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Review

Prognostic significance of multidrug resistance-related proteins in childhood acute lymphoblastic leukaemia

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ABSTRACT

An important problem in the treatment of children with acute lymphoblastic leukaemia (ALL) is pre-existent or acquired resistance to structurally and functionally unrelated chemotherapeutic compounds. Various cellular mechanisms can give rise to multidrug resistance (MDR). Best studied is the transmembrane protein-mediated efflux of cytotoxic compounds that leads to decreased cellular drug accumulation and toxicity. Several MDR-related efflux pumps have been characterised, including P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), breast cancer resistance protein (BCRP) and lung resistance protein (LRP). P-gp expression and/or activity has been associated with unfavourable outcome in paediatric ALL patients, whereas MRP1 and BCRP do not seem to play a major role. LRP might contribute to drug resistance in B-lineage ALL, but larger studies are needed to confirm these results. The present review summarises the current knowledge concerning multidrug resistance-related proteins and focuses on the clinical relevance and prognostic value of these efflux pumps in childhood ALL.

1. Introduction

The prognosis of children diagnosed with acute lymphoblastic leukaemia (ALL) has improved markedly during the past decades. However, approximately 25% of affected children relapse and cannot be cured with current chemotherapy [1]. Intrinsic or acquired resistance to a wide variety of structurally and functionally unrelated chemotherapeutic compounds is one of the most important causes of treatment failure in childhood ALL. A variety of cellular mechanisms can give rise to multidrug resistance (MDR), including enhanced expression of cellular transporters, reduced drug uptake, alterations in detoxifying mechanisms, enhanced DNA repair processes, downregulation of drug targets, changes in cell cycle regulation and alterations in apoptotic pathways [2,3].

Classical multidrug resistance is associated with transmembrane protein-mediated efflux of cytotoxic compounds

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leading to a decreased cellular drug accumulation and toxicity. Several MDR-related drug efflux pumps have been characterised. Most of them belong to the superfamily of ATP-binding cassette (ABC) transporters.

P-glycoprotein (P-gp) is the best-characterised MDR pump [4]. Various neoplastic agents, such as anthracyclines, mitoxantrone, taxanes, vinca alkaloids and epipodophyllotoxins, are P-gp substrates. During the past 10 years, several new drug resistance-related proteins have been identified. One of these is the multidrug resistance-associated protein 1 (MRP1), an ATP-dependent efflux pump which extrudes glutathione conjugated compounds out of the cell [5]. Currently, the MRP family consists of nine MRP homologues (MRP1–MRP9). The breast cancer resistance protein (BCRP) is evolutionarily distinct from the other ABC transporters [6]. BCRP is a half-molecule, and formation of homo- or heterodimers is essential for its function as an active transporter [7,8]. Anthracyclines, topoisomerase I inhibitors, topoisomerase II inhibitors, cell-cycle inhibitors and methotrexate are BCRP substrates. Another MDR-related protein, named lung resistance protein (LRP), is involved in the nuclear-cytoplasmic transport and/or sequestration of cytotoxic compounds. LRP is identified as the major vault protein (MVP) and confers resistance against vincristine, doxorubicin and etoposide [9]. Unlike P-gp, MRP and BCRP, LRP is not a member of the ABC transporter family.

In the present review, current knowledge concerning the clinical relevance and prognostic value of multidrug resistance-related proteins in childhood ALL is summarised.

2. P-glycoprotein

Juliano and Ling were the first to isolate P-gp from resistant Chinese hamster ovary cells [4]. P-gp is encoded by the *MDR1* gene, located on the long arm of chromosome 7 (7q21.1) [10,11]. It is a 170-kDa protein consisting of two structurally homologous domains, each containing six hydrophobic transmembrane segments and a highly conserved ATP binding site [12,13]. The two times two domain organisation is most likely the result of an internal gene duplication [14]. The presence of two ATP binding sites defines P-gp as a member of the ATP binding cassette (ABC) superfamily of transport proteins [15].

The mechanism by which P-gp decreases the intracellular accumulation of anthracyclines, mitoxantrone, taxanes, epipodophyllotoxins and vinca alkaloids, is poorly understood. There are at least three possible mechanisms of action. The first hypothesis assumes that amphiphatic and lipophilic substrates are removed from the cytoplasm through a channel formed by the transmembrane segments [16,17]. Furthermore, a 'vacuum cleaner' model has been suggested, in which P-gp transports compounds from either the inner or the outer leaflet of the lipid bilayer into the external medium [18,19]. Alternatively, P-gp might function as a 'flippase', transporting drugs from the inner to the outer leaflet of the bilayer after which the compounds will leave the plasma membrane by diffusion [20,21].

P-gp is expressed in various normal tissues with secretory or barrier functions, including lung, placenta, testes, adrenal gland, kidney, liver, pancreas, colon, jejunum and brain [22–24]. In addition, P-gp is expressed by haematopoietic precursors and lymphocytes [25,26]. These findings suggest that P-gp plays a major role in the excretion and/or transport of cytotoxic xenobiotics.

P-gp mediated multidrug resistance can be reversed by various inhibitors. Competitive binding experiments showed that most modulators compete with cytotoxic substrates for P-gp binding sites [27]. Such agents include calcium channel blockers (e.g. verapamil), calmodulin inhibitors (e.g. pimozide), immunosuppressive agents (e.g. cyclosporin A, PSC 833), quinolones (e.g. chloroquine and quinine), indole alkaloids (e.g. reserpine), antibiotics (e.g. erythromycin), detergents (e.g. cremophor EL), steroids and anti-oestrogens (e.g. tamoxifen) [28,29]. Other agents, such as MDR1 anti-sense oligonucleotides, interference RNA and protein kinase C inhibitors (e.g. staurosporine), modulate P-gp activity through the transcriptional regulation of the MDR1 gene [30–32]. In addition, P-gp specific monoclonal antibodies, such as MRK16, UIC2 and HYB-241, can be used to inhibit the P-gp mediated drug efflux [33–35]. To our knowledge, no clinical trials with P-gp antagonists in paediatric ALL patients have been performed. Clinical trials in paediatric and adult acute non-lymphoblastic leukaemia patients yielded disappointing results [36–39]. In early trials the efficacy of first-generation inhibitors, such as verapamil and tamoxifen, was evaluated. Variability in drug absorption, excessive protein binding, unpredictable plasma levels or unacceptable toxicity limited their clinical development [40]. The most promising first-generation compounds were quinine and cyclosporin A [41,42]. These P-gp antagonists could be administered at doses high enough to yield pharmacologically active serum concentrations. However, clinical trials failed to prove a role for P-gp inhibitors in clinical drug resistance. One of the major reasons for this failure was the lack of confirmation of P-gp expression in the malignant cells. Careful selection of patients is essential in facilitating positive outcomes, as those deriving benefit from P-gp antagonists may be obscured in trials failing to identify the appropriate target population. Furthermore, the effectiveness of P-gp modulators in patients was not confirmed.

Second-generation agents, such as PSC833 and VX-710, were more potent, specific and less toxic compared with the first-generation inhibitors. Moreover, these compounds had a higher P-gp binding affinity. However, results from clinical trials were disappointing. Pharmacokinetic interactions between the P-gp inhibitor and the anticancer agent resulted in delayed anticancer drug clearance necessitating dose reduction. However, it is now felt that these dose reductions may have compromised drug concentrations in malignant cells.

Highly specific third-generation P-gp inhibitors, such as tariquidar, zosuquidar and laniquidar, display minimal activity against other members of the ABC transporter family, lack interaction with cytochrome P450 and act as non-competitive P-gp modulators [43]. Consequently, pharmacokinetic interactions are negligible. The next generation of P-gp modifiers is emerging from efforts to delineate structural interactions with P-gp and transcriptional regulators of MDR1 [38]. Future clinical trials will

evaluate the clinical benefit of these recently discovered compounds.

Various techniques have been developed to study P-gp expression and function. MDR1 mRNA can be detected and/or quantified by reverse transcriptase polymerase chain reaction (RT-PCR) [44]. P-gp expression can also be determined using different monoclonal antibodies. Some recognise internal P-gp epitopes (e.g. C219 and JSB-1), others interact with external antigens (e.g. MRK16, 4E3 and UIC2) [45,46]. Monoclonal antibodies binding to external epitopes are preferred because they are superior in detecting low and variable levels of P-gp [47]. The P-gp activity is usually studied using functional flow cytometric assays. The intracellular accumulation of P-gp substrates such as rhodamine 123, JC-1, 3,3′-diethyloxacarbocyanine iodide ($DiOC_2$) or daunorubicin, is measured in the presence or absence of a P-gp specific inhibitor [48–50].

Several contradictory reports about the clinical importance of P-gp in childhood acute leukaemia have been published [44,51–83]. Table 1 summarises the reported data.

Approximately 30% of all diagnostic ALL samples are P-gp positive. Some authors reported more P-gp positive patients at relapse [54,58,63,67] whereas others were unable to find a significant difference in the number of P-gp positive samples taken at initial diagnosis or at relapse [62,73]. When P-gp expression levels in initial ALL samples were compared with those in relapse samples, several authors reported a higher P-gp expression at relapse, i.e. especially at multiple relapses [53,60,61]. However, these results were not confirmed by others [44,51,66].

Tafuri and colleagues found an increased P-gp activity in samples from relapsed ALL patients [61]. However, Pieters and co-workers evaluated the daunorubicin and vincristine accumulation in the presence or absence of a P-gp inhibitor and found no difference in P-gp function between initial and relapse samples [55].

The P-gp expression did not differ between infant or older ALL patients [80,81]. Kanerva and colleagues found a significantly higher P-gp expression in T-ALL compared with B-ALL patients [67]. Other authors were unable to confirm these results [59,74,79,83].

In some studies no relationship between P-gp status and event free survival (EFS) or overall survival (OS) was found [68,74,75,82,83]. However, other authors were able to demonstrate a significant association between P-gp expression and/or function and clinical outcome in childhood ALL. Brophy, Tafuri and Kanerva investigated the relationship between the response rate and P-gp expression [44,61,67]. Brophy and Tafuri reported a correlation between clinical response and absence of P-gp expression. By contrast, Kanerva did not find a relation between early response and P-gp status.

Goasguen and co-workers found no difference in the first complete remission rate between P-gp positive or P-gp negative patients [57]. However, relapses occurred more frequently in children with P-gp positive blasts and the survival rate was significantly higher in P-gp negative patients. The EFS-curve followed the same trend but did not reach statistical significance. In accordance with Goasguen, Sauerbrey and co-workers found a significantly lower probability of remaining in continuous complete remission and a tendency for an increased relapse rate in P-gp positive patients [59]. In a study published by Dhooge and co-workers, a significant correlation between EFS and P-gp status was found [70]. The OS-curve followed the same trend but reached borderline statistical significance. These results were confirmed by Casale and colleagues [79]. De Moerloose and co-workers reported that the combination of P-gp expression and activity was a statistically significant parameter predicting relapse in childhood ALL [77].

In conclusion, the clinical importance of P-gp in childhood ALL remains unclear. One of the reasons for the variability in published data is the lack of standardised detection techniques. Moreover, the variety in detection methods hampers the comparison of data. Although this problem has been studied by a number of international workshops, implementation of consensus recommendations has been difficult [84–88]. Large and well-controlled clinical studies, using standardised detection techniques, are indispensable to determine the prognostic significance of P-gp in childhood ALL.

3. Multidrug resistance-associated protein

The multidrug resistance-associated protein (MRP1), which Cole and colleagues isolated from the doxorubicin-resistant small cell lung cancer cell line H69AR, is also a member of the ATP-binding cassette transporter protein family [5]. The *MRP1* gene, mapped on chromosome 16 (16p13.1), encodes a 190-kDa N-glycosylated hydrophobic anion pump localised on both the plasma and intracytoplasmic membranes, including the endoplasmic reticulum and Golgi apparatus [89].

The amino acid homology between MRP1 and P-gp is 15%. Compared with P-gp, MRP1 has an additional N-terminal membrane-spanning domain, which is linked via a cytoplasmic loop to a P-gp-like core [90].

The spectrum of resistance caused by MRP1 overexpression is very similar to that of P-gp [91]. In vitro, enforced MRP1 expression confers resistance to anthracyclines, vinca alkaloids, epipodophyllotoxins, campothecins and methotrexate, but not to taxanes and mitoxantrone [91–94]. Various glutathione, glucuronate or sulphate conjugates, such as cysteinyl leukotriene LTC4, an important mediator of inflammatory responses, are also transported by MRP1 [95,96]. In addition, Rigato and colleagues reported that the transport of unconjugated bilirubin is mediated by MRP1 [97].

Glutathione (GSH) plays an important role in the MRP1 transport process. However, the exact mechanism by which GSH participates in the MRP1-mediated efflux is not yet fully understood. GSH interacts with MRP1 by different mechanisms [98]. First, GSH appears to be a direct, low-affinity substrate for MRP1. Evidence for GSH transport by MRP1 was provided by studies utilising MRP1 overexpressing cells [89,99–102]. MRP1 overexpression is associated with a significant decrease in the intracellular GSH levels, consistent with GSH efflux by MRP1. Studies with Mrp1 knockout mice confirmed these results [103,104].

Second, GSH is required for the transport of certain MRP1 substrates such as daunorubicin, vincristine and rhodamine [89,100]. When intracellular GSH levels are decreased, transport of daunorubicin, doxorubicin, etoposide and vincristine in MRP1 overexpressing cells is reduced [99–101,104–107].

Table 1 – Clinical importance of P-gp in childhood acute lymphoblastic leukaemia (ALL)

Author	Method	Samples	Conclusions
Ubezio et al., 1989 [51]	MDR1 gene expression:	5 I ALL	MDR1 gene expression level and intracellular DOX concentration did not differ between initial and relapse samples
	RNA blot	5 R ALL	
	P-gp function:		
	DOX Acc by FC		
Mizuno et al., 1991 [52]	P-gp expression:	9 I ALL	P-gp was not detected in any of the samples
	FC	4 R ALL	
Gekeler et al., 1992 [53]	MDR1 gene expression:	7 I ALL	No difference in MDR1 mRNA levels between initial and relapsed ALL
	RNA slot blot	14 1st R ALL	Significant increase in MDR1 expression levels in multiple relapsed ALL
		11 ⩾ 2nd R ALL	
Kingreen et al., 1992 [54]	P-gp expression:	48 I ALL	At Dx: 6% P-gp expression; At relapse: 23% P-gp expression
	IC	47 R ALL	No relation between P-gp expression and clinical outcome in the relapsed patients
Pieters et al., 1992 [55]	P-gp expression:	28 I ALL	P-gp was not detected in any of the tested samples
	IC	14 R ALL	DNR and VCR accumulation did not differ between resistant and sensitive cells
	P-gp function:		Resistance modifiers did not enhance the in vitro cytotoxicity of DNR or VCR
	DNR/VCR Acc + Mod		
	In vitro drug resistance:		
	MTT assay		
Fenneteau et al., 1993 [56]	P-gp expression:	35 I ALL c	At Dx: 4% P-gp expression
	IC	16 I ALL a	
Goasguen et al., 1993 [57]	P-gp expression:	36 I ALL	At Dx: 33% P-gp expression
	IC		The rate of first complete remission did not differ between P-gp positive and P-gp negative patients
			Survival rate was significantly higher in P-gp negative compared to P-gp positive patients
			EFS curve followed this trend
Brophy et al., 1994 [44]	MDR1 gene expression:	Cell lines from:	No difference in P-gp expression level between initial and relapsed ALL
	RT-PCR and slot blot	16 I ALL	Complete clinical response to therapy correlated with absence of MDR1 expression
	P-gp expression:	20 R ALL	
	IC and ISH		
Dhooge et al., 1994 [58]	P-gp expression:	33 I ALL	At Dx: 12% P-gp expression; At relapse: 55% P-gp expression
	IC	11 R ALL	4 out of 6 relapsed patients with P-gp expression experienced a rapid unfavourable outcome
Sauerbrey et al., 1994 [59]	P-gp expression:	104 I ALL	At Dx: 35% P-gp expression
	IC		P-gp positive patients had a significantly lower probability of remaining in first continuous complete remission
			Tendency for an increased relapse rate in P-gp positive patients
			P-gp expression was independent of sex, FAB type, immunological subtype and initial WBC count
Beck et al., 1995 [60]	MDR1 gene expression:	27 I ALL	No difference in MDR1 mRNA levels between initial and relapsed ALL
	RT-PCR	18 1st R ALL	Significant increase in MDR1 expression levels in multiple relapses
		7 ⩾ 2nd R ALL	

Table 1 – *continued*

Author	Method	Samples	Conclusions
Tafuri et al., 1995 [61]	P-gp expression: FC P-gp function: Rho 123 + Mod by FC	19 I ALL 14 R ALL	Significant difference in P-gp expression levels and activity between initial and relapsed patients Patients in complete remission showed a significant lower P-gp expression and function compared to those who failed to respond
Ivy et al., 1996 [62]	P-gp expression: FC P-gp function: Rho 123 + Mod by FC	30 I ALL 38 R ALL 22 I AML 8 R AML 5 Others	ALL + AML + Others: No difference in number of patients with P-gp expression at initial Dx or at relapse Significant increase in P-gp function at relapse
Volm et al., 1996 [63]	P-gp expression: IC	111 I ALL 28 R ALL	At Dx: 35% P-gp expression; At relapse: 54% P-gp expression At Dx, P-gp was more frequently expressed in patients who relapsed under therapy
Srinivas et al., 1997 [64]	P-gp expression: IC	88 I ALL	At Dx: 68% P-gp expression
Volm et al., 1997 [65]	P-gp expression: IC	104 I ALL	At Dx: 36% P-gp expression Patients with P-gp expression had a significant lower median relapse-free interval
den Boer et al., 1998 [66]	P-gp expression: FC In vitro drug resistance: MTT assay	112 I ALL 22 1st R ALL 7 ⩾ 2nd R ALL 20 I AML 6 1st R AML 1 2nd R MLL	No difference in P-gp expression levels in initial or relapsed patients No significant correlation between P-gp expression and in vitro drug resistance P-gp expression did not relate to age, initial WBC count or unfavourable immunophenotype P-gp expression did not differ between AML and ALL patients
Kanerva et al., 1998 [67]	P-gp expression: FC	103 I ALL 15 R ALL	At Dx: 53% P-gp expression; At relapse: 73% P-gp expression No correlation between P-gp expression and early response at day 15 No relation between P-gp expression and age, sex, initial WBC count, FAB type or karyotype changes P-gp expression was significantly higher in T-ALL compared to B-ALL patients
Gurbuxani et al., 1998 [68]	MDR1 gene expression: RT-PCR	32 I ALL	No correlation between MDR1 gene expression levels and treatment outcome
den Boer et al., 1999 [69]	P-gp expression: FC P-gp function: DNR acc and efflux In vitro drug resistance: MTT assay	60 I ALL 25 R ALL	After correction for cell volume: Intracellular DNR concentration was lower in relapsed compared with initial ALL patients Inverse correlation between intracellular DNR concentration and in vitro DNR resistance No relation between P-gp expression and intracellular DNR concentration or in vitro DNR resistance
Dhooge et al., 1999 [70]	P-gp expression: IC	102 I ALL 35 R ALL	At Dx: 14% P-gp expression; At relapse: 34% P-gp expression P-gp positive patients at relapse had a 1,9-fold greater risk for adverse clinical outcome EFS was significantly higher in patients without P-gp expression

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Table 1 – *continued*

Author	Method	Samples	Conclusions
			OS curve followed the same trend but did not reach statistical significance P-pg expression was independent of WBC count, age, sex and karyotype P-gp expression was not induced by exposure to chemotherapy
Kakihara et al., 1999 [71]	MDR1 gene expression: RT-PCR	40 I ALL	No correlation between MDR1 gene expression and age or WBC count No difference in MDR1 expression levels between high- and low-risk patients
Ogretmen et al., 2000 [72]	MDR1 gene expression: RT-PCR	12 I ALL	MDR1 mRNA was undetectable in tested samples
Gurbuxani et al., 2000 [73]	MDR1 gene expression: RT-PCR	80 I ALL 9 R ALL	At Dx: 16% MDR1 gene expression; MDR1 was not more frequently expressed at relapse Mean MDR1 mRNA levels were significantly higher for patients not achieving complete remission No association between EFS and MDR1 gene expression
Wuchter et al., 2000 [74]	P-gp expression: FC P-gp function: Rho 123 + Mod by FC	102 I ALL	At Dx: 10% P-gp function No difference in P-gp expression or function between T-ALL and B-ALL Neither P-gp function, nor P-gp expression correlated with response to induction chemotherapy or OS No significant difference in P-gp expression or function between patients who relapsed and those in CCR
Kanerva et al., 2001 [75]	P-gp expression: FC	103 I ALL	No association between P-gp expression and EFS and OS No correlation between P-gp expression and coexpression of myeloid antigens
Dhooge et al., 2002 [76]	P-gp expression: IC	102 I ALL 37 R ALL	At Dx: 14% P-gp expression; At relapse: 35% P-gp expression P-gp expression at diagnosis was associated with an increased risk for relapse Association between P-gp expression and EFS P-gp positive patients at relapse had a 2,2-fold greater risk for adverse clinical outcome
De Moerloose et al., 2003 [77]	P-gp expression: IC P-gp function: Rho 123 + Mod by FC	52 I ALL	At Dx: 46% P-gp expression and 23% P-gp function EFS and OS were significantly lower in patients with P-gp expression and function Combination of P-gp expression and function is a statistically significant parameter predicting relapse

Table 1 – *continued*

Author	Method	Samples	Conclusions
Plasschaert et al., 2003 [78]	MDR1 gene expression: RT-PCR P-gp function: Rho 123 + Mod by FC	36 I ALL c 35 I ALL a	Children + adults: P-gp activity was higher in T-ALL than in B-ALL No correlation between P-gp activity and age, haemoglobin or platelet count at Dx Negative correlation between P-gp activity and WBC count In T-ALL: association between P-gp activity and EFS or OS In B-ALL: no impact of P-gp activity on EFS or OS
Casale et al., 2004 [79]	P-gp expression IC and FC P-gp function Rho 123 + Mod by FC	85 I ALL	At Dx: 47% P-gp expression and 30% P-gp function EFS was significantly lower in the P-gp positive population EFS was independent of age, WBC count, immunophenotype, FAB subtype and prednisolone response
Ramakers-van Woerden et al., 2004 [80]	P-gp expression: FC	469 I ALL	No difference in P-gp expression between infants and older common/pre B-ALL patients ALL patients with MLL rearrangements had a median 1,3-fold higher P-gp expression than MLL negative cases
Stam et al., 2004 [81]	MDR1 gene expression: real time RT-PCR In vitro drug resistance: MTT assay	26 I ALL	No difference in MDR1 gene expression in infants compared to older children MDR1 expression levels did not correlate with in vitro drug resistance
Swerts et al., 2004 [82]	P-gp expression: FC P-gp function: Rho 123 + Mod by FC JC-1 + Mod by FC	19 I ALL 2 R ALL 5 I AML 3 R AML 1 Other	ALL + AML + Other: Reversible JC-1 efflux in 20% and reversible Rho 123 efflux in 27% of all samples No association between P-gp expression or activity and clinical outcome
Valera et al., 2004 [83]	MDR1 gene expression: RT-PCR	30 I ALL	MDR1 gene expression did not correlate with age, WBC count, race, immunophenotype, FAB type, CNS infiltration, MRD on day 28, EFS and OS

DOX, doxorubicin; DNR, daunorubicin; VCR, vincristin; FC, flow cytometry; I ALL, Initial ALL; R ALL, Relapsed ALL; IC, immunocytochemistry; RT-PCR, reverse transcription polymerase chain reaction; ISH, in situ hybridization; Acc, accumulation; Rho l23, rhodamine 123; Mod, modulator; EFS, event-free survival; OS, overall survival; c, children; a, adults; WBC, white blood cell; CCR, continuous complete remission.

These compounds do not conjugate with GSH, suggesting that GSH is required as a possible co-transporter or cofactor. However, co-transport does not apply to all substrates requiring GSH for MRP1-dependent transport. Certain compounds such as daunorubicin, estrone 3-sulphate, and 4-(methylnitrosamino-)-1-(3-pyridyl)-1-butanol-glucuronide require GSH for transport but do not decrease intracellular GSH levels [100,101,108,109]. Additionally, GSH efflux can be enhanced by certain compounds (e.g. verapamil and some biflavanoids), which are not themselves MRP1 substrates [102,110–113]. Finally, compounds such as LTC4 and DNP-SG do not require GSH to be transported by MRP1 [95].

Based on these findings, Salerno and co-workers proposed a new working model [114]. They assumed that MRP1 is composed of two interlocked wheels. The first wheel binds GSH, LTC4 and DNP-SG and functions as the power unit that turns when a substrate is bound and energy is provided by the hydrolysis of ATP. The second wheel is inert and turns only when it is connected to the first wheel. In the presence of saturating amounts of GSH and daunorubicin, both wheels are able to turn and expel one molecule of GSH and one molecule of daunorubicin, respectively [107].

MRP1 is expressed in most tissues in the human body, especially in lung, testes, kidney, skeletal muscle, epithelial

Table 2 – MRP 1

Author	Method	Samples	Conclusions
Beck et al., 1995 [60]	MRP1 gene expression: RT-PCR	27 I ALL 18 1st R ALL 7 ⩾ 2nd R ALL	No difference in MRP1 mRNA levels between initial and relapsed ALL patients Significant increase in MRP1 expression levels in multiple relapses
den Boer et al., 1998 [66]	MRP1 expression: FC In vitro drug resistance: MTT assay	112 I ALL 22 1st R ALL 7 ⩾ 2nd R ALL 20 I AML 6 1st R AML 1 2nd R AML	No difference in MRP1 gene expression levels between initial and relapsed ALL patients No significant correlation between MRP1 expression and in vitro drug resistance MRP1 expression did not relate to age, initial WBC count or unfavourable immunophenotype MRP1 expression did not differ between AML and ALL patients
Kakihara et al., 1999 [71]	MRP1 gene expression: RT-PCR	40 I ALL	No correlation between MRP1 gene expression and age or WBC count No difference in MRP1 gene expression between high- and low-risk patients
Ogretmen et al., 2000 [72]	MRP1 gene expression: RT-PCR	12 I ALL	MRP1 was overexpressed in most children with pre-B-ALL MRP1 mRNA was undetectable in most children with T-ALL
Sauerbrey et al., 2002 [117]	MRP1 gene expression: RT-PCR	58 I ALL 28 R ALL	No difference in MRP1 gene expression levels between initial and relapsed ALL patients MRP1 overexpression was not associated with unfavourable outcome MRP1 expression was independent of age, sex, initial blast count, FAB-type and immunological subtype
Plasschaert et al., 2003 [78]	MRP1 gene expression: RT-PCR MRP1 function: CFDA + Mod by FC	36 I ALL c 35 I ALL a	Children + adults: No difference in MRP1 activity between T-ALL and B-ALL or adults and children High MRP1 activity did not influence EFS or OS in ALL patients No correlation between MRP activity and age, haemoglobin or platelet counts at diagnosis
Valera et al., 2004 [83]	MRP1 gene expression: RT-PCR	30 I ALL	At Dx: 16.6% MRP1 overexpression MRP1 gene expression did not correlate with age, WBC count, race, immunophenotype, FAB-type, CNS infiltration, MRD on day 28, EFS and OS

and haematopoietic cells [5,115]. MRP1 is also expressed in the endothelial cells that form the blood-brain barrier [116]. These findings suggest that MRP1 plays an important role in the elimination and sequestration of cytotoxic drugs, leading to decreased concentrations at their target sites.

MRP1 does not seem to play a major role in multidrug resistance in childhood ALL (Table 2). Beck and co-workers did not find a difference in *MRP1* gene expression levels in samples taken at diagnosis or at first relapse [60]. However, the *MRP1* gene expression increased significantly in multiple relapse samples. Den Boer and colleagues evaluated the MRP1 expression in peripheral blood or bone marrow samples from 141 children with ALL [66]. In addition, the in vitro cytotoxicity of daunorubicin, vincristine, etoposide, prednisolone and L-asparaginase was evaluated. The *MRP1* expression did not differ between samples taken at diagnosis or at relapse and no relation between MRP1 positivity and in vitro resistance was found. The *MRP1* expression did not correlate with a prognostically unfavourable immunophenotype, white blood cell count or age. These results were confirmed by others [71,72,117]. In several studies, no correlation between *MRP1* expression at diagnosis and EFS was found [83,117]. Plasschaert and colleagues analysed samples from 36 children and 35 adults with de novo ALL and concluded that MRP1 activity had no prognostic impact on OS and EFS [78].

4. Breast cancer resistance protein

Chen and co-workers detected a 95-kDa ABC transporter in the human breast cancer cell line MCF-7/AdrVp [118]. RNA fingerprinting led to the identification of a 2.4-kb mRNA encoding a 655 amino acid protein, which was termed breast cancer resistance protein (BCRP) [119]. The transporter is also known as ABCG2, placental transporter or mitoxantrone resistance protein. BCRP is encoded by the *ABCG2* gene which was mapped on chromosome 4 (4q22) [120]. The transporter is a half-molecule with a C-terminal transmembrane segment and a N-terminal ATP-binding site [7]. The formation of homo- or heterodimers bridged by disulphide bonds is essential for its function as an active transporter [8]. In vitro, high

Table 3 – Clinical importance of breast cancer resistance protein (BCRP) in childhood ALL

Author	Method	Samples	Conclusions
Sauerbrey et al., 2002 [126]	BCRP gene expression	47 I ALL	BCRP gene expression was lower in T-ALL than in precursor B-ALL patients
	RT-PCR	20 R ALL	No difference in BCRP gene expression levels between initial and relapsed ALL patients No relationship between BCRP gene expression and age, sex, initial blast count, prednisolone response or bone marrow response on day 15 and day 33 No association between BCRP overexpression and unfavourable outcome
Stam et al., 2004 [81]	BCRP gene expression RT-PCR	26 I ALL	Infants expressed 2.4-fold less BCRP mRNA than older children with ALL

I ALL, initial ALL; R ALL, relapsed ALL; RT-PCR, reverse transcription polymerase chain reaction.

BCRP expression causes resistance to anthracyclines (e.g. doxorubicin and daunorubicin), topo-isomerase I inhibitors (e.g. topotecan), topo-isomerase II inhibitors (e.g. bisantrene, etoposide and mitoxantrone), cell-cycle inhibitors (e.g. flavopiridol) and antifolates (e.g. methotrexate) [7,121–123]. However, a mutation in a single amino acid can change the substrate specificity and thereby alter the drug resistance profile. Cell lines overexpressing BCRP with an arginine at position 482 are able to transport mitoxantrone but not rhodamine 123 or doxorubicin, whereas cells with threonine or glycine at position 482 extrude rhodamine 123, doxorubicin and mitoxantrone [124].

BCRP overexpression has been described in resistant ovary, breast, colon and gastric cancer, fibrosarcoma cell lines, placental tissue, liver canalicular membranes, ducts and lobules of the breast, endothelium of veins and capillaries, epithelium of colon and small intestine and bile canaliculi [125].

Since children with ALL are treated with BCRP substrates such as methotrexate and doxorubicin, BCRP overexpression could be responsible for MDR. A few studies investigated the role of BCRP in childhood ALL (Table 3). In a retrospective study, Sauerbrey and colleagues analysed the BCRP expression by TaqMan real-time RT-PCR in samples from 67 ALL patients (47 initial stage and 20 relapses) [126]. Children with T-ALL showed a lower BCRP expression than patients with precursor B-ALL. No relationship between BCRP expression and age, sex, initial blast count, prednisolone response or bone marrow response on day 15 and 33 was found. BCRP expression levels at relapse were not significantly different from those at diagnosis and BCRP overexpression was not associated with unfavourable outcome. Stam and co-workers reported a lower BCRP expression in infants than in older ALL patients [81].

BCRP does not seem to play a role in drug resistance in paediatric ALL patients. However, larger studies are needed to confirm these results.

5. Lung resistance protein

The lung resistance protein (LRP) was initially identified in an anthracycline-resistant, non-small cell lung cancer cell line that lacked *P-gp* overexpression [127]. The *LRP* gene is located on chromosome 16 (16p11.2), close to the *MRP1* and protein kinase *C-β* gene, and encodes a 110-kDa protein [128]. Based on the LRP amino acid sequence, no transmembrane fragments or ATP-binding sites, characteristic for ABC transporters, were identified. Therefore, LRP is not considered to be a member of the ABC transporter family. Screening of an expression library identified LRP as the major vault protein (MVP) [9]. Vaults are highly conserved ribonucleoprotein organelles that are found in all higher eukaryotes. They are localised in cytoplasmic vesicles and nuclear membranes and form the transporter core of the nuclear pore complex. Vaults are composed of the major vault protein, vault poly (ADP-ribose) polymerase, telomerase-associated protein 1 and small untranslated RNA [129]. The functional role of vaults in MDR is still unclear but it was proposed that they act by transporting drugs away from their subcellular targets by mediating the extrusion of cytostatics from the nucleus and/or the sequestration of drugs into vesicles.

By immunocytochemistry, LRP has been found to be widely distributed in normal human tissues [130]. LRP overexpression has been observed in epithelia of the bronchus and digestive tract as well as in keratinocytes, adrenal cortex and macrophages. These results suggest that vaults play a role in detoxification processes.

The enforced expression of LRP in an ovarian carcinoma cell line led to increased numbers of vault particles, but failed to confer drug resistance to etoposide, doxorubicin and vincristine [9,131]. These findings are in accordance with those published by van Zon and colleagues [132]. They found no relationship between vault expression and efflux or sequestration of anthracyclines in vesicles. Moreover, the absence of vaults in MVP–/– murine cells did not induce hypersensitivity to cytostatic drugs [133]. Huffman and Corey reported that decreased MVP expression did not alter the ability of resistant cells to remove doxorubicin from the nucleus [134]. Conversely, upregulation of MVP did not confer drug resistance on chemosensitive cells. By contrast, in MVP/vaults overexpressing colon carcinoma cells, anthracyclines are cleared from the nucleus in a MVP-dependent way [135]. These contradictory findings suggest the need for additional studies investigating the possible link between upregulation of vaults and multidrug resistance.

Table 4 – LRP

Author	Method	Samples	Conclusions
Volm et al., 1997 [136]	LRP expression: IC	38 I ALL 25 R ALL	At Dx: 47% LRP expression; At relapse: 68% LRP expression Patients without LRP expression had a higher probability of remaining in continuous first remission
den Boer et al., 1998 [66]	LRP expression: FC In vitro drug resistance: MTT assay	112 I ALL 22 1st R ALL 7 ⩾ 2nd R ALL 20 I AML 6 1st R AML 1 2nd R AML	No difference in LRP expression levels between initial or relapsed patients LRP expression was 1.6-fold higher in multiple relapse samples than in initial or first relapse samples LRP expression was 2-fold higher in AML than in ALL patients LRP expression was 1.4-fold lower in T-ALL compared with common/pre-B-ALL patients LRP expression was weakly associated with in vitro resistance to daunorubicin LRP expression did not relate to age or initial WBC count
den Boer et al., 1999 [69]	LRP expression: FC LRP function: DNR Acc and efflux In vitro drug resistance: MTT assay	60 I ALL 25 R ALL	After correction for cell volume: Intracellular DNR concentration was lower in relapsed compared with initial ALL patients Inverse correlation between intracellular DNR concentration and in vitro DNR resistance Accumulated DNR concentration inversely correlated with expression of LRP
Kakihara et al., 1999 [71]	LRP gene expression: RT-PCR	40 I ALL	No correlation between LRP gene expression and age or WBC count No difference in LRP expression levels between high- and low-risk patients LRP gene expression was increased in CD 10-positive ALL
Ogretmen et al., 2000 [72]	LRP gene expression: RT-PCR	12 I ALL	LRP was overexpressed in most children with pre-B-ALL LRP mRNA was undetectable in most children with T-ALL
Sauerbrey et al., 2002 [117]	LRP gene expression: RT-PCR	58 I ALL 28 R ALL	No difference in LRP gene expression between initial and relapsed ALL patients Patients without LRP expression exhibited a higher tendency of remaining in continuous first remission LRP expression was independent of age, sex, initial blast count, FAB-type and immunological subtype
Ramakers-van Woerden et al., 2004 [80]	LRP expression: FC	469 I ALL	No difference in LRP expression between infants and older common/pre-B-ALL patients LRP expression was 1.4-fold higher in pro-B-ALL than in common/pre-B-ALL

Information about the clinical relevance of LRP in childhood ALL is limited (Table 4). In most studies, no difference in LRP expression between initial and relapsed ALL patients is found [72,117,136]. However, LRP expression was significantly higher in multiple relapse samples compared with diagnostic or first relapse samples [66]. Furthermore, LRP expression levels do not seem to differ between risk groups identified by WBC count, sex or age [66,71,117]. Ogretmen and colleagues found a high LRP expression in most children with pre-B ALL [72]. By contrast, LRP expression was much lower in T-ALL patients. These results are in accordance with those published by den Boer and co-workers [66]. Ramakers-van Woerden and colleagues found a high LRP expression in pro-B-ALL patients [80].

Volm and co-workers reported a significant association between LRP expression and long-term survival in 38 children with de novo ALL [136]. The relationship between LRP expression and in vitro resistance to daunorubicin, vincristine, etoposide and prednisolone was also studied [66]. The LRP expression was weakly but significantly related to the in vitro resistance to daunorubicin. In addition, LRP expression correlated inversely with the intracellular accumulated daunorubicin concentration [69]. These findings suggest that LRP might contribute to drug resistance in children with ALL.

6. Other multidrug resistance proteins

During the past few years, several new members of the ABC transporter family have been identified, including eight new MRP1 homologues (MRP2 or cMOAT, MRP3, MRP4, MRP5, MRP6, MRP7, MRP8 or ABCC11 and MRP9 or ABCC12), the sister of P-gp (sP-gp) and the transporter associated with antigen processing (TAP) [137–139].

Plasschaert and colleagues evaluated MRP2 and MRP3 mRNA expression in childhood ALL [78]. In 89% and 24% of the samples, respectively, MRP2 or MRP3 transcripts were detected. Steinbach and co-workers assessed the clinical relevance of MRP2–MRP5 and SMRP, a splice variant of MRP5, in 103 children with previously untreated ALL [140]. All five genes were expressed with great variability. However, only MRP3 expression was associated with a significantly worse prognosis. The median MRP3 levels were 10-fold higher in T-ALL compared with precursor B-ALL patients and 4-fold higher in boys than in girls. These findings suggest than MRP3 overexpression might account for the poor prognosis of male and T-ALL patients. However, larger studies are needed to confirm the prognostic importance of MRP3 in childhood ALL.

The clinical importance of the other transport proteins in childhood ALL remains to be elucidated.

7. Conclusion

One of the most important causes of treatment failure in childhood ALL is the emergence of multidrug resistance. Various mechanisms can give rise to clinical drug resistance, but best studied is the overexpression of transmembrane transport proteins, such as P-gp, MRP1, BCRP and LRP. In the present review, we focused on the clinical relevance and prognostic significance of these MDR-related efflux pumps in childhood ALL. Several authors found a relationship between P-gp expression and/or function and clinical outcome in paediatric ALL patients. Other studies, however, contradicted these findings. Data on the prognostic significance of other MDR proteins are scarce. LRP and BCRP might contribute to drug resistance in B-lineage ALL, but larger studies are needed to confirm these results. MRP1 does not seem to play a major role in MDR in childhood ALL and of all newly identified ABC transporters (e.g. MRP2–MRP9, sP-gp, TAP), only MRP3 expression was associated with a worse prognosis.

Despite profound research, the clinical importance of MDR-related proteins in childhood ALL remains controversial. One of the major causes of the variability in published data is the lack of standardised, sensitive and specific detection techniques. Although this problem has been addressed by a number of international workshops, development of consensus recommendations has been difficult [84–87,141]. Generally, flow cytometry is preferred to immunoblots, Northern blots and immunocytochemical assays. Especially, the use of functional flow cytometric tests assessing modulator-induced changes in fluorophore retention and/or efflux has been promoted because they allow, in contrast to immunological or molecular tests, the evaluation of protein activity. Moreover, functional assays are characterised by a high sensitivity, specificity and reproducibility. We believe an international effort should be made to develop, optimise and standardise a sensitive, specific and reproducible functional detection technique.

Comparison of data is also hampered by the heterogeneity in patients groups (e.g. pooled data from ALL and AML patients, initial and relapse samples, adults and children) and differences in treatment protocols. In our opinion, proper study design and selection of patients, treated according to a standardised protocol, is indispensable in order to determine the prognostic significance of MDR-related proteins in childhood ALL in a reliable way. Consequently, large quality-controlled multicentre studies are needed to elucidate the clinical relevance of MDR-related proteins.

Detailed information on the clinical relevance of MDR-related efflux pumps is needed before the potential of transporter-specific modulators can be studied. So far, phase 3 clinical trials with first- and second-generation P-gp antagonists have yielded conflicting results. This may be explained by the functional redundancy between different drug resistance efflux pumps. Moreover, limitations in the design of early resistance reversal trials contribute to the disappointing results.

It is also important to keep in mind that multidrug resistance is a multifactorial process. In addition to transmembrane transport proteins, other resistance mechanisms, such as alterations in detoxification processes, apoptosis, DNA repair, cell cycle progression and drug uptake, might contribute to clinical drug resistance. Tools such as oligonucleotide-based or cDNA-based microarrays are relevant methods to screen for multifactorial mechanisms since they allow the determination of the expression profile of many genes in a single hybridisation experiment [142–144]. Microarrays may open new avenues for the diagnosis of MDR in clinical samples. In addition, they will help us to obtain a clear picture of how to optimise treatment schedules in leukaemia.

Conflict of interest statement

None declared.

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