Multidrug Resistance in Prostate Cancer

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Sponsoren:

Cover:

Print: Optima Grafische Communicatie, Rotterdam

Multidrug Resistance in Prostate Cancer

Multidrug resistentie in prostaatkanker

Proefschrift

ter verkrijging van de graad van doctor aan de

Erasmus Universiteit Rotterdam

op gezag van de

rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 16 november 2005 om 15.45 uur

door

Jérôme Patrick van Brussel

geboren te Leiden

PROMOTIECOMMISSIE

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C'est le courage qui compte

Ben Vautier

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CHAPTER 1

General introduction

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

In our ageing society , with a life expectancy of 79 years in Dutch man, prostate cancer plays and will play a predominant role, with regard to cancer related morbidity and mortality. It is traditionally stated that one does not die from, but that one dies with prostate cancer. Nonetheless, prostate cancer has become the second cause of male cancer-related deaths, after lung cancer. At diagnosis, the risk of dying from the disease is 40%[1]. Fortunately, many men with prostate cancer have limited disease, that can be adequately treated surgically, radiotherapeutically or with a "wait and see" policy. More advanced tumours can be treated with anti-hormonal therapy. However, a significant number of men have, or have acquired, advanced hormone independent prostate cancer. It is in this group of men that prostate cancer is a killer and efficient therapy is lacking. For systemic disease local therapy is insufficient. Chemotherapy has a long history in the treatment of (disseminated) cancer, but its use has not been generally advocated in advanced prostate cancer, due to disappointing treatment outcomes, certainly with regard to survival. It has easily been concluded that chemotherapy is ineffective for the treatment of prostate cancer, although the cause of failure of chemotherapy has not been determined. Clarification of the underlying cause(s) of its ineffectiveness appears to be an obligation towards prostate cancer patients, before disregarding chemotherapy as a treatment option, thus leaving a patient with no alternative, except purely palliative, treatments. Unveiling of mechanisms that render prostate cancer cells resistant to cytotoxic drugs may justify omitting chemotherapy as a treatment option for prostate cancer patients. However, insight into the causing factors of resistance may help to determine how to use chemotherapy adequately in prostate cancer patients. Importantly, increased understanding may even result in the development of treatment strategies, based on chemotherapy, that ameliorates the anti-tumour effect of chemotherapy with regard to palliation and even cure.

It has been from this point of view that the present research has emerged. The role of Multidrug Resistance (MDR), which is a phenotype characterized by cross-resistance against a wide range of chemotherapeutic agents, has been investigated

in vitro (human prostate cancer cell lines), *in vivo* (using human prostate cancer xenografts on athymic nude mice) and on clinical prostate cancer material.

In **Chapter 2** a literature overview is given over the most important MDR mechanisms in relation to prostate cancer including transmembrane pumps, detoxifying pathways, apoptosis and topoisomerases. Also, strategies to challenge these mechanisms are reviewed.

In **Chapter 3** expression of transmembrane pumps P-glycoprotein (Pgp) and Multidrug resistance associated protein1 (MRP1), of Glutathione-S-transferase-π (GST-π), which plays part in a detoxifying pathway, of Bcl-2 and Bax, involved in programmed cell death and of Topoisomerase (Topo) I, IIα and IIß, which are targets for cytotoxic drugs is assessed in human prostate cancer cell lines by immunocytochemistry and Western blotting. Proliferative activity of the cell lines was assessed by immunocytochemistry. MTT assays were used to determine the sensitivity to etoposide, doxorubicin and vinblastine.

In **Chapter 4** immunohistochemistry was used for detection of P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP), lung resistance protein (LRP), glutathione-S-transferase-π, (GST-π), p53, Bcl-2, Bax, topoisomerase (Topo) I, IIα and IIβ and Ki-67 in clinical prostate cancer. Paraffin-embedded formalin fixed prostate cancer specimens from archival sources of three groups of patients which were clearly distinct with regard to pathological stage and responsiveness to anti-hormonal therapy, representing early prostate cancer (pathological stage (p) T2N0M0 tumours) early advanced prostate cancer (disseminated prostate cancer before receiving anti-hormonal therapy) and late advanced prostate cancer (disseminated prostate cancer that relapsed despite anti-hormonal treatment).

In **Chapter 5** the involvement of the multidrug resistance associated protein (MRP1) and the glutathione (GSH) pathway in the multidrug resistant (MDR) phenotype of prostate cancer is assessed *in vitro*. Human prostate cancer cell lines PC3 and DU145 were selected with etoposide (VP-16), resulting in the cell lines PC3-R and DU-R. Resistance against etoposide, doxorubicin and vincristine and its reversal with leukotriene D4 antagonists MK-571 and zafirlukast, and buthioninesulfoximine (BSO) was studied in MTT assays. Western blot analysis of MRP1 expression

and GSH content measurements were performed. MRP1 function was studied in fluorescence assays.

In **Chapter 6** the objective was to assess the *in vivo* role of MRP1 in prostate cancer and its modulation aiming at chemosensitization. Expression of MRP1 was determined with Western blotting in a panel of human prostate cancer xenografts and xenograft derived cell line PC346C. Chemosensitization of PC346C to VCR, DOX and VP-16 with MK-571 was assessed in MTT assays. Carboxyfluorescein efflux blocking with MK-571 in PC436C, GLC4 and GLC4/ADR was measured in fluorescence assays. Toxicity and efficacy of VCR and VCR combined with MK-571 was determined *in vivo* with human prostate cancer xenografts.

In **Chapter 7** (general discussion) the results of all chapters are discussed, conclusions are given and implications for the clinical situation are evaluated.

In **Chapter 8** a English and Dutch summary of the thesis is given.

The research that has resulted in this thesis has been carried out at the Erasmus Medical Centre Rotterdam, Department of Urology, Pathology and Hematology.

1. Aus G, Pileblad E, and Hugosson J. Impact of competing mortality on the cancer-related mortality in localized prostate cancer. Urology 1995; 46: 672-5.

CHAPTER 2

Multidrug Resistance in Prostate Cancer – review article

van Brussel JP, Mickisch GHJ.

Onkologie 2003;26:175-81

SUMMARY

Advanced hormone refractory prostate cancer remains a therapeutic challenge, because all available pharmaceutical concepts have been proven to be ineffective in improving on cancer specific survival. Among those, a multitude of chemotherapeutic approaches did not offer a superior life expectancy. Failure of chemotherapy may be caused by multidrug resistance (MDR) mechanisms protecting cancer cells against cytotoxic drugs, and the question arises whether prostate cancer is also using MDR principles to develop resistance against chemotherapeutic agents. In consequence, an array of diverse pathways known to lead to MDR such as MDR1, MRPs, Glutathione, Apoptosis, and Topoisomerases have been examined and partially established at varying degrees in hormone-refractory prostate cancer. Thus, evidence keeps accumulating for the involvement of some MDR mechanisms in the chemoresistance of prostate cancer *in vitro* and *in vivo*. For some of them, e.g. MRP-1, functional expression appears to be probable. This lends credit to the idea that reversal, circumvention or overcoming of MDR pathways in advanced prostate cancer may be feasible and well lead to new avenues with improved treatment efficacy in otherwise untreatable disease.

INTRODUCTION

Prostate cancer is the most common cancer in men and one of the leading causes of cancer death[1; 2]. Localized prostate cancer has a good prognosis and can be managed by watchful waiting, early versus deferred hormonal treatment[3] external radiotherapy, brachytherapy or by surgery. However, metastatic prostate cancer remains a major oncological problem. Metastatic disease is incurable, although palliation can be achieved in 75% of all patients with hormonal ablation therapy[4]. Despite initial response, eventually the patients will relapse and die of progressive prostate cancer after an average period of 40 weeks[5]. Failure of androgen ablation is likely to be caused by the heterogeneous character of prostate cancer, which is composed of clones of both androgen dependent and independent prostatic cancer cells[6]. The life span of patients with metastatic disease may be determined by the hormone-independent cell populations. To increase survival of men with metastatic prostate cancer, treatment strategies that effectively targets androgen independent cancer cells are required.

Chemotherapy is the principal strategy to systemically challenge metastasized cancers. Unfortunately, prostate cancer is resistant to a broad range of antineoplastic agents[7-11]. Over the past decade new and more effective treatments have been developed based on an increased understanding of the morphological and functional characteristics of prostate cancer. Although several studies have reported favourably on response rates to chemotherapeutical regimens, patient selection, tumour heterogeneity, stage migration and definition of response have decisively confused evaluation of survival[12]. Altogether, results of chemotherapy in prostate cancer, single- as well as multiple agent regimens, have been disappointing, so far. At present, none of them have exhibited a clear survival advantage, although two recent randomized clinical trials have reported on a two to four month survival benefit with combination therapies of docetaxel and prednisone or docetaxel and estramustine as compared to mitoxantrone and prednisone, respectively[13; 14].

Resistance to anti-proliferative agents, which kill dividing cells, may be caused by large proportions of prostate cancer cells that are in interphase[15]. Importantly,

involvement of the multidrug resistance (MDR) phenomenon may well play a role in progressive therapy resistant prostate cancer and may offer an explanation for resistance to a variety of structurally- and functionally distinct cytotoxic agents. The resistance to cytotoxic chemotherapy is a major problem in the clinical management of cancer patients. Malignancies frequently respond to antineoplastic treatment initially, but eventually relapse and become progressive despite chemotherapy. Resistance has developed, not towards the applied drug alone, but often to a variety of structurally and functionally unrelated drugs as well. The MDR phenomenon of cross-resistance to different cytotoxic agents was first described three decades ago[16]. Apart from tumours with acquired or induced MDR, malignancies can display intrinsic MDR: they are primarily unresponsive to chemotherapeutic regimens. The mechanisms of these types of resistance appear to be similar. The multidrug resistance phenotype of cancer cells can be caused by different mechanisms and various MDR pathways have been identified over the years. Knowledge of the biological and molecular mechanisms of MDR is increasing. This is essential to develop new approaches to tackle the problem of chemotherapy-unresponsive tumours more successfully and find modalities by which MDR can be overcome, circumvented or reversed. Such strategies are termed chemosensitization.

In this article we discuss the most important MDR mechanisms in relation to prostate cancer. Several mechanisms for MDR have been detected, including transmembrane pumps, detoxifying pathways, changes in apoptosis and drug targets. Involvement of MDR mechanisms in prostate cancer implicates that development of strategies to overcome MDR is essential. Therefore, we will review strategies to challenge these mechanisms in multidrug resistant prostate cancer, aiming at improvement of the treatment of advanced hormone refractory prostate cancer.

MDR1 / P-GLYCOPROTEIN (PGP)

Overexpression of the MDR gene MDR1[17] and its product P-glycoprotein (Pgp) has received a great deal of attention. Pgp acts as a transmembrane efflux pump by extruding different classes of natural-product cytotoxic drugs from the cell in an energy-dependent manner.

The role of P-glycoprotein mediated multidrug resistance in prostate cancer is questionable with studies reporting conflicting results. Expression of Pgp in the human prostate cancer cell lines PC3 and DU145 has been reported in one study[18], albeit at low levels. Doxorubicin resistant cell lines derived from the Dunning R3327 rat prostate carcinoma model[19] expressed Pgp as well[20]. In contrast, using Pgp targeted-immunoconjugates harbouring Pseudomonas exotoxin, cell lines LNCaP and DU145 were described not to express Pgp at significant amounts[21]. Results obtained by Western blotting and immunocytochemistry appear to confirm the latter observation[22; 23]. Other groups were also unable to detect Pgp in clinical prostate cancer or prostate cancer cell lines[24; 25]. Although chemotherapeutic agents, such as vinblastine, doxorubicin and etoposide, are substrates for Pgp, resistance of prostate cancer to these products may not be caused by Pgp as this drug efflux pump is barely if not expressed in prostate cancer. Reversal of multidrug resistance by Pgp targeting chemosensitizers may therefore not be beneficial. In contrast to this, Siegsmund et al.[26] found MDR1 expression at low level in 8 of 11 prostate carcinoma tissues by RT-PCR and advocated the use of Ketoconazole as MDR reversing agent in prostate cancer. This antifungal imidazole has been demonstrated to repress steroid synthesis, to have direct cytotoxic effect on tumour cells and to potentiate the efficiency of chemotherapeutic drugs as it blocks Pgp. According to the authors, this agent could be useful in relapsing hormone sensitive prostate cancer with its antihormonal and cytotoxic characteristics and its potency to revert Pgp-mediated resistance. However, Pgp expression in the prostate cancer samples in this study is questionable, as with the method applied it is not possible to distinguish epithelial MDR1 mRNA expression from stromal MDR1 expression. Furthermore, functional involvement of MDR1 was not proven.

Chemosensitization

Much effort has been put in chemosensitization of cancer cells expressing the MDR1 phenotype. Drug-resistant cells have been exposed to a variety of alternate substrates for Pgp, which ideally in themselves are only slightly cytotoxic, if at all. Pgp modulators, which include calcium channel blocking agents, calmodulin

antagonists, cyclic peptides and steroids, interfere with drug binding to Pgp and may be competitive substrates for Pgp-mediated transport. In phase I and II clinical studies of several first-generation MDR modulators, the achievement and maintenance of biologically –relevant concentrations were barely feasible because of the development of adverse effects, such as cardiovascular toxicity with verapamil and immunosuppression with cyclosporin[27; 28]. The first observations were done by Tsuruo et al.[29] who showed that verapamil was able to enhance drug accumulation of vincristine and vinblastine in the P388/VCR drug-resistant cell line *in vitro* and *in vivo*.

The first generation of MDR reverters were existing drugs which appeared to have MDR reversal activity *in vitro*, but they were originally designed for other pharmacological properties. Levels necessary *in vivo* for efficient modulation of MDR could often not be obtained because of prohibitive toxicity. Numerous compounds have been described which efficiently inhibit the Pgp efflux pump[30; 31]: calcium channel blockers (e.g. amiodarone, verapamil), cyclic peptides (e.g. cyclosporine-A), protein kinase C inhibitors (e.g. staurosporine), calmodulin antagonists (e.g. trifluoperazine), steroidal agents (e.g. progesterone, tamoxifen, megestrol acetate), Vinca alkaloid analogues, and miscellaneous compounds (e.g. quinidine).

Analogues of these modulators, that are devoid of these adverse effects are currently in clinical development[33-34]. These new generation of compounds (e.g. dexniguldipine, PAK-200, AHC-52, PSC 833, SDZ 280-446, dexverapamil) are especially selected for MDR reversal activity. They distract the drug-efflux pump, leading to decreased extrusion and increased intra-cellular accumulation of antineoplastic drugs. Efficacy and toxicity of modulators can be evaluated in xenograft-bearing-mouse models of human tumours: multidrug resistant tumours are implanted in mice and the activity of a cytotoxic agent in the presence of a chemosensitizer is assessed.

The potential disadvantage of many chemosensitizers resides in increased toxicity as a result of higher doses of intracellular cytotoxins in normal tissue or cells. Myelotoxicity has often been and remains the dose limiting factor in chemotherapy. Strategies for preventing bone marrow toxicity have therefore become a high priority of research on dose escalation protocols. A promising approach was

the construction of transgenic mice[35]. Constructs of cDNA encoding full-length human MDR1 in a plasmid carrier were injected into fertilized mouse embryos. A homozygous line was obtained in which the expression of the MDR1 gene was limited to the bone marrow and the spleen[36]. The level of MDR1 expression in bone marrow cells was clinically significant and comparable with that seen in an *in vitro* selected cell line (KB-8-5) that exhibits 3- to 18-fold resistance, depending on the drug. Expression of Pgp was of particular significance, because the MDR1 gene expressing bone marrow was protected against chemotherapy induced toxicity in comparison to normal bone marrow. This protective advantage of the MDR1 gene could be transferred to recipient animals by bone marrow transplantation. Subsequently, protection of bone marrow cells by virtue of expression of the drug resistance MDR1 gene was also clinically investigated using a gene therapeutic approach[140]. Due to technical limitations at the gene transduction level, no significant dose escalation of chemotherapy in patients could be detected.

Use of drugs that are not susceptible to the Pgp efflux mechanism (non-MDR drugs) is another option to bypass Pgp mediated MDR. However, tumours do not tend to be sensitive to many different anticancer drugs. Modification of known active MDR drugs at the biochemical level in such a way that they are less sensitive to the Pgp extrusion mechanism, but retain their cytotoxic activity can be done by conjugation to other structures, such as conjugation of doxorubicin or methotrexate to albumin[37; 38]. This results in prolonged intracellular accumulation of the drug and increased cytotoxicity. An alternate method of chemosensitization relies on circumventing, rather than distracting the activity of Pgp via false substrates. Liposomal encapsulation of drugs reduce the binding affinity to Pgp[39]. Liposomes themselves may inhibit the pump function of Pgp, modify the phospholipid membrane structure and subsequently introduce functional and/or steric alterations of Pgp. Alternatively, delivery of drugs encapsulated in liposomes may bypass the cell membrane, directly discharging the drug into the cytoplasm.

Another approach is to use the drug transporter Pgp as target for immunotherapy by monoclonal antibodies. The monoclonal antibody MRK16 specifically binds human Pgp and has moderate direct cytotoxic activity in xenografted MDR tumour models[40]. Anti-Pgp monoclonal antibodies like HYB-241 and MRK16 also act as

chemosensitizers by binding to Pgp, thus changing its action[41; 42]. Alternatively the Pgp related MDR can be challenged by selectively killing cells that express Pgp on their surfaces. This goal can be achieved experimentally by using protein toxins, such as Pseudomonas exotoxin, that are chemically linked to or recombinantly attached to certain anti-Pgp antibodies, such as MRK16[43]. Recently Ihnat et al.[44] reported that application of sub-chemotherapeutic doses of DNA cross-linking agents could be used to modulate multidrug resistance through suppression of P-glycoprotein, prior to treatment with a second cytotoxic agent. These findings should be further explored *in vivo*.

Several studies were performed aiming at chemosensitization of MDR1 driven MDR of prostate cancer. Rat mdr1b mRNA was found in hormone-insensitive sublines of Dunning rat prostate carcinoma cells. Reversal of resistance against Vinblastine and Taxol was achieved with verapamil[45]. Experiments in a three dimensional *in vitro* model for prostate cancer revealed resistance against doxorubicin, expression of Pgp and modulation of resistance with MDR1 modulators[46]. Downregulation of Pgp by reactive oxygen species in multicellular prostate tumour spheroids was observed[47]. In summary, clinical relevance of MDR1 expression in prostate cancer remains questionable and chemosensitization approaches in experimental and clinical prostate cancer models have to be further investigated for a potential benefit in prostate cancer patients .

Figure 2.1 represents a schematic overview of multidrug transporter related multidrug resistance and chemosensitization.

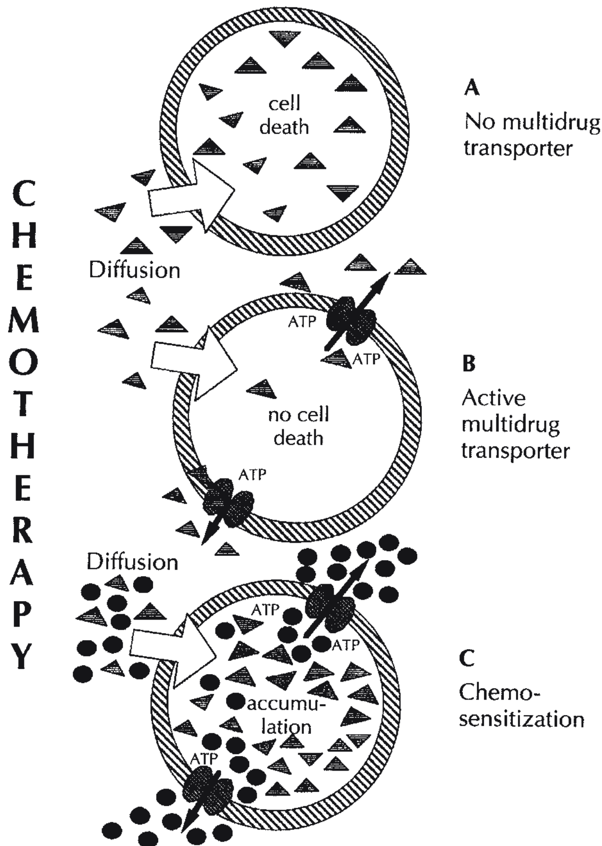


Figure 2.1.

MULTIDRUG-TRANSPORTER-RELATED MULTIDRUG RESISTANCE

Schematic representation of mechanisms of (**A**) action of a chemotherapeutic drug, (**B**) energy dependent extrusion of drugs by an active multidrug transporter, and (**C**) chemosensitization to chemotherapy by distracting the multidrug resistance pump with a chemical compound that competes for active transport with the chemotherapeutic drug. In **A**, chemotherapeutic agents (triangles) enter the cell and cause drug-induced cell death. In **B**, decreased accumulation of the drug through active transport out of the cell by the multidrug resistance pump results in survival of the cell. In **C**, concomitant accumulation of a chemosensitizing agent (black dots) has enhanced intracellular accumulation of chemotherapeutic agents by competing for active transport by the multidrug resistance efflux pump.

Multidrug resistance associated protein (MRP)

The role of the multidrug resistance associated protein in MDR of prostate cancer is the subject of recent and current investigations.

In 1992 the MDR-associated protein (MRP)[48] was found to mediate drug resistance in distinct MDR tumour cell lines lacking Pgp. Since then five more members

of the MRP family have been described[49; 50]. MRP1-3 transport MDR drugs at varying degree and are active as carriers for transport of glutathione S-conjugated endo- and xeno-biotics[51; 52]. The role of MRP4-6 as MDR transporter remains subject of investigation.

Pgp and MRP1 confer resistance to doxorubicin, vincristine, and etoposide, but only Pgp confers high levels of resistance to paclitaxel and cholchicine[53-55]. The mechanism of MRP1-mediated drug resistance is less well defined than that conferred by Pgp. Plasma membrane-associated MRP1 may lead to drug accumulation defects, whereas the presence of MRP1 in the endoplasmic reticulum or post-Golgi vesicles may sequester anticancer drug away from cellular targets[53; 56]. The specific determinants of whether a cell will acquire drug resistance through increased expression of MRP1 are not yet known; however, overexpression of Pgp and MRP is not mutually exclusive and increased levels of both proteins may occur in the same cell line[57].

Whereas Pgp transports substrates in an unmodified form, MRP1 overexpression is associated with an increased ATP-dependent glutathione S-conjugate transport activity [51; 52]. Experiments with inside-out membrane vesicles indicated that MRP1 is able to transport a range of substrates that are conjugated to gluthathione (GSH), glucuronide, or sulfate[51; 54; 58]. Transporters with these characteristics are known as GS-X pumps[57], multispecific organic anion transporters (MOAT; [60]) or leukotriene C4 (LTC4) transporters[61]. Besides the transport of glutathione S-conjugates, it was demonstrated that MOAT's cause transport of amphipathic anti-cancer drug vinblastine. This raises the possibility that MOAT's are ABC-transporters that potentially could be involved in multidrug resistance in mammalian cells. Vinca alkaloids are not known to be converted into negatively charged conjugates, and it is not yet clear how organic anion transporters, such as MRP1 or other MOAT's, transport these compounds. Evidence that GSH is a prerequisite for MRP1-mediated MDR in intact cells was obtained by depleting cells of GSH, which resulted in the loss of resistance to vincristine[62]. Loe et al.[54] showed that high GSH concentrations are required for MRP1-mediated vincristine uptake into vesicles. Furthermore, direct binding of GSH to MRP1 was shown in a vanadate induced trapping experiment[63]. It is not known, however, whether

vincristine is transported as a short-lived complex with GSH, cotransported with GSH, or whether glutathione is only required allosterically[62].

This clearly raises the possibility that mechanisms which interfere with either the leukotriene C4 transport or the glutathione metabolism may also effectively interfere with the MRP1 driven efflux system.

MRP1 expression is found in all major organs analysed including prostate and all cell types from peripheral blood[64]. MRP1 is a Mr.190.000 protein that is encoded by the MRP1 gene, which is located on chromosome 16p 13[48]. Some other members of a putative 21 gene family[65] have been identified[55; 66; 67], some of which show expression in the prostate like MRP1, MRP2 (cMOAT)[67], MRP3 (cMOAT-2) and MRP4 (MOAT-B). Nevertheless, an exact analysis of expression pattern of MRP-genes other than MRP1 is still lacking and will be subject of future studies.

Chemosensitization

To date, strategies aimed at reversing MDR have principally focused on inhibition or modulation of Pgp activity. Various MDR reversal agents have been described that restore intracellular accumulation of drugs in MDR1-overexpressing cells by the inhibition of the drug efflux activity of Pgp[17; 27; 68] .

Compounds with proven MRP1-associated MDR modulating capacity are rare at present. Some agents, however, which originally were found to circumvent a Pgp-mediated MDR effectively show some activity, such as verapamil[69], its L-stereoisomer[70], cyclosporin A and its non-immunosuppressive derivative SDZ PSC 833[69], and dihydropiridines[69]. In addition, depletion of glutathione via the glutathione synthesis inhibitor D,L-buthionine-(S,R)-sulfoximine (BSO)[72], was successful in modulating MRP1 related MDR. Circumvention of MRP1-related MDR was achieved in Etoposide resistant prostate cancer cell lines by a dihydopyridine derivative NIK250[73].

Particular attention is paid, however, to the effective inhibition of MRP1 induced by the leukotriene D4 receptor antagonist MK571[74], a specific MRP1 blocker. MK571 is clinically used for relieving bronchoconstriction in asthma patients [75], and can be safely administered in high doses[76] either intravenously or orally.

Quite recently a similar leukotriene D4 receptor antagonist (ONO-1078) was also described to circumvent MRP1 modulated MDR in cancer cell lines[77]. Zafirlukast, another leukotriene inhibitor, was demonstrated to have MDR modulation potential in prostate cancer cell lines [78]. The compounds MK-571, ONO1078 and zafirlukast would bind to leukotriene C4 and D4 receptors thus interfering with the MRP1 driven efflux of leukotrienes[61]. Functional testing by visualisation of MRP1 mediated efflux via specific fluorescent substances with and without MRP1 modulator MK-571 has also been achieved in acute myeloid leukemia blasts from patients and in several AML cell lines[79; 80]. MRP1 activity was a prognostic factor for achievement of complete remissions suggesting that MRP1 contributes significantly to drug resistance in AML.

In prostate cancer, MRP1 is expressed even in early stages[81]. MRP1 expression increases in late disease stages[23] and is consistently present in all prostate cancer cell lines examined[22]. MRP2 expression has been found in prostate cancer cell lines and xenografts (van Brussel; unpublished results). MRP3 in prostate cancer cell lines[67] and MRP4 in prostate cancer[66]. MRP5 and MRP6 expression in prostate cancer has not been reported thus far. A functional role for the members of the MRP family in prostate cancer is subject of current investigations. Clinically, establishment of the role of MRP in multidrug resistant prostate cancer would offer a potential to apply MRP modulators in combination with chemotherapeutic regimens to treat patients with advanced hormone refractory prostate cancer. To elucidate the role of the multidrug resistance associated protein MRP1in the resistance of prostate cancer against chemotherapy multidrug resistant *in vitro* prostate cancer models were developed from the human prostate cancer cell lines PC3 and DU145 [78]. Increased expression of MRP1 in multidrug resistant prostate cancer cells is functionally related to resistance against chemotherapy. Effective blockade of MRP1 pump function by leukotriene receptor antagonists, such as MK-571 and Zafirlukast, resulted in the intracellular accumulation of MRP1 substrate and increased sensitivity to cytotoxic drugs. This provides some evidence for a functional role of MRO1 in drug resistance of prostate cancer, however, results have to be interpreted cautiously. MRP homologs other than MRP1 and other MDR mechanisms may well be involved in the MDR phenotype of prostate cancer cells.

Studies on functional involvement of MRP2-6 in prostate cancer will be initiated. Furthermore, the role of MRP1 in clinical prostate cancer requires further study. Also, approaches to inhibit MRP1 clinically must be carefully designed, because MRP1 is ubiquitously expressed[49]. Fine-tuning of dosages schedules of chemotherapy and reversal agent is necessary to limit toxicity. For instance, possible myelotoxicity may be reduced by chemoprotection of the bone marrow, achieved with retrovirus-mediated gene transfer of MRP cDNA[82].

GLUTATHIONE

Several studies have demonstrated that glutathione is an important factor in the sensitivity of cancer cells to cytotoxic drugs. Glutathione and glutathione-S-tranferase detoxification systems protect cancer cells against the lethal effects of chemotherapy. Changes in the anticancer drug detoxifying anti-oxidant glutathione metabolism[83] and in expression or activity of Glutathione-S-transferase intracellular transport proteins (GST) have been described in relation to MDR. GSTs catalyze the conjugation of hydrophobic, electrophilic xenobiotics by glutathione, resulting in conjugated or transformed metabolites that are more easily excretable and less toxic. Some GST isozymes may participate in the repair of oxidative damage to membrane lipids and DNA. Furthermore, GSTs are high capacity intracellular binding proteins which may serve in the storage, transport, or sequestration of many hydrophobic compounds. The relation to the multidrug resistance associated protein has been extensively reported (see above, MRP section). These properties suggest that GSTs may function as important cellular defences against the cytotoxic effects of antineoplastic agents.

Interestingly, expression of GST-π, a detoxifying enzyme and part of the glutathione detoxifying pathway, is significantly increased in advanced hormone independent prostate cancer[23]. Expression of GST-π in advanced clinical prostate cancer is a novel finding. Previous studies have only reported the lack of its expression in locally confined prostate cancer obtained from radical prostatectomy samples [81; 84; 85] and suggested a role of GST-π inactivation in the early steps of prostatic carcinogenesis[86]. Accordingly, in our series of tumours locally confined prostatic

carcinomas were entirely GST-π negative, whereas the disseminated cancers clearly expressed GST-π, especially in hormone-independent progressive disease[23]. Concomitant overexpression of MRP and GST-π may synergistically result in increased drug resistance of advanced prostate cancer.

Another study reports expression of drug-metabolizing enzymes cytochrome P450 subfamilies CYP1A, CYP2C, CYP3A, GST-α and GST-μ[85] in prostate cancer.

Chemosensitization

In prostate cancer reversal of MDR by challenging the glutathione pathway has been successfully performed *in vitro* in only few studies. Ripple et al.[87] reported reversal of melphalan resistance of MDR LNCaP sublines by depletion of glutathione with D,L-buthionine-(S,R)-sulfoximine (BSO). Van Brussel et al. demonstrated chemosensitization of MDR PC3 and DU145 derivatives with a similar approach [78]. Further *in vivo* and eventually clinical studies are warranted to clarify the importance of glutathione-aiming strategies in prostate cancer.

Reversal of MDR that is related to increased glutathione- and glutathione-S-transferase content and activity can be achieved by lowering intracellular concentrations of GSH by blocking the GSH biosynthesis with glutathione synthesis inhibitors, such as diethylmaleate, vitamin K3, sodiumselenite and BSO. BSO, which does not inhibit the glutathione-S-transferase but gamma-glutamyl-cystein-synthetase, occurring earlier in the GSH metabolism, may be advantageous as it has fewer neurotoxic effects. BSO has been used successfully *in vivo*[70] and clinically in a phase I trial[88]. A putative role for the glutathione metabolism in prostate cancer is of importance, especially in the context of MRP expressing prostate cancer. Chemosensitization strategies combining GSH depletion and MRP blocking are the subject of current investigations and may ameliorate the results of chemotherapy in clinical prostate cancer.

Figure 2.2 represents a schematic overview of glutathione-synthesis-related multidrug resistance.

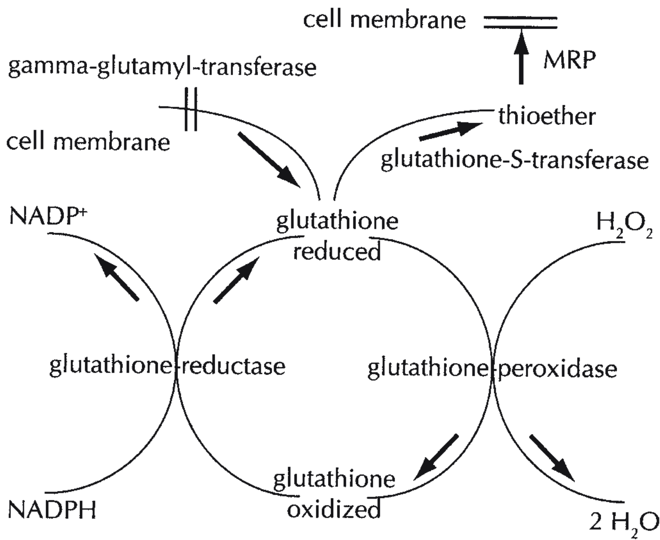


Figure 2.2.

Glutathione-synthesis-related multidrug resistance

Model of the glutathione-mediated detoxification pathway. Oxidizing compounds are conjugated to reduced glutathione. Glutathione-S-transferases mediate the energy-dependent extrusion of cytotoxic compounds by the multidrug resistance associated protein, MRP. Different approaches can be applied to reverse multidrug resistance. Detoxification can be blocked by depleting intracellular glutathione, with, for example, buthionine sulfoximine (BSO). BSO irreversibly inhibits the gamma-glutamyl-cysteine synthetase enzyme, which is essential for synthesis of glutathione. Another approach would be to inhibit the action of MRP. Such strategies will eventually lead to intracellular accumulation of cytotoxic drugs, resulting in cell death. NADP+, nicotinamide adenine dinucleotide phosphate, oxidized form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form.

APOPTOSIS

The phenomenon of apoptosis, also called programmed cell death, has received a great deal of attention. It is widely appreciated that changes in the regulatory processes of apoptosis contribute to the malignant phenotype of cancer cells. Importantly, as many cytotoxic agents act through induction of apoptosis, alterations in apoptotic processes contribute to the multidrug resistant phenotype of malignancies[89]. Many molecular regulators of cell death have been discovered and several mechanisms have been identified in prostate cancer. Alterations of proteins regulating the apoptotic cascade, such as Bcl-2[90] and Bax[91] may cause a broad

spectrum of drug resistance. Upregulation of Bcl-2 and/ or downregulation of its counterpart Bax prevent cells from entering programmed cell death. Wild-type p53 is a suppressor of cell growth and transformation, causing a proliferation-inhibiting G1 block and regulating apoptosis. Mutation of the p53 gene is the most common molecular alteration in cancers resulting in decreased apoptosis and possibly resistance to apoptosis-inducing cytotoxic agents[92]. Bcl-2, which has been identified as a powerful inhibitor of apoptotic cell death, appears to be expressed at low levels in low grade prostate carcinoma and at higher levels in high grade tumours[93]. Accordingly, Bcl-2 expression was increased in clinical hormone independent prostate cancers as compared to organ confined cancers[23]. Bcl-2 expression in prostate cancer has previously been related to the androgen independent phenotype[94; 95]. Relatively high Bcl-2 levels may promote cell survival and protect androgen independent prostate cancer cells from drug-induced apoptosis[96-98]. Bax, a pro-apoptotic protein, has been found to be well expressed in prostate cancer [93]. In a study on clinical prostate cancer Bax is equally and homogeneously expressed in normal prostate and prostate cancer cells in all phases of progression[24]. Correlation between p53 mutations and tumour progression has been presented in several studies, with mutations occurring as a late event in the development of prostate cancer[99-101]. This is in concordance with our finding of more frequently occurring p53 expression in advanced disease[23], which may explain the decreased susceptibility to apoptosis-inducing agents. We observed a significantly increased number of p53 positive patients in hormone independent cancers. Also, p53 expression was statistically significantly related to a higher histological grade. These findings should be interpreted cautiously as immunohistochemical expression of p53 does not necessarily represent p53 mutation[102]. Interestingly, transfection of a temperature sensitive p53 mutant into the LNCaP human prostate cancer cells produced a time-dependent increase in MRP1. Mutant p53 and increased MRP1 were related to drug accumulation and decreased drug sensitivity[103] . Apart from bcl-2 and p53, several other molecular alterations have been documented in prostate cancer, as reviewed by Bruckheimer [104] involving the androgen receptor and cell-cycle regulatory genes such as the retinoblastoma suppressor gene, c-myc and p21. Ras gene family mutations may

contribute to the pathogenesis of prostate cancer. Loss of heterozygosity and alterations in newly identified tumour suppressor genes such as PTEN/MMAC1 may influence apoptosis in prostate cancer. The implication of these genetic changes for MDR of prostate cancer remains to be determined. Another important factor in the apoptotic chain is Fas, a member of the nerve growth factor/tumour necrosis factor receptor superfamily. It is present in benign and malignant prostate[105] and induces cell death. Disruption of the Fas/Fas ligand signaling pathway may result in acquired MDR[105]. Activation of protein kinase C (PKC) induces apoptosis in prostate cancer cells [106] and therefore pharmacological manipulation of PKC may be of interest in MDR prostate cancer.

Chemosensitization

Countering of Bcl-2 related MDR may become applicable in the future possibly by the development of small-molecule pharmaceuticals that can disrupt Bcl-2/Bax interactions and thus abrogate Bcl-2 protein function[107]. Sequence-specific down-regulation of Bcl-2 expression has been reported *in vitro* in lymphoma cell lines and AML cells using synthetic antisense oligonucleotides, showing that decreased expression of the Bcl-2 protein can result in markedly enhanced sensitivity to chemotherapeutic drugs[108]. Antisense approaches to down-regulation of Bcl-2 function in prostate cancer are feasible. Reduction in Bcl-2 protein levels in LNCaP cells by Bcl-2 antisense oligonucleotides resulted in enhanced sensitivity to the cytotoxic drug etoposide[109]. Another study treated Shionogi tumour cells with antisense bcl-2 oligonucleotides resulting in downregulation of bcl-2 expression and chemosensitization to taxanes in this model for androgen independent prostate cancer[110]. These findings demonstrate that agents that diminish Bcl-2 levels could possibly be used as chemosensitizers in the treatment of prostate cancer. Alternatively, it has been observed that certain lymphokines and retinoids can regulate the expression of the Bcl-2 gene and sensitize to antineoplastic drugs (reviewed by Reed[90]), rendering cancer cells more prone to induction of apoptosis by chemotherapeutic drugs. Paclitaxel, vinblastine and docetaxel induces bcl-2 phosphorylation-associated apoptosis in bcl-2 expressing PC3 prostate cancer

cells [111; 112]. Other chemotherapeutic agents, such as mitomycin, actinomycin D, doxorubicin and etoposide do not induce bcl-2 phosphorylation[113]. The combination of 13-cis-retinoic acid (CRA) and interferon-α (IFN-α) is cytotoxic in tumour cells that overexpress bcl-2 and reduces the expression of bcl-2, overcoming bcl-2 mediated resistance [114; 115]. Clinical studies on this regimen combined with taxanes are currently underway[118]. Bax, Akt/P13K, BH3 peptides, Fas, NF-κB are targets in the apoptotic pathway that are under investigation[117]. With increased knowledge of apoptosis at the molecular level, it may be possible to develop novel approaches for cancer therapy that specifically aim at modulating the physiologic programmed cell death pathway. Apoptosis is likely to play an important role in prostate cancer and the development of apoptosis stimulating techniques may be of particular importance for the treatment of advanced prostate cancer.

Figure 2.3 represents a schematic overview of Bcl-2 related multidrug resistance.

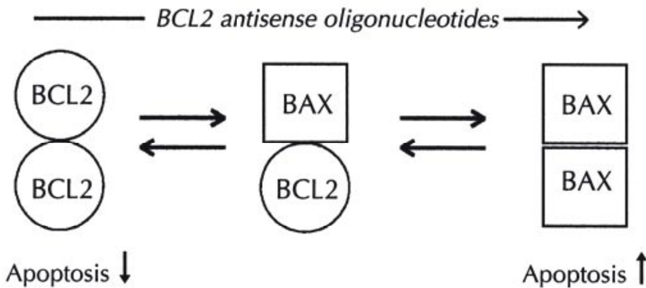


Figure 2.3.

Bcl-2 related multidrug resistance.

Scheme of modulation of apoptosis (programmed cell death). Bcl-2 and bax form heterodimers. The ratio of Bcl-2 qnd bax determines the relative sensitivity of cells to apoptosis induced by chemotherapy. When the balance is shifted to Bcl-2, cell death is less likely to occur. Excess of bax leads to induction of apoptosis. Reversal of inhibition of apoptosis by increased levels of Bcl-2 can be achieved by Bcl-2 antisense oligonucleotide treatment. This results in enhanced apoptosis induced chemotherapy.

TOPOISOMERASES

The nuclear topoisomerase enzymes are targets for several cytotoxic drugs. Topoisomerases are involved in multidrug resistance through down-regulation of ex-

pression or decreased enzymatic activity[118].This type of resistance was termed altered- or attenuated topoisomerase MDR[119] (at-MDR).

Changes in expression and activity of DNA topoisomerases may underlie the multidrug resistance phenotype of prostate cancer and further investigations will have to clarify this issue. Topoisomerase isoforms are at least partially regulated by testosteron in rat ventral prostate and may play a role in cellular proliferation[120]. Topoisomerase IIα expression is correlated with Gleason score in clinical prostate cancer. In our studies on clinical prostate cancer[23] expression of Topo IIα is significantly increased in progressive disseminated prostate cancer and tumours with a higher histological grade. The Topo IIα enzyme is known to be related to cell proliferation as well[121]. We found strong correlation between Topo IIα expression and expression of the proliferation marker Ki-67. This marker was demonstrated to be significantly increased in the group of advanced prostate cancers and in tumours with a higher histological grade. A relation between proliferative activity and a more progressive phase of disease has been reported previously[122-124]. Increased expression of Ki-67 and Topo IIα in advanced prostate cancer possibly reflects the more aggressive character of these tumours. Expression of the isoforms topo I and Topo IIβ is high and homogeneous in every phase of prostate cancer progression and is slightly increased in advanced disease. Topo IIβ negative tumours had a significantly lower histological grade. Increased expression of topoisomerases does not directly point towards a role for at-MDR in advanced prostate cancer. High expression of topoisomerases in end-stage prostate cancer may favour the introduction of topoisomerase targeting agents. However, efficacy of such drugs may be hampered by increased drug efflux and detoxification through MRP and GST-π and by inhibition of apoptosis through the presence of p53 mutations and an increased Bcl-2/Bax ratio.

Chemosensitization

Sensitivity of the cancer cells to topoisomerase targeting drugs is thought to be related to the level- and activity of topoisomerase in the nucleus and the relation between Topoisomerase quantity and -activity and drug resistance has been shown

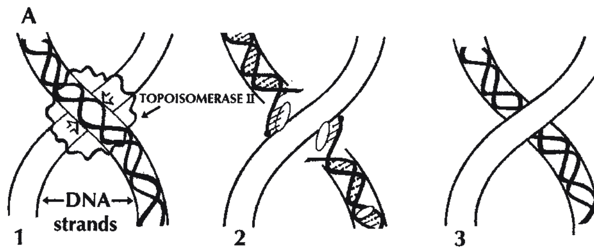
in several tumours[125; 126]. Several options for challenging Topoisomerase related drug resistance and improvement of topoisomerase directed chemotherapy have been proposed: increasing efficacy of established topo directed drugs by prolonged drug exposure or combination therapy and modulations leading to upregulation of topoisomerase levels and activity resulting in increased susceptibility to topoisomerase targeting drugs. Selection of a prostate cancer cell line for 9-nitrocamptothecin resistance resulted in altered topoisomerase IIα activity and increased sensitivity to etoposide[127]. Topoisomerase I and II targeting drugs have been applied in prostate cancer therapy. Pienta et al.[128] reported inhibition of prostate cancer growth by estramustine and etoposide (a topoisomerase II drug), acting synergistically at the nuclear matrix. Topotecan (a topo I drug) had limited activity in patients with hormone refractory prostate cancer. Alternative schedules of topotecan (e.g., prolonged infusion) or other camptothecin analogs with more potent topoisomerase inhibitory activity should be investigated[129]. Husain et al.[130] reported elevated Topo I quantity and activity in samples of prostatic adenocarcinoma compared to normal tissue, implicating that Topoisomerase I may be a feasible target for chemotherapy and that Topoisomerase I directed drugs may be selectively cytotoxic to prostate cancer cells. Currently chemotherapy with mitoxantrone and prednisone is applied in patients with symptomatic hormone-resistant prostate cancer to provide palliation. Two studies using mitoxantrone and a glucocorticoid demonstrated this regimen to be importance in prostate cancer patients. In the first study[131], 38% of the mitoxantrone plus prednisone group had a palliative response versus 21% of the group receiving prednisone alone. There was no significant difference in median survival between the two groups. In 39% of the patients with available PSA data the mitoxantrone arm had evidence of response. In the second study[132] there was improvement in pain control in the chemotherapy arm as well as a delay in time-to-treatment failure and disease progression in the chemotherapy arm, but no difference in overall survival between the two arms.

Alternatively, estramustine and either a microtubule inhibitor such as paclitaxel or docetaxel, or a topoisomerase inhibitor such as etoposide is applied to treat patients with advanced metastatic prostate cancer[133]. Estramustine consists of

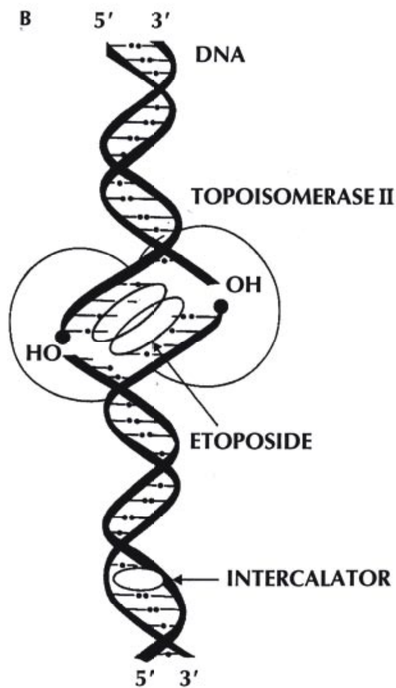
nornitrogen mustard bound to estradiol and was initially developed to deliver the nornitrogen mustard to estrogen receptor-positive prostate tumour cells. It was found to work primarily by inhibiting microtubules synergistically with taxanes or vinca alkaloids. Combination with taxanes appears to be the first choice. Estramustine has also been examined in combination with etoposide, a topoisomerase II inhibitor[134]. It shows a 50% tissue response and a 52% PSA response. A three-drug regimens was also studied combining estramustine, etoposide, and paclitaxel, 45% of patients had a measurable disease response and 65% had a PSA response[135]. Two recent randomized clinical trials have reported on a two to four month survival benefit with combination therapies of docetaxel and prednisone or docetaxel and estramustine as compared to topoisomerase I inhibitor mitoxantrone and prednisone, respectively. Taxane docetaxel, phosphorylates Bcl-2 *in vitro*. This results in its inactivation and, eventually, to programmed cell death[136]. Interestingly, anti-apoptotic Bcl-2 is increased in advanced prostate cancer. Docetaxel was studied earlier *in vivo* on doxorubicin resistant multidrug resistance protein expressing HT1080/DR4 tumour xenografts (sarcoma). It showed significant anti-tumour activity and it was suggested that docetaxel may not as readily be transported by the multidrug resistance protein as paclitaxel. Therefore, docetaxel potentially has therapeutic advantages in the treatment of multidrug resistance protein expressing tumours[137].

Topoisomerase drug action finally triggers mechanisms leading to programmed cell death or apoptosis. Factors that inhibit apoptosis include p53 mutation and /or Bcl-2 overexpression and can at least partially be responsible for the ineffectiveness of several topoisomerase directed cytotoxic agents in prostate cancer. However, several authors[138; 139] reported that β-Lapachone effectively induced apoptosis in prostate cancer cell lines and that apoptosis is independent of p53 expression. Furthermore, overexpression of Bcl-2 did not confer significant resistance to β-Lapachone. Altogether, β-Lapachone may be of importance for the treatment of advanced prostate cancer. However, no clinical data on this approach have been published until now.

Figure 2.4 represents a schematic overview of attenuated- or altered topoisomerase mediated multidrug resistance (at-MDR).



2.4a



2.4b

Figure 2.4.

Attenuated- or altered topoisomerase mediated multidrug resistance (at-MDR)

(A) The mode of action of topoisomerase enzymes: 1) binding to and cleaving of strands of DNA; 2) passage of another strand of DNA through the gap; 3) resealing of the broken DNA. The conformational changes of DNA are essential for many nuclear processes. (**B**) A topoisomerase-targeting drug can either irreversibly stabilize complexes of toposiomerase and the broken DNA (as done by etoposide), or directly inhibit the enzymatic action of topoisomerase (as done by an intercalator). Approaches to increase the efficacy of anti-topoisomerase poisons are prolonged drug exposure, combination therapy and modulations resulting in upregulation of topoisomerase levels and activity, which increases susceptibility to targeting drugs. Using drugs that directly inhibit topoisomerase catalytic activity when at-MDR is due to low topoisomerase expression is another option.

CONCLUSION

Metastatic hormone-independent prostate cancer remains a major oncological challenge. However, basic and clinical research have provided insight into underlying mechanisms of drug resistance and further study of possibilities to circumvent this resistance, innovative use of established drugs and development of more potent antineoplastic agents will remain of the utmost importance. Modulation of MDR in prostate cancer is the subject of current studies and promising results were obtained *in vitro* and *in vivo*. Clinical relevance of modulation strategies are and will be the subject of investigation. Attempts at challenging progressive hormone independent prostate cancer more successfully are still experimental and should be carried out within the setting of controlled clinical studies.

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CHAPTER 3

Chemosensitivity of prostate cancer cell lines and expression of multidrug resistance related proteins

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Eur J Cancer 1999;35:664-71

SUMMARY

Purpose: The aim of this study was to obtain insight in the role of the multidrug resistance (MDR) phenomenon in hormone-independent progressive prostate cancer.

Material and methods: Using immunocytochemistry and Western blotting we determined expression of P-glycoprotein (Pgp), Multidrug resistance associated protein (MRP), Glutathione-S-transferase-π (GST-π), Bcl-2, Bax, Topoisomerase (Topo) I, IIα and IIß in the human prostate cancer cell lines PC3, TSU-Pr1, DU145 and LNCaP derivatives -R, -LNO and -FGC. Proliferative activity was assessed by immunocytochemistry. MTT assays were used to determine the sensitivity to Etoposide, Doxorubicin and Vinblastine.

Results: Pgp was not expressed in any of the cell lines. MRP was variably expressed. GST-π was expressed in TSU-Pr1, PC3 and DU145. Expression of Bcl-2 was restricted to TSU-Pr1, whereas Bax was found in all cell lines. Topo IIα was expressed at the highest level in the rapidly proliferating cell lines TSU-Pr1 and DU145. Topo I and IIß were equally expressed. Resistance profiles varied among the cell lines, with TSU-Pr1 being the most sensitive and LNCaP-LNO relatively resistant.

Conclusions: Multiple MDR proteins are expressed in prostate cancer cell lines and may well influence response to chemotherapy. Future functional studies, using chemo-selected MDR models, may further help to determine the mechanism or combination of mechanisms underlying the resistance of prostate cancer to chemotherapy.

INTRODUCTION

Prostate cancer is the most common cancer in men and one of the leading causes of cancer death [1]. Metastatic disease is a major oncological problem and is not curable, but palliation can be achieved by hormonal deprivation therapy [2]. However, prostate cancer patients will relapse at some stage in time, in majority within one to two years after start of endocrine therapy. After failure of hormonal therapy, there are no successful treatment strategies available with respect to prolongation of survival and patients will die of progressive, hormone independent disease after an average period of 40 weeks after relapse [3].

Hormone-independent prostate cancer is resistant to a broad range of anti-neoplastic agents [4], which may be caused by the fact that large proportions of prostate cancer cells are in interphase [5]. Also, multidrug resistance (MDR), the resistance of cancer cells to a variety of structurally and functionally distinct cytotoxic agents, may play an important role in progressive therapy resistant prostate cancer. However, the role of MDR in prostate cancer remains to be elucidated. A better understanding of mechanisms underlying in the resistance of prostate cancer to chemotherapy may lead to novel approaches to challenge hormone unresponsive prostate cancer more successfully.

We determined the expression of eight multidrug resistance associated proteins in prostate cancer cell lines derived from patients with progressive disease: the drug transporter molecules P-glycoprotein (Pgp) [6] and Multidrug Resistance associated Protein (MRP) [7], the detoxifying enzyme Glutathione-S-Transferase-π (GST-π) [8], modulators of apoptosis Bcl-2 [9] and Bax [10] and the enzymes Topoisomerase (Topo) I, IIα and IIβ, which are related to one form of MDR: atypical- or attenuated MDR [11].

Furthermore, we measured response of the cell lines to treatment with the cytotoxic agents Etoposide, Doxorubicin and Vinblastine, which are commonly used in the treatment of several malignancies and have been used to challenge prostate cancer in clinical trials.

MATERIALS AND METHODS

Cell lines and culture conditions

The human prostate cancer cell lines PC3 [12], TSU-Pr1 [13], DU145 [14] and LNCaP sublines that were established from the original LNCaP line [15]: the androgen dependent FGC (available through the ATCC), the hormone unresponsive R line [16] and the hormone independent LNO line [17] were maintained at 37oC in 5% CO2-95% air atmosphere. PC3, TSU-Pr1, DU145, LNCaP-R and LNCaP-FGC were cultured in RPMI 1640 medium, supplemented with 7,5% foetal calf serum (FCS), penicillin 1 unit/ml, streptomycin 1 µg/ml and glutamine 2 mM (GibcoBRL). The culture medium for LNCaP-LNO contained 5% Dextran coated charcoal (DCC) treated (androgen depleted) serum, instead of FCS. All cell lines were repeatedly tested for Mycoplasma by polymerase chain reaction and proven to be free of infection.

Antibodies

For detection of Pgp the mouse monoclonal antibody JSB1 (Sanbio, the Netherlands) was used. MRP was detected with the rat monoclonal antibody MRPr1 (Sanbio), GST-π with the rabbit polyclonal antibody NCL-GST-π (Novocastra, United Kingdom), Bcl-2 with mouse monoclonal antibody Bcl-2 (100) (Santa Cruz Biotechnology, Inc, CA, USA), Bax with rabbit polyclonal antibody Bax (P-19) (Santa Cruz Biotechnology), Topo I with the human Scl-70 antibody (Topogen, Inc, Columbus, Ohio), Topo IIα with the mouse monoclonal antibody Ki-S1 (Boehringer Mannheim, Germany). Topo IIβ was detected with a polyclonal rabbit antibody (Biotrend, Köln, Germany) for Western blotting and the monoclonal mouse antibody 3H10 (a kind gift of Dr. I. Hickson, Cambrigde, United Kingdom) for immunocytochemistry. BrdU was detected with the mouse monoclonal antibody 2B5 (Eurodiagnostics, the Netherlands). Horseradish peroxidase (HRP)-conjugated secondary antibodies were all purchased from Dako (Glostrup, Denmark).

Antineoplastic drugs

For *in vitro* testing, drug solutions were freshly prepared in culture medium. The following compounds were tested: Etoposide (VP-16) (Pharmachemie BV, Haarlem, the Netherlands), Doxorubicin (DOX) (Farmitalia Carlo Erba, Italy) and Vinblastine (VBL) (Lilly, France).

MTT assay

Sensitivities of all cell lines to the antineoplastic drugs were assessed by the MTT assay [18]. Cell lines were plated in 96-well plates (Costar Corp. Cambridge, MA, USA) at densities allowing logarithmic growth throughout the experiments. After allowing cells to attach for 24 hours, proliferating cells were incubated with culture medium containing a range of exponentially increasing concentrations of cytotoxic agents for a period of 72 hours. Subsequently, 30 µl of MTT (Sigma Chemical Co., St. Louis, MO, USA) solution (5 mg/ml in PBS) was added to each well and incubated for 4 hours at 37oC. The supernatant was carefully aspirated and 100 µl of dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany) with alkalinizing buffer (0.1 M Glycin, 0.1 M NaCl, pH 10.5) was added to each well. The plates were shaken for 5 minutes in order to dissolve the formazan crystals. The absorbance was measured photometrically at 570 nm using a Bio-Rad Microplate Reader (Model 450, Bio-Rad, CA, USA). The percentage of viable cells was calculated relative to untreated cells. All assays were done in triplicate. IC50 values for the separate drugs were calculated from the dose response curves by interpolation. Standard errors were calculated from the square root of the variance as determined with the Delta method [19].

Western blotting

Whole cell lysates were prepared from near-confluent cell cultures (1 x 106 cells). Separate optimalized protocols were used for preparation of lysates for detection of Pgp and MRP, GST-π, Bcl2 and Bax and for Topoisomerase I, IIα and IIß. For topoisomerase detection cells were scraped and resuspended in lysis buffer (10

mM Tris-HCl, 1 mM MgCl2, 0.1 mM CaCl2, pH 7.8) containing protease inhibitors (phenylmethylsulfonylfluoride 1mM, benzamidine 1 mM, Soy bean trypsin inhibitor 10 µg/ml, leupeptin 50 µg/ml, pepstatin 1 µg/ml, aprotinin 20 µg/ml; all from Sigma) and put on ice for 20 minutes. Then 120 units of DNase I (Sigma) were added and incubated for 20 minutes at 37oC. For Bcl-2 and Bax detection cells were scraped and resuspended in another lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X100, 1% deoxycholate, 0.1% SDS, 5 mM EDTA, pH 7,4) supplemented with the fore-mentioned protease inhibitors. The suspension was kept on ice for 15 minutes, subsequently centrifuged (14000 rpm) for 20 minutes at 4oC, whereafter the supernatant was recovered. For Pgp and MRP detection harvested cells were resuspended in lysis buffer (10 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl, 0.5 (w/v) SDS, pH 7.4) supplemented with protease inhibitors. DNA was sheared by sonification. For GST-π, near-confluent cultures where trypsinized and homogenized in 2.5 mM Tris-HCl buffer (pH 7.5) containing 0.25 mM EDTA, 1.25 mM EGTA and 31.3 mM sucrose. After two serial centrifugation steps of 10 minutes at 1000 g and 1 hour at 105000 g the supernatant was recovered. Protein concentrations were measured (Bio-Rad Protein assay, Bio-Rad). All cell lysates were stored at -80oC. Whole cell lysate samples (40 µg protein) with Laemmli buffer [20] (15 µl) were fractioned onto a 10% SDS-polyacrylamide gel after boiling for 2 minutes (except the Pgp and MRP lysates) and electrophoresed for 45 minutes at 200 volts. Prestained markers (Novex, San Diego, CA, USA) were used as size standards. Proteins were transferred to a nitrocellulose membrane (Protan Nitrocellulose, Schleicher & Schuell, Dassel, Germany) by electroblotting at 100 volts. Membranes were blocked for 1 hour with Tris buffered saline (TBS, pH 7.5) containing 1% blotting substrate (Boehringer Mannheim). Separate membranes were incubated overnight at 4oC with antibodies directed to the specific MDR proteins. After washing with TBS containing 0.1% Tween 20 (Sigma), the membranes were incubated with the appropriate peroxidase-conjugated secondary antibodies (see antibodies section in materials and methods). Protein bands were visualized by chemiluminescence. After incubation with detection solution (Boehringer Mannheim) X-ray film (Fuji photo film, Tokyo, Japan) was exposed and developed.

Immunocytochemistry

Cells were grown on 3-aminopropyl-trietoxysilane (Sigma) coated glass slides until semi-confluent cultures were obtained. For BrdU staining, cells were incubated with bromo-deoxy-uridine (BrdU, Sigma) for 2 hours. After fixation in acetone (Pgp), 3.7% paraformaldehyde (MRP, GST-π, Bcl-2, Bax, Topo I, IIα and IIβ) or 70% ethanol (BrdU), endogenous peroxidase activity was blocked with 1% hydrogen peroxide (Merck) in 100% methanol. For BrdU detection, slides were incubated with 2N HCl followed by 0.1M Borax buffer, whereafter the slides were placed in a humid incubator (Sequenza, Shandon, United Kingdom) and incubated with normal goat- or rabbit serum (Dako) diluted 1:10 in PBS/ 5% bovine serum albumine (Sigma). Subsequently, slides were incubated overnight with antibodies directed against Pgp, MRP, GST-π, Bcl-2, Bax, Topo I, Topo IIα ,Topo IIβ and BrdU, respectively. For Pgp-, Bcl-2, Topo IIα and Topo IIβ the peroxidase-anti-peroxidase (mouse-PAP, Dako) method was applied to increase staining sensitivity. Slides were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (all from Dako) and the antigen-antibody binding was visualized with 0.075% 3,3'-diaminobenzidine-tetrahydrochloride (Fluka, Germany) and 0.25% hydrogen peroxide in PBS. The slides were counterstained with hematoxylin, dehydrated and covered. Negative controls were included for all stainings by replacing the primary antibody by PBS.

RESULTS

Chemosensitivity assay

Drug-induced inhibition of human prostate cancer cell growth by the anticancer agents Etoposide, Doxorubicin and Vinblastine was established *in vitro*. Growth of the cell lines during the assay, defined as the optical density (OD) value after 72 hours divided by the OD value after plating of the cells, was 5.4, 2.6, 3.3, 1.5, 3.3 and 3 for TSU-Pr1, LNCaP-FGC, PC3, LNCaP-LNO, LNCaP-R and DU145, respectively.

This indicates that drug testing was performed under the condition of cell lines in proliferative growth. Dose response curves of each cell line for the three drugs tested are shown in **figure 3.1** and IC50 values are given in **Table 3.1**. The cell lines showed a variable response to a 72-hour exposure to Etoposide: TSU-Pr1 was the most sensitive, followed by DU145 and LNCaP-FGC. PC3 had an intermediate pattern of response, whereas LNCaP-LNO and -R were relatively insensitive (fig 3.1a). The sensitivity profiles of the cell lines with Doxorubicin were similar to those found for Etoposide, as shown in figure 3.1b. The MTT assay with Vinblastine (fig 3.1c) showed that TSU-Pr1 is the most sensitive followed by PC3 and DU145. The LNCaP derivatives were less sensitive: LNCaP-FGC was nearly as sensitive as DU145, but LNCaP-R was two-fold more resistant and LNCaP-LNO was relatively insensitive to Vinblastine.

Table 3.1. IC50 values of human prostate cancer cell lines as determined with the MTT assay.*

	TSU-Pr1	LNCaP-FGC	PC3	LNCaP-LNO	LNCaP-R	DU145
Etoposide (µg/ml)	0.48 ± 0.05	1.9 ± 0.14	6.1 ± 0.15	21 ± 0.4	16 ± 0.6	3.6 ± 0.1
Doxorubicin (ng/ml)	7.2 ± 0.2	16.7 ± 0.2	176 ± 7.4	272 ± 4.5	222 ± 10	41 ± 2.5
Vinblastin (ng/ml)	0.5 ± 0.01	1.8 ± 0.2	0.8 ± 0.02	35 ± 2	2.4 ± 0.2	1.2 ± 0.1

* IC50 values are calculated by interpolation, standard errors of the mean are calculated by taking the square root of the variance of the IC50 value as determined by the Delta method [19].

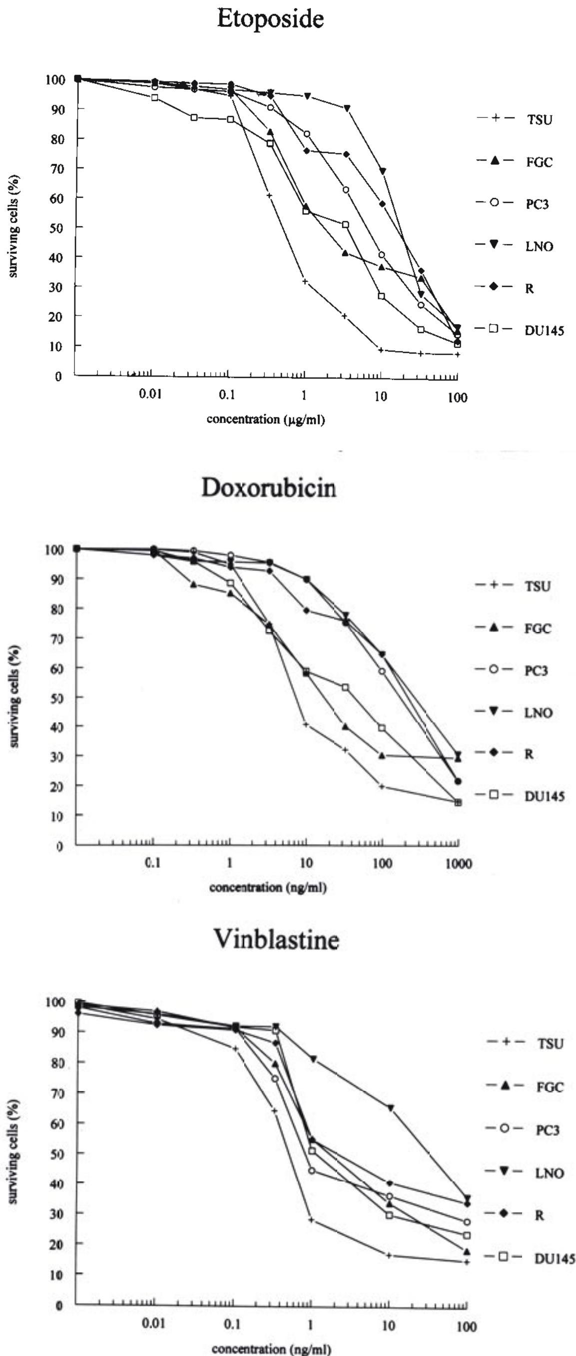

Figure 3.1. Dose response curves of human prostate cancer cell lines TSU-Pr1, LNCaP-FGC, PC3, LNCaP-LNO, LNCaP-R and DU145 after 72 hours of exposure to (a) Etoposide, (b) Doxorubicin and (c) Vinblastine. Values are given as percentage of the untreated controls. Y-axis, percentage of viable cells as calculated relative to untreated controls (n=24), x-axis, concentrations of the drug solutions on a log scale.

Western Blotting

Pgp, MRP, GST-π, Bcl-2, Bax, Topo I, IIα and IIß were detected by immunoblotting of total cell lysates. All experiments were performed in triplicate and reproducible results were obtained. Representative protein bands are shown in **figure 3.2**. Expression of Pgp was below detection level in all cell lines. The Pgp positive, multidrug resistant cell line RC21E [21], served as positive control and was run on the same gel. MRP was variably expressed, but at much lower level than in the control sample, the human multidrug resistant lung cancer cell line GLC4/ADR [22] (not shown). GST-π is clearly expressed in the cell lines TSU-Pr1, PC3 and DU145 , whereas the LNCaP-FGC, -LNO and -R had no detectable levels of GST-π, with the colon carcinoma cell line HT-29 [23] serving as positive control. Expression of Bcl-2 was restricted to TSU-Pr1, whereas Bax was found in all cell lines. Bcl-2 expression in TSU-Pr1 was low in comparison to Chinese hamster ovary cells transfected with Bcl-2 (kindly provided by Dr H. Burger, Department of Oncology, Erasmus University, the Netherlands), which was run on the same gel. The topoisomerase proteins were expressed in all cell lines. The topoisomerase IIα isoform was most clearly expressed in the rapidly proliferating cell lines TSU-Pr1 and DU145. The IIβ isoform was uniformly expressed in all cell lines. which also applied to the Topoisomerase I protein.

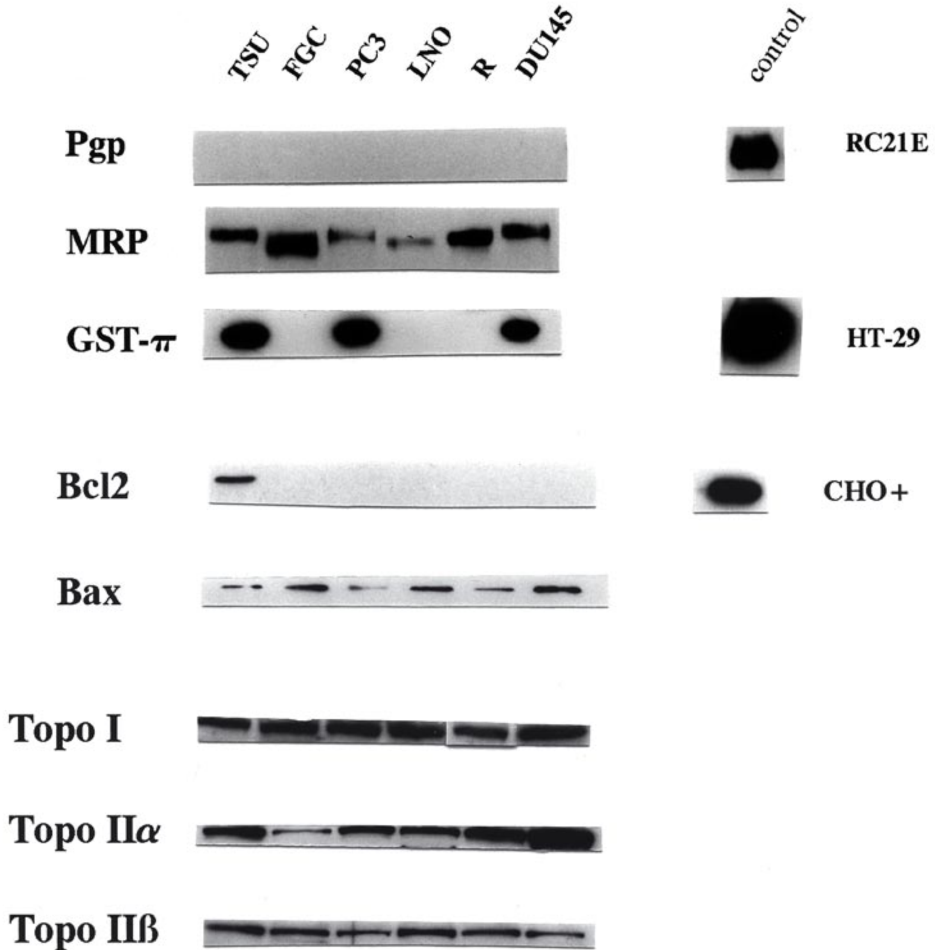

Figure 3.2. Expression of multidrug resistance proteins P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP), glutathione-S-transferase-π (GST-π), Bcl-2, Bax, topoisomerase (Topo) I, IIα and IIβ in the human prostate cancer cell lines TSU-Pr1, LNCaP-FGC, PC3, LNCaP-LNO, LNCaP-R and DU145 as determined by Western blotting of total cell lysates. Bands represent protein expression. Positive controls are shown for Pgp (RC21E), GST-π (HT-29) and Bcl-2 (CHO-Bcl-2) detection and were run on the same gel.

Immunocytochemistry

In addition to Western blot analysis, cultured prostate cancer cells were analyzed for the expression of the same multidrug resistance proteins by immunocytochemical staining. Nearly confluent cultures of all cell lines were stained with antibodies directed against the MDR proteins. A selection of the immunocytochemical stainings is shown in **figure 3**.3 The immunocytochemical stainings matched the Western Blotting results well. All prostate cancer cell lines were negative for Pgp (**A**), whereas RC21E control cells were clearly Pgp positive, with evident cell membrane-bound localization (not shown). MRP was expressed in all cell lines, demonstrated as a cytoplasmatic staining (**B**). GST-π stained positively in cell lines TSU-Pr1, PC3 (**C**) and DU145 and showed a reticular cytoplasmatic pattern and nuclear staining. Bcl-2 was only expressed at a low level in TSU-Pr1. The other cell lines showed some staining of mitotic cells (**D**). All cell lines were Bax positive (**E**). Topo I was present in the nuclei of all cell lines, with prominent nucleolar staining (**F**). Also, a light cytoplasmatic background staining was observed. Nuclear and nucleolar topoisomerase IIα was present in all cell lines but varied between cell lines. DU145 (**G**) and TSU-Pr1 cells stained intensely in comparison to the other cell lines. Topo IIα expression was compared to bromo-deoxy-uridine incorporation of cells (**I**) during the S phase of the cell cycle (see **table 3.2**). The BrdU staining results corresponded well with the proliferative status of cells as observed during the MTT assay. Topo IIα expression appears to be related to the proliferative activity of most of the prostate cancer cell lines. Topo IIβ was expressed in all cell lines with predominant granular staining of the nuclei and absence of staining of nucleoli (**H**) and was expressed in the majority of the cells.

Table 3.2. Percentages of cells staining positively for Topoisomerase IIα (Topo IIα) and bromo-deoxy-uridine (BrdU) as determined by immunocytochemistry.

	TSU-Pr1	LNCaP-FGC	PC3	LNCaP-LNO	LNCaP-R	DU145
Topo IIα	68	40	80	48	33	87
BrdU	41	17	32	18	27	43

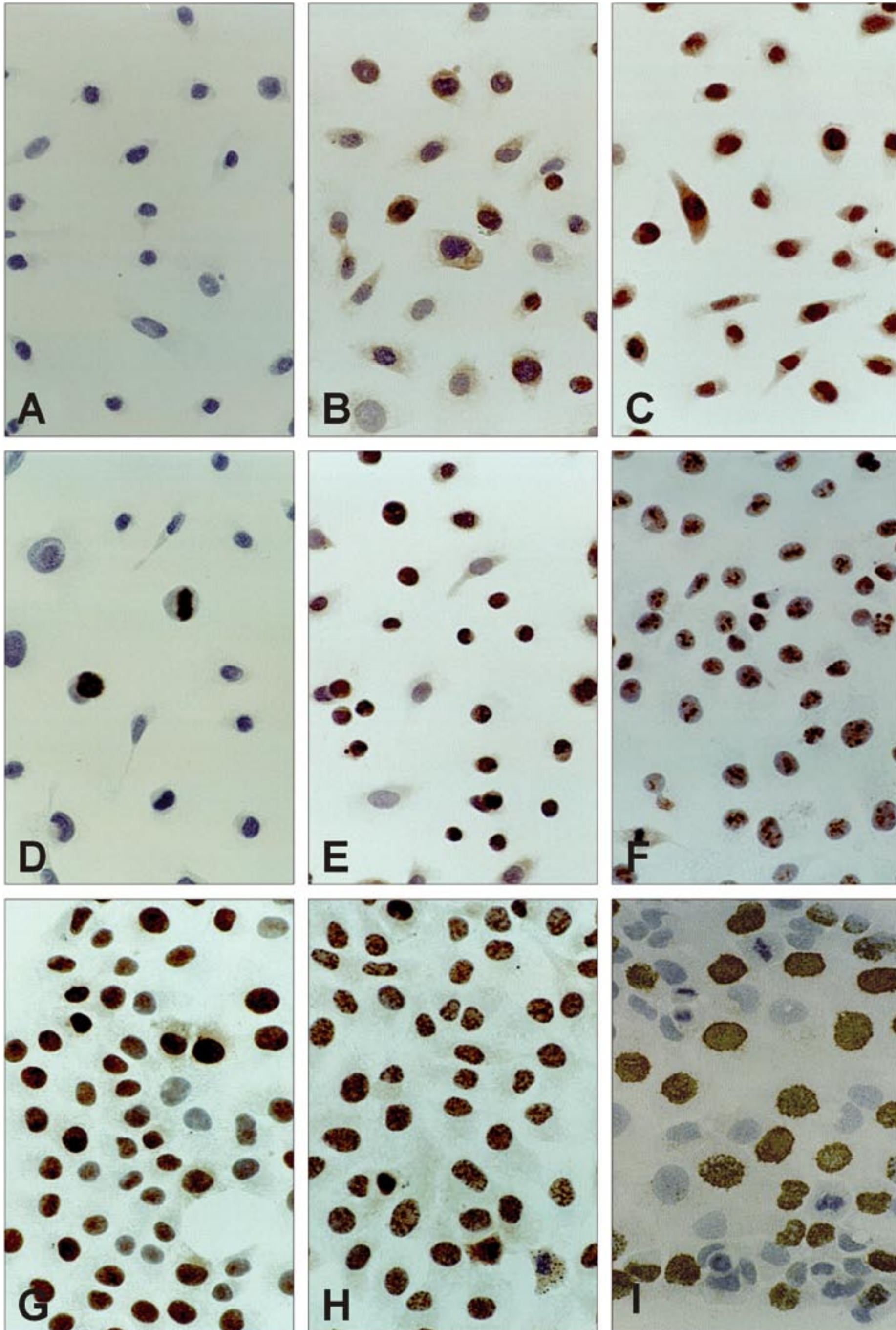

Figure 3.3. Immunocytochemical staining of P-glycoprotein (Pgp) in PC3 (A) , multidrug resistance-associated protein (MRP) in PC3 (B), glutathione-S-transferase-π (GST-π) in PC3 (C), Bcl-2 in PC3 (D), Bax in PC3 (E), topoisomerase (Topo) I in DU145 (F), Topo IIα in DU145 (G), Topo IIβ in DU145 (H) and bromodeoxyuridine (BrdU) in DU145 (I) (magnification 400x).

DISCUSSION

To gain insight into the mechanisms involved in the resistance of metastatic hormone independent prostate cancer against chemotherapy we determined the expression of multidrug resistance proteins, which may lead to the MDR phenotype, in prostate cancer cell lines. Furthermore, we assessed the resistance profiles to three commonly used antineoplastic agents, Etoposide, Doxorubicin and Vinblastine, which action is affected by multidrug resistance.

MDR proteins expression

We found that expression of Pgp, the energy-dependent drug transporter molecule, was below detection level in all cell lines. Expression of Pgp in the human prostate cancer cell lines PC3 and DU145 has been reported in one study [24], albeit at low levels. Doxorubicin resistant cell lines derived from the Dunning R3327 rat prostate carcinoma model [25] expressed Pgp as well [26]. In contrast, Mickisch et al. [27] reported that MDR1 mRNA was undetectable in prostate cancer cell lines LNCaP and DU145. Exposure of non-MDR1 expressing prostate carcinoma cells to Pgp targeted-immunoconjugates harbouring Pseudomonas exotoxin did not affect these cells, whereas Pgp expressing renal cell cancer cell lines were killed. Goldstein et al. [28] were also unable to detect Pgp in clinical prostate cancer or prostate cancer cell lines. The generally negative results for Pgp suggest that Pgp is probably not significantly involved in MDR of prostate cancer.

MRP, the multidrug resistance associated protein, which acts as a transporter molecule for glutathione-S-conjugates [22], was variably expressed at low levels compared to the MRP overexpressing cell line GLC4/ADR. Little is known about expression and function of the drug-transporter MRP in prostate cancer. Low expression levels have been reported in clinical prostate cancer samples by Nooter et al. [29] The expression of MRP in our models indicates that this MDR protein may play a functional role in prostate cancer.

GST-π was expressed in TSU-Pr1, PC3 and DU145. Among the glutathione-S-conjugates, which participate in the detoxification of xenobiotics by conjugation

to reduced glutathione, the GST-π isoenzyme is especially associated with the resistance of tumours against alkylating agents and cisplatin [30]. GST-π was found in normal prostate tissue, but was absent in locally confined prostate cancer [31, 32]. Our results with GST-π were in line with the observations of Lee et al., who reported that cell lines TSU-Pr1, PC3 and DU145 expressed GST-π, but the LNCaP cells did not. This lack of expression in LNCaP is possibly due to hypermethylation of GST-π promotor sequences [31]. The expression of GST-π in several of our cell lines suggests a potential role of this enzyme in drug resistance of advanced prostate cancer.

Bcl-2 [9], has cell survival promoting capacity and blocks cancer cells in their ability to undergo drug-induced apoptosis (e.g. by Etoposide). In our cell lines, Bcl-2 was only expressed in TSU-Pr1. Conflicting data have been reported about the expression of Bcl-2 in LNCaP, but generally expression of this protein was low or undetectable. Expression of Bcl-2 in PC3 has been reported by Sinha et al. [33], which is in contrast to our findings. Differences between cell lines originating from one parental cell line may have arisen after culturing in different laboratories. In clinical prostate cancer Bcl-2 appears to be expressed at relatively low levels in low grade carcinomas and at higher levels in high grade tumours [34]. The expression of Bcl-2 in one of our prostate cancer cell lines together with the results of several studies [33, 35] reporting modulation of drug resistance with changed Bcl-2 expression, support the idea that Bcl-2 contributes to the MDR phenotype of prostate cancer. The role of Bcl-2 in MDR of prostate cancer clearly requires further investigation.

Bax [10] is an apoptosis promoting protein and acts as opponent of Bcl-2. Bax was expressed in all cell lines tested, as it was also found to be well expressed in most cancers including prostate cancer [34]. The ratio of Bcl-2 and Bax in prostate cancer cells may be of importance to determine if the apoptotic cascade will be triggered by treatment with chemotherapy.

The Topo IIα enzyme was expressed in all cell lines. The highest expression was found in the rapidly proliferating cell lines TSU-Pr1 and DU145. Topo I and IIß were equally expressed in all cell lines. This is in line with the findings of Boege et al. [36] that topoisomerase I and IIβ are constantly expressed during the cell cycle, whereas IIα is proliferation-associated. The topoisomerase enzymes are essential for several

cellular processes and constitute targets for a number of clinically important drugs, which induce lethal DNA damage by irreversibly stabilizing topoisomerase-DNA complexes. Topoisomerase directed drugs, such as Etoposide and Doxorubicin, are generally believed to target the IIα isotype [36], although Topo I [37] and IIβ [38] may constitute targets as well. Changes in amount or activity of topoisomerases have been described in relation to one form of MDR: atypical- or attenuated MDR [11]. The relation between the resistance of prostate cancer to chemotherapy and expression of topoisomerase enzymes is still unclear. However, drug resistance to topoisomerase poisons may arise from decreased expression or activity of the drug targets Topo I, IIα and/or IIβ or reduced proliferative activity related to Topo IIα.

Drug resistance

The presence of several MDR proteins in the prostate cancer cell lines suggests that drug resistance may arise by up- or down regulation of one- or multiple MDR proteins. In our experiments, the cell lines responded variably to exposure to cytotoxic drugs (figure 3.1a-c, table 3.1). It is likely that this response is influenced by a combination of MDR mechanisms, rendering interpretation of the dose response curves difficult. However, certain patterns can be distinguished. The cell line TSU-Pr1 is sensitive to all three drugs. Its sensitivity to Vinblastine, a mitotic spindle blocker, can be expected, as TSU-Pr1, together with DU145, is one of the most rapidly proliferating cell lines among our models, as measured by BrdU incorporation (table 3.2). The sensitivity of TSU-Pr1 to Etoposide and Doxorubicin, both topoisomerase II targeting drugs, relates well to the high expression of topo IIα en β enzymes. TSU-Pr1 expresses Bcl-2, which may protect these cells from Etoposide mediated apoptosis [35]. However, immunocytochemistry shows an inhomogeneous expression of Bcl-2 and the majority of cells do not express the protein, suggesting that only a small proportion of the cells may be relatively resistant. DU145, which has high expression of topo IIα, is also sensitive to Etoposide and Doxorubicin. The sensitivity of LNCaP-FGC, having a lower expression of Topo IIα and a lower proliferative activity, is nearly identical to that of DU145. Possibly GST-π, the detoxifying enzyme, induces a certain degree of drug resistance in DU145,

PC3 and TSU-Pr1, whereas LNCaP-FGC, which does not express GST-π, remains relatively sensitive, resulting in similar resistance profiles of these cell lines. The LNCaP derivatives -LNO and -R, which in contrast to LNCaP-FGC, are both hormone independent, are relatively resistant to Etoposide, Doxorubicin and Vinblastine. This may be in agreement with the finding that transition of prostate cancer cells to a hormone independent state concomitantly leads to resistance to chemotherapy [35, 39]. Proliferative activity of cell line LNCaP-R is higher than that of LNCaP-LNO and its sensitivity to Vinblastine is comparable to that of PC3, DU145 and FGC. The role of MDR in this cell line is questionable as it lacks Pgp, significant amounts of MRP, GST-π and Bcl-2 and expresses Bax and all topoisomerases. The slow growth rate of LNCaP-LNO may explain its resistance to Vinblastine.

CONCLUSIONS

This study shows that the resistance of prostate cancer cells to chemotherapy may be determined by MDR mechanisms and proliferative activity. Furthermore, drug resistance is potentially multifactorial, as several MDR proteins are expressed in our models. As the provided evidence of the contribution of MDR associated proteins to drug resistance in prostate cancer is indirect, the development of multidrug resistant models from relatively sensitive prostate cancer cell lines is an obligatory step for future investigations. Such models offer the possibility of further functional studies of regulation of activity and expression of MDR proteins *in vitro*. Also, modulation and circumvention of MDR could be investigated. Furthermore, a detailed search for expression of MDR proteins in clinical prostate cancer samples at different stages of disease and progression may help elucidate the role of the various MDR-related factors, which were described in the present study of experimental systems. Eventually, a better understanding of mechanisms causing resistance of prostate cancer to antineoplastic agents may lead to new approaches to treat hormone independent metastatic prostate cancer more successfully.

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CHAPTER 4

Expression of multidrug resistance related proteins and proliferative activity is increased in advanced clinical prostate cancer.

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J Urol 2001;165:130-135

SUMMARY

Purpose: Advanced disseminated prostate cancer is highly resistant to cytotoxic chemotherapy. In this study we aimed at identifying proteins that may be involved in multidrug resistance in clinical prostate cancer. Expression of these proteins was examined in the context of tumour progression.

Materials and Methods: Paraffin-embedded formalin fixed prostate cancer specimens from archival sources of three distinct patient groups were examined. These groups were clearly distinct with regard to pathological stage and responsiveness to anti-hormonal therapy. Group 1 consisted of patients with organ confined prostate cancer, treated by radical prostatectomy (early prostate cancer, pathological stage (p) T2N0M0 tumours). Group 2 had disseminated prostate cancer and was treated with transurethral resection of the prostate (TURP) for urinary obstruction before receiving anti-hormonal therapy (early advanced prostate cancer). Group 3 had disseminated prostate cancer and relapsed despite anti-hormonal treatment (late advanced prostate cancer) and were treated with TURP to relieve symptoms of urinary obstruction. Immunohistochemistry was used for detection of P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP), lung resistance protein (LRP), glutathione-S-transferase-π, (GST-π), p53, Bcl-2, Bax, topoisomerase (Topo) I, IIα and IIβ and Ki-67.

Results: Advanced tumours could be distinguished from locally confined tumours because they exhibited a significantly higher histological grade and proliferative activity than organ confined prostate cancer. Expression of MRP, p53, Topo IIα, Ki-67 and Topo IIβ was significantly related to a higher Gleason sum score. The number of patients expressing MRP, LRP, GST-π, p53, Bcl-2, Topo IIα and Ki-67 was significantly increased in the group with advanced disseminated prostate cancer. Topo I and Topo IIβ were homogeneously and highly expressed in all stages of prostate cancer progression. Pgp was not expressed in any of the tumours, regardless of the patient group.

Conclusions: Upregulation of expression of drug transporters MRP and LRP, detoxifying enzyme GST-π and apoptosis inhibiting proteins Bcl-2 and P53 may offer an explanation for the resistance of disseminated progressive prostate cancer to

chemotherapy. Increased proliferation, as shown by upregulation of Ki-67 and Topo IIα, reflects the aggressiveness of metastatic prostate cancer. Research on agents that counteract multidrug resistance mechanisms and that may sensitize prostate carcinomas to cytotoxic chemotherapy, will possibly lead to more effective treatment of patients with progressive disseminated prostate cancer.

INTRODUCTION

Disseminated prostate cancer that has relapsed despite anti-hormonal treatment has an unfavorable prognosis. The treatment of choice for many metastasized cancers is systemic chemotherapy. Unfortunately, metastatic prostate cancer is highly resistant to treatment with cytotoxic agents[1].

Resistance to functionally- and structurally distinct cytotoxic drugs is termed multidrug resistance (MDR) and several mechanisms are held responsible for the MDR phenotype of various malignancies. Decreased intracellular concentration of cytotoxic agents may be caused by active drug extrusion by drug transporter molecules. These include the multidrug resistance (MDR1) gene product P-glycoprotein (Pgp)[2], the multi resistance associated protein (MRP)[3] and the Lung resistance protein (LRP)[4]. Increased detoxification by GST-π, which is part of the glutathione detoxifying mechanism, generally causes resistance to several cytotoxic drugs[5]. Furthermore, glutathione-S-conjugates are transported by MRP in an energy-dependent manner[6]. Mutations in p53[7], overexpression of the anti-apoptotic protein Bcl-2[8] and decreased expression of the pro-apoptotic protein Bax[9] may protect cancer cells from chemotherapy-induced programmed cell death and lead to drug resistance. Downregulation of nuclear proteins Topo I, IIα and/or IIβ, which are targets for several cytotoxic agents, is associated with multidrug resistance[10] and is termed altered- or attenuated topoisomerase MDR (at-MDR).

Recently, involvement of several MDR mechanisms in organ confined prostate cancer has been demonstrated[11-13]. In prostate cancer patients treated by radical prostatectomy high proliferative activity, as determined by Ki-67 labelling index, was related to unfavorable prognosis[14]. Prostate cancer is characterized by heterogeneity and clones of tumour cells expressing different MDR-associated

proteins and exhibiting high proliferative activity may very well determine the clinical behaviour of organ confined and advanced prostate cancer and its response to therapy.

We hypothesize that MDR mechanisms that are present in locally confined prostate cancer are upregulated in advanced prostate cancer and may cause failure of treatment with cytotoxic drugs in this stage of disease. To address this issue we examined and compared the expression of the above-mentioned MDR-associated proteins and proliferation markers in three clearly distinct patient groups, each group representing a particular phase in prostate cancer progresssion. These groups cover the range from clinically curable disease, prostate cancer with effective palliative treatment options and aggressive cancers, that have escaped both curative and palliative approaches.

MATERIALS AND METHODS

Tissue specimens

Formalin-fixed, paraffin embedded prostate tissue collected from archival sources available at our institutions of 53 prostate cancer patients was examined.

Patient groups

Three distinct patient groups were studied.

Group 1 consisted of 19 consecutive radical prostatectomy specimens obtained from all patients who were recently operated for locally confined (pT2N0M0) prostate cancer from April to November 1998 and represents the group with early phase prostate cancer.

Group 2 consisted of seventeen transurethral resection of the prostate (TURP) specimens of patients with disseminated prostate cancer treated for urinary obstruction prior to anti-hormonal therapy, representing an intermediate phase of prostate cancer. Group 3 consisted of seventeen TURP specimens of patients with disseminated prostate cancer that relapsed during anti-hormonal treatment, rep-

resenting the group of advanced hormone-independent prostate cancer. Group 2 and 3 comprised all archival material from patients that matched the definition of these groups.

These groups represent three, clinically different, phases of prostate cancer and were chosen by review of medical records: group 1 consists of non-metastatic disease (pathological stage N0M0) and group 2 and 3 of metastatic disease (N+/M+), with the difference that group 3 patients are unresponsive to anti-hormonal therapy. **Table 4.1** shows the patient characteristics of the groups: patient age, extent of disease, Gleason sum score, prostate specific antigen (PSA) and prior therapy. In groups 2 and 3, which were obtained from archival sources, PSA values were only known in a limited number of patients (6 and 5, respectively). Therefore, a relation between PSA levels and other parameters was only studied in group 1.

Table 4.1. Patient characteristics.

Group No.	Mean Pt. Age (range)	Min./Max. 1992 International Union Against Cancer Stage	Mean Gleason Score Sum (range)	Mean PSA (ng/ml) (range)	Previous Systemic Therapy
1	63.7 (56 –71)	T2aN0M0-T2cN0M0	6.5 (5 – 7)	6.4 (n=19) (1.6 – 21)	None
2	75.2 (65 – 89)	T1N0M1-T4N0M1	9 (8 – 10)	112 (n=5) (5.6-199)	None
3	71 (54 – 94)	T2N2M0-T4N2M1	9.1 (7 – 10)	13.3 (n=6) (0.1 – 46)	Androgen deprivation

Antibodies

Antibodies were used that recognize antigenic epitopes which are well preserved in formalin-fixed, paraffin embedded tissue. Optimal dilution of an antibody was determined using human tissues reported to have distinct and relatively high expression of a particular antigen. All antibodies were diluted in 5% bovine serum albumin (Sigma Chemical Co, St. Louis, USA) in phosphate buffered saline (BSA/PBS) (**Table 4.2**).

Table 4.2. Antibodies, applied dilution, control tissue used for detection of the studied proteins.

Antigen	Antibody	Source	control	Dilution (times)
P-glycoprotein	JSB1	M	ileum	100x
Multidrug resistance associated protein	MRPr1	M	ileum	100x
Lung resistance protein	LRP-56	M	ileum	20x
Glutathione-S-transferase-π	NCL-GST-pi	N	liver	500x
p53	Clone DO-7	D	bladder ca.	25x
Bcl-2	Clone 124	D	tonsil	60x
Bax	sc-526	SC	ileum	50x
Topoisomerase I	Anti-Topo I	I.B.	ovary ca.	10x
Topoisomerase IIα	Ki-S1	BM	tonsil	250x
Toposiomerase IIβ	3H10	I.H.	-	900x
Ki-67	MIB-1	I	tonsil	3000x

M.:Monosan, Sanbio, the Netherlands; N.: Novocastra, United Kingdom;
D.: Dako, Glostrup, Denmark; SC.: Santa Cruz biotechnology, USA;
I.B.: Dr. Igor Bronstein, Department of Chemistry, University of York, England;
BM.: Boehringer Mannheim, Germany; I.H.: Dr Ian Hickson, Institute of Molecular Medicine, University of Oxford, United Kingdom;
I.: Immunotech, France.

Immunohistochemistry

Staining conditions were optimized using positive control tissues (**table 4.2**). For all stainings negative controls were included by substituting PBS for the primary antibody. Tissue sections were cut at 4µm and mounted on amino-trietoxy-silane (Sigma) coated glass slides. After overnight incubation at 37oC, slides were dewaxed in xylene and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked in 10% H2O2 in methanol. Antigen retrieval was performed for Pgp, MRP, Bcl-2, Bax, p53, Topo I, Topo IIα, Topo IIβ and Ki-67 in a 700W microwave for 10 to 12 minutes, by boiling slides in citrate buffer (pH 6.0). For Pgp antigen retrieval in EDTA buffer (pH 8.0) was used to achieve detection with the JSB1 antibody. After rinsing in PBS, the avidin-biotin complex method[15] was applied for detection of all antigens, except for Pgp, which was detected through the peroxidase-anti-peroxidase method[15], to reduce background staining. All compounds were diluted in 5% BSA/PBS. Antibody-antigen binding was visualized with diaminobenzidine/hydrogen peroxidase. Nuclei were counterstained with hematoxyline, dehydrated in graded alcohols and xylene and mounted with coverslips.

Scoring and statistics

Representative sections were examined for expression of every antigen. Scoring was performed by a pathologist (Th. vd K.). The pathologist was masked to the clinical categories of the specimens. In case of scoring by counting positive cells, one thousand cells were counted. A tumour was scored positive if more than 5% of the cells in a tumour area were stained.

Statistical analysis was performed using the SPSS and STATA statistical computer packages. Differences in histological grade between the three patient groups as reflected by Gleason sum score were examined using the Kruskal Wallis H test. Differences in expression of the studied proteins between patient groups were assessed using the chi-square test for trend (Fisher's exact test) for Pgp, MRP, LRP, GST-π, p53, Bcl-2, Bax, Topo I and TopoIIβ, Topo IIα and Ki-67. Correlation between Topo IIα and Ki-67 expression, between MDR-and proliferation related proteins and Gleason sum score, between PSA and histological grade (group1) and between PSA and MDR-and proliferation related proteins (group 1) was determined by the Pearson's test and Spearman's test.

RESULTS

The expression of MDR related proteins and proliferation related proteins was studied immunohistochemically in tissue specimens from patients with locally confined, hormonally untreated disseminated, and hormone independent disseminated prostate cancer. Differences between these clinically distinct patient groups with regard to MDR- and proliferation related protein expression were analyzed. The relation between proliferation related proteins Ki-67 and Topo IIα was calculated. The relation between the groups and histological grade was studied. Also, the relation between histological grade and expression of MDR proteins and proliferation associated proteins was assessed. Furthermore, the relation between PSA value and MDR- and proliferation related protein expression and between PSA value and histological grade was assessed in patients with locally confined prostate cancer (group 1).

Differences in expression of MDR proteins and proliferation associated proteins between the patient groups.

Figure 4 (**A** to **V**) shows representative stainings for all studied proteins in locally confined (left side of the figure) and hormone independent disseminated prostate cancer (right side of figure).

Pgp. The MDR1 gene product Pgp was not expressed in any of the normal prostatic (luminal and/or basal) or prostate cancer cells, whatever the stage of disease as shown in **figure 4A** and **B**.

MRP. Prostate cancer cells homogeneously expressed MRP in the cytoplasm and cell membrane (**figure 4C** and **1D**) in 53% of locally confined prostate carcinomas, 100% of hormonally untreated disseminated prostate carcinoma and 100% of the hormone independent disseminated prostate carcinoma (**table 4.3**). This difference is stastically significant ($p<0.001$; **table 4.4**).

LRP. LRP was clearly expressed in the cytoplasm of prostate cancer cells in all three patient groups (**figure 4E** and **F**; **table 4.3**). The number of patients expressing LRP was significantly increased in disseminated hormone independent prostate cancer ($p<0.05$; **table 4.4**).

GST-π. Prostate cancer cells of locally confined disease did not express GST-π, although it was clearly expressed in the cytoplasm of the basal cells of benign prostatic epithelia (**figure 4G**). Hormonally untreated disseminated prostate carcinoma cells expressed GST-π in 18% of the tumours and hormone independent disseminated prostate carcinoma expressed GST-π in 53% (**figure 4H**, **table 4.3**), which is a significant increase in hormone independent disseminated disease ($p<0.001$; **table 4.4**), compared to organ confined disease.

Bcl-2. Bcl-2 was expressed in the cytoplasm of the basal cells of normal prostatic glands. Locally confined tumours (**figure 4I**) and hormonally untreated disseminated prostate cancer equally expressed Bcl-2. A significantly higher number of tumours with expression of Bcl-2 ($p<0.05$; **table 4.4**) (**figure 4J**) was found in hormone independent metastatic prostate cancer (82%, **table 4.3**).

Bax. Bax (**figure 4K-L**) was equally and homogeneously expressed in the cytoplasm of normal prostatic tissue and in the tumours of all three patient groups regardless of the phase in prostate cancer progression (**table 4.3**).

P53. A significantly larger number of tumours from the group with progressive disseminated disease were p53 positive (**figure 4M-N**) in comparison to locally confined disease ($p<0.001$; **table 4.4**).

Topo I. Topo I was highly and homogeneously expressed in the nuclei and nucleoli of the prostate cancer cells (**figure 4O-P**) in all three patient groups. The number of patients expressing Topo I was increased (**table 4.3**), although not significantly, in the two groups with metastatic prostate cancer (**table 4.4**).

Topo IIα. Prostate cancer cells heterogeneously expressed the nuclear enzyme topo-IIα (**figure 4Q and R**). Compared to locally confined prostate cancers, the number of prostate carcinomas with topo IIα expression was significantly increased in the tumours of both groups with metastatic disease ($p<0.05$; **table 4.4**). Similar differences in the number of positive prostate cancer cells were also observed: 3 percent (range: 0 – 11), 10 percent (range: 2-27) and 11 (range: 1 – 37) percent, respectively in locally confined, early metastatic and progressive prostate cancer.

Topo IIβ. Expression of topo IIβ (**figure 4S-T**) was high and homogeneous in nuclei of the prostate cancer cells of all three patient groups. Expression of topo IIβ was more frequently seen in disseminated prostate cancer specimens (100% of the specimens were positive; **table 4.3**), although this increase was not statistically significant (**table 4.4**).

Ki-67. Expression of the nuclear Ki-67 antigen (**figure 4U-V**), reflecting proliferative activity, was significantly increased in advanced prostate cancer (non-hormonally treated disseminated disease and hormone independent disseminated prostate cancer: $p< 0.05$ and $p<0.001$, respectively; **table 4.4**). Nuclear expression of Ki-67 displayed a heterogeneous pattern within tumour areas and between different tumour foci. Ki-67 was expressed in 4 percent (range: 1-12), 6 percent (range: 1-26) and 9 percent (range: 1-49) of the cells in locally confined, early metastatic and progressive prostate cancer, respectively. Expression of Ki-67 was closely correlated to Topo IIα expression (correlation coefficient: 0.89; $p<0.01$).

Relation between histological grade and patient groups

Patients with either hormonally untreated disseminated- or hormone independent disseminated prostate cancer had significantly higher Gleason sum scores than patients with locally confined tumours (mean 9.0 and 9.1 versus 6.5 respectively (**table 4.1**); $p<0.001$). The difference in Gleason sum score between the two groups with disseminated disease was not statistically significant.

Relation between histological grade and expression of MDR proteins and proliferation associated proteins

In all groups histological grade was significantly higher in patients expressing proliferation associated proteins Topo IIα ($p=0.02$) and Ki-67 ($p=0.008$). Also, tumours of patients had a significantly higher Gleason sum score when expressing Topo IIβ ($p=0.007$), p53 ($p=0.001$) and MRP ($p=0.01$).

Relation between PSA value and MDR- and proliferation related proteins and between PSA value and histological grade in patients with locally confined prostate cancer (group 1)

In this group of patients MDR- and proliferation related proteins and histological grade were not correlated to PSA value.

Table 4.3. Percentages of tumours expressing the studied proteins p-glycoprotein (Pgp), multidrug resistance associated protein (MRP), glutathione-S-transferase-π (GST-π), Bcl-2, bax, p53, topoisomerase (Topo) I, II, β and Ki-67.

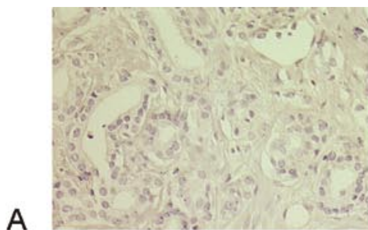
	Pgp	MRP	LRP	GST-π	Bcl-2	Bax	p53	Topo I	Topo IIα	Topo IIβ	Ki-67
1	0	53	32	0	47	100	5	79	16	89	16
2	0	100	47	18	47	100	29	94	59	100	65
3	0	100	71	53	82	100	65	94	65	100	82

Group 1: Locally confined prostate cancers (pT2N0M0, radical prostatectomy specimens; n=19). Group 2: disseminated prostate cancer before anti-hormonal therapy (transurethral resection of the prostate (TURP); n=17). Group 3: progressive disseminated prostate cancer, relapsed after anti-hormonal treatment (TURP; n=17).

Table 4.4. Statistical analysis of differences in expression of the studied proteins in three prostate cancer groups.

	Group 1 vs 2 vs 3 (No. of tumours)	group 1 vs 2	group 1 vs 3	group 2 vs 3
P-glycoprotein	1 (0-0-0)	1	1	1
Multidrug resistance protein	0.00001 (10-17-17)	0.0006	0.0003	1
Lung resistance protein	0.09 (6-8-12)	0.29	0.03	0.27
Glutathione-S-transferase-π	0.0007 (0-3-9)	0.05	0.0003	0.04
Bcl-2	0.09 (9-8-14)	0.59	0.08	0.04
Bax	1 (19-17-17)	1	1	1
P53	0.002 (1-5-11)	0.1	0.0006	0.05
Topoisomerase I	0.35 (15-16-16)	0.5	0.15	1
Topoisomerase II α	0.006 (3-10-11)	0.002	0.016	0.99
Topoisomerase IIβ	0.15 (17-17-17)	0.18	0.15	1
Ki-67	0.001 (3-11-14)	0.007	0.0009	0.23

p-values are shown. The absolute number of positive tumours for respectively group 1, group 2 and group 3 are shown between brackets. Group 1 (n=19): Locally confined prostate cancers (pT2N0M0, radical prostatectomy specimens). Group 2 (n=17): disseminated prostate cancer before anti-hormonal therapy (transurethral resection of the prostate (TURP)). Group 3 (n=17): progressive disseminated prostate cancer, relapsed after anti-hormonal treatment (TURP).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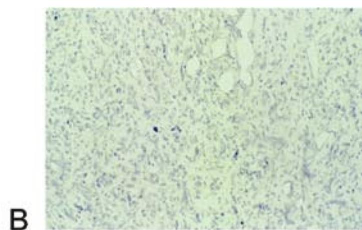
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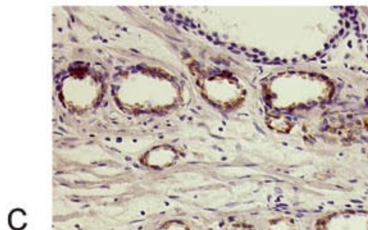
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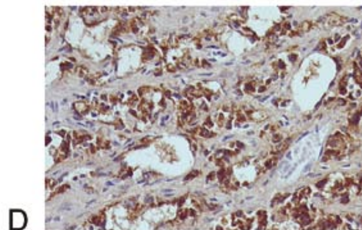
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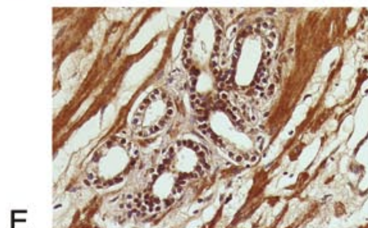
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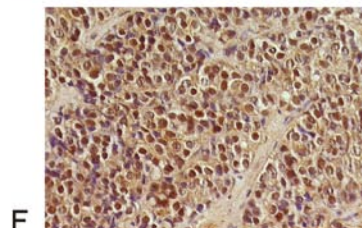
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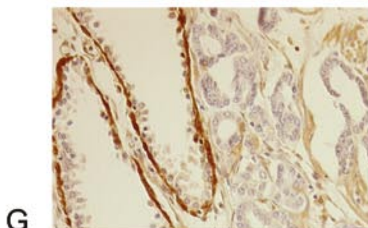
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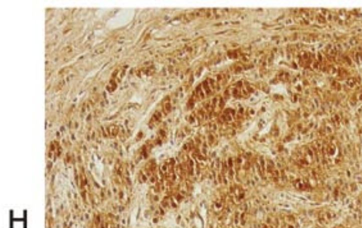
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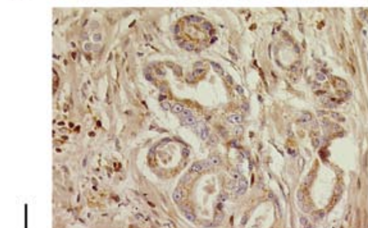
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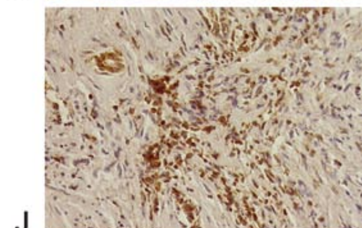
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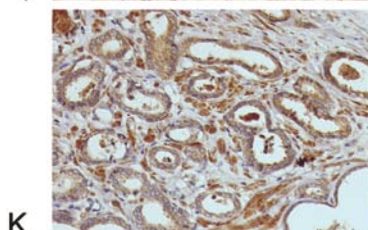
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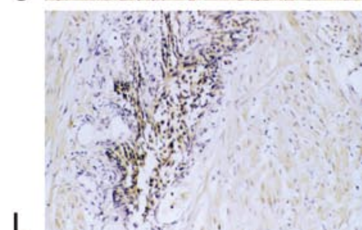
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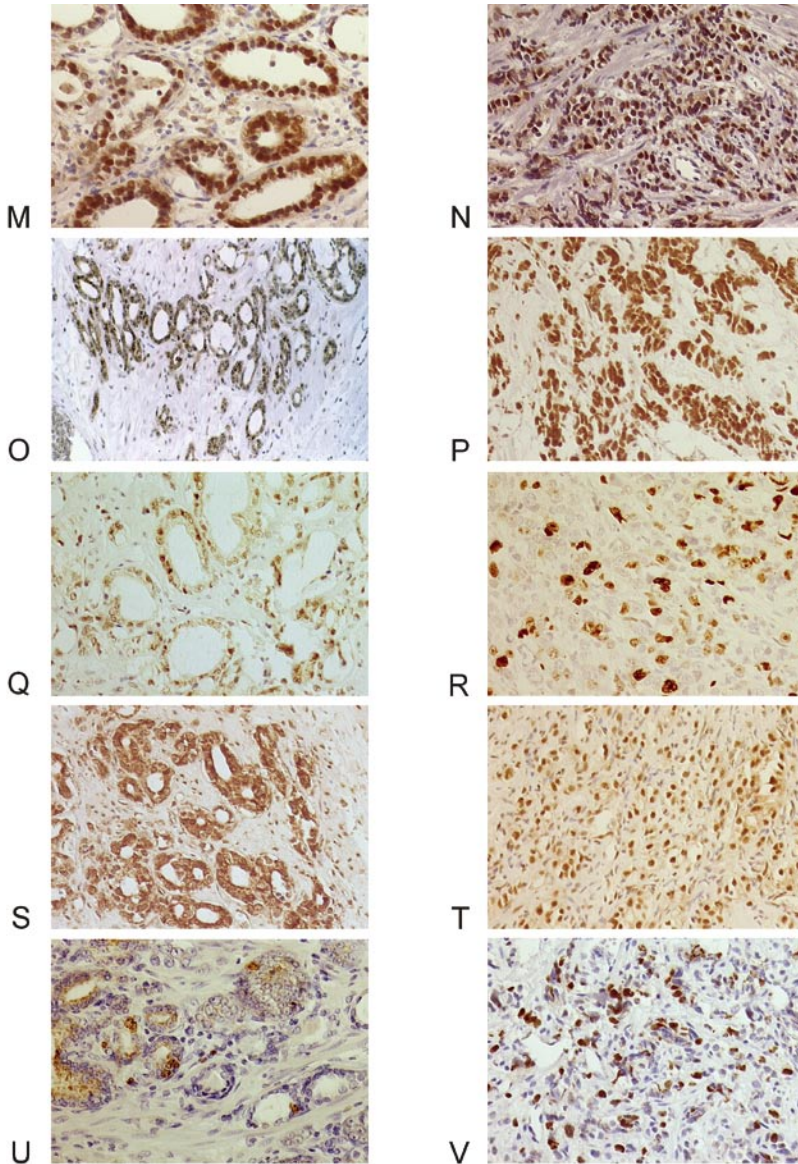

Figure 4.

Representative examples of immunohistochemical analysis of paraffin-embedded formalin fixed prostate cancer samples. Tumours of patients with locally confined prostate cancer (pT2N0M0, radical prostatectomy specimens) are shown on the left side; tumours of patients with progressive disseminated prostate cancer, relapsed after anti-hormonal treatment (treated by transurethral resection of the prostate (TURP)) are shown on the right side of the figure . A and B: P-glycoprotein; C and D: Multidurg resistance associated protein; E and F: Lung resistance protein; G and H: Glutathione-S-transferase-π; I and J: Bcl-2; K and L: Bax; M and N: p53; O and P: Topoisomerase I; Q and R: Topoisomerase IIα; S and T: Topoisomerase IIβ; U and V: Ki-67. Magnification 200x.

DISCUSSION

Systemic chemotherapy has generally been ineffective in the treatment of hormone independent prostate cancer[1]. Advanced stage prostate cancer appears to have an intrinsic resistance to chemotherapeutic drugs. Resistance to structurally- and functionally distinct chemotherapeutic agents has been described in many tumours and termed multidrug resistance (MDR), which can be caused by several mechanisms. Although reports on mechanisms underlying MDR in prostate cancer are scarce and such studies were often limited to a small number of MDR proteins[5, 11-13, 16-18], together they offer support for the hypothesis that failure of treatment of advanced prostate cancer with systemic chemotherapy may be caused by MDR mechanisms.

To address this issue, we studied the expression of a large panel of multidrug resistance-related proteins in three groups of prostate carcinoma patients, which are clearly distinct with regard to progression of disease. Patients with prostate cancer ranging from locally confined to progressive therapy resistant disease were allocated to the different groups by reviewing medical records and were obtained from archival material. The clinical differences between these groups were reflected by a significantly higher histological grade in the advanced tumours.

We investigated the presence and expression pattern of proteins involved in drug transport (including Pgp, MRP and LRP) and drug detoxification (GST-π), the expression of proteins involved in modulation of apoptosis (p53, Bcl-2 and Bax), the nuclear drug targets (Topo I, IIα and IIβ) and the cell proliferation associated proteins (Ki-67 and also Topo IIα). We found that expression of several of these proteins was significantly increased in the groups of more advanced types of carcinomas as shown in **table 4.3** and **table 4.4**. Upregulation of the expression of multiple multidrug resistance related proteins may play a role in chemotherapy resistance of advanced prostate cancer. Emergence or upregulation of several of the studied proteins was also associated with a significantly higher histological grade. These proteins may provide cancer cells with protective mechanisms, resulting in more progressive- and multidrug resistant tumours. However, although we demonstrated expression of a panel of MDR related proteins, a functional role of these proteins in advanced prostate cancer remains to be established.

Remarkably, Pgp, the MDR1 gene product and member of the ATP-binding cassette (ABC) superfamily of membrane transporter proteins, which is held responsible for multidrug resistance in numerous tumours[19], is not expressed in the prostate carcinomas of the three patient groups, whatever the phase of progression. Most reports studying Pgp expression in prostate cancer had negative results as well[11, 16]. In contrast, MRP, which is a second member of the membrane transporter proteins family, was clearly expressed in the prostate cancer samples analyzed and we could demonstrate a significantly increased expression in advanced disease. Also, MRP positive tumours had a significantly higher histological grade. These findings suggest that during tumorogenesis and progression amplification and/or overexpression of the MRP gene[3] occurs which may contribute to the multidrug resistant phenotype of advanced disease by decreasing the intracellular drug content. Expression of MRP was previously reported in locally confined prostate cancer[11, 17] and a relation with progression has been suggested[11]. This is confirmed in the present study showing expression of MRP in all patients with metastasized disease.

Apart from being a transmembrane drug pump, MRP is a glutathione S-conjugate carrier[6]. Interestingly, expression of GST-π, a detoxifying enzyme and part of the glutathione detoxifying pathway, is also significantly increased in advanced hormone independent prostate cancer. Concomitant overexpression of MRP and GST-π may synergistically result in increased drug resistance of advanced prostate cancer. Expression of GST-π in clinical prostate cancer is a novel finding. Previous studies have only reported the lack of its expression in locally confined prostate cancer obtained from radical prostatectomy samples[5, 11]. Indeed, in our series of tumours locally confined prostatic carcinomas were entirely GST-π negative, whereas the disseminated cancers clearly expressed GST-π, especially in hormone-independent progressive disease.

LRP, which has been identified as the human major vault protein (MVP)[20] and is involved in transmembrane transport of various substrates, was the third drug transporter studied. To our knowledge, this is the first study reporting expression of LRP in prostate cancer. This protein was expressed in nearly one third of the organ confined prostate cancer samples and its expression was significantly increased

in hormone independent disseminated prostate cancer. Altogether, three out of four proteins of the panel of drug-transporter and detoxifying proteins are more frequently expressed in patients with hormone independent prostate cancer. This may indicate that failure of chemotherapy in progressive prostate cancer could be caused by these MDR mechanisms.

Additionally, we have studied the expression of proteins involved in the apoptotic process. Wild-type p53 is a suppressor of cell growth and transformation, causing a proliferation-inhibiting G1 block and regulating apoptosis. Mutation of the p53 gene is the most common molecular alteration in cancers resulting in decreased apoptosis and possibly resistance to apoptosis-inducing cytotoxic agents. We observed a significantly increased number of p53 positive patients in hormone independent cancers. Also, p53 expression was statistically significantly related to a higher histological grade. These findings should be interpreted cautiously as immunohistochemical expression of p53 does not necessarily represent p53 mutation[21]. However, correlation between p53 mutations and tumour progression was presented in several studies, with mutations occurring as a late event in the development of prostate cancer[22-24]. This is in concordance with our finding of more frequently occurring p53 expression in advanced disease, which may explain the decreased susceptibility to apoptosis-inducing agents. This may also be caused by expression of the Bcl-2 protein, which has been identified as a powerful inhibitor of apoptotic cell death, and which was increased in the group of hormone independent prostate cancer. Bcl-2 expression in prostate cancer has previously been related to the androgen independent phenotype[25, 26]. Relatively high Bcl-2 levels may promote cell survival and protect the androgen independent prostate cancer cells from drug-induced apoptosis. The Bax protein, which forms heterodimers with Bcl-2 and promotes apoptosis[9], is equally and homogeneously expressed in normal prostate and prostate cancer cells in all phases of progression. It has been suggested that the ratio of Bcl-2 and Bax determines cells to enter the apoptotic process. With the observed unchanged levels of Bax, increased number of patients with Bcl-2 and p53 positive tumours in the group with hormone independent disease, apoptosis of cancer cells is possibly less likely to occur when challenged with drugs that act through triggering apoptosis, resulting in increased resistance.

The nuclear topoisomerase enzymes are targets for a number of cytotoxic drugs. They are involved in multidrug resistance through down-regulation of their expression or decreased enzymatic activity (at-MDR)[27]. Expression of the isoforms topo I and Topo IIβ is high and homogeneous in every phase of prostate cancer progression and is slightly increased in advanced disease. Topo IIβ negative tumours had a significantly lower histological grade. Expression of Topo IIα is significantly increased in progressive disseminated prostate cancer and tumours with a higher histological grade. Increased expression of topoisomerases does not point towards a role for at-MDR in advanced prostate cancer. Interestingly, the Topo IIα enzyme is known to be related to cell proliferation as well[28]. We report that this holds true for prostate cancer, as was confirmed by the strong correlation between Topo IIα expression and expression of the proliferation marker Ki-67. This marker was demonstrated to be significantly increased in the group of advanced prostate cancers and in tumours with a higher histological grade. A relation between proliferative activity and a more progressive phase of disease has been reported previously[14, 29, 30]. The differences in proliferative activity between the three patient groups are in agreement with their clinical phase of prostate cancer and increased expression of Ki-67 and Topo IIα in advanced prostate cancer possibly reflects the more aggressive character of these tumours.

High expression of topoisomerases in end-stage prostate cancer may favour the introduction of topoisomerase targeting agents. However, efficacy of such drugs may be hampered by increased drug efflux and detoxification through MRP, LRP and GST-π and by inhibition of apoptosis via p53 mutations and an increased Bcl-2 to Bax ratio.

CONCLUSION

This study demonstrates that advanced disseminated prostate cancer overexpresses MDR-, anti-apoptotic- and proliferation-related proteins. Three out of four proteins of the panel of drug-transporter and detoxifying proteins (MRP, LRP and GST-π) are more frequently expressed in patients with hormone independent prostate cancer. This indicates that failure of chemotherapy in progressive pros-

tate cancer could be caused by these MDR mechanisms. Furthermore, increases in Bcl-2/ Bax ratio and p53 positive tumours in hormone independent prostate cancer, may inhibit cell death when challenged with drugs that act through triggering apoptosis. Overexpression of topoisomerases in advanced prostate cancer may favour the application of topoisomerase targeting agents. Further studies will have to provide evidence for a functional role of the MDR proteins in advanced chemotherapy resistant disseminated prostate cancer. Such functional studies, using prostate cancer models, could include chemo-sensitization by MRP blocking agents and glutathione-depleting agents or agents which down-regulate Bcl-2 in combination with topoisomerase-targeting drugs that act independently of p53 and Bcl-2. If this functional role could be established, application of approaches to overcome drug resistance may become an important strategy to clinically challenge advanced prostate cancer.

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CHAPTER 5

Identification of MRP1 and glutathione as multidrug resistance mechanisms in human prostate cancer cells: chemosensitization with leukotriene D4 antagonists and buthioninesulfoximine

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Br J Urol Int 2004;93:1333-38

SUMMARY

Purpose: To assess the involvement of the multidrug resistance associated protein (MRP1) and the glutathione (GSH) pathway in the multidrug resistant (MDR) phenotype of prostate cancer *in vitro*.

Materials and methods: chemo-selection of human prostate cancer cell lines PC3 and DU145 with etoposide (VP-16), resulted in the cell lines PC3-R and DU-R. Resistance against etoposide, doxorubicin and vincristine and its reversal with leukotriene D4 antagonists MK-571 and zafirlukast, and buthioninesulfoximine (BSO) was studied in tetrazolium-dye viability (MTT) assays. Western blot analysis of MRP1 expression and GSH content measurements were performed. MRP1 function was assessed in fluorescence assays.

Results: MRP1 was increased in the MDR models. The GSH content was significantly higher in PC3-R. No increase in GSH was found in DU-R. Adding non-toxic doses of MK-571, zafirlukast or BSO significantly increased the sensitivity of the MDR models to cytotoxic drugs. Inhibition of MRP1 function was inhibited with MK-571 in the MDR models.

Conclusion: MRP1 and glutathione mediate MDR in newly developed prostate cancer models.

INTRODUCTION

Resistance against chemotherapeutic regimens has hampered treatment of clinically advanced metastatic prostate cancer[1]. Data have been provided regarding the functional role in resistance against chemotherapy of MRP1 and glutathione in acute myeloid leukemia cells[2] and human colorectal cells, respectively[3]. The role of both MRP1 and the glutathione pathway in drug resistant prostate cancer, remains to be established. Several studies have recently reported the expression of the multidrug resistance related protein1 (MRP1) in experimental prostate cancer models and in specimens of prostate cancer tissues of patients with organ-confined prostate cancer and metastatic prostate cancer[4-6]. It was found that the number of patients expressing MRP1 was clearly increased in metastatic prostate cancers, as compared to organ-confined prostate cancers. Recently, we also described increased expression of glutatione-S-transferase-π (GST-π), a key-protein in the glutathione pathway[7], in advanced clinical prostate cancer[6].

Confirmation of a functional role for MRP1 and glutathione in the resistance against cytotoxic drugs in advanced prostate cancer would be of great interest for the development of novel strategies for treatment of patients suffering from this disease. We therefore have addressed the roles of MRP1 and glutathione using *in vitro* prostate cancer models.

MATERIALS AND METHODS

Cell lines

The human prostate cancer cell lines PC3[8] and DU145[9] were maintained at 37oC in a 5% CO2-95% air atmosphere and cultured in RPMI 1640 medium, with 7.5% foetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine (all GibcoBRL, Paisley, United Kingdom). Drug resistant derivatives of PC3 and DU145 were cultured under identical conditions. The cell lines were regularly tested for Mycoplasma infection by a sensitive PCR-based assay.

Selection and cloning of drug resistant cell lines

Etoposide (VP-16; Pharmachemie BV, Haarlem, The Netherlands) was dissolved in culture medium and PC3 and DU145 were incubated in cycles of 72 hours with cumulative concentrations of VP-16. Between cycles cultures were allowed to recover in fresh medium without VP-16 until confluent. After 12 and 13 cycles for DU145 and PC3 respectively, the chemoselected cell lines were cloned using the limiting dilution technique: single cell solutions were plated on 96-well plates (Costar Corp., Cambridge, Massachusetts) and allowed to form monocultures in culture medium without VP-16. Viable clones were harvested and the limiting dilution technique was repeated. Clones were cultured in the absence of VP-16 and stored in liquid nitrogen. Low passages of the newly obtained cell lines were used in further experiments. Cells cultured without VP-16 maintained their level of resistance against chemotherapeutic drugs for up to three months.

MRP1 detection by Western blot analysis

Cell lines were grown to near-confluency, harvested by scraping in PBS and resuspended in lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5 mM EDTA supplemented with protease inhibitors (1 mM phenylmethylsulphonylfluoride, 1 mM benzamidine, 10 µg/mL soy bean trypsin inhibitor, 50 µg/mL leupeptin, 1 µg/mL pepstatin, 20 µg/mL aprotinin)]. The cell lysates were stored on ice for 15 minutes, centrifuged (14000 x g) for 20 minutes at 4oC, after which the supernatant was removed and further processed. Cell lysate samples (20 µg protein) in Laemmli buffer[10] (15 µl) were heated at 100oC for three minutes and loaded on a 10% SDS-polyacrylamide gel and electrophoresed for 45 minutes at 200 Volts. Prestained markers (Novex, San Diego, California) were used as size standards. The *in vitro*-selected adriamycin-resistant human small-cell lung-carcinoma cell line GLC4/ADR[11], expressing MRP1 at high levels, served as positive control and 2 µg protein was loaded. Subsequently, the proteins were transferred to a nitrocellulose membrane (Protan Nitrocellulose, Schleicher & Schuell, Dassel, Germany) by electroblotting at 100 V for 45 minutes.

The remaining protein binding sites were blocked by incubating the blot for 1 hour in Tris buffered saline (TBS, pH 7.5) containing 1 % blotting substrate (Boehringer Mannheim, Germany). The membranes were then incubated overnight at 4oC with the MRPr1 (Sanbio, the Netherlands) antibody at 1 : 3000 dilution in TBS, pH 7.5, containing 1 % blotting substrate. After washing with TBS containing 0.1% Tween 20 (Sigma), the membranes were incubated with rabbit-anti-rat immunoglobulines conjugated to HRP (1/4000; Dako). Immune complexes were detected by chemiluminescence (Boehringer Mannheim, Germany) and visualised on Hyperfilm ECL (Amersham Pharmacia Biotech, Uppsula, Sweden).

Measurement of glutathione content

The glutathione content of parental and selected drug resistant cell lines was measured. One million cells were plated and incubated without or with 50 µM buthioninesulfoximine during 24 hours. Afterwards the cells were harvested and resuspended in 250 µl PBS and 250 µl 2 M HClO4 / 4 mM EDTA. After centrifugation, the supernatant was neutralised with 250 µl 2M KOH / 0.3 M MOPS. Glutathione content was then measured in 25 µl of this cell extract supplemented with 1 ml 2 M HClO4 / 4 mM EDTA, 50 µl NADPH, 20 µl DTNB (5,5-dithio-bis(2-nitrobenzoicacid) and 20 µl glutathione reductase after 30 minutes. The absorbance was measured photometrically at 412 nm using an Ultrospec 3000 (Amersham Pharmacia Biotech, Roosendaal, the Netherlands). All measurements were performed in triplicate.

MDR1 detection by Western blot analysis and FACS

Western blotting experiments were performed as described for MRP1, without boiling the samples. The monoclonal antibody JSB1 (Sanbio, the Netherlands) was used for MDR1 detection in a 1 : 100 dilution in TBS, pH 7.5.

Also, fluorescence assay was used for MDR1 detection. Cells were fixed by resuspending in 2% formaldehyde (37%) in acetone for 10 seconds. After washing, cells were incubated with 0.2 µg/µl JSB1 antibody diluted in PBS with 1% rabbit serum, 0.2% goat serum and 0.02% BSA or mIgG1 control antibody for 45 minutes at room

temperature. Cell-bound antibodies were detected by fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse immunoglobulin antibodies (Dako). Fluorescence was measured using the FACScalibur (Becton Dickinson). The MDR1 expressing drug resistant cell line 8226 D6[12] was used as positive control.

Function of MRP1 and MDR1

MRP1 function was studied using a modification of a previously described functional assay[2]. The fluorescent molecule carboxyfluorescein diacetate (DCFDA) (Sigma) was used as an MRP1 substrate. Rhodamine 123 (Sigma) was used as a P-glycoprotein substrate. Cells were incubated at 37oC for 1 hour in the absence or presence of 100 µM of the MRP1 modulator MK-571 (Alexis Biochemicals) or 2 µM of the MDR1 modulator PSC 833 (Novartis Pharma, Basel, Switzerland). Following this incubation, 0.1 µM carboxyfluorescein diacetate or 200 ng/mL rhodamine 123 was added to the cells. A sample was taken at 60 minutes to assay MRP1 function or at 75 minutes to assay MDR1 function via intracellular carboxyfluorescein diacetate or rhodamine accumulation, respectively, after optimalization of experimental protocols. Cells were incubated with 0.1 µM TO-PRO-3 to identify dead cells that were subsequently excluded from further calculations. Fluorescence was measured using a FACScalibur. GLC4/ADR, served as positive control in the DCFDA experiment, whereas the cell line 8226 D6 served as positive control in rhodamine 123 accumulation assay and 8226 S as negative control[12]. All experiments were done in triplicate.

Measurement of response to chemotherapy with the MTT assay

Sensitivities of all cell lines to the antineoplastic drugs with or without addition of MK-571, were assessed by the MTT assay[13]. Cell lines were plated in 96-well plates (Costar Corp. Cambridge, MA, USA) at densities allowing logarithmic growth throughout the experiments. After allowing cells to attach for 24 hours, cells were incubated with culture medium containing a range of exponentially increasing concentrations of cytotoxic agents for a period of 72 hours. Etoposide (VP-16;

Pharmachemie BV, Haarlem, the Netherlands), doxorubicin (DOX; Farmitalia, Carlo Erba, Italy) and vincristine (VCR; Pharmacia, the Netherlands) were tested. MK-571, zafirlukast and BSO dose-response curves were obtained by exposing cells to exponentially increasing concentrations of these drugs. Then, cells were incubated with a maximum non-toxic concentration of MK-571 together with the cytotoxic drugs for 72 hours. This same procedure was followed with zafirlukast and BSO and the combination of MK-571 and BSO. Subsequently, 30 μl of MTT (3-[4,5-dimethyl thiazol-2-4]-2,5 diphenyl tetrazoliumbromide, Sigma Chemical Co., St. Louis, MO, USA) solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours at 37oC. The supernatant was carefully aspirated and 100 μl of dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany) with alkalinizing buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) was added to each well. The plates were shaken for 5 minutes in order to dissolve the formazan crystals. The absorbance was measured photometrically at 570 nm using a Bio-Rad Microplate Reader (Model 450, Bio-Rad, CA, USA). The percentage of viable cells was calculated relative to untreated cells. All assays were done in triplicate.

Statistical analysis

We calculated 95% confidence intervals to determine statistically significant differences between IC50 values in the cell viability assays. Statistically significant differences are indicated in **table 5.1** with a *. Statistically significant modulation of drug resistance is indicated in **table 5.1** with #. IC50 values and standard errors of the mean were calculated from three representative experiments with the Delta method[14].

RESULTS

Induction of multidrug resistance by chemoselection with etoposide

Culturing PC3 and DU145 in the presence of increasing concentrations of etoposide and cloning as described resulted in the selection of drug resistant clones

PC3-R and DU-R. PC3-R is significantly more resistant to etoposide, doxorubicin and to a lesser extend to vincristine than PC3. Resistance of DU-R to doxorubicin and vincristine is significantly increased compared to DU145. Resistance against etoposide was increased in DU-R, although not significantly (**Table 5.1**).

Table 5.1. Sensitivity of prostate cancer cell lines to various cytotoxic drugs and modulation of drug resistance by MK-571, zafirlukast and buthioninesulfoximine (BSO).

	DU145	**DU-R**	**PC3**	**PC3-R**
	IC50 (SE) **RF**	**IC50 (SE)** **RF**	**IC50 (SE)** **RF**	**IC50 (SE)** **RF**
VP-16 [μM]	9.3 (2.5)	16 (3.9) 1.7	13.6 (2.2)	85.6 (10.7)* 6.3
VP-16 + 30 μM MK-571	9.2 (2.7) 1	5.9 (0.34) # 0.6	15.3 (4.6) 1.1	57.3 (7.1) 4.2
Dox [nM]	81 (11.6)	313 (82.8)* 3.9	137 (15.3)	935 (187)* 6.8
Dox + 30 μM MK-571	28.5 (2.2) # 0.4	100 (9.9) # 1.3	99 (27.2) 0.7	740 (92.4) 5.4
VCR [nM]	7.1 (0.12)	8.4 (0.12)* 1.2	3.6 (0.12)	4.1 (0.12)* 1.1
VCR + 30 μM MK-571	0.36 (0.02) # 0.05	0.24 (0.01) # 0.03	0.12 (0.02) # 0.03	0.097 (0.01) # 0.03
VCR + ZLK 25 μM			3.5 (0.1) 1	3.9 (0.2) 1.1
ZLK 100 μM	4.0 (0.2) # 0.6	5.0 (0.3) # 0.7		
VCR + 50 μM BSO	3.8 (0.2) # 0.5	8.4 (0.7) 1.2	3.6 (0.6) 1	0.8 (0.04) # 0.2

The *in vitro* sensitivity to cytotoxic drugs of two prostate cancer cell lines (DU145; PC3) and their drug resistant derivatives (DU-R; PC3-R) was examined using MTT assays. The values are the calculated IC50 values with standard error of the mean (SE) for etoposide (VP-16); doxorubicin (DOX) and vincristine (VCR), either in the absence or presence of the multidrug resistance associated protein-1 (MRP1) modulators and leukotriene inhibitors MK-571 and zafirlukast (ZLK) and the glutathione depleting agent buthioninesulfoximine (BSO).
Also shown are the resistance factor values which indicate changes in IC50 values after chemo-selection and/or after exposure to a modulator and a chemotherapeutic drug. Note that the resistance factor in the parental cell lines DU145 and PC3 is set at 1.
Abbreviations: VP-16: etoposide; Dox: doxorubicin; VCR: vincristine; RF: resistance factor
*: statistically significant increase in resistance between parental and chemo-selected cell lines.
#: Statistically significant modulation of drug resistance by MK-571
Note that differences are considered statistically significant if 95% confidence intervals for IC50 values did not overlap.

Expression of MRP1 and MDR1

Figure 5.1 shows a representative Western blotting experiment demonstrating the expression of MRP1 in PC3, PC3-R, DU145 and DU-R. MRP1 expression in PC3 and DU145 is relatively low. MRP1 expression is clearly increased in the resistant chemo-selected PC3-R and DU-R. GLC4/ADR expresses MRP1 at a high level.

MDR1 could not be detected in any of these cell lines in Western blotting experiments or in experiments involving the fluorescent tagging of MDR1 expressing cells with JSB1 and fluorescently labelled secondary antibody (data not shown). However, both procedures clearly detected MDR1 expression in the positive control 8226 D6.

Figure 5.1. MRP1 expression in prostate cancer cell lines
Cell extracts were prepared from the drug sensitive parental prostate cancer cell lines PC3 and DU145 and their drug resistant derivatives PC3-R and DU-R. A cell extract from the MRP1 overexpressing small cell lung cancer cell lines GLC4/ADR was used as a positive control. Twenty µg of total protein, except for GLC4/ADR where 2 µg was loaded, was subjected to SDS-PAGE and blotted onto nitrocellulose filters. MRP1 expression is clearly increased in PC3-R and in DU-R as compared to the parental cell lines PC3 and DU145.

Glutathione content

The concentration of glutathione, measured with or without a 24-hour exposure to the glutathione depleting agent buthioninesulfoximine, was 40 µM and 55 µM in PC3 and DU145, respectively, and was reduced to 10 µM and 12.5 µM, respectively, after treatment with BSO. Glutathione concentration was increased to 80 µM in

the selected drug resistant cell line PC3-R. After BSO treatment glutathione was reduced to 27.5 µM. In DU-R glutathione concentration was not increased (40 µM), but markedly decreased after BSO treatment: 7.5 µM.

MRP1 function; inhibitory effect of MK-571

The activity of MRP1 was tested in cells by measuring the ability to extrude DCFDA in the absence or presence of the MRP1 inhibitor MK-571. Fluorescence histograms, showing fluorescence intensity and reflecting carboxyfluorescein content, with or without MK-571, are shown in **Figure 5.2** for DU145 and DU-R, PC3 and PC3-R, GLC4 and GLC4/ADR. Ratios of DCFDA content with and without MK-571 are shown in **Figure 5.2**, representing the relative inhibition of MRP1 function (blocking factor). MRP1 function was inhibited by MK-571 resulting in a higher DCFDA content in all cell lines exposed to MK-571. No toxicity was observed of the 100 µM MK-571 used during the course of the experiment. This was verified with TO-PRO-3 to exclude non-viable cells, revealing a survival of 98 to 99% of cells during the experiment in every cell line.

The absence of MDR1 function in prostate cancer cell lines was confirmed by flow cytometry using and a rhodamine retention assay in conjunction with PSC833. MDR1 function was clearly detected in the drug resistant plasma cell line 8226 D6, which served as positive control. The blocking factor (increased rhodamine accumulation) was 5.6 for the positive control and 0.96 for the negative control 8226 S.

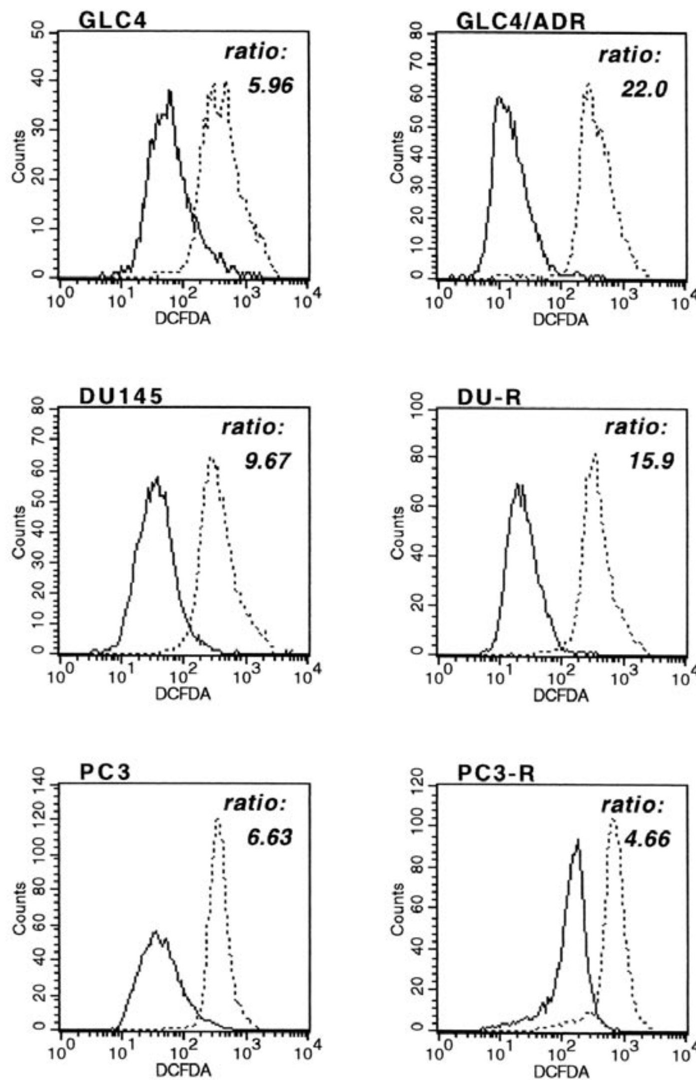

Figure 5.2. Modulation of MRP1-mediated carboxyfluorescein transport by MK-571.

MRP1 function was measured using carboxyfluorescein diacetate (DCFDA) in the presence (dashed line) or absence (solid line) of MK-571. Fluorescence histograms show fluorescence intensity and reflect carboxyfluorescein content for cell lines GLC4 and GLC4/ADR (control), DU145, DU-R, PC3 and PC3-R. Exposure of cells to MK-571 results in accumulation of carboxyfluorescein in all cell lines. Values shown in the upper right hand corner of the histograms represent the ratio of the carboxyfluorescein content of the cells determined in the presence of MK-571 and the carboxyfluorescein content of the cells detemined in the presence of MK-571 and that of the cells with no MK-571.

Decreased multidrug resistance with MK-571

PC3, DU145, PC3-R and DU-R, were exposed to etoposide, doxorubicin and vincristine. In these experiments, 30 μM of MK-571 was added. MK-571 dose-response experiments revealed that this concentration was not cytotoxic in our cell lines (data not shown). Significant reversal of drug resistance against etoposide was observed in DU-R (IC50 values for all experiments are shown in **table 5.1**). Significant reversal of doxorubicin resistance was seen in DU145 and DU-R. The combination of vincristine and MK-571 enhanced cytotoxicity significantly and most strongly in both parental and chemo-selected cell lines PC3, DU145, PC3-R and DU-R.

Decreased vincristine resistance with zafirlukast

Zafirlukast (ZLK), another leukotriene inhibitor, was tested for its ability to enhance vincristine toxicity in the cell lines PC3, PC3-R, DU145 and DU-R.

Maximum non-toxic doses of zafirlukast were titrated to 25 μM for PC3 and PC3-R and 100 μM in DU145 and DU-R. The combination of vincristine and 25 μM zafirlukast did not increase cytotoxicity in PC3 and PC3-R. However, 100 μM zafirlukast in combination with vincristine significantly increased cytotoxicity in DU145 and DU-R (**Table 5.1**).

Decreased drug resistance with BSO

A significant reduction in glutathione level was achieved by incubating cell lines with BSO for 24 hours (see result section, glutathione content). Addition of a non-toxic dose of 50 μM BSO in combination with vincristine reduced IC50 values significantly in DU145 and PC3-R, respectively (**Table 5.1**).

Combination of 50 μM BSO with 3.4 μM etoposide or 36.8 nM doxorubicin reduced the viable cell count of DU145 by 15% and 0%, respectively and the viable cell count of DU-R by 46% and 36%, respectively. In PC3 and PC3-R no sensitization occurred with the BSO – etoposide / doxorubicin combination.

DISCUSSION

Intrinsic or acquired resistance to cytotoxic drugs may cause failure of chemotherapy in prostate cancer patients. In this report, we study the role of MRP1 and glutathione in *in vitro* prostate cancer resistance against chemotherapy and its reversal targeting these proteins.

Extensive laboratory studies on mechanisms of drug resistance have revealed that MDR is a complex phenomenon, reflected by a vast array of genetic, molecular and biochemical alterations in resistant tumour cells and tissues. These include reduced intracellular drug uptake, increased drug efflux, drug sequestration into cytoplasmic compartments, alterations in drug metabolism and/or target enzymes, increased detoxification, enhanced ability to repair DNA damage and failure to undergo apoptosis[15-17].

Evidence is accumulating to support the view of involvement of MRP1 and the glutathione metabolism in clinically relevant drug resistance[18, 19]. Expression of MRP1 and GST-π in *in vitro* and clinical prostate cancer has been reported in several publications[4-6]. Their expression appears to be increased in advanced prostate cancer, but a functional role remains uncertain.

To investigate the role of MRP1 and glutathione in prostate cancer, experiments were performed in human prostate cancer cell lines. Chemo-selection of cell lines PC3 and DU145 with etoposide resulted in the resistant cell lines PC3-R and DU-R. Etoposide, which is used clinically in combination therapy for advanced prostate cancer[20] is a well-known MDR drug and may up-regulate different MDR mechanisms, and was therefore chosen for selection. Resistance was significantly and reproducibly increased with a factor 1.7 and 6.3 in DU-R and PC3-R, respectively (**table 5.1**). Resistance against doxorubicin was increased with a factor 3.9 and 6.8 in DU-R and PC3-R, respectively. Resistance against vincristine was significantly increased although resistance factors were quite low: 1.2 and 1.1 in DU-R and PC3-R, respectively. In a clinical setting, a comparable increase of resistance to cytotoxic regimens could result in treatment failure due to unacceptable toxicity of higher dosages of cytotoxic drugs. Cross-resistance against doxorubicin and vincristine implies involvement of the multidrug resistance phenomenon in our cell lines.

We observed MRP1 expression in parental prostate cancer cell lines PC3 and DU145. Chemoselection resulted in increased MRP1 expression in PC3-R and DU-R. Glutathione content was increased in the selected cell line PC3-R, but not in DU-R. It is known that MRP1-mediated transport requires glutathione[21]. Upregulation of MRP1 and glutathione in PC3-R explains increased resistance against etoposide and doxorubicin. In DU-R, a moderate increase in MRP1 expression and a slightly decreased glutathione content may result in a relatively low increase of resistance. Resistance against vincristine is only slightly increased. One can speculate that vincristine toxicity is already efficiently countered by MRP1 and glutathione in the non-selected cell lines. An increase in expression of these MDR factors may then not further increase vincristine resistance.

To further clarify the possibility of MRP1 and glutathione mediated resistance, experiments with putative MRP1 and glutathione mediated-MDR reverting agents were performed. An inhibitor of MRP-mediated transport[2], leukotriene D4 receptor antagonist MK-571, and another leukotriene inhibitor, zafirlukast[22] which is clinically applicable, were used to study reversal. Furthermore, cellular depletion of glutathione with BSO was performed to study glutathione mediated multidrug resistance.

Complete reversal of resistance in the MK-571 experiments was seen in DU-R with etoposide and vincristine and nearly complete reversal with doxorubicin. Complete reversal in PC3-R was seen with vincristine and partial reversal with etoposide and doxorubicin (**table 5.1**). A less efficient reversal of resistance in PC3-R may be caused by upregulation of both MRP1 and glutathione, resulting in enhanced drug transport. If MRP1 were not totally blocked by MK-571, the remaining active MRP1 could continue to extrude cytotoxic drugs, supported by an increased glutathione content. In DU-R, a lower glutathione content may facilitate the inhibiting action of MK-571 on MRP1, resulting in reversal of drug resistance.

Significant reversal of resistance with zafirlukast in combination with vincristine was seen in DU-R, but not in PC3-R (**table 5.1**). Zafirlukast appears to be less effective than MK-571 and its action may be influenced by MRP1 and glutathione synergy as proposed for MK-571. Also, the highest achievable non-toxic concentration of zafirlukast (25 μM) may be too low to block MRP1 in PC3-R.

Significant reduction of vincristine resistance by glutathione depletion with BSO was achieved in PC3-R (**table 5.1**). Significant reduction of etoposide and doxorubicin resistance was observed in DU-R (see results section). Depletion of glutathione with BSO was significant in all cell lines, but BSO does not consistently reduce resistance. It is speculative whether different cell lines require different glutathione concentrations for MRP1 mediated drug transport. Combination of MK-571 and BSO led to markedly increased cell death without addition of a cytotoxic drug (data not shown). Apparently, blocking of MRP1 and depletion of glutathione is highly lethal to our models. This may support the idea of MRP1 mediated transport of glutathione S-conjugated endo- and xenobiotics in prostate cancer cells.

MRP1 function and blocking of MRP1 by leukotriene inhibitor MK-571 was further studied in flow cytometric assays. Increased MRP1 expression was related to a more pronounced efflux of carboxyfluorescein. Exposure to MK-571 resulted in accumulation of the MRP1 substrate carboxyfluorescein, demonstrating that MRP1 can be blocked by a leukotriene inhibitor in prostate cancer cells (**Figure 5.2**). Blocking MRP1 in PC3-R was less effective, which is in concordance with a less efficient reversal of drug resistance in PC3-R. Increased MRP1 expression combined with an increased glutathione content may result in enhanced drug transport, which is more difficult to overcome by MK-571. In contrast, DU-R cells contain less glutathione and blocking of MRP1 results in a more profound accumulation of MRP1 substrate.

We observed accumulation of MRP1 substrate by MK-571 in both chemo-naive and chemo-selected cells and enhanced cytotoxicity in chemo-naive and chemo-selected cells in the chemo-modulation experiments with MK-571 and BSO, which appears to reflect a role of MRP1 and glutathione in MDR of prostate cancer cells. The possibility to modulate drug resistance in the chemo-naive parental prostate cancer cell lines is of importance because most patients have not previously received chemotherapy. Applied concentrations of MK-571 in the MTT assay are 20 times less than clinically achievable peak plasma levels: the concentration of MK-571 in our experiments was 30 μM, whereas peak plasma level can reach as high as 610 μM in man[23]. BSO has been used in clinical trials[24] and concentrations used in this study are clinically achievable as well.

CONCLUSION

Active drug transport by MRP1 and glutathione metabolism mediated detoxification appears to influence the response to cytotoxic drugs in our prostate cancer models and may cause the multidrug resistant phenotype of prostate cancer cells. Although we have ruled out involvement of MDR1, upregulation of other MDR mechanisms may also play a role.

As MRP1 can be blocked and glutathione can be depleted *in vitro*, further *in vivo* experiments are imperative as a step towards clinical application of MDR modulation strategies aiming at increased efficacy of chemotherapy with respect to palliation or even prognosis of prostate cancer patients with progressive disseminated prostate cancer.

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CHAPTER 6

In vitro- and in vivo reversal of resistance against chemotherapy with leukotriene D4 antagonist MK-571 in MRP1-expressing human prostate cancer cell line PC346C and xenografts PC339 and PC346BI.

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Submitted for publication

SUMMARY

Purpose: efficacy of chemotherapy has been limited in advanced prostate cancer, possibly as a result of the multidrug resistance phenomenon (MDR). The multidrug resistance associated protein (isoform 1; MRP1) mediated MDR can be selectively modulated with the leukotriene inhibitor MK-571. The objective of the present study was to investigate the possibility to chemosensitize prostate cancer *In vitro* and *in vivo* aiming at modulation of MRP1-related MDR.

Methods: expression of MDR associated proteins P-glycoprotein (MDR1), MRP isoforms 1 and 2 was determined with Western blotting in a panel of human prostate cancer xenografts and in the xenograft derived cell line PC346C. The effect of chemosensitization of PC346C to vincristine (VCR), doxorubicin (DOX) and etoposide (VP-16) with MK-571 on cell growth was assessed with cell viability assays. Efficiency of blocking the MRP1 pump with MK-571 was investigated in PC346C by measuring the carboxyfluorescein efflux in fluorescence assays. Toxicity and antitumour effect of VCR and VCR combined with MK-571 was determined *in vivo* using the human prostate cancer xenografts PC339 and PC346BI.

Results: MRP1 and MRP2 were expressed in all xenografts and in PC346C. No expression of MDR1 was found. Significant chemosensitization with MK-571 was achieved *In vitro* in PC346C. MRP1-mediated carboxyfluorescein efflux was demonstrated in PC346C which was significantly inhibited with MK-571. *In vivo* application of tolerable doses of intravenous VCR to PC339 tumour-bearing mice, with relatively high MRP1 expression, resulted in significant although transient reduction of tumour volumes. Intravenous VCR combined with intraperitoneal MK-571 resulted in significant *in vivo* chemosensitization to VCR to the xenograft PC346BI, which had relatively low MRP1 expression.

Conclusions: effective chemosensitization with MK-571 was demonstrated, both *in vitro* and *in vivo*, indicating that MRP1 plays a role in the *in vitro* and *in vivo* MDR phenotype of prostate cancer. These findings may encourage clinical chemosensitization strategies to improve results of chemotherapy in advanced prostate cancer patients.

INTRODUCTION

Clinically, metastasized prostate cancer can progress to a hormone-independent state. Failure of androgen deprivation to halt tumour growth necessitates alternative treatment modalities to challenge advanced prostate cancer. Chemotherapy results have been disappointing, with limited response rates in metastatic prostate cancer[1, 2]. Recently, two randomized clinical trials have reported on a two to four month survival benefit with combination therapies of docetaxel and prednisone or docetaxel and estramustine as compared to mitoxantrone and prednisone, respectively[3, 4]. Resistance against a variety of chemotherapeutic agents- multidrug resistance-(MDR), has been well described in many studies, among which several have focussed on prostate cancer[5]. *In vitro* experiments have demonstrated the expression of MRP1 in human prostate cancer cell lines, among other multidrug resistance related proteins[6, 7]. Further experiments have pointed out a role of the multidrug resistance associated protein 1 (MRP1) in *in vitro* prostate cancer, which was upregulated in cells treated with etoposide resulting in increased resistance against etoposide, doxorubicin and vincristine. Reversal of resistance of prostate cancer cells was achieved with leukotriene inhibitor MK-571, which blocked the function of MRP1, namely ATP-dependent extrusion of MRP1 substrates, comprising several chemotherapeutic drugs, including vincristine, doxorubicin and etoposide[8]. In clinical prostate cancer, increased expression of MRP1 in advanced prostate cancer was found[9] , as compared to locally confined disease, possibly explaining the clinical resistance of disseminated progressive prostate cancer against chemotherapy.

Elaborating on these findings, the goals of the present study were to confirm the relevance of *in vitro* expression of MRP1 and modulation of drug resistance by targeting MRP1 for an *in vivo* situation. We have studied a xenograft derived prostate cancer cell line PC346C[10] to determine the possibility to modulate etoposide, doxorubicin and vincristine response with MK-571. After verifying the concept of chemosensitization in PC346C, we proceeded to *in vivo* experiments using athymic nude mice bearing human hormone independent prostate tumours: xenograft models PC-346BI and PC-339 established in our laboratory [10, 11] were used to

investigate modulation of vincristine response with leukotriene inhibitor MK-571 *in vivo*.

Application of chemo-sensitization strategies in advanced prostate cancer have been successful in cell cultures. Therefore, these *in vivo* experiments aim at providing the important link between *in vitro* reversal of multidrug resistance and the clinical application of chemo-modulation. To proceed to clinical studies, information on efficacy and, particularly, on toxicity of such strategies in a biological setting is of the utmost importance. Patients with metastatic hormone-independent prostate cancer, in whom, at present, effective therapy is lacking, may benefit from chemo-sensitization.

MATERIALS AND METHODS

Cell lines

The human prostate cancer cell line PC-346C was established from the PC-346 xenograft [10] derived from primary tumour tissue from a transurethral resection of the prostate (TURP) from a non-progressive prostate cancer patient. Both the xenograft and the cell line PC-346C are androgen responsive, secrete prostate specific antigen (PSA) and express the wild type androgen receptor. PC-346C cells were cultured in a modified medium based on Dulbecco's modified Eagle's medium/Ham's F12 (1:1) (Invitrogen, Breda, The Netherlands) supplemented with 2% (v/v) foetal calf serum, 1% Insulin-Transferrin-Selenium-general (ITS-G) (Invitrogen, Breda, The Netherlands), 10 ng/ml epidermal growth factor (EGF, Sigma Aldrich, St Louis, MO, USA), 0.1 nM of the synthetic androgen R1181 (NEN, Boston, MA, USA). Serving as control cell lines in the MRP1 experiments, the human small-cell lung-carcinoma cell line GLC4 and the *in vitro*-selected adriamycin-resistant GLC4/ADR[12] were cultured in RPMI 1640 medium, supplemented with 7.5% (v/v) foetal calf serum, 100 units/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine (all Gibco-BRL, Paisley, United Kingdom). The multiple myeloma cell line 8226 S (MDR1 negative control cell line) and its doxorubicin selected variant 8226 D6 (MDR1 positive control cell line) [13] were cultured in RPMI 1640 (Invitrogen – Life Technologies,

Paisley, Scotland) supplemented with 10% foetal calf serum, 2 mM L-glutamin, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO2 in air at 37oC. The 8226 D6 cell line was routinely cultured in the presence of 60 nM doxorubicin, however at least two days before use in an experiment the cells were cultured in medium without doxorubicin. The cell lines were regularly tested for mycoplasma infection by a sensitive PCR-based assay.

Xenografts

The PC-339 and PC-346BI human prostate tumour xenografts were selected from a panel of 13 human prostate tumour xenograft models [10, 11] on the basis of their MRP1 and MRP2 expression levels. The origin and main characteristics of PC-339 and PC-346BI have previously been published: PC-339 was derived from a patient who underwent a transurethral resection and who was progressive under hormonal therapy whereas PC-346 was derived from an untreated patient who underwent a TURP. PC-346BI is an androgen unresponsive variant of the original PC-346 and although androgen independent, still exhibits characteristics of differentiation such as secretion of PSA (van Weerden, personal communication). The androgen independent PC-339 model lacks PSA expression and histologically shows an anaplastic nature, representing late stage, hormone refractory disease.

MDR1, MRP1 and MRP2 expression - Western blotting

PC346C cells were harvested by trypsinization and washed three times with 4oC pre-cooled phosphate buffered saline (pH 7.4). Snap frozen (-80oC) material of xenografts PC339 and PC346BI were disrupted in a Polytron blender (Kinematica, Switzerland), washed three times with 4oC pre-cooled phosphate buffered saline pH 7.4 and finally pelleted in 0.5 ml polypropylene Eppendorf tubes at 13.000 rpm and 4oC. Supernatants were removed without disturbing the cell pellets. Pellets of 5-50x106 cells were solubilized in 100 µl 20 mM tris-HCl pH7.4 containing 1 mM MgCl2, 2% (v/v) Triton X-100, 0.1% (w/v) SDS and protease inhibitors (Complete tablets, Roche Diagnostics GmbH, Mannheim, Germany), by end over end rotation

on a Westbart RM10A roller apparatus overnight at 4oC. After the cell lysates were centrifuged at 13.000 rpm for 30 minutes at 4oC the resulting supernatants were filtrated over an Acrodisc LC13 PVDF 0.45 µM filter (Gelman Sciences, USA). Each filtrate was mixed with 3 volumes of SDS-PAGE (4x) sample buffer, pH 6.8, containing 0.25 M Tris-HCl, 40% (v/v) glycerol, 8% (w/v) SDS, 20% (v/v) â-mercaptoethanol and 0.02% (w/v) pyronin G. Cell lysate samples (4 µg protein for MDR1, 20 µg for MRP1 and 4µg for MRP2) in Laemmli buffer[14] (15 µl) were heated for three minutes (100 oC) and loaded on a 10% SDS-polyacrylamide gel and electrophoresed for 60 minutes at 100 Volts. Prestained markers (Novex, San Diego, California) were used as size standards. Subsequently, the proteins were transferred to a nitrocellulose membrane (Protan Nitrocellulose, Schleicher & Schuell, Dassel, Germany) by electroblotting at 100 V for 60 minutes. The blotting paper was blocked by incubating the blot for 1 hour in Tris buffered saline (TBST, pH 7.5) containing 5% (v/v) NFDM (non fat dry milk protifar plus; Nutricia, the Netherlands). The membranes were then incubated overnight in TBST, pH 7.5, containing 5 % NFDM at 4oC with the C219 MDR1 antibody (Signet, Dedham, MA; dilution 1:1000), the MRPr1 (Sanbio, the Netherlands) MRP1 antibody and the M2III-6 MRP2 antibody (Sanbio, the Netherlands) at 1 : 1000 dilution in TBST, pH 7.5, containing 5 % NFDM blotting substrate. After washing with TBS containing 0.1% Tween 20 (Sigma), the membranes were incubated with rabbit-anti-rat immunoglobulines conjugated to HRP (1/1000; Dako). Protein bands were visualised by chemiluminescence.

In vitro response to chemotherapy with or without the MRP1-modulator MK-571 - MTT assay

Sensitivity of PC346C to antineoplastic drugs with or without addition of MK-571, was assessed by the MTT assay [15]. Cells were plated in 96-well plates (Costar Corp. Cambridge, MA, USA) at densities allowing logarithmic growth throughout the experiments. After allowing the cells to adhere for 24 hours, cells were then incubated with culture medium containing a range of exponentially increasing concentrations of cytotoxic agents for a period of 72 hours. For *in vitro* testing, drug solutions were freshly prepared in culture medium. The following compounds

were tested: etoposide (VP-16; Pharmachemie BV, Haarlem, the Netherlands), doxorubicin (DOX; Farmitalia Carlo Erba, Italy) and vincristine (VCR; Pharmacia, the Netherlands). MK-571 dose-response curves were obtained by exposing cells to exponentially increasing concentrations of these drugs in an identical manner as the cytotoxic drugs. In addition, cells were incubated with a maximum non-toxic concentration of MK-571 together with the cytotoxic drugs. After 72 hours, 30 ml of MTT (Sigma Chemical Co., St. Louis, MO, USA) solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours at 37oC. The supernatant was carefully aspirated and 100 ìl of dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany) with alkalinizing buffer (0.1 M glycin, 0.1 M NaCl, pH 10.5) was added to each well. The plates were shaken for 5 minutes in order to dissolve the formazan crystals. The absorbance was measured photometrically at 570 nm using a Bio-Rad Microplate Reader (Model 450, Bio-Rad, CA, USA). The percentage of viable cells was calculated relative to untreated cells. All assays were done in triplicate.

Measurement of MRP1 and MDR1 function in a fluorescence assay

For measurement of the function of MRP1 in cell lines PC346C, GLC4 and GLC4/ADR, the fluorescent molecule DCFDA (Sigma Chem Co. St Louis, MO, USA) was used as an MRP1 substrate[16]. For measurement of the function of MDR1 (P-glycoprotein) in cell lines PC346C, 8226 D6 and 8226 S, Rhodamine 123 (Sigma) was used as a MDR1 substrate[17] . Cells were incubated at 37oC for 1 hour at 5% CO2 in the absence or presence of 100 µM of the MRP1 modulator MK-571 (Alexis Biochemicals) or 2 µM of the MDR1 modulator PSC 833[18] (Novartis Pharma, Basel, Switzerland). Following this incubation, 0.1 µM DCFDA or 200 ng/mL rhodamine 123 was added to the cells. A sample was taken at 60 minutes to assay MRP1 function or at 75 minutes to assay MDR1 function via intracellular DCFDA or rhodamine accumulation, respectively, after optimalization of experimental protocols. Cells were incubated with 0.1 µM TO-PRO-3 (Molecular Probes, Inc. OR, USA) to exclude non-viable cells. Fluorescence was measured using a FACScalibur (Becton Dickinson, USA). The *in vitro*-selected adriamycin-resistant human small-cell lung-carcinoma cell line GLC4/ADR, expressing MRP1 at high levels, as well as parental cell line GLC4, served

as positive control. Cell line 8226 D6 served as positive control in rhodamine 123 accumulation assay and 8226 S as negative control[13]. All experiments were done in triplicate.

In vivo treatment of human prostate cancer cells with Vincristine and/ or MK-571

- Mice

Intact male NMRI athymic nude mice [19] were obtained from Harlan Holland (Horst, The Netherlands). Mice were kept under specific pathogen-free conditions with water and food ad libitum. The study was authorized by the Institutional Committee for the Use of Experimental Animals in research in compliance with the Dutch Law on Animal Experiments.

- Drug treatment

Treatment of non-tumour bearing (control) mice with VCR and MK-571 was performed to ascertain the relative safety, in terms of toxicity, in the strain of athymic nude mice used (NMRI) prior to application to tumour-bearing mice. Maximum tolerated dosages (MTD) were identified for a single dose schedule. Dosages were chosen according to literature data[20-22] and based on tolerated dosages of the chemotherapeutics and modulators as resulting from the outcome of previous *in vitro* studies[8]. Criteria of toxicity are based on guidelines of the "Code of Practice of the use of experimental animals in cancer research".

- Experimental study

PC-339 and PC-346BI tumour fragments were implanted subcutaneously in the right shoulder of intact male athymic nude mice. Treatment was started when tumours were established. Maximum tolerated dose (MTD) of vincristine intravenously (i.v.) and MK-571 intraperitoneally (i.p.) were used as was first determined in non-tumour-bearing animals. PBS (control) was administered intraperitoneally. Mice were assigned to the following treatments: a PBS control group, a group in which MK-571 was administered intraperitoneally at a 40 mg/kg concentration, a group in which MK-571 was administered at a 80 mg/kg concentration (in PC346BI

only), a group in which vincristine was administered intravenously at a 0.8 mg/kg concentration, a group in which vincristine (0.8 mg/kg intravenously) was administered in combination with MK-571 (40 mg/kg intraperitoneally) and a group in which vincristine (0.8 mg/kg) was administered in combination with a 80 mg/kg concentration of MK-571 (in PC346BI only).

- Tumour measurement and body weight

Tumour growth was followed twice weekly by caliper measurements of tumour nodules. Tumour volume was estimated using the following formula: $TV = \pi/6 * (d1*d2)\ 3/2$, with d1 and d2 being two perpendicular tumour diameters. Body weight of mice was followed concomitantly.

- Antitumour activity

Tumour growth curves (tumour volume versus time) were determined for the different treatments. Slopes of the growth curves, defined as the regression line of tumour volume versus time, were calculated. Antitumour activity was determined by comparing slopes to PBS control experiments and calculating significant differences ($p< 0.05$) between treatments.

Statistical analysis

We calculated 95% confidence intervals to determine statistically significant differences between IC50 values in the cell viability assays. IC50 values and standard errors of the mean were calculated from three representative experiments with the Delta method[14].

Analyses of xenograft experiments was done with SAS procedure PROC MIXED, version 8.02 (SAS Institute Inc, Cary, NC, USA). In order to calculate the doubling time of tumour volume, the value was converted to 2Log(tumour volume). Using this logarithmic value, the slope of tumour volume rise was calculated. The doubling time was calculated by taking 1/slope. p-Values were calculated for the comparison of the doubling time of the different treatment groups among each other and with the placebo group.

RESULTS

MRP1 and MRP2 expression in xenografts and xenograft derived cell line PC346C.

MRP1 and MRP2 were variably expressed in prostate cancer xenografts PC82, PC133, PC135, PC295, PC310, PC324, PC329, PC339, PC346, PC346I, PC346B, PC346BI, PC374, PC374F and cell line PC346C.

Figure 6.1 shows a representative Western blotting experiment with detection of MRP1 and MRP2 in prostate cancer xenografts PC339, PC346BI and cell line PC346C. MRP1 and MRP2 had similar expression patterns in the xenografts.

PC339, with relatively high MRP1 expression and PC346BI, with relatively low MRP1 expression, were chosen for further *in vivo* experiments.

MDR1 could not be detected in Western blotting experiments, although the positive control cell line 8226 D6 showed MDR1 expression (data not shown) .

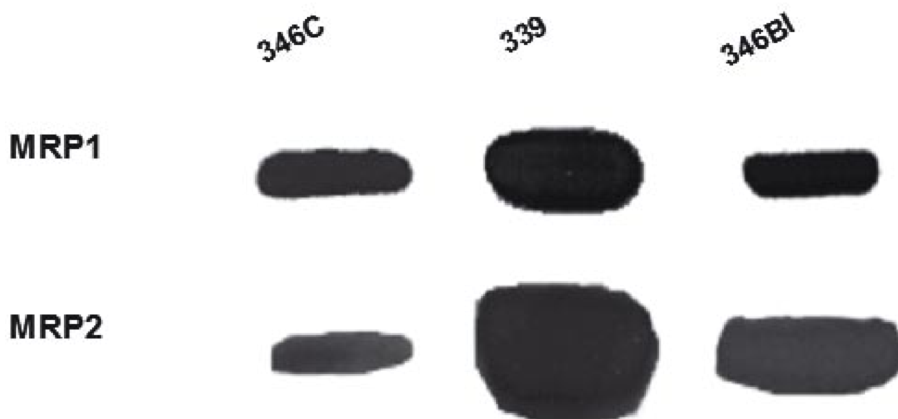

Figure 6.1. Western blotting

MRP1 expression in prostate cancer xenografts PC339 and PC346BI and in prostate cancer cell line PC346C.

Cell extracts (20 µg for MRP1 and 4µg for MRP2) were loaded and subjected to SDS-PAGE and blotted onto nitrocellulose filters.

Chemosensitization of cell line PC346C to etoposide, doxorubicin and vincristine with MK-571.

Table 6.1 shows the results of exposure of PC346C to etoposide, doxorubicin and vincristine (IC50 values). Significant sensitization of PC346C was achieved in the presence of a non-toxic dose of 5 μM MK-571 (as determined in preliminary experiments, data not shown), reflected by 2.4-, 3.6- and 7.7-fold decreased resistance factors (RF) for etoposide, doxorubicin and vincristine, respectively.

Table 6.1. Sensitivity of prostate cancer cell line PC346C to various cytotoxic drugs and modulation of drug resistance by 5 μM MK-571.

	RF	IC50 (Molar)
VP-16	1	8.7 x 10-7 ± 0.9 x 10-7
VP-16 + MK-571	0.41 ± 0.1*	3.6 x 10-7 ± 0.4 x 10-7
DOX	1	5.6 x 10-8 ± 0.15 x 10-8
DOX + MK-571	0.28 ± 0.013*	1.6 x 10-8 ± 0.04 x 10-8
VCR	1	5.0 x 10-10 ± 0.3 x 10-10
VCR + MK-571	0.13 ± 0.04*	6.6 x 10-11 ± 0.4 x 10-11

The *in vitro* sensitivity to cytotoxic drugs of xenograft derived prostate cancer cell line PC346C was examined using the MTT assay. The values are the calculated mean IC50 values ± standard error of the mean (SEM) for etoposide (VP-16), doxorubicin (DOX) and vincristine (VCR) either in the absence or presence of MRP1 modulator MK-571. Also shown are the resistance factor values which indicate changes in IC50 values after exposure to MK-571 and a chemotherapeutic drug.
RF= resistance factor.
IC50= drug concentration which inhibits the cell growth by 50%.
* significant modulation: 95% confidence intervals do not overlap.

DCFDA efflux blocking with MK-571 in PC436C, GLC4 and GLC4/ADR.

The possibility to modulate MRP1 activity was studied in cells by measuring their ability to extrude DCFDA in the absence or presence of MK-571. Ratios of DCFDA content with and without MK-571 (blocking factor) representing the relative inhibition of MRP1 function by MK-571 were 7.2 in PC346C, 12.2 in GLC4 and 28.9 in GLC4/ADR. **Figure 6.2** shows fluorescence histograms, representing fluorescence intensity and reflecting DCFDA content, with and without MK-571. No toxicity was observed of the 100 μM MK-571 used during the course of the experiment. This was verified with TO-PRO-3 to exclude non-viable cells, revealing a survival of 98 to 99% of cells during the experiment in every cell line.

Flow cytometry using a rhodamine retention assay with MDR1 substrate PSC833 showed no MDR1 function in cell line PC346C. MDR1 function was clearly detected in the drug resistant plasma cell line 8226 D6, which served as positive control. The blocking factor (increased rhodamine accumulation) was 5.6 for the positive control and 0.96 for the negative control 8226 S.

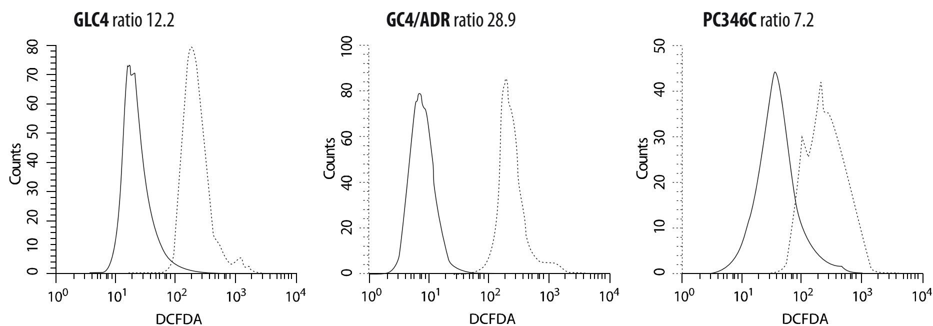

Figure 6.2. Modulation of MRP1-mediated carboxyfl uorescein transport by MK-571

Significant tumour reduction with the vincristine – MK-571 combination compared to vincristine alone - in vivo experiments

To investigate adequate bio-activity of our *in vitro* chemosensitization strategy, combinations of vincristine and MK-571 were tested in athymic nude mice bearing either the human prostate tumour models PC339 (relatively high expression of MRP1) or PC346BI (relatively low expression of MRP1).

In these xenografts, vincristine dosages were tolerated equally well: maximum tolerated dose (MTD) of vincristine i.v. was determined at 0.8 mg/kg in PC339 and PC346BI. The MTD of MK-571 was higher in PC346BI than in PC339 (80 mg/kg versus 40 mg/kg). No major side effects (neurotoxic or gastro-intestinal) were observed for single drug administration or combined treatments. In PC339 we measured no weight loss at all, whereas in PC346BI we observed a weight loss of 17,8% in 7 days in the group treated with vincristine and MK-571, but mice recovered to a weight loss of 6% over a 14 days period. In both xenografts, tumourgrowth was highest in

the group treated with PBS. Treatment with MK-571 did not alter tumour growth during the course of the experiments.

For PC339, the average growth curves are shown in **figure 6.3a** for the treatment with 0.8 mg/kg vincristine and for 0.8 mg/kg vincristine combined with 40 µg/kg MK-571. A significant anti-tumour effect of vincristine alone ($p= 0.0002$) and of vincristine combined with MK-571 ($p< 0.0001$) is seen as compared to placebo (PBS) over a 14 day interval. We observed a trend for a stronger anti-tumour effect in the vincristine combined with MK-571 group, although this did not reach significance ($p= 0.16$). Tumour volumes decreased during the course of the experiment, without significant regrowth until the end of this experiment at 14-days.

For PC346BI the average growth curves are shown in **figure 6.3b** for the treatment with 0.8 mg/kg vincristine and for 0.8 mg/kg vincristine combined with 80 µg/kg MK-571. An anti-tumour effect is observed when vincristine is administered, although not significant ($p= 0.081$ at 7 days and $p= 0.29$ at 14 days). However, addition of MK-571 to vincristine resulted in a significant anti-tumour effect after 7 days, when compared to PBS ($p=0.004$ at 7 days and $p= 0.058$ at 14 days) and when compared to vincristine alone ($p= 0.039$ at 7 days; $p= 0.12$ at 14 days). Growth curves show maximum tumour regression around the seventh treatment day, followed by regrowth of tumours, reflected by increased slopes of the growth curves. Three mice were again treated with the vincristine and MK-571 regimen at day 14, without significant anti-tumour effect, although in one mouse a 28% tumour volume reduction was measured at day 21.

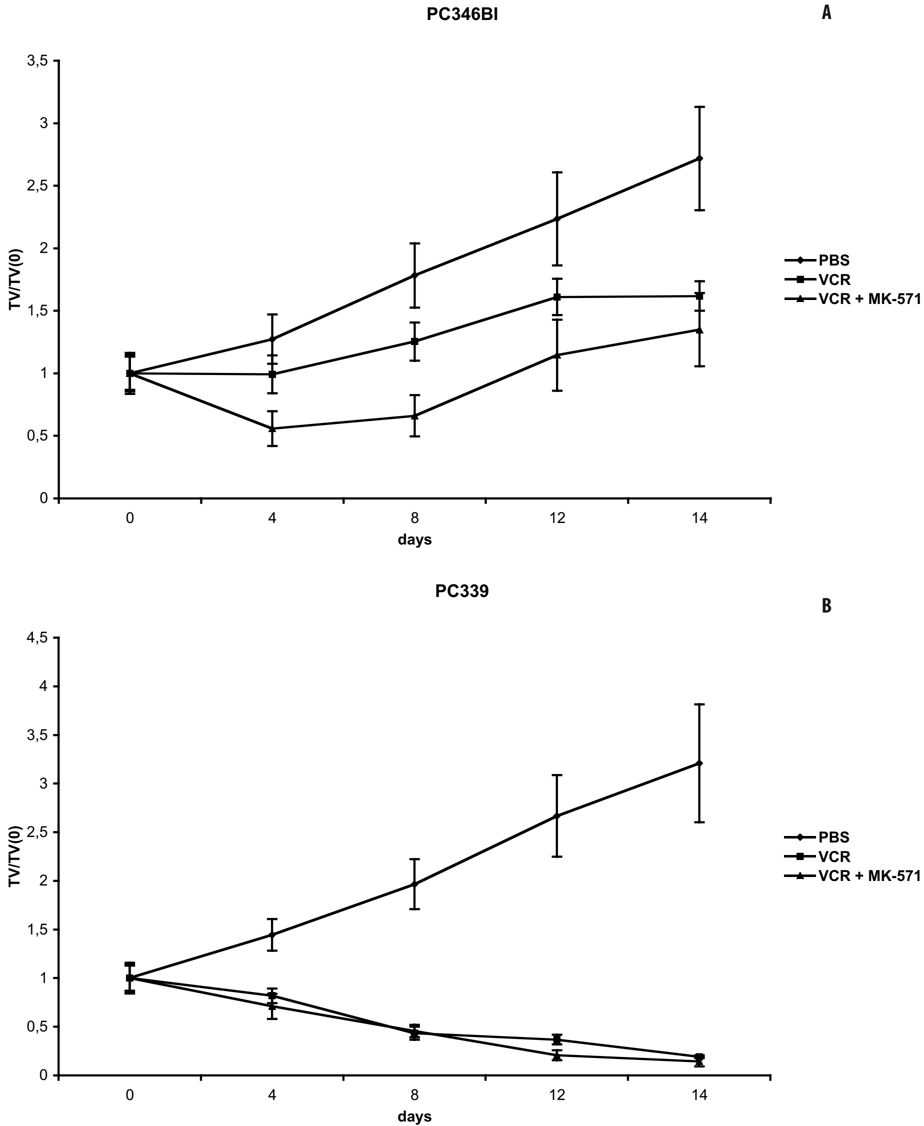

Figure 6.3.
For PC339, the average growth curves are shown in **figure 6.3a** for the single dose treatment with 0.8 mg/kg vincristine and for 0.8 mg/kg vincristine combined with 40 µg/kg MK-571. For PC346BI, the average growth curves are shown in **figure 6.3b** for the treatment with 0.8 mg/kg vincristine and for 0.8 mg/kg vincristine combined with 80 µg/kg MK-571.

Table 6.2a summarizes the slopes of the growth curves of tumours in different treatments, reflecting the regression line of tumour volume in time, representing the anti-tumour effect of the treatment. The anti-tumour effects of the treatments

are compared in **table 6.2b** and p-values are given. A higher slope reflects a more aggressively growing tumour.

Table 6.2a: Anti-tumour effects of phosphate buffered saline (PBS), MRP1 modulator MK-571, cytotoxic drug vincristine and the combination of MK-571 and vincristine on *in vivo* prostate cancer models PC339 and PC346BI.

	PC339 slope 14 days	**PC346BI slope 7 days**	**PC346BI slope 14 days**
PBS	98.8	65.2	64.2
MK-571 40 mg/kg	98.8		
MK-571 80 mg/kg		58.8	61.2
vincristine 0.8 mg/kg	-38.5	12.6	44.3
vincristine 0.8 mg/kg + MK-571 40 mg/kg	-58.7		
vincristine 0.8 mg/kg + MK-571 80 mg/kg		-48.1	12.9

Anti-tumour effects are reflected as slopes, which is defined as the regression line of tumour volume versus time. Xenografts PC339 and PC346BI are treated with placebo (PBS), respectively 40 and 80 mg/kg of MK-571, 0.8 mg/kg of vincristine and the combination of vincristine and MK-571.

Table 6.2b: Comparison of anti-tumour effects of phosphate buffered saline (PBS), MRP1 modulator MK-571, cytotoxic drug vincristine and the combination of MK-571 and vincristine (VCR) in *in vivo* prostate cancer models PC339 and PC346BI.

		MK 40 mg/kg	**MK80 mg/kg**	**VCR 0.8 mg/kg**	**VCR 0.8 mg/kg + MK 40 mg/kg**	**VCR 0.8 mg/kg + MK 80 mg/kg**
PC339	PBS	0.88		0.0002*	<0.0001*	
(14 days)	VCR 0.8				0.16	
PC346BI	PBS		0.89	0.081		0.004*
(7 days)	VCR 0.8					0.039*
PC346BI	PBS		0.91	0.29		0.058
(14 days)	VCR 0.8					0.12

p-values reflect differences in anti-tumour effects. * indicates a significantly stronger anti-tumour effect.

DISCUSSION

Advanced prostate cancer patients that have failed on hormonal therapy have a poor prognosis. Chemotherapy, the treatment modality of choice in many disseminated malignancies, has provided very little help for these patients, due to the resistance of most prostate cancer cells to cytotoxic drugs[1-4]. The multidrug resistance phenomenon has received a great deal of attention and several papers

have reported on its relevance for prostate cancer based on prostate cancer cell lines and material obtained from clinical prostate cancer[6, 7, 9]. Also, strategies to modulate multidrug resistance in prostate cancer in an *in vitro* setting have been reported and reviewed[5, 8]. The present research aims at further determining the relevance of the multidrug resistance phenomenon in clinical prostate cancer, in particular MRP1 mediated multidrug resistance. To achieve this *in vivo* experiments are essential because such experiments may form a bridge from the *in vitro* results to future clinical application of strategies to improve results of chemotherapy in prostate cancer. However, *in vivo* experiments, using models for human prostate cancer, have not been described. Therefore, experiments to determine efficacy and toxicity of the *in vivo* application of a MDR reversal strategy were performed. Although the nude mouse model used in our experiments differs from man in a variety of pharmacological, immunological and endocrine factors, these models offer the possibility to study the behaviour of human prostate cancer tissue in a physiological setting.

Based on prior studies, showing that MRP1 is expressed in *in vitro* and clinical prostate cancer and in which chemosensitization was achieved *in vitro* by modulation of MRP1 function with MK-571 [8], this research was focussed on the role of MRP1 related MDR *in vivo*.

In the present study MRP1 expression, as well as MRP2 and MDR1 expression were assessed in a panel of human prostate cancer xenografts. In concordance with earlier reports[7-9, 23], MDR1 was not found in the *in vivo* prostate cancer models. Therefore, MDR1 probably does not play a role in prostate cancer chemotherapy resistance. MRP1 and MRP2 are expressed in all xenografts, as well as in xenograft derived cell line PC346C, at varying levels. MRP2 expression appears to follow MRP1 expression relatively closely. It has been described that kinetic transport properties of MRP1 and MRP2 are different but substrate specificities of MRP1 and MRP2 are quite similar [24]. This may indicate that MRP2, next to MRP1, may be of importance in MDR of prostate cancer. Further study should be carried out to confirm this hypothesis. The non chemo-selected MRP1 expressing cell line PC346C, derived from xenograft PC346[10] was studied in a cell viability assay (MTT). We found that the viable cell count was significantly reduced in the presence of chemotherapeu-

tic agents etoposide, doxorubicin and vincristine when putative MRP1 inhibitor MK-571 was added. This may be a result of accumulation of the chemotherapeutic agents caused by decreased MRP1 pump activity through inhibition by MK-571, leading to increased cell death. This was further studied in fluorescence assays, which revealed significantly increased concentration of MRP1 substrate DCFDA in the presence of MK-571. Apparently, MK-571 inhibits MRP1 mediated DCFDA cellular efflux in PC346C, as well as in the control cell lines. This clearly points at a functional role of drug transporter MRP1 in prostate cancer, represented by cell line PC346C. Furthermore, MK-571 may be a valuable tool for chemosenstization of prostate cancer.

To proceed towards effective and safe clinical application of MDR reversal strategies *in vivo* experiments were initiated to determine efficacy and toxicity of experimental regimens. Two human prostate cancer *in vivo* models were selected with clear MRP1 expression for further experiments, PC339, with relatively high MRP1 expression and PC346BI, with relatively low MRP1 expression. Treatment of non-tumour bearing NMRI mice with VCR and MK-571 was performed to ascertain maximum tolerated dosages according to criteria of toxicity, based on guidelines of the "Code of Practice of the use of experimental animals in cancer research" for a single dose schedule (data not shown). These dosages were further applied in PC339 and PC346BI. In PC339, vincristine and vincristine combined with MK-571 produced a significant anti-tumour effect compared PBS over a 14 day interval. The combination regimen of VCR and MK-571 appeared to have a stronger anti-tumour effect than VCR alone, with a lower p-value ($p<0.0001$ and $p=0.0002$, respectively), when comparing to the control (PBS). Although this additive effect of MK-571 was measured it did not reach significance ($p= 0.16$). Tumour volume decreased during the course of the experiment, without significant regrowth in the 14-day duration of the experiment. After this time interval mice were sacrificed. In PC346BI an anti-tumour effect was observed when vincristine was administered, although this effect was not significant ($p=0.29$). The combination of VCR and MK-571 resulted in a significant anti-tumour effect after 7 days ($p=0.004$). Importantly, when comparing VCR alone versus VCR + MK-571 at day 7, the latter regimen had a significantly stronger anti-tumour effect than VCR alone ($p=0.039$). Growth curves showed max-

imum tumour regression around the seventh treatment day, followed by regrowth of tumours, reflected by increased slopes of the growth curves. Although regrowth occurred, at the 14 days measurement the combination of VCR and MK-571 appeared to have a superior anti-tumour effect over VCR alone, with a lower slope (44,3 versus 12,9; **table 6.2a**) and a lower p-value than VCR alone (p=0.058 and p=029, respectively). However this difference in anti-tumour effect did not reach significance at day 14. Re-treatment at day 14 resulted in a 28% tumour volume reduction in only one mouse.

When comparing both xenografts, PC339 showed a stronger response to vincristine alone, although it had higher MRP1 expression. Apparently, PC339 is more sensitive to VCR. It is speculative if other mechanisms of MDR limit the effect of VCR in PC346BI. MRP2 is expressed in both xenografts and may play a similar role in both PC339 and PC346BI as its expression follows MRP1 expression quite closely. MDR1 is not likely to cause the difference in VCR response as it is not expressed in the xenografts. Data on expression and function of other MRP isoforms or other MDR factors in the xenografts remain to be obtained. Addition of MK-571 resulted in a more moderate anti-tumour effect in PC339 than was seen in PC346BI. This may have been a consequence of administration of a lower dose of MK-571 in PC339, due to dose-limiting toxicity. Also, the achievable anti-tumour effect of MK-571 may have been related to the level of MRP1 expression, resulting in a relatively stronger effect in PC346BI as compared to the effect in PC339. During the course of the 14 day experiments, no tumour re-growth was seen in PC339 and mice were sacrificed after 14 days. However, in PC346BI re-growth became apparent after 7 days. Half-life in man for vincristine is one day with normal liver function and two to three hours for MK-571. Although half-life in mice is unknown, decreased functional levels of VCR and/or MK-571 may have explained the regrowth of surviving tumour cells after a week, as observed in PC346BI. As NMRI mice formed the carrier for both PC339 and PC346BI, this does not explain the differences observed in these xenografts. One may speculate that during exposure to vincristine and MK-571 multidrug resistance mechanisms may have been up-regulated in PC346BI, resulting in increased resistance against treatment regimens. This may also explain the limited effect of a second treatment that was given in PC346BI.

CONCLUSION

These results demonstrate expression of MRP1 and MRP2 in *in vitro* and *in vivo* models for human prostate cancer. Also, chemosensitization *in vitro* and, importantly, the possibility to modulate the response to chemotherapy of prostate cancer by inhibiting MRP1 in an *in vivo* setting was demonstrated. Further fine-tuning of regimens and use of alternative chemotherapeutic agents and MDR modulators may improve anti-tumour activity and reduce toxicity. Therefore, further study on the metabolism of chemotherapeutic agents and modulators in an *in vivo* system, as well as assessment of expression of MDR mechanisms other than described in this paper, is required. Upregulation of MDR mechanisms during or after treatment of xenografts with chemotherapeutic agents with or without modulators should be investigated. Also, in the absence of treatment options for patients with hormone-refractory prostate cancer, clinical application of combination therapy of a cytotoxic drug and an MRP1 modulator should be investigated and its curative or palliative value should be evaluated in a clinical trial.

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CHAPTER 7

General discussion

BACKGROUND

Failure of chemotherapy as treatment modality for patients with advanced prostate cancer that have progressive disease despite hormone deprivation therapy has elicited the present study in which two main questions were addressed.

Primarily, which mechanism causes or which mechanisms cause failure of chemotherapy in prostate cancer? Secondly, can results of chemotherapy be improved by challenging such mechanisms, aiming at an increased cytotoxic effect on prostate cancer cells?

Metastatic prostate cancer is resistant to a broad range of antineoplastic agents. Although several studies have reported favourably on response rates to chemotherapeutical regimens, results of chemotherapy in prostate cancer, single- as well as multiple agent regimens, have been disappointing, so far[1-5]. At present, none of them have exhibited a clear survival advantage, although two recent randomized clinical trials have reported on a two to four month survival benefit with combination therapies of docetaxel and prednisone or docetaxel and estramustine as compared to mitoxantrone and prednisone, respectively. Taxane docetaxel, phosphorylates Bcl-2 *in vitro*. This leads to its inactivation and to eventually to cell death by apoptosis[41]. This Bcl-2 pathway may play a role in clinical prostate cancer. It is known that anti-apoptotic protein Bcl-2 is increased in advanced prostate cancer. Interestingly, the taxoid docetaxel was studied earlier *in vivo* on doxorubicin resistant multidrug resistance protein expressing HT1080/DR4 tumour xenografts (sarcoma). Docetaxel showed significant anti-tumour activity and it was suggested that docetaxel was not as readily transported by the multidrug resistance protein as paclitaxel. Docetaxel may therefore have therapeutic advantages in the clinical treatment of multidrug resistance protein expressing tumours[42]. The (limited) survival advantage in advanced prostate cancer treated with docetaxel, its *in vitro* effect on Bcl-2 and the *in vivo* finding that docetaxel has superior anti-tumour activity in an multidrug resistance protein expressing sarcoma model indicate that MDR pathways may influence the response of prostate cancer to chemotherapy.

The multidrug resistance (MDR) phenomenon may play a role in progressive therapy resistant prostate cancer and may explain resistance against a variety of structurally- and functionally distinct cytotoxic agents. Tumours may have acquired- or induced MDR and can display intrinsic MDR: they are primarily unresponsive to chemotherapeutic regimens. Various MDR pathways have been identified over the years. Approaches to overcome MDR of chemotherapy-unresponsive tumours have been developed or are the subject of present investigations (reviewed in **chapter 2**).

RESEARCH SET-UP

Clearly, the final goal of our field of research is to decrease prostate cancer morbidity (improvement of quality of life) and mortality. Before reaching this ultimate step with clinical application of strategies to overcome resistance of prostate cancer against chemotherapy, many questions have to be answered, as little data exist on this matter.

Therefore, we have chosen a straightforward approach towards the development of a strategy to overcome resistance of prostate cancer against chemotherapy leading from experimental- to clinical prostate cancer.

Our research was started at the basic level of *in vitro* prostate cancer, using established cell cultures of human prostate cancer. After identification of several proteins in *in vitro* prostate cancer that are known for their role in the multidrug resistance phenomenon we proceeded to researching clinical prostate cancer material. Identification of several multidrug resistance factors in clinical prostate cancer samples confirmed the relevance of the *in vitro* expression of certain resistance proteins. However, this provided only indirect evidence of contribution of these proteins to the failure of chemotherapy. Consequently, we developed *in vitro* models of resistant prostate cancer cells in order to perform functional studies to provide direct evidence of involvement of chemotherapy resistance factors in prostate cancer cells. Also, such models could provide tools to study strategies to overcome resistance against chemotherapy. After confirming a functional role of several previously detected chemotherapy resistance factors, successful applica-

tion of such a strategy to overcome chemotherapy resistance was demonstrated in *in vitro* prostate cancer. In sight of clinical application of this treatment in prostate cancer patients, it is clear that testing of this approach in *in vivo* models for human prostate cancer is essential to confirm safety and efficacy. Therefore, we performed a study using *in vivo* models, that have been developed in our laboratory. This showed the possibility to safely apply a strategy to overcome resistance against chemotherapy of clinical prostate cancer and bio-activity was demonstrated.

Our attempt to develop a treatment for hormone refractory advanced prostate cancer has put us on the doorstep to clinical application of a strategy to overcome resistance against chemotherapy. However, for optimal outcome, fine-tuning of treatment regimens should be carried out and clinical safety and efficacy should be assessed in a controlled clinical trial.

This discussion will elaborate on the findings and conclusions of our studies, and future perspectives will be set out.

IN VITRO STUDIES – CELL LINES; IMMUNOCYTOCHEMISTRY

To obtain insight into the role of the multidrug resistance (MDR) phenomenon in hormone-independent progressive prostate cancer the *in vitro* study as described in **chapter 3** was initiated. The expression of various multidrug resistance related proteins was determined using immunocytochemistry and Western blotting. Furthermore, the intrinsic resistance against chemotherapy was determined. Established human prostate cell lines PC3[6], TSU-Pr1[7], DU145[8], LNCaP[9] derivatives LNCaP-R[10], LNCaP-LNO[11] and LNCaP-FGC were used for this purpose.

Based on a literature study (described in **chapter 2**) an inventory was made of several MDR related proteins including drug transporter proteins Pgp[12] and MRP1[13], detoxifying enzyme Glutathione-S-transferase-p (GST-π)[14], which is part of the glutathione metabolism, proteins that are important in programmed cell death or apoptosis Bcl-2[15] and Bax[16], and topoisomerases I, IIα and IIβ[17] that may cause multidrug resistance when their expression and/or activity is altered.

Pgp, which has long been considered as the predominant protein responsible for MDR in malignancies, was not expressed in the human prostate cancer cell lines, whereas our chemo-resistant positive control cell line[18] RC21E (renal cell cancer) showed a clear signal in the Western blotting experiments. This result is in line with our finding in further experiments, in which Pgp was expressed neither in clinical prostate cancer samples at different stages of progression (**chapter 4**), nor in chemo-selected multidrug resistant prostate cancer cell lines (**chapter 5**), nor in prostate cancer xenografts (**chapter 6**). A role for Pgp (MDR1) is therefore not likely in chemo-resistance of prostate cancer.

In contrast to Pgp, the multidrug resistance associated protein MRP1, another drug transporter protein, which was detected in MDR cell lines that lacked Pgp[13], was variably but clearly expressed in the prostate cancer cell lines. The relevance of the MRP1 expression for the resistance against chemotherapy in prostate cancer has played a central role in our further research (**chapters 4**, **5** and **6**), especially because encouraging results of preliminary experiments, aiming at reversal of putative MRP1 mediated drug resistance in prostate cancer cell lines, were obtained.

GST-π was expressed in PC3, TSU-Pr1 and DU145, but not in the LNCaP derivatives which may be due to hypomethylation or hypermethylation of GST-π promotor sequences, respectively[19]. Further studies were performed regarding clinical GST-π expression (**chapter 4**) and expression of GST-π in chemo-selected prostate cancer cell lines and modulation of glutathione mediated drug detoxification by cellular depletion of glutathione by buthioninesulfoximine (**chapter 5**).

Bcl-2, the protein that inhibits apoptosis and may cause resistance against apoptosis-inducing chemotherapeutics, was expressed in only one cell line in our experiments, namely TSU-Pr1. In contrast to our findings, other studies reported Bcl-2 expression in LNCaP and in a drug resistant variant of PC3 [20]. We have elaborated on Bcl-2 by determining expression in clinical prostate cancer samples (**chapter 4**), but we have not carried out functional studies, because of conflicting data on Bcl-2 expression in prostate cancer. However, experiments with sequence-specific down-regulation of Bcl-2 using antisense oligonucleotides may be worthwhile [21].

Bcl-2 counterpart Bax was variably expressed in prostate cancer cell lines. The balance between Bax and Bcl-2 may determine the response of prostate cancer

cells to chemotherapy. As for Bcl-2, Bax expression in clinical samples was assessed, but functional studies were not done.

Expression of topoisomerase I, IIα and IIβ was determined in the prostate cancer cell lines. The topoisomerases I and IIβ were equally expressed in all cell lines and expression of topoisomerase IIα was related to BrdU stainings, which reflects proliferative activity. Topoisomerase enzymes are essential for several cellular processes and constitute targets for a number of clinically important drugs, which induce lethal damage by irreversibly stabilizing Topo-DNA complexes. Topoisomerase directed drugs, such as etoposide and doxorubicin are generally believed to be targets to the IIα isotype[22], although Topo I[23] and IIβ[24] may constitute targets as well. Expression of the topoisomerases was further studied in clinical samples at different stages of progression and it was concluded that increased expression in more progressive disease could not be matched with resistance to chemotherapy (see below and **chapter 4**). We could not detect changes in topoisomerase activity in preliminary studies (data not shown), although an other group found that selection of a prostate cancer cell line for 9-nitrocamptothecin resistance resulted in altered topoisomerase IIα activity and increased sensitivity to etoposide [25]. Table 7.1 summarizes the expression of MDR factors in cell lines studied in **chapter 3**.

IN VITRO STUDIES – CELL LINES; RESPONSE TO CYTOTOXIC DRUGS

To obtain insight in the relation between expression of multidrug resistance associated factors and response to chemotherapy we determined the intrinsic resistance against three chemotherapeutic agents which are commonly used in the treatment of several malignancies, whose action is known to be affected by MDR: etoposide, doxorubicin and vinblastine. Vinblastine, etoposide and doxorubicin are substrates for the drug transporters Pgp and MRP1. Doxorubicin and etoposide (and not vinblastine) are conjugated to less toxic and more easily excretable metabolites by glutathione. Bcl-2 may block etoposide and doxorubicin induced apoptosis. Changes in expression or activity of Topoisomerases may decrease etoposide or doxorubicin induced toxicity. The cell lines were in proliferative growth when tested and responded variably to exposure to cytotoxic drugs.

Possibly, response to chemotherapy is influenced by a combined action of MDR factors, which makes interpretation of dose response curves versus expression of MDR factors speculative and difficult. Also, proliferative activity, measured with BrdU incorporation in our experiments may influence response of cells to chemotherapy. This is illustrated by cell lines TSU-Pr1 and DU145, which are rapidly proliferating and the most sensitive to chemotherapy.

To further determine the importance of one specific MDR factor in the resistance of prostate cancer cells, experiments are needed with cells that either express only one MDR factor or in which one MDR factor is specifically challenged. In our opinion, the former approach would not closely match a clinical situation, in which one can expect multiple MDR factors to be of influence. Therefore, we have chosen the latter approach as described in **chapter 5**.

	TSU	FGC	PC3	LNO	R	DU145	Group 1 (%)	Group 2 (%)	Group 3 (%)
P-glycoprotein	-	-	-	-	-	-	0	0	0
Multidrug resistance associated protein 1	+	+	+	+	+	+	53	100	100
Lung resistance protein	nd	nd	nd	nd	nd	nd	32	47	71
Glutahtione-S-transferase-π	+	-	+	-	-	+	0	18	53
P53	nd	nd	nd	nd	nd	nd	5	29	65
Bcl-2	+	-	-	-	-	-	47	47	82
Bax	+	+	+	+	+	+	100	100	100
Topoisomerase I	+	+	+	+	+	+	79	94	94
Topoisomerase IIα	+	+	+	+	+	+	16	59	100
Topoisomerase IIβ	+	+	+	+	+	+	89	100	100
Ki-67	nd	nd	nd	nd	nd	nd	16	65	82

Table 7.1 summarizing the expression of MDR factors in cell lines studied in **chapter 3** and the percent of tumours expressing the MDR factors in clinical prostate cancer studied in **chapter 4**: locally confined (group 1), hormonally untreated disseminated prostate carcinoma (group 2) and hormone independent prostate carcinoma (group 3). Nd= not determined.

IN VITRO STUDIES –CLINICAL HISTOLOGICAL MATERIAL

In **chapter 4**, we addressed the question of clinical relevance of the MDR factors that were detected in human prostate cancer cell lines. The purpose of this research was to identify proteins that may be involved in the multidrug resistance of clinical prostate cancer and assess their expression in the context of tumour progression using immunohistochemistry. Three different prostate cancer patient groups, that were clearly distinct with regard to disease progression, were examined. Group 1 consisted of paraffin embedded prostate tissue of patients that underwent surgery (radical prostatectomy) for locally confined disease (pT2N0M0). This group represented early phase prostate cancer. Group 2 consisted of transurethral resection of the prostate material obtained from patients with metastatic prostate cancer that were treated for urinary problems, before receiving antihormonal therapy. This group represented intermediate phase prostate cancer. Group 3 consisted of hormonally-treated metastatic prostate cancer patients that received a palliative transurethral resection because of progressive disease despite antihormonal therapy. This group represented advanced hormone independent prostate cancer. The proliferative activity, as reflected by Ki-67 immunohistochemical stainings, was significantly increased in the non-locally confined patient groups, in concordance to the clinical phase of tumour progression and this was correlated with topoisomerase IIα expression, which is known to be a proliferation marker, as well. Pgp (MDR1) was not expressed in any clinical prostate cancer sample, whatever the phase of progression. Therefore, it seems unlikely that Pgp plays a role in the resistance of clinical prostate cancer.

In contrast to Pgp, MRP1 is clearly expressed in the clinical prostate cancer samples and, interestingly, we found a significantly increased expression in advanced disease, with a significantly higher histological grade of MRP1 positive tumours. MRP1 was detected in all cases of metastatic prostate cancer. A relation of MRP1 expression with tumour progression has been suggested[26] and is confirmed in our study. MRP1 overexpression in advanced tumours may contribute to the MDR phenotype of advanced prostate cancer. These results in clinical prostate cancer samples, together with the *in vitro* results (**chapter 3**), prompted us to pursue in-

vestigations concerning the relevance of MRP1 in prostate cancer focussing on its functional importance. This issue was addressed *in vitro* (**chapter 5**) and *in vivo* (**chapter 6**).

We assessed the expression of glutathione-S-transferase-π, a detoxifying enzyme which is part of the glutathione detoxifying pathway. We found a significant increase in the number of patients expressing this protein in the group with advanced hormone independent prostate cancer. GST-π was not expressed in organ confined prostate cancer, in concordance with earlier reports[19, 26]. MRP1 is a glutathione-S- conjugate carrier[27]. Possibly, over expression of MRP1 and GST-π in advanced prostate cancer may result in increased drug resistance.

The lung resistance protein (LRP) [28] is thought to be involved in the transmembrane transport of several substrates. Although its clinical relevance for MDR in cancer remains to be proven, we assessed its expression in our panel of clinical prostate cancer. We found LRP expression in one third of the organ confined prostate cancers and its expression was significantly increased in metastatic disease. This finding may be of interest in the context of MDR of advanced prostate cancer.

Altogether, three out of four members of the panel of drug transporter and detoxifying proteins were more frequently expressed in hormone independent prostate cancer, possibly reflecting that chemotherapy failure in progressive disease is caused by these mechanisms of multidrug resistance.

Programmed cell death (apoptosis) is triggered by several cytotoxic drugs. Changes in proteins that regulate apoptosis can induce resistance against chemotherapy. In our immunohistochemical research, three important apoptosis related proteins were investigated: Bax, Bcl-2 and p53.

Bax[16] is thought to be a pro-apoptotic protein. Expression of Bax was homogeneous in normal prostate tissue and in prostate cancer, regardless of the phase of prostate cancer progression.

In contrast, its counterpart Bcl-2[15], an anti-apoptotic protein, is significantly more often expressed in the patient group with hormone independent prostate cancer, as compared to organ confined prostate cancer and hormonally untreated metastatic prostate cancer. Bcl-2 expression in hormone independent disease may

be one of the factors influencing the resistance of this patient group against chemotherapy.

Furthermore, p53 expression was significantly increased in hormone independent disease and its expression was significantly related to a higher histological grade. This finding matches previous publications, with mutations of p53 occurring as a late event in prostate cancer [29]. Wild-type p53 promotes apoptosis. P53 mutation, reflected by overexpression in immunohistochemistry, results in decreased apoptosis and may cause resistance to apoptosis inducing cytotoxic agents. In the group with advanced hormone independent disease, p53 over expression may therefore result in a decreased susceptibility to apoptosis inducing cytotoxic drugs. However, one should bare in mind that immunohistochemical expression of p53 does not necessarily represent p53 mutation[30]. The monoclonal p53 antibody DO7 detects both wild type and mutated p53, but mutated p53 protein has a longer half-life.

Altogether, anti-apoptotic factors appear to be present in hormone independent disease, and these may cause MDR of progressive disease.

Because the nuclear topoisomerases are targets for several cytotoxic drugs and decreased expression and/ or change in enzymatic activity causes multidrug resistance, we investigated the presence of topoisomerases I, IIα and IIβ in clinical prostate cancer after confirming expression in *in vitro* prostate cancer. We found that expression of topoisomerases I, IIα and IIβ was increased in progressive disease. Increased expression does not point at a role for topoisomerases in MDR of prostate cancer, although we have not determined changes in enzymatic activity. On the other hand, increased expression of topoisomerases may favour the application of topoisomerase targeting drugs in advanced prostate cancer, although the efficacy of such drugs may be limited by increased drug efflux, detoxification and decreased apoptosis by MRP1, LRP, GST-π, p53 mutations and decreased Bax/ Bcl-2 ratio, respectively.

FUNCTIONAL IN VITRO STUDIES

The presence of several MDR factors in prostate cancer and their altered expression in advanced clinical prostate cancer is an indication for their role in the failure of chemotherapy. Functional studies will have to clarify this issue. For this reason, we developed *in vitro* models for prostate cancer (**chapter 5**).

To obtain *in vitro* models of chemotherapy resistant prostate cancer we applied chemo-selection: the prostate cancer cell lines PC3 and DU145 were exposed to etoposide. In our opinion, etoposide was the most suitable drug for selection, although it is not commonly used in the treatment of prostate cancer. Etoposide is know to be a substrate for Pgp and MRP1, it is detoxified by the glutathione metabolism, its function is impaired by increased Bcl-2 expression and, finally it is a topoisomerase targeting drug. Exposure to and selection with etoposide may induce cellular changes that involve the MDR factors which are subject of our investigations. After multiple cycles of exposure to cumulative concentrations of etoposide, increasingly resistant clones were obtained with the limiting dilution technique. Finally, this resulted in two chemo-selected *in vitro* prostate cancer models: PC3-R (from PC3) and DU-R (from DU145), which were used for further experiments. For these cell lines we determined the resistance against etoposide, doxorubicin and vincristine. We found that the multidrug resistance phenomenon occurred: cells had not only acquired resistance against etoposide but also to doxorubicin and vincristine.

In further experiments we investigated the roles of MRP1 and glutathione, because increased expression of both MRP1 and GST-π in PC3-R and DU-R was found in preliminary immunocytochemical stainings (data not shown). Also, preliminary functional studies aiming at these proteins produced encouraging results in terms of reversal of resistance against chemotherapy. Increased MRP1 expression in the multidrug resistant cell lines PC3-R and DU-R was confirmed in Western blotting experiments. Furthermore, glutathione content was increased in PC3-R in contrast to DU-R. Subsequently, with the assumption that MRP1 causes drug resistance in the prostate cancer cells, we attempted to block MRP1 function. Using the cell viability assay (MTT)[31], cells were exposed to an inhibitor of MRP1-mediated

transport, leukotriene D4 antagonist MK-571[32], as well as another leukotriene inhibitor, zafirlukast[33]. From the chemical structure of MK-571 and the fact it was designed to bind to cysteinyl leukotriene receptors, it is generally presumed that MK-571 competes with the LTC4 binding site(s) of MRP1 which overlaps or is the same as the drug binding site(s)[43]. Application of MK-571 resulted in significant to complete reversal of acquired resistance in our *in vitro* models. Furthermore, non-chemoselected MRP1 expressing cells were sensitised. The possibility to decrease drug resistance in chemo-naïve cells is important, because most prostate cancer patients have not been exposed to chemotherapy, previously. Zafirlukast appeared to be less effective, although significant reversal of resistance was achieved in cell line DU-R when combined with vincristine. Application of buthioninesulfoximine (BSO) significantly reduced cellular glutathione levels. However BSO did not consistently reduce resistance. In PC3 there was a significant reduction of vincristine resistance when BSO depleted glutathione. Etoposide and doxorubicin resistance decreased with application of BSO in cell line DU-R. Possibly, different cell lines require different glutathione concentrations for MRP1-mediated drug transport. The relation between the multidrug resistance protein and the glutathione metabolism has been reviewed in **chapter 2**. In preliminary studies we blocked MRP1 and simultaneously depleted glutathione which led to strongly increased cell death, even without addition of a cytotoxic drug. This may be a result of MRP1-mediated transport of glutathione-S-conjugated endo- and xenobiotics in prostate cancer cells.

To actually measure MRP1 mediated cellular transport in our prostate cancer models we used a modification of a previously described assay[34] in which carboxyfluorescein was used as a MRP1 substrate. This allowed us to measure fluorescence intensity, and calculate cellular substrate content at different time intervals. This showed efflux of carboxyfuorescein in untreated cells. When MK-571 was added, efflux was significantly reduced, reflecting blocking of the MRP1 pump. In analogy to this finding, it is likely that impaired MRP1 function results in intracellular drug accumulation in prostate cancer cells that are exposed to cytotoxic drugs that are MRP1 substrates. This will eventually lead to increased cell death. Again, MDR1 did not play a role in our chemo-selected prostate cancer models as

measured with a rhodamine-retention assay, using PSC833 as a substrate. MDR1 could neither be detected in Western blotting experiments nor in experiments with fluorescent tagging of MDR1-expressing cells with specific antibodies. MDR1 function and expression in positive control experiments, confirmed the validity of our experiments. For future clinical application, it is important to note that MK-571 concentrations used in our experiments are 20 times less than clinically achievable peak plasma levels[35]. Also, BSO levels as used in our experiments are lower than clinically achievable[36]. The experiments described in chapter 5 offer evidence of involvement of MRP1 and the glutathione metabolism in the resistance of prostate cancer against chemotherapy. Although we have demonstrated that involvement of MDR1 is unlikely, we have not ruled out a role of other MDR mechanisms. This remains a subject for future studies.

FUNCTIONAL IN VIVO STUDIES

Focussing further on MRP1 as an important factor of drug resistance in prostate cancer, important questions remained to be addressed: can the strategy to block MRP1 effectively be applied in a physiological environment and can such a strategy safely be applied? A confirmative answer is a prerequisite to proceed to clinical studies. Therefore, we studied efficacy and toxicity in the biological setting of NRMI athymic nude mice bearing human hormone independent prostate cancers (**chapter 6**). Unfortunately, efforts to implant and culture our newly established chemo-selected multidrug resistant human prostate cancer cell lines into xenografts failed, due to lack of growth after subcutaneous injection on the shoulders of NRMI athymic nude mice. Alternatively, we used established human prostate cancer xenografts, that have been developed in our laboratory.

First of all, we determined expression of MDR1, MRP1 and MRP isoform MRP2 in a panel of human prostate cancer xenografts and in xenograft derived cell line PC346C with Western blotting experiments. As in our other experiments (**chapter 3** to **5**) MDR1 was not expressed. In contrast, both MRP1 and MRP2 were expressed at varying level in xenografts and cell line. MRP2 expression appears to follow MRP1 expression relatively closely. It has been described that kinetic transport properties of MRP1 and

MRP2 are different but substrate specificities of MRP1 and MRP2 are quite similar[37]. This may indicate that MRP2, next to MRP1, may be of importance in MDR of prostate cancer. Further study should be carried out to confirm this hypothesis.

Cell line PC346C, which is derived from xenograft PC346 [38] was used to a confirm a role of MRP1 in our experiments. The cell line strongly resembles clinical prostate cancer as it expresses androgen receptors and produces PSA. In concordance with the experiments performed in **chapter 5** cell viability assays were used to study chemosensitization of PC346C to etoposide, doxorubicin and vincristine with a non-toxic dose of MK-571. This resulted in sensitisation of PC346C for all chemotherapeutic drugs, which may be a result of intracellular accumulation of the cytotoxic drugs caused by MRP1 pump activity inhibition by MK-571. Further studies with fluorescence assays revealed a significantly increased concentration of MRP1 substrate carboxyfluorescein in the presence of MK-571. MK-571 inhibits MRP1 mediated carboxyfluorescein cellular efflux in PC346C, as well as in the control cell lines. This clearly points at a functional role of drug transporter MRP1 in prostate cancer, represented by cell line PC346C. Furthermore, MK-571 appears to be valuable for chemosensitization of prostate cancer. We also examined the leukotriene D4 (LTD4) receptor antagonist ONO-1078, which is used for the treatment of allergic asthma and other immediate hypersensitivity diseases (data not shown). ONO-1078 has been applied as MRP inhibitor *in vitro*[39] and could be of interest for our studies because it is already in clinical use, as described above. ONO-1078 did not decrease the viable cell count of prostate cancer cells, exposed to vincristine in MTT assays, nor did it cause accumulation of DCFDA in FACS analyses, performed as described for MK-571. Therefore, it is not likely that ONO-1078 has clinical value for the treatment of advanced prostate cancer.

After confirming the feasibility of our strategy in the xenograft derived cell line, we proceeded to functional studies in a physiological setting: the NMRI athymic nude mice transplanted with a human prostate cancer. As stated previously, proof of efficacy and acceptable toxicity of chemosensitization strategies *in vivo* is an imperative step towards their clinical application. NRMI athymic nude mice xenografted with PC339 and PC346BI (derived from human adenocarcinoma of the prostate) were used in *in vivo* experiments and these tumours expressed relatively high and

relatively low levels of MRP isoforms (MRP1 and MRP2), respectively. Vincristine was selected as cytotoxic drug, because combination of this drug with MK-571 produced the most significant *in vitro* chemosensitization. Toxicity of vincristine alone, MK-571 alone or combinations of vincristine and MK-571 was assessed in non-tumour bearing mice. Toxicity for mice has been defined in guidelines of the "Code of Practice of the use of experimental animals in cancer research". Defined dosages were used in xenografts PC339 and PC346BI.

In PC339 both vincristine alone and vincristine in combination with MK-571 resulted in significant tumour volume reduction. The effect appeared to be stronger in the combination therapy, but not significantly stronger. In PC346BI vincristine alone did not have a significant anti-tumour effect. However, the combination of vincristine and MK-571 resulted in significant tumour volume reduction, and therefore it is likely that MK-571 chemosensitized xenograft PC346BI, although regrowth occurred after 7 days. It is speculative whether during exposure to vincristine and MK-571 multidrug resistance mechanisms may have been up-regulated in PC346BI, resulting in increased resistance against further treatment regimens.

Importantly in **chapter 6**, the possibility to modulate the response to chemotherapy of prostate cancer by inhibiting MRP1 in an *in vivo* setting was demonstrated. Further fine-tuning of treatments and application of alternative chemotherapeutic drugs and MDR modulators, may improve anti-tumour efficacy and reduce toxicity.

FUTURE DIRECTIONS

As a result of the studies, which are described in this thesis, several mechanisms have been identified that potentially cause failure of chemotherapy in clinical prostate cancer. Also, we have successfully increased the cytotoxic effect of chemotherapy on prostate cancer cells by challenging multidrug resistance mechanisms.

Investigation into expression and function of described and newly emerging factors that may cause multidrug resistance is of importance, as well as further refinement of *in vitro* and *in vivo* chemosensitization strategies: chemotherapeutic

drugs and chemosensitization strategies may be combined, depending on expression patterns of MDR factors in prostate cancer cells. Newly emerging cytotoxic drugs and chemosensitizers could be tested in our *in vitro* and *in vivo* prostate cancer models. Upregulation of MDR mechanisms during or after treatment with chemotherapeutic drugs with or without modulators should be investigated. Furthermore, studies on the *in vivo* metabolism of cytotoxic drugs and modulators are needed, to achieve optimal treatment.

In the absence of successful treatment options for patients with hormone-refractory prostate cancer, clinical application of combination therapy of a cytotoxic drug and a chemosensitizer (for instance a MRP1 modulator) should be investigated and its curative or palliative value should be evaluated in a clinical trial. One could tailor this treatment to the individual patient by making an inventory of the expression of MDR factors in the patient's prostate cancer tissue samples, using appropriate (combinations of) cytotoxic drugs and chemosensitizers, after assessing the safety of their application.

Preceding animal experiments will be crucial to decide on the proper selection of chemotherapeutic drug and modulator as well as on timing and mode of application. In our studies, the combination of vincristine and MK571 was demonstrated to effectively circumvent MRP1 related chemoresistance. Peak plasma levels of vincristine are reached under a continuous intravenous regimen and amount to nanomoles (>10-9 M) [44]. In our *in vitro* experiments vincristine concentrations needed for a cytotoxic effect were 2ng/ml (≈10-9 M), which is in agreement with clinically achievable peak plasma concentration. As such vincristine appears to be suitable to be used in a clinical trial. In clinical trials , MK571 has been used orally at 750 mg (once) or split over 3 equal doses over 24 hours [45] or intravenously at 160 mg once [46]. In healthy volunteers the achievable peak serum levels amount to 313 ng/ml [47], which is 20 times higher than the concentrations used effectively in our *in vitro* studies (15.4 µg/ml or 30 µM) and which is not toxic by itself to prostate cancer cells. As such, the leukotriene D4 receptor agonist MK 571 again appears to be a suitable modulator in a clinical trial. We have shown that *in vivo* toxicity of the combination of vincristine and M-571 is limited.

Such a clinical trial should aim at improving on standard chemotherapy and could be performed in symptomatic hormone unresponsive metastatic prostate cancer patients with MRP1 positive tumours. The aim of this study would be to assess safety and efficacy of the combination Vincristine and MK-571. Clinical endpoints of this trial would be: cancer specific and overall survival, number and duration of objective and prostate specific antigen (PSA) responses, and quality of life.

The study design would be a sequential phase II trial (Gehan design[40]) in which 14 patients should be recruited in this trial first. In this scheme the trial would have to be discontinued if no response had been observed after 14 patients, with further enrolment depending of the number of patients needed to estimate the real response rate with a required precision of 10% in terms of the standard error (maximum 25 patients).

The therapy would consist of vincristine, followed by vincristine and MK-571 in non-responders. The duration of treatment would be until progressive disease, unacceptable toxicity or until patient's refusal occurred. A tumour response is defined as an objective response: either complete disappearance of all known tumour deposits for a minimum of one month (complete response) or reduction of all bi-dimensionally measurable tumour deposits by more than 50% for a minimum of one month (partial response), or a PSA response consisting of a PSA reduction to ≤ 50% of the pre-treatment value, confirmed by one control measurement, together with an improvement of WHO performance score ≥ 1 scale or an improvement at a 5 step pain score by ≥ 2 scales. Progression is defined as any increase in number of metastatic deposits (new lesions) or increase of the volume of all bi-dimensionally measurable tumour deposits by ≥ 25%, or a PSA increase of ≥ 50% of the pre-treatment value, confirmed by one control measurement together with a decrease of WHO performance score ≥ 1 scale or a decrease of a 5 step pain score by ≥ 2 scales. In addition, a general stopping rule for the trial applies. In case of 2 or more unexplained serious adverse events (SAE, definition in accordance with GCP guidelines) the investigational trial has to be halted and, if left unresolved, to be discontinued. Safety of the trial and trial conduct will be monitored in accordance with GCP guidelines.

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CHAPTER 8

Summary and conclusions

Samenvatting en conclusies

SUMMARY AND CONCLUSIONS

Advanced hormone refractory prostate cancer constitutes a therapeutic challenge, because all available treatment strategies have failed to substantially increase cancer specific survival. Among these strategies, a multitude of chemotherapeutic approaches did not offer a superior life expectancy longer than a few months. Failure of chemotherapy may be caused by multidrug resistance (MDR) mechanisms protecting cancer cells against cytotoxic drugs. The question arises whether the MDR phenomenon plays a role in prostate cancer. Several pathways known to lead to MDR such as the P-glycoprotein (Pgp; MDR1), isoforms of the multidrug resistance associated protein (MRP), glutathione, apoptosis and topoisomerases have been examined and are reviewed in **chapter 2**. Evidence for the involvement of several MDR mechanisms in the chemoresistance of prostate cancer *in vitro* and *in vivo* is accumulating. Reversal, circumvention or overcoming of MDR pathways in advanced prostate cancer may be feasible and will lead to new strategies with improved treatment efficacy in otherwise untreatable disease.

The aim of the study described in **chapter 3** was to obtain an inventory of MDR factors in human prostate cancer cell lines representing progressive prostate cancer and to assess their response to chemotherapy.

Using immunocytochemistry and Western blotting expression of P-glycoprotein (Pgp), Multidrug resistance associated protein (MRP), Glutathione-S-transferase-π (GST-π), Bcl-2, Bax, Topoisomerase (Topo) I, IIα and IIß was determined in the human prostate cancer cell lines PC3, TSU-Pr1, DU145 and LNCaP derivatives -R, -LNO and -FGC. Proliferative activity was assessed by immunocytochemistry. MTT assays were used to determine the sensitivity to Etoposide, Doxorubicin and Vinblastine.

Pgp was not expressed in any of the cell lines. MRP was variably expressed. GST-π was expressed in TSU-Pr1, PC3 and DU145. Expression of Bcl-2 was restricted to TSU-Pr1, whereas Bax was found in all cell lines. Topo IIα was expressed at the highest level in the rapidly proliferating cell lines TSU-Pr1 and DU145. Topo I and IIß were equally expressed. Resistance profiles varied among the cell lines, with TSU-Pr1 being the most sensitive and LNCaP-LNO relatively resistant. It was concluded

that multiple MDR proteins are expressed in prostate cancer cell lines and may well influence response to chemotherapy.

The study described in **chapter 4** aimed at identifying proteins that may be involved in multidrug resistance in clinical prostate cancer. Expression of these proteins was examined in the context of tumour progression. Paraffin-embedded formalin fixed prostate cancer specimens from archival sources of three distinct patient groups were examined. These groups were clearly distinct with regard to pathological stage and responsiveness to anti-hormonal therapy. Group 1 consisted of patients with organ confined prostate cancer, treated by radical prostatectomy (early prostate cancer, pathological stage (p) T2N0M0 tumours). Group 2 had disseminated prostate cancer and was treated with transurethral resection of the prostate (TURP) for urinary obstruction before receiving anti-hormonal therapy (early advanced prostate cancer). Group 3 had disseminated prostate cancer and relapsed despite anti-hormonal treatment (late advanced prostate cancer) and were treated with TURP to relieve symptoms of urinary obstruction. Immunohistochemistry was used for detection of Pgp, MRP1, lung resistance protein (LRP), GST-π, p53, Bcl-2, Bax, Topo I, IIα and IIβ and Ki-67. Advanced tumours could be distinguished from locally confined tumours because they exhibited a significantly higher histological grade and proliferative activity than organ confined prostate cancer. Expression of MRP, p53, Topo IIα, Ki-67 and Topo IIβ was significantly related to a higher Gleason sum score. The number of patients expressing MRP, LRP, GST-π, p53, Bcl-2, Topo IIα and Ki-67 was significantly increased in the group with advanced disseminated prostate cancer. Topo I and Topo IIβ were homogeneously and highly expressed in all stages of prostate cancer progression. Pgp was not expressed in any of the tumours, regardless of the patient group. It was concluded that up-regulation of expression of drug transporters MRP and LRP, detoxifying enzyme GST-π and apoptosis inhibiting proteins Bcl-2 and P53 may offer an explanation for the resistance of disseminated progressive prostate cancer to chemotherapy. Increased proliferation, as shown by up-regulation of Ki-67 and Topo IIα, reflects the aggressiveness of metastatic prostate cancer.

The objective of the study described in **chapter 5** was to assess the involvement of MRP1 and the glutathione (GSH) pathway in the MDR phenotype of prostate cancer *in vitro*. Chemo-selection of human prostate cancer cell lines PC3 and DU145 with etoposide, resulted in the cell lines PC3-R and DU-R. Resistance against etoposide, doxorubicin and vincristine and its reversal with leukotriene D4 antagonists MK-571 and zafirlukast, and buthioninesulfoximine (BSO) was studied in MTT assays. Western blot analysis of MRP1 expression and GSH content measurements were performed. MRP1 function was studied in fluorescence assays. MRP1 was increased in the MDR models. The GSH content was significantly higher in PC3-R. No increase in GSH was found in DU-R. Addition of non-toxic doses of MK-571, zafirlukast or BSO significantly increased sensitivity of the MDR models to cytotoxic drugs. Inhibition of MRP1 function was achieved with MK-571 in the MDR models. It was concluded that MRP1 and glutathione mediate MDR in newly developed prostate cancer models.

The objective of the study described in **chapter 6** was to investigate the possibility to chemosensitize prostate cancer *in vivo* and *in vitro* aiming at modulation of MRP1-related MDR. Expression of MDR1, MRP1 and MRP2 was determined with Western blotting in a panel of human prostate cancer xenografts and in the xenograft derived cell line PC346C. Chemosensitization with MK-571 of PC346C to vincristine (VCR), doxorubicin (DOX) and etoposide (VP-16) was assessed in MTT assays. Carboxyfluorescein efflux blocking with MK-571 in PC346C was measured in fluorescence assays. Toxicity and efficacy of VCR and VCR combined with MK-571 was determined *in vivo* with human prostate cancer xenografts PC339 and PC346BI. MRP1 and MRP2 were expressed in all xenografts and in PC346C. No expression of MDR1 was found. Significant chemosensitization was achieved with MK-571 in PC346C. MRP1-mediated carboxyfluorescein efflux was demonstrated in PC346C and significantly inhibited with MK-571. *In vivo* application of tolerable doses of intravenous VCR resulted in significant reduction of tumour volumes in xenograft PC339 *in vivo* experiments. Intravenous VCR combined with intraperitoneal MK-571 resulted in significant *in vivo* chemosensitization to VCR in xenograft PC346BI. It was concluded that effective chemosensitization with MK-571 was demonstrated,

both *in vitro* and *in vivo*, indicating that MRP1 plays a role in the *in vitro* and *in vivo* MDR phenotype of prostate cancer.

Altogether, these findings may encourage clinical chemosensitization strategies aiming at improving the results of chemotherapy in advanced prostate cancer patients.

SAMENVATTING EN CONCLUSIES

Hormoonongevoelige prostaatkanker in een vergevorderd stadium van progressie vormt een therapeutische uitdaging aangezien alle behandelingsstrategieën ineffectief blijken te zijn. Chemotherapie verbetert de levensverwachting bij patiënten met een dergelijke prostaatkanker niet langer dan enkele maanden. Het falen van chemotherapie wordt mogelijk veroorzaakt door multidrug resistentie (MDR) mechanismen, die kankercellen tegen chemotherapeutica beschermen. De vraag doet zich voor of het MDR fenomeen een rol speelt bij prostaatkanker. Verscheidene mechanismen, waarvan bekend is dat zij MDR veroorzaken, zoals P-glycoproteine (Pgp; MDR1), varianten van het multidrug resistentie geassocieerde eiwit (MRP), glutathione, apoptose en topoisomerases zijn onderzocht en beschreven in het overzichtsartikel in **hoofdstuk 2**. Bewijs voor de rol die diverse MDR factoren *in vitro* en *in vivo* bij prostaatkanker spelen is in meerdere studies geleverd. Het omkeren, omzeilen of overwinnen van MDR mechanismen bij patiënten met uitbehandelde prostaatkanker komt in zicht en zal naar nieuwe behandelingsvormen leiden met een verbeterde effectiviteit.

Het doel van de studie, beschreven in **hoofdstuk 3**, was het inventariseren van het voorkomen van MDR factoren bij cellijnen van humane prostaatkanker en het vaststellen van het effect van chemotherapie op deze prostaatkankercellen.

Met immunocytochemie en Western blotting werd de expressie van Pgp, Multidrug resistance associated protein (MRP), Glutathione-S-transferase-π (GST-π), Bcl-2, Bax, Topoisomerase (Topo) I, IIα en IIß bepaald in cellijnen PC3, TSU-Pr1, DU145 en LNCaP afgeleiden -R, -LNO and -FGC. Proliferatie activiteit werd bepaald met immunocytochemie. Met MTT proeven werd de gevoeligheid voor etoposide, doxorubicine en Vinblastine vastgesteld. Pgp kwam in geen enkele van de cellijnen tot expressie. MRP expressie was variabel. GST-π kwam tot expressie in TSU-Pr1, PC3 en DU145. Expressie van Bcl-2 was beperkt tot TSU-Pr1, Bax kwam in alle cellijnen voor. Topo IIα kwam het meest tot expressie in snel delende cellijnen TSU-Pr1 en DU145. Topo I en IIß kwamen gelijkwaardig tot expressie. Resistentie profielen varieerden tussen de cellijnen, waarbij TSU-Pr1 het meest gevoelige en LNCaP-LNO

relatief resistent tegen chemotherapie was. Concluderend werd gesteld dat meerdere MDR eiwitten tot expressie komen in prostaatkankercellijnen en dat deze mogelijk de effectiviteit van chemotherapie beïnvloeden.

Het doel van de studie, beschreven in **hoofdstuk 4**, was het identificeren van eiwitten die mogelijk betrokken zijn bij multidrug resistentie bij het klinische prostaatcarcinoom. Expressie van deze eiwitten werd onderzocht in relatie tot tumour progressie. Paraffine coupes van met formaline gefixeerd prostaatkanker materiaal uit het pathologisch archief van drie verschillende patiëntengroepen werden onderzocht. Deze groepen onderscheidden zich duidelijk wat betreft pathologische stagering en reactie op antihormonale therapie. Groep 1 bestond uit patiënten met prostaatkanker die beperkt was tot de prostaat. Deze patiënten werden met een radicale prostatectomie behandeld ("vroege" prostaatkanker, pathologische stagering (p) T2N0M0). Groep 2 bestond uit patiënten met uitgezaaide prostaatkanker en werd behandeld met een transurethrale resectie van de prostaat (TURP) vanwege obstructieve mictieklachten, alvorens behandeld te worden met antihormonale therapie ("intermediaire" prostaatkanker). Groep 3 had uitgezaaide prostaatkanker en was progressief onder antihormonale therapie ("late" prostaatkanker) en werd met een TURP behandeld omwille van verlichting van symptomen van obstructieve mictieklachten. Immunohistochemie werd toegepast om Pgp, MRP1, lung resistance protein (LRP), GST-π, p53, Bcl-2, Bax, Topo I, IIα en IIβ en Ki-67 te detecteren. Uitgezaaide kanker kon onderscheiden worden van tot de prostaat beperkte carcinomen omdat uitgezaaide kanker een significant hogere histologische graad en proliferatie activiteit had dan niet uitgezaaide ziekte. Expressie van MRP, p53, Topo IIα, Ki-67 en Topo IIβ was significant gerelateerd aan een hogere Gleason score. Het aantal patiënten met tumoren die MRP, LRP, GST-π, p53, Bcl-2, Topo IIα en Ki-67 tot expressie brachten was significant hoger in de groep 3. Topo I en Topo IIβ kwamen gelijkelijk en in hoge mate tot expressie in alle drie de groepen. Pgp kwam in geen enkele tumour tot expressie, ongeacht de progressie van prostaatkanker. De conclusie luidde dat verhoogde expressie van de (chemotherapie) transport eiwitten MRP en LRP, ontgiftend enzym GST-π en apoptose remmende eiwitten Bcl-2 en p53 de resistentie tegen chemotherapie van uitgezaaide

klinische progressieve prostaatkanker kunnen verklaren. Toegenomen celdeling, aangetoond door verhoogde expressie van Ki-67 en Topo IIα, weerspiegelen het progressieve karakter van uitgezaaide prostaat kanker.

Het doel van de studie, beschreven in **hoofdstuk 5**, was het vaststellen van betrokkenheid van MRP1 en het glutathione (GSH) metabolisme bij het MDR fenotype van *in vitro* prostaatkanker. Selectie van humane prostaatkankercellijnen PC3 en DU145 door blootstelling aan het chemotherapeuticum etoposide, resulteerde in het ontstaan van MDR cellijnen PC3-R en DU-R. Resistentie tegen etoposide, doxorubicine and vincristine en omkeren van resistentie met leukotriene D4 antagonisten MK-571 and zafirlukast, en met buthioninesulfoximine (BSO) werd bestudeerd door middel van MTT proeven. Western blot analyses van MRP1 expressie en GSH concentratie metingen werden uitgevoerd. MRP1 functie werd bestudeerd met fluorescentie experimenten. Expressie van MRP1 was verhoogd in de MDR cellijnen PC3-R en DU-R. De GSH concentratie was significant toegenomen in PC3-R. Toename van GSH concentratie werd niet gevonden in DU-R. Toevoeging van niet-toxische doses van MK-571, zafirlukast of BSO resulteerde in een significant hogere gevoeligheid van de cellijnen PC3, PC3-R, DU145 en DU-R voor de chemotherapeutica. Inhibitie van MRP1 functie werd bereikt met MK-571 in alle cellijnen. Er werd geconcludeerd dat MRP1 en glutathione MDR veroorzaken bij de bestaande en nieuw ontwikkelde prostaatkankercellijnen.

Het doel van de studie, zoals beschreven in **hoofdstuk 6**, was het bestuderen van de mogelijkheid om *in vivo* en *in vitro* prostaatkanker te chemosensitizeren, gericht op het beïnvloeden van MRP1-gerelateerde MDR. Expressie van MDR1, MRP1 en MRP2 werd vastgesteld met Western blotting in een panel van prostaatkankerxenografts (heterotransplanteerbare humane prostaatcarcinomen; modellen waarbij stukjes humane prostaatkanker onderhuids in naakte immuundeficiënte (thymusloze) muizen geïmplanteerd worden, die vervolgens uitgroeien tot tumoren) en in xenograft-afgeleide cellijn PC346C. Chemosensitizering met MK-571 van cellijn PC346C voor vincristine (VCR), doxorubicine (DOX) en etoposide (VP-16) werd vastgesteld met MTT proeven. Blokkering van carboxyfluorescein (DCFDA)

efflux met MK-571 in PC346C werd gemeten met fluorescentie experimenten. Toxiciteit en effectiviteit van VCR en VCR in combinatie met MK-571 werd *in vivo* bestudeerd bij *in vivo* humane prostaatkankermodellen PC339 en PC346BI. Zowel MRP1 als MRP2 kwamen tot expressie in alle *in vivo* modellen en in cellijn PC346C. MDR1 kwam niet tot expressie. Significante chemosensitizering werd met MK-571 bereikt in cellijn PC346C. MRP1-gemedieerde DCFDA efflux werd aangetoond in PC346C en kon in significante mate geremd worden met MK-571. *In vivo* toepassing van niet-toxische doses intraveneuze VCR op model PC339 resulteerde in een significante afname van tumour volumes. Intraveneuze toediening van VCR in combinatie met intraperitoneaal gespoten MK-571 resulteerde in een significante *in vivo* chemosensitizering voor VCR in model PC346BI. De conclusie luidde dat het mogelijk is, zowel *in vivo* als *in vitro*, met MK-571 effectief voor chemotherapie te sensitizeren, hetgeen een rol aanduidt voor MDR eiwit MRP1 in het *in vivo* en *in vitro* MDR fenotype van prostaatkanker.

De bevindingen, beschreven in dit proefschrift, brengt het toepassen van klinische chemosensitizeringsstrategieën dichterbij, met als doel het verbeteren van de behandelingsresultaten van gemetastaseerde prostaatkankerpatiënten met chemotherapie.

List of publications

LIST OF PUBLICATIONS

this thesis:

van Brussel JP, Mickisch GHJ. Multidrug Resistance in Prostate Cancer – review article. Onkologie 2003;26:175-81

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Nawoord

NAWOORD

De in het woord "doctorandus" besloten belofte ben ik bij deze nagekomen!
Het verliezen van de "s" is mooi. Maar, het doet me met name veel goed dat dit promotieonderzoek, ondanks de veranderingen in mijn loopbaan, afgerond is. Dat was ik aan mijzelf verplicht en aan allen die mij op enigerlei wijze geholpen hebben met dit werk.

Professor Schröder, u belde mij om halfnegen op een zaterdagochtend met het aanbod om onderzoek te doen, in afwachting van een plaats in de kliniek. Hoewel er aanvankelijk geen sprake was van een promotieonderzoek, liep het al snel uit de hand. Dank voor de mogelijkheid die u mij gaf in het urologisch lab te werken en het project op congressen te presenteren. En dank dat u mijn promotor wilt zijn. Ik heb uw sportieve reactie zeer gewaardeerd, toen ik schoorvoetend kwam vertellen dat ik toch chirurg wilde worden.

Dit geldt zeker ook voor professor Mickisch, tevens mijn promotor. Beste Gerald, met jou had ik de eerste gesprekken over het onderzoek. Na een doordringende blik van jouw kant, begon je te vertellen over multidrug resistentie – toen nog nooit van gehoord – en dat gesprek is tot op heden niet beëindigd. Jij liet mij de vrijheid om de onderzoekslijnen uit te zetten, af en toe sturend en motiverend en altijd enthousiast. Ondanks de indrukwekkende stapels werk op je bureau, kon ik immer bij je terecht. Je was voor mij een onuitputtelijke bron van klinische- en literatuurkennis en wist bovendien overal ter wereld de beste kroegen. Helaas is de afstand tussen ons nu groter. De ontmoetingen met jou en Marianne, met goeie recepten en "Le trou Normand", waren altijd bijzonder gezellig. Alle reden om eindelijk eens een keer naar Bremen te gaan.

De leden van de leescommissie, hoogleraren Scheper, Sonneveld en van der Kwast dank ik voor de beoordeling van het manuscript. Theo, bedankt voor de commentaren en je komst uit Canada.

Dr. van Steenbrugge, beste Gert Jan, ik werd bij jou in het lab geplaatst en jij werd mijn directe begeleider, waarvoor dank. Wekelijks hadden we besprekingen over het onderzoek, ondanks de enorme klus die je had aan de verhuizing van de 10e naar het Josephine Nefkens Instituut. Je stond altijd klaar om voor de medewerkers van het lab in de bres te springen. Naast een groot talent als organisator, ga je voor mij ook door het leven als bestrijder van Mycoplasma, hetgeen mogelijk de redding was voor meerdere laboratoria.

Zo'n periode in het lab is een bijzondere ervaring. Ik ben ervan overtuigd dat de basis voor vooruitgang in de Geneeskunde in het experimentele werk ligt. Bewondering heb ik voor de wetenschappers, voor hun nieuwsgierigheid naar het onbekende en hun gevecht met de wet van Murphy, onder druk van het "publish or perish". Het is de kunst om de kloof tussen de werelden van het lab en de kliniek te overbruggen. Dat dit geen makkelijke opgave is heb ik ondervonden. Er worden verschillende talen gesproken en er is meer dan een tweetalige opvoeding voor nodig om die te begrijpen. Mijn gebrek aan opleiding voor het doen van labwerk speelde me geregeld parten. Gelukkig kon ik dan aankloppen voor hulp en advies bij de collegae in het lab. Met name Wilma Teubel, Monique Oomen, Marja Verleun, Corrina de Ridder en Cindy Bolder waren onmisbaar bij het praktische werk. Robert Kraaij was mijn vraagbaak, Hans Romijn, Wytske van Weerden, Arjen Noordzij en Hans Scheltema gaven goeie adviezen. Ries Kranse en Mark Wildhagen waren behulpzaam wat betreft de statistiek. In het hematologisch lab werkte ik met plezier samen met Erik Wiemer, Paula Vossebeld en Arie Prins. De medewerkers van de pathologie waren behulpzaam met antilichamen en coupes, en Frank van de Panne hielp mij regelmatig met het fotowerk.

Naast degenen die in het onderzoek zelf betrokken waren, zijn er meerdere mensen die mij stimuleerden en prikkelden (is je boekje al af?), als mijn gedachten afdwaalden van het onderzoek.

Mijn opleiders en de stafleden van het St. Elisabeth ziekenhuis hielpen mij de bal aan het rollen te houden door mij enkele keren "schrijfvrij" te geven.

Dr. Roukema, beste Rouk, als we samen aan tafel stonden vroeg je steevast: "heb je al een datum?". Als ik dan mompelde dat ik ermee bezig was, liet je me fijntjes weten dat je dat niets interesseerde, alleen die datum wilde je horen. Zestien november 2005! Natuurlijk was jij wel de eerste die de voorlopige versie van dit proefschrift van commentaar voorzag. Ook gaf jij Jan op andere fronten meerdere keren het zetje dat hij nodig had…veel dank!

De leden van de Rotterdam Love Express, Wouter Vles, Jacques van der Meulen, Erik Walbeehm en Jur Vellema wil ik danken voor de ruim 200.000 veilige kilometers inclusief ontbijt. En dat met een totale vertraging van slechts vier uur. Met veel plezier denk ik in dit kader terug aan het achteruitrijdend aankomen bij het ziekenhuis, met de knalpijp slepend over het wegdek. De wijze lessen in de RLE zijn van onschatbare waarde geweest, ook voor dit proefschrift!

Mijn collegae in Tilburg moesten een stapje extra doen als ik aan het schrijven was, dank hiervoor!

Prof.dr. H.J. Bonjer en prof.dr. R.P. Bleichrodt maakten het mij, onder andere, mogelijk om de eindsprint in te zetten.

Jaren geleden werd de promotie-wisseltrofee ingesteld. Dit begerenswaardig voorwerp valt mij nu ten deel, al is het maar van korte duur (1 fles Whisky). Joris Meijaard, Stephan Slingerland en Jorrit Hoff, onze gesprekken over promoveren veranderden meestal snel via gymnasiale discussies in vrij associëren. Inmiddels wordt er meer en meer over de volgende generatie gesproken!

Het thuisfront, zowel in Rotterdam, in Leiden als in Kamperland is ongelofelijk belangrijk voor het totale plaatje, waar dit proefschrift ook lange tijd deel van uit heeft gemaakt.

Papa, jij liet me kennismaken met de wetenschap, toen ik als jochie met je meeging naar de Nonnensteeg en door de hortus zwierf. Daar snoof ik de geur van

historie, het laboratorium en viltstiften op. Toen had ik niet gedacht zelf ook met kweekkasten en centrifuges aan de slag te gaan. Met je adviezen en je grote (soms wat bezorgde) interesse naar de vorderingen van het onderzoek, heb jij mij veel geholpen.

Maman, tu as toujours été là, pour moi et mes frères Pieter et Nicolas et maintenant aussi pour Veerle et Luc. Tu es vraiment fantastique!

Rien en Lia, veel dank voor alle steun, ik zal m'n computer niet meer meenemen naar Zeeland…

Nicolas en Jean, paranimfen, ik ben er trots op door jullie geflankeerd te worden tijdens het uur U!

Lieve Caroline, over jou kan ik een boek schrijven en dat is zo veel leuker en mooier dan het schrijven van dit boekje! Wat jij allemaal voor me betekent…blijft tussen ons. Je t'aime!

Rotterdam, 16 november 2005

Curriculum Vitae

CURRICULUM VITAE

23 oktober 1968	Jérôme Patrick van Brussel, geboren te Leiden
1981 – 1987	Gymnasium β, Stedelijk Gymnasium Leiden
1987	Aanvang studie Geneeskunde, Rijksuniversiteit Leiden
27 januari 1995	Artsexamen
Februari 1995	House Officer, Department of Surgery and Urology, Bolton Royal Infirmary, Bolton, Lancashire, United Kingdom
1995 –1996	AGNIO chirurgie, Rijnland ziekenhuis, Leiderdorp
1996-1998	AIO, Erasmus Universiteit Rotterdam, afdeling urologie, onderzoek resulterend in dit proefschrift: Multidrug resistance in Prostate Cancer Promotores: Prof.dr. F.H. Schröder en Prof.dr. G.H.J. Mickisch
1999 – 2000	AGIO heelkunde (vooropleiding urologie), St. Elisabeth ziekenhuis Tilburg, opleider Dr. J.A. Roukema
2001	AGIO urologie, AZR Dijkzigt, opleider Prof. Dr. F.H. Schröder
2002 – 2004	AGIO chirurgie, St. Elisabeth ziekenhuis Tilburg, opleiders Dr. J.F. Hamming en Dr. C.J.H.M. van Laarhoven
2005	AGIO chirurgie, ErasmusMC, Rotterdam, opleider Prof.dr. J.N.M. IJzermans

Opgroeiend onder het toeziend bronzen oog van Boerhaave, genietend van een Nederlands-Franse opvoeding, doorkruiste ik gedurende mijn middelbare schoolperiode per fiets Leiden, op weg naar het Stedelijk. Tijdens deze inspirerende periode ontmoette-, en kort erna ontdekte- ik Caroline met wie ik het leven deel, samen met onze kinderen Veerle en Luc.

Met nummer 2513 uitgeloot voor Geneeskunde, begon ik aan de studie Biologie, maar werd na een maand alsnog geplaatst voor Geneeskunde aan de Vrije Universiteit te Amsterdam, om na drie weken weer te worden overgeplaatst naar de Rijksuniversiteit Leiden. Gedurende een verder vlot verlopende studie, roeide ik wedstrijd bij de K.S.R.V. Njord.

De doctoraalfase werd in 1991 afgesloten met een afstudeerproject in het lab van Prof.dr. H.E. ter Keurs, aan de University of Calgary, Alberta, Canada, waarbij ik ritmestoornissen in rattenhartspieren onder invloed van ouabaïne onderzocht.

De wachttijd voor de co-schappen (doorlopen in regio Leiden) bracht ik, samen met Caroline, door in Lagdo, Noord-Kameroen, waar we, in het kader van keuze co-schap, de prevalentie van Schistosoma Hematobium infecties in de regio in kaart brachten, onder supervisie van Dr. A.M. Polderman, afdeling Parasitologie van de Rijksuniversiteit Leiden.

Meteen na het artsexamen, reisde ik af naar Bolton, Lancashire, United Kingdom, waar ik gedurende een korte periode mijn eerste schreden als House Officer zette, op de afdeling chirurgie en urologie van het Bolton Royal Infirmary. Vervolgens werkte ik gedurende een jaar als AGNIO chirurgie in het Rijnland ziekenhuis, te Leiderdorp.

Na deze klinische periode, startte ik, op uitnodiging van Prof.dr. F.H. Schröder en onder supervisie van Prof.dr. G.H.J. Mickisch met experimenteel onderzoek in het urologisch lab. De resultaten worden in dit proefschrift gepresenteerd.

Na het verkrijgen van een opleidingsplaats Urologie, doorliep ik de vooropleiding Chirurgie in het St. Elisabeth ziekenhuis te Tilburg. Daar raakte ik zeer geënthousiasmeerd door de Chirurgie. Hoewel ik de opleiding Urologie in het Dijkzigt ziekenhuis begon, besloot ik van specialisme te veranderen. In 2002 verkreeg ik een opleidingsplaats Chirurgie en deed mijn perifere opleiding in het St.Elisabeth ziekenhuis. Gedurende mijn laatste opleidingsjaar ben ik werkzaam in het Erasmus medisch centrum. Vanaf januari 2006 zal ik in het Erasmus MC als CHIVO vaatchirurgie werken.

I love fools' experiments. I am always making them.

Charles Darwin