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Reversal of MDR1/P-glycoprotein associated drug resistance in human Hepatoblastoma

Research Article

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Abbreviations: α-fetoprotein (AFP); ATP-binding cassette (ABC); breast cancer resistance protein (BCRP)Cisplatin (CDDP); hepatoblastoma (HB); multi drug resistance (MDR); Multidrug Resistance Gene 1 (MDR1); P-glycoprotein (P-gp); RNA interference technology (RNAi)

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Summary

Despite impressive improvements of treatment results in children suffering from hepatoblastoma (HB), advanced tumor stages still provide unsolved problems for treating physicians. A major factor for this phenomenon is the phenotype of drug resistance. The mechanism related to the Multidrug Resistance Gene 1 (MDR1) and its product P-glycoprotein (P-gp) has been identified in experimental studies as major factor contributing to drug resistance in HB. P-gp is an ATP dependant membrane channel, which pumps cytotoxic agents out of the tumor cells. Modulation of P-gp using atoxic chemosensitizers improved treatment results in several tumor types. There is a correlation between MDR1 gene expression levels and the amount of applied chemotherapy courses in resected HB. Relapses and metastases show the highest expression followed by primary tumors after neoadjuvant chemotherapy. Lowest expression levels are found in tumors that were not pre-treated before surgery. We also observed significant treatment improvements of chemosensitizers compared to respective mono-therapies both, in cell lines and in xenotransplanted HB. In cell cultures, cell viabilities decreased significantly without an increase of MDR1 gene expression levels. In xenotransplanted HB, tumor growth and serum alpha-fetoprotein levels were decreased significantly. Again, there were no effects of chemosensitizers on MDR1/P-gp expression levels. Our data underline the hypothesis that chemosensitizers may represent a promising tool for the treatment of advanced types of HB.

I. Introduction

Hepatoblastoma (HB) is the most common primary malignant pediatric liver tumor in children of the western hemisphere (Ishak and Glunz, 1967; Ishak, 1976; Ross and Gurney, 1998). Treatment results of children with HB have been improved remarkably during the last twenty years through treatment strategies developed by several national and international trials in which surgery is combined with perioperative chemotherapy (Ortega et al, 2000; Fuchs et al, 2002; Haberle et al, 2003; Perilongo et al, 2004). Complete surgical resection of the tumors is the main goal of the treatment and is essential for survival of the patients. It also represents a major prognostic factor. Most HB show a good response to chemotherapy. Nevertheless, chemotherapy alone can not eradicate primary tumors (Fuchs et al, 2002; Perilongo et al, 2004). Some tumors remain unresectable after chemotherapy. Also, advanced and metastasized HB may become drug resistant after a certain number of chemotherapy courses (Lockwood et al, 1993; von Schweinitz et al, 1995, 1997).

P-glycoprotein, the product of the human *MDR1* gene, belongs to the ATP-binding cassette (ABC) family of proteins and is physiologically localized in epithelial cells of the gastrointestinal tract, liver, kidney, and capillaries of the brain, testes, and ovaries. P-gp acts as a barrier to the uptake of xenobiotics, and promotes their excretion in the bile and urine. Some tumor cells show enhanced MDR1/P-gp expression after chemotherapy. P-gp actively pumps cytotoxic agents out of tumor cells thus increasing their resistance against cytotoxic agents. P-gp has been proposed as a major factor for the development of drug resistance in experimental models of HB (Bader et al, 1998; Minemura et al, 1999).

The P-gp related resistance has been antagonized using chemosensitizers in various malignancies under experimental and clinical conditions (Mistry et al, 2001;

Lee et al, 2003; Limtrakul et al, 2005; Kankesan et al, 2006). We present the analyses of MDR1/P-gp expression in resected HB specimen in order to reveal a correlation between chemotherapy administration and MDR1/P-gp related drug resistance. Also we investigated the effects of P-gp modulation on the treatment of HB *in vitro* and *in vivo*.

II. Patients and Methods

A. Patients

Clinical data were registered according to the questionnaires of the prospective multi center trials HB 89 and HB 94 of the German Society for Pediatric Oncology and Hematology (Fuchs et al, 2002a,b). In this study cytotoxic agents were administered following the listed schemes:

i. Standard Therapy (IPA)

1. Ifosfamid: 1g/m²/24h for 96h (total dose 3g/m²) together with Uromitexan in equal dosage (day 1-3).
2. Cisplatin: 1x/d 20mg/m² over 1 hour (total dose 100mg/m², day 4-8).
3. Doxorubicin: 60mg/m² over 48h (day 9 and 10).

ii. CARBO/VP-16

1. Carboplatin: 800 mg/m² as continuous infusion (day 1-4)
2. Etoposid: 400mg/m² as continuous infusion (day 1-4)

iii. High dose CARBO/VP-16

1. Carboplatin: 500mg/m²/day over 96h
2. Etoposid: 500mg/m²/day over 96h

Histological analyses were performed by the local pathologist as well as by the Study reference pathology (Prof. Harms, PD Dr. med. Leuschner, Kiel, Germany). Additionally, all tumor specimens were processed and analysed for expression of P-gp against negative controls using antibodies C219 and MM 4.17. A qualitative evaluation was performed using light microscopy. P-gp expression on representative sections was considered negative (no positive signal), low (<10% positive), moderate (10-25% positive), strong (25-50% positive), or very strong (>50% positive).

All tumor specimens were also assessed for MDR1 gene expression using rt-PCR as described earlier (Mosmann, 1983; Warmann et al, 2003). GAPDH served as internal standard, and samples from regular liver tissue was analysed simultaneously to determine relative gene expression levels. The relative expression levels of the MDR1 gene were calculated according to the formula:

$$(\Delta MDR^{T}-\Delta MDR^{L}) / \Delta MDR^{T}$$

with ΔMDR^{T} representing the MDR1 gene expression of the tumors against GAPDH and MDR^{L} representing the MDR1 gene expression of the liver against GAPDH.

All studies were approved by the Regional Government's Ethical Committtee in Hannover, Germany.

B. Cell lines

For invitro analyses we used the cell lines HepT1, HUH6, and HepT3 which have been described previously (Doi, 1976; Pietsch et al, 1996; Warmann et al, 2003). HepT1 was derived from a HB of embryonal subtype, HUH6 originated from a mixed HB, and HepT3 from an embryonal/fetal HB. The cell lines were cultured in DMEM + 10%FCS at 37°C in a humidified atmosphere containing 5% CO_2. Viability analyses were performed using 96 well plates with 20.000 cells per well. For gene expression analyses we used 12 well plates with 300.000 cells per well. All used cells were Mycoplasma negative.

Cell viabilities were assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid) -assay (Mosmann, 1983). The IC_{50} of Cisplatin (CDDP) or Doxorubicin (DOXO) was used for chemotherapy. As chemosensitizers we used ascending concentrations (**Table 1**) of verapamil, PSC 833, a non immunosupressine derivate of cyclosporin, and GG 918, an acridine-carboxamide (Tai, 2000; Thomas and Coley, 2003; Ward and Azzarano, 2004). Incubation with the different agents was carried out for 72 hours.

C. Animal studies

For *in vivo* analyses we used a cell line which was established by our group (nude mouse hepatoblastoma No. 1, NMHB#1) (Warmann et al, 2002) as well as the HepT1 cell line (NMHB#2). Nude mice NMRI (nu/nu) were xenotransplanted according to the model of Fuchs and colleagues, 1996. Animals bearing subcutaneous HB xenografts were treated with CDDP or DOXO +/- verapamil or PSC 833. All agents were administered intraperitoneally. CDDP (9mg/m² days 1-3 and 15-17) and DOXO (15mg/m² days 1-4 and 15-18) were given in cumulative doses equitoxic to the regimens in the HB99 Study. The concentrations for verapamil (5mg/kg) and PSC 833 (5mg/kg) were taken from previously described studies (Todd and Abernethy, 1987; Colombo et al, 1996; Fuchs et al, 1998). The chemosensitizers were administered 10 minutes before the cytotoxic agents.

Tumors were measured every five days (length (a), height (b) and width (c)) and tumor volumes were calculated according to the formula:

$$V_{Tumor} = \frac{a}{2} * \frac{b}{2} * \frac{c}{2} * \frac{4}{3\pi}$$

For statistical analysis we used relative volumes:

$$V_{relativ} = V_{dx} / V_{d0}$$, where V_{dx}: volume on day x; V_{d0}: initial volume

Before and after treatment we determined serum α-fetoprotein (AFP) levels in the mice using a radioimmunoassay (CIS biointernational, Behring Institute, Germany). Samples from xenografts after treatment were assessed for MDR1 gene expression using rT-PCR.

All animal studies were approved by the Regional Government's Ethic Committees for animal studies in Hannover and Tuebingen, Germany.

III. Results

A. Patients

i. Patients' data

From the studies HB 89, HB 94, and HB 99 we analysed 28 resected tumor specimens. However, only 13 of them were of sufficient quality to be used for immunohistochemistry and PCR against P-gp/MDR1. One

Table 1. Chemosensitizer concentrations.

	1	2	4	3
GG 918	0.01µM	0.05µM	0.5µM	0,1µM
Verapamil	1µM	3µM	30µM	10µM
PSC 833	0.1µM	0.3µM	3µM	1µM

child had a relapse and one had metastases. Therefore, 15 tumor specimens were evaluated. The mean age at operation was 39.2 months (1.5-144). Patients' specific data are shown in **Table 2**.

ii. Histology and clinical course

14/15 Tumor specimen were of epithelial origin (2 embryonal, 4 fetal and 8 embryonal/fetal HB). One was a teratoid HB. From the 4 patients who died, 2 had an embryonal tumor, one a teratoid and one an embryonal/fetal. Three of these 4 children had a stage IV tumor and one a stage II tumor. Data of histological analyses after chemotherapy and immunohistochemistry are shown in **Table 3**.

iii. MDR1 gene expression

In operated patients we found a close correlation between MDR1 gene expression levels (as compared to the internal control) and the amount of administered chemotherapy (**Figure 1**). Lowest levels were seen in tumor specimens without chemotherapy. Higher levels were found in resected tumors after neoadjuvant chemotherapy and highest levels were present in tumor relapses and metastases (**Figure 1**).

B. Cell lines

i. Chemosensitizer effects on DOXO treated cells.

Addition of chemosensitizers lead to a relevant growth reduction in all cell lines compared to the respective monotherapy. When added to DOXO in the highest concentration (**Figure 2**), strongest effects were seen for PSC 833 (growth reduction 66.3-75.7%) followed by verapamil (53.2-66%) and GG 918 (10.2-41.3%).

Table 2: Patient specific data; (x) †=died after (x) months. Two patients had relapses/metastases

Pat.-No.	Age at surgery (months)	Tumor-Stage	Preoperative Chemotherapy	Follow up (months)
1	20,5	I	2 x IPA	87
2	12	IV	5 x IPA,3 x Carbo/VP 16	127
3	20	I	3 x IPA	43
4	30,5	I	2x IPA	42,5
5	17	I	3xIPA	32
6	35	IV	3x IPA	28
7	49	IV	3 x IPA, 2 x Carbo/VP 16	(36) †
8	16,5	IV	3 x IPA	(5) †
9	98,5	I	2 x IPA	32
10	1,5	I	none	22
11	32	IV	4 x IPA, 3 x Carbo/VP 16	(98) †
12a	33	II	3 x IPA	(36) †
12b		IV	3x Carbo/VP 16-HD	
13a	144	I	2 x IPA	53
13b		I	2-VP 16/Carbo	

Table 3. Clinical data of outcomes, tumor staging, histology, and P-gp expression levels. Antibody MM 4.17 was used for P-gp detection in immunohistochemistry.

Pat.-No.	Tumor Staging	Histology	Outcome	P-gp-Expression (MM4.17)	Preop Chemotherapy
1	I	e/f, vital	alive	strong	2 x IPA
2	IV	e/f, vital	alive	very strong	5 x IPA,3 x Carbo/VP 16
3	I	e/f, regressive	alive	strong	3 x IPA
4	I	e/f	alive	strong	2 x IPA
5	I	f, necrotic	alive	strong	3 x IPA
6	IV	e/f, necrotic	alive	negative	3 x IPA
7	IV	teratoid, vital	died	strong	3x IPA, 2 x Carbo/VP 16
8	IV	e/f, necrotic	died	strong	3 x IPA
9	I	e/f, regressive	alive	moderate	2 x IPA
10	I	f	alive	moderate	none
11	IV	e, vital	died	strong	4x IPA, 3 x Carbo/VP 16
12a	II	e, vital	died	strong	3 x IPA
12b	IV	e/f, vital		very strong	3 x Carbo/VP 16-HD
13a	I	f	alive	strong	2 x IPA
13b	I	f		strong	2 x VP 16/Carbo

Histology: e = embryonal, f = fetal, n.m. = not measurable. For explanation of chemotherapy regimens see text.

ii. Chemosensitizer effects on CDDP treated cells.

When added to CDDP in the highest concentration (**Figure 3**), strongest effects were seen for PSC 833 (growth reduction 14.3-67.5%) followed by GG 918 (31.3-43.8%) and verapamil (8.2-41.5%).

iii. MDR1 gene expression

Enhanced MDR1 gene expression levels were only detected in HepT1 cells after treatment with DOXO. There were no differences between untreated control cells and cells incubated with chemosensitizers only (p 0.076-0.38). Mono therapy with DOXO resulted in enhanced MDR1 gene expression against control (p=0.018). Addition of chemosensitisers did not further increase gene expression compared to cells after mono therapy (p 0.07-0.29). In all other cells and treatment regimens there were no differences regarding the MDR1 gene expression (all p>0.05).

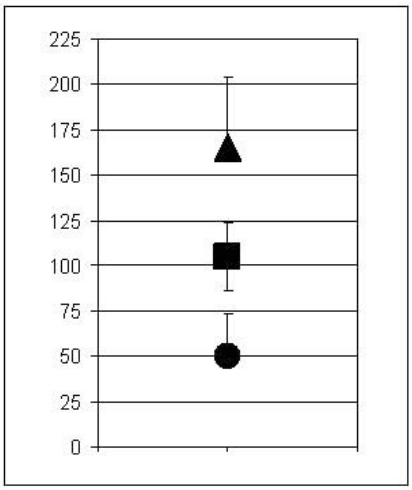

Figure 1. MDR1 Expression levels in tumor specimen after resection [%].Circle: tumors without preoperative chemotherapy; square: primary tumors after standard chemotherapy (IPA); triangle: Relapses and metastases (mean, sd).

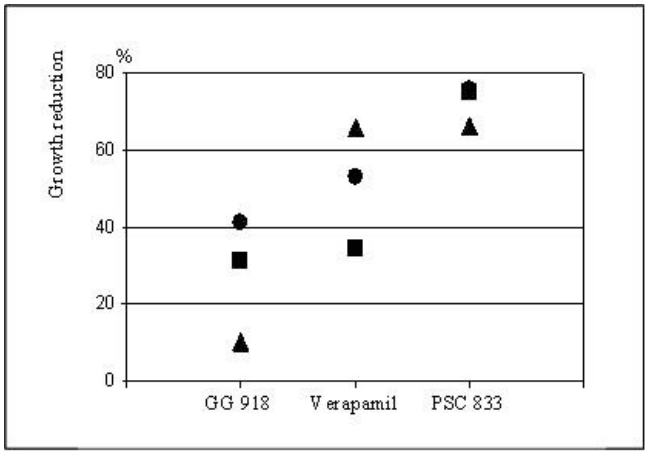

Figure 2. Additional effects of chemosensitisers on tumor cells treated with DOXO. The related DOXO mono therapy was set as 0%. Triangles: HepT3; squares: HUH6; circles: HepT1.

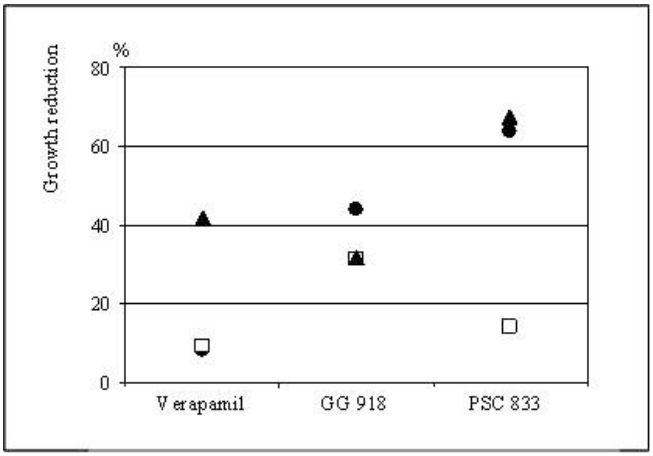

Figure 3. Additional effects of chemosensitisers on tumor cells treated with CDDP. The related CDDP mono therapy was set as 0%. Triangles: HepT3; squares: HUH6, circles: HepT1.

C. Animal studies

i. Tumor volumes

In NMHB#1 there were no differences between tumor volumes in control animals and animals that received chemosensitizers only. Treatment with DOXO and CDDP led to significantly decreased tumor volumes (p<0.001 for both). Addition of chemosensitizers further improved treatment results; PSC 833 was more effective than verapamil (p 0.0004-0.011, **Figure 4**).

In NMHB#2 results were comparable; however, chemosensitizers were less effective than in NMHB#1. Improvements were observed in all regimens but reached significance only in one case (DOXO+PSC 833, p=0.007, **Figure 5**).

ii. Serum AFP levels

In NMHB#1 serum AFP was significantly decreased after treatment with CDDP (p=0.002) and DOXO (p=0.01). Addition of chemosensitizers further decreased AFP levels in the animals' sera (p 0.0001-0.0023). Chemosensitizers alone had no influence on the tumor vitality (**Figure 6**).

In NMHB#2 serum AFP was significantly decreased after treatment with CDDP (p=0.013) and DOXO (p=0.011). Addition of chemosensitizers further decreased AFP levels in the animals' sera, however, differences were not significant (p 0.1-0.31). Chemosensitizers alone had no influence on the tumor vitality (**Figure 7**).

iii. MDR1 gene expression

MDR1 gene expression levels in untreated NMHB#1 was 22.8 (+/- 8.6). Gene expression was enhanced after treatment with DOXO (38.4 +/- 10.3, p=0.01) and CDDP (47.9 +/- 11.3, p=0.003). Chemosensitizers had no influence on the MDR1 gene expression in control cells as well as in combination with cytotoxic agents (all p>0.05). In NMHB#2 results were identical: untreated tumors: 36.6 +/- 12.5, increased expression levels after chemotherapy with DOXO (47.9 +/- 11.3, p=0.024) and CDDP (52.1 +/- 14.4, p=0.0013), and no significant differences through addition of chemosensitizers (all p>0.05).

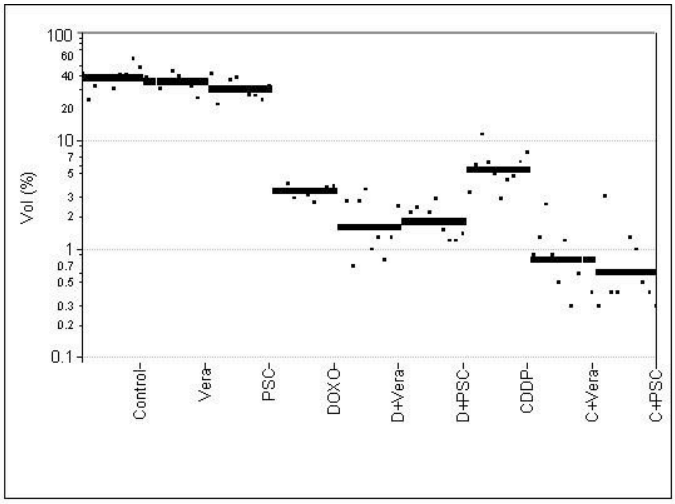

Figure 4. Relative tumor volumes NMHB#1 after treatment (log, n=10, expected and observed).

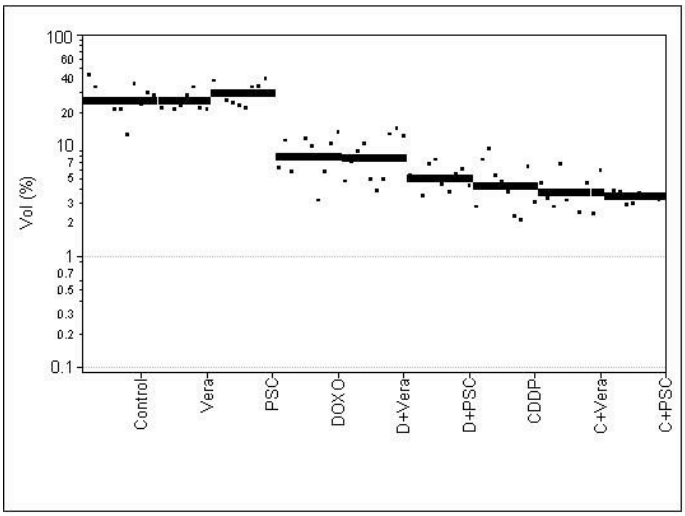

Figure 5. Relative tumor volumes NMHB#2 after treatment (log, n=10, expected and observed).

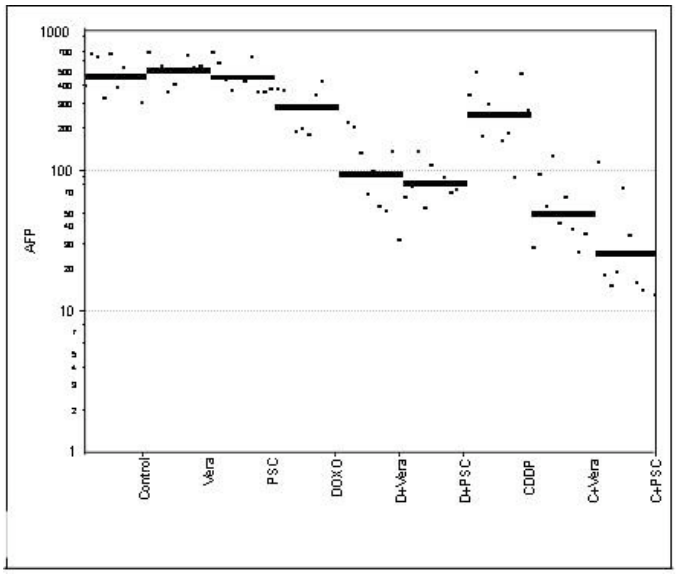

Figure 6. Serum AFP levels NMHB#1 after treatment (log, n=10, expected and observed).

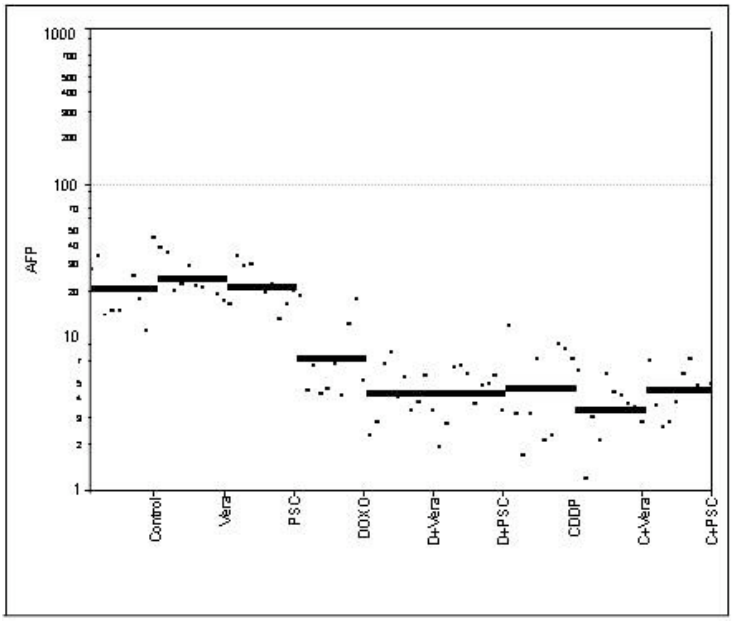

Figure 7. Serum AFP levels NMHB#2 after treatment (log, n=10, expected and observed).

IV. Discussion

P-gp is a membrane-bound glycoprotein (170 kDA, 1280 amino acids), which transports substances out of cells in an active process. It belongs to a number of proteins that are encoded by the MDR-gene family. In humans there exist two genes (MDR1 and MDR 2/3), however, only the MDR1 gene contributes to the phenotype of multi drug resistance (MDR) (Gros et al, 1986; Ueda et al, 1987; Devault and Gros, 1990). P-gp is physiologically localized in excretory tissues such as the liver and kidneys. It's existence in capillaries of the CNS, testicles and placenta indicates the relevance of P-gp in the functioning of the blood-brain barrier, the conservation of the germline and of the intrauterine embryo (Sugawara et al, 1988; Cordon Cardo et al, 1989; Sasongko et al, 2005). Relating observations could be verified using nock-out models (Schinkel et al, 1994; Van Asperen et al, 1996). P-gp shows enhanced expression levels in cell line presenting the MDR phenotype. The P-gp concentration hereby correlates with the grade of MDR. This predominantly affects anthracyclines, epipodophyllotoxines, vinca-alcaloids and taxanes (Juliano and Ling, 1976; Kartner et al, 1983, 1985; Ueda et al, 1986; Di Nicolantonio et al, 2005). The relevance of MDR1/P-gp as major factor for MDR has also been demonstrated for HB (Bader et al, 1998; Minemura et al, 1999).

Analysing the the resected tumor specimen, we found a correlation between MDR1 gene expression levels and the amount of administered chemotherapy: Highest levels were found in tumor relapses and metastases compared to tumors after neoadjuvant standard treatment. Lowest levels were found in tumors that were not treated before operation. This observation is in concordance with clinical findings, where most tumors develop multi drug resistance after a certain number of chemotherapy courses (von Schweinitz et al, 1997). It also affirms the experimental data and emphasizes the transfer to the clinical conditions.

In HB cell lines we observed significant improvements of chemotherapy through MDR1 modulation. PSC 833 proved to be the strongest *in vitro* P-gp inhibitor. Highest effects were observed in combination with DOXO. This fact underlines the observation that DOXO acts as substrate of P-gp. However, we also observed positive effects of chemosensitizers in combination with CDDP. In other studies the same observation has been made (Baekelandt et al, 2001). Since CDDP is not considered to belong to the P-gp substrates, the exact mechanisms of this interaction remain unclear. Nevertheless, we found significant evidence for a positive effect both, *in vitro* and *in vivo*. Expression levels of the MDR1 gene were not altered *in vitro* with one exception. But in this case also, there were no effects of chemosensitizers on the MDR1 gene expression. For a more reliable conclusion, we would, however, prefer the data drawn from patients' or *in vivo* studies.

In xenotransplanted HB there were significant improvements of chemotherapy through combination with chemosensitizers. According to the *in vitro* findings, the treatment with CDDP could be improved *in vivo* also. The histologically more favorable HB cell line responded better to the P-gp directed approach. There was a significant increase of MDR1 gene expression levels after chemotherapy in all xenotransplants. These findings were discordant to the *in vitro* studies but seem in our view more appropriate because of the solid formation of the tumors. Also the higher amount of RNA for assessment studies seems to play a role here. Finally, these results resemble the clinical observations as described above. In every case, P-gp inhibitors did not further enhance MDR1 gene expression, which seems to indicate that there is no increased drug resistance through co-administration of chemosensitizers. The importance of the described observations makes MDR1/P-gp a promising target for the future treatment of HB (Schnater et al, 2003).

The development of P-gp inhibitors underwent several Steps in recent years. First generation chemosensitizers were not specifically developed to inhibit MDR. They had low affinities to MDR transporters (Perez-Tomas, 2006). Second generation agents, to which PSC 833 belongs, were more promising in preclinical studies and have been investigated in clinical studies already. Unfortunately, most of them did not display promising results (Nobili et al, 2006; Perez-Tomas, 2006). Third generation inhibitors were developed to specifically block efflux capacities of ABC transporters. Clinical studies analysing the potential of these agents (e.g. VX 710, LY 35979, XR 9576) are ongoing and some of them seem more favourable than the preceding substances. Besides the specifity one major reason for their superiority seems to be caused by simultaneous effects against several transporters such as P-gp, the breast cancer resistance protein (BCRP), and the multi drug resistance protein (MRP-1) (Minderman et al, 2004; Mahadevan and Shirahatti, 2005; Jekerle et al, 2006; Nobili et al, 2006). Another important observation has been recently reported in which P-gp enhances TRAIL-triggered apoptosis in MDR cancer cells via the death receptor DR5 (Park et al, 2006).

Lately, the RNA interference technology (RNAi) has been introduced as experimental strategy to reverse MDR. RNAi is a physiological double stranded RNA-triggered mechanism resulting in gene-silencing in a sequence-specific manner. Different RNAi strategies have been successfully applied to silence the MDR1 gene *in vitro* and *in vivo* (Stege et al, 2004; Chen et al, 2006; Lage, 2006; Shi et al, 2006). We could recently demonstrate that the technique of RNAi can be successfully applied to HB *in vitro* (Warmann et al, 2006).

Due to it's important value, MDR1/P-gp remains an important and promising target for the improvement of chemotherapy results in HB. Our data demonstrate that overcoming P-gp related MDR might improve treatment results, especially in advanced tumors. Last generation chemosensitizers as well as the RNAi technique seem to be the most promising developments in this regard.

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