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Multidrug Resistance and the Role of P-glycoprotein Knockout Mice

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Drug resistance, be it intrinsic or acquired, is a major problem in cancer chemotherapy. *In vitro*, one well characterised form of resistance against many different cytotoxic drugs is caused by the MDR1 P-glycoprotein, a large plasma membrane protein that protects the cell by actively pumping substrate drugs out. Available evidence suggests that this protein may cause drug resistance in at least some clinical tumours. Drugs inhibiting the MDR1 P-glycoprotein activity are, therefore, co-administered during chemotherapy of these tumours. To predict the biological and pharmacological effects of the blocking of this protein, we have generated mice with a genetic disruption of the drug-transporting mdr1a P-glycoprotein. These mice are overall healthy, but they accumulate much higher levels of substrate drugs in the brain, and have markedly slower elimination of these drugs from the circulation. For some drugs, this leads to dramatically increased toxicity, indicating that P-glycoprotein inhibitors should be used with caution in patients.

Key words: multidrug resistance, P-glycoprotein, reversal agents, blood–brain barrier, cyclosporin A, neurotoxicity

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INTRODUCTION

UPON SELECTION with a single cytotoxic drug, mammalian tumour cells can develop resistance against a range of drugs with different structures and intracellular targets. This phenomenon, called multidrug resistance (MDR), can be caused by P-glycoproteins. These large (140–170 kDa), glycosylated proteins consist of two similar halves, each containing six putative transmembrane segments and an intracellular ATP binding site. They are mainly localised in the plasma membrane where they can actively extrude a wide range of amphiphilic hydrophobic drugs from the cell, thus providing protection from the toxic action of these drugs [1]. Many of the transported drug substrates are toxic compounds of natural or semisynthetic origin that are extensively used in the chemotherapy of cancer (e.g. *Vinca* alkaloids, anthracyclines, actinomycin D, epipodophyllotoxins, taxanes). It is, therefore, thought that the P-glycoprotein could be one of the causes of the intrinsic or acquired multidrug resistance observed during chemotherapy of many clinical cancers [2]. However, several important anticancer drugs, such as cisplatin and its analogues, 5-fluorouracil, cytarabine, bleomycin and alkylating agents such as melphalan, cyclophosphamide and ifosphamide, are apparently not affected by P-glycoprotein activity, allowing some room for alternative chemotherapy for tumours resistant due to P-glycoprotein.

Although P-glycoprotein was the first identified cause of multidrug resistance in cultured cells, recently Cole and Deeley's group identified another, distantly related protein that is also associated with multidrug resistance [3, 4]. This protein, called MRP (multidrug resistance-associated protein) also has two intracellular ATP binding sites and two regions containing putative transmembrane regions, although in this protein eight transmembrane segments are postulated in the N-terminal half and four in the C-terminal half. Further analysis of this protein indicates that, like P-glycoprotein, it is mainly localised in the plasma membrane, and that it confers drug resistance by active extrusion of substrate drugs [5, 6]. MRP has been shown to confer resistance to anthracyclines, vincristine, and etoposide (VP-16), and is, therefore, a true multidrug resistance protein. However, unlike P-glycoprotein, MRP does not confer high levels of resistance to vinblastine or paclitaxel, indicating clear differences in the substrate spectrum of both proteins. MRP does not confer resistance to cisplatin. Whether this protein can confer resistance to other drugs that are not affected by P-glycoprotein (see above) awaits further systematic analysis. Intriguingly, cells that overexpress MRP also demonstrate increased transport of glutathione *S*-conjugates, and the transported glutathione conjugate leukotriene C_4 specifically binds to MRP [7, 8]. These findings suggest that MRP is a glutathione *S*-conjugate transporter.

From the available data, both P-glycoprotein and MRP can be expected to contribute to some form of clinical multidrug resistance, although direct proof for such an involvement from clinical studies is still limited. In the remainder of this paper, we concentrate on P-glycoprotein-mediated multidrug resistance, as considerably more basic and clinical data are available relating to this type of resistance. The knowledge we have gained from mice in which P-glycoprotein gene(s) are disrupted will be

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particularly discussed, in relation to clinical trials that aim to circumvent P-glycoprotein-mediated multidrug resistance.

TISSUE DISTRIBUTION OF P-GLYCOPROTEIN IN HUMANS AND MICE

Humans have one drug-transporting glycoprotein, MDR1, which is prominent in the brush border of renal proximal tubules, in the biliary membrane of hepatocytes, in the apical membrane of mucosal cells in the intestine, in capillary endothelial cells of brain and testis, in the adrenal gland and in placental trophoblasts [9, 10]. These findings, in combination with the known drug-transporting ability, suggested that MDR1 P-glycoprotein can protect the organism against toxic xenobiotic compounds, by excreting these compounds into urine, bile and the intestinal lumen, and by preventing their accumulation in critical organs such as the brain or testis. Moreover, the high expression of P-glycoprotein in the adrenal gland and (pregnant) uterus, and the demonstrated ability of the MDR1 P-glycoprotein to transport steroid hormones, such as hydrocortisone and aldosterone [11], suggested a role in steroid secretion.

In contrast to humans, mice have two genes encoding drug-transporting P-glycoproteins, *mdr1a* (also called *mdr3*) and *mdr1b* (also called *mdr1*), respectively [12, 13]. The mouse *mdr1a* gene is predominantly expressed in intestine, liver, and blood capillaries of brain and testis, whereas the *mdr1b* gene is predominantly expressed in the adrenal, placenta, ovary and (pregnant) uterus. Similar levels of *mdr1a* and *mdr1b* expression are found in kidney [14–17]. Table 1 gives an updated comparison of the expression detected for the human *MDR1* and the mouse *mdr1a* and *mdr1b* genes. These distribution data suggest that *mdr1a* and *mdr1b* in the mouse together fulfil the same function as *MDR1* in humans.

*Table 1. Tissue distribution of human and mouse drug-transporting P-glycoproteins**

Tissue	MDR1 (human)	mdr1a (mouse)	mdr1b (mouse)
Digestive tract			
Oesophagus	●		
Stomach	●	○	●
Jejunum/Ileum	●●●	●●●	●
Colon	●●●	●●●	●
Liver	●●●	●	●
Endocrine organs			
Thyroid	○		
Adrenal	●●●●	●	●●●●
Ovary	●	●	●●
Testis	●	●	○
Urogenital tract			
Kidney	●●●	●	●●
Bladder	●		
Uterus	●	●	●
Uterus in pregnancy	●●	●	●●●●
Placenta		○	●●●
Prostate	●		
Central nervous system	●●	●	○
Other tissues			
Skeletal muscle	●	●	●
Heart muscle		●	●
Lung	●	●●	●
Spleen	●	●	●

*Modified and updated after Borst and associates [30], mainly compiled from RNA analyses. The relative expression level is indicated by filled circles, very low or undetectable mRNA levels by open circles. No circle indicates that data are not available.

REVERSAL OF P-GLYCOPROTEIN-MEDIATED DRUG RESISTANCE

Studies of MDR1 P-glycoprotein (RNA) levels in clinical tumour samples indicate that this protein may be relevant for intrinsic or acquired MDR in a range of tumour types [2, 18, 19]. This led to an extensive search for P-glycoprotein inhibitors. Many compounds with low cytotoxicity can inhibit P-glycoprotein activity, for instance verapamil, quinidine, cyclosporin A and its non-immunosuppressive analogue PSC833 [20, 21]. There is currently great interest in the co-administration of these so-called reversal agents with anticancer drugs to patients to reduce P-glycoprotein-mediated drug resistance of tumours during chemotherapy. Many phase I and II clinical trials to test the feasibility of this approach are now in progress (for overviews see [22, 23]). One major concern in this approach is the effect that P-glycoprotein inhibitors will have on the normal function of the drug-transporting P-glycoproteins. To learn more about the physiological and pharmacological role of these proteins, we have recently generated a mouse strain with a genetic disruption of the *mdr1a* gene (*mdr1a*(−/−) mice [17]).

THE PHENOTYPE OF *mdr1a* P-GLYCOPROTEIN-DEFICIENT MICE

The *mdr1a* (−/−) mice provided a striking confirmation of the protective role of P-glycoprotein. By a chance discovery, these mice turned out to be 100-fold more sensitive to the centrally neurotoxic pesticide, ivermectin (oral administration). Further analysis showed that wild-type mice have high levels of mdr1a P-glycoprotein at the blood–brain barrier, whereas *mdr1a* (−/−) mice did not have detectable P-glycoprotein there. This resulted in approximately 100-fold higher levels of the neurotoxin in the brain 24 h after administration, thus explaining the increased sensitivity of these mice [17]. Apparently, mdr1a P-glycoprotein actively pumps ivermectin out of the brain. 4 h after oral administration, the level of [^{3}H]ivermectin was almost 50-fold higher in the brains of *mdr1a* (−/−) mice, whereas the overall levels in plasma and other tissues were 3- to 4-fold higher (Table 2). The *mdr1a* (−/−) mice were also 3-fold more sensitive to the anticancer agent, vinblastine (intravenous administration), and they accumulated much higher levels of this drug in their brains. As with ivermectin, they displayed overall increased accumulation and decreased elimination of vinblastine in tissues and plasma [17]. We attribute the more limited increase in toxicity of vinblastine compared to ivermectin to vinblastine being rather toxic to organs other than the brain (intestine?). Together, these data demonstrated that P-glycoprotein can have a very marked effect on the tissue distribution, pharmacokinetics and excretion of suitable substrate drugs, including anticancer agents. In view of the efforts to block P-glycoprotein activity in human cancer patients [22], it was reassuring to find that, apart from the pharmacological effects, *mdr1a* (−/−) mice appeared to be completely healthy. This suggests that P-glycoprotein activity, at least in some organs, is not essential for life. However, mdr1b P-glycoprotein is still present in *mdr1a* (−/−) mice. A more reliable prediction of possible effects of complete blocking of all P-glycoprotein activity should come from the analysis of mice in which both the *mdr1a* and *mdr1b* P-glycoprotein genes are disrupted. Efforts in this direction are currently ongoing in our group.

Table 2. Tissue concentrations of [^{3}H]*ivermectin in* mdr1a (+/+) *and* (−/−) *mice 4 h after oral injection of a dose of 0.2 mg/kg*

Tissue	*mdr1a* (+/+)	*mdr1a* (−/−)	Ratio (−/−):(+/+)
Brain	0.9 ± 0.5	41 ± 6	46
Muscle	14 ± 8	55 ± 15	3.9
Heart	27 ± 10	128 ± 45	4.7
Kidney	52 ± 19	182 ± 54	3.5
Liver	126 ± 38	460 ± 125	3.7
Gall bladder	110 ± 22	235 ± 104	2.1
Lung	32 ± 15	120 ± 31	3.8
Stomach	238 ± 100	303 ± 146	1.3
Small intestine	247 ± 18	638 ± 229	2.6
Colon	75 ± 27	95 ± 25	1.3
Fat (neck)	57 ± 17	241 ± 71	4.2
Fat (organ)	16 ± 5	60 ± 15	3.8
Testis	4.6 ± 4.0	18 ± 3	3.9
Epididymis	10 ± 4	41 ± 25	4.1
Spleen	20 ± 8	58 ± 26	2.9
Thymus	24 ± 3	75 ± 28	3.1
Plasma	22 ± 11	70 ± 17	3.2

Results are expressed as means ± s.d. ($n-1$) in ng/g tissue. Three male mice were analysed in each group.

RELATION TO CLINICAL P-GLYCOPROTEIN MODULATION

One of the main findings in clinical trials combining P-glycoprotein substrate anticancer drugs and P-glycoprotein inhibitors, such as cyclosporin A, was that the overall pharmacokinetics of the cytostatic drugs were altered [22, 23]. Specifically, the "area under the curve" (AUC), a measure for the systemic exposure of tissues (and tumours) to a circulating (anticancer) drug, was clearly increased in the presence of inhibitors. This is probably due to a combination of altered tissue distribution, and decreased drug elimination, as a consequence of P-glycoprotein blocking in normal tissues. In some cases, this meant that the dose of cytostatic drug had to be lowered to prevent increased toxicity. These effects were also clearly observed in the *mdr1a* (−/−) mice with vinblastine, indicating that the pharmacokinetic alterations observed in patients could indeed be mainly the consequence of blocking the activity of P-glycoprotein. Nevertheless, in patients, compounds such as cyclosporin A or PSC833 may also interfere with other drug-transporting or degrading enzymes, for instance the cytochrome P450–3A family of enzymes, that share many substrates with P-glycoprotein [24].

The question remains whether effective P-glycoprotein modulation in patients will be really successful in selectively increasing the penetration of the cytostatic drug in the target tumour cells that express P-glycoprotein, relative to the increased overall exposure of other tissues that are dose-limiting for the therapy regimen. It is even possible that bone marrow stem cells, dose-limiting for many anticancer drugs, are also protected to some degree by P-glycoprotein [25, 26]. We are currently addressing this question in a *mdr1b* knockout mouse model system. If this turns out to be true, P-glycoprotein inhibition in patients might be a self-defeating strategy, at least for some cytostatics. It should be realised that positive results obtained in combination treatment might be attributable to increased overall exposure of the tumour to the drug (dose intensification), rather than to increased permeability of the tumour cells. In that case, dose intensification of the cytostatic drug(s) alone may be a better strategy than combining drugs with P-glycoprotein inhibitors [22, 23].

OTHER DRUGS AFFECTED BY P-GLYCOPROTEIN

Apart from anticancer agents, many other drugs are known to be substrates of P-glycoprotein *in vitro* [1, 20]. These include, for instance, morphine [27], dexamethasone, an anti-inflammatory and glucocorticoid drug [11], digoxin, a heart glycoside that is widely used in the treatment of congestive heart failure [28], and cyclosporin A, an agent that has revolutionised organ transplantation by its ability to suppress allograft rejection [29], but which is also used to inhibit P-glycoprotein activity *in vivo*. Preliminary results obtained with *mdr1a* (−/−) mice indicate that the tissue distribution and elimination of dexamethasone, digoxin and cyclosporin A are also affected by P-glycoprotein activity. Alternatively, the tissue distribution of several other drugs (including some anticancer agents), that are clearly affected by P-glycoprotein activity *in vitro*, was apparently not affected by the absence of mdr1a P-glycoprotein *in vivo*. This demonstrates that further analysis is required before we will be able to predict from *in vitro* data the extent to which a certain drug is affected by P-glycoprotein *in vivo*. The *mdr1a* (−/−) mice provide a useful model system for addressing these questions.

P-GLYCOPROTEIN IN THE GASTRO-INTESTINAL TRACT

MDR1 and mdr1a P-glycoprotein are highly abundant in the apical membrane of mucosal cells of the intestine [9, 17]. In principle, this may affect the oral bioavailability of certain P-glycoprotein substrate drugs, as their uptake could be delayed, or even incomplete, due to the continuous back-transport into the intestinal lumen by P-glycoprotein. As mouse intestine epithelial cells contain predominantly mdr1a P-glycoprotein, and hardly any mdr1b P-glycoprotein ([17], Table 1), this can be tested using *mdr1a* (−/−) mice, although the overall altered tissue distribution does complicate such an analysis to some extent (Table 2).

PERSPECTIVE

To extend our insight into the role of P-glycoproteins, we are currently analysing mice with disruptions of the *mdr1b*, and both the *mdr1a* and *mdr1b* P-glycoprotein genes. These mice should provide additional information on the biological and pharmacological roles of P-glycoprotein. Hopefully, the knowledge currently gained by the extensive analysis of P-glycoprotein-mediated drug resistance both *in vitro*, in *in vivo* model systems, and in clinical studies, will improve the chemotherapy of cancer patients. In addition, it may speed up the elucidation of (the contribution of) other mechanisms of clinical drug resistance, and the identification of new methods to circumvent it.

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