MODULATION OF P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE IN THE CC531 RAT COLON TUMOR MODEL


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MODULERING VAN MULTIDRUG RESISTENTIE GEMEDIEERD DOOR P-GLYCOPROTEINE IN HET COLON TUMOR MODEL CC531 IN DE RAT

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GENERAL INTRODUCTION

1.1 THE PROBLEM OF DRUG RESISTANCE IN CANCER THERAPY

About half of the patients that come to the physician with cancer have a localized stage of the disease and can be cured by surgery or radiotherapy. The remaining cancers have spread systemically because the primary tumor has metastasized or because they are systemic cancers by nature. The only hope for cure for patients with these cancers lies in systemic treatment such as chemotherapy or immunotherapy. Cure can be obtained by intensive chemotherapy in childhood acute leukemia and sarcoma, in adult testicular cancer and choriocarcinoma, and, to a lesser extent, in lymphomas. In other malignancies like breast cancer adjuvant chemotherapy after curative surgical ablation has proven beneficial in a minority of the patients by reducing the likelihood of disease recurrence. In these patients residual microscopic disease, which would have resulted in disease recurrence, has been eradicated by chemotherapy. However, only 5%-10% of the patients with systemic cancer can be cured by chemotherapy to day.[1,2] A still much smaller percentage of the cancers responds to various forms of immunotherapy.

Anticancer drugs are not specifically directed against tumor cells; they merely take advantage of some tumor characteristics, especially accelerated cycle of cell division. Dose intensification of a drug leads to increased antitumor activity, but is hampered by the inherent enhanced toxicity to normal cells. To overcome this problem combination chemotherapy has been introduced in which various drugs that are effective against a certain cancer, but that differ in their toxicity to normal cells, are combined. This approach has greatly increased the effectiveness of chemotherapy.[2,3] Nevertheless, many cancers are still not curable by this approach, because they do not react to the treatment from the start and are said to be intrinsically resistant to chemotherapy. Examples of these tumors are hepatocellular carcinoma, carcinoma of the biliary tree, non-small-cell lung cancer, renal cell cancer and glioblastoma multiforme. It is striking that many of these cancers originate from duct cells or cells lining excretory organs. This suggests that these tumors have retained the ability to detoxify, excrete, and eliminate noxious compounds and exploit these mechanisms to resist chemotherapeutic agents. Other cancers that were initially responsive to anticancer drugs may become refractory to treatment or recur after an initial response. This is called acquired or induced drug resistance.[4]

Although originating from one mutated clone of cells, a tumor is a heterogeneous group of cells. Numerous additional mutations occur during tumor growth, some leading

to new characteristics of the tumor, like metastasizing potential and altered susceptibility to anticancer drugs.[5] Drug treatment may accelerate the selective outgrowth of mutations that are responsible for drug resistance and which help the tumor to survive. On the other hand, drug pressure on tumor cells can also induce upregulation and development of defense and repair mechanisms that function in normal cells.[2,6]

The mechanisms underlying resistance to anticancer drugs are manyfold. Tumor cells may defend themselves against chemotherapy by diminishing drug accumulation in the cell through decreased influx or increased efflux. The drug metabolism can be altered leading to lowered turnover of prodrugs into active metabolites or by increased metabolism of drugs leading to inactivation. Targets for drugs in the tumor cell can be altered quantitatively or qualitatively. Drug resistance may be caused by activation of repair mechanisms for DNA damage which results in diminished cell kill. Gene expression may be altered by DNA mutation, gene amplification, deletion and other mechanisms leading to altered tumor characteristics. Some drug resistance mechanisms are only found in certain kinds of tumors or are unique for a group of drugs, while others are expressed ubiquitously in various tumors and are active against many anticancer drugs.[6,7] An example of the last group is multidrug resistance (MDR) which is the subject of the studies described in this thesis.

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1.2

IN VIVO MODEL SYSTEMS IN P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE

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(submitted for publication)

Summary

In this article we will review the *in vivo* model systems that have been developed for studying P-glycoprotein-mediated multidrug resistance (MDR) in the preclinical setting. Rodents have two *mdr* genes that both confer the MDR phenotype: *mdr1a* and *mdr1b*. At gene level they show strong homology to the human *MDR1* gene and the tissue distribution of their gene product is very similar to P-glycoprotein expression in humans. *In vivo* studies have shown the physiological roles of P-glycoprotein among which protecting the organism from damage by xenobiotics. Tumors with intrinsic P-glycoprotein expression, induced MDR or transfected with an *mdr* gene can be used as syngeneic or xenogenic tumor models. Ascites, leukemia, and solid MDR tumor models have been developed. Molecular engineering has resulted in transgenic mice that express the human *MDR1* gene in their bone marrow, and in knockout mice missing murine *mdr* genes. The data on pharmacokinetics, efficacy and toxicity of reverters of P-glycoprotein *in vivo* are described. Results from studies using monoclonal antibodies directed against P-glycoprotein and other miscellaneous approaches for modulation of MDR are mentioned. The importance of *in vivo* studies prior to clinical trials is being stressed and potential pitfalls due to differences between species are discussed.

1. Introduction: the problem of multidrug resistance in human cancer

A major obstacle for successful chemotherapy of cancer is the resistance of tumors to anticancer drugs. Resistance may be caused by an intrinsic resistance to anticancer drugs or by the emergence of drug-resistant cells during chemotherapeutic treatment. In recent years several mechanisms causing drug resistance have been elucidated: decreased uptake or increased efflux of drugs by tumor cells, alterations in target proteins, cellular drug metabolism or drug binding, and enhancement of DNA repair mechanisms. Some mechanisms affect only a specific drug, while others cause resistance to a wide variety of drugs.

An important mechanism, which has been observed in many different malignancies and which affects various groups of unrelated anticancer drugs is called multidrug resistance (MDR). Sometimes the prefix classical is added to distinguish classical MDR from other forms of pleiotropic drug resistance. In classical MDR the mechanism of drug resistance is an energy-dependent, unidirectional transmembrane efflux pump, called P-glycoprotein or P-170, that extrudes drugs and other xenobiotics out of the cell. Thus intracellular levels of these compounds can be kept under a non-cytotoxic level. The efflux pump has a broad substrate specificity affecting drugs as anthracyclines, epipodophyllotoxins, *Vinca* alkaloids, taxanes, colchicine, topotecan, and actinomycin D. Therefore, tumors expressing the MDR phenotype are cross-resistant to a wide variety of structurally unrelated drugs. Many compounds that have no antineoplastic activity can also interact with the P-glycoprotein efflux pump and block its function. This leads to increased intracellular levels of cytotoxins that are substrates for P-glycoprotein and to enhanced cell death. Compounds that can block P-glycoprotein are termed MDR modulators, reverters, or chemosensitizers.[1-3]

Studies on the expression of the *mdr* gene or its product P-glycoprotein in human tumors are difficult to interpret as various methods with varying sensitivity and specificity, often leading to conflicting results, have been employed by the investigators. Molecular methods for the detection and measurement of the *mdr* DNA and mRNA are generally sensitive and quantitative, the most sensitive being the reverse transcriptase polymerase chain reaction. However, contamination of the tumor sample with normal cells and heterogeneous expression of the *mdr* gene within the tumor can not be detected. Immunohistochemical assays with specific monoclonal antibodies against P-glycoprotein can detect P-glycoprotein expression at the individual cell, but the sensitivity is generally less than in molecular techniques and the measurement of P-

glycoprotein level is semi-quantitative. Functional assays measure the actual activity of the P-glycoprotein efflux pump in tumor cells. This technique is currently only available for hematological malignancies.[4,5] Recently, recommendations have been published for standardization of methods to detect P-glycoprotein-associated MDR.[6]

In general, it can be stated that tumors originating from tissues with high expression levels of P-glycoprotein have clear P-glycoprotein expression as well and are often intrinsically refractory to chemotherapy. Among these are colorectal cancer, renal cell carcinoma, hepatoma, adrenocortical carcinoma and pancreatic cancer.[7-9] Results of P-glycoprotein expression in breast cancer and soft tissue sarcoma are variable,[8,9] while in lung cancer, ovarian cancer, and melanoma levels of *MDR* expression are low to absent.[8,9] For some of these solid tumors higher levels of *MDR* expression have been reported after relapse or failure of chemotherapy with MDR substrates, *e.g.* breast cancer,[8,10] ovarian cancer,[11] and neuroblastoma.[12] For more extensive information about the expression of *MDR* in solid tumors, the reader is referred to some specific reviews.[13,14]

In hematological tumors *MDR* overexpression is frequently observed in acute myeloid leukemia, while its expression in acute lymphoblastic leukemia is generally low.[8,9,15,16] Secondary acute myeloid leukemia and disease recurrence after chemotherapy are associated with a markedly higher frequency of *MDR* expression.[16,17] In lymphoma and myeloma P-glycoprotein expression is infrequent in newly diagnosed cases, but common in recurrent disease after chemotherapy.[18,19] In specific reviews the results of P-glycoprotein expression in hematological malignancies are summarized.[20,21]

Several studies have brought forward evidence that P-glycoprotein expression has prognostic significance in certain malignancies. In neuroblastoma and childhood sarcoma P-glycoprotein expression is associated with poor response to chemotherapy, increased chance of relapse, and decreased survival.[22,23] In acute leukemia there is a correlation with reduced frequency and duration of complete remission.[15,16,24] However, for many tumors studies with results contradicting other reports have been published and the exact significance of P-glycoprotein-mediated MDR remains to be defined.[25]

The remainder of this review will concentrate on *in vivo* studies on the physiological functions of P-glycoprotein and on *in vivo* model systems that have been developed for studying modulation of P-glycoprotein-mediated MDR. The similarities and differences in MDR-related P-glycoproteins between species will be outlined. The validity of the animal models for studying MDR and their relevance to the clinical situation in humans

will be discussed.

2. *Mdr* gene expression and function across species

2.1. The *mdr* superfamily of genes

The human P-glycoprotein is a 170-kilodalton protein which consists of 1280 amino acids. The putative structure of P-glycoprotein consists of two homologous halves each containing 6 transmembrane regions and a large intracytoplasmatic loop encoding an ATP-binding site. Together they form the functional multidrug transporter.[26] The gene encoding for P-glycoprotein is highly conserved during evolution and belongs to a superfamily of membrane-associated transport proteins: the ATP-binding cassette (ABC) family. This family includes, amongst others, bacterial transporters, the STE-6 transporter in yeast, the *Plasmodium* chloroquine resistance gene, the *Leishmania* resistance gene, *Drosophila* genes, and the cystic fibrosis gene CTFR. Proteins of the ABC family are transporters of various nutrients, peptides, polysaccharides, toxins and drugs.[27-29]

In humans two *mdr* genes have been detected, from which only *MDR1* encodes for the MDR-related P-glycoprotein efflux pump and confers the MDR phenotype.[26,30-32] The function of the second *mdr* gene product in humans, *MDR3* or *MDR2* (for nomenclature see Table 1), has only recently been elucidated, but it is not a transporter of drugs used in chemotherapy. Rodents have 3 *mdr* genes, from which class 1 and 2 have been shown to confer the MDR phenotype.[33-35] In mouse and rat these genes are called *mdr1a* and *mdr1b* (in mice also designated as *mdr3* and *mdr1* respectively, and in rat as *pgp1* and *pgp2* respectively);[35,36] in hamster they are named *pgp1* and *pgp2*.[27,37] Table 1 gives an outline of the *mdr* genes in various species and the nomenclature. Comparison of coding sequences of the various *mdr* genes shows high homology and sequence identity. The homology is higher between genes of the classes 1 and 2 versus the genes of class 3, consistent with the different abilities of their products to transport drugs. The mouse *mdr1a* and *mdr1b* genes show 83% identity to each other, while they have 73% and 71% identity with the *mdr2* gene respectively.[35] The human *MDR1* and *MDR3* coding sequences are over 75% identical. The higher identity within classes is retained when *mdr* genes of the same class are compared across species. Sequence identity of human *MDR1* and mouse *mdr1a* is 82% and of human *MDR1* and mouse *mdr1b* 79%. Similar homology is found with hamster and rat class 1 and 2 *mdr*

Table 1. Classification of the *mdr*/P-glycoprotein genes

	P-glycoprotein gene		
species	class 1	class 2	class 3
human	*MDR1*	-	*MDR3*[*]
mouse	*mdr1a*[‡]	*mdr1b*[@]	*mdr2*
hamster	*pgp1*	*pgp2*	*pgp3*
rat[¶]	*mdr1a*	*mdr1b*	*mdr2*

[*] *MDR3* is also known as *MDR2*
[‡] *mdr1a* is also known as *mdr3*
[@] *mdr1b* is also known as *mdr1*
[¶] The rat *mdr* genes are designated *pgp1*, *pgp2*, and *pgp3* in some studies, like the nomenclature of the hamster *mdr* genes

genes.[37,38] Within class 3 identity of human *MDR3* and mouse *mdr2* at the amino acid level is even 91%.[39] This suggests that early in evolution a primordial *mdr* gene gave rise to the ancestral class 1/2 and class 3 genes. A second gene duplication event occurred in rodents, but not in humans, and resulted in the *mdr1a/pgp1* and *mdr1b/pgp2* genes.[37,40] Regions with the greatest homology are the ATP-binding/utilization regions, the 2nd, 4th, and 11th transmembrane domains, and the 1st and 2nd intracytoplasmatic loops in each half of the molecule. Among the least conserved regions are the 1st extracytoplasmatic loop, the connecting region between the two halves of the molecule, and both terminal ends.[41,42]

2.2. Tissue distribution of the *mdr* genes

The expression of the *mdr* genes in rodents and human is tissue specific and *mdr* genes of the same classes show a comparable pattern of distribution in different species. In mice the *mdr1a* gene is mainly expressed in intestine, lung, liver and blood capillaries of brain and testis. The predominant isoform in the adrenal, uterus in pregnancy, placenta and kidney is *mdr1b*.[43-46] In hamsters a similar pattern of distribution of the *pgp1* and *pgp2* genes is shown.[47,48] Although minor differences in class 1 and 2 *mdr* expression between the various studies are found, the overall pattern concurs very well. The tissue distribution of *mdr1a/pgp1* and *mdr1b/pgp2* together matches very neatly to that of the human *MDR1*, as can be seen in table 2. In humans *MDR1* is mainly expressed in the adrenal, kidney, intestine, liver, uterus in pregnancy,

Table 2. Tissue expression of the *mdr* class 1 and 2 genes in human and rodents

	human *MDR1*	murine *mdr1a*	hamster *pgp1*	murine *mdr1b*	hamster *pgp2*
heart muscle		+	+	+	
lung	+	+ +	+ +	+	
liver	+ + +	+	+ /pi	+	
stomach	+	0		+	
jejunum-ileum	+ + +	+ + +	pi	+	
colon	+ + +	+ + +	pi	+	
spleen	+	+	+	+	
kidney	+ + +	+	+	+ +	
adrenal	+ + + +	+	+	+ + + +	pi
testis	+	+	+ + + /pi	0	
ovary	+	+	+ + /pi	+ +	
uterus	+	+	+ + + /pi	+	
uterus (in pregnancy)	+ +	+		+ + + +	pi
placenta		0		+ + +	
skeletal muscle	+	+	+	+	
brain	+ +	+	+ + /pi	0	

+ - + + + + represents the relative expression level of mRNA; 0 = very low to undetectable mRNA; pi = predominant isoform in the particular tissue.
Data on human *MDR1* were compiled from references 7,9,46,49; data on murine *mdr1a* and *mdr1b* from 44-46; data on hamster *pgp1* from 47 (all mainly RNA analyses); non-quantitative data on hamster *pgp1* and *pgp2* from 45 are designated as predominant isoform (pi)

and is the predominant isoform in the capillaries of brain and testis.[7,9,46,49] Diverse human hematopoietic differentiation lineages show *MDR1* expression as well.[50]

P-glycoprotein class 3 genes are the predominant isoforms in mouse and rat liver, spleen, heart muscle and striated muscle,[39,44,45,51] and in hamster heart and striated muscle.[48] There are several studies on class 3 *mdr* expression in mice and overall most studies show comparable results. Only two studies on rat *mdr2* and one on hamster *pgp3* expression have been published and some differences are notable. Two different

cDNA clones have been reported for the rat *mdr2*, with a mismatch in nucleotide bases between the two sequences resulting in nucleotide differences for four amino acids.[51,52] Work with the first DNA sequence derived from the Fischer 344 rat strain revealed a distribution pattern of *mdr2* in the rat comparable to that in the mouse.[51] With the second *mdr2* cDNA cloned from the Sprague-Dawley rat strain however, high expression levels were detected in liver, but also in gastrointestinal tract, low levels in brain, heart and kidney, and undetectable levels in spleen and striated skeletal muscle in this rat strain.[52] If these results are confirmed with additional studies, this would indicate that strain differences exist within species in expression level of the various P-glycoproteins. Seen the similarity in distribution of the various *mdr* genes across species and the differences in function between class 1 and 2 versus class 3 P-glycoproteins (*vide infra*) this is not very likely. In hamsters the predominant isoform in the liver would be *pgp1*,[45] while in other species this is class 3 *mdr*. As both isoforms are found in the liver, the differences may be based on factors as differences in investigational techniques. These inconsistencies left aside, the distribution of rodent and human class 3 P-glycoprotein is quite similar. See table 3. In humans high expression levels of *MDR3* are found in the liver,[39,49] and with specific monoclonal antibodies only in this organ expression of the *MDR3* P-glycoprotein is shown.[39,53] Low expression levels of *MDR3* mRNA are found in human adrenal, spleen, heart, and striated muscle.[39,49]

Immunohistochemical and *in situ* hybridization studies have shown that within the tissues the P-glycoproteins have specific subcellular localizations, with a similar pattern of distribution in human and rodents. In epithelial cells with a polarized excretion or absorption function P-glycoprotein is mainly expressed at the apical surface that lines the lumen. The *MDR1* product is demonstrated in the brush border of the proximal tubules of the kidney, the biliary canalicular surface of the hepatocytes, the apical surface of columnar epithelial cells in small and large intestine, and luminal surface of the cells in the pancreatic ductules.[54,55] In rat kidney, liver, and intestine a comparable subcellular distribution of P-glycoprotein is detected, and in the pancreas acinar cells were stained with a specific monoclonal antibody (C219).[56] In mouse and hamster class 1 P-glycoprotein is expressed in colonic epithelial cells in a polarized manner.[48,53,57] In the gravid uterus of the mouse *mdr1b* is expressed at high levels in the secretory epithelial cells of the endometrium.[43,53] At blood-tissue barriers of the central nervous system and testis, and in the papillary dermis, *MDR1* is expressed at high level in endothelial cells.[55,56,58] P-glycoprotein expression in endothelial cells of the brain is

Table 3. Tissue expression of the *mdr* class 3 genes in human and rodents

	human *MDR3*	murine *mdr2*	rat *mdr2*[f]	hamster *pgp3*
heart muscle	+	+ +	+ +	pi
lung	0	+	+	
liver	+ + +	+ + + +	+ + + +	
stomach	0	0		
jejunum-ileum		0	0[f]	
colon	0	0		
spleen	+	+ +	+ +	
kidney	0	0	0	
adrenal	+	+		
testis	0	0		
ovary	0			
uterus		0		
uterus (in pregnancy)		0		
placenta	0	0		
skeletal muscle	+	+ +	+ +	pi
brain	0	0	0	

+ - + + + + represents the relative expression level of mRNA; 0 = very low to undetectable mRNA; pi = predominant isoform in the particular tissue

Data on human *MDR3* were compiled from references 39,49; data on murine *mdr2* from 39,44,45; data on rat *mdr2* from 51 (studies are mainly RNA analyses); non-quantitative data on hamster *pgp3* from 45 are designated as predominant isoform (pi)

[f] Furuya *et al.*[52] cloned *mdr2* cDNA from the Sprague-Dawley rat strain and detected high expression levels in liver and gastrointestinal tract, low levels in brain, heart and kidney, and undetectable levels in spleen and striated skeletal muscle in this rat

shown in rat and mouse, in the latter being *mdr1a*.[56,59] In hamsters a similar expression of *pgp1* is demonstrated in endothelial cells of brain and testis, but also of ovary and uterus.[48] Staining of adrenal cortical cells for P-glycoprotein is homogeneous and not polarized.[54,55,57]

Human *MDR3*, and mouse and rat *mdr2* is shown to be localized in the biliary

canalicular membrane of hepatocytes but not in epithelial cells of the bile ductules.[39,53]

2.3 Physiological roles of the P-glycoproteins

The similarities between species in tissue distribution and subcellular localization suggest that the P-glycoprotein isoforms perform fundamentally important physiological functions in cells and that these functions are retained across species. The localization of P-glycoprotein at the apical side of cells that line luminal surfaces in kidney, liver and intestine is consistent with a putative detoxification role for P-glycoprotein mediating excretion or preventing (re)absorption of degradation products, xenobiotics, carcinogens and drugs.[60] Additional evidence for such a role comes from studies that show that *mdr* RNA levels are upregulated in rodents in response to stressing situations like acute cytotoxic insults and partial hepatectomy.[61,62]

In murine kidney cells a basal to apical transepithelial transport of vinblastine has been shown.[63] Secretion into urine of vincristine and vinblastine in dogs and colchicine in mice is diminished by inhibitors of P-glycoprotein like cyclosporin A and PSC 833, which strongly suggests a P-glycoprotein-dependent transport mechanism.[64-66] In the *mdr1a* knockout mouse, that has no functional *mdr1a* expression (*vide infra*), digoxin plasma levels are raised 2-fold compared to normal mouse, probably because of diminished renal elimination.[67]

Evidence for a physiological function of P-glycoprotein in the intestine comes from studies with rat everted gut sacs and segments of rat intestine in which transport of the P-glycoprotein substrates from serosal to mucosal side is shown.[68,69] Intriguingly, in the last study differences in transport of various substrates depending on the intestinal site, whether duodenal, jejunal or colonic, were observed. This might suggest that P-glycoprotein-mediated efflux systems exist with different substrate specificities depending on the intestinal site.[69]

In vitro studies with rat liver tissue have shown that canalicular membrane vesicles, but not sinusoidal (basolateral) membrane vesicles of the liver have a P-glycoprotein efflux pump which is ATP-dependent and can be inhibited by MDR modulators.[70] In mice, canalicular membrane vesicles express *mdr1a* and *mdr2*, while sinusoidal membrane vesicles do not express P-glycoprotein.[53] Biliary clearance of vinblastine, colchicine, and adriamycin is blocked by P-glycoprotein inhibitors *in vivo*.[65,71,72] Induction of cholestasis by ligation of the bile duct or by administering a cholestatic agent resulted in upregulation of *mdr1a* and *mdr1b* expression in the rat. This upregulation of P-glycoprotein caused a significant increase in biliary excretion of vinblastine. In

monkeys the cholestatic agent resulted in upregulation of both *MDR1* and *MDR3*.[71] Together, these data strongly support the idea that P-glycoprotein plays an important role in the secretion of xenobiotics and other compounds into the bile.

The hypothesis that P-glycoprotein has a protective function at the blood-brain barrier has recently been confirmed. Cultured mouse brain endothelial cell lines that expressed *mdr1b* have been shown to transport vincristine from basal to apical side, that is from the brain to the blood luminal side.[73] In most rodent studies the predominant isoform of *mdr* in the brain capillaries was found to be *mdr1a*, although minor expression of *mdr1b* has also been observed.[44,73] The enhanced expression of *mdr1b* in brain endothelial cells in cultures is possibly an *in vitro* phenomenon.[74] *In vivo* evidence for functional P-glycoprotein at the blood-brain barrier comes from the group of Borst, who have generated mice homozygous for a disruption of the *mdr1a* gene, so called *mdr1a* knockout mice.[59] No functional expression of the *mdr1a* gene could be shown in these mice, and especially in gut epithelium and brain capillaries all detectable *mdr1a* was lost and no increased expression of *mdr1b* was observed. The mice appeared normally healthy and fertile. However, they displayed a markedly increased sensitivity to the neurotoxic effects of the pesticide ivermectin, an acaricide and anthelmintic drug, and to the carcinostatic drug vinblastine. Brain tissue levels of ivermectin were 87-fold and vinblastine 22-fold higher in *mdr1a* knockout mice compared to normal mice, while plasma levels of the drugs were only 3.3-fold and 2-fold higher respectively.[59] The neurotoxic effects of ivermectin were also observed in experiments with the MDR reverters PSC 833 and SDZ 280-446 in normal mice showing that potent inhibitors of P-glycoprotein are able to block its normal function at the blood-brain barrier *in vivo*.[75] Coadministration of cyclosporin A significantly increased the distribution of rhodamine-123, a dye that is transported by P-glycoprotein, to the brain in rats, without altering the plasma disposition of rhodamine-123.[76] These data show that P-glycoprotein is an important component of the functional blood-brain barrier and protects the central nervous system against deleterious effects of endogenous and exogenous toxins.

The homogenous distribution of P-glycoprotein in cells of the adrenals and in the placental trophoblast suggests a role in steroid transport. Evidence of *in vitro* studies support this putative role. It has been shown that various steroids as cortisone, dexamethasone, and possibly aldosterone can be transported by rodent and human P-glycoprotein.[77,78] Progesterone binds strongly to P-glycoprotein and is an efficient inhibitor of its transport function, but is not transported itself by P-glycoprotein.[77-79] Together with estrogen, progesterone seems to induce the expression of P-glycoprotein

in the uterus during gravitation in mice.[80] In mouse adrenocortical Y1 cells, steroid secretion is blocked by high concentrations of inhibitors of P-glycoprotein function *in vitro*.[81] And in *mdr1a* knockout mice intracerebral uptake of radiolabeled dexamethasone is moderately enhanced, which suggests a potential role for P-glycoprotein in transport of dexamethasone *in vivo*.[67] The importance of P-glycoprotein as a steroid transporter is however questioned by preliminary results of *mdr1b* knockout mice and *mdr1a* + *mdr1b* double knockout mice that show no gross disturbances in the corticosteroid metabolism and have normal fertility. This suggests that both *mdr1a* and *mdr1b* P-glycoprotein have no essential function in the normal metabolism of the adrenals and pregnant uterus.[82]

The function of the class 3 mdr genes has long puzzled investigators. Despite numerous experiments, involvement in MDR has never been observed.[53,83] A breakthrough came when the *mdr2* knockout mouse was generated, that has no detectable functional *mdr2* P-glycoprotein.[84] Homozygous *mdr2* (-/-) mice develop a severe liver disease that is caused by the complete inability of the liver to secrete phospholipid into the bile. Heterozygous *mdr2* (+/-) mice have approximately half of the normal level of the major component of biliary phospholipids, phosphatidylcholine, in their bile. The output of bile salt is not affected.[84] Studies in transgenic mice carrying the human *MDR3* gene crossed with the *mdr2* knockout mice show that this human gene product can fully replace the function of murine *mdr2*: phosphatidylcholine levels in bile were normal and no liver pathology was observed.[82] The *mdr2* P-glycoprotein is supposed to function as a flippase which translocates actively phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane.[84,85] In the *mdr2* knockout mouse elevated levels of *mdr1a* and *mdr1b* were observed. However, as their gene products do not transport the same substrates, they cannot replace *mdr2* functionally and the upregulation is probably an expression of reactions of the organism to the adverse condition.[84]

2.4. Substrate specificity for cytotoxins?

cDNA transfection experiments have shown that *mdr* genes of class 1 and 2, human *MDR1* and mouse *mdr1a* and *mdr1b*, can confer the complete MDR phenotype.[31,35,86] Class 3 genes, *MDR3* and *mdr2*, can not confer MDR.[53,83] As different localizations, possibly with specialized functions, of the two rodent *mdr* class 1 and 2 genes have been shown, an important question is whether these genes also have substrate specificity and different binding properties. In other words, focused to the subject of this review, has the duplication in rodents of the *mdr1* gene into *mdr1a* and *mdr1b* lead to

different abilities to extrude cytotoxins?

Induction of MDR by drug pressure in rodent tumor cell lines has led to various cell lines that overexpressed either *mdr1a*, or *mdr1b*, or both.[41,87,88] Expression of either *mdr1a* or *mdr1b* seems to be controlled in the first place by the specific tissue cells in which drug resistance is induced and not by the selecting drug.[87,89] In some studies *mdr1b* was first expressed in the lower-resistant cell line, while at later stages of induction at increased drug resistance levels *mdr1a* was expressed.[89-91] This suggests a switch to a more efficient drug transporter. Better efficiency of the *mdr1a* P-glycoprotein was observed in transfection studies, in which at similar levels of protein expression the mouse *mdr1a* product seemed to be a more efficient drug efflux pump, as the *mdr1a* transfectant showed the highest level of resistance.[92,93] In the *mdr1a* knockout mouse a reactive upregulation of expression of *mdr1b* in kidney and liver was observed.[59] Whether this means that part of the physiological excretion function of *mdr1a* in these organs can be replaced by the *mdr1b* product, or that this is only an expression of a reaction of the organism to stress, remains to be elucidated.

Several differences in resistance patterns in the transfected cell lines with murine and human *mdr* products suggest possible substrate specificity. The *mdr1a* and *mdr1b* P-glycoproteins showed no relative preference for either doxorubicin, vinblastine, or colchicine and for all drugs the resistance level of the *mdr1a* product was about 2-fold higher.[93] Resistance to doxorubicin and vinblastine of the *MDR1* transfectant was 1- to 3-fold lower compared to *mdr1b* and 3- to 5-fold compared to *mdr1a*. For colchicine resistance was much lower (13- to 28-fold). The *mdr1a* product conferred much higher resistance levels for actinomycin D compared to *mdr1b* (over 25-fold), while *MDR1* was intermediate resistant (6-fold).[93] Apart from variations in the cross-resistance pattern, differential affinity for various chemosensitizers has been reported for the two rodent *mdr* products in these transfectants.[93,94] These studies suggest that the two rodent drug transporters of *mdr1a* and *mdr1b* have a large overlapping substrate affinity and transporter activity, but also some distinct substrate specificity.

Induction of drug resistance by drug pressure usually results in cell lines that show the highest levels of resistance to the agent used in the selection procedure. The pattern of cross-resistance to other drugs can be extremely variable, even when P-glycoprotein has been shown to be induced.[95] This effect is found in human and rodent cell lines and can therefore not solely be explained by the difference in number of genes that confer MDR.[95,96] Several other mechanisms that can explain the variability in MDR phenotype have been proposed.

Point mutations in the *mdr* genes strongly influence substrate specificity. A mutation of Gly^{185} to Val^{185} in human *MDR1* caused a decrease in the resistance to vinblastine and an increase in the resistance to colchicine.[97] A point mutation in transmembrane zone 6 of hamster *pgp1* diminished the resistance to colchicine, vincristine, and daunorubicin, while actinomycin D resistance was elevated.[95] A single amino acid substitution (Ser to Phe) within a coding region for transmembrane part 11 of murine P-glycoprotein strongly modulated the activity and substrate specificity of the *mdr1a* and *mdr1b* products in a transfection study.[98] Interestingly, mutations at the homologous position (transmembrane part 11) of the *pfmdr1* gene of *Plasmodium falciparum* is associated with chloroquine resistance.[99] The mutation in the murine cDNA had happened by accident during construction of *mdr1b* and was due to a polymerase error. Introduction of this mutation into *mdr1b* as well as into *mdr1a* changed the resistance pattern compared to the wild-type cDNA.[98] Resistance to vinblastine was mildly reduced, to adriamycin and colchicine strongly reduced in both cell lines transfected with either mutant *mdr1a* or mutant *mdr1b*. However, effects on other drugs were distinct: in the *mdr1a* transfectant sensitivity to actinomycin D was not changed, while this was decreased 5- to 10-fold in the *mdr1b* transfectant. Exactly opposite results were obtained for Gramicidin D.[92] Additional studies on this mutation of *mdr1a* and *mdr1b* showed that drug binding and transport of the mutant P-glycoproteins were altered.[92] Modulators of P-glycoprotein were also affected by the mutation: the introduced mutations either produced no effect, or enhanced, or reduced the potency of the particular modulator. These studies indicate that the recognition and transport of the structurally heterogeneous compounds by P-glycoprotein involves several determinants within the transmembrane domains of the transport proteins, which form together a complex binding pocket.[94] Differential effects of P-glycoprotein inhibitors in a wild-type and a mutant *MDR* transfectant were also observed with human material.[100]

An additional explanation for the variable drug resistance patterns lies in the differences in assays used to test drug sensitivity *in vitro*, which may not be directly comparable. And, very importantly, the induction of MDR in tumor cells by drug pressure is not a clean process and additional drug resistance mechanisms may be induced alongside P-glycoprotein-mediated MDR.[93,95] Other proposed mechanisms as allele polymorphism or alternative gene splice variants have not been observed in rodents sofar.

The studies described in this chapter show that the rodent *mdr1a* and *mdr1b* genes

show high homology and sequence identity to the human *MDR1* gene. Similar cross-species homology is found for class 3 *mdr* genes (rodent *mdr2* and human *MDR3*). The tissue distribution of the *mdr* genes is very similar across species: *mdr1a* and *mdr1b* expression in rodents together resembles human *MDR1* expression, as does rodent *mdr2* and human *MDR3*. Studies in rodents have revealed the various physiological functions, which confirmed roles proposed on the action of P-glycoprotein and its localization in tissues. A matter of concern are differences between the *mdr1a*, *mdr1b* and *MDR1* gene products in activity as drug transporter dependent on drug and transporter. Nevertheless, the transporter activity of the *mdr* gene products is largely overlapping and therefore, the rodent P-glycoproteins seem suitable tools for studying function and modulation of MDR.

3. *In vivo* MDR model systems

We will not discuss all reported *in vivo* models in extenso. The most important characteristics of the MDR tumors and cell lines that have been described for *in vivo* use are summarized in the tables 4-8. The MDR phenotype consisting of the typical cross-resistance pattern to cytotoxins in cytotoxicity assays and diminished accumulation of MDR drugs or dyes, together with reversibility of these features with P-glycoprotein modulators has been shown for most reported cell lines. The human intrinsic MDR tumors are less well characterized in these respects. In the tables is indicated whether the MDR character is further proved by *mdr* gene expression, and for rodent cell lines whether this is *mdr1a* or *mdr1b*, and by P-glycoprotein expression with monoclonal antibodies. For the overall picture, when possible the level of resistance of the MDR subline relative to the parental cell line is being mentioned for anthracyclines. Other features summarized in the tables are the kind of tumor and the specific *in vivo* model, which concerns with how tumors are grown and tumor growth is determined. Here, we will discuss the general features of *in vivo* models with their merits and limitations.

An ideal model would consist of a drug-sensitive parental tumor and a derived drug-resistant tumor that only differ in the enhanced expression of P-glycoprotein. The tumors should not differ with respect to other drug resistance mechanisms, and preferably other mechanisms should not be active at all. *In vivo* growth characteristics like tumorigenicity, growth rate, invasive and metastatic potential should be similar.

The same holds good for histopathological features. And the tumor model should represent the characteristics of a frequently occurring human tumor. It will be shown that none of the existing *in vivo* models for MDR at the moment matches this ideal model.

3.1. Spontaneous and induced MDR tumors

Spontaneous tumors in animals may function as tumor models for MDR. Lymphomas in dogs closely mimic the clinical situation in men: histopathology, tumor behaviour, and response to chemotherapy are quite comparable with aggressive, high-grade non-Hodgkin's lymphoma in humans.[153,154] The majority of de-novo canine lymphomas do not express P-glycoprotein but after relapse expression of P-glycoprotein is increased. Like in men, in dogs P-glycoprotein expression before drug treatment is an independent negative predictor of overall survival.[154] However, the model is not well defined and other drug resistance mechanisms apart from MDR may be operative as well. The low numbers of dogs with lymphoma are another reason why this is not a useful model for drug testing. Papillomas in mice induced by DMBA are intrinsically resistant to doxorubicin and express P-glycoprotein, while virally induced tumors do not.[155] Conditions in this mouse model are more controllable and MDR tumors can be reproduced reliably.

Serial transplantable rodent tumors or cell lines with tumorigenic potential that intrinsically express MDR can become well-characterized with respect to the MDR genotype and phenotype, and other features. Examples are the murine C-26 and rat CC531 colon carcinoma,[115-117,122] and many human xenografted tumors (see tables 4,5,7 and 8). A drawback of these intrinsically MDR tumors is that they lack a P-glycoprotein negative parental for comparative studies and represent only one unique tumor. For CC531 an MDR negative cell line has been developed *in vitro*: CC531^{rev}, a revertant cell line.[156] *In vivo* growth characteristics of CC531^{rev} however, were altered and did not allow meaningful comparisons with the parental, intrinsically MDR cell line CC531. (W. van de Vrie, R.L. Marquet and A.M.M. Eggermont, unpublished observations)

Tumor pairs consisting of a drug-sensitive parental tumor and a P-glycoprotein expressing drug-resistant tumor can be used to compare efficacy of anti-MDR therapy more reliably. Various rodent tumor cell lines have been described, from which the P388 and L1210 murine leukemias with several MDR sublines are best known (tables 4 and 5). Paired human cell lines used as xenografts are described in the tables 7 and 8. In the ideal situation, reverters of MDR do not enhance cytotoxicity in parental tumors,

Table 4. Murine MDR tumor cell lines *in vivo*

cell line	MDR subline	*mdr*/Pgp expression	RR *in vitro*	tissue	*in vivo* model	references
P388 [@]	P388/ADR	*mdr1a* / Pgp	DOX 68-150x	lymphoblastoid leukemia	ip - ascites, survival; iv - disseminated, survival; bone marrow purging	87,101-108
	P388/VCR	*mdr1a* / Pgp	VCR 12-30x DOX 6x			
L1210	$L1210_{DOX}$	*mdr1b* / Pgp	DOX 45x	leukemia	ip - ascites; sc - solid, tumor size	87,91, 109,110
	$L1210_{DNR}$	*mdr1b* / Pgp	DNR 30x DOX 57x			
S 180	$S\ 180_{DOX}$	*mdr1b* / Pgp	DOX 340x	sarcoma	ip - ascites	111
	$S\ 180_{DNR}$	nr / Pgp	DNR 73x DOX 275x			
EA/DS	EA/DR	nr / nr	DNR 2.4x	Ehrlich ascites carcinoma	ip - ascites, survival	112
B16V	B16V/DXR	*mdr* [*] / nr	DOX 200x	melanoma	sc - solid, tumor size	113,114
-	C26 [@]	*mdr1b* / Pgp	-	colon carcinoma	sc - solid, tumor size	115-117

[@] intrinsic MDR expression
[*] *mdr* gene undetermined as no specific probe is used

abbreviations:
Pgp = P-glycoprotein
RR = relative resistance
nr = not reported
ip = intraperitoneal
iv = intravenous
sc = subcutaneous
ADR = adriamycin
DNR = daunorubicin
DOX = doxorubicin
VBL = vinblastine
VCR = vincristine

while enhanced antitumor effects can be observed in MDR tumors. It should be noted that even in the most often used tumor model, the P388 leukemia, this is not the case, because the parental P388 cell line expresses low levels of P-glycoprotein and is sensitive to reversal activity.[157] Because in most of the tumors MDR is induced by exposure to cytotoxins, other drug resistance mechanisms may be induced as well. In a doxorubicin-induced drug-resistant rat mammary carcinoma cell line (MatB) *mdr* RNA is elevated, but also glutathione *S*-transferase activity, while glutathione levels are decreased.[121] Similar observations have been done in a doxorubicin-resistant human MCF-7 breast carcinoma cell line.[136] Most *in vivo* used tumors are not well characterized with respect to other drug resistance mechanisms like altered glutathione *S*-transferase isoenzymes, decreased topoisomerase activity, and expression of the multidrug resistance-associated protein (MRP).

Transfected cell lines (table 6, and various cell lines in the tables 7 and 8) have the advantage that no other drug resistance mechanisms are introduced and in this respect deliver a 'pure' MDR tumor model. Another advantage is that human *MDR* can be used. This bypasses possible substrate specificities or differences in efflux efficiency of the *mdr1a* and *mdr1b* products of rodents. Results of studies on the efficacy of modulators may be more comparable to the clinical situation. As human *MDR1* is used, immunological reactions to foreign protein may be induced which can have influence on results *in vivo*. In both induced and transfected MDR cell lines alterations in tumorigenicity and growth rate and growth pattern have been observed. This will be described in the next section.

The advantage of human xenografts, whether intrinsically MDR or paired tumors, is that they are human tumors with their own pathological characteristics and that they express human *MDR1*. Virtually all kinds of human tumors can be grown in immunocompromised rodents and are available for therapeutic experiments. The results of cytotoxin experiments in xenografts will be more relevant to the clinical practice of those particular tumors than standard tumor models like the murine P388 leukemia, which has low predictive value in screening new anticancer drugs.[158] Human xenograft can readily be grown in immunodeficient rodents like nude mice, nude rats, or SCID (severe combined immunodeficient) mice. SCID mice lack both functional B and T cells and are more severely immunocompromised than the nude mouse. They have a greater propensity for transplantation of hematopoietic and lymphoid tissue and generally do not develop graft versus host reaction.[151,152]

Table 5. Rat MDR tumor cell lines *in vivo*

cell line	MDR subline	*mdr*/Pgp expression	RR *in vitro*	tissue	*in vivo* model	references
DHD/K12	DHD/K12/TR	*mdr* ˚/ Pgp	nr	colon adenocarcinoma	peritoneal carcinomatosis, survival, tumor weight	118-120
	DHD/K12/PRObb	*mdr* ˚/ Pgp	nr			
MatB 13762	(Adr^R)MatB	*mdr* ˚/ nr	DOX 166-200x	breast adenocarcinoma	sc - solid, tumor size	121
-	CC531 ®	*mdr1a* / Pgp	-	colon adenocarcinoma	subrenal capsule, tumor weight	122,123
AH66P	AH66DR	nr / Pgp	DOX 200x	hepatoma	ip - ascites, survival	124
AT2/P	$AT2/Dox^{1800}$	nr / Pgp	DOX 25x	prostate carcinoma	sc - solid, tumor size	125
AT3/P	$AT3/Dox^{1800}$	nr / Pgp	DOX 25x	"	"	"
MAT-LyLu/P	$MAT\text{-}LyLu/Dox^{1800}$	nr / Pgp	DOX 76x	"	"	"

® intrinsic MDR expression
˚ *mdr* gene undetermined as no specific probe is used
abbreviations: see legend of Table 4

3.2. Growth characteristics of MDR tumors *in vivo*

Induction of drug resistance not only alters the sensitivity of cells to cytotoxins, but other characteristics of the tumor may be changed as well. Several investigators have reported that drug-resistant cell lines are less tumorigenic than their parental cell line.[159,160] Resistant variants of the human osteosarcoma cell line U-2 OS showed a progressive loss of tumorigenic potential in athymic mice associated with increasing levels of *MDR1* expression.[160,161] In contrast however, overexpression of P-glycoprotein did not effect the tumorigenicity of human leukemia (CEM) cells.[148] A drug-resistant variant of the human multiple myeloma cell line 8226 showed enhanced tumorigenicity compared to its parental, as a lower inoculation dose was necessary to achieve a 100% take rate.[151] Alterations in tumorigenicity are not unique for MDR or for induction of MDR with a particular drug. We found that a subline of the CC531 rat colon adenocarcinoma in which cisplatin resistance (non-MDR) was induced *in vitro*[156] had totally lost its tumorigenic potential *in vivo* in syngeneic WAG/RIJ rats. A colchicine induced CC531 cell line with MDR characteristics showed a tumor take of over 80%, but further growth was not consistent and some tumors seemed to be rejected. (W. van de Vrie, R.L. Marquet and A.M.M. Eggermont, unpublished observations)

It is well-known that fast-growing tumors in general are more sensitive to cytotoxins than slower-growing tumors and therefore similar growth rates are a prerequisite for meaningful comparative studies. Introduction of drug resistance sometimes results in alterations in growth rate. MDR cell lines derived from various rat prostatic carcinoma cell lines showed no difference in tumor take, but tumor growth rate *in vivo* was decreased.[125] A doxorubicin-resistant subline of the MCF-7 human breast cancer grew twice as slow *in vivo* as wild-type xenografts.[136] For most parental and drug-resistant cell lines however, comparable growth rates and patterns have been reported which allows meaningful testing.

Alterations in growth rate could in some instances be explained by increased immunogenicity. Enhanced immunogenicity proved by immunization experiments, has been shown in P388 tumors in a drug resistance (MDR and non-MDR) induction study *in vivo*.[162] In another study immunogenic properties of drug-resistant murine fibrosarcoma and colonic adenocarcinoma (CT-26) tumors did not correlate with expression of the MDR phenotype.[163]

Yet another feature of tumors is their metastasizing potential. Diminished development of metastases has been described for doxorubicin-resistant variants of a murine melanoma.[164,165] The Dunning rat prostatic cancer cell lines that were rendered MDR

Table 6. Murine MDR tumor cell lines *in vivo* transfected with human *MDR1*

cell line	MDR subline	*mdr*/Pgp expression	RR *in vitro*	tissue	*in vivo* model	references
P388/S	P388/VMDRC.04	*MDR1* / Pgp	DOX 19x	monocytic leukemia	ip - ascites, survival	126
L1210	L1210/VMDRC.06	*MDR1* / Pgp	DOX 7x	leukemia	ip - peritoneal carcinomatosis, survival	127
parental L1210	resistant L1210	*MDR1* / nr	DOX 32x	leukemia	ip - peritoneal carcinomatosis, survival	128
B16/F10	B16/hMDR-1	nr / nr	VBL >570x DOX 30x	melanoma	ip - peritoneal carcinomatosis, tumor weight	129

abbreviations: see legend of **Table 4**

lost their metastasizing potential, but no direct correlation was observed between MDR level and ability to metastasize.[125] The U-2 OS MDR sublines also exhibited a decreased metastasizing ability in athymic mice. *In vitro* migration, invasion, and homotypic adhesion abilities were diminished. Changes in adhesion molecules or integrins could not explain these features as levels of the adhesion proteins ICAM-1, LFA-3 and A-CAM were not altered and expression of some integrins was even highly increased.[160] In other rodent test models however, higher P-glycoprotein expression has been observed in spontaneous lung metastases compared to the primary liver tumor and in lung metastases produced by intravenous injection of tumor cells versus subcutaneously produced tumors.[117,166] Similar observations have been done in a human neuroblastoma cell line xenografted in nude mice. In this cell line which produced metastases *in vivo*, gradual and significant increases in the *MDR1* gene transcript level leading to functional P-glycoprotein expression were associated with the metastatic process.[146] An explanation for the findings in these studies could be that metastasizing potential and P-glycoprotein expression both indicate a more aggressive phenotype of the tumor. This does not concur however with the aforementioned studies. An alternative explanation comes from the studies by Dong *et al.*[117] who showed in crossover experiments that P-glycoprotein expression could be induced by the organ environment. Lung metastases had higher expression levels of P-glycoprotein than subcutaneously grown tumors of the same cell line. When cells from lung tumors were grown subcutaneously in other mice, their P-glycoprotein level decreased to the same level as originally subcutaneously grown tumor cells. Vice versa, cells from subcutaneously grown tumors got an increased level of P-glycoprotein when grown as lung metastases.[117]

Together, the data on growth characteristics indicate that induction of MDR in experimental tumors results in an altered tumor phenotype which is often less aggressive. However, no consistent pattern has been observed and these features are not confined to MDR, but are also found in other drug-resistant tumors. Most investigators concluded that tumorigenicity, metastasizing potential, and other growth characteristics are not directly correlated with P-glycoprotein expression and may be co-induced in the process of development of drug resistance.

More direct evidence for the hypothesis that MDR does not induce changes in growth characteristics might come from studies with transfectant cell lines. Theoretically, only the *MDR* gene is introduced in transfectant sublines, and no other drug resistance mechanisms or cell surface markers. However, alterations in growth qualities

Table 7. Human MDR tumor cell lines xenografted in rodents, solid tumors

cell line	MDR subline	*mdr*/Pgp expression	RR *in vitro*	tissue	*in vivo* model	references
HT-29^{par}	HT-29^{mdr1} [f]	nr / Pgp	VCR 6x	colon carcinoma	ip - peritoneal carcinomatosis, survival	130
HCT-116	HCT-116/VM46	*MDR1* / nr	DOX 13x	colon carcinoma	sc - solid, tumor size	131
-	HCT-15 [@]	*MDR1* / Pgp	- [+]	colorectal carcinoma	sc - solid, tumor size	132
-	SW480 [@]	*MDR1* / Pgp	- [+]	"	"	"
-	SW1417 [@]	*MDR1* / Pgp	- [+]	"	"	"
-	DLD-1 [@]	*MDR1* / Pgp	- [+]	"	"	"
-	COK-36LN [@]	*MDR1* / nr	- [¶]	mucineus colon carcinoma	sc - solid, tumor size	133
-	COK-28LN [@]	*MDR1* / nr	- [¶]	colon adenocarcinoma	"	"
-	SW948 [@]	*MDR1* / Pgp	-	colon carcinoma	sc - solid, tumor size	134
-	LS174T [@]	*MDR1* / Pgp	-	"	"	"
SW480	SW480^{DXR}	*MDR1* / nr	DOX 6x	colon carcinoma	sc - solid, tumor size	135

[f] transfectant
[@] intrinsic MDR
[+] P-glycoprotein expression is relatively clear in HCT-15, SW480, and SW1417, and moderate in DLD-1 compared to other colon carcinoma cell lines (COLO 205 and KM20L2), and correlates with resistance to vincristine and, to a lesser extent, doxorubicin
[¶] *MDR1* expression level is 3-fold lower in COK-28LN compared to COK-36LN
abbreviations: see legend of **Table 4**

have been observed in several studies. Doubling time of the transfected HT-29 human colon carcinoma was 36 h compared to 24 h for the parental HT-29^{PM} *in vitro*, but *in vivo* survival times of mice xenografted with HT-29^{mdr1} or HT-29^{PM} were similar (39 versus 37 days).[130] In two independent experiments in which the human *MDR1* gene was transfected into L1210 murine leukemia cells, the resultant MDR cell lines had an altered growth pattern *in vivo*. While the parental L1210 cell lines produced copious ascites and rapidly killed host mice, the transfected cell lines grew more slowly and as solid tumors that were often limited to the peritoneal cavity.[126,128] In another experiment 9 out of 10 subclones of the transfected P388 tumor grew at a slower rate *in vivo* and without producing significant amounts of ascites, while only one had growth characteristics similar to the parental P388 tumor.[126] There are some possible explanations for these findings. First, procedures used in the transfection process may be responsible for additional alterations in tumor cells. For example, a low level of a cytotoxin is added to the growth medium in order to select transfected cells in culture and to maintain drug resistance. These drugs might be responsible for additional changes in the growth characteristics. Immunological mechanisms may play a role when a xenogenic *MDR* gene is transfected and no immune-deprived host rodents are used. A second possible conclusion is that expression of P-glycoprotein does alter growth characteristics of tumors. The mechanism by which this could happen is not understood, and possible explanations do not follow logically from the physiological functions of P-glycoprotein. As stated before, there is no consistent pattern of growth alterations associated with MDR tumors and therefore a role for P-glycoprotein expression in growth qualities remains speculative.

3.3 Retention of the MDR phenotype *in vivo*

A very important question in *in vivo* studies is whether the MDR genotype and phenotype are retained during *in vivo* passages, especially when tumor cell lines are used that are cultured *in vitro* in the presence of a low level of cytotoxin in order to maintain their resistance level. Broxterman *et al.* have shown that this is not the case in all tumors.[142] Although *in vitro* and *in vivo* determined drug resistance levels are not directly comparable due to different techniques of measuring drug sensitivity, the authors were able to show that the KB-8-5 drug-resistant subline of a human epidermoid carcinoma had a lower level of resistance *in vivo*. The xenografted cells were less sensitive to the modulating effect of an MDR reverter and had lower levels of *MDR1* RNA expression. The doxorubicin-resistant subline 2780^{AD} of a human ovarian

Table 7. Human MDR tumor cell lines xenografted in rodents, solid tumors (continued)

cell line	MDR subline	*mdr*/Pgp expression	RR *in vitro*	tissue	*in vivo* model	references
MCF-7 (WT)	MCF-7/ADR	*MDR1* / Pgp	DOX >150x	breast carcinoma	sc - solid, tumor size	131,136
MDA435/LCC6	MDA435/LCC6^{MDR1} [f]	nr / Pgp	DOX ±45x	breast carcinoma	ip - ascites, survival; mammary fat pad - solid, tumor size	137
HXL55	HXL55/VCR	*MDR1* / Pgp	DOX 3.8x [1]	epidermoid lung carcinoma	sc - solid, tumor size	138
	HXL55/AD	*MDR1* / Pgp	DOX 3.2x [1]			
-	HXL54 [e]	*MDR1* / Pgp	- [1]	"	"	"
SCLC-6 [e]	SCLC-6T	*MDR1* / nr	nr	small cell lung carcinoma	sc - solid, tumor size	139
SCLC-41 [e]	SCLC-41T	*MDR1* / nr	nr	"	"	"
-	SCLC-74T	*MDR1* / nr	-	"	"	"
-	SCLC-75 [e]	*MDR1* / nr	-	"	"	"
Alex 0 [e]	Alex 0.5	nr / Pgp	DOX 25x	hepatocellular carcinoma	hepatic - solid, tumor weight, HBsAg level	140

[f] transfectant
[e] intrinsic MDR expression
[1] drug resistance determined *in vivo*; HXL54 is ±9.5x more resistant to doxorubicin *in vivo* compared to HXL55
abbreviations: see legend of **Table 4**

carcinoma generally showed reduced P-glycoprotein activity, but a minority of the cells seemed to have retained the high resistance level. Regrowth of these cell lines *in vitro* confirmed these observations.[142] In another study a 17-fold loss of resistance level was observed after *in vivo* passage of 2780^{AD} cells.[143] In contrast, xenografts of the transfected *MDR1* BRO melanoma cell line had a comparable expression level of the *MDR1* gene and similar functional activity of P-glycoprotein as the original cell line.[141,142] Retention of the MDR genotype and phenotype has also been found after *in vivo* passage of resistant CEM leukemia cells.[148] For other MDR tumors these features have not been studied as extensively as for the tumors described above, but the differences between parental and drug-resistant tumors in drug sensitivity *in vivo* and the efficacy of MDR reverters show indirectly that at least part of the MDR mechanism is retained during the process of *in vivo* passage. The drug resistance level of an induced or transfected MDR tumor tends to decrease when tumor cells are grown for a long time in the absence of their selecting drug, giving rise to so-called reverted cell lines with lowered expression levels of P-glycoprotein. This phenomenon is observed *in vitro* as well as *in vivo*.[126,165] Therefore, in most studies selecting drugs are only withheld from the culture medium a short time before and during the testing period.

3.4. *In vivo* tumor models

In ascites models the tumor is grown in the peritoneal cavity and the drugs are administered intraperitoneally (ip), the so-called ip-ip model. Efficacy of antitumor agents is determined by scoring prolongation of survival. The well-known P388 cell lines are grown this way and this has become a sort of standard *in vivo* model for anti-neoplastic drug screening.[167] Survival time is approximately 10 days for parental and MDR cell lines. Advantages of the ascites tumor models like the murine P388 and L1210 leukemia are the ease of *in vitro* and *in vivo* maintenance, and the ability to perform reproducible and rapid testing of drugs.

The ascites model can be criticized for being artificial and it is said that relatively high therapeutic effects are observed. In the first place, this is partly inherent to the standard procedure in which treatment is started on the same day as tumor inoculation, which means that the tumor is not yet established. Secondly, drugs are most often administered intraperitoneally. This may result in a chemical peritonitis that contributes non-specifically to the antitumor effect. Thirdly, direct administration of drugs at the site of the tumor bypasses the vascular route. In the clinical situation most of the drugs are administered intravenously because most human tumors do not grow as ascites

Table 7. Human MDR tumor cell lines xenografted in rodents, solid tumors (continued)

cell line	MDR subline	*mdr*/Pgp expression	RR *in vitro*	tissue	*in vivo* model	references
BRO	BRO/mdr1.1 '	*MDR1* / Pgp	DOX 89x	melanoma	sc - solid, tumor size	141,142
A2780	2780^{AD}	*MDR1* / Pgp	DNR 190x	ovarian carcinoma	sc - solid	142,143
-	Kgg2 ®	*MDR1* / Pgp	-	renal cell cancer	sc - solid, tumor size	144
KB-3-1	KB-8	*MDR1* / Pgp	DNR 3.6x	epidermoid carcinoma	sc - solid, tumor size	142
	KB-8-5	*MDR1* / Pgp	DNR 3.4x			
KB	KB-V1	*MDR1* /Pgp	DOX 420x	epidermoid carcinoma	sc - solid, tumor size	145
-	IGR-N-91 ®	*MDR1* / nr	-	neuroblastoma	sc - solid, tumor size	146
BE(2)-C	BE(2)-C/CHC	nr / Pgp	DOX 40x	neuroblastoma	sc - solid, tumor size	147

' transfectant
® intrinsic MDR expression
abbreviations: see legend of **Table 4**

tumors, but are localized solid tumors. Tumor vasculature and the ability of drugs to penetrate into tumors through multiple cell layers are major factors that determine the drug levels that can be obtained within the tumor and consequently tumor cell kill.[168]

For P388 leukemia apart from the ascites model, two other models have been reported. Tumor cells are inoculated intravenously (iv) in the iv-iv model, as are the drugs. This model seems to reproduce in mice the pathological features of clinical leukemia, but the model is not well defined for MDR P388 tumors.[107] Mixed parental and MDR P388 leukemia cells have also been used in an *in vivo* model for autologous bone marrow transplantation to show the feasibility of using MDR reverters in *ex vivo* bone marrow purging in order to eliminate MDR cells.[106]

Solid tumors are most often grown subcutaneously. The tumor is readily available for measurement of size and serial observations can be made, which makes that efficacy of antitumor treatment can be readily assessed. However, there are some differences between transplanted solid tumors and spontaneous solid tumors. Tumors, especially xenografts, grown from subcutaneously injected cells tend to grow well-encapsulated and invasive growth is only a late feature. The vascularization of these tumors is moderate and does not represent the vasculature of spontaneous tumors.[169] It was found for example that mouse host tissues accumulated 6- to 12-fold more doxorubicin than xenografts of the subcutaneously grown human mammary carcinoma MCF-7, most likely because of better vascular perfusion.[136] Evidence is accumulating that the microenvironment in which tumors grow, can profoundly influence their characteristics. It has already been mentioned that P-glycoprotein levels can vary, dependent on the tissues in which the tumors grow.[117] The metastasizing potential may be influenced: distant metastases from a subcutaneously xenografted tumor are a rare occurrence as opposed to metastases obtained with orthotopic grafting.[170] Other sites for solid tumor grafts are the subrenal capsule assay in which a tumor piece is implanted under the capsule of the kidney and peritoneal carcinomatosis models for which tumor cell suspensions are seeded intraperitoneally.[119,122] Serial measurements are not possible and animals must be sacrificed to determine tumor burden.

Recently, some interesting new models have been reported in which serial quantification of tumor burden can be made indirectly by measuring products secreted by the tumor. In two multiple myeloma tumor models grown in SCID mice human monoclonal light chain excretion in urine is directly related to tumor growth. The 8226 cell lines grow heterotopically in SCID mice.[151] The ARH myeloma cell lines exhibit an orthotopic growth pattern with the development of osteolytic lesions, which closely

Table 8. Human MDR tumor cell lines xenografted in rodents, hematologic tumors

cell line	MDR subline	*mdr*/Pgp expression	RR *in vitro*	tissue	*in vivo* model	references
CEM	CEM/VLB$_{100}$	*MDR1* / Pgp	VBL 286x	T-cell leukemia	sc - solid	148
Namalwa	Namalwa/mdr-1 '	*MDR1* / Pgp	DOX 6x	B-cell Burkitt's lymphoma	iv - disseminated	149,150
8226	8226/C1N	nr / Pgp	nr	multiple myeloma	ip - peritoneal carcinomatosis, light-chain excretion in urine	151
ARH-77	ARH-D60	*MDR1* / Pgp	DOX 76x	multiple myeloma	iv - disseminated, light-chain excretion in urine	152
	ARM-80	*MDR1* / Pgp	DOX 10x			

' transfectant
abbreviations: see legend of **Table 4**

mimics the pathophysiology of human myeloma.[152] A human hepatoma cell line (Alex 0) and its MDR subline grow as an intrahepatic xenograft after intrasplenic injection. The tumor cells produce HBsAg and serum levels correlate with tumor burden.[140] The measurement of secreted tumor products allows starting of treatment at a determined tumor burden and permits a direct comparison of the effectiveness of drugs used at the same extent of disease in each animal.

Novel endpoints to measure functional MDR *in vivo* come from radio-imaging techniques. Drug-resistant and sensitive tumor xenografts have been shown to be distinguishable by differences in uptake of radiolabeled colchicine.[171] *In vivo* quantification of P-glycoprotein has been performed with the radiolabeled monoclonal antibody MRK16 that specifically recognizes P-glycoprotein.[147] And imaging studies in rats bearing wild-type and drug-resistant tumors showed that the imaging agent 99Tcm-sestamibi, which is transported by P-glycoprotein, was washed out of resistant tumors three times the rate of wild-type tumors.[172] These studies suggest potential use of radio-imaging techniques to evaluate MDR *in vivo*.

Two other models should be mentioned here: the *mdr* knockout mice and *MDR1* transgenic mice. The *mdr1a* (-/-) knockout mouse has no functional *mdr1a* P-glycoprotein,[59] and the *mdr1a* + *mdr1b* (-/-) double knockout mouse totally lacks P-glycoproteins that are involved in MDR.[82] These mice have been engineered by disrupting the *mdr1a* and/or *mdr1b* genes in the germ lines of mice, which resulted in mice heterozygous for the disrupted gene. Mice homozygous for the disrupted gene were obtained by inbreeding techniques.[59,82] The features of these mice have already been described in chapter 2.3. *Mdr* knockout mice are excellent tools for studying the physiological role of P-glycoprotein and toxic effects of drugs due to the absence of functional P-glycoprotein.

Transgenic mice that express the human *MDR1* gene in their bone marrow have been engineered by the group of Gottesman and Pastan. cDNA constructs encoding full-length human *MDR1* in a plasmid carrier were injected into fertilized mouse embryos. A homozygous line was obtained of mice in which the expression of the *MDR1* transgene was limited to the bone marrow and spleen.[173] *MDR1* heterozygous animals were obtained by backcrossing with *MDR1*-negative mice and these mice were used in MDR modulation studies. The mice are resistant to the myelosuppressive effects of drugs that are influenced by the MDR mechanism like anthracyclines, *Vinca* alkaloids, etoposide, and taxol. The level of *MDR1* expression in the bone marrow is comparable to that found in many human cancers. The effect of drugs and combination therapy with

chemosensitizers on the bone marrow can easily be measured by peripheral white blood cell count. This makes it an efficient model for testing efficacy of MDR reverters *in vivo*.[173-175] A problem with the model is that after many generations of breeding the *MDR1* expression is not kept at its initial level.[167] It should be mentioned that the transgenic mouse model is not a tumor model as the *MDR1* gene is expressed in normal bone marrow.

4 Modulation of MDR with reverters *in vivo*

Most attempts to circumvent MDR have used the possibility to inhibit the P-glycoprotein efflux pump, which results in increased intracellular drug concentrations and enhanced cell death. In this chapter we will review studies on modulation of MDR *in vivo* with so-called chemosensitizers or reverters. In the next chapter other approaches to circumvent MDR will be described.

4.1 Pharmacokinetics

Combination treatment of cytotoxins with reverters altered the pharmacokinetics of MDR related drugs in phase I/II clinical trials in humans. Changes induced by reverters are a decrease in drug clearance and an increase of the area under the curve (AUC) of the cytotoxin. These pharmacokinetic interactions have been obtained with the reverters verapamil, dexverapamil, nifedipine, cyclosporin A, and PSC 833 and the drugs doxorubicin, epirubicin, vincristine, etoposide, and paclitaxel in various combinations, showing that this phenomenon is not limited to certain drugs.[176,177] There are few studies on plasma levels and AUC of drugs in animals. Plasma levels of drugs in animals are often not much increased by combination treatment with reverters,[143,176-181] The AUC of drugs can be elevated,[139,157,182] which is due to prolonged elimination.[139,182] But in other studies elimination of doxorubicin was not found to be altered.[180,181]

Animal studies have shown the various mechanisms by which MDR modulators can change the pharmacokinetics of drugs. The decreased elimination of drugs by reverters is caused by alterations in intestinal, biliary and renal absorption and excretion. The absorption from the gut of orally administrated etoposide is higher in quinidine pretreated rats and the intestinal secretion (exsorption) of intravenously administered etoposide is diminished by quinidine, compatible with inhibition of the P-glycoprotein transporter in intestinal cells.[183] Reverters partly inhibit the active biliary excretion of

colchicine, doxorubicin and etoposide by the liver.[65,72,184,185] In the kidneys a net secretion of MDR-related drugs is observed by the luminal membrane of renal cells. Cyclosporin A inhibits the renal secretion of vincristine and vinblastine in a dose-dependent manner in dogs.[66] Similar observations have been reported for colchicine by cyclosporin A and PSC 833 in rats.[64,184] These studies show that the physiological function of P-glycoprotein which is prevention of (re)absorption and elimination of xenobiotics also affects drug transport and can be blocked by reverters *in vivo*.

The extent by which drug pharmacokinetics are altered by a reverter will depend on the fraction of drug that is normally eliminated by the P-glycoprotein efflux mechanism. This has not been studied extensively *in vivo*. The conflicting results of pharmacokinetic studies with doxorubicin have been mentioned above.[120,139,180,181] Of note is the influence of route of administration of drugs. In combination with PSC 833 the AUC of etoposide is much more elevated in case of oral administration of etoposide compared to intravenous dosing. Apparently, etoposide absorption from the gut is normally largely inhibited by intestinal P-glycoprotein, while the relative role of P-glycoprotein in elimination of etoposide seems smaller.[182]

A consistent observation in animal studies is the altered distribution of drugs over the various tissues in mice and rats. In Sprague-Dawley rats cyclosporin A and PSC 833 significantly increased tissue levels of doxorubicin in liver, kidney, small intestine, and adrenals. A smaller increase was also observed in the heart, while cyclosporin A had no effect on doxorubicin concentration in the brain. In these studies the increases in drug tissue levels did not appear to be the result of changes in drug metabolism or elimination, as plasma levels and elimination of drugs were not significantly altered.[180,181] Other investigators reported elevation of doxorubicin levels in liver and kidney caused by amiodarone and cinchonine,[120,186] and elevation of vincristine levels in liver, kidney and small intestine by verapamil.[178] The alterations in drug levels are compatible with relatively high expression levels of P-glycoprotein in these tissues. Increase of drug levels by reverters in tissues with lower expression levels of P-glycoprotein like lung and spleen have been noted in some studies.[120,157] The pattern of distribution of drugs in the *mdr1a* knockout mouse is different from the pattern described above. In the *mdr1a* knockout mice a marked elevation of drugs like vinblastine, cyclosporin A, digoxin and ivermectin is observed in the brain and, to a lesser extent, in the testis. Elevation of these drugs in other tissues was less marked.[59,67] At the blood-tissue barriers of brain and testis predominantly *mdr1a* is expressed and disruption of the *mdr1a* gene in the *mdr1a* (-/-) knockout mouse leads to total absence of functional P-

glycoprotein at these sites. In other tissues *mdr1b* is also expressed and probably the *mdr1b* product can, at least partly, replace the function of the *mdr1a* product. This is also suggested by the increased expression of *mdr1b* in kidney and liver of the *mdr1a* knockout mouse.[59] It is apparently not easy to break the blood-brain barrier by MDR reverters as cyclosporin A. More potent reverters of MDR like PSC 833 and SDZ 280-446 have the capacity to enhance the neurotoxic effects of drugs, suggesting that these reverters are able to block P-glycoprotein at the blood-brain barrier, but drug levels in the brain have not been measured in this study.[75]

4.2. Efficacy of MDR reverters *in vivo*

The first observations that verapamil could reverse drug resistance were done by Tsuruo *et al.* 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*.[187] Since then, numerous compounds have been described which efficiently inhibit the P-glycoprotein efflux pump: calcium channel blockers (e.g. verapamil, dexniguldipine, PAK-200, AHC-52), cyclic peptides (e.g. cyclosporin A, PSC 833, SDZ 280-446), calmodulin antagonists (e.g. trifluoperazine), protein kinase C inhibitors (e.g. staurosporine), steroidal agents (e.g. progesterone, tamoxifen, megestrol acetate), *Vinca* alkaloid analogues, and miscellaneous compounds (e.g. amiodarone, quinidine).[188,189] The first generation MDR reverters were existing drugs which appeared to have MDR reversal activity, but had other pharmacological effects as well. Levels necessary *in vivo* for MDR reversal could often not be obtained because of prohibitive toxicity. For example, the target concentration of verapamil in mice could not be reached by bolus injection because this dose was acutely lethal.[178] In a human study cardiovascular effects as hypotension and cardiac arrhythmias prevented adequate dosing of verapamil.[190] Cyclosporin A is a potent blocker of P-glycoprotein but its immunosuppressive potential and nephrotoxic side-effects are matters of concern. A new generation of compounds, often analogues of known reverters, but devoid of their primary pharmacological effects and especially selected for MDR reversal activity, have been developed.[189]

Almost all chemosensitizers have first been tested in the drug-resistant P388 ascites tumors. With verapamil an increase in life span of 25%-45% was obtained in P388/ADR and in P388/VCR tumors.[187,191] For newer reverters survival increases of ±40%-100% have been reported in the P388/VCR ascites tumor. In the more drug-resistant P388/ADR reverters were less effective.[102,105,108,179,192] Impressive survival increases (up to 300% increased life span) have been reported for treatment with the

reverters PSC 833 and SDZ 280-446 in the P388/ADR tumor cell line.[104,116,193] In all studies mentioned above drugs were administered intraperitoneally, while chemosensitizers were given intraperitoneally, orally or intravenously. Intravenous administration of drugs in combination with PSC 833 in the P388 model was not effective against the P388/ADR tumors, but highly effective against the less resistant P388/VCR.[157,194] In the intravenously disseminated P388 leukemia model doxorubicin and etoposide, but not vincristine, in combination with a reverter (AHC-52) increased survival of P388/VCR bearing mice. In intravenously inoculated P388/ADR bearing mice reversal of MDR was not obtained.[107]

Chemosensitizers also reverse MDR in solid tumor models. In most studies established tumors were used: tumors were first allowed to grow to a certain volume before drug treatment was started. In intrinsic MDR tumors the addition of a reverter to ineffective treatment schedules with cytotoxins resulted in significant tumor growth delays.[116,122,179] In a study with subcutaneously grown C26 murine colon tumors the reverter PSC 833 even induced some cures.[194] Reversal studies in solid tumor pairs with a parental and an MDR tumor show specific enhancement of antitumor activity against MDR tumors.[131,133,141,143]

These studies demonstrate that reversal of MDR *in vivo* is feasible and can be obtained in ascites models, as well as in more difficult solid tumor models and the intravenously disseminated leukemia model. Possibilities for reversal are dependent on the level of drug resistance and reversal is not always obtained in highly drug-resistant tumors. Comparison of the *in vivo* efficacy of reverters is not possible with the current data because of differences between studies in tumors, dosing schedules of drugs and reverters, and experimental designs in the different studies. Comparative studies with several reverters within the same model and study design are scarce and only available for new potent reverters versus first generation chemosensitizers like verapamil and cyclosporin A, and show a clear higher potential of the novel reverters *in vivo*.[104,116]

4.3. Toxicity

The reverse of enhanced efficacy of cytotoxins by chemosensitizers is the possible increase in toxicity. As described above, many reversal studies have shown that reverters can enhance survival. However, several studies in animals that specifically investigated adverse effects of MDR reversal have shown that the combination of a chemosensitizer with a high dose cytotoxin results in increased toxicity leading to accelerated death. These observations have been reported for verapamil, cyclosporin A,

and PSC 833 in combination with doxorubicin and etoposide.[157,180-182,195,196] The nature of the increased toxicity has been investigated in few studies. Myelotoxicity is often the dose limiting factor in chemotherapy. Combination treatment of PSC 833 and etoposide caused increased leucopenia in mice,[182] and cyclosporin A with doxorubicin induced a transient doxorubicin-dose-dependent leucopenia and thrombopenia in rats.[197] Mice treated with PSC 833 and doxorubicin showed transient spleen hypoplasia, with a general decrease in all leucocyte lineages (B cell, T cell, and myeloid lineages). Changes were dependent on dose of doxorubicin and increased by addition of PSC 833. In the bone marrow only a persistent fall in the number of B cells was observed.[198] In two studies all deviations in blood parameters, and abnormal pathological findings at autopsy and at light-microscopic examination, could be attributed to known toxicities of the cytotoxins (doxorubicin, daunomycin and vinblastine). The reverter only accentuated these abnormalities, while the pattern of organ toxicity was not altered and no signs of new toxicity were found, especially not in organs with known high expression levels of P-glycoprotein.[175,197] In another histopathological study, cardiotoxicity of doxorubicin was enhanced by verapamil in mice.[196]

Possibly, studies with novel, potent reverters of P-glycoprotein will be different, as their capacity to block P-glycoprotein is more effective. In a recent study potent chemosensitizers as PSC 833 and SDZ 280-446, but not cyclosporin A, were able to break the P-glycoprotein-dependent component of the blood-brain barrier. High doses of these reverters in combination with the neurotoxic agent ivermectin caused acute dysfunction of the central nervous system (convulsion, paralysis, coma and death).[75] Interestingly, within one day after administration of the reverter the increased sensitivity of mice for the neurotoxic effects of ivermectin disappeared. This can be explained by dissociation of the reverter from the P-glycoprotein, or new expression of P-glycoprotein by the blood-brain barrier, or the emergence of alternative mechanisms causing ivermectin resistance.[75]

4.4. Specific modulation of MDR at the tumor level?

An important question to be answered in *in vivo* studies is whether combination treatment of cytotoxins with chemosensitizers merely alters pharmacokinetics and must be considered as a method of dose-intensification, or if specific interaction with P-glycoprotein at the tumor level results in increase in intratumoral drug levels and antitumor activity. Possibly, both mechanisms do contribute. Alterations of drug levels in the tumor caused by reverters have seldom been determined. In a study with a drug-

resistant sarcoma xenograft intratumoral vincristine levels were not significantly altered by verapamil. In this study the combination showed no antitumor activity too and no proof of MDR expression is given.[178] Different results were obtained in a study with effective reversal in MDR tumors. The reverter PAK-200 induced a 4.6-fold increase in intratumoral doxorubicin concentration in the P-glycoprotein expressing KB-8-5 xenografts, while PAK-200 did not significantly alter doxorubicin levels in the parental KB-3-1 drug-sensitive tumors. In COK36LN, a P-glycoprotein expressing colon carcinoma, PAK-200 elevated intratumoral levels 1.5-fold, and in xenografts of another colon carcinoma, COK28LN, which expressed little P-glycoprotein the reverter had no influence. The doxorubicin levels in the MDR tumors with the modulator exceeded the doxorubicin levels in the drug-sensitive tumors.[133] This study suggests that specific modulation of P-glycoprotein in the tumor has resulted in enhanced antitumor activity.

Reverters of P-glycoprotein are no magic bullet since P-glycoprotein in other tissues than the tumor is also inhibited resulting in increased cytotoxicity in these tissues. The possibilities for MDR reversal will depend on the sensitivities of tissues and tumors for the cytotoxic effects of certain drugs and on their relative content of P-glycoprotein. The question is whether the therapeutic index can be increased. In some *in vivo* reversal studies the dose of the cytotoxin had to be lowered because of increased toxicity caused by addition of a reverter. Nevertheless, effective antitumor activity was at least retained.[104,128] In the *MDR1* transgenic mouse model, that has bone marrow with a 10-fold increased resistance to MDR-associated drugs, the maximal tolerable dose of cytotoxins in combination with a chemosensitizer was 20%-45% lower. In experiments with a chemosensitizer most drugs caused a 44%-78% decrease in white blood cell count suggesting that there is a possibility to increase the therapeutic index of P-glycoprotein sensitive agents by concomitant administration of MDR reverters.[175]

5. Alternative approaches for modulation MDR *in vivo*

The most direct way to circumvent MDR is to utilize drugs that are not susceptible to the P-glycoprotein efflux pump mechanism. This is often not possible, because tumors do not tend to be sensitive to many different anticancer agents. Dose-intensification as a means of overcoming MDR is prevented by toxicity of the drugs. An alternative approach is to modify known active MDR drugs at the biochemical level in such a way that they are less sensitive to the P-glycoprotein drug extrusion mechanism but retain

their cytotoxic potential. This can be done by chemical reactions, by conjugation to other structures or by encapsulation of a drug in liposomes. An example of a chemically altered cytotoxin is ME2303, a fluorine-containing doxorubicin derivative, which has prominent antitumor activity against a wide variety of tumors *in vitro* and *in vivo*, also against MDR tumors. ME2303 caused a 57%-96% increased life span of P388/VCR-bearing mice, while doxorubicin or vincristine had only a marginal therapeutic effect (maximum increased life span of 24% and 8%, respectively). The mechanism of the enhanced effectiveness has not been investigated in the study.[199] Partial lack of cross-resistance in MDR tumors *in vitro* and *in vivo* was found for the anthracycline annamycin, mediated by increased drug accumulation and retention.[145] Conjugation of doxorubicin to albumin resulted in a prolonged intracellular accumulation of doxorubicin, and increased its cytotoxic efficacy against MDR tumors *in vivo*.[124] Doxorubicin encapsulated in liposomes effectively lowered the white blood cell count in the *MDR1* transgenic mouse model, whereas free doxorubicin alone or in combination with free liposomes was not or only marginally effective respectively.[200] Annamycin entrapped in small liposomes showed markedly increased activity against the KB-V1 human xenograft compared to free annamycin and doxorubicin.[145]

Other attempts on MDR modulation have used the drug transporter P-glycoprotein as a target for immunotherapy by monoclonal antibodies. Antibodies directed against P-glycoprotein can be used for modulation of MDR in various ways. The monoclonal antibody MRK16 specifically binds human P-glycoprotein and has direct cytotoxic activity in xenografted MDR tumor models. The antitumor activity is probably mediated by immune mechanisms like complement-dependent cytotoxicity and antibody-dependent cell-mediated cytolysis.[132,140,201] Monoclonal antibodies like MRK16 and HYB-241 are also able to act as a chemosensitizer by their binding to P-glycoprotein and thus inhibiting its function.[130,132,202,203] In later studies it has been shown that the efficacy of the reverter MRK16 could be augmented by interferon-α treatment.[204] Enhanced killing can be achieved by targeted toxin therapy: conjugation of *Pseudomonas* exotoxin to MRK16 resulted in dose-dependent specific killing of bone marrow cells in *MDR1* transgenic mice.[203] *In vitro* studies showed that the monoclonal antibody UIC2 is a more efficient blocker of P-glycoprotein than other externally binding antibodies and reverses resistance to a wide variety of drugs, where MRK16 and HYB-241 could reverse vincristine and actinomycin D resistance, but not doxorubicin resistance.[202,205,206] MRK16 and UIC2 only recognize the human *MDR1* product and do not cross-react with rodent P-glycoprotein.[205,206] This may lead to favourable outcome in

murine studies with xenografted human tumors and disguise possible side-effects as the monoclonal antibodies do not react with normal tissues in these models. HYB-241 is not species specific and reacts with murine P-glycoprotein.[207]

Transfer of the *MDR1* gene into bone marrow cells of mice has resulted in a test model for the activity of MDR reverters.[173,174] Bone marrow transfected with *MDR1* can also be utilized in a different way. In the treatment of non-hematological tumors the bone marrow toxicity of several drugs is dose-limiting and prevents adequate dosing. Insertion of the *MDR1* gene into bone marrow may provide resistance to the myelosuppressive effects of drugs and allow higher dosing of these cytotoxins. The feasibility of this approach has recently been shown in murine transplantation models.[208-210]

6. Discussion

In vitro studies play a prominent role in the development of new anticancer drugs in the clinical situation.[211,212] This allows massive screening of numerous compounds. The predictive value of *in vitro* cytotoxicity tests for *in vivo* effectiveness is still under debate. Massive screening in animals is considered less ethical for reasons of animal well fare. The standard screening method *in vivo* in the P388 ascites leukemia model has been criticized for being a quite artificial model in which favourable results are readily obtained. As a leukemia model, it may be a poor predictor of effectiveness of drugs in other malignancies, especially solid tumors.[211] Various ways to test compounds in more relevant model systems *in vivo* are being carried out, like multicenter collaborative screening in human tumor xenografts.[158] Testing of drugs in animals is an indispensable step to be taken before drugs can be tried in the clinical situation.[212,213] Important issues to be tested in *in vivo* model systems are for example uptake, metabolism, excretion routes and excretion rate of the drug, volume of distribution, protein binding, availability of the drug at the tumor site, interactions with other drugs, side-effects including carcinogeneity and teratogeneity. Not all pharmacokinetic findings in animal studies can directly be translated to the clinical situation, as cross-species differences in drug metabolism and elimination do exist, but many important problems associated with *in vivo* use of new drugs can be investigated in animal models.

Sofar, there is no ideal *in vivo* model for studying MDR. The various models have their value at certain stages in the research of MDR. Syngeneic intrinsic MDR and paired rodent tumor models can be used to explore *in vivo*-related factors in MDR

modulation in relatively cheap and readily available models. Xenografted human tumors represent more clinically relevant tumors. *MDR1* transfected tumors are valuable because they are more 'clean', as other resistance mechanisms are not introduced in the drug-resistant tumors. The transgenic MDR mice model is a valuable model for screening new chemosensitizers in modulation studies.[167] Development of MDR tumor models should be directed towards clinically relevant models: orthotopically growing tumors that represent frequently occurring human malignancies, express relevant low levels of P-glycoprotein, and allow serial measurement of tumor burden. Some interesting models have been described recently.[140,152] Wild-type tumors exploit various defense mechanisms against cytotoxic insults. The other drug resistance mechanisms, apart from MDR, should be investigated as well. Modulation of several drug resistance mechanisms will be necessary to overcome clinical drug resistance. Clear model systems are indispensable for investigation of these complicated matters. A future modulator may be represented by BIBW 22 which can block the P-glycoprotein efflux pump, and inhibits nucleoside transport resulting in enhancement of 5-fluorouracil cytotoxicity.[189] Another example is 5'-deoxy-5-fluorouridine, a prodrug of 5-fluorouracil, which has antitumor activity in its own, and is a P-glycoprotein reverter.[214]

The *mdr* genes of rodents and humans are not identical. Substrate specificity and differences in transport efficacy have been reported for the various *mdr* gene products and for gene mutations. Although these differences remain a matter of concern, and warrant further investigations, they have not disqualified the rodent MDR tumors as models for studying MDR. We have shown in this review that the expression of the *mdr1a* and *mdr1b* gene products in normal tissues in rodents have a very similar distribution as the *MDR1* gene product in humans. The physiological functions of P-glycoprotein in rodents correspond to the putative roles that were proposed on their localizations and are especially related to defense against xenobiotics and transport of valuable compounds. Modulation studies of MDR have shown very similar effectiveness of the P-glycoprotein reverters in human and in rodent tumor cell lines *in vitro* and in rodent MDR tumors, *MDR1* transfected tumors, and human xenografts *in vivo*.

The studies in rodents in various ascites and solid tumor models have shown the feasibility of reversal of MDR by chemosensitizers *in vivo*. Modulation of MDR by reverters does alter pharmacokinetics and must be considered as a means of dose-intensification. But additional P-glycoprotein modulation at tumor level does also seems to take place, as has been shown by increased intratumoral levels of a cytotoxin by a reverter in MDR tumors and increase in therapeutic index.[133,175] The evidence for P-

glycoprotein reversal at tumor level is scant however, due to few studies on this subject. This is partly caused by a lack of suitable *in vivo* models. Studies in the *mdr1a* knockout mice have shown the deleterious effects that total elimination of this P-glycoprotein may have. These observations warn of the possible side-effects that very potent reverters of P-glycoprotein may have. On the other hand, this may lead to novel strategies for treating tumors and other diseases in sanctuaries like the central nervous system.

Modulation of MDR by chemosensitizers has entered the clinic and has yielded promising results in clinical trials in multiple myeloma and acute leukemia.[215,216] However, in clinical studies with solid tumours like colon carcinoma and renal cell cancer results with chemosensitization were disappointing.[217,218] Many other phase I/II trials have been conducted with various chemosensitizers largely without remarkable response rates.[219-221] The question whether this is the result of inadequate levels of reverter or cytotoxin, has to be answered by ongoing trials with adequate doses of potent P-glycoprotein reverters like PSC 833.

P-glycoprotein-mediated MDR is a powerful resistance mechanism, which may play an important role in clinical drug resistance. Most tumors however, exploit various mechanisms to resist antitumor treatment, and research on the contribution of additional drug resistance mechanisms should be continued.

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1.3. AIMS OF THE THESIS

When we started our investigations back in 1990 few *in vivo* models for studying reversal of MDR were available. Most studies were done in ascites models like the P388 leukemia model. We felt that these models might not be relevant for the situation in solid tumors and could be inadequate for predicting efficacy of reversal in solidly growing cancers. Further, little was known about the possibilities to overcome MDR in solid tumors *in vivo* by reverters. Therefore, it was decided to investigate the possibilities of developing a relevant solid MDR *in vivo* model in which reversibility of MDR could be tested. The objectives of the investigations can be summarized as follows:

1. development of an *in vivo* model of a solid MDR tumor with relevance for the clinical situation;

2. investigation of the feasibility of reversal of MDR in a solid tumor *in vivo* by reverters of MDR;

3. study of pharmacokinetics of MDR reverters;

4. exploration of the side-effects of MDR reversal by concomitant treatment of anticancer drugs and reverters;

5. investigation of the influence of drug resistance on sensitivity for immunotherapy.

2

ORIGINAL STUDIES

2.1

IN VITRO AND IN VIVO CHEMOSENSITIZING EFFECT OF CYCLOSPORIN A ON AN INTRINSIC MULTIDRUG-RESISTANT RAT COLON TUMOR

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Summary

Colon tumors are intrinsically resistant to chemotherapy and most of them express the multidrug transporter P-glycoprotein. Whether this P-glycoprotein expression determines their resistance to anticancer agents in patients is not known. We report here on the reversibility of intrinsic MDR in a syngeneic, solid tumor model. CC531 is a rat colon carcinoma that expresses P-glycoprotein, as was shown with the monoclonal antibody C219. *In vitro* the sensitivity to doxorubicin, daunorubicin and colchicine was enhanced by the addition of the chemosensitizers verapamil and cyclosporin A, while the sensitivity to cisplatin was not enhanced. In a daunorubicin accumulation assay verapamil and cyclosporin A enhanced the daunorubicin content of CC531 cells. *In vivo* cyclosporin A was injected intramuscularly for 3 consecutive days at a dose of 20 mg kg^{-1} day^{-1}. This resulted in whole blood cyclosporin A levels above 2 μmol/l, while intratumoral cyclosporin A levels amounted to 3.6 μmol/kg. In a subrenal capsule assay the maximal tolerable dose of doxorubicin (4 mg/kg) significantly reduced tumor growth. Doxorubicin at 3 mg/kg was not effective, but in combination with cyclosporin A this dose was as effective as 4 mg/kg doxorubicin. These experiments show that adequate doses of the chemosensitizing drug cyclosporin A can be obtained *in vivo*, resulting in increased antitumoral activity of doxorubicin *in vivo*. The *in vitro* and *in vivo* data together suggest that the chemosensitization by cyclosporin A is mediated by P-glycoprotein. This finding may have implications for the application of cyclosporin A and cyclosporin A-like chemosensitizers in the clinical setting.

Introduction

The phenomenon of multidrug resistance to anticancer agents can be an intrinsic characteristic of tumors, or can be acquired by tumors during the course of chemotherapy. Among the tumors that intrinsically have a very low response rate to chemotherapy are colon cancer, renal cell cancer, hepatocellular cancer and adrenocortical cancer. These tumors have high expression levels of the gene for MDR, *MDR1*, at a high frequency.[1] The tissues from which they arise also have a high level of *MDR1* expression.[2] In these tissues the gene product of *MDR1*, P-glycoprotein, may function as an efflux pump for xenobiotics. It is striking that organs with the highest expression of *MDR1* all have excretory functions and that within these organs P-glycoprotein is principally found in cells lining excretory lumina.[3] Whether *MDR* expression in intrinsic MDR tumors is the most important factor determining their resistance to chemotherapy, and whether blocking of P-glycoprotein or suppression of *MDR* expression can result in enhancement of cytotoxicity of anticancer drugs in the clinical situation, are still under study.

Several drugs have been reported to reverse MDR *in vitro*. One of the most effective reverters is the immunosuppressive drug cyclosporin A.[4] Many *in vitro* studies have shown an increase in cytotoxicity to MDR cell lines when cyclosporin A is added to drugs that are affected by the MDR cross-resistance pattern, like doxorubicin, vincristine and colchicine.[5-7] Cyclosporin A, like other reverters, acts as a chemosensitizer almost only against MDR cell lines; the cytotoxicity to parental cell lines that do not express P-glycoprotein is not influenced. On some cell lines cyclosporin A alone has antiproliferative and/or cytotoxic effects, especially at higher doses of cyclosporin A.[8]

Compared to the abundance of *in vitro* data on the role of P-glycoprotein and the reversal of MDR by chemosensitizers, very few data on their value *in vivo* have been published, especially concerning their role in solid tumors. Most *in vivo* studies have been carried out with ascites tumors. In these models intraperitoneally floating tumor cells are treated with intraperitoneal injections of drugs. In solid tumors a prerequisite is achieving effective drug concentrations at the tumor site by a vascular route.

We investigated the question of intrinsic MDR in a syngeneic, solid tumor model. We report here on the chemosensitizing effects of cyclosporin A *in vitro* and *in vivo* on an intrinsic MDR rat colon carcinoma.

Materials and methods

Animals

Male rats of the inbred WAG/RIJ (RT1^u) strain were obtained from Harlan-CPB (Austerlitz, The Netherlands). Animals were bred under specific-pathogen-free conditions and fed standard rat chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. In the experiments rats 12-18 weeks old, weighing 220-280 g, were used.

Tumors

CC531 is a colon carcinoma, which was induced chemically in the WAG rat with 1,2-dimethylhydrazine. The tumor, a moderately differentiated adenocarcinoma, is weakly immunogenic and transplantable in syngeneic rats.[9] *In vitro* the cell line grows as a monolayer in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, aspartic acid (0.1 m*M*), and glutamic acid (0.3 m*M*), all obtained from Gibco (Paisley, UK), in a humidified atmosphere of 5% CO_2/95% air at 37°C. Regular screening for *Mycoplasma* infection was performed. Cells were isolated by trypsinization; viability, determined by trypan blue exclusion, was over 90% in all experiments.

The human ovarian carcinoma cell lines A2780 and 2780^{AD} were grown in complete medium. 2780^{AD}, an MDR cell line with a high level of P-glycoprotein expression, was grown in the presence of 1 μM doxorubicin.[10] This cell line was used as a positive control in immunofluorescence studies, while the parental line, A2780, was used as a negative control.

Chemicals

Cyclosporin A was obtained from Sandoz, Basel, Switzerland; doxorubicin (Adriablastina) from Farmitalia, Nivelles, Belgium; daunorubicin, colchicine, *cis*-diaminedichloroplatinum (cisplatin), verapamil and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) from Sigma Chemical, St Louis, Mo., USA; and dimethylsulphoxide from Merck, Darmstadt, Germany.

In vitro *cytotoxicity assay*

We determined chemosensitivity *in vitro* by the MTT assay, essentially carried out as described by Carmichael *et al.*[11] In brief, in 48-well culture plates (Costar, Cambridge, Mass., USA) 1500 cells were plated in 500 μl complete medium. Drugs were dissolved in 0.9% NaCl. To each well 250 μl drug solution was added, using a fixed concentra-

tion of the chemosensitizer and graded concentrations of the drugs. Cells were grown at 37°C in 5% CO_2 humidified air. After 4 days 150 μl MTT, dissolved in phosphate-buffered saline (PBS) at a concentration of 2 mg/ml was added to each well. After an incubation period of 4 h the supernatant was carefully removed and 250 μl dimethylsulphoxide was pipetted to each well. Plates were placed in a microplate shaker for 5 min. The content was pipetted into 96 wells plates in order to read the absorbance at 570 nm in an automatic microtiter reader (EAR-400). Survival was calculated using the formula: survival = (test well/control) x 100%. The drug concentration reducing the absorbance to 50% of control (IC_{50}) was determined from the graphs. Sensitization ratios were determined by dividing the IC_{50} in the absence of the reverter by the IC_{50} in the presence of the chemosensitizer.

Drug accumulation

In order to determine the accumulation of daunorubicin, cells were incubated with 1 μg/ml daunorubicin for 1 h at 37°C. The content of the fluorescent drug in individual cells was measured on the FACStar Plus flow cytometer (Becton Dickinson, Mountain View, Calif., USA), equipped with a 4-W argon-ion laser tuned to 488 nm with 300 mW power. Orange fluorescence pulses were collected through a 575/26 nm bandpass filter. Results were calculated using the FACStar Plus research software.[12] Enhancement of daunorubicin accumulation was tested by adding cyclosporin A (5 μM) and/or verapamil (6.6 μM) to the incubation medium. Results are presented as mean fluorescence intensity.

Immunofluorescence

P-glycoprotein expression was determined with the specific anti-P-glycoprotein monoclonal antibody C219, which recognizes an internal epitope of P-glycoprotein.[13] Single cell suspensions of A2780, 2780^{AD}, and CC531 were fixed with methanol 70% for 10 min at -20°C. Cells were washed three times in PBS and resuspended in PBS with 1% bovine serum albumin (Centocor, Leiden, The Netherlands). Next, cells were incubated with the monoclonal antibody C219-FITC (fluorescein isothiocyanate conjugate; P-glyco-CHEK, Centocor) diluted 1:100, for 60 min on ice. IgG2a-FITC was used as a control antibody, to determine the aspecific and/or autofluorescence of the cells. After three washings in PBS, cells were analysed on the FACStar Plus flow cytometer using green fluorescence pulses, collected through a 530/30 nm bandpass filter.

In vivo *assay*

Solid tumors of the CC531 cell line were obtained by intraperitoneal inoculation of $5x10^6$ tumor cells. After 30-40 days a rat carrying a large tumor mass was sacrificed and a viable tumor part was excised and divided into small pieces. In a subrenal capsule assay tumor pieces weighing 6-8 mg were implanted under the capsule of the kidneys. Rats were matched for implanted tumor weight in the different treatment groups. On the same day cyclosporin A treatment was started. Cyclosporin A, dissolved in olive oil, was injected intramuscularly into the hind leg daily, for 3 consecutive days at a dose of 20 mg/kg, in order to generate sustained high levels of cyclosporin A. On day 3, rats were injected with 3 mg/kg or 4 mg/kg doxorubicin, or PBS in control rats. After 10 days animals were sacrificed and tumors were enucleated and weighed. All experimental groups consisted of six rats and all animals were evaluable.

Cyclosporin A levels

Cyclosporin A levels *in vivo* were determined with the Emit cyclosporin assay (Syva, Palo Alto, Calif.) on the ELAN analyser (Eppendorf, Hamburg, Germany). This homogeneous enzyme immunoassay is designed for measuring cyclosporin A levels in whole blood. Blood samples were taken 24 h after the third injection of cyclosporin A. Whole blood samples (100 μl) were mixed with 200 μl 100% methanol, which solubilizes cyclosporin A. The samples were centrifuged and aliquots of the supernatant containing the cyclosporin A were diluted with Emit cyclosporin diluent before assaying. In order to measure intratumoral cyclosporin A levels, tumors were grown under the renal capsule for 10 days. On days 7, 8 and 9 cyclosporin A (20 mg/kg) was administered intramuscularly. On day 10 rats were sacrificed and tumors were enucleated without renal tissue. Tumors were crushed in a small tube in 300 μl methanol with a pestle for 3 min. Then 200 μl solution was mixed with 100 μl normal rat whole blood and assayed as a blood sample. The results were corrected for dilution steps and calculated per kilogram tumor tissue, while the plasma and whole blood levels are presented per liter. As the weight of 1 l whole blood is 1.06 kg, the results of the tissue levels and the blood levels are comparable. Because the cyclosporin A levels appeared to be very high, an additional dilution step was necessary to reach the measurable range of the assay. Blood samples and tumor samples were taken from three rats and are represented individually in the graph.

Statistics

Statistical significance was determined with SPSS/PC+, using the Mann-Whitney *U*/Wilcoxon rank-sum *W* test. $P < 0.05$ was considered significant. Results are presented as means with standard deviations.

Ethical approval

The experimental protocols adhered to the rules laid down in *"The Dutch Animal Experimentation Act"* (1977) and the *"Guidelines on the Protection of Experimental Animals"* published by the Council of the E.C. (1986). Specific protocols were approved by the Committee on Animal Research of the Erasmus University, Rotterdam.

Table 1. Chemosensitizing effect of verapamil on growth inhibition by drugs in CC531 cells

drug	IC^{50} - verapamil[a]	IC^{50} + verapamil[a]	sensitization ratio[b]
colchicine	0.081 (± 0.021)	0.028 (± 0.001)	2.9
daunorubicin	0.200 (± 0.113)	0.030 (± 0.002)	6.7
cisplatin	0.600 (± 0.120)	0.865 (± 0.087)	0.7

[a] The drug concentration that results in a 50% reduction of the absorbance in the MTT assay; the values in parentheses are standard deviations

[b] The mean sensitization ratio is shown

All experiments were carried out at least three times

Results

In vitro *chemosensitizing effect*

In the first tests we used the best-known chemosensitizer, verapamil. At a concentration of 6.6 μM growth inhibition of the cell line CC531 by drugs like daunorubicin and colchicine was enhanced. Verapamil was not able to amplify the growth-inhibiting effect of cisplatin. In contrast some growth enhancement was observed (Table 1). At lower concentrations the efficacy of verapamil diminished rapidly: at a concentration of 1 μM only a small chemosensitizing effect was seen. Next we tested the chemosensitizing potential of cyclosporin A; in Fig. 1 a representative experiment is shown. Cyclosporin A had a concentration-dependent chemosensitizing effect on growth inhibition by doxorubicin and was active at concentrations as low as 0.1 μM. The mean sensitization ratio of 0.5 μM cyclosporin A to doxorubicin cytotoxicity was 6.6.

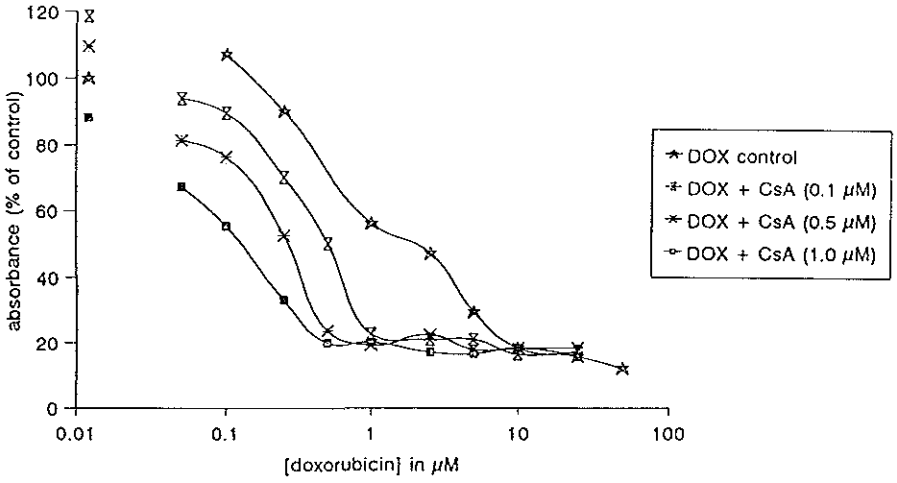

Figure 1. Dose/response curves of the cell line CC531 to doxorubicin (DOX) in the absence or presence of various concentrations of cyclosporin A (CsA) determined in the MTT assay. Cell number, measured as absorbance in the colorimetric assay, is represented as a percentage of the control cell growth on the *y* axis

Enhancement of drug accumulation

Table 2 shows the results of the daunorubicin accumulation study. The mean fluorescence intensity, a measure for the intracellular daunorubicin quantity, calculated from the histograms, increased under the influence of 5 *μM* cyclosporin A 2.1 times. At this concentration cyclosporin A was as effective as 6.6 *μM* verapamil.

Table 2. Effect of chemosensitizers on daunorubicin accumulation

drugs	daunorubicin content*
daunorubicin	100
daunorubicin + cyclosporin A	211 ± 38
daunorubicin + verapamil	193 ± 20

* Numbers represent the mean fluorescence intensity of daunorubicin in CC531 cells after incubation with and without chemosensitizers. Daunorubicin: 1 *μg*/ml; cyclosporin A 5 *μM*; verapamil 6.6 *μM*. The fluorescence of daunorubicin alone is arbitrarily defined as 100

The experiments were repeated at least twice

P-glycoprotein expression

Ideally immunofluorescence studies should be carried out with a monoclonal antibody

that recognizes an external epitope of a membrane protein. Using an antibody that recognizes an internal epitope requires membrane disturbance by fixation with the drawback of possible loss of intensity because of epitope loss by this fixation. However, for rat P-glycoprotein there is no monoclonal antibody available that recognizes an external epitope, as MRK16 is human-specific and the antibodies HYB-241 and 265\F4 do not recognize rat P-glycoprotein (unpublished observations). Therefore, we tested with C219, an antibody widely used to determine P-glycoprotein expression. In Fig. 2 a distinct difference is seen between the non-P-glycoprotein-expressing A2780, and the P-glycoprotein-expressing 2780^{AD}. The fluorescence peak of CC531 cells bound to C-219-FITC lies between the two control cell lines, just to the right of A2780, demonstrating the P-glycoprotein expression in CC531 cells.

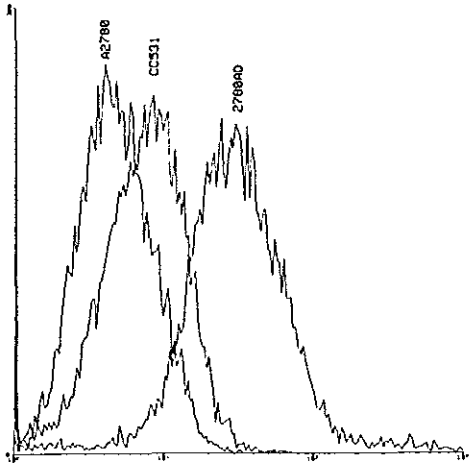

Figure 2. P-glycoprotein expression in A2780, 2780^{AD} and CC531 cells determined by flow cytometry with the monoclonal antibody C219. The figure shows cell count on the *x* axis (linear scale) and fluorescence intensity (fluorescein-isothiocyanate-labelled C219) on the *y* axis (logarithmic scale)

In vivo *cyclosporin A levels*

Because we had no data on the bio-availability of cyclosporin A in tumors, we chose to administer cyclosporin A at a higher dose than necessary to reach a chemosensitizing effect on CC531 cells *in vitro*. With the injection of 20 mg kg^{-1} day^{-1} on 3 consecutive days, an intramuscular depot was generated, which gave sustained high levels of cyclosporin A. Whole blood levels, determined 24 h after the last injection of cyclosporin A (about 2 h before the administration of doxorubicin), amounted to 2.1 (± 0.2) μmol/l. In a separate experiment blood and intratumoral cyclosporin A levels were compared. The cyclosporin A level in whole blood was again 2.1 μmol/l. Intratumoral cyclosporin A levels, measured with the same method, amounted to 3.6 μmol/kg, which is higher than in whole blood. See Fig. 3.

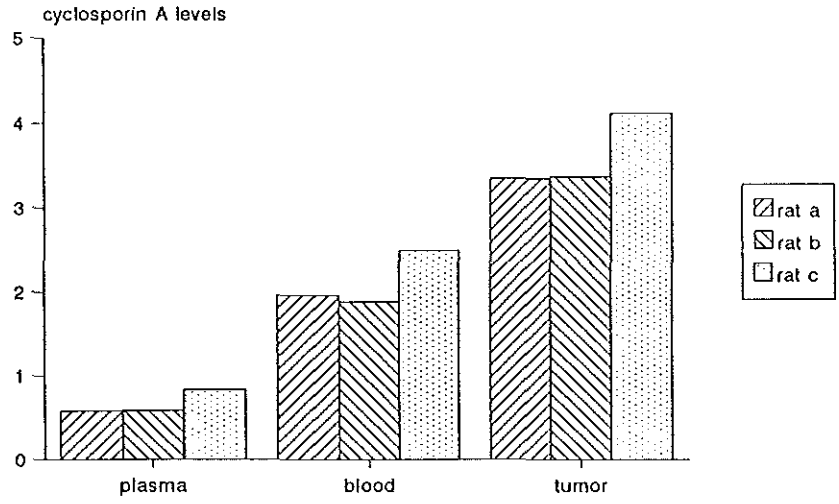

Figure 3. Cyclosporin A levels in plasma, whole blood and in CC531 tumors determined with the Emit cyclosporin assay. The results of three animals are represented individually. The units used for cyclosporin A levels in plasma and whole blood are μmol/l, in tumor tissue μmol/kg

In vivo *chemosensitizing effect*

In the subrenal capsule assay doxorubicin alone, administered at the maximal tolerable dose of 4 mg/kg, significantly inhibited tumor growth (Fig. 4). A lower dose of 3 mg/kg doxorubicin had no significant growth-retarding effect. In combination with cyclosporin A both doses of doxorubicin were significantly more effective compared to treatment with the respective doses of doxorubicin alone. Treatment with cyclosporin A alone had no effect on tumor growth. A repeat experiment with 4 mg/kg doxorubicin showed a significant chemosensitizing effect of cyclosporin A also at the 4 mg/kg doxorubicin dose level.

Discussion

In this study we show the feasibility of overcoming intrinsic MDR in a rat colon adenocarcinoma *in vivo*. We believe that the CC531 tumor provides a good, syngeneic, solid tumor model for studying intrinsic MDR. *In vivo* CC531 is very resistant to most anticancer drugs and only a moderate sensitivity to cisplatin has been described.[14] In our experiments a significant growth retarding effect could be obtained by using doxorubicin, but only when used at the maximal tolerable dose of 4 mg/kg, while no

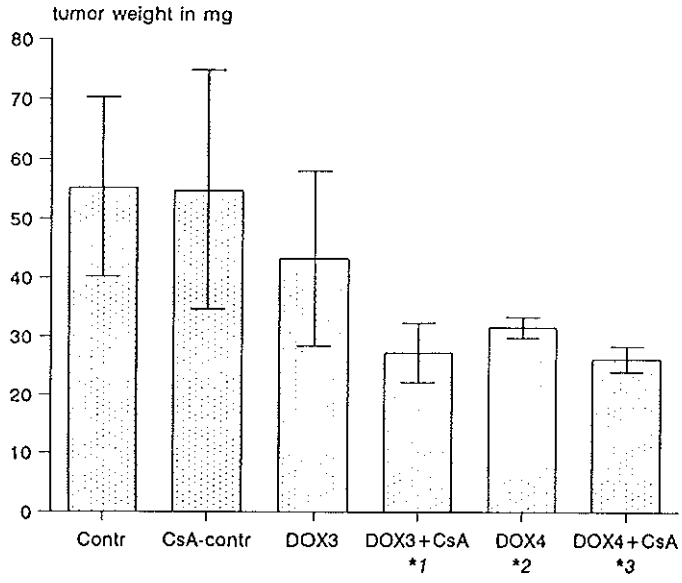

Figure 4. Effect of doxorubicin and/or the chemosensitizer cyclosporin A (CsA) on weights of CC531 tumors in the subrenal capsule assay experiment. Contr, control, no drug treatment; CsA-contr, only treatment with cyclosporin A; DOX3, rats treated with 3 mg/kg doxorubicin; DOX3 + CsA, rats treated with 3 mg/kg doxorubicin, and cyclosporin A; DOX4, rats treated with 4 mg/kg doxorubicin; DOX4 + CsA, rats treated with 4 mg/kg doxorubicin, and cyclosporin A. The following groups were significantly smaller compared to other groups (determined with the Mann-Whitney *U*/Wilcoxon rank sum *W* test): *1: DOX3 + CsA versus Contr ($P = 0.0039$), CsA-contr ($P = 0.0039$) and DOX3 ($P = 0.0163$); *2: DOX4 versus Contr ($P = 0.0039$) and CsA-contr ($P = 0.0039$); *3: DOX4 + CsA versus Contr ($P = 0.0039$), CsA-contr ($P = 0.0039$), DOX3 ($P = 0.02$) and DOX4 ($P = 0.0039$)

significant growth inhibition was observed at lower dosage. In *in vitro* cytotoxicity tests the addition of chemosensitizers enhanced the growth-inhibiting effect of daunorubicin, doxorubicin and colchicine, drugs of the cross-resistance pattern of MDR, whereas the cytotoxicity of cisplatin was not enhanced by cyclosporin A. We demonstrated by flow cytometry that co-incubation of daunorubicin with chemosensitizers like cyclosporin A and verapamil results in an enhanced accumulation of daunorubicin in cells. A distinct expression of P-glycoprotein was shown by immunofluorescence. These data indicate that CC531 has the typical MDR phenotype.

Of the drugs that are able to reverse MDR *in vitro,* verapamil is the best known and most intensely studied. However, levels of verapamil necessary to reverse MDR *in vitro,* about 5 μM, can not be reached in patients because of prohibitive cardiovascular toxicity.[15] We chose cyclosporin A as a chemosensitizer because several investigators

reported a higher effectivity on an equimolar basis of cyclosporin A over verapamil,[5,7,16] a result we also found with the CC531 cell line. The second reason we chose cyclosporin A was that the concentration of cyclosporin A necessary *in vitro* to overcome drug resistance is achievable without intolerable side-effects *in vivo* in humans.[17,18] However, one can not translate *in vitro* concentrations directly into doses required *in vivo*, as pharmacological aspects may play an important role in the bioavailability of the drug,[19] *e.g. in vivo* over 95% of cyclosporin A is bound to proteins or cells. Therefore we decided to administer cyclosporin A at a higher dose than required to achieve a chemosensitizing effect in our *in vitro* experiments. After 3 days of intramuscular administration, cyclosporin A levels in whole blood obtained in the *in vivo* experiments were above 2 μmol/l. Interestingly, measured with the same cyclosporin assay, intratumoral levels of cyclosporin A amounted to 3.6 μmol/kg, which suggests that in solid tumors cyclosporin A levels may be even higher than in whole blood. This means that adequate levels of the chemosensitizer cyclosporin A can be reached at the tumor site by a vascular route. *In vivo* this dose had neither a growth-retarding, nor a growth-stimulating effect on the tumor: in the subrenal capsule assay, tumors in the control group or tumors treated with cyclosporin A alone had similar weights.

In this intrinsic MDR model we show that cyclosporin A can have an effective chemosensitizing effect on doxorubicin *in vivo*. In the subrenal capsule assay experiment rats treated with the combination of cyclosporin A and doxorubicin had significantly smaller tumors compared to all other groups and also compared to the rats treated with doxorubicin alone. Adding cyclosporin A to doxorubicin rendered a suboptimal dose of 3 mg/kg doxorubicin effective, and the activity of an effective dose of 4 mg/kg was enhanced by combination with cyclosporin A. The differences in standard errors between the experimental groups, with larger errors in the control groups and less variation in results in the treated groups, is a phenomenon that is often seen in experimental pharmacology. Probably this is due to the logarithmic growth of tumors, which is attenuated for some time in effectively treated groups, resulting in smaller tumors with inherently smaller standard errors.

Together with the *in vitro* data, demonstrating P-glycoprotein expression, enhancement of drug uptake and increased growth inhibition in CC531 cells by the addition of cyclosporin A, these findings furnish evidence for direct MDR reversal at the cellular tumor level. However, an alternative explanation, cyclosporin A altering drug pharmacokinetics,[20] can not be ruled out. As P-glycoprotein expression is found in the kidneys, especially in the renal tubules,[3] blocking of this efflux pump may diminish excretion of

anticancer drugs and, via higher and more prolonged blood levels, cause indirect enhanced exposure of cells to drugs.[20]

In vivo reversibility of drug resistance by cyclosporin A has been reported in other, mostly non-solid tumor models. In ascites tumor models, Slater *et al.*[21] found a correction of daunorubicin resistance by cyclosporin A on a daunorubicin-resistant subline of the Ehrlich ascites tumor *in vivo*, and they also described enhancement of the cytotoxicity of daunorubicin by cyclosporin A to the parental Ehrlich ascites and hepatoma 129.[22] Boesch *et al.*[23] however, did not find any effect of cyclosporin A on vincristine cytotoxicity to an MDR variant of the murine monocytic leukemia P388. Only Osieka *et al.*[24] published a study about a solid tumor and showed enhancement of etoposide cytotoxicity by cyclosporin A to a human embryonal carcinoma in nude mice. We feel that our tumor model is, therefore, a valuable one, as it is both a syngeneic, and a solid, MDR positive tumor.

Although our experiments suggest potential use for cyclosporin A in the clinical setting, results in the few trials reported so far have not yet substantiated its role, as they are not unequivocal. Sonneveld and Nooter[25] showed the possibility of eliminating *MDR1* positive acute myeloid leukemia cells by adding cyclosporin A to an ineffective treatment schedule. In a very recent report they described reversal of clinical drug resistance in patients with multiple myeloma after addition of cyclosporin A to the combination chemotherapy.[18] Response was correlated with P-glycoprotein and *MDR1* expression. Steady-state plasma levels of cyclosporin A were about 1000 µg/l. Verweij *et al.*[17] tested cyclosporin A as a reverter in combination with epidoxorubicin in colorectal cancer. In four out of four tumor samples they showed the ability of cyclosporin A to enhance daunorubicin uptake by flow cytometry. Despite this observation, only 1 patient out of 24 had a partial response, while 2 of the patients who showed enhanced daunorubicin uptake *in vitro* had progressive disease. The cyclosporin A levels they reported seem quite high and adequate: peak levels of about 6000 ng/ml and levels around 1000 ng/ml 18 h later. Similar cyclosporin A levels were achieved in a clinical trial in renal cell cancer patients with the combination cyclosporin A plus vinblastine. No response was found in 15 patients.[26] Although *MDR* levels or P-glycoprotein expression were not determined in these studies, it is likely that a substantial number of these 39 patients expressed P-glycoprotein in their tumors, as in other studies colon carcinoma as well as renal cell cancer was found to be *MDR*- or P-glycoprotein-positive in the majority of the patients.[27-31] Moreover, Kanamaru *et al.*[29] and Mickisch *et al.*[30] showed that P-glycoprotein expression in renal cell carcinomas

correlated with resistance in primary cell cultures *in vitro* to doxorubicin and vinblastine, which could be reversed by chemosensitizers. The clinical trials by Rodenburg *et al.*[26] and Verweij *et al.*[17] are the only studies that dealt exclusively with tumors from organs that inherently have a high expression level of P-glycoprotein. Other studies were carried out in heavily pretreated patients or patients with advanced disease. In these studies, a clear response was found in a trial with patients with myeloma or lymphoma resistant to vincristine/doxorubicin/dexamethasone: 3 out of 8 patients responded to the addition of verapamil to the drug regimen, while 6 of these 8 patients exhibited P-glycoprotein expression on their tumor cells.[32] So this last study and the study by Sonneveld *et al.*[18] clearly suggest a beneficial role of MDR reverters.

So far only clinical trials with hematological disorders have been successful in reversing MDR. In solid tumors no responses were found. We cannot compare our experimental data with clinical trials, but we show in our model the feasibility of reversing intrinsic MDR in a solid tumor *in vivo*. Adequate levels of the chemosensitizer cyclosporin A could be obtained at the tumor site by a vascular route and this rendered a suboptimal dose of doxorubicin effective. Our results, obtained in a syngeneic, solid tumor model therefore suggest that there may still be a place for chemosensitizers in the chemotherapy of MDR solid tumors.

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2.2

MODULATION OF MULTIDRUG RESISTANCE WITH DEXNIGULDIPINE HYDROCHLORIDE (B8509-035) IN THE CC531 RAT COLON CARCINOMA MODEL

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Summary

The chemosensitizing potency of dexniguldipine hydrochloride (B8509-035) on epidoxorubicin was assessed in an MDR tumor model, the intrinsically MDR rat colon carcinoma CC531. *In vitro* in the sulphorhodamine B cell-viability assay the cytotoxicity of epidoxorubicin was increased approximately 15-fold by co-incubation with 50 ng/ml dexniguldipine. *In vivo* concentrations of dexniguldipine 5 h after a single oral dose of 30 mg/kg were 72 (±19 sd) ng/ml in plasma and 925 (±495 sd) ng/g in tumor tissue. Levels of the metabolite of dexniguldipine M-1, which has the same chemosensitizing potential, were 26 (±6 sd) ng/ml and 289 (±127 sd) ng/mg respectively. The efficacy of treatment with 6 mg/kg epidoxorubicin applied intravenously combined with 30 mg kg^{-1} day^{-1} dexniguldipine administered orally for 3 days prior to epidoxorubicin injection was evaluated on tumors grown under the renal capsule. Dexniguldipine alone did not show antitumor effects *in vivo*. Dexniguldipine modestly, but consistently, potentiated the tumor-growth-inhibiting effect of epidoxorubicin reaching statistical significance in two out of four experiments. In conclusion, these experiments show that dexniguldipine has potency as an MDR reverter *in vitro* and *in vivo* in this solid MDR tumor model.

Introduction

Multidrug resistance is an important mechanism in clinical drug resistance and expression of the *MDR* gene is found in a variety of tumors.[1,2] In MDR a transmembrane efflux pump, P-glycoprotein, confers drug resistance on a group of chemically unrelated anticancer drugs by increasing the efflux. The P-glycoprotein pump can be blocked reversibly by so-called chemosensitizers, which are substrates for the protein themselves. As a result, higher intracellular levels of anticancer drugs are achieved and enhanced cell death occurs. Among the various compounds that can function as chemosensitizers are verapamil, cyclosporin A and its non-immunosuppressive analogue PSC 833, quinidine, tamoxifen, and many others.[1] Previous studies have been carried out with verapamil, but levels necessary for modulation of MDR *in vivo* appeared too high, resulting in severe cardiovascular side-effects. In a clinical trial with verapamil, dose-limiting side-effects were hypotension and cardiac arrhythmias at levels of exposure anticipated to be inadequate for MDR reversal.[3] This has led to a search for related compounds that are devoid of the cardiovascular side-effects.

Stereoisomers of verapamil and related drugs vary in calcium-channel-blocking activity. For example, the (+)stereoisomer of verapamil is a 10-fold less potent calcium antagonist than the (-)isomer,[4] but has approximately the same chemosensitizing effectiveness.[5] Of the other calcium antagonists, the dihydropyridine drug niguldipine, was found to be a very effective chemosensitizer in MDR.[6] The (-)stereoisomer, dexniguldipine hydrochloride (B8509-035), displays a 45-fold lower affinity for calcium-channel-binding sites compared to the (+)isomer, while both have the same MDR-modulating potency.[6] In various preclinical models *in vitro* the chemosensitizing potency of dexniguldipine was either equal to or up to 50 times more effective than verapamil.[6-11]

We tested the activity of dexniguldipine in the CC531 MDR tumor model. CC531 is a colon carcinoma, derived from and transplantable in the WAG/RIJ rat, that intrinsically expresses the multidrug-resistant phenotype. In a previous report the potency of cyclosporin A as a modulator of resistance to doxorubicin was shown in this model.[12] Here we report on the chemosensitizing effect of dexniguldipine *in vitro* and *in vivo* and on levels of dexniguldipine and its active metabolite M-1 in plasma and tumors.

Materials and Methods

Animals

Male rats of the inbred WAG/RIJ ($RT1^v$) strain were obtained from Harlan-CPB (Austerlitz, The Netherlands). Animals were bred under specific-pathogen-free conditions and fed standard rat chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. In the experiments rats 12-18 weeks old, weighing 220-280 g, were used.

Tumor and cell line

CC531 is a colon carcinoma, which was induced chemically in the WAG/RIJ rat with 1,2-dimethylhydrazine. The tumor, a moderately differentiated adenocarcinoma, is weakly immunogenic and transplantable in WAG/RIJ rats.[13] *In vitro* the cell line grows as a monolayer. CC531 is an intrinsically multidrug-resistant tumor as it expresses the MDR phenotype. At the mRNA level expression of *mdr1a*, and not *mdr1b*, has been detected by the polymerase chain reaction.[14] A low level of P-glycoprotein expression has been shown with the monoclonal antibody C219 by Western blotting and by immunofluorescence techniques.[12,14] Intracellular accumulation of daunorubicin can be enhanced by chemosensitizers like verapamil and cyclosporin A.[12,15] Cytotoxicity assays have shown the typical drug resistance pattern of MDR and enhancement of cytotoxicity by chemosensitizers.[12,15]

The cell line was grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, aspartic acid (0.1 m*M*), glutamic acid (0.3 m*M*), penicillin (111 IU/ml) and streptomycin (111 μg/ml), all obtained from Gibco (Paisley, UK), in a humidified atmosphere of 5% CO_2/95% air at 37°C. Regular screening for *Mycoplasma* infection was performed. Cells were isolated by trypsinization; viability, determined by trypan blue exclusion, was over 90% in all experiments.

Chemicals

Dexniguldipine hydrochloride (B8509-035), the metabolite M-1 (B8909-008) and B9003-001 (internal standard for dexniguldipine in the HPLC) were kindly provided by Byk Gulden, Konstanz, Germany; epidoxorubicin (Farmorubicin) was obtained from Farmitalia, Carlo Erba, Italy; sulphorhodamine B was purchased from Sigma Chemicals, St. Louis, Mo., USA; deionized Milli-Q water was from Millipore, Etten Leur, The Netherlands; trichloroacetic acid from J.T. Baker, Deventer, The Netherlands; and dichloromethane/hexane/isobutyl alcohol (40:60:0.5) from Rathburn, Walkerburn,

Scotland.

In vitro *cytotoxicity assay*

Chemosensitivity *in vitro* was determined with the sulphorhodamine B cell-viability assay, essentially carried out as described by Skehan *et al.*[16] In brief, 2 x 10^3 trypsinized tumor cells/well in 200 μl complete medium were plated into 96-well flat-bottomed microtitre plates (Costar, Cambridge, Mass., USA). Tests were carried out in quadruplicate. The plates were incubated for 24 h at 37°C, 5% CO_2/95% air to allow the cells to adhere. Then the old medium was replaced by medium containing the test drug in graded concentrations; in the interaction studies epidoxorubicin together with a fixed concentration of dexniguldipine was added. On day 7 the incubation was terminated by washing the plates twice with PBS. Subsequently the cells were fixed with 10% trichloroacetic acid in deionized Milli-Q water and placed for 1 h at 4°C. After five washings with tap water, the cells were stained for 15 min with 0.4% sulphorhodamine B dissolved in 1% acetic acid, and subsequently washed with 1% acetic acid to remove the unbound stain. The plates were air-dried and bound protein stain was dissolved in 150 μl 10 mM TRIS base. The absorbance was read at 540 nm using an automated microplate reader (Titertek, Flow Laboratories Ltd., Irvine, Scotland).

In vivo *assay*

Subcutaneously grown solid tumors of the CC531 cell line were used 20-30 days after implantation. In a subrenal capsule assay tumor pieces weighing 6-8 mg were implanted under the capsule of the kidneys. In the pharmacokinetic experiment, treatment with dexniguldipine was given 10 days after implantation. Rats were restrained from food 12 h prior to administration of the drug. A single dose of 30 mg/kg dexniguldipine was administered orally through a thin metal cannula. Five hours later rats were sacrificed, a blood sample was taken and the tumors were collected for analysis of dexniguldipine and M-1 levels.

In the pharmacodynamic experiment dexniguldipine treatment was started 24 h after implantation of the tumor. In contrast to the single dosing in the pharmacokinetic experiment, dexniguldipine was administered for 3 consecutive days orally at a dose of 30 mg kg^{-1} day^{-1}. On day 4, 5 h after the last dexniguldipine dose, rats were injected intravenously with 6 mg/kg epidoxorubicin, or PBS in control rats. On day 10 the animals were sacrificed, tumors were enucleated and weighed.

All experimental groups consisted of 6-8 rats.

Apparatus for dexniguldipine and M-1 measurement

Dexniguldipine was determined in plasma and tumor tissue with an automated reverse-phase isocratic high-performance liquid chromatography (HPLC) assay with UV detection at 230 nm. A model 710B WISP autosampler and a model M510 pump were used (all Waters Assoc., Milford, Mass., USA). The detector was a UV2000 (Spectra Physics, San José, Calif., USA). The data were processed with a Shimadzu CR3A integrator (Shimadzu Corp., Kyoto, Japan). The column was a Shandon Hypersyl CPS, 3 μm 150 x 4.6 mm (LC Services, Emmen, The Netherlands). The eluent consisted of a 5 m*M* phosphate buffer (pH 7.5) with 60% acetonitrile. The flow rate was 1.5 ml/min and the column temperature 40°C. Sample size was 100 μl for each analysis.

Sample preparation for dexniguldipine and M-1 measurement

A volume of 150 μl plasma was collected, to which 50 μl 2000 ng/ml solution of internal standard (B9003-001) in methanol was added. Next, 800 μl deionized Milli-Q water was added and the sample was mixed on a whirl mixer for 15 s. For extraction of the test chemicals 7 ml dichloromethane/hexane/isobutyl alcohol (40:60:0.5) was added. The mixture was mixed for 30 min on a whirl mixer and subsequently centrifuged for 10 min at 4000 *g*. The organic layer was collected and evaporated to dryness at 50°C under vacuum. The residue was reconstituted in 150 μl eluent.

Tumor tissue was homogenized with a Turrax homogenizer (Boom, Meppel, The Netherlands) in 1 ml of Milli-Q water. The homogenizer was flushed twice with 250 μl Milli-Q water. A 50 μl volume of a 2000-ng/ml internal standard solution in methanol and 7 ml dichloromethane/hexane/isobutyl alcohol (40:60:0.5) were added. Further handling of the tissue sample was as described for the plasma sample.

The recovery of dexniguldipine, M-1, and the internal standard was determined relative to direct injection of the individual dissolved compounds.

Statistics

Statistical analysis was carried out with SPSS/PC+, using the Mann-Whitney *U*/Wilcoxon rank-sum *W*-test. $P<0.05$ was considered significant.

Ethical approval

The experimental protocols adhered to the rules laid down in *The Dutch Animal Experimentation Act* (1977) and the *Guidelines on the Protection of Experimental Animals* published by the Council of the E.C. (1986). Specific protocols were approved

by the Committee on Animal Research of the Erasmus University, Rotterdam.

Results

In vitro *chemosensitizing effect of dexniguldipine on epidoxorubicin*

Dexniguldipine up to 1000 ng/ml had less than 10% growth-inhibiting effect on CC531 cells *in vitro*. The median inhibitory dose (ID_{50}) for dexniguldipine was approximately 5000 ng/ml. Epidoxorubicin showed a concentration-dependent growth-inhibiting effect with an ID_{50} of 62 ng/ml. The chemosensitizer dexniguldipine at a concentration of 50 ng/ml enhanced cytotoxicity of epidoxorubicin approximately 15 times (Fig. 1). Higher concentrations of dexniguldipine were equally effective (500 ng/ml) or too toxic for CC531 cells by themselves (5000 ng/ml) (data not shown).

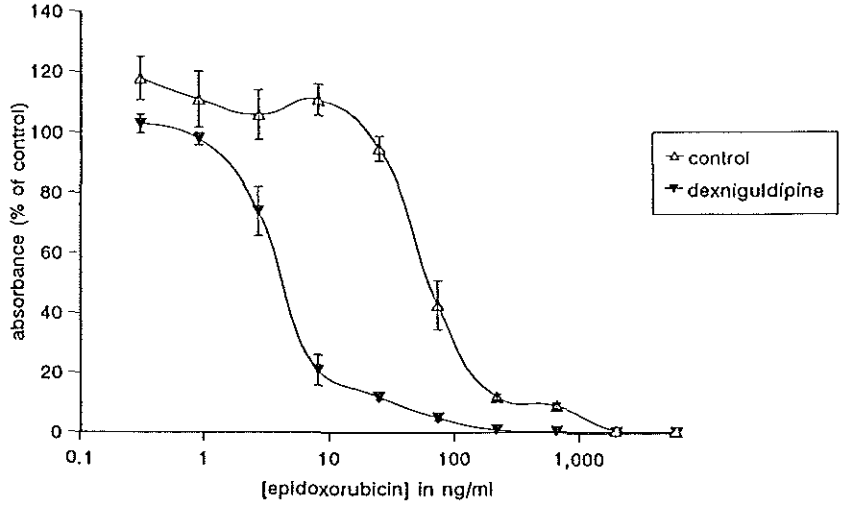

Figure 1. Dose/response curves of the cell line CC531 to incubation with graded concentrations of epidoxorubicin in the absence (▲) or presence (▼) of the chemosensitizer dexniguldipine (50 ng/ml) determined in the sulphorhodamine cell viability assay. Sulphorhodamine B absorbance is expressed as a percentage of the control absorbance on the *y* axis and represents the percentage cell viability

Tissue concentrations of dexniguldipine

In this report we show some data on the levels of dexniguldipine and M-1 in plasma and in tumor tissue. In a separate report more elaborate studies on dexniguldipine and

M-1 pharmacokinetics are presented.[17] Dexniguldipine was readily absorbed after oral administration reaching levels in plasma after 5 h of 72 (±19 sd) ng/ml (see Fig. 2). Levels in tumor tissue were much higher: 925 (±495 sd) ng/g. A similar pattern was observed for the metabolite M-1: in plasma 26 (±6 sd) ng/ml and in tumor tissue 289 (±127 sd) ng/gr. The level of M-1 was approximately one-third of the level of dexniguldipine.

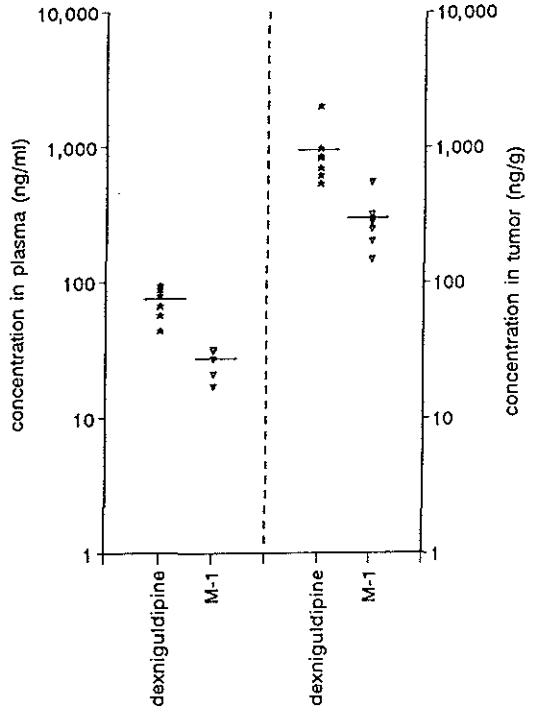

Figure 2. Dexniguldipine (*) and M-1 (▾) levels in plasma (ng/ml) and tumor tissue (ng/g) determined by HPLC 5 h after a single oral dose of 30 mg/kg. Results from rats are represented individually on a logarithmic scale, with lines indicating the mean values. Left data from plasma; right data from tumor tissue

In vivo *chemosensitizing effect of dexniguldipine on epidoxorubicin*

Pilot experiments with epidoxorubicin alone showed that a dose of 6 mg/kg had a moderate but consistent growth-inhibiting effect on CC531 tumors grown in the subrenal capsule assay. Dexniguldipine treatment alone had no influence on growth of the tumor. In all experiments tumors treated with the combination dexniguldipine and epidoxorubicin were the smallest and statistical significance was reached in two experiments. The results are shown in Table 1. Dexniguldipine had no additive effect in combination with a lower dose of 4 mg/kg epidoxorubicin (data not shown).

The experiments were not specifically designed for assessment of toxicity, but some

effects on the body weight of rats were observed. Data on body weights were available from three experiments. All rats lost some weight during the experiment: control rats 1%-5%, dexniguldipine-treated rats 2%-6%, rats treated with epidoxorubicin alone 8%-12%, and rats treated with the combination dexniguldipine and epidoxorubicin 9%-13%. The total weight loss was significantly higher in the rats treated with epidoxorubicin compared to control groups. In one of the three experiments, rats treated with the combination dexniguldipine and epidoxorubicin had significantly more weight loss compared to epidoxorubicin-treated rats. In this particular experiment (number 3) no difference was observed in tumor weights between these groups.

Table 1. Results of experiments on tumor growth inhibition *in vivo* with epidoxorubicin and the chemosensitizer dexniguldipine

treatment group	experiment 1	2	3	4
control	45.1 (±15.6)	34.4 (±7.8)	20.8 (±6.5)	41.3 (±9.2)
dexniguldipine	38.6 (±17.9)	35.1 (±9.0)	16.4 (±10.6)	40.6 (±14.9)
epidoxorubicin 6 mg/kg	23.2 (±2.5)*1	20.9 (±3.2)*2	12.3 (±4.2)*1	27.2 (±5.1)*1
epidoxorubicin 6 mg/kg + dexniguldipine	18.5 (±10.2)*2	13.9 (±4.7)*2,*3	9.9 (±4.1)*1	19.6 (±5.2)*2,*3

Tumor weight in mg; standard deviations are shown in parentheses
Statistically significant ($P<0.05$) results: *1 epidoxorubicin or epidoxorubicin + dexniguldipine versus control; *2 epidoxorubicin or epidoxorubicin + dexniguldipine versus control and versus dexniguldipine; *3 epidoxorubicin + dexniguldipine versus epidoxorubicin

Discussion

Dexniguldipine is a novel modulator of MDR that has low intrinsic calcium antagonist activity. *In vitro* studies have shown its efficacy as a chemosensitizer in various cytotoxicity tests and accumulation assays.[8-11] Effective chemosensitizing was shown in experimental and in human tumor cell lines, and in induced as well as in intrinsic MDR. On a molar basis dexniguldipine was shown to be at least as effective as verapamil. Most studies report a superior drug-modulating effect of dexniguldipine over

verapamil of 2.5- to 50-fold.[7,9-11]

This study expands these *in vitro* studies with *in vivo* data. After oral administration, dexniguldipine is readily absorbed and distributed into various tissues. Dexniguldipine has a very lipophilic nature and its volume of distribution is high (in animals 20-40 l/kg).[18] In the present study intratumoral levels of dexniguldipine were 925 ng/g tissue, which is more than ten times the plasma levels. Compared to the *in vitro* level of 50 ng/ml, which was effective in MDR modulation, these *in vivo* levels are high. The data on dexniguldipine levels were obtained after a single oral dosing. In the antitumor experiment dosing was tripled by administration on 3 consecutive days. Because of the lipophilic nature of the drug this will probably have resulted in even higher intratumoral levels. The wide variation in the results of the levels of the chemosensitizer is not readily explained. Wide interindividual variation in pharmacokinetics has been reported by others for dexniguldipine after oral administration, as well as for other dihydropyridine compounds.[19]

Part of the activity of dexniguldipine *in vivo* is mediated by the active metabolite M-1, which is shown to have the same MDR-modulating potency as dexniguldipine.[6,9] The pharmacokinetics of M-1 followed the results of dexniguldipine closely. The M-1 level was approximately one-third of the level of dexniguldipine in plasma and in tumor tissue 5 h after administration. Recently, comparable levels of dexniguldipine and M-1 have been published from a phase I trial in patients.[19]

Dexniguldipine showed no direct antitumor activity against CC531 cells. Antiproliferative effects of dexniguldipine have been reported for some tumors, possibly tumors with a neuroendocrine differentiation depending on autocrine stimulating factors.[20,21] The chemosensitizing potency of dexniguldipine on the MDR CC531 tumor was observed in all *in vivo* experiments. Dexniguldipine had a significant potentiating effect on growth inhibition of CC531 tumors by epidoxorubicin in two out of four experiments, while in the other experiments the observed differences did not reach statistical significance. The results with dexniguldipine are comparable to those with earlier published experiments in the CC531 tumor model, which revealed the chemosensitizing effect of cyclosporin A in combination with doxorubicin.[12]

Levels of epidoxorubicin in plasma and tumor have not been measured in these experiments. Therefore, we can not rule out the possibility that altered pharmacokinetics of epidoxorubicin contribute to the chemosensitizing effect apart from direct modulation of MDR at the cellular level. The fact that combination treatment resulted in a small enhancement of toxicity in one experiment, as measured by body-weight loss,

suggests that at least some systemic enhancement of epidoxorubicin activity may have occurred. This is in agreement with previous studies that showed enhancement of doxorubicin toxicity caused by combined treatment with cyclosporin A.[22] Other investigators, however, have also furnished evidence for a direct effect of chemosensitizers on the tumor. Niwa *et al.*[23] showed that PAK-200, like dexniguldipine a dihydropyridine analogue, enhanced the accumulation of doxorubicin in solid tumors *in vivo*. The effect of PAK-200 on doxorubicin accumulation in the tumors was dependent on the level of P-glycoprotein expression: only the tumors with a clear expression of P-glycoprotein had a higher doxorubicin content in the presence of the chemosensitizer. Furthermore, in patients with refractory multiple myeloma, addition of cyclosporin A to the chemotherapeutic regimen vincristine, doxorubicin and dexamethasone resulted in enhancement of the response rate.[24] Additional studies showed that the effect was probably achieved by specific killing of the plasma cells expressing P-glycoprotein.[25] The studies clearly suggest drug modulation directly at the cellular level by a P-glycoprotein-dependent mechanism.

The results in our study are comparable to results obtained with dexniguldipine and doxorubicin in a nude mouse xenograft model. Here partial reversal of resistance to doxorubicin was observed in solid tumors of the *MDR1*-overexpressing KB-8-5 cell line grown subcutaneously.[26] Dexniguldipine has entered clinical studies now and promising results have been obtained in trials in acute myeloid leukemia and multiple myeloma.[27,28]

The present study confirms the chemosensitizing potency of dexniguldipine on MDR cells *in vitro*. It shows that *in vivo* relatively high levels of dexniguldipine in plasma and tumor tissue can easily be achieved by oral administration. *In vivo* this resulted in a strong trend towards a significant enhancement of the antitumor effect of epidoxorubicin in the solid MDR tumor CC531.

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2.3

PHARMACOKINETICS OF THE MDR-REVERSING DRUG DEXNIGULDIPINE AND ITS PYRIDINE METABOLITE M-1 IN PLASMA, TUMOR AND RENAL TISSUE IN TUMOR BEARING WAG/RIJ RATS

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Summary

The pharmacokinetics of oral dexniguldipine, a new MDR reverter under clinical evaluation, and its pyridine metabolite M-1 were determined in plasma, tumor and renal tissue in WAG/RIJ rats bearing an MDR CC531 colon adenocarcinoma under the renal capsule. The pharmacokinetics were studied in 4 experiments. After a single administration of dexniguldipine (30 mg/kg) tumors and kidneys were collected after 5 h, 24 h and 48 h in separate experiments. In the fourth experiment dexniguldipine was administered once daily for 3 consecutive days. The dose was 30 mg/kg. In all experiments plasma samples were collected at regular intervals.

The concentrations of dexniguldipine and M-1 could be determined in plasma in most of the rats up to 32 h after drug administration. The area under the curve (AUC) of dexniguldipine and M-1 varied 2- to 6-fold in the four experiments. High tumor tissue concentrations of dexniguldipine were observed. The concentrations were highest in the multiple dose experiment (2014 ± 1005 ng/g tissue). High correlations (>0.8) were established between the concentrations of dexniguldipine in plasma and tumor and renal tissue. Tumor tissue concentrations of M-1 were overall one third of the dexniguldipine concentrations.

Introduction

Dexniguldipine is the (-) enantiomer of niguldipine, a dihydropyridine derivative. In *in vitro* studies dexniguldipine was found to bind to P-glycoprotein and to enhance the cytotoxicity of chemotherapeutic agents such as doxorubicin and etoposide in several cell lines resistant to these agents.[1-3] The synergistic effects may well be associated with reversal of MDR related to the activity of P-glycoprotein. Also, the pyridine metabolite M-1 demonstrated pharmacological activity.[1-4] Dexniguldipine is extensively metabolised by the cytochrome P450 system and most likely by CYP 3A.

In addition, other *in vitro* studies revealed that dexniguldipine itself has potent and selective cytotoxic activity against several tumor cell lines. The mechanism of cytotoxic action has not been fully elucidated, but interaction with protein kinase C and other parts of the intracellular signal transduction pathway have been proposed.[5,6]

Many MDR modifying agents have been applied in the clinic, such as verapamil, cyclosporin A, quinidine, tamoxifen and others.[7-9] The results obtained with verapamil revealed serious cardiovascular side-effects at levels of exposure which are presumably insufficient to achieve MDR reversal.[10] In addition, lack of information about tumor tissue concentrations of the MDR modifier limited the optimal design of clinical studies with an MDR modifier and a P-glycoprotein-dependent anticancer agent.[11,12] The affinity of dexniguldipine for the calcium channel receptor site is relatively low. This enables clinical administration of high doses of the drug. However, the resulting concentration range of the drug in tumor tissues has not been established. At present, dexniguldipine in combination with anticancer agents is in phase I/II of clinical testing, *e.g.* in small cell lung cancer.

The aim of the present studies was to explore the pharmacokinetics of dexniguldipine and pyridine metabolite M-1 in plasma, tumor and renal tissue of WAG/RIJ rats bearing an intrinsic MDR CC531 colon adenocarcinoma, grown as a solid tumor under the renal capsule.[13,14]

Materials and Methods

Experiments were approved by the Animal Ethics Board of the University of Rotterdam.

Assay of dexniguldipine and M-1 in plasma, tumor and renal tissue

Apparatus

Dexniguldipine and M-1 were determined in plasma, tumor and renal tissue with an automated reverse-phase isocratic high-performance liquid chromatography (HPLC) assay with UV detection at 230 nm. A model 710B WISP autosampler and a model M510 pump were used (all Waters Assoc., Milford, Mass., USA). The detector was a UV2000 (Spectra Physics, San José, Calif., USA). The data were processed with a Shimadzu CR3A integrator (Shimadzu Corp., Kyoto, Japan). The column was a Shandon Hypersyl CPS, 3 μm 150 x 4.6 mm (LC Services, Emmen, The Netherlands). The eluent consisted of a 5 m*M* phosphate buffer (pH 7.5) with 60% acetonitrile. The flow rate was 1.5 ml/min and the column temperature 40°C. Sample size was 100 μl for each analysis.

Chemicals

Dexniguldipine hydrochloride (B8509-035, batch 292-349), the metabolite M-1 (B8909-008, batch U1 29/071) (chemical name 3-acetyl-2,6-dimethyl-4-nitrophenyl-5-((5-(4,4-diphenyl-1-piperidinyl)-pentanoyl)pyridine fumarate) and the internal standard for the assay (B9003-001, batch Zi 04/106) were obtained from Byk Gulden, Konstanz, Germany; deionized Milli-Q water was from Millipore, Etten Leur, The Netherlands; trichloroacetic acid from J.T. Baker, Deventer, The Netherlands; and dichloromethane/-hexane/isobutyl alcohol (40:60:0.5) from Rathburn, Walkerburn, Scotland. All chemicals were of analytical grade.

Plasma sample preparation

A volume of 150 μl plasma was collected, to which 50 μl 2000 ng/ml solution of internal standard (B9003-001) in methanol was added. Next, 800 μl deionized Milli-Q water was added and the sample was mixed on a whirl mixer for 15 s. For extraction of the test chemicals 7 ml dichloromethane/hexane/isobutyl alcohol (40:60:0.5) was added. The mixture was mixed for 30 min on a whirl mixer and subsequently centrifuged for 10 min at 4000 *g*. The organic layer was collected and evaporated to dryness at 50°C under vacuum. The residue was reconstituted in 150 μl eluent. Calibration curves were constructed up to 2000 ng/ml. The recovery of dexniguldipine, M-1 and internal standard was determined relative to direct injection of the individual dissolved compounds.

Tumor tissue extraction

Tumor tissue was homogenized with a Turrax homogenizer (Boom, Meppel, The Netherlands) in 1 ml of Milli-Q water. The homogenizer was flushed twice with 250 µl Milli-Q water. A 50 µl volume of a 2000-ng/ml internal standard solution in methanol and 7 ml dichloromethane/hexane/isobutyl alcohol (40:60:0.5) were added. The mixture was vortexed for 30 min. Subsequently, the mixture was centrifuged for 10 min at 4000 *g*. The organic layer was collected and evaporated to dryness at 50°C under vacuum. The residue was reconstituted in 150 µl eluent. The recovery of dexniguldipine, M-1 and internal standard was determined relative to direct injection of the individual dissolved compounds.

Construction of calibration curves up to 500 ng of dexniguldipine in tumor tissue

Dexniguldipine hydrochloride (B8509-035) and M-1 (B8909-008) were added to clean test tubes and the samples were evaporated under vacuum. A volume of approximately 100 mg tumor tissue was added. Subsequently, the procedure was carried out as outlined above.

Tumor model and in vivo *experiments*

Solid tumors of the CC531 colon adenocarcinoma tumor model were used according to previously described method.[14,15] Tumors of the intrinsically MDR cell line (CC531) were grown in donor WAG/RIJ rats. Tumor particles of 6-7 mg were prepared and implanted under the renal capsule of both kidneys of the rats (subrenal capsule model). In all pharmacokinetic experiments 2 particles were implanted per kidney. For each experiment (*i.e.* the 5 h, 24 h, 48 h and repeated administration experiment) a new tumor batch was grown in donor rats. In all rats the right jugular vein was cannulated in the pharmacokinetic experiments to obtain blood samples at regular intervals. The experiments were started on day 1 with tumor implantation. The rats were canulated, after full recovery, on day 3 or 4. During the experiment di-ethyl ether anaesthesia was applied. After termination of the experiments rats were sacrificed by cervical dislocation.

Pharmacokinetic experiments

An oral solution of 30 mg/kg dexniguldipine (1.5 ml/kg) was administered, through a thin metal oral cannula of 23 gauge, on day 8 (single administration) or day 8,9 and 10 (repeated administration) after tumor implantation. Eight rats per treatment group were

used. The solution consisted of undiluted 2% dexniguldipine micro-emulsion (batch LSc 1974). The rats were restrained form food the night prior to the experiment. They had free access to drinking water. Blood samples were collected up to 5 h (5 h experiment), 24 h (24 h experiment) or 48 h (48 h experiment). The time points were: 0, 10, 20, 30, 60, 120 min and 4, 6, 10, 24, 32, 48 h. In the 5 h and 24 h experiments the sampling time ended at 5 h and 24 h respectively. Tumors and kidneys were collected immediately at the end of the sampling period. In the multiple dose experiment peak and trough whole blood samples were collected. Tumors and kidneys were collected 5 h after the final dose on day 10. Plasma samples were collected after centrifugation of whole blood (5 min at 5000 rounds per minute).

The AUC was calculated with the lin-log trapezoidal method in all experiments. The Pearson correlation coefficient was calculated where appropriate.

Results

Assay of dexniguldipine and M-1 in plasma, tumor and renal tissue

Results of the analysis of dexniguldipine and M-1 in plasma

Calibration curves were linear up to the studied concentration of 2000 ng/ml. Correlation coefficients were better than 0.999. The lower limit of quantitation of dexniguldipine and M-1 was 25 ng/ml. The between-run coefficient of variation at the lower limit of quantitation for dexniguldipine was 15.1% and for M-1 24.7%. At almost all concentrations higher than the lower limit of quantitation the coefficient of variation was around 5% or lower.

Results of the tumor tissue extraction

Calibration curves are linear up to the studied concentration of 500 ng. The correlation coefficients are >0.999. The lower limit of quantitation of dexniguldipine and M-1 in tumor and renal tissue was 25 ng/g.

Pharmacokinetic experiments

In the 5 h experiment 7 of the 8 rats were evaluable for plasma kinetics of dexniguldipine and M-1 and all rats were evaluable for tumor and renal tissue uptake. The plasma concentration-time curves of the 48 h experiment are given in Fig. 1. The AUC data are summarized in Table 1. The AUC of M-1 is always lower than of the parent drug (see

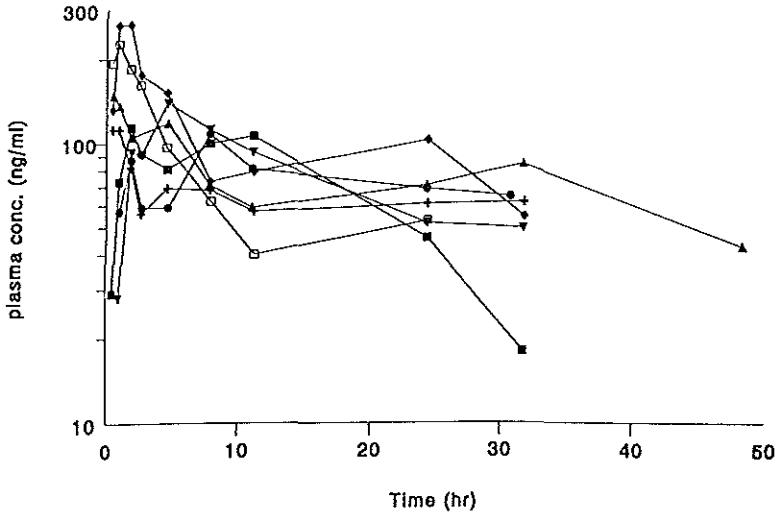

Figure 1. Plasma concentration-time curves up to 48 h of dexniguldipine in 7 WAG/RIJ rats after single oral administration of 30 mg/kg dexniguldipine. Rat 8 was not evaluable

Table 1. AUC data of dexniguldipine and M-1 in WAG/RIJ rats after single oral administration of a dose of 30 mg/kg dexniguldipine (DNIG)

	5 h experiment		24 h experiment		48 h experiment	
	$AUC_{0\text{-}5\,h}$ (μg.h/ml)		$AUC_{0\text{-}24\,h}$ (μg.h/ml)		$AUC_{0\text{-}48\,h}$ (μg.h/ml)	
rat	DNIG	M-1	DNIG	M-1	DNIG	M-1
mean	0.67	0.16	2.17	0.64	2.49	0.54
sd	0.80	0.15	1.03	0.34	0.64	0.43
range	0.22-2.47	0.03-0.49	1.15-4.31	0.12-1.26	1.76-3.49	0.16-1.19
n	7	7	7	7	7	7

sd = standard deviation
The AUC data were calculated in all experiments up to the latest measured time point. In the 48 h experiment the AUC was determined up to the latest measurable concentration which was 31.7 h in all except one rat (11.2 h)

Table 1). The concentration-time curves of the 48 h experiment reveal that the plasma concentration at 48 h could only be determined in one of the rats. In almost all rats the concentration-time curves could be determined up to 32 h after administration. The $AUC_{0\text{-}48\,h}$ was calculated up to the latest measurable data point. The mean plasma

concentration-time profiles of the 24 h and 48 h experiments are superimposable, which illustrates that the pharmacokinetics are reproducible.

In the repeated administration experiment only limited plasma kinetic data became available. The limited plasma concentration data do not show major differences in the kinetics on day 1, 2 and 3.

Table 2. Concentrations of dexniguldipine in tumor and renal tissue and of M-1 in tumor tissue

tumor concentrations of dexniguldipine (ng/g tissue)				
	5 h experiment	24 h experiment	48 h experiment	repeated dose experiment
mean	1463	595	386	2014
sd	1470	404	330	1005
sem	264	78	58	193
CV (%)	101	68	85	50
tumor concentrations of metabolite M-1 (ng/g tissue)				
	5 h experiment	24 h experiment	48 h experiment	repeated dose experiment
mean	389	262	146	692
sd	293	182	107	309
sem	53	36	21	59
CV (%)	75	69	73	45
renal tissue concentrations of dexniguldipine (ng/g tissue)				
	5 h experiment	24 h experiment	48 h experiment	repeated dose experiment
mean	2707	2632	463	5284
sd	1028	1811	444	3129
sem	285	523	111	836
CV (%)	38	69	96	59

sd = standard deviation
sem = standard error of the mean
CV (%) = coefficient of variation

The tumor concentrations of dexniguldipine and M-1 in the 4 experiments are given in Table 2. The concentrations of M-1 are 20-30% lower than of the parent drug.

The concentrations of dexniguldipine in renal tissue were higher than in tumor tissue (see Table 2). Also, the M-1 concentrations in renal tissue are of the order of 30% of the dexniguldipine concentrations, which resembles the relationship in tumor tissue. The order of magnitude of the renal concentrations in the individual experiments was the same as in tumor tissue. The decline of the plasma concentration-time curves in the 24 h and 48 h experiment parallels the decline of the tumor tissue concentrations of dexniguldipine, which illustrates the close relationship between plasma and tumor kinetics of dexniguldipine (see Fig. 2). In this figure the tumor concentrations of the 5 h, 24 h and 48 h experiments have been combined in order to construct a concentration-time curve. The estimated terminal half-life in plasma is of the order of 20 h (see Fig. 2). Furthermore, high correlation coefficients were found between the tumor tissue concentrations at the end of the experiment and the plasma AUC in the 5 h experiment ($R = 0.98$, $n = 7$) and 24 h experiment ($R = 0.84$, $n = 7$) (see Fig. 3). The relationship between the tumor tissue concentration and the $AUC_{0\text{-}48\,h}$ was not calculated, because the $AUC_{0\text{-}48\,h}$ could not be determined in most of the rats, as is outlined above.

In addition, high correlation coefficients were found between tumor and renal tissue concentrations in all experiments (see Fig. 4).

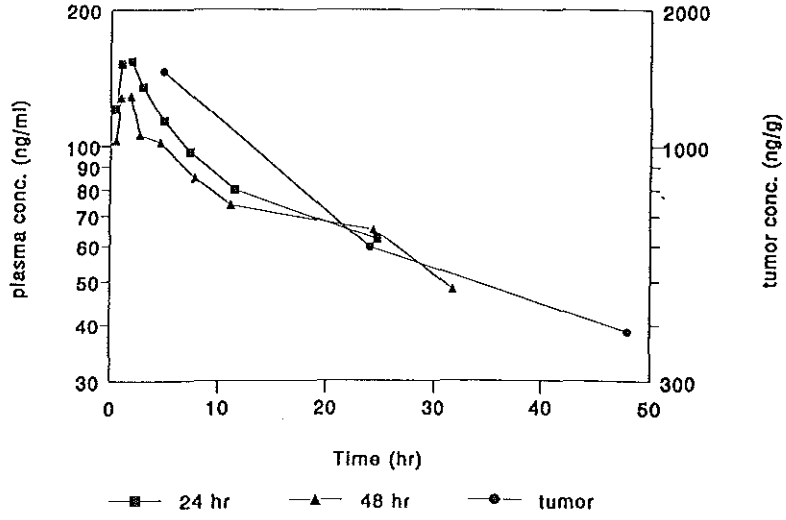

Figure 2. Mean plasma concentration-time curves of the 24 h and 48 h experiment combined with the mean tumor concentrations obtained in the 5 h, 24 h and 48 h experiments

The tumor weights in the 4 experiments varied and were 63 ± 19 mg (5 h experiment), 57 ± 15 mg (24 h experiment), 131 ± 42 mg (48 h experiment) and 81 ± 29 mg (repeated administration experiment).

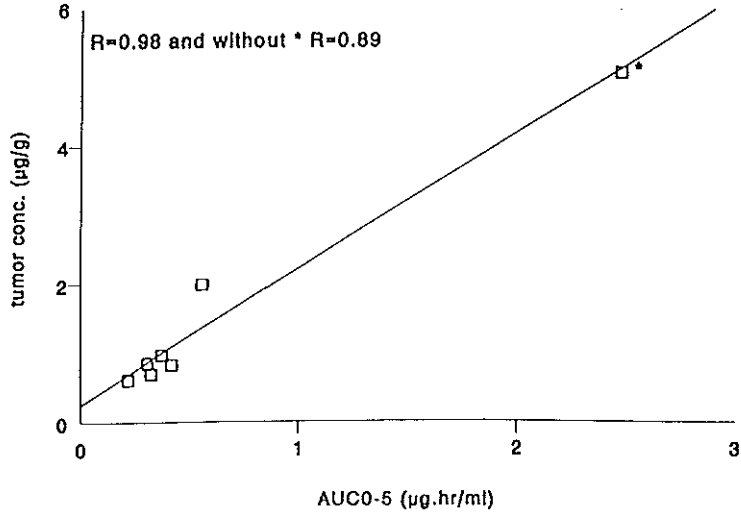

Figure 3. Correlation between the tumor tissue concentrations of dexniguldipine and the $AUC_{0\text{-}5\,h}$ in the 5 h experiment. The correlation coefficient has also been calculated without the extreme observation (*)

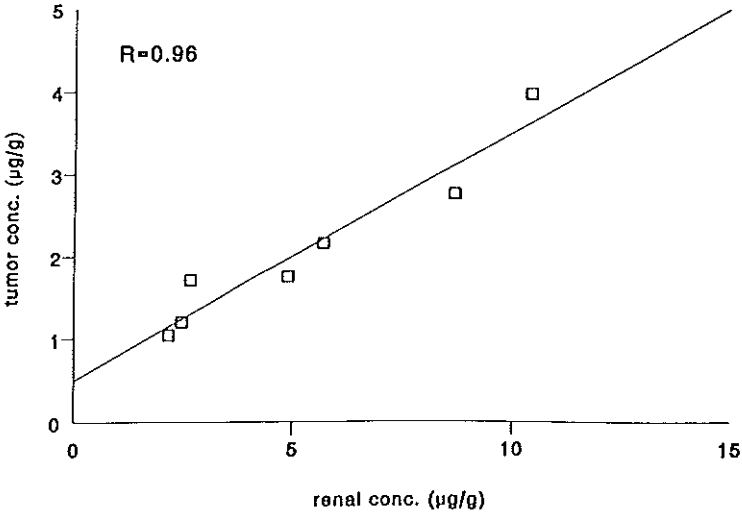

Figure 4. Correlation between tumor and renal tissue concentrations of dexniguldipine in the experiment with the repeated administration once daily on 3 consecutive days

Discussion

The presented data describe the plasma, tumor and renal tissue kinetics of dexniguldipine and metabolite M-1 after single or repeated oral administration of 30 mg/kg dexniguldipine to tumor bearing WAG/RIJ rats. The concentrations of dexniguldipine and metabolite M-1 were determined in tumor tissue at 5, 24, and 48 h to evaluate the uptake kinetics in tumor tissue. In addition, the tumor concentrations were determined after steady state had been reached after 3 days of drug administration, which is based on the estimated terminal half-life of approximately 20 h.

The plasma concentration-time curves of the 5 h experiment only showed a moderate decline. No rapid distribution phase was visible in these curves. The kinetics in plasma were highly variable. The AUC range in the 24 h experiment was 1.15 to 4.31 μg.h/ml. In addition, the time to maximal plasma concentration (T_{max}) was highly variable (see Fig. 1). Also a study in man revealed that pharmacokinetics are highly variable.[16] The elimination phase in the 48 h experiment can not be described sufficiently long enough to calculate the total AUC.

In the experiment with the repeated administration limited plasma data became available. This was due to plugging of 3 cannulas during the experiment. The plasma concentration data in the 4 evaluable rats revealed that the concentrations did not further increase after 2 days of dosing, indicating that near steady state had been reached. The administration of once daily 30 mg/kg dexniguldipine, during 3 subsequent days was feasible, except in one rat. No significant cumulative toxicity in the remaining animals was observed. A previous pilot experiment revealed that this dose was the highest feasible dose upon repeated administration in this model (data not shown).

The tumor concentrations of dexniguldipine and M-1 also showed wide variation (see Table 2). It is unlikely that this variation is due to differences in the distribution kinetics of dexniguldipine, regarding the high correlations between AUC and tumor concentrations of dexniguldipine. The concentrations of the parent drug and M-1 are highest after the repeated administration. This indicates that the drug accumulates in the peripheral tissues. The high correlations between plasma and tumor tissue concentrations enables the prediction of tumor concentrations using plasma samples in this *in vivo* model. The high variability of the plasma concentrations may be due to variations between the rats in bioavailability.

High correlations were found between tumor and renal tissue concentrations of

dexniguldipine. The renal tissue concentrations were always a factor 1.5 to 4 higher than the tumor tissue concentrations, dependent on the sampling time (see Table 2). Dexniguldipine is a dihydropyridine derivative. This class of drugs is known to have a high tissue distribution. Differences in lipophilicity between tissues, such as renal and tumor tissue, may therefore contribute to the differences in tissue concentration after exposure to dexniguldipine, in particular at steady state. The ratio dexniguldipine and M-1 was constant in all experiments.

The tumor weights between the experiments showed variation. The mean tumor weight in the 48 h experiment is clearly higher than in the other experiments, which may have been due to tumor batch differences. As a consequence, tumor growth inhibition experiments should be carried out with the same tumor batch.

The *in vitro* results of the MDR-modifying effect of dexniguldipine revealed that dexniguldipine was highly active at a concentration as low as 50 ng/ml.[17] In that experiment the same CC531 cell line was used. It is hazardous to extrapolate results of *in vitro* studies to *in vivo* tumor models. However, regarding the high tumor tissue concentrations of dexniguldipine *in vivo*, it may be anticipated that these concentrations are high enough to reverse MDR *in vivo*. Results of the pharmacodynamic study with dexniguldipine and epidoxorubicin in this model reveal a moderate synergistic antitumor effect, which was statistically significant in 2 out of 4 experiments.[17]

In the present study high concentrations were achieved in intrinsically MDR solid tumor tissue implanted under the renal capsule after single oral dosing of the MDR reverter dexniguldipine. In addition, high correlations between plasma and tumor tissue concentrations enables prediction of tumor concentrations in this model by simply measuring plasma concentrations.

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2.4

THE CHEMOSENSITIZER CYCLOSPORIN A ENHANCES THE TOXIC SIDE-EFFECTS OF DOXORUBICIN IN THE RAT

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1994; 120: 533-8

Summary

The feasibility of using chemosensitizers in the circumvention of P-glycoprotein-mediated MDR has been shown in many studies. We recently reported on the chemosensitizing effect of cyclosporin A on doxorubicin in a rat solid tumor model. Using the same experimental design we investigated the side-effects of the combination treatment. During the 35-day experiment doxorubicin treatment caused dose-dependent weight loss, which was enhanced by combination treatment with cyclosporin A. The main doxorubicin-related side-effects were myelosuppression (transient leucopenia and thrombopenia) and nephrotoxicity. Damage to the kidney was severe, leading to a nephrotic syndrome and resulting in ascites, pleural effusion, hypercholesterolemia and hypertriglyceridemia. These toxicities were enhanced by the addition of the chemosensitizer cyclosporin A. Mild doxorubicin-related cardiomyopathy and minimal hepatotoxicity were seen on histological examination. There were no signs of enhanced toxicity of the combination treatment in tissues with known high expression levels of P-glycoprotein, like the liver, adrenal gland and large intestine. Cyclosporin A had a low toxicity profile, as it only caused a transient rise in bilirubin. In conclusion, the chemosensitizer cyclosporin A enhanced the side-effects of the anticancer drug doxorubicin, without altering the toxicity pattern. There was no evidence of a therapeutic gain by adding cyclosporin A to doxorubicin, compared to single agent treatment with doxorubicin in 25%-33% higher doses, because of the enhanced toxicity of the combination treatment.

Introduction

Multidrug resistance is an important mechanism of resistance of tumors to anticancer drugs. In MDR an efflux pump, P-glycoprotein, expels drugs from the cell by active transport.[1] P-glycoprotein expression has been found in many tumors. High expression levels of P-glycoprotein were demonstrated in colon cancer, renal cell cancer, hepatocellular carcinoma, and adrenocortical cancer, while intermediate levels were found in sarcomas and breast cancer.[2,3] In hematological malignancies, like several leukemias, lymphomas and in multiple myeloma, expression of P-glycoprotein was found in untreated, and, to a greater extent, in treated tumors.[3-5] However, this protein is also expressed in normal tissues. Organs with a high expression level of P-glycoprotein are the adrenal gland, liver, kidney, colon and pancreas,[2,6] and the protein is mainly localized in cells lining excretory lumina, which suggests a detoxification function.[6]

One way of disturbing the P-glycoprotein-mediated resistance mechanism is by blocking the efflux pump with so-called chemosensitizers. Numerous *in vitro* studies have shown the efficacy of drugs like verapamil, cyclosporin A, quinine/quinidine, tamoxifen, and others in enhancing the sensitivity of MDR tumor cell lines to anticancer drugs.[7] *In vivo* studies have confirmed the feasibility of reversal of MDR by chemosensitizers in ascites tumor models[8,9] and in solid tumor models.[10,11] In clinical trials promising results have been observed in patients with multiple myeloma, lymphoma, and leukemia.[4,12-14] In studies with solid tumors chemosensitizers showed less efficacy with responses in a minority of the patients only.[15-18] Besides, some authors have reported on enhancement of toxic side-effects, like myelosuppression by the addition of chemosensitizers to the therapeutic regimen.[17,19,20] Therefore, the question is raised whether the use of chemosensitizers in combination with anticancer drugs enhances the toxic side-effects of these drugs, apart from enhancing the efficacy of the anticancer treatment. A second question is whether other toxic effects will appear, especially in P-glycoprotein-expressing tissues. Third, chemosensitizers themselves may have adverse effects.

We recently published our results on chemosensitizing in a rat MDR tumor model.[11] The chemosensitizer cyclosporin A was shown to enhance the cytotoxic efficacy of doxorubicin *in vitro* and *in vivo*. A suboptimal dose of doxorubicin (3 mg/kg) was rendered effective against the solid growing CC531 rat colon carcinoma *in vivo* by the addition of cyclosporin A. Drugs were administered intramuscularly and intravenously, which means that, unlike in ascites tumor models, drugs were transported to the tumor

and other tissues by a vascular route. Because this is close to the clinical situation, the same model was used to study the toxic effects of the combination treatment on normal tissues in rats. In this study we show that the chemosensitizer cyclosporin A enhances the specific toxic effects of doxorubicin on normal tissues, resulting in myelosuppression, severe nephrotoxicity, and mild cardiotoxicity. There were no signs of additive toxic damage in tissues with a high expression level of P-glycoprotein, nor of severe cyclosporin A-induced toxicity.

Materials and methods

Animals

Male rats of the inbred WAG/RIJ ($RT1^v$) strain were obtained from Harlan-CPB (Austerlitz, The Netherlands). Animals were bred under specific-pathogen-free conditions and fed standard rat chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. In the experiments rats were 12-18 weeks old and had a body weight of 220-280 g.

Chemicals

Cyclosporin A was obtained from Sandoz, Basel, Switzerland; doxorubicin (Adriablastina) from Farmitalia, Nivelles, Belgium.

Experimental design

Animals were randomly allocated to the experimental groups. The two control groups consisted of eight animals, while the five experimental groups contained four animals each. The experiment was repeated once. Intravenous injection and blood sampling were done under anaesthetic conditions using ether. Rats were weighed weekly. On day 3, 7, 14, 21, and 28 a blood sample of 0.75 ml was taken by bleeding from the tail vein. On the 35th day the experiment was terminated and all rats were sacrificed. If an animal was critically ill such that it was not supposed to survive 48 h, or if it had lost approximately 20% body weight, the animal was sacrificed earlier than day 35.

Drug treatment

The chemosensitizer cyclosporin A, dissolved in olive oil, was injected intramuscularly into the hind leg daily for 3 consecutive days at a dose of 20 mg/kg body weight.

Animals in groups not to be treated with cyclosporin A were injected with the vehicle of cyclosporin A: olive oil and 6.25% alcohol. Treatment was given on days -2, -1 and 0. Doxorubicin was administered intravenously on day 0 as a single dose at a concentration of 3 mg/kg, 4 mg/kg or 6 mg/kg body weight. Control rats were injected with PBS. This resulted in the following groups: Control (treatment with PBS and vehicle), CsA-con (PBS + cyclosporin A), DOX3 (3 mg/kg doxorubicin + vehicle), DOX3+CsA (3 mg/kg doxorubicin + cyclosporin A), DOX4 (4 mg/kg doxorubicin + vehicle), DOX4+CsA (4 mg/kg doxorubicin + cyclosporin A), and DOX6 (6 mg/kg doxorubicin + vehicle).

Hematological and biochemical studies

Blood was collected in lithium/heparin microtubes (Sarstedt, Germany). The hemoglobin content was determined on the TOA hemoglobin counter HB-100, leucocytes on the Sysmex microcell counter CC-108 and platelets on the TOA platelet counter PL-100 (all Sysmex, TOA Medical Electronics, Hamburg, Germany). The remaining blood sample was centrifuged and serum was collected. Biochemical values of creatinine, urea, aspartate aminotransferase, *r*-glutamyltransferase, total bilirubin, cholesterol and triglyceride were determined on the ELAN-Analyzer (Eppendorf, Hamburg, Germany) with reagents from Merck (Merck Diagnostica, Darmstadt, Germany).

Histology

On day 35 all animals were sacrificed and an autopsy was performed. Ascites and pleural effusion, if present, were aspirated in a syringe and measured. Specimens of the following organs were taken for histological examination: heart, lung, liver, spleen, kidney, large intestine, pancreas and adrenal gland. The organs were removed immediately, fixed in 10% buffered formalin and embedded in paraffin. Sections were cut at 5 μm, stained with hematoxylin and eosin and periodic-acid/Schiff. Microscopic sections were coded and scored blindly. The following histological parameters were evaluated: edema, necrosis, inflammation, accumulation of fat, fibrosis, glycogen storage (liver), and degenerative changes. The extent of damage in kidney and liver was graded semiquantitatively on a 0 to 2+ scale (0 = absent, 1+ = slightly damaged, 2+ = severely damaged). The histopathological changes in the heart were assessed according to the scoring system of Bristow *et al.*,[21] which scale runs from 0 to 3.0+.

Statistics

Statistical significance was determined with SPSS/PC+, using the Mann-Whitney *U*/Wilcoxon Rank Sum *W* Test. $P < 0.05$ was considered significant. Results are presented as means with standard deviations. In the line diagrams standard deviations are omitted for readability reasons.

Ethical approval

The experimental protocols adhered to the rules laid down in "The Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the Council of the EC (1986). Specific protocols were approved by the Committee on Animal Research of the Erasmus University, Rotterdam.

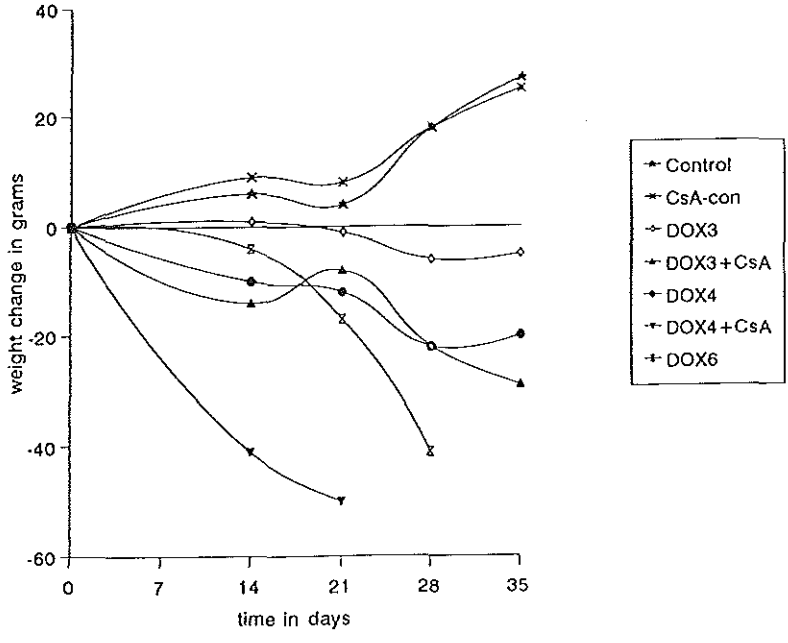

Figure 1. Body weight changes of rats under treatment with doxorubicin (DOX) with or without cyclosporin A (CsA) (see Materials and methods for definitions of the groups). Statistically significant differences determined on day 21 are: DOX6 versus DOX4+CSA, DOX4, DOX3+CsA, DOX3, CsA-con, and Control; DOX4+CsA versus DOX4, DOX3+CsA, DOX3, CsA-con, and Control; DOX4 versus DOX3, CsA-con, and Control; DOX3+CsA versus DOX3, CsA-con, and Control (all $P<0.025$)

Results

Weight

Drug treatment had a profound influence on the body weight of the animals. In the first experiment rats treated with the highest doses of doxorubicin and doxorubicin plus cyclosporin A did not survive for 35 days (Fig. 1), whereas in the repeat experiment under the same conditions none of the rats died before the end of the experiment. The maximal tolerable dose in WAG rats is 4 mg/kg. We purposely chose to administer a higher dose for the comparison of toxic side-effects. Rats treated with the highest doses of doxorubicin developed ascites, which made body weight a less reliable parameter towards the end of the experiment for comparison of groups. Therefore, significance of the differences is determined for day 21. All control rats continued to grow during the experiment, while rats treated with doxorubicin lost weight. This effect was most pronounced in the groups DOX6 and DOX4 + CsA. The weight curves of the groups DOX3 + CsA and DOX4 ran down in parallel, while the DOX3 group fared better.

Table 1. Blood parameters determined on day 7

group	leucocytes	thrombocytes	bilirubin
Control	7.76 (±0.75)	545 (±39)	2.39 (±0.28)
CsA-con	6.96 (±0.66)	497 (±33)	3.49 (±0.45)*8
DOX3	5.90 (±1.80)*1	575 (±50)*1	1.60 (±0.88)
DOX3 + CsA	4.68 (±1.06)*2	414 (±98)*5	2.35 (±0.10)
DOX4	5.40 (±1.68)*3	415 (±74)*6	1.65 (±0.17)
DOX4 + CsA	2.93 (±0.25)*4	246 (±38)*4	2.08 (±0.61)
DOX6	3.20 (±0.50)*4	129 (±10)*7	1.58 (±0.30)

The groups are defined in Materials and methods

*1-*8 Significance was determined at the $P<0.05$ level: *1 not significantly lower than control groups; *2 significantly lower than both control groups; *3 significantly lower than Control; *4 significantly lower than both control groups and DOX3, DOX3 + CsA and DOX4; *5 significantly lower than Control and DOX3; *6 significantly lower than both control groups and DOX3; *7 significantly lower than all other groups; *8 significantly higher than all other groups

Hematological parameters

Drug treatment had no effect on hemoglobin during the first 14 days (data not shown). The nadir for leucocytes and thrombocytes was reached around day 7 (Table 1). A significant drop in leucocyte and platelet count was observed in all drug treated groups except DOX3. In the groups DOX3+CsA and DOX4 approximately equal levels of leucocytes and platelets were found. The same holds good for leucocytes in the groups DOX4+CsA and DOX6. The DOX4+CsA group had a significant lower nadir compared to the DOX4 group for both hematological parameters.

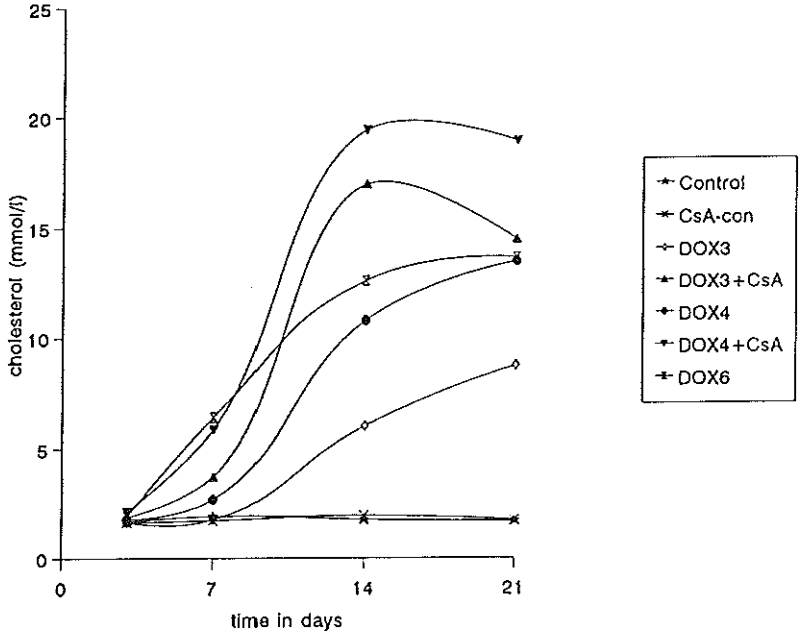

Figure 2. Cholesterol concentrations in rats treated with doxorubicin with or without cyclosporin A. Statistical significance between the groups is determined for day 14. All treated groups are significantly different from both control groups ($P<0.01$); DOX4+CsA versus DOX6, DOX4 and DOX3 ($P<0.025$); DOX3+CsA versus DOX3 and DOX4 ($P<0.05$); DOX6 versus DOX3 ($P<0.025$).

Lipid biochemical values

Doxorubicin had a profound influence on the lipid metabolism in WAG rats. All treated groups had increased levels of serum cholesterol and triglyceride (Fig. 2 and 3). The rise continued for 14-21 days and was amplified by cyclosporin A: in the DOX4+CsA and DOX3+CsA groups the highest levels were observed. Cyclosporin A alone had no influence on cholesterol and triglyceride levels.

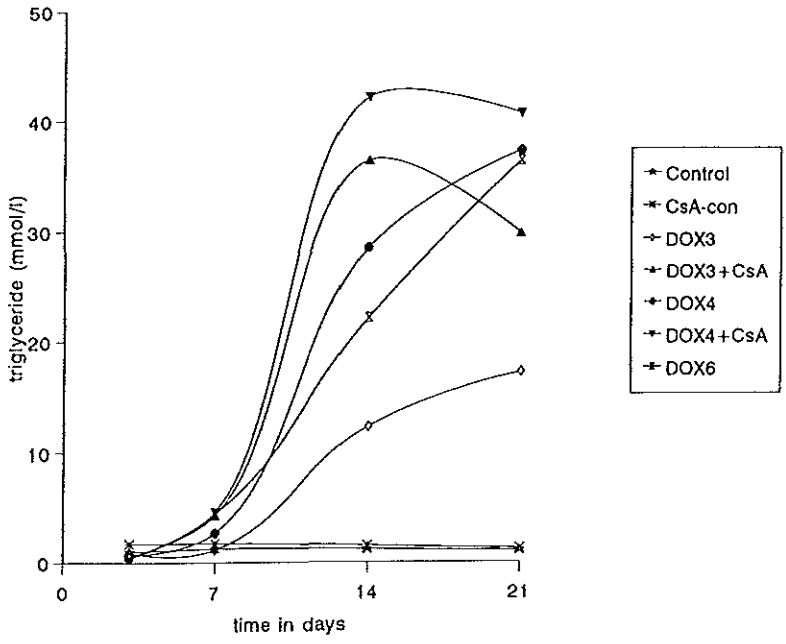

Figure 3. Triglyceride concentrations in rats treated with doxorubicin with or without cyclosporin A. Statistical significance between the groups is determined for day 14. All treated groups are significantly different from both control groups ($P<0.01$); DOX4+CsA versus DOX6, DOX4 and DOX3 ($P<0.05$); DOX3+CsA versus DOX3 ($P<0.05$).

The high levels of lipids disturbed the measurement of other hematological and biochemical values from 14 days onwards. Therefore, we were only able to determine the short-term effects of the treatment on these values.

Renal biochemical values

During the first 14 days no significant changes in creatinine and urea were observed (data not shown).

Liver biochemical values

Doxorubicin treatment had no short-term effect on the liver function parameters aspartate aminotransferase and γ-glutamyltransferase (data not shown). A significant rise in bilirubin was observed in the cyclosporin-control group (Table 1). This cyclosporin-related effect seemed to be mitigated by combination treatment with doxorubicin.

Autopsy

Pathology data presented here are from the second experiment, in which all animals survived. On day 35 rats in the groups DOX4 + CsA and DOX6 were critically ill. They had lost body weight and subcutaneous fat. During the experiment rats had not had diarrhoea. At autopsy a large amount of ascites and hemorrhagic pleural effusion was found in rats treated with DOX4 + CsA and DOX6. Rats in the DOX4 + CsA group had 12.4 (± 12.4) ml ascites, rats in the DOX6 group 15.3 (± 6.9) ml, while only a small amount of 2.1 (± 2.4) ml was found in the DOX3 + CsA group and no ascites in the other rats. Pleural effusion was 6.5 (± 3.1) ml and 6.3 (± 4.4) ml in the DOX4 + CsA and DOX6 groups respectively. In the DOX3 + CsA group 0.8 (± 1.5) ml pleural effusion was found and none in the other rats. The differences in ascites an pleural effusion were statistically significant for the DOX4 + CsA and DOX6 groups compared to all other groups except for pleural effusion in DOX6 versus DOX3 + CsA. In addition, edema of the pancreas and paleness of the liver, kidneys and adrenal glands were observed in rats of the DOX4 + CsA and DOX6 groups. In the other groups all these macroscopic findings were minimal or absent.

Microscopic study

Light microscopic examination of the kidney showed severe damage (2+) in all rats treated with doxorubicin or the combination doxorubicin and cyclosporin A, while rats injected with PBS or cyclosporin A had normal kidneys. Injured kidneys showed increased glomerular mesangial cellularity, lipid accumulation in macrophages, thickening of basement membranes of glomerular capillaries and Bowman's capsule with in some glomeruli focal adhesions (Fig. 4). The tubules epithelium showed degenerative changes, focal regenerative activity (mitotic figures) and some showed protein casts. In the interstitial space of injured kidneys focal lymphocytic infiltrates were seen. Blood vessels had normal morphology.

The myocardium of rats treated with doxorubicin and the combination doxorubicin plus cyclosporin A showed minimal morphological changes with edema in the interstitial space, slight vacuolization of myocytes, and sporadic focal inflammation. Necrosis or fibrosis was not observed. The maximal score according to Bristow *et al.*[21] was 1.5. Increased doses of doxorubicin revealed the same degree of damage, however, a greater percentage of rats in each group was affected with higher doses (DOX3 25%; DOX3 + CsA 50%; DOX4 75%; DOX4 + CsA and DOX6 100%).

Minimal hepatotoxic changes were demonstrated. In rats treated with DOX4 and

DOX6 mononuclear inflammation and spotty necrosis were observed with a reduced amount of glycogen (score 2+). Rats treated with the combination doxorubicin and cyclosporin A showed slight morphological changes (score 1+) with minor inflammation and sporadic necrotic hepatocytes.

Histological examination of the colon demonstrated edema in the mucosa of rats treated with doxorubicin and the combination doxorubicin plus cyclosporin A, while inflammation or necrosis was absent in this experiment. The lung parenchyma showed some focal inflammatory aggregates not related to the bronchial tree in rats treated with DOX4 and DOX6. The spleen showed slight hypoplasia of the white pulpa in the groups with DOX6 and the combination doxorubicin plus cyclosporine A. Pancreas and adrenal glands showed normal histology. The findings in colon, lung and spleen, however, were not observed consistently in all rats within the same treatment group, and differences between the groups were minimal.

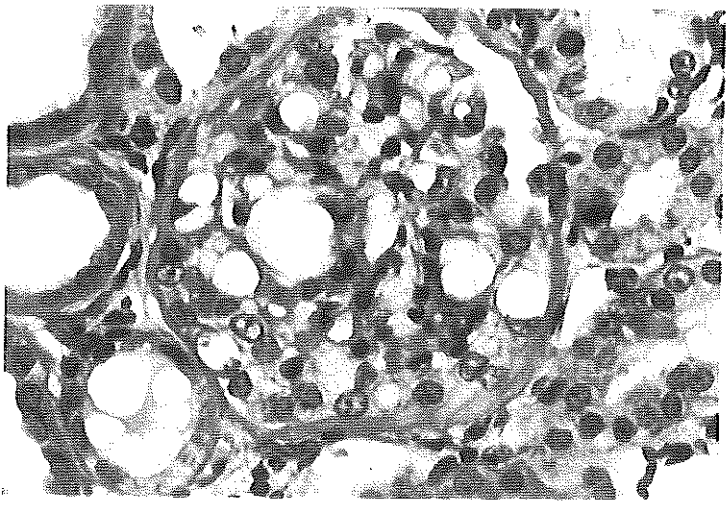

Figure 4. Nephrotoxicity caused by doxorubicin treatment: thickening of the basement membrane, mesangial hypercellularity, accumulation of lipids, and adhesion to Bowman's capsule. Original magnification 400x; hematoxylin and eosin staining

Discussion

The addition of the chemosensitizer cyclosporin A to the anticancer drug doxorubicin

clearly enhances its toxicity. In a previous study we demonstrated that the addition of cyclosporin A made a suboptimal dose of 3 mg/kg doxorubicin as effective as 4 mg/kg doxorubicin.[11] In the present study we show data (body weight change curves, hematological parameters, and autopsy findings) indicating that the combination of doxorubicin with cyclosporin A is about as toxic as a 25%-33% higher doxorubicin dose alone. We found therefore no therapeutic window, in contrast to Mickisch *et al.*[22] in their transgenic mouse model. They had to reduce the dose of most anticancer drugs by 20%, while these doses in combination with *D*-verapamil reduced MDR cell populations by 44%-78%. The results of the combination treatment were favourable compared to results with full doses of the drugs alone. Boesch *et al.*[23] reported similar favourable results of a combination treatment of vinblastine and doxorubicin with the cyclosporin A analogue PSC 833 in a survival model of mice with MDR tumors.

The toxicity pattern of doxorubicin is not altered by the addition of cyclosporin A. Doxorubicin, like most other cytotoxic agents, causes severe damage to cell-renewal systems, which are highly proliferative in postfetal life.[24] In our experiments the main acute side-effect was myelosuppression with significant leucopenia and thrombopenia. This effect was reversible. No signs of enterocolitis were observed. Unique toxic actions of anthracyclines, especially doxorubicin, are cardiovascular toxic effects, nephrotoxicity and toxic effects on the skeletal system. The last two effects can be observed in several experimental models, while cardiotoxicity is also found in humans.[25] In man cardiomyopathy leading to congestive heart failure is dependent on the total cumulative dose administered.[26] Our study was not designed specifically for studying the toxic effects of doxorubicin on cardiac tissue. The study was short-term, lasting only 35 days, and involved a single dose treatment schedule for doxorubicin, which made it more apt for studying acute toxic effects than chronic damage. Nevertheless, on microscopic examination mild damage to the cardiac tissue was observed in the most intensely treated rats. Other investigators have found severe cardiomyopathy in rats from 35 days onwards after administering multiple low doses of doxorubicin instead of a single high dose, and reaching higher cumulative values for doxorubicin than we did.[27,28] Results in studies with dogs and mice suggest that the addition of the chemosensitizer verapamil potentiates the cardiotoxic effects of doxorubicin.[29,30]

WAG rats proved to be very sensitive to the nephrotoxic effects of doxorubicin, eventually developing a full-blown nephrotic syndrome.[25,28] We were unable to measure proteins and renal parameters after 14 days because of disturbance of the assays caused by turbidity of the hyperlipidemic serum, but the ascites and pleural effusion

indicate hypoproteinemia. Levels of cholesterol and triglyceride were significantly raised by doxorubicin. Addition of cyclosporin A raised the levels of cholesterol and triglyceride even higher, in such a way that combination treatment produced the highest levels. Meanwhile, significant amounts of ascites and pleural effusion were only found in the two most intensely treated groups. This indicates that the nephrotoxic effects of doxorubicin were amplified by the chemosensitizer cyclosporin A.

Tissues with known high expression levels of P-glycoprotein, like liver, large intestine and adrenal gland, were monitored for toxic effects. No signs of major toxicity were observed. This suggests that these tissues are not susceptible to the cytotoxic effects of doxorubicin and that raising its intracellular concentration either by administering a higher dose or by adding a chemosensitizer, does not make these tissues sensitive to doxorubicin, despite the presence of P-glycoprotein. Other researchers have come to the same conclusion in a pathological study using the chemosensitizer *D*-verapamil and the drugs vinblastine, doxorubicin, and daunomycin.[22] In contrast, Horton *et al.*[31] found enhanced concentrations of vincristine in P-glycoprotein-expressing normal tissues, like small intestine, kidney and liver, caused by the addition of high doses of the chemosensitizer verapamil. Toxicity was enhanced eight-fold and symptomatic of vincristine-related neurotoxicity. They did not describe the functional and morphological effects of the raised concentration of intracellular vincristine in these normal tissues. Genne *et al.*[32] also reported enhanced doxorubicin accumulation in kidney and liver in combination treatment with the chemosensitizer amiodarone. Combination treatment accelerated doxorubicin-induced death. In clinical studies with chemosensitizers, however, no toxicities, apart from those attributable to the drug or the chemosensitizer, have been observed so far.

Cyclosporin A seems to produce few toxic effects in the concentrations used for chemosensitizing. In clinical trials steady state levels from 1000 μg/l up to 5000 μg/l were reported.[13,14,17,20] The cyclosporin A concentration of 1000 μg/l suffices *in vitro* for MDR reversal. Side-effects of cyclosporin A observed were an early and transient rise in serum bilirubin, without increases in liver enzymes, and hypomagnesemia.[13,14,20,33] In our rat study the transient hyperbilirubinemia appeared to be a purely cyclosporin A-dependent feature, which was not enhanced by the addition of doxorubicin. We found no evidence in our rat model for the hypothesis that bilirubin is raised as a consequence of competition between doxorubicin, cyclosporin A, and bilirubin at the excretion level, and thus might be used as a marker for P-glycoprotein modulation *in vivo*.[14,20]

From our studies with doxorubicin and cyclosporin it can be concluded that the addition of a chemosensitizer seriously enhances the toxic side-effects of the anticancer drug without altering the pattern of toxicity. As the toxicity patterns of anticancer drugs are known, side-effects can be anticipated in the planning of clinical trials. However, it remains unclear from this study whether therapeutic gains can be made by the application of a chemosensitizer.

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2.5

CYCLOSPORIN A ENHANCES LOCOREGIONAL METASTASIS OF THE CC531 RAT COLON TUMOR

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Summary

The immunosuppressive drug cyclosporin A has been evaluated recently in phase II trials in cancer therapy as a reverter of P-glycoprotein-mediated MDR. As an immunosuppressive agent, cyclosporin A potentially can enhance tumor growth. We investigated this potency of cyclosporin A in the weakly immunogenic CC531 colon adenocarcinoma model, using the same dose that had previously been shown to intensify the antitumor activity of doxorubicin *in vivo*. *In vitro* cyclosporin A caused no growth acceleration and only at high doses was growth inhibition of CC531 cells observed. *In vivo* no evidence of growth enhancement was found in short-term assays, but, after 4 weeks, rats treated with cyclosporin A had a significantly higher tumor load, mainly consisting of locoregional metastases. These experiments in the CC531 tumor model show that cyclosporin A, used as a reverter of MDR, may produce short-term improvement of antitumor activity but may also induce enhancement of tumor metastasis.

Introduction

Cyclosporin A is an immunosuppressive drug that is widely used in transplantation programmes. Since the introduction of cyclosporin A, graft survival and patient survival have increased considerably.[1] A new application in anticancer therapy is its use as a reverter of P-glycoprotein-mediated MDR. Cyclosporin A is an efficient blocker of the P-glycoprotein efflux pump, resulting in higher intracellular levels of drugs and enhanced cell death.[2] The efficacy has been shown in numerous *in vitro* and *in vivo* studies.[3-8] Compared to other modulators of MDR the potency of cyclosporin A is high.[3] In a clinical trial with refractory multiple myeloma, cyclosporin A, in combination with standard chemotherapy, resulted in improvement of the response rate. Additional studies showed that the effect was probably obtained by specific killing of the plasma cells expressing P-glycoprotein.[7,8]

By its immunosuppressive properties, however, cyclosporin A might also enhance growth of tumors that are susceptible to immunocompetent cells. It is known that any form of severe and sustained immunosuppression can lead to the development of certain cancers. This is a complication of the intensity of the immunosuppression and not a side-effect of certain agents.[9,10] In animals, rats and non-human primates, development of lymphoproliferative lesions, especially lymphomas, was seen after immunosuppressive doses of cyclosporin A.[11,12] In humans the incidence of lymphomas (all non-Hodgkin's type) under severe immunosuppression may be raised 28- to 49-fold.[13] An aetiological role for the Epstein-Barr virus is strongly suspected in these cases. Other tumors that are reported to have a raised incidence under immunosuppression are skin cancer of the squamous cell type, Kaposi's sarcoma, primary liver cell cancer, and, probably, carcinoma of the kidney and melanoma.[13,14] Little is known about the effect of immunosuppression on the *growth rate* of already-existing tumors in humans. A study in animals showed an increase of metastasis but not of growth of the primary tumor following treatment with cyclosporin A in immunogenic tumors.[15]

We investigated the effect of immunosuppression by cyclosporin A on tumor growth in the CC531 model. CC531 is a weakly immunogenic rat adenocarcinoma that expresses low levels of P-glycoprotein.[16,17] We have recently shown the efficacy of cyclosporin A as a modulator of drug resistance to doxorubicin *in vitro* and *in vivo* in this intrinsic MDR model.[6] In these short-term experiments no evidence of growth enhancement was observed. In the present study longer-lasting experiments are

presented that show tumor growth enhancement by cyclosporin A in the same dosage as used in the experiments with MDR modulation. These observations caution that the use of cyclosporin A in the clinical setting might induce similar tumor growth enhancement.

Materials and Methods

Animals

Male rats of the inbred WAG/RIJ ($RT1^u$) strain were obtained from Harlan-CPB (Austerlitz, The Netherlands). Animals were bred under specific-pathogen-free conditions and fed standard rat chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. In the experiments rats 10-18 weeks old, weighing 200-280 g, were used.

Tumor and cell line

CC531 is a colon carcinoma, which was induced chemically in the WAG rat with 1,2-dimethylhydrazine. The tumor, a moderately differentiated adenocarcinoma, is weakly immunogenic and transplantable in WAG/RIJ rats.[16] *In vitro* the cell line grows as a monolayer. CC531 is intrinsically MDR: at the mRNA level, expression of *mdr1a* has been detected by the polymerase chain reaction; Western blotting with the monoclonal antibody C219 shows P-glycoprotein expression;[17] MDR reverters can enhance intracellular drug accumulation and reduce drug resistance in cytotoxicity assays.[6,18] The cell line was grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, aspartic acid (0.1 m*M*), glutamic acid (0.3 m*M*), penicillin 111 IU/ml and streptomycin 111 μg/ml, all obtained from Gibco (Paisley, UK), in a humidified atmosphere of 5% CO_2/95% air at 37°C. Regular screening for *Mycoplasma* infection was performed. Cells were isolated by trypsinization; viability, determined by trypan blue exclusion, was over 90% in all experiments.

Chemicals

Cyclosporin A was obtained from Sandoz, Basel, Switzerland; MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma Chemical, St Louis, Mo., USA; and dimethylsulphoxide from Merck, Darmstadt, Germany.

In vitro *cytotoxicity assay*

Chemosensitivity *in vitro* was determined by the MTT assay, essentially carried out as described by Carmichael *et al.*[19] In brief, 5 x 10^3 trypsinized tumor cells/well in 100 µl complete medium were plated into 96-well flat-bottomed microtitre plates (Costar, Cambridge, Mass., USA). The plates were incubated for 24 h at 37°C, 5% CO_2/95% air to allow the cells to adhere. Then 100 µl medium, containing the test drug cyclosporin A in graded concentrations, was added. Cyclosporin A was dissolved in pure ethanol and diluted in complete medium. The concentration of ethanol in the test wells did not exceed 0.2%. After 4 days 30 µl MTT, dissolved in PBS at a concentration of 5 mg/ml, was added to each well. After an incubation period of 3.5 h the supernatant was carefully removed and 200 µl dimethylsulphoxide was pipetted into each well. Plates were placed in a microplate shaker for 5 min. The absorbance was read at 540 nm on an automated microplate reader (Titertek, Flow Laboratories Ltd., Irvine, Scotland). Cell survival was calculated using the formula: survival (%) = (test well/control) x 100. The drug concentration reducing the absorbance to 50% of the control (IC_{50}) was determined from the graph.

In vivo *assays*

For the intraperitoneal *in vivo* experiments viable pieces of a solid CC531 tumor, weighing 12-15 mg, were implanted in the fat flap of the testis. Four days after implantation treatment was started. Cyclosporin A was injected intramuscularly into the hind leg daily, on 3 consecutive days, at a dose of 20 mg/kg. Control rats received injections of the vehicle of cyclosporin A (olive oil and 6.25% ethanol). After 4 weeks, animals were killed and primary tumors and locoregional metastases were counted, enucleated and weighed. Groups consisted of 8 rats each. The experiment was repeated once.

Statistics

Statistical significance was determined with SPSS/PC+, using the Mann-Whitney *U*/Wilcoxon rank-sum *W* test. $P < 0.05$ was considered significant. Results are presented as means with standard deviations.

Results and discussion

In vitro, in the MTT assay, cyclosporin A had a growth-retarding effect on CC531 cells in concentrations above 2.5 *μM*. The IC_{50} of cyclosporin A was at approximately 6-7 *μM*; at 25 *μM* there was no evidence of surviving CC531 cells. In Fig. 1 a representative experiment is shown. Several other studies have shown that cyclosporin A is able to retard the growth of some tumor cell lines *in vitro*. Leukaemic T cells in particular, but not B cells, are sensitive to the inhibitory effects of cyclosporin A. This is compatible with the observations that cyclosporin A inhibits the T-cell-mediated immune reaction *in vivo*.[20] Some tumor cell lines of lung carcinoma and gastrointestinal tumors are also retarded in growth by cyclosporin A *in vitro* at concentrations similar to those used by us.[21-24]

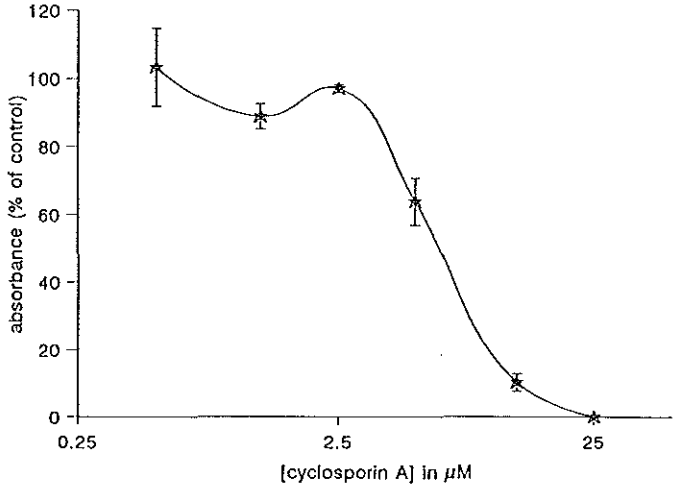

Figure 1. Dose/response curve of the cell line CC531 to incubation with graded concentrations of cyclosporin A, determined in the MTT cytotoxicity assay. MTT absorbance is expressed as a percentage of the control absorbance on the *y* axis and represents the percentage cell viability

In an earlier study we determined the cyclosporin A levels after intramuscular injection of 20 mg kg^{-1} day^{-1} for 3 consecutive days, a regimen that was also used in the intraperitoneal *in vivo* model in this study. This resulted in whole blood levels of 2520 (±240) ng/ml.[6] This level of cyclosporin A was able to raise the efficacy of doxorubicin on CC531 tumors grown for 10 days under the renal capsule and, therefore, cyclosporin A was judged to function as a reverter of MDR in this model. In those

studies, no signs of growth acceleration or retardation by cyclosporin A were observed. In the present study, tumors were implanted in the fat flap of the testes lying intraperitoneally, which gave the tumors more space to grow and enabled longer-lasting experiments. In this model, cyclosporin A enhanced the growth of CC531 (see Table 1). Tumors treated with cyclosporin A alone were larger than control tumors, a difference that was nearly statistically significant ($P=0.06$). The total tumor load was significantly increased. This was caused by increase of the number and size of the intraperitoneal metastases. These data concur with those of Eccles *et al.*[15] for immunogenic tumors. They showed that cyclosporin A exerts its effects via immunosuppression. The immunosuppressive treatment was only given for 3 days in our experiments.

Table 1. Tumor growth enhancement *in vivo* under cyclosporin A treatment

parameter	control	cyclosporin A	statistical significance, *P*
total tumor load (mg)	864 (±249)	1486 (±593)	0.02
primary tumor load (mg)	840 (±236)	1197 (±404)	0.06
metastases load (mg)	24 (±40)	289 (±376)	0.02
metastases number	1.0 (±1.4)	7.9 (±7.4)	0.01

Results are means with standard deviations in parentheses

In some *in vivo* studies, cyclosporin A retarded the growth of tumors, like the murine colon cancer MC26.[23] In human studies cyclosporin A has been used with variable success in some hematological malignancies. Promising results have been described in cutaneous T cell disease (the Sézary syndrome) and in Hodgkin's lymphoma.[25,26] No objective responses were observed in a clinical trial involving 17 patients with colorectal cancer.[27]

Tumor growth in phase I/II oncological studies is considered as primary resistance to the experimental drug. Therefore, tumor growth enhancement by an experimental drug can remain unnoticed in these studies. As an immunosuppressive agent, cyclosporin A potentially can accelerate growth of tumors that are restrained in their growth by the immune system. Although experimental tumors in animals are more often immunogenic than are human tumors, the immune system may play a beneficial role in various

human malignancies, like renal cell cancer and malignant melanoma. In clinical trials these tumors, and to a lesser extent colorectal cancer, responded to treatment with interleukin-2 and lymphokine-activated killer cells in up to 30% of the cases.[28] In these trials parts of the immune system are activated that are suppressed by cyclosporin A. There are no studies on a potential tumor-growth-enhancing effect of immunosuppression on existing tumors in humans. Only one study has measured the effect of cyclosporin A on T cell levels in the setting of MDR reversal. Significant decreases in total lymphocyte counts and in CD19, DC3, DC4 and CD8 subpopulations were observed, which were totally reversible.[29]

Our experiments in an animal model show that a short course of cyclosporin A administration that was able to modulate MDR was also able to enhance the locoregional metastatic growth of the weakly immunogenic CC531 colon carcinoma. It is important to realise that, with the novel utilisation of cyclosporin A as a reverter of MDR in cancer therapy, growth enhancement might be induced in some tumors. Non-immunosuppressive analogues of cyclosporin A, such as PSC 833, or other compounds that are at least equally potent reverters of MDR should, therefore, be preferred in anticancer chemotherapy.

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2.6

DRUG RESISTANCE IN RAT COLON CANCER CELL LINES IS ASSOCIATED WITH MINOR CHANGES IN SUSCEPTIBILITY TO CYTOTOXIC CELLS

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Summary

The development of resistance to anticancer drugs urges the search for different treatment modalities. Several investigators have reported the concomitant development of drug resistance and resistance to natural killer (NK), lymphokine-activated killer (LAK) or monocyte/macrophage cell lysis, while others described unchanged or even increased susceptibility. We investigated this subject in the rat colon carcinoma cell line, CC531-PAR, which is intrinsically MDR, and in three sublines derived from this parental cell line: a cell line with an increased MDR phenotype (CC531-COL), a revertant line from CC531-COL (CC531-REV) which demonstrates enhanced sensitivity to anticancer drugs of the MDR phenotype, and an independently developed cisplatin-resistant line (CC531-CIS). In a 4 h ^{51}Cr-release assay we found no differences in susceptibility to NK cell lysis. No significant differences in lysability by adherent LAK (aLAK) cells were observed in a 4 h assay. In a prolonged 20 h ^{51}Cr-release assay an enhanced sensitivity to aLAK cell-mediated lysis was observed in the revertant, P-glycoprotein-negative cell line and in the cisplatin-resistant cell line (CC531-CIS). None of the cell lines was completely resistant to lysis by aLAK cells. Therefore, a role for immunotherapy in the treatment of drug-resistant tumors remains a realistic option.

Introduction

One of the major problems in cancer chemotherapy is the development of resistance to drugs. Several mechanisms of drug resistance have been elucidated; some operate against a particular drug, while others affect a group of structurally unrelated anticancer agents, as in MDR. Doxorubicin, vincristine and etoposide are examples of drugs subjected to the MDR mechanism. MDR cells express an efflux pump, P glycoprotein, which expels anticancer drugs from the cells. Expression of the multidrug transporter can be demonstrated by anti-P-glycoprotein monoclonal antibodies, or at the DNA and RNA level by blotting techniques.[1] Resistance to a specific drug may be caused by several mechanisms. For instance, resistance to cisplatin is related to reduced drug accumulation, increased detoxification, and increased DNA repair.[2] The development of resistance to anticancer drugs urges the search for alternative treatment modalities, for example immunotherapy.

Adoptive immunotherapy using interleukin-2 (IL-2) and cytotoxic cells (lymphokine activated killer (LAK) cells) has proven to be effective in renal cell cancer. Response rates up to 35% have been reported. In melanoma, responses up to 21% were found. Response rates in colon carcinoma were lower (13%).[3] All these tumors are very resistant to currently available drugs. Of these tumors, renal cell cancer and colon carcinoma intrinsically express the MDR phenotype at a high frequency.[4]

Before immunotherapy can be used as an alternative treatment after failure of chemotherapy, it is important to know whether there might be a correlation between drug resistance and sensitivity or resistance to immunotherapy. Reports in the literature have yielded conflicting data about a possible correlation between drug resistance, especially MDR, and resistance to NK and LAK cell lysis.[5,6] We investigated this subject in four cell lines of a rat colon carcinoma with different mechanisms and levels of drug resistance. In our model drug resistance was not associated with changes in sensitivity to NK-cell-mediated lysis. Only minor alterations in sensitivity to IL-2/adherent LAK (aLAK) cell lysis were observed.

Materials and methods

Animals

Male rats of the inbred WAG/RIJ ($RT1^u$) strain were obtained from Harlan-CPB

(Austerlitz, The Netherlands). Animals were bred under specific-pathogen-free conditions and fed standard rat chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. In the experiments, rats of 12-18 weeks old, weighing 220-280 g, were used.

Cell lines

CC531 is a rat colon adenocarcinoma, which was induced chemically in the WAG rat with 1,2-dimethylhydrazine. The moderately differentiated tumor is weakly immunogenic, as determined by the method described by Prehn and Main,[7] and transplantable in syngeneic rats.[8] Following subcutaneous implantation and subsequent resection, the tumor metastasizes to the lungs. *In vivo* the tumor is resistant to most anticancer drugs and only at the maximal tolerable dose were significant growth-retarding effects observed.[9] (and unpublished observations) Cisplatin was reported to be one of the most effective drugs against CC531.[9] Moderate sensitivity to immunotherapeutic agents like interferon-γ and tumor necrosis factor-α *in vitro* and *in vivo* has been shown in previous studies.[8,10-12] *In vitro* CC531, the parental cell line (CC531-PAR), grows as a monolayer in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal calf serum, L-aspargine (50 mg/l), glutamic acid (2 m*M*), 100 IU/ml penicillin and 100 μg/ml streptomycin, all obtained from Gibco (Paisley, UK), in a humidified atmosphere of 5% CO_2/95% air at 37°C. CC531-PAR intrinsically expresses the MDR phenotype. We recently reported the reversibility of MDR *in vitro* and *in vivo* in this model.[13] *In vitro* drug-resistant sublines were induced by continuous incubation with colchicine (CC531-COL) and cisplatin (CC531-CIS). Established cell lines were maintained in the presence of 0.2 μM colchicine and 0.75 μM cisplatin (*cis*-diaminedichloroplatinum) respectively.[14] Compared to the parental line, CC531-COL shows enhanced resistance to drugs of the MDR phenotype (colchicine 33-fold, daunorubicin 10-fold) and to cisplatin (5.5-fold). CC531-CIS is resistant to cisplatin (9.8-fold), while it has approximately the same sensitivity to other drugs as the parental line. From CC531-COL a revertant line (CC531-REV) was isolated that is more sensitive to drugs of the MDR phenotype than the parental line (resistance to colchicine 0.6-fold, to daunorubicin 0.25-fold), but the resistance to cisplatin is maintained (4-fold) as in the CC531-COL line.[14,15] See Table 1 for characteristics.

YAC-1, a mouse T cell lymphoma, sensitive to NK cell lysis, was used as a positive control in the NK cell experiments. P815, a mouse mastocytoma, NK cell lysis resistant but sensitive to LAK cell lysis, was used as a negative control in NK cell experiments and as a positive control in aLAK cell cytotoxicity tests. Both cell lines were grown in

Table 1. Characteristics of the CC531 cell lines

characteristics	CC531-PAR	CC531-COL	CC531-REV	CC531-CIS
RF[a]-daunorubicin	1	10	0.25	0.2
RF-colchicine	1	33	0.6	0.94
RF-vinblastine	1	4.4	0.3	1.0
RF-cisplatin	1	5.5	4	9.8
SR[b]-verapamil	6.6	31.6	2.0	5.0
daunorubicin-accumulation[c]	100	64	102	nd[d]
P-glycoprotein expression[e]	+	+ +	±	nd

[a] RF = resistance factor. Drug sensitivity was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric cell growth-inhibition-assay for different drugs. In the table the relative sensitivity of the cell lines compared to the parental line is given. The sensitivity of CC531-PAR is arbitrarily defined as 1

[b] SR = sensitization ratio. The daunorubicin concentration that causes 50% growth inhibition (IC_{50}) in the MTT assay divided by the daunorubicin concentration in the presence of 6.6 μM verapamil that reaches the IC_{50}

[c] The percentage intracellular fluorescent daunorubicin determined in an accumulation assay by flow cytometry. The fluorescence of daunorubicin in the parental line is defined as 100%

[d] nd = not determined

[e] P-glycoprotein expression in cells was determined by flow cytometry with the monoclonal antibody C219. The immunofluorescence staining is indicated with + + + for bright staining, + + for clear, + for moderate, ± for feeble, and - for no staining

suspension in RPMI-1640 medium, (Dutch modification; Gibco) supplemented with 10% fetal calf serum, 2 m*M* glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Cell lines were grown in drug-free medium for at least 72 before being used in tests. Adherent growing cells were isolated by trypsinization. Viability, determined by trypan blue exclusion, was more than 90% in all experiments (85% for YAC-1 and P815 cells). Regular screening for *Mycoplasma* infection was performed.

The NK and aLAK cell experiments were conducted in complete RPMI medium (10% fetal calf serum) without HEPES buffer. Complete LAK medium for the generation of aLAK cells consisted of complete RPMI medium plus 1000 EuroCetus units IL-2/ml and 50 μM 2-mercaptoethanol.

Chemicals

2-Mercaptoethanol was obtained from J. T. Baker, Deventer, The Netherlands; interleukin-2 (IL-2) from EuroCetus, Amsterdam, The Netherlands; 1% sodium dodecyl sulphate from Merck, Darmstadt, Germany; sodium [^{51}Cr]chromate from Amersham, Aylesbury, UK.

Preparation of effector cells

Spleens were removed aseptically from the rats and crushed with the hub of a syringe in complete medium. Spleen cells were incubated for 5 min at 37°C in a buffered ammonium chloride solution to lyse the erythrocytes. For the NK cytotoxicity tests cells were rested for 1 h in complete medium at 37°C in 25 cm^2 culture flask (Costar, Cambridge, Mass., USA) to remove the macrophages by adherence to the plastic. The remaining free-floating cells were aspirated, counted, and added to 96-well round-bottomed microtiter plates (Costar). In the NK cell lysis tests effector:target (E:T) cell ratios were 200:1, 100:1, 50:1 and 25:1.

For the aLAK cell cytotoxicity tests cells were passed over nylon-wool columns to remove monocytes/macrophages and B cells.[16] Samples containing 2x10^8 spleen cells were added to a syringe containing 0.6 g sterile nylon wool (Cellular Products, Buffalo, N.Y., USA) and incubated for 1 h at 37°C. The nonadherent cells were carefully washed out with 50 ml medium. These cells were cultured at a concentration of 2x10^6 cells in 75 cm^2 culture flasks in LAK medium for 24 h. Then only the cells adherent to the plastic of the flasks were cultured further in conditioned medium to make the aLAK cell bulk culture. Conditioned medium was prepared by decanting the medium from the flasks, removing the nonadherent cell by centrifugation and passing the supernatant through a 0.45-μm Millipore filter.[17] After 72 h all cultured cells were collected; the adherent cells by adding EDTA and scraping the flask with a rubber policeman. In the aLAK cell lysis experiments E:T cell ratios were 50:1, 25:1, 12.5:1 and 6.25:1.

Cytotoxicity assay

Sensitivity to NK and aLAK cells was tested in the ^{51}Cr-release cytotoxicity assay. Samples containing 1x10^6 target cells were incubated for 1 h with 200 μCi ^{51}Cr in 200 μl medium. Cells were washed three times with complete medium and counted, and 1x10^4 cells in 100 μl complete medium were added to the effector cells (100 μl) in the plates. Spontaneous release was tested in wells containing target cells and medium (100 μl) only; maximal release was obtained by adding 100 μl 1% sodium dodecyl

sulphate to target cells. Tests were performed in triplicate and all tests were repeated at least twice. Plates were incubated for 4 h at 37°C. The supernatant was harvested using the Skatron supernatant collection system (Skatron, Lier, Norway). The release of ^{51}Cr was determined by counting radioactivity in a gamma counter (LBK Wallace Ultragamma II 1280, Wallace Instruments, Stockholm, Sweden). The percentage specific cytotoxicity was calculated by the following formula:

specific cytotoxicity (%) = 100 x [(experimental release - spontaneous release)/(total release - spontaneous release)].

Lytic units (LU) were calculated according to the method described by Pross *et al.*[18] The calculations of lytic units and of statistical significance were done on the compilations of different tests. In figures, representative experiments are shown.

Statistics

Statistical significance of the results was determined at the highest E:T ratios: 200:1 for NK cell lysis and 50:1 for aLAK cell lysis. The Student *t*-test (paired *t*-test) was used and a *P* value of less than 0.05 was considered significant.

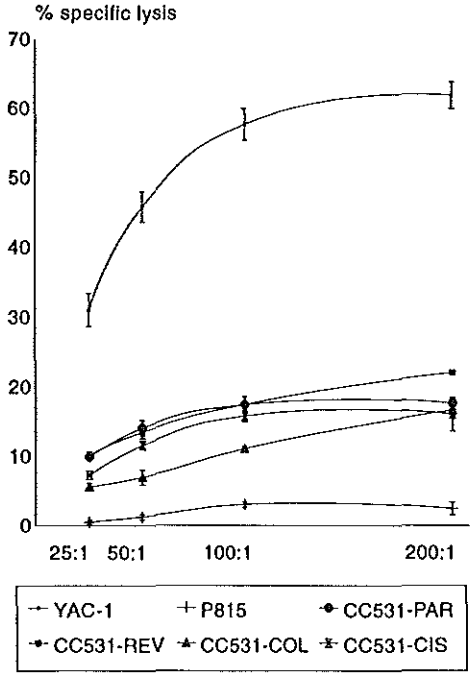

Figure 1. Natural killer (NK) cell lysis determined in a 4-h ^{51}Cr release assay. Mean specific lysis by NK cells of YAC-1 (positive control), P815 (negative control) and the cell lines CC531-PAR, CC531-REV, CC531-COL and CC531-CIS at E:T ratios of 25:1, 50:1, 100:1 and 200:1 is presented in the graph. *Error bars* are standard errors of the mean

Results

NK cells of the WAG rat were highly effective against the NK-sensitive YAC-1 cell line; up to 60% lysis was observed. The NK-resistant cell line P815 was not lysed at all: less than 5% lysis was recorded at the 100:1 and 200:1 E:T cell ratios. The CC531 cell lines were rather resistant to NK cell lysis. At the highest (200:1) E:T cell ratio 15%-22% specific lysis was found. See Fig. 1. There were no significant differences in sensitivity between the CC531 cell lines.

We used aLAK cells instead of LAK cells because the adherent cells were proven to be the most effective cytotoxic cells in bulk cultures with IL-2.[17] A representative experiment for the lysis by aLAK cells in a standard 4 h ^{51}Cr-release assay is shown in Fig. 2. Maximal lysis of P815 was more than 60%, while lysis of the CC531 cell lines did not exceed 20%. At the maximal E:T ratio of 50:1 only minor differences in lysis were observed between some cell lines. CC531-CIS was significantly more sensitive to aLAK lysis compared to the least lysable cell line CC531-COL.

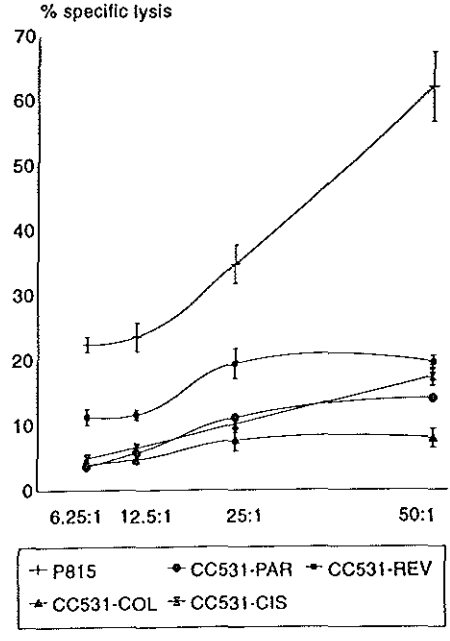

Figure 2. Adherent lymphokine-activated killer (aLAK) cell lysis determined after 4 h. Mean specific lysis (± sem) by aLAK cells of P815 (positive control) and the cell lines CC531-PAR, CC531-REV, CC531-COL and CC531-CIS at E:T ratios of 6.25:1, 12.5:1, 25:1 and 50:1 is presented in the graph. Statistical significance at the 50:1 E:T ratio was found between CC531-CIS and CC531-COL (P = 0.019)

As there were indications that the resistance to lysis by cytotoxic cells could be overcome by prolonging the incubation period, we extended the aLAK tests to 20 h. In

these prolonged tests P815 cells appeared to be fragile as the spontaneous release was more than 25%. Specific lysis of P815 by aLAK cells was not enhanced by longer exposure to the cytotoxic cells in comparison with 4 h exposure. In all CC531 cell lines, spontaneous release after 20 h was about 20%; CC531-PAR: 19.7 ± 2.6% (sd), CC531-COL: 19.8 ± 3.7%, CC531-REV: 20.1 ± 7.3%, and CC531-CIS: 20.6 ± 6.0%. In this prolonged assay none of the CC531 cell lines proved resistant to lysis by aLAK cells; specific lysis was more than 25% in all cell lines. Differences in sensitivity between the sublines became clearer (Fig. 3). CC531-COL appeared the most resistant line, and at the 50:1 E:T ratio, less than 30% lysis was found. CC531-REV and CC531-CIS proved to be the most lysable with more than 50% lysis after 20 h. The differences between CC531-REV and CC531-COL, and CC531-CIS and CC531-COL were significant. The same significant difference was found for CC531-REV compared to CC531-PAR, while CC531-CIS was found significantly more sensitive to aLAK lysis than the parental line in three out of four tests, but not in the compilation of four tests. Lytic units as values of cytotoxicity show the same order of sensitivity to aLAK-cell-mediated lysis in the 20-h assay, but differences between the cell lines are small. See Table 2.

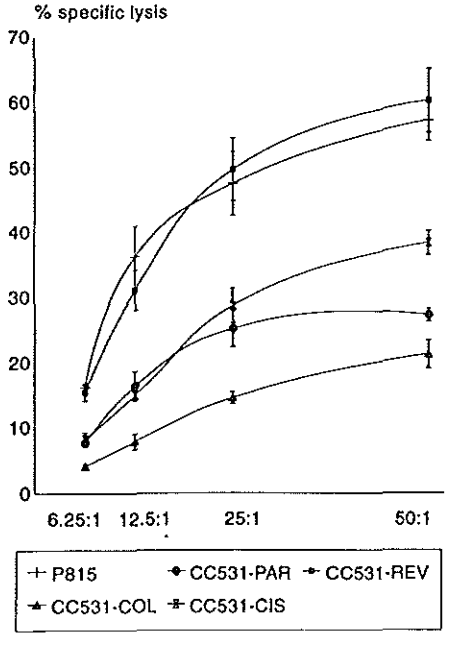

Figure 3. Adherent lymphokine-activated killer (aLAK) cell lysis determined after 20 h. Mean specific lysis (± sem) by aLAK cells of P815 (positive control) and the cell lines CC531-PAR, CC531-REV, CC531-COL and CC531-CIS at E:T ratios of 6.25:1, 12.5:1, 25:1 and 50:1 is presented in the graph. Statistically significant differences at the 50:1 E:T ratio: CC531-CIS versus CC531-COL, $P = 0.023$; CC531-REV versus CC531-COL, $P = 0.002$; CC531-REV versus CC531-PAR, $P = 0.02$

Table 2. Activity of natural killer and adherent lymphokine-activated killer (aLAK) cells

Cell line	Activity (LU/10^6 cells)*		
	NK	aLAK 4 h	aLAK 20 h
YAC-1	5.5		
P815	0	10.4	21.7
CC531-PAR	0	0	10.1
CC531-REV	0.6	3.2	13.9
CC531-COL	0.3	0	7.1
CC531-CIS	1.5	6.0	14.4

* Lytic units/10^6 effector cells based on 15% lysis

Discussion

CC531 cells appeared to be rather resistant to lysis by cytotoxic cells. NK cell lysis was below 22% in all cell lines even at an E:T ratio of 200:1. No differences in specific lysis were observed between the CC531 cell lines. Lysis by IL-2 induced cytotoxic cells, aLAK cells, still was very low at a 50:1 E:T ratio, but some difference between the cell lines became apparent. We studied these differences in a prolonged assay. None of the cell lines was found to be completely resistant to aLAK lysis. The most drug sensitive cell line, CC531-REV, with the least expression of P-glycoprotein was the most sensitive to aLAK lysis, while the most drug resistant cell line, CC531-COL, seemed the most resistant to aLAK lysis.

So far conflicting results have been reported about a possible correlation between drug resistance and sensitivity to NK cell lysis. Various reports about doxorubicin-induced drug resistance showed enhanced resistance to NK-cell-mediated lysis in doxorubicin-resistant sublines.[19-22] In MDR cell lines, both no change in sensitivity,[23-26] and also enhanced sensitivity to NK cell lysis compared to the parental cell lines has been described.[27] In contrast, Woods *et al.* reported diminished NK sensitivity of an MDR subline with enhancement of sensitivity in a revertant cell line.[28] Treichel *et al.* only found this relationship for MDR cell lines that express P-glycoprotein, and not for non-P-glycoprotein MDR cell lines.[29]

With regard to LAK cell sensitivity, various studies show no difference in lysability between drug-resistant cell lines and their parental lines.[22,24-26,30-32] Enhanced sensitivity as well as enhanced resistance of MDR cell lines to LAK cell lysis has been reported by others.[27,29,32-35] An inverse relationship has also been found: the induction of LAK resistance in melanoma cell lines rendered these more sensitive to doxorubicin.[36] Kimmig *et al.* found that LAK resistance correlated with the level of P-glycoprotein expression. A revertant cell line that was as drug sensitive as the parental line was found to be as lysable by LAK cells as the parental cell line.[35] We did a comparable observation with the CC531 cell lines in the prolonged aLAK lysis assay.

Explanations for the phenomenon of differences in sensitivity to cytotoxic cells between drug-sensitive and drug-resistant cells have been sought in differences in the expression of cell membrane molecules. In most studies no correlation between MDR expression, NK or LAK cell resistance and expression of MHC class I and II antigens was observed.[26,33,37] With regard to adhesion molecules a correlation between enhanced LAK cell lysis and ICAM-1 and LFA-3 expression in MDR cell lines was described,[37] but other authors could not confirm these results using other cell lines.[26]

To our knowledge only few reports about the association between cisplatin resistance and NK/LAK cell resistance have been published. In most experiments no influence of cisplatin resistance on sensitivity to LAK-cell-mediated lysis was observed.[25,30,31,36] In one of their cisplatin-resistant cell lines Ohtsu *et al.* found enhanced sensitivity to LAK cell lysis.[31] Allevana *et al.* reported significant lysis by LAK cells of freshly isolated tumor cells from ovarian cancer patients that were refractory to chemotherapy with cisplatin.[30] In a recent article short-term pretreatment of cancer cells with cisplatin was reported to render these cells more sensitive to cytotoxic cells.[38] We observed enhanced lysis by aLAK cells of the cisplatin-resistant cell line CC531-CIS. In CC531-COL and CC531-REV cells a moderate resistance to cisplatin was found, about half the resistance of CC531-CIS. In these two cell lines an association between their MDR phenotype and LAK cell resistance can be supposed, while the resistance to cisplatin seems of no importance. It is very possible that the colchicin induced MDR cell line CC531-COL and its revertant CC531-REV have a different mechanism of cisplatin resistance from CC531-CIS, as this resistance was induced differently.

It is clear from our results and those of others that basic research has not yet provided coherent data on the relationship between drug resistance and sensitivity to immunotherapy. The lack of coherence in the results may be inherent to the different

cell lines and the diversity of methods used to induce resistance. Possibly this reflects the heterogeneity of sensitivity and resistance to drugs and immunotherapy in the clinical situation. Our results seem to indicate that drug resistance does not preclude the use of immunotherapy with IL-2 and LAK cells, but enhancement of the efficacy of immunotherapy is necessary. With regard to P-glycoprotein-expressing MDR tumors, an attractive approach might be to turn the strength of MDR cells into their weakness. This can be done by using antibodies against the multidrug transporter P-glycoprotein. Immunotoxin therapy using the anti-P-glycoprotein antibody MRK16 coupled to *Pseudomonas* exotoxin has been shown to be effective against MDR cells *in vitro* and *in vivo*.[39-41] Another possible method of targeted immunotherapy is the use of bispecific monoclonal antibodies directed against P-glycoprotein on one hand and against an antigen on cytotoxic cells on the other.[42-43] Further research in this direction is warranted.

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3

DISCUSSION AND SUMMARY

3.1 GENERAL DISCUSSION

Our studies go back to 1990 when the mechanism of P-glycoprotein-mediated MDR had only recently been elucidated. The feasibility of blocking the P-glycoprotein efflux pump, thereby increasing intracellular drug levels and enhancing cell death had been shown in numerous *in vitro* studies, but few studies had shown the possibilities of MDR reversal *in vivo*. At this point we started collaborative investigations with the group of Prof. Dr. A.T. Van Oosterom and Dr. E.A. De Bruijn of the Laboratory of Cancer Research and Clinical Oncology of the University of Antwerp in Belgium. They had developed several drug-resistant cell lines from the CC531 rat colon adenocarcinoma, among which two MDR cell lines.[1] Their work was the basis on which further investigations *in vivo* could be done. Major contributions from their part were the phenotypic characterization of the CC531 cell lines by *in vitro* cytotoxicity tests and drug accumulation studies, and genotyping of the cell lines for drug resistance markers.[1-3]

Our part of the investigations dealt with the development of *in vivo* models to test the feasibility of MDR reversal. It appeared not possible to obtain consistent *in vivo* growth of the sublines of CC531 that had been manipulated by cytotoxins *in vitro*. The cell line CC531-COL which has an increased MDR phenotype, had a tumor take rate of over 80%, but its growth pattern was not consistent: some tumors regressed, while others continued to grow. A revertant cell line of CC531-COL, CC531-REV, which showed a diminished level of MDR, even compared to the parental CC531 line, had a more consistent tumor take and growth pattern. However, the differences in sensitivity to drugs like doxorubicin between CC531 and CC531-COL were too small to obtain discriminating results. The cell line manipulated with cisplatin, CC531-CIS, non-MDR, was not tumorigenic at all. (W. Van de Vrie, R.L. Marquet and A.M.M. Eggermont, unpublished observations)

We have used the parental CC531 cell line and tumor as a model for an intrinsic MDR expressing solid cancer. The CC531 tumor appeared to express P-glycoprotein, shown at protein and gene level, and to exhibit the MDR phenotype (see chapter 2.1 and 2.2).[1,3] The level of P-glycoprotein expression is low compared to that in the induced MDR cell line 2780^{AD}, but closely resembles the situation in humans, which contributes to the relevance of the model. A tumor cell line without a control cell line is not an ideal model as only effects between different treatments can be compared and results can not be validated by comparison with another, non-MDR cell line. A model

like the CC531 intrinsically MDR tumor remains however useful to study various aspects of drug resistance.[4] In chapter 1.2, we argued that an ideal model should be an orthotopically growing tumor that represents a frequently occurring human malignancy, expresses low levels of P-glycoprotein and allows serial measurement of tumor burden. Further, the model should consist of a parental and a drug-resistant subline, only differing in the expression of P-glycoprotein and not with respect to other resistance mechanisms and growth characteristics. No such model exists at the moment. Gottesman *et al.* have described the advantages and disadvantages of various *in vivo* models, and shown their value at various stages of research of MDR.[4]

We have used the CC531 tumor as a model to test the feasibility of reversal of intrinsic MDR in a solid tumor *in vivo* and study the side-effects of combined treatment. We observed a clear enhancement of doxorubicin efficacy *in vivo* against the CC531 tumor by addition of the reverter cyclosporin A (chapter 2.1), while with dexniguldipine a nearly significant result was obtained (chapter 2.2). We have no direct proof that the modulation of MDR in the CC531 tumors was obtained by blocking of P-glycoprotein at the tumor level. It can not be excluded that pharmacokinetic interactions at the level of drug metabolism and elimination have contributed to the increase in doxorubicin activity, as we did not measure levels of doxorubicin in blood and tumors. The fact that in toxicity experiments (chapter 2.4) the addition of the reverter cyclosporin A clearly increased the known doxorubicin-related toxicity points in this direction. Others have found evidence for specific modulation of MDR at the tumor level.[5] The efficacy of MDR reversal in our experiments was moderate. No disappearance of the tumors was observed. We have not searched for an optimal dosing schedule and possibly more efficient MDR reversal could have been obtained by repeated dosing. Increased efficacy might also be obtained by more potent reverters of MDR.

The plasma levels of the reverters in our experiments were assessed to be adequate for reversal of MDR, as they were much higher than effective levels *in vitro*. It has recently been reported that the efficacy of reverters *in vitro* is considerably lower in the presence of serum proteins, to which these compounds are bound *in vivo*.[6] Our *in vitro* experiments had not been carried out in serum-free conditions, and are therefore not subject to this bias. Moreover, we measured tumoral levels of the reverters, which were about 6-fold higher for cyclosporin A and over 10-times higher for dexniguldipine compared to plasma levels (chapter 2.1 and 2.2). It should also be mentioned that levels of dexniguldipine in normal kidney tissue were even higher, possibly due to differences in lipophilicity between the tissues (chapter 2.3). Differences in levels of

reverters in various tissues may contribute to efficacy and toxicity of MDR reversal.

Using a first generation chemosensitizer, cyclosporin A, in combination with doxorubicin we observed that the known side-effects of doxorubicin were enhanced (chapter 2.4). In tissues that were not affected by the toxic effects of doxorubicin, no evidence of enhanced damage caused by coadministration of the reverter was observed, irrespective of P-glycoprotein expression in the tissues. Recently, new data have been published on this subject showing that P-glycoprotein-dependent toxicity can be enhanced by stronger reverters than cyclosporin A. With new potent chemosensitizers like PSC 833 and SDZ 280-446, but not with cyclosporin A, it was shown that P-glycoprotein at the blood-brain barrier could be blocked, leading to neurotoxicity of coadministered ivermectin.[7] PSC 833 also enhanced the brain penetration of vincristine.[8] On one hand this shows the limits for reversal in the clinical situation, as unwanted neurotoxicity and other new side-effects are inherent to the use of potent reverters. On the other hand this may open novel ways to treat tumors and metastases in sanctuaries by opening blood-tissue barriers.

In later experiments we observed an important side-effect of cyclosporin A on the CC531 tumor, which was enhancement of tumor growth, especially locoregional metastasis (chapter 2.5). This is likely mediated by the weakly immunogenic character of the tumor. Human tumors are less immunogenic than experimental tumors in animals, and, therefore, this observation has possibly not much relevance for the situation in humans. But, in tumors like malignant melanoma, renal cell cancer, and, possibly, colorectal cancer a beneficial role of the immune system has not been ruled out.

Clinical trials with chemosensitizers have shown the feasibility of modulation of MDR in hematological malignancies, like multiple myeloma and acute myeloid leukemia.[9-11] Attempts to modulate MDR in solid tumors have yielded disappointing results.[12] *E.g.* in clinical trials in colorectal cancer with the combinations epidoxorubicin plus cyclosporin A and vinblastine plus bepridil only one partial response was obtained in 39 treated patients.[13,14] The levels of the chemosensitizer reached in patients were at least as high as effective levels in *in vitro* studies, and comparable to those used in the clinical studies in hematological malignancies. The only favourable MDR reversal study in solid tumors is a recently published study on intraocular retinoblastoma. Neuroblastoma is normally responsive to chemotherapy and P-glycoprotein expression is a negative prognosticator for response to drug treatment.[15] In this phase I/II trial the addition of

cyclosporin A to the therapeutic regimen of vincristine, teniposide, and carboplatin enhanced its efficacy compared to historical controls.[16] The mere fact that a benefit was shown as compared to results reported in historical controls must caution against too much optimism.

There are some possible explanations for the resistance of solid tumors, especially those with intrinsic P-glycoprotein expression, to MDR modulation. At first, the solid nature of the tumor makes it more difficult for drugs to reach all cells, especially those in the centre of the tumor that are less well perfused and here conditions may exist that are unfavourable for the activity of cytotoxic drugs. Therefore, despite adequate levels in blood, this may still be inadequate to modulate drug resistance effectively. Secondly, it is possible that P-glycoprotein-mediated MDR is not a major drug resistance mechanism in solid tumors and that other mechanisms play a more important role. The significance of MDR expression in colorectal cancer is also questioned by the fact that *MDR1* in tumors is not upregulated as *MDR1* levels do not differ significantly between normal tissue and carcinomas in colorectal tissues.[17] Thirdly, it is striking that trials with reverters have only proven effective in tumors that are usually sensitive to anticancer drugs, while results in trials with intrinsic chemoresistant tumors were negative. There is no evidence from basic research that intrinsically expressed MDR is qualitatively different from induced P-glycoprotein, provided no mutations have occurred. Probably, in induced MDR tumors the P-glycoprotein mechanism is upregulated as a last defense, that may help the tumor cells to survive. This mechanism is for example active in lymphomas where increase in MDR expression levels has been shown by sequential biopsies during chemotherapy with epidoxorubicin.[18] In intrinsically MDR tumors like colorectal cancer current chemotherapy is far from effective and the blocking of P-glycoprotein alone is not sufficient to render these cells drug-sensitive. Therefore, it is likely that MDR reversal will only be effective in tumors that are almost sensitive to chemotherapy.

Some other items deserve discussion. In tumors for which an effective chemotherapy is available even a small drug-resistant subpopulation may already determine treatment failure. It has been shown that P-glycoprotein expressing cells in numbers as low as 1%-5% are a risk factor for refractory disease in acute myeloid leukemia.[19] Possibly, early intervention by adding a reverter to the cytotoxic regimen from the start of treatment may prevent the selective outgrowth of MDR subclones. Reverters of MDR may also prevent the activation or upregulation of MDR. In an *in vitro* study in which

drug resistance was induced by doxorubicin, the addition of the reverter PSC 833 suppressed the emergence of *MDR1* mutants.[20]

P-glycoprotein-mediated MDR is only one of the mechanisms tumor cells exploit to defend themselves against toxic insults. Various other types of drug resistance active against more than one group of cytotoxins have been detected: another multidrug transporter named the MDR-associated protein (MRP); the major vault protein LRP which probably works by vesicular sequestration of drugs; alterations in drug targets such as DNA topoisomerase II expression and activity; increased detoxification of compounds, e.g. by the glutathione system; and dysfunction of the genes involved in apoptosis: apoptosis-MDR.[21] In most tumors various mechanisms contribute to clinical drug resistance. Will chemotherapy be successful in these tumors, all mechanisms that contribute to the resistance to cytotoxins will have to be identified and circumvented. It is to be expected, that in the future apart from the histopathological typing of a tumor, a typing of drug resistance mechanisms will be performed on each tumor before and during chemotherapeutic treatment and at relapse. Reverters of the drug resistance mechanisms that are active, or drugs that are not susceptible to those resistance mechanisms can than be added to the therapeutic regimen. Future research has to show whether this ideal will ultimately be attainable without intolerable side-effects. For this goal new *in vitro* and *in vivo* models have to be developed in which several drug resistance mechanisms are active and the feasibility of reversal of more than one drug resistance mechanism can be investigated.

P-glycoprotein-mediated MDR is an important drug resistance mechanism which has been elucidated by intensive basic research over the last 10-15 years. Although current data on the significance of P-glycoprotein expression in various tumors and on MDR reversal in patients indicate that few patients will benefit from the addition of an MDR reverter to the cytotoxic regimen, its discovery has had an enormous impact in oncology. The detection of the MDR mechanism and of the possibilities to overcome P-glycoprotein-mediated MDR has greatly stimulated the research on other drug resistance mechanisms. Ultimately, modulation of several drug resistance mechanisms will be necessary to overcome clinical drug resistance.

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3.2 SUMMARY

In this thesis studies on P-glycoprotein-mediated multidrug resistance (MDR) in an experimental rat tumor model are described. MDR is a mechanism in tumors that makes them resistant to a wide variety of cytotoxic drugs. In a general introduction the magnitude of the problem of drug resistance in chemotherapy of cancer, hindering effective drug treatment in many cancers, is outlined.

Chapter 1.2 is a review of *in vivo* model systems that have been developed for studying P-glycoprotein-mediated MDR. First, the mechanism of MDR and its clinical relevance are delineated. In short, in MDR a membrane efflux pump called P-glycoprotein expels a wide variety of anticancer drugs out of the cell and thus prevents their cytotoxic actions. Overexpression of P-glycoprotein has been observed in many human cancers, hematological as well as solid tumors. Rodents have two *mdr* genes that both confer the MDR phenotype: *mdr1a* and *mdr1b*. At the gene level they show strong homology to the human *MDR1* gene and the tissue distribution of their gene product is very similar to P-glycoprotein expression in humans. It is argued that, albeit human and rodent P-glycoprotein show some differences in substrate affinity and specificity, rodent MDR tumors are relevant models for studying MDR. *In vivo* studies have shown the physiological roles of P-glycoprotein among which protecting the organism against damage by xenobiotics. An extensive overview of experimental *in vivo* tumors is given. Tumors with intrinsic P-glycoprotein expression, induced MDR or transfected with an *mdr* gene can be used as syngeneic or xenogenic tumor models. Ascites, leukemia, and solid MDR tumor models have been developed. Molecular engineering has resulted in transgenic mice that express the human *MDR1* gene in their bone marrow, and in knockout mice missing murine *mdr* genes. The function of P-glycoprotein can be blocked by so called reverters. The data on pharmacokinetics, efficacy and toxicity of reverters of P-glycoprotein *in vivo* are described. Results from studies using monoclonal antibodies directed against P-glycoprotein and other miscellaneous approaches for modulation of MDR are mentioned. The importance of *in vivo* studies prior to clinical trials is being stressed and potential pitfalls due to differences between species are discussed.

In **chapter 1.3** the aims of the thesis are outlined.

Part 2 of the thesis contains the original studies. **Chapter 2.1** introduces the CC531 rat colon adenocarcinoma as a model for studying intrinsic MDR expression. In CC531 cells P-glycoprotein expression was shown with the monoclonal antibody C219. In cytotoxicity

studies *in vitro* the sensitivity to doxorubicin, daunorubicin and colchicine was enhanced by the addition of the chemosensitizers verapamil and cyclosporin A while the sensitivity to cisplatin was not enhanced. In a daunorubicin accumulation assay verapamil and cyclosporin A increased the daunorubicin content of CC531 cells. These data show that CC531 intrinsically expresses the MDR phenotype. *In vivo* intramuscular administration of cyclosporin A (20 mg/kg for 3 days) resulted in whole-blood levels superior to 2 μmol/L, while intratumoral levels amounted to 3.6 μmol/kg. This dose of cyclosporin A rendered an ineffective dose of 3 mg/kg doxorubicin into an effective antitumor treatment. The experiments show that adequate levels of the chemosensitizing drug cyclosporin A can be obtained *in vivo*, resulting in increased antitumoral activity of a cytotoxin *in vivo*. The *in vitro* and *in vivo* data together suggest that the chemosensitization by cyclosporin A is mediated by P-glycoprotein.

In **chapter 2.2** comparable experiments to those in chapter 2.1 are described, using another reverter of MDR, dexniguldipine. Dexniguldipine is a dihydropyridine derivative with low calcium-channel-blocking activity, which makes it more suitable for MDR reversal than other calcium antagonists because adequate dosing is probably not limited by toxic cardiac effects. *In vitro* coincubation with 50 ng/ml dexniguldipine increased the cytotoxicity of epidoxorubicin approximately 15-fold. *In vivo* concentrations of dexniguldipine 5 h after a single oral dose of 30 mg/kg were 72 (± 19 sd) ng/ml in plasma and 925 (± 495 sd) ng/g in tumor tissue. Dexniguldipine alone did not show antitumor effects *in vivo* against CC531. Pretreatment for 3 days with dexniguldipine modestly, but consistently, potentiated the tumor-growth-inhibiting effect of epidoxorubicin (6 mg/kg) reaching statistical significance in 2 out of 4 experiments. Although the results *in vivo* in this tumor with dexniguldipine are less clear than those with cyclosporin A in separate experiments, it is concluded that dexniguldipine has potency as an MDR reverter *in vitro* and *in vivo*.

In **chapter 2.3** pharmacokinetic data of dexniguldipine and its pyridine metabolite M-1 that has the same MDR-reverting activity are reported. After single oral dosing, concentrations of dexniguldipine and M-1 could be determined in plasma in most of the rats up to 32 hours after administration. High tumor tissue concentrations of dexniguldipine were observed, but levels in normal renal tissue were even higher. The concentrations of dexniguldipine were highest in the multiple dose experiment: 2 μg/g tumor tissue. High correlations (> 0.8) were established between the concentrations of dexniguldipine in plasma and tumor and renal tissue. Tumor tissue concentrations of M-1 were overall one third of the dexniguldipine concentrations.

In **chapter 2.4** side-effects of the addition of a reverter to a cytotoxin are explored.

Using the same experimental design as in chapter 2.1, we investigated the side-effects of combination treatment of cyclosporin A and doxorubicin in an experiment lasting 35 days. The main doxorubicin-related side-effects were dose-dependent weight loss, myelosuppression (transient leucopenia and thrombopenia) and nephrotoxicity. Damage to the kidney was severe, leading to a nephrotic syndrome and resulting in ascites, pleural effusion, hypercholesterolemia and hypertriglyceridemia. These toxicities were enhanced by the addition of the chemosensitizer cyclosporin A. Mild doxorubicin-related cardiomyopathy and minimal hepatotoxicity were seen on histological examination. There were no signs of enhanced toxicity of the combination treatment in tissues with known high expression levels of P-glycoprotein, like liver, adrenal gland and large intestine. Cyclosporin A had a low toxicity profile. It was concluded that the chemosensitizer cyclosporin A enhanced the side-effects of the anticancer drug doxorubicin without altering the toxicity pattern. The increased toxicity observed in these experiments casted doubt whether a therapeutic gain could be obtained in this intrinsically MDR tumor model by adding a reverter to a cytotoxin.

Chapter 2.5 describes a 'side-effect' of the reverter cyclosporin A caused by the immunosuppressive action of the compound. *In vitro* cyclosporin A caused no growth acceleration and only at high doses growth inhibition of CC531 cells was observed. *In vivo* no evidence of growth enhancement was found in short term assays, but after 4 weeks rats treated with cyclosporin A had a significantly higher tumor load, mainly consisting of locoregional metastases. These experiments in the CC531 tumor model show that cyclosporin A used as a reverter of MDR may produce short-term improvement of antitumor activity, but also induce enhancement of tumor metastasis, at least in this weakly immunogenic tumor.

In chapter 2.6 the feasibility of immunotherapy in drug-resistant cell lines is investigated in *in vitro* studies. Several investigators have reported on concomitant development of drug resistance and resistance to natural killer (NK), lymphokine-activated killer (LAK) or monocyte/macrophage cell lysis, while others described unchanged or even increased susceptibility. We investigated this subject in the rat colon carcinoma cell line, CC531-PAR, which is intrinsically MDR, and in three sublines derived from this parental cell line: a cell line with an increased MDR phenotype (CC531-COL), a revertant line from CC531-COL (CC531-REV) which demonstrates enhanced sensitivity to anticancer drugs of the MDR phenotype, and an independently developed cisplatin-resistant line (CC531-CIS). In a 4 h ^{51}Chromium-release (^{51}Cr) assay we found no differences in susceptibility to NK cell lysis. No significant differences in lysability by adherent-LAK (aLAK) cells were observed

in a 4 h assay. In a prolonged 20 h ^{51}Cr-release assay an enhanced sensitivity to aLAK-cell-mediated lysis was observed in the revertant, P-glycoprotein negative cell line and in the cisplatin-resistant cell line (CC531-CIS). None of the cell lines was completely resistant to lysis by aLAK cells.

In the general discussion, chapter 3.1 the results of the experiments are discussed together with new data from later reports in the literature. Possible explanations for the low activity of MDR reverters in solid tumors are brought forward. Future directions for research on additional drug resistance mechanisms are suggested.

3.3 SAMENVATTING

In dit proefschrift worden onderzoeken beschreven op het gebied van multidrug resistentie (MDR) veroorzaakt door P-glycoproteine. MDR staat voor een mechanisme in tumoren dat resistentie veroorzaakt tegen meerdere cytostatische middelen. In een algemene introductie wordt de grootte van het probleem van resistentie voor cytostatica in de behandeling van kanker uiteengezet.

Hoofdstuk 1.2 geeft een overzicht van de *in vivo* modellen die zijn ontwikkeld om MDR te bestuderen. Eerst wordt het mechanisme van MDR en de klinische betekenis geschetst. Kort samengevat is MDR een resistentie mechanisme waarbij een pomp in de celmembraan die P-glycoproteine wordt genoemd, de capaciteit heeft een groot aantal verschillende cytostatica de cel uit werken zodat deze onvoldoende tijd krijgen voor hun celdodende activiteit. In meerdere hematologische en solide tumoren in de mens is verhoogde expressie van dit mechanisme aangetoond. Knaagdieren (de meest gebruikte proefdieren) hebben twee *mdr* genen die allebei het MDR fenotype overdragen: *mdr1a* en *mdr1b*. Op gen niveau blijken deze genen sterke homologie te vertonen met het humane *MDR1* gen; de verdeling van de expressie van P-glycoproteine over de normale weefsels toont eveneens een sterke overeenkomst. Er wordt beargumenteerd dat, hoewel er meerdere verschillen zijn tussen het humane P-glycoproteine en de dierlijke P-glycoproteines, onder andere in affiniteit en specificiteit voor diverse substraten, de overeenkomsten zo groot zijn dat dierlijke tumoren die P-glycoproteine tot expressie brengen relevante modellen zijn om MDR te bestuderen. Onderzoeken *in vivo* hebben de fysiologische functies van P-glycoproteine in normale weefsels aangetoond, waaronder met name de bescherming van het organisme tegen schadelijke stoffen. In het review wordt een uitgebreid overzicht gegeven van de verschillende experimentele MDR tumoren die voor *in vivo* gebruik zijn beschreven. Deze behelzen tumoren met een intrinsieke expressie van P-glycoproteine, tumoren waarin de expressie van P-glycoproteine is opgewekt en tumoren waarin dit door transfectie met een *mdr* gen is verkregen. Er zijn zowel syngene als xenogene modellen en MDR tumoren kunnen als ascites, leukemie of als solide tumoren groeien. Met behulp van moleculair-biologische technieken zijn transgene muizen ontwikkeld die het humane *MDR1* gen in hun beenmerg tot expressie brengen, en 'knockout' muizen die *mdr* genen missen. De resultaten van studies in deze modellen met 'reverters' (stoffen die de functie van P-glycoproteine blokkeren) op het gebied van farmacokinetiek, effectiviteit en toxiciteit worden beschreven. Tevens worden andere vormen van modulering van het MDR mechanisme genoemd, waaronder het gebruik van monoclonale antilichamen gericht tegen

P-glycoproteine. In de discussie wordt het belang van proefdier studies benadrukt als noodzakelijke stap alvorens klinische trials kunnen worden verricht. De mogelijke valkuilen door de verschillen tussen species worden bediscussieerd.

In **hoofdstuk 1.3** worden de doelstellingen van het in dit proefschrift beschreven onderzoek uiteengezet.

Deel 2 van het proefschrift bevat de originele studies. **Hoofdstuk 2** introduceert het CC531 colon adenocarcinoom in de rat als een model voor de bestudering van de betekenis van intrinsieke MDR expressie. De expressie van P-glycoproteine in CC531 werd aangetoond met behulp van een specifiek monoclonaal antilichaam (C219). In cytotoxiciteits studies *in vitro* werd de gevoeligheid van CC531 cellen voor doxorubicine, daunorubicine en colchicine versterkt door toevoeging van de 'reverters' verapamil en cyclosporine A, terwijl de sensitiviteit voor cisplatinum niet veranderde. Verapamil en cyclosporine A verhoogden eveneens de accumulatie van daunorubicine in tumorcellen. Deze gegevens tonen aan dat CC531 een funktioneel MDR mechanisme tot expressie brengt. Intramusculaire cyclosporine A toediening (20 mg/kg gedurende 3 dagen) aan ratten resulteerde in cyclosporine A spiegels in vol bloed boven de 2 μmol/l, terwijl in tumoren hogere spiegels van 3,6 μmol/kg werden gemeten. Cyclosporine A in deze dosering versterkte het effect van een ineffectieve dosering van doxorubicine (3 mg/kg) dusdanig dat een significant antitumor effect werd verkregen. Deze experimenten toonden aan dat het mogelijk was om in ratten voldoende hoge spiegels van een 'reverter' zoals cyclosporine A te verkrijgen die zorgden voor versterking van het antitumor effect van een cytostaticum. De resultaten van de *in vitro* en *in vivo* studies samen suggereren dat dit effect werd behaald door blokkering van de P-glycoproteine effluxpomp.

In **hoofdstuk 2.2** worden vergelijkbare experimenten beschreven met een andere stof die P-glycoproteine kan blokkeren, namelijk dexniguldipine. Dexniguldipine is een dihydropyridine derivaat met een zwakke calcium antagonerende activiteit. Dit maakt de stof meer geschikt als 'reverter' dan andere calcium antagonisten omdat adequate dosering waarschijnlijk niet wordt verhinderd door toxiciteit op cardiovasculair gebied. Dexniguldipine in een dosering van 50 ng/ml versterkte het cytotoxische effect van epidoxorubicine *in vitro* met een factor 15. De spiegels van dexniguldipine in ratten 5 uur na een eenmalige orale toediening van 30 mg/kg waren 72 ($\pm$19 sd) ng/ml in plasma en 925 ($\pm$495 sd) ng/g in tumoren. Dexniguldipine zelf vertoonde geen cytotoxische activiteit *in vivo* tegen de CC531 tumor. Voorbehandeling gedurende 3 dagen van ratten met dexniguldipine potentieerde het tumorgroeiremmende effect van epidoxorubicine (dosering

6 mg/kg). Potentiëring werd gevonden in alle 4 de uitgevoerde experimenten, en was statistisch significant in 2 van de 4. Hoewel de resultaten *in vivo* in dit tumor model met dexniguldipine minder sterk zijn dan die met cyclosporine A die zijn verricht in afzonderlijke experimenten, wordt geconcludeerd dat dexniguldipine potentieel heeft als een 'reverter' van MDR *in vitro* en *in vivo*.

In **hoofdstuk 2.3** worden gegevens gerapporteerd over de farmacokinetiek van dexniguldipine en de belangrijkste pyridine metaboliet M-1 die even actief is als MDR 'reverter'. Na eenmalige orale dosering konden in de meeste ratten plasmaspiegels van dexniguldipine en M-1 worden gedetecteerd gedurende de eerste 32 uur. In tumorweefsel werden hoge spiegels gemeten, maar de spiegels in weefsel van de nier waren nog hoger. De concentraties van dexniguldipine waren het hoogst na herhaalde dosering: 2 μg/g tumorweefsel. De correlaties tussen de concentraties van dexniguldipine in plasma, tumor- en nierweefsel waren hoog (>0,8). De weefsel concentratie van M-1 was in het algemeen een derde van de dexniguldipine concentratie.

In **hoofdstuk 2.4** worden de bijwerkingen van toevoeging van een 'reverter' aan een cytostaticum onderzocht. In een experiment dat langer duurde (35 dagen), maar verder dezelfde opzet had als de experimenten waarmee in hoofdstuk 2.1 effectiviteit was aangetoond, werden nu de bijwerkingen van de combinatiebehandeling van cyclosporine A en doxorubicine bestudeerd. De belangrijkste bijwerkingen die te wijten waren aan doxorubicine waren dosisafhankelijk en betroffen gewichtsverlies, beenmergsuppressie (voorbijgaande leucopenie en trombopenie) en nefrotoxiciteit. De schade aan de nier was ernstig en veroorzaakte een nefrotisch syndroom resulterend in ascites, pleuravocht, hypercholesterolemie en hypertriglyceridemie. Toevoeging van cyclosporine A versterkte deze toxiciteit. Een milde, aan doxorubicine gerelateerde cardiomyopatie en minimale hepatotoxiciteit werden gezien bij histologisch onderzoek. Er waren geen aanwijzingen voor toegenomen toxiciteit door combinatiebehandeling in weefsels met een hoog niveau van P-glycoproteine expressie zoals de lever, bijnieren en dikke darm. Cyclosporine A zelf veroorzaakte weinig toxiciteit. Er wordt geconcludeerd dat toevoeging van de 'reverter' cyclosporine A aan het cytostaticum doxorubicine de bijwerkingen van doxorubicine versterkte maar het toxiciteitsprofiel niet veranderde. De toegenomen toxiciteit van de combinatiebehandeling was dusdanig dat deze de therapeutische winst te niet leek te doen van de toevoeging van de 'reverter' aan het cytostaticum .

Hoofdstuk 2.5 beschrijft een 'bijwerking' van de 'reverter' cyclosporine A veroorzaakt door de immunosuppressive werking van de stof. *In vitro* veroorzaakte cyclosporine A in een celgroei-assay geen groeiversnelling en groeivertraging werd eerst gezien bij zeer hoge

concentraties van cyclosporine A. *In vivo* werden bij kort durende experimenten geen aanwijzingen gevonden voor een effect op groeisnelheid van tumoren door cyclosporine A alleen. In experimenten die 4 weken duurden hadden ratten die behandeld werden met cyclosporine A echter een significant hogere tumormassa, voornamelijk bestaande uit toename van locoregionale metastasen. Deze experimenten met de CC531 tumor toonden aan dat cyclosporine A als een 'reverter' van MDR kort durende versterking van de antitumor activiteit van een cytostaticum kan geven, maar eveneens metastasering van deze zwak immunogene tumor kan bevorderen.

In **hoofdstuk 2.6** worden de mogelijkheden van immunotherapie in verschillende cellijnen die resistent zijn gemaakt voor cytostatica onderzocht in *in vitro* experimenten. Verscheidene onderzoekers hebben gerapporteerd dat bij het induceren van resistentie voor cytostatica in tumorcellijnen, eveneens resistentie voor immunocompetente cellen zoals natural killer (NK) cellen, lymphokine-activated killer (LAK) cellen en monocyten en makrofagen zou ontstaan. Andere onderzoekers meldden een onveranderde of juist toegenomen gevoeligheid. Wij hebben dit onderwerp bestudeerd in de ratte coloncarcinoom cellijn CC531-PAR, die intrinsiek MDR tot expressie brengt en in drie sublijnen die van de parentale CC531 cellijn zijn afgeleid: CC531-COL, een cellijn met een toegenomen MDR expressieniveau; CC531-REV, een revertante lijn van CC531-COL met een toegenomen gevoeligheid voor cytostatica van het MDR mechanisme; en CC531-CIS, een onafhankelijk gekweekte sublijn van CC531 die resistent is gemaakt voor cisplatinum. Met behulp van de 4 uurs ^{51}Chromium-release assay vonden wij geen verschil tussen de cellijnen in gevoeligheid voor NK gemedieerde celdood. Eveneens werd geen verschil gevonden tussen de cellijnen in gevoeligheid voor adherente LAK (aLAK) cellen gemedieerde celdood na 4 uur. In een langer durende assay met aLAK cellen van 20 uur kwamen kleine verschillen naar voren met toegenomen gevoeligheid van CC531-REV, die P-glycoproteine negatief is, en de cisplatinum resistente CC531-CIS. Geen van de cellijnen was totaal resistent tegen LAK gemedieerde cytotoxiciteit.

In de algemene discussie, **hoofdstuk 3.1** worden de resultaten opnieuw bediscussieerd samen met nieuwe data van latere verslagen uit de literatuur. Mogelijke verklaringen voor de zeer matige activiteit van 'reverters' van MDR in solide tumoren worden geopperd. Er worden suggesties gedaan voor verder onderzoek naar additionele resistentie mechanismen tegen cytostatica.

4

APPENDICES

4.1 ABBREVIATIONS

ADR	adriamycin
aLAK	adherent lymphokine-activated killer (cells)
AUC	area under the curve
CsA	cyclosporin A
CV	coefficient of variation
DNIG	dexniguldipine
DNR	daunorubicin
DOX	doxorubicin
E:T ratio	effector cell : target cell ratio
HPLC	high-performance liquid chromatography
IC_{50}	inhibitory concentration (50%)
ICAM	intercellular adhesion molecule
ID_{50}	inhibitory dose (50%)
IL-2	interleukin-2
ip	intraperitoneal
iv	intravenous
LAK	lymphokine-activated killer (cells)
LFA	leucocyte function antigen
LU	lytic units
MDR	multidrug resistance
MDR	human multidrug resistance gene
mdr	rodent multidrug resistance gene
MHC	major histocompatibility complex
MTT	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NK	natural killer (cells)
nd	not determined
nr	not reported
PBS	phosphate-buffered saline
Pgp	P-glycoprotein
pgp	rodent multidrug resistance gene
pi	predominant isoform
RF	resistance factor
RR	relative resistance
sc	subcutaneous
SCID	severe combined immunodeficiency (mouse)
sd	standard deviation
sem	standard error of the mean
SR	sensitization ratio
VBL	vinblastine
VCR	vincristine

cell lines

CC531	parental CC531 rat colon adenocarcinoma
CC531-PAR	parental CC531
CC531-COL	colchicine-induced MDR subline
CC531-REV	revertant cell line derived from CC531-COL, expresses less P-glycoprotein
CC531-CIS	cisplatin-induced resistant cell line, non-MDR

4.2 NASCHRIFT

Wetenschappelijk onderzoek doe je niet alleen en dat maakt het mede zo boeiend en leerzaam. Graag wil ik iedereen bedanken die op een of andere wijze heeft bijgedragen aan het tot standkomen van dit boekje, dat ik als bijnamen ook wel 'mijn levenswerk' of 'mijn molensteen' heb gegeven.

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En Marian, voor alle geduld en steun en liefde en nog héél véél meer.

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4.4 CURRICULUM VITAE AUCTORIS

Wim van de Vrie werd op 8 oktober 1960 geboren in Kattendijke. In 1979 legde hij het eindexamen Gymnasium-ß af aan het Christelijk Lyceum voor Zeeland te Goes. Hierna studeerde hij gedurende een jaar Psychologie aan de Vrije Universiteit in Amsterdam en werd het propedeutisch examen afgelegd.

Van 1980 tot 1989 volgde hij de studie Geneeskunde aan dezelfde universiteit. In deze periode was hij gedurende enige jaren studentlid van de faculteitsraad en de facultaire onderwijscommissie en bestuurslid van de medische faculteitsvereniging. Hij werkte in 1986 gedurende drie maanden mee aan medisch wetenschappelijk onderzoek in Ndoungué, Kameroen. Tevens werd een cursus Medische Antropologie gevolgd aan de Universiteit van Amsterdam. Op 8 september 1989 werd de artsenbul behaald.

In 1990 werd hij voor twee jaar wetenschappelijk onderzoeker aan de Dr. Daniël den Hoed Kliniek te Rotterdam, gedetacheerd in het Laboratorium voor Experimentele Chirurgie aan de Erasmus Universiteit Rotterdam, begeleiders Dr. A.M.M. Eggermont en Dr. R.L. Marquet. In deze periode werd een belangrijk deel van de experimenten verricht die beschreven zijn in dit proefschrift.

In 1992 werd hij assistent-geneeskundige niet-in-opleiding op de afdeling chirurgische oncologie in Dr. Daniël den Hoed Kliniek te Rotterdam. In 1993 was hij gedurende drie maanden gedetacheerd op de afdeling algemene heelkunde in het Zuiderziekenhuis te Rotterdam. In september 1993 volgde een assistentschap inwendige geneeskunde in het IKAZIA ziekenhuis te Rotterdam.

Op 1 januari 1994 werd hij assistent-geneeskundige in opleiding tot internist in het IKAZIA ziekenhuis, opleider Dr. R.J.Th. Ouwendijk. De opleiding is vanaf mei 1995 voortgezet in het Academisch Ziekenhuis Dijkzigt te Rotterdam bij de opleider Prof. J.H.P. Wilson.