. The monoclonal antibody has been previously described in detail. Similar antibodies have been produced in other laboratories. The antibody adheres to its targets intracellularly. It has been shown to cross-react with human EMBP and produces a similar staining of purified rat EMBP and EMBP in DU 145 human prostate cancer cells. The antibody used in the experiment was tested by the supplier by western blotting, and it showed high affinity to the relevant epitopes. [107, 138]

7.2 Radiolabelling

Estramustine phosphate

EMP was labelled using Iodogen® (1,3,4,6-tetrachloro-3 α ,6 α -diphenyl glycoluril) (Pierce, Rockford, IL) as an oxidizing agent in phosphate buffer 0.15 mol/l, pH 7.4. 1 mg EMP was iodinated using 75 MBq Na-I-125 (Amersham, Little Chalfont, UK). After 30 min incubation the supernatant was transferred from the iodination vessel, incubated for another 30 min and filtered with 2 ml of saline.

Estramustine binding protein antibody

In experiment I, EMBP-AB was iodinated with solid lactoperoxidase as follows: Lactoperoxidase suspension was first diluted 1:5 with acetate buffer 0.1 mol/l, pH 6.0. The iodination was started by adding 75 MBq of Na-I-125 and 20 μ l of peroxide, 0.88 mmol/l, followed by two 10 μ l additions of peroxide during 30 min. After the incubation solid lactoperoxidase was centrifuged down and the supernatant was filtered with 2 ml of saline.

The labelling was controlled with thin layer chromatography (ITLC Gelman Sciences, Ann Arbor, MI). The specimens were incubated against a high resolution photostimulable plate (Fuji-III, Fuji Co., Japan) and read by an image reader digiscan (Siemens Corp., Erlangen, Germany) using a linear fixed scale program. The solutions of RI-EMP and RI-EMBP-AB contained less than 1% and less than 5% of free RI, respectively.

In experiment II, EMBP-AB was iodinated using lactoperoxidase sorbent (LPS) as follows: 10 µl of LPS suspension, 25 µl of stock EMBP-AB solution and 40 µl of acetate buffer, 0.1 mol/l pH 6.0, were mixed. 2 mCi of Na I-125 was added to the mixture and iodination was started with 20 µl of hydrogen peroxide dilution (1:1000 of perhydrol). After iodination the precipitate was centrifuged and the supernatant diluted with isotonic sodiumchloride and filtered through a silver disc (Millipore). Tested by thin layer chromatography (ITLC Gelman Sciences, Ann Arbor, MI), the labelling efficiency was 60-70% and purity over 90% after the filtering.

Experiment II was repeated using I-131-labeled EMBP-AB. This time, labelling was performed like in experiment I, with solid lactoperoxidase.

Fluoromisonidazole

¹⁸F-labelled fluoromisonidazole, 1H-1-(3-[¹⁸F]fluoro-2-hydroxypropyl)-2-nitroimidazole ([¹⁸F]FMISO), was prepared in one step, starting from 1-(2'-nitro-1'-imidazolyl)-2-O-tetrahydropyranyl-3-O-toluenesulfonyl-propanediol (NITTP) using an automatic fluorine-18 fluorodeoxyglucose (FDG) synthesis module made by IBA (Ion Beam Applications, Belgium). The synthesis procedure was based on the method by Lim & Berridge [136], slightly modified by us. N.C.A. aqueous ¹⁸F-fluoride was transferred to the synthesis vessel containing 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo (8.8.8) hexacosane

(Kryptofix [2.2.2]) (13.5 mg) and Sodium carbonate (K_2CO_3) (1.7 mg) in acetonitrile/water (8:1). The solvents were evaporated under an argon-flow by adding 1 ml of acetonitrile (CH_3CN) two times. Next, NITTP in 2 ml of CH_3CN was added, and the reaction was performed at 100 °C during 10 min. Subsequently, 10 ml of diethyl ether was added, and the product was transferred through two silica Sep Pak cartridges (Waters, Milford, MA, USA) to a second vessel (in two 5-mL-portions). The ether was evaporated, and 2 ml of 1 N HCl was added to the residue for hydrolysis at 100 °C for 3 min. Then, 1 ml of 2 N NaOH was added to neutralize the solution. The solution was then transferred, through a C-18 Sep Pak cartridge (Waters), an Alumina Sep Pak cartridge (Waters) and a Millipore filter (Millipore Oy, Espoo, Finland) connected in series, to the product vial containing 1 ml of 1 N $NaHCO_3$. Finally, the column was rinsed with 4 ml of 10%-ethanol and used subsequently for the animal experiment. No extra purification step was needed. [139, 140]

The synthesis time was about 50 min and the radiochemical yield for the [^{18}F]FMISO was 40% (End of Bombardment; EOB) on an average after a synthesis time of 96 min. The identity of the intermediates and the final product were confirmed by comparing the chromatograms with unlabelled reference materials. The radiochemical purity of the final product was over 97%, confirmed by thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

7.3 Experimental animals

The study was approved by the ethical committee of the hospital and the committee for animal experiments. A total of about two hundred 6-12-week-old

male Balb/c mice were used in the experiments. Their weight ranged from 18 to 22 g. The animals had free access to food and water. The mice in experiments III and IV were of the nude variant, with deficient cell-mediated immunity, to facilitate human xenograft transplantation. The nude mice were kept in an isolated room, in cages equipped with air filters.

7.4 Tumour xenografts

Human prostate cancer cells of the line DU 145 were cultured in modified Eagle's medium supplemented with 10% fetal bovine serum in a 5% CO₂ atmosphere and synchronised to an exponential growth phase. Two million cells (in II, 3.4 million cells) in 0.2 ml of saline were inoculated intracutaneously into each flank of the Balb/c nude mice. The tumours were allowed to grow for three to four weeks, reaching diameters of about 2 mm to 2 cm. Most mice had two tumours, one on each side. The larger one of the two tumours was taken as a specimen for tissue analysis. The tumour xenografts had no other visible effect on the animals. Three mice in IV had no tumour and were excluded from the study.

To ascertain the effectiveness of the treatment, the changes in tumour size were measured. The volumes of tumours were calculated from the length, width and height of each tumour, measured with a calliper at the beginning of the treatment and 1 and 3 weeks thereafter. The relative size of the tumour was calculated by dividing the volume of the tumour at 1 and 3 weeks by the volume of the same tumour in the first measurement.

7.5 Determination of biodistribution

In **experiment I**, RI-EMP, RI-EMBP-AB or pure iodine-125 (RI) was administered to each animal in an intravenous injection of 0.1 ml of saline. RI was used as a control to exclude the effect of free RI and RI dissociated from the other substances. The average dose of RI-EMP was 50 µg and that of RI-EMBP-AB 100 µg. The activities of the syringes were measured before and after the injection, yielding an average injected activity of 150 kBq for RI-EMP, 75 kBq for EMBP-AB and 37 kBq for RI. The mice in RI-EMP and RI-EMBP-AB groups were decapitated 1, 3, 7, 15 or 31 h from the injection, while in the RI-control group, only 1, 7 and 31 h periods were used. Three to four mice per time point were dissected in each group and specimens of 14 different organs were obtained. The specimens were weighed and their radioactivities were measured with a gamma counter (LKB 1282 Compugamma, Wallac Oy, Turku, Finland).

In **experiment II**, the mice received an injection of RI-EMBP-AB. The average injected dose of I-125-EMBP-AB was 243 µCi (9.00 MBq). In the group treated with I-131-EMBP-AB, the average injected dose was 19 µCi (0.70 MBq). The mice were decapitated and dissected 4, 24 or 48 h from the injection and measured as in experiment I.

In **experiment IV**, the mice were sacrificed three weeks from the beginning of the treatment. One hour before decapitation, about 5.6 - 7.4 MBq (150-200µCi) of [¹⁸F]FMISO was injected intraperitoneally to each animal. The activities of the syringes were measured before and after the injection. After decapitation, samples of the tumours, testes and hearts were weighed, and the activity of each sample was measured with the gamma counter as in I and II. Care was taken to include in the specimen a representative portion of all parts of the tumour, deep and superficial, excluding the entirely necrotic portion when present. The

decrease in the activity of ^{18}F ($T_{1/2} = 109.8$ min) during the time between the injection and the measurement of each sample was taken into account. To evaluate the uptake of [^{18}F]FMISO, the decay-corrected activity of ^{18}F in the samples was correlated with the injected activity of the individual animal and divided by the weight of the tissue sample, yielding the percentage of the injected activity per gram of tissue (%ID/g). The [^{18}F]FMISO uptake ratio was obtained by dividing this value of a tissue sample with that of the heart from the same animal.

7.6 Treatment with estramustine

In experiments III and IV, estramustine phosphate was diluted with a solution containing 5% glucose into a concentration of 1 mg/ml. A daily dose of 0.2 mg estramustine phosphate (EMP) was injected intraperitoneally on 9 to 14 consecutive days to each mouse randomised to get the treatment; the other mice received a daily injection of the same amount of the solution without EMP.

7.7 Radiotherapy

During the second week of estramustine therapy, the mice in groups R and ER were submitted to fractionated external beam radiotherapy (3 or 6 fractions of 6 Gy within one or two weeks, total dose 18 and 36 Gy, respectively). The mice were placed in cylindrical polyethylene containers with inner diameter of 36 mm and wall thickness of 0.9 mm with conical ends so that the area to be treated rested congruently against the inner surface of the container. The containers were then placed into tightly fitting holes in a polymethylmetacrylate (PMMA) phantom to obtain adequate fixation of the mice and to get a sufficient build-up

layer for the superficial tumours. The phantom consisted of five cylindrical cone-ended holes to irradiate five mice at a time. The dimensions of the phantom were 29 cm × 5 cm × 6.5 cm (width, depth and height, respectively), which enabled the irradiation of five mice at a time with a treatment field size of 28 cm × 4.5 cm. Due to proper fixation and tranquility of the animals, the localization of the treatment areas was competent and sedation unnecessary.

The mice were irradiated with a daily dose of 6 Gy with 6 MV photons produced by Varian Clinac 600 C linear accelerator (Varian Medical Systems Inc., Palo Alto, CA, USA). The field of radiation was limited to the caudal part of the animals, covering both of the tumours and the testes.

7.8 Southern-blot analysis of apoptotic DNA fragmentation

To assess the presence of apoptosis, samples of one tumour and one testis per mouse were examined. Tissue samples were snap-frozen in liquid nitrogen and stored at -70°C until DNA isolation. Genomic DNA was extracted using the Apoptotic DNA Ladder Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions, with some modifications. Briefly, the carcinoma and testis samples were homogenised and incubated for 10 min at room temperature in a binding/lysis buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% TritonX-100, pH 4.4). The samples were then mixed with isopropanol (final proportion of isopropanol 25%), loaded into polypropylene tubes and centrifuged for 1 min at 8000 rpm. The tubes were washed twice with washing buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5), and the bound DNA was eluted from the tubes with 10 mM Tris, pH 8.5. Finally, the samples were incubated with RNase (2.5 $\mu\text{g}/\text{ml}$, Roche Molecular Biochemicals) for 20 min at room temperature. After quantification, the DNA samples were 3'-end-labeled

with digoxigenin-dideoxy-UTP (Dig-dd-UTP; Roche Molecular Biochemicals) by the terminal-transferase (Roche Molecular Biochemicals) reaction, subjected to electrophoresis on 2% agarose gels, and blotted onto nylon membranes overnight. Next day, the DNA was crosslinked to the membranes by UV irradiation. The membranes were then washed and blocked with 1% Blocking reagent (Roche Molecular Biochemicals) in maleic buffer (100 mmol/L maleic acid, 150 mmol/L NaCl, pH 7.5) for 30 min at room temperature. The 3'-end labelled DNA on the membranes was localised with alkaline phosphatase-conjugated anti-digoxigenin antibody (Anti-Digoxigenin-AP; Roche Molecular Biochemicals), and the bound antibody was detected by the chemiluminescence reaction (CSPD, Roche Molecular Biochemicals). The x-ray films exposed to chemiluminescence were scanned with a tabletop scanner (Hewlett Packard ScanJet 6300C) and the digital image was analysed with Scion Image beta 4.0.2 (Scion Corporation) analysis software. The digitised quantification of the low-molecular-weight DNA fragments (< 1.3 kB) of the samples was expressed in relation to a standard amount (20 ng) of a commercial DNA marker (DNA Pnix, Amersham).

7.9 Histological and immunohistochemical analysis

Pieces of tumours were snap-frozen in liquid nitrogen and cut to 5 µm for histological studying. After staining with hematoxylin and eosin, the specimens were evaluated for cellularity and necrosis by an experienced pathologist, unaware of the treatment groups of the samples. Monoclonal antibody PP-67 against proliferating cell nuclear protein Ki-67 (Sigma, St Louis, MO) was used to assess proliferative capacity of the xenografts. For this purpose frozen sections were fixed with acetone at -20°C and the sections were exposed to fluorescein isothiocyanate (FITC)-coupled goat anti-mouse immunoglobulin

(Jackson Laboratories, West Grove, PA) for 30 min. After washing the specimens were exposed to DAPI (Riedel-de Haen, Hannover, Germany), to detect nuclei, and after washing were embedded and examined by using Leica Aristolan microscope equipped with appropriate filters.

7.10 Calculation of the dose of radiation

In experiments I and II, the effective half-lives of radioactivity were calculated by fitting the biodistribution data to an exponential curve. To calculate the absorbed dose of radiation in the tumour and in the prostate we used the effective half-lives, and the S-factors calculated earlier for the mouse testis, assuming the activity to be evenly distributed within a 100 mg sphere, with a radius of 3 mm.

In experiments III and IV, the 6 Gy dose was calculated to the depth of 2.5 cm in the phantom which was the average depth of the tumours, at a source - phantom distance of 100 cm and a dose rate of 1.9 Gy/min in experiment III and 3.8 Gy/min in experiment IV. The testicle dose in the average depth of 4 cm was 5.6 to 5.8 Gy per fraction, depending on the position of the mouse. The variation of dose within the tumours was $\pm 5\%$ or less.

7.11 Statistical methods

In IV, statistical analyses were performed using analysis of variance (ANOVA) to compare ratios (testis/heart and tumour/heart) between groups (O, E, R, ER). Paired t-test was used to compare ratios (testis/heart and tumour/heart) within groups for possible difference. ANOVA was also used to compare groups in

difference between ratios (difference between ratios testis/heart and tumour/heart). Pairwise comparisons in ANOVA were calculated comparing other groups to control (adjusted using Dunnett's method). P-value less than 0.05 was considered as significant. The statistical analyses were carried out using SAS/STAT® software, Version 9.1.3 SP4 of the SAS System for Windows.

8. RESULTS

8.1 Radiolabelling

The labelling of EMP and EMBP with radioactive iodine was successful; only small amounts of free RI were present in the solutions. [¹⁸F]FMISO was prepared in about one hour with an average radiochemical yield of 40% decay-corrected to end of bombardment. The radiochemical purity of the final product exceeded 97%, determined by radiochromatographic methods.

8.2 Animals

No side effects appeared after the injection in experiments I and II. The tail, which was the injection site, contained no significant radioactivity in any of the animals. In experiment III, the untreated mice gained about 10% of weight during the follow-up. The mice in the six-day radiation groups had diarrhea starting on the fourth to fifth day of irradiation. They lost 25% of their weight rapidly and several of them died, leading to early decapitation of the rest of the mice in the six-day radiation groups, and to the conclusion that a 36 Gy total dose is too high in this setting. About half of the mice in the three-day radiation group had mild diarrhea, and they lost on average 10% of their weight but all survived. In experiment IV, no side effects were encountered.

8.3 Tumour size

In study III, the size of an untreated tumour after four weeks was on average 8.96 (+- 10,75) times the original size. Tumours treated with estramustine only were 3.40 (+-3.58) times the original size. Those treated with radiation only (18 Gy) had grown to 1.21 (+-0.61) times and those treated with estramustine and radiation (18 Gy) had diminished to 0.46 (+-0.54) times the original size. The higher dose of radiation (36 Gy) was associated with high mortality and the tumour sizes in these groups were measured three weeks after the beginning of the trial: the sizes were 1.59 and 0.59 (+-0.13) times the original sizes for the radiation only and the estramustine and radiation groups, respectively.

The relative size of the tumours in study IV increased constantly in the untreated group and, in this case, also in the group treated with estramustine. The groups treated by irradiation showed a decrease in the relative tumour size between weeks 1 and 3, with the combined treatment group decreasing more rapidly after an initial increase.

8.4 Biodistribution

Radioactive iodine

In study I, RI was present in relatively high concentrations in almost all organs 1 h after the injection: the prostate contained 11.9% ID/g, and most other organs 2 to 4% ID/g. After that, the activities rapidly decreased. The prostate contained 0.9% ID/g 7 h after the injection and most other organs less than 0.3%. The kidney, the lung and the gallbladder each contained about 1% ID/g after 31 h, while after 7 h, the gallbladder contained over 29.7% ID/g and the kidney and the lung 0.2% ID/g.

RI was found to concentrate in the thyroid gland, 5.2% ID/g being present after 7 h and 0.8% after 31 h from the injection. The thyroid gland also contained the most activity per weight in the RI-EMBP-AB group parenchymal organs. 7 h after the injection, the activity was 18.4% ID/g. The activity had decreased to 0.1% after 31 h. In the RI-EMP group, the activity of the thyroid gland after 7 h and 31 h was 11.0% and 1.9% ID/g, respectively.

Radioiodinated estramustine phosphate

In study I, RI-EMP was found to accumulate in the liver and the gallbladder of the mouse. 1 h after the injection, the liver contained 21.4% of the injected dose (ID) / 1 g tissue. After 7 h, the liver contained 2.9% ID/g and the gallbladder 332.0% ID/g. 31 h from the injection, the liver contained only 0.9% ID/g while the gallbladder still had 9.3% ID/g. The lung presented a diphasic accumulation of RI-EMP, containing 2.3% ID/g 7 h after the injection, while most other parenchymal organs contained 0.3-0.6% ID/g at the same time. The prostate was found to have more RI-EMP than most other organs at 1 to 7 h after the injection (6.4 to 2.6% ID/g, respectively). The activity in the prostate and in most other organs had practically disappeared after 15 h. The prostate/blood -ratio of the proportion of ID/weight of sample at 7 h was 3.3. The activities of most other organs were close to that of the blood. No organ seemed to retain significant activity longer than 31 h.

Radioiodinated estramustine binding protein antibody

RI-EMBP-AB accumulated in the prostate of the mouse. In study I it contained 5.9 to 2.9% ID/g 1 and 7 h after the injection, respectively, while the liver contained 1.5 to 1.0% ID/g, as did most other parenchymal organs. The gallbladder contained 6.5% and 0.8% ID/g 7 h and 31h after the injection.

Prostate / blood ratio of activity at 7 h was 3.2. In study II, the prostate contained 2.4% ID/g after 4 h while the testis contained 0.95% ID/g.

In study II, the amount of RI-EMBP-AB in the tumour graft (0.65% ID/g) was slightly higher than in blood (0.45% ID/g) and most other organs. The prostate contained 5.2 times and the tumour 1.4 times the amount in the blood at four hours. The organs which contained most RI-EMBP-AB at 4 h, the prostate, testis and the tumour, showed very short half-lives of radioactivity: 8.6, 10.4 and 15.8 h, respectively. Half-lives in organs not accumulating much RI-EMBP were as follows: 37.6 h in the lung, 44.9 h in the blood, 50.6 h in the liver and 94.7 h in the kidney. The doses of radiation absorbed by the prostate and the tumour, assuming the injected dose to be 1 mCi, were 1.81 and 0.92 cGy, respectively. The experiment was repeated with another group of Balb/c nude mice with similar DU-145 tumours, this time using I-131-labeled EMBP-AB. The prostate-blood ratio of activity was 4.5, but the tumour contained no more activity than the blood at 4 h.

[¹⁸F]FMISO

The distribution of [¹⁸F]FMISO was studied in experiment IV. In the control group testes the mean uptake value of [¹⁸F]FMISO was 1.14 ± 0.05 and that of tumours 1.73 ± 0.18 . After treatment the values for testes in groups E, R and ER were 1.05 ± 0.19 , 1.10 ± 0.15 and 1.33 ± 0.14 , and for tumours 1.76 ± 0.38 , 2.30 ± 0.68 and 2.64 ± 0.58 , respectively. [¹⁸F]FMISO uptake ratio values of tumours were significantly higher than those of testes, being of statistical significance in all groups ($p < 0.001$) and the difference was significantly higher in groups R ($p=0.019$) and ER ($p=0.012$) than in the control group.

8.5 Apoptosis

In study III, the relative amount of low-molecular-weight fragments of DNA after 24 h, consistent with apoptosis, was significantly higher in the DU 145 tumours treated with radiation only or EMP only than in untreated tumours or those treated with the combination of EMP + radiation. In the testes, treatment with radiation only was associated with a significantly higher level of DNA-fragmentation than in all other groups.

After one week, the amount of DNA-fragmentation in the tumours of all groups was about the same, and thereafter the EMP group seemed to demonstrate higher levels, followed later by the untreated group. In the testes, the initial proneness to DNA-fragmentation in the radiation-treated group still persisted 18 days after therapy, and the group treated with EMP + radiation showed rising levels from 1 week after treatment.

8.6 Histology and Immunohistochemistry

The results of the histological and immunohistochemical studies in experiment IV are shown in *Table 2*. There was more necrosis in the tumours of group ER than in the other groups and almost none in group O. The groups that had received radiotherapy (R and ER) had less mitoses and less proliferation (less Ki-67).

9. DISCUSSION

9.1 Biodistribution of radioiodinated estramustine phosphate

In study I, the distributions of RI-EMP and RI-EMBP-AB were relatively similar, except for the higher uptake of RI-EMP in the liver, gallbladder and lung. The distribution of RI, however, was totally different, which supports the assumption that the solutions did not contain excessive amounts of free iodine. The apparent uptake of RI-EMP and RI-EMBP-AB by the thyroid gland is possibly due to *in vivo* dehalogenation. The uptake of RI by the thyroid gland can easily be blocked by iodine.

RI-EMP was found in the prostate in higher concentrations than in most other organs 7 h from the injection. The initial high concentration of RI-EMP in the liver decreased to the level of the prostate by 7 h. By that time, the concentration in the gallbladder had raised substantially: the gallbladder contained 3.3 times the injected activity per gram, which amounts to 3.3% of the injected activity in a gallbladder weighing 10 mg. The gallbladder retained a relatively high activity 31 h after the injection. The hepatic uptake and biliary secretion of a steroid derivative is not surprising. The activity of the lung raised back to the level of 3.6% ID/g 15 h after the injection, which equals the activity after 1 h. After that, the activity started to decrease again. The activity of the thyroid gland had decreased to 1.9% ID/g by 31 h.

The prostate seems to be a target organ for RI-EMP, containing 2,6 % ID/g after 7 h. However, the lung is found to have a roughly equal ability for uptake than the prostate, and to retain the radioactivity longer. The liver has a somewhat higher initial uptake, which decreases as RI-EMP is secreted in bile.

An earlier study on the distribution of tritiated EMP in rats had similar results: retention of activity in the prostate as compared to blood, and high accumulation into liver [28]. The accumulation of RI-EMP in the prostate and liver has also been observed in scintigraphic images of man [31].

In clinical use, the pulmonary and hepatic uptake and biliary secretion may not be significant, but if excessive toxicity or irradiation on these organs presents a problem, methods for local administration may have to be considered. Temporary surgical drainage of the biliary system could diminish irradiation of the bowel and other organs during the intestinal transit. The accumulation in the liver and lung may prove beneficial when RI-EMP is used to treat tumours in these organs.

The absorbed radiation dose in the prostate was calculated as described earlier. We obtained 3.0 mGy/MBq for RI-EMP and 2.3 mGy/MBq for RI-EMBP-AB. The calculations are based on prostatic uptakes at 7 h (2.6% and 2.9% ID/g, respectively) and mean residence times ($T_{1/2} : \ln 2$) of 9.23 h and 6.35 h, respectively. This calculation does not take into account intracellular distribution and Auger-electrons. With Auger-electrons of I-125 the relative biological effectiveness (RBE) can be 7.9 for I-125-UdR [33], and thus also the absorbed dose can be higher in the range of one order of magnitude, because we assume RBE=1.

The absorbed radiation dose with these tracers is feasible in view of their clinical use. When combined with the radiosensitizing effect of EMP, the radiation effect can be further enhanced. Whether the metabolites of RI-EMP have the same radiosensitising effect as those of EMP is not presently known and should be investigated. These studies are essential in determining the potential of RI-EMP in cancer treatment.

9.2 Biodistribution of radioiodinated estramustine binding protein antibody

RI-EMBP-AB was found to accumulate in the prostate, which contained 2.9% ID/g after 7 h, the thyroid gland and the gallbladder. In an earlier study with rats receiving 10-50 µg of antibody L6, the proportion of activity at 6-24 h was 1.5-0.7% ID/g in the liver, 1.6-1.2% ID/g in the lung and 1.4-0.8% ID/g in the kidney [33]. These figures are slightly higher than those of RI-EMBP-AB at 7-31 h in our study: 0.7-0.1% ID/g in the liver, 1.0-0.1% ID/g in the lung and 0.9-0.2% ID/g in the kidney.

RI was found in much higher concentrations in the gallbladder after 7 h, but significantly less in the thyroid gland than RI-EMBP-AB. Antibodies may be removed from circulation through degradation; the rise of activity in the gallbladder is possibly due to RI freed in the process, or a result of *in vivo* dehalogenation. After 31 h, RI-EMBP-AB had disappeared from all organs.

The accumulation of RI-EMBP-AB in the prostate was higher than that of antibody L6 in a heterotransplanted human tumour in a rat, even though this antibody showed excellent tumour uptake [34]. A study with antibody CE7 in mice demonstrated higher uptake in the liver, kidney and blood than that of RI-EMBP-AB in our study [35]. In another study, radioiodinated antibody 17-1A was found to remain longer in most organs of nude mice than RI-EMBP-AB in our study, and the activity in the blood was about four times that in the liver [36].

Experiment I showed that RI-EMBP-AB is mostly taken up by the prostate. A known target for the antibody, the tumour was expected to accumulate activity at least as much as the prostate. Contrary to the expectations, experiment II

showed that the concentration of activity at its highest was not much higher in the tumour than in the blood. The dose of radiation in the prostate was two times higher than in the tumour. This is an interesting finding, as it means that the antibody could function as a carrier of radioiodine into the prostate but not into the tumour, both containing the relevant antigen. The reason for this could be a difference in the affinity of EMBP-AB to the antigen between the mouse prostate and the human tumour. Produced in mice against rat prostatic EMBP, the antibody has nevertheless been shown to have high affinity to human EMBP [107]. The relative affinities between species are not known and should be studied. Another explanation to our finding might be that the labelling of EMBP-AB affects its ability to be incorporated into cells in healthy tissue and tumour cells.

In experiment II, the male reproductive glands and the tumour, containing high amounts of EMBP, initially accumulated RI-EMBP-AB but then lost it rapidly, while the descent of activity in other organs took place slower. The half-lives of activity in the blood and liver were about three-fold, and in the kidney about six-fold, as compared with the tumour graft, while the concentrations of RI-EMBP-AB in these organs at 4 h were only slightly lower than in the tumour. Thus most organs received doses of radioactivity comparable with that of the tumour, which would cause intolerable side effects when using I-125-EMBP-AB in therapeutic doses. Other agents should be studied to find better carrier substances for the treatment of hormone refractory prostate cancer, perhaps strontium-89, (177)Lu-DOTA-8-AOC-BBN (7-14)NH(2) and antibodies against prostate-specific antigen (PSA).

9.3 Apoptosis

Studies on EMP-induced apoptosis have had follow-up times of 4 to 96 hours. This seems practical, since the plasma half-life of EMP in humans is 10-20 hours. The possible long-term apoptotic effect of EMP has not been studied. In this study, we extended the follow-up time from 24 hours to 18 days from the end of all treatments to find out longer-term effects on apoptosis and to be able to verify the radiosensitising effect of EMP by tumour regression. The ability of EMP to potentiate the effect of radiation on prostate cancer xenograft growth has been demonstrated earlier in a similar setting [86]. Our results are in accordance with the previous findings: the tumours that were treated with the combination of EMP and radiation tended to diminish in size, while radiation alone seemed only to retard growth and EMP alone had little effect.

DNA fragmentation analysis was used as an indicator of apoptosis. In apoptosis, the DNA is divided by a process in the cell itself into fragments with a typical molecular weight distribution. This study uses the quantitative analysis of the typical molecular weight sequence. The measured values are compared to a standard sample, and the measurements in the charts can only be compared within the same chart, not so reliably between different charts and time points. For this reason, an untreated control group was included in the study. The amount of DNA-fragmentation 24 hours from the end of treatment was high in DU 145 tumours that were treated with either radiation or EMP alone. The amount was lower in tumours treated with the combination of EMP and irradiation than after a single-treatment regimen, although a greater diminution of tumours was noted after 2 weeks in the combined treatment group. This is in opposition to our hypothesis of an increase in apoptotic rate with the combined treatment, or an additive or potentiating effect on the separate apoptotic effects of radiotherapy and estramustine. Rather, both EMP and radiation seem to

prevent apoptosis caused by the other treatment modality. Therefore, the radiosensitising effect of EMP must be due to enhancement of some other mechanism of action of radiotherapy. Ischaemia due to damage on small blood vessels is a known effect of radiotherapy. Hypoxia, on the other hand, decreases radiosensitivity. EM has been shown to increase blood flow in tumours [17] and, theoretically, this could temporarily reverse radiation-induced ischaemia and return the cancer cells into a well-oxygenated, more radiosensitive state for the following irradiation sessions. This, however, is inconsistent with our finding of reduced apoptosis after the combined treatment. The mechanism of radiosensitisation may be based on other cellular level effects of radiation. As stated earlier, the mitotic arrest of the dividing cancer cells seems to play a role in radiosensitisation.

The longer-term levels of DNA-fragmentation in all groups of tumours are rather similar, with a rise in the EMP-treated group after 2 weeks and the untreated group after 18 days. These results, showing no clear pattern, are probably of no greater significance.

In the testis, radiotherapy had a significant increasing effect on DNA-fragmentation from 24 hours to 18 days from treatment. EMP, while causing fragmentation in the tumours, did not have this effect on healthy testes. This is consistent with previous findings in malignant gliomas and healthy brain tissue [34]. In the testes, as in the tumours, the combination of EMP and radiation reversed the supposed apoptotic effect of both treatment modalities. In the longer-term follow-up, however, the testes treated with EMP + radiation showed DNA-fragmentation levels comparable with those of radiation. This is contrary to the observation in the tumours. The reason for this difference is not clear.

The amounts of DNA-fragmentation between 7 to 18 days from the end of treatment are comparable with the amounts after the first day and show considerable variation. This may be due to biological diversity in the tumours but may also have an impact on the long-term growth or regression of tumours. Long term effects on apoptosis should be taken into account in further studies and follow-up carried over a longer period than 1 to 3 days, even in cell culture and xenograft studies.

9.4 Tumour hypoxia, proliferation and necrosis

In experiment IV, most tumours had a necrotic centre despite neovascularisation, which was present superficially. In the untreated group (group O), the tumours had a relative [^{18}F]FMISO uptake of 1.73 as compared with the heart, indicating tumour hypoxia. Tumours in groups O and E had more mitoses and more Ki-67, indicating stronger proliferation than in groups R and ER. The main finding of experiment IV was that as compared with tumours treated with EMP or radiation only, those in the group treated with both EMP and radiation showed more uptake of [^{18}F]FMISO, indicating hypoxia. This group (ER) also had more necrosis in the histological samples.

EMP concentrates less effectively in tissues with poor blood supply and it has a limited effect in the slowly dividing hypoxic cells. The effectiveness of EMP against cancer is believed to be mainly based on stabilising microtubule dynamics and preventing microtubule formation, causing mitotic arrest in the G2/M-phase of the cell cycle. [141, 142] Cells arrested in that phase are more sensitive to radiation. Because of the slow cell cycle, the radiosensitising effect is expected to be weaker in poorly oxygenated tissues.

The tissue specimen was taken from the viable layer of the tumour and no necrotic core was included. With the well-oxygenated superficial layer and the intermediate layer included and the central necrotic region excluded, the samples represent an average of the viable tumour in respect to tissue oxygenation. The trend towards increased tumour hypoxia after radiotherapy observed in this study and the increased amount of necrosis are attributable to radiation-induced injury to tissue and microvasculature. The radiation-induced hypoxia and necrosis in the tumours seemed to be accentuated by presensitization with EMP. The finding that the uptake of [¹⁸F]FMISO in the testes remained the same after the different treatment regimes suggests that EMP and irradiation do not induce as much damage to healthy tissue as to a malignant tumour.

Another proposed explanation for the ability of EMP to sensitise tumours to radiation is that it increases their blood flow. This has been shown to occur in a rat brain glioma model [143]. By improving the oxygenation status of the intermediately oxygenated cells, EMP may widen the portion of the tumour with an oxygen tension sufficient for radiotherapy to be effective. EMP has been shown to counteract the anti-angiogenic effect of radiotherapy and to increase blood vessel size and density and the expression of VEGF [57, 143]. The presence of these phenomena in DU-145 cells was not addressed in our study and should be investigated by a different experimental approach. However, tumour blood flow does not necessarily directly correlate to the oxygenation status of all cells in a tumour; the increased flow may be directed mainly to the well vascularised portion of the tumour and leave the overall oxygenation status unchanged. In this study, estramustine alone did not slow down the growth of the tumours, had no effect on tumour proliferation and did not improve tumour oxygenation, but it did induce tumour necrosis when used either alone or in combination with irradiation.

10. CONCLUSION

I - Radioiodinated estramustine phosphate has a biodistribution similar to non-labelled estramustine phosphate. It accumulates in the prostate, the liver and the lung. The prostate-specific targeting of RI-EMP may promote its use as a combined radiating-radiation sensitising-cytotoxic agent against prostate cancer.

The radioiodinated antibody against estramustine binding protein accumulates in the prostate, with no significant uptake in other organs.

II - The uptake of ^{125}I -EMBP-AB in DU-145 xenografts compared to most other organs is too low to make it feasible for targeted treatment of prostate cancer.

III - Estramustine potentiates radiotherapy but not by enhancing radiation-induced apoptosis.

IV – Estramustine alone or in combination with radiotherapy was not shown to improve oxygenation of DU-145 xenografts.

11. SUMMARY

The results of the study are summarized in the following tables:

Table 1:

Relative accumulation of radio iodinated estramustine (RI-EMP), radio iodinated estramustine binding protein antibody (RI-EMBP-AB) and radio iodine (RI) in different organs of the mouse.

+ mild accumulation
++ moderate accumulation
+++ strong accumulation
NT not tested

	RI-EMP	RI-EMBP-AB	RI
Prostate	+++	+++	+
Testis	+	+	+
Kidney	+	+	+
Liver	+++	+	+
Lung	+++	+	+
Rectum	++	++	+++
Heart	+	+	+
Spleen	+	+	+
Tumour	NT	+	NT
Bone	+	+	+
Blood	+	+	+

Table 2:

Relative effect of radiotherapy and estramustine on the mouse testis and DU-145 human prostate cancer tumours implanted in nude mice. Roman numerals refer to experiments I–IV of this study.

O no treatment
 E estramustine treatment
 R radiotherapy
 ER radiotherapy with neoadjuvant estramustine therapy

o absent or minimal
 + mild
 ++ moderate
 +++ strong
 - mild regression
 -- moderate regression
 --- strong regression

	O		E		R		ER	
Tumour growth (III)	++		+		o		--	
Tumour necrosis (IV)	o		o		o		++	
Apoptosis (III)	24 h	7 d	24 h	> 7 d	24h	> 7 d	24 h	> 7 d
Tumour	+	++	++	++	++	+	+	+
Testis	+	+	+	+	++	++	+	++
Proliferation (IV)								
Mitosis	+++		+++		++		++	
Ki 67	+++		+++		+		+	
Hypoxia (IV)								
Tumour	+		+		++		+++	
Testis	o		o		o		o	

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