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PERSPECTIVES IN MEDICAL SCIENCES

Drug Resistance in Acute Leukaemia and Reversion

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Introduction

Haematological neoplasms are usually primarily sensitive to chemotherapy, but the relapse rate is still high, except for Hodgkin's lymphoma and childhood acute lymphoblastic leukaemia (ALL). Drug resistance is therefore a major cause of chemotherapeutic failure and patient death in haemato-oncology.

A mathematical model for the development of drug resistance in tumours was proposed in 1979 by Goldie and Coldman (1). This model was based on the postulate that cancer cells have a high spontaneous mutation rate, leading over time to the emergence of cells resistant to chemotherapeutic drugs. In accordance with this hypothesis, it was suggested that a reduction in the rate of emergence of resistant cells could be achieved by the simultaneous administration of multiple drugs with different targets. However, despite combination chemotherapies, treatment failures were observed in lymphoid and myeloid malignancies, and relapses occurred in more than half of the cases in acute myeloid leukaemia (AML) and adult ALL.

It is therefore clear that a better understanding of the mechanisms involved in drug resistance is still warranted for the development of new therapeutic strategies.

Measurement of Drug Resistance in Clinical Samples

In vitro assays have been developed to test the drug resistance of clinical samples (mainly acute leukaemia) (2). Clonogenic assays offer the advantage of testing the drug sensitivity of the progenitor cells, and have been successfully correlated with the clinical outcome in AML: resistance to both anthracyclines and cytosine arabinoside (Ara-C, cytarabine) was highly correlated with clinical failure in adult AML, and was one of the best predictive variables in a multivariate analysis (3). Unfortunately, clonogenic assays are time-consuming and cannot be automated. For these reasons, other viability tests have been developed. The most commonly used assay is the MTT assay, which relies on the ability of the mitochondria of living cells to convert a soluble tetrazolium salt (MTT) into an insoluble formazan. The formazan precipitate is purple, can be dissolved, and its extinction can be read on a 96-well plaque reader. The extinction is linearly correlated with the number of viable cells in suspension. The 4-day MTT assay is an efficient tool for large-scale drug-resistance testing, and results have shown good correlation with prognosis in childhood ALL (4).

Classification of Drug Resistance

The efficacy of cytostatic antineoplastic therapy is determined by a sequential cascade of events, including drug delivery, drug–target interaction and the induction of cellular damage. The first part of this cascade corresponds to the pharmacological resistance, and up to now has been the most widely studied mechanism of resistance.

Classically, resistance is divided into extrinsic and intrinsic causes.

- *Extrinsic resistance* corresponds to the inability of the drug to reach the tumour cell: this is the case when the bioavailability of the oral form varies greatly from patient to patient, as with 6-mercaptopurine in ALL (5). Defects in tumour vascularisation, frequently observed in solid tumours, are also probably relevant for haematological malignancies (6).
- *Intrinsic resistance* is directly due to the properties of the tumour cell. This phenomenon can be observed in vitro, and can be classified as *simple resistance*, when the cells are resistant to only one drug, or as *multidrug resistance*, when a cross-resistance is observed for chemostatic drugs with different biochemical targets. This latter type of resistance is mainly observed in patients, and can be due to several mechanisms. The underlying pharmacological mechanism corresponds mainly to an active efflux of the drugs out of the tumour cells. Molecular profiles giving rise to broader forms of resistance are now under investigation, and it is believed that a defect in drug-induced apoptosis is at least partly responsible. This could be due to increases in anti-apoptotic signals (survival signals from the micro-environment) and/or increases in anti-apoptotic proteins (ex: bcl-2) or decreases in pro-apoptotic proteins (ex: bax) (Figure 1).

Pharmacological Drug Resistance

ABC proteins

MDR cells are resistant to several naturally occurring plant or microbial products, but are not resistant to synthetic compounds such as cisplatine, or to nucleotide analogues such as cytosine arabinoside (Ara-C) (Table). Resistant tumour cells maintain lower intracellular drug concentrations than their sensitive counterparts (7), and in the majority of cases express transport proteins that are responsible for active efflux of the drugs.

The first – and probably most important – protein described in cell models of MDR resistance is P-glycoprotein (P-gp), encoded by the ABCB1 (*MDR1)* gene. This trans-membrane protein belongs to a super-family of ABC ('ATP Binding Cassette') proteins, specialised for energy-dependent cellular transport (8). The genes coding for these proteins are highly conserved between species, from bacteria to man. Besides *MDR1*, members of the ABCC family (*MRP1-6* 'multidrug resistance-associated protein'), with low homology with

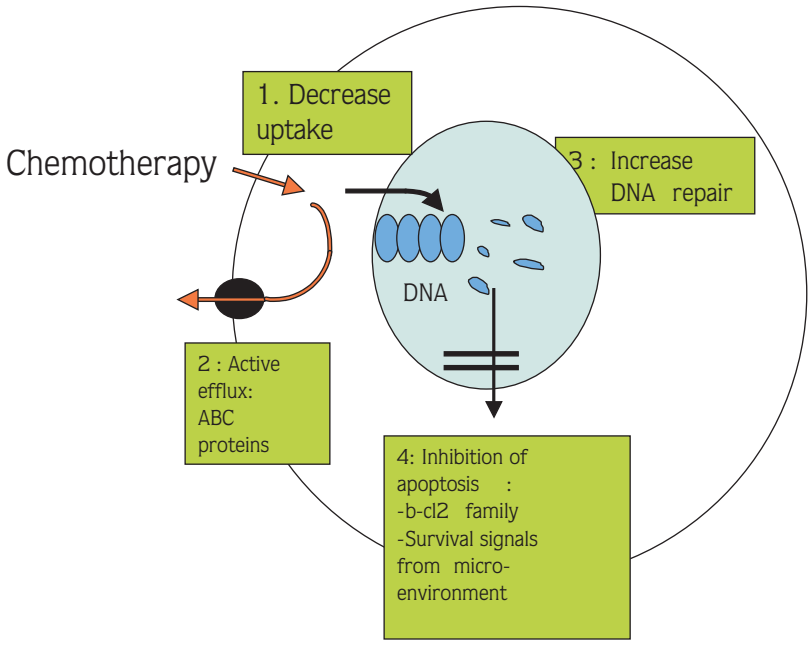

Figure 1. Main mechanisms of drug resistance in leukaemia.

Table. ABC proteins involved in multi-drug resistance phenotype: international nomenclature.

Nomenclature	Name	Localisation/Structure[1]	Chromosomal location[2]	MDR phenotype after transfection[3]
ABCB1	P-gp/MDR1	Plasma Membrane (TMD-ABC)2	7q21	++
ABCC1	MRP1	Plasma Membrane TMD0(TMD-ABC)2	16q13.1	+ (if GSH)
ABCC2	MRP2(cMOAT)	Plasma Membrane TMD0(TMD-ABC)2	10q24	+/-(if GSH)
ABCG2	BCRP/MXR1/ABCP	Plasma Membrane (works as dimer) (TMD-ABC)	4q22	+

Previous name(s): [1]Protein localisation, [2]Gene localisation, [3]cDNA transfection result.

MDR1, have been described as other proteins of this super-family involved in drug resistance (for a review see reference 9).

Recently, a small ABC protein, the ABCG2 (or BCRP), was described simultaneously by 3 different groups. It was described as a "specific" ABC protein of the placenta ("ABCP"), as a new ABC protein expressed in a MDR1 (-) MRP1(-) breast cell line resistant to anthracycline ("BCRP"), and in a colon carcinoma cell line resistant to mitoxantrone ("MXR1") (8).

The expression of these genes is believed to confer resistance – the evidence for this being that the transfection of their cDNA in sensitive cell lines gives rise to the MDR phenotype (10-12).

P-gp, MRPs and BCRP are not unique to drug-resistant cells, and are expressed by tissues with excretory–secretory functions. In haematopoietic cells, progenitor cells express P-gp and BCRP, but not MRP. These localisations suggest that these proteins are involved in the protection of organisms (and cells of vital importance) against natural xenobiotics (13).

A few years ago, several workshops (14,15) proposed a consensus on technical recommendations for measuring P-gp. Protein detection and, more importantly, functional test by flow cytometry are recommended for leukaemic samples. Several monoclonal antibodies, recognising an external epitope, are available for protein expression, and several fluorescent probes could be used, with and without specific inhibitors, for measuring drug efflux.

P-gp in acute leukaemia

Several recent studies have reported a high frequency of *MDR1* gene expression in AML. Large multi-centric studies (review: 16) showed between one-third and one-half of positive cases at diagnosis, whatever the technique used, and usually a higher proportion of positive cases were described in elderly patients (17). The MDR phenotype is associated with markers of bad prognosis, such as CD34 or CD7.

Except for the promyelocytic subtype (AML3), which is devoid of P-gp expression (18), all other AML subtypes are able to express the MDR phenotype. In the majority of cases, the functional tests (dye efflux) were correlated with P-gp expression, except in $CD34^-$ cells, like myelo-monocytic and monoblastic leukaemias. For that reason, dye efflux appears more informative than the quantification of P-gp itself in AML.

The MDR phenotype is also frequent in myelodysplastic syndromes (19) and blast crisis of chronic myeloid leukaemia (CML) – both of which are known to be diseases with particularly bad prognoses.

The correlation between *MDR1* gene expression and treatment outcome is well documented in AML (for a review see reference 16): many studies have reported a relationship between either the absence of remission (refractory disease and death during aplasia) and the overexpression of P-gp, or the overexpression of P-gp and refractory disease.

More discordant results have been published concerning the incidence of MRP expression in AML (20): the range of MRP expression is narrow compared with P-gp, and a basal expression is found in all cases. One-third of patients presented with high expression. The prognostic value of MRP itself is not yet clearly determined, but a functional test measuring efflux due to P-gp and MRP1 (using calcein AM and modulators) clearly indicated that both P-gp and MRP1 functions are of prognosis importance in adult AML (21). The role of other MRPs is, to date, under investigation (22).

Discordant results were published concerning the incidence of expression and the prognostic value of ABCG2 in leukaemia. Frequency of expression ranges from 5% (23) to 30% (24,25) in adult AML, though until now, no large series have been published concerning functional assay and the prognostic value of such a protein in adult acute leukaemia. In pediatric AML, ABCG2 was linked to a worse prognosis (26).

Acute lymphoblastic leukaemia

Except for a few studies, the incidence of *MDR1* overexpression in untreated patients with ALL has generally been found to be low (<10%) at diagnosis and even at relapse, except during the last stage of the disease, when clinical drug resistance is usually observed (for a review see reference 16).

The MDR phenotype in ALL is found only in ALL subgroups with a bad prognosis: adult ALL (often $Ph1^+$) and a subtype of $CD7^+CD4^-CD8^-$ ALL, which is thought to originate from a haematopoietic stem cell.

P-gp expression was not predictive for induction treatment failure in childhood ALL. This could be explained by the predominant role of corticosteroids, which are not expelled by P-gp, as the major drugs used to treat ALL. In adult ALL, a recent study showed that protein expression, but not function(?), predicted CR achievement in patients treated in the GIMEMA ALL0496 protocol (27).

Few publications concern MRP in ALL (20), but all have shown a measurable level of MRP at diagnosis – comparatively higher than in AML cells, and some cases showed an increase after treatment. The prognostic value of MRP expression is at present not clearly demonstrated.

To date only one publication described a low incidence of ABCG2 expression in pediatric ALL, with no prognostic significance (28).

Lung resistance protein/major vault protein

An additional protein was described in a wide variety of P-gp-negative multidrug-resistant cancer cell lines and in clinical samples. This protein is a non-ABC, MDR-associated protein originally termed lung resistance-related protein (LRP), and now identified as the major vault protein (MVP) (for a review see reference 29). Vaults are highly conserved among species, supporting the notion that their function is essential to eukaryotic cells. It was hypothesised that vaults mediate the bidirectional transport of a variety of substrates between the nucleus and the cytoplasm. To date, the cDNA of LRP/MVP is unable to confer the MDR phenotype; therefore, proof of the involvement of LRP/MVP in the MDR phenotype could not be demonstrated.

In the majority of cases of AML, LRP/MVP is low (30), but remains detectable by RT-PCR. The clinical significance of LRP expression is still controversial (31), and functional tests specific for LRP need to be developed.

Pharmacological resistance to Ara-C

Ara-C plays a key role in the achievement and consolidation of complete remission in AML, and is also widely used at high doses as a rescue treatment in refractory ALL and AML. This nucleotide analogue is not expelled by ABC proteins, and is of particular interest in patients expressing P-gp or MRP. In vitro resistance to Ara-C has been given as a main parameter for predicting treatment failure (32-33) or relapse (34) after conventional doses of Ara-C and anthracycline in AML.

After transport into the cell, Ara-C must be phosphorylated to its active triphosphate form (Ara-CTP) by a series of kinase enzymes (Figure 2). The ability of fresh human leukaemic cells to retain the cellular concentration of Ara-CTP has been correlated with the duration of remission in patients treated with Ara-C and anthracycline (35). Although Ara-CTP is an inhibitor of DNA polymerase a, it is also a substrate, and is in competition with dCTP (the natural compound) for DNA incorporation. It is thought that the incorporation of Ara-CTP into DNA is responsible for the lethal effect of this drug. Factors that reduce the intracellular concentration of triphosphorylated cytarabine (ara-CTP), the active form of cytarabine (ara-C), may induce chemoresistance in AML patients. These factors include reduced influx of ara-C by the hENT1 transporter, reduced phosphorylation by deoxycytidine kinase (dCK), and increased degradation by high Km cytoplasmic 5'-nucleotidase (5NT) and/or cytidine deaminase (CDD). Recent papers suggest that the expression of DNA POL, 5NT and hENT1 at diagnosis may be a resistance mechanism to ara-C in AML patients (36-38).

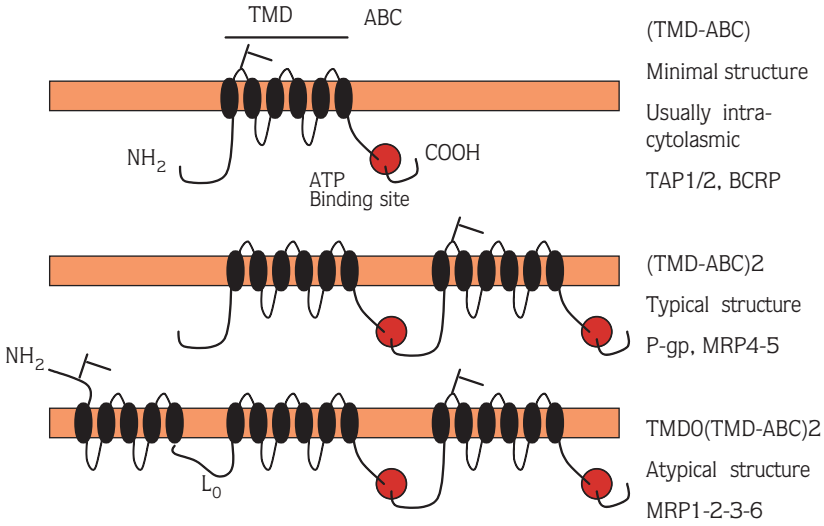

Figure 2. Different structures of ABC proteins involved in multi-drug resistance.

TMD: trans-membrane domain

ABC: ATP-binding cassette

Distal Drug Resistance

p53 and proteins regulating the cell cycle

The ultimate success of genotoxic anticancer agents is determined by the ability of malignant cells to initiate an apoptotic response to induced DNA damage (39). Among the numerous factors known to modulate cancer-related apoptosis, p53 and the bcl-2 family are the most extensively characterised proteins. p53 is activated in response to DNA damage, and stops the cell in the G_1 phase (via p21), permitting DNA repair, whereas apoptosis can be considered to be a fail-safe mechanism to rid the organism of cells with severely damaged DNA. In cases of non-functional p53, the threshold of DNA damage leading to apoptosis increases, and this could contribute to drug resistance (40). In haematological malignancies, p53 is usually functional at diagnosis, but mutations have been described during progression of the disease in both lymphoid and myeloid leukaemias as well as in lymphomas (41), and this progression corresponds mainly to highly resistant tumours.

Genetic alterations affecting the $p16^{INK4a}$ and cyclin D1 proteins, which govern phosphorylation of the retinoblastoma protein (pRb) and control exit from the G_1 phase of the cell cycle (42), are frequent in cancer, including ALL and mantle cell lymphoma, and are associated with an aggressive course of the disease.

Anti-apoptosis proteins and signals

Analysis of the t(14;18) translocation of follicular lymphoma has permitted the description of the *bcl-2* (B-cell lymphoma 2) oncogene. The anti-apoptosis properties of bcl-2 and bcl-x_L, another member of the same family, are now well documented, and their overexpression has been shown to protect tumour cell lines from the toxicity of several chemotherapeutic agents (for a review see reference 43). The clinical impact of bcl-2/bcl-x_L overexpression on drug resistance has not been demonstrated, but correlations have been described between high bcl-2 cell content and clinical drug resistance in AML (44), but not in ALL (45), The bax/bcl2 ratio was correlated with spontaneous apoptosis of AML blasts in adult AML, and was found to be highly predictive of outcome in a recent study on 255 adult AML patients (46), while relapse in childhood ALL has been associated with a decrease in the Bax/Bcl-2 ratio (47).

Signals from the micro-environment could be strong stimulators for tumoural cell survival and resistance to chemotherapy. In AML, the role of bone marrow endothelial cells is beginning to be explored, and has revealed paracrine loops involving VEGF and VEFG receptors (48).

Reversal of the Mdr Phenotype

P-gp inhibition

Numerous compounds have been identified that inhibit the efflux activity of P-gp and reverse cellular resistance in experimental systems (for a review see reference 49). This has led to the strategy of concomitant administration of chemotherapy and an 'MDR modulator to reverse clinical drug resistance. These compounds act mainly as competitive or non-competitive inhibitors, by binding to similar drug substrate binding sites, or to other P-gp sites that cause allosteric changes of the molecule, resulting in a decrease in cytotoxic drug binding.

The most commonly used modulators in randomised phase 3 in leukaemia are quinine and cyclosporines (CsA and PSC833, an analogue of cyclosporin D). More recently, other compounds, specially designed and developed for modulation of drug resistance, entered phase 1 : VX710, LY335979, XR9576.

The first large randomised study of a MDR modulator in haemato-oncology tested the usefulness of the addition of quinine to a combination of mitoxantrone and high doses of Ara-C in 315 bad-prognosis adult leukaemia cases (relapsing/refractory/secondary) (50). Global results showed no difference between the groups with or without quinine, but it was noted that (i) clinical drug resistance was higher in the control group and (ii) toxic death rate was higher in the quinine group. The clinical toxicity of quinine could have masked the clinical benefit of MDR reversal. Recently, the same dose of quinine was randomised in de novo untreated patients receiving induction treatment in 425 patients: the addition of quinine benefits only patients with functionally P-gp positive AML cells, but does not influence the overall survival of such patients (51).

Similar results were observed with PSC833 (10-fold more potent than CsA and without any renal or immunosuppressive toxicity) in elderly untreated patients (52). The CALG B phase 3 trial consisted of Standard doses of cytarabine, with daunorubicin 60 (40 if PSC833) mg/m^2 and etoposide 100 (60 if PSC833)mg/m^2 daily for 3 days without (ADE) or with PSC-833 (10 mg/kg daily by 3-day infusion) (ADEP). The ADEP arm was closed after the randomisation of 120 patients because of excessive early mortality. Nevertheless, disease-free survival (median 7 vs. 8 months; P = 0.38) and overall survival (approximately 33% alive at 1 year) did not differ and were similar to historical results. For patients with PSC-833-modulated efflux, median disease-free survival was 5 months with ADE and 14 months with ADEP (P = 0.07).

Several other phase 3 clinical trials in relapsed myeloid leukaemia with PSC833 are now completed, with globally negative results.

On the other hand, the South-West Oncology Group conducted a randomised phase 3 study on 226 patients, randomly assigned to sequential treatment with cytarabine 3 g/m^2 daily for 5 days, followed by DNR 45 mg/m^2 per day (continuous infusion) on days 6 to 8, with or without intravenous CsA (16 mg/kg daily) (53). The addition of CsA significantly reduced the frequency of resistance and increased relapse-free and overall survival. The effect of CsA on survival was greatest in patients with Pgp(+) leukaemic cells. The same cooperative group performed another randomised trial with the same arms in CML blast crisis, but in this disease CsA was unable to improve the rate of CR or survival (54).

Other P-gp inhibitors are now in phase 2 studies, mainly in solid tumors. LY335979 is a quinoline derivative specific to P-gp, is delivered orally and has a low effect on the pharmacokinetics of the drug co-administered (55). Phase 2 is now completed in AML, and a phase 3 is now beginning in the USA. The XR9576, an anthranilic acid derivative, delivered orally or intravenously, is now in phase 1-2 trials (56).

Reversion of other ABC pumps

GG120918, a derivative of acridonecarboxamide, was developed as an inhibitor of P-gp, but was recently described as a potent inhibitor of BCRP (57). Phase 2 trials resumed recently for solid tumours.

VX-710 (Biricodar®), a pipecolinate derivative, is a modulator of P-gp and MRP1. It has no effect on doxorubicin pharmacokinetics, although an increase in bilirubin was noted in a phase 1 study (58).

Reversal of Other Kinds of Resistance

Resistance to Ara-C

High-dose (1–3 g/m^2 per 12 h x 8 to 12) infusion (2–3 h) of Ara-C is a very potent treatment in secondary/relapsing/refractory AML and in ALL. Resistance to standard doses of Ara-C (100–200 mg m^2/d x 7 d) is usually overcome by increasing the dose,

because of the competition between this nucleoside and the natural deoxycytidine (dC): more Ara-C will enter the cell, more will be phosphorylated, and more will be incorporated into DNA. The cell concentration of Ara-CTP of circulating leukaemic cells during the high-dose infusion of this drug is highly correlated with the clinical outcome (59). confirming the importance of (i) Ara-C uptake and (ii) Ara-C phosphorylation.

It has also been proposed to prime leukemic myeloid progenitors with growth factors (G-CSF, GM-CSF) before Ara-C treatment. After several large trials, the value of this approach is unclear (for a review see reference 60).

Inhibitors of farnesyl transferase

The membrane-associated G proteins encoded by the Ras family of proto-oncogenes are a potential target for new therapeutic agents. Ras proteins are activated downstream of protein tyrosine kinases and, in turn, trigger a cascade of phosphorylation events through sequential activation of Raf, MEK-1 and ERKs. To be active, Ras proteins have to be farnesyled. Therefore, several inhibitors of farnesyl transferase were recently developed. A phase 1 study in refractory/relapsing acute leukaemia with R115777 (Janssen) demonstrated a significant inhibition of ERK phosphorylation in bone marrow after treatment, and clinical response occurred in 29% of the patients (including 2 CR in 34 evaluable patients) (61).

Restoring apoptosis: *Bcl*-2 anti-sense therapy

Restoration of drug-induced apoptosis could be obtained in vitro with bcl-2 anti-sense. A phase 1 study of Bcl-2 anti-sense oligonucleotide was performed in relapsed Bcl-2 (+) NHL, using 14 days of sub-cutaneous infusion of G3139, an 18-mer oligonucleotide complementary to the first codons of *bcl*-2 (62). An achievable concentration of anti-sense was obtained without major toxicity, and few responses were observed. Another phase 1 study was performed in refractory/relapsed acute leukaemia, treated with 10 days of G3139 (continuous infusion) together with fludarabine, aracytine and G-CSF. A steady-state concentration of G3139 was achieved after 24 h without major toxicity, and 6/20 patients achieved CR (63). A randomised phase 3 trial is now running in untreated elderly AML.

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