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MDR1/P-glycoprotein Expression in Colorectal Cancer

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Drug resistance to multiple chemotherapeutic agents is considered a major cause of chemotherapy failure. An extensively studied and relatively well understood type of cellular drug resistance is P-glycoprotein (Pgp)-mediated multidrug resistance (MDR). Pgp acts as an energy-dependent drug efflux pump, thereby decreasing the intracellular drug concentration and causing drug resistance, in *in vitro* experiments. Colorectal cancer and colorectal mucosa generally express high levels of Pgp, and this may contribute to the general unresponsiveness of colorectal cancer to natural product, anticancer drugs. The controversies concerning the prognostic role of Pgp expression and its contribution to tumour aggressiveness, and possible reasons for the dissapointing results of clinical MDR reversal trials in colorectal cancer are discussed.

Key words: colorectal cancer, *MDR1*
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INTRODUCTION

COLORECTAL CANCER is a leading cause of death in Europe and the U.S.A. and each year over 300 000 new cases are diagnosed [1]. The primary treatment is surgery, and approximately 45% will be cured by local resection [2]. Of the group not cured by primary surgery, only 10% will be cured by a second surgical intervention [2]. The other 90% may receive palliative chemotherapy with a (5-fluorouracil) 5-FU-containing regimen to prolong short term median survival (11 instead of 5 months) [1], but ultimately all these patients will die of their disease.

Drug resistance to multiple chemotherapeutic agents is considered a major cause of chemotherapy failure in colorectal cancer. Drug resistance can be divided into pharmacokinetic resistance (e.g low drug concentration at the tumour site; poor tumour vascularisation, high intratumoral pressure), physiological resistance (tumour sanctuaries; influence of pH at the tumour site), tumour cell kinetic resistance (low tumour growth fraction), and cellular resistance [3]. In recent years, most research has focused on mechanisms of cellular resistance. An extensively studied and relatively well understood type of cellular drug resistance is P-glycoprotein (Pgp)-mediated multidrug resistance (MDR); tumour cells *in vitro* can become cross-resistant to a broad variety of structurally unrelated, natural product, anticancer drugs (i.e. anthracyclines, epipodophyllotoxins, vinca alkaloids, colchicine, actinomycin D, and paclitaxel), after having been grown in the presence of only one of them. Pgp is a plasma membrane protein of 170 kDa, encoded by the *MDR1* gene, which acts as an energy-dependent drug transporter [3]. Pgp decreases the intracellular drug concentration by active extrusion of natural product, anticancer drugs out of the tumour cell, thereby causing drug resistance.

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Pgp expression appears to be highest in tumours originating from normal Pgp expressing tissues [4–6] (Table 1), including liver, kidney, adrenal gland, and colon, and, interestingly, these tumours are all intrinsically drug resistant. Other normal Pgp-expressing tissues include endothelial cells at several blood–tissue barrier sites (brain, placenta, testis) [4], and secretory and gestational cells of the endometrium [8]. Recently, it has been reported that Pgp is expressed in natural killer cells, lymphocytes, granulocytes, monocytes, and in a minority of CD34+ haematopoietic stem cells [9]. The pattern of distribution of Pgp in normal humans suggests that its physiological role is to protect cells against xenobiotics and endogenous toxins, but actually little is known about the normal function of Pgp. A steroid transporting role has been suggested in the gravid uterus [8] and the adrenal [10], and an active transport of cortisol, aldosterone and dexamethasone has been demonstrated in porcine cells transfected with human *MDR1* cDNA isolated from the human adrenal gland [10].

Of potential clinical interest was the finding in 1981 that verapamil, a calcium channel blocker, could reverse the MDR phenotype *in vitro* [3]. Since then, numerous other resistance

Table 1. P-glycoprotein expression in cancer [7]

High	Intermediate	Low
Renal cell	Breast	Lung
Adrenal	Sarcoma	Melanoma
Hepatocellular	Oesophageal	Prostate
Colorectal	Gastric	Bladder
Carcinoid	Glioma	Head and neck
Pancreatic	Neuroblastoma	Ovarian
Retinoblastoma	Cervical	Meningioma
Myeloma	Lymphoma	Thyroid
	Leukaemia	Wilms' tumour

modifying agents (RMAs) have been identified [11]. Among the intrinsically drug resistant cancers with high Pgp expression, colorectal cancer is an interesting candidate for clinical MDR reversal studies, and results of several clinical trials combining RMAs with MDR-related cytotoxins in colorectal cancer are now available. Results of these studies are reviewed here together with recent *in vitro* and *in vivo* data from studies on MDR in colorectal cancer. Furthermore, the role of Pgp as a marker of tumour aggressiveness and its possible prognostic relevance are discussed.

MDR1/P-GLYCOPROTEIN EXPRESSION IN COLORECTAL CANCER

Tumour cell lines

Most *in vitro* studies have been carried out with tumour cell lines that have been made resistant by serial exposure to increasing concentrations of a single anticancer drug. This situation more closely resembles the clinical picture of acquired drug resistance. Although it is unknown whether intrinsic (*de novo*) resistance is caused by different mechanisms from acquired resistance, MDR reversal appears more successful in selected sublines, than in intrinsically resistant, unselected cell lines. One study addressed the question of whether sensitivity to chemotherapy could be enhanced by verapamil (5 μg/ml) in colon cancer cell lines with low-level *MDR1*/Pgp expression [12]. Comparison of parental and selected sublines, with similar *MDR1*/Pgp expression levels, showed less sensitisation in the parental cell lines [12]. Another study evaluated the ability of verapamil (1.0, 5.0, and 10.0 μg/ml) to modulate the effect of doxorubicin and vinblastine on the *in vitro* cloning of fresh tumour cells [13]. Six colon cancers were tested among 53 tumours. Drug resistance in these colon tumours could not be reversed by the addition of verapamil. In fact, only a subset of 13 tumours from patients who had previously showed a response to doxorubicin, but were resistant at the time the specimen was obtained, could be modulated by verapamil *ex vivo* [13]. This observation suggests that *de novo* (intrinsic) drug resistance is mediated by multiple resistance mechanisms, in addition to Pgp-mediated MDR [12–14].

An important issue is whether the Pgp molecule, once detected in a tumour cell, is also functionally active [14]. Kramer and associates investigated Pgp biosynthesis in 19 wild type human colon carcinoma cell lines and compared these levels with *MDR1* RNA levels, daunomycin accumulation +/− verapamil, and doxorubicin cytotoxicity +/− verapamil in the same cell lines [14]. They concluded that only the mature 170 kDa Pgp molecule, but not the 140 kDa precursor, can be phosphorylated and located at the cell surface. Furthermore, they observed a correlation between the phosphorylation status and membrane association of Pgp with the MDR phenotype, emphasising that both of these factors are important in establishing cellular drug resistance [14]. Interestingly, one cell line, with appreciable levels of expression and demonstrated Pgp activity, appeared equally resistant to vincristine as Pgp-negative colon cell lines. Probably other resistance mechanisms (such as topoisomerase II, MRP (multidrug resistance-associated protein), glutathione peroxidase) play a role in these Pgp-negative colon cell lines [14].

Cell line studies remain simplified models of the clinical situation, which is far more complex, dealing with problems such as tumour heterogeneity, sampling error, admixture of normal cells, interplay with the tumour environment, and tumour host factors.

P-glycoprotein, prognosis and tumour aggressiveness

Pgp expression has been associated with a poor prognosis in neuroblastoma [15], sarcoma and acute myeloid leukaemia [3]. Its prognostic value in colorectal cancer is less clear (Table 2). Weinstein and associates [16] reported a significant correlation between Pgp-positive tumour cells at the leading edge of the tumour and increased incidence of tumour vessel invasion and lymph node metastases. They suggested that Pgp-positive tumour cells had an increased metastatic potential, and hypothesised a role for Pgp in enhancement of tumour cell locomotion and adhesion [16]. Another study reported Pgp to have prognostic value in stage B2 colon cancers [19]. The number of patients studied was, however, small ($n = 52$), and the P-value of marginal significance ($P = 0.04$). Two other reports could not demonstrate a relationship between Pgp expression and shorter (disease-free) survival, using either immunohistochemistry [17] or an RNA slot blot technique [18] to assess Pgp expression. In conclusion, more research is needed to determine the prognostic relevance of Pgp expression in colorectal cancer.

Of potential interest is the possible relationship between *TP53* mutations and *MDR1* expression. The *TP53* tumour suppressor gene regulates genomic stability [20], and inactivation of its product by several mechanisms, including point mutation, gene deletion, overexpression of *MDM-2*, and binding to proteins encoded by DNA tumour viruses, is at present the most common event identified in human cancers [20]. Recently, *in vitro* experiments have demonstrated that mutant p53 can stimulate the *MDR1* promoter [21], and may be involved in chemoresistance, as wild type p53 is required for the efficient activation of apoptosis following treatment with anticancer drugs [22, 23]. The prevalence of *TP53* mutations in colorectal cancer is around 50% [20]. The role of *TP53* mutations in the occurrence of intrinsic drug resistance in colorectal cancer has not yet been investigated.

CLINICAL TRIALS OF MULTIDRUG RESISTANCE REVERSAL

Table 3 summarises MDR reversal trials in colorectal cancer. The rationale for these studies was based on: (a) the presence of intrinsic drug resistance of colorectal cancer to natural product anticancer drugs; (b) the generally high Pgp expression of colorectal cancer; and (c) the *in vitro* evidence of successful MDR reversal in colon cancer cell lines when combining natural product, anticancer drugs with RMAs.

MDR reversal studies with verapamil as an RMA have been hampered by the occurrence of prohibitive cardiotoxicity before adequate verapamil plasma concentrations, equivalent to those

*Table 2. Pgp/*MDR1 *expression and prognostic value in colorectal cancer*

Reference	*n*	Technique	*P*-value	Relation with
Weinstein *et al.* [16]	95	IHC	<0.001	Vessel invasion
			<0.01	Lymph node metastases
Mayer *et al.* [17]	82	IHC	NS	Survival
Pirker *et al.* [18]	103	RNA slot blot	NS	Disease-free survival
			NS	Survival
Sinicrope *et al.* [19]	52	IHC	0.04	Disease-free survival

IHC, immunohistochemistry; NS, not significant.

Table 3. Clinical MDR modulation in colorectal cancer

RMA	Anticancer drug	RMA route	RMA median plasma level*	(Dose-limiting) toxicity	Response CR	PR	NR	Ref.
Verapamil	Doxorubicin	p.o.	656 (1.44 μM) (6–10 μM)†	Cardiac arrhythmia, hypotension, dizziness	0	2	19	[24]
D-Verapamil	Doxorubicin	p.o.	2180 (4.75 μM) (6–10 μM)	Cardiac arrhythmia, hypotension, oral mucositis, granulocytopenia, thrombocytopenia, anaemia	0	1	14	[25, 26]
Bepridil	Vinblastine	i.v.	1320 (3.6 μM) (4 μM)	Cardiac arrhythmia, hypotension, constipation	1	0	14	[27]
Cyclosporin-A	Epidoxorubicin	i.v.	6248 (peak) 1012 (at 18 h) (6000)	Flushing‡, leucopenia	0	1	23	[28]

RMA, resistance modifying agent; CR, complete remission; PR, partial remission; NR, no response.
*In ng/ml unless stated otherwise; † In parentheses: the optimal *in vitro* concentration for MDR reversal; ‡ Probably related to the vehicle of cyclosporin-A; p.o., oral; i.v., intravenous.

needed *in vitro* to modulate MDR, could be reached. Although higher plasma concentrations were achieved with D-verapamil and bepridil, median plasma concentrations were still below the optimal *in vitro* concentrations needed for MDR reversal (Table 3). With cyclosporin-A, satisfactory peak levels could be reached, but plasma levels at 18 h appeared suboptimal. Dose-limiting toxicities of these studies were cardiovascular side effects (D-verapamil, bepridil), and increased myelosuppression, when an anthracycline was combined with an RMA. Increased myelosuppression might have been a result of altered pharmacokinetics of the anticancer drug (increased area under the plasma concentration × time curve (AUC)), or of increased vulnerability of Pgp-positive CD34+ haematopoietic stem cells to MDR reversal therapy.

All MDR reversal studies published so far (for recent overview, see ref. [29]) have limitations that do not allow firm conclusions. Some of these limitations are mentioned in Table 4. For instance, Pgp tumour status of most patients entered into MDR reversal trials has not been assessed. One reason is the lack of a standardised detection technique for Pgp expression, making interpretation of results difficult, and comparison between studies impossible. In addition, it has been demonstrated that cells can express Pgp, while the protein is not functionally active [9]. Therefore, a functional assay would be the best way to assess Pgp tumour status. Investigations of non-invasive scanning techniques are underway, using radiolabelled substrates for Pgp, reflecting Pgp transport activity (scintigraphy and positron emission tomography) (reference in [29]). Another problem is that most patients have not been demonstrated to be resistant to the natural product anticancer drug only, before participating in a MDR reversal trial. Responses could erroneously be attributed to effective MDR reversal, while in fact the tumour is just Pgp-negative and sensitive to the anticancer drug [30]. Furthermore, a pharmacokinetic interaction has been demonstrated for the addition of a RMA to a natural product, anticancer drug. An increase in the AUC of 1.5–2-fold had been reported for etoposide (reference in [29]) when cyclosporin-A was added to the regimen, and for (epi)doxorubicin after addition of (D-)verapamil [26]. This dose intensification effect should be taken into account before a response is attributed to MDR reversal. Properly designed phase II, and randomised phase III trials might circumvent this problem, matching AUCs of the anticancer drug by changing the anticancer drug dose when an RMA is added to the regimen.

Table 4. Pitfalls in MDR reversal studies

1. Unknown Pgp expression and function in tumour cells
2. Unknown sensitivity to anticancer agents
3. Response attributed to MDR reversal, dose intensification effect neglected
4. Blood and tumour cell kinetics of RMA and anticancer drug unknown
5. Presence of other cellular resistance mechanisms

An important drawback of most pharmacokinetic studies is the assessment of plasma drug concentrations, instead of tumour drug concentrations. High RMA tumour concentrations, equal to effective *in vitro* concentrations for MDR reversal, have only been reported for bepridil and quinidine (references in [27] and [7]). Non-invasive imaging techniques, using radiolabelled anticancer drugs and RMAs, are now being developed (*vide supra* and reference in [29]) to address this issue.

Finally, other cellular resistance mechanisms may play a role in the occurrence of drug resistance. Several mechanisms are being unravelled, both in the laboratory and clinical setting, such as those mediated by topoisomerase II and MRP [29]. Furthermore, less specific resistance mechanisms, including those mediated by glutathione S transferases and proteins involved in the blockade of the apoptotic pathway (such as altered p53, overexpression of bcl-2) [29], may be of importance.

CONCLUSION

Colorectal cancer belongs to a group of intrinsically drug resistant cancers. Most of these cancers originate from normal human cells located at the border between self and non-self (such as lung, renal cell, gastric, and head and neck cancers). One of the functions of these cells is to protect the body against many different toxic compounds. Probably multiple detoxification mechanisms are involved in this protection. Therefore, Pgp-mediated MDR may only be one of those mechanisms present in colorectal cancer, causing resistance to anticancer drugs derived from natural products. The presence of additional cellular resistance mechanisms makes colorectal cancer less suitable for MDR reversal studies, and may be one of the reasons for disappointing results reported so far.

The prognostic role of Pgp expression in colorectal cancer remains controversial. Additional, prospective studies on a large patient population are warranted. A possible relationship with the *TP53* tumour suppressor gene should be investigated and might clarify the suggested role of Pgp in tumour aggressiveness.

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