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Review

An overview of cancer multidrug resistance: a still unsolved problem

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Abstract. Although various mechanisms involved in anticancer multidrug resistance (MDR) can be identified, it remains a major problem in oncology. Beyond that, the introduction of new "targeted" drugs have not solved the problem. On the contrary, it has been demonstrated that the "classical" MDR-associated mechanisms are similar or identical to those causing resistance to these novel agents. These mechanisms include the enhanced activity of drug pumps, i.e. ABC- or alternative transporters; modulation of cellular death pathways; alteration and repair of target molecules; and various less common mechanisms. Together they build a complex network of cellular pathways and molecular mechanisms mediating an individual MDR phenotype. Although the application of new high throughput "-omics" technologies have identified multiple new gene-/protein expression signatures or factors associated with drug resistance, so far none of these findings has been useful for creating improved diagnostic assays, for prediction of individual therapy response, or for development of updated chemosensitizers.

Keywords. Cancer, drug resistance, chemotherapy, targeted therapy, ABC-transporters, death pathways, repair pathways.

Introduction to the clinical problem

When, due to the pioneering studies of Paul Ehrlich, chemotherapy was introduced as the "magic bullet" against infectious diseases, clinical obstacles promptly arose as a result of the development of drug-resistant bacterial strains. The same story happened following the introduction of chemotherapeutic treatment for neoplastic diseases with cytotoxic agents. Unless tumor cells already exhibit primary resistance to anticancer drugs, they immediately acquire resistance to those compounds during chemotherapy. Due to this phenomenon the efficacy of a chemotherapeutic regimen varies from patient to patient. The tumors of some patients show a complete or partial response (CR, PR) to chemotherapy; they seem to be clinically drug-sensitive. On the other hand, malignancies can manifest as stable or progressive disease (SD, PD). Depending on the tumor entity, these cancers are designated as clinically drug-resistant. However, it is noteworthy that SD following chemotherapeutic treatment of primary drug-resistant tumor entities, such as malignant melanoma, clinically appears to be drug-sensitive. The obvious strategy to overcome clinical drug resistance by employing multiple cytotoxic drugs with different chemical structures and mechanisms of action is not the solution to this problem, because tumors appear able to develop resistance simultaneously to many different antineoplastic agents. This phenomenon is commonly designated as pleiotropic resistance or the multidrug resistance (MDR) of cancer, whereby the original

concept of this occurrence was introduced into the scientific literature in 1970 [1]. Likewise, the introduction of new "targeted" anticancer drugs such as small molecule protein kinase inhibitors or therapeutic antibodies resulted in the emergence of drug resistance. Despite recent advances in the treatment of cancer, drug resistance, in particular MDR, is still the major cause of anticancer chemotherapy failure in clinics. Thus, the clinical outcome of cancer patients is still far from expectation.

Mechanisms of drug resistance

In clinical practice, the phenomenon of drug resistance becomes a crucial problem when toxicity of drugs at dosages necessary to kill cancer cells increase to a non-manageable clinical situation. Since most antineoplastic agents have a low therapeutic index – i.e. a poor ratio of the amount of the drug that causes the therapeutic effect to the amount that causes toxic effects – even a weak decrease in the sensitivity of cancer cells can manifest a clinically drug-resistant phenotype. The biological mechanisms underlying this therapy failure can be classified into two broad categories, into pharmacological and into cellular factors.

Pharmacological mechanisms of drug resistance. Chemotherapy should be given at the maximum tolerated dose (MTD) to kill a maximum number of tumor cells. A tumor is defined as being clinical resistant to the MTD if the effective drug dosage in the tumor, given as the area under the curve (AUC = drug concentration × time of drug exposure), is not effective in achieving a clinically ascertained CR or PR. This therapeutically insufficient drug dosage may be due to different physiological mechanisms that can be summarized as pharmacological mechanisms of drug resistance. These mechanisms include (i) the application of the drugs, e.g. inadequate infusion; (ii) low metabolic activation in the case of utilization of prodrugs, e.g. the conversion of cyclophosphamide by cytochrome P450 oxidases in the liver to the active metabolite 4-hydroxycyclophosphamide; (iii) the pharmacokinetics in the plasma, i.e. metabolisms and excretion of the drugs; (iv) the tumor microenvironment, e.g. vascularization, diffusion, hypoxia; and (v) the availability, i.e. the architecture of barriers such as the blood brain barrier (BBB). However, here we will focus on the cellular mechanisms of drug resistance.

Cellular mechanisms of drug resistance. In addition to these pharmacological mechanisms of drug resistance, various cellular mechanisms taking place directly within the tumor cell have been described. These multiple mechanisms involved in drug resistance, especially in MDR, may occur simultaneously and/or sequentially and may be switched on and off during the establishment of a drug-resistant phenotype.

In the last four decades dramatic progress has been made in understanding cellular drug resistance-associated genes, proteins and their mechanisms of action [2–5]. Due to the inherent difficulties of the investigation of the biological mechanisms leading to drug resistance directly in the patient, various *in vitro* models have been developed. These models were commonly established by application of the principle of mithridatism, the practice of protecting oneself against a poison by gradual self-administration of non-lethal amounts of the poison. This strategy derives from Mithridates VI, the last king of Pontus in what today is Anatolia, who so feared being poisoned that he regularly ingested small doses to accustom himself to the poison. Transferred to laboratory practice, mithridatism stands for the exposure of drug-sensitive cancer cells to sub-lethal dosages of a defined anticancer compound increased stepwise. The result is an acquired drug-resistant phenotype, often with cross resistance to a variety of structurally and functionally unrelated drugs, in other words a multidrug-resistant phenotype. Following the isolation of the first MDR cell lines, i.e. daunorubicin-selected rodent cell lines [6] forty years ago, a huge number of MDR *in vitro* models derived from multiple human cancer tissues have been established and characterized. These cell models provide the basis for the current knowledge of anticancer drug resistance. However, in this context it should be mentioned that the phenomenon of the acquired drug resistance of neoplastic cells was already described in 1950, when mouse leukemic cells were passaged in mice treated with 4-amino-N^{10}-methyl-pteroylglutamic acid [7]. Today, the experimental search for drug resistance mechanisms, as well as for unknown mechanisms that are clinically relevant targets whose circumvention can improve cancer therapy, is still ongoing.

Resistance to conventional cytotoxic drugs

ABC-transporters. The first factor that was identified as mediating a multidrug-resistant phenotype in an *in vitro* model was what, in the meantime, has become the well-known membrane-embedded drug extrusion pump MDR1/P-glycoprotein (MDR1/P-gp or ABCB1). This transporter was originally isolated from the plasma membranes of Chinese hamster ovary cells displaying a "classical" MDR phenotype,

Table 1. Selected drugs of the "classical" MDR spectrum and "classical" MDR modulators.

Drugs transported by MDR1/P-gp	"Classical" MDR modulators
Anthracyclines: daunorubicin, doxorubicin	*Calcium channel blockers:* verapamil, nifedipine, azidopine, dihydropyridines
Vinca alkaloids: vinblastine, vincristine, vindesine	*Immunosuppressants and derivatives:* cyclosporin A, valspodar (PSC833), tacrolimus
Epipodophyllotoxines: etoposide, teniposide	*Antiarrhythmics:* quinine, quinidine, amiodarone
Antibiotics: actinomycin D, dactinomycin, mitomycin C	*Antihypertensives:* reserpine, yohimbine
Taxanes: paclitaxel	*Antibiotics:* hydrophobic cephalosporins
Others: colchicine, topotecan, valinomyycin, puromycin, emetine, digoxin, imatinib	*Steroid hormones and derivatives:* progesterone, tamoxifen
Many other hydrophobic amphipatic drugs and derivatives	*HIV protease inhibitors:* sequinavir, indinavir, retanavir
	Herbal constituents: curcumin
	Others: elacridar (GF120918), zosuquidar (LY335979), tariquidar (XR9576), laniquidar (R101933)

in 1976 [8]. The "classical" MDR phenotype is characterized by a typical cross resistance pattern against natural product-related anticancer agents, such as Vinca alkaloids, Epipodophyllotoxins, anthracyclines, or taxanes, and the reversibility by verapamil and cyclosporin A derivatives (Table 1). Based on the different biological mechanisms, this "classical" MDR can be distinguished from so-called "atypical" or "MDR1/P-gp-independent" MDR phenotypes. The cross resistance pattern of a "classical" MDR and an "atypical" MDR can be identical, similar, or different [2–4, 9]. MDR1/P-gp was purified in 1979 [10], found to be encoded by the MDR1 gene or, according the HUGO (human genome organisation) gene nomenclature committee (HGNC), the ABCB1 gene [11], and was also found to be over-expressed in a huge number of other multidrug-resistant human and mammalian cells [12, 13]. In these cells, MDR1/P-gp acts as a drug efflux pump with the consequence that the intracellular drug concentration is dramatically decreased. For this reason, the extruded antineoplastic agents can no longer interact with their target molecules. Beside the sub-cellular localization of ABC-transporters in the cytoplasmic membrane, there are also data available showing that these drug efflux pumps can be expressed in intracellular membranes, e.g. the nuclear membrane [14] or vesicular membranes [15]. In the first case, it prevents the interaction of the drug with target molecules in the nucleus, in the other case the drug will be trapped in membrane-surrounded compartments.

MDR1/P-gp is, arguably, the most prominent and best characterized member of the superfamily of adenosine triphosphate (ATP)- binding cassette (ABC) transporters. The designation "ABC-transporters" was introduced by Higgins [16] and pertains to the common structural elements of these proteins. The most characteristic feature of this protein family is a highly conserved, approximately 215-amino acids consensus sequence designated as ATP-binding cassette (ABC) or nucleotide binding domain (NBD). The ABC domain contains two short peptide motifs, a glycine-rich Walker A- and a hydrophobic Walker B-motif [17], both involved in ATP binding and commonly present in all nucleotide-binding proteins. A third consensus sequence is named ABC signature [18] and is unique in ABC domains. NBD containing proteins couple the phosphate bond energy of ATP hydrolysis to many cellular processes and are not necessarily restricted to transport functions. However, the proper meaning of the term ABC-transporter is achieved when the NBD containing protein is, in addition, associated with a hydrophobic, membrane-embedded transmembrane domain (TMD) usually composed of at least six transmembrane (TM) α-helices. The TMDs are believed to determine the specificity for the substrate molecules transported by the ABC-transporter protein. The minimal structural requirement for a biologically active ABC-transporter seems to be two TMDs and two NBDs $[TMD\text{-}NBD]_2$. This structural arrangement may be formed by a single polypeptide chain or in multiprotein complexes by more than one polypeptide chain (Fig. 1). In some genes the different domains are fused into higher structural units, e.g. the so-called "half-transporters" [TMD-NBD] or "full-transporters" $[TMD\text{-}NBD]_2$. However, the different domain combinations are commonly distributed in one or two genes encoding "half- or full-transporters".

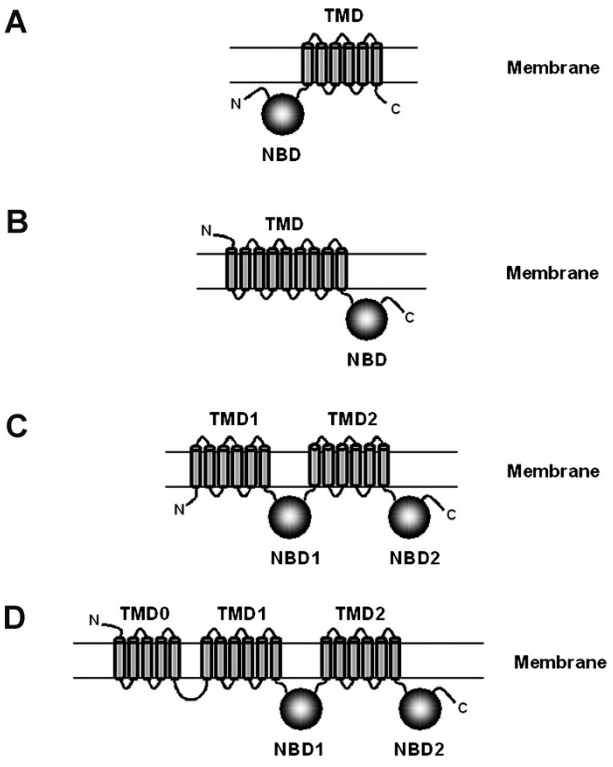

Figure 1. Human ABC-transporters mediating drug-resistant phenotypes. Schematic representation of the predicted domain arrangement of (*A*), (*B*) "half-size transporters" having only one TMD fused to one NBD. (*A*) BCRP posses a TMD of 6 α-helices forming a [NBD-TMD] structure; (*B*) MTABC3 and both TAP molecules are arranged reversed, forming a [TMD-NBD] structure, whereby TAP1 posses a 10 α-helices TMD (not shown), and TAP2 contains a 9 α-helices TMD. "Half-size transporters" dimerize to form a biologically active ABC-transporter. In the case of BCRP, a homodimeric complex is formed, in the case of TAP, a heterodimer consisting of TAP1 and TAP2. (*C*), (*D*) Predicted domain arrangement of "full-size transporters", whereby (*C*) shows the predicted structure of MDR1/P-gp, MDR3/P-gp, MRP4, 5, 8 and 9 $[TMD\text{-}NBD]_2$; and (*D*) the structure of MRP1, 2, 3, 6 and 7 containing an additional TMD (TMD0) forming a $[TMD0(TMD\text{-}NBD)_2]$ structure. TMD, transmembrane domain consisting of 6 to 10 α-helices; NBD, nucleotide binding domain. It should be noted that the orientation of BCRP is reverse that of TAP, MDR1/P-gp and the MRPs.

Since completion of the human genome sequence [19, 20], 48 different ABC-transporters have been identified in humans and divided by their phylogenetic characteristics into seven subfamilies, ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG [21]. Besides the MDR1 gene encoded drug pump MDR1/P-gp mediating the "classical" MDR phenotype, ABC-transporters play important roles in "atypical" forms of MDR. At least 17 other human ABC-transporters have been described to be associated with drug transport *in vitro* (Table 2). Thus, it seems likely that different ABC-transporter expression patterns play an important role in interindividual differences in drug sensitivity.

MDR1/P-gp (ABCB1). Structurally, the human MDR1/P-gp consists of 1280 amino acids residues forming a $[TMD\text{-}NBD]_2$ configuration (Fig. 1). Physiologically, the 170 kDa transporter protein has been found to be over-expressed in tissues with excretory or secretory function. [22, 23]. In tumors derived from those tissues, such as colon, kidney, adrenocortical or hepatocellular carcinomas, MDR1/P-gp is intrinsically over-expressed. The result is a primary multidrug-resistant phenotype of these cancers [9, 24, 25]. In various other cancer types, including acute myeloid leukemia (AML), various childhood tumors and local-regionally advanced breast cancer MDR1/P-gp can be over-expressed, whereby the level of expression correlates with poor outcome in patients treated with chemotherapy [26–31]. These data indicate that "classical", MDR1/Pgp-mediated MDR is the cause of poor treatment outcome in MDR1/P-gp-positive tumors.

ABC-transporters of the MRP (ABCC) subfamily. The second ABC-transporter that was found to mediate a multidrug-resistant phenotype in *in vitro* models was identified in 1992 [32]. This drug pump was originally designated as "multidrug resistance-associated protein" (MRP) and is currently known as MRP1 or ABCC1. It is the founding member of the ABCC subfamily consisting of 12 transporter proteins. In addition to the $[TMD\text{-}NBD]_2$ core of MDR1/P-gp, MRP1, 2, 3, 6, and 7 have an additional NH_2-proximal TMD0 domain consisting of 5 TM α-helices forming a $[TMD0\text{-}(TMD\text{-}NBD)_2]$ configuration (Fig. 1). In contrast, MRP4, 5, 8, and 9 lack the TMD0 domain (the remaining ABCC members are no MRPs; they are known as CFTR, SUR1, and SUR2). So far, MRP1–8 but not MRP9 have been demonstrated to be able to transport any type of anticancer compound. In contrast to MDR1/P-gp which transports unmodified neutral or positively charged hydrophobic compounds, MRPs also act as pumps for organic anions and Phase II metabolic products.

The antineoplastic drug substrates for MRP1 are similar to those of the classical MDR spectrum of MDR1/P-gp. They include anthracyclines, Vinca alkaloids, Epipodophyllotoxins, and the clinical important non-MDR1/P-gp agent methotrexate [33] (Table 2). The physiological substrates of MRP1 appear to be glutathione-, glucuronide-, and sulfate conjugated compounds [34]. Furthermore, MRP1 is able to co-transport natural product-derived drugs with glutathione without covalent conjugation. Although, MRP1 expression was analyzed in various human cancers, e.g. breast cancer [35] or ovarian cancer [36], studies have both confirmed and rejected a correlation between clinical outcome and

Table 2. Human ABC-transporters associated with drug resistance.

ABC-transporter		Drugs	Physiological substrates	References
HUGO nomenclature	Common names			
ABCA2	ABC2	estramustine, mitoxantrone	steroids	[76, 207–209]
ABCA3	ABC3	doxorubicin	surfactant production	[77]
ABCB1	MDR1-P-gp, P-170, P-gp, MDR1, PGY1	"classical" MDR spectrum (Table 1)	phospholipids, neutral and cationic organic compounds	[3, 5, 10, 12, 210]
ABCB2	TAP1	mitoxantrone, Epipodophyllotoxins	peptides	[3, 78, 79]
ABCB3	TAP2	mitoxantrone, Epipodophyllotoxins	peptides	[3, 78, 79]
ABCB4	MDR3-P-gp, MDR3, PGY3	paclitaxel, Vinca alkaloids	phosphatidylcholine	[2, 80]
ABCB5		doxorubicin, camptothecin, 5-fluorouracil	pigment transport ?	[88, 89, 93]
ABCB6	MTABC3	cisplatin, camptothecin	mitochondrial porphyrin uptake	[83, 84]
ABCB11	BSEP, SPGP, ABC16, PGY4	Paclitaxel	bile salts	[5, 81, 82]
ABCC1	MRP, MRP1	anthracyclines, Vinca alkaloids, Epipodophyllotoxins, methotrexate	glutathione-, and other conjugates, organic anions, leukotrienes	[32–34, 211]
ABCC2	MRP2, cMOAT	platin-drugs, anthracyclines, Vinca alkaloids, Epipodophyllotoxins, camptothecins, methotrexate	glutathione-, and other conjugates, organic anions, leukotriene C_4	[33, 37, 38, 212, 213]
ABCC3	MRP3, MOAT-D, MLP2	Vinca alkaloids, Epipodophyllotoxins, methotrexate, cisplatin	glucuronides, bile salts, peptides	[41, 214–216]
ABCC4	MRP4, MOAT-B	nucleotide analogues, methotrexate	organic anions	[41, 217–219]
ABCC5	MRP5, MOAT-C	nucleotide analogues	organic anions, cyclic nucleotides	[41, 220, 221]
ABCC6	MRP6	anthracyclines, Epipodophyllotoxins, cisplatin	glutathione conjugates	[5, 52]
ABCC10	MRP7	taxanes, Vinca alkaloids	organic anions, cyclic nucleotides, bile salts, leukotriene C_4	[48, 56]
ABCC11	MRP8	5-fluorouracil	organic anions, cyclic nucleotides, leukotriene C_4	[48, 49]
ABCG2	BCRP, MXR, ABCP	mitoxantrone, anthracyclines, camptothecins, topotecan	prazosin	[58–61, 222]

expression. Thus, the role of MRP1 in clinical MDR remains to be elucidated.

MRP2 or ABCC2 was originally identified as a platinum drug transporter [37, 38], but it can also extrude typical MRP1 substrates such as anthracyclines, Vinca alkaloids, Epipodophyllotoxins, and methotrexate [33] (Table 2). Physiologically, MRP2 is expressed on the apical membranes of hepatocytes and transports bilirubin glucuronide into the bile canaliculi of the liver. Accordingly, disorders of MRP2 activity result in hyperbilirubinemia due to accumulation of bilirubin glucuronide and unconjugated bilirubin in the liver. These symptoms are known as Dubin-Johnson syndrome (DJS). MRP2 was found to be expressed in different tissue samples prepared from cancer patients, e.g. in breast cancer [39] or ovarian carcinoma [14, 40]. In breast cancer MRP2 did not correlate with response rates or survival time, but in ovarian cancer absence of MRP2 mRNA expression was associated with prolonged progression-free survival time of platinum-treated stage FIGO III patients [40]. Considering the subcellular localization of MRP2, it could be demonstrated that no or weak expression of MRP2 on the nuclear membranes of the ovarian carcinoma cells before treatment with platinum drugs was associated with significantly longer overall and progression-free survival [14]. However, for final assessment of the clinical impact of MRP2, further studies investigating clinical samples are necessary.

Although MRP3 (ABCC3) has the most pronounced structural resemblance to MRP1 among all ABCC subfamily members, it has less affinity to anticancer agents than MRP1 and 2. It confers merely low levels of resistance to Epipodophyllotoxins and Vinca alkaloids [41] (Table 2). In contrast to MRP1 and 2,

MRP3 does not require glutathione for drug transport. In view of the fact that MRP3 is usually expressed at the basolateral surface of hepatocytes [42], it may be physiologically involved in the extrusion of organic anions into the sinusoidal blood. Since high levels of MRP3 expression were also found in kidney and gut, a role for this ABC-transporter in the enterohepatic circulation of bile salts was suggested [43]. Although MRP3 was found to be expressed in some cancer tissues, i.e. ovarian cancer [44], breast cancer [45], pancreatic carcinoma [46], and glioma [47], so far no link between MRP3 and clinical parameters has been demonstrated.

Three of the four MRPs without an N-terminal TMD0 domain, MRP4, 5, and 8, (ABCC4, ABCC5, ABCC11) are nucleotide analogue transporters, whereas the fourth, MRP9, has not been associated with drug transport so far [41, 48, 49] (Table 2). Accordingly, their physiological substrates are purine and pyrimidine nucleotides, including their cyclic derivatives. The transcripts of MRP4 and 5 can be detected in various tissues [50], whereas MRP4 is predominantly localized in prostate and kidney and MRP5 in urogenital tissue [51]. MRP8 appears difficult to detect but is assumed to be expressed widely, including human cancer tissues [48].

In vitro studies have demonstrated that MRP6 (ABCC6) can act as a drug extrusion pump and, for this reason, mediates low levels of resistance to some Epipodophyllotoxins, anthracyclines and cisplatin [5, 52] (Table 2). Physiological substrates are hydrophobic organic anions including cyclic peptides and glutathione conjugates but not glucuronate conjugates. Interestingly, genetic deficiencies of MRP6 are the basis for a rare autosomally inherited systemic connective tissue disorder, pseudoxanthoma elasticum (PXE) [53]. This disease is characterized by dystrophic elastin fibers in retina, skin, and large blood vessels leading to clinical manifestations such as loss of vision, baggy skin and calcification of large blood vessels. Early studies indicated that MRP6 is expressed primarily in the liver and, to a lesser extent, in the kidney, but more recently a widespread distribution pattern has been suggested [54]. In frozen sections of human tumor samples prepared from tissue of different histogenetic origins, so far no clear MRP6 levels could be detected, indicating that the contribution of MRP6 to drug-resistant phenotypes in human tumors may be rather limited [55].

MRP7 or ABCC10 can mediate resistance against taxanes and Vinca alkaloids [48, 56] (Table 2). Although MRP7 can be found in cancer cell lines, its detection in human tissues, including neoplastic specimens, appears to be difficult [48, 57].

BCRP (ABCG2). Of the remaining ABC-transporters, BCRP (breast cancer resistance protein) or ABCG2 appears as the most important drug extrusion pump in the context of anticancer drug resistance. It was simultaneously identified by three independent studies [58–60]. The pump protein of 72 kDa is a so-called "half-transporter" with a [NBD-TMD] configuration (Fig. 1), probably forming homodimers to produce an active transport complex [61]. BCRP expression was found in various normal tissues including placenta, especially in synctiotrophoblastic cells, colon, small and large intestine, sebaceous glands, islet and acinar cells of the pancreas, hepatocytes and biliary canaliculi, alveolar pneumocytes, breast tissue, venous endothelium, and in capillaries, zona reticularis of the adrenal gland, cortical tubules of the kidney, and prostate epithelium, suggesting a protective role for ABCG2 [62, 63]. The observation that BCRP is physiologically induced in the mammary gland during lactation may have health risk consequences for breast-fed infants [64]. It was shown that BCRP secreted xenotoxins, including carcinogens and anticancer drugs, into milk.

BCRP was detected in various *in vitro* models, in particular in atypical multidrug-resistant cancer cell lines selected to mitoxantrone [65], as well as in cancerous samples prepared from tumors of different origins. These neoplasms include hematological diseases as well as solid tumors. Firstly, BCRP was found to be expressed in AML and acute lymphoblastic leukemia (ALL). Unfortunately, the BCRP expression levels varied widely between different studies with relative high levels in some AML studies [66, 67] and merely low levels in others [68–70]. Likewise, contradictory results were obtained in ALL. BCRP was associated with prognosis [71] or no correlation was found between expression of BCRP and response in childhood ALL [72]. Furthermore, BCRP was frequently expressed in solid tumors of different origin, in particular in tumors from the digestive tract, endometrium, lung and melanoma [73]. As in hematological malignancies, BCRP expression data and clinicopathological data are not in concordance between different investigations. For example, an RT-PCR-based investigation of breast carcinoma samples found correlations between therapy response in patients treated with anthracyclines and BCRP expression [39]. Other RT-PCR studies did not find any correlations [74, 75]. Before a final assessment can be made regarding the contribution of BCRP to clinical drug resistance, more and larger studies are needed for the future.

Other ABC-transporters. Additional human ABC-transporters were found to transport antineoplastic

agents in vitro (Table 2). However, merely weak correlations between the expression level of the ABC-transporter and a drug-resistant phenotype could be demonstrated. Thus, over-expression of ABC2 (ABCA2) contributes to estramustine resistance [76]. ABC3 (ABCA3) was associated with resistance to doxorubicin [77]. Over-expression of both sub-units of the dimeric peptide transporter TAP (transporter associated with antigen presentation), TAP1 (ABCB2) and TAP2 (ABCB3), results in increased resistance to mitoxantrone or etoposide [78, 79]. Likewise, in vitro investigations showed that MDR3/P-gp (ABCB4) can contribute to resistance to taxanes and Vinca alkaloids [5, 80] as well as BSEP (ABCB11) that may be involved in resistance to taxanes [5, 81, 82]. MTABC3 (ABCB6), a mitochondrial porphyrin transporter, was found to be over-expressed in cell lines resistant to cisplatin [83] or camptothecin [84]. Since it was demonstrated that MTABC3 can be localized to both, the outer mitochondrial membrane and the cytoplasm membrane [84], it is obvious that MTABC3 contributes to resistance to those compounds. Although these transporters can be detected in tumor samples [77, 85–87], so far no data of their potential role in clinical drug resistance has been found.

ABCB5, an ABC-transporter expressed in two splicing variants, ABCB5α and ABCC5β [88], was found to mediate doxorubicin transport and chemoresistance in human melanoma cells [89]. Most interestingly, ABCB5 expression was identified in a subpopulation of tumor-initiating cells exhibiting cancer stem cell (CSC)-like characteristics (see paragraph "cancer stem cell concept" in this review) in human malignant melanoma [90]. ABCB5-positive tumor cells detected in melanoma patients showed a primitive molecular phenotype and correlated with clinical melanoma progression. Furthermore, in serial human-to-mouse xenotransplantation experiments, ABCB5 expressing melanoma cells possess greater tumorigenic capacity than cells negative for ABCB5 and re-establish clinical tumor heterogeneity. Since systemic administration of a monoclonal antibody directed against ABCB5 induced antibody-dependent cell-mediated cytotoxicity in ABCB5-expressing melanoma subpopulations and the capability of ABCB5 to mediated drug resistance, this transporter may have important implications for clinical management of melanoma patients.

Simultaneous expression of multiple ABC-transporters. Many studies have been done to detect the expression of single members of the ABC-transporter gene family in cell culture models as well as in tissue specimens prepared from cancer patients. The exact role of many members of the ABC-transporter family in drug resistance is still unknown or not fully understood. To explore the role of different transporters in drug resistance in more detail, the simultaneous expression of multiple ABC-transporters has also been investigated. Since the concept of mithridatism did not clarify whether the same ABC-transporter is always activated from the beginning of drug treatment to the end of drug exposure, the expression of all three well-known mitoxantrone extrusion pumps, i.e. MDR1/P-gp, MRP1 and BCRP, was continuously measured during the mithridatism period using mitoxantrone [91]. It was demonstrated that increased expression of all three transporters was induced by the drug but in the end, merely a single, arbitrarily selected extrusion pump was dominant.

Global gene expression analyses of cancer cell lines using array technologies showed that, besides the expected expression of well characterized ABC-transporters, alternative, non-inevitably drug resistance-associated transporters can also be expressed, suggesting that several ABC-transporters of unknown function can, in fact, influence the response of cancer cells to drug treatment [92–95]. So far only a few studies are available that have applied global ABC-transporter gene expression analyses to clinical samples. In patients suffering from acute lymphoblastic leukemia (ALL), the expression of no or a single well-characterized drug resistance-associated ABC-transporter, e.g. MRP1, and the expression of different alternative members of this family could also be observed [96]. Similar results were obtained in breast cancer patients, whereby the expression of the majority of the ABC-transporters was a clear feature of breast tumors, and the comparison of drug treated and untreated tumors showed an unexpected similarity of ABC-transporter expression levels [97]. However, the *in vitro* data as well as the investigations with clinical specimens indicate that a drug-resistant phenotype may be primarily mediated by a single dominant transporter, but it may also be facilitated by the activity of additional ABC-transporters of less characterized function.

Non-ABC-transporters. *In vitro* studies have provided evidence that cellular transporter proteins other than ABC-transporters may also contribute to anticancer drug resistance. Thus, RALBP1 (RLIP76), a 76 kDa Ral-binding, Rho/Rac-GAP and Ral effector protein can act as a transporter of structurally unrelated amphiphilic chemotherapeutic drugs such as doxorubicin and glutathione-electrophile conjugates [98–100]. Analogous to ABC-transporters, RALBP1 mediates transport of xenobiotics in an ATP-dependent manner, but it has no

Table 3. Cellular death pathways triggered by antineoplastic agents.

Type of death pathway	Morphological characteristics	Biochemical features	Detection methods
Apoptosis	Chromatin condensation; DNA fragmentation; blebbing of cell membranes; formation of apoptotic bodies	Caspase-dependent	DNA laddering; TUNEL staining; annexin-V staining; caspase activity assays; electron microscopy, flow cytometry
Autophagy	Cellular self digestion leads to formation of autophagic vesicles; blebbing of cell membranes; no DNA fragmentation	Caspase-independent; p53-independent; elevated lyosomal activity	Monodansylcadaverine (MDC) staining; electron microscopy; exclusion of vital dyes (trypan blue); protein degradation assays
Mitotic catastrophe	Multiple micronuclei; multinucleate cells; giant cell formation	Caspase-independent; p53-independent; abnormal CDK1/cyclin B activation	Assays for mitotic markers (MPM2); light or electron microscopy for detection of micronuclei and multinucleate cells; TUNEL staining
Necrosis	DNA degradation; atypical nuclear shape with vacularization; swelling of mitochondria and cell membrane	Not genetically determined	Permeability to vital dyes (trypan blue); electron microscopy; flow cytometry
Senescence	Increased cell size; distinct heterochromatic structure	Activity of senescence-associated β-galactosidase (SA-β-gal); p53-dependent	SA-β-gal staining

sequence homology with the ABC-transporter family and differs from the ABC-transporters in several other important aspects. These include lack of the characteristic Walker domains, membrane integration without obviously defined transmembrane domains and its role as a link between Ras/Ral/Rho and EGF-R signaling pathways. The transporter consists of multiple motifs including two distinct ATP-binding domains, a H(+)-ATPase domain, a Rho/Rac GAP domain, a Ral effector domain binding motif, PKC and tyrosine kinase phosphorylation sites and the ability to undergo fragmentation into various smaller fragments which may participate as components of macromolecular functional complexes. Although a current study demonstrates that RALBP1 can be expressed in human tumors such as ovarian cancer [101], so far no data are available as to whether this non-ABC-transporter has an impact on clinical drug resistance.

Cellular uptake and efflux of platinum-containing drugs such as cisplatin, carboplatin or oxaliplatin appear to be mediated by specific transporter molecules. During recent years it has been shown that these roles can be adopted by the copper transporters CTR1, ATP7A and ATP7B [102, 103]. The 190 amino acid CTR1 is the main copper influx transporter in human cells. The two structurally similar copper efflux transporters, ATP7A and ATP7B, possess ATPase activity. Defects in these transporters cause clearly defined copper accumulation syndromes, Menke's disease in the case of ATP7A disorders, while defects in ATP7B cause Wilson's disease. A positive correlation has been described between the expression level of these copper transporters and the degree of resistance to platinum-containing drugs in cancer cell lines and in breast [104] and ovarian [105] tumor tissues [102, 106]. These observations indicate that the metal homeostasis-mediated copper transporters may be involved in a complex mechanism leading to platinum drug resistance in cancer cells. However, for conclusions concerning clinical drug resistance more and larger studies are necessary.

Modulation of cellular death pathways

The primary cellular effects mediated by antineoplastic agents finally trigger cell cycle arrest or cellular death pathways which are the underlying pharmacological causes of the cytotoxicity in tumor cells. Thus, alterations of these secondary effects causing modifications in these pathways can play an important role in the development of resistance to anticancer drugs (Fig. 2). In particular, given the fact that defects in cell death pathways are a hallmark of cancer, alterations of the homeostasis of these pathways appear to be of major impact for anticancer drug resistance. Both apoptotic and non-apoptotic mechanisms, such as autophagy mitotic catastrophe, necrosis and senescence, may contribute to drug-resistant phenotypes of cancer cells (Table 3) [107, 108].

Apoptosis is one of the main types of programmed cell death and is characterized by typical morphologic changes including blebbing, loss of cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. These events are commonly associated with activation of caspases, cystein-containing proteases that mediate the apoptotic cascade by cleavage of defined cellular substrates. However, apoptosis can also be caspase-independent [109]. Solid tumors, in particular those derived from

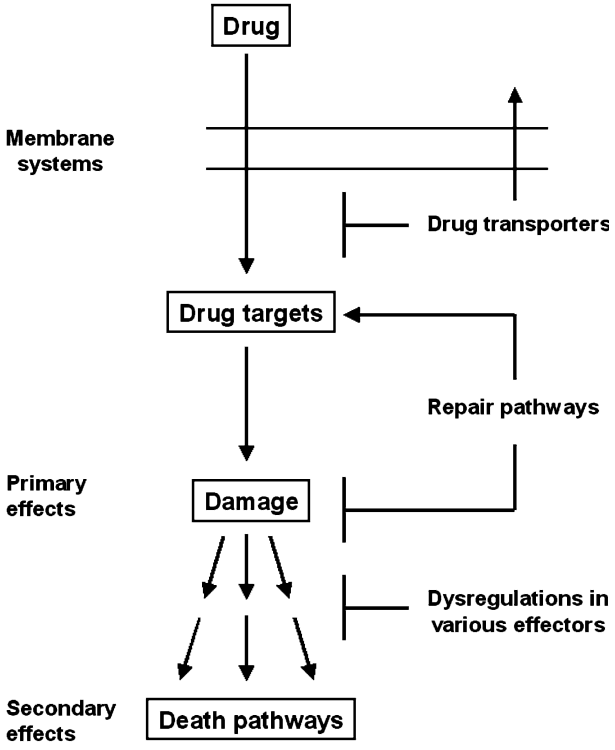

Figure 2. Main cellular pathways leading to chemotherapeutic resistance of cancer cells. Penetration of antineoplatic agents into the cancer cell induces the primary pharmacological effect by interaction with the target molecules. Membrane-embedded drug efflux pumps, such as ABC-transporters or non-ABC-transporters, can inhibit the primary effect by impeding drug-target interactions. The anticancer drug-mediated damage of the target molecules, the primary effect, can trigger various cellular signal transduction pathways leading to cell death or cell cycle arrest. These secondary effects include downstream effects resulting in apoptosis as well as non-apototic cell deaths including autophagy mitotic catastrophe, necrosis and senescence. Consequently, modifications of these pathways can mediate anticancer drug resistance.

epithelial tissues, are often primarily or intrinsically resistant to chemotherapeutic treatment. However, in the case of response to drug treatment, apoptotic cell death commonly occurs after one or more cell divisions and appears to be cell cycle dependent. On the other hand, in leukemia as well as in childhood tumors anticancer drug treatment is commonly efficient and results in the triggering of apoptotic pathways, which in these cases also appear to be cell cycle independent [110]. Although apoptotic machinery is constitutively present in both drug-sensitive as well as drug-resistant neoplasms, the pathways leading to apoptosis seem to by dysregulated in non-responsive cancers. Accordingly, each of the cellular proteins involved in maintenance of the homeostasis of pro-apoptotic and anti-apoptotic factors may contribute to the development of a drug-resistant phenotype. Since the detailed discussion of all these factors is beyond the scope of this paper, the principle will be discussed by taking the representative example of the Bcl-2 (B-cell lymphoma-2) proteins family (Table 4) (detailed overviews in [110–112]).

Table 4. The Bcl-2 protein family.

Pro-apoptotic	Anti-apoptotic
Bad	A1/Bfl-1
Bak	Bag-1
Bar	Bcl-2
Bax	Bcl-B
Bcl-GL	Bcl-W
Bcl-GS	Bcl-X_L
Bcl-rambo	Bhrf-1
Bcl-X_s	Brag-1
Bfk	Diva/Boo
Bid	Mcl-1
Bik/Nbk	NR-13
Bim/Bod	
Blk	
Bmf	
Bnip3/Nip3	
Bok/Mtd	
BRCC2	
Hrk/DP5	
MAP-1	
Nix/Bnip3L	
Noxa	
Puma	

The family of Bcl-2 proteins comprises more than 30 different members with anti- or pro-apoptotic features (Table 4) [113, 114]. The founding member Bcl-2 was originally identified as a gene with a t(14;18) chromosome translocation breakpoint in B-cell follicular lymphomas, where its transcription becomes excessively driven by the immunoglobulin heavy chain gene promoter and enhancer on chromosome 14. The resulting over-expression of Bcl-2 was associated with inhibition of cell death. With this finding the phenomenon of apotosis was revealed as a fundamental biological mechanism of tumor suppression. All members of the Bcl-2 protein family contain at least one of four conserved Bcl-2 homology domains, BH1-4. The multidomain proteins of Bcl-2 family also contain a C-terminal transmembrane domain (TM) that anchors them to cellular membrane systems such as mitochondrial, nuclear or ER membranes. Whereas the BH3 domain appears to be necessary for pro-apopototic effects, the BH4 domain has been suggested as essential for anti-apoptotic activities. The pro-apoptotic Bcl-2 proteins are distributed to two subfamilies, the Bax subfamily (Bax, Bak, and Bok), whose members contain BH1-BH3 domains, and the "BH3-only" subfamily (Bid, Bad, Bim, Bik, Blk, Hrk, Noxa, and Puma). All of these pro-apoptotic proteins are key regulators of the apoptosis-mediating mitochondrial cytochrome c release. In the end, their function is regulated by anti-apoptotic Bcl-2 proteins such as Bcl-2, Bcl-X_L, and Mcl-1. Since apoptotic signaling is a result of the homeo-

stasis of the Bcl-2 proteins, it is not surprising that modifications to the Bcl-2 family members will influence the response of tumor cells to chemotherapeutic treatment. Down regulation of Bcl-2 family proteins can contribute to anticancer drug resistance, and increased expression of anti-apoptotic Bcl-2 proteins or dysfunction of the pro-apoptotic Bcl-2 members can be associated with a decreased susceptibility of cancer cells to trigger apoptosis in response to drug exposure [115–117]. Although an increase in the Bcl-2/Bax ratio can be frequently observed in cancer cells and over-expression of Bcl-2 and/or Bcl-X_L has been found to correlated with poor clinical outcome in various different solid tumor types as well as in hematological malignancies, the prognostic impact of Bcl-2 in the response to chemotherapy could not be confirmed in all tumor types [118–120]. Thus, dysfunctions of members of the Bcl-2 family should be taken into consideration for predicting the chemotherapy response, but they do not seem to be the single cause for resistance to chemotherapy. Bcl-2 family members and other proteins which are part of an apoptotic pathway rather appear to be important items of a complex cellular network mediating drug resistance.

Moreover, a causal link between expression and activity of ABC-transporters, e.g. MDR1/P-gp, and cellular apoptotic pathways was shown. Cancer cells expressing MDR1/P-gp are more resistant to caspase-dependent apoptosis-triggering agents but not to caspase-independent cell death stimuli [121]. This phenomenon can be associated with the fact that MDR1/P-gp-expressing cells exhibit a block in the release of mitochondrial cytochrome c into the cytosol and, because of this, they seem protected from undergoing apoptosis [122]. This antiapoptotic feature of MDR1/P-gp-expressing cancer cells seems to be dependent on the over-expression of Bcl-X_L. Likewise, antiapoptotic roles were also suggested for other ABC-transporters including BCRP [123]. On the other hand, it has been reported that the non-steroidal anti-inflammatory drug indomethacin, an inhibitor of MRP1, induces apoptosis in MRP1-expressing lung cancer cells through an MRP1-dependent mechanism [124]. Thus, possible pro-apoptotic features of ABC-transporters should also be considered.

DNA repair pathways

Since DNA is the main target of many classical cytotoxic anticancer drugs including anthracyclines, alkylating agents or platinum-containing compounds, the activation of DNA repair pathways represents one of the most important target repair mechanisms causing drug resistance [125–129]. However, alternative cellular repair mechanisms were also suggested to be involved in resistance to cell target-damaging drugs, e.g. heat shock proteins, the "molecular chaperones" which are involved in the maintenance of the correct three-dimensional folding of polypeptide chains [130]. For simplification, merely a short overview about DNA repair pathways involved in anticancer drug resistance is shown in Table 5. In addition to the factors participating in the DNA repair pathways, there are proteins involved in regulation of the activity of these pathways. These regulation factors include ATM, ATR, PARP, p53 or BRCA1. Accordingly, such regulators of DNA repair may contribute to drug resistance.

MGMT (O^6-methylguanine-DNA methyltransferase) is the most important cause of the resistance of cancer cells to methylating agents. This ubiquitously expressed DNA repair protein is physiologically involved in the maintenance of the integrity of cellular DNA, but this protective effect also mediates resistance to the pharmacological effects of methylating drugs, as damage induced by these antineoplastic agents provides a perfect substrate for MGMT [129]. Unlike other DNA repair mechanisms, MGMT does not trigger a pathway, but instead recognizes and repairs adducts at the O^6 position of guanine residues in a suicidal stoichiometric non-catalytic reaction. The inactivated protein is then ubiquitinated and degraded by the proteosome system. It was demonstrated that MGMT has major clinical impact. Silencing the MGMT expression in gliomas by epigenetic mechanisms, i.e. methylation of the MGMT promoter, was associated with clear survival benefit in patients treated with the methylatiung agent temozolomide [131]. Thus, MGMT appears to be an interesting drug resistance-associated factor useful for prediction of therapy response as well as a pharmacological target for application of MGMT inhibitors for reversal of drug resistance.

Alternative drug-induced DNA adducts can be recognized by members of other DNA repair pathways (Table 5) and therewith can trigger a repair reaction, or the DNA repair pathway can be involved in attenuation of the cellular willingness to trigger a cell death pathway. As a consequence, decreased cell deaths by defects in activation of death pathways mean drug resistance. This phenomenon was described for the DNA mismatch repair system (MMR). Down regulation of proteins of the MMR pathway is associated with resistance to clinically important drugs including platinum-containing compounds, anthracyclines, alkylating agents, antimetabolites and epipodophyllotoxins [126]. This phenomenon appears to be paradoxical. An explanation is that MMR

Table 5. DNA repair pathways.

DNA repair mechanism	Corresponding DNA repair pathways	Participating proteins
Reversion repair	Single-step repair by MGMT	MGMT
	Repair by AlkB homologous	ABH1; ABH2; ABH3
Base excision repair (BER)	Short patch repair (SPR)	Glycosylases I, II; Polβ; XRCC1; PARP-1; Lig III
	Long patch repair (LPR)	Glycosylases I, II; Polβ; RF-C; FEN1; Pol δ; Pol ε; PCNA
Nucleotide excision repair (NER)	Global genomic repair (GGR)	DDB1; DDB2; RPA; HR23B; ERCC1; XPA; XPB (ERCC3); XPC; XPD (ERCC2); XPE; XPF; XPG; GTFH1; GTFH2; GTFH3; GTFH4; CDK7; CCNH; MNAT1; Pol δ; Pol ε; PCNA; Lig I
	Transcription-coupled repair (TCR)	GTFH1; GTFH2; GTFH3; GTFH4; CDK7; CCNH; MNAT1; XPB (ERCC3); XPD (ERCC2); FFIIS; CSA; CSB; XPF; XPG; Pol δ; Pol ε; Lig I
Mismatch repair (MMR)	Mismatch repair (MMR)	hMSH2; hMSH6; hMLH1; hPMS2; Pol δ; Exo I; Lig
Double-strand break repair (DSB)	Homologous recombination (HR)	MRE11; NBS1; Rad50; Rad51; Rad51B; Rad51C; RAd51D; Rad52; RPA; XRCC2; XRCC3
	Non-homologous end-joining (NHEJ)	Ku70; Ku80 (XRCC5); DNA-PKCs; FEN1; MRE11; NBS1; Rad50; XRCC4; XRCC7; Artemis; Lig IV

proteins try to excise DNA adducts that were induced by anticancer drugs. In a case where the number of adducts exceeds a critical threshold, the MMR system is no longer able to repair the DNA damage. These continuous attempts to repair lead to futile cycles of DNA synthesis past the DNA lesion, followed by recognition and removal of the newly synthesized strand by an active MMR system. This event results in the generation of gaps or DNA strand breaks that induce cell death pathways. A decrease of MMR activity means less triggering of cell death or, in other words, drug resistance. However, the clinical impact of the MMR pathway to resistance to anticancer treatment is not elucidated. In some studies these *in vitro* findings, i.e. the down regulation of members of the MMR system was associated with poor response to chemotherapy, were confirmed [132, 133]. In other reports no correlations were observed [134]. They even found a benefit to survival by inactivation of the MMR system [135, 136].

Repair of platinum-containing drug-induced DNA damage is predominantly performed by the nucleotide excision repair (NER) pathway [137]. However, the NER system was also associated with resistance to alternative drugs including alkylating agents [138]. The NER machinery is a complex biological process that acts via two different pathways: global genomic repair (GGR) and the transcription-coupled repair (TCR) pathway. Both pathways involve more than 20 different proteins (Table 5), whereby only a few rate-limiting components of the NER pathways appoint the cellular repair capacity. The most known rate-limiting factor of NER is ERCC1 (excision repair cross-complementing protein 1). Various *in vitro* studies have demonstrated that enhanced activity of ERCC1 has been associated with platinum drug resistance of cancer cells [139, 140]. Furthermore, it has been reported that high levels of ERCC1 in tissue samples of tumors of different origins correlated with poor response to platinum-based chemotherapy [141–143].

In vitro investigations also linked alternative DNA repair pathways with anticancer drug resistance, i.e. the base excision repair (BER) [144], and the double-strand break repair (DSB) pathway [145]. However, data demonstrating clinical impact are missing.

All in all, the different DNA repair pathways consist of very complex biological systems of interacting proteins. Although it is obvious that DNA repair mechanisms contribute to anticancer drug resistance *in vitro* as well as in clinical settings, further investigations are necessary to clarify whether single factors of DNA repair pathways may be useful for predicting clinical drug resistance or whether they may appear as "drug-able" targets for modulation of drug resistance.

Various mechanisms

In addition to these well investigated and clinical important mechanisms of antineoplastic drug resistance, various alternative biological mechanisms involved in MDR of cancer have been described. The

most familiar of these mechanisms is the decrease of DNA topoisomerases II (Topo II) activity [146].

DNA topoisomerases are enzymes that isomerise the tertiary structure or rather the topology of DNA without changing its primary structure. Cellular DNA is under torsional stress resulting in multiplex twisting of the molecule. DNA replication or gene expression requires relaxation and untangling of intertwined DNA strands. This change is the typical task of Topo. The enzyme relieves torsional strain by creation and religation of DNA single strand breaks. In humans, two classes of Topo are well characterized, type I and type II, whereby Topo II interacting drugs have become central parts of chemotherapy regimens in neoplastic diseases. Two Topo II isoforms, the 170 kDa Topo IIα and the 180 kDa Topo IIβ exist as homodimers. Topo II binds to DNA, cleaves both strands, passes a second strand of DNA through the cleaved site in an ATP-depended manner and rejoins the strands at the original site of cleavage. This reaction results in a DNA molecule altered in its topological configuration. During a breakage-reunion reaction, Topo II can form a cleavable complex with DNA with the covalent linking of each Topo II subunit to each 5'-phosphoryl end of the cleaved DNA molecule through a phosphotyrosyl bond [147].

Classical Topo II-targeting drugs such as Epipodophyllotoxins or anthracyclines interfere with the breakage-reunion reaction of Topo II by stabilizing this cleavable complex. The stabilization of the cleavable complex and not the inhibition of the Topo II activity is presumed to play the decisive role in the cytotoxic effect of the classical Topo II interacting agents. The stabilized cleavable complex leads to DNA breaks, which trigger cellular death pathways. Accordingly, resistance to these agents can result from any process that leads to a reduced formation of cleavable complexes. Thus, decreased Topo II catalytic activity can mediate drug resistance to cancer cells. Since these drug-resistant tumor cells showed cross resistance to other drugs, this phenotype was designated as altered Topo II multidrug resistance (at-MDR) [148]. The decrease in Topo II activity can be caused by diminished expression levels of both Topo II isoforms as well as by mutations within the Topo II encoding genes.

Although *in vitro* studies have clearly demonstrated the important role of Topo II for drug-resistant phenotypes of various cancer cell lines of different origins, the clinical impact of Topo II appears to be tissue-dependent. Different investigations with samples prepared from breast cancer showed that a high Topo II expression level was associated with favorable treatment response to chemotherapeutic treatment and, in reverse, a low Topo II expression level was detected in the drug-resistant breast cancers with poor response [149–151]. However, in malignant tissues derived from different types of lung cancers, a high Topo II expression level was linked to poor chemotherapeutic response and prognosis [152, 153]. These findings are completely contrary to the *in vitro* data. Thus, the clinical impact of Topo II for clinical drug resistance is finally not clarified.

In addition to the presently discussed factors, various alternative cellular proteins were found to be associated with a drug-resistant phenotype in cell culture models as well as in clinical samples, e.g. glutathione-S-transferases [154] and metallothioneins [155]. Although, the clinical impact of these factors has not been conclusively elucidated, these factors may also be essential parts of a complex cellular multi-component system that causes an individual multidrug-resistant phenotype of a distinct malignancy.

Resistance to new targeted drugs

Due to the manifold side-effects and inadequate response rates of many neoplasms to "classical" cytotoxic anticancer drugs, the development of new targeted, "tumor cell-specific" drugs has been intensified in recent years. The concept is that these new drugs should interact with molecules whose expression is predominantly restricted to tumor cells. The era of targeted therapy of cancer patients started with the approval of the therapeutic antibody rituximab by the regulatory authority FDA in the United States in 1997 [156]. This chimeric monoclonal antibody is directed against the B-non-Hodgkin's lymphoma (B-NHL)-associated surface antigen CD20 expressed on the surface of both, normal and malignant B cells. It produces antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity (CDC), and triggers apoptosis. Although many patients benefited considerably from rituximab therapy, a subset of patients did not initially respond to rituximab treatment. Other patients acquired resistance to rituximab during the therapy [157]. The exact cellular mechanisms of rituximab resistance are not known.

Due to the fact that targeted drugs mediate their antineoplastic effects by interaction with different target molecules, the mechanisms of resistance have been suggested to be different from "classical" MDR-associated mechanisms and actually restricted to a given target. In the case of rituximab, the pharmacological effect was shown to be mediated by triggering cell death via CD20. Although the exact function of CD20 is not completely understood, *in vitro* studies demonstrated that this effect was conveyed by inhib-

ition of p38 MAPK (mitogen-activated protein kinase), NF-κB (nuclear factor-κB), the ERK 1/2 (extracellular signal-regulated kinase 1/2) and AKT-depending antiapoptotic survival pathways. These events result in chemosensitization of drug-resistant B-NHL cell lines by downregulation of antiapoptotic gene products, in particular Bcl-2, Bcl-X_L and Mcl-1 [157]. In other words, this is the link between "classical" MDR caused by modulation of cellular death pathways, in particular Bcl-2-dependent apoptotic pathways (see above), and resistance to new targeted drugs, in this case resistance to the chimeric antibody rituximab. Accordingly, resistance to other therapeutic antibodies which finally trigger cellular death pathways may also be mediated by alterations of components in these pathways. These alterations may be identical or similar to those which are well-known from "classical" MDR mechanisms.

This is true for new small molecule targeted drugs, e.g. the tyrosine kinase inhibitor imatinib. This drug, a 2-phenylaminopyrimidine derivative, was approved for first-line treatment of chronic myeloid leukemia (CML) in 2003 [158]. Imatinib potently inhibits all ABL tyrosine kinases including the CML-associated BCR-ABL fusion kinase. Since CML cells are reliant on the activity of the BCR-ABL kinase, the proliferation of these neoplastic cells is deeply affected by the drug. In contrast, normal cells express additional ABL-independent signal transduction pathways. Thus, they will be impaired merely marginal by the compound.

It was not astonishing that malignant cells also developed resistance to this tyrosine kinase inhibitor. In leukemia cell line models exhibiting resistance to imatinib, it was shown that enhanced activity of the "classical" MDR-mediating ABC-transporter MDR1/P-gp caused the drug-resistant phenotype [159]. Furthermore, it was demonstrated that imatinib is a substrate for BCRP [160] but not for MRP1 [161]. Interestingly, imatinib has also been demonstrated to be an inhibitor of BCRP [162]. Hence there is the potential for imatinib to modulate the pharmacology of other drugs that are BCRP substrates.

Commonly, patients with CML in the chronic phase receive initial treatment with imatinib. A minority of these patients never achieves a CR. An additional proportion of patients appear initially to respond by achieving either cytogenetic or major molecular effects, but then lose their response. These resistance phenomena were associated with amplification of the BCR-ABL gene and over-expression of the corresponding oncoprotein, or due to the over-expression of MDR1/P-gp [163]. Likewise, the mechanisms leading to MDR to "classical" cytotoxic anticancer drugs and the mechanisms causing resistance to novel targeted drugs, small molecules as well as therapeutic antibodies, appear to be identical or at least overlapping, but not fundamentally different.

Cancer stem cell concept

Although already proposed in the 1960s [164], only in recent years has the cancer stem cell (CSC) hypothesis come into greater focus. CSCs have been defined as "a small subset of cancer cells within a cancer that constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor" [165]. CSCs have been identified in a variety of human neoplasms including ALL [166], breast cancer [167] and glioma [168], as assayed by their ability to initiate tumor growth in immunocompromised mice. They represent approximately 1% of the tumor cells and were also a minor population of cells in well established cancer cell lines with some of the same properties as CSCs [169]. The derived CSC concept assumes that all types of neoplasms consist of a mixture of CSCs and proliferative cancer cells with a limited lifespan [170]. CSCs can be selected based on the expression of cell surface markers associated with immature cell types. Most interestingly, CSCs can express high levels of specific MDR-associated ABC-transporters. For example, MDR1/P-gp and BCRP appear to be expressed in normal stem cells as well as in most CSCs [171]. Drug transporter expressing CSCs were originally identified through the transport of fluorescent dyes including Hoechst 33342 or rhodamine. In fluorescence-activated cell sorter (FACS) analyses, they were separated from normal neoplastic cells and have been termed side-population (SP) cells, as they were found on the side of the cell distribution pattern in the FACS plot.

Tumor cells exhibiting CSC-like characteristics including MDR1/P-gp and BCRP expression were identified in various types of cancers, e.g. brain tumors [172], lung cancer [173], melanomas [174], or pancreatic cancer [175]. These observations suggest that the presence of ABC-transporter-containing CSCs may be relatively common in tumors consisting of heterogeneous cell populations, particularly within established tumors. Thus, future chemotherapeutic strategies should consider coupling identification and targeting of these potential CSC populations with standard treatment regiments.

Basing on the CSC concept, the conventional models of MDR, the somatic mutation model of MDR [176], and the model of intrinsic cellular MDR were expanded with an alternative point of view, the CSC model of MDR [170]. The first traditional model, the

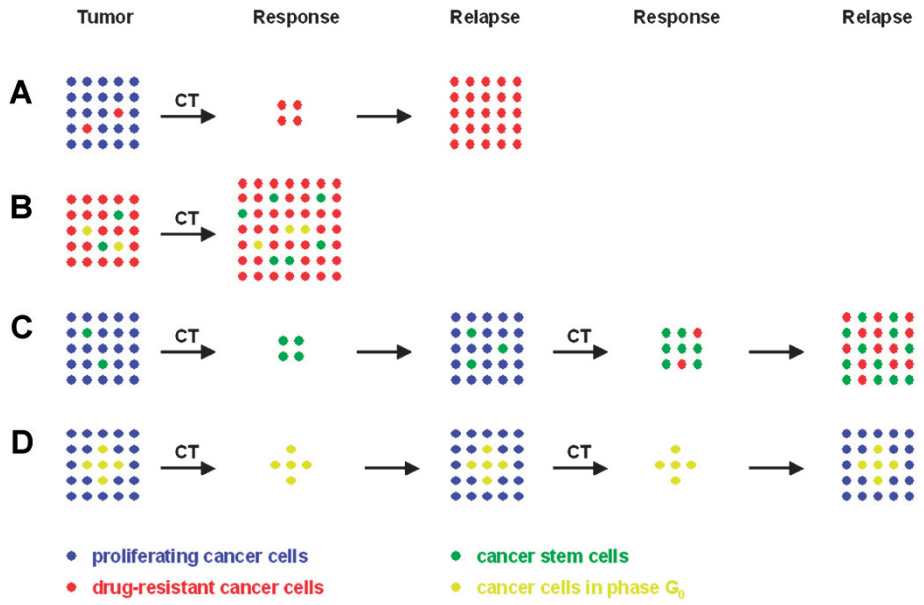

Figure 3. Models of cellular drug resistance. (*A*) The conventional somatic mutation model supposes that genetically changed cancer cells exhibiting a drug-resistant phenotype (red) can persist in the proliferating, drug-sensitive tumor cell population (blue) or they can occur following chemotherapy. (*B*) In the model of intrinsic cellular drug resistance, the tumor mass consists of different drug-resistant cell types, proliferating intrinsic drug-resistant cancer cells (red), CSCs (green) and cells in phase G_0 (yellow). (*C*) In the stem cell model, the tumor consists of CSCs (green) exhibiting a drug-resistant phenotype due to their ABC-transporter content, their capacity for DNA repair, and the high proportion of cells in phase G_0, as well as conventional drug-sensitive cycling cancer cells (blue). (*D*) The model of quiescent cells starts from the assumption that cancer cells in phase G_0 (yellow) show a drug-resistant phenotype because cytotoxic anti-cancer drugs commonly attack proliferating cells (blue). In reality, probably all processes occur simultaneously, but the relative percentages vary from tumor to tumor. Thus, an individual clinical response to chemotherapy and an individual pattern of relapse may be determined. CSC, cancer stem cell; CT, chemotherapy.

somatic mutation model (Fig. 3A), presumed that MDR develops as a result of progressively gained somatic mutations and epigenetic alterations in the tumor over time. In a single or multiple cancer cell clones genetic changes are acquired that confer a multidrug-resistant phenotype by activation of various molecular mechanisms. These genetic alterations can be coincidentally present within the tumor cell population or can be induced by drug exposure. The genetically altered cancer cells have a selective advantage that helps them withstand treatment with anticancer drugs. In the model of intrinsic cellular MDR (Fig. 3B), the malignancy primarily consists of different multidrug-resistant cell types. These intrinsic drug-resistant cancer cells already express one or more drug resistance-mediating proteins or pathways. Furthermore, the tumor consists of cells in quiescent phase G_0. As a result, chemotherapy has no effect and the cells can proliferate.

The CSC model of MDR (Fig. 3C) supplements the model of intrinsic MDR. It assumes that the malignancy contains a small subpopulation of CSCs. These cells are intrinsically multidrug-resistant through their ABC-transporter content as well as their capacity for DNA repair. Thus, drug treatment does not kill CSCs and these cells support relapse of the tumor. For completeness, a further concept of MDR has to be mentioned briefly, the model of quiescent cells [177]. This model takes into account that anti-cancer drugs primarily attack proliferating cells. A tumor is heterogeneous and consists of cycling cells and a significant contingent of cells in a quiescent state. Thus, these cancer cells in phase G_0 can tolerate a higher dosage of anticancer drugs relative to proliferating tumor cells. The non-dividing cancer cells can survive chemotherapy and the malignancy can relapse (Fig. 3D). This model can explain the observation that patients who relapse after first-line chemotherapy can also be retreated with the same chemotherapeutic regimen and achieve a CR.

In clinics, the processes of all four models may occur simultaneously and in concurrence, but the relative percentages vary from tumor to tumor. In this way, differences in the clinical response to chemotherapeutic treatment and an individual pattern of progression and relapse may be determined.

Search for new drug resistance-associated factors

For human malignancies, it was hypothesized that the histological and clinical progression including the

response to treatment with anticancer drugs corresponds to the accumulation of genetic alterations and, therefore, to changes in gene expression patterns of the tumors [178]. In recent years, powerful new techniques for large scale gene expression investigations have been developed to detect modifications in genome-wide gene expression profiles or protein expression pattern. These new approaches, commonly designated as transcriptomics and proteomics, have been broadly applied to studying the biology of many types of cancer. The hope is that the newly obtained data will allow deeper insights into these diseases on the molecular basis, and finally facilitate a systematic search for new diagnostic and therapeutic targets that will help to design new individually-tailored chemotherapeutic treatment options. Thus, in recent years various complex data sets have been described, including potential new drug resistance-associated factors of global gene expression analyses [179–182] and protein expression signatures [183–187].

Although many studies have identified drug resistance-specific expression profiles in various *in vitro* models as well as in specimens prepared from cancer patient tissues, so far no new factors or expression signatures with clinical impact have been identified. Further difficulties arise from the observation that the comparability and reproducibility of gene and protein expression data from different studies is not satisfactory. Merely the altered expression a few genes could be identified in different independent studies. Moreover, the majority of these factors is obviously not functionally involved in anticancer drug resistance. These factors may be co-regulated during the development of a drug-resistant phenotype, or they may represent coincidental events. Although the use of gene expression signatures appeared to be applicable for the molecular classification of a given tumor [188] or for the prediction of response to chemotherapy [189–191], so far no diagnostic test has been approved for clinical use.

Clinical consequences of MDR research, reversal strategies and MDR diagnostics

For clinical exploitation of the knowledge of anticancer drug resistance research, various chemosensitizers have been identified over the last years. The hope was that these "drug resistance factor-targeting" compounds would inhibit a specific cellular pathway leading to drug resistance and, therewith, allow the ongoing antineoplastic efficacy of the actual anticancer drugs. Predominantly, these agents were developed as ABC-transporter inhibitors, in particular MDR1/P-gp-inhibiting compounds [5, 192].

The first-generation MDR1/P-gp inhibitors, i.e. verapamil and ciclosporin A, are themselves substrates of MDR1/P-gp and compete with anticancer drugs to the binding site. The result is an impaired drug efflux leading to an increased intra-cellular drug concentration. Although these inhibitors are utmost effective in cell culture models, they failed in clinical settings. Reasons for this include the lack of pre-therapeutic analysis of MDR1/P-gp expression in the tumor, no consideration of the activity of alternative MDR-associated mechanisms, the necessity of extremely high inhibitor concentrations including unwanted side effects, and unpredictable pharmacokinetic interactions with the anticancer drugs when they are co-administered [193]. Interestingly, it was observed that the first-generation MDR modulator, verapamil, triggers apoptosis through stimulation of glutathione extrusion mediated by MRP1 [194, 195] indicating that verapamil may represent a novel approach in the selective treatment of MRP1-positive drug-resistant tumors. Furthermore, it must be considered that the two enantiomers of verapamil have different effects on MRP1 activity. (S)-verapamil induces cell death in MRP1-expressing MDR cells, whereas (R)-verapamil sensitizes MRP1-over-expressing cells to chemotherapeutics [196].

The second-generation chemosensitizers, e.g. the cyclosporin derivative valspodar (PSC833), were designed to show less toxicity and more efficacy for MDR1/P-gp inhibition. Like the first-generation sensitizers, they act as competitive substrates to the transporter binding sites. Although phase I and II trials with the second-generation inhibitor valspodar showed hopeful results in different tumor entities [5], no benefit of valspodar in addition to different chemotherapeutic regiments in different types cancers could be demonstrated in phase III clinical trials [197–199]. Similar to problems with first-generation MDR modulators, the unsatisfactory results were mainly due to disregarding alternative drug resistance mechanisms and a need for dose reduction of the anticancer agent in context with the transporter inhibitor as well as unpredictable pharmacological interactions and toxic side effects.

Third-generation inhibitors were developed specifically for low pharmacokinetic interaction and, of course, high target affinity. In particular, the inhibition of cytochrome P450 oxidases, which are responsible for many adverse pharmacokinetic effects with first and second-generation inhibitors, has been avoided. These compounds include laniquidar (R101933), zosuquidar (LY335979), elacridar (GF-120918), tariquidar (XR9576) or oc144–093 (ONT-093). Likewise, phase I and II trials with a third-generation inhibitor, i.e. tariquidar, showed positive results [200–202]. The

first phase III trial with tariquidar was done in combination with first-line chemotherapy for patients with NSCLC (non-small cell lung cancer) but, due to problems with toxicity, the trial was abandoned [5]. Further trials are on-going and will provide more information in the future.

In addition to these classical attempts to overcome MDR with the application of low molecular weight pharmacologically active compounds, preclinical studies have been performed with experimental therapeutics. These efforts include the development of monoclonal antibodies directed against extra cellular epitopes of MDR1/P-gp. It has been demonstrated *in vitro* that this antibody inhibits the drug efflux activity of this ABC-transporter [203]. Furthermore, strategies based on RNA-technology to overcome drug resistance have been developed, including ribozyme and RNA interference (RNAi) approaches [204]. *In vitro* as well as *in vivo* experiments demonstrated that MDR could be completely reversed by RNA technologies [205]. Although these preclinical data are promising, no clinical trials are planned so far.

To design individually tailored treatment regimens for cancer patients, exact molecular diagnostics of individual patients is necessary. These diagnostics include a detailed analysis of the drug resistance status of a patient. They are a basis to predict the response of each individual tumor following application of conventional or "targeted" anti-cancer drugs. Although numerous investigations of cancer MDR provided manifold information on the biological mechanisms and their clinical impact, so far no satisfying diagnostic tests have been developed. Although functional assays for detection of drug resistance, e.g. on the basis of inhibition of cell proliferation, or expression analyses, e.g. immunohistological detection of drug resistance-associated factors, were established, none of these tests was introduced in clinical routine.

The reasons for the problems of MDR detection were already discussed extensively in the 1990 s, drawing on the example of the diagnostics of MDR1/P-gp [206]. Although some of these problems have been solved in the meantime and new techniques have been introduced into laboratory praxis, e.g. the development of quantitative real time RT-PCR technologies, the establishment of methods for global gene expression analyses or the improvement of DNA sequencing technologies, the problems for the clinical introduction of MDR diagnostics are similar to those of 10 years ago. These problems include no standardization of the diagnostic methods and no harmonization of patient samples that should be analyzed (using whole tumor specimens does not allow differentiation of adjacent normal epithelial cells, stroma cells, and tumor cells).

Concluding remarks

Cancer MDR is still a major cause of therapy failure in the clinical management of cancer patients. Notwithstanding that in the last decades dramatic progress has been made in understanding the molecular mechanisms leading to cellular resistance to anticancer drugs and the clinical introduction of new "targeted" antineoplastic agents, drug resistance still represents an unsolved clinical problem.

Reasons for this include the not yet clarified roles of the identified biological mechanisms in therapy resistance in clinical settings. Besides, the lack of validated diagnostic tests, reliance on the clinical trial process to define the exact roles of the targeted drug resistance factor, and the possibility that alternative mechanisms may be present, are confounding clinical trial results. Furthermore, the problem of drug resistance was more or less misconceived due to an oversimplification. Certainly, a drug-resistant phenotype is not the result of a single mechanism, it is rather the consequence of a complex network of various cellular pathways and molecular mechanisms that can be switched on and off and temporarily simultaneously active during the development of anticancer drug resistance.

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