Resistance to Targeted Anti-Cancer Therapeutics 4

Thomas Efferth *Editor*

Resistance to Targeted ABC Transporters in Cancer

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Preface

The development of resistance is a major obstacle in cancer chemotherapy since decades. Drug resistance may develop during repeated treatment cycles after initially successful therapy (acquired or secondary resistance). Alternatively, tumors may be resistant from the beginning (inherent or primary resistance). The failure of chemotherapy is a major reason for the fatal outcome of tumor diseases in many patients. Even worse, tumors frequently develop resistance not only to single drugs but also to many others at the same time. This phenomenon was termed **multidrug resistance** and decreases the success rates of therapy regimens with combinations of structurally and functionally different drugs.

The pioneering research of Victor Ling, Michael M. Gottesman, and others led to the discovery of the drug efflux transporter **P-glycoprotein** and its encoding gene, ***MDR1***. This membrane protein expels a large array of different drugs and xenobiotic compounds out of the tumor cell leading to sublethal intracellular drug concentration and ultimately survival of tumor cells.

The initial cross-resistance profile of P-glycoprotein (P-gp) comprises **anthracyclines** (**doxorubicin**, **daunorubicin**), ***Vinca*-alkaloids** (**vincristine**, **vinblastine**), **epipodophyllotoxins** (**etoposide**, **teniposide**), **taxanes** (**paclitaxel**, **docetaxel**), and others.

P-gp/*MDR1* belongs to the family of ATP-binding cassette (ABC) transporters which are widely distributed in nature from bacteria to humans. The human genome consists of 48 ABC transporter genes, with P-gp/*MDR1* as the best analyzed one. Other drug resistance mediating ABC transporters are the multidrug resistance-related proteins (MRPs), breast cancer resistance protein (BCRP), and others. These ABC transporters are also characterized by specific cross-resistance profiles, which partly differ from the one of P-gp. They can also confer resistance to **camptothecin derivatives** (**topotecan**, **irinotecan**), **Mitoxantrone**, **sterols**, **tyrosine kinase inhibitors**, **compounds used in photodynamic therapy antimetabolites,** and others.

The uncommonly broad spectrum of anticancer agents that are transported by ABC transporters makes these proteins exquisite targets to search for compounds that inhibit their transport function. The idea is to block ABC transporter-mediated

drug efflux by specific inhibitors and thereby to overcome multidrug resistance. This concept was introduced by Takashi Tsuruo, who described that verapamil is able to inhibit P-gp's transport function. Subsequently, a huge amount of compounds from many pharmacologically established drug classes (e.g., **calcium channel blockers**, **calmodulin antagonists**, **cyclosporines**, **dipyridamole**, **and other hydrophobic**, **cationic compounds**) were observed to inhibit P-gp and to reverse multidrug resistance. Interestingly, many natural compounds derived from plants or marine organisms were also found to block ABC transporters' function.

All these fascinating results from basic cancer research were complemented by investigations from clinical oncology. A plethora of analyses have shown that P-gp/*MDR1* is of predictive value for success or failure of chemotherapy and of prognostic value for the survival time of cancer patients. Since certain **radiopharmaceuticals** are also transported by ABC transporters, they can be used for radiological diagnosis of multidrug-resistant tumors.

The importance of ABC transporters for drug resistance in tumors and the thriving development of research in this area can also be documented by the number of papers appearing every year during the past three decades (Fig. 1). ABC transporters have been a hot topic in cancer research for many years and still are. This motivated me to edit a book on this topic to keep scientists and physicians updated with the latest development in this exciting research area. I was fortunate to team up a panel of international experts with renowned expertise in the field of ABC transporters in drug-resistant tumors. The book covers most currently relevant topics in

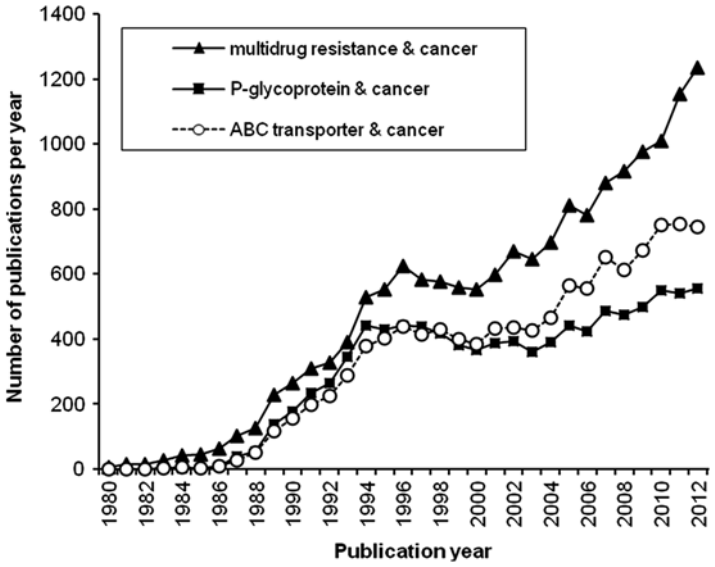

Fig. 1 Survey of the literature deposited in the PubMed database from 1980 to 2012 with the indicated keywords

the field reaching from the clinical relevance of ABC transporters for resistance to novel and established anticancer drugs and prognosis of patients to compounds to modulate multidrug resistance, compounds used in photodynamic therapy, tyrosine kinase inhibitors, and others. Furthermore, the potential of radiopharmaceuticals for diagnosis of multidrug-resistant tumors will be discussed.

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Contents

Chapter 1
Role of P-Glycoprotein for Resistance of Tumors to Anticancer Drugs: From Bench to Bedside

Manfred Volm and Thomas Efferth

Abstract Success of cancer chemotherapy is limited by simultaneous resistance towards many anticancer drugs making clinical combination therapy protocols less efficient. P-glycoprotein represents an efflux pump of the ABC transporter family, which recognizes and extrudes anticancer drugs of diverse chemical classes and biochemical functions. The P-glycoprotein-mediated profile of cross-resistance has been termed multidrug resistance (MDR). In our investigations, we focused on MDR of in vivo tumor lines maintained in mice. The development of in vivo resistance towards anthracyclines (doxorubicin, daunorubicin) in L1210 and S180 ascites tumor lines was accompanied with decreased uptake and increased efflux of the fluorescent dye rhodamine 123, overexpression of P-glycoprotein as well as *MDR1* mRNA overexpression and *MDR1* gene amplification. In addition to acquired multidrug resistance in these syngeneic mouse tumor lines, we investigated inherent drug resistance in human lung xenograft tumors transplanted to nude mice. Drug resistance in these xenografts was also associated with overexpression of P-glycoprotein and *MDR1* mRNA, but without *MDR1* gene amplification. Furthermore, we explored P-glycoprotein expression in clinical biopsies of diverse tumor entities (leukemia, lung cancer, breast cancer, cervical carcinoma, endometrial carcinoma. nephroblastoma, renal cell carcinoma) and found that high levels of P-glycoprotein expression correlated with pretreatment with chemotherapy, drug resistance, and failure to achieve complete remission. During the past years, a wealth of publications worldwide confirmed a role of the P-glycoprotein for clinical treatment refractoriness and as an unfavorable prognostic factor for survival time of patients.

Keywords ABC transporter • Anthracyclines • Cancer • Chemotherapy • Drug resistance • Prognostic factor • Rhodamine 123 • Survival time • Xenograft tumor

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1.1 Introduction

The development of resistance to anticancer drugs has dogged clinical oncology since the very early days of chemotherapy. Since the establishment of nitrogen mustard-derived compounds and methotrexate in the 1940s and 1950s, it became clear that tumors have efficient escape mechanisms preventing the cure of many cancer patients [1, 2]. A surprising observation was that over the decades until today it was not possible to develop drugs without appearance of resistance phenomena in tumors. The development of combination therapy improved the situation compared to monotherapy, but sustainable treatment success leading to reliable cure of patients could also not be reached [3–5]. A main problem is that the side effects of most cytotoxic and cytostatic drugs to proliferating normal tissues (e.g. bone marrow, gastrointestinal mucosa, hair roots, reproductive organs) are significantly such that sufficient doses of drugs cannot be applied to kill tumor cells Suboptimal drug concentrations facilitate the survival of inherently more resistant cells in a heterogeneous tumor population. Depending of the type of tumor, the clinical situation is that a fraction, but not all patients, benefits from chemotherapy leading to improved survival times. It was unexpected at that time that combination regimens were also subject to resistance development, since drugs with different cellular and molecular modes of action were used. The clinical phenomenon of unresponsiveness to multiple drugs was reproducible under cell culture conditions in the laboratory. It was David Kessel who described in 1968 that cell lines display cross-resistance to different drugs [6]. This observation was also reported in hundreds of publications in subsequent years and laid the bases for a new area of research in cancer biology. Strikingly, tumors that are initially responsive to chemotherapy can develop resistance during treatment. Resistant tumor cells can acquire cross-resistance to a wide range of compounds that have no obvious structural or functional similarities, e.g. alkaloids (colchicine, vinblastine, vincristine), anthracyclines (doxorubicin, daunorubicin), taxanes (paclitaxel, docetaxel), epipodophyllotoxins (etoposide, teniposide), and antibiotics (actinomycin D, mitomycin C) [7, 8]. This phenomenon has been designated as pleiotropic or multidrug resistance (MDR).

MDR of cancer cells is associated with decreased net cellular drug concentrations and has been attributed to alterations in the plasma membrane [9–11]. A drug efflux pump termed P-glycoprotein (P for permeability) has been unraveled as an underlying mechanism [10, 12]. The *MDR1/ABCB1* gene encoding the P-glycoprotein has been cloned in 1986 and was found to be amplified in many multidrug-resistant cell lines in vitro [13]. P-glycoprotein was the first member of the ATP-binding cassette (ABC) transporter family identified in cancer. Its discovery has enormously stimulated cancer research in subsequent years and numerous in vitro cell lines were characterized to overexpress P-glycoprotein and *MDR1* mRNA as well to carry amplified copy numbers of the *MDR1* genes in their genomes [14].

1.2 Architecture and Function of the P-Glycoprotein

This efflux transporter belongs to a gene family of ATP-binding cassette (ABC) transporters. The human genome consists of 48 members of this gene family. About one dozen ABC transporters have been suggested to transport anticancer drugs, although the P-glycoprotein is the best understood. The P-glycoprotein consists of 1,258 amino acids which are organized as two duplicated halves. It spans the cell membrane with 123 transmembrane segments and two intracellular ATP-binding domains. The extracellular glycosylation of P-glycoprotein is not of functional relevance.

Several hypotheses have been discussed on the biochemical mode of action (Fig. 1.1):

The partitioning model: It has been speculated that the P-glycoprotein does not transport drugs by itself but affects drug distribution indirectly by altering the pH value or membrane potential [15]. This model was supported by the fact that some anticancer drugs are positively charged and that the pH in multidrug-resistant cells differs from sensitive cells. It is now generally accepted that the P-glycoprotein is primarily not an ion channel [16].

The hydrophobic vacuum cleaner hypothesis: Many P-glycoprotein substrates are hydrophobic and are better soluble in the lipid bilayer of the cell membrane than

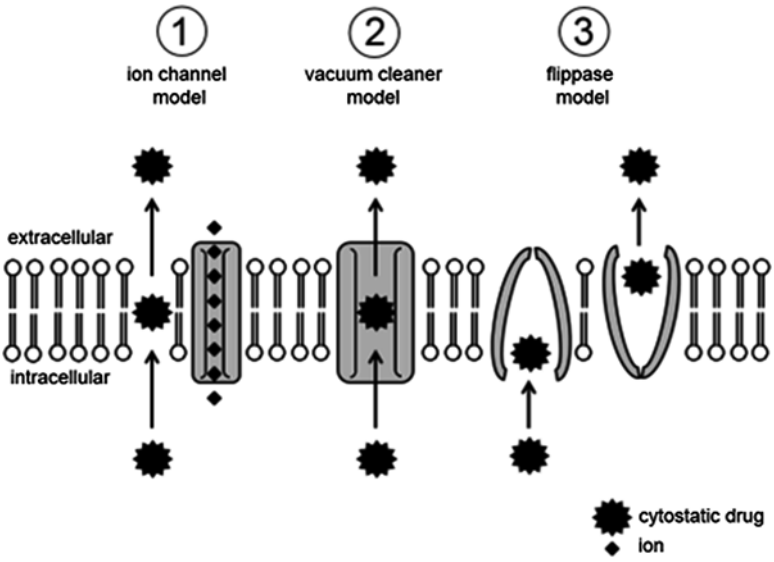

Fig. 1.1 Models on the function of P-glycoprotein

in the extracellular aqueous phase. Anticancer drugs are thought first to interact with the cell membrane and then bind to a binding site of the P-glycoprotein leading the drug to the internal channel of P-glycoprotein and effluxes out of the cell [17, 18]. This model may not only explain the increased efflux of multidrug-resistant cells, but also the frequently observed decreased drug influx [19, 20]. Hydrophilic drugs do not enter the P-glycoprotein from the lipid bilayer but first they passively diffuse into the cytosol and then reach the P-glycoprotein from the intracellular side.

The translocase or flippase model postulates that according to the higher intracellular drug concentration gradient, drug molecules bind to the intracellular binding domains of the P-glycoprotein, which changes conformation by an energy-driven process (i.e. ATP cleavage) and flips drugs to the extracellular space [17].

1.3 Acquired Drug Resistance in Tumor Lines In Vivo

Instead of studying multidrug resistance in vitro, our own research efforts were directed to investigate drug resistance in animals to get closer to the clinical situation. One reason for that decision was that drug-resistant cell lines have been described to exhibit 1,000- to 10,000-fold resistance to drugs such as methotrexate [21–23]. These high degrees of resistance do usually not occur in the clinical situation and might be laboratory artifacts.

We established doxorubicin-resistant L1210 leukemia cells, which express the MDR phenotype. The tumor cell line was generated in vivo in mice—an approach which might be more analogous to the clinical development of drug resistance. Mice bearing L1210 ascites cells were treated with doxorubicin i.p. weekly. Doxorubicin was applied at a concentration of 2 mg/kg body weight. The pretreatment was carried out for at least 20 passages. The test for resistance was carried out by injecting groups of animals with either untreated (sensitive) or drug-pretreated (resistant) tumor cells. Then, doxorubicin was injected i.p. on the fourth and fifth days after tumor cell transplantation. The effects of doxorubicin (different concentrations) on the tumor cells were measured 2 days after the last injection. Under these conditions, tumor-bearing animals were killed, the ascites cells were removed by puncture and counted using a coulter counter. L1210 ascites tumor cells preconditioned with doxorubicin were more resistant in vivo to this drug than the parental cells (Fig. 1.2, *left side*).

An in-vitro short-term test was also used to detect the resistance of the pretreated tumor cells and to define the degree of resistance. The basic feature of this test is the measurement of inhibition of incorporation of radioactive nucleic acid precursors into tumor cells after the addition of cytostatic agents [24]. The tumor cells were incubated in vitro with doxorubicin for 3 h. The radioactive precursor was added during the third hour of incubation. Aliquots of the cell suspensions were pipetted onto filter discs, the acid-soluble radioactivity extracted, and the incorporated activity measured by scintillation counting. As shown in Fig. 1.2 (*right side*), the resistance of the pretreated cells found in vivo could be confirmed in vitro. The maximum

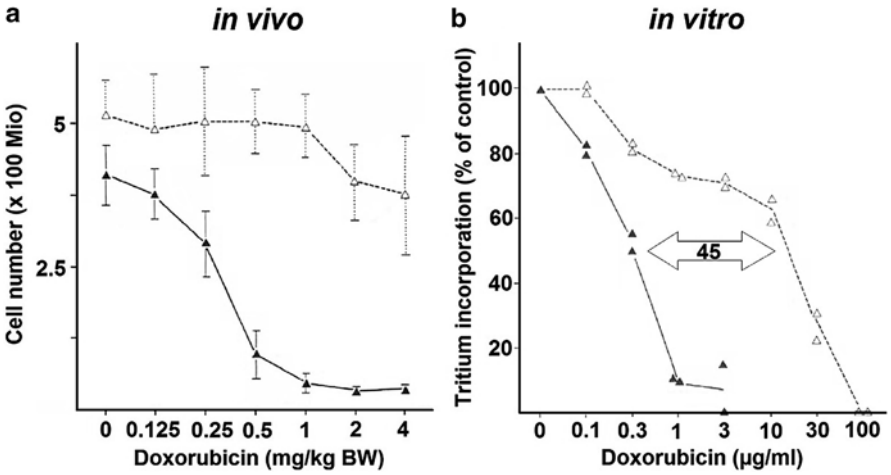

Fig. 1.2 Detection of resistance of doxorubicin-preconditioned L1210 ascites tumor cells in vivo and In vitro. Abscissa: different dose levels of doxorubicin. Closed symbols indicate parental (sensitive) ascites tumor cells and open symbols drug-pretreated (resistant) ones. Data were taken from Volm et al. [25]

degree of resistance was 45-fold for the cells preconditioned with doxorubicin. Cross-resistance of these cell lines was found to doxorubicin and daunorubicin, but not to cytosine-arabinoside and cyclophosphamide [25].

Cross-resistance of anthracyclines to the cytotoxic fluorescent dye rhodamine 123 has been described [26]. Therefore, we determined rhodamine 123 accumulation in the resistant and sensitive L1210 ascites cells by fluorescence microscopy. We found that resistant cells needed more time to accumulate rhodamine 123 than their sensitive parental cells. Sensitive cells showed a significant rhodamine 123 fluorescence, whereas daunorubicin-resistant cells did not (Fig. 1.3).

For the quantification of rhodamine 123 fluorescence, we used an ORTHO 30L cytofluorescence analyzer equipped with two argon ion lasers and an INTERTECHNIQUE Plurimed spectrum analysis computer system. Figure 1.3 shows representative biparametrical fluorescence histograms of intracellular rhodamine 123 fluorescence intensity in resistant and sensitive L1210 ascites tumor cells. These flow cytometric data confirmed the microscopic observation that resistant cells accumulated less rhodamine 123.

The rhodamine 123 accumulation was measured in a time- and concentration-dependent manner. Drug-sensitive parental L12010 cells accumulated high amounts of rhodamine 123 depending on the concentration and the incubation time (Fig. 1.3c), whereas rhodamine 123 was taken up at much lower amounts in the resistant L1210 cells (Fig. 1.3d). These accumulation kinetics showed the intracellular accumulation, which was the net sum of both the uptake and the efflux. Therefore, the question remains about the relevance of drug efflux, since P-glycoprotein functions as an efflux transporter. Sensitive and resistant L1210

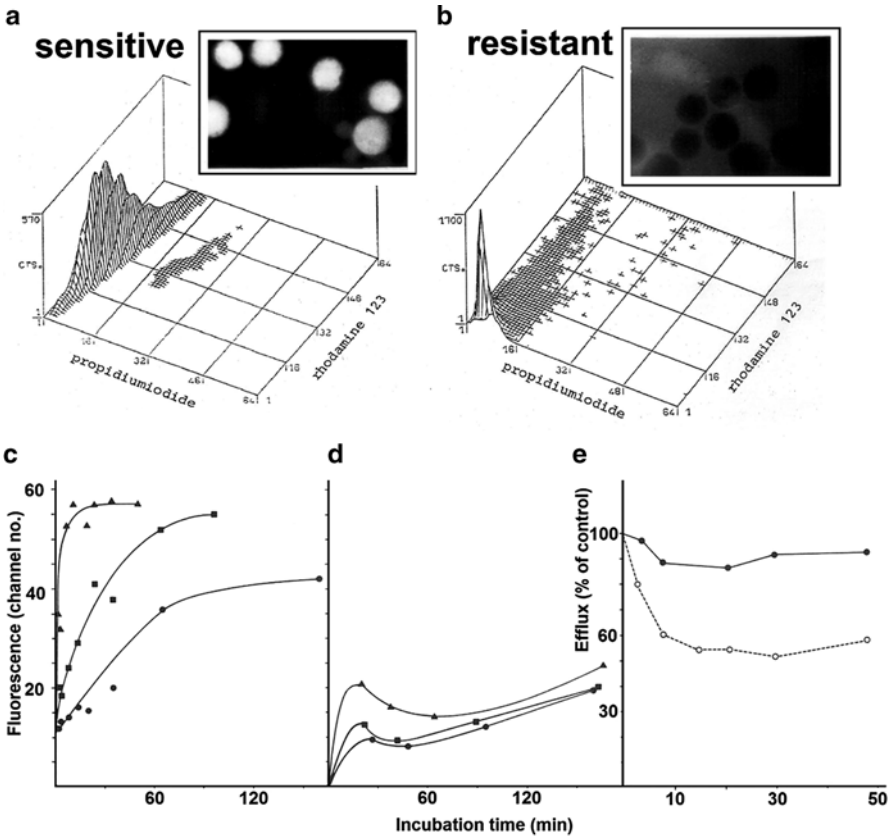

Fig. 1.3 Accumulation of rhodamine 123 in sensitive and daunorubicin-resistant L1210 ascites tumor cells as measured by flow cytometry. Biparametrical histograms of cells stained with (**a**) rhodamine 123 (4 mg/ml, 60 min incubation) and (**b**) propidium iodide (35 μg/ml). At least 20,000 cells were measured per histogram. Propidium iodide was used to exclude dead cells. Time and concentration kinetics of rhodamine 123 (2, 4, and 8 μg/ml) in sensitive (**c**) and resistant L1210 cells (**d**). (**e**) Determination of rhodamine 123 efflux (1 h preincubation with 8 μg/ml) in sensitive (*closed symbols*) and resistant L1210 cells (*open symbols*). Data were taken from Efferth [27] and Efferth et al. [28]

cells were loaded with rhodamine 123, centrifuged and transferred into rhodamine 123-free medium. As shown in Fig. 1.3e, sensitive cells retained rhodamine 123, whereas resistant cells showed a considerable efflux of this fluorescent dye. To further analyze whether rhodamine 123 is pumped out in an energy-dependent process (presumably by the P-glycoprotein), resistant cells were incubated with and without 2-deoxy-glucose. Cells use glucose for energy metabolism to generate ATP and 2-deoxy-glucose is an efficient inhibitor of ATP production. Hence, 2-deoxy-glucose is expected to inhibit the ATP-dependent efflux of P-glycoprotein. Resistant cells loaded with 2-deoxy-glucose were incubated with rhodamine 123

and then transferred into rhodamine 123-free Hanks's balanced salt solution. 2-Deoxy-glucose-treated resistant cells did not show a considerable efflux of rhodamine 123, whereas untreated resistant cells did [27, 28]. We conclude that the ATP-dependent efflux of rhodamine 123 was blocked by 2-deoxy-glucose and that ATP is necessary for the energy-dependent efflux function of the P-glycoprotein.

1.4 Development of Resistance by Repeated Drug Treatments

To monitor the development of drug resistance during consecutively repeated doxorubicin treatments, mice bearing L1210 tumors were once weekly treated with 0.5 mg doxorubicin/kg body weight i.p. The development of doxorubicin resistance was analyzed during eight treatments in vivo by using the in vitro short term test. Sensitive (S) and established resistant L1210 ascites tumor cells (R) with a resistance factor of 45 were used as negative and positive controls respectively (Fig. 1.4). Resistance increased with the number of treatments and the level of resistance achieved after eight treatments is similar to that of the established tumor line (Fig. 1.4). This suggests that a maximum level of resistance was already reached after only eight treatments. In order to prove this result, we increased the dosage of doxorubicin in the established tumor line from 2 to 8 mg /kg body weight for ten treatments and indeed did not observe a further increase of the level of resistance.

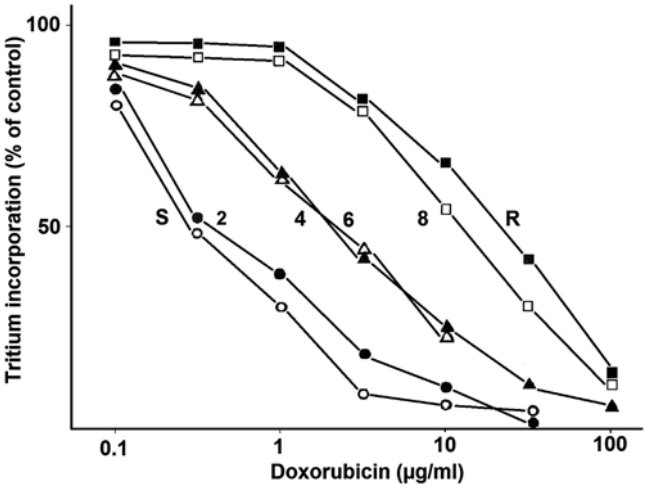

Fig. 1.4 Development of resistance to doxorubicin in L1210 ascites tumor cells in vivo. Dose–response curves of doxorubicin after treatments (2–8×) with 0.5 mg/kg doxorubicin. S, sensitive (parental) tumor line; R, doxorubicin-resistant line after more than 20 treatments with 2 mg/kg body weight doxorubicin. Each point represents mean of four measurements. Data were taken from Volm et al. [32]

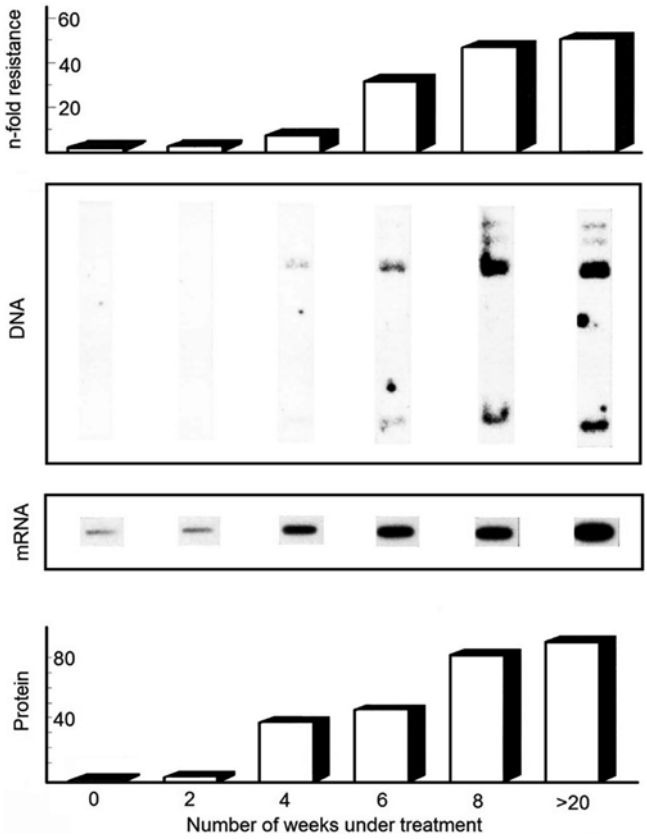

Fig. 1.5 Development of doxorubicin resistance, MDR1 gene amplification, MDR1 and P-glycoprotein overexpression in L1210 tumor cells after treatment (2–8×) with 0.5 mg/kg doxorubicin in vivo. S, sensitive (parental) tumor line; R, doxorubicin-resistant cell line after more than 20 treatments with 2 mg/kg body weight doxorubicin. Figure was taken from Volm [33]

In order to find out whether the P-glycoprotein increases according to both the number of treatments and the increase of doxorubicin resistance, we investigated *MDR1* gene amplification and mRNA expression as well as P-glycoprotein expression. After four treatments many tumor cells expressed P-glycoprotein and after eight treatments nearly all tumor cells showed increased expression of the P-glycoprotein as shown by immunohistochemistry (Fig. 1.5). Southern-blot analysis during eight treatments with doxorubicin showed that the development of resistance was associated with *MDR1* gene amplification and correlated with the degree of drug resistance. A comparison of the intensity of the bands in the established line (L1210/Dox) with that in the tumors with eight treatments indicated similar degrees of amplification. Slot-blots probing *MDR1* mRNA expression confirmed these results (Fig. 1.5). The gene amplification did not increase by further treatments with a higher concentration of doxorubicin.

1.5 Reversal of Resistance After Cessation of Chemotherapy

The question arises as to whether or not the resistance that developed over 20 passages would persist after cessation of doxorubicin treatment. Remarkably, resistance decreased over time and disappeared after 20 passages without treatment (Fig. 1.6). This decrease in resistance was accompanied by a loss of P-glycoprotein as well as by a loss of *MDR1* mRNA expression and gene amplification (Fig. 1.7; [32]). These results in mouse tumors fit to the clinical situation. It has been observed clinically that the longer the duration of the disease free intervals following chemotherapy, the more likely is the response of the tumors to the same chemotherapy.

Corresponding to the development of resistant L1210 leukemia ascites cell lines, we developed doxorubicin- or daunorubicin-resistant sarcoma S180 ascites cell lines. These resistant S180 cell lines also displayed cross-resistance to various other drugs [29, 30]. As shown in Fig. 1.8, these two resistant S180 ascites tumor lines also displayed decreased uptake of rhodamine 123 (as shown by flow cytometry), amplification of the *MDR1* gene (as shown by Southern blot), as well as overexpression of *MDR1* mRNA (as shown by Northern blot) and protein overexpression (as shown by Western blot, immunofluorescence and immunohistochemistry). Hence, multidrug resistance in these S180 tumor lines can also be explained by the P-glycoprotein in a comparable fashion as observed in the multidrug-resistant L1210 tumor lines.

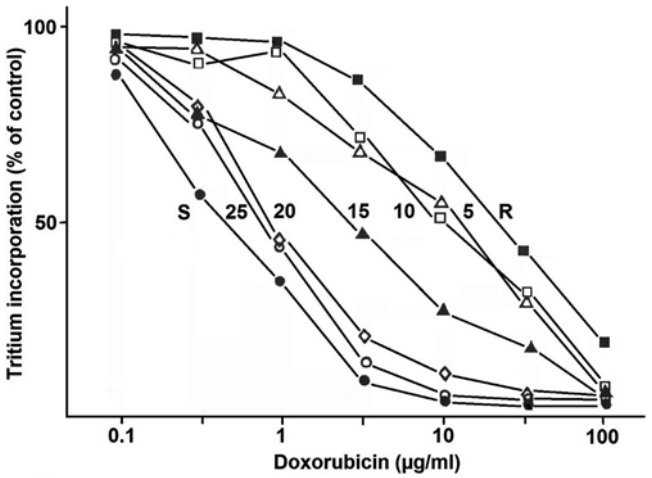

Fig. 1.6 Dose response curves of doxorubicin after cessation of doxorubicin treatment (5–25 passages). S, sensitive (parental) tumor line; R, doxorubicin-resistant tumor line. Each point represents mean of four measurements. Data were taken from Volm et al. [32]

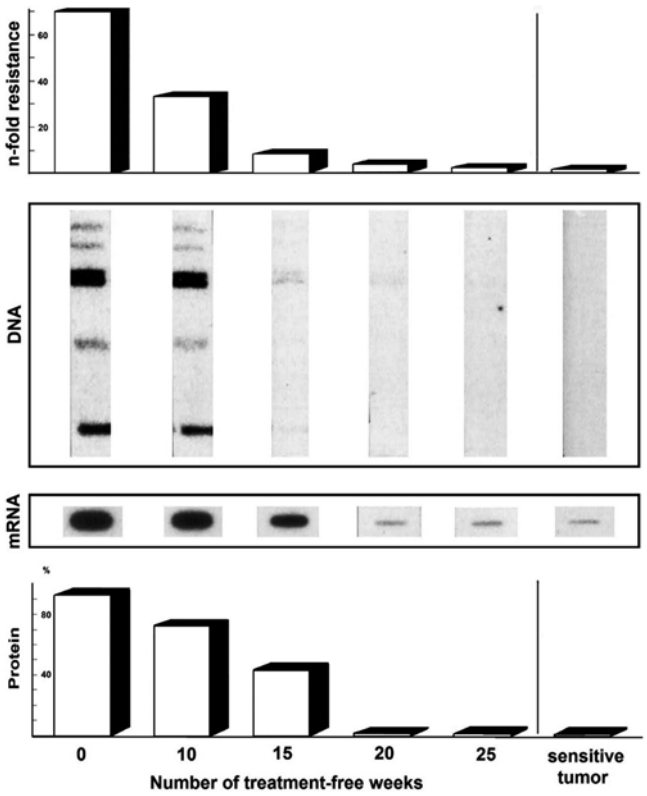

Fig. 1.7 Reversion of doxorubicin resistance, P-glycoprotein and MDR1 overexpression and MDR1 gene amplification in L1210 tumor cells after cessation of treatment for 20 passages in vivo. Each point represents mean of four measurements. Data were taken from Volm [33]

1.6 Inherent Drug Resistance in Human Lung Tumor Xenografts In Vivo

Transplantable murine tumors have the advantage that they can be maintained in mice with an intact immune system. Chemotherapy depresses the immune functions of the body—a process that takes place both in mice and men. In this respect, syngeneic mouse tumors reflect the situation in human cancer patients. Another animal model utilizes human xenograft tumors. These tumors grow in nude mice with a deficient immune system and do, therefore, not reject human cells. The advantage of this animal model is that the human tumors can be investigated in living mouse models without performing experiments in patients. Hence, this is an attractive model to analyze the chemotherapy effects in human tumors.

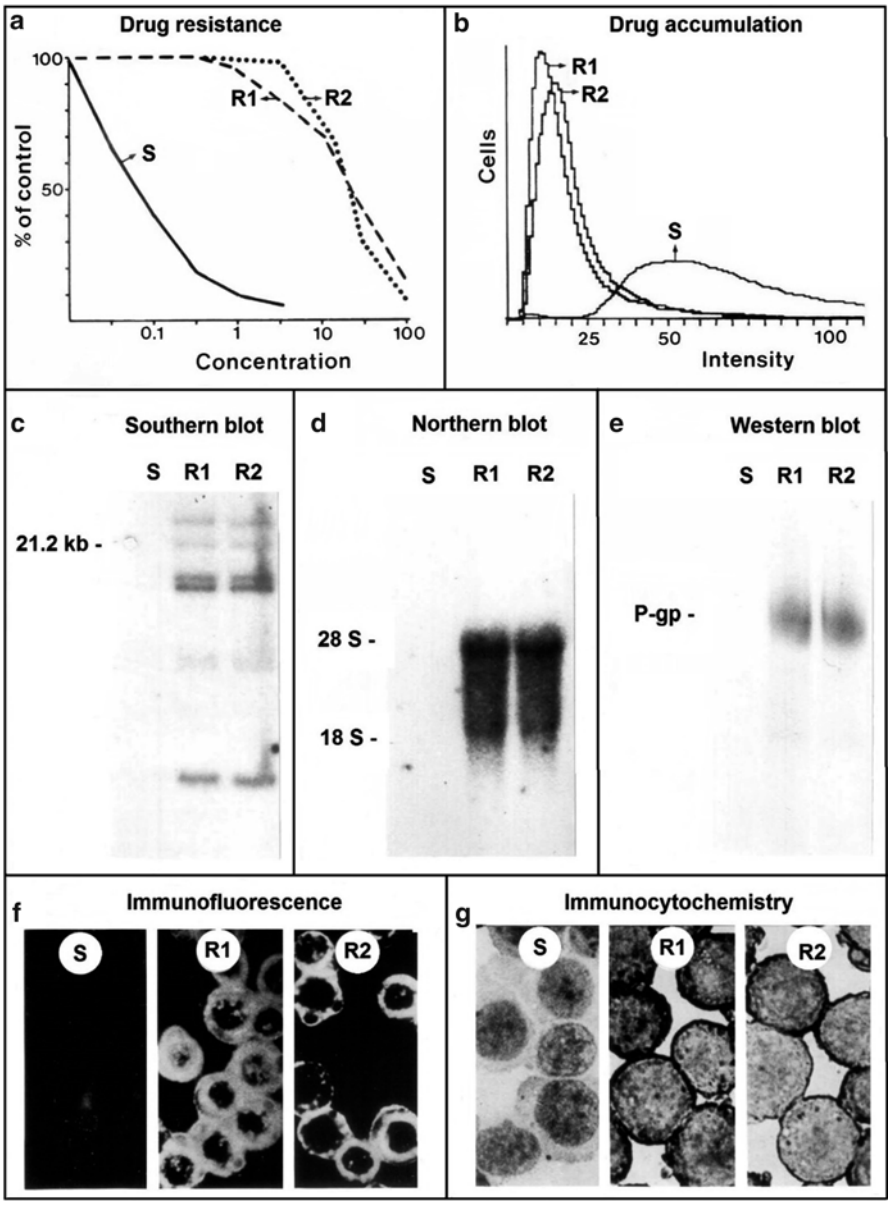

Fig. 1.8 Different methods for detecting multidrug resistance. Shown are results in sensitive parental cells (S) and multidrug-resistant cell lines (R1, R2). Resistance was developed in response to doxorubicin (R1) and daunorubicin (R2). (**a**) In vitro short-term test to determine the degree of resistance. (**b**) Flow cytometry to measure accumulation of rhodamine 123. (**c**) Southern blotting to detect gene amplification of the MDR1 gene. The hybridization was performed with the pcDR 1.5 cDNA probe. (**d**) Northern blotting to detect mRNA overexpression of the MDR1 gene (Hybridization with pcDR 1.5). (**e**) Western blotting to detect overexpression of P-glycoprotein (with mAb C219). (**f**) Immunofluorescence and (**g**) immunohistochemistry to detect overexpression of P-glycoprotein (with mAb C219). Taken from Volm et al. [31]

In addition to the development of resistance during repeated treatments with anticancer drugs (termed acquired or secondary drug resistance), resistance can also occur in untreated tumors. This form of resistance is characterized by unresponsiveness to anticancer drugs even if the tumors had never been challenged with chemotherapy. This form is termed inherent or primary resistance. Clinically, both forms lead to the failure of chemotherapy and the question arises, whether the P-glycoprotein/*MDR1* is also of relevance for the inherent drug resistance. Lung cancer is frequently characterized by inherent drug resistance, which is one reason for the observed survival times of lung cancer patients.

We investigated the intrinsic resistance of a panel of eight human epidermoid lung cancer xenografts to vincristine and daunorubicin and tested the cross-resistance pattern to a variety of other agents. Our results demonstrated that xenograft lines derived from human lung tumors, not previously treated with chemotherapy exhibited a comparable pattern of cross-resistance as observed in the multidrug-resistant L1210 or S180 cell lines [34, 35]. By means of fluorescence microscopy, we found that drug-resistant epidermoid lung carcinoma xenograft tumors showed an intensive immunostaining for P-glycoprotein, whereas no immunoreactivity could be seen in drug-sensitive xenografts (Fig. 1.9). Northern and Southern blot analyses revealed

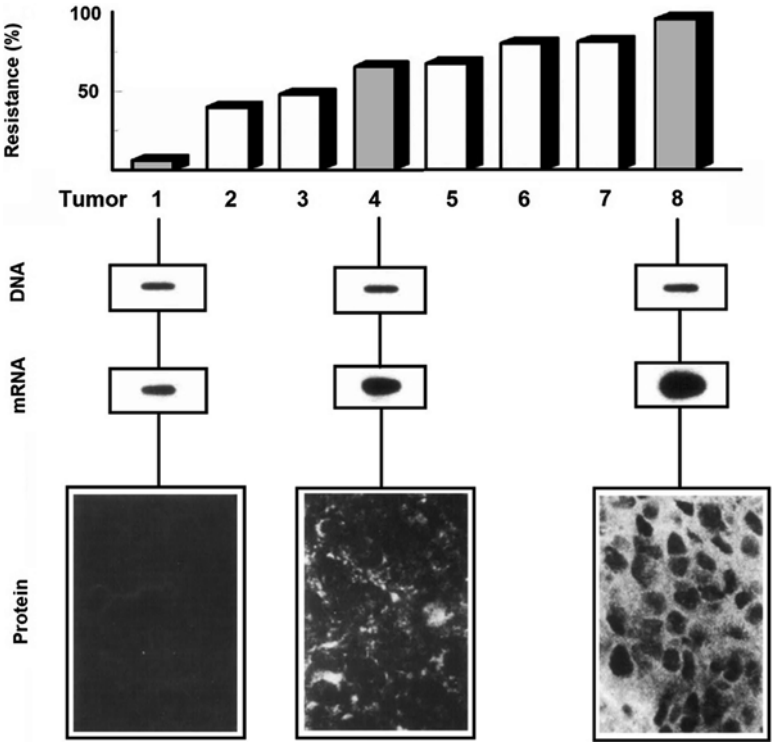

Fig. 1.9 Effect of a single dose of 2 mg/kg vincristine on tumor size (*top*), MDR1 gene amplification and mRNA expression (*middle*) and P-glycoprotein expression (*bottom*) of eight different epidermoid lung cancer xenografts. MDR1 hybridization was performed with the genomic pcDr 1.5 DNA probe. P-glycoprotein was detected by immunofluorescence and mAb 265/F4. Data are taken from [33, 35]

that the *MDR1* mRNA expression was increased according to P-glycoprotein expression and the degree of drug resistance, whereas *MDR1* gene amplification was not detectable (Fig. 1.9).

1.7 P-Glycoprotein in Clinical Tumor Biopsies

After studying transplantable tumors with either acquired or inherent drug resistance in animals, we were interested to explore the relevance of P-glycoprotein in human tumors. Patients with previously untreated non-small cell lung carcinomas (NSCLC) were surgically treated and specimens of the tumors for resistance testing (in vitro-short-term test) and detection of P-glycoprotein (immunohistochemistry) were used.

Of the 91 lung tumors, 43 were P-glycoprotein positive and 48 were negative. There was a significant correlation between the resistance and P-glycoprotein expression (Fig. 1.10) Of the 64 resistant tumors 39 were P-glycoprotein positive (61 %), whereas of the 27 sensitive tumors only four were P-glycoprotein positive (15 %) [38]. Furthermore, a significant correlation between the oncoproteins c-Fos

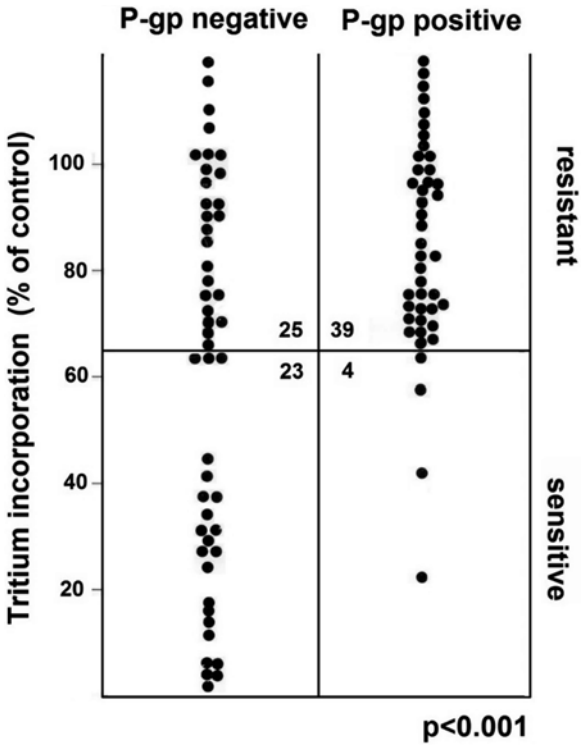

Fig. 1.10 Relationship between the response to doxorubicin as determined by the in vitro short-term test (ordinate) and immunohistochemical detection of P-glycoprotein (abscissa) in human lung cancer biopsies [38]

and c-Jun and the P-glycoprotein was found [36, 37]. Both proteins form a protein complex, which functions as transcription factor (AP-1). These results and the fact that the *MDR1* gene promoter carries AP-1 binding motifs may be taken as a clue that the P-glycoprotein may be regulated by the transcription factor AP-1.

Investigations by us and others on liver carcinogenesis revealed a connection between P-glycoprotein expression and resistance to liver-carcinogenic compounds [39–41]. As smoking is widely accepted to cause lung cancer, we hypothesized an association between smoking habits, resistance to doxorubicin, and expression of P-glycoprotein. Biopsies of 94 non-small cell lung cancers were analyzed for their doxorubicin resistance using an in vitro short-term assay as well as their P-glycoprotein expression by immunohistochemistry. These results have been correlated to the smoking habits of the patients [42]. As shown in Fig. 1.11, lung tumors from smokers were more frequently resistant to doxorubicin and expressed higher levels of P-glycoprotein than lung tumors from nonsmokers ($p = 0.007$ and $p = 0.0001$, respectively). These data may be taken as a hint that smoking contributes not only to lung carcinogenesis but also to drug resistance of lung tumors. This is an interesting result, since non-small cell lung cancer is a tumor entity well known for its unresponsiveness to chemotherapy and short survival times of patients.

Another question raised was whether chemotherapy of tumors may induce P-glycoprotein expression. Accordingly, we analyzed a total number of 162 tumor biopsies by means of immunohistochemistry [43–54]. The tumors were divided into three groups according to their treatment. The first group consisted of untreated tumors

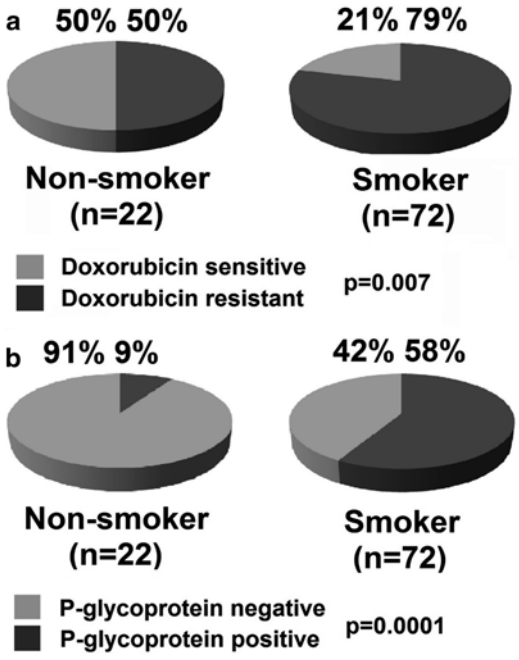

Fig. 1.11 Relationship between smoking habits of patients and response to doxorubicin as determined by the in vitro short-term test and immunohistochemical detection of P-glycoprotein in 94 human non-small cell lung cancer biopsies [42]

Table 1.1 Expression of P-glycoprotein in 162 human tumor biopsies

Tumor type	Without therapy	Non-MDR therapy	MDR therapy	Sum
Acquired resistance				
Leukemia	1/4	1/5	3/11	20
Breast cancer	0/12	0/4	4/7	23
Ovarian carcinoma	2/25	0/0	3/3	28
Nephroblastoma	0/8	0/0	12/23	31
Sum	*3/49 (=6 %)*	*1/9 (=11 %)*	*22/44 (=50 %)*	*102*
Inherent resistance				
Endometrial carcinoma	20/20	0/0	3/3	23
Cervix carcinoma	15/26	0/0	0/0	26
Renal carcinoma	8/9	0/0	2/2	11
Sum	*43/55 (=78 %)*	*0/0 (=0 %)*	*5/5 (=100 %)*	*60*

The numbers indicate P-glycoprotein-positive cases in comparison to the total tumor number. Extended data based on Volm et al. [31]

and the second group of tumors was treated with anticancer drugs not inducing MDR. The third group consisted of tumors, which were pretreated with drugs known to cause MDR (e.g. doxorubicin, vincristine). Biopsies from leukemia, breast cancer, ovarian carcinoma, or nephroblastoma which have not been pretreated displayed P-glycoprotein expression in a few cases (3 of 49=6 %). Among the tumors treated with non-MDR-drugs only one out of nine was P-glycoprotein positive (=11 %). By contrast, biopsies from 44 tumors treated with MDR-inducing drugs exhibited P-glycoprotein in 22 cases (=50 %) (Table 1.1). This pattern of P-glycoprotein expression speaks for the expression of P-glycoprotein during chemotherapy and indicates that P-glycoprotein is one responsible factor for the development of acquired (secondary) drug resistance (Table 1.1). Another group of tumors (renal, cervical, and endometrial carcinoma) showed a completely different profile. These tumors showed P-glycoprotein expression in a high number of biopsies, although these tumors have not been pretreated (43 out of 55 tumors=78 %). A few tumors were nevertheless treated with MDR-relevant drugs and they were all P-glycoprotein positive (5/5=100 %). These three tumor types are known to be relatively unresponsive towards chemotherapy. This indicates that P-glycoprotein expression in these tumor types is tightly correlated with inherent (primary) resistance towards anticancer drugs. Exemplary immunostainings of P-glycoprotein in human tumors are depicted in Fig. 1.12.

The results of our own investigations were compared with data from the literature. In a previous meta-analysis, a total number of 6.248 tumors were investigated for their P-glycoprotein and *MDR1* mRNA expression as well as *MDR1* gene amplification [55]. While *MDR1* gene amplification is a very rare event in clinical tumor samples, overexpression of MDR1 mRNA frequently occurred even in untreated tumors (Fig. 1.13). Comparable results were found for the expression of P-glycoprotein in untreated tumors (Fig. 1.14). These results in a large number of clinical tumors indicate that *MDR1* gene expression does not account for multidrug

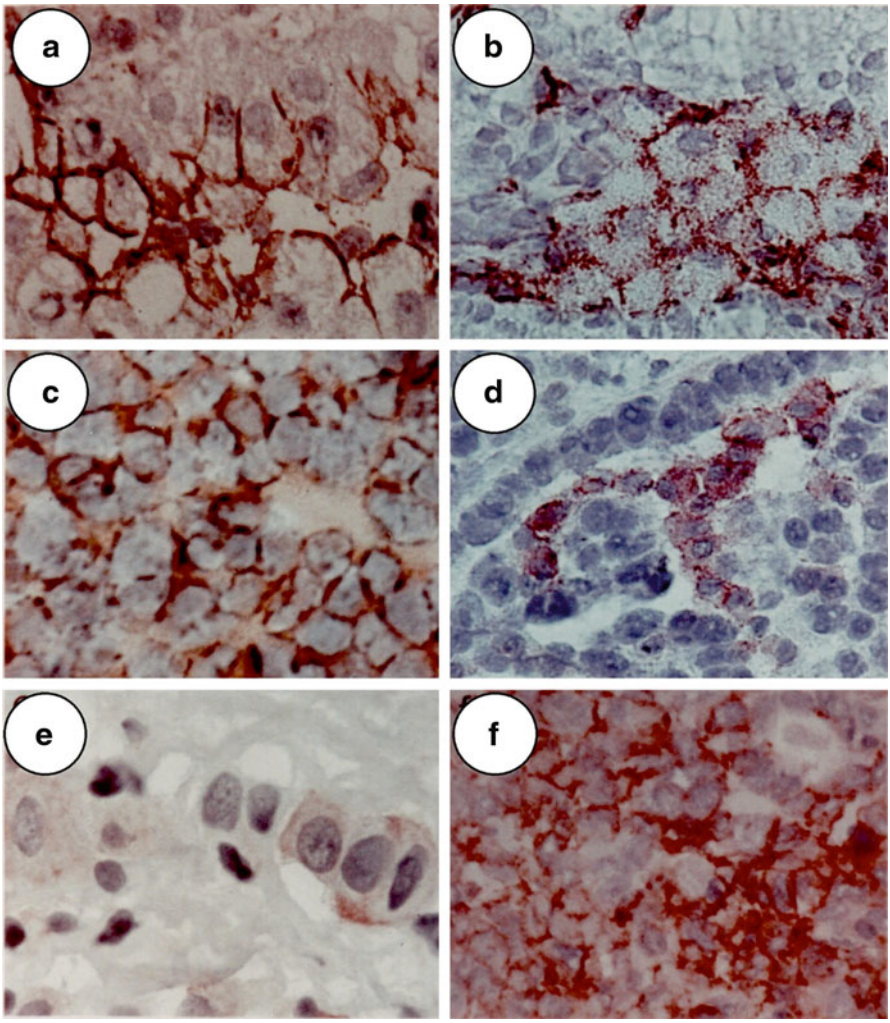

Fig. 1.12 Immunohistochemical detection of P-glycoprotein in human tumors by mAb C219. (**a**) Renal cell carcinoma, (**b**) endometrial carcinoma, (**c**) multiple myeloma, (**d**) ovarian carcinoma, (**e**) breast carcinoma, and (**f**) control tumor (daunorubicin-resistant solid carcinoma 180 of the mouse). Taken from Volm et al. [31]

resistance in the clinic. Nevertheless, overexpression of *MDR1* mRNA and P-glycoprotein represent a frequent event. Their occurrence even in untreated tumors point to the relevance of P-glycoprotein/*MDR1* for inherent or primary drug resistance.

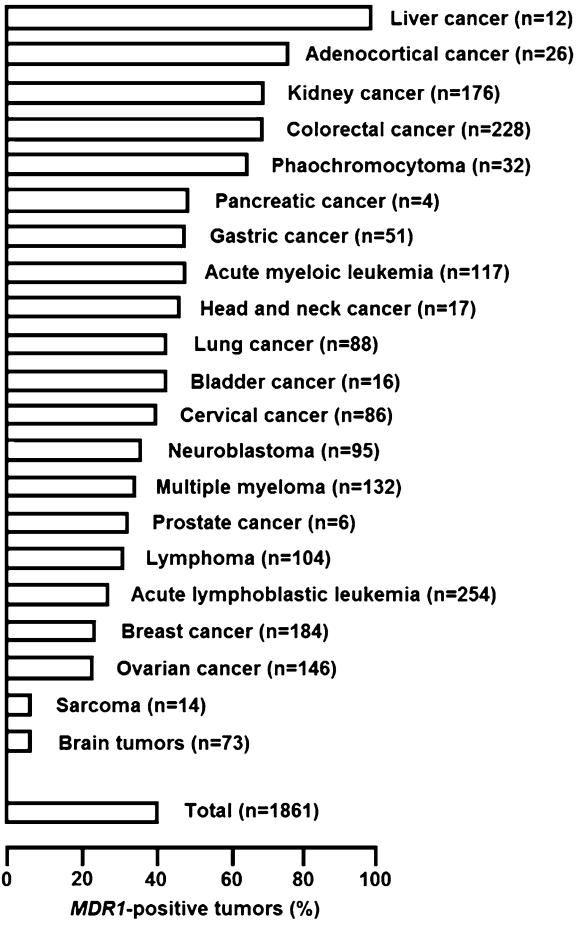

Fig. 1.13 Expression of MDR1 mRNA in untreated human tumors as determined by Northern blotting, slot blotting, in situ hybridization, RNAse protection assay or RT-PCR. Taken from Efferth and Osieka [55]

The next question raised was whether the increase of P-glycoprotein/*MDR1* in animal tumors in vivo after treatment with anticancer drugs could also be observed in a clinical setting. Therefore, it was evaluated whether the expression of P-glycoprotein/*MDR1* in human tumors was higher after chemotherapy as compared to untreated tumors before chemotherapy. As shown in Fig. 1.15, the mRNA and protein expression levels considerably increased after chemotherapy. This indicates that P-glycoprotein/*MDR1* also plays a role for acquired or secondary drug resistance.

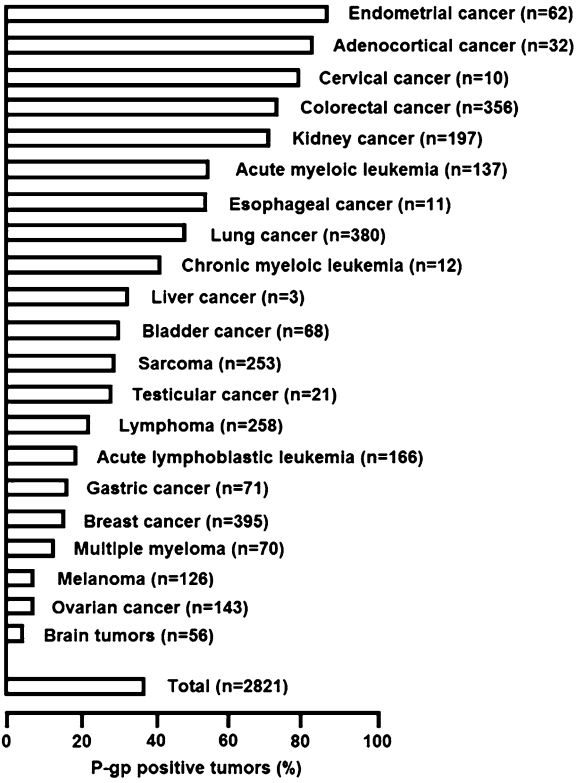

Fig. 1.14 Expression of P-glycoprotein in untreated human tumors. P-glycoprotein was detected either by Western blotting, immunohistochemistry, immunofluorescence, or flow cytometry. Taken from Efferth and Osieka [55]

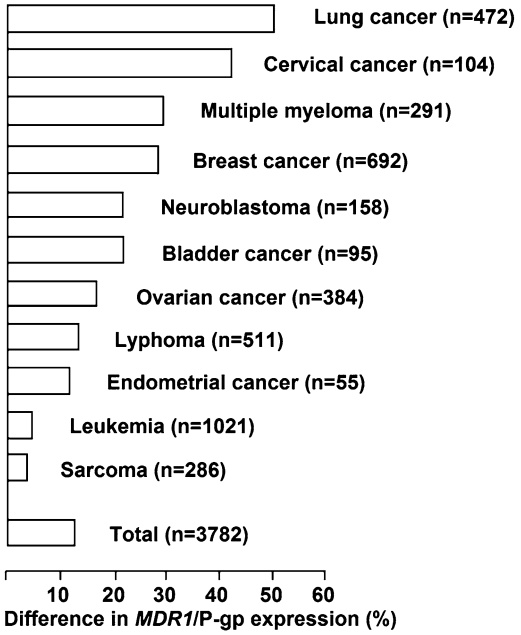

Fig. 1.15 Increase of P-glycoprotein/MDR1 expression in treated as compared to untreated tumors. Taken from Efferth and Osieka [55]

1.8 Prognostic Relevance of P-Glycoprotein for Treatment Outcome

To address the question whether P-glycoprotein expression is of prognostic relevance for the survival of patients, a total number of 104 pediatric patients with untreated non-B-type acute lymphoblastic leukemia are analyzed using immunohistochemistry for the expression of P-glycoprotein in relationship to their therapy outcome. As response criteria, we used the relapse rate and the relapse-free interval. P-glycoprotein expression was detected in 36 out of 104 leukemia (35 %). We found a significant lower probability of remaining in first continuous complete remission (CCR) ($p = 0.05$) and a tendency for an increased relapse rate in patients with P-glycoprotein-positive blast cells (Fig. 1.16; [56]).

Since the pioneering work on the role of P-glycoprotein for multidrug resistance by us and others in the 1980s and 1990s, several hundred clinical papers appeared on the prognostic role of P-glycoprotein. It seems that the scientific fruits of the early years of P-glycoprotein fell on a fertile ground leading to flowering clinical research. It is beyond the scope of the present article to give a comprehensive overview of the prognostic role of P-glycoprotein vis-a-vis survival time of patients with all different tumor types. Nevertheless, we have focused on few tumor entities to highlight the clinical value of P-glycoprotein. For that purpose, we have chosen acute myeloic leukemia and acute lymphoblastic leukemia as hematopoietic tumors and breast cancer as example of a solid tumor.

As can be seen in Table 1.2, most but not all clinical investigations on P-glycoprotein expression in acute myeloid or acute lymphoblastic leukemia are significantly correlated with shorter survival times of patients. The prognostic significance of P-glycoprotein has also been reported in detail by Marie and Legrand [73]. While most studies used immunohistochemistry or flow cytometry, *MDR1*

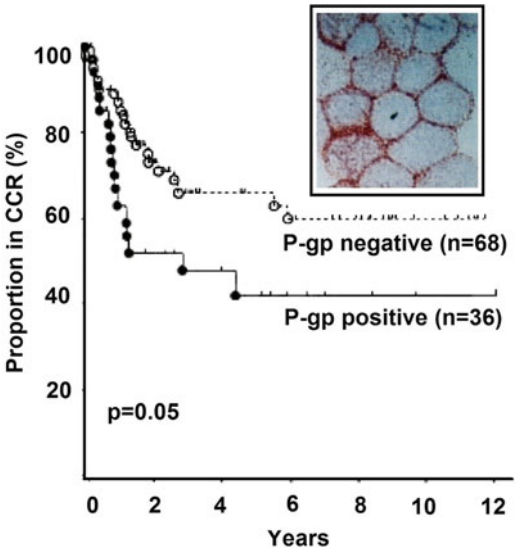

Fig. 1.16 Curves of relapse-free intervals in children with P-glycoprotein-positive versus -negative acute lymphoblastic leukemia (ALL) obtained by Kaplan-Meier statistics. Insert: P-glycoprotein immunostaining in ALL. Data taken from Sauerbrey et al. [56]

Table 1.2 Prognostic significance of P-glycoprotein (P-gp) and its encoding MDR1 (ABCB1) gene in acute myeloic leukemia (AML), acute lymphoblastic leukemia (ALL), and breast cancer

Disease	N	Method	Antibody	Result	Reference
Adult AML	150	Immunofluorescence	MRK16	Survival was shorter in P-gp positive patients	[69]
Adult AML	79	RT-PCR	N/A	*MDR1* expression was associated with shorter survival ($p=0.02$)	[75]
Adult AML	54	Immunohistochemistry	JSB1	Overall survival of Pgp-positive patients was significantly shorter ($p=0.004$)	[77]
Adult AML	52	Immunohistochemistry	C219 JSB-1	P-gp expression was associated with shorter survival ($p=0.007$)	[76]
Childhood AML	130	Flow cytometry	MRK16	No correlations of P-gp expression to event-free survival or overall survival	[74]
Adult AML	158	Flow cytometry	C219 JSB-1	Shorter survival times of P-gp positive patients ($p<0.001$)	[70]
Adult AML	230	RT-PCR flow cytometry	UIC2	Pgp/*MDR1* expression was a poor prognostic factor	[68]
Adult AML	121	Flow cytometry flow cytometry	MRK16, 4.E3, UIC2 rhodamine 123	Prognostic impact of P-gp expression on overall survival. Rhodamine 123 efflux-negative patients had worse survival prognosis ($p=0.0013$)	[72]
Childhood ALL	36	Immunohistochemistry	JSB1, C219	P-gp-positive patients had shorter survival rates ($p=0.05$)	[71]
Adult ALL	23	Immunohistochemistry	JSB1, C219	P-gp-positive patients had shorter survival rates ($p=0.03$)	[71]
Adult ALL	37	RT-PCR flow cytometry	UIC2	Pgp/*MDR1* expression was a poor prognostic factor	[68]
Childhood ALL	137	Immunohistochemistry		Pgp-positive patients had a higher relapse rate ($p=0.02$)	[66]

Childhood ALL	102	Flow cytometry flow cytometry	MRK16, 4.E3, UIC2 rhodamine 123	No correlation between P-gp expression and survival rates rhodamine 123-efflux-negative patients had worse but no significant survival prognosis.	[72]
Adult ALL	95	Flow cytometry		P-gp-positive patients had shorter disease-free survival duration ($p=0.01$)	[67]
Adult ALL	203	Flow cytometry	MRK16	Pgp expression predicted achievement of complete response after chemotherapy, but not survival time	[65]
Adult ALL	115	Flow cytometry	C219, JSB-1	C219-positive patients had shorter overall survival ($p=0.011$) and disease-free survival ($p=0.0006$) after 2 years	[64]
Breast Ca (locally advanced)	17	Immunohistochemistry	C494	Pgp- positivity was associated with shorter progression free survival ($p<0.02$)	[57]
Breast Ca	31	Immunohistochemistry	NCL-p-GLYp	Pgp overexpression associated with shorter survival ($p=0.0059$)	[58]
Breast Ca (primary and metastatic)	104	Immunohistochemistry	JSB1, C219, MRK16	Pgp expression was associated with shorter overall survival ($p<0.05$)	[59]
Breast Ca	94	Immunohistochemistry	UIC2	Correlation between Pgp expression and disease relapse (0.0322). Pgp positive patients had shorter disease-free period	[63]
Breast Ca	177	Immunohistochemistry	Clone 6/1C	No association between Pgp/MDR1 expression and relapse-free survival or overall survival	[61]
Breast Ca (invasive)	104	Immunohistochemistry	C219	High Pgp expression was associated with shorter survival ($p<0.0001$) and progression-free time ($p<0.0001$)	[62]
Breast Ca (locally advanced)	25	RT-PCR	N/A	High *MDR1* expression was associated with shorter disease-free survival ($p=0.0004$) and shorter overall survival ($p=0.0025$)	[60]

N/A not applicable

mRNA or P-glycoprotein efflux function have been addressed in only a few studies. These investigations confirm the prognostic value of P-glycoprotein found in protein expression analyses.

Most investigations on P-glycoprotein expression in breast cancer were also performed using immunohistochemistry. Similar to acute leukemia, P-glycoprotein expression in breast cancer was significantly associated with shorter progression-free survival and overall survival of patients in most clinical studies (Table 1.2).

1.9 Conclusion and Perspectives

Analysis of the drug resistance phenomenon revealed that P-glycoprotein is not only important to explain multidrug resistance of tumors in laboratory animals, but also in cancer patients. Hence, this efflux transporter represents a determinant leading to the failure of chemotherapy and ultimately to the death of many patients.

A consequence drawn from these research efforts during the past three decades may be the development of diagnostic tools to detect P-glycoprotein in tumors before chemotherapy to predict treatment success or failure. Indeed, there have been considerable efforts to develop methods making P-glycoprotein detection possible for routine diagnostics in clinical laboratories [78, 79]. It turned out that the standardization of P-glycoprotein detection assays for routine diagnostics seems not to be trivial, but nevertheless is a reachable task. For leukemia, flow cytometric assays based on double labeling with a fluorescent-labeled anti-P-glycoprotein antibody and a fluorescent P-glycoprotein substrate such as rhodamine 123 have been reported as reliable techniques to determine even low amounts of P-glycoprotein expression and activity [80, 81]. For the detection of P-glycoprotein in solid tumors, radiolabeled substrates of P-glycoprotein (e.g. sestamibi) represent an interesting approach [82–84].

The pretherapeutic determination of P-glycoprotein expression in human tumors may be a valuable tool for personalized cancer therapy. In the case of high P-glycoprotein expression, anticancer drugs involved in the MDR phenotype may be ceased and other non-cross-resistant drugs or treatment modalities (e.g. immunotherapy) may be used. Another well-known approach is the use of inhibitors, which specifically inhibit the efflux function of P-glycoprotein, thereby, re-sensitizing tumors to standard antitumor drugs. This concept will be discussed in more detail in Chap. 7 of this book.

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Chapter 2
Clinical Relevance of Multidrug-Resistance-Proteins (MRPs) for Anticancer Drug Resistance and Prognosis

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Abstract Chemoresistance in cancer is frequently associated with elevated levels of multidrug transporters. While P-glycoprotein is the best known, the majority of multidrug transporters belong to the Multidrug Resistance Protein (MRP) family, also known as the ABCC family, which includes MRP1–9. These proteins are typically found in the plasma membrane of cells, where they efflux a broad range of both physiological substrates and xenobiotics, including anticancer drugs. Consistent with the removal of chemotherapeutics from the cancer cell, high expression of several MRPs has been linked to poorer outcome in a variety of cancer types, suggesting these proteins represent targets for therapy. In this review we will describe the range of reported substrates for MRP1–9 in vitro, discuss associations between the MRP family and patient outcome, and investigate the evidence that MRP family members contribute directly to drug resistance based on in vivo models and patient data. We will also discuss the value of MRP expression as a prognostic marker and its potential role in selecting treatment protocols and examine existing and novel strategies to target MRPs.

Keywords Cancer • MRP • ABCC • Multidrug resistance • Chemotherapy

Abbreviations

ABC	ATP binding cassette
ABCC	ATP binding cassette sub-family C
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia

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BCRP/ABCG2	Breast cancer resistance protein
CSC	Cancer stem-like cell
CLL	Chronic lymphoblastic leukaemia
ESCC	Esophageal squamous cell carcinoma
EFS	Event-free survival
ESFT	Ewing's sarcoma family of tumours
GSH	Glutathione
HER2	Human epidermal growth factor receptor 2
MDR	Multi-drug resistance
miRNA	MicroRNA
MRP	Multidrug resistance protein
NSCLC	Non-small cell lung cancer
OS	Overall survival
P-gp	P-glycoprotein
RNAi	RNA interference
SCLC	Small cell lung cancer
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNPs	Single nucleotide polymorphisms

2.1 Introduction

The net uptake of drugs into cells is regulated by both import and export mechanisms, with the latter commonly mediated by ABC transporters. Not surprisingly, elevated levels of ABC transporters are frequently observed in cancer cells compared to their normal counterparts and the role of ABC transporters in chemoresistance has been an area of major research interest more than three decades. P-glycoprotein (P-gp, MDR1, ABCB1) stands out for conferring the most extensive resistance to the broadest range of compounds [152] and has received by far the most attention, followed by the Breast Cancer Resistance Protein (BCRP, ABCG2). However, at least a dozen other family members have been demonstrated to efflux chemotherapeutics and mediate chemoresistance in vitro, the majority of which belong to the ABCC subfamily, commonly referred to as the Multidrug Resistance Protein (MRP) family. For the majority of MRP family members, the ability to confer drug resistance in vitro is well established, and a large number of reports describe associations between their expression in tumours and patient outcome. To date, however, compelling evidence for a clinically relevant role in multidrug resistance is very limited. This chapter will examine the ability of MRPs to efflux chemotherapeutics in vitro, the available evidence for their importance in clinical drug resistance and mediating therapeutic responses, their potential prognostic value, and possible approaches to more clearly define their roles.

2.2 The ABCC/MRP Subfamily

The ABCC (or MRP) subfamily of ABC transporters consists of 12 members, 9 of which function as energy-dependent transporters, including MRP1–6 (ABCC1–6) and MRP7–9 (ABCC10–12). The remaining members of the ABCC subfamily, CFTR (ABCC7), SUR1 (ABCC8) and SUR2 (ABCC9), are either ion channels or ion channel regulators. The MRPs are often further subdivided into the short MRPs (MRP4, MRP5, MRP8, MRP9), which contain two membrane-spanning domains and two nucleotide binding domains, and the long MRPs (MRP1, MRP2, MRP3, MRP6, MRP7), which contain an additional *N*-terminal membrane-spanning domain. The long MRPs are mostly closely related to MRP1, with 46, 56 and 45 % identity for MRP2, MRP3 and MRP6, respectively, although the identity of MRP7 to long and short MRPs is comparable [35, 142]. The short MRPs are also generally more closely related, with MRP5 and MRP8 exhibiting 42 and 46 % identity with MRP9, while MRP4 is less closely related (31 %) [142]. For more detailed discussion of the structure and evolutionary relationships between family members, the reader is referred to previous comprehensive reviews [33, 35, 142]. Of the MRPs, all except MRP9 [118] have been shown to efflux chemotherapeutics and, therefore, have the potential to contribute to drug resistance in cancer. Drugs established as MRP substrates are listed in Table 2.1. As with P-gp and ABCG2, transporters of the MRP family are also present in a range of pharmacological barriers including in the gastrointestinal tract, the blood–brain barrier and the proximal tubules of the kidney where they contribute to the vectorial transport of their substrates [153]. Consequently, they are key contributors to the absorption, distribution and elimination of cancer drugs. Table 2.2 lists the localisation of MRP family members in different pharmacological barriers and cell types.

2.3 Specificity of MRPs for Chemotherapeutic Agents

A variety of compounds with differing mechanisms of action have been identified as MRP substrates (Table 2.1), ranging from traditional DNA damaging cytotoxics (doxorubicin) to antifolates (methotrexate) and microtubule damaging agents (vincristine). In contrast to P-gp, which effluxes unmodified neutral and positively charged hydrophobic drugs, members of the MRP family also efflux a range organic anions and drugs modified by Phase II metabolism, including glutathione (GSH), glucuronide and sulphate conjugates, or (in the case of MRP1) efflux some drugs in a GSH-dependent manner. There is significant overlap in the substrate profiles of the family members, particularly within the long and short MRP subfamilies. Each of the long MRPs, for instance, effluxes etoposide (Table 2.1), while each of the short MRPs effluxes nucleoside analogues. Interestingly, MRP7, which has comparable sequence identity to both short and long MRPs, effluxes both these drug classes.

To date there are few examples of targeted agents as substrates of MRP family members. Sorafenib was shown to be an MRP2 substrate [141] while sunitinib has

Table 2.1 Anticancer substrates of MRP1–9

	Class	Drug	Experimental method	References
MRP1	Alkylating agents	Chlorambucil (GSH conjugate)	Stable transfectant	[5, 104]
		Melphalan (GSH conjugate)	Membrane vesicles	[5, 69]
	Anthracenedione	Mitoxantrone	Drug-resistant cell line	[14, 105, 143]
			Membrane vesicles	
			Stable transfectant	
	Anthracyclines	Daunorubicin (GSH conjugate)	Drug-resistant cell line	[14, 27, 128, 126]
			Knockout cell line	
			Stable transfectant	
		Doxorubicin (GSH conjugate)	Drug-resistant cell line	[14, 27, 52, 128, 126]
			Knockout cell line	
			Stable transfectant	
		Epirubicin	Stable transfectant	[27]
	Antifolates	Methotrexate	Membrane vesicles	[61]
			Stable transfectant	
		ZD1694	Stable transfectant	[61]
	Arsenic-based	Arsenate (conversion to arsenite)	Stable transfectant	[27, 93, 128]
			Knockout cell line	
		Arsenite (GSH conjugate)	Knockout cell line	[27, 93, 128]
			Stable transfectant	
	Epipodophyllotoxins	Etoposide (glucuronide conjugate; stimulated by GSH)	Drug-resistant cell line	[14, 27, 52, 128, 69, 133]
			Membrane vesicles	
			Knockout cell line	
			Stable transfectant	
		Teniposide	Knockout cell line	[128]
	Camptothecins	Irinotecan	Drug-selected cells	[23, 150]
		SN-38	Drug-selected cells	[23, 150]
			Membrane vesicles	
	Microtubule damaging	Colchicine	Drug-selected cells	[27, 75, 78]
			Stable transfectant	
	Taxane	Taxol	Stable transfectant	[27]
	Vinca alkaloids	Vinblastine	Drug-resistant cell line	[14, 27, 52]
			Stable transfectant	
		Vincristine (GSH co-transport)	Drug-resistant cell line	[14, 27, 52, 99, 128]
			Knockout cell line	
			Stable transfectant	

(continued)

Table 2.1 (continued)

	Class	Drug	Experimental method	References
MRP2	Anthracyclines	Doxorubicin	Stable transfectant	[31, 77]
		Epirubicin	Stable transfectant	[31]
	Antifolates	GW1843	Stable transfectant	[61]
		Methotrexate	Membrane vesicles	[4, 61]
			Stable transfectant	
		ZD1694	Stable transfectant	[61]
	Arsenic oxoanion	Arsenite	Stable transfectant	[37, 93]
	Camptothecins	Irinotecan	Animal studies	[22]
		SN-38	Animal studies	[22]
	Epipodophyllotoxins	Etoposide (stimulated by GSH)	Stable transfectant	[31]
	Platinum-containing	Cisplatin (GSH dependent)	Stable transfectant	[31, 77]
	Taxanes	Docetaxel	Stable transfectant	[67]
		Paclitaxel	Stable transfectant	[67]
	Vinca alkaloid	Vinblastine (stimulated by GSH)	Stable transfectant	[42, 158]
		Vincristine (stimulated by GSH)	Stable transfectant	[31, 77]
MRP3	Antifolates	Methotrexate	Stable transfectant	[81]
	Epipodophyllotoxins	Etoposide	Membrane vesicles	[81, 173]
			Stable transfectant	
		Teniposide	Stable transfectant	[81]
	Vinca alkaloid	Vincristine	Stable transfectant	[175]
MRP4	Alkylating agent	Cyclophosphamide	Stable transfectant	[156]
	Camptothecins	10-hydroxy-camptothecin	Stable transfectant	[156]
		Irinotecan	Stable transfectant	[114, 156]
		Rubitecan	Stable transfectant	[156]
		SN-38	Stable transfectant	[114, 156]
	Purine analogues	6-mercaptopurine, Azathioprine	Stable transfectant	[20]
		6-thioguanine	Stable transfectant	[20]
		Methotrexate	Stable transfectant	[89, 156]
MRP5	Anthracyclines	Doxorubicin	Stable transfectant	[125]
	Antifolates	Methotrexate	Membrane vesicles	[125, 165]
			Stable transfectant	
		Pemetrexed	Stable transfectant	[125]
	Platinum-based	Cisplatin	Stable transfectant	[125]
		Oxaliplatin	Stable transfectant	[125]
	Purine analogues	5-fluorouracil, 5′-deoxy-5′-fluoridine	Membrane vesicles	[125]
			Stable transfectant	
		Azathioprine, 6-mercaptopurine	Stable transfectant	[168]
		6-thioguanine	Stable transfectant	[125, 168]

(continued)

Table 2.1 (continued)

	Class	Drug	Experimental method	References
MRP6	Actinomycines	Actinomycin D	Stable transfectant	[9]
	Anthracyclines	Daunorubicin	Stable transfectant	[9]
		Doxorubicin	Stable transfectant	[9]
	Epipodophyllotoxins	Etoposide	Stable transfectant	[9]
		Teniposide	Stable transfectant	[9]
	Platinum-containing	Cisplatin	Stable transfectant	[9]
MRP7	Anthracyclines	Daunorubicin	Stable transfectant	[63]
	Camptothecins	SN-38	Stable transfectant	[63]
	Epipodophyllotoxins	Etoposide	Stable transfectant	[63]
	Epothilones	Epothilone B	Stable transfectant	[63]
	Platinum-containing	Cisplatin	Stable transfectant	[151]
	Pyrimidine analogues	Cytarabine	Animal studies	[62, 63]
			Stable transfectant	
		Gemcitabine	Stable transfectant	[63]
	Taxanes	Docetaxel	Stable transfectant	[62, 63]
		Paclitaxel	Stable transfectant	[62, 63]
	Vinca alkaloids	Vincristine	Stable transfectant	[62, 63]
		Vinblastine	Stable transfectant	[62]
		Vinorelbine	Drug selected cells	[13]
MRP8	Antifolates	Methotrexate	Stable transfectant	[21]
	Purine analogues	5-fluorouracil	Stable transfectant	[53]

Stable transfectant: cells transfected with vector containing specific MRP construct, resulting in specific overexpression of the MRP
Membrane vesicles: inside out membrane vesicles prepared from cell lines, with uptake of substrates into vesicles determined
Drug-resistant cell line: cell line selected for high transporter expression in vitro by continuous culture in the presence of a cytotoxic agent
Knockout cell line: cells derived from knockout mice lacking a particular transporter, or cells expressing an RNAi construct targeting a transporter
Animal studies: studies performed in whole animals

been identified as a weak substrate of MRP4 [66]. However, since a range of tyrosine kinase inhibitors has been identified as MRP inhibitors, including imatinib, nilotinib and cediranib (MRP1 inhibitors [38, 58, 155]) and lapatanib, erlotinib and masatinib (MRP7 inhibitors [72, 85]), it remains possible that some of these agents may be MRP substrates.

While the list of established substrates for MRP family members is quite extensive, it should be recognised that the majority of these were determined to be substrates using cell lines selected for high transporter expression in vitro by continuously culturing in the presence of a cytotoxic drug, cell lines overexpressing individual transporters or as "inside-out vesicles" derived from these cell lines [50]. These systems, while often well controlled, do not necessarily reflect typical endogenous transporter expression levels. Furthermore, transport of a substrate in vitro does not necessarily imply physiological relevance in vivo, with the latter dependent on both the affinity of the substrate for the transporter and the achievable concentration of the substrate

Table 2.2 Localisation of MRP family members relevant to drug disposition

	Pharmacological barrier/cell	Cellular localization	References
MRP1	Blood–brain barrier	Apical (luminal)	[149, 177]
	Blood–cerebrospinal fluid barrier	Basolateral	[127]
	Blood–testis barrier	Basolateral	[157]
	Placenta	Apical	[146]
	Bronchial epithelium	Basolateral	[137]
MRP2	Intestinal epithelium	Apical (luminal)	[48]
	Hepatocytes	Apical (canalicular)	[15, 122]
	Kidney proximal tubules	Apical (luminal)	[136]
MRP3	Intestinal epithelium	Basolateral	[138]
	Hepatocytes	Basolateral (sinusoidal)	[79]
MRP4	Hepatocytes	Basolateral (sinusoidal)	[129]
	Blood–brain barrier	Apical (luminal)	[90, 109, 177]
	Blood–cerebrospinal fluid barrier	Basolateral	[90]
	Kidney proximal tubules	Apical (luminal)	[159]
MRP5	Blood–brain barrier	Apical (luminal)	[109, 177]
MRP6	Hepatocytes	Basolateral (sinusoidal)	[102]
	Kidney proximal tubules	Basolateral	[51, 139]

in vivo. Furthermore, the transport of some substrates is dependent on or modulated by endogenous molecules, as exemplified by the glutathione dependence of both doxorubicin and vincristine transport by MRP1 [14, 27, 52, 128, 99] (Table 2.1). Validation of drugs as transporter substrates using MRP knockout mice has the potential to be informative, although care is required with the interpretation of these data as the affinity of some MRP substrates varies markedly between the mouse and human transporters. Human MRP1, for example, confers resistance to anthracyclines, whereas mouse MRP1 does not [147], while cGMP has a very low affinity for mouse MRP4 compared to human MRP4 [32]. On the other hand, Mrp1$^{-/-}$ mice show increased sensitivity to etoposide [100, 166], Mrp2$^{-/-}$ mice show elevated plasma methotrexate, Mrp4$^{-/-}$ mice have higher levels of plasma and cerebrospinal fluid topotecan [90] and Mrp7$^{-/-}$ mice are more sensitive to paclitaxel [64], these findings validated these drugs as physiologically relevant substrates of their respective transporters.

2.4 Association of MRPs With Cancer Survival

2.4.1 MRP1

MRP1 was the first of the family to be identified [26] and as a result, it has been the focus of the majority of clinical and in vitro studies. MRP1 expression has been reported in a variety of cancer types, including chronic lymphoblastic leukaemia (CLL) [111], non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [12], prostate cancer [160], gastric carcinoma [41], esophageal squamous cell

carcinoma (ESCC) [112], colorectal cancer [46], endometrial carcinoma [82], glioma [1], retinoblastoma [19], acute myeloid leukaemia (AML) [91], acute lymphoblastic leukaemia (ALL) [7], breast cancer [44], Ewing's Sarcoma Family of Tumours (ESFT) [131] and neuroblastoma [55, 113]. The presence of MRP1 in this broad range of adult and childhood cancer tissues is consistent with the hypothesis that MRP1 may have an important role in cancer biology. Supporting this, MRP1 expression has been associated with the time to a first event (event-free survival, EFS) and overall survival (OS). More specifically, MRP1 mRNA expression has been linked with poor survival rates in NSCLC [95, 96], neuroblastoma [55, 113], childhood and adult AML [134] and ALL [40, 124]. Similarly, protein expression of this ABC transporter has been linked with an adverse outcome in both adult breast cancer [44] and nasopharyngeal carcinoma [87] and childhood cancers (ESFT; [131]). In contrast, although expressed, MRP1 protein levels have been reported as showing no association with EFS in breast cancer [17], ovarian carcinomas [68] and ALL [148] or OS in AML [43].

Consistent with increased ABC transporter protein expression as a mechanism by which cells develop multidrug resistance, MRP1 mRNA and protein expression have been identified as increased in post-treatment and metastatic samples compared to normal and diagnosis tissues, respectively, in a variety of solid tumours [1, 19, 80]. Similarly, MRP1 expression has been linked with adverse tumour grade in primary epithelial ovarian carcinoma [3]. Taken together, these data suggest a role for MRP1 in the progression of cells from a normal to a malignant phenotype. However, despite this, very few studies have described a significant link between MRP1 expression and patient response to therapy [12, 65, 68, 119], although this likely reflects the complexity of the chemotherapeutic response in patients.

2.4.2 MRP2

Similar to studies investigating MRP1, both MRP2 protein and mRNA expression have been reported as predictive of a worse OS in oesophageal squamous cell carcinoma [172], gall bladder carcinoma [74], breast cancer [101], NSCLC [45], AML [145], and adult and child ALL [124]. An association with a shorter EFS in breast cancer [101] and both adult and child ALL [124] has also been reported. Furthermore, MRP2 also appears to be involved in the response of cells to therapy. For example, in oesophageal squamous cell carcinoma tumour samples, MRP2 mRNA and protein expressions were increased after treatment with MRP2 substrates [172].

2.4.3 MRP3

Unlike MRP1 and MRP2, the prognostic significance of MRP3 expression at the protein level is yet to be evaluated. However, increased mRNA expression levels have been linked to a poor OS and EFS in ALL [124, 145], AML [145] and

pancreatic carcinoma [80]. In contrast, high MRP3 mRNA has been correlated with better outcome in neuroblastoma [59].

2.4.4 MRP4

Although MRP4 expression in cancer has been described [124, 145], studies investigating the clinical relevance of MRP4 are limited and inconsistent. Although a correlation between high MRP4 expression and poor patient survival has been described in epithelial ovarian carcinoma [3] and neuroblastoma [114], low MRP4 protein expression in prostate cancer was linked to a worse EFS and prostate-specific Gleason score [103]. Interestingly, MRP4 expression has been shown to be significantly higher in colorectal and pancreatic carcinoma tumour specimens compared with normal tissue [60], and in polyps from *ApcMin*/+ mice compared with normal mucosa [60, 80], consistent with a role in tumour development. Furthermore, *Abcc4* deficiency reduces systemic exposure to oral Dasatinib [49], possibly through reducing oral absorption.

2.4.5 MRP5

A correlation between MRP5 mRNA expression and both overall and EFS has been described in adult and child ALL [124] and, consistent with a causative role in cancer development, mRNA is increased in pancreatic carcinoma compared to normal tissue [80] and in lung cancer patients exposed to platinum drugs [117]. However, no association between MRP5 mRNA levels and patient survival was described in AML [145], suggesting the role of MRP5 is not consistent across all cancer types.

2.4.6 MRP6

To our knowledge only one study has evaluated MRP6 expression in cancer tissues; MRP6 mRNA was expressed in 98 % of ALL patients and was predictive of EFS and OS in both adult and child ALL [124], suggesting a role in the biology of ALL. However, since MRP6 overexpression induced only low-level resistance to chemotherapeutics in vitro (less than threefold increase in IC_{50} [9]), its ability to contribute to chemoresistance in vivo would seem limited.

2.4.7 MRP7

Although MRP7 has been well characterised in vitro (Table 2.1; [62, 63]), the prognostic significance of this protein is yet to be investigated. However, incubation with MRP7 substrates increased expression of the transporter in NSCLC [13] and in

salivary gland adenocarcinoma [106] cell lines, and MRP7 expression was inversely correlated with paclitaxel sensitivity in 17 non-small cell lung cancer (NSCLC) cell lines [117], suggesting MRP7 is important for the response of cells to specific substrates in cancer.

2.4.8 MRP8

The prognostic significance of MRP8 has been examined exclusively in breast cancer and the data are conflicting. Although MRP8 has been associated with a poor prognosis in all subtypes except luminal A [171], mRNA and protein expression were decreased in cancer compared to normal breast tissue [144]. In addition, SNPs within MRP8 have also been linked with cancer risk in 270 Japanese patients [120] but this association was not observed in European cohorts [8, 86]. Therefore, it is likely that MRP8 is similar to MRP3 and MRP6, since despite an association with patient survival in some cases, there is no clear evidence that MRP8 has a specific role in cancer development or the response of cancer cells to therapy.

2.5 Validation of Multidrug Resistance Proteins as Therapeutic Targets

Despite the multitude of clinical correlations between the expression of MRP family members and outcome, evidence of a causative link between MRP expression and clinical multidrug resistance is largely absent. A number of factors contribute to the difficulty of establishing causality subsequent to validation as a therapeutic target. Firstly, drug resistance in the clinic is frequently multifactorial. In additional to enhanced drug efflux, which may be mediated by multiple transporters, alterations in the ability of cancer cells to take up drugs, and alterations affecting the ability of drugs to kill cells, including increased drug metabolism activity, increased DNA repair capacity, alterations to the cell cycle, and increased resistance to apoptosis, can all contribute to multidrug resistance. Secondly, many of the clinical correlations described above have been determined in the context of combination chemotherapy that may include both substrates and non-substrates for a given transporter. Thirdly, the cohorts examined frequently contain multiple cancer subtypes and/or consist of patients treated under differing chemotherapeutic protocols, and have been conducted retrospectively rather than prospectively, or have been conducted solely with diagnosis samples with no measure of MRP expression at relapse. Finally, few if any of the studies described above have been able to account for the possibility of tumour heterogeneity, which may limit the ability to detect MRP expression in the tumour cell subpopulations that allow relapse [148]. This consideration is also relevant for cancer stem-like cell (CSC) populations, which express a variety of ABC transporters [34, 73, 178, 135], that may allow them to remain viable after therapy, leading to re-population of the tumour and patient relapse.

In the absence of compelling clinical data, the knockout of MRPs in genetically modified mouse cancer models can provide opportunities to formally assess their role in drug resistance. Mice deficient in MRP1 [100, 167], MRP2 [24, 108, 161], MRP3 [10, 174], MRP4 [11, 90, 98], MRP5 [32], MRP6 [51, 76] and MRP7 [64] have all been previously described, however to date, only the MRP1 knockout mouse has been crossed to a genetic cancer mouse model. In these experiments, the TH-MYCN mouse, a clinically relevant model of the paediatric solid tumour neuroblastoma [164] was crossed with an Mrp1 knockout ($Mrp1^{-/-}$) mouse [18]. To examine tumour intrinsic effects, as opposed to pharmacokinetic effects, tumours were harvested from $Mrp1^{+/+}$ or $Mrp1^{-/-}$ mice and engrafted into secondary recipients. In tumours lacking Mrp1, the response to the MRP1 substrates vincristine and etoposide was significantly enhanced, with a two to threefold delay in tumour growth, while response to cisplatin and cyclophosphamide, neither of which are effluxed by MRP1, was unaffected by MRP1 status [18]. These data provide direct evidence that MRP1 mediates chemoresistance in vivo. Comparable approaches may be of value for determining the role of other MRPs in chemoresistance, although as mentioned in Sect. 2.3 above, the affinity of some MRP substrates varies markedly between the mouse and human transporters, limiting the ability to extrapolate to humans.

2.6 Can Knowledge of MRP Expression Improve Patient Outcome?

2.6.1 MRP Expression as a Marker of Patient Prognosis

While MRP expression in tumours is frequently associated with outcome, it is less clear whether their expression has additional prognostic value beyond standard predictors of outcome. In breast cancer and ovarian cancer, where histological subtypes are more clearly defined, MRP expression appears to correlate with a more aggressive phenotype. MRP1 was more frequently expressed in both high-risk resistant triple-negative (Human Epidermal Growth Factor Receptor 2 (HER2), oestrogen and progesterone receptor negative) breast cancer [171] and stage 1C endometrial carcinoma [82] than in other subtypes. Furthermore, expression of MRP1 predicted a worse OS in these groups [82, 171], consistent with the classical markers of adverse prognosis in these subtypes [30, 36]. Similarly, MRP8 was more common in HER2 enriched and luminal A subtypes, where it predicts an adverse outcome [171], consistent with the poor survival associated with these patient groups [57]. Furthermore, increased MRP2 mRNA expression has been linked with poor survival in oestrogen receptor negative patients [101] which is linked to an adverse survival compared to receptor positive patients [39]. Therefore, since increased MRP expression identifies a similar population to that of the current well-established markers of patient outcome in breast and ovarian cancer, a role for MRP expression in improving patient prognosis in this scenario is unclear. However, more generally, while associations between MRP expression and clinical outcome have been frequently described, only relatively few studies have addressed whether MRP

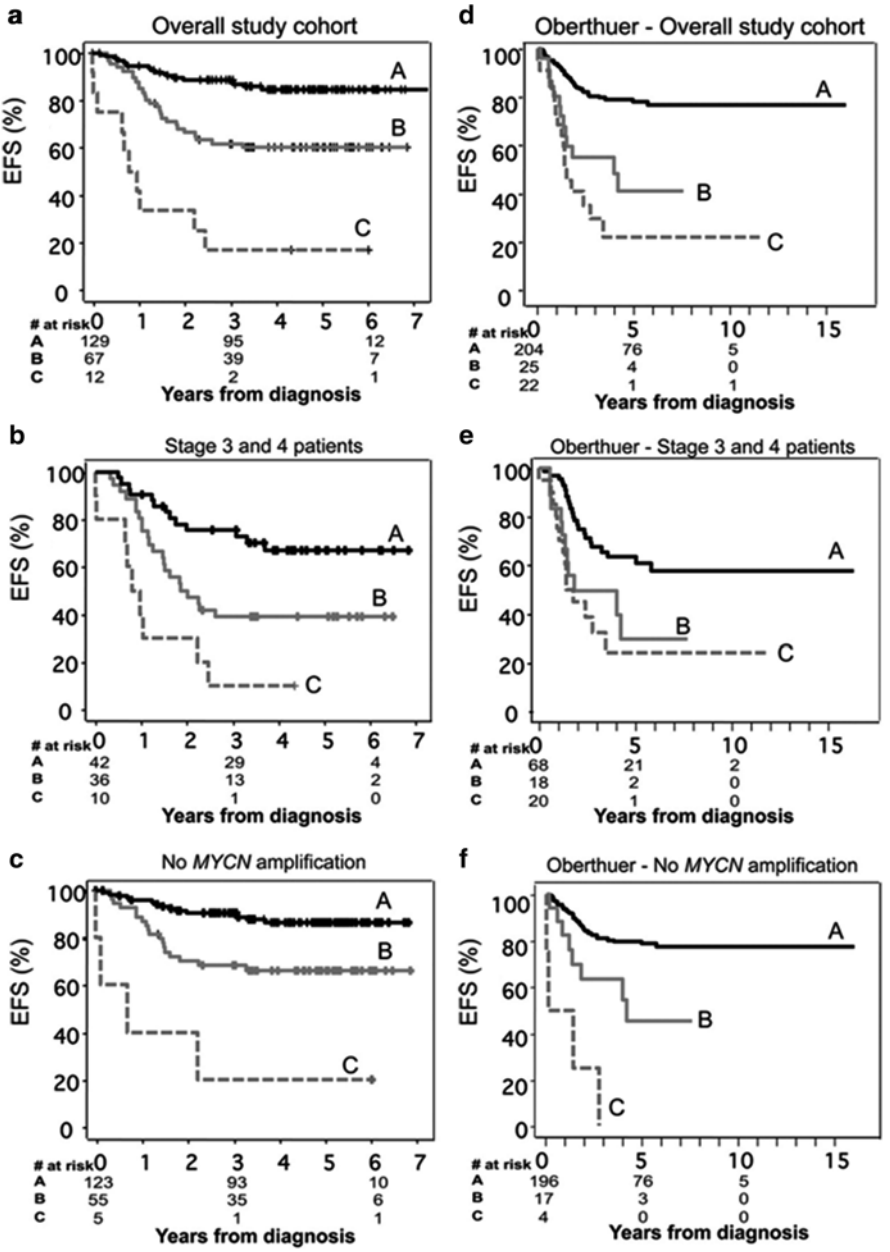

Fig. 2.1 Prognostic significance of ABCC gene expression in neuroblastoma. (**a**) Combined expression of the ABCC1, ABCC3 and ABCC4 genes and cumulative event-free survival (EFS) in 209 patients with neuroblastoma. Patients were categorized into eight clusters on the basis of their combined ABCC1, ABCC3, and ABCC4 expression pattern. Kaplan–Meier survival analysis of these clusters revealed three statistically distinct groupings (Groups A, B, and C), which were associated with the risk of relapse associated with individual ABCC gene expression. Group A

expression is informative independent of established predictors of outcome [3, 87, 95, 114, 134]. The prognostic value of the MRP family, therefore, remains to be firmly established in any cancer, at least at the level of a single gene. It is possible, however, that the combined expression of several MRPs may be of prognostic value. The combined expression of MRP1 (ABCC1), MRP3 (ABCC3), and MRP4 (ABCC4) stratified neuroblastoma patients into groups having excellent, intermediate, or poor outcome (Fig. 2.1), and is one of the most powerful independent prognostic markers yet identified for this disease [59].

2.6.2 MRP Expression as a Method to Stratify Patients for Therapy

If we assume that an MRP family member contributes to response to therapy, expression of that MRP might be exploited to select more effective therapeutic options. For instance, patients with high expression of MRP may be treated with a non-MRP substrate chemotherapeutic, thereby avoiding the resistance generated by efflux of the agent.

In addition to expression at the protein and mRNA levels, specific single nucleotide polymorphisms (SNPs) in genes of the MRP family (particularly MRP1) have also been associated with patient survival [2, 16, 154, 121] and response to therapy [154, 163], including alterations in drug plasma levels [2], response to exogenous and endogenous substrates [28, 29, 94, 84], and increased chemotherapy-induced side effects (cardiotoxicity) [84, 140, 169, 162]. Therefore, similar to the basic expression of MRPs in cancer, it would be advantageous to stratify patients with a SNP conferring a more aggressive phenotype, to more extensive therapy, and/or include those substrates unaffected by the expression of a specific SNP. However, as we have described, the response of cells to chemotherapy is dependent on multiple factors and so it is unlikely that the use of a non-MRP substrate in patient treatment would completely bypass the resistance of tumours to therapy.

Fig. 2.1 (continued) included only those patients whose tumours displayed low levels of ABCC1 and ABCC4 and high levels of ABCC3, reflecting "favourable" ABCC gene expression. Group B consisted of patients whose tumours displayed only one unfavourable risk factor with respect to the three ABCC genes analysed (i.e., ABCC1 high, ABCC4 high, or ABCC3 low), and Group C comprised patients whose tumours exhibited two or more unfavourable ABCC risk factors. Similar associations between combined ABCC gene expression and increasingly poor outcome were also observed in subgroups of patients (**b**) with stage 3 or 4 disease or (**c**) whose tumours lacked MYCN amplification. (**d**) Combined expression of the ABCC1, ABCC3, and ABCC4 genes and cumulative EFS in 251 neuroblastoma patient samples analysed by Oberthuer et al. [116]. Patients in the Oberthuer et al. cohort were categorized into eight groups as described for panel (**a**) above. These groupings were also strongly predictive of EFS in subgroups of patients (**e**) with unfavourable (stages 3 and 4) disease or (**f**) with non–MYCN-amplified disease. At 0, 3, and 6 years from diagnosis [Panels (**a**), (**b**), and (**c**)], or 0, 5, and 10 years from diagnosis [Panels (**d**), (**e**), and (**f**)], the number of patients at risk of relapse are shown. Figure reproduced from [59], with permission

2.7 MRPs as Targets for Therapy

2.7.1 Small Molecule Modulators

Assuming that MRP transporters contribute significantly to clinical multidrug resistance, it will be critical to establish whether MRPs can be successfully targeted to either reverse drug resistance, or at least allow a broader therapeutic window for drugs that are at or approaching their maximum tolerated dose. Therefore, a variety of strategies have been employed to abolish the resistance caused by MRPs. The most common of these is the use of small molecule inhibitors which, prevent binding or efflux of substrates, thus enhancing the efficacy of chemotherapeutics [152]. Although many small molecule inhibitors of MRPs have been evaluated in vitro and shown good inhibition of efflux activity and enhanced the effects of MRP substrates (Table 2.1), sulindac, an inhibitor of MRP1 ([115], clinicaltrials.gov) is the only modulator to progress through in vivo studies to clinical trials. Several hypotheses have been suggested as to why a lack of correlation exists between the responses observed using in vivo models and behaviour in clinical studies, including differences in pharmacokinetics of combination therapies in mice and humans, leading to unpredicted toxicities. Furthermore, the cellular heterogeneity that exists in patient tumours, in addition to differences in basal MRP expression and pharmacokinetic activity, cannot be modelled effectively using in vitro cell line and in vivo mouse models.

Inhibitors of P-glycoprotein (discussed in detail elsewhere in this volume) have been extensively examined in clinical studies and several P-gp inhibitors, also reported to inhibit MRP1, have been evaluated in Phase I clinical trials [123, 132]. However, only one MRP inhibitor, sulindac, has been examined in a Phase II clinical trial, given in combination with epirubicin in patients with advanced melanoma, where no unacceptable toxicity was observed (ClinicalTrials.gov). Despite the limited MRP inhibitor trials, extensive studies modulating P-gp have identified a range of challenges associated with ABC transporter protein inhibition. For instance, pharmacokinetic interactions between modulators and chemotherapeutics often limit the normal efflux, absorption and metabolism of therapeutics, leading to unacceptable plasma levels. Therefore, it is essential to appreciate the normal physiological role of these proteins when developing inhibitors.

An ideal modulator would have high specificity and potency, good bioavailability, absence of toxicity in combination with chemotherapeutics and be screened/designed specifically for MRP inhibition. As with early generation P-gp inhibitors, MRP inhibitors have typically been drugs previously identified for other purposes. Sulindac, for example, is a non-steroidal anti-inflammatory drug [115], while MK-571 was developed as a cysteinyl leukotriene receptor antagonist [92]. Modulators optimised specifically for MRP inhibition would be expected to be more effective. In addition to non-specific modulators, the only clinical trial evaluating sulindac enrolled advanced cancer patients, after failure of initial chemotherapy protocols [115]. As a result, tumours in this cohort may have acquired multiple alternative

mechanisms of resistance, which may also contribute to clinical outcome. Ideally, clinical studies with more stringent criteria and objectives should be employed, including evaluating modulators in ABC transporter expressing cancer types at diagnosis, using MRP substrate chemotherapeutics, effective monitoring of patient side effects/toxicity and effect on MDR phenotype [152].

2.7.2 Monoclonal Antibodies

In addition to the traditional inhibition of MRP efflux activity by small molecule modulators, several strategies to prevent MRP activity are currently under investigation. Targeting general cancer cell-specific mechanisms using monoclonal antibodies has been a successful strategy in a variety of leukaemias and solid tumours [88]. However, although a large number of MRP antibodies are currently available and have shown activity in vitro, an antibody targeting an MRP is yet to be evaluated in vivo. In the case of MRP1, in addition to ineffective inhibition, the lack of progression to in vivo studies has also been linked with the intracellular binding of currently available antibodies and subsequent inability to generate antibodies to the extracellular domains [25].

2.7.3 RNA Interference

Although RNA interference (RNAi) targeting MRPs has proved valuable in vitro, there are limited studies investigating the use of RNAi in vivo. Significant alterations in tumour mass or substrate accumulation after treatment with substrates and MRP1 siRNA [170] or MRP2 shRNA by adenovirus [107] in vivo have been described, suggesting this is a plausible strategy for MRP inhibition. In addition, several microRNAs have been associated with expression of MRPs, particularly MRP1 in vitro [54, 83, 97]. However, as a single miRNA has multiple mRNA targets [6], it is likely that the addition of an MRP inhibitory miRNA to a cell will induce effects on additional mRNA species and pathways. Therefore, to confirm inhibition of MRP by a particular RNA species as a viable strategy, more extensive validation in vivo is required.

It must be remembered that MRPs are also expressed on the surface of non-cancer cells and so targeting the malignant cell specifically by RNAi represents a challenge. However, recent studies have suggested the use of bio-nanocapsules, which display a molecule capable of recognising a tumour cell-specific target, such as HER2 in breast and ovarian cancer. Consequently, capsules deliver RNAi only to abnormal cells, inducing target protein knockdown [110].

2.8 Are There Roles for MRPs in Cancer Progression Other Than in Drug Resistance?

Although the focus of most studies has been the role of MRPs in drug resistance and the subsequent effects on cancer patient prognosis, there is some suggestion that MRPs may influence patient outcomes by alternative mechanisms [47]. The efflux of endogenous substrates, such as glutathione and sulphur conjugates [56, 176], leukotriene C_4 [92], bilirubicin, bile salts [70], 17-beta-D-glucuronide, cAMP and cGMP [20, 71] by MRPs is well described and the ability of cells to successfully maintain cellular homeostasis in conditions of stress, such as chemotherapy insult, may also represent a targetable mechanism. Furthermore, MRPs have also been linked with normal [130] and cancer [59] cell migration in vitro and also tumour formation in a transgenic mouse model of neuroblastoma [59], suggesting a possible secondary role for these proteins in cancer development in addition to traditional efflux activity.

2.9 Summary

In this chapter we have described the wide range of known anticancer substrates of MRPs, and the correlations between expression and patient outcome in a variety of cancer types. However, whether these associations will improve the prediction of patient prognosis above existing markers is yet to be fully examined. Furthermore, there is some difficultly in confirming a causative role for MRPs in cancer, since although some substrates induce expression of MRPs in tissues, multiple factors contribute to drug resistance. In addition, clinical correlative studies routinely involve patients who have been treated with differing chemotherapeutic protocols, both between and within a cancer type. A better understanding of the role of MRPs in patient outcome may lead to expression of MRPs being usefully employed to stratify patients with increased expression for more intensive therapy or treatment with a non-MRP substrate. However, only one MRP inhibitor has been evaluated in clinical trials, although monoclonal antibodies and RNAi techniques are currently showing promise as novel strategies for MRP inhibition. Therefore, until these multidrug transporter proteins are shown to have clear causative roles in drug resistance, we must question whether MRP inhibition will be a viable therapy in combination with traditional therapeutics.

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Chapter 3
Role of Breast Cancer Resistance Protein (BCRP, ABCG2) in Cancer Outcomes and Drug Resistance

Karthika Natarajan, Maria R. Baer, and Douglas D. Ross

Abstract The breast cancer resistance protein (BCRP), formally known as ATP-binding cassette protein G2 (ABCG2), is an efflux transporter that plays a significant role in altering absorption, distribution, metabolism, and excretion (ADME) of most extant and emerging molecular cancer therapeutics. BCRP expressed by neoplastic cells may also contribute to the resistance of these cells to chemotherapeutic agents. Although the expression of BCRP in human cancers has often correlated with adverse outcomes, to date therapeutic strategies utilizing the inhibition of BCRP function to improve the ADME of cancer chemotherapeutics or to sensitize cancer cells that express BCRP to chemotherapy have not been fruitful. This review will examine the most current literature probing BCRP's role in ADME of cancer therapeutic agents and drug resistance.

Keywords ATP binding cassette transporters • ABCG2 • BCRP • MDR • Cancer chemotherapy • ADME • Gene polymorphisms

Abbreviations

ABCG2	ATP-binding cassette protein G2
ADME	Absorption, distribution, metabolism, and elimination
BCRP	Breast cancer resistance protein
MDR	Multidrug resistance

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3.1 Introduction

The breast cancer resistance protein, (BCRP), formally known as ATP-binding cassette protein G2 (ABCG2), is an efflux transporter that plays a significant role in altering absorption, distribution, metabolism, and excretion (ADME) of most extant and emerging molecular cancer therapeutic agents. BCRP expressed on neoplastic cells may also contribute to the resistance of these cells to chemotherapeutic agents. BCRP was cloned for the first time in our laboratories at the University of Maryland from multidrug resistant human breast cancer cells [1]. Although the expression of BCRP in human cancers has often correlated with adverse outcomes, to date therapeutic strategies utilizing the inhibition of BCRP function to improve the ADME of cancer chemotherapeutics or to sensitize cancer cells that express BCRP to chemotherapy have not been fruitful. This review will examine the most current literature probing BCRP's role in cancer ADME and drug resistance.

This chapter will be a sequel to two previous reviews [2, 3], the more recent published in January of 2012. Except for background information on BCRP, the literature review for the present paper will be from July 2011 to August 15, 2013. The present chapter will reprise the basic structure of the most recent review published in Biochemical Pharmacology in 2012 [3]. Prior to August 15, 2013, there were 2860 articles listed in PubMed concerning BCRP or ABCG2. In the interval from July 1, 2011 to Aug 15, 2013, PubMed identified 919 articles relating to BCRP or ABCG2.

3.2 Background Review: BCRP Functional Dynamics in Normal Tissues

The past two decades have seen an accumulation of information on BCRP structure, function, expression, and regulation, thereby providing novel features/targets for altering BCRP expression and function.

3.2.1 BCRP Structure and Function

BCRP, a half-transporter, is synthesized as a monomer and subsequently dimerizes and translocates to the plasma membrane to function as a drug efflux pump for its substrates. The BCRP monomer has six transmembrane domains (TMDs) and a single nucleotide binding domain (NBD) (Refs. 15–17 in our most recent review [3]). It is now established that the amino acid at position 482 (R/G/T) in TMD3 is essential for BCRP substrate binding and specificity [4, 5]. Recently, specific mutagenesis of proline residues on BCRP demonstrated that the proline residue at position 485, but not at 480, in TMD3 is also essential for substrate specificity, while the

proline residue at 392 was essential for transport activity of BCRP in general [6]. In addition to the substrate binding site around R482, a sterol binding site (SBE) encompassing amino acids 555–558 (LXXL) exists in the cytoplasmic loop between TMD4 and TMD5 in BCRP [7]. The data on the role of BCRP in sterol transport are contradictory [8–11], but cholesterol was found to be essential for optimal functioning of BCRP in vitro [12]. Another study confirmed sterol dependence of BCRP function, with sterols such as estradiol, testosterone, progesterone, and androstenedione inhibiting BCRP transport noncompetitively [13], suggesting that these hormones bound to an allosteric site, possibly the SBE. Mutation analysis of L558 in the SBE indeed rescued BCRP from cholesterol dependence, confirming the role of cholesterol as a regulator of BCRP function. Until recently functional BCRP was thought to localize primarily to the plasma membrane, but recent studies have shown that BCRP can also localize to the mitochondria [14, 15] as well as to the nucleus [16]. The function of nuclear-localized BCRP is yet unclear, but BCRP localized to the mitochondria regulates heme metabolism by modulating protoporphyrin IX (PPIX) transport and accumulation in the mitochondria [14, 15]. BCRP, a half-transporter, in addition to forming functional homodimers or oligomers, could possibly also heterodimerize or heteromultimerize. Indeed several interacting partners of BCRP have been reported, including CD147 (extracellular matrix metalloproteinase inducer) [17], F-actin, Eps8 (epidermal growth factor receptor pathway substrate 8) and rp3 (actin-related protein 3) [18, 19].

3.2.2 BCRP Expression

Studies have reported BCRP expression in the side population (SP) cells of every organ and tumor studied so far. BCRP determines the symmetric cell division and the G1-S transition of the cell cycle in cardiac side population progenitor cells [20]. Besides the more primitive side population cells, BCRP is also expressed in the well-differentiated surface epithelial cells of all organs involved in drug transport, as well as in fully mature erythrocytes. Since different organs and specific tissues within an organ express BCRP, to enhance specificity and decrease toxicity, it is critical to target and inhibit BCRP only in the specific tumor/cancer tissue or pharmacokinetically relevant organ. To achieve this goal, it is necessary to characterize BCRP expression and understand its function in various organs. Recent studies have focused on characterizing BCRP expression within specific cell types of a particular organ. BCRP was found to be expressed in the cortex of the adrenal gland and the plasma membrane of adipocytes, while its expression in the pituitary, pancreas, ovary, and testis was limited to capillaries [13]. However, during spermatogenesis in mouse, rat, and bull testis, BCRP expression in the spermatogonia and the acrosome of the spermatozoa has also been reported [21]. A more comprehensive characterization of BCRP expression in the rat testis during spermatogenesis revealed that the apical ectoplasmic specialization (ES), which is the spermatid–Sertoli cell interface, expresses BCRP selectively in stages VI–VIII. BCRP formed a complex with

F-actin, Eps8, and rp3 at the apical ES in Sertoli cells. The basal ES forms the blood–testis barrier (BTB) but BCRP was not associated with any of the molecules of the basal ES, suggesting that BCRP might not regulate drug transport across BTB [18, 19]. BCRP expression and complex formation with F-actin, Eps8, and rp3 were essential for spermatid polarity as well as properly timed release of mature spermatids from the seminiferous epithelium. This is the first report of a role of BCRP other than its efflux transport activity. Irrespective of the stage of the seminiferous epithelial cycle, myoid peritubular cells, and capillaries in the testis expressed BCRP, the function of which is yet unclear.

While BCRP expression and function in the BTB is now questionable based on the above study, the blood–brain barrier (BBB) expresses BCRP [22]. Besides the BBB, a blood–cerebrospinal fluid (CSF) barrier also exists in the brain. In a recent study in mice, BCRP was expressed in the arachnoid cells that form part of the blood–CSF barrier. The arachnoid cells express BCRP on both their basal and apical surfaces, the dura- and CSF-facing membranes respectively, indicating that BCRP is also a component of the blood–CSF barrier. Drug transporters on the arachnoid barrier cells contribute to the blood–CSF barrier [23].

Limited availability of human tissues restricts human BCRP expression studies. Since our last review on BCRP, only two studies have analyzed BCRP expression in human tissues. One study analyzed regional differences in BCRP expression in the human placenta, revealing uniform BCRP expression in different regions of the human placenta [24]. The other study demonstrated BCRP expression in the human retina, both in the nerve fibers and in the retinal pigment epithelium (RPE) [25]. The function of BCRP in the RPE is yet unclear, but the RPE is the active site of photoreceptor phagocytosis as well as melanin synthesis. Further studies are required to elucidate the role of BCRP in the RPE.

3.2.3 BCRP Regulation

Methylation status, alternative promoters, microRNAs (miRs), dimerization/multimerization, and degradation pathways regulate BCRP expression and function. Any of these mechanisms or a combination can be therapeutically targeted to alter BCRP expression and function.

3.2.3.1 Pretranscriptional and Transcriptional Regulation

Pretranscriptionally, BCRP promoter methylation can be enhanced and hence its expression decreased by treatment with melatonin [26]. We and others have reported tissue-specific alternative promoter usage in normal tissues of both humans and mice [27–29]. We also showed that alternative promoter usage varied between normal human tissues and BCRP-overexpressing resistant cancer cell lines, suggesting that cancer cell-specific alternative promoters and transcription factors can be targeted to overcome BCRP-mediated resistance [27]. For example, non-Down syndrome-associated pediatric acute megakaryoblastic leukemia [French-American-British (FAB)

subtype M7] is associated with E1U isoform-specific high BCRP mRNA expression with functional activity for the promoter upstream of the E1U 5′ UTRs, while all other subtypes of pediatric AML and normal tissues expressed the E1C mRNA isoform [30]. Since pediatric patients with M7-AML have poor treatment outcomes, targeting the E1U promoter or E1U mRNA isoform might help overcome drug resistance specifically in FAB M7 AML with minimal side effects [30]. However, therapeutic targeting of tissue-/cancer-specific BCRP isoforms requires proof of concept data. We and others have indeed shown modulation of specific BCRP mRNA isoform expression by treatment with specific agonists/antagonists of BCRP alternative promoter-specific transcription factors. We have shown specific binding and activation of a BCRP alternative promoter upstream of the novel mouse testis-specific E1u isoform by the steroidogenic factor-1 (SF-1) [31]. Also, with two Aryl hydrocarbon Response Elements (AhRE) in the genomic region between the 5′ UTRs of human BCRP A and B/C transcript variants, the Aryl hydrocarbon Receptor (AhR) agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) activated expression of the B/C variant, but not the A variant [32]. Two separate groups have reported interaction of AhR with the AhRE in the BCRP promoter [33, 34].

Chromatin immunoprecipitation (ChIP) assays have confirmed direct interaction of several transcription factors with their respective transcription factor binding elements on the BCRP E1B/C promoter [3]. Recently, in SP cells isolated from the lung cancer cell line A549, Sp1 and Sp3 transcription factors interacted with Sp1 cis-elements on the BCRP promoter and regulated BCRP expression [35].

Correlation studies to identify specific upregulated or downregulated genes during development of chemoresistance or an aggressive phenotype have identified numerous transcriptional BCRP modulators. The transcription factors regulating BCRP expression other than those reported in our previous reviews are described. Estrogen receptor alpha (ERα) regulates BCRP transcription by binding to the estrogen response element (ERE) [36] in the BCRP promoter, but the role of estrogen receptor beta (ERβ), which also binds to estrogen response elements on its target genes, is unclear [37]. In human ERα-/Progesterone receptor (PR)-human breast cancer samples ERβ expression correlated positively with BCRP expression. Further analysis showed that ERβ upregulated BCRP expression in ERα-/PR- breast cancer cells by interacting directly with the ERE [36, 37] in the BCRP promoter, suggesting a potential role of ERβ in regulating BCRP expression in cells devoid of ERα. Prolactin, essential for normal mammary development, induced both BCRP mRNA and protein by activation of the Janus kinase/signal transducer and activator of transcription 5 (JAK/STAT5) pathway in breast cancer cells. The activated STAT5 interacted with the gamma-interferon activation sequence (GAS element) in the BCRP promoter, but required binding of coregulators activated by the mitogen-activated protein kinase (MAPK) and phosphoinositol 3-kinase (PI3K) pathways in order to induce BCRP transcription [38].

A positive correlation between the E2F transcription factor 1 (E2F1) and BCRP expression has been demonstrated in human lung cancers. Microarray analysis of E2F1-expressing human osteosarcoma (Saos-2) cells revealed BCRP as the main downstream target of E2F1 (a G1-S phase transition regulator) [39]. E2F1 binds to the E2F1 binding site on the BCRP promoter, upregulating its expression [39]. Interestingly, in

progenitor cardiac side population cells from BCRP knockout mice, the G1-S transition was defective and the SP cells underwent asymmetric cell division [20], raising the possibility that BCRP is a downstream effector of E2F1 for G1-S cell transition.

Cigarette smoke is a predisposing factor for esophageal squamous cell carcinoma (ESCC), while ABCG2 expression is a negative prognostic factor. Cigarette smoke contains AhR agonists and AhR interaction with its xenobiotic response element on the BCRP promoter induces BCRP expression and chemoresistance in cisplatin-resistant ESCC [40]. Furthermore, cigarette smoke condensate (CSC) was found to enhance resistance to chemotherapy in lung cancer cells by upregulating both phospho-Akt (p-Akt) and BCRP, suggesting CSC's- posttranscriptional regulation of BCRP [41]. However, in another study, exposure to CSC altered BCRP mRNA transcription as well [42]. Direct binding of Sp1, AhR, and nuclear factor erythroid 2-like 2 (NRF2) transcription factors to their respective elements on the ABCG2 promoter mediated this transcriptional increase in BCRP expression. Mithramycin, a DNA binding transcriptional inhibitor, decreased the effect of CSC on transcription factor binding to the ABCG2 promoter [42].

The constitutive androstane receptor (CAR) interacts with a direct repeat motif separated by five nucleotides (DR5) on the BCRP promoter to increase BCRP mRNA expression in normal hepatocytes [43]. The CAR agonist 6-(4-chlorophenyl) imidazo[2,1-b][1, 3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl)oxime (CITCO) increases BCRP expression in the BBB via the CAR receptor [44]. Peroxisome proliferator-activated receptor (PPAR)-α directly interacts with the peroxisome proliferator response element (PPRE) on the BCRP promoter in a cerebral microvascular endothelial system. Treatment with PPARα ligands and pharmacological inhibitors, respectively, increased and decreased BCRP mRNA/protein expression and accumulation of BCRP substrates [45, 46]. Changes to CAR levels would possibly alter the pharmacokinetics of BCRP substrate chemotherapeutics.

The circadian rhythm of BCRP expression was altered by binding of ATF4 to the cyclic AMP response element (CRE) in the exon 1B promoter in mouse cell lines [47]. We had previously shown binding and activation of the CRE by phospho-cAMP response element-binding protein (p-CREB), with the CRE unique to the exon 1B promoter [28].

Based on the above pharmacological transcriptional activators and repressors of BCRP expression, as proposed by To et al., antagonists or compounds that decrease the stability of positive transcription factors that upregulate BCRP or agonists of the transcription factors that downregulate BCRP would help overcome BCRP-mediated chemoresistance [40].

3.2.3.2 Post-transcriptional Regulation

Binding of IMP3, an insulin-like growth factor II mRNA binding protein, stabilizes BCRP and increases its expression in ER-/PR-/human epidermal growth factor receptor 2 (HER2)- triple negative breast cancers. Since IMP3 expression is absent in normal breast cells, it might be a novel target to overcome BCRP-mediated drug resistance in triple negative breast cancer cells [48].

BCRP mRNA stability is decreased by binding of mir-487a [49], mir-142-3p [50]), mir-181a [51], mir-519c [52], mir-520h [53], mir-199a [54], mir-328 [55, 56], and mir-212 [56] to the BCRP 3′ UTR. mir-487a, mir-142-3p, mir181a, mir-519c, mir-199a, mir-520h, mir-328, mir-212, and mir-9 expression levels negatively correlate with ABCG2 expression. Moreover, in a study of gliomas, mir-21 and mir-7 expression correlated positively with glioma grade IV and mir-222 with grade I–II, possibly correlating with expression of BCRP, as well as other genes [57].

3.2.3.3 BCRP Dimerization/Multimerization

Once translated, BCRP monomers undergo glycosylation [58], dimerize/multimerize [59–61], and translocate to the cell surface where BCRP functions as a drug efflux pump. BCRP phosphorylation at T362 by the serine/threonine kinase Pim-1 is essential for its dimerization and surface translocation [62]. Pim-1 inhibition with SGI-1776 or Pim-1 knockdown with shRNA overcomes BCRP-mediated chemoresistance by decreasing BCRP phosphorylation and cell surface expression [63]. Phospho-Akt, essential for surface translocation of BCRP, is also a target for modulation of BCRP function [64, 65]. Pathways that positively and negatively regulate phosphorylation of Akt indirectly regulate BCRP expression. Knockdown of epidermal growth factor receptor (EGFR) inactivates the PI3K/AKT pathway, decreasing BCRP expression [66]. EGFR inhibitors including gefitinib and PD158780 decrease BCRP expression and drug resistance in breast cancer cells [67].

There is a negative correlation between phosphatase and tensin homolog (PTEN) expression and p-Akt expression. Downregulation of PTEN increases BCRP expression in adult acute leukemia, while PPARγ agonists that increase PTEN levels decrease p-AKT and hence decrease surface localization and function of BCRP [68]. The abstract of an article in a Chinese journal reported that metformin, an antidiabetic drug, inhibited BCRP expression in a hepatocellular carcinoma cell line by upregulating PTEN and downregulating p-Akt [69].

In acute lymphoblastic leukemia (ALL) cell lines the MAPK/extracellular signal-regulated kinase (ERK) pathway downregulates BCRP expression, while activation of the JNK pathway increases BCRP expression [70]. Activation of JNK1/c-jun also upregulates BCRP in human colon cancer cells [71]. The PI3K/Akt and β-catenin/TCF pathways are involved in c-kit-mediated activation of BCRP transcription [72].

In addition to identifying the promoter elements, the interacting transcription factors and the signal transduction pathways involved in regulating BCRP expression and function, it is essential to delineate the mechanism underlying induction of BCRP overexpression by standard chemotherapeutic agents. Doxorubicin treatment-induced BCRP expression negatively correlates with expression of Hu antigen R (HUR), protein kinase C delta (PKC-delta) and DNA topoisomerase 2-alpha [73]. Rescue of either HUR or PKC-delta restores the chemosensitivity of doxorubicin-treated cells, suggesting that BCRP might function in concert with other proteins to cause resistance to doxorubicin [73]. BCRP expression in rat placental trophoblast cells was induced by upregulation of ERα levels by mitoxantrone treatment [74]. As described earlier, ERα directly interacts with the ERE on the BCRP promoter,

regulating its expression [36]. Camptothecin upregulated BCRP, multidrug resistance-associated protein 2 (MRP2) and B-cell lymphoma 2 (Bcl-2) by enhancing phosphorylation of ataxia telangiectasia mutated (ATM), which in turn induced phosphorylation of NF-kappa-B inhibitor alpha (IkBα) and nuclear factor NF-kappa-B p65 subunit (p65(A)), as well as translocation of p65 in the (Nuclear Factor-kappaB (NF-kB) pathway [75].

Inhibitors of BCRP recently reported in the literature are shown in Table 3.1.

Table 3.1 Inhibitors of BCRP recently reported in the literature

Inhibitor	BCRP substrate	Effect on BCRP	BCRP substrate modulated	Reference
MBL187	Not known	Protein inhibition	Irinotean	[188]
Elacridar	Yes	Protein inhibition	ABT-888 Crizotinib Sunitinib Vemurafenib	[189] [190] [191] [192]
Genistein	Yes	Decreased protein expression	5-fluorouracil, cisplatin	[193]
Symmetric bis-chalcones 1p	No	Protein inhibition	Mitoxantrone	[194]
Chromone 1	No	Protein inhibition		[195]
Telatinib	Not known	Protein inhibition	Mitoxantrone, doxorubicin	[196]
Low molecular weight heparin	Not known	Decreased protein expression	Cisplatin	[78]
Quinazoline compound 20	Not known	Protein inhibition		[197]
Cannabidiol	Not known	Protein inhibition	Mitoxantrone	[198]
Falcarinol type dietary polyacetylenes	Not known	Protein inhibition	Mitoxantrone, methotrexate	[199]
YHO-13351	Not known	Protein inhibition	Irinotecan	[200, 201]
Emodin	Not known	Decreased protein expression	Doxorubicin	[202]
Terrien	Not known	Decreased surface expression		[203]
3,5,3′,4′-tetramethoxy trans-stilbene	No	Protein inhibition	Mitoxantrone	[204]
Methoxylated aurones	No	Protein inhibition	Mitoxantrone	[205]
Purvalanol A	No	Protein inhibition	Mitoxantrone	[206, 207]
Olomoucine II	Yes	Protein inhibition	Mitoxantrone	[206, 207]
Poloxamines	Not known	Decreased protein expression, protein inhibition	Rhodamine 123	[208, 209]
Oncostatin M	Not known	Decreased mRNA and protein expression		[210]
Gefitinib	Yes	Decreased mRNA and protein expression	5-aminolevu linic acid	[211]

3.2.3.4 BCRP Degradation

Mature, properly folded, plasma membrane-localized BCRP is degraded in the lysosome, while misfolded BCRP, the Q141K mutant form, is degraded in the proteasome [3]. Enhancing BCRP degradation might serve as a mechanism of decreasing BCRP function. Sorafenib [76], a tyrosine kinase inhibitor (TKI), and human cathelicidin cationic peptide [77], an endogenously expressed natural host defense peptide, were indeed found to overcome BCRP-mediated resistance by increasing its lysosomal degradation. Interestingly, low molecular weight heparin (LMWH) was found to enhance proteasomal degradation of BCRP in cisplatin-resistant lung cancer cells [78]. Recently calpain-dependent proteolytic cleavage and degradation of BCRP not mediated by lysosomal or proteasomal processes was reported when the BCRP-overexpressing the human colon carcinoma cell line S1-M1-80 was treated with secalonic acid D, a mycotoxin [79].

3.3 Impact of Non-neoplastic Expression of BCRP on Cancer Treatment Outcomes

3.3.1 BCRP Polymorphisms

The major functional single nucleotide polymorphism (SNP) reported for BCRP is the 421C>A SNP, translating into the Q141K BCRP variant [80]. Meta-analysis studies of literature revealed a significant association between the 421C>A SNP and a decrease in susceptibility to cancer [81]. Mechanistic studies showed that the fully processed Q141K mutant is retained in aggresomes where misfolded proteins aggregate [82], but other studies suggest that it has mild processing and folding defects as well as reduced activity [83]. Treatment of Q141K-expressing cells with colchicine or histone deacetylase inhibitors could restore membrane localization and function of the aberrant BCRP Q141K variant protein [82]. Another group is of the opinion that the Q141K mutation is in the NBD, resulting in decreased stability of the protein [84]. However, all studies agree that the Q141K mutant BCRP has reduced activity compared to the nonmutant. A recent study also showed that the 421C>A SNP is associated with lower expression of BCRP in the human liver [85].

The role of the Q141K mutation in determining molecular response has been widely studied. One study suggested absence of correlation between imatinib therapy response and the Q141K mutation in chronic phase CML patients, but a correlation between high BCRP mRNA levels and decreased rate of achievement of major molecular response (MMR), as well as poor response to subsequent second-generation tyrosine kinase inhibitor therapy [86]. The 421C>A genotype was associated with a good molecular response to imatinib treatment, whereas the BCRP diplotype G34A421C>A correlated well with good response of patients to imatinib therapy [87]. After 6 months on imatinib therapy at 400 mg/day dose, the

MMR was associated with the 421C>A mutation [88]. Another study confirmed that the 421C>A polymorphism was an independent positive predictor of complete molecular response to imatinib therapy in chronic phase CML patients [89]. In addition, the BCRP haplotype (G–G, rs12505410, and rs2725252) was positively associated with cumulative incidence of MMR in patients treated with 400 mg per day imatinib dose but this association was overcome by increasing the dose to 600 mg per day [90], suggesting that a higher dose of imatinib could overcome the influence of this haplotype. These studies suggest better outcome for CML patients with the Q141K mutant treated with imatinib.

In adult acute myeloid leukemia (AML) patients treated with idarubicin-based chemotherapy, the Q141K mutant was associated paradoxically with poor prognosis. A positive correlation existed only between better disease-free survival and low wild type BCRP expression [91].

Besides 421C>A, the 34GA/AA genotype (rs2231137) has also been associated with altered prognosis. 34GG was associated with poor prognosis in Chinese patients with acute leukemia undergoing treatment including bone marrow transplantation (BMT) [92]. The G34A (V12M) mutation is more frequent in a Spanish population when compared to the hapmap data, and better response to immunosuppressants as well as higher toxicity profiles were suggested in these patients [93].

A lower risk for developing B-cell Non-Hodgkin's lymphoma (B-NHL) was associated with the presence of the BCRP rs6857600 SNP minor allele [94], and a lower risk for developing CLL was associated with the BCRP rs2231142 variant.

The influence of BCRP SNPs has also been studied in both colorectal cancer and non-small cell lung cancer (NSCLC). In colorectal cancer patients receiving oxaliplatin therapy, the BCRP wild type rs3114018 A/A was associated with a higher incidence of peripheral neuropathy [95]. The rs3114018 SNP has also been shown to be associated with the heritable dental caries phenotype $DMSFS_5$ and $DMFS_{5mand}$ [96]. Hence, investigation of the association of the rs3114018 SNP in BCRP with occurrence of dental caries with oxaliplatin therapy is warranted. For patients with metastatic colorectal cancer treated with folic acid, 5-fluorouracil and irinotecan (FOLFIRI), the BCRP (−15622C>T) mutation and the rs7699188 mutation were associated with lower and higher tumor response rates [97], respectively. In patients with metastatic colorectal cancer undergoing treatment with folic acid, 5-fluorouracil and oxaliplatin (FOLFOX), low BCRP expression was associated with a better response [98]; however, in another study the BCRP 421C>A mutation was associated with a good response rate [99].

In non-small cell lung cancer (NSCLC) patients receiving gefitinib therapy, the 34G>A mutation was associated with occurrence of grade 2 or worse skin rash [100]. In an overall comparison of first-line platinum-based chemotherapy to taxane-platinum and gemcitabine-platinum treatment groups in unresectable NSCLC patients, there was an association of overall survival (OS) of the overall and taxane-platinum group with BCRP htSNP rs2725264 and of the gemcitabine-platinum group with rs4148149 [101]. The systemic exposure and enhanced CSF penetration of erlotinib and OSI-420 was higher in NSCLC patients harboring the 421C>A mutation, with more frequent adverse effects as well [102].

In metastatic renal cell carcinoma (RCC) patients, the heterozygous BCRP 421C>A allele was associated with higher systemic exposure of sunitinib, while presence of the homozygous BCRP 421C>A was associated with sunitinib-related toxicity [103]. In ovarian cancer patients receiving platinum-taxane-based therapy, the 421C>A variant allele was associated with longer progression-free survival (PFS) [104]. In breast cancer patients receiving docetaxel therapy, the BCRP rs2231142 C/C mutant allele was associated with the development of neutropenic fever [105].

3.3.2 BCRP Effects on Absorption, Distribution, Metabolism, and Elimination (ADME) of Antineoplastic Drugs

BCRP limits brain penetration of sorafenib [106], erlotinib [107] and methotrexate [108], and penetration is further inhibited by P-glycoprotein (Pgp, ABCB1), as shown in BCRP- and (Pgp-double knockout mice. BCRP knockout by itself increased systemic exposure of all these compounds significantly. However, for dantrolene [109] and CYT387 [110], BCRP knockout significantly increased brain exposure but not systemic exposure. In addition, the presence of homozygous C421 or A421 significantly altered brain penetration of the tyrosine kinase inhibitors, which are substrates and modulators of both Pgp and BCRP [111].

Urate excretion is both renal and extra-renal (intestinal). In the 5/6 nephrectomy rat model of chronic kidney disease, BCRP expression in the ileum increased to compensate for the loss of BCRP expression and efflux of uric acid in the kidney [112]. Hence blocking ABCG2 activity at one organ/tissue site might increase its expression and activity at another site as a compensatory mechanism, and compensatory changes in expression should be considered when studying tissue-specific inhibitors of BCRP expression and function.

3.3.3 BCRP Novel-Drug Delivery Mechanisms

BCRP-mediated chemoresistance can be overcome by formulating cancer chemotherapeutics in dosage forms that override BCRP-mediated drug resistance, such as pegylated topotecan [113] or nanostructured lipid-dextran sulfate hybrid carriers for delivering mitoxantrone [114], or targeting BCRP with anti-BCRP antibody in conjunction with paclitaxel nanoparticles for treating multiple myeloma [115]. The bioavailability of elacridar, the combined inhibitor of BCRP and Pgp, the two major drug efflux transporters in the BBB, is improved when it was administered as a microemulsion [116]. Alternatively, novel stable formulations of BCRP siRNA can be prepared to target and knock down BCRP mRNA expression [117, 118].

3.4 BCRP Expression in Human Cancers: Roles in Drug Resistance and Prognosis

3.4.1 BCRP Expression and Hematologic Malignancies

3.4.1.1 Brief Summary of Findings and Controversies as of the Last Paper

Our group last summarized the literature on BCRP in hematologic malignancies in our 2012 publication [3]. BCRP is frequently expressed on malignant hematopoietic and lymphoid cells, and, importantly, on stem cells in these malignancies. Moreover, some of the chemotherapeutic agents used to treat hematologic malignancies are substrates of BCRP. Of additional note, tyrosine kinase inhibitors (TKIs) are being incorporated into treatment of several hematologic malignancies, and an evolving literature focuses on interactions between tyrosine kinase inhibitors and BCRP. Finally, an evolving literature associates BCRP SNPs with both incidence of hematologic malignancies and treatment response.

The most recent literature, summarized here, has included novel insights into regulation of BCRP in hematologic malignancies. There is also additional recent work on interactions of BCRP with TKIs used in the treatment of hematologic malignancies and on the significance of BCRP SNPs.

3.4.1.2 Regulation of ABCG2 in Hematologic Malignancies

Recent literature has focused on novel mechanisms of regulation of BCRP in hematologic malignancies.

Sorrentino's group at St. Jude Children's Research Hospital identified a new tissue-specific BCRP promoter selectively expressed in pediatric acute megakaryoblastic leukemia (AML FAB-M7) [30]. Analyzing BCRP transcript isoforms from 85 pediatric AML samples, they found uniformly higher levels of BCRP transcripts in samples from children with AML FAB-M7 not associated with Down syndrome, compared to other AML subtypes. AML FAB-M7 samples contained a novel 5′ UTR identified 90 kb upstream of the exon 2 translation, and an associated upstream promoter fragment was selectively expressed in these leukemia samples. Finally, these pediatric AML FAB-M7 samples had a relatively high incidence of BCRP mRNA expression, which might contribute to the relatively poor prognosis of children with this AML subtype.

A variety of signal transduction pathways have been implicated in regulation of BCRP expression in recent publications. Activation of the MAPK/ERK pathway was found to downregulate BCRP expression but upregulate Pgp expression in the T-acute lymphoblastic leukemia (ALL) cell line CCRF-HSB-2 and the B-ALL cell line

YAMN90, while activation of the JNK pathway upregulated BCRP gene expression [70]. Inactivation of PTEN protein was found to upregulate BCRP via activation of the PI3K/Akt pathway in acute leukemia [119] and chronic myelogenous leukemia (CML) [120] cells. c-Myc was also found to upregulate BCRP as well as other ABC proteins in CD34+ CML cells, and c-Myc regulation in CD34+ CML cells is enabled by hypomethylation of the c-Myc binding site in the BCRP promoter [121]. Additionally, an activated hedgehog (Hh) signaling pathway was found to upregulate BCRP transcription in diffuse large B-cell lymphoma via binding of GLI transcription factors to a single binding site in the BCRP promoter, and Hh was shown to be upregulated by paracrine signaling from stromal cells [122]. Finally, in primary effusion lymphoma, caused by Kaposi's sarcoma-associated herpesviruses and characterized by chemoresistance, BCRP was found to colocalize with the protein emmprin (CD147) which modulates leukocyte transmigration, and with the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) on the cell surface, and to be upregulated by emmprin [123].

3.4.1.3 Interactions with Kinase Inhibitors

Diverse TKIs are playing increasing roles in the management of hematologic malignancies, and interactions with ABC proteins including BCRP have been a focus of recent studies and were the subject of several recent review articles [124, 125]. The BCR-ABL TKIs imatinib, nilotinib, and dasatinib interact with BCRP, as well as Pgp, with interactions that are concentration-dependent, so it is important to perform in vitro studies with TKIs at pharmacologically relevant concentrations [126]. Both imatinib and nilotinib are potent inhibitors of BCRP, and may also be BCRP substrates, while dasatinib is a substrate of BCRP, but likely does not inhibit it at clinically relevant concentrations [126]. Statins were found to increase intracellular levels of imatinib via inhibition of Pgp and BCRP transport function, without an effect on their expression [127]. ABC transporters also decrease TKI penetration of the BBB, with brain accumulation of dasatinib mainly restricted by Pgp, that of the multikinase inhibitor sorafenib mainly by BCRP, and that of the multikinase inhibitor sunitinib equally by Pgp and BCRP [111]. Moreover both Pgp and BCRP restrict brain accumulation of the active sunitinib metabolite *N*-desethyl sunitinib [128]. Both sorafenib and sunitinib can be used as fms-like tyrosine kinase 3 (FLT3) inhibitors in AML with FLT3 mutations. In addition, the FLT3 inhibitor tandutinib inhibits BCRP function, and may thus be active in AML stem cells [129]. Finally, quizartinib or AC220, the most potent and specific FLT3 inhibitor tested to date, inhibits BCRP at pharmacologically relevant concentrations, and may thus sensitize cells to chemotherapy, but also increases intestinal absorption of orally administered BCRP substrate drugs, including some that may potentiate the QT prolongation that is the dose-limiting toxicity of quizartinib [130].

Other novel kinase inhibitors under study in hematologic malignancies also interact with BCRP, including BI 2536, a potent and selective inhibitor of Polo-like kinase 1 [131] and the aurora-B kinase inhibitor barasertib-hQPA [132], which are substrates.

3.4.1.4 BCRP Polymorphisms

In a study investigating the frequency and clinical significance of BCRP SNPs in malignant blasts from 184 Chinese patients with de novo acute leukemia, a novel SNP at exon 16 (13561218 C/T) and five known SNPs at exon 2 (13608835 G/A), exon 5 (13600044 C/A), intron 10 (13576005 C/T), intron 13 (13564503 C/T), and intron 14 (13563578 A/G) occurred with frequencies of 1.1 %, 64.1 %, 30.4 %, 21.2 %, 39.7 %, and 28.8 %, respectively, and the 34GG genotype was associated with longer disease-free survival (DFS) ($P<0.001$) and overall survival (OS) ($P<0.001$) than the 34GA/AA genotypes [92]. A subsequent study evaluated the frequency of the Q141K variant (421C>A polymorphism, exon 5), associated with decreased BCRP protein expression, in a cohort of Caucasian AML patients, finding the Q141K polymorphism in 29 of 163 patients (18 %), with no association with attainment of complete remission, but longer disease-free survival in patients with low BCRP expression and wild-type gene, compared to those with Q141K or high BCRP expression [91].

Several recent studies examined the importance of BCRP SNPs in CML therapy. In one study of 82 patients with CML who had been treated with 400 mg imatinib mesylate daily for over 6 months, the CC, CA, and AA genotypes in BCRP 421C>A did not correlate with differences in imatinib trough levels, but were associated with significantly different frequencies in MMR, with the AA genotype associated with a significantly higher frequency of MMR than the CC and CA genotypes [88]. In another study, the frequency distribution of ABCG2 421 CC, CA, and AA genotypes was significantly different between imatinib good response and resistant groups ($P=0.01$), with the AA diplotype significantly correlated with good response and MMR [87]. Thus genotyping of this SNP may be useful in predicting imatinib response in CML patients. Based on in vitro data, this SNP is also relevant for dasatinib, and nilotinib [133].

BCRP polymorphisms were also implicated in risk for B-cell malignancies, possibly via altered exposure to environmental toxins. In a recent study, carriers of the BCRP rs2231142 variant (exon 5, Q141K, as above) were found to have a decreased risk of chronic lymphocytic leukemia (CLL) ($P=0.0004$), while carriers of the BCRP SNP rs6857600 minor allele (intron 1) had a decreased risk of B-cell lymphoma (B-NHL) ($P<0.001$) [94].

Hyperuricemia is a frequent complication of hematologic malignancies. An accumulating literature has demonstrated a role of BCRP polymorphisms in modifying renal uric acid excretion. Two nonsynonymous BCRP SNPs, 421C>A (major) (discussed above, resulting in Q141K) and 376C>T (minor), result in impaired transport activity, owing to ubiquitination-mediated proteasome degradation and truncation of BCRP, respectively; both are associated with hyperuricemia and gout [134].

3.4.2 BCRP Expression and Solid Tumors (Summarized in Table 3.2)

3.4.2.1 Brief Summary of Findings, Controversies Since the Previous Paper

Our most recent review [2] described a growing number of reports that BCRP is expressed in a wide variety of human cancers in sub-populations of cells that possess stem cell properties, particularly those that are able to exclude Hoechst 33342 dye, termed side-population (SP) cells. SP cells generally display multidrug resistance manifested by multiple mechanisms of resistance acting in concert, including diminished programmed cell death, expression of drug resistance transporters, upregulated cellular repair mechanisms and anti-oxidant defenses, and commitment to self-renewal. Such reports have continued to be published since our last review: in many of these papers the focus is on the characterization of the cancer stem cell, with BCRP expression reported in the context of other genes that are commonly expressed in SP or stem cell populations. Papers that focus on documentation of neoplastic cells with stem cell properties that do not describe the specific functionality or relationship of BCRP to resistance or aggressiveness have not been included in the present review.

3.4.2.2 Gastrointestinal Cancers

A number of recent studies investigated regulation of BCRP expression in colorectal cancers. Inhibition of the JNK1 signaling pathway reversed the expression of BCRP in cultured human colon cancer cells (SW1116) that were made resistant to hydroxycamptothecin [71]. miR expression profiling studies found that miR-328 was a regulator of the SP in colorectal cancer cell lines, with lower expression of miR-328 in SP cells; furthermore, it was found that miR-328 directly regulates the expression of BCRP [135]. Expression of the novel gene schlafen-3 (Slfn-3) has been shown to correlate with intestinal epithelial cell differentiation [136]. Transfection of human colorectal HCT-116 cells resistant to 5-fluorouracil and oxaliplatin with Slfn-3 reduced mRNA levels of cancer stem cell markers, including BCRP [137].

Some studies looked at the influence of BCRP on colon carcinogenesis. Butyrate, an HDAC-inhibiting short chain fatty acid important for normal colonic epithelial homeostasis, was found to be a substrate for BCRP, but not for Pgp or MRP. It was proposed that increased expression of BCRP may play a carcinogenetic role in colon cancer by reducing intracellular levels of butyrate and hence thwarting butyrate's differentiating and reactive oxygen species (ROS) protective effects [138]. In contrast, other investigators found downregulation of BCRP expression in 29 colonic adenomas from 21 patients and eight adenomas from four ApcMin mice (which develop spontaneous intestinal adenomas) compared to normal adjacent colonic epithelial cells [139]. Another group found that mice given the dietary

Table 3.2 Impact of BCRP/ABCG2 expression on drug resistance and outcomes in selected clinical translational studies of human solid tumors

Lead author, year, citation	Disease	Number of patients studied	Parameter, methodology	Findings/conclusions
Hang, 2012 [141]	Esophageal squamous cell carcinoma	166	BCRP expression, Immunohistochemistry	BCRP expression was an independent adverse prognostic factor
Lee, 2012 [144]	Pancreatic ductal adenocarcinoma	67	BCRP expression, Immunohistochemistry	BCRP expression predicted early relapse and poorer survival
Vizio, 2012 [145]	Pancreatic ductal adenocarcinoma	37	BCRP expression, Immunohistochemistry	BCRP expression did not correlate with tumor grade or disease stage
Zeng, 2011 [146]	Pancreatic cancer	211	Genotyping of cases in PanScan database	Improved survival of patients who harbored the inactivating rs2231164 allele of BCRP
Larbcharoensub, 2011 [150]	Intrahepatic cholangiocarcinoma	60	BCRP expression, Immunohistochemistry	BCRP expression was associated with a favorable prognosis
Litviakov, 2013 [151]	Breast cancer, before and after neoadjuvant therapy	84, clinical stage IIA-IIIC	qPCR	BCRP and other ABC transporter mRNA transcript levels increased in stable or progressive disease cases
Britton, 2012 [152]	Breast cancer, chemotherapy naïve	49—fine needle aspirations; 25 had concomitant biopsies	Hoechst 33342 dye accumulation; 25 also had immunohistochemistry for BCRP	SP size was proportional to BCRP expression by IHC; SP and BCRP expression were associated with ER-negative and "triple negative" breast cancers
Wu, 2012 [153]	Breast cancer, invasive ductal	95	Immunohistochemistry	Expression of BCRP was inversely correlated with progesterone receptor and estrogen receptor α
Xiang, 2011 [157]	Breast cancer, invasive ductal	196	Immunohistochemistry	BCRP expression was found to correlate with expression of HER2, lymph node metastasis, and clinical stage
Xiang, 2011 [160]	Breast cancer, invasive ductal	196	Immunohistochemistry	Hypoxia-inducible factor 2a expression correlated with BCRP and Ki62 expression, and with histologic grade

Rijavec, 2011 [162]	Lung cancer nodal metastases, previously untreated	14 SCLC 7 NSCLC	qPCR	BCRP mRNA was significantly decreased in metastatic cells from SCLC compared to NSCLC patients
Liu, 2012 [164]	NSCLC, metastatic	242	Genotyping	The BCRP 421C>A inactivating polymorphism did not predict the effectiveness of erlotinib in a clinical trial
Gottschling, 2013 [165]	NSCLC, resected early stage	80 cases with neuroendocrine differentiation	Immunohistochemistry	BCRP expression was detected in 30% of cases, but BCRP, CD117 and CD133 expression were not of prognostic significance
Herpel, 2011 [166]	NSCLC, completely resected stage I/II	133	Immunohistochemistry	Expression of cancer stem cell antigens, including BCRP were not reflective of prognosis
Merk, 2011 [167]	NSCLC	Xenografts derived from 24 NSCLC patients	qPCR and immunoblotting of xenografts	Xenograft chemosensitivity to etoposide, carboplatin, gemcitabine, paclitaxel, cetuximab, or erlotinib generally did not correlate with the expression of multidrug resistance markers
Li, 2011 [168]	NSCLC, Stage I	144	Immunohistochemistry	Co-expression of CD133 and BCRP was predictive of early postoperative recurrence
Shen, 2011 [170]	Laryngeal squamous cell carcinoma	98	Immunohistochemistry	Expression of P-glycoprotein or BCRP were independent adverse prognostic factors
Feng, 2012 [171]	Oral erythroplakia	34	Immunohistochemistry	Co-expression of podoplanin and BCRP was highly predictive of developing oral cancers
Liu, 2012 [172]	Oral leukoplakia	135	Immunohistochemistry	Expression of BCRP and BMI-1 (a stem cell marker) was associated with a high risk of developing oral cancer
Cheng, 2012 [54]	Ovarian cancer	Cancer initiating cells isolated from tumor from nine patients	qPCR, Northern, Western blotting	Downregulation of CD44 by miR-199a sensitized the ovarian cancer stem cells to cisplatin, paclitaxel, and doxorubicin, and r educed BCRP expression
Furman, 2012 [185]	Neuroblastoma, high risk newly diagnosed	23 children enrolled	Clinical trial	Clinical trial of gefitinib (a tyrosine kinase and BCRP inhibitor) with irinotecan (a BCRP substrate) showed little useful clinical activity

carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-β] (PhIP, a BCRP substrate) accumulated higher levels of PhIP and PhIP-DNA adducts in colonic adenomas than in normal colonic epithelium, suggesting that the loss of BCRP expression could play a carcinogenetic role by leading to carcinogen accumulation and adenoma formation.

In three gastric cancer cell lines, expression levels of BCRP, Pgp, and CD133 measured by immunohistochemistry were found to correlate with the grade of malignancy; these findings were corroborated in a study of biopsy specimens from 90 patients with gastric cancer: poorly differentiated cancers expressed more BCRP, Pgp, and CD133 than did well differentiated ones [140]. In esophageal cancer, the prognostic value of the expression of CD133 and BCRP, putative "stem cell" markers, was evaluated by immunohistochemistry in paraffin-embedded biopsy specimens from 110 patients with esophageal squamous cell carcinoma [141]. BCRP expression was found to be an independent adverse prognostic factor, using multivariate analysis. CD133 expression predicted the degree of tumor differentiation, but did not correlate with survival. In another study, esophageal carcinoma cell lines with acquired resistance to cisplatin displayed constitutive activation of the aryl hydrocarbon receptor, AhR, which bound to a xenobiotic response element in the BCRP promoter, upregulating BCRP and causing drug resistance [40].

In pancreatic ductal adenocarcinoma—a highly drug-resistant neoplasm—samples of tumor from 32 patients had upregulation of transcript levels of seven ABC transporters including BCRP, compared to normal pancreatic tissue [142]. Other investigators examining inhibition of hedgehog signaling in pancreatic cancer stem cells (isolated from PANC-1 tumorspheres) observed that hedgehog signaling inhibition decreased self-renewal and expression of BCRP [143]. A study of BCRP expression in 67 surgically resected pancreatic ductal adenocarcinomas revealed that BCRP expression was a predictor of early relapse and poor survival [144]. Expression of BCRP in biopsy specimens from 37 patients with pancreatic ductal adenocarcinomas did not correlate with tumor grade or disease stage [145]. Germline inactivating mutations of BCRP (rs2231164) were associated with improved survival in patients with pancreatic cancer [146]. The homeobox gene MSX2 was found to regulate BCRP expression in pancreatic cancer cell lines via the SP1 binding elements in the BCRP promoter [147].

In hepatocellular carcinoma cell lines, isocorydine, an investigational anticancer drug, diminished the SP and BCRP expression and sensitized these cells to doxorubicin [148]. In primary tumor specimens from patients with cholangiocarcinoma, and in cholangiocarcinoma cell lines, the transcriptional coactivator "amplified in breast cancer 1" (AIB1) was found to be frequently upregulated, compared to normal bile duct tissue [149]. AIB1 upregulation was found to activate the Akt and Nrf2 pathways, resulting in an increase in BCRP mRNA and functional expression.

Intrahepatic cholangiocarcinoma (ICC) is a tumor derived from hepatic cholangiocytes. Bile ducts are known to exhibit high expression of ABC transporters, including BCRP. A recent study in Thailand evaluated the expression of Pgp, MRP1 and BCRP in tumor specimens from 60 patients with ICC tumors, using

immunohistochemical techniques [150]. Paradoxically, low expression of BCRP in these tumors was correlated with worse prognosis and higher tumor grade. The authors suggest that BCRP plays a role in cholangiocarcinogenesis.

3.4.2.3 Breast Cancer

Investigators in Siberia used qPCR to examine transcript levels of genes associated with multidrug resistance (ABCB1, ABCC1, ABCC2, ABCC3, ABCC5, ABCG1, ABCG2, GSTP1, and major vault protein) in 84 clinical stage IIA to IIIC breast cancers, before and following neoadjuvant chemotherapy with 5-FU-, doxorubicin- or taxane-containing regimens [151]. Nine patients achieved a complete remission, and hence paired comparisons were not possible; in the remaining patients with tumor available pre- and post-neoadjuvant therapy, an increase in the transcript levels of all of these genes was observed following neoadjuvant chemotherapy in patients who showed progression or stabilization of disease, whereas reductions in mRNA levels were seen in patients who showed a partial response to therapy.

Some controversy exists regarding the influence of HER2 on BCRP expression and side population stem cells. As will be discussed, many investigators report that HER2 can regulate the SP and promote BCRP expression; moreover, many studies find that stem cells identified as the SP are associated with breast cancer cells with a "luminal" phenotype, and not a "basal" phenotype. However, one recent study which analyzed fine needle aspirations of breast tumors for presence of SP cells found that the size of the SP correlated with BCRP expression that was measured in paraffin-embedded tissue from the same patients, and that the SP presence in the fine needle aspirations was associated with estrogen receptor-negativity, and with "triple negative" breast cancers [152]. Other investigators examined the expression of BCRP, progesterone receptor (PR), estrogen receptor α (ERα), androgen receptor (AR), and HER2 in 95 breast cancer samples from patients, using immunohistochemical methods [153]. Expression of BCRP was inversely correlated with those of PR and ER α. These studies contrast with earlier findings that HER2 regulated BCRP expression in SP cells, that the SP was associated with a "luminal" breast cancer phenotype (generally HER2-, estrogen, and progesterone receptor-positive), and the observation that "triple negative" cell lines generally lacked a side population [154]. Breast cancer cell lines selected for resistance to aromatase inhibitors were found to overexpress HER2 and BCRP and to exhibit enhanced tumor-initiating cell properties [155]. This paper confirmed that HER2 regulated BCRP expression in breast cancers, as shown previously [154]. Other investigators provided further confirmation of HER2 regulation of BCRP expression in breast cancer cells by enforced expression of HER2 in MCF-7 cells: BCRP expression and drug resistance were enhanced by HER2 stimulation of the Akt/PI3K and NF-kB pathways [156]. In tumor tissue from 196 patients with invasive ductal breast cancer, expression of BCRP was found to correlate with expression of HER2, lymph node metastasis and clinical stage [157].

BCRP was found to cause drug and photoactive dye accumulation in BCRP-rich extracellular vesicles and intracellular lysosomes in human breast cancer cells, which can result in cellular destruction with photodynamic treatment [158]. The BCRP-rich vesicles were disrupted by inhibition of the PI3K/Akt signaling pathway in human breast cancer cells [159].

Hypoxia-inducible factor 2a (Hif-2a) expression detected by immunohistochemistry correlated with BCRP expression, histologic grade, and Ki62 expression in a study of biopsy samples from 196 patients with invasive ductal breast cancer [160]. Other investigators found that prolactin induced the expression of BCRP in human breast cancer cells, mediated, at least in part, by the JAK2/STAT5 pathway [38].

BCRP may confer resistance to tamoxifen, based on in vitro studies of MCF-7 cells [161].

3.4.2.4 Lung Cancers

Small Cell Lung Cancer (SCLC)

Metastatic cells obtained by fine needle aspiration biopsy of enlarged mediastinal lymph nodes from 14 patients with SCLC and 7 patients with previously untreated non-small cell lung cancer (NSCLC) were evaluated for the expression of topoisomerase IIα and BCRP mRNA. The expression of topoisomerase IIα mRNA was significantly increased and the expression of BCRP mRNA was significantly decreased in the nodal metastatic cells from SCLC compared to NSCLC patients, suggesting to these investigators that *de novo* metastatic cells from SCLC are more chemosensitive than those from NSCLC patients [162].

In vitro studies by investigators in Japan found that HER2 is more frequently upregulated in SCLC cell lines derived from Japanese patients compared to cell lines derived from Caucasian patients. One Japanese-derived cell line, SBC-3, was selected for resistance to etoposide, SN-38, or cisplatin. All three drug-resistant sublines displayed further upregulation of HER2; the subline made resistant to SN-38 had upregulated BCRP. Treatment with a HER2 inhibitor, lapatinib, sensitized these cells to SN-38 both in vitro and in a murine xenograft model; however, silencing of HER2 by RNA interference methods did not affect BCRP function in vitro, suggesting that HER2 did not regulate BCRP expression in this line, but rather that lapatinib acted as an inhibitor of BCRP to exert its effect on reversing resistance to SN-38 [163].

Non-small Cell Lung Cancer (NSCLC)

A number of clinical translational studies have been completed recently: one study examined the BCRP polymorphism 421C>A, which results in diminished transporter function. Presence of this polymorphism did not predict the effectiveness of erlotinib (a BCRP substrate/inhibitor) in a clinical trial of 242 patients with advanced

NSCLC who underwent genotyping for BCRP and other gene polymorphisms [164]. Another study found that BCRP protein expression (as measured by immunohistochemistry) was present in 33 % of 80 cases of resected, early-stage NSCLC with neuroendocrine differentiation; however, expression of BCRP as well as other stem cell markers (CD117, CD133) did not correlate with prognosis or clinicopathologic characteristics of these patients [165]. Another investigation looked at the expression of cancer stem cell antigens, including BCRP, in biopsies from 133 patients with completely resected stage I/II non-small cell lung cancer with a median follow-up time of 53.8 months. These cancer stem cell markers were not reflective of prognosis [166]. Other investigators examined the drug sensitivity of xenografts derived from 24 human NSCLC cases, compared with the mRNA and protein expression of a variety of multidrug resistance markers, including BCRP. With only one exception, there was no association of marker expression with drug sensitivity, leading these investigators to conclude that multidrug resistance must be a multifactorial process [167]. In contrast, a separate study of tumor samples from 145 patients with stage I NSCLC found that coexpression of CD133 and BCRP was predictive of early postoperative recurrence [168].

Studies evaluating the role of BCRP in drug resistance in lung cancer cell lines have also been completed recently. One group of investigators observed that low-dose cisplatin treatment of human NSCLC adenocarcinoma cell lines enriched the percentage of cells expressing the stem cell marker CD133, accompanied by an increase in BCRP expression and activity of the NOTCH signaling pathway [169]. Other workers found that exposure of cultured human lung cancer and esophageal cancer cells to cigarette smoke condensate caused an increase in BCRP expression, as well as an increase the side population of stem cells. This effect was thought to be mediated via elements in the BCRP promoter; mithramycin exposure caused downregulation of BCRP [42]. Another study found that low molecular weight heparin induced proteasomal degradation of BCRP in SP cells from cisplatin-resistant lung adenocarcinoma A549/DDP cells in vitro, and restored the cisplatin sensitivity of these lung cancer cells in vitro and in vivo [78].

3.4.2.5 Head and Neck Cancers

Paraffin-embedded samples from 98 cases of laryngeal squamous cell cancer were probed for expression of BCRP and Pgp, using immunohistochemical techniques. Expression of either protein was found to be an independent adverse prognostic factor in this disease [170].

Oral erythroplakia is an aggressive premalignant lesion with high potential for developing into oral cancer. Investigators in China evaluated samples from lesions of 34 patients with erythroplakia for expression of podoplanin (a transmembrane glycoprotein) and BCRP. More than 90 % of patients whose lesions expressed both podoplanin and BCRP subsequently developed oral cancers [171].

A long-term follow-up study of 135 patients with oral leukoplakia was performed at a Chinese institution. The duration of follow-up averaged 5.5 years,

during which time 32 patients developed oral cancer. Expression of BCRP and BMI-1 in the leukoplakia was found to be associated with a high risk of developing oral cancer in this group [172].

Purification of BCRP-expressing nasopharyngeal carcinoma cells from the 5-8F NPC cell line revealed that some, but not all, BCRP-expressing cells have a stem cell phenotype [173]. Constitutive activation of the aryl hydrocarbon receptor (AhR) was observed in three human cell lines derived from tumors from patients with squamous cell head and neck cancers; AhR activation was found to be increased in the cancer cell lines than in human epidermal keratinocytes (HEK). Treatment with AhR antagonists decreased the aggressive phenotype of these cell lines by inhibiting cell migration, invasion, and benzo[a]pyrene induction of BCRP [174].

3.4.2.6 Ovarian Cancer

A clinical trial of the combination of lapatinib (a tyrosine kinase inhibitor as well as an inhibitor of BCRP) with topotecan (a BCRP substrate) in patients with ovarian cancer in first relapse was reported in 2012 [175]. No clinical benefit was observed compared to topotecan alone; furthermore, no correlation was seen between BCRP expression and clinical outcomes, suggesting involvement of other resistance mechanisms in addition to BCRP. In a similar study (LapTop) from 2011 in refractory/relapsed ovarian cancer, the combination of lapatinib and topotecan was found to lack sufficient activity and to have substantial hematologic toxicity [176]. Both studies concluded that mechanisms other than, or in addition to, BCRP are involved in topotecan resistance.

In contrast to studies in NSCLC patients harboring the 412C>A polymorphism of BCRP (which has impaired transporter activity) who were treated with erlotinib, in whom no benefit was observed [164], patients with advanced ovarian or primary peritoneal cancers and the 412C>A polymorphism had longer progression-free survival following treatment with platinum- and taxane-containing regimens [177].

Ovarian cancer stem cells that expressed BCRP, CD44, and CD117 were diminished by exposure of primary ovarian tumor tissues to miR-199a, which downregulates its target gene CD44. This sensitized these ovarian cancer stem cells to cisplatin, paclitaxel, and doxorubicin, and reduced expression of BCRP [54]. It is of interest that paclitaxel is a substrate for Pgp, but not BCRP.

Lysosomal sequestration of photosentizing imidazoacridinone dyes mediated by ABC transporters (including BCRP) resulted in selective destruction of ovarian cancer xenografts in a chorioallantoic membrane model following photodynamic therapy [178].

3.4.2.7 Glioblastoma

Malignant gliomas were induced in wild type or Mdr1a/b$^{-/-}$ or BCRP$^{-/-}$ mice, then treated with oral dasatanib (an inhibitor of BCR/Abl and Src family tyrosine kinases), which is a substrate for Pgp and BCRP. Survival of the knockout mice was

twice that of the wild-type mice following dasatinib therapy. Human gliomas were also found to express Pgp and BCRP, and xenografts from cell lines were sensitized to dasatanib by treatment with a Pgp/BCRP inhibitor [179].

Sonodynamic therapy employs a "sonosensitizer" which kills cancer cells (usually by ROS activation) when activated by ultrasound. BCRP was found to be a key determinant in the effectiveness of the photo/sonosensitizer photofrin in cultured U251 glioma cells [180].

BCRP expression was found to localize to the nuclear membrane in 6 of 7 glioblastoma multiforme cell lines studied, and BCRP inhibitors or RNAi to BCRP sensitized the cell lines to mitoxantrone cytotoxicity [15]. In a similar vein, Pgp has been found previously to be expressed on nuclear membranes of certain multidrug-resistant cell lines using immunohistological methods [181].

3.4.2.8 Prostate Cancer

Exposure of PC-3 or DU145 human prostate cancer cells to stem cell factor or G-CSF increased their expression of the stem cell markers CD117, BCRP, and CD44, accompanied by increased clonogenicity and tumor sphere formation, suggesting that such conditions may create a favorable bone marrow niche for prostate cancer cells [182]. PC-3 and DU145 cultured under hypoxic conditions exhibited upregulation of Hif1a, Hif2a, and developed stem cell-like characteristics, including expression of BCRP [183]. Cells with BCRP expression induced by hypoxia tended to coexpress CD44, another protein often expressed in stem cells.

3.4.2.9 Other Cancers

In malignant pleural mesothelioma (MPM), the PI3K pathway was found to be a key player in maintaining the side-population (SP) phenotype and BCRP expression in three cell lines [184]. In a clinical trial in children with newly diagnosed high-risk neuroblastoma, the TKI gefitinib (a BCRP inhibitor) was combined with irinotecan (a BCRP substrate): the combination was tolerable but insufficiently efficacious to warrant further study [185].

The human osteosarcoma cell line MNNG/HOS has cell subpopulations with high expression of Pgp and BCRP which are resistant to chemotherapy [186]. For patients with malignant melanoma, vemurafenib is a clinically effective inhibitor of mutant BRAF (V600E), however, resistance develops rapidly. A recent study found that vemurafenib is a substrate/inhibitor of ABCG2 [187].

3.5 Summary

Transport mediated by BCRP is crucial to multiple cellular processes, including the intracellular transport of heme and other porphyrins involved in the heme synthesis pathway, protection of the organism from natural dietary toxins and carcinogens,

protection of the fetus, brain, and stem cells from potential toxins, and importantly, the disposition of many pharmaceuticals. Evidence is growing that the BCRP physiologic function as well as BCRP gene polymorphisms can have an impact on cancer development and cancer treatment outcomes. Numerous chemotherapy drugs are substates or inhibitors of BCRP, including recently developed novel therapeutics targeting the cancer kinome. Evidence continues to accrue relating tumor expression of BCRP with adverse prognosis. Since BCRP is expressed in stem cells, including many so-called cancer stem cells, BCRP could be part of a cassette of genes whose expression is increased in neoplastic cells with embryonic properties programmed for self-renewal and immortality. As such, BCRP may be a contributing factor (but not in itself sufficient) to drug resistance in cancer stem cells, and hence serve as a sentinel for these more aggressive or resistant cancers. Future research into the mechanisms that regulate BCRP expression in specific normal tissues, in cancers, and in normal and neoplastic stem cells may enable precise targeting BCRP expression in cancers and cancer stem cells to weaken neoplastic cellular resistance to therapy.

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Chapter 4
A New Strategy of ALA-Photodynamic Cancer Therapy: Inhibition of ABC Transporter ABCG2

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Abstract Photodynamic therapy (PDT) is a clinical tool for treating various tumors. PDT is achieved by a photon-induced physicochemical reaction that is induced by excitation of porphyrins by exposure to light and the subsequent generation of singlet oxygen ($^1O_2$) and other reactive oxygen species. Recently, 5-aminolevulinic acid (ALA)-based PDT has been developed as an anticancer treatment whereby ALA is orally administered as the precursor of protoporphyrin IX (PpIX) to induce the biosynthesis and accumulation of PpIX in cancer cells. Recent studies, however, provide evidence that the ABC transporter ABCG2 plays a pivotal role in regulating the cellular accumulation of PpIX in cancer cells and thereby affects the efficacy of ALA-based PDT. In response to the photoreaction of porphyrin leading to oxidative stress, the NF-E2-related transcription factor (Nrf2) can transcriptionally upregulate many target genes, including those for metabolizing enzymes and transporters essential for cellular defense. Whereas Nrf2 upregulates transcription of the *ABCG2* gene to confer cancer cells resistance, several protein kinase inhibitors reportedly interfere with the transport function of ABCG2. In fact, gefitinib inhibits ABCG2-mediated porphyrin efflux from cancer cells to enhance the efficacy of PDT in vitro. Thus, it is of great interest to develop ABCG2-specific

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inhibitors that are clinically applicable to photodynamic cancer therapy. Hitherto, we have performed high-speed screening, quantitative structure–activity relationship (QSAR) analysis, and in vivo validation to identify potent ABCG2-inhibitors. This chapter addresses such a new approach to improve ALA-based photodynamic cancer therapy.

Keywords ABCG2 • Brain tumor • Cancer stem cell • Nrf2 • Photodynamic therapy • Porphyrin • Gefitinib

Abbreviations

ALA	5-Aminolevulinic acid
PDT	Photodynamic therapy
PpIX	Protoporphyrin IX
Nrf2	NF-E2-related transcription factor
$^1O_2$	Singlet oxygen
ROS	Reactive oxygen species
QSAR	Quantitative structure-activity relationship
HO-1	Hemeoxigenase
PKC	Protein kinase C

4.1 Introduction

Porphyrins and heme are important components of diverse biological processes, such as respiration and oxidative metabolism [41, 76]. Both porphyrin biosynthesis and its intracellular concentration are tightly regulated by multiple biochemical mechanisms. The biosynthesis of porphyrin molecules begins with 5-aminolevulinic acid (ALA) synthesis from glycine and succinyl Co-A, and is followed by multiple enzymatic reactions that are spatially shared among mitochondria and cytoplasmic compartments (Fig. 4.1). Different from the endogenous biosynthesis of ALA within cells, exogenous ALA administration short circuits the first step of porphyrin biosynthesis, whereby ALA is transported into cancer cells by the oligopeptide transporter 1 or 2 (PEPT1 or PEPT2) [17, 50, 79, 61]. Exogenously administered ALA induces high levels of PpIX biosynthesis and accumulation in cancer cells, rendering them photosensitive. ALA-induced endogenous PpIX accumulation, thus, constitutes a photosensitization process in which the tendency of neoplastic cells to synthesize and/or accumulate PpIX may be exploited to enhance the efficacy of photodynamic therapy (PDT).

PDT utilizes porphyrin derivatives to generate singlet oxygen ($^1O_2$) and other reactive oxygen species (ROS) through visible light irradiation in cancerous tissues [16, 18] (Fig. 4.2, upper panel). Multiple signaling cascades are concomitantly activated in

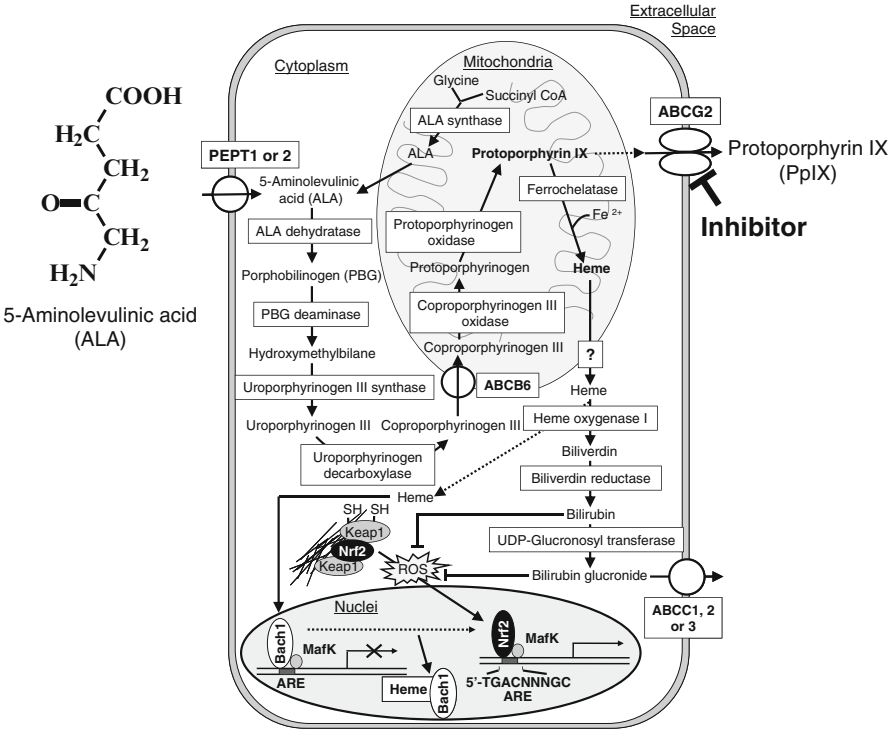

Fig. 4.1 Biosynthesis and metabolism of porphyrins and heme. Porphyrins are synthesized from glycine and succinyl Co-A via multiple enzymatic reactions in the cytosol and mitochondria. *Rectangles* indicate the enzymes involved in heme metabolism: *ALA* 5-aminolevulinic acid, *PBG* porphobilinogen. ABC transporter ABCB6 is responsible for the import of coproporphyrinogens into mitochondria, whereas ABCG2 transports porphyrins across the plasma membrane to maintain porphyrin homeostasis. Heme formed from protoporphyrin IX is catabolized to biliverdin by the microsomal enzyme heme oxygenase 1 (HO-1). Biliverdin is subsequently metabolized to bilirubin by biliverdin reductase. Bilirubin is a potent antioxidant that quenches reactive oxygen species (ROS), whereas ROS activates Nrf2, a transcription factor regulating the transcription of various target genes, such as HO-1, ABCG2, ABCC1, ABCC2, and ABCC3

cancer cells exposed to photodynamic stress. Dependent upon the subcellular localization of cytotoxic ROS, those signals are transduced into adaptive or cell death responses [10]. Currently available evidence indicates that photodynamic therapy can kill cancer cells directly by the efficient induction of both apoptotic and non-apoptotic cell death pathways [16]. The identification of molecular effectors that regulate the cross-talk between cell death and cell protection pathways is an area of intense interest in the field of photodynamic killing of cancer cells.

In response to oxidative stress, nuclear factor erythroid-derived 2 (NF-E2)-related factor (Nrf2) is known to play a role in transcriptional upregulation of many target genes essential for cellular defense [47, 75]. Nrf2 is a basic region-leucine zipper (bZip)-type transcription factor [31, 46], which targets the antioxidant responsive

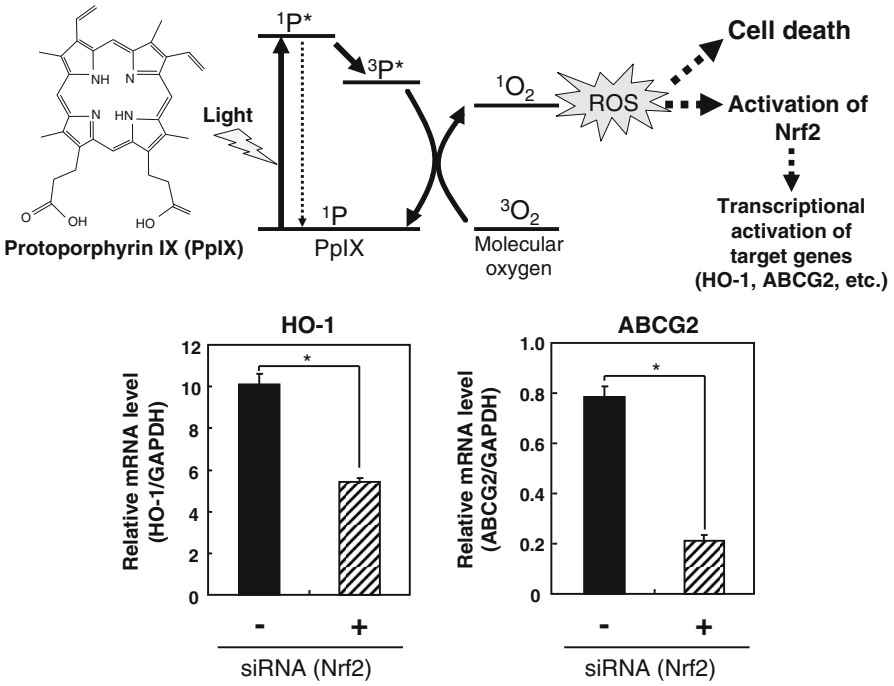

Fig. 4.2 Schematic illustration of porphyrin-mediated photodynamic therapy (*upper panel*), and the effect of Nrf2-specific siRNA on HO-1 and ABCG2 in HepG2 cells incubated with PpIX and subsequently exposed to visible light (*lower panel*). To study the role of Nrf2 on the transcriptional activation of *HO-1* and *ABCG2* genes, HepG2 cells, treated with (+) or without (−)200 nM Nrf2-specific siRNA for 68 h, were cultured with 10 μM PpIX in the dark for 4 h and then exposed to visible light for 1.5 h. The mRNA levels of HO-1 and ABCG2 were determined by quantitative RT-PCR at 8 and 28 h after the start of exposure to PpIX, respectively. Data are expressed as the means ± S.D. of triplicate determinations. $*p < 0.05$ as compared between the indicated groups. See Hagiya et al. [19] for experimental details

element (ARE) containing the consensus sequence of 5′-A/GTGACNNNGC-3′ [49]. On the other hand, the Kelch-like ECH-associated protein 1 (Keap1) acts as a negative regulator of Nrf2 by retrieving it from the cytoplasm. Oxidative stress and/or electrophilic attack leads to the dissociation of Nrf2 from Keap1 and thereby activates Nrf2 for transcriptional regulation of ARE-dependent genes. The induction of ARE-regulated genes is under the control of Nrf2. Indeed, many genes encoding detoxifying and antioxidant enzymes were found to be regulated by Nrf2 [36, 38]. It has recently been reported that mRNA levels of ABC transporters ABCC1, ABCC2, ABCC3, and ABCG2 were significantly elevated under oxidative stress and that translocation of Nrf2 into the nucleus was associated with the induction of ABCG2 [1]. Nrf2 interacts with the ARE located in the human *ABCG2* gene promoter region. Nrf2-specific siRNA treatments suppressed the induction of ABCG2 and hemeoxigenase (HO-1) expression after the photoactivation of porphyrins in vitro (Fig. 4.2, lower panel). These findings strongly suggest that the induction of ABCG2 expression under oxidative stress is mediated, at least in part, by the Nrf2/Keap1 system.

The activation and nuclear translocation processes of Nrf2 seem to be more complex. Activation of the Nrf2 protein may involve at least three distinct pathways [30]. Pathway 1: Oxidation of critical cysteinyl residues of the Keap1 protein with concomitant inhibition of the ubiquitination activity of Keap1 [38, 80]. Pathway 2: Phosphorylation of the Nrf2 protein via protein kinases, such as $p38^{MAPK}$, phosphoinositol-3-kinase (PI3K), protein kinase C (PKC), and RNA-dependent protein kinase (PKR)-like ER kinase (PERK) [44, 37, 4, 6, 12, 13, 33, 32]. Pathway 3: Direct binding of heme to Bach1 and the facilitation of Nrf2/small Maf heterodimer formation [23, 53, 68, 69, 21, 34, 64, 52, 71, 82, 57]. Thus, it is important to understand the molecular mechanisms underlying the activation of Nrf2 in cancer cells so as to understand the nature of ABCG2-mediated drug resistance of human cancer.

4.2 Key Role of ABCG2 in Photodynamic Therapy

The ABC transporter ABCG2 was originally identified as a breast cancer resistance protein (BCRP) [15]. The *ABCG2* gene encodes a half ABC transporter protein, which forms a homodimer [78, 27] (Fig. 4.3). Reportedly, ABCG2 is overexpressed in many cancer cells and functions to transport a wide variety of anticancer agents [60, 5], including cytotoxic agents (e.g., mitoxantrone, topotecan, flavopiridol, methotrexate), fluorescent dyes (e.g., Hochest 33342), and various toxic compounds found in normal food (e.g., 2-amino-1-methyl-6-phenylimidazo[4,5-β]pyridine). Typical ABCG2 substrates are shown in Fig. 4.4.

ABCG2 is responsible for the cellular homeostasis of porphyrins and their related compounds [39, 77, 28]. Hitherto, evidence has been accumulating to show that ABCG2 transports porphyrins in an ATP-dependent manner [72–74]. Robey et al. [59] reported that ABCG2-mediated transport of photosensitizers has the potential to impact photodynamic therapy. ABCG2 has recently been recognized to have physiologically important roles in porphyrin homeostasis, photosensitivity, and photodynamic therapy [59, 19, 9, 35, 20, 29]. Genetic polymorphisms of ABCG2 and its inhibition by certain drugs may be associated with photosensitivity [72, 73]. ABCG2 expression in tumor cells is associated with resistance to multiple chemotherapeutic agents [5, 26]. ABCG2 also protects cells against phototoxicity by mediating the efflux of porphyrins from them [59]. Clinical photosensitizers, such as protoporphyrin, 2-(1-hexyloxethyl)-2-devinyl pyropheophorbide-a (photochlor), and benzoporphyrin derivative monoacid ring A (verteporfin) were also transported out of cells by the action of ABCG2, whereas this effect was abrogated by the co-administration of imatinib mesylate in vitro [43]. It is suggested that, by increasing intracellular photosensitizer levels in ABCG2-positive tumors, imatinib mesylate or other ABCG2 transport inhibitors could enhance the efficacy of clinical photodynamic therapy [43]. In fact, cellular phototoxicity was evoked through the inhibition of ABCG2 function by several protein kinase inhibitors, including imatinib and cyclin-dependent kinase (CDK) inhibitors in vitro [73, 3, 29].

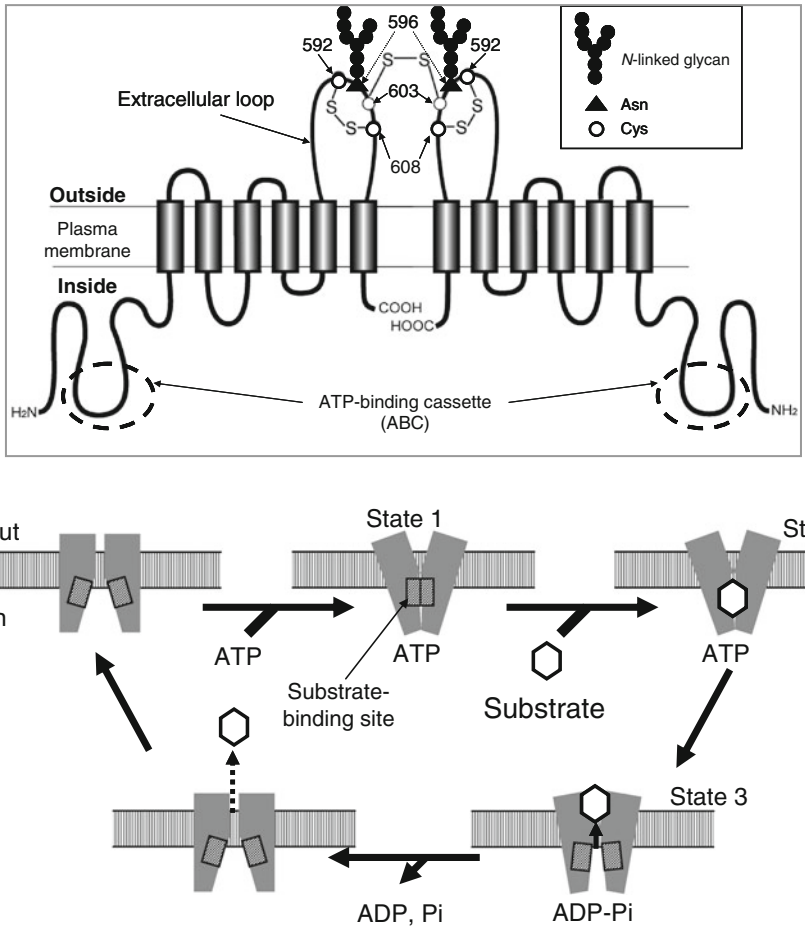

Fig. 4.3 Schematic illustrations of ABCG2 protein structure (*upper panel*) and transport mechanism (*lower panel*). The ABCG2 protein expressed in the plasma membrane is a homodimer linked via a cysteinyl disulfide bond. The cysteine residue corresponding to Cys603 of human ABCG2 is involved in the homodimer formation. The substrate-binding site is formed when ATP is bound to the ATP-binding cassettes (ABC) of the ABCG2 homodimer. Disulfide bond formation at Cys603 does not appear to be prerequisite for exerting the transport activity of ABCG2. The substrate-binding site is formed when ATP is bound to the ATP-binding cassettes of the homo dimmer of ABCG2. Gefitinib is thought to be bound to the substrate-binding site

Interestingly, gefitinib was found to inhibit not only the transport function of ABCG2 but also its expression [3]. When brain tumor cell lines were incubated in vitro with ALA (1 mM) and gefitinib at different concentrations (0.01–1.0 μM), gefitinib enhanced the intracellular accumulation of protoporphyrin IX in a dose-dependent manner [70]. Concomitantly, incubation of brain tumor cells with gefitinib resulted in decreased levels of ABCG2 mRNA. Moreover, gefitinib significantly decreased ABCG2 protein levels expressed at the plasma membrane surface [70]. These observations suggest that ABCG2 expression is regulated via a protein

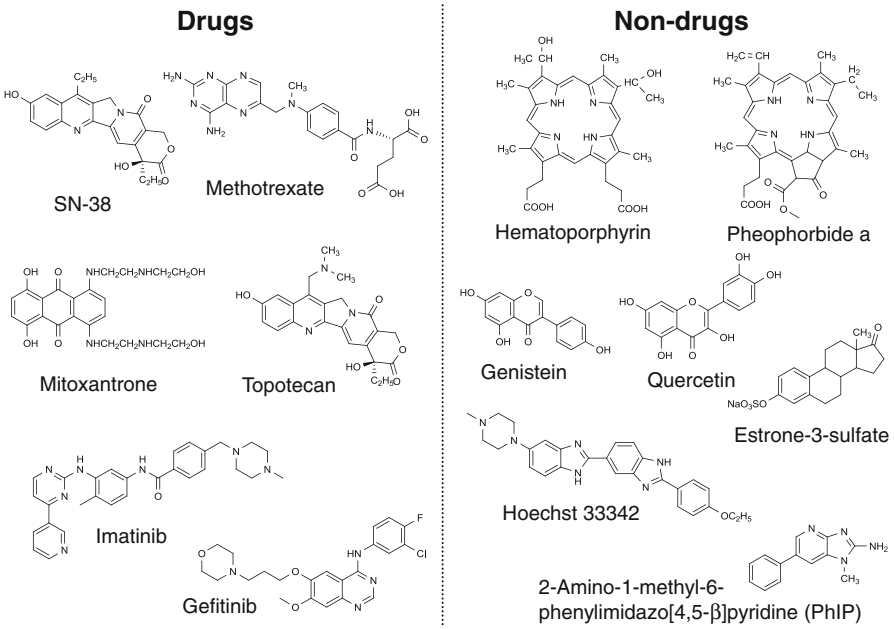

Fig. 4.4 Chemical structures of ABCG2 substrates. These substrates are classified into two groups; namely, drugs and nondrugs

kinase-mediated pathway and that ABCG2 expression is regulated by the EGFR-tyrosine kinase cascade [45, 22] as well as by the Nrf2-Keap1 pathway [65, 30].

4.3 Mechanism of ABCG2 Inhibition by Gefitinib

Özvegy-Laczka et al. [54] provided the first evidence that gefitinib, imatinib, and N-[4-[(3-bromophenyl)amino]-6-quinazolinyl]-2-butynamide (EKI-785) interacted with ABCG2 at submicromolar concentrations in vitro [54]. At low concentrations, gefitinib, imatinib, and EKI-785 inhibited ABCG2-dependent active drug extrusion and significantly affected drug resistance patterns in cells expressing ABCG2 [54]. In ABCG2-overexpressing cancer cells in vitro, gefitinib potently reversed resistance to SN-38 [81]. In mice, gefitinib treatment dramatically increased the oral bioavailability of irinotecan after simultaneous oral administration and enhanced the accumulation of SN-38, an active metabolite of irinotecan, in cancer [24]. Thus, it is expected that gefitinib modulates SN-38 activity at the cellular level to reverse tumor resistance mediated by ABCG2 through inhibition of the oral drug efflux and, thus, might potentially be used in humans to modulate the oral bioavailability of poorly absorbed camptothecins such as irinotecan [67, 24].

It was originally speculated that gefitinib would compete with the ATP-binding site on ABC transporters, because it was designed to interact with the conserved

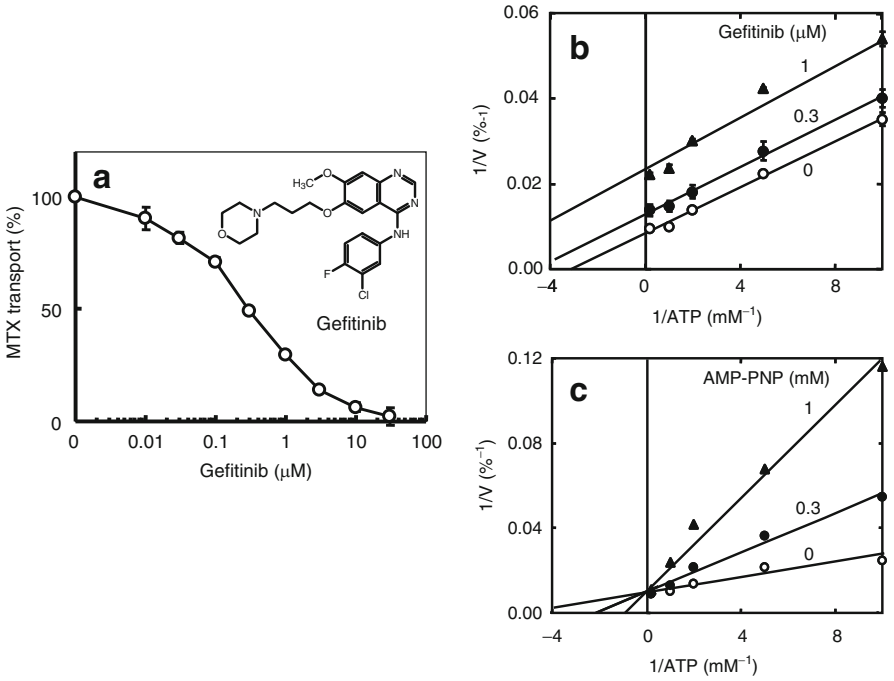

Fig. 4.5 Inhibition of ABCG2-mediated methotrexate transport by gefitinib. (**a**) ATP-dependent [^{3}H]methotrexate (MTX) transport was measured at 37 °C for 20 min in the presence of gefitinib at different concentrations in the standard incubation medium (0.25 M sucrose and 10 mM Tris/Hepes, pH 7.4, 10 mM creatine phosphate, 100 μg/ml creatine kinase, 10 mM $MgCl_2$). MTX transport is expressed as relative values as compared with the transport activity measured without gefitinib (100 % of MTX transport). Data are expressed as mean values ± S.E.M. ($n = 4$). (**b**) membrane vesicles were incubated with 200 μM [^{3}H]MTX in the presence of 0.1, 0.2, 0.5, 1, or 5 mM ATP in the standard incubation medium at 37 °C for 20 min, where gefitinib was added in the reaction mixture at a concentration of 0 μM (*circle*), 0.3 μM (*filled circle*), or 1 μM (*filled triangle*). Data are expressed as mean values ± S.E.M. ($n = 3$). (**c**) Membrane vesicles were incubated with 200 μM [^{3}H]MTX in the presence of 0.1, 0.2, 0.5, 1, or 5 mM ATP in the standard incubation medium at 37 °C for 20 min, where AMP-PNP was added to the reaction mixture at a concentration of 0 mM (*circle*), 0.3 mM (*filled circle*), or 1 mM (*filled triangle*). MTX transport was quantified by measuring the amount of MTX transported into membrane vesicles as described in Saito et al. [62]

kinase domain [8]. Therefore, a question was raised about whether gefitinib might inhibit ABCG2-mediated drug transport by occupying the ATP-binding cassette of ABCG2. To answer this question, we carried out transport experiments using ABCG2-expressing membrane vesicles, where methotrexate (MTX) was used as a substrate of ABCG2 [62]. Contrary to our expectation, gefitinib did not interfere with ATP at all. As clearly demonstrated in Fig. 4.5b, inhibition of ABCG2 by gefitinib was "uncompetitive" with ATP, whereas AMP-PNP, an ATP analogue, competed with ATP (Fig. 4.5c). These results have revealed that gefitinib is bound to an ABCG2-ATP complex; namely gefitinib behaves as a substrate for ABCG2 (Fig. 4.2).

4.4 QSAR Analysis of ABCG2 Inhibitors

Hitherto we performed quantitative structure–activity relationship (QSAR) analysis and showed that a structure having one amine bonded to one carbon of a heterocyclic ring is an important component for interaction with the ABCG2 protein [62, 63]. In addition, fused heterocyclic ring(s) and two substituents on a carbocyclic ring of the fused heterocyclic ring(s) have been noted to be important chemical moieties for the interaction with ABCG2 [62, 63]. Interestingly, many protein kinase inhibitors carry such structural components within their molecules [62]. To gain further insights into drug–ABCG2 interactions and the three-dimensional (3D) structures of protein kinase inhibitors, we performed ab initio molecular orbital (MO) calculations based on the restricted Hartree-Fock (RHF) level of theory [3]. It has become clear that, like gefitinib, purvalanol A and WHI-P180 have a planar structure, whereas bohemine, roscovitine, and olomoucine do not [3]. As compared with bohemine, roscovitine, and olomoucine, both purvalanol A and WHI-P180 were stronger inhibitors of ABCG2-mediated porphyrin transport in membrane vesicles. Thus, it is suggested that the planar structure is an important factor for interactions with the active site of ABCG2. Furthermore, the highest occupied molecular orbital (HOMO) of protein kinase inhibitors may play a significant role for stronger interaction with a substrate-binding site(s) of the ABCG2 protein [63]. Indeed, gefitinib, purvalanol, and WHI-P180 have very similar HOMO structures. Among them, however, gefitinib was the strongest inhibitor for ABCG2.

4.5 The Effect of Gefitinib on ALA-PDT in Brain Tumor U87MG Cells In Vitro

It is of great interest to experimentally examine the inhibition of ABCG2 by gefitinib and its effect on ALA-PDT in vitro. Since the human brain tumor U87MG cell line is capable of efficiently synthesizing PpIX from ALA, we used this cell line to evaluate the effect of gefitinib on cellular accumulation of PpIX [25]. U87MG cells were incubated with 2 mM ALA in the absence or presence of gefitinib (1 nM to 100 μM). During the incubation (4 h), ALA was taken up by peptide transporters PEPT1/2 into the cell, wherein PpIX was then synthesized. While PpIX was released from U87MG cells by ABCG2, gefitinib inhibited the ABCG2-mediated PpIX efflux in a dose-dependent manner and, consequently, greatly affected cellular PpIX accumulation (Fig. 4.6). It is suggested that cellular PpIX accumulation levels are significantly enhanced by gefitinib at concentration ranges over 1 μM, reaching approximately fivefold enhancement when cells were treated with 100 μM gefitinib.

As shown in Fig. 4.6, the intracellular accumulation of PpIX was significantly enhanced through ABCG2 inhibition by gefitinib. We therefore evaluated the effect of gefitinib on ALA-PDT in brain tumor U87MG cells in vitro. After a 24 h-incubation with ALA and gefitinib, cells were exposed to the light with a maximal intensity at 635 nm from a light-emitting diode (LED). Figure 4.7 depicts the relationship

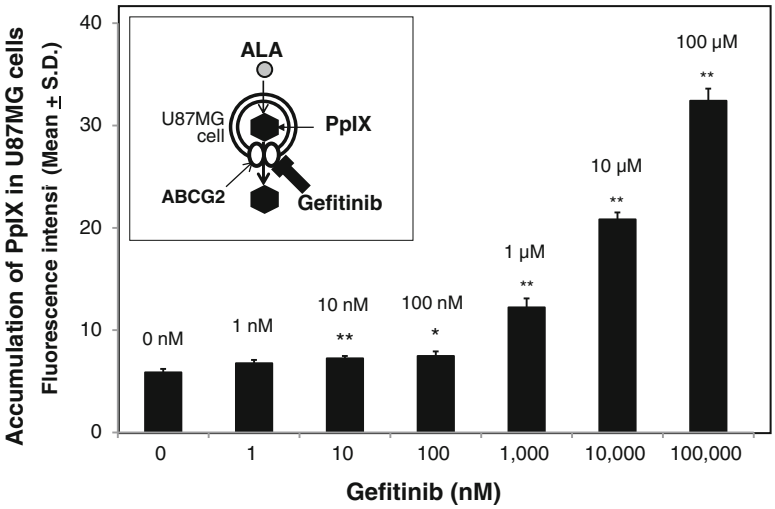

Fig. 4.6 Effect of gefitinib on accumulation of PpIX in human brain tumor U87MG cells after incubation with ALA. U87MG cells were incubated with 2 mM ALA and gefitinib at different concentrations for 4 h under the standard incubation conditions. The fluorescence of PpIX, which was synthesized and accumulated in individual cells, was measured by FACS in the EPICS ALTRA flow cytometry system with excitation at 325 nm; detection at 575 nm. The mean fluorescence intensity and standard deviation were calculated from measurement results. Data are expressed as mean ± S.D. in triplicate experiments [25]

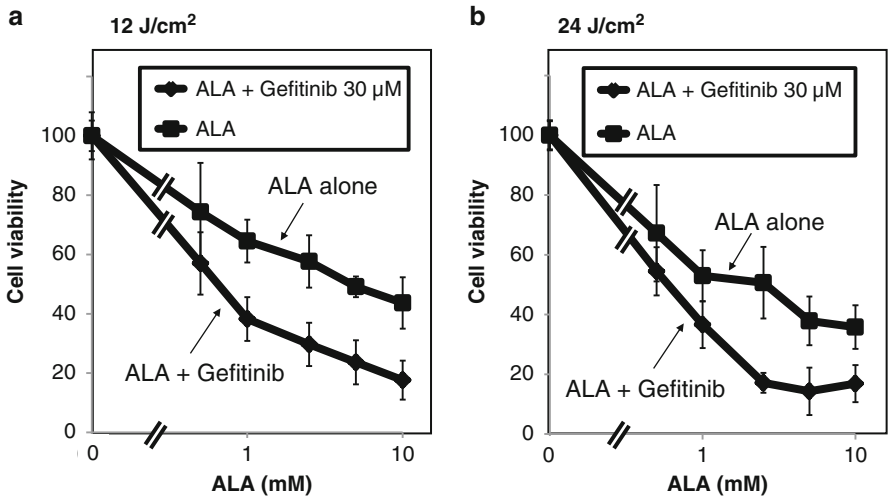

Fig. 4.7 Effect of gefitinib on ALA-PDT of human brain tumor U87MG cells in vitro. U87MG cells were first seeded into 96-well plates (1,000 cells/well) and cultured at 37 °C. After 24 h, ALA was added to the culture medium at varying concentrations of 0–10 mM with or without gefitinib, and cells were cultured at 37 °C for 4 h in the dark. Cells were then exposed to LED (635 nm) at a power of 12 J/cm^2 (**a**) or 24 J/cm^2 (**b**). Surviving cells were detected by MTT assay. Data are expressed as means ± S.D. in triplicate experiments [25]

between the ALA concentration and the viability of U87MG cells incubated without or with 30 μM gefitinib. We performed PDT at two different light intensities, i.e., 12 J/cm^2 (a) or 24 J/cm^2 (b). Gefitinib sensitized the brain tumor cells to ALA-PDT in vitro. It is suggested that inhibition of ABCG2-mediated PpIX efflux by gefitinib may be effective for ALA-PDT in vivo.

4.6 The Effect of Gefitinib on ALA-PDT in a Xenograft Model

Using a xenograft model, i.e., BALB/c-nu/nu nude mice, we next examined the effect of gefitinib on ALA-PDT in vivo [25]. Human brain tumor U87MG cells were subcutaneously inoculated into the right flank of each mouse (5×10^6 cells/0.1 ml/mouse). Three days after this injection, the mice were orally administered ALA (30 mg/kg body weight, p.o.) and/or gefitinib (100 mg/kg body weight, p.o.). Three hours later, the tumor tissue was exposed to LED light (maximal intensity at 635 nm) for 30 min.

Figure 4.8 demonstrates the effect of ALA, gefitinib, or ALA + gefitinib on the growth of brain tumor U87MG cells in BALB/c-nu/nu nude mice after LED irradiation. When nude mice were administered with both ALA and gefitinib and exposed to the LED light, the tumor growth rate appeared to slow down, as compared with the control; however, this difference was very modest and lacked statistical significance [25]. While experimental conditions could be changed to obtain better results, we concluded that gefitinib is not potent enough to inhibit the ABCG2 function for ALA-PDT. PpIX has a high affinity to human ABCG2. We need to explore more potent ABCG2 inhibitors that are clinically applicable to photodynamic cancer therapy.

4.7 Conclusion and Future Perspectives

Hitherto, cancer research has focused on the molecular and cellular analyses of the bulk tumor mass. There is overwhelming evidence in some malignancies, however, that the tumor clone is heterogeneous with respect to proliferation and differentiation. Recently, cancer stem cells are regarded to be a factor in causing chemo/radioresistance of many neoplasms [40, 14]. Cancer stem cells represent a new paradigm in tumor biology. These cells have been demonstrated to initiate and sustain tumor growth in cancers of the hematopoietic system [7], breast [2], brain [66, 48], prostate [11, 55], head and neck [56], pancreas [42], and colon [51, 58]. The discovery of cancer stem cells within certain tumor types has launched a new discipline within cancer research and therapy. Cancer stem cells are defined as those cells within a tumor that can self-renew and drive tumorigenesis. For the next step, we will elucidate whether ALA-PDT administered together with an ABCG2 inhibitor is

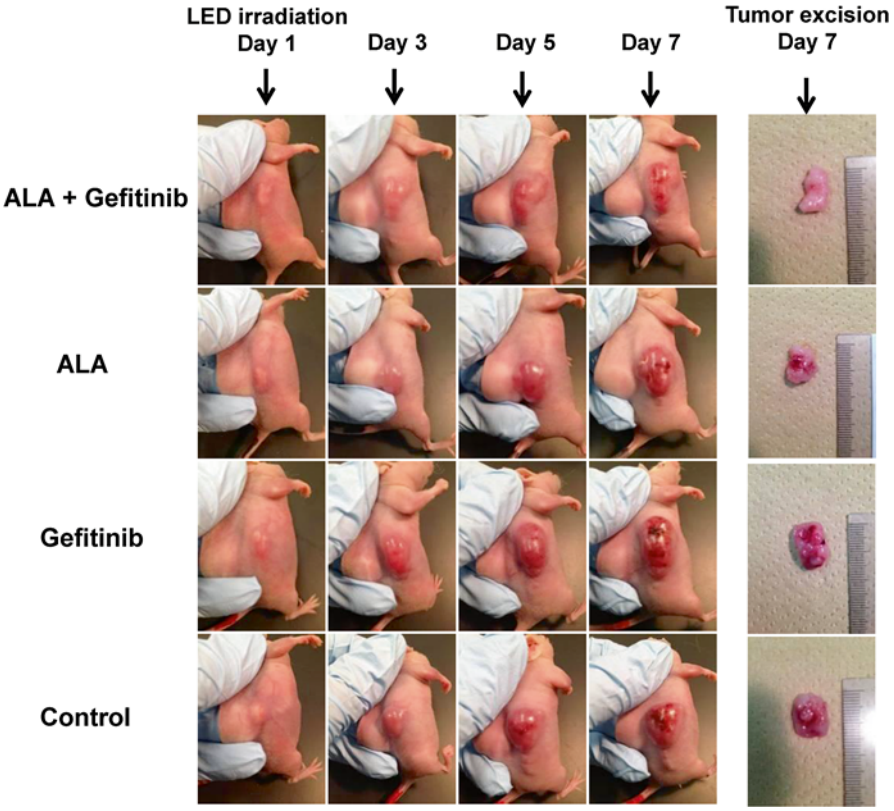

Fig. 4.8 Effect of gefitinib on ALA-PDT of human brain tumor U87MG cells in the xenograft model. ALA-PDT in the xenograft model. U87MG cells were injected subcutaneously into the right flank of each mouse (5×10^6 cells/0.1 ml/mouse). Under ether anesthesia, the tumor tissue was irradiated with LED light (100 J/cm^2) for 30 min. After the LED light irradiation, tumor growth in each mouse was observed for 1 week. On day 7, the mice were killed and each tumor was surgically excised to measure its size. Data are from Inoue et al. [25]

clinically useful for killing cancer stem cells. The new strategy of achieving ALA-photodynamic cancer therapy by inhibiting ABC Transporter ABCG2 is presently in progress.

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Chapter 5
ATP Binding Cassette Transporters in Cancer Stem-Like Cells

Paola Perego

Abstract A fraction of tumor cells designated as cancer stem cells (CSC) has been identified in various tumor types. Such cells appear to be capable of initiating and sustaining the growth of a tumor, being responsible for tumor initiation, invasive growth, metastasis, and drug resistance. The isolation of CSC is not easy to achieve due to the need for proving phenotypic and functional features; thus, under many circumstances "putative CSC" is the most appropriate designation. Like normal stem cells, CSC appear to exhibit increased expression of ABC transporters as compared to their nonstem counterparts. Here, the cancer stem cell hypothesis is described with particular reference to the timeline of its development, together with the acquired knowledge on ABC transporters that may be instrumental for therapeutic targeting of CSC.

Keywords Cancer stem cells • ABC transporters • Drug resistance

Abbreviations

CSC	Cancer stem cells
ABC	ATP binding cassette
HSC	Hematopoietic stem cells
SP	Side population
BCRP	Breast cancer resistance protein
EMT	Epithelial to mesenchymal transition
Pg-p	P-Glycoprotein
MRP	Multidrug resistance-related protein
miRNAs	microRNAs
MRP1	Multidrug resistance-related protein 1

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PI3K	Phosphoinositide-3-kinase
Akt	V-Akt murine thymoma viral oncogene homolog
MAPK	Mitogen-activated protein kinases
ERK	Extracellular signal-regulated kinases
LMWH	Low molecular weight heparin
ALDH1	Aldehyde dehydrogenase 1

5.1 Introduction

In all differentiated mammalian normal tissues there are cells that can differentiate in response to environmental stimuli, because they maintain stem cell features [77]. Such cells are abundant in regenerating tissues, but represent a small fraction of the tissue cells in nonregenerating tissues. The existence also in tumors of a cell fraction endowed with self-renewal, differentiating and tumor initiating properties is supported by old and recent studies. Such cells designated as cancer stem cells (CSC) have been proposed to represent a population of cancer cells which initiates and sustains the growth of a tumor, being responsible for tumor initiation, invasive growth, and metastasis [90, 6, 16]. CSC have been identified in different tumor types, but only in a few diseases (e.g., breast and brain tumors) the precise phenotypic and functional features of CSC have been well defined [20]. A better definition of the role of CSC in various tumor types and molecular subtypes will need additional efforts, specifically in establishing refined markers for CSC. Moreover, extensive in vivo work, in particular limiting dilution assays will be required to establish the tumor-initiating capability of the used experimental models. Such approaches are expected to reduce over-interpretation of results that has been a frequent risk in the field of CSC.

Expression of proteins contributing to drug resistance, and in particular of the ATP-binding cassette (ABC) transporters, in putative CSC versus differentiated cancer cells are being regarded as a major feature of CSC that could be pharmacologically targeted in an attempt to improve the efficacy of treatment and to achieve durable responses [22]. It has been reported that normal and tumor stem cells exhibit increased expression of ABC transporters as compared to their nonstem counterparts [5, 103]. Indeed, ABC transporters have been documented to be involved in the regulation of stem cell physiology in studies regarding normal hematopoietic stem cells (HSC, see below).

Here, the cancer stem cell hypothesis is described with particular reference to the timeline of its development, together with the acquired knowledge on ABC transporters that may be instrumental for therapeutic targeting of CSC. The significance of ABC transporters in the biology of CSC is presented by considering the specific features of those transporters that have been implicated in phenotypes related to CSC, such as the side population (SP) phenotype. In addition, the interrelation between ABC transporters and other markers for CSC is examined, as well as the regulation of ABC transporters expressed in CSC or cancer stem-like cells by specific factors or pathways. Moreover, clinical trials involving CSC have been

reported. In summary, the available evidence on CSC supports the need for strong basic science efforts before fully translating the knowledge generated in the preclinical context to the clinical setting. Several modulators of ABC transporters seem to have promising therapeutic potential, as shown in preclinical studies. The scientific community is already facing a few clinical observations that may be useful to interpret recurrence and that will be hopefully exploited to define improved therapies to cure cancer patients.

5.2 The Cancer Stem Cell Hypothesis

5.2.1 Rationale and Historical Notes

The CSC hypothesis proposes a hierarchical model for tumors in which cells "at the apex of tumor hierarchy" can be identified. Such a hypothesis is apparently in contrast with the clonal evolution model or stochastic model of tumorigenesis, which is based on increased proliferative potential of the clone with the best fitness among tumor cells. The model of a dominant clone where clonal selection relies on genetic mutation does not appear real, or at least exclusive. In fact, epigenetic mechanisms and also the tumor microenvironment contribute to tumor heterogeneity. In this context, nonmutational mechanisms of drug resistance have been described (i.e., polygenic clinical drug resistance) [39], that may be useful to interpret the real nature of tumor heterogeneity and may be in line with a relevant role for CSC.

The first report that started to build the CSC hypothesis was published in 1937 by Furth and Kahn [89], who documented that a single tumor cell can initiate leukemia in mice. "The transmission of leukemia of mice with a single cell" was subsequently confirmed in several studies [108]. Remarkably, in 1997, Bonnet and Dick reported that human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Such a study shed light on the mechanism underscoring the phenotypic, genotypic, and clinical heterogeneity of acute myeloid leukemia, given the debate about the target cell, within the hematopoietic stem cell hierarchy, that is susceptible to leukemic transformation [8]. The study suggested that normal primitive cells, rather than committed progenitor cells, are the target for leukemic transformation, and that such cells could differentiate in vivo into leukemic blasts, a phenomenon supporting that the leukemic clone is organized as a hierarchy [8]. The first evidence of the existence of CSC in solid tumors was provided in 2003, when the CSC hypothesis was tested starting from considering the phenotypically diverse population of breast cancer cells [1]. In this context, it was found that only a small number of breast cancer cells were able to form new tumors in immune-deficient mice, and tumor initiating cells were distinguished from noninitiating cells based on surface marker expression [1]. Indeed, starting from patient-derived material, tumorigenic cells that were found to be positive for CD44, negative for CD24 or expressing low levels of CD24 and negative for lineage markers could be serially transplanted and generated heterogeneous tumors [1].

5.2.2 Tumor Heterogeneity and Tumor Initiating Capability of CSC

At present, the available evidence supports that a tumor can be regarded as a heterogeneous aberrant tissue, possibly originating from a single cancer stem cell, and maintained by the surrounding niche which contains stromal cells and other components of the microenvironment (i.e., immune cells). Thus, the definition of CSC has been précised over time and expanded to comprise tumor cells capable to regrow the tumor from which they were isolated [115]. In such a view, several laboratories have directed their efforts toward the isolation of CSC from tumor biopsies and from tumor cell lines with the final goal to discover druggable targets expressed by CSC. In principle, CSC are endowed with tumor initiating capability—supported by in vivo testing—and with differentiation properties. In addition, CSC express a set of markers that allow researchers to accomplish their identification and isolation. Using multiple tools, it has been shown that subpopulations of CSC may account for tumor initiation, invasive growth, and dissemination to distant organs [90]. It has been documented that a rare population of CSC is more easily detectable in hematological malignancies than in solid tumors, like for example in leukemia [64]. Indeed, Lapidot and colleagues identified an acute myeloid leukemia cell initiating human acute myeloid leukemia after transplantation into severe combined immune-deficient mice. Such cells that were CD34 positive and CD38 negative, displayed leukemic cell morphology and produced a pattern of dissemination similar to what observed in the original patients [64]. No information about the expression of ABC transporters, in particular of BCRP, was provided in this study for historical reasons. Indeed, the gene coding for BCRP was cloned in 1998 and subsequent studies revealed the expression of ABC transporters (see below).

5.2.3 CSC and the Cell of Origin

A matter of debate in the field of CSC is the relationship between CSC and cell of origin of tumors. It is important to note that a clear distinction between the cancer stem cell and the cell of origin of a tumor has recently been proposed [107]. In this perspective, the cell of origin would be the tumor initiating cell and CSC would be tumor propagating cells. Such distinct cell categories would have different phenotypes. In chronic myeloid leukemia, the cell of origin has been recognized as the hematopoietic stem cell containing the BCR-ABL mutation; however, subsequent genetic lesions in progenitor cells downstream of the hematopoietic stem cell produce leukemia stem cells [107]. Additional evidence has shown that cancer can arise from differentiated cells (e.g. T-cell acute lymphoblastic leukemia) [76]. In particular, the *LMO2* oncogene was reported to induce a subset of human T cell acute lymphoblastic leukemias, by promoting the self-renewal of pre-leukemic thymocytes; thus, committed T cells appear to accumulate additional genetic mutations required

for leukemic transformation [76]. Moreover, whether in solid tumors the stem or progenitor cells appear to be candidates for tumor initiation, the exact mapping of the cell of origin is far from being completed, and further effort is needed in this field.

5.2.4 CSC and Differentiation

The capability of putative CSC to differentiate can be easily proven in specific tumor types, whereas in others it is not clear which differentiation markers should be considered. In fact, tumors present phenotypic plasticity and dedifferentiation properties that may result in changes of markers and molecular features during disease progression. Some diseases provide examples of clearly assessable differentiation markers (e.g., neuroblastoma, melanoma). In this regard, melanospheres containing tumor initiating cells when cultured in differentiating media for the mesenchymal lineages (adipocytes, chondrocytes, osteocytes) were shown to be capable of differentiating into all cell types [84]. In addition to their differentiation ability, like normal stem cells, CSC must be capable of self-renewal, which is the ability to undergo an unlimited number of replicative cycles, while maintaining the stemness properties. The cancer stem cell produces one cancer stem cell and a cell which differentiates by asymmetric division. This phenomenon allows the maintenance of the pool of CSC (Fig. 5.1).

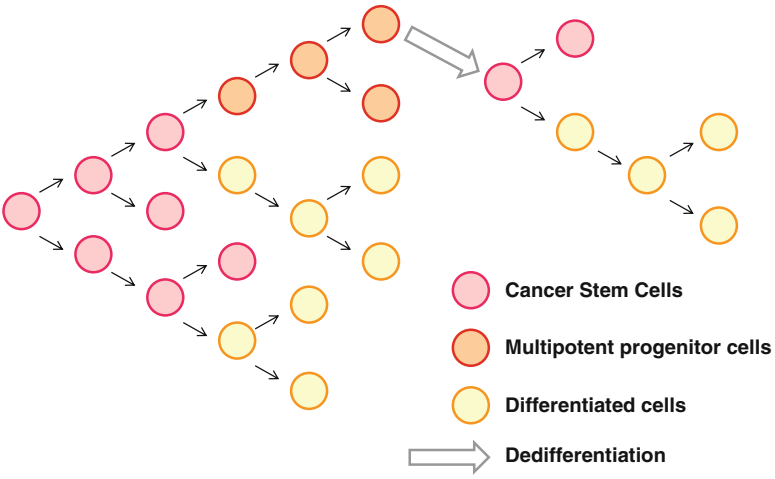

Fig. 5.1 Schematic representation of cell division of cancer stem cells. Cancer stem cells (CSC) can divide symmetrically producing two daughter cells with the same characteristics of the cancer stem cell of origin, or can divide asymmetrically, thereby generating differentiated cells. Over the different rounds of division, cells with decreased stemness features as compared to the cancer stem cell of origin can be generated. Such cells are designated as pluripotent progenitors. Cells that have lost the stemness phenotype can eventually dedifferentiate and reacquire some stemness features

5.2.5 CSC and Metastasis

Increasing evidence supports the notion that CSC play a role in tumor progression and may be responsible for tumor growth as well as for metastatic spread, a multistep process in which epithelial to mesenchymal transition (EMT) occurs [73]. Of note, the process of EMT—in which cells lose the expression of epithelial markers and acquire a mesenchymal phenotype—has been shown to be able to produce cells with stem cell-like features [74]. The coexistence of a stationary phase population embedded in the epithelial tissue that cannot disseminate (stationary CSC) and a migratory population of mobile cells located at the tumor microenvironment interface (migratory CSC) has been proposed [10]. In keeping with a tight relation between EMT and stemness, when tumor cells undergo EMT, a number of properties and processes (invasiveness, drug resistance, angiogenesis, and metastasis) appear to be increased in parallel, thus generating a tumor with more aggressive characteristics. The acquisition of a drug-resistant phenotype is associated with the expression of ABC transporters (see below).

5.2.6 Tumor Initiating Capability of CSC and Markers

The demonstration of the tumor initiating capability is a critical aspect in the CSC field because in vivo experiments are required and the use of immune-compromised mice is hampered by the need for properly equipped animal facilities as well as by the costs. Besides being capable of self-renewal and asymmetric division, CSC should have tumor initiating capability (Fig. 5.2). However, not all the literature published on CSC contains proofs about the tumor initiating capability of the studied CSC. This is the reason why, the designation of cancer stem-like cells should be preferred in general to that of CSC, and specifically in experimental work not including assessment of the tumor initiating capability. Indeed, CSC are very often identified (a) on the basis of the expression of multiple markers (CD24, CD29, CD44, CD133, ALDH1, etc.) that, however, are not necessarily unique to CSC [53, 56, 103] or (b) on their ability to grow independently of anchorage (i.e., as spheres) in serum-free medium added with growth factors. Of note, such growth factors are recognized

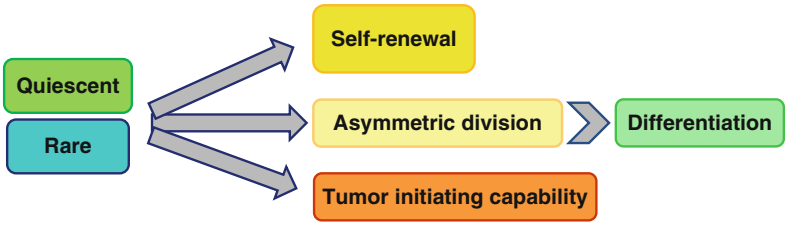

Fig. 5.2 Hallmarks of cancer stem cells. The main features of cancer stem cells (CSC) are represented

as being capable of activating survival pathways, a feature that has rendered complex the set up of comparisons between the growth characteristics of differentiated cells, cultured in serum-containing media, and those of CSC cells cultured in peculiar media, under circumstances often difficult to standardize among different laboratories due to patent issues and/or to complex procedures.

5.3 ATP-Binding Cassette Transporters

Membrane proteins of the ABC super-family have been documented to participate in energy-dependent efflux of a variety of structurally unrelated antitumor agents [26]. Such a phenomenon is known as multidrug resistance and besides overexpression of efflux pumps it can involve other interrelated or independent mechanisms. ABC transporters play a relevant physiological role in protection against xenotoxins. In fact, they decrease the intestinal uptake and tissue penetration representing important physiological barriers (brush border membrane of intestinal cells and epithelium of the blood-brain barrier) and mediate excretion of their substrates [3]. Based on sequence similarity, all ABC transporters can be grouped in seven classes (A–G) and members of at least four classes (A, B, C, G) have been clearly implicated in conferring resistance to antitumor agents [33]. All ABC transporters share an ABC domain, but the organization of the domains is different in various transporters with diverse numbers and location of trans-membrane domains. The structural characteristics may influence folding of the transporter as well as substrate accessibility, thereby regulating transport properties. Among the 49 members of the ABC super-family, different transporters play a major role in multidrug resistance. The first identified transporter for which a contribution to multidrug resistance has been shown is the P-glycoprotein (Pg-p) encoded by the *ABCB1* gene [59]. Pg-p is the best known ABC transporter and is a 170 kDa protein which transports neutral, cationic, and hydrophobic compounds, including antitumor agents commonly used in the clinical setting (anthracyclines, camptothecins, epipodophyllotoxins, Vinca alkaloids). The MRP (multidrug resistance-related protein) family, which comprises ten members has been implicated in conferring resistance to several antitumor agents. The first member of the family, MRP1, has shown transport specificity similar to that of Pg-p, but it transports drugs conjugated with glutathione or anionic compounds [17]. BCRP, the second member of the ABCG family, is encoded by *ABCG2*. It is a 72 kDa protein which transports unmodified drugs and xenobiotics [23]. It is an organic anion pump very efficient in transport of sulfate, glucuronic acid, and glutathione conjugates. ABCB5 codes for a protein of 90 kDa that has been implicated in the efflux of the DNA topoisomerase II inhibitor doxorubicin and more recently in reduced sensitivity to 5-fluorouracil [28, 110].

At the time the above mentioned transporters were identified and cloned, studies regarding the hypothesis on CSC were already ongoing, but the two lines of research were somehow parallel till the SP assay was set up and innovative technical approaches allowed fine molecular studies (Fig. 5.3). The clinical relevance of

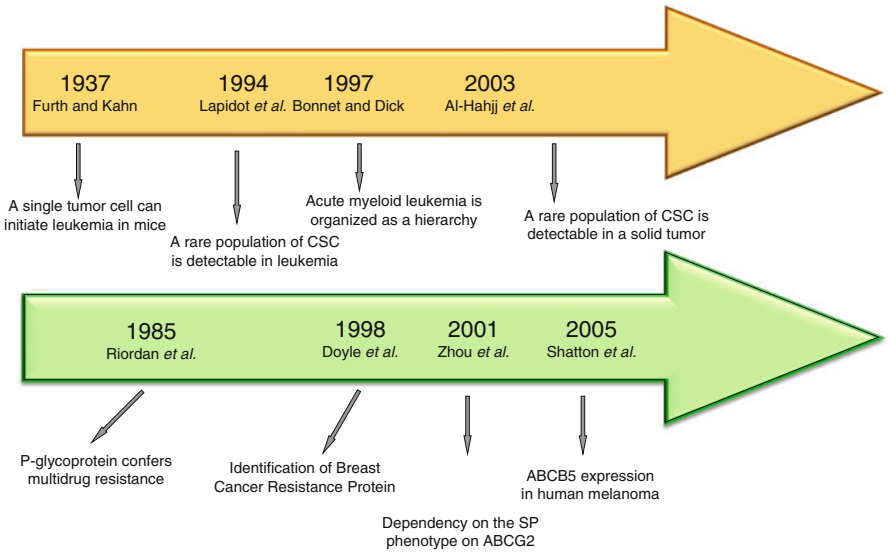

Fig. 5.3 Time frame of research on CSC and on ABC transporters. Some relevant years for research about CSC and ABC transporters are indicated together with the published findings. The two lines of research were parallel and then began being interrelated when the side population (SP) phenotype was described

increased expression of ABC transporters in conferring resistance in patients is still a matter of debate, but it is possible that the ABC transporter super-family plays still not well defined roles in CSC.

5.3.1 Profiling of ABC Transporters

The advent of genome-wide approaches as well as of quantitative methods for examining expression of gene families have allowed researchers to improve the molecular characterization of tumors, with particular focus on drug-resistant tumors. Expression profiling of ABC transporter super-family genes has been carried out both in drug-resistant cell lines and in tumor specimens, including posttreatment tumors [47, 87] to identify genes that are potential regulators of drug resistance or modifiers of progression and/or response. More refined analyses have included an evaluation of specific transporters at the mRNA and protein levels. The concomitant analysis of the transcript and protein appears a wise strategy, if considering the layers of regulation occurring between mRNAs and proteins. In addition to a variable stability of the different mRNA molecules, the regulation by microRNAs (miRNAs) is an important aspect that may explain discrepancies between mRNA and protein levels. In fact, miRNAs, endogenous, noncoding-single stranded RNAs of

19–25 nucleotides that can modulate gene expression, play an important role in regulating different aspect of the biology of CSC (proliferation, differentiation), and are therefore expected to participate in a complex network that also regulates ABC transporters. In general, miRNAs target specific mRNAs, thereby causing their degradation [101]. The levels of several genes of the ABC super-family have been shown to be modulated by miRNAs, which are expected to be involved in CSC biology [33]. Of note, the available literature already supports the complex transcriptional regulation of the ABCG2 transcript that in cellular studies has been reported to be downregulated by various miRNAs such as miR-328, miR519c and miR-520h, miR-487a, miR-181a [33, 71, 66, 58, 97, 109].

An analysis of the expression of ABC transporters has been undertaken also in different models of CSC, as these transporters or at least some of them are considered phenotypic markers of CSC and are regarded as functional regulators. For example, putative prostate stem cells and prostate CSC in benign and malignant tumors have been defined by the expression of BCRP and concomitant lack of the androgen receptor [50]. According to the findings, BCRP may protect prostate CSC from androgen deprivation, hypoxia, or chemotherapy, thus favoring recurrence of prostate cancer [50].

A relevant evidence emerging from the recent literature is the link between the ABC transporter activity and radiation resistance [52]. In this regard, sensitization to radiation was found in pediatric medulloblastoma cells upon treatment with the ABC transporter inhibitors verapamil or reserpine. Of note, radiation tolerant cells displayed stem cell-like behavior (e.g., increased tumorigenic potential). In medulloblastoma specimens, selected ABC transporter super-family members were found to be associated with specific molecular subtypes (high ABCA8 and ABCB4 in Sonic/Hedgehog-driven tumors) [52]. The mechanism for increased ABC transporter expression in radiation resistant cells is not clear, but it is likely that their upregulation results from a stress response, from a pro-survival response, or from activation of regulators of ABC transporters expression. In this regard, it has been shown that both hypoxia and oxidative stress can upregulate or stimulate ABC transporters [54, 95].

In addition, gene expression profiles of normal cells should be taken into account (e.g., melanocyte). In fact, two mRNA isoforms of the *ABCB5* gene, *ABCB5alfa* and *ABCB5beta* have been shown to be expressed in melanoma, but also in melanocytes, their expression being pigment-cell specific, thereby suggesting their possible involvement in melanogenesis [14]. The expression of ABCB5 and other transporters of the ABC super-family has been linked to the resistance of melanoma cells to structurally unrelated drugs, but also to the resistance of melanocytes to toxic intermediates of melanin metabolism, supporting that the melanogenic pathway may provide therapeutic targets [15].

The profiling of ABC transporters in cancer cells including cancer stem-like cells has been simplified by the availability of quantitative Real time PCR platforms providing standardized assays [37]. However, further effort is needed to set up routine high-throughput analysis for detecting protein levels of the specific transporters.

5.3.2 ABCG2/Breast Cancer Resistance Protein

ABCG2 is a gene included in the super-family of ABC transporters that codes for a protein member of the White subfamily. As briefly mentioned above, the protein is referred to as a breast cancer resistance protein and it acts as a xenobiotic transporter. ABCG2 is a widely studied transporter, which has been characterized in terms of substrate specificity and for its role in drug resistance. Its significance in CSC and in normal stem cells (e.g., placental trophoblasts, neural stem cells, hematopoietic progenitors) physiology is mainly related to its expression in side population cells; indeed, its expression is fundamental for the capability of a cell population to give rise to a side population (see below) [118, 25, 55, 61, 93]. ABCG2 expression in human embryonic stem cells has been debated as conflicting results have been published [83]. However, recent results obtained with sensitive methods indicate that ABCG2 may be regarded as a late stage differentiation marker in cultured human embryonic stem cells.

Leukemic CD34 positive and CD38 negative stem cells are considered relevant to cure acute myeloid leukemia as incomplete eradication of these cells may be responsible for disease relapse. BCRP was found to be expressed by these cells [88]. Inhibition of mitoxantrone extrusion by a specific BCRP inhibitor (the fumitremorgin C analog, KO143) produced increased drug accumulation in cells obtained from different patients, but drug efflux still occurred in the presence of KO143, thereby suggesting that additional transporters including Pg-p and MRP1 are expressed by leukemic stem cells. Consistently, KO143 could not increase chemosensitivity of leukemic stem cells. Such a study supports the need for broad-spectrum inhibition of different mechanisms/transporters [88].

Although BCRP was originally identified in breast cancer cells [23], such a transporter plays a role in a variety of tumor types comprising colorectal cancer, brain tumors, etc.

5.3.3 ABCB5

In addition to BCRP, the ABCB5 transporter has been implicated in CSC biology, with particular reference to malignant melanoma; in such a disease ABCB5 has been proposed as a marker of melanoma-initiating cells [94]. Indeed, ABCB5 has been shown to mark CD133-expressing progenitor cells among human epidermal melanocytes, and to positively regulate the propensity of this subpopulation to undergo cell fusion, a process contributing to culture growth and differentiation [29]. ABCB5 has also been involved in doxorubicin efflux transport and it has been already exploited as a therapeutic target by the development of a specific antibody [28].

High ABCB5 expression has been recently associated with progression of oral squamous cell carcinoma and tumor recurrence [44]. Interestingly, double labeling immunofluorescence and immunohistochemistry experiments indicated that

ABCB5 was expressed by CD44 positive cells. Unfortunately, in this study, there was no in vitro or in vivo characterization of the stem cell properties of the ABCB5 positive cells. Thus, although the results are statistically sound as ABCB5 was an independent prognostic factor in multivariate analyses, further studies will be needed to establish if such cells are endowed with features of CSC.

ABCB5 positive melanoma cells have been shown to be targeted by parthenolide, a natural sesquiterpene lactone described as an NF-kB inhibitor, endowed with anti-microbial, anti-inflammatory and anticancer effects [19]. Of note, cell survival after treatment exhibited an immunophenotype different from that of control cells. In spite of its limited penetration capacity, parthenolide could target both CSC like cells and bulk tumor cells [19].

Recent evidence suggests that ABCB5 together with CD133 play a critical function in promoting vasculogenic mimicry and the morphogenesis of the perivascular niche in melanoma [63]. In fact, loss of function approaches based on RNA interference could prove that knockdown of CD133 produced an impairment in the cell ability to form vascular mimicry-like channels, a phenomenon associated with the depletion of the ABCB5 positive population [63]. Thus, co-expression of CD133 and ABCB5 in melanoma cells seems to be important for the generation of a vascular-mimicry-dependent perivascular niche, although the specific role of each one of the markers, in particular that of ABCB5, is not clear. Based on the available data, it is uncertain whether the transport capability of ABCB5 is used by CD133/ABCB5 double positive cells to regulate the content of the cellular or extracellular level of molecules which regulate vascular mimicry [63]. This process appears complex and has been shown to involve VEGF-A signaling which stimulates the expression of vascular mimicry associated genes such as *CD144*, a marker reported to be preferentially expressed by ABCB5 positive cells in colorectal cancer [110]. In this context, ABCB5 appeared to be responsible for resistance to 5-fluorouracil. ABCB5 was expressed only on rare cells within normal intestinal tissue, whereas increased levels of ABCB5 were found in colorectal cancer specimens. The abundance of ABCB5 positive cells appeared increased after treatment in residual disease. Thus, ABCB5 has been proposed as a novel molecular marker of therapy-refractory tumor cells in colorectal cancer patients. Targeting of ABCB5 positive cells is proposed to eradicate such tumors. Moreover, additional evidence is available in melanoma where, by highlighting the role of the immune system in tumor progression, it has been shown that ABCB5 positive melanoma initiating cells induce T regulatory cells via a B7.2-dependent pathway [94].

In keeping with a relevant role for ABCB5 in the biology of CSC and with the drug-resistant phenotype of melanoma cells, melanoma chemotherapy has recently been shown to lead to the selection of ABCB5-expressing cells [12]. In addition, increased expression of the ABCB5 protein from benign nevi to invasive melanoma has been reported in a study in which immunohistochemistry was used [96]. ABCB5 should not be regarded, however, as a transporter playing a role only in melanoma, because an evaluation of its expression in hematological malignances suggested that it may be involved in both the progression and the resistance of acute leukemia [112].

5.3.4 ABCB1/P-Glycoprotein

Breast cancer stem cells have been reported to express high levels of Pg-p [21]. A recent report suggests that the commercially available anti-alcoholism drug disulfiram may be useful in reversing drug resistance of CSC by virtue of its pleiotropic effects on factors expressed by CSC [67]. In fact, disulfiram has been shown to produce persistent inhibition of Pg-p activity by covalent modification of cysteine residues localized in the nucleotide binding domain of the transporter [70]. Besides this effect, disulfiram is capable of inhibiting the activity of ALDH, a marker for CSC [38, 79, 102]. Of note, in a triple negative drug-resistant breast cancer cell line endowed with CSC features (slow cycling, high transporter expression, high levels of embryonic stem cell markers), disulfiram was shown to target CSC characteristics leading to reversal of resistance [67]. This evidence supports the value of drugs that are already available and that may fit with a drug repositioning program.

In addition, although the relevance of Pg-p in conferring resistance in the clinics is still reported as uncertain, likely because in real tumors overexpression is not easily achieved like in cultured cells [9], the clinical relevance of Pg-p might be linked, in principle, to its expression in selected subpopulations of tumors cells present in the tumor (e.g., CSC), that may finally underlie recurrence. Further studies are needed to clarify these aspects.

5.3.5 Multidrug Resistance-Related Protein 1 (MRP1)

MRP1 has also been shown to be increased in SP cells [118], although it does not appear to be a major determinant of the SP phenotype (see below, Sect. 5.4.1). Thus, BCRP appears to be the most relevant determinant of the SP phenotype, but other transporters expressed by SP cells may cooperate with it to efflux drugs, thereby underscoring resistance. Specific inhibitors of each one of the transporters should, therefore, be used to better examine if more than one SP phenotype exists and which transporters are implicated.

5.3.6 ABCA5

Using an approach based on multiple markers, it has been shown that osteosarcoma cell populations enriched for putative CSC are characterized by high ABCA5 expression. Of note, in this study, ABCA5 was proposed as a putative biomarker of CSC [91].

Although ABCA5 is still a poorly understood transporter, its correlation with the differentiation state has been reported in human colon cancer, thereby suggesting a possible role in the CSC biology [82]. Of note, in the same study also ABCB1 was shown to be correlated with the differentiation state, a phenomenon that may be an indicator of common regulation.

5.3.7 *Regulation of the Function of Drug Transporters*

Increasing evidence supports that cellular survival pathways, in particular the PI3K/Akt pathway, play a role in the biology of CSC. The PI3K/Akt pathway has been shown to be important for maintaining the pluripotency of embryonic stem cells [4]. Indeed, transcriptional analysis and a functional assay have shown that PI3K/Akt together with the MAPK/ERK and NF-kB pathways are down regulated during differentiation of these cells [51]. The PI3K/Akt pathway has been proposed to be required in the maintenance of CSC in the brain, breast, prostate cancer, and glioma [116].

The regulation of CSC by the PI3K/Akt pathway is supported by a variety of studies pointing out (a) a reduced SP cells in the bone marrow of Akt1-null mice, and (b) increased SP cells in the bone marrow of mice following enforced expression of Akt. Moreover, inhibition of the PI3K/Akt pathway has been associated with BCRP internalization, a phenomenon that suggests a regulation of distribution of BCRP by the PI3K/Akt signaling. The precise mechanisms involved are still poorly understood [51]. The relationship between cell survival pathways and ABC transporters is also supported by the association between ABCG2 and HER-2 expression in breast invasive ductal carcinoma [111]. Of note, the Hedgehog pathway had been previously shown to regulate also ABCB1 besides ABCG2, although the molecular determinants of the regulation were less characterized; the pharmacological relevance of the Hedgehog pathway inhibition was proved as its targeting reversed resistance to structurally unrelated antitumor agents [98]. Activated Hedgehog signaling has been implicated in sustaining high ABCG2 expression in diffuse large B cell lymphoma, a disease in which high expression of this transporter was shown to inversely correlate with disease-free survival [99]. A molecular analysis indicated that the ABCG2 promoter contains a binding site for the GLI1 transcription factor; since high ABCG2 and GLI1 expression was found in tumors with lymphnodes involvement, it has been proposed that the stroma microenvironment might regulate ABCG2 and GLI1 [99]. This hypothesis was supported by in vitro assays including coculture experiments in which tumor cells upregulated ABCG2 as a result of stroma cell-induced Hedgehog signaling. In this experimental model, ABCG2 was not the only resistance factor induced by Hedgehog signaling as also anti-apoptotic proteins were upregulated, but ABCG2 was characterized in detail in terms of the transcriptional regulation [99]. An indirect regulation of ABC transporters levels by E2F involving p73 has also been described [2], further corroborating the view of a complex regulatory network acting to favor the survival of CSC.

5.4 The Side Population

ABC transporters are not straightforward markers for CSC, but it has been documented that CSC can express ABC transporters. This characteristic is shared by CSC and normal stem cells. Both normal and CSC can be identified in the so called SP in a dot plot from flow cytometry analysis. In fact, the term “side” refers to the position at the side of the plot. The isolation of SP cells has been carried out and

described from different types of normal tissues including the bone marrow and tumors. Indeed, a small fraction of bone marrow cells that can be evidenced by flow cytometry for the ability to efflux the fluorescent dye Hoechst 33342 and are enriched for HSC, has been identified in the hematopoietic compartments of different organisms including humans and in non-hematopoietic tissues [42, 104]. SP cells from murine bone marrow can self-renew and generate both lymphoid and myeloid lineages [42]. Normal HSC express at least two ABC transporters, but the complexity of the ABC transporter family suggests that other members could be present [100]. Studies carried out in mice more than a decade ago have indicated that BCRP, but not MDR1, is responsible for the HSC phenotype [104, 117, 93]. Accordingly, in Abcb1-knockout mice, the SP is not depleted [11]. Expression of the murine orthologue of ABCG2 appears a constant feature of murine stem cells from different sources such as bone marrow, skeletal muscle, primary mammary tissue, and embryos. In murine HSC, Abcg2 is highly expressed and is downregulated during differentiation. The dependency on Abcg2 of the SP phenotype has been clearly defined by gain and loss of function studies [118].

Moreover, flow cytometry approaches applied to the CSC field have allowed researchers to define the existence of a tumor cell fraction that is enriched for drug efflux transporters, specifically ABC transporters. Again, this tumor cell population is functionally defined based on its capability to extrude the specific fluorescent dye Hoechst 33342, an activity that produces a shift of the fluorescence of the cells belonging to this population in a dot plot obtained by flow cytometry analysis. In particular, cells expressing ABC transporters recognizing the fluorescent dye, decrease their fluorescence becoming clearly separated from the rest of the cells. In this kind of assay, verapamil, a calcium channel blocker which binds with high affinity to Pg-p and with less affinity to BCRP, is used, as the SP disappears upon transporter inhibition in the presence of verapamil. Thus, the SP is identifiable as reported in Fig. 5.4. It is important to note that evaluation of the SP is not an easy procedure and should be carried out with a well set up protocol and adequate gating procedures.

The SP assay is routinely carried out in the presence of verapamil, a well known inhibitor of ABCB1. This is surprising as the SP phenotype is thought to be due to the expression of ABCG2. However, verapamil can also inhibit, although less strongly, ABCG2. Indeed, it has been shown that in bone marrow cells, ABCB1 contributes in part to the SP phenotype [118].

A causal link between the expression of ABCG2 and SP has been proposed in a report on leukemia in which over-expression of ABCG2 in Jurkat and HL60 cells was shown to increase the SP; such a phenomenon was concomitant with upregulation of the phosphorylated forms of PI3K and Akt [49]. Conversely, treatment with PI3K or Akt inhibitors downregulated ABCG2 expression, phospho-PI3K, phospho-Akt and SP. Activation of Akt appears to occur via inactivation of PTEN, a lipid phosphatase which has been implicated in preventing leukemogenesis [114]. Thus, ABCG2 regulation by the PI3K/Akt pathway appears a likely phenomenon in leukemia, similarly to what was described in glioma and esophageal cancer [7, 65].

SP cells from bladder cancer were found to be characterized by increased levels of ABCG2 together with phospho-ERK1/2 activation [46]. Accordingly, inhibition of MEK1/2, the upstream regulator of ERK1/2, resulted in inhibition of the SP phenotype.

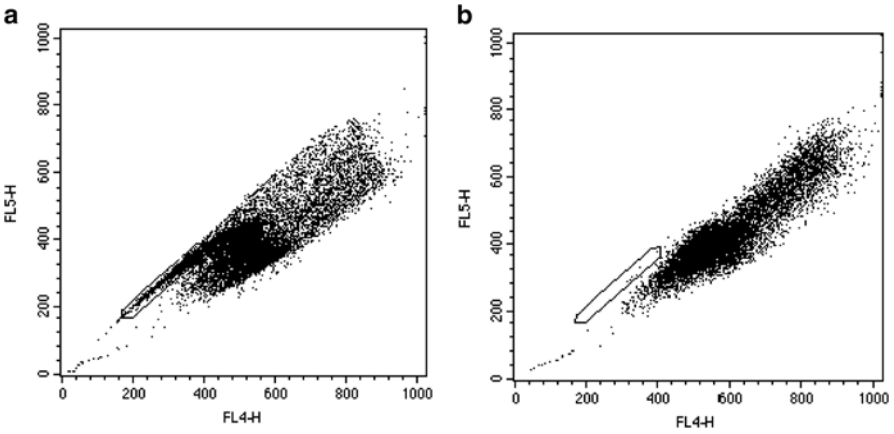

Fig. 5.4 Representative plots of side population. The rat C6 glioma cells were used and incubated with Hoechst 33342 (**a**, **b**). The so-called side population evidenced in the gate in plot **a** displays low fluorescence because ABC transporters efflux the dye. If transporters are blocked by verapamil (plot **b**), the dye is not extruded and the cell fraction which expresses ABC transporters kept inactivated retains the fluorescence of the main cell population. Viable cells were gated and blue (FL-4) and red (FL-5) fluorescence of viable cells are reported. The shown plots are a courtesy of Dr. Emilio Ciusani (Fondazione IRCCS, Istituto Neurologico C. Besta)

An important finding of this study is the observation that, in tumor specimens, ABCG2 and pERK1/2 were positively correlated and their expression correlated with decreased progression-free survival [46].

The application of the SP approach to the study of CSC has some limitations for subsequent analysis of the tumor initiating capability of the non-SP cell fraction. Such a fraction that in principle should be devoid of tumor initiating capability by virtue of its intrinsic nature, i.e. the lack of stemness, may result devoid of the capability due to the fact that it is treated with a fluorescent dye in the SP assay. In fact, because the dye is a DNA binding agent, it might finally affect the proliferative potential of the non-SP cells simply because it targets the DNA (Fig. 5.5). Again, the use of SP an indicator of stemness should be regarded with caution. Indeed, it has been reported that not all SP populations diplay increased tumorigenic potential as compared to non-SP cells [113].

An important step in the study of the SP has been represented by the isolation of SP cell from biopsies [40]. Using glioblastoma samples grown orthotopically in immune-deficient mice, SP cells of human glioblastoma were found to be stroma-derived and nonneoplastic [40]. Indeed, tumor cells did not exhibit efflux properties which were present in brain-derived endothelial cells and in astrocytes.

In summary, a SP fraction has been demonstrated in different tumor types and the SP has been shown to be endowed with tumor initiating ability. SP cells can divide asymmetrically, generating SP and non-SP cells, and can form spheres when grown in serum-free media. Due to the toxic effects of the dye used in the SP assay, the identification of CSC by the SP assay has major limitations.

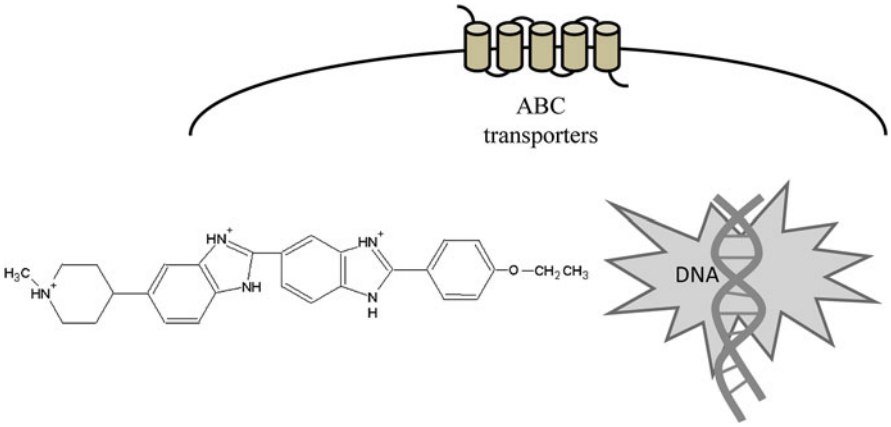

Fig. 5.5 Chemical structure of the fluorescent dye Hoechst 33342 and its mechanism of action. The dye is a DNA targeting agents which binds the minor groove of DNA. In the side population assay, it accumulates in viable cells, when not effluxed by ABC transporters

5.4.1 Approaches to Modulate the Side Population

Various strategies are being employed at the cellular level to hit the SP in an attempt to discover therapeutic options selective for CSC. Some examples taken from the recent literature are provided below. It has to be considered when examining the literature available on targeting CSC by interference with the SP that, although several of the preclinically tested compounds display an effect on ABC transporters, a direct cause-effect relationship between treatment and ABC transporter inactivation or downregulation cannot always be defined.

A promising approach has exploited the capability of CSC to efflux dyes and to be identified as SP in a high-throughput screening platform in which hit compounds were selected based on decrease of SP after treatment. Thus, in an attempt to hit CSC in breast cancer, in a recent study [41], a combination of an inhibitor of NF-kB (IMD-0354) and nanoparticle-encapsulated doxorubicin has been employed. A reduction in the SP and in ABC transporters (ABCB1, ABCG2) was associated with a decrease of self-renewal genes (*Oct4*, *Sox2*, *Nanog*). The NF-kB inhibitor produced cell death also in non-CSC cells. Of note, targeted delivery to hypoxic cells could be achieved, a feature that allowed the administration of a well tolerated treatment as normal nonhypoxic tissues were spared.

It has been recently reported that low-molecular weight heparin (LMWH), which is approved for anticoagulant therapy, can inhibit survival of lung cancer SP cells, as it decreases their colony forming abilities [81]. Interestingly, it also decreases ABCG2 protein levels by interference with its proteasomal degradation; in fact, LMWH-induced ABCG2 downregulation could be rescued by proteasome inhibition. Treatment with LMWH has been reported to ablate lung cancer cisplatin resistance [81]. However, a clear synergistic interaction between cisplatin and LMWH

could not be proven, in keeping with the fact that cisplatin is not a substrate for ABCG2. Thus, it is likely that the combination of cisplatin and LMWH is additive although, in principle, cells downregulating ABCG2 may display reduced fitness when treated with a variety of antitumor agents, even if not substrates, due to the possible transport by ABCG2 of molecules indirectly affecting the cell response to drugs [85]. Among the compounds recently tested on CSC-like models, secalonic acid D, the main toxic metabolite of several strains of *Penicillium oxalicum*, has shown antiproliferative activity on tumor cells over-expressing ABCB1, ABCC1, and ABCG2 as well as its capability to decrease SP cells in lung cancer cells [48]. Modulation of ABCG2 mRNA levels may occur via epigenetic events induced by pharmacological treatments. For example, the indolamine melatonin which contributes to regulate endocrine functions and has been reported to exhibit cytotoxic and antioxidant effects, appears capable to induce methylation of the ABCG2 promoter [75]. Such a phenomenon—which is prevented by an inhibitor of DNA methylation—has been proposed to underscore the synergism observed between melatonin and antitumor agents in brain tumor stem-like cells [75].

The search for drugs selectively killing CSC has lead to the identification of promising compounds which, however, under most circumstances, are endowed with their activity versus CSC and bulk tumors. Most of the compounds have been tested in preclinical studies, mainly in vitro, and only a fraction of the tested compounds are proposed to act by virtue of their interference with ABC transporters. Among them, salinomycin, a polyether ionophore antibiotic isolated from *Streptomyces albus* has shown promising results [80]. Such a compound known to be endowed with antibacterial activity was shown to be capable of killing CSC in a murine model of breast cancer [45]. Subsequently, additional studies reported that this biomolecule can kill a variety of human tumor cells [32], thereby providing evidence that it acts both on CSC and the tumor bulk [31]. The drug is already undergoing clinical evaluation [80].

5.5 Markers for CSC Other Than ABC Transporters

Several markers are used to identify CSC in different tumor types, and the available literature suggests unexpected links between some of these markers and ABC transporters. To identify such links, the concomitant expression of ABC transporters and of other markers has to be taken into account. For example, aldehyde dehydrogenase 1 (ALDH1) is a NAD(P)-dependent enzyme which detoxifies endogenous or exogenous aldehydes [38, 56] because it has been implicated in the physiology of normal and CSC, ALDH1 is being used as a marker. Indeed, since the SP allows a functional identification of stem cells, ALDH1 activity has been proposed as a functional marker of potential interest in different tumor types. In 1990, Kastan and colleagues showed that ALDH displays increased activity in human hematopoietic progenitor cells [60]. Since then, a variety of studies have reported the isolation of stem cells from normal and cancer tissues on the basis of ALDH activity [56].

Recent studies have shown that ALDH1 appears to be an appropriate marker for stemness also in human sarcomas [69]. Indeed, the subpopulations characterized by high ALDH1 activity are also endowed with increased proliferation rate, colony forming ability, increased expression of ABC transporters and stemness markers, as well as by reduced sensitivity to antitumor agents as compared to cells with low ALDH1 activity [69].

The concomitant expression of ALDH1 and ABCG2 or other transporters in CSC appears to support the need for the presence in CSC of multiple independent mechanisms for detoxication. Indeed, because stem cells are rare it is reasonable that they try to activate a complex pro-survival response in which different factors can eventually cooperate to improve chances of survival.

A very interesting study has shown that ABC transporters can be transcriptionally regulated by the transcription factor Oct1, which is fundamental in self-renewal [72]. Thus, in drug-resistant cells, there are genes that act as hubs by coregulating multiple processes finally leading to drug resistance. For example, exposure of breast cancer cells to TGF beta or Twist over-expression have been shown to lead to enhanced expression of ABC transporters [105]. Conversely, knockdown of Twist and Zeb, besides reversing EMT, also results in reversal of drug resistance [73]. In addition, a positive correlation between ABCG2 and Oct4 has been reported in cellular models of liver CSC, in which the efflux transporter and the transcription factors involved in self-renewal appeared to be highly expressed in CD90/CD133 positive cells [57].

5.6 Clinical Implications of the CSC Hypothesis

The translation of the CSC hypothesis toward the clinics is far from being accomplished also in view of the skepticism regarding the biology of CSC. There is a wide heterogeneity in the experimental models used for CSC, especially in vitro, where research for appropriate 3D culture systems is still ongoing [13]. In spite of this, some clinical studies may already offer positive results in terms of validation of the CSC hypothesis in the clinical setting. In fact, as recently reviewed [36], enrichment for tumor cells with a CSC phenotype has been reported in minimal residual disease of different tumor types. Thus, the CSC hypothesis may explain why patients cannot be cured in spite of initial responses; some studies support this concept. For example, in breast cancer, residual tumor cells after conventional treatment have been shown to display tumor initiating cell features [18]. In addition, Huff and colleagues have shown that a correlation between clonogenic growth of CSC and clinical outcomes occurs in multiple myeloma [36]. The expression of a stem cell phenotype by minimal residual disease in acute myeloid leukemia has also been documented [34].

The association between breast cancer stem cells and resistance to paclitaxel-epirubicin based chemotherapy has been reported in a case material of primary breast cancer patients [102]. In such a study, breast CSC identified as ALDH1-positive, but

not CD44 positive/CD24 negative cells, have been proposed to play a role in resistance to chemotherapy. This study underscores the variability that may result from considering different markers. Although it is likely that ALDH positive cells express ABC transporters, this aspect was not considered in the study.

Many of the ongoing clinical trials in which ABC transporters relevant in the CSC biology, in particular ABCG2, have been taken into account, deal with pharmacokinetics and pharmaco-dynamics issues in an attempt to establish the role of single nucleotide polymorphisms of drug transporters in the efficacy of therapies based on the use of substrates of transporters. Moreover, a certain number of trials focuses on the assessment of the feasibility of isolation and characterization of CSC (NCT01641003), on the set up of reliable drug sensitivity assays (Feasibility Study on Stem Cells Sensitivity Assay, STELLA, NCT01483001), and on the characterization of CSC of different tumor types. A few studies are already directed at evaluating the anti-cancer stem cell activity of treatment, for example by measuring the amount of ALDH1 positive cells before and after treatment (NCT01190345), or in an attempt to target CSC for prevention of relapse (NCT01579812). When the results of these clinical studies will be available, it will be possible to consider the opportunity to translate positive achievements to the routine clinical analysis, following further validation in further studies.

5.7 Discussion

In spite of the technical progress and of the intellectual knowledge acquired in the molecular characterization of tumors and in the processes leading to tumorigenesis, multidrug resistance still represents an obstacle to the cure of cancer. ABC transporters are implicated in multidrug resistance of tumor cells mainly because of their capability to extrude toxic compounds including antitumor agents from cells, but also through indirect mechanisms as recently reviewed by Fletcher and colleagues [26].

If CSC are indeed the tumor cells that maintain the tumor and they express ABC transporters, a successful therapeutic option tempting to cure patients or at least to improve disease-free survival should include drugs targeting transporters of the ABC super-family, in particular ABCG2, because the product of this gene is the most frequently reported transporter in CSC. However, it is evident that the expression of ABC transporters is not an exclusive characteristic of CSC, as their expression in tumors might also be related to the tissue of origin of the tumor. In addition, also normal stem cells express ABC transporters. Thus, a wise therapeutic strategy would need to spare normal cells. Accordingly, selectivity of therapies remains an important issue and there is an effort towards testing of selective approaches at the preclinical level, like for example in a study in which targeting of hypoxic cells was undertaken [41].

The effects of natural compounds on cancer stem-like cells have been recently reviewed, highlighting the variety of pathways that can be targeted in an attempt to kill CSC [24, 68]. Different pathways including self-renewal pathways, Wnt/β--catenin, Sonic Hedgehog, and Notch signaling are implicated in the biology of CSC

and they can favor high self-renewal potential, survival, invasion and the metastatic behavior of CSC and their progeny [78, 106]. Thus, the expression of ABC transporters is only one of the mechanisms by which CSC evade the effects of therapies (Fig. 5.6). Because ABC transporters do not appear to be simply in charge of efflux of drugs, but they participate in a complex molecular network in which other mechanisms, in part even coregulated mechanisms, contribute to cell survival, the inhibition of their function could be regarded as a sort of multi-targeting strategy. Indeed, it has been reported that transcription factors that participate in EMT can positively regulate ABC transporters [72, 92]. Moreover, it has been reported that the PI3K/Akt pathway can modulate the function of transporters through different mechanisms [51]. In addition, in keeping with the wide current interest in metabolic alterations of tumor cells, SP cells have been shown to exhibit increased glycolytic activity than non-SP cells [68].

In summary, research on CSC is a fast-moving field, but translation of results in the clinics is still at an early stage. Additional studies are required to establish a precise link between expression of BCRP or other ABC transporters and stem cell-like features and behavior. Prospective studies are required to establish the utility of less characterized transporters as therapeutic targets for CSC. The evidence that protection of CSC against drugs and toxins is mediated by expression of several ABC transporters continues to provide therapeutic opportunities to overcome resistance. However, it is true that a careful consideration of the specific literature should be made when facing the field of CSC, also considering that CSC have been correctly designated as a moving target [27]. With specific reference to the analysis of levels of ABC transporters in addition to modulation of mRNA levels, also protein levels should be considered. Again, not only ABCG2 should be taken into account, given the complexity of the ABC super-family. In an attempt to generate experimental models for studying CSC, enrichment for CSC by therapy has been proposed [30]. However, this approach cannot be exclusive, but it should be complementary to the others reported above. With such caveats and trying to control the complexity of the biology of CSC, it will be easier to establish also the predictive and prognostic significance of CSC, as recently proposed for Non-small Cell Lung Cancer [43, 86]. The complexity of the ABC transporter super-family suggests that it will be a difficult task to clearly define the specific role of ABC transporters in CSC biology and resistance. The role of ABCG2 as well as of a few other transporters has been in part defined, but we are far from effective strategies to target them for modulation of antitumor therapy.

In conclusion, among the transporters involved in CSC biology, ABCG2 appears to play a remarkable role, because it is expressed by SP cells, and its expression is associated with the activation of cell survival pathways and with the expression of self-renewal genes in specific models of CSC. Indeed, BCRP is very likely to play relevant physiological functions because of its expression by normal stem cells and by CSC or cancer stem-like cells. In addition, the less known ABCB5 transporter, besides playing a role in melanoma resistance, may be of relevance also in colon carcinoma and leukemia (see above). It is therefore expected that CSC-related research will provide knowledge useful for the development of novel therapeutic strategies involving targeting of ABC transporters. Even if the contribution of different transporters to drug resistance of CSC remains to be clarified, it is evident

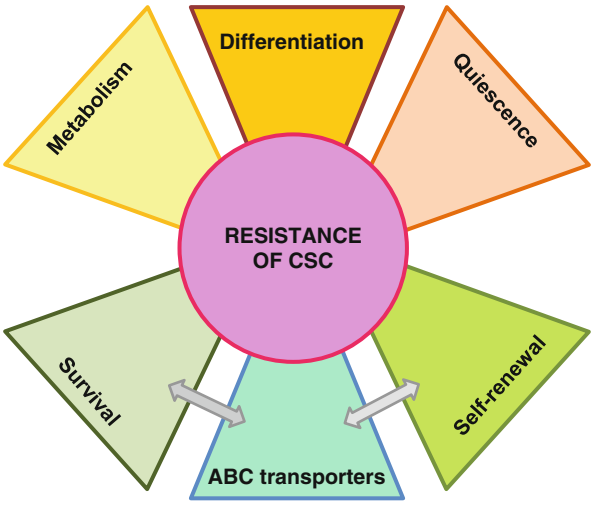

Fig. 5.6 Multifactorial nature of the drug-resistant phenotype of cancer stem cells. Some characteristics of cancer stem cells (CSC) that can underlie their drug-resistant phenotype are shown. The increased expression of ABC transporters is one of the drug resistance mechanisms of CSC that could produce resistance to drugs of different classes. ABC transporters can be regulated by survival pathways and transcription factors acting in self-renewal can up-regulate ABC transporters

that a lot of information about transporters is already available from the in vitro and in vivo preclinical studies carried out using cell cultures or murine models and xenografts. For instance, the mutational status of specific transporters can affect their interaction with substrate and the reversal activity of all modulators may be influenced by the gene status of the transporters. An efficient targeting of CSC will be possibly achieved also considering the complexity of the tumor niche and of all the processes favoring the maintenance and survival of CSC [35, 62].

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Chapter 6
Radiopharmaceuticals for the Imaging of ABC-Transporter-Mediated Multidrug Resistance in Cancer

Sabina Dizdarevic and Adrien Michael Peters

Abstract ATP-binding cassette (ABC) transporter proteins, the most widely studied of which is P-glycoprotein (P-gp), function to translocate a wide range of xenobiotics across biological membranes, especially those exposed to the external environment. Radiopharmaceuticals that are substrates for ABC-transporter proteins are used to image their expression. They can be divided into single photon-emitters, especially technetium-99 m, and positron emitters, e.g. carbon-11 and fluorine-18. Tc-99m-MIBI has been most frequently used to image ABC transporters. Following passive diffusion into cytoplasm, it accumulates in mitochondria at a rate dependent on tissue perfusion and cellularity. Tissue retention of Tc-99m-MIBI correlates inversely with P-gp expression and can be modified with P-gp antagonists. Inter-individual variability of P-gp expression is linked to C3435T polymorphism of the human *ABCB1* gene. C/C, T/C and T/T genotypes are associated with increased, intermediate and low P-gp expression, respectively. Functional up-regulation of ABC proteins results from exposure to inhibitors, resulting in acquired multi-drug resistance (MDR). Tc-99m-MIBI has been used for imaging MDR in several cancers. Early compounds developed to reverse MDR have not shown any benefit in patient

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outcome due to toxicity or interactions with chemotherapeutic agents. Newer MDR modulators, however, are less toxic, more specific for P-gp and do not interact with anti-cancer drugs. Combinations of chemotherapeutics to reverse MDR with tumour targeting agents are being evaluated. In conclusion, clinical trials optimally tailored to tumour types, genetic polymorphism and adequate dosing regimens need to be conducted and based on imaging for selecting patients whose cancers express MDR primarily though ABC-mediated mechanisms.

Keywords Radiopharmaceuticals • ABC-transporters • P-glycoprotein • Positron emission tomography • Single photon emission tomography • Cancer • Imaging

Abbreviations

ABC-transporters	ATP-binding cassette transporters
MDR	Multi drug resistance
P-gp	P-glycoprotein
PET	Positron emission tomography
SPECT	Single photon emission tomography
MIBI	Tc-99m-hexakis-methoxy-isobutyl isonitrile

6.1 Introduction

ATP-binding cassette (ABC) transporter proteins belong to the largest transporter gene family and are so called because they generally use energy derived from ATP hydrolysis for translocation of their substrates across biological membranes. They are classified into seven sub-families based on phylogenetic analysis and designated as ABCA to ABCG [1]. ABC proteins that confer drug resistance include P-glycoprotein (P-gp; gene symbol *ABCB1*), the multidrug resistance protein 1 (MRP1, gene symbol *ABCC1*), MRP2 (gene symbol *ABCC2*), and breast cancer resistance protein (BCRP; gene symbol *ABCG2*).

Collectively, these proteins are capable of transporting a large and chemically diverse range of toxins that include bulky lipophilic cationic, anionic and neutrally charged molecules, many drugs in routine clinical use, as well as conjugated organic anions, that encompass dietary and environmental carcinogens, pesticides, metals, metalloids, and lipid peroxidation products [2].

P-gp is the most widely studied transporter protein. It is a 170-kDa plasma membrane protein that serves as an energy-dependent adenosine-5′-triphosphate (ATP) efflux pump [3]. It has been termed a molecular “hydrophobic vacuum cleaner” because it extracts substrates from the membrane and expels them to promote multiple drug resistance [3, 4]. By protecting tissues from toxic xenobiotics and endogenous metabolites, P-gp fulfils an important physiological role. Along with other

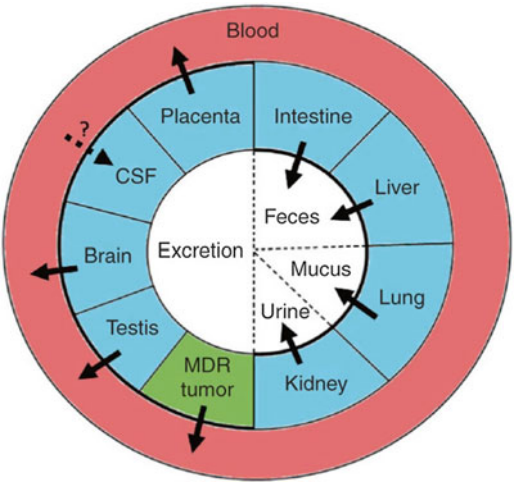

Fig. 6.1 Direction of substrate transport by P-glycoprotein (P-gp) located in various organs of the human body. The *bold solid arrows* indicate the known direction of transport, whereas the *broken-line arrow* indicates unclear direction of transport. P-gp is located in the lipid bilayer (*thick black line*) that forms a barrier between various organs; *red* indicates vasculature, *blue* represents tissue, and *white* indicates excreta. Reprinted with permission from Kannan P, John C, Zoghbi S, Halldin C, Gottesman M, Innis R, et al. Imaging the Function of P-Glycoprotein With Radiotracers: Pharmacokinetics and In Vivo Applications. Clin Pharmacol Ther. 2009; 86(4): 368–377 (Fig. 2) modified from Szakács G, Paterson JK, Ludwig JA, et al. Targeting multidrug resistance in cancer. Nat Rev Drug Discov. 2006;5:219–234

proteins in the family, it regulates the transport of various structurally unrelated substrates, such as anticancer agents and toxins [5].

Many tissues express P-gp physiologically (Fig. 6.1) [6, 7], including the broncho-pulmonary epithelium, hepato-biliary epithelium, renal tubular epithelium, gastro-intestinal tract, blood–brain barrier and choroid plexus. P-gp in the apical border of foetus-derived epithelial cells faces the maternal circulation and is therefore optimally placed to protect the foetus against toxins [8]. So it can be appreciated that these tissues share the common property of a strategic location where they protect against the passage of xenobiotics from the external environment. P-gp is also expressed in cells of the haematopoietic system, including natural killer cells, antigen-presenting dendritic cells, human peripheral blood mononuclear cells (PBMCs) and subpopulations of T and B lymphocytes, implying diverse physiological and pharmacological roles [8, 9].

In addition to their role in drug resistance, there is compelling evidence that in tissue defence, ABC transporter proteins have overlapping physiological functions. Single nucleotide polymorphisms (SNPs) in ABC drug-efflux pumps may play a role in responses to drug therapy and disease susceptibility. The effects of various genotypes and haplotypes (combinations of single nucleotide polymorphisms) on the expression and function of these proteins are not yet entirely clear [10].

6.2 Radiopharmacueticals for the Diagnosis of Multidrug Resistance

Radiopharmaceuticals that are substrates for ABC transporter proteins can be used to image their expression. Radiopharmaceuticals for the diagnosis of ABC-transporter-mediated multidrug resistance in cancer can be divided into single photon agents, generally labelled with technetium-99m (Tc-99m), and agents that emit positrons, generally labelled with carbon-11 (C-11), gallium-68 (Ga-68) or fluorine-18 (F-18). They share the general functional property of being substrates or inhibitors for ABC transporters.

The term 'radiopharmaceutical' implies the association of a radionuclide with a pharmaceutical. The biological properties of the pharmaceutical determine the tissues in the body that are targeted by the radiopharmaceutical, while the radionuclide emits single photons or positrons that are externally detected and used to construct a functional image. Images are constructed from the detection of single photons using planar gamma camera imaging or single photon emission computed tomography (SPECT), and from the detection of positrons using positron emission tomography (PET).

6.3 SPECT

A radioactive element has an unstable nucleus which when it emits a neutron and/or proton becomes a new element. The disintegration process is accompanied by the emission of electromagnetic radiation (photons) of a specific energy measured in KeV. Most radioactive elements used for routine imaging emit single gamma photons as discrete events in association with nuclear disintegration. Such photons can be detected using equipment that comprises firstly a transparent crystal, which absorbs the photon, and an array of photomultiplier tubes that detect the resulting flash of light (scintillation) and its location in the crystal that is imparted by the photon when its kinetic energy is converted into visible light within the crystal. Gamma cameras get their name by analogy with light cameras, because visible light is replaced by gamma radiation. The focusing device of a gamma camera is its collimator, a lead disc perforated with hundreds of parallel holes of sufficient length-to-radius ratio to permit the passage only of photons travelling perpendicular to the collimator face. Like light cameras, gamma cameras generate 2-dimensional images. However, by rotating the camera slowly around the subject and obtaining an image every 6° of rotation, 3-dimensional images can be re-constructed.

6.4 PET

Some radionuclides emit positrons from the nucleus when it is unstable. A positron is positively charged and has the mass of an electron. Following emission from the nucleus, the positron is immediately attracted to a negatively charged electron

with the result that the two particles collide and undergo mutual annihilation. Their energies are converted into two gamma photons of 511 keV energy that leave the atom at exactly 180° to each other. These co-incident photons are then simultaneously detected by a ring of detectors that encircle the subject. The opposing directions of the emitted pair of photons is random and their simultaneous detection allows the construction of a 3-dimensional tomographic image.

SPECT and PET provide images of function. Structural details of tissues are portrayed with relatively poor anatomical resolution. Modern SPECT and PET cameras, therefore, have 'built-in' CT machines so that the functional image generated by the gamma radiation can be precisely registered on to a high-resolution structural image ('hybrid' imaging). The latest technological development is a PET-MRI hybrid camera.

6.4.1 Single Photon-Emitting Radiopharmaceuticals

Tc-99m-hexakis-methoxy-isobutyl isonitrile (Tc-99m-MIBI) is the radiopharmaceutical that has most frequently been used to image ABC transporters. Tc-99m-tetrofosmin and several other Tc-99m-Q complexes that are closely related to Tc-99m-MIBI with respect to their clinical applications are also transport substrates for P-gp and MRP [11], although Tc-99m-tetrofosmin is recognised by fewer transporters than Tc-99m-MIBI. Nevertheless, the available data suggest that the clinical imaging and in vivo modulation of multi-drug resistance can be performed with either Tc-99m-tetrofosmin or Tc-99m-MIBI [12], even though they do not have identical physiological properties. Both agents were originally introduced for imaging myocardial perfusion, although from an early stage, Tc-99m-MIBI was also, and continues to be, used for imaging tumours, such as parathyroid adenomas [13] and assessment of thyroid nodules [14] rather than characterising their ABC transporter status. Tc-99m-MIBI and Tc-99m-tetrofosmin have also been used for diagnosing breast cancer in patients with indeterminate mammography and dense breasts [15].

6.4.1.1 Tc-99m-MIBI

Tc-99m-MIBI is a substrate for P-gp, MRP1, MRP2 and BCRP [16]. It is a lipophilic, stable monovalent cation with a central Tc(I) core surrounded by six identical MIBI ligands, coordinated through the isonitrile carbons in an octahedral geometry [4]. Tc-99m–MIBI is taken up by passive diffusion into the cytoplasm and accumulates in the mitochondria. Its tissue uptake rate following intravenous injection is broadly dependent on tissue blood flow and cellularity. Cellular transport of Tc-99m-MIBI is affected by apoptosis, cellular proliferation and angiogenesis. Tc-99m-MIBI is therefore used to image cellular metabolism in tumours [17, 18]. Tissue retention is variable and markedly influenced by tissue expression of P-gp [4, 19, 20]. The mechanism of Tc-99m-MIBI cellular uptake is clearly different from the mechanism of elimination, which specifically reflects activity of drug transporters, such as P-gp.

Tc-99m-MIBI has been validated as a transport substrate for P-gp in cultured multidrug-resistant rodent [20, 21] and human tumour cells [4, 22], as well as in cells over-expressing the recombinant human *mdr1* gene [23]. Piwnica-Worms et al. first demonstrated that Tc-99m-MIBI is a substrate of P-gp and that it can be used as a functional imaging agent for P-gp in tumour xenografts in nude mice [4]. They and others have shown that tumour retention of Tc-99m-MIBI correlates inversely with the degree of P-gp expression and can be modified in vitro with P-gp antagonists [24].

In rodent models, tumours that express P-gp eliminate Tc-99m-MIBI faster than those that do not [20, 22]. The hepatic and renal excretion pathways of Tc-99m-MIBI are mediated by P-gp and can be modulated in humans following administration of cytotoxic drugs. Thus, intravenous administration of a P-gp modulator delayed excretion of Tc-99m-MIBI from the liver and kidney in patients investigated for MDR [25]. In vitro Tc-99m-MIBI studies have shown that P-gp inhibitors, such as varapamil and ciclosporin, can reverse P-gp expression in adenocarcinoma cells if given shortly before the administration of cytotoxic drug [26].

Additional mechanisms of cell resistance, mainly involving alterations of apoptosis, may also affect tumour Tc-99m-MIBI uptake. In particular, over-expression of the anti-apoptotic protein, Bcl-2, prevents tumour cells entering apoptosis and inhibits Tc-99m-MIBI accumulation in mitochondria. So, whilst absent or reduced early tracer uptake in breast cancer reflects the existence of a defective apoptotic programme, an enhanced tracer clearance in Tc-99m-MIBI-positive lesions reflects the activity of drug transporters, such as P-gp. The existence of two different mechanisms underlying the predictive role of Tc-99m-MIBI scan may be important to establish whether individual patients may benefit from P-gp inhibitors or Bcl-2 antagonists [27].

In general and in relation to a range of malignancies, patients whose tumours showed Tc-99m-MIBI uptake or retention responded well to chemotherapy, whereas those whose tumours showed little or no uptake, or a rapid rate of Tc-99m-MIBI washout, responded poorly [6].

6.4.1.2 Tc-99m-Tetrofosmin

Similar to Tc-99m-MIBI, Tc-99m-tetrofosmin is a widely available lipophilic cationic complex routinely used for imaging myocardial perfusion. Also similar to Tc-99m-MIBI, it has been explored as a tumour-seeking agent in the evaluation of a diverse range of human malignancies or for predicting anti-cancer treatment response [28].

Tc-99m-tetrofosmin has been shown to be a transport substrate for P-gp and MRP in vitro and in vivo. Its properties are similar but not identical to those of Tc-99m-MIBI and, hence, the two should probably not be used interchangeably [12].

The potential response to chemotherapy in lung carcinoma has been investigated by both Tc-99m-tetrofosmin and Tc-99m-MIBI. A low baseline uptake was correlated with a poor response to chemotherapy in non-small and small cell lung carcinomas

likely due to MDR expression in tumour cells. However, a few false positive results were also recorded with Tc-99m-tetrofosmin, which could be due to other mechanisms of resistance and decreasing image specificity. In addition, Tc-99m-MIBI imaging correlates more closely with P-gp immunohistochemistry (IHC) than Tc-99m-tetrofosmin in patients with lung carcinoma suggesting that besides the expression of P-gp, other MDR-related proteins or other mechanisms and factors may contribute to the cellular elimination of Tc-99m-tetrofosmin [11].

6.4.2 Tc-99m-HIDA for MRP Imaging

Although many studies are currently focussing on functional imaging of P-gp, other ABC drug transporters have also attracted interest. Thus, Tc-99m-HIDA (Hepatic IminoDiacetic Acid) is transported only by MRP1 and MRP2 (MRP). Hepatic P-gp and MRP could therefore be assessed by sequential use of both Tc-99m-MIBI (or Tc-99m-tetrofosmin) and Tc-99m-HIDA [11].

6.4.3 Positron-Emitting Radiopharmaceuticals

Several C-11-labelled P-gp-avid radiopharmaceuticals developed for PET, including C-11-colchicine, C-11-verapamil, C-11-daunorubicin, C-11-paclitaxel, and C-11-loperamide, have been evaluated in animals, but only C-11-verapamil and C-11-loperamide [29] have been extended to humans to investigate multidrug resistance and quantify P-gp expression in the blood–brain barrier [8]. Other compounds that have been developed include (Ga-67/68-[3-ethoxy-ENBDMPI])(+) tracers [30], 4-F-18-Fluoropaclitaxel (Fig. 6.2) [31] and the positron-labelled P-gp inhibitor, C-11-Tariquidar [32]. Recent studies using the positron-emitter, Tc-94m-MIBI and parallel previous studies with Tc-99m-MIBI show essentially identical performance, thereby providing validation for microPET [33].

Leukotrienes are substrates for MRP, so N-C-11-acetyl-leukotriene E4 could possibly be used to noninvasively image MRP expression [11].

The development of new radiotracers for PET and SPECT imaging is challenging. Effective radiotracers to visualise molecular targets need to fulfil certain key criteria, such as high affinity and selectivity for the molecular target of interest, a low degree of nonspecific binding in tissue where the molecular target is located and absence of radiolabelled metabolites taken up into tissue. To date, three different kinds of imaging probes have been described for visualisation of ABC transporters in vivo: (1) radiolabelled transporter substrates, (2) radiolabelled transporter inhibitors and (3) radiolabelled pro-drugs that are enzymatically converted into transporter substrates in the organ of interest (e.g. brain) [34].

SPECT and PET radiotracers used in clinical practice and research and their relationship to genotypes are shown in Table 6.1.

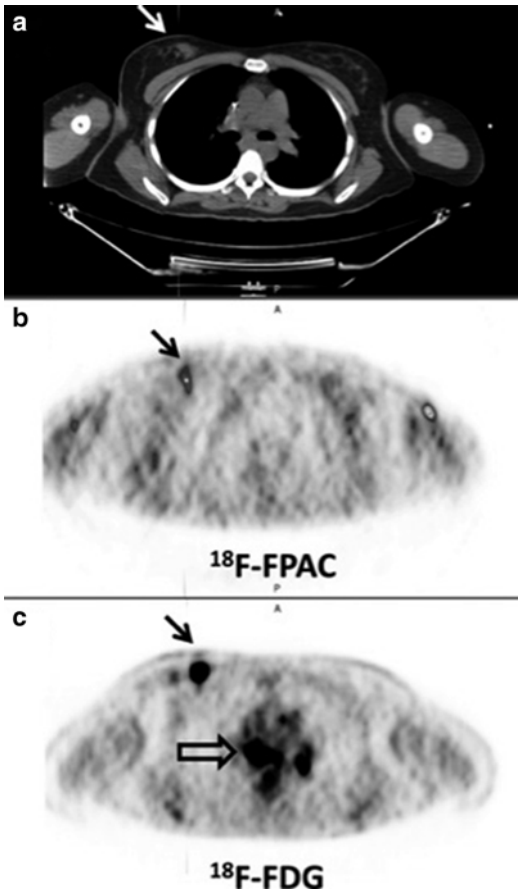

Fig. 6.2 Images of a breast cancer patient with biopsy-proven tumor in the right breast mass (*arrows* on CT [**a**] and PET [**b** and **c**] scans) and biopsy-proven sarcoid in the mediastinum or hila. Increased F-18-fluoropaclitaxel (**b**; Standardised uptake value (SUV) corresponding to tumor and no F-18-fluoropaclitaxel uptake corresponding to mediastinum/hilar lesions) seen on F-18-FDG scan (**c**; *open arrow*). F-18-FDG and F-18-fluoropaclitaxel images are scaled to SUVmax of 2.0. Tumor SUVmax on F-18-fluoropaclitaxel (**b**) at 78 min was 0.9; 18F-FDG (**c**) SUVmax at 123 min was 10.0. Uptake in anterior portion of left arm on F-18-fluoropaclitaxel image (**b**) is residual tracer within vessel wall. Reprinted with permission from the Society of Nuclear Medicine from Kurdziel KA, Kalen JD, Hirsch JI et al. Human Dosimetry and Preliminary Tumor Distribution of 18F-Fluoropaclitaxel in Healthy Volunteers and Newly diagnosed breast cancer patients using PET-CT. J Nucl Med. 2011;52: 1339–1345 (Fig. 6)

6.4.4 Genetics in Relation to Cancer Imaging with ABC Transporter Substrates

Genetically determined responses to some anticancer drugs may influence anticancer treatment. It has been shown that imaging the liver with Tc-99m-MIBI may provide pre-treatment indicators of ABCB1–mediated hepatic drug clearance in

Table 6.1 SPECT and PET ABC substrates (s) and inhibitors (i) and their relationships to genes

Imaging modality	Radiopharmaceuticals	ABC transporter	Gene symbol
SPECT	Tc-99m-MIBI (s)	P-gp	*ABCB1 (mdr1)*
		MRP1,2 BCRP	*ABCC1,2*
			ABCG2
	Tc-99-m-tetrofosmin (s)	P-gp	*ABCB1(mdr1)*
		MRP1,2	*ABCC1,2*
	Tc-99m-HIDA (s)	MRP1,2	*ABCC1,2*
	67Ga-[3-ethoxy-ENBDMPI] (s)	P-gp	*ABCB1(mdr1)*
		MRP1	*ABCC1*
PET and micro PET(μ)	Tc-94m-MIBI (s, μ)	P-gp	*ABCB1 (mdr1)*
		MRP1,2	*ABCC1,2*
		BCRP	*ABCG2*
	C-11-colchicines (i, μ)	P-gp	*ABCB1 (mdr1)*
	C-11-verapamil (i)	P-gp	*ABCB1 (mdr1)*
	C-11-loperamide (i)	P-gp	*ABCB1 (mdr1)*
	C-11-paclitaxel (i, μ)	P-gp	*ABCB1 (mdr1)*
	C-11-daunorubicin (i, μ)	P-gp	*ABCB1 (mdr1)*
	4-F-18-Fluoropaclitaxel (i, μ)	P-gp	*ABCB1 (mdr1)*
	C-11-Tariquidar (i, μ)	P-gp	*ABCB1 (mdr1)*
	68-Ga-[3-ethoxy-ENBDMPI] (s)	P-gp, MRP1	*ABCB1(mdr1);ABCC1*
	N-C-11-acetyl-leukotriene E4 (s)	MRP 2	*ABCC2*

cancer patients. Tc-99m-MIBI hepatic elimination (kH) was significantly reduced in patients with SNPs in exons 21 and 26. The mean Tc-99m-MIBI kH was respectively 1.90 times and 2.21 times higher in subjects homozygous for the wild-type alleles compared with those homozygous for these SNPs [35].

The inter-individual variability of P-gp expression is linked to C3435T polymorphism of the human *ABCB1* gene, which is located on the long arm of 7th chromosome at q21.1 band position. The C/C, T/C and T/T genotypes are associated with increased, intermediate and low P-gp expression, respectively [5] (Fig. 6.3).

C3435T polymorphism plays a significant role in ADME processes (absorption, distribution, metabolism, and excretion) and drug-drug interactions. Variations in the *ABCB1 (MDR1)* gene product may directly affect therapeutic effectiveness; thus, over-expression of P-gp results in increased efflux of anticancer drugs and development of drug resistance. The ABCB1 (*MDR1*) gene is highly polymorphic and numerous single nucleotide polymorphisms (SNPs) have been identified, some of which influence MDR1 expression levels [5]. Polymorphism in exon 26 at C3435T (silent polymorphism) influences P-gp expression.

In the analysis of MDR1 variant genotype distribution in a large sample of Caucasian subjects, Cascorbi et al. demonstrated that C3435T occurred in 53.9 % of heterozygous individuals (T/C), while 28.6 % were homozygous (T/T) carriers and 17.5 % were homozygous (C/C) carriers [31]. In general, the prevalence of the T/T genotype in Caucasian individuals has been shown to range between 24 % and 29 % [36, 37].

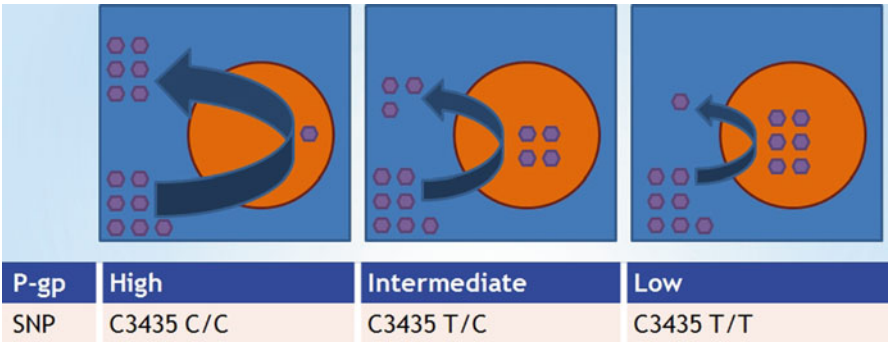

Fig. 6.3 Genetically determined P-glycoprotein (P-gp) expression: Polymorphism in exon 26 at C3435T (silent polymorphism) influences the P-gp expression. The C/C, T/C, and T/T genotypes are associated with *increased*, *intermediate*, and *low* P-gp expression, respectively. High P-gp expressors (C3435C/C) are linked to MDR. Low P-gp expressors (C3435T/T) are prone to drug toxicity. *SNP* single-nucleotide polymorphism (Fig. 6.3 is our own Figure no permission required)

6.4.5 T/T Genotype: Link with Drug Toxicity and Susceptibility to P-gp-Mediated Disease

As the T/T genotype is associated with low P-gp expression, and therefore lower protection against specific P-gp-dependent xenobiotics and carcinogens, individuals with T/T genotype have a reduced efficiency to eliminate toxins. This results in higher intracellular concentrations of mutagens and susceptibility to DNA damage and accumulation of mutations. The reduced capacity of detoxification may therefore have implications for disease risk and therapeutic outcome arising from the development of drug toxicity. For instance, T/T individuals were found to be at increased risk of CML [5], acute childhood lymphoblastic leukaemia (ALL) [38], renal epithelial tumours [39], colorectal cancer, glioblastoma, breast cancer [5] and inflammatory bowel disease [37].

With respect to gender, the T/T genotype is more frequent in males. Tumour development in response to exposure to carcinogens was found to be higher in males compared with females. The association with gender is illustrated by male glioblastoma in relation to the T/T genotype as well as by a greater risk of developing chronic myeloid leukaemia (CML) in males [5].

In the post-genomic era of individualized medicine, ABC transporter imaging may be helpful to adjust the treatment dose in individual patients. More research is needed to identify patients, ideally non-invasively by functional imaging, who are susceptible to the side effects of drug toxicity, to provide information concerning dose adjustment and to allow better decision making when considering therapy with anti-cancer drugs that are substrates for ABC transporters.

6.4.6 C/C Genotype: Link with Multidrug Resistance (MDR) and Poor Risk Prognosis

The C/C genotype is associated with MDR. In cancer therapy, for example, a high expression of MDR1 makes cancer cells refractory to treatment with agents that are P-gp substrates.

The functional significance of MDR1 C3435T polymorphism with respect to *Imatinib* treatment was studied in terms of haematological and cytogenetic responses. The frequency of the C/C genotype was significantly increased in cytogenetic non-responders to an extent that was inversely proportional to the degree of cytogenetic response. As a result of MDR, the C/C genotype is also associated with poor prognosis in acute lymphoblastic and myeloid leukaemias [5, 38].

The effects of *ABCB1* polymorphism on the handling of drugs that are P-gp substrates have also been shown to vary among races [40]. Racial variability within C3434T has been demonstrated. Thus, there is a significantly higher frequency of the C/C genotype in West Africans and African Americans (83 % and 61 %, respectively [41]), compared with Caucasians (17.5 % [12] and 26 %; $p<0.0001$ [41]). This could affect treatment with drugs, in addition to anti-cancer drugs, that are also P-gp substrates, such as HIV-1 protease inhibitors and ciclosporin, in African populations [41] in whom there is a higher prevalence of the relevant diseases. The development of MDR not only reflects multiple genetic and epigenetic changes in cells under cytotoxic conditions, but is also a normal physiological response displayed by cells in their struggle to survive. The challenge of translating the concept of MDR modulation in vivo involves a complex cellular interplay between both malignant and normal cells [42].

6.4.7 Imaging Multidrug Resistance in Cancer

Functional up-regulation of ABC transporter proteins often results from prolonged exposure to inhibitors [43]. As many inhibitors are also modulators, initial down-regulation may be followed by up-regulation, resulting in acquired drug resistance. Thus, treatment of patients with malignant tumours using a variety of structurally unrelated classes of drugs that include anthracyclines, taxanes and epipodophyllotoxines to which the tumour had previously been sensitive is sometimes rendered inadequate because of the activation of cellular biochemical mechanisms that result in MDR.

In tumour cell lines, MDR is associated with an ATP-dependent decrease in cellular drug accumulation attributable to over-expression of ABC transporter proteins. Non-invasive imaging techniques have been developed that can identify such MDR. For example, Tc-99m-MIBI has been used for imaging MDR in lung cancer [44], brain tumours [45, 46], gastric cancer [47], head and neck cancer [48], hepatobilliary cancer [49] and haematological malignancies [50].

6.4.8 Examples of Individual Cancers

6.4.8.1 Breast Cancer

It has been demonstrated that pre-operative elimination rates of Tc-99m-MIBI from primary breast tumours correlated with levels of P-gp semi-quantified by immunohistochemistry in the surgically resected specimens (Fig. 6.4). Imaging demonstrated that the elimination rates from cancers over-expressing P-gp were three-fold faster than those from cancers not expressing P-gp [51, 52].

6.4.8.2 Lung Cancer

In lung cancer, the sensitivity, specificity and accuracy of Tc-99m-MIBI for identifying responders to chemotherapy are 94 %, 90 % and 92 %, respectively [48]. There is evolving evidence that Tc-99m-MIBI is cost-effective in predicting the response to chemotherapy in patients with lung cancer [44].

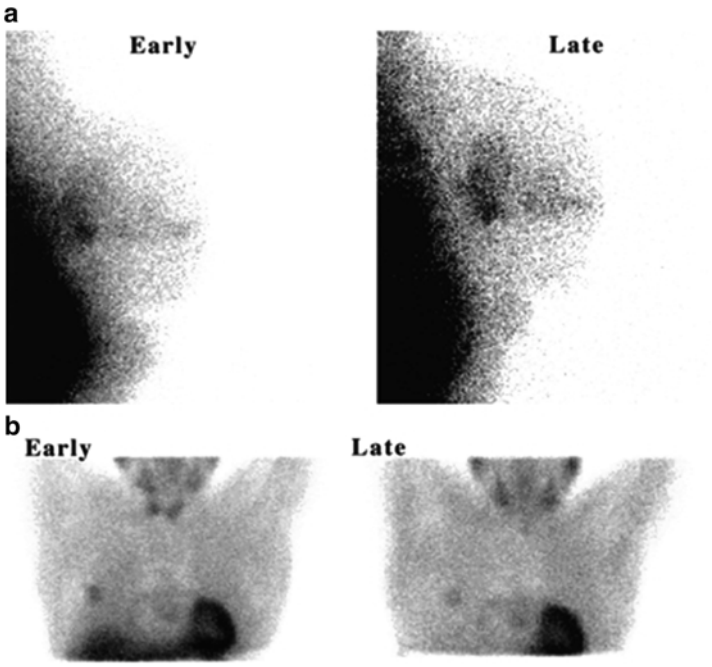

Fig. 6.4 Images of breast cancer obtained 20 min (*early*) and 120 min (*late*) after injection of Tc-99m-MIBI. (**a**) A patient with tumour displaying immunohistochemically negative P-gp expression showing Tumour/Background (T/B) that increased from 1.65 to 1.99. (**b**) A patient with tumour displaying strongly positive P-gp expression showing T/B that decreased from 2.25 to 1.52. Reprinted with permission from the Society of Nuclear Medicine from Mubashar M, Harrington KJ, Chaudhary KS, et al. ^{99m}Tc-Sestamibi Imaging in the Assessment of Toremifene as a Modulator of Multidrug Resistance in Patients with Breast Cancer. J Nucl Med. 2002;43(4): 519–525 (Fig. 2)

6.4.8.3 Thyroid Cancer

In thyroid imaging, Tc-99m-MIBI scintigraphy can be used to reliably exclude thyroid cancer when ultrasound-guided fine needle aspiration cytology (US-FNAC) is reported as non-diagnostic, and hence avoid more invasive surgery and reduce costs [53]. It has, however, also been demonstrated that semi-quantitative Tc-99m-MIBI scintigraphy may pre-operatively predict the malignant behaviour of non-oncocytic follicular thyroid nodules indeterminate at US-FNAC. Moreover, a good correlation was found between Tc-99m-MIBI scintigraphy and immuno-histochemical apical expression of MRP1. A negative Tc-99m-MIBI retention index correlated strongly with high MRP1 expression. So Tc-99m-MIBI scinitgraphy may provide information on the molecular expression of MRP1 in the thyroid gland [54].

6.4.8.4 Haematological Malignancies

A potential role for Tc-99m-MIBI scintigraphy has been investigated in the management of haematological malignancies, particularly multiple myeloma, in which it has been shown that the rate of Tc-99m-MIBI elimination can predict response to chemotherapy. Patients showing disease progression at re-staging had higher elimination rates (19.3 ± 9.8 % vs. 12.8 ± 6.9 %, $p < 0.05$) than patients in remission. Disease-free survival was significantly longer in patients with lower elimination rates. When patients treated with melphalan were excluded from the analysis, 87.5 % of patients in remission displayed slow elimination [55].

6.4.9 MDR Modulation

Based on the premise that blockade of ATP-dependent drug efflux pumps will enhance the effect of chemotherapy, there has been an intense search for compounds able to reverse MDR in cultured cells, animal models, and patients. There have been many attempts to image these pumps using both SPECT and PET tracers [34]. However, administration of P-gp or MRP1 modulators has failed to show any significant clinical benefit in patient outcome, mainly due to toxicity (first generation) or interaction with anticancer drugs and alterations in pharmacokinetics of the chemotherapy agents (second generation). These MDR tracers have not, therefore, found routine clinical use.

Promising clinical trials have been conducted in acute myeloid leukaemia, breast cancer, and non-Hodgkin's lymphoma, all of which are known to express P-gp. Paradoxically, several studies focused on MDR reversal in cancers in which resistance may not be ABC transporter-mediated. Several clinical trials, including the phase III trials of tariquidar, unsurprisingly yielded negative results in cancers in which P-gp expression is generally low, such as small-cell lung cancer and non-small-cell lung cancer [6]. Poor study design, regarding either dosing regimens or patient selection, and genetic polymorphism of P-gp were further major reasons for negative results in clinical trials using third generation P-gp modulators.

The two major phase III trials of tariquidar in patients with non-small-cell lung cancer were terminated prematurely due to toxicity as a result of higher doses of chemotherapy than recommended. Furthermore, the prevalence of various genetic polymorphisms of P-gp may have influenced results (both negatively and positively). Some single-nucleotide polymorphisms and haplotypes of the *ABCB1* gene have been shown to alter P-gp expression and activity both in vitro and in vivo. For example, patients with ovarian cancer who express the wild-type allele for P-gp had a mean progression-free survival of 19 months when treated with chemotherapy, whereas in those expressing the G1199A polymorphism, corresponding survival was only 2 months [6]. Other studies have shown a relation with C3435T polymorphism. All the above-mentioned factors may influence imaging outcome in an individual patient, leading to controversial results. In general, functional imaging of MDR remains under-utilised in clinical practice.

6.4.10 Third Generation MDR Modulators

On-going research has led to the development of a third generation of MDR modulators, some of which have demonstrated encouraging results compared to earlier modulators. They are less toxic, more specific for P-gp and do not affect the pharmacokinetics of anti-cancer drugs. Some MDR-reversing strategies aim to destroy mRNAs for ABC drug transporters, inhibit transcription of ABC transporter genes, or block ABC transporter activity using monoclonal antibodies. There is an optimistic view that much more can be achieved in developing agents for reversing ABC transporters [56]. It is therefore likely that with the development of more potent P-gp inhibitors, effective imaging agents that are analogues of ABC transporters will emerge.

6.4.11 Chemotherapeutic Drug Combinations

It has emerged that combinations of chemotherapeutic drugs aimed to reverse MDR with the so-called targeting agents may improve patient outcome. Molecular imaging can be used to visualise the targets for these agents, such as HER2/neu, and angiogenic factors, such as vascular endothelial growth factor (VEGF). Visualisation of molecular drug targets in the tumour could serve as biomarkers to facilitate treatment decisions for the individual patient [11]. Simultaneous combined imaging using both MDR and target analogues may also evolve initially in the clinical trial setting, a potential role yet to be explored.

6.4.12 Imaging MDR in Drug Trials

Clinical trials optimally tailored to tumour types, genetic polymorphism and adequate dosing regimens need to be conducted. Imaging in such trials may be useful for selecting patients whose cancers express MDR primarily though

ABC-mediated mechanisms. For accurate assessment of tumour P-gp levels, patients should be scanned twice with a P-gp radiolabelled substrate: firstly at baseline and again after P-gp inhibition. Patients whose tumours show enhanced uptake of the radiotracer following P-gp blockade would be suitable candidates for P-gp inhibitor trials [6, 57] (Fig. 6.5). Two studies using Tc-99m-sestamibi have shown the potential value of this approach with respect to the administration of tariquidar or valspodar. Another ongoing study is using Tc-99m-MIBI to monitor progress throughout the trial [6].

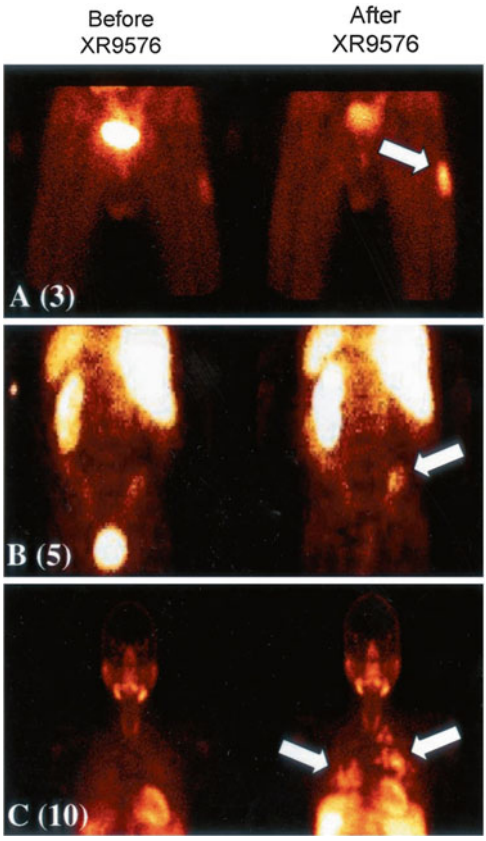

Fig. 6.5 Tc-99m-sestamibi images at baseline and after administration of XR95767 for patients 3, 5, and 10. Patient numbers are shown in *parentheses*. (**a**) *Arrow* identifies a left thigh mass that had gone undetected until the whole body Tc-99m-sestamibiscan was performed (patient 3, renal cell carcinoma, 263 % increase in tumour:heart area under curve (AUC) 0–3 h ratio). (**b**) *Arrow* indicates a soft tissue mass invading the iliac bone (patient 5, renal cell carcinoma, 18 % increase in tumour:heart AUC0–3 h ratio). (**c**) *Arrows* indicate numerous bilateral lung metastases that are all more readily visualized after the administration of XR9576 (patient 10, adrenocortical carcinoma, 76–191 % increase in tumour:heart AUC0–3 h ratios). Reprinted with permission from the American Association for Cancer Research from Agrawal M, Abraham J, Balis FM et al. Increased 99mTc-Sestamibi Accumulation in Normal Liver and Drug-Resistant Tumors after the Administration of the Glycoprotein Inhibitor, XR9576. Clinical Cancer Research. 2003;9: 650–656 (Fig. 2)

6.5 Conclusions

Multidrug resistance and specific ABC transporters may be imaged with either single photon or positron emitting radiopharmaceuticals that are MDR substrates, inhibitors or radiolabelled pro-drugs that are converted into transporter substrates. The most studied of these, and the first to be used in clinical practice, is the single photon emitting radiopharmaceutical, Tc-99m-sestamibi, which is a substrate for P-gp, MRP1, MRP2 and BCRP, and can therefore be used to image their expression in vivo. Tc99m-tetrofosmin and several other Tc-99m-Q complexes that are closely related to MIBI with respect to their clinical applications are also transport substrates for P-gp and MRP. Recent studies using the positron-emitter, Tc-94m-MIBI and parallel previous studies with Tc-99m-MIBI show essentially identical performance, thereby providing validation for microPET.

Several C-11, F-18 and Ga-68-labelled P-gp-avid radioligands developed for PET have been evaluated in animals and some have been extended to humans to investigate MDR and quantify P-gp expression in the blood–brain barrier. Although many studies are currently focussing on functional imaging of P-gp, other ABC drug transporters, particularly MRP, have also attracted interest. Thus, Tc-99m-HIDA is transported only by MRP1, MRP2. Leukotrienes are substrates for MRP, so N-C-11-acetyl-leukotriene E4 could possibly be used to noninvasively image MRP function. Clinical trials better tailored to tumour types, genetic polymorphism and adequate dosing regimens need to be conducted because imaging is proving to be useful for selecting patients whose cancers express MDR primarily though ABC-mediated mechanisms. More research is needed to identify patients, through imaging, who are susceptible to multidrug resistance, but also drug toxicity side effects, to provide information concerning dose adjustment and to allow better decision making when considering therapy with anti-cancer drugs that are ABC transporter substrates. In the post-genomic era of individualised medicine, ABC imaging may be helpful to adjust the treatment dose in individual patients.

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Chapter 7
Modulation of P-Glycoprotein-Mediated Multidrug Resistance by Synthetic and Phytochemical Small Molecules, Monoclonal Antibodies, and Therapeutic Nucleic Acids

Thomas Efferth, Maen Zeino, and Manfred Volm

Abstract Multidrug resistance of malignant tumors severely hampers their successful treatment frequently leading to fatal consequences for affected patients. During the past three decades, many efforts have been spent to develop strategies to overcome multidrug resistance. Many chemical compounds have been shown to inhibit the drug efflux of the multidrug-resistance-mediating P-glycoprotein. Chemical P-glycoprotein inhibitors are from the classes of calcium channel antagonists, calmodulin inhibitors, cyclosporins, antiarrhythmics, hormones, antimalarials, antibiotics, detergents, beta-blockers, antidepressants, blood pressure lowering indol alkaloids, aerobic glycolysis inhibitors, HIV-protease inhibitors, antimycotics, and others. More recently, chemical compounds from medicinal plants or food were also identified as potent P-glycoprotein inhibitors. P-glycoprotein-inhibiting phytochemicals are from diverse classes, such as flavonoids, nonflavonoid polyphenols, alkaloids, steroids, stilbenes, monoterpenoids, dipterpenoids, triterpenoids, triterpene saponines, lignans, flavolignans, polyketides, carotenoids, and others. In addition to chemical synthetic or natural small molecules several other therapeutic strategies have been devised, e.g. monoclonal antibodies blocking drug efflux, immunotoxins specifically targeting and killing multidrug-resistant cells and therapeutic nucleic acids downregulating the P-glycoprotein encoding the *MDR1* gene and resensitizing tumor cells to anticancer drugs. We give an overview of our own research with in vitro and in vivo tumor models in the context of the worldwide efforts to overcome multidrug resistance.

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Keywords ABC transporter • Antibody therapy • Antisense therapy • Cancer • Chemotherapy • Drug resistance • Natural products • P-glycoprotein • Small molecules • Xenograft tumor

Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
Calcein-AM	Calcein-acetoxymethylester
CHO	Chinese hamster ovary
CLO	Clomipramine
Cpm	Counts per million
DOX	Doxorubicin
EGC	Epigallocatechin
EGCG	Epigallocatechin-gallate
HIV	Human immunodeficiency virus
MDR	Multidrug resistance
MDR1	Multidrug resistance gene 1
NBD	Nucleotide binding domain
PBCEC	Porcine brain capillary endothelial cells
P-gp	P-glycoprotein
S180	Sarcoma 180
TAM	Tamoxifen
TFP	Trifluperazine
VER	Verapamil

7.1 Introduction

Multidrug resistance (MDR) of tumor cells to cytostatic agents is a major limiting factor in successful clinical chemotherapy. Therefore, over the past several years there has been considerable interest in devising strategies to combat MDR. This could be achieved either by using non-cross-resistant drugs and thereby bypassing resistance or by the use of specific inhibitors of the MDR-mediating drug efflux pump, P-glycoprotein. Numerous synthetic and natural agents have been described to block P-glycoprotein and thereby mediate sensitivity to standard cytostatic drugs which are transported by the P-glycoprotein. Chemical classes of P-glycoprotein inhibitors are derived from many different pharmacological classes such as antibiotics, antidepressants, calcium channel blockers, calmodulin antagonists, cyclosporins, detergents, hormones, and many others [1–4] (Table 7.1). More recently, phytochemicals have also been found to inhibit P-glycoprotein, e.g. flavonoids, alkaloids

Table 7.1 Synthetic small molecules that inhibit P-glycoprotein

Compounds	Class
Lonidamine	Aerobic glycolysis inhibitor
Amiodarone, quinidine, lidocain	Antiarrhythmics
Erthromycin, cefoperazone, ceftriaxone, clarithromycin	Antibiotics
Clomipramine, yohimbine, trimethoxyyohimbine	Antidepressants
Chloroquine, quinine, quinacrine, cinchonine	Antimalaria drugs
Ketonazole, Itraconzole	Antimycotics
Propanolol	Beta-blocker
Reserpine, rescin amine	Blood pressure lowering indol alkaloids
Verapamil, (dextroverapamil), nifedipin, diltiazene, tiapamile, nicardipine, nimodipine, caroverine, felodipine, nitrendipine	Calcium channel inhibitors:
Trifluoperazine, thioridenzine, chlorpromazine, trifluopromazine, perphenazine, clomipramine, fluphenazine, cis/transflupenthixol, cis/trans-chlorprothixene, cis/trans-clopenthixol	Calmodulin inhibitors
Cyclosporin A, PSC-833, cyclosporin C, cyclosporin G, cyclosporin 4	Cyclosporins
Tween 80, Cremophor EL	Detergents
Indinavir, nelfinavir, ritonavir, saquinavir	HIV-protease inhibitors
Progesterone, deoxycorticosteroide, tamoxifen, toremifene, testosterone, ethinylestradiol	Hormones
Biricodar (VX-710), S9788, elacridar (GF-120918), Zosuquidar (LY335979), tariquidar (XR9576), dofequidar (MS-209)	Others (3rd generation drugs)
Dipyridamole, B1BW22BS	Platelet anticoagulant

and terpenoids [5, 6] (Table 7.2). In spite of these numerous substances that overcome MDR in vitro and in vivo, the question was as to which of these potentially wide assortment of chemicals might be the most suitable candidates for use in cancer patients. The ideal compound should reveal high affinity and specificity to bind to the P-glycoprotein and should be of proven low toxicity.

In addition to overcoming multidrug resistance by small molecules–either chemically synthesized or derived from natural resources such as plants, monoclonal antibodies have been used. Antibodies targeting external epitopes of the P-glycoprotein are able to plug the efflux channel, thereby inhibiting the extrusion of anticancer drugs. Furthermore, such antibodies can also be used for coupling with toxins. P-glycoprotein-directed immunotoxins are capable of highly specific targeting and killing of P-glycoprotein expressing tumor cells.

Moreover, multidrug resistance may be modulated by downregulating P-glycoprotein in MDR cells and thereby resensitizing them to anticancer drugs. This goal may be achieved by therapeutic nucleic acids (antisense oligodeoxynucleotides, siRNA, ribozymes), which target MDR1 mRNA and thereby initiate the degradation of MDR1 mRNA and P-glycoprotein.

Table 7.2 Phytochemical small molecules that inhibit P-glycoprotein

Phytochemical	Class	Plant
(−)Epigallocatechin gallate	Flavonoid	*Camelia sinensis*
(R)-(+)-citronellol	Monoterpenoid	*Cymgopogon nardus*
(S)-(−)-beta-citronellol	Monoterpenoid	*Cymbopogon nardus*
[6]-gingerol	Catechol	*Zingiber officinale*
Acetogenin	Polykeside	*Sinomonium acutum*
Apigenin	Flavonoid	*Petroselinum crispum* and others
Astragalosides	Triterpene saponin	*Astragalus membranaceus*
Auraptene	Monoterpenoid	*Citrus* × *aurantium*
Baicalin	Flavonoid	*Scutellaria baicalensis*
Berbamine	Alkaloid	*Cephalotaxus harringtonia*
Berberine	Alkaloid	*Coptis chinensis*
Bergamottin	Flavonoid	*Citrus* × *aurantium*
Biochanin A	Flavonoid	*Trifolium pratense*
Canadine	Alkaloid	*Coptis chinensis*
Capsaicin	Alkaloid	*Capsicum annuum*
Capsanthin	Carotenoid	*Capsicum annuum*
Capsorubin	Carotenoid	*Capsicum annuum*
Carmosic acid	Diterpenoid	*Rosmarinus officinalis*
Chrysin	Flavonoid	*Passiflora caerulea* and others
Coptisine	Alkaloid	*Coptis chinensis*
Cryptotanshinone	Diterpenoid	*Salvia milthiorrhiza*
Curcumin	Nonflavonoid polyphenol	*Curcuma longa*
Dauricine	Alkaloid	*Menispermum canadense* and others
Daurigoline	Alkaloid	*Cephalotaxus harringtonia*
Ephedrine	Alkaloid	*Angelica sinensis*
Fangchinoline	Alkaloid	*Stephania tetrandra*
Fisetin	Flavonoid	*Rheum palmatum*
Galangin	Flavonoid	*Alpinia officinarum*
Genistein	Flavonoid	*Genista tinctoria*
Ginkgolides A and B	Diterpenoid	*Ginkgo biloba* and others
Ginsenosides	Triterpene saponin	*Panax ginseng*
Glabridin	Flavonoid	*Glycorrhiza glabra*
Glycyrrhetinic acid	Triterpene saponin	*Glycorrhiza glabra*
Guggulsterone	Seroid	*Commiphora mukul*
Hesperitin	Flavonoid	*Citrus spec.*
Homoharringtonine	Alkaloid	*Cephalotaxus harringtonia*
Honokiol	Lignan	*Pseudolarix kaempferi*
Hypericin	Anthraquinone	*Hypericum perforatum*
Icariin	Flavonoid	*Epimedium koreanum*
Jatrorrhizine	Alkaloid	*Coptis chinensis*
Kaempherol	Flavonoid	*Brassica oleracea* and others
Lutein	Carotenoid	*Spinacia oleracea* and others

(continued)

Table 7.2 (continued)

Phytochemical	Class	Plant
Lycopene	Carotenoid	*Solanum lycopersicum* and others
Matairesinol	Lignan	*Glycine max*
Morin	Flavonoid	*Morus tinctoria*
Naringenin	Flavonoid	*Citrus spec.*
Neostenine	Alkaloid	*Stemona tuberosa*
Neotuberostermonine	Alkaloid	*Stemona tuberosa*
Nobiletin	Flavonoid	*Citrus spec.*
Paeoniflorin	Monoterpenoid	*Paeonia lactiflora*
Palmatine	Alkaloid	*Coptis chinensis*
Phellamurin	Flavonoid	*Phellodendron acutum*
Phloretin	Flavonoid	*Pyrus communis* and others
Procyanidine	Flavonoid	*Pinus massoniana*
Pseudolaric acid B	Diterpene acid	*Pseudolarix kaempferi*
Pyranocoumarins	Courmarins	*Peucadanum praeruptorum*
Quercetin	Flavonoid	*Ginkgo biloba*
Resveratrol	Stilbene	*Vitis vinifera*
Rhinacanthin C	Naphthoquinone	*Rhinacanthus nasutus*
Rotenone	Flavonoid	*Derris eliptica* and others
Rutin	Flavonoid	*Morus alba* and others
Sakuranetin	Flavonoid	*Polymnia fruticosa*
Schisandrins (=Gomisins)	Lignan	*Schisandra chinensis*
Sesamin	Lignan	*Sesamum indicum*
Silibinin	Flavolignan	*Silybum marianum*
Silymarin	Flavonoid	*Silybum marianum*
Sinomenine	Alkaloid	*Sinomonium acutum*
Spiraeoside	Flavonoid	*Filipendula ulmaria*
Tamarixetin	Flavonoid	*Ginkgo biloba* and others
Tangeretin	Flavonoid	*Citrus tangerina*
Tanshinone B	Diterpenoid	*Salvia milthiorrhiza*
Tenacissimoside A	Steroid	*Marsdenia tenacissima*
Tetramethylpyrazine	Alkaloid	*Cephalotaxus harringtonia* and others
Tetrandrine	Alkaloid	*Stephania tetrandra*
Triptolide	Diterpenoid	*Triterygum wilfordii*
Ursolic acid	Triterpenoid	*Rosmarinus officinalis*

7.2 Function of P-Glycoprotein

As a starting point of a discussion, we questioned how the P-glycoprotein can be inhibited with the aim to overcome multidrug resistance of tumors, what are the different models and hypotheses, and how the P-glycoprotein extrudes anticancer drugs out of cancer cells should be addressed. There are two classes of P-glycoprotein substrates. Hydrophobic compounds (e.g. vinblastine) enter the

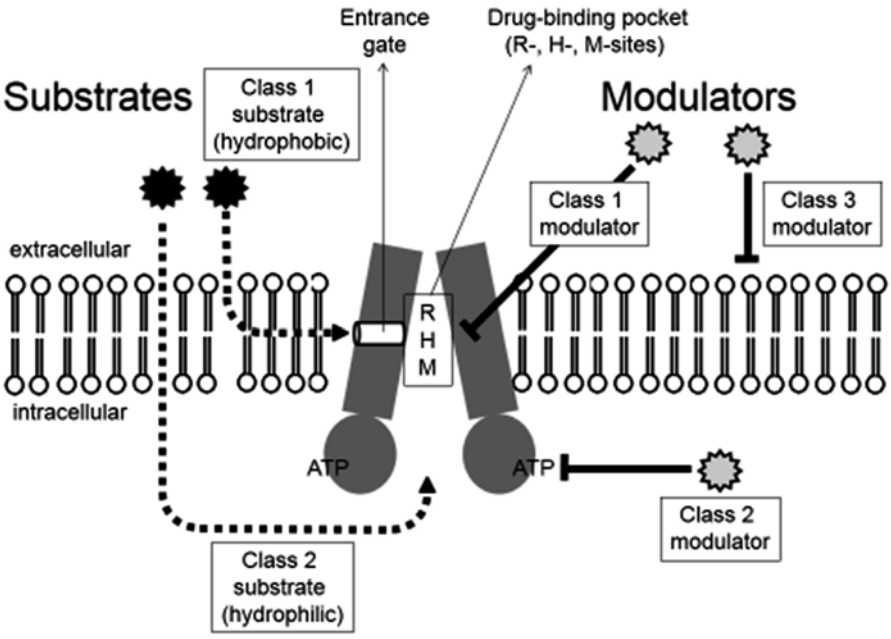

Fig. 7.1 Schematic illustration of different classes of substrates and modulators of P-glycoprotein function

outward channel of the P-glycoprotein through the lipid bilayer by an entrance gate in the transmembrane domains of the P-glycoprotein. Hydrophobic compounds (e.g. rhodamine 123) enter the cell by passive diffusion and bind to the efflux channel of the P-glycoprotein from the intracellular side (Fig. 7.1). There are also different classes of inhibitors of P-glycoprotein efflux. The number of binding sites at the P-glycoprotein has been a matter of long-lasting discussions ranging from several defined pharmacophores to a substrate-induced fit model [7]. Three overlapping binding sites in the transmembrane region of the P-glycoprotein have been suggested [8, 9, 19]. They have been termed the R-site (rhodamine 123 binding site), H-site (Hoechst 33342 binding site), and M-site (modulator-binding site). Modulators interact with substrates at these overlapping sites leading to competitive efflux inhibition. A second class of inhibitors (e.g. flavonoids) bind to the ATP binding domain, thereby inhibiting ATP hydrolysis and allocation of energy, which is necessary for the efflux process (Fig. 7.1). This type of P-glycoprotein inhibition occurs in a non-competitive manner. A third type of drug resistance modulators have been described not to (e.g. Tween 80, cremohor EL) interact with the P-glycoprotein at all. Rather, these surfactants alter the membrane fluidity and thereby membrane permeability for anticancer drugs (Fig. 7.1) [10]. In addition to small molecule inhibitors of P-glycoprotein resistance-modifying detergents, monoclonal antibodies have been used to overcome multidrug resistance. Some of them bind to the extracellular loops (e.g. MRK16), thereby inhibiting drug efflux, whereas others bind to a cytoplasmic domain (e.g. C219).

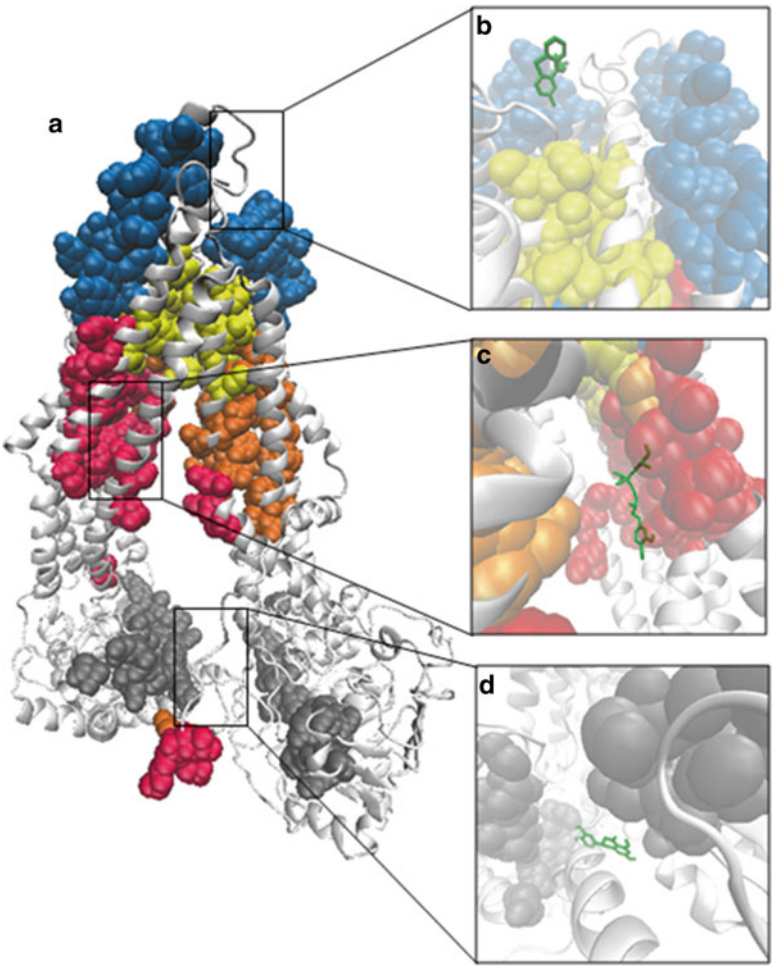

Fig. 7.2 Different binding pockets on P-gp: (**a**) Graphical presentations of the different potential binding sites on P-gp: P-gp molecule has been depicted in new cartoon format (*white*), whereas different binding sites in CPK format: MRK16 epitope (*blue*), M-site (*yellow*), R-site (*orange*), H-site (*red*), and amino acids involved in ATP interaction (*gray*). (**b**, **c**, and **d**) Molecular docking of chlorpromazine (lowest binding energy mode [−6.14 kcal/mol]), R-verapamil (lowest binding energy mode [−4.96 kcal/mol]) and hesperitin (cluster with highest number of conformations [−6.01]) respectively. All ligands are depicted in *green*

The binding sites of competitive and noncompetitive small molecule inhibitors as well as of monoclonal antibodies have been illustrated in a three-dimensional homology model of the human P-glycoprotein (Fig. 7.2), which is based on the X-ray structure or murine P-glycoprotein [11]. Representative small molecules at the MRK16 epitope, transmembrane drug binding site, and the nucleotide binding domain (NBD) are also displayed in this figure as found from our dockings (refer to Tables 7.3 and 7.4).

Table 7.3 Molecular docking of selected synthetic compounds on homology modeled human P-glycoprotein: for each compound the cluster with the lowest binding energy and with highest number of conformations (in case different than the latter), respective binding energies, amino acids involved in H-bond, number of interacting amino acids and the distribution of amino acids at the multiple interaction sites are shown

Compound	Number of conformations in cluster	Binding energy (kcal/mol)	AA involved in H bond	Number of interacting AA	Amino acids found at:				
					M-site	R-site	H-site	NBD	MRK16 epitope
Bamipine	4	−6.29	–	8	–	2	–	–	–
	19	−5.63	–	6	–	–	5	–	–
Chlorpormazine	7	−6.14	–	7	–	–	–	–	5
	10	−5.16	–	4	2	1	–	–	–
Chlorphenoxamine	2	−6.14	–	6	1	2	–	–	–
	16	−5.22	–	9	1	–	–	–	–
Clomipramine	3	−6.59	–	11	2	1	–	–	–
	9	−5.49	–	5	4*	1*	–	–	–
Hycanthone	4	−6.15	GLN347	6	2	3*	1*	–	–
	7	−5.67	ALA343	6	1	2	–	–	–
Tamoxifen	7	−5.5	–	5	3	–	–	–	–
Trifluorperazine	4	−6.51	ASP87	6	–	–	–	–	3
	18	−5.79	–	7	–	–	7	–	–
R-verapamil	1	−4.96	–	7	–	–	7	–	–
	2	−3.91	–	4	4*	2*	–	–	–
S-verapamil	1	−4.82	–	7	5	–	–	–	–
	3	−4.48	–	11	–	–	8	–	–

A star (*) indicates that an amino acid shared between the multiple sites

Table 7.4 Molecular docking of selected phytochemicals on homology modeled human P-glycoprotein: for each compound the cluster with the lowest binding energy and with highest number of conformations (in case different than the latter), respective binding energies, amino acids involved in H-bond, number of interacting amino acids and the distribution of amino acids at the multiple interaction sites are shown

Compound	Number of conformations in cluster	Binding energy (kcal/mol)	AA involved in H bond	Number of interacting AA	Amino acids found at:				
					M-site	R-site	H-site	NBD	MRK16 epitope
4-methoxy(2,3-b) quinoline	1	–5,57	–	7	–	–	–	–	–
	24	–5,1	–	5	1	–	–	–	–
Acetylmarinobufogenin	9	–8,46	Lys934, His145	7	–	–	3	–	–
	31	–7,5	–	6	–	–	–	–	–
Biacalin	3	–5,68	Ser931	6	–	–	4		–
	–	–	–	–	–	–	–	–	–
Bufalin	4	–7,28	Ile663	7	–	–	–	–	–
	13	–6,82	–	8	–	1	–	–	–
Bufatalin-3-acetate	1	–6,91	Gln629	6	–	–	–	1	–
	4	–5,84	–	8	–	–	–	–	–
Deoxyserofendic acid	3	–7,01	–	8	2	1	–	–	–
	8	–6,44	–	8				–	–
Diosmetin	13	–6,2	Leu225	6	1	1	–	–	–
	–	–	–	–	–	–	–	–	–
Ent-15-atisen-19-oic acid	14	–7,79	–	4	–	3	–	–	–
	32	–7,07	–	6				–	–
Ent-16-atisen-19-oic acid	17	–7,98	Lys271	7	–	–	–	–	–
	26	–6,82	Lys826	4	–	4	–	–	–
Glybomin A	38	–6,22	Ser222	8	2	–	–	–	–
	–	–	–	–	–	–	–	–	–

(continued)

Table 7.4 (continued)

Compound	Number of conformations in cluster	Binding energy (kcal/mol)	AA involved in H bond	Number of interacting AA	Amino acids found at:				
					M-site	R-site	H-site	NBD	MRK16 epitope
Glybomin B	19	−6,78	–	7	2	1	–	–	–
	–	–	–	–	–	–	–	–	–
Hesperitin	5	−6,09	Lys234	6	1	1	–	–	–
	8	−6,01	–	6	–	–	–	2	–
Proscillaridin	12	−7,84	–	9	–	–	4	–	–
	–	–	–	–	–	–	–	–	–
Quinidine	9	−7,18	PHE303	9	1	2	1*	–	–
Quinine	14	−6,91	–	7	1	1	–	–	–
Resibufogenin	5	−7,85	–	10	–	1*	1*	–	–
	17	−7,81	–	8	–	–	3	–	–
Scillaren A	1	−5,83	–	9	6*	3*	–	–	–
	3	−2,81	–	5	–	–	–	–	–
Shogaol	5	−5,99	–	4	1	–	–	–	–
	–	–	–	–	–	–	–	–	–

A star (*) indicates that an amino acid shared between the multiple sites

7.3 Modulation of Resistance by Synthetic Drugs

As a starting point we made a comparison of the most active effects for reversing multidrug resistance in vitro. The effects of the modifying substances were determined by the nucleoside incorporation assay [12, 13]. As a test model, we used parental L1210 ascites tumor cells, a doxorubicin-resistant subline thereof, which expresses the MDR-phenotype (30-fold resistance to doxorubicin) and a cytosine-arabinoside-resistant subline without the MDR phenotype as a negative control (2,500-fold resistance). The leukemic L1210 cells were grown intraperitoneally in ascites form and were transplanted at 7-day intervals (1.5×10^7 cells/mouse). For experiments on MDR reversal, cells were taken on the 5th to 7th day after tumor cells inoculation. The maximal concentration of each drug was selected by exhibiting only a slight inhibitory effect on both resistant and parental L1210 ascites cell lines.

The MDR-reversing activity of eight chemical substances derived from different pharmacological classes is shown in Fig. 7.3. All tested substances enhanced the cytotoxicity of doxorubicin in the MDR-resistant cells in vitro [14]. MDR resistance was partly circumvented by bamipine, chlorophenoxamine, and verapamil (10- to 30-fold) compared to hycanthone, tamoxifen, and trifluoperazine which completely reversed doxorubicin resistance. All these agents were ineffective in the sensitive (parental) L1210 ascites cells. Furthermore, none of these drugs had any significant sensitizing effects on L1210 ascites cells resistant to cytosine-arabinoside (data not shown). The chemical structures of all these modulators had two features in common: a lipophilic aromatic ring-system with a hydrophilic N-alkyl chain.

To prove whether the resistance reversal is restricted to agents with these structural properties, we tested substances lacking an aromatic ring and an N-alkyl-group

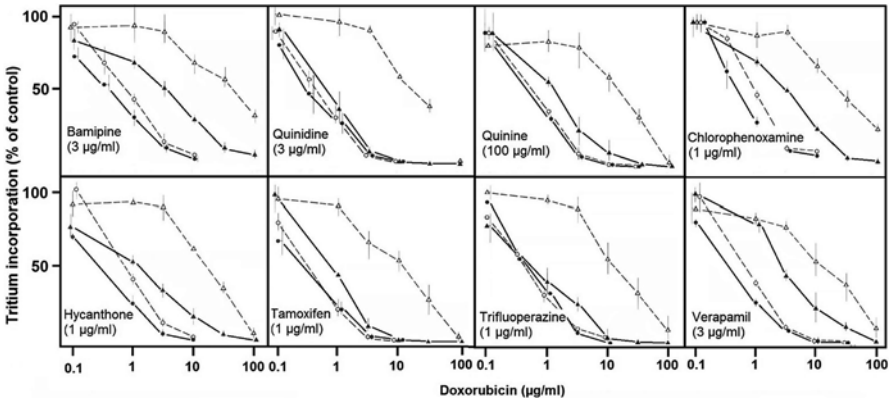

Fig. 7.3 Dose-response curves of doxorubicin on doxorubicin-resistant (*triangle*, *filled triangle*) and parental (*circle*, *filled circle*) L1210 ascites tumor cells with (*closed symbols*) or without (*closed symbols*) modulators. Ordinate: Nucleoside incorporation (% of control). The short-term test was carried out as described [12, 13]. Abscissa: doses of doxorubicin. Mean and range of 6 determinations of three independent experiments are shown. Data were taken from [14]

(e.g. testosterone), having a N-alkyl-group but no aromatic ring (methylpiperazine), or aromatic substances lacking an N-alkyl group (ß-estradiol). Indeed, all these substances could not overcome the resistance in doxorubicin-resistant L1210 ascites cells (data not shown). In contrast to estradiol, estracit which has both an aromatic ring and an N-alkyl group showed a significant MDR reversal.

In further investigations, we analyzed the reversal of doxorubicin resistance in vivo. As shown in Fig. 7.4b, a strong reduction of doxorubicin-resistant solid L1210 tumors was observed following treatment with doxorubicin plus verapamil compared to both compounds, if applied alone to animals. In contrast, this effect was not observed in sensitive solid L1210 tumors (Fig. 7.4a) [16].

As clinical pilot studies with verapamil have been ceased due to intolerable high cardiovascular effects [18, 19], the stereoisomer (R)-verapamil might be more suitable as a P-glycoprotein inhibitor, since it revealed considerable less cardiovascular effects compared to (S)-verapamil and racemic (R/S)-verapamil [20, 21]. Therefore, we compared the effects of (R)-verapamil (Fig. 7.4c) in comparison to racemic (R/S)-verapamil (Fig. 7.4b) in doxorubicin-resistant solid L1210 tumors in mice [15]. While (R)-verapamil did not reveal significant inhibition of tumor growth compared to untreated control tumors, doxorubicin significantly inhibited tumor growth. Similar to R/S-verapamil (Fig. 7.4b), the combination of doxorubicin plus (R)-verapamil synergistically increased the inhibitory effects of the compounds applied alone (Fig. 7.4c). During treatment, the body weight of the animals were recorded as a parameter of toxicity. (R)-verapamil-treated animals did not reveal a weight loss in contrast to (R/S)-verapamil treated mice.

Since our in vitro experiments showed that tamoxifen reversed doxorubicin resistance (Fig. 7.3), we were interested to see whether reversal of resistance can also be obtained in vivo [16]. Figure 7.4e depicts that the combination of doxorubicin with tamoxifen led to a significant decrease of growth of doxorubicin-resistant solid L1210 tumors compared to both drugs alone. No significant effect was found in the sensitive solid L1210 tumors (Fig. 7.4d).

Finally, the effect of clomipramine in doxorubicin-resistant solid L1210 tumors in mice was investigated [17]. A significant inhibition of tumor growth was observed after treatment with clomipramine and doxorubicin combined when compared to doxorubicin alone ($p = 0.014$). Clomipramine or doxorubicin alone showed only marginal effects compared to untreated control (Fig. 7.4f).

In addition to measuring the effects of P-glycoprotein modulators on tumor growth in vitro and in vivo, we also analyzed these compounds by flow cytometry and the rhodamine 123 accumulation assay. Figure 7.5 shows representative

Fig. 7.4 (continued) L1210 tumors within 4 days in nontreated animals (controls), after treatment with doxorubicin (3×2 mg/kg body weight), R/S-verapamil or R-verapamil (3×30 mg/kg), tamoxifen (3×20 mg/kg), and clomipramine (4×0.3/kg) alone or doxorubicin in combination with the modulators. The animals were treated daily (4 consecutive days). Each bar represents mean values of tumor volumes of 6–8 animals. *The data were taken from Pommerenke et al.* [15, 16], *Pommerenke and Volm* [17]

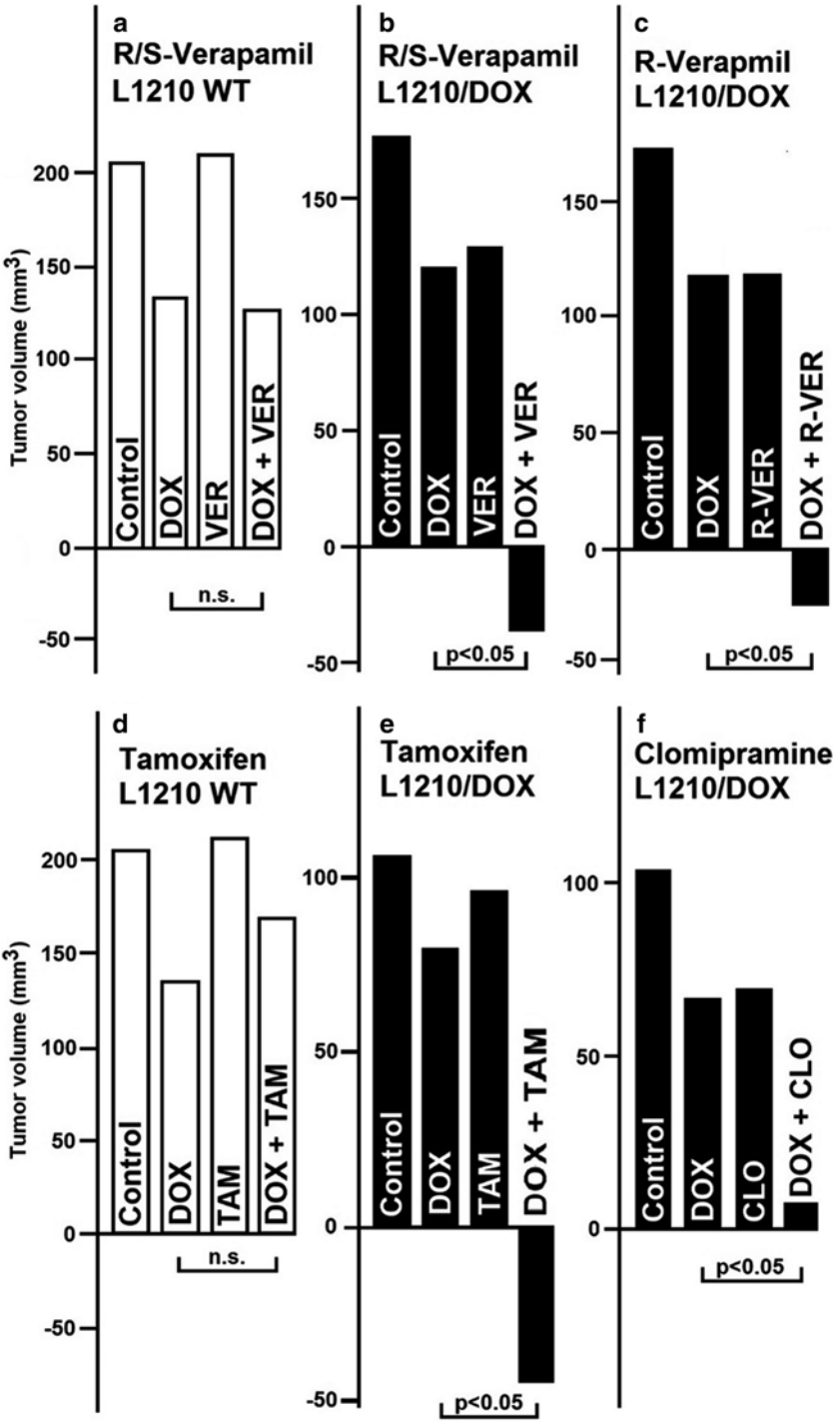

Fig. 7.4 Reversal of doxorubicin resistance in solid L1210 tumors in vivo. P-glycoprotein-expressing doxorubicin-resistant and P-glycoprotein-negative sensitive L1210 WT tumors were treated with doxorubicin and resistance-reversing agents (R/S-verapamil, R-verapamil, tamoxifen, clomipramine) either alone or in combination. Increase in tumor volumes (mm^3) of doxorubicin-resistant solid

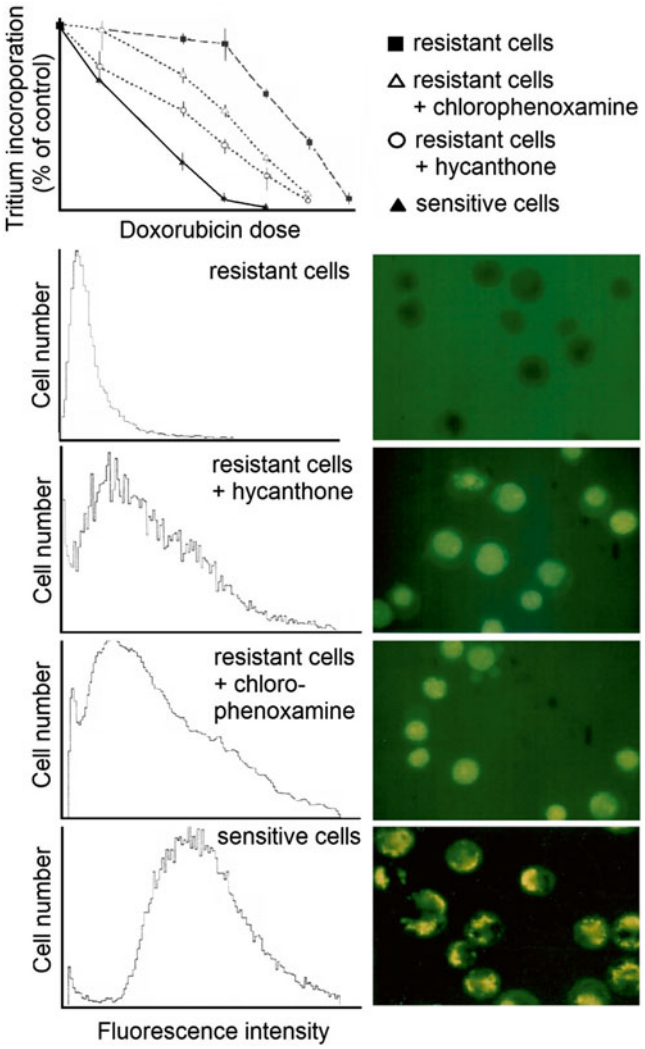

Fig. 7.5 Modulation of rhodamine 123 accumulation in multidrug-resistant L1210 ascites tumor cells. Synthetic compounds (hycanthone, chlorophenoxamine) able to partially modulate doxorubicin cytotoxicity in the short-term test (*figure at the top*) increase the accumulation of the fluorescent dye, rhodamine 123 in resistant cells. The low rhodamine 123 accumulation in multidrug-resistant cells (*second figure*) was increased by the addition of hycanthone (*third figure*) or chlorophenoxamine (*fourth figure*). The high rhodamine 123 accumulation of sensitive cells (without P-glycoprotein expression) was, however, not reached. Data were taken from Efferth et al. [22]

biparametric fluorescence histograms of intracellular rhodamine 123 fluorescence. The fluorescence intensity was significantly higher in cells that were treated with modulators. Propidium iodide (PI) staining was used to stain nuclei of dead cells, which nonspecifically accumulate rhodamine 123. PI-stained dead cells were

electronically excluded and fluorescence signals of rhodamine 123-stained living cells were plotted onto monoparametric histograms. The flow cytometric data of the rhodamine 123 accumulation assay shown in Fig. 7.5 confirmed the results found by the short-term test.

We have performed molecular docking using AutodockTools-1.5.6rc3 and Autodock4 on a homology modeled human P-glycoprotein for a selection of the synthetic compounds mentioned throughout the text above. The results are presented in Table 7.3, where information about binding energies, number of conformations in the lowest binding energy cluster and the cluster containing the highest number of conformations, interacting amino acids and their assignment to the previously described interaction sites on P-glycoprotein are listed. It is worth mentioning that estimation of binding energies in regards of P-glycoprotein has been unsatisfactory and molecular docking was more reliable in identifying the binding mode [23]. As may be noticed, most compounds were found to be docked in the transmembrane region (M-, R-, and H-site). Interestingly, chlorpromazine and Trifluorperazine have shown to bind close to the MRK16 epitope, which might indicate for the first time that a small molecule may possibly interact on the extracellular domain as some monoclonal antibodies do. This represents a new aspect of a small molecule interaction with the P-glycoprotein.

7.4 Modulation of Resistance by Phytochemicals

In evolution of life, P-glycoprotein-type transporters served as detoxification mechanisms, which expel natural xenobiotic substances taken up by food or inhaled by breath. P-glycoprotein is a phase 3 protein in liver metabolism transporting substrates oxidized by phase I enzymes (e.g. cytochrome P450 monooxigenases) and conjugated by phase II enzymes (e.g. glutathione S-transferases). Moreover, the P-glycoprotein is expressed in many normal organs, which are in contact with xenobiotic substances, e.g. the gastrointestinal tract, kidneys, and pancreas. In brain and placenta, the P-glycoprotein contributes to the blood–brain and blood–placenta barriers preventing the entry of potentially harmful substances into the brain or the fetus. It has been discussed that secondary plant metabolites were useful weapons in the evolutionary arms race between plants and herbivores and that phase I and II detoxifying enzymes and phase III transporters in mammalian herbivores were effective defense lines against toxic xenobiotic compounds from plants [24]. Having this background in mind, it comes as no surprise that a huge number of natural compounds are either substrates or inhibitors of the P-glycoprotein.

Consumption of green tea made from unfermented leaves of *Camellia sinensis* has been shown to afford protection against carcinogenesis of the esophagus, stomach, duodenum, colon, liver, and lung in humans. The main responsible components of green tea are some polyphenols, especially flavonols from the catechin-type, in particular epigallocatechin gallate (EGCG) and epigallocatechin (EGC). We proved whether these compounds modulate the activity of antineoplastic drugs. We found a

modulating effect of EGCG and EGC on the sensitivity of human SW620 and murine S180 doxorubicin-resistant cells to doxorubicin [25].

In addition to quinine and quinidine, shown in Fig. 7.3, which have shown along with hycanthone, tamoxifen, and trifluoperazine to cause a complete reversal of MDR, a screening of phytochemicals with a special focus on medicinal plants used in traditional Chinese medicine was carried out. We identified a number of compounds that are able to inhibit the P-glycoprotein either in tumor cells or at the blood–brain barrier [26–30]. The P-glycoprotein acts not only as a drug transporter in tumor cells, but also at the blood–brain barrier to prevent penetration of harmful substances from the blood circulatory system into the brain tissue [31].

Using immunofluorescence, we showed that the P-glycoprotein revealed a luminal localization at membranes of freshly isolated porcine brain capillaries (Fig. 7.6a). We isolated porcine brain capillary endothelial cells (PBCECs), cultured them in vitro, and performed P-glycoprotein staining. P-glycoprotein expression was detected not only in cell membranes, but also in intracellular vesicular-like structures (Fig. 7.6a).

Living cell confocal microscopy was used for functional investigations of the P-glycoprotein in brain capillaries. Brain capillaries were maintained in vitro and incubated with fluorescent NBD-cyclosporin A. Whereas control brain capillaries transported NBD-cyclosporin A into the capillary lumen indicating functional P-glycoprotein, the addition of the PSC-833 inhibited luminal accumulation of the fluorescent compound (Fig. 7.6b). NBD-cyclosporin A accumulated only in endothelial cells, but was not secreted anymore into the capillary lumen due to effective P-glycoprotein inhibition. Likewise, one of the test compounds, 4-methoxy [2,3,-b] quinolone, inhibited P-glycoprotein transport (Fig. 7.6b).

A total of 57 chemical compounds from plants used in traditional Chinese medicine and three control phytochemicals (biochanin A, diosmetin, hesperitin) were investigated in PBCECs, P-glycoprotein-positive CEM/ADR5000, and P-glycoprotein-negative CCRF-CEM leukemia cells [29]. For measuring P-glycoprotein transport, another P-glycoprotein fluorescent probe was used, calcein-acetoxymethylester (calcein-AM). Inhibition of P-glycoprotein reduces calcein-AM efflux and cytosolic esterases rapidly cleave ester bonds, leading to the formation of highly fluorescent organic anion calcein, which is intracellularly trapped and detectable by confocal microscopy and flow cytometry. As shown in Fig. 7.6c, six phytochemicals derived from traditional Chinese medicine and the three control compounds (biochanin A, diosmetin, hesperitin) increased calcein fluorescence in PBCEC and CEM/ADR5000 cells, but not in CCRF-CEM cells.

Fig. 7.6 (continued) (two independent experiments with each two parallel measurements). The bar diagram shows intracellular fluorescence (E_{max}) values a concentration of 5 μg/ml for PBCECs and 50 μg/ml for CCRF-CEM and CEM/ADR5000 cells. The *inset* exemplarily shows three dose response curves used for E_{max} calculation. ***$p<0.001$; **$p<0.01$; *$p<0.05$. (**d**) Inhibition of P-glycoprotein by bufadienolides and bufatrienolides showing a concentration-dependent increase of intracellular fluorescence in freshly isolated PBCECs (two independent experiments with each 6 parallel measurements). The data were taken from Mahringer et al. [29]

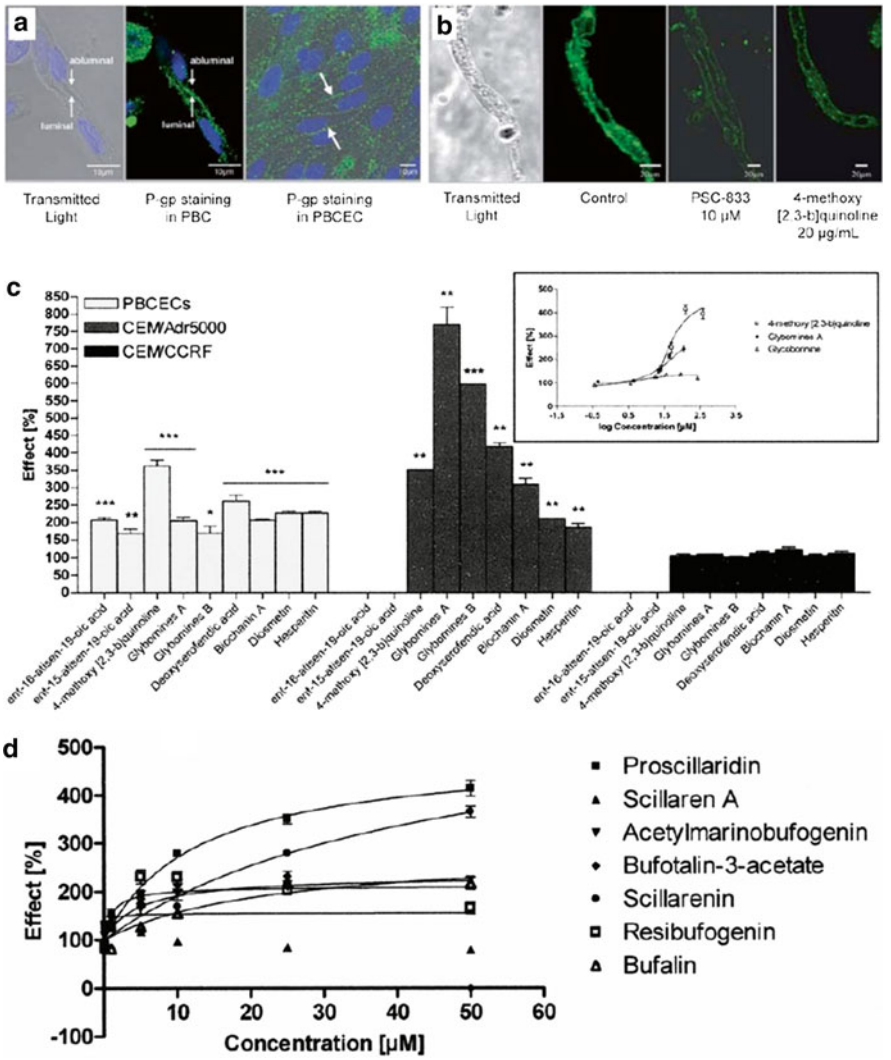

Fig. 7.6 Inhibition of P-glycoprotein by phytochemicals from traditional Chinese medicine. (**a**) Immunostaining for P-glycoprotein in porcine brain capillaries shows luminal localization of the efflux transporter. In cultivated PBCECs, P-glycoprotein is also localized at the membrane, but also in cytoplasmic vesicles. The left image shows a transmitted light image of a porcine capillary discriminating between luminal and basolateral endothelial membrane side. (**b**) Freshly isolated brain capillaries were incubated with the fluorescent NBD-cyclosporin A and left untreated (control), or treated for 30 min with PSC-833 or 4-methoxy [2,3-b]quinolone. Images are representative for two separate capillary isolations. (**c**) Concentration-dependent increase of intracellular fluorescence in P-glycoprotein-expressing b rain capillary endothelial cells sub-cultures as monolayer, P-glycoprotein expressing human CEM/ADR5000 leukemia cells and P-glycoprotein-negative CCRF-CEM leukemia cells. Intracellular calcein fluorescence in PBCECs was determined in a fluorescence reader (two independent experiments with each 6 parallel measurements), whereas fluorescence in CEM/ADR5000 and CCRF-CEM cells was determined by flow cytometry

They also increased calcein fluorescence and expressed as E_{max} values, which were calculated from concentrations of intracellular calcein fluorescence (see inset in Fig. 7.7c). The E_{max} values tended to be higher in CEM/ADR5000 cells than in PBCECs, indicating a higher modulatory activity. The E_{max} values of CCRF-CEM cells were not increased as compared to untreated controls.

Next, we analyzed a panel of chemically related bufadienolides and bufatrienolides for their interaction with P-glycoprotein in cultivated PBCECs [29]. The results of the calcein assays are shown in Fig. 7.6d. Proscillaridin and scillaren A revealed the most pronounced P-glycoprotein inhibition, Acetylmarinobufogenin, bufotalin-3-acetate, and scillarenin showed intermediate and bufalin exhibited the weakest P-glycoprotein inhibition.

Molecular docking on phytochemicals discussed above has also been conducted in the same approach used for the synthetic compounds represented in Table 7.3. Results are displayed in Table 7.4 below. We noticed here as well that most of the compounds showed preference to the transmembrane region. Two compounds, namely bufotalin-3-acetate and hesperitin, were shown to probably interact at the nucleotide binding domain (NBD). Hesperitin belongs to flavonoids, which have been described throughout literature to interact with the nucleotide binding domain [34], but they also as well might interact at the common binding site within the transmembrane region [35].

The investigation of phytochemicals in the context of P-glycoprotein has two important implications:

1. A large number of cancer patients takes herbal remedies in addition to chemotherapy with or without informing their physicians [36]. As herbs interfere with Phase I–III detoxification proteins, they may alter pharmacokinetics and –dynamics of standard drugs by either increasing or decreasing the activity of Phase I–III proteins. This has been not only shown for anticancer drugs but also for many drugs used for diverse diseases [37–41]. One well-known example is St. John's Wort (*Hypericum perforatum*). Cancer patients are frequently depressive about their disease and take antidepressive preparations. St. John's Wort indeed reveals considerable antidepressive activity, but also induces the expression of cytochrome P450 monooxigenases and P-glycoprotein in the liver [38]. This leads to an increased metabolization and detoxification of anticancer drugs potentially leading to reduced response of tumor cells to chemotherapy or even therapy failure. As yet, the potential harm of herb-drug interactions has not been fully uncovered and needs to be studied in more detail to prevent unwanted effects during cancer chemotherapy.
2. The inhibition of P-glycoprotein may be specifically exploited to overcome multidrug resistance. Many phytochemicals effectively inhibit P-glycoprotein function in a comparable manner to well-known P-glycoprotein inhibitors such as verapamil or cyclosporin A [5, 6, 42–44]. A number of phytochemicals also reveal cytotoxic activity towards cancer cells. Both features—P-glycoprotein activity and cytotoxicity towards cancer cells—may lead to synergistic tumor killing rates if used in combination therapy.

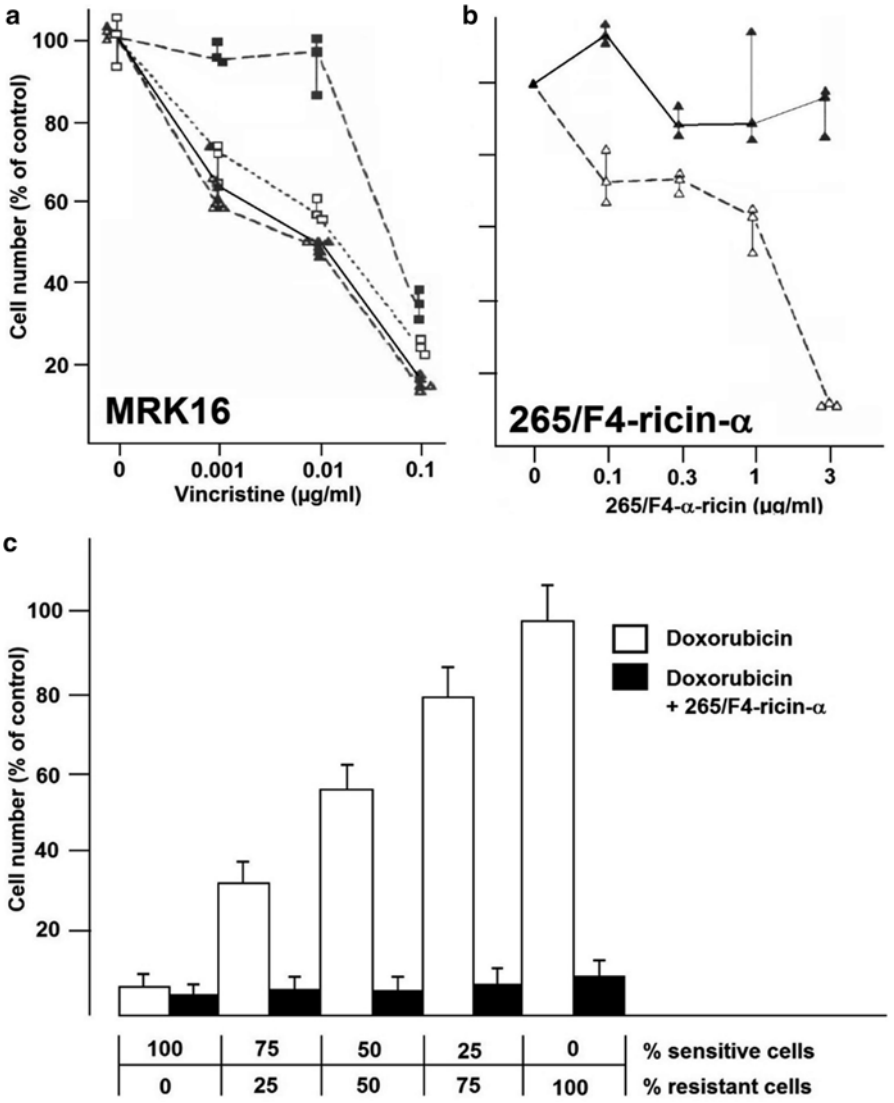

Fig. 7.7 Antibody-directed therapy of multidrug-resistant tumors. (**a**) Enhancement of vincristine by anti-P-glycoprotein monoclonal antibody MRK16. Primary cell cultures of kidney carcinoma were incubated with vincristine without or with addition of MRK16 (1 µg/ml). Cell number was determined after 7 days incubation. (**b**) Selective growth inhibition by immunotoxin 265/F4-ricin-alpha of P-glycoprotein expressing CHO-C^{5}R cells (*open symbols*, *dashed line*), but not of P-glycoprotein negative parental CHO cells (*closed symbols*, *solid line*). (**c**) Combination treatment with doxorubicin and immunotoxin 265/F4-ricin-alpha of mixed cultures of sensitive and multidrug-resistant CHO cells. Doxorubicin was applied at 1 µm/mL and the immunotoxin at 3 µg/mL. In contrast to doxorubicin alone, the combination of doxorubicin plus immunotoxin killed all cell cultures with comparable efficacy. The diagrams were taken from Efferth et al. [32] and Efferth and Volm [33]

7.5 Modulation of Multidrug Resistance by Monoclonal Antibodies and Immunotoxins

In addition to small molecule inhibitors of P-glycoprotein, multidrug resistance can also be overcome by a specific eradication of multidrug-resistant cells with monoclonal antibodies directed against P-glycoprotein. Tsuruo and colleagues raised a monoclonal antibody, MRK16, which binds to an external epitope of P-glycoprotein and thereby, inhibits drug efflux [45]. Figure 7.7a shows that cotreatment of MRK16 and vincristine inhibited growth of P-glycoprotein-expressing primary kidney carcinoma cells in a similar fashion as non-P-glycorotein-expressing primary kidney carcinoma cells, whereas the antibody did not affect the cytotoxicity of vincristine in cells without P-glycoprotein expression [46]. These results indicate that MRK16 blocked the outward channel of P-glycoprotein and thereby inhibited the efflux of vincristine out of multidrug-resistant cells.

Furthermore, we coupled another monoclonal antibody, 265/F4, which also binds to an external epitope of P-glycoprotein to the highly potent toxin ricin-alpha [32]. This immunotoxin was used to treat P-glycoprotein-negative CHO cells and P-glycoprotein-positive CHO-C^5R cells. As depicted in Fig. 7.7b, the growth of P-glycoprotein-expressing cells was inhibited in a dose-dependent manner, whereas P-glycoprotein-negative cells remained unaffected.

Tumor heterogeneity is a considerable problem to successful chemotherapy, since small subpopulations which differ in their biological features from the main population may evade drug therapy and overgrow the tumor, which ultimately leads to refractory tumors and treatment failure. As P-glycoprotein expression in human tumors is also rather heterogeneous, immunotoxins may not kill all malignant cells in a tumor. To illustrate the dimension of this problem, we mixed P-glycoprotein-negative CHO and P-glycoprotein-positive CHO-C^5R cells in various proportions. These mixtures artificially yielded heterogeneous cell cultures, which were then treated either with doxorubicin alone (to kill the drug-sensitive P-glycoprotein-negative cells) or with a combination of doxorubicin plus immunotoxin. Doxorubicin effectively inhibited sensitive CHO cells, whereas resistant cells were not affected (Fig. 7.7c) [33]. Mixed cultures were killed according to their fraction of P-glycoprotein-negative sensitive cells. Combination therapy with doxorubicin plus immunotoxin optimized cell killing independent of their proportion of P-glycoprotein-positive resistant cells (Fig. 7.7c). Therefore, this combination treatment may be suitable to overcome multidrug resistance in tumors with heterogeneous P-glycoprotein expression.

7.6 Modulation of Multidrug Resistance by Albumin-Doxorubicin Conjugates

Coupling anticancer drugs to protein carriers may improve tumor targeting and overcome multidrug resistance. We analyzed the reversing effect of bovine serum albumin-conjugated doxorubicin on doxorubicin-resistant solid tumors in vivo.

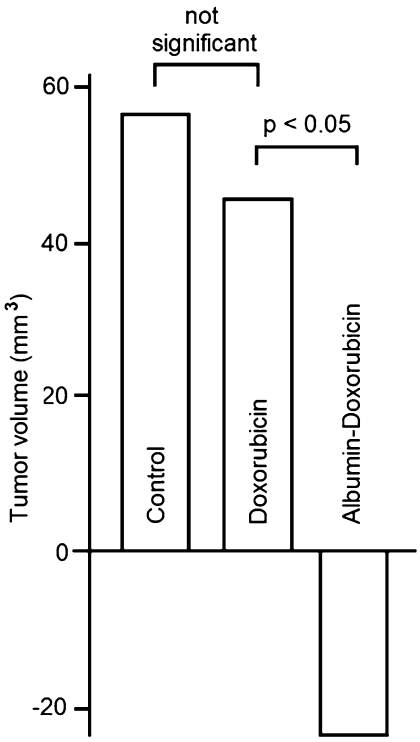

Fig. 7.8 Inhibition of solid multidrug-resistant S180 sarcoma in mice by albumin-doxorubicin conjugates. Increase/decrease in tumor volumes (From day 0 to day 4 after treatment). After the solid tumors had reached an average of 5 mm, the animals were treated with doxorubicin alone (8 mg/kg or bovine serum albumin-conjugated doxorubicin at equivalent dose). The control animals were treated with 0.9 % NaCl solution. Four days after treatment, the tumor volumes were determined. Mean values of each 10 tumors per treatment group are shown ($n=40$ mice). Data were taken from Pommerenke et al. [47]

Coupling of doxorubicin to bovine serum albumin was carried out using a method described previously [47]. Mice bearing doxorubicin-resistant solid S180 sarcoma tumors were treated with a single intraperitoneal injection of uncoupled doxorubicin (8 mg/kg), bovine serum albumin-conjugated doxorubicin (conjugate at equivalent doses of doxorubicin) or 0.9 % NaCl solution (each group 10 mice) as mock control. Tumor growth was measured daily using calipers and the tumor volume was calculated by the formula ($a^2 \times b/2$). The in vivo effect on 4th day after treatment is shown in Fig. 7.8. Mice treated with the albumin-doxorubicin conjugate had a significant reduction in tumor volume compared to mice treated with doxorubicin alone ($p=0.023$; Kruskal Wallis test) Mice treated with doxorubicin alone had no significant changes in tumor volume compared to tumors of control mice. In contrast to unconjugated doxorubicin, the conjugate was well tolerated.

7.7 Inhibition of P-Glycoprotein in Tumor and Normal Cells

In general, cancer chemotherapy exerts a number of severe side effects such as myelosuppression, gastrointestinal mucositis, nausea, vomiting, cardiotoxicity (anthracyclines), neurotoxicity (*Vinca* alkaloids, taxanes), sterility, alopecia, and others. Therefore, the question arises, whether modulation of P-glycoprotein-mediated

multidrug resistance would also provoke side effects. The dimension of this problem becomes clear, if one recalls that P-glycoprotein is not a tumor-specific protein but is also expressed in many normal organs such as kidney, adrenal, colon, liver, blood-placenta-barrier, and blood–brain barrier [31, 48, 49]. Therefore, it can be expected that any strategy targeting P-glycoprotein would affect both P-glycoprotein-expression tumor and normal tissues. To prove this hypothesis, we selected matched pairs of primary cell cultures derived from kidney cancer or normal kidney with different P-glycoprotein expression levels [50]. The P-glycoprotein expression has been determined by three different anti-P-glycoprotein monoclonal antibodies (C219; 265/F4, JSB-1) (Fig. 7.9a).

To investigate the effect of the calmodulin inhibitor, trifluperazine, on the sensitivity of the primary cell cultures to doxorubicin, trifluperazine was added 30 min before doxorubicin. As can be seen in Fig. 7.9b, trifluperazine increased the effect of doxorubicin both in kidney tumor and normal kidney primary cell cultures. The extent of inhibition was associated with the expression level of P-glycoprotein, i.e. cell cultures with low P-glycoprotein expression were less affected by the combination of trifluperazine and doxorubicin than cell cultures with high P-glycoprotein expression. These results also indicate that the inhibitory effects of trifluperazine occurred independent of whether the cell cultures were of malignant or normal origin.

As a next step, we investigated whether this phenomenon is restricted to P-glycoprotein inhibition only by chemical compounds or whether antibody-directed approaches would also affect both normal and tumor cells. This set of experiments is shown in Fig. 7.9c. We observed that monoclonal antibody 265/F4 alone or coupled to ricin-alpha inhibited the growth of both normal kidney and kidney carcinoma cells according to their P-glycoprotein expression.

Therapeutic nucleic acids (antisense oligodeoxynucleotides, ribozymes, siRNA) are gene therapeutic approaches, which can specifically target *MDR1* and P-glycoprotein. We also investigated the effect of antisense oligodeoxynucleotides directed against *MDR1*. Normal kidney and kidney carcinoma derived cell cultures with either low (patient 1), intermediate (patient 2) or high P-glycoprotein expression (patient 3) were used for this set of experiments (Fig. 7.10a). The antisense oligodeoxynucleotides partially downregulated P-glycoprotein expression in both normal and tumor cell cultures. Then, primary cell cultures of patients 1 and 3 were incubated with antisense or sense phosphorothioate-labeled oligodeoxynucleotides

Fig. 7.9 (continued) and radioimmunoassay. Mean values and standard deviations of 6–8 determinations are shown. (**b**) Reversal of inherent doxorubicin resistance by trifluoperazine in kidney carcinoma and normal kidney primary cells. In vitro effects (% of control) of 3H-uridine incorporation after addition of doxorubicin (DOX, 1 μg/ml, 3 h) alone or in combination with trifluoperazine (TFP, 1 μg/ml). TFP was added 30 min before DOX. Mean values and range of four determinations are shown. (**c**) Selective growth inhibition by monoclonal antibody 265/F4 (30 μg/ml, left side) and 265/F4-ricin-alpha immunotoxin (3 μg/ml, right side) in kidney carcinoma and normal kidney primary cells (% of control). Three independent determinations with each four parallel measurements are shown. Data were taken from Volm et al. [50] and Efferth and Volm [65]

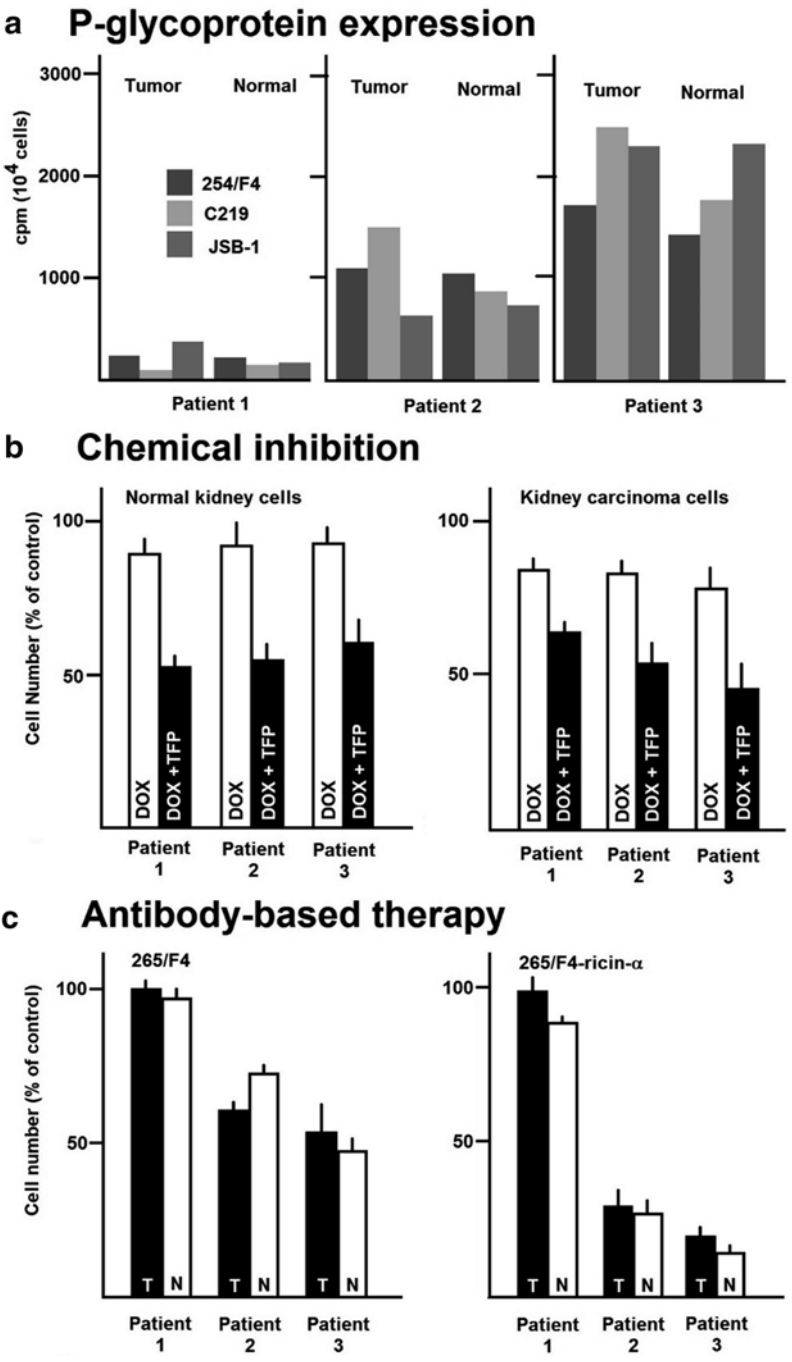

Fig. 7.9 Inhibition of P-glycoprotein in kidney carcinoma and normal kidney primary cell cultures of three patients by small molecules and antibodies. (**a**) Expression of P-glycoprotein in kidney carcinoma and normal kidney primary cells using three monoclonal antibodies (265/F4, C219, JSB-1)

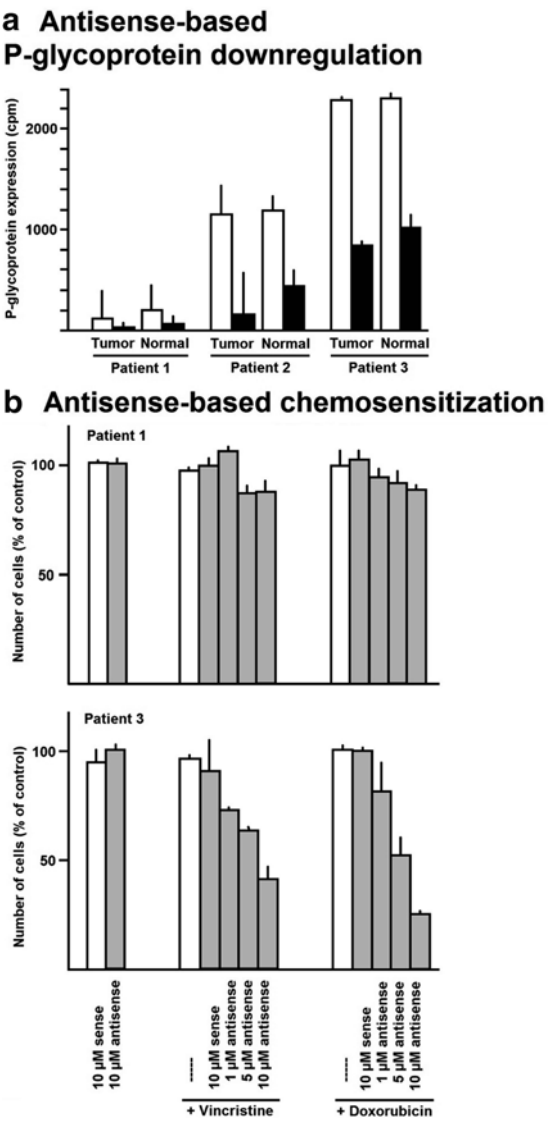

Fig. 7.10 Inhibition of P-glycoprotein in kidney carcinoma and normal kidney primary cell cultures of three patients by antisense oligodeoxynucleotides. (**a**) Downregulation of P-glycoprotein expression by antisense oligodeoxynucleotides in primary cell cultures. Effect with (*filled square*) or without (*square*) 10 µM antisense oligodeoxynucleotides as measured by radioimmunoassay (cpm/10^4 cells). In control experiments kidney carcinoma cells of patient 3 were treated with sense oligodeoxynucleotides and a P-glycoprotein expression of 2,072 (1856–2297) cpm/10^4 cells was measured. Median and range of 2–5 determinations are shown. (**b**, **c**) Effect of antisense oligodeoxynucleotides on vincristine or doxorubicin cytotoxicity of (**b**) kidney cells and (**c**) normal kidney cells of patient 3. Cells were treated with vincristine or doxorubicin alone or in combination with phosphorothioate-labeled sense and antisense oligodeoxynucleotides. Details see (**a**). Data were taken from Efferth and Volm [65]

at different concentrations or left untreated. Cells were additionally treated with vincristine or doxorubicin. With increasing antisense oligodeoxynucleotide concentrations, kidney tumor cells of patient 3 with high P-glycoprotein expression were sensitized to vincristine and doxorubicin (Fig. 7.10c), but not cells of patient 1 with low P-glycoprotein expression (Fig. 7.10c) [65].

7.8 Conclusions and Perspectives

The reversal of multidrug resistance is possible in vitro by different strategies directed against P-glycoprotein, i.e. synthetic and natural small molecules, monoclonal antibodies, and immunotoxins, as well as therapeutic nucleic acids (e.g. oligodeoxynucleotides). A still unresolved problem is that the P-glycoprotein does not represent a tumor-specific marker of multidrug resistance. Its expression in several normal organs and tissues raise the possibility that attempts to overcome P-glycoprotein-mediated multidrug resistance in a clinical setting may lead to unwanted side effects in these normal tissues. Clinical trials with several P-glycoprotein modulators were not successful due to intolerable toxicity and other problems [51, 52].

Therefore, in addition to further optimizing clinical trial designs to overcome multidrug resistance in the clinic, other treatment strategies should be explored for their potential to kill P-glycoprotein-expressing, multidrug-resistant tumors:

1. Collateral sensitivity: In the search for P-glycoprotein inhibitors, it was underestimated that multidrug resistance comprises many but not all anticancer drugs. P-glycoprotein-expressing tumor cells retain sensitivity to alkylating agents, platin compounds, and some antimetabolites. This finding opens the possibility to treat multidrug-resistant tumors by non-cross-resistant established or novel cytotoxic and thereby to bypass multidrug resistance [53, 54]. Some of the non-cross-resistant drugs are even hypersensitive, i.e. they kill resistant cells at low IC_{50} values than their drug-sensitive counterparts [55]. This phenomenon has been termed collateral sensitivity. Molecular and cellular mechanisms of collateral sensitivity have been investigated in recent years opening avenues for the specific targeting of multidrug-resistant tumor cells by collateral sensitive drugs [56, 57].
2. Immunogenic cell death: One of the most threatening side effects of standard cancer chemotherapy is myelosuppression. Thereby, drugs kill not only cancer cells but also proliferating bone marrow cells leading to a severe damage of the immune system. It is known that immune cells of the body help to eradicate cancer cells. Treated patients lack this favorable effect, which might ultimately lead to refractory cancers. Recently, it has been proposed to use low dose chemotherapy instead of the standard high doses of anticancer drugs. Cell death can be immunogenic. Some drugs, e.g. anthracyclines stimulate dendritic cells to take up and process tumor antigens of dying cells. Antigen presentation by dendritic cells prime cytotoxic T lymphocytes, thus eliciting a tumor-specific cognate immune response [58–60]. Immunogenic cell death can also target multidrug-resistant cancer cells ultimately leading to tumor eradication independent of P-glycoprotein [61].
3. Drug holidays: Frequently, tumors develop drug resistance after several cycles of chemotherapy. Therefore, intermittent chemotherapy may be more beneficial than continuous drug treatment [62–64]. The reduced cumulative toxic effects of

chemotherapy improve quality of life of cancer patients. As repeatedly shown, intermittent chemotherapy may bear the danger of shorter survival times for patients due to suboptimal tumor killing compared to continuous treatment. In some patients, intermittent chemotherapy even improves the response rates to chemotherapy. Genetic aberrations and epigenetic changes in resistant tumor subpopulations may disappear during treatment holidays leading to a resensitization of the tumor. This point of view has been substantiated by in vivo experiments with revertant multidrug-resistant tumor lines. Withdrawal of drug treatment for 25 weeks led to a reversion of amplification of the MDR1 gene and downregulation of MDR1 mRNA and P-glycoprotein expression in L1210 tumors in mice (see Chap. 1 of this book).

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Chapter 8
ABC Transporter Modulatory Drugs from Marine Sources: A New Approach to Overcome Drug Resistance in Cancer

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Abstract Fighting this unconquerable mammoth of a disease that is cancer has been increasingly difficult from its discovery over a century ago. A multitude of agents have been identified in the past to conquer the battle against cancer. Nevertheless, controlling it from its onset has been very difficult, the reason being its ability to evade the toxic insults, and to develop mechanisms to survive in the presence of cancer drugs. Of the several factors responsible, the phenomenon of multidrug resistance (MDR) has contributed essentially for the cancerous cells to survive. This phenomenon is characterized by the ability to impart immunity to several classes of drugs with different structural and mechanistic traits. The association of ATP-binding cassette (ABC) efflux transporters with the development of MDR has been a major impediment toward attaining an efficient chemotherapeutic outcome in cancer patients. Overcoming this pathway of resistance with the use of modulators to block the drug efflux transporters has shown some promise in recent years. However, there is still room for improvement in designing the clinical strategy and developing newer agents to overcome MDR. Nature has provided researchers

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amazing treatment options in the past for numerous diseases. Hence, it is now time to look into nature and find answers to effectively modulate the function of ABC drug transporters to overcome resistance to anticancer drugs. Here we discuss some lead molecules isolated from marine organisms that have shown promising results in overcoming MDR associated with ABC transporters.

Keywords ABC transporters • Marine drugs • Modulators • Multidrug resistance • Drug efflux • P-glycoprotein • Anticancer drugs

Abbreviations

ABC	ATP-binding cassette
ABCP	ABC transporter expressed in the placenta
BCRP	Breast cancer resistance protein
DX	Doxorubicin
MDR	Multidrug resistance
MRP1	Multidrug resistance protein 1
MRP7	Multidrug resistance protein 7
MXR	Mitoxantrone resistance protein
P-gp	P-glycoprotein
VCR	Vincristine
VLB	Vinblastine

8.1 Introduction

Two centuries ago a 21 year old German, Friedrich Sertürner, while serving as a pharmacist's apprentice isolated morphine, the first pharmacologically active compound from a natural plant source, it was isolated from opium produced by the cut seeds of poppy, *papaver somniferum* [1]. With this started an era where compounds extracted from natural sources began being explored for their medicinal values. Research in the pharmaceutical industry heightened soon after the Second World War. By the early 1990s, scientists and clinicians were left inspired and enlightened by the amount of medicinally active ingredients nature had to provide. About 80 % of the available drugs, then, were either discovered from natural origin or were synthetic analogs of the same. Antibiotics (e.g., penicillin, tetracycline, erythromycin), antiparasitics (e.g., ivermectin), antimalarials (e.g., quinine, artemisinin), lipid control agents (e.g., lovastatin and its analogs), immunosuppressants for organ transplants (e.g., cyclosporine, rapamycin), and a majority of anticancer drugs (e.g., paclitaxel, doxorubicin) revolutionized medicine. Life expectancy in much of the world lengthened from about 40 years early in the twentieth century to more than 77 years today.

Cancer, however, has always been a hard-fought disease from its discovery centuries ago. The treatment of cancer has been controversial due to its highly

robust and impervious mechanisms to resist chemotherapy. Although the neoplastic process has been recognized for centuries, little was known about the biological mechanisms of transformation and tumor progression until the advent of molecular medicine in the latter half of the twentieth century. Getting acquainted with this vastly disseminating disease is now no longer based on the empirical observations of tumor growth by surgeons but has now become increasingly dependent on the understanding of tumor biology and its genetic makeup. Surgery and radiation therapy remained the only source of treatment options in the past. Unfortunately, however, they were unable to eradicate the metastatic cancers. It was soon understood that the treatment needed to reach every organ in the body, and hence, drugs biologically tailored molecules and immune therapy have become the focus of mainstream chemotherapy. From the introduction of the first chemotherapeutic strategy by Louis Goodman and Alfred Gilman in the 1940s there has been immense progress in the field of anticancer drug discovery with the introduction of several mainline chemotherapeutic drugs to specific target modeled drugs. Throughout time, nature has been an important tool in providing key lead molecules to combat this highly proliferative and invasive disease.

8.1.1 Plant Sources of Anticancer Drugs

Traditional Native Americans have been using the plant extracts over a long period of time providing them relief from several diseases such as skin cancers and venereal warts. The main constituent of that was podophyllotoxin, the forerunner of the drugs now known as etoposide and teniposide. Soon as the importance of these medicinal extracts was realized, scientists at the National Cancer Institute in the 1960s began an intensive large-scale screening of antitumor agents from natural origin. After screening of over 35,000 plant extracts a wonder drug "paclitaxel" (taxol) was introduced to the world of cancer chemotherapy. The extract from the bark of the Pacific yew tree, *Taxus brevifolia*, was used widely to fight cancer. It soon became the best selling drug in the United States for refractory breast and ovarian cancer. Very soon a plethora of molecules were isolated and identified for their use in cancer chemotherapy, drugs such as vincristine, etoposide, and camptothecin are among various others that are a part of several mainline chemotherapeutic strategies; together accounting to more than 60 % of the chemotherapeutic regimens. Thus, the modern pharmaceutical discovery programs owe much debt to natural products.

8.1.2 Marine Sources of Anticancer Drugs

Interest in natural products isolated from marine sources, however, awaited refinements in technologies (mainly scuba diving) to collect the source organisms. The medicinal value of the marine environment attracted much interest even in the late 1950s. Beginning in 1951, Werner Bergmann published three reports of unusual arabino- and

ribo-pentosyl nucleosides obtained from marine sponges collected in Florida, USA [2–4]. The compounds eventually led to the development of the chemical derivatives ara-A (vidarabine) and ara-C (cytarabine), two nucleosides with significant anticancer properties that have been in clinical use for decades. Although the marine environment puts forth a few challenges toward acquiring source materials, technological advances in analytical chemistry, spectroscopy, and high-throughput screening have aided in isolating and identifying valuable constituents. This being an essential driving force due to the broad realization that competing technologies, such as combinatorial chemistry, have failed to deliver new drug leads in significant numbers. The major impediment for this flourishing natural marine resource library was obvious from its outset, the procurement and manufacture of quantities of rare compounds from marine sources to ensure a sustainable supply. For example, the chemically versatile marine sponges, the source of many developmental compounds such as discodermolide, a natural promoter of tubulin assembly and hemiasterlin, a natural disruptor of tubulin [5, 6], are primitive metazoans that live almost exclusively in marine habitats. Sponges and their microbial fauna are largely un-culturable under laboratory conditions, and the valuable compounds they produce must be extracted and purified from specimens collected by hand with the help of scuba diving from shallow to deep waters, or sometimes with the aid of submersibles equipped with robotic arms. Both of these techniques are expensive endeavors that are unwieldy and foreign to the modern pharmaceutical industry. Nevertheless, interest in the remarkable properties of marine natural products remained high enough, inspiring modern techniques such as aquaculture of marine invertebrates to semi-synthesizing these molecules.

8.2 Cancer and Drug Resistance

Along with the evolution of technology and advances in isolation of novel molecular targeted anticancer drugs, cancer, the disease, has evolved to resist these anticancer drugs and lived on to co-exist in their presence. A diverse range of host specific and molecular mechanisms have been implicated in drug resistance; these include but not limited to host-specific factors such as poor absorption, rapid metabolism, patient tolerance, drug delivery, and molecular mechanisms such as increased rates of drug efflux, alterations in drug metabolism, increased DNA damage repair, and mutation of drug targets (Fig. 8.1) [7–11]. With this, cancer cells gain cross immunity against the cytotoxic insults of multiple chemotherapeutic agents, a phenomenon widely accepted as multidrug resistance (MDR) that is characterized by resistance to a multitude of structurally and mechanistically unrelated drugs [8, 12]. Tumors are highly adaptable, and the activation of survival signaling pathways and the inactivation of downstream death signaling pathways can also lead to drug resistance [13, 14]. Moreover, the increasingly recognized molecular and genetic heterogeneity that is present in many tumors is another major problem that can contribute substantially to drug resistance [15]. Several interestingly detailed reviews enumerate and elaborate on the mechanisms of resistance in cancer cells [12, 8, 16–18].

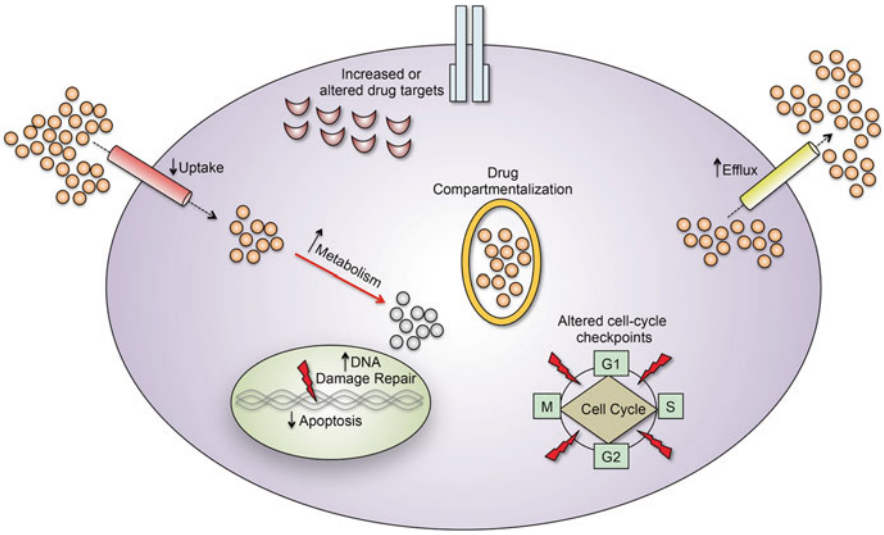


Fig. 8.1 List of different mechanisms used for acquiring drug resistance in cancer cells. Over the years, a great amount of research has been conducted to understand the pathology and to uncover the backbone that this disease thrives upon. The progress made thus far has helped unveil the resistance mechanisms adopted by the cancer cells to avoid the cytotoxic effects of anticancer agents and grow even in their presence. Here we enlist the various mechanisms that the cancer cells embrace to overcome chemotherapeutic insults

8.3 ABC Transporters and MDR

Comprised of 48 members over seven subfamilies from A through G, human ABC proteins work together to help maintain homeostasis within the body. These evolutionarily conserved and widely expressed transporters are one of the largest family of transporter proteins in bacteria and eukaryotes [19]. Genetic defects in the ABC transporters give rise to several disease phenotypes. Some important ones include the well-known cystic fibrosis, characterized by a defect in the chloride channel function of CFTR, and the autosomal recessive disorder called progressive familial intrahepatic cholestasis. These are characterized by an inability to transport bile salts and phospholipids from and to the liver, due to a dysfunctionally mutated *ABCB11* and *ABCB4* genes, respectively [20–23]. Dubin–Johnson syndrome, another autosomal recessive disorder, has its roots embedded in the disruption of functionally important ABC domains in MRP2 (ABCC2), a hepatobiliary anionic conjugate exporter [24–26]. Another intriguing disorder is Pseudoxanthoma elasticum (PXE), a heritable disorder characterized by calcification of elastic fibers in skin, arteries, and retina, which results in dermal lesions with associated laxity and loss of elasticity, arterial insufficiency, and retinal hemorrhages leading to macular degeneration [27–30]. A list of ABC transporters and the associated diseases are enlisted in Table 8.1.

Table 8.1 ABC transporters involved in diseases

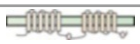
ABC transporter	Disease associated	Implication
ABCA1	Tangier disease	Inability to transport HDL
	Familial HDL deficiency	
ABCA4	Stargardt disease	Inability to transport *N-retinyl-phosphatidylethanolamine* in turn causing degeneration of photoreceptors
	Age-related macular degeneration	
	Retinitis pigmentosa	
ABCB1	Cancer	Overexpression of ABCB1 causing MDR
ABCB4	Progressive familial intrahepatic cholestasis	Inability to transport phospholipids
ABCB7	Sideroblastic anemia and ataxia	Inability to produce heme
ABCB11	Progressive familial intrahepatic cholestasis	Reduction of bile salt transport
ABCC1	Cancer	Overexpression of MRP1
	Chronic obstructive pulmonary disease	Reduced MRP1 expression levels in lung
	Cystic fibrosis	
ABCC2	Dubin–Johnson syndrome	Reduced expression of ABCC2 levels in liver
ABCC6	Pseudoxanthoma elasticum	Reduced expression of ABCC6
ABCC7	Cystic fibrosis	Absence or reduced expression of ABCC7 in liver respiratory and intestinal tract
ABCC8	Persistent hypoglycemia of infancy	Increased and continuous insulin secretion
ABCC9	Persistent hypoglycemia of infancy	Increased and continuous insulin secretion
ABCD1	Adrenoleukodystrophy	Inability to transport fatty acids into peroxisomes
ABCG2	Cancer	Overexpression of ABCG2 causing MDR
	Gout	Reduced expression of ABCG2 in renal tubules

More importantly, the association of ABC transporters with cancer was just like a curve ball thrown at the scientists, when it was discovered that cancer cells have been utilizing these efflux transporters to vault themselves to the toxic insults of chemotherapeutic agents. P-glycoprotein (P-gp), also known as multidrug resistance 1 (MDR1) and ABCB1, is an ATP-dependent efflux transporter that was among the first of the superfamily of ABC transporters to have been linked to MDR in cancer cells. ABCG2, the second member of the G subfamily of ABC transporters, also known as BCRP (breast cancer resistance protein) is another efflux transporter to have been linked to MDR. Also contributing to MDR is the first member of the C subfamily of ABC transporters ABCC1 also known as multidrug resistant protein 1 (MRP1). ABCC10 the tenth member of the C subfamily of transporters, or multidrug resistance protein 7 (MRP7), is a recently identified and characterized transporter to contribute toward the development of MDR in cancer cells. These transporters are overexpressed in cancer cells and they all have broad, overlapping substrate specificity and promote the elimination of various hydrophobic compounds, including major cancer chemotherapeutics such as taxanes, topoisomerase inhibitors, and antimetabolites.

8.4 Clinical Significance of ABC Transporters

P-gp was the first ABC transporter to be identified; it is a membrane-bound glycoprotein that is expressed in almost all tissues at low levels, but is found at much higher levels on the surface of epithelial cells that have excretory roles, such as those lining the colon, small intestine, pancreatic ducts, bile ducts, and kidney proximal tubules [31]. The *MDR1* gene is innately overexpressed in many tumors (thus causing intrinsic drug resistance) and the expression of *MDR*1 can also be induced by chemotherapy (thus also resulting in the acquired development of MDR) [32]. The overexpression of *MDR*1 or *ABCB*1 has been associated with chemotherapy failure in many cancers, including kidney, colon, and liver cancers, as well as leukemias and lymphomas. More recently, the overexpression of MRP1 has also been correlated with chemoresistance in prostate, lung, glioblastoma, and breast cancer [27, 33–35]. BCRP, which was the third major MDR drug efflux pump to be identified, has been associated with chemoresistance in breast cancer and leukemia [36, 37]. Recent reports have suggested that molecularly targeted therapeutics such as imatinib, erlotinib, sunitinib, and nilotinib are also substrates and modulators of P-gp and BCRP [38]. Cancer stem cells, which are inherently drug resistant, also display higher levels of drug efflux proteins [39]. CD44 is a cancer stem cell marker that exhibits strong negative correlations with patient survival [40] and has been associated with expression of MDR proteins, most notably BCRP. Recent reports have suggested that the ABCC10 expression level is elevated in non-small cell lung cancer (NSCLC) compared with normal lung, with ABCC10 expression in adenocarcinoma correlated with tumor grade and stage [41]. A list of anticancer drug substrates of the corresponding MDR-linked ABC transporters is given in Table 8.2.

Table 8.2 ABC transporters overexpressed in MDR cancers, their localization, topology, and substrate anticancer drugs

MDR transporter	Alternate nomenclature	Localization	Topology model	Anticancer substrates
P-gp	ABCB1, MDR1	Apical		Multiple, including Vinca alkaloids, anthracyclines, etoposide, taxanes, imatinib, irinotecan, methotrexate, mitoxantrone
BCRP	ABCP, ABCG2, MXR	Apical		Multiple, including anthracyclines, etoposide, imatinib, flavopiridol, irinotecan, methotrexate, mitoxantrone
MRP1	ABCC1	Basolateral		Vinca alkaloids, anthracyclines, etoposide, imatinib, irinotecan, methotrexate, mitoxantrone
MRP7	ABCC10	Basolateral		Vinca alkaloids, taxanes, nucleoside analogs, gemcitabine, Ara-C

8.5 Overcoming MDR in Cancer Cells

The clinical prevalence of these ABC transporters in rendering the cancer cells MDR was soon realized and strategies to block this detestable transporter were being prepared. The strategies involved searching within the huge library of drugs to effectively block these MDR transporter efflux systems and soon a vast majority of drugs were identified such as verapamil, cyclosporine A for P-gp blockade [42–45] to newly identified molecular target specific drugs such as nilotinib, lapatinib, erlotinib for P-gp, BCRP, MRP1 and MRP7 [46–57]. Figure 8.2 depicts the different modalities of overcoming MDR in cancer cells. A total of three generations of modulators for ABC transporters were developed in order to elucidate their functions and attain a better outcome of blocking these ABC transporters in cancer patients. Some showed promising results in clinical trials achieving better overall survival (OS) rates; these include the addition of quinine to mitoxantrone and cytarabine therapy for patients with high-risk myelodysplastic syndromes resulting in improved OS rates among P-gp positive patients [58]. The addition of cyclosporine to daunorubicin and cytarabine therapy for patients with poor-risk acute-myeloid leukemia (AML) also resulted in improved OS rates [59] and cyclosporine plus daunorubicin increased the complete remission (CR) rate for patients with AML [60]. These results encourage clinicians and researchers around the world to search for drugs with improved ability to modulate the efflux function of these crucial ABC transporters.

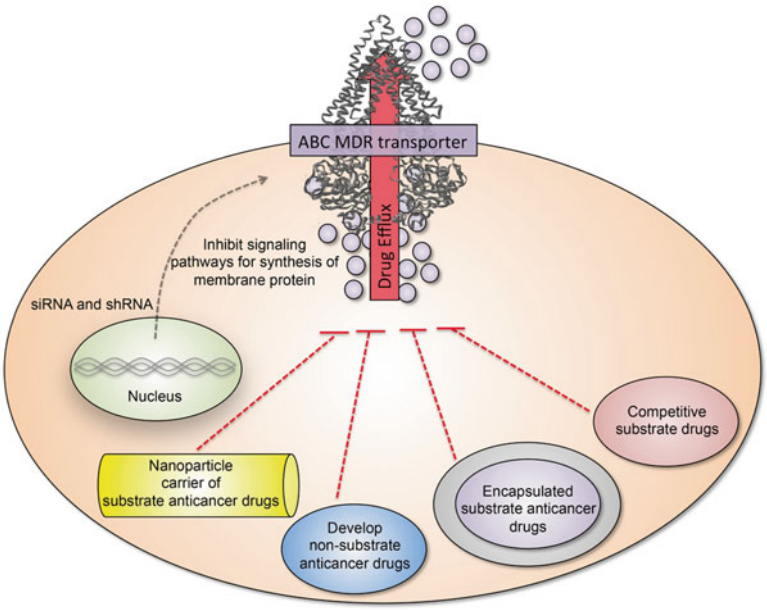


Fig. 8.2 ABC transporters have been used by cancer cells to overcome chemotherapeutic insults and to impart immunity to the cancer cells against anticancer drugs. The figure describes the modalities used in the past to successfully overcome the phenomenon of ABC transporters-mediated MDR. The structure of mouse P-gp [137] is shown as a representative MDR-linked ABC transporter

Another strategy involves the ability of lead molecules to diminish the protein expression of ABC transporters. A number of studies have shown that targeted agents inhibiting specific pathways may induce downstream effects on MDR as a consequence of signaling inhibition. In a recent report, the cyclooxygenase-2 (COX-2) inhibitor SC236 and the nonsteroidal anti-inflammatory drug (NSAID) indomethacin were shown to inhibit P-gp and MRP1 expression and, thus, to enhance doxorubicin cytotoxicity in an MDR hepatocellular carcinoma cell line [61]. Multiple cell line studies have demonstrated that numerous novel compounds have the ability to inhibit MDR protein function, although no recent clinical studies have been reported. Curcumin, an active ingredient in the plant *Curcuma longa,* showed inhibitory activity against P-gp expression in leukemia cells from patients [62] and the combretastatin A-4 analog 4-(4-bromophenyl)-2,3-dihydro-*N*,3-bis(3,4,5-trimethoxyphenyl)-2-oxoidmidazole-1-carboxamide (MZ3) overcame MDR in leukemia cells by downregulating *MDR1* transcription and antiapoptotic protein expression [63].

Another interesting approach is to use small interfering RNAs (siRNAs), including short hairpin RNAs (shRNAs), targeted at MDR genes that were shown to be effective in a number of studies [64]. shRNAs/siRNAs targeting *MDR1* were shown to be effective in inhibiting P-gp expression and resensitizing cells to harringtonine and curcumin when they were transfected into MDR HT9 leukemia cells [65], and they were shown to downregulate P-gp expression and to increase drug sensitivity in MDR K562/Adr leukemia cells [66]. A combination of daunorubicin-conjugated magnetic Fe_3O_4 nanoparticles and shRNA expression vector aimed at P-gp mRNA overcame resistance in MDR K562/AO2 leukemia cells [67]. Alternative approaches to gene silencing, including the use of antisense oligonucleotides [68], transcriptional regulation [69], and targeted ribozymes [70], also have been studied [64].

An alternative approach to circumvent MDR was to develop chemotherapeutic agents that are not substrates of ABC transporters. New therapeutic agents might be designed to avoid these efflux mechanisms and, thus, to achieve high drug concentrations in cancer cells, which might result in enhanced cell death. Amonafide, a novel topoisomerase II inhibitor considered for the treatment of AML, was shown to be neither a substrate nor an inhibitor of P-gp [71, 72]. Also increasing the lipophilicity by encapsulation of agents in liposomes as in the case of pegylated liposomal doxorubicin [73] or stealthy liposomal encapsulation of vincristine and quinacrine [74] may increase the lipid permeability and improve their passive diffusion in turn abolishing the large concentration gradients and thereby alleviating the resistance attributed to efflux transporters.

8.6 ABC Transporter Modulators and the Lack of Clinical Benefit

Although many MDR modulators have been discovered, there has been more failure of outcome than success since several clinical trials then sought to evaluate their ability to improve the therapeutic index in patients with advanced cancer. Most of the agents tested were not evaluated for their ability to improve the therapeutic

index when used in rodents with established macroscopic tumors. In addition, modulators of ABC transporters were soon identified as substrates of the cytochrome P450 systems thereby compromising the action of these biotransformation enzymes, which are necessary for the clearance and/or metabolism of co-administered anticancer drugs [75, 76]. While ABC transporter modulator development being in its nascent stages, patients chosen for preclinical and clinical trials had undergone several chemotherapeutic regimens making MDR multifactorial and not primarily due to ABC transporters. Thus, the failure of modulators of ABC transporters was attributed to the inability of such agents to account for the full spectrum of MDR found in the clinic [77–79]. Modulators identified were also not selective to the action on one ABC transporter, thereby increasing the unexpected side effects. Taking into consideration these multitudes of factors and the lack of a successful clinical trial in the recent past warrants the need to search for newer chemical agents to overcome this seemingly invincible drug efflux system.

Nature in the past has provided scientists answers in the most difficult times giving us wondrous chemical agents to fight several diseases; hence there is no better place to look for modulatory agents than nature itself. Here we discuss the several efforts made in the past to isolate and characterize agents isolated from marine origin having potential to overcome MDR in cancer cells.

8.7 Marine Modulators of ABC Transporters

Succeeding the failure of several agents in the past to qualify for clinical trials and others that have failed to impart improved survival in MDR cancer patients, scientists took a detour and started to look for answers in nature. The challenge further lies in finding a way to improve the MDR phenotype by supporting the chemotherapeutic agents with clinically useful ABC transporter inhibitors. These efforts resulted in the identification of a number of marine compounds that are able to reverse MDR, such as lamellarins, agosterol A, ecteinascidin 743, sipholane triterpenoids, welwitindolinones, and parguerenes among others (Fig. 8.3, Table 8.3). In this review, we discuss marine compounds, their activity as MDR reversal agents, and their impact on the chemotherapy over various malignancies. Recently, Lopez and Martinez-Luis [80] reported in a review that patellamide d, kendarimide A, bryostatin 1, ISA, ISA B, nocardioazines, discodermolide, and polyoxygenated steroids are also MDR reversal agents (these will not be discussed here).

8.7.1 Lamellarins

Lamellarins are polyaromatic alkaloids previously isolated from *Lamellaria* sp. a prosobranch mollusc collected from tunicates belonging to the genus*, Didemnum chartaceum* from the Great Barrier Reef [81, 82]. Most probably the reason for the

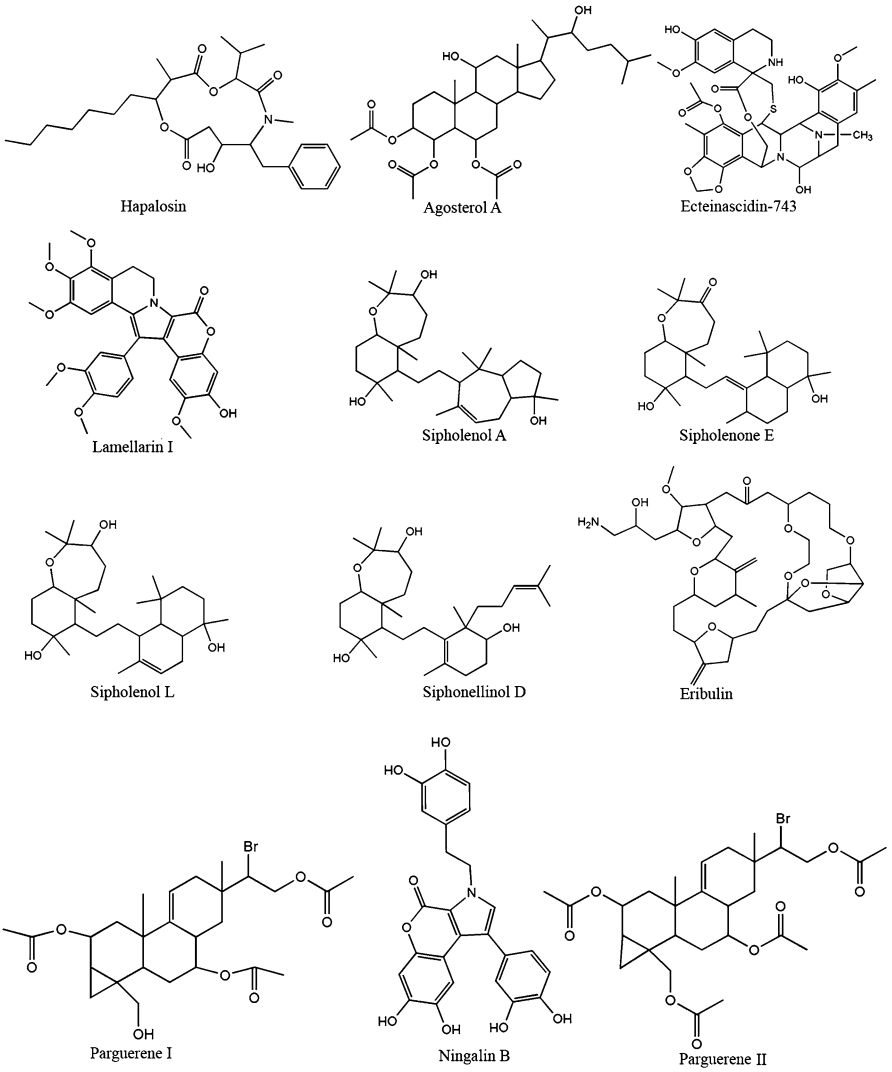


Fig. 8.3 Structures of active constituents isolated from marine natural sources identified as having potent activity toward reversing the MDR-linked ABC transporters in cancer cells

presence of these compounds in the mollusc is the use of these ascidians as a food source. Quesada et al. in 1996 identified their potential for reversing MDR mediated by P-gp in cancer cells [83]. The results obtained depicted two independent modalities in which lamellarins could be useful for the treatment of cancer: cytotoxicity against cancer cells and enhancement of the cytotoxicity of doxorubicin (DX) against MDR cells restoring in them the levels of sensitivity to those of the parental cells.

Table 8.3 Marine modulators of MDR-linked ABC transporters

Name	Origin	P-gp	BCRP	MRP1	MRP7	Reference
Lamellarin I	*Didemnum chartaceum*	+	Undetermined	Undetermined	Undetermined	[71]
Agosterol A	*Spongia sp.*	+	Undetermined	+	Undetermined	[73]
ET-743	*Ecteinascidia turbinate*	+	Undetermined	Undetermined	Undetermined	[88]
Hapalosin	*Hapalosiphon welwitschii*	+	Undetermined	Undetermined	Undetermined	[91]
Ningalin	*Didemnum sp.*	+	Undetermined	Undetermined	Undetermined	[94–98]
10		+	Undetermined	Undetermined	Undetermined	
11		+	Undetermined	Undetermined	Undetermined	
13		+	Undetermined	Undetermined	Undetermined	
14		+	Undetermined	Undetermined	Undetermined	
Triterpenoids	*Callyspongia siphonella*	+	–	–	–	[104, 105]
Sipholenone E		+	–	–	–	
Sipholenol L		+	–	–	–	
Siphonellinol D		+	–	–	–	
Eribulin (Synthetic analog of Halichondrin B)	*Halichondria okadai*	+	Undetermined	Undetermined	Undetermined	[119]
N-Methylwelwitindolinone C isothiocyanate	*Hapalosiphon welwitschii*	+	Undetermined	Undetermined	Undetermined	[121]
Parguerenes I	*Laurencia sp.*	+	–	+	Undetermined	[122]
Parguerenes II	*Laurencia sp.*	+	–	+	Undetermined	

The anticancer activity was tested in cells derived from murine leukemias (p388), human colon adenocarcinoma (LoVo), lung carcinoma (A549), human colon carcinoma (HT29), and melanoma (MEL28). Five of the lamellarins tested (lamellarins D-triacetate, K, K-triacetate, M and N-triacetate) displayed considerable cytotoxic activity against all the tumor cell lines tested showing IC_{50} in the nanomolar to submicromolar range. This cytotoxic activity among the five lamellarins tested in these cells was not affected by the efflux activity of P-gp. Among the lamellarins isolated, lamellarin I completely sensitized the DX resistant cells LoVo/DX cells to the cytotoxic effects of DX, vinblastine (VLB), and daunorubicin at concentrations up to 2 μM. Also lamellarin I reduced the efflux of rhodamine 123, a fluorescent P-gp substrate in Lo Vo/DX cells. The increase in accumulation of rhodamine 123 in MDR cells after the addition of lamellarin I supports its P-gp modulatory activity.

8.7.2 Agosterols

In 1998 Aoki et al. described the potential anticancer activity of agosterol A, a novel polyhydroxylated sterol acetate from a marine sponge of *Spongia* sp. collected in Mie Prefecture, Japan [84–86]. The ethyl acetate-soluble extract sensitized the KB-C2 epithelial carcinoma cells overexpressing P-gp to the cytotoxic effects of colchicine. Later upon isolating the extract, Aoki et al. identified six different sterol analogues, agosterol A, B, C, D2, A5, and C6 [85]. Of them, agosterol A showed the strongest activity by completely reversing the colchicine resistance in KB-C2 and vincristine resistance in KB-CV60 cells, at 1 μg/mL concentration. Even at 10 μg/mL concentration, agosterol A was not cytotoxic to parental cells. Subsequent biological evaluation of agosterol A was published 2 years later [87]. Agosterol A completely reversed the resistance to vincristine, colchicine, doxorubicin, and etoposide in both KB-C2 and KB-CV60 cells at a 3 μM concentration. Functional assays conduced then showed that agosterol A inhibited the ATP-dependent active efflux of vincristine in both KB-C2 and KB-CV60 cells and increased the intracellular levels of vincristine. Moreover, agosterol A inhibited both the photolabeling of P-gp with [^{3}H]-azidopine and the uptake of [^{3}H]-S-(2,4-dinitrophenyl) glutathione (DNP-SG) in inside-out membrane vesicles derived from KB-CV60 cells. These data indicated that agosterol A inhibited the drug efflux activity of P-gp and MRP1 by directly binding to the drug-binding pocket of these transporters. Agosterol A inhibited MRP1-mediated transport of typical amphipathic substrates, increased the drug accumulation of vincristine in MRP1-transfected cells, and reduced intracellular glutathione levels [88]. This suggests that agosterol A diminished MRP1-mediated drug resistance by both directly inhibiting the ability of the pump to transport drugs and reducing the levels of the cellular component glutathione required for drug efflux.

8.7.3 Ecteinascidin-743 (ET-743)

ET-743 is a unique isolate from the Caribbean tunicate sea squirt *Ecteinascidia turbinate* possessing remarkable antiproliferative activity and modulatory activity against P-gp. It was discovered in the late 1960s; however the purification and the chemical structure elucidation of the active constituents were not completed until 1990 [89, 90]. To perform basic studies for the mechanism of action and preclinical in vivo studies, large amounts of the tunicate had to be collected. Currently, ET-743 is obtained by a semisynthetic process using cyanosafracin B obtained in bulk through fermentation of the marine bacterium *Pseudomonas fluorescens* [91]. ET-743 has been reported to bind to the minor groove of DNA, thus bending the DNA helix toward the major groove [92]. Its mechanism of action also includes interference with cellular transcription-coupled nucleotide excision repair to induce cell death and cytotoxicity [93]. Phase II/III clinical trials demonstrated the potency of ET-743 against human solid tumors and soft tissue sarcomas [94–96]. ET-743 being a potent inhibitor of (nuclear transcription factor-Y) NF-Y, (transcription factor) sp1, and (steroid and xenobiotic receptor) SXR transcription factors, ET-743 in turn blocks the transcription of certain genes including the *MDR*1 promoter [97–99]. Also reported was the fact that P-gp was overexpressed in the human ovarian carcinoma IGROV-1 cell line selected for resistance to ET-743 in vitro and that this resistance was attenuated by the P-gp reversing drug agent PSC833 (valsopodar), a cyclosporine A analog [100]. Kanzaki et al. investigated the activity of ET-743 in two well-characterized P-gp overexpressing cell lines, KB-8-5 and KB-C-2, both derived from exposing KB-3-1 cells to increasing concentrations of colchicine up to 10 ng/mL and 2 μg/mL, respectively, and they found that neither cell line was cross-resistant to ET-743 [101]. Furthermore, they found sub-toxic concentrations of ET-743 reversed resistance to DX and VCR in these cells. In addition, human colon carcinoma HCT116 cells made resistant to ET-743 at concentrations up to 15 nM did not upregulate P-gp nor did they show any resistance toward paclitaxel or VCR. Pretreatment with ET-743 increased the accumulation of these two drugs, by downregulating the expression of P-gp. These results suggest the usefulness of ET-743 alone or in combination with other agents in P-gp-overexpressing tumors. Currently, ET-743 has received orphan drug status for the treatment of soft tissue sarcoma in the United States and for recurrent ovarian cancer in both the United States and Europe [102].

8.7.4 Hapalosin

In screening extracts of blue–green algae (cyanobacteria) for activity against MDR, about 1 % of the extracts exhibited MDR reversing activity. The lipophilic extracts of two strains of *Hapalosiphon welwitschii* W. & G. S. West (UH IC-52-3 and UTEX B1830) reversed MDR in a P-gp overexpressing, VLB-resistant cell line

(SKVLB1) derived from a human ovarian adenocarcinoma cell line (SKOV3) [103]. Hapalosin was found to have better MDR reversing activity than verapamil. In an independent accumulation study of [^{3}H]-paclitaxel, a substrate of P-gp, hapalosin at concentrations up to 20 μM significantly and dose dependently enhanced the accumulation of [^{3}H]-paclitaxel in the SKVLB1 cells. Breast cancer cells MCF-7/ADR, a P-gp overexpressing subline derived from the MCF-7 cells, resistant to VLB were more sensitive to the cytotoxic effect of VLB when combined with hapalosin than VLB alone treatment. The MCF-7 cells showed no change in sensitivities to VLB either in combination with hapalosin or alone, thereby attributing the changes in sensitivities to the ability of hapalosin to sensitize MCF-7/ADR cells to VLB due to the inhibition of P-gp transport function. Further efforts were made to synthesize hapalosin and its analogs with the aim of obtaining compounds with similar or superior MDR reversal activity [104]. Twenty-six structural analogs were synthesized with partial or less than desired MDR reversal activity, thus making hapalosin and its structural modification open for research and the potential to develop agents with more potent MDR reversal abilities.

8.7.5 Ningalin

Ningalin B is the member of a newly described family of marine natural products isolated by Fenical (1997) from an ascidian of the genus *Didemnum* collected in western Australia near Ningaloo Reef which appear to be derived from condensation of 3,4-dihydroxyphenylalanine (DOPA) [105]. Ningalin A-D compounds are the newest members of a family of DOPA-derived *o*-catechol metabolites that include the tunichromes. Boger et al. in 1999 were successfully able to isolate and then synthesize the complete ningalin B structure along with precursors to ningalin B [106–108]. Of these precursors 10 (Dimethyl 3-(4,5-Dimethoxy-2-(methoxymethoxy) phen-yl)-4-(3,4-dimethoxyphenyl)-1-[2-(3,4-dimethoxyphenyl)-ethyl]pyrrole-2,5-dicarboxylate), 11 (Methyl 7,8-Dimethoxy-3-(2-(3,4-dimethoxyphenyl)ethyl)-1-(3,4-dimethoxyphenyl)-[1]-benzopyrano[3,4-*b*]pyrrol-4(3*H*)-one-2-carboxylate), 13 (Hexamethyl Ningalin B), and 14 (9,10-Dihydro-12,13-dimethoxy-1-(3′,4′-dimethoxyphenyl)-3,4-dimethoxy-[4,3-*d*]-[1]-benzopyrano-15*H*-benzaze-pino[3,2-*a*]-[3]-pyrrol-7,15(18*H*)-dione) significantly sensitized the HCT116/VM46 human colorectal carcinoma cells overexpressing P-gp to VLB and DX but not to ningalin B. Thus, ningalin B characterized by a highly functionalized tetra- or penta-substituted pyrrole, which is ideally suited to construction using this strategy, inspired scientists to modulate its structure to come up with more active MDR reversal agents. These compounds lacked intrinsic cytotoxic activity and, in turn, were potentially safe MDR reversal agents. Furthermore, ningalin B analogs, namely, N1–N6 showed reversal ability toward HCT116/VM46 cells. These compounds contain a common 3,4-diaryl-substituted pyrrole nucleus bearing 2- or 2,5-carboxylates. Of them, N3 showed the greatest potential, reversing the MDR phenotype at 1 μM concentration [108]. N3 at 10 μM showed about a 4,000-fold increase in sensitivity against the

VLB resistant MDR-leukemia cell line CCRF-CEM/VLB_{100} against VLB compared to the sensitive cells whose sensitivity against VLB remained relatively unaffected by the presence of N3 [109]. N3, in turn, demonstrates that it competes with [^{3}H]-azidopine for the P-gp binding site. At the cellular level, N3 increases the intracellular accumulation and retention of the MDR substrates [^{3}H]-VLB and [^{3}H]-paclitaxel. Furthermore, in nude mice-xenograft models, combination of suboptimal doses of paclitaxel with N3 not only led to shrinkage of HCT116 tumor size but also achieved a complete therapeutic remission, without increasing toxicity toward the host. Later in 2010, Zhang et al. designed and synthesized ningalin B analogs around the permethyl ningalin B structure and demonstrated remarkable enhancement of MDR reversal abilities of a synthetic analog compound 25 (1-[2-(4-Methoxyphenyl)-2-oxoethyl]-3, 4-bis (3,4-dimethoxyphe-nyl)-1H-pyrrole-2, 5-dione) that reversed P-gp-mediated MDR at concentrations up to 1 μM [110]. Thus, these findings lead to opening up a whole new potential of marine isolates and analogs for safer use as MDR reversal agents.

8.7.6 Sipholane Triterpenoids

The Red Sea sponge *Siphonochalina siphonella* when isolated yielded about 30 triterpenoids that have been isolated, possessing four different skeletons, namely, the sipholane, siphonellane, neviotane, and dahabane [111–114]. Sipholenol A and sipholenone A are the major sipholane triterpenoids. The sipholanes contain a perhydrobenzoxepine and a bicyclodecane system, linked together through an ethylene bridge. Jain et al. reported for the first time the ability of sipholane triterpenoids to reverse MDR mediated via P-gp [115]. The sipholane triterpenoid sipholenol A at a concentration of 5 μM significantly sensitized the P-gp overexpressing KB-C2 cells to the cytotoxic effects of colchicine, a substrate of P-gp, while having no reversal ability toward MRP1 when tested for the same sensitization in KB-CV60 cells (a subline resistant to vincristine overexpressing MRP1 derived from KB-3-1). Also surprising was the selectivity since it had no effect toward ABCG2 overexpressing S1-M1-80 colorectal cancer cells derived from the parental S1 cells [116]. Delving into the mechanism of action of reversal it was found that this marine natural product increased the accumulation of paclitaxel by directly inhibiting P-gp-mediated drug efflux, stimulated ATPase activity, and inhibited the photolabeling of P-gp with its transport substrate [^{125}I]-iodoarylazidoprazosin (IAAP). Treatment of KB-C2 and KB-V1 cells with sipholenol A for 36 and 72 h had no effect on P-gp expression. These data indicate that sipholenol A inhibited the function of P-gp through direct interactions and attested the potential of sipholane triterpenoids as a new class of P-gp reversing agents. Further studying the isolates from the same sponge sipholenone E, sipholenol L, and siphonellinol D were later found to inhibit the function of P-gp [117]. They enhanced the cytotoxicity of P-gp substrate anticancer drugs and reversed the MDR phenotype in KB-C2 cells in a similar fashion to sipholenol A. These sipholanes had no effect on the response to cytotoxic agents in MRP1-, MRP7- and BCRP-overexpressing cells [117]. They increased the accumulation of

[^{3}H]-paclitaxel and calcein by inhibiting the drug efflux function of P-gp. All three triterpenoids stimulated P-gp ATPase activity and inhibited the photolabeling of this transporter with IAAP, suggesting that they directly interact with P-gp. In silico molecular docking analysis identified the ligand binding sites of these compounds. These findings provided evidence of marine derived isolated structures demonstrating specific and satisfactory reversal of MDR mediated by P-gp.

8.7.7 Eribulin

Eribulin mesylate is a fully synthetic analog of the polyether macrolide natural product halichondrin B, which was isolated from *Halichondria okadai* and other marine sponges [118–121]. The US Food and Drug Administration recently approved eribulin for the treatment of locally advanced and metastatic breast cancer based on results of a Phase III clinical trial [122, 123]. Several ongoing trials have shown promising results using eribulin for the treatment of sarcoma, non-small cell lung cancer and prostate cancer [124, 125]. Eribulin acts by targeting the microtubule dynamics as an antimitotic agent. However, distinct in its mode of action from paclitaxel and vinblastine, it inhibits the microtubule growth, with little or no effect on microtubule shortening [126–130]. With its high potency and wide therapeutic window in preclinical studies and combined with its great physicochemical and pharmacokinetic properties, eribulin mesylate was selected for further development. However, eribulin was seen to be a substrate for P-gp [131]. This was averted by uniquely modulating the structure of the parent eribulin to yield structures that were not recognized by the P-gp pump and also had good antitumor activity. Altogether, these compounds also showed higher plasma concentrations in mice after intravenous administration after 3–4 h of dosing. These structural modifications assist in developing analogs that are equally cytotoxic and do not succumb to the efflux activity of P-gp.

8.7.8 Welwitindolinones

Smith et al. started a wide search for compounds holding potential to reverse the MDR phenotype mediated by P-gp [132]. These compounds were isolated from close to 1,500 strains of blue–green algae (cyanobacteria), with some initial success they delved deeper into finding a strong candidate. Soon after a novel alkaloid was discovered that thrived to reverse the P-gp-mediated MDR, they were named welwitindolinones. These were isolated from the extracts of *Hapalosiphon welwitschii* W. & G .S. West [133]. The activity of these compounds was tested on SK-VLB-1, an ovarian cancer adenocarcinoma cell line derived from the parental SKOV3 cells at concentrations up to 5 μM. *N*-Methylwelwitindolinone C isothiocyanate had reversing efficacy similar to that of verapamil in two different MDR cell lines. *N*-Methylwelwitindolinone C increased the cytotoxicity of actinomycin D and

daunorubicin in SK-VLB-1 cells. It also decreased the IC_{50} values of vinblastine, paclitaxel, actinomycin D, colchicine, and daunorubicin in the drug-resistant breast carcinoma (MCF-7/ADR) cells. While welwitindolinone C isothiocyanate exhibited a weaker reversing activity, an analog of the former compound with the isothiocyanate group replaced by an isonitrile group was inactive. The *N*-Methylwelwitindolinone C isothiocyanate derivative seemed to give the most activity toward inhibiting the P-gp efflux activity. In addition, results obtained from photoaffinity labeling indicated that this compound could inhibit the labeling of P-gp with [^{3}H]-azidopine in MDR cells. As a result, replacement of the isothiocyanate by the isonitrile group seems to influence the activity and ability of these agents to interact with P-gp.

8.7.9 Parguerenes

First reported by Capon et al. in 1996 from a southern Australian marine red alga, *Laurencia filiformis*, parguerenes I (15-bromoparguer-9(11)-ene-2,7,16,19-tetrol-2,7,16-triacetate) and parguerene II (15-bromoparguer-9(11)-ene-2,7,16,19-tetrol-2,7,16,19-tetraacetate) belong to a rare class of brominated diterpene unique to marine alga of the genus *Laurencia* [134]. Another study by Huang et al. demonstrated the potential of parguerene I and II to reverse MDR mediated by P-gp and MRP1 [135]. Parguerene I and II at concentrations of 10 μM resensitized the cells overexpressing P-gp (SW620/ADV300, CEM/VLB100, and HEK293/ABCB1) to its substrate chemotherapeutic agents when used in combination. In addition, this effect also extended toward MRP1 when it was combined with calcein-AM a fluorescent substrate. It increased the intracellular accumulation of calcein in the ovarian cancer 2008/MRP1 cells overexpressing MRP1 compared to the parental 2008 cells. However, accumulation analysis performed along with a fluorescent substrate of BCRP pheophorbide A (PhA) revealed no change in accumulation of the fluorescent substrate either in the presence or the absence of parguerene I and II. Thereby, the reversal effect of parguerene I and II is only toward P-gp and MRP1. Drug accumulation and efflux studies in the presence of fluorescent and radioactive substrates of P-gp on SW620/ADV300 cells directly portrayed the ability of parguerene I and II to inhibit the efflux activity of P-gp. Further, there was no change in the expression profile of P-gp in the presence of parguerene I and II at concentrations up to 10 μM. This discovery and characterization of the interaction between parguerenes and P-gp and MRP1 reveal a new inhibitory pharmacophore, deserving further investigations.

8.8 Conclusions

The discussion entailed herein enumerates the great amount of potential and valuable information held in nature and the interest of scientists in further delving into the depths of nature to fight cancer. The therapeutic potential of marine products has

driven innovative methods for its procurement, and also elaborated on newer methods such as aquaculture, semi-synthesis, and molecular modeling to yield large amounts of the therapeutically active ingredients. While scientists in an academic setting have been inspired to isolate the marine natural products and drug discovery in the past, novel approaches to translational medicine including preclinical studies unite marine natural products chemists and pharmacologists. This is an important step in accelerating the progression of marine natural products from their discovery to the laboratory bench and later to the clinic. Successful collaborations between academic institutes and pharmaceutical companies will continue to provide mutual benefits that each party seeks. Academic programs gain access capacities for sophisticated screening, pharmacological evaluation, and advancement of leads to in vivo models, whereas industry gains high-value leads while evading the high risk associated with marine drug discovery. The importance of naturally originated therapeutics remains in the fact that the cure for cancer needs newer chemicals having high potency and less toxic side effects. Some may argue that the combinatorial libraries are capable of providing us with these lead molecules; however, these structures are intentionally imbued with high randomness. This is due to the fact that the structures of drugs used in the modern medicine today are merely a reflection in some way or other of their natural counterparts.

The constant failure of a majority of MDR reversal agents in the past warrants a closer look at the modulators that are currently available. Isolation and identification of lead structures for the reversal of the MDR phenotype is currently very critical for overcoming this overpowering resistance factor. Not only do these natural marine isolates provide an effective solution to this impediment to successful chemotherapy, but they also provide scaffolds for rational drug design of analogs having higher potency and fewer pharmacokinetic interactions. These make it hard to ignore the usefulness of chemicals of marine origin as modulators of ABC transporters. Furthermore, recently, Shipp and Hamdoun [136] described the use of sea urchins as an alternative model to screen for modulators of ABC transporters. Sea urchins embryos are reported to express *ABCB, ABCC,* and *ABCG* transporter genes in their first 58 h of development and thus make them a valuable experimental marine model.

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Chapter 9
The Role of ABC Multidrug Transporters in Resistance to Targeted Anticancer Kinase Inhibitors

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Abstract Currently the main treatment option for generalized or metastatic cancer is chemotherapy. Besides conventional chemotherapeutics, small molecule targeted kinase inhibitors (TKIs), which are specifically capable of eliminating key pathways driving cancer growth and metastasis, are also applied in cancer treatment. The hydrophobic TKI molecules need to pass the cell membrane to reach their intracellular targets, and in many cases become substrates of ABC multidrug (MDR) transporters. These large membrane proteins, by using the energy of cellular ATP, actively extrude a wide variety of xeno- and endobiotics from the cells. Tumor cells, and especially cancer stem cells, abuse this promiscuous transporter capacity to protect themselves against therapeutic molecules, including many TKIs. Importantly, the interaction/extrusion by MDR-ABC transporters is not related to the specific, targeted mechanism of TKI action. In this review, we present the key TKIs currently used in cancer therapy, and discuss their interactions with MDR-ABC transporters. We also describe the methods for studying various forms of direct MDR-ABC and TKI interactions, and present a framework for understanding a complex regulation of transporter expression and function by these therapeutic molecules.

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Abbreviations

ABC	ATP-binding cassette
ADME-Tox	Absorption, distribution, metabolism, excretion, and toxicity
EGFR	Epidermal growth factor receptor
MDR	Multidrug resistance
P-gp	P-glycoprotein
SP	Side population
TKI	Targeted kinase inhibitor

9.1 Introduction

Targeted kinase inhibitors (TKIs) have already provided a major clinical success and are promising to bring further breakthroughs in treating various malignancies. These treatments are based on targeting well-defined aberrant signal transduction mechanisms in selected cancer types, thus requiring personalized diagnostics for selecting the most appropriate, stratified treatment strategies. Based on properly characterized tyrosine kinases and their mutant variants involved in the development of uncontrolled cell division, an array of new small molecules have been developed and many more are currently in the pipeline of the biotech and pharmaceutical companies. Reversible and irreversible, competitive and noncompetitive types of inhibitory molecules are devised; single- or multispecific, high-affinity agents are selected in high-throughput methodologies. These efforts should provide many more clinically applicable kinase inhibitors for the targeted treatment of even those types of cancers which are currently untreatable by conventional chemotherapy.

Still, the occurrence of inherent drug resistance and the treatment-dependent development of cancer resistance against initially successful TKIs are major clinical problems. Resistance to TKIs often develops through secondary mutations of the target enzymes, activation of alternative pathways, or the occurrence of additional signaling mutations. These alterations require the adaptation of treatment modalities to the resistant cancer phenotype, in many cases by the application of TKIs with modified target specificities.

In addition to these specific alterations, cancer cells may develop nonspecific (target independent) resistance against various TKIs by protective cellular responses, including, e.g., increased DNA or protein repair mechanisms, or decreased apoptotic response. One of these protective mechanisms is the upregulation of MDR-ABC transporters, protecting the cancer cells against a wide range of drugs or xenobiotics. Since small molecule TKIs have intracellular targets, they have to pass the cell membrane, and thus may become the substrates of the transporters capable for an active extrusion of these molecules. It is important to note that this nonspecific extrusion of drugs by MDR-ABC transporters is in contrast to the specific, targeted mechanism of kinase inhibitor action. That is, independent of the well-defined

mechanism of action of a TKI compound, the biologically active molecule may or may not be a substrate for the drug-extrusion mechanisms.

An important aspect of cancer drug resistance is related to the cancer initiating cell populations, often called "cancer stem cells." These cells with a less differentiated phenotype have high potential for regenerating the tumor, and are also inherently drug resistant. This drug resistance in many cases is caused by the overexpression of MDR-ABC transporters, experimentally observed as the so-called side population (SP) of cancer stem cells. Thus, small molecule TKI and MDR-ABC transporter interactions may have a special importance in finding kinase inhibitors capable of selectively killing the cancer stem cells.

In this chapter, we focus on the role of the key ABC transporters involved in multidrug resistance (MDR), ABCB1-MDR1, ABCC1-MRP1, and ABCG2-ABCP/BCRP/MXR in resistance to TKIs. We discuss the basic features of the abovementioned MDR-ABC transporters and describe the basic methodologies for assessing their interactions with the TKIs. We also provide a detailed description of the in vitro and in vivo observed interactions of several clinically applied TKIs with the MDR-ABC transporters. Interestingly, the hydrophobic xenobiotic-like TKI molecules may significantly modify the complex drug handling properties of the tumor cells. Therefore, in addition to discussing the direct MDR-ABC transporter-kinase inhibitor interactions, we give an overview of the potential regulation of the MDR-ABC transporter expression and cellular localization by these therapeutic agents.

Based on reviewing these data in the literature, we discuss potential methodologies to overcome MDR-ABC transporter-based TKI resistance at the clinic. In the past decades numerous efforts for a direct, specific inhibition of one or more transporters failed at the clinical trials, because the general inhibition of the physiological drug and xenobiotic transport in multiple tissues, including important barriers, caused a major toxicity in the patients. We suggest that the most successful strategy in this regard could be based on the detailed knowledge of the actual, experimentally established TKI-transporter interactions. Development of new, efficient TKI small molecules which can avoid pump-dependent extrusion can be one of the solutions for avoiding cancer TKI resistance. Interestingly, an even more successful attempt could be to select small molecule TKIs which, in addition to their kinase targeted effect, efficiently modulate transporter functions. This strategy should be devised to focus on kinases and transporters especially overexpressed in the cancer stem cells; thus TKIs could provide a selective lethality in the tumor initiating cellular compartments.

9.2 Targeted Kinase Inhibitors (TKIs) in Cancer Therapy

9.2.1 Targeted Cancer Therapy

Conventional chemotherapeutics target universal cellular functions such as DNA replication, DNA uncoiling, nucleic acid metabolism, or microtubule function, and therefore cannot distinguish between rapidly dividing normal and cancer cells.

Accumulating evidence suggests that the proliferation and survival of cancer cells often depend on a single, or at least a small number of activated oncogene(s). This phenomenon is currently believed to render cancer cells especially susceptible to interference with the function of the oncogene and also the oncogene-associated signaling pathways, and has recently been termed as "oncogene addiction." The addiction of cancer cells to certain oncogenes has provided a rationale for the design and clinical application of molecularly targeted cancer therapeutic strategies, which could enable the specific targeting and elimination of the malignant cells [1–4].

Oncogenes which confer oncogene addiction most frequently code for kinases [2, 5–7]. The human kinome has been shown to contain 518 putative kinase genes which constitute about 1.7 % of the human genes [8]. Chromosomal mapping of the human kinase genes revealed that 164 kinases are located to amplicons frequently appearing in tumors [8]; furthermore, an unexpectedly large number of putative driver (playing a causal role in oncogenesis) and passenger somatic mutations in kinomes of various tumor origins has recently been described [9, 10]. Several kinases have been validated as major contributors to oncogenesis, and therefore have already served as promising drug targets.

9.2.2 Bcr-Abl Signaling in Cancer and Its Small Molecule Inhibitors

Bcr-Abl is an oncogenic non-receptor tyrosine kinase whose kinase activity is required for the pathogenesis of chronic myeloid leukemia (CML), a clonal myeloproliferative disorder of hematopoietic stem cell origin. The genetic hallmark of CML is the Philadelphia chromosome, which is generated by the t(9;22)(q34;q11) reciprocal translocation. The Philadelphia chromosome (actually a shortened chromosome 22) can be detected in approximately 95 % of CML patients. The above-mentioned chromosomal translocation fuses the *Abl* proto-oncogene physiologically located on chromosome 9 to the *Bcr* (breakpoint cluster region) gene on chromosome 22, thus generating the *Bcr-Abl* hybrid gene. Depending on the exact location of the breakpoints in *Bcr* (designated as major, minor, and micro breakpoint cluster regions; M-*bcr*, m-*bcr*, and μ-*bcr*, respectively) and on alternative splicing of the different transcripts, four fusion protein variants can be generated, with molecular weights of 190, 210, or 230 kDa. Most CML patients carry the 210 kDa Bcr-Abl kinase [11, 12]. The fusion-mediated loss of the N-terminal myristoylation site of Abl and the Bcr coiled-coil motif-mediated interference with the kinase autoinhibitory domains (Src homology domains, SH2 and SH3) of Abl results in constitutive tyrosine kinase activity and leukemogenic potential of Bcr-Abl [13, 14]. The autophosphorylation of Bcr-Abl at Tyr177 links Bcr-Abl to mitogenic Ras signaling through its direct interaction with the adaptor protein Grb2 (growth factor receptor-bound protein 2), a phenomenon which is required for the transforming potential of

the fusion kinase [15–17]. Bcr-Abl also transduces pro-survival and anti-apoptotic signals, and signals resulting in altered cell adhesion and migration [13, 18].

Since Bcr-Abl plays a pivotal role in leukemogenesis, it was soon recognized as a promising molecular drug target. The first identified small molecule inhibitor showing potent in vitro and in vivo Abl inhibitory activity was imatinib (Gleevec/Glivec/imatinib-mesylate/CGP57148/STI-571) [19]. Imatinib was soon reported to suppress proliferation and induce apoptosis of Bcr-Abl positive primary and model cells, setting the stage to its translation to clinical use [20–23]. Imatinib also displays an additional inhibitory activity on c-KIT (stem cell factor receptor, CD117) and PDGFR (platelet-derived growth factor receptor) kinases [24]. Imatinib received accelerated FDA (US Food and Drug Administration) approval in 2001, and 5- and 6-year follow-up studies reported impressive response rates and outstanding estimated overall survival rates in CML patient cohorts receiving imatinib as initial therapy [25, 26]. Nevertheless, imatinib is observed to be less effective in the advanced phases of CML as disease persistence is detected in the majority of patients thus indicating that imatinib is unable to eradicate all of the malignant cells, and resistance to imatinib also develops in many cases [27].

To overcome the problems experienced with imatinib, a second generation small molecule inhibitors of Bcr-Abl have been developed. The novel inhibitors include the selective Abl inhibitor nilotinib (Tasigna/AMN107), and the dual inhibitors of the Abl and Src kinases dasatinib (Sprycel/BMS-354825) and bosutinib (Bosulif/SKI-606). Similarly to imatinib, nilotinib and dasatinib also inhibit c-KIT and PDGFR [24]. Nilotinib and dasatinib are currently approved as a frontline therapy of CML, and as a second-line treatment option in CML patients resistant or intolerant to imatinib. Bosutinib has recently been approved to treat CML patients who failed on prior treatments with multiple Bcr-Abl inhibitors [28, 29]. Novel TKIs which are active against the Bcr-Abl kinase are also emerging, including ponatinib [30], danusertib, and saracatinib. Ponatinib (AP24534) is a pan-Bcr-Abl inhibitor and has been shown to be active against the T315I gatekeeper mutant. In addition, ponatinib showed inhibitory activity on Src kinase, members of the VEGFR, FGFR, and PDGFR families, and FLT3 [30, 31]. Danusertib (PHA-739358) is also active against several resistance causing Bcr-Abl mutant variants (including T315I), and inhibits Aurora kinases as well [32, 33], while saracatinib (AZD0530) is a dual Src/Abl kinase inhibitor [34, 35]. Resistance against several representatives of the new generation inhibitors of Bcr-Abl has also been reported to occur [27].

9.2.3 EGFR Signaling in Cancer and Its Small Molecule Inhibitors

The Epidermal Growth Factor Receptor (EGFR/ErbB1/HER1) belongs to the ErbB subclass of the receptor tyrosine kinase (RTK) superfamily. ErbB receptors are physiologically expressed in various tissues of epithelial, mesenchymal, and

neuronal origins and play an essential role in embryogenesis. ErbBs are single-pass transmembrane receptors having an extracellular domain responsible for the binding of polypeptide ligands of the EGF (epidermal growth factor) family, and an intracellular region containing a tyrosine kinase catalytic domain. ErbB receptors homo- or heterodimerize upon ligand binding that results in activation of the intrinsic kinase domain and subsequent phosphorylation of tyrosine residues in the cytoplasmic tail. The phosphotyrosine residues serve as docking sites for adaptor proteins or enzymes with Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains, recruitment of which mediates the activation of multiple downstream signaling cascades involved in various cellular programs, such as proliferation, differentiation, survival, migration, and adhesion. Under physiological conditions, the ErbB receptor activity is tightly controlled by the spatial and temporal ligand accessibilities [36, 37].

EGFR (and ErbB2/HER2/Neu as well) has been reported to be constitutively active in numerous cancer types, including glioma, breast cancer, ovarian cancer, colorectal cancer (CRC), squamous-cell carcinoma of head and neck (SCCHN), and non-small cell lung cancer (NSCLC). Aberrant activation of EGFR was reported to result from an autocrine ligand production or overexpression or mutation of the receptor itself and was associated with poor clinical outcomes [36]. Accordingly, EGFR has intensively been pursued as a molecular drug target. Therapeutic inhibition of EGFR can be achieved by monoclonal antibodies (mAbs) that either neutralize ligands or target the extracellular ligand binding domain of the receptor thus preventing dimerization-mediated activation and causing receptor internalization. Still, the major therapeutic option is the administration of small molecule inhibitors which target the intracellular tyrosine kinase domain of the receptor [37, 38].

The first generation of EGFR inhibitors includes gefitinib (Iressa/ZD1839) and erlotinib (Tarceva/OSI-774), which reversibly bind to and inhibit EGFR [37]. Second generation EGFR inhibitors are multikinase specific and/or irreversibly bind to the target receptor that is believed to enable augmentation of drug efficacy and the targeting of multiple types of solid tumors. The second generation of EGFR inhibitors includes lapatinib (Tykerb/GW572016), vandetanib (Zactima/ZD6474), pelitinib (EKB-569), and neratinib (HKI-272) [4, 37, 39]. Lapatinib is a reversible inhibitor of EGFR and is also active against another HER2, another member of the ErbB receptor family. Vandetanib is a potent inhibitor of vascular endothelial growth factor receptor 2 and 3 (VEGFR-2,3), and shows additional inhibitory activity against EGFR [40]. Pelitinib covalently binds to and inhibits EGFR [41]. Neratinib, another irreversible inhibitor of EGFR, was synthesized on the chemical scaffold of pelitinib, and has additional inhibitory activity against ErbB2/HER2/Neu [42]. These EGFR inhibitors are presently under clinical evaluation or being used either as monotherapy or in combination for a histologically diverse range of tumors, including lung cancer [39], breast cancer [43–45], and colorectal cancer [46]. Notably, resistance to small molecule inhibitors of EGFR has also been reported to occur in several cases and represents a major impediment to the successful management of the relevant solid tumors [39, 47, 48].

9.2.4 *Other Kinases Important in Cancer Cell Signaling and Their Small Molecule Inhibitors*

Several other kinase enzymes have been shown to play an important role in cancer cell signaling, including the c-Kit, FLT3, PDGFR, VEGFR receptor tyrosine kinases [5, 49], and also the Ser/Thr kinase BRAF whose oncogenic mutations are the most common genetic events detected in melanoma [50]. Inhibitors targeted against these kinases include the multikinase inhibitors sunitinib (Sutent/SU11248) and sorafenib (Nexavar/BAY-43-9006) which are active against c-Kit/FLT3/PDGFR/VEGFR [51, 52]; apatinib (YN968D1) [53], telatinib (BAY 57-9352) [54], and axitinib [55] which all inhibit VEGFR and other kinase targets such as Ret, c-Kit, c-Src, PDGFR; tandutinib (MLN518/CT5351) [56] and quizartinib (AC220) [57] with primary inhibitory activity against FLT3; and vemurafenib (PLX4032) that inhibits BRAF (V600E), the most common mutant BRAF variant in melanoma [50].

9.3 Potential Mechanisms of Tumor Resistance Against TKIs: The Role of Cancer Stem Cells

9.3.1 *Resistance to TKIs*

Target-dependent and target-independent molecular mechanisms causing resistance to small molecule TKIs have both been described. These include secondary mutations in the targeted kinase enzymes, overexpression of the targeted kinases, or activation of alternative signaling molecules (such as increased signaling by Lyn kinase or the MET receptor kinase in CML and lung cancer, respectively) [4, 24, 27, 37–39, 47, 48, 58]. Increased DNA repair mechanisms, decreased apoptosis, and altered transport of drugs might also account for the emergence of drug resistance [59]. Here we do not discuss these mechanisms in detail.

9.3.2 *Relevance of MDR-ABC Transporters in Resistance to Anticancer TKIs*

As small molecule TKIs have to pass the cell membrane to exert their intracellular kinase inhibitory action, involvement of the MDR-ABC efflux transporters has also been implicated in the emergence of drug resistance. Cancer cells which overexpress MDR-ABC proteins exploit the physiological protective function of these transporters and can develop resistance against a wide array of chemically and target-wise unrelated compounds. Therefore, the MDR-ABC transporter function can significantly modify the anticancer efficacy of the administered agents in the

targeted cancer cells. On the other hand, as MDR-ABC transporters are physiologically expressed at pharmacologically important tissue barriers, they might also significantly modify the biodistribution of the orally administered small molecule kinase inhibitors [60]. Furthermore, accumulating evidence suggests that the ABCG2 multidrug transporter might also be a molecular marker of cancer stem cells (CSCs). Thus, the specific protective function of ABCG2 might also protect the cancer stem cell population, and therefore might significantly contribute to the replenishment of the tumor. The expression and the role of MDR-ABC transporters in cancer stem cells are discussed below.

9.3.3 The Cancer Stem Cell Hypothesis: SP Cells and Overexpression of MDR-ABC Transporters

The cancer stem cell hypothesis, originally formulated by Bonnet and Dick and Reya et al. [61, 62], suggests that cancer growth is driven by rare cancer stem cells. These cancer stem cells are suggested to possess especially high self-renewal capacity, and play a key role in tumor initiation, progression, and metastasis. Moreover, these cells are highly resistant to chemo- or radiotherapy, thus are protected against these medical interventions. Cancer stem cells (CSCs) have been suggested to be present in various hematological and solid tumors, including retinoblastoma, melanoma, tumors of the breast, brain, liver, pancreas, colon, or the lung [63, 64].

The underlying mechanisms of cancer stem cell resistance have been attributed to a greater capacity for DNA repair, activation of survival pathways [65, 66], and the overexpression of MDR-ABC transporters [67]. It is by now well documented that many kinds of stem cells preferentially express the ABCG2 multidrug transporter. Regarding tissue-derived normal stem cells, the first finding was that a high-level expression of the ABCG2 protein and its fluorescent dye extrusion function could identify hematopoietic stem cells [68, 69]. In the following studies, the so-called side population (SP) of progenitor cells, actively extruding the fluorescent Hoechst 33342 dye, has been reported to represent stem cells in a variety of tissues.

The activity of ABCG2 may protect stem cells against drugs and xenobiotics, and these functions become important under unfavorable conditions. Zhou et al. first showed that ABCG2 null hematopoietic stem cells (HSCs) were significantly more sensitive to mitoxantrone in vivo, thus ABCG2 expression in HSCs may provide protection from cytotoxic substrates [70]. The SP cells are also more resistant against hypoxic challenges [71], or the accumulation of free radicals [72].

A key approach in the CSC identification thus became the flow cytometry-based determination of the side population (SP) cells. Several reports indicate that SP cells obtained from a number of tumor cells have especially high tumorigenicity and proliferation capacity and also show higher resistance to a number of anticancer agents [73–78]. Thus, the overexpression of the ABCG2 protein may serve as a biomarker of tumor stem cells. Clearly, ABCG2 expression and active drug extrusion in the

CSCs may impede their effective eradication and result in long-term cancer reappearance after chemotherapy [79]. The special tumorigenic potential of the ABCG2 positive subpopulation of cancer cells, however, is still controversial [80].

9.4 Basic Features of the Key MDR-ABC Transporters Involved in Resistance TKIs

In this book, several chapters deal with the structure and function of multidrug (MDR) transporter ABC proteins. Therefore, here we provide only a basic description of their structural and functional aspects, general features of drug recognition and transport, and the important pharmacological and physiological functions. For further details see the reviews [49, 59, 60, 81–86].

MDR-ABC transporters are large, glycosylated proteins residing in the plasma membrane and capable of actively extruding a large variety of xenobiotics and endobiotics. All these proteins contain major membrane-spanning domains composed of alpha helices, and the functioning units contain two cytoplasmic nucleotide binding, often called ATP-binding cassette (ABC) domains. Recent structural data indicate that the membrane-spanning and the ABC domains are connected by specific alpha helices which provide the transfer of conformational information between the two major domains. Binding and hydrolysis of ATP in the ABC domains drive the conformational changes leading to drug extrusion performed by the transmembrane regions.

The ABCB1 (MDR1/Pgp) multidrug transporter and its closely related homologs contain two ABC domains and 12 transmembrane helices within one polypeptide; the ABCC type multidrug transporters contain the same basic elements and in many cases an additional 5 helix N-terminal transmembrane regions within one large polypeptide. The (ABCP/BCRP/MXR) protein is called a “half transporter,” as it has only six transmembrane helices and one ABC domain, but the functional unit is a homodimer [81].

All MDR-ABC transporters have the feature of promiscuity in drug recognition and transport, which is believed to be based on their relatively large and flexible transmembrane domains which can accommodate (probably more than one) drug molecules with chemically different cores and side chains. This promiscuity is the explanation for the fact that even the specifically targeted signaling inhibitors are nonspecifically recognized and potentially extruded by the MDR-ABC transporters. Most of the MDR-ABC inhibitors are also similar to transported substrates, but are unable to cross the transporting mechanism and thus fix the protein in a state when further transport cycles cannot be performed. This substrate to inhibitor switch is in many cases drug concentration-dependent, thus higher substrate concentrations block further transport cycles. In addition, a competitive inhibition of the transport of one substrate may occur by another substrate of the transporter.

In addition to their key role in cancer multidrug resistance, MDR-ABC transporters have major physiological roles in the xenobiotic defense mechanism. As described in Chap. 7, in this regard, these proteins are major "effectors" in a chemodefense system protecting our body against harmful, mostly hydrophobic chemicals. Thus, MDR-ABC transporter and drug interactions may also affect these physiological functions.

As to the ABCB1 proteins, there is no well-described other physiological functions than its role in the xenobiotic defense mechanism. Some close relatives (ABCB4/MDR3 and ABCB11/BSEP) are more specific lipid or bile acid transporters, while their role in xenobiotic extrusion is less defined. Still, TKI interactions may affect these proteins and some major side effects of targeted inhibitors may result in reduced liver functions [87].

Some of the ABCC type proteins have well-defined physiological functions, especially in exporting partially detoxified compounds from the cells. The key role of the ABCC proteins ABCC2/MRP2, ABCC3/MRP3, and ABCC4/MRP4 in the Phase III metabolic step [84] in the liver and kidney functions is well accepted in this regard. ABCC1, which has a key role in cancer multidrug resistance, may have only a minor role in endogenous transport processes.

In the case of ABCG2, in addition to xenobiotic extrusion, there are known physiological functions related to the transport of endogenous metabolites. Human ABCG2 is an efficient uric acid transporter, and mutations or polymorphic variants of this transporter are causative in the development of gout [88–90]. ABCG2 is preferentially expressed in stem cells, where it may be involved in protection against hypoxic challenges [71]. Progenitor cells obtained from Abcg2 knockout mice showed a reduced ability to form colonies under hypoxia, and blocking of Abcg2 function in normal progenitor cells reduced survival under hypoxic conditions. The primary cause of this hypoxic susceptibility was the accumulation of cellular heme and/or porphyrins. Heme was shown to be specifically bound by ABCG2, and drug transport by ABCG2 was significantly modulated by heme. It has been further demonstrated that ABCG2 overexpression permits enhanced stem cell survival in oxygen-poor environments by reducing the accumulation of toxic heme metabolites [91].

9.5 Methods for Studying Interactions of MDR-ABC Transporters and TKIs

Several methods have been established for the evaluation of the drug interaction profiles of MDR-ABC transporters. The white paper published by the International Transporter Consortium has recently highlighted the importance of the transporter studies in drug development, and has also provided workflows and decision trees supposed to aid efficient screening of the transporter-interaction profiles [82]. In the following sections, we provide a brief overview of the in vitro assays and the in vivo model systems which are widely used to assess drug interactions of the MDR-ABC transporters.

9.5.1 Membrane-Based In Vitro Assays to Assess Drug Interactions of the MDR-ABC Transporters

The MDR-ABC transporters utilize the energy of ATP hydrolysis to facilitate the active transport of their substrates across the plasma membrane, and the translocation of substrate molecules is strictly coupled to ATP hydrolysis. The effect of drug molecules on the ATPase activity of MDR-ABC proteins can be measured using isolated transporter-expressing membranes. Heterologous expression of human MDR-ABC proteins in *Spodoptera frugiperda* (*Sf9*) cells by the baculovirus expression system is the most common method of choice to yield membranes highly enriched in functional transporters [92–94]. Recent findings, however, suggest that the lipid composition, especially the cholesterol content of the plasma membrane, significantly influences the function of MDR-ABC transporters [95–100]. Therefore, cholesterol-loading of the cholesterol-poor insect membranes is suggested to achieve enhanced MDR-ABC function and therefore greater sensitivity of the insect membrane-based MDR-ABC functional assays [95, 101].

The effect of test molecules on the vanadate-sensitive ATPase activity of the transporters expressed in insect membranes can be measured by determining the liberation of inorganic phosphate in a colorimetric reaction [92]. In general, ATP hydrolyzing activity of MDR-ABC proteins is stimulated in the presence of transported substrate molecules. However, especially in the case of ABCG2, a relatively high basal ATPase activity exerted in insect membranes in the absence of drugs might mask the ATPase stimulatory effect of ABCG2 substrates. This relatively high basal ABCG2 ATPase activity had previously been believed to represent either an uncoupled state of the transporter or its ATPase stimulation by an unknown substrate resident in the insect membrane [94]. An uncoupled ATPase activity was also suggested to be important for the promiscuous drug recognition capability of multidrug transporters [102]. Studies performed on purified and reconstituted functional ABCG2 proposed that the basal ABCG2 ATPase is dependent on the lipid environment and especially on the presence of cholesterol but may not be coupled to any transport activity [97]. It is widely accepted that drugs which can stimulate the relatively high ABCG2 ATPase in Sf9 insect membrane preparations are most probably transported substrates, whereas transporter inhibitors significantly decrease the basal ABCG2 ATPase. However, known ABCG2 substrates, most probably transported at a lower rate, have also been described to decrease or not to affect the basal ATPase activity of the transporter; thus, results of the ATPase assay do not always predict the drug-transporter interaction and the nature of the interaction [60, 101]. Therefore, it is important to highlight that a combination of in vitro measurements is required to determine whether a drug interacts with ABCG2 and other MDR-ABC proteins and to further distinguish between transporter substrates and inhibitors.

Intact MDR-ABC transporter-containing membrane vesicles isolated from insect or mammalian cells can also be applied to study the direct transport of the test molecules. Inside-out membrane vesicles, in which the nucleotide-binding domains of the MDR-ABC transporters are oriented extra-vesicularly whereas the

substrate off-sites are facing the intravesicular space, allow the determination of the ATP-dependent vesicular accumulation of drugs. However, this experimental setup requires either fluorescently or radioactively labeled test drugs that allow for detection of the test molecules [101]. Detection of TKIs by sensitive analytical methods (e.g., HPLC-MS) has also been successfully applied and represents another option for the direct evaluation of the vesicular accumulation of TKIs [103–107].

Both the ATPase and the transport measurements can be performed in an indirect setup, that is, when the modulatory effect of the TKI on the drug-stimulated ATPase activity or on the vesicular transport of a simultaneously applied MDR-ABC substrate molecule is followed. In the indirect ATPase assay, MDR-ABC interacting molecules are expected to decrease the substrate-stimulated ATPase activity of MDR-ABC transporters. Similarly, drugs that interact with the transporters most probably inhibit the accumulation of MDR-ABC substrates in inside-out membrane vesicles. Purified MDR-ABC proteins incorporated into liposomes, containing fluorescent reporter molecules, and measuring ATP-dependent, transport-related changes in vesicular fluorescence may provide additional, more specific methodologies for drug interaction screening [108]. It is important to note, however, that while these indirect measurements predict drug-transporter interactions, they cannot distinguish between MDR-ABC substrates and inhibitors.

The MDR-ABC protein expressing membranes can also be used to investigate whether a test drug interacts with the nucleotide binding domain or the substrate binding site of a given transporter. This experimental setup is based on the photoaffinity labeling of the transporters with nucleotide or substrate analogue molecules. Co-incubation of transporter-expressing membranes or of isolated MDR-ABC proteins with the nucleotide analogue [α-^{32}P]-8-azidoATP or the substrate analogue [^{125}I]Iodoarylazidoprazosin (IAAP) followed by UV-irradiation results in covalent binding of these compounds [109]. [α-^{32}P]-8-azidoATP is supposed to bind to the nucleotide binding domain, whereas [^{125}I]IAAP binds to the substrate binding sites of these MDR-ABC transporters. The solubilized proteins are then separated by gel electrophoresis and binding of the photolabels is visualized by autoradiography. Competitive inhibition of the binding and photoaffinity labeling of the MDR-ABC transporters with [α-^{32}P]-8-azidoATP or [^{125}I]IAAP by the TKI indicates that the TKI interacts with the nucleotide binding domain or the substrate binding sites of the given transporter, respectively [101, 110].

9.5.2 Cell-Based In Vitro Assays to Assess Drug Interactions of MDR-ABC Transporters

Numerous cellular assays have been developed to investigate the drug interactions of MDR-ABC transporters. Cellular toxicity assays are commonly applied to assess whether the presence of a functional MDR-ABC transporter can significantly alter the effect of the investigated TKI on cell viability. If proliferation and survival of the

model cells depend on the function of the kinase targeted by TKI of interest, the direct cytotoxic effect of the TKI can be compared in parental and transporter-expressing cells. If the TKI is a transporter substrate, the presence of a functional MDR-ABC protein can reduce its intracellular concentration below the drug efficacy threshold, resulting in increased IC50 values in the transporter-expressing cells. Changes in key signaling events (e.g., phosphorylation status of the targeted kinase enzyme or downstream kinase molecules) can also be compared in parental and MDR-ABC-expressing cells exposed to the TKI of interest, which can further confirm whether the presence of a functional transporter can limit the access of the TKI to its intracellular kinase targets.

The specific contribution of MDR-ABC transporters to the cellular TKI resistance can be evaluated by co-incubation of the TKI with a specific pharmacological inhibitor of the given transporter, which is supposed to restore TKI sensitivity of the transporter-expressing cells. Notably, direct cellular toxicity measurements allow for the investigation of a relatively narrow TKI concentration range. Intracellular accumulation or vectorial transport across cell monolayers of TKIs can also be compared in parental and transporter-expressing cells, in cases of radioactively or fluorescently labeled compounds or an appropriate analytical method are available for the detection of the TKI. Both the cellular toxicity and the transport measurements can be performed in an indirect setup, where the effect of the tested TKIs on the toxicity or the transport of a simultaneously administered MDR-ABC substrate "reporter" drug is followed. MDR-ABC-interacting TKIs are supposed to inhibit the transport of MDR-ABC substrates and, on the same basis, they can reverse transporter-mediated cellular drug resistance. Similarly to the indirect membrane-based assays mentioned above, the indirect cell-based transport and cytotoxicity measurements only indicate drug-transporter interactions and cannot distinguish between transporter substrates and inhibitors [49, 101].

In the presence of interacting drugs, MDR-ABC proteins undergo conformational changes which can be followed by monoclonal antibodies that recognize an extracellular epitope and bind to the transporters in a conformation-sensitive fashion. UIC2, an ABCB1-specific antibody [111], and 5D3, an ABCG2-specific antibody [68, 112], have both been shown to be sensitive to drug-induced conformational changes of the transporters, and therefore have been used to identify transporter-interacting compounds, including TKIs [113–115].

9.5.3 In Vivo Models to Assess Drug Interactions of MDR-ABC Transporters

Accumulating evidence suggests that MDR-ABC transporters, which are physiologically expressed on pharmacologically important tissue barriers, can significantly modify the ADME-Tox (absorption, distribution, metabolism, excretion, and toxicity) parameters of drug molecules, including TKIs [60]. The ADME-Tox properties of drugs can be evaluated in animals, mostly rodent models; a series of

MDR-ABC transporter knockout (KO) mouse models have been generated and successfully used for the assessment of the in vivo impact of MDR-ABC transporters on the biodistribution and toxicity of drugs [60, 116]. Although MDR-ABC KO mice have proved to be useful tools in evaluating the transporter effects on the tissue distribution of several drugs, it has to be mentioned that species differences in the substrate recognition of MDR-ABC transporters might limit the applicability of these rodent models [60, 117, 118]. In order to circumvent this problem, humanized ABC transporter mouse models (that is the stable, heritable expression of the human ortholog of a transporter in an endogenous transporter knockout mouse) have already been developed [119, 120].

9.6 Interactions of Clinically Applied TKIs with MDR-ABC Transporters

MDR-ABC transporters have been shown to interact with several conventional chemotherapeutic agents and mediate cancer cell resistance against these drugs [59, 83]. As MDR-ABC proteins display multispecific substrate recognition profiles, it could be suspected that they might also interact with the novel, molecularly targeted anticancer kinase inhibitor drugs. Indeed, several studies have thus far reported an interaction of MDR-ABC transporters with small molecule TKIs. Both in vitro and in vivo data are now available showing that MDR-ABC transporters are capable of recognizing and transporting a wide array of clinically relevant kinase inhibitors, and thus have the potential to confer cancer cell resistance against these agents and can also modify their pharmacokinetic profiles. Interestingly, accumulating evidence also suggests that TKI molecules, especially when applied at higher doses, can efficiently block the function of MDR-ABC proteins and thereby can enhance the intracellular accumulation of MDR-ABC substrate cytotoxic drugs and reversing the transporter-mediated multidrug resistance phenotype of cancer cells. In the following sections, we give a detailed overview on the current literature about the in vitro findings and the in vivo relevance of the interaction between MDR-ABC transporters and clinically relevant anticancer TKIs.

9.6.1 In Vitro Interaction of MDR-ABC Transporters with Bcr-Abl Inhibitors

Imatinib, the prototype of the targeted kinase inhibitor molecules, has been extensively characterized concerning its interaction with MDR-ABC transporters. In the first published studies, imatinib was reported to modulate the ATPase activity of both ABCB1 [121] and ABCG2 [122], which strongly suggested that these transporters recognize and interact with imatinib. However, initially imatinib was

recognized either as a transported substrate [123] or as a transporter inhibitor [124]. In subsequent reports, the modulatory effect of imatinib on the transporter ATPase activity was confirmed [110, 125] and both ABCB1 and ABCG2 were shown to actively transport imatinib and were demonstrated to specifically confer in vitro cellular imatinib resistance [114, 123, 125–133]. ABCB1 and ABCG2 were also shown to mediate retained phosphorylation of CRKL, a downstream protein target of Bcr-Abl, which further suggested that these transporters efficiently prevent imatinib from reaching its intracellular target kinase and inducing apoptosis of Bcr-Abl positive cells [125, 128, 133].

Parallelly, imatinib was also reported to inhibit the transport of calcein-AM [127], rhodamine 123 [127, 133], mitoxantrone [123], pheophorbide a [134, 135], and Hoechst 33342 [122, 125], which are well-established substrates of ABCB1 or ABCG2. Accordingly, imatinib was also capable of reversing transporter-mediated cellular resistance to simultaneously applied ABCB1 or ABCG2 substrates, such as mitoxantrone [122], topotecan, SN-38 [124], vincristine, paclitaxel, and etoposide [136]. Notably, the chemo-sensitizing effect of imatinib occurred, when it was administered at higher concentrations compared to the relatively narrow concentration range, when efficient transport of imatinib by ABCB1 or ABCG2 could be measured.

Following the extensive studies on the MDR-ABC transporter interactions of imatinib, information about the interaction of ABCB1 and ABCG2 with second generation Bcr-Abl inhibitors soon started to emerge. Nilotinib, dasatinib, and bosutinib, the Bcr-Abl kinase inhibitors which are already approved by the FDA, have all been shown to interact with both ABCB1 and ABCG2. Several reports suggested that nilotinib and dasatinib are actively transported by ABCB1 and ABCG2, a phenomenon that ultimately resulted in cellular resistance against these agents [103, 104, 125, 126, 133, 137, 138]. However, whether ABCB1 affects the cellular disposition of nilotinib still remains a controversial issue [103, 130, 139–141]. Interestingly, current reports suggest that neither ABCB1 nor ABCG2 is capable of actively transporting bosutinib and the function of these transporters does not confer cellular bosutinib resistance [103, 141, 142]. Therefore, bosutinib might also be efficient in the targeting and elimination of transporter-expressing multidrug resistant cancer cells or cancer stem cells.

The second generation Bcr-Abl inhibitors were also probed regarding their ability to block the MDR-ABC transporter function. When administered at higher concentrations, nilotinib, dasatinib, and bosutinib could all inhibit the transport of MDR-ABC substrates such as calcein-AM, rhodamine 123, and Hoechst 33342 [103, 125, 133]. Efficient reversal of transporter-dependent cellular resistance to conventional chemotherapeutics, like mitoxantrone, doxorubicin, colchicine, vincristine, and paclitaxel, by nilotinib has been described as well [143, 144]. Moreover, an increased anticancer effect by treatment with nilotinib in combination with imatinib has been observed [145]. This synergy was at least partly explained by an increase in the intracellular accumulation and retention of nilotinib through imatinib-mediated blockade of the MDR-ABC transporter function [146]. These findings highlight that co-treatment with multiple TKIs might be a promising treatment option also for overcoming MDR-ABC transporter-mediated drug resistance.

Emerging novel inhibitors of Bcr-Abl are now in clinical trials, such as ponatinib, danusertib, and saracatinib, and have also been shown to interact with ABCB1 and ABCG2. Ponatinib and danusertib have been suggested to be MDR-ABC substrates [147, 148], while all of these three compounds were shown to efficiently inhibit transporter function and to overcome transporter-dependent resistance to various conventional chemotherapeutic agents [147–149].

9.6.2 In Vitro Interaction of MDR-ABC Transporters with EGFR Inhibitors

Similarly to imatinib, the first successful EGFR inhibitor gefitinib was subject to extensive studies concerning its in vitro interaction profiles with MDR-ABC transporters. In the first studies addressing this issue, stimulation of both the ABCB1 and the ABCG2 ATPase activities by gefitinib was shown, thus implying the interaction of this drug with the proteins [122, 150, 151]; however, there was no consensus whether gefitinib is a substrate or an inhibitor of these transporters [150–154]. Subsequent studies confirmed active transport of gefitinib by ABCB1 and ABCG2 and also demonstrated the emergence of gefitinib resistance in cells expressing MDR-ABC transport proteins which could be reverted by simultaneous administration of specific transporter inhibitors [115, 151, 152, 155–157]. These findings further confirmed that the MDR-ABC transporter function can efficiently reduce gefitinib concentrations below the intracellular drug efficacy threshold and prevent cellular toxicity by limiting drug access to the target kinase EGFR [115, 151]. Notably, the active transport of gefitinib by MDR-ABC transporters proved to be strictly concentration-dependent, and higher doses of gefitinib rather inhibited ABCB1 and ABCG2 transport functions [115, 122, 153], resulting in the restoration of cellular mitoxantrone, topotecan, SN-38, and paclitaxel, and docetaxel sensitivities [122, 150, 152, 153].

Several representatives of the new generation of EGFR inhibitors (some of them also acting on multiple kinase targets), such as erlotinib, lapatinib, pelitinib, neratinib, and vandetanib, have already been shown to interact with MDR-ABC transporters. Although some controversies still exist regarding the ability of MDR-ABC transporters to transport and confer resistance against these drugs, the currently available literature data agree that relatively higher concentrations of all of these TKIs can antagonize the function of ABCB1 and ABCG2 [107, 115, 156, 158–165]. Therefore, in MDR-ABC transporter expressing cells, these TKIs can enhance the cytotoxic effect of various drugs, such as vincristine, vinorelbine, docetaxel, paclitaxel, mitoxantrone, flavopiridol, doxorubicin, SN-38, and topotecan [160–164, 166, 167]. If efficiently recognized by MDR-ABC transporters, these TKIs are presumably only transported only in a narrow concentration range, which could account for the current discrepancies between the thus far published results.

9.6.3 *In Vitro Interaction of MDR-ABC Transporters with Other Clinically Relevant TKIs*

Novel TKI molecules with various target spectra are currently emerging and represent potential therapeutic approaches. Continuous interest has produced a large set of in vitro experimental data about the MDR-ABC transporter interaction profiles of several novel TKIs. For example, the multikinase (c-Kit/FLT3/PDGFR/VEGFR) inhibitors sunitinib and sorafenib [113, 139, 168–170], as well as the VEGFR inhibitors apatinib [53, 171], telatinib [172], and axitinib [173]; the FLT3 inhibitor tandutinib [56] and quizartinib [174]; or the BRAF inhibitor vemurafenib [175–177] have been investigated in this regard, by similar experimental approaches as detailed in the previous sections. Collectively, all of the abovementioned agents have been implicated to interact with at least one of the MDR-ABC proteins, and therefore can be transported by and/or can inhibit these transporters. Notably, rather than displaying decreased cellular toxicity, quizartinib showed an enhanced toxic effect in K562 cells expressing ABCB1 or ABCG2 [174]. Although the molecular background of the collateral sensitivity of the transporter-expressing cells to quizartinib remains to be explored, quizartinib might be a promising agent which selectively targets and exploits MDR-ABC transporter overexpression of cancer cells [83, 178–180].

9.6.4 *In Vivo Relevance of the Interaction of MDR-ABC Transporters with Clinically Applied TKIs*

As detailed in the previous sections, numerous in vitro data support that clinically relevant TKI molecules interact with MDR-ABC transporters. Various genetic knockout animal models have been generated to assess whether MDR-ABC transporter functions also have significant in vivo impact on the tissue distribution and anticancer effect of TKIs. Chemical knockout mice, in which the transporter function was blocked by administration of specific pharmacological transporter inhibitors, have also been utilized for experiments studying the in vivo interactions between MDR-ABC transporters and TKIs.

In mice, both ABCB1 and ABCG2 were shown to affect the bioavailability, especially the brain penetration of several TKIs including the Bcr-Abl inhibitors imatinib [181–187], dasatinib [188–191]; the EGFR inhibitors gefitinib [157, 192], erlotinib [158, 193, 194], lapatinib [159, 195], and vandetanib [165]; the multikinase inhibitors sunitinib and sorafenib [168, 169, 190]; and the BRAF inhibitor vemurafenib [175, 176].

In vitro data suggest that TKI molecules can inhibit the transport of simultaneously applied MDR-ABC substrates and therefore can significantly modify their pharmacokinetic and efficacy profiles. Indeed, numerous TKIs have been reported to alter the in vivo tissue distribution and anticancer efficiency of conventional chemotherapeutics. Gefitinib was reported to enhance the oral bioavailability and

antitumor activity of irinotecan [152, 154], increased the bioavailability and decreased the clearance of topotecan [155], and also enhanced topotecan penetration of gliomas in mice [196]. The antitumor activity of topotecan in rhabdomyosarcoma cell xenografts was shown to be highly potentiated by imatinib [197]. Lapatinib was found to strongly enhance the antitumor effect of paclitaxel on ABCB1 overexpressing KBv200 xenografts in mice [160], while co-administration with neratinib or apatinib significantly increased the antitumor effect of paclitaxel in the same xenograft model [53, 164]. Also, co-treatment of ABCG2 overexpressing xenografts in mice with telatinib and doxorubicin was reported to significantly decrease the growth rate and size of the transporter-expressing tumors [172].

The issue whether MDR-ABC transporters display a major impact on the biodistribution and toxicity of TKIs in vivo in humans is usually addressed by investigating the genotype-specific influence of the transporters on these parameters. ABCB1 and ABCG2 polymorphic variants have been associated with altered pharmacokinetics of imatinib and were also shown to cause significant differences in the clinical response during imatinib treatment [198–202]. The impact of the genetic variants of these MDR-ABC transporters on the plasma levels and toxicity of gefitinib and sunitinib has also been suggested [156, 203–205].

9.7 Regulation of MDR-ABC Transporters by TKIs

9.7.1 Potentially Coordinated Regulation of Transporters and Drug Metabolizing Enzymes by TKIs

In addition to the above described direct interactions of TKIs with the MDR-ABC transporters, the hydrophobic xenobiotic-like TKI molecules may also significantly modify the complex drug handling properties of the tumor cells. These effects may be treated within the conceptual framework of “chemoimmunity,” regarding the cellular defense mechanisms against xenobiotics as a complex network function [84]. In this system, the drug-metabolizing enzymes (Phase I and Phase II), the MDR-ABC transporters preventing the cellular entry of hydrophobic xenobiotics (Phase 0), and similar transporters promoting the extrusion of partially detoxified compounds (Phase III) provide the effectors of chemodefense. A sensor system for this defense is provided by an array of nuclear receptors, and potentially other signal transduction pathways. This “chemoimmune network” is coordinately regulated, resembling in many aspects to the classical immune system [84, 206].

TKI molecules are hydrophobic molecules and recognized as harmful chemicals similar to many hydrophobic xenobiotics. Thus, TKIs, similarly to other xenobiotics and drugs, may activate these complex cellular resistance mechanisms in many different ways depending on type and state of the cell they enter. As described above, numerous TKIs are directly recognized as transported substrates and their cellular accumulation is limited by MDR-ABC transporters. In other cases, TKIs may directly inhibit the transporters.

After entering the cells, most of the TKIs are exposed to the action of drug metabolizing enzymes, oxidized by specific cytochrome P450 isoforms and conjugated by various glutathione-S transferases to yield a less toxic product [207, 208]. The transporters and metabolic enzymes share the common property of a promiscuous interaction with chemically diverse compounds. Although they exhibit overlapping substrate specificities, they also exhibit characteristic differences in substrate recognition. For example, ABCB1 and CYP3A4 recognize large, hydrophobic, neutral or weakly positively charged compounds, whereas ABCC1 and CYP2C9 also recognize negatively charged chemicals [209, 210]. The synthesis of these proteins is concomitantly regulated to overproduce those transporters and enzymes that are capable of the detoxification of the given xenobiotics entering the cell. This regulation is mainly provided by transcription factors that, together with the transporters and metabolic enzymes, form the cellular chemoimmune system [84, 208].

9.7.2 Transcriptional Regulatory Pathways for Transporter and Metabolic Enzyme Expression: Role of Nuclear Receptors

The expressional regulation of transporters and metabolic enzymes includes both setting the amount and type of the chemoimmunity defense proteins. When the cell is exposed to a specific type of xenobiotic (e.g., a positively charged hydrophobic compound), it is advantageous if the levels of those transporters and enzymes increase that they are able to recognize and convert the toxic compound (e.g., ABCB1, CYP3A4 for detoxifying hydrophobic and positively charged compounds). The lipophilic xenobiotics are recognized in the cytoplasm by promiscuous transcriptions factors that translocate to the nucleus upon ligand binding and activate the transcription of the appropriate chemoimmune genes [207, 208]. Some of these transcription factors have been called orphan receptors as no physiological ligands were known to activate them. These proteins include the Aryl Hydrocarbon Receptor (AHR) and nuclear receptors such as the Pregnane X receptor (PXR) and the Constitutive Androstane Receptor (CAR) [211, 212].

AHR, a basic helix-loop-helix transcription factor, is associated with chaperons and binds its ligands (small, planar hydrophobic drugs, such as Polycyclic aromatic hydrocarbons (PAHs)) in the cytoplasm [213]. Upon binding, it is translocated to the nucleus where the dissociation of chaperons is followed by dimerization with ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator). This heterodimer is able to bind AHR-, dioxin- or xenobiotic-responsive elements (AHRE, DRE, or XRE) in the promoter regions of target genes. The xenobiotic sensing nuclear receptors (such as PXR and CAR) are members of the Type II nuclear receptor subfamily that bind ligands in the cytoplasm, enter the nucleus, where heterodimerization occurs with RXR (Retinoid X Receptor) [211]. The heterodimer activates genes with specific hormone response elements in their promoter region. The structural background of their multispecific recognition, which might exhibit similar molecular

features as MDR-ABC transporters and metabolic enzymes, has been extensively studied [212, 214, 215], but the details of this mechanism still remain to be elucidated.

The Aryl Hydrocarbon Receptor has been described to enhance the transcription of various ABC transporters. Several AHR agonists were reported to enhance the expression of ABCG2. Recently, this transcription induction has been shown to be directly coupled to AHR signaling by gene promoter analysis and experimental demonstration of an active dioxin-responsive element [216, 217]. The other AHR-regulated ABC protein is MRP4/ABCC4, which is upregulated by oxidative stress in cholestasis, as shown in human and animal models [218]. Bioinformatics analysis revealed XRE and Maf response elements in the mrp4 promoter that was demonstrated also by a luciferase reporter assay [218]. These results suggest an ABCC4 regulation mediated by both AHR and Nrf2 and that the stimulation of these transcription factors may have a positive effect on cholestasis. Interestingly, there is no known dioxin response element in the promoter of ABCB1, but its functional expression has been shown to be increased by dioxin in the blood–brain and blood-spinal cord barriers [219, 220].

PXR and CAR have been the first and main reported promiscuous nuclear receptors shown to activate the transcription of MDR-ABC transporters [221–223]. A DR4 motif in the upstream enhancer at about −8 kbps from the PXR binding site was shown to be responsible for ABCB1 induction by rifampin [221]. It has also been demonstrated that CAR can also affect ABCB1 transcription via binding to the same DR4 motif [224]. These nuclear receptors have not been shown to regulate ABCC1/MRP1, while they strongly influence the expression of the Phase III ABCC2/MRP2 transporter [225]. Recent reports indicate that PXR is also a regulator of the ABCG2 transcription [226, 227].

The transcriptional regulation of the MDR-ABC proteins is still largely unexplored in its complexity. These regulatory interactions exhibit strong tissue and cell type specificity; thus the same MDR-ABC transporter may be regulated by different nuclear receptors in different tissues. For example, in contrast to PXR-dependent ABCG2 regulation in the blood–brain barrier [226], imatinib treatment of liver cells did not influence ABCG2 expression through neither CAR nor PXR [228]. In the latter study, Caco2 cells reacted immediately to imatinib exposure by inducing ABCG2, but the mRNA level of this MDR-ABC transporter decreased again when ABCB1 expression was increased. This observation suggests a coordinated regulation of various chemoimmune during detoxification.

Interestingly, recent studies indicate AHR regulation of ABCB1, ABCC2, and ABCG2 in both the blood–brain and blood-spinal cord barrier [220, 229], while no other reports indicate an AHR-dependent ABCB1 transcriptional activation. These studies employed the dioxin TCDD and suggested that it promotes ABCB1 promoter activation through AHR. However, alternative dioxin responsive, AHR-independent pathways [230] could be responsible for this regulation. Moreover, AHR can act as an ubiquitin ligase and affect the degradation of other nuclear

receptors and transcription regulators, thus cross-talk with other signaling pathways [231, 232]. Other complex regulatory patterns can also be imagined such as a positive feedback of AHR activation increases the expression of ARNT that dimerize with Sp1 leading to enhanced expression of ABCB1 through Sp1-binding sites on its promoter [233].

The exploration of the pathways is even more difficult because of species differences. It has been shown that while the human ABCG2 is regulated by AHR, the murine ABCG2 is not under AHR or PXR control [234]. Experiments, including high-throughput drug-screening studies, may be misleading when using nonhuman nuclear receptors as targets or recombinant human proteins in a nonhuman cell line or transgenic animal. In the latter cases, nonhuman coregulators may result in a completely different transporter expression pattern than in human cells. This phenomenon highlights the role of coregulator proteins also in the xenobiotic signaling pathways and explains differences in expression patterns when the drug binds to the same nuclear receptor in different tissues or under different conditions.

Another challenge in understanding the regulation of the chemoimmune system is a potential xenobiotic activation of nuclear receptors with primary function in lipid sensing (e.g., PPAR, LXR, FXR) and their further activation of genes involved in upregulation of proteins involved in detoxification [235]. The vitamin D receptor (VDR), although being more specific than CAR and PXR, still shares target genes such as that of ABCB1 with the two promiscuous nuclear receptors [236, 237]. Detoxification function of VDR may be related to its ability to bind bile acids, thus mediate their elimination [238]. Interestingly, in tissue barriers with low CAR and PXR expression (e.g., the placenta), VDR can substitute the function of these nuclear receptors and activate ABCB1 and metabolic enzyme transcription [239, 240].

Activation of these regulatory pathways by TKIs may have different features in different cell types and under different conditions. The ABCG2 substrate TKI, gefitinib, has been reported not to increase ABCG2 expression in one study [241], while gefitinib-resistant cell lines were reported to show increased ABCG2 expression [115, 242, 243]. One possible explanation for these observations is the translocation of EGFR to the nucleus where it may act as a transcription factor [244].

The apparently nonspecific transcriptional activation of ABCG2 and other multidrug transporters by Nrf2 [245–247] suggests the possibility for a general stress response type activation of the xenobiotic response. In this scenario, the drugs accumulating in the cell and oxidized by metabolic enzymes may result in an oxidative stress signal. Under normal conditions, Nrf2 is bound by Kelch like-ECH-associated protein 1 (Keap1) and Cullin 3 and degraded following ubiquitination [248]. Oxidative or electrophilic stress alters cysteine residues in Keap1 leading to abrogated ubiquitination and translocation to the nucleus to upregulate oxidative stress genes including multidrug transporters. According to these findings, the first xenobiotic defense activation signal involves promiscuous nuclear receptors in a relatively specific manner, while the increased oxidized drug species may trigger a further response through the Nrf2 pathway.

9.7.3 Posttranslational Regulation of Transporter Activity by TKIs

In addition to transcriptional regulation, the function of multidrug transporters may be significantly modulated by posttranslation modifications, trafficking to the cell surface, or by modulation of their degradation. Detailed studies have been performed in this regard for several MDR-ABC transporters including ABCB1/MDR1, ABCC2/MRP2, ABCB11/BSEP, etc. [249, 250]. Although the role of TKIs has been less conclusive in this regard, in the case of ABCB1 it has been convincingly demonstrated that substrate binding can significantly increase the membrane trafficking and insertion of this protein [251]. In the case of ABCG2, ER-retained variants have been delivered to the plasma membrane when treated by a transported substrate, mitoxantrone [252]. These observations suggest that MDR-ABC protein substrate TKIs can enhance the cell surface level of the MDR-ABC proteins acting as pharmacological chaperons, thus increasing their own clearance from the cell.

Recently, the Ser/Thr kinase Pim-1 has been shown to affect drug resistance mediated by ABCB1 and ABCG2 [253–255]. Both ABCB1 and ABCG2 were reported to contain Pim-1 phosphorylation sites. ABCG2 was demonstrated to be phosphorylated by Pim at Thr362, and exhibited impaired ABCG2 multimer formation resulting in the restoration of drug sensitivity in prostate cancer cells in which expression of the Pim-1 kinase was knocked-down [254]. Similarly, the cell surface expression of ABCB1 was suggested to be maintained by Pim-1 kinase activity [255]. In accordance, Pim-1 kinase inhibition by the small molecule Pim kinase inhibitor SGI-1776 resulted in decreased ABCG2 and ABCB1 expression [253]. It is important to note that even though SGI-1776 did not alter the expression of ABCB1 or ABCG2 in cells which do not express its target kinase Pim-1, it still enhanced the cellular uptake of ABCB1 or ABCG2 substrates in Pim-1-negative cells. This observation suggests that similarly to SGI-1776, TKI compounds might modulate drug resistance not only via affecting their specific kinase targets but also by interacting with and acting as "nonspecific" inhibitors of MDR-ABC proteins.

The plasma membrane localization of ABCG2 has also been suggested to be regulated by the PI3K/Akt signaling axis. Inhibition of Akt signaling was reported to provoke a rapid translocation of ABCG2 from the plasma membrane to intracellular compartments in several cell types [256–261], a phenomenon which has also been associated with attenuated ABCG2 function [256, 258, 260, 261] and subsequent reversal of drug resistance caused by the transporter [260, 261]. Nevertheless, opposing results have also been published showing that inhibition of Akt signaling downregulated overall ABCG2 protein levels rather than affecting only its plasma membrane localization in human leukemia cells [132]. Involvement of the downstream mTOR kinase in regulating plasma membrane insertion of ABCG2 has also been controversial [258, 262]. In the above referenced studies, several pharmacological inhibitors of the PI3K and mTOR enzymes have been applied to manipulate cellular signal transduction, such as wortmannin, LY294002, and rapamycin.

Notably, LY294002 and rapamycin, inhibitors of the PI3K and the mTOR kinases, respectively, have recently been shown to interact with and inhibit the function of ABCG2, which could also account for the reversal of transporter-mediated cellular drug resistance irrespectively of alterations in PI3K/Akt/mTOR signaling and subsequent potential changes in functional ABCG2 protein levels [263, 264]. The versatile PI3K and ABCG2 inhibitory potential of LY294002 has also been proposed to be exploited to design novel therapeutic strategies for the targeting of ABCG2-expressing drug resistant cancer cells or cancer stem cells which rely on Akt signaling for survival [265].

9.8 Conclusions: Potential Solutions for Overcoming Clinical Drug Resistance

Several therapeutic approaches have been proposed to circumvent cancer multidrug resistance caused by the expression of MDR-ABC proteins. These include the administration of MDR-ABC inhibitors in order to allow enhanced intracellular accumulation and cytotoxic action of simultaneously applied MDR-ABC substrate molecules; the use of drugs that are not recognized by MDR-ABC transporters and therefore can bypass MDR-ABC-mediated efflux, and also the application of drugs to which MDR-ABC transporter expressing tumor cells show hypersensitivity (collateral sensitivity) [83].

Multispecific MDR-ABC transporter inhibitors showing both in vitro and in vivo activity are already available, such as elacridar (GF120918) and tariquidar (XR-9576) which inhibit ABCB1 and ABCG2; or cyclosporine A (CSA) and biricodar (VX-710) which inhibit ABCB1, ABCC1, and ABCG2 [266]. Most of these multispecific inhibitors have already had history as potential (ABCB1 mediated) MDR reversal agents applied in clinical trials, which unfortunately all ended with disappointing results. Inefficiency and/or toxicity of the MDR-ABC inhibitors observed are currently believed to have resulted from insufficient dosing and previously unexpected pharmacokinetic interactions of the MDR-ABC inhibitors, from recruitment of patient cohorts not biased for MDR-ABC transporter expression and also from interference with the physiological xenoprotective function of the targeted transporters [83, 266, 267]. Accumulating evidence suggests that most of the clinically relevant TKI molecules can efficiently block MDR-ABC transporter functions in a certain concentration range. Choosing a therapeutic window where off-the-shelf kinase inhibitors, with well-established safety and toxicity profiles, can parallelly inhibit their specific kinase targets and also MDR-ABC proteins might lead to an enhanced anticancer effect of the kinase inhibitors themselves and also with simultaneously administered conventional chemotherapeutics (see Fig. 9.1).

MDR-ABC transporters can recognize a wide array of chemically and target-wise unrelated compounds, although the molecular mechanism of the polyspecific drug binding is yet to be explored. As detailed in the previous sections, all of the

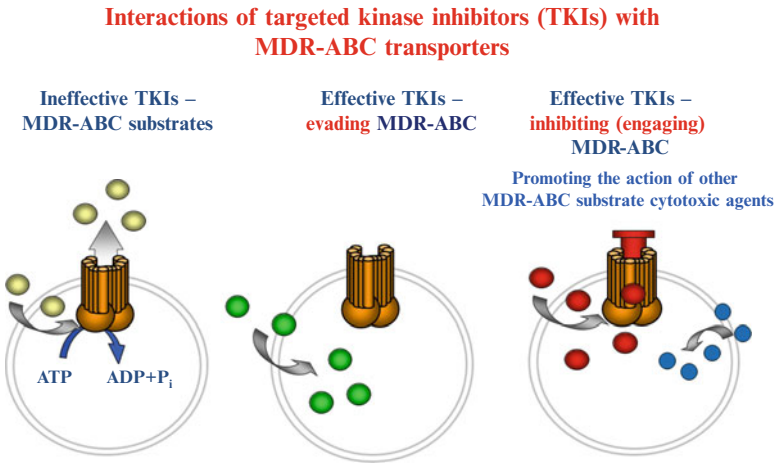

Fig. 9.1 Interactions of targeted kinase inhibitors (TKIs) with MDR-ABC transporters

investigated clinically relevant TKI molecules can interact with at least one of the MDR-ABC proteins and can act as transporter substrates and/or inhibitors. TKI molecules which are not efficiently transported and therefore evade the MDR-ABC-mediated efflux can retain their intracellular kinase inhibitory action even in a multidrug resistant background. Such TKIs thus can also successfully target and eliminate kinase-addicted cancer cells with inherent or acquired MDR-ABC transporter expression, as was suggested in the case of the Bcr-Abl inhibitor bosutinib [103, 141, 142].

Recently, several compounds have been identified to which drug-resistant cancer cells overexpressing ABCB1 showed an unexpected hypersensitivity (collateral sensitivity) [178–180]. Whether drugs that selectively kill ABCC1 or ABCG2-expressing cancer cells exist is yet to be elucidated. Nevertheless, novel promising therapeutic strategies seem to emerge that aim to selectively target and exploit MDR-ABC transporter overexpression of cancer cells. Molecules that show a favorable inhibitory activity against certain kinases and in addition show significantly enhanced specific toxicity in cancer cells which express MDR-ABC transporters, as it was reported in the case of the FLT3 inhibitor quizartinib [174], might therefore also represent promising novel therapeutic options.

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Chapter 10
Nanotechnology to Combat Multidrug Resistance in Cancer

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Abstract Multidrug resistance (MDR) in cancer is a prime obstacle toward successful cancer chemotherapy which is the combination of the complicated mechanisms involving abnormal vasculature, localized area of hypoxia, upregulated ABC transporters, aerobic glycolysis, elevated apoptotic threshold, and increased interstitial fluid pressure. Nanomedicines in targeted cancer chemotherapy hold great promise as an effective approach to prevail over MDR. Extensive research has been conducted to get success in development of Nanomedicines against MDR that introduced many of them as personalized medicine and in different clinical stages. Nanomedicines can be preferentially accumulated in tumor areas by EPR and by active targeting of upregulated processes such as ABC transporters of cancer cells. In this review, we aimed to discuss different nanomedicines that showed promises against MDR in cancer and improved the chemotherapeutic efficacy in the last decade. Moreover, different cellular and physiological factors that underlie MDR in cancer will also be discussed.

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Abbreviations

ABC	ATP binding cassette
ASOs	Antisense oligonucleotides
Au-NPs	Gold nanoparticles
BCRP	Breast cancer resistance protein
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine
DOXO	Doxorubicin
EPC	Egg phosphatidylcholine
EPR	Enhanced permeation and retention
FDA	Food and drug administration
HCC	Hepatocellular carcinoma cells
HIF	Hypoxia-inducible factors
MDR	Multidrug resistance
MNSP	Mesoporous silica nanoparticles
MRP	Multidrug resistance-associated protein
MX-LPG	Mitoxantrone incorporated liposome
NBD	Nucleotide binding domain
NFκB	Nuclear factor kappa B
NOS	Nitric oxide synthase
NP	Nanoparticle
PAX	Paclitaxel
PCL	Poly(ε-caprolactone)
PEG	Poly(ethylene glycol)
P-gp	P-Glycoprotein
pHe	Extracellular pH
PHSM/f	pH-sensitive micelles with folate
PLGA	Poly(lactic-co-glycolic acid)
PSS	Protonation, sequestration, and secretion
RES	Reticuloendothelial system
ROS	Reactive oxygen species
siRNA	Small interfering RNA
SLN	Solid lipid nanoparticle
TBD	Transmembrane domain
Tf-R	Transferrin receptor
TGF-β	Transforming growth factor beta
TNF	Human tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
VEGF	Vascular endothelial growth factor

10.1 Introduction

Cancer is possibly the most complex and challenging disease ever known to mankind and an unavoidable health concern of this millennium [1]. Even though many efforts have been made to cure cancer, it remains a challenging task. Besides surgery, radiation therapy and chemotherapy are the frontline strategies for the treatment of cancer [2]. However, development of multidrug resistance (MDR) poses a major obstruction to the successful outcome of chemotherapy. MDR is a state of resilience against structurally and mechanistically unrelated drugs with different targets and chemical structures [3, 4]. It mediates a process of inactivation of the drug or diverts it from the target tumor cells and causes a substantial obstacle for the treatment of cancer [5].

It is anticipated that, annually, approximately five million new cases of cancer will eventually exhibit the MDR phenotype. Such phenotype can either be acquired from drug treatment, be intrinsic or pre-existing in the cancer cells [6]. This defense mechanism can be responsible for therapeutic failure and tumor relapses in over 90 % of patients [7]. Higher doses or dosing frequency of the chemotherapeutic agents is required to overcome MDR, which results in fatal adverse effects or toxicities [8]. This is a complex process that can result from various mechanisms, which may be grouped into different categories viz. decreased drug influx, increased drug efflux, activation of DNA repair, altered drug metabolism and detoxification, secondary mutations in drug targets, and activation of downstream or parallel signal transduction pathways [9, 10]. In some drug resistant cancer cells, the intracellular pH gradient, altered rates of vesicular transport, exocytosis, higher interstitial fluid pressure, low pH environment, hypoxic region in the tumor core, and irregular tumor vasculature are key factors regulating MDR [11].

Even though numerous strategies which emerged over the past 30 years helped us to understand mechanisms of resistance in a great deal, there is still a need for an effective clinical tool to overcome resistance. Current strategies to overcome drug resistance include modification of chemotherapeutic regimens, targeted inactivation of MDR-related mRNAs, use of monoclonal antibodies against an extracellular epitope of an MDR efflux transporter, development of new chemotherapeutics that are not recognized as transport substrates by MDR efflux pumps, use of MDR chemosensitizers or modulators, and development of targeted nanomedical approaches that evade MDR pumps and combination of two or more of the above approaches [12–17].

From the perspective of drug delivery, MDR in cancer can be suppressed by improving delivery of chemotherapeutics to the tumor sites and reducing MDR-based drug efflux. A rational way for efficient cancer drug delivery is to amalgamate chemotherapeutic approaches with nanotechnology [18]. According to the US FDA, nanotechnology-based formulation refers to those that are at least one dimension in the length scale between 1 and 100 nm and show functional behavior in relation to their nanosized properties [19]. In the last few decades, the progression of nanotechnology has made it possible to synthesize drug delivery vehicles of nanoscale which are biocompatible and biodegradable in nature. Various types of nanocarriers including liposomes, nanomicelles, polymeric nanoconjugates, nanoparticles, carbon nanotubes,

dendrimers, etc. have been established for delivery of a number of anticancer drugs [20, 21]. These nanocarriers have shed light on desirable drug delivery characteristics such as prolonged systemic circulation, reduced nonspecific cellular uptake, targeting potential, controlled drug release, and multidrug encapsulation for combinatorial treatment. In addition to passive targeting, nanomedicines also provide an alternative strategy to avoid MDR by active targeting through simultaneous encapsulation and attachment of multifunctional components like ligands, antibodies against MDR tumor cells, chemotherapeutic drugs, nucleic acid, and inhibitors of MDR-causing enzymes [5, 8, 12, 22–24].

This chapter illustrates different contributors of MDR in cancer cells and shows the impetus of nanotechnology for the reversal of this challenging situation.

10.2 MDR in Oncology

The specificity of drug, tumor, and host, collectively, characterizes resistance to chemotherapy which consequently results in the ineffectiveness of anticancer drugs in treating tumor without excessive toxicity [25]. Therefore, there is a special need for a cytotoxic agent that can cause selective destruction of neoplastic cells along with protecting the host cells and their functions. The two aspects that mark the necessary distinction between cancer chemotherapy and antibacterial chemotherapy consist of the resistance of human cancer to anticancer drugs and the lack of selectivity of anticancer agents whilst the antibacterial chemotherapy is characterized by complete eradication of infection. The principal barrier to the efficacy of anticancer therapy is the acquisition of natural (inherited) and acquired resistance [5, 9, 25]. Natural resistance is the primary unresponsiveness of a tumor toward an antineoplastic drug whereas the acquired resistance is unresponsiveness of tumors that develop after continuous exposure to successful anticancer drug therapy. Importantly, the resistance to chemotherapy can be classified as kinetic, biochemical, and pharmacological resistance [12, 22, 25]. In kinetic resistance, only a few cells are in a plateau growth phase with a minor growth fraction which is frequently observed in many human tumors. The kinetic resistance can be overcome by minimizing the tumors bulk via surgery or radiotherapy, applying combinations of drugs that can influence the resting populations (G_0 cells), and scheduling of drugs in a way to avoid the phase escape or synchronizing cell populations and increasing tumor cell elimination. Unfortunately, the exact mechanism of biochemical resistance is not very clearly understood [5, 25]. However, the chief causes of biochemical resistance are supposed to be the incapability of the tumor to convert the drug into its active form and the upregulation of the tumor enzymatic repair systems that counteract the tumoricidal action. Moreover, biochemically resistant cells can reduce the uptake of drug, enhance the drug efflux activity, alter the configuration of the intracellular target, decrease intracellular activation, improve inactivation of the drug, or enhance the rate of repair of damaged DNA [22–25]. For instance, MDR termed as pleiotropic drug resistance is the development of resistance during treatment with an anticancer agent that confers

resistance to that drug and other(s) of its class in addition to several other unrelated agents [12, 14, 25]. The third category of resistance, which is pharmacological resistance, emerges from reduced tumor blood supply, erratic absorption, augmented excretion or catabolism, and drug interactions which eventually result in the inefficient systemic availability of the anticancer drugs [3, 5, 8, 12, 25]. Examples of pharmacologic resistance include inadequate transport of anticancer drugs into body tissues and tumor cells [12, 25]. In this context, an optimal concentration of an anticancer agent in the brain tissue, which is effective against the tumor cell type, is essential to treat tumors of the central nervous system.

10.3 Factors Accountable for MDR

Factors responsible for the MDR can be mainly divided into cellular factors and physiological factors [23]. Cellular factors include altered molecular targets, genetic defects like polymorphism and gene deletion, overexpression of efflux pumps, reduced apoptosis, and increased drug metabolism. Physiological factors include cell–cell interaction, higher interstitial fluid pressure, low pH environment, hypoxic region in the tumor core, irregular tumor vasculature, and the presence of cancer cells in areas difficult to penetrate [5, 8, 12, 22–25]. Most of these factors lead to the requirement of higher doses of chemotherapeutic agents, which demonstrate systemic toxicity [24].

10.3.1 Cellular Factors

Resistance to anticancer drugs can develop by several cellular mechanisms. The important contributing cellular factors include increased efflux pumps activity, e.g., ATP-dependent transporters and/or reduced influx of drugs requiring intracellular carriers (or endocytosis). In cases where the influx/efflux is unaffected, resistance can occur due to activation of detoxifying proteins like cytochrome P450 etc. Additionally, disruptions in the apoptotic machinery can also render cells resistant. Heterogeneity and mutations of cancer cells play an important role in all of these mechanisms [23]. Out of many possible mechanisms, those encountered frequently in the clinic and having a significant impact on the outcome of chemotherapy are discussed below.

10.3.1.1 ATP Binding Cassette (ABC) Transporters

The ABC transporter superfamily consists of several members subdivided into several subfamilies based on their structure. ABC transporters are membrane proteins with a nucleotide binding domain (NBD) and a transmembrane domain (TBD)

which function to efflux molecules out of cells against the concentration gradient by hydrolyzing ATP at the NBD [26, 27]. This leads to reduced intracellular concentration of a chemotherapeutic agent, thus requiring administration of higher doses [27–29]. Researchers have identified various substrates for drug efflux proteins of the ABC superfamily such as taxols, anthracyclines, mitoxantrone, topotecan, and etoposides [29, 30]. Among all the members of the ABC superfamily, ABCB1 (MDR1), ABCC1 (MRP1), and ABCG2 (BCRP) are the most frequently overexpressed proteins in cancer cells and play a vital role in demonstrating the drug resistant phenomenon [27–29, 31].

10.3.1.2 Defective Apoptotic Machineries

Apoptosis is characterized by the process of development of cell membrane blebs, DNA condensation, and DNA fragmentation leading to cell death [32, 33]. Apoptosis plays a significant role in the life of a cell and is mediated by proapoptotic and antiapoptotic molecules [34]. Two interlinked pathways have been identified for apoptosis, i.e., the extrinsic pathway is activated by binding of ligands to cell surface receptors and the intrinsic pathway is activated by stimuli at mitochondria [35]. The extrinsic pathway is always caspase-dependent, whereas the intrinsic pathway may or may not be mediated by caspases. Due to the complex nature of the apoptotic machinery cancer cells have evolved several adaptations against apoptosis. Most common mechanisms include the upregulation of antiapoptotic proteins like Bcl-2, Bcl-XL, and Mcl-1 as well as the downregulation of Bcl-2 family proteins like bax all of which avoid apoptosis [36, 37]. Endogenously, the inhibitor of apoptosis (IAP) and the PI3K/Akt pathway are common mechanisms against apoptosis and leading to resistance. IAP is a family of proteins identified as endogenous inhibitors of apoptosis whereas upregulation the PI3K/Akt pathway contributes to cell growth especially in breast cancer and non-small-cell lung cancer via NFκB upregulation and Bad downregulation [38]. Exogenously, upregulation of mutated death receptors can also occur at the surface of resistant cancer cells. Apart from death receptors, genetic mutation of the p53 genes and overexpression of p53 can also render cancer cells resistant to drugs like paclitaxel and cisplatin [39].

10.3.1.3 Altered DNA Repair Pathways

There are numerous drugs, like alkylating agent, anthracycline analogues, and epipodophyllotoxin, which exert their anticancer action by causing direct or indirect DNA damage. Thus, DNA repair mechanisms serve as an important target for drug resistance. There are five most common DNA repair mechanisms viz. reversion repair, base excision repair, nucleotide excision repair, mismatch repair, and double-strand break repair [40]. It is important to note that DNA damage caused by an alkylating agent is a direct substrate for reversion repair whereas the nucleotide excision repair is responsible for resistance to platinum-based compounds and alkylating agents [41].

10.3.2 Physiological Factors

The physiological factors that are primarily responsible for the MDR under investigation are high interstitial fluid pressure (IFP), hypoxia, and low extracellular pH (pHe) [42]. These factors are interrelated and considered to affect one another.

10.3.2.1 Interstitial Fluid Pressure (IFP)

In normal tissue, pro-angiogenesis and anti-angiogenesis factors regulate the process of blood vessel formation leading to efficient vasculature which meets nutritional and oxygen requirements. In case of tumor cells, this regulation of angiogenesis is lost and unorganized vasculature away from the cells is generated [43]. This further causes leakage of protein from blood vessels into the interstitium leading to a rise in the interstitial fluid pressure (IFP > 100 mm of Hg) [44]. The unorganized vasculature and high IFP obstruct the delivery of intravenously administered chemotherapeutic agents [45]. The underlying mechanisms for obstruction to delivery of such drugs include decreased blood flow, transcapillary fluid flow, and the convective transport. Further, deep localization of cancer cells beyond blood vessels also makes it difficult for the delivery of drug to target cells [43].

10.3.2.2 Hypoxia and Low Extracellular pH

Hypoxia is another important factor for drug resistance in cancer cells which occurs in more than 50 % of tumors [46]. As mentioned previously irregular blood vessels are formed in the cancer tissue which lack sufficient oxygen supply whereas oxygen demand in cancer cells is more than normal cells, thus causing hypoxia. Hypoxia also develops due to deep localization of cancer cells. The mechanism of hypoxia can be classified into three classes, i.e., perfusion limited oxygen delivery due to abnormality in microvessels, diffusion limited oxygen delivery due to altered diffusion mechanism, and anemic hypoxia due to decline in oxygen carrying capacity of the blood [47]. Reduced oxygen availability at the cellular level in tumor tissue forces cells to rely on glycolysis for ATP supply. Furthermore, anaerobic respiration leads to production of high amounts of lactate and carbonic acid [48]. These acidic products accumulate in the tumor due to the absence of the lymphatic system and the decrease of extracellular pH to 5.8–7.2 [49]. Hypoxia-associated drug resistance can be categorized into four mechanisms. First, therapies that require oxygen to generate free radicals like reactive oxygen species (ROS), e.g., radiation therapy [50]. Second, drug that depend on partial pressure of oxygen, e.g., melphalan, bleomycin, etoposide, etc. [51]. Third, induction of hypoxia-inducible factors (HIF1) resulting in the expression of vascular endothelial growth factor (VEGF), nitric oxide synthase (NOS), transforming growth factor beta (TGF-β), and interlukin-8 and causing resistance to drugs, e.g., cisplatin and doxorubicin [52]. Under the harsh environment of anoxia, the cell cycle may get arrested in either the G1/G2 or the S phase as well as induces an increase in the DNA repair enzymes resulting in

resistance to the cycle-selective cytotoxic drugs (5-FU, paclitaxel) and to the DNA-damaging agents (alkylating agents, cisplatin) [51]. Fourth, the most important indirect effect of hypoxia is the induction of the ABC transporters described previously [53]. Additionally, the presence of acidic products of glycolysis also leads to a pH gradient which causes the "ion trapping" phenomenon, characterized by permeability difference between ionized and non-ionized forms of an anticancer drug. This mechanism is of particular importance for weakly basic drugs like doxorubicin and vincristine which remain ionized and therefore trapped in the acidic extracellular environment [54]. This ion trapping is thus dependent on the pHe as well as the pKa of the drugs.

10.3.3 Other Factors

Cancer cells have an extraordinary tendency to adapt to their environment, due to its survival and growth. Besides most commonly encountered phenomenon such as overexpression of efflux transporter pumps and defective apoptotic machineries, numerous researches have shown other adaptive mechanisms responsible for the MDR. Recent finding demonstrated that overexpression of survivin was associated with increased tumor grade and its profound impact on inhibition of apoptosis makes it an interesting target to overcome the MDR phenomenon [55]. Further, mutations of folate transporters and tyrosine kinase have also been shown to be responsible for MDR [56, 57].

10.4 Nanotechnology Overcome the Barrier of MDR in Cancer

In the current scenario, nanotechnology is explored profoundly in the active area of chemotherapeutics to overcome MDR and thereby improving drug delivery, toxicity profile, and clinical efficacy of anticancer drugs. The discovery of novel nanostructures leads to a better understanding of their potential as drug carriers. The pathophysiology of tumor blood vessels is markedly different from that of normal blood vessels such that they exhibit rather a large fraction of proliferating endothelial cells, augmented tortuosity, deficit pericyte, and unusual basement membrane. Furthermore, lymphatic drainage is also impaired in tumors, which is attributable to a greater retention of extravasated macromolecules. Such a series of events is collectively denoted as the "enhanced permeation and retention (EPR) effect" [12, 58]. Of interest, this effect favors the uptake of nanocarriers by accelerating the passive targeting in tumors. In contrast, active targeting implicates binding of nanocarriers to the receptors present on the surface of tumor cells or tumor blood vessels. In a noteworthy way, inconsistent tumor vasculature and impeded lymphatic flow generate a high interstitial fluid pressure leading to an augmented hydrophilic ambience

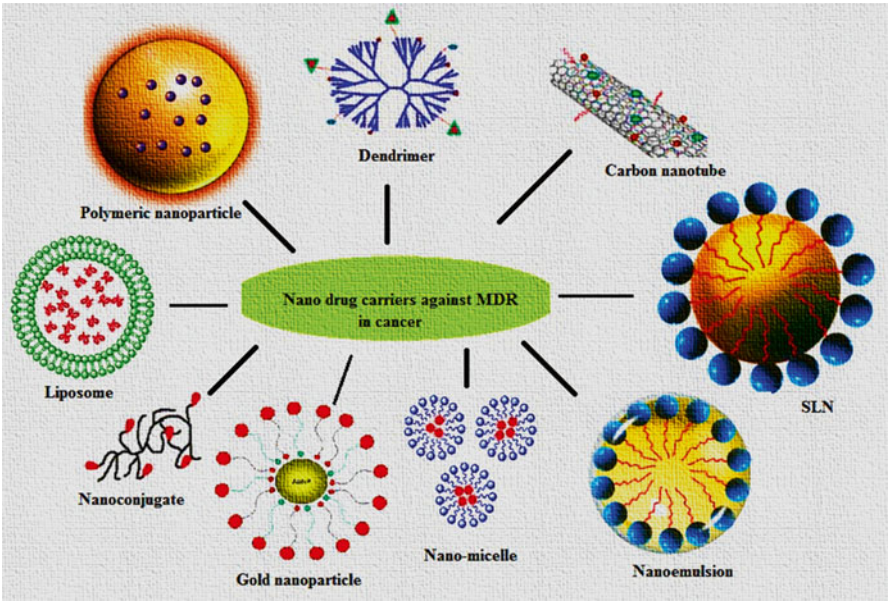

Fig. 10.1 Illustration showing different types of nanotechnology-based drug carriers (called as nanoparticles) that have been investigated for chemotherapeutics delivery in MDR cancer

that restricts the access of drugs to solid tumors [12]. In addition, the extracellular matrix of tumors, fibrillar collagen, and necrotic non-supporting regions are other significant hindrances affecting the clinical efficacy of anticancer agents [6–9]. In this regard, anti-angiogenics are considered to be promising agents for improving anticancer drug therapy by normalizing the blood flow in tumors and consequently minimizing the hypertensive interstitial condition [59]. More recently, the field of nanoparticles is gaining a lot of consideration, essentially for its safety and efficacy aspects. Therefore, different nano-delivery systems have been developed and evaluated both in vitro and in vivo, as discussed herein. A variety of nanotechnology-based drug carriers (called as nanoparticles), which come from diverse classes based on their nature, have been investigated for chemotherapeutics delivery in MDR cancer. We illustrated them in family in Fig. 10.1 and a summary table (Table 10.1) is also given wherever in this article indicating the potential outcome researchers find out while using nanoparticles for cancer targeting in MDR cases against free drug.

10.4.1 Vesicular Nanocarriers

Different vesicular nanocarriers like liposomes, micelles, and nanoemulsions have been utilized to overcome the MDR in cancer. These carriers have shown significant contributions to overcome the limitations of tumor-associated MDR.

Table 10.1 Examples nano-drug carriers that showed anticancer improved efficacy of chemotherapeutics in in vitro and in vivo studies against MDR

Nanomedicine	Drug	Type of cancer	Model	Outcome	Ref.
Liposome	Doxorubicin	Human colon cancer	HT29 cells and HT29-dx cells	Doxorubicin encapsulated liposome (Lipodox) more effective than free doxorubicin in HT29-dx cells	[66]
	Mitoxantrone	Hepatocellular carcinoma (HCC)	HCC Huh-7 cells and MDCKII/BCRP cells; HCC xenograft BALB/c mice	2.3-fold higher cytotoxicity in Huh-7 cells and 14.9-fold increase in mitoxantrone accumulation in MDCKII/BCRP cells; furthermore improved antitumor activity in vivo in orthotopic HCC xenograft BALB/c mice	[68]
	Doxorubicin	Small-cell lung cancer (SCLC)	SBC 3/ADM cells	3.5 times more cytotoxicity than free doxorubicin	[69]
Polymeric micelles	FG020326	Nasopharyngeal carcinoma	KB(v200) cells	Folate-functionalized and FG020326-loaded polymeric micelles approximately five times more resensitized the human KB(v200) cells treated with vincristine. Moreover, significantly inhibited the P-gp drug efflux function	[72]
	Doxorubicin	Colorectal carcinoma	SW480 cells	The co-administration of DOX and TRAIL in P(MDS-co-CES) micelles resulted in increased cytotoxicity against resistant tumor cells	[73]
	Doxorubicin	Breast cancer	MCF-7/DOX(R) cells and MCF-7/DOX(R) xenografts model	Doxorubicin-loaded pH-sensitive micelles decorated with folate showed more than 90 % cytotoxicity of DOX resistant MCF-7 cells in vitro. Moreover, in the MCF-7/DOX(R) xenograft model accumulated doxorubicin level in solid tumor was 20 times higher than free doxorubicin	[113]
Nanoemulsion	Paclitaxel and Curcumin	Human ovarian adenocarcinoma	SKOV3 and SKOV3(TR) cell	Co-therapy of paclitaxel and curcumin, in the nanoemulsion formulations, was very effective in enhancing the cytotoxicity in wild-type and resistant cells by promoting the apoptotic response	[75]
Polymeric nanoparticles	Paclitaxel	Human colon adenocarcinoma	HCT-15 cells and HCT-15 mouse xenograft model	Significant inhibition in tumor growth was observed in HCT-15 mouse xenograft model	[77]
	Doxorubicin	Osteosarcoma	KHOS and KHOSR2 cells; U-2OS and U-2OSR2 cells	Nanoparticle loaded with doxorubicin showed increased apoptosis in osteosarcoma cells as compared with doxorubicin alone	[79]
	Doxorubicin and curcumin	Chronic myeloid leukemia(CML)	K562 cells	Coformulation of doxorubicin and curcumin in nanoparticle inhibits the development of multidrug resistance in K562 cells by the enhancement of antiproliferative activity	[80]
	Doxorubicin	Ovarian and uterine carcinoma	SKOV-3 and MES-SA/Dx5 cells	Cellular uptakes of drug from nonconjugated and HER-2 conjugated PLGA NPs were higher compared with both free drug and nonconjugated PLGA NPs	[81]

Dendrimers	Doxorubicin	Colon carcinomas	C-26 cells and C-26 tumors bearing BALB/c mice	Dendrimer-doxorubicin was ten times more toxic than free doxorubicin toward C-26 cells. Moreover, its tumor uptake was ninefold higher than i.v. administered free doxorubicin in C-26 bearing BALB/c mice	[86]
Cyclodextrin nanoparticle	Doxorubicin	Breast cancer	MCF-7/ADR cells	There was threefold decrease in IC50 value by doxorubicin-loaded cyclodextrin nanoparticle as compared to free doxorubicin in resistant MCF-7/ADR cells	[87]
Gold nanoparticle	Doxorubicin	HCC	HepG2R cells	Extent of intracellular drug uptake of gold nanoparticle was increased in contrast to free doxorubicin in resistant HepG2R cells confirmed by confocal imaging and plasma mass spectrometry	[93]
	Doxorubicin	Breast cancer	MCF-7/ADR cells	Doxorubicin-tethered gold nanoparticles enhanced drug accumulation and retention in multidrug resistant MCF-7/ADR cells and induced elevated apoptosis of cancer cells	[94]
Magnetic nanoparticle	Doxorubicin	CML	K562 cells	Remarkable synergistic effect of Fe(3)O(4) nanoparticles on drug uptake of daunorubicin in leukemia K562 cells	[97]
Silica nanoparticle	Doxorubicin and siRNA	Nasopharyngeal carcinoma	KB-V1 cells	The dual delivery of doxorubicin and siRNA in drug resistant KB-V1 cells was capable of increasing the intracellular as well as intranuclear drug concentration to levels exceeding that of free doxorubicin	[99]
Carbon nanotube	Doxorubicin	CML	K562 cells	2.4-fold higher cytotoxicity and significant cell proliferation suppression toward multidrug resistant K562 leukemia cells as compared with free doxorubicin	[104]
Solid lipid nanoparticle	Doxorubicin	Breast cancer	MCF-7/ADR cells	Efficiently enhanced apoptotic cell death through the higher accumulation of doxorubicin in resistant MCF-7/ADR cells in comparison with free doxorubicin	[106]
	Doxorubicin and Mitomycin C	Breast cancer	MDA435/LCC6 cells	Hybrid systems, solid polymer-lipid NPs were effective in killing MDR cells at 20- to 30-fold lower doses than the free drugs	[107]
Polymer conjugate	Doxorubicin	Lymphoma	Mice bearing T cell lymphoma EL4 or B cell lymphoma 38C13	The best antitumor effects were produced by conjugates with 10–13 wt% of bound doxorubicin and free doxorubicin up to 4.6 % relative to total drug content had no impact on the treatment efficacy	[109]

10.4.1.1 Liposomes

Liposomes are vesicular structures having an aqueous core surrounded by a lipid bilayer shell. The basic method of their preparation comprises hydrating the mixture of natural or synthetic phospholipids, cholesterol, and tocopheryl acetate [1]. Liposomes encapsulate drugs either in an aqueous compartment or in the lipid bilayer depending on their nature. Passive targeting by the EPR effect is responsible for delivery of liposomes to cancer cells that necessitate leaky tumor vasculature [60]. Recently, many liposomal formulations and their advanced forms with targeting ligands, such as the mAb 2C5 with Doxorubicin and an anti-HER2 mAb with Paclitaxel, have been tested in the preclinical phase, while many others are undergoing clinical trials [61, 62]. Additionally, long circulating liposomes are also being prepared by adding polyethylene glycol. Nevertheless, novel approaches of triggered release of the drug once internalized have also been tried by investigators such as hyperthermia for example ThermoDOXO® which is currently in Phase III trials [60, 63, 64]. Interestingly, Mangala et al. developed a neutral DOPC (1,2-dioleoyl-sn-glycero-3-phosphatidylcholine) liposome-siRNA delivery system for siRNA-dependent silencing of the cisplatin resistance transporter mRNA of ATP7B [65]. The findings demonstrated this system to be highly efficacious in vivo in reducing ATP7B expression, and thereby retarding tumor growth in conjunction with non-encapsulated cisplatin. Such encouraging findings favor the encapsulation of important anticancer agents such as paclitaxel and Doxorubicin (DOXO) as DOPC liposome-siRNA delivery systems. Nevertheless, modulation of Pgp by liposomes marks another important mean of enhancing the therapeutic efficacy of anticancer drugs. In this context, Riganti et al. designed an anionic liposomal formulation of DOXO (LipoDOX). The results indicated LipoDOX to be much more effective in resistant HT29-dx cells in comparison to free DOXO [66]. These authors suggested that the alteration in the normal functioning of P-gp by LipoDOX was attributable either to the interaction between liposomes and cell membrane that leads to a change in the composition of lipid and P-gp localization, or to the direct inhibition of ATPase activity. The multiple mechanisms of MDR demand the drug delivery system to be effective through all possible MDR pathways. Therefore, Minko et al. formulated a multifaceted liposome system which comprised an anticancer agent-DOXO; an antisense oligonucleotides (ASOs) targeting MDR1 mRNA; and ASOs targeting BCL-2 mRNA [67]. It was demonstrated that the developed formulation was more toxic in vitro in resistant A2870/AD human ovarian carcinoma cells than free DOXO, DOXO liposomes, and DOXO liposomes with ASOs. Moreover, their study revealed that this complex liposomal formulation was internalized into the cancer cells both in vitro and in vivo and was able to penetrate deep inside the nucleus. Though the exact mechanisms were unidentified, however, it was presumed that membrane fusion and endocytosis might be the possible mechanisms of internalization of the liposome into the tumor cells. Zhang et al. investigated a dual-functionalized liposome of mitoxantrone utilizing synthetic polymeric nano-biomaterial (Gal-P123) for their efficiency in targeting cancer cells and reversal of MDR in hepatocellular carcinoma (HCC) cells [68]. Their findings

demonstrated a significantly increased cytotoxicity by 2.3-fold in Huh-7 cells and 14.9-fold enhanced intracellular accumulation in MDCKII/BCRP cells by means of mitoxantrone incorporated liposome (MX-LPG) in comparison to free mitoxantrone. The results of the pharmacokinetic study in rats confirmed a markedly increased circulation time along with a significantly improved bioavailability of mitoxantrone via the liposome. In addition to this, MX-LPG resulted in improved antitumor activity and enhanced selectivity in BALB/c mice bearing orthotopic HCC xenograft tumors. The above investigators suggested that a combined effect of active targeting and chemosensitization by Gal-P123 were the main contributors for the increased suppression of tumor growth by MX-LPG. In another study, Kobayashia et al. studied the potential of transferrin receptor (Tf-R)-targeted liposomes in overcoming MDR by by-passing P-gp-mediated drug efflux via delivery of DOXO into MDR cells (SBC-3/ADM) through Tf-R-mediated endocytosis [69]. They formulated four types of liposomes which include untargeted and Tf-R-targeted using either egg-PC/cholesterol (EPC) or hydrogenated egg PC/cholesterol. The results demonstrated significant enhanced cytotoxicity by 3.5-fold via targeted EPC-liposome in comparison to free DOXO. In addition, there were increased intracellular and intranuclear DOXO concentration by targeted liposomes in both drug-sensitive and MDR cells. They hypothesized that the targeted liposomes rapidly got internalized via Tf-R-mediated endocytosis following a subsequent release of their contents into the cytoplasm which was facilitated by the higher fluidity of the EPC-based liposomes. It was concluded that Tf-R-targeted EPC-liposomes possess a great potential as a drug delivery system to circumvent P-gp-mediated MDR of tumors.

10.4.1.2 Polymeric Micelles

The polymeric micelles represent a potential nanocarrier system for efficient delivery of anticancer agents. In the early 1990s, Kataoka's and associates designed doxorubicin-conjugated block copolymer micelles which resulted in benchmark outcomes and compelled a great deal of interest of researchers in this area [70, 71]. A polymeric micelle is principally formed when the hydrophobic part of a block copolymer is driven to the interior which can encapsulate a poorly soluble drug, whereas the hydrophilic portion of the block copolymer faces outward to form a shell. Currently, numerous modified pendant polymeric micelles targeting anticancer agents are in preclinical and clinical phases of development. In one report, Yang et al. formulated folate-functionalized polymeric micelles from diblock copolymers of poly (ethylene glycol) (PEG) and biodegradable poly (ε-caprolactone) (PCL), co-encapsulating FG020326 and vincristine. They tested this combination for P-gp blocking of the imidazole derivative, FG020326 and vincristine in resistant KB-V200 cells [72]. The results demonstrated approximately fivefold higher re-sensitization of KB-V200 cells in vitro by folate-functionalized FG020326-loaded micelles than their folate-free counterparts. Further, the prepared folate-functionalized micelles were shown to obstruct the P-gp dependent-rhodamine 123 effluxes.

Further, Lee et al. co-administered human TNF-related apoptosis inducing ligand (Apo2L/TRAIL) and self-assembled micelles of DOXO with a cationic copolymer of poly{*N*-methyldietheneaminesebacate)-co-[(cholesteryloxocarbonyl-amido ethyl) methyl bis(ethylene) ammonium bromide]sebacate} (P(MDS-co-CES)) [73]. The co-administration of DOXO and TRAIL in P(MDS-co-CES) micelles resulted in increased cytotoxicity against resistant tumor cells.

10.4.1.3 Nanoemulsion

Nanoemulsions are principally oil-in-water dispersion with fine oil droplets of 10–200 nm diameter evenly distributed in a continuous aqueous phase which is stabilized with surfactants and co-surfactants. Many clinically important anticancer drugs such as taxanes (Paclitaxel, Docetaxel), etoposides, tamoxifen, and dacarbazine with highly lipophilic nature have been successfully delivered using nanoemulsions as carrier system [74].

Ganta and Amiji conducted a study to investigate the effect of co-administration of paclitaxel and curcumin on overall therapeutic efficacy. They selected curcumin as it inhibits NFκB and causes downregulation of ABC transporters in wild-type SKOV3 and resistant $SKOV3_{TR}$ human ovarian adenocarcinoma cells [75]. They formulated a nanoemulsion by incorporating paclitaxel and curcumin in flaxseed oil. Their findings demonstrated an efficient delivery of encapsulated drugs within SKOV3 and $SKOV3_{TR}$ cells. In addition, co-administration of curcumin resulted in inhibition of NFκB activity and downregulation of P-glycoprotein expression in resistant cells. It was concluded that the combined effect of paclitaxel and curcumin therapy exclusively by means of their nanoemulsion formulations significantly improved the cytotoxicity in wild-type and resistant cells by stimulating apoptosis. Such skillful co-administration of therapeutic agents seems to be promising in future endeavors of curing refractory diseases particularly ovarian cancer.

10.4.2 Particulate Nanocarriers

Various particulate nanocarriers such as polymeric nanoparticles, solid lipid nanoparticles, inorganic nanoparticles, polymeric conjugates, carbon nanotubes, and dendrimers have been widely explored to overcome the MDR in cancer. These carriers have been found to be very efficacious to overcome the limitations of MDR in tumors.

10.4.2.1 Polymeric Nanoparticles

Polymeric nanoparticles are extensively explored nowadays for their remarkable potential as a drug delivery system for anticancer compounds. They are prepared either by encapsulation, dissolution, and entrapment of the drug in biodegradable

polymers or by embedding the drug in polymeric matrix. The binding of drugs to hydrophilic polymers increases their circulation time and minimizes toxicity to normal tissues [15]. Therefore, long circulating nanoparticles are more frequently formulated using PEG which avoids opsonization. Furthermore, advanced forms of nanoparticles are being developed with the use of targeting ligands and pH-sensitive or hypothermic polymer conjugates. Presently, polylactide and poly (lactidecoglycolide) (PLGA) are the most widely used biodegradable polymers for synthesis of FDA-approved nanomedicines, while many more are undergoing clinical trials [76]. Koziara et al. have investigated the in vivo efficacy of paclitaxel (PAX) NPs in a PAX-resistant human colorectal tumor HCT-15 xenograft model [77]. Their findings revealed a marked inhibition of tumor growth in mice treated with PAX-NPs in contrast to free PAX by overcoming PAX resistance and the antiangiogenic effect. In another study, Yang et al. formulated chitosan NPs containing shRNA targeting MDR1 which showed significant reversal of paclitaxel resistance in A2780/TS cells, in a time-dependent manner [78]. These NPs were not carrying the cytotoxic drug paclitaxel. Therefore, upcoming "future generation" of these chitosan NPs integrating paclitaxel may result in a synergistic antitumor activity.

Susa et al. have designed stearylamine-modified dextran nanoparticles of DOXO which resulted in markedly increased accumulation of DOXO in the nucleus of resistant osteosarcoma cells in contrast to free DOXO [79]. The study demonstrated that fluorescence of free DOXO was confined to the cytoplasm in resistant cells, whereas nanoparticle-loaded DOXO fluorescence was in the nucleus of resistant cells. These findings suggested that DOXO was able to get into the nucleus of resistant cells via its incorporation into nanoparticle formulations. However the mechanism behind this finding is not clearly understood; it was assumed that minimization of P-gp efflux might be the reason for enhanced accumulation of DOXO nanoparticles in resistant cells. In another study, Misra et al. developed PLGA nanoparticles by co-incorporating DOXO and curcumin [80]. The integration of curcumin in nanoparticles facilitated the retention of DOXO in the nucleus in addition to downregulating the expression of P-gp and BCL-2 in K562 cells. This co-incorporation of curcumin in nanoparticles formulations of DOXO resulted in better in vitro cytotoxicity in comparison to DOXO nanoparticles. Moreover, Lei formulated HER2 antibody-conjugated DOXO-loaded PLGA nanoparticles and compared their cellular uptake and cytotoxicity to free DOXO and non-targeted nanoparticles in resistant ovarian SKOV-3 and uterine MES-SA/Dx5 cells [81]. The results revealed increased cellular uptake of targeted nanoparticles as compared to free DOXO and non-targeted nanoparticles in SKOV-3 cells. It was concluded that receptor-mediated endocytosis was the chief mechanism for the enhanced uptake of targeted PLGA NPs. Further, Shieh et al. designed a complex form of DOXO-loaded nanoparticles in which they co-encapsulated DOXO and a photosensitizer in 4-armedporphyrin-polylactide nanoparticles and coated the surface of these nanoparticles with d-α-tocopheryl polyethyleneglycol 1000 succinate (TPGS) which is a potential P-gp inhibitor [82]. The findings indicated that such a combination of agents exhibited a marked synergistic effect that resulted in enhanced transport of DOXO to the nucleus in resistant MCF-7/ADR cells.

10.4.2.2 Dendrimers

Dendrimers represent ideal candidates that link molecular chemistry to polymer science [83]. They possess distinct structures characterized by a central core, an inner dendritic structure of highly branched polymers, and an outer surface of multivalent functional groups. The functional groups present on their surface can incorporate charged polar compounds by electrostatic interaction while their hydrophobic interior is able to efficiently lodge uncharged, nonpolar compounds. Due to the presence of both hydrophobic and hydrophilic sections in their structures, a variety of drug molecules can be successfully encapsulated in dendrimers based on their solubility criterion. Nevertheless, the exterior functional groups enable modulated drug release which is governed by a specific pH, specific enzymes, or by targeting moieties, such as the RGD peptide or mAbs. In the area of chemotherapeutics, many hydrophobic drugs such as Doxorubicin and Paclitaxel are frequently targeted as dendrimers [84]. Several clinical trials are undergoing to deliver Paclitaxel using amphiphilic diblock copolymer forming micelles for treating breast, non-small-cell lung cancer and advanced pancreatic cancer [85]. Lee et al. designed DOXO dendrimers through hydrazone linkage [86]. They demonstrated that DOXO-dendrimer exhibited a controlled drug-loading via multiple attachment sites, and modulated solubility profile through PEGylation along with characteristic drug release which is influenced by pH-sensitive hydrazone dendrimer linkages. The developed polyester dendrimer–PEO–doxorubicin conjugate significantly blocked the growth of DOXO-insensitive C-26 tumor. The cell culture studies indicated that DOXO-dendrimers were more than 10 times less toxic than free DOXO against C-26 colon carcinoma cells. Further, in vivo studies in tumor bearing mice, via intravenous delivery, showed ninefold higher uptake of DOXO-dendrimers by tumor cells in comparison to free DOXO with complete tumor regression.

10.4.2.3 Cyclodextrin Nanoparticles

Qiu and coworkers developed advanced cyclodextrin-based formulation of DOXO (sPEL/CD) for which they reacted methoxy polyethylene glycol and poly lactic acid to obtain linear mPEG-PLA which entrenched like the arms of core β-cyclodextrin [87]. This delivery system was found to exhibit higher drug loading and entrapment efficiency of 18 % and 84 %, respectively, attributable to the presence of poly lactic acid which increased the hydrophobic interaction between the polymer and DOXO along with allowing adequate lodging of DOXO by enlarging β-cyclodextrin inter spaces. Their findings revealed that there was a threefold decrease in IC50 value by DOXO-loaded sPEL/CD as compared to free DOXO in resistant MCF-7/ADR cells. They concluded that the mPEG-PLA block segment of sPEL/CD complex was having identical activities as pluronic in averting MDR in cancer cells owing to their contrasting structural resemblance [88]. Moreover, it was presumed that alteration of the normal functioning of P-gp by polymers might also be a probable mechanism of the reversal of MDR by sPEL/CD.

10.4.2.4 Gold Nanoparticles

Gold nanoparticles (Au-NPs) are being extensively implicated for biomedical imaging and biosensing. Their biocompatibility, high stability, and tissue permeability made them a promising carrier system for efficacious delivery of anticancer drugs. The anticancer agents can be formulated ad Au-NPs by physical adsorption, ionic bonding, and/or covalent bonding [89, 90]. Au-NPs can be also utilized for delivery of small molecules such as proteins, DNA, or RNA. The gold core of these NPs does not exhibit any toxicity. The photo-physical properties of Au-NPs are considered to influence the release of loaded drugs from the conjugate. In order to improve the stability and prolong the circulation time, PEG and/or other targeting moieties can be attached onto the surface of these metallic nanoparticles similar to polymeric nanoparticles [91]. More recently, TNFα-integrated colloidal gold is used for treating advanced solid tumors; for example, sarcomas and melanomas are under phase I clinical trials [92]. Gu et al. prepared Au-NPs of DOXO by integration of DOXO in PEGylated Au-NPs via a disulfide bond (Au-PEG-SS-DOXO), which resulted in increased intracellular drug uptake in contrast to free DOXO in resistant HepG2-R cells, as shown by confocal imaging and plasma mass spectrometry (ICP-MS) [93]. Of interest, it was observed that DOXO uptake was confined to the cytoplasm only which reflected that the cytotoxic activity of Au-PEG-SS-DOXO was not due to its interaction with nuclear DNA. The investigators hypothesized that NP caused an MDR reversal via alteration of cell membrane properties and by interrupting with the normal functioning of mitochondria resulting in cell apoptosis. Wang et al. designed DOXO Au-NPs using a hydrazone linker (DOXO-Au-Hyd-NPs). The study demonstrated uptake of nanoparticles via active caveolae- and clathrin-mediated endocytosis and subsequent release of DOXO from the nanoparticles to the cytoplasm and the nucleus [94]. The DOXO-Au-Hyd-NPs resulted in a markedly increased DOXO intracellular uptake and minimum efflux, in addition to a significantly enhanced cytotoxicity in comparison to free DOXO in resistant MCF-7/ADR cells.

10.4.2.5 Magnetic Nanoparticle

The magnetic nanoparticles are formulated by either encapsulating drug into magnetic micro/nanosphere or implanting as a magnetically active disc. In magnetic targeting, a strong magnetic field is applied in the tumor area and controls the release of drug in the blood circulation. Various magnetic materials with a wide range of magnetic properties are available such as magnetite, iron, nickel, cobalt, neodymium-iron-boron, and samarium-cobalt. Moreover, there are liquids which get intensely magnetized in the presence of a magnetic field known as ferrofluids. Ferrofluids are basically colloidal suspensions of nano-dimension ferromagnetic particles. At present, the most frequently applied magnetic NPs are of iron oxide owing to its biodegradability, biocompatibility, superparamagnetic effects, and ability to serve as a contrast agent in MRI. These nanoparticles, after getting internalized within the lysosomes of RES cells dissociate to ferritin and/or hemosiderin [95].

Currently, superparamagnetic iron oxide (Fe3O4) nanoparticles which involve local hyperthermia or oscillation strategies to deliver conjugated drugs are being profoundly investigated. Nevertheless magnetic fields can also be utilized for targeted delivery of drugs inside the body. However, due to the acute in vivo toxicity, their therapeutic potential is not explored to the fullest [96]. Though, they are comprehensively investigated in fields of imaging and theranostics. Chen et al. studied the role of Fe3O4 magnetic nanoparticles in Daunorubicin-mediated prevention of MDR in vitro, in sensitive and resistant K562 cells [97]. The coating of tetraheptylammonium (THA) on the NPs was purposely done to improve the interaction between NPs and lipid portion of cell membrane. The study findings revealed the significant interaction of THA-coated Fe3O4 NPs with the cell membrane and a substantial increment of the uptake of Daunorubicin in resistant K562 cells. The control formulation composed of comparable size of THA capped Ni magnetic nanoparticles did not have much influence on Daunorubicin cellular uptake in sensitive and resistant cells, which suggests the exclusivity of THA-capped Fe3O4 nanoparticles in accelerating the Daunorubicin uptake.

10.4.2.6 Silica Nanoparticles

The use of inorganic carriers in nanoformulation for delivery of anticancer agents is expanding nowadays. One of the most important is silica which is well utilized as a nonmetallic inorganic carrier in chemotherapeutics. Huang et al. designed DOXO silica nanoparticles (MNSP) by covalently linking DOXO in mesoporous silica by hydrazone bonding (DOXO-Hyd-MNSP) [98]. The prepared DOXO-Hyd-MNSP revealed considerable apoptosis in vitro and in vivo in resistant MES-SA/Dx-5 cells in contrast to controls. They proposed that circumventing of the P-gp efflux was the reason behind the enhanced intracellular uptake of MNSP through endocytosis. In another study, Meng et al. formulated MNSP by co-incorporating DOXO and MDR1 siRNA [99]. They activated MNSP surface with a phosphonate group which facilitated DOXO binding within the MNSP by electrostatic action and the coating of cationic polyethylenimine (PEI) on this functional group aided complexation with anionic MDR1 siRNA. The co-delivery of DOXO and MDR1 siRNA by means of MNSP notably improved intracellular and intranuclear DOXO uptakes than free DOXO or DOXO MNSP without siRNA in resistant KB-V1 cells. They hypothesized that the release of DOXO from lysosomes was through a proton sponge mechanism as evident by inhibition of DOXO release and access to the nucleus by addition of NH_4Cl. Further, Shen et al. also formulated DOXO MNSP which exhibited remarkably eight times greater potency and significantly increased intracellular and intranuclear concentrations of DOXO in comparison to free DOXO in vitro in resistant MCF-7/ADR cells [100]. Authors claimed that the findings established for the first time that MNSP inhibited P-gp expression by itself owing to its capability of downregulating P-gp levels. It was proposed that intracellular uptake of MNSP was through micropinocytosis and after getting entrapped inside the cell, MNSP was able to circumvent P-gp due to its extremely large size.

In a more comprehensive study, Chen et al. co-engineered DOXO and BCL-2 siRNA as MNSP by entrapment of DOXO inside MNSP pores and complexation of BCL-2 siRNA with modified polyamidoamine dendrimers of MNSP [101]. The study findings revealed an excellent 132-fold increase in cytotoxicity by MNSPs in contrast to free DOXO in resistant A2780/AD human ovarian cancer cells. It was suggested that suppression of BCL-2 mRNA and perinuclear localization of DOXO by means of MNSP possibly resulted in such noticeably improved antitumor efficacy.

10.4.2.7 Carbon-Based Nanoparticles (Carbon Nanotube)

Carbon nanotubes are allotropes of carbon and members fullerenes family [102]. Fundamentally, they are thin sheets of benzene ring carbons evenly rolled to form a smooth, seamless rod-like tubular structure. They exhibit a characteristic feature of "needle-like penetration" while entering into the cells and subsequently delivering molecules into the cytoplasm. They possess very large surface area offering abundant attachment sites targeting ligands along with an interior void for incorporating therapeutic or diagnostic agents. Interestingly, these carbon nanotubes have electrical and thermal conductivity, which may be advantageous in chemotherapeutics for instance thermal ablations. The critical parameters in designing carbon nanotubes are their length and diameter which also influence inflammogenic effect. Recent strategies in context of nanotubes comprise the integration of important anticancer drugs such as Doxorubicin and Paclitaxel, nucleic acids including antisense oligonucleotides and SiRNAs [103]. Li et al. have designed DOXO carbon nanotubes by linking a P-gp antibody onto functionalized carbon nanotubes by a diimide-activated amidation reaction followed by loading DOXO through physical adsorption [104]. This physical adsorption of DOXO in nanotubes kept the molecular integrity intact by preventing chemical bonding. Moreover, modified DOXO release from nanotubes was observed while exposure of DOXO nanotubes was under near-infrared radiation. They suggested that the controlled and sustained release of DOXO via near-infrared radiation and specific P-gp targeting were major contributors in addressing MDR in resistant human leukemia K562R cells. Further, it was concluded that coupling of the P-gp antibody on nanotubes offers enormous stereo hindrance for P-gp recognition of DOXO which resulted in inhibition of its P-gp-mediated efflux.

10.4.2.8 Solid Lipid Nanoparticles (SLNs)

Currently, SLNs have gained huge notice for delivery of drugs, particularly poorly water soluble drug candidates. They offer improved properties by combining the benefits of liposomes, NPs, and fatty emulsions. They are usually prepared by high-pressure homogenization or microemulsification techniques whereby the drug is efficiently entrapped in a lipid matrix [105]. SLNs overcome the general limitations of polymeric systems by exhibiting low toxicity due to the presence of

biodegradable lipid, and their extremely small size which facilitate circumventing of the RES. Kang et al. formulated DOXO-loaded SLNs using glycerylcaprate (Capmul MCM C10) as the lipid phase, polyethylene glycol 660 hydroxystearate (Solutol HS15) as the surfactant, and curdlan as the shell forming material [105, 106]. The developed DOXO SLNs showed 17.1- and 21.6-fold increased cellular uptake at 1 h and 2 h, respectively. In addition, the was a higher apoptotic cell death as assayed by crystal violet staining, in comparison to free DOXO in resistant MCF-7/ADR cells. Moreover, the SLNs did not produce hemolysis in human erythrocytes which confirmed their safety. They proposed that DOXO SLNs can potentially overcome MDR. In another study, Shuhendler et al. prepared a polymer-lipid hybrid SLN by co-incorporating DOXO and mitomycin C using myristic acid, HPESO, pluronic F68, PEG100SA, and PEG40SA [107]. The developed SLNs showed 20- to 30-fold increased toxicity in resistant MB435/LCC6/MDR1 cells than free DOXO.

10.4.2.9 Polymeric Nanoconjugates

The hydrophilic polymers conjugated to proteins and anticancer drugs are one of the most extensively explored approaches for drug delivery which establishes polymer therapeutics as one of the first classes of anticancer nanomedicine. The prospects of using more sophisticated polymer-based vectors in chemotherapeutics are expanding day by day [108]. The protein drugs conjugation with synthetic polymers, predominantly PEG, by covalent linkage enhance their residence in plasma, decrease protein immunogenicity, and widen their therapeutic index. Nowadays, many PEGylated enzymes such as L-asparaginase and cytokines that include interferon-α and granulocyte colony-stimulating factor are being utilized very frequently. Nevertheless, polymer conjugation plays a crucial role in anticancer drug delivery in terms of alteration of the biodistribution of low-molecular-weight drugs, facilitation of tumor-specific targeting with minimization of toxicity by avoiding unspecific passive delivery. Polymeric conjugates can be delivered either via passive targeting by the enhanced permeability for lysosomotropic delivery following the EPR effect or actively by binding cell-specific ligand for receptor-mediated targeting. One such example of polymeric conjugates in chemotherapeutics includes polyglutamic acid–paclitaxel which is undergoing phase III trials for non-small-cell lung cancer in females. At present, novel approaches in the field of polymeric conjugates make it promising for drug delivery systems against new molecular targets (e.g., anti-angiogenics), and the co-delivery of polymer conjugates with low-molecular-weight anticancer drugs, tailor-made prodrugs, or radiotherapy all of which have potential prospects for success. Furthermore, the polymeric conjugates expand therapeutic options for delivery of combination of drugs from a single carrier; for instance, the combination of endocrine therapy with chemotherapy in breast cancer demonstrated its great preclinical potential. The linear architecture of polymers has been used clinically so far. The new hyperbranched dendrimers and dendritic polymer architectures are also being introduced with the emerging principles

for the engineering of polymer therapeutics. However, establishing the safety of new polymers, mainly in terms of general toxicity, immunogenicity, and metabolic fate before its clinical evaluation, is very crucial. In a study, Sirova M et al. investigated the in vivo efficacy and safety of HPMA-based copolymers of DOXO through a spacer containing pH-sensitive linkage in murine tumor models bearing T cell lymphoma EL4 or B cell lymphoma 38C13 [109]. Their findings revealed that conjugates with 10–13 % weight of bound DOXO produced remarkable antitumor effects. However, free DOXO (4.6 % relative to total drug content) did not have any influence on the efficacy of tumors and had acute toxicity. The results confirmed complete cure of mice and development of specific antitumor resistance on regular treatment-dependent basis via conjugates. Further, there were no reports of myelosuppression or organ damage.

10.4.3 pH-Sensitive Nanocarriers

Drug delivery via pH-sensitive nanocarriers represents an active targeting approach leading to higher accumulation of these vehicles in non-diseased cells attributable to basal expression of antigens, carbohydrates, and receptors. As the name suggests, these nanocarriers exhibit stimuli sensitive intracellular release of drug. In stimulus-responsive drug delivery, drug release is governed by stimuli such as pH. These nanocarriers are likely to have benefitting outcomes in MDR reversal as well. An extensive work has been done in exploiting the mechanism to overcome efflux-dependent MDR via acidic pH activation. The probable mechanisms as reported in this context include disruption of the endosomal membrane and burst release of nanocarrier-loaded drugs into the cytoplasm [110]. Thus, a pH difference between the extracellular (not < 5.7) and lysosomal milieus (not > 5.4) can be explored to design a specific pH-responsive drug release nanocarrier system targeting lysosomal instead of endosomal compartments, and thus avoiding undesirable drug release in the tumor stroma [111, 112]. Therefore, it is extremely important to design lysosomal pH-sensitive pKb of polymers sensitive to accomplish the precise pH-sensitive drug release. The stability of these nanocarriers in slightly acidic environment such as the extracellular tumor stroma is also important. In order to trigger drug release, these nanocarriers should be sufficiently sensitive to structural transformations and solubility changes in the more acidic lysosomal compartments. Nevertheless, the pH-responsive polymers cause disruption of endosomal membranes most probably by proton absorption and by interacting with these membranes [113, 114]. The proton absorption leads to osmotic swelling and rupturing of the membranes. The interaction of nanocarriers with endosomal membranes generates defects in them by creating pores or channels in lipid membranes. For instance, in the acidic milieu of endosomes/lysosomes, Doxorubicin (DOXO) immediately got protonated and turned hydrophilic and positively charged. This water soluble charged DOXO was unable to get across the endosomal/lysosomal membrane, and thus became entrapped there. The similar phenomenon accounted for resistant

cancer cells which sequester cytosolic DOXO and formed the basis of the proposed protonation, sequestration, and secretion (PSS) model. The PSS model possibly explains the sensitivity of some tumor cells and resistance of MDR cells toward weakly basic chemotherapeutic drugs. Therefore, DOXO-incorporated nanoparticles must escape the endosome/lysosome membranes by causing their intact disruption.

Lee ES et al. engineered pH-sensitive polymeric micelles of Doxorubicin targeting resistant solid tumors [113]. The surface of these DOXO-loaded pH-sensitive micelles was coated with folate (PHSM/f). PHSM/f were designed using a mixture of two block copolymers of poly (L-histidine)-*b*-PEG-folate (75 wt.%) and poly(L-lactic acid)-*b*-PEG-folate (25 wt.%). The results demonstrated greater than 90 % cytotoxicity in DOXO resistant MCF-7 (MCF-7/DOXO^R) via PHSM/f. They presumed that a series of events lead to enhanced cytotoxicity including active internalization of PHSM/f through folate-receptor-mediated endocytosis, ionization of His residues resulting in micelle destabilization, and disruption of endosomal membranes. These were the possible reasons behind circumventing P-gp efflux and sequestration of DOXO in acidic intracellular compartments which resulted in increased cytotoxicity. Further, tumor regression evaluation in mice bearing s.c. MCF-7 or MCF-7/DOXO^R xenografts demonstrated substantial reduction in tumor volumes of mice administered with PHSM/f in comparison to free DOXO or identical micelles formulation without folate (PHSM). In addition, the accumulation of PHSM/f DOXO was 20 times greater in solid tumors as compared to free DOXO. The study concluded that PHSM/f possesses potential as a carrier system for treating multidrug resistant tumors. Similarly, He Q et al. formulated pH-responsive nano-multidrug delivery systems via in situ co-self-assembly of DOXO, surfactant micelles-CTAB (chemosensitizer), and silica. They designated these drugs-surfactant micelles-co-loaded mesoporous silica nanoparticles as DOXO-CTAB-MSNs [115]. The developed nano-multidrug delivery systems DOXO-CTAB-MSNs showed extremely precise pH-responsive drug release both in vitro and in vivo and significant anticancer and overcoming MDR. The mechanism of overcoming MDR was demonstrated to be a synergistic cell cycle arrest/apoptosis-inducing effect as a consequence of the chemosensitization property of the surfactant CTAB. The results showed nano-multidrug delivery systems to be highly effective in overcoming MDR in cancer via a pH-responsive controlled drug release.

In another study, Kim D et al. have evaluated the DOXO-loaded second generation of pH-sensitive micelles composed of poly(L-histidine-*co*-L-phenylalanine (16 mol%))(MW: 5 K)-*b*-PEG(MW: 2 K) and poly(L-lactic acid)(MW: 3 K)-*b*-*PEG*(MW: 2 K)-folate (80/20 wt/wt%) for early endosomal pH targeting (pH 6.0) using in vivo MDR ovarian tumor-xenografted mouse models [116]. Their findings demonstrated prolonged circulation of the drug carrier, higher tumor-selective accumulation, as well as an increased intracellular drug delivery. Moreover, the prepared micelle formulation successfully suppressed the growth of existing MDR tumors in mice. They concluded that micelle formulation was better than its first generation formulation targeting pH 6.8 and the folate receptor.

10.5 Future Outlook and Conclusive Remarks

Nanotechnology-based chemotherapeutic targeting approaches are gaining much interest in cancer drug therapy more particularly in multidrug resistant cases. Poorly vascularized tumor cells/tissues is one of the main noncellular factor for drug resistance that easily can be overcome by passive targeting by cancer nanomedicines of sizes less than 200 nm and have the characteristics of long circulation. Doxil®, a doxorubicin-loaded long circulating liposome used in clinical practice, is the proof of concept for this principle. Furthermore, the high concentration of chemotherapeutics can be attained by the active mean of targeting or the combination of active and passive targeting simultaneously by functionalizing the nanocarrier of a size less than 200 nm for the overexpressed targets (e.g., P-gp efflux transporter, drug-resistance proteins, HER2, Vasoactive intestinal peptide in breast cancer, etc.) in the cancerous cells. We have summarized diverse nanotechnology-based formulations in different stages of clinical development for cancer therapy, theranostics, and diagnostics in our recently published review [1]. Ongoing research and clinical status clearly indicate that applying nanotechnology for therapeutic delivery in cancer is very beneficial and has excellent future potential while dealing with MDR.

Recently, researchers are trying to explore multi-targeting nanosystems that may have four important components in one: (1) a selective targeting ligand, (2) a chemosensitizer/efflux pump inhibitor/siRNA oligonucleotide that can overcome drug resistance including the suppression of resistance gene, (3) a small molecule or biological anticancer drug or self-therapeutic, and (4) an imaging probe that can facilitate tumor diagnostics along with chemotherapy. Such a complex nanosystem (we can say; tetratheranostic nanomedicines) could be an excellent nanoapproach for MDR-resistant cancer. However, the development, stability, reproducibility, and in vivo performance of such a complex system remain a concern. Our view is that for the effective and unidirectional irreversible killing of MDR cancer cells, nanotechnology should be more focused (along with the above established approaches) as follows:

1. Targeting the communication between cancer cells with their microenvironment and their supporting stroma and/or vasculature.
2. In case of B-cells-based malignances which are in most of the cases unaffected by the therapy, targeting of the pathways between the malignant B-cells and their stroma is a good approach for therapy.
3. Another important therapeutic interest is to target the cancer stem cells that have the property of self-renewal and initiation of tumor. These stem cells are normally resistant to chemotherapy and radiotherapy.

Overall, nanotechnology is in a distinguishing status to transform cancer chemotherapy and diagnosis to produce a new generation of cancer therapeutics/theranostics (categorized as nanomedicines) with high sensitivity and precision for cancerous cells, and thus leading to overcome the MDR and reduce the conventional therapeutics-associated predictable toxicity.

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Chapter 11
Drugs Affecting Epigenetic Modifications of ABC Transporters for Drug Resistance

Kazuhiro Satake, Yu Toyoda, and Hiroshi Nakagawa

Abstract Drug resistance in the cancer cells is a leading cause of mortality during the cancer treatment. ABC transporters expressed in the cancer cells have been found to extrude anticancer drugs from the inside of the cells to their outside for the protection of the cells from the drugs, resulting in the drug resistance. However, the mechanisms of how cancer cells acquire ABC transporter-mediated drug resistance have not yet been fully understood. Therefore, numerous strategies have been tested or proposed to control the functions and the expressions of ABC transporter genes so far. The first strategy utilized to inhibit ABC transporter function relied on the identification of nonchemotherapeutic agents as competitors. Other approaches have included the use of hammerhead ribozymes against the ABC transporter genes and the gene-targeted antisense oligonucleotides. On the other hand, recently, epigenetic modifications of the genes have been emerging as a part cause of ABC transporter-mediated drug resistance. In this chapter, therefore, epigenetic modifications of the ABC transporter genes are covered, where the outlines of the regulatory mechanisms and factors involved in the epigenetic modifications are described, focusing on the best known mechanisms in human such as DNA methylation/demethylation and posttranslational modifications of histone proteins. Furthermore, drugs affecting epigenetic modifications are also introduced and described from a viewpoint of ABC transporter-mediated drug resistance in this chapter. We hope that this chapter can provide new insights into the understanding of cancer cells to acquire drug resistance, which could lead us to conquering the ABC transporter-mediated drug resistance.

Keywords Epigenetics • Epigenetic modification • DNA methylation • Histone acetylation • Epidrug • Drug resistance • ABC transporter

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Abbreviations

ABC	Transporter
CpG	Cytosine guanine
DNMT	DNA methyltransferase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
MBD	Methyl-CpG binding domain
TET	Ten-eleven translocation

11.1 Epigenetic Modifications of the Genome in Gene Expression

All the cells within an organism, including diversely differentiated somatic cells, differentiating cells, and undifferentiated cells such as stem cells, have basically identical genetic information in their genome. However, these cells neither necessarily play the same role in the organisms nor exhibit the same pattern of gene expression. Even monozygotic twins, clones, or the cells from the same tissues or organs that share a common genotype are neither identical nor always show the same gene expression pattern [1].

During the last 10–20 years, a new academic field "Epigenetics" has been developed, where the heritable and age-related modifications of the genome, called "epigenetic modifications," that occur without a change of the primary DNA sequence have been found to determine the gene expression patterns in the cells by changing the state of chromatin, the packaging of DNA with histone proteins (Fig. 11.1). The epigenetic modifications which register, signal, or perpetuate altered activity states of genome [2] can allow the cells having identical genetic information to differently express genes and play different physiological roles. Thus, epigenetic modifications have important roles as major contributors to the regulation of gene transcription [3] and their regulation is crucial for maintenance of cell identity, proliferation, development, and differentiation [4–6]. Since strict regulation of gene expression patterns is crucial for the normal cell function, in particular during development and differentiation, inappropriate controls of epigenetic modifications are involved in many human diseases including neurological disorders [7] and cancer [3, 8–18].

In the cells, chromosomal DNAs are stored within their nucleus by forming a structure called "chromatin" together with histone proteins, where nucleosomes, the basic repeating units of chromatin, are formed by a histone octamer that is composed of two copies of the four core histones (H2A, H2B, H3, and H4) around which 145–147 bp of DNA are wrapped [19–21]. Based on condensation level of DNA, the chromatins are classified into euchromatin and heterochromatin, where

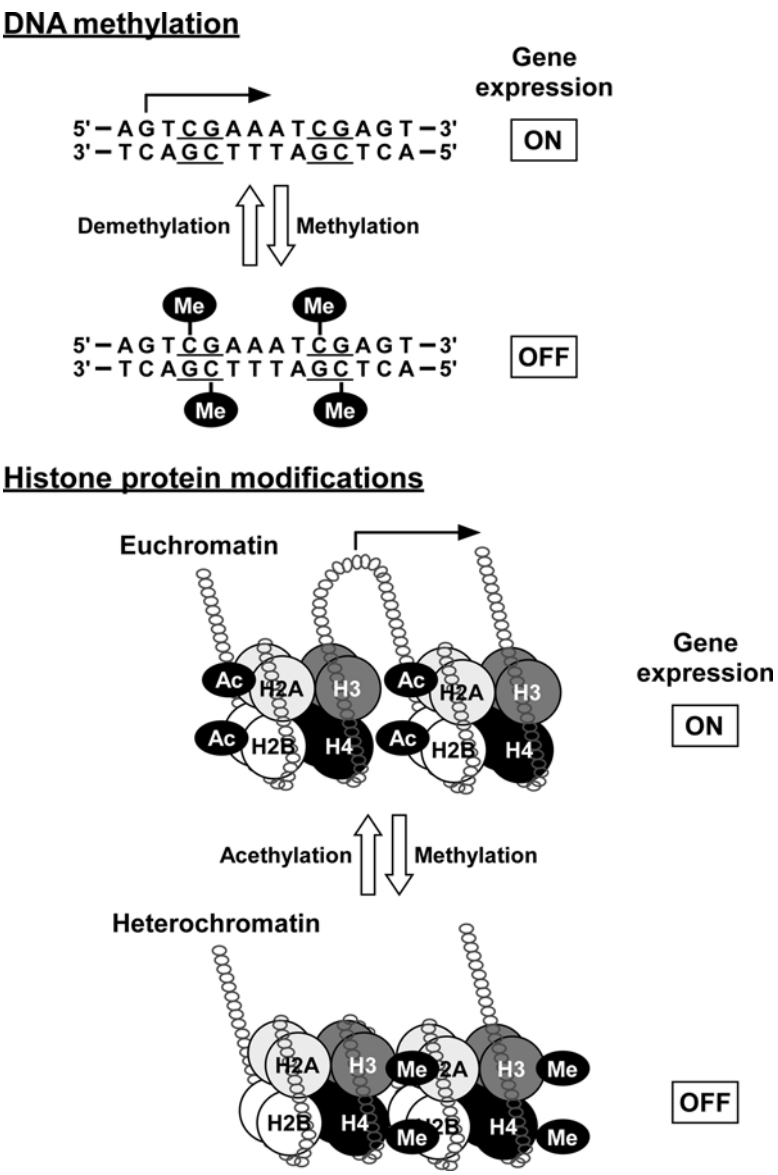


Fig. 11.1 Epigenetic gene regulations

DNA is more loosely and more tightly packaged, respectively. Since these states of chromatins allow or prohibit transcription factors and RNA polymerase II to access their recognition sequences and transcription start sites, respectively, DNA in euchromatin and heterochromatin are actively and hardly transcribed, respectively. Furthermore, the chromatin states can also allow or prohibit regulatory proteins to bind their recognition sequences or proteins.

The condensation level of DNA in chromatin is widely acknowledged to be determined by both DNA methylation and histone modifications, one of the most characterized epigenetic modifications. Since these epigenetic modifications act in concert to regulate gene expression by controlling chromatin state, these modifications have been suggested to play major roles in the maintenance of cell identity, proliferation, development, and differentiation by maintaining differential patterns of gene expression [3, 22].

11.2 DNA Methylation and Its Regulation

The chromosomal DNA methylation is an important epigenetic mark and one of the most characterized epigenetic modifications controlling gene expression and cell differentiation [23], where the transfer of a methyl group to the 5 position of a cytosine is the key feature. The resulting 5-methylcytosine (5mC) typically occurs in a cytosine guanine (CpG) dinucleotide (Fig. 11.1), where 70–80 % of cytosine is methylated [24–27]. On the other hand, regions of high CpG dinucleotide density, called "CpG islands" that locate in 60–70 % of promoter region of genes, are usually free of methylation in normal cells [23, 24, 28, 29] and the hypermethylation of CpG islands located in the promoter regions of certain genes, such as most imprinted genes (with exclusive expression of the paternal or maternal allele) and the X chromosome in normal (XX) females, has been shown to result in inactivation of the genes [30, 31]. Similarly, it has also been demonstrated that methylation of CpG islands in the promoter region of genes leads to suppression of transcriptional activity in normal and malignant cells [11, 28, 32–35]. Thus, DNA methylation in CpG islands is a well-established mechanism mediating epigenetic silencing of gene expression and is a prerequisite in vertebrate development and tissue-specific gene expression [28, 36].

DNA methylation is catalyzed by enzymes known as DNA methyltransferases (DNMTs), where a methyl group from S-adenosyl-L-methionine is transferred to the cytosine. Five kinds of proteins—DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L—are major members of DNMT family [37–44]. The functions of DNMT in DNA methylation can be divided into maintenance and de novo methylations. DNMT1 is involved in maintenance methylation, which refers to the process of copying DNA methylation profiles to the daughter strands during DNA replication, whereas DNMTs 3a and 3b effect de novo DNA methylation [41]. As DNMT1 acts primarily on hemimethylated DNA, it is often associated with DNA replication machinery [40], whereas DNMTs 3a and 3b are primarily expressed during development [37]. DNMT3L has no catalytic activity but can assist DNMTs 3a and 3b by improving their ability of binding to DNA and stimulating their activity [39, 43, 44]. Instead of methylating DNA, DNMT2 was shown to methylate the anticodon loop of aspartic acid transfer RNA at cytosine-38 [42].

The main mechanism for the transcriptional repression that results in silencing of genes is believed to be the binding of methyl-CpG-binding domain (MBD) proteins

(MeCP2, MBD1, MBD2, MBD3, and MBD4) to the hypermethylated DNA to promote the recruitment of both histone-modifying enzymes and transcription repressors (e.g., SIN3A and EZH2) to the methylated genomic loci for the development of a repressive chromatin environment [45–53]. In mammals, these MBD proteins help DNMTs to recognize and bind to 5mCs in CpG islands [53] and recruit chromatin-remodeling enzymes, such as histone deacetylases and mSin3, to the DNA with their transcriptional repression domains, creating an inactive chromatin configuration [45–47].

In addition to 5mC, there exists another kind of methylated cytosine, 5-hydroxymethylcytosine (5hmC), a product of the oxidation of 5mC by the 10–11 translocation (TET)1, TET2, and TET3 proteins [54–56]. For example, the presence of 5hmC epigenetic marks in chromosomal DNA was demonstrated to positively correlate with gene expression in mouse embryonic stem cells [56]. In addition, it was also reported that the depletion of TET1 in mouse embryonic stem cells resulted in activation of 851 genes although that also resulted in repression of 556 genes [55]. Therefore, the conversion of 5mC to 5hmC is supposed to contribute to DNA demethylation that, in most cases, associates with gene activation.

11.3 Histone Protein Modifications and Its Regulation

Histones H2A, H2B, H3, and H4 are the basic packaging units for chromosomal DNA and they undergo posttranslational modifications at the amino-terminal tails of them which unstructurally protrude from the nucleosome core. Histone-modifying enzymes have been identified for acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, deamination, and proline isomerization [57–64]. It is now well established that histones are acetylated at lysines, biotinylated at lysines, methylated at lysines or arginines, phosphorylated at serines and threonines, SUMOylated at lysines, and ubiquitinated at lysines [65–70]. These histone modifications are considered to serve two main purposes. The first purpose is to provide or remove recruitment signals for nonhistone proteins involved in transcriptional activation and silencing. The second purpose is to change chromatin structure via alteration of charge that controls the physical interactions between histones and chromosomal DNA. Among these histone modifications, the ones which are involved in gene expression are called “histone code” [65]. Acetylation and methylation of histones are important and one of the most characterized histone codes controlling gene expression (Fig. 11.1) [67, 71].

Normally, histone acetylation leads to an increased negative charge of the histones and is thus believed to loosen the interaction between the histone and the negatively charged DNA, which is associated with euchromatin, a more open chromatin configuration that is permissive for transcription [72]. Consequently, highly acetylated histones are associated with transcriptionally active sequences, where ε-amines of lysines on the *N*-terminal tails of the core histones (H2A, H2B, H3, and H4) are acetylated [73]. On the other hand, deacetylation of histones in nucleosome is usually associated with heterochromatin and transcriptional repression. The acet-

ylation states of histones that control chromatin condensation and result in alterations of gene transcription as mentioned above are maintained by histone acetyltransferases (HATs) and histone deacetylases (HDACs) [74]. HATs are organized into families based on primary-structure homology, and the GNAT, the p300/CBP, the MYST, and the Rtt109 families have been studied extensively among the families [75–77]. These HATs catalyze the transfer of an acetyl group from acetyl-CoA to lysine residues in histones for activation of gene expressions, whereas HDACs remove it to inactivate the gene expressions [78–80].

In addition to histone acetylation, histone methylations are also occurred without changing the charge of itself, which can be normally occurred on all basic amino acid residues [81–86]. In fact, lysines can be monomethylated (me1) [81], dimethylated (me2), or trimethylated (me3) [82] on their ε-amine group. Arginines can be monomethylated (me1) [84], symmetrically dimethylated (me2s), or asymmetrically dimethylated (me2a) on their guanidinyl group [85]. Histidines can be monomethylated [83, 85]. Among these amino acid residues, in general, methylations of histone mainly occur on arginine and lysine residues [81, 82, 84, 87–89]. Since the first report of histone methyltransferase, several protein families have been found to be able to catalyze the addition of methyl groups to histones using S-adenosylmethionine as a methyl donor [81, 90, 91]. The SET-domain-containing proteins and DOT1-like proteins have been shown to methylate lysines [90, 92], and members of the protein arginine *N*-methyltransferase (PRMT) family have been shown to methylate arginines [93]. These histone methyltransferases have been shown to methylate histones that are incorporated into chromatin and also free histones and nonhistone proteins [90, 94]. Histone lysine methylation has predominantly been found within the tails of histone H3 and H4, which include histone H3 lysine 4 (H3K4), H3K9, H3K27, H3K36, H3K79, and H4K20, and histone H3 arginine 2 (H3R2), H3R8, H3R17, H3R26, and H4R3. Methylations of H3K4, H3K36, and H3K79 found in regions with transcriptional activity are often associated with transcriptionally active euchromatin. By contrast, H3K9me2/me3, H4K20me3, and H3K27me2/me3 are considered to be associated with transcriptionally silenced heterochromatin. In particular, monomethylation of H3K9 (H3K9me1) has been reportedly implicated in heterochromatic gene expression whereas H3K9me2 and H3K9me3 have been implicated in euchromatic gene silencing [95], where a direct causal role for H3K9 di- and tri-methylations in euchromatic silencing has been demonstrated [96–101]. In addition, several studies have identified that H3K9 methylation levels were dynamically regulated at some genes in response to activation stimuli [102, 103]. Furthermore, it has been reported that there is a very strong correlation between the degree of H3K4me3 at the 5′ ends of genes and their transcription rate, RNA polymerase II occupancy, and histone acetylation [68, 104, 105]. Thus, there is increasing evidence of cross talk between histone modifications in the regulation of gene transcription.

11.4 Interrelation Between DNA Methylation and Histone Modification

A complex interplay of different combinations of epigenetic modifications (including acetylation, methylation, ubiquitination, and ADP-ribosylation) has been established to play significant roles in determining the transcriptional status of a gene. It has recently become apparent that DNA methylation and histone modification events are tightly interrelated and can be dependent on one another, and that this cross talk can be mediated by biochemical interactions between SET domain of histone methyltransferases and DNMTs [45, 50, 106–109]. In fact, it was indicated that histone modifications such as deacetylation promoted DNA condensation and contributed to DNA methylation [110], and that the establishment of the basic DNA methylation profile during early development might be mediated through histone modification [43]. It has also been reported that histone-modifying enzymes can also recruit DNMTs to the genomic loci bearing an epigenetic modification. In turn, MBDs, which bind to methylated DNA sequences, have been demonstrated to recruit HDACs and histone methyltransferases to the hypermethylated loci, thus providing the opportunity for combined DNA/histone epigenetic marks to be established [45, 50, 106–109]. On the other hand, contact between DNMT3L and the nucleosome is reportedly inhibited by all forms of methylated H3K4 while DNMT3L recruits the methyltransferases to DNA by binding to histone H3 in the nucleosome [43]. Furthermore, there is also evidence that DNA methylation inhibits H3K4 methylation [111, 112].

Certain forms of histone methylation cause local formation of heterochromatin, which is readily reversible. In contrast, DNA methylation tends to be a more stable modification than histone methylation, but it can undergo changes during embryogenesis and aging. The turnover rates of histone lysine acetylation and methylation are reportedly estimated to be 2–40 min and 0.3–4 days, respectively [113, 114].

11.5 Epigenetics of Cancer/Tumor

It is widely recognized for many years that tumor initiation, development, and progression (tumorigenesis) are caused by genetic mutations, which result in aberrant patterns of gene expression, a key characteristic of many types of cancer. In recent years, on the other hand, it has become clear that tumorigenesis is also caused by epigenetic alteration and that a wide variety of epigenetic changes are prevalent in cancer [18]. In fact, cancers can display global DNA hypomethylation while exhibiting hypermethylation of genomic regions responsible for the expression of tumor-suppressor genes at the same time. Therefore, the epigenetic alterations are considered to be an early event during tumorigenesis and one of the hallmarks of cancer, leading to the activation of oncogenes and the loss of function of tumor-suppressor genes [18, 115]. It was also shown that global histone modification levels are predictive of

cancer recurrence [116]. On the other hand, exome sequencing studies of primary cancers have revealed that genes coding for chromatin-associated and epigenetic factors are frequently mutated in multiple types of cancer [117]. Thus, epigenetic alterations have been firmly demonstrated to play a role in cancer.

While DNA methylation is essential for mammalian development [12], distortion of the genome-wide DNA methylation profile is responsible for aberrant methylation patterns that are generally marked by global hypomethylation and promoter-associated hypermethylation [13]. The distortion of the genome-wide DNA methylation profile also causes silencing of tumor-suppressor genes, resulting in the loss of proliferation control in the cells and their genomic instability leading to tumorigenesis [3, 8–18]. In tumors, in recent years, it has been found that CpG islands are often hypermethylated, whereas other regions, such as centromeres and heterochromatin, generally exhibit hypomethylation during tumorigenesis [118]. The global hypomethylation but local hypermethylation in CpG islands and consequent gene silencing in cancer/tumor have been found to be mediated by the deregulation and inappropriate activity of DNMTs, particularly DNMT1 and DNMT3a/b, which are responsible for maintenance and de novo methylations, respectively [119]. As well as in vivo and clinical experiments, there have also been evidences of overexpression of these enzymes in various cultured cancer cells [120–123]. In animal experiments, furthermore, transgenic mice with 90 % decreased DNMTs reportedly develop widespread tumorigenesis and growth defects [124]. On the other hand, the mice injected TET-deficient stem cells also reportedly form significantly more aggressive teratomas than their wild-type counterparts [125], suggesting that 5hmC have an important role in cancer progression as functional component of the epigenetic machinery, distinct from the role of 5mC.

While many studies have shown that cancer cells are subject to abnormal de novo methylation compared with their normal counterparts, it has been suggested that this process may be linked to histone modification [117, 126, 127]. In fact, HDAC1 and 2 are reportedly overexpressed in a number of tumors, including breast, colon, esophageal, gastric, lung, and prostate cancers, and cervical, colorectal, and gastric cancers, respectively, where they correlate with reduced patient survival and likely contribute to tumorigenic gene silencing [128–136]. In addition, HDAC3 is reportedly overexpressed in colorectal, gastric, and prostate cancers [137], and expression of HDAC11 is reportedly increased in rhabdomyosarcoma [79, 138, 139]. It has also been found that genes encoding epigenetic regulators were mutated in the high-throughput sequencing projects, where mutations of HDAC2 and 4 have been identified in human epithelial cancer cell lines and breast cancer samples, respectively [117, 126, 127]. Furthermore, overexpression of HDACs and alterations in histone acetylation patterns have often been found in many tumors [79, 128–134, 137–143]. Furthermore, it has also been reported that diffuse large B-cell lymphomas (DLBCL) and peripheral T-cell lymphomas exhibited the overexpressions of HDAC1, 2, and 6 while Hodgkin's lymphomas displayed increased expression levels of HDAC1, 2, and 3 [142, 143]. Therefore, decreased activities of HDACs are associated with suppressed tumor cell development and growth as previously reported [144, 145]. On

the other hand, a considerable amount of researches have reported deregulations of histone lysine methylation in tumorigenesis [146–148].

In breast, gastric, kidney, lung, pancreatic, and prostate cancers, altered genome-wide acetylation levels of H3K9, H3K18, H4K12, and H4K16 have been found [116, 149–151]. Among them, in particular, H3K18 acetylation was shown to be a prognostic factor in several studies, where high acetylation level of it reportedly correlated with better prognosis [151]. In contrast, a study involving non-small-cell lung cancer patients found that higher levels of H3K9 acetylation correlated with shorter survival [152]. These contrasting findings suggest that acetylation on various lysine residues may have different functions in different types of cancer.

11.6 Epidrugs for Cancer Chemotherapy

DNMTs and histone-modifying enzymes can be drug targets for epigenetics therapy as inhibitors for DNMTs, HDACs, and histone methyl transferases have been the most extensively studied and a considerable number of small-molecule inhibitors of them have been identified so far (Fig. 11.2) [72, 74, 153–170]. These compounds command serious attention as candidates for epigenetic drugs, so-called "epidrugs." For example, several drugs which can inhibit the activity of DNA methyltransferase and cause genomic hypomethylation have been discovered so far [72]. The first of these inhibitors was the cytidine analog 5-azacytidine (Vidaza) and 5-aza-2′-deoxycytidine (Dacogen/Decitabine), which have been shown to effect demethylation in numerous cancer cell lines and already approved by the US Food and Drug Administration (FDA) for the treatment of acute myelogenous leukemia and myelodysplastic syndromes [153–157, 166]. In addition, several HDAC inhibitors have also been tested in clinical trials and approved for the clinical use [72, 74, 169]. For example, trichostatin A, suberoylanilide hydroxamic acid (Vorinostat), and FK228 (Nomifensine/Romidepsin) have been approved by the FDA for the treatment of relapsed and refractory cutaneous T-cell lymphoma (CTCL) [158, 159, 161–163, 165, 168]. Also, the well-known antiepileptic and mood-stabilizing drug valproic acid has been approved for the treatment of leukemia, breast cancer, and ovarian cancer since its HDAC inhibitory activity has been discovered [167], and MS-275 (Entinostat), isoform-selective inhibitor of HDAC I, has been recently approved for the treatment of breast cancer [160, 164, 170]. On the other hand, curcumin, a substance in turmeric, was discovered to inhibit histone H3 and histone H4 acetylation by p300/CBP [171]. Furthermore, several histone methyl transferase inhibitors have been discovered, where H3K9 methyl transferase G9a and H3K79 methyltransferase DOT1L have been shown to be inhibited by BIX-01294 and EPZ004777, respectively [172, 173]. These findings suggest that the development of epidrugs to target specific epigenetic enzymes or epigenetics-related proteins is a promising and rapidly growing field of modern pharmacology.

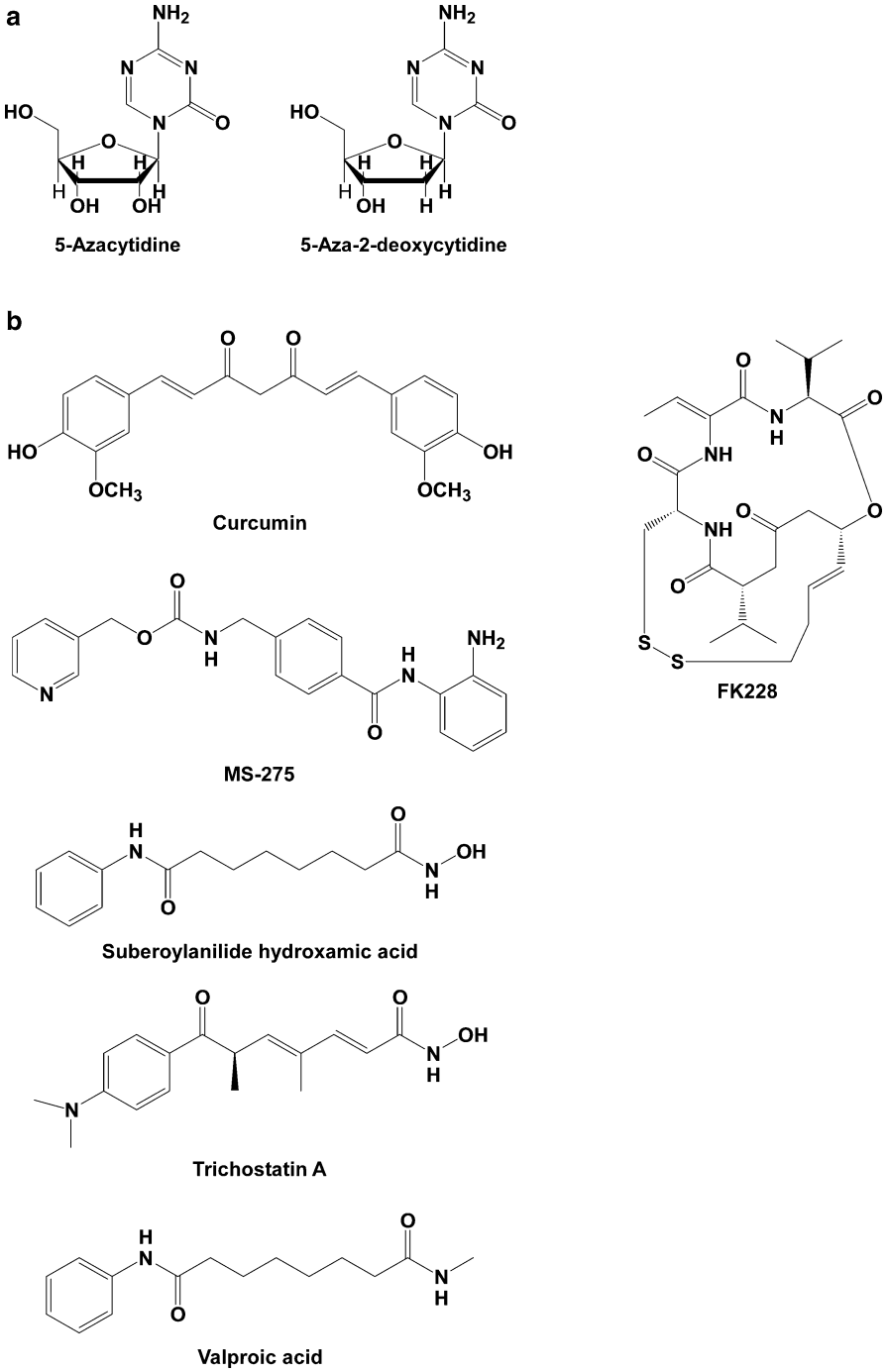
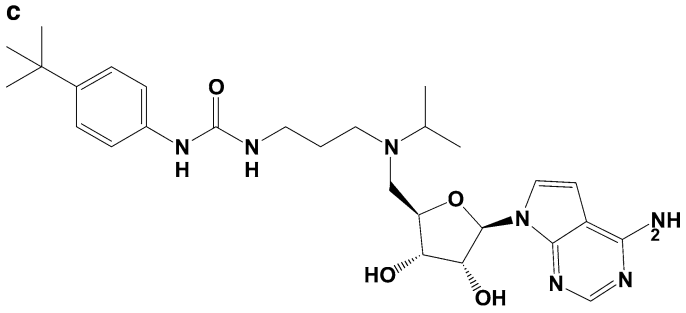
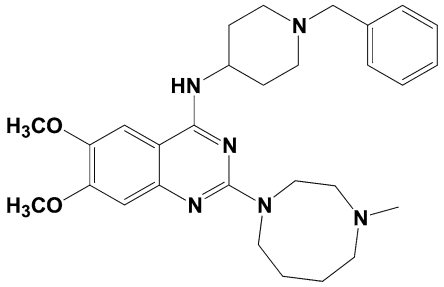


Fig. 11.2 Structures of epidrugs. (**a**) DNA methylation inhibitors. (**b**) Histone acetylation inhibitors. (**c**) Histone methylation inhibitors



EPZ004777



BIX-01294

Fig. 11.2 (continued)

11.7 Epigenetics and the Expression of ABC Transporter Genes

Epigenetic changes in tumors are associated not only with cancer development and progression, but also with resistance to chemotherapy. Aberrant DNA methylation at CpG islands and associated epigenetic gene silencing have been observed during the acquisition of drug resistance [174–176]. For example, Dai et al. have analyzed methylation changes associated with acquired cisplatin resistance in isogenic ovarian cancer cell lines and showed that hypermethylation of CpG islands was prevalent during the acquisition of drug resistance [175]. Similarly, it has been found that global acetylation levels of histones were observed in drug-resistant MCF7-KCR and OV1/VCR multidrug-resistant ovarian carcinoma cells [174, 176].

The MDR phenotype, the best known mechanism of acquired drug resistance and observed in various types of cancer, is mainly due to overexpression of the ABC transporters such as ABCB1 (MDR1), ABCC1 (MRP1), ABCC2 (MRP2), and ABCG2 (BCRP) that can extrude anticancer drugs from the cells [177]. Among these ABC transporters, the roles of ABCB1 and ABCG2 in MDR during chemotherapy have been extensively studied. ABCB1, a 1,280 amino-acid transmembrane protein encoded by the *MDR1* gene located on chromosome 7q21.1, is the first

transporter which was identified and characterized as a member of the ABC transporter family [178]. ABCG2, also called breast cancer resistance protein (BCRP), is a 655 amino-acid transmembrane protein encoded by the gene located on chromosome 4q22.1, and forms a homodimer [179]. Since these ABC transporters are endogenously expressed in several normal tissues such as capillary endothelial cells in brain, gastrointestinal tract, kidney, liver, ovary, and testis [180–184], intrinsic transporter-mediated multidrug resistance in cancer is not surprising, generally associated with tissues that endogenously express them. However, ABCB1 expression in the absence of drug treatment has also been observed in various cancers such as acute and chronic leukemia, astrocytoma, chronic myelogenous leukemia in blast crisis, non-Hodgkin's lymphoma, neuroblastoma, non-small-cell lung cancer with neuroendocrine properties, and sarcoma although normal cells of these cancer origins are not expressing ABCB1 [185].

A growing number of studies have demonstrated that the expression of ABC transporters was clearly affected by changes in DNA methylation patterns that are characteristic of cancers/tumors [186–192]. In drug-resistant and -sensitive cancer/tumor cells and samples from cancer patients, the density of *MDR1* promoter methylation has been found to be inversely correlated with the basal gene expression, and the methylation of *MDR1* promoter has been shown to transcriptionally silence basal ABCB1 expression in cell line models and clinical samples [186–190, 193–202]. Similarly, it has also been shown that expression of ABCG2 was regulated at least in part by promoter methylation both in cell lines and in plasma cells from patients [203]. In addition, the increased or acquired ABCB1 expression has been observed in a range of cell lines including leukemia (CCRF-CEM and HL60), epidermoid (KB3-1), and uterine sarcoma cells (MES-SA) during chemotherapy treatment due to the epigenetic modifications in its promoter [186, 188, 193, 204]. Furthermore, it was reported that ABCG2 was not expressed in human small-cell lung cancer PC-6 cells but was overexpressed in the SN-38-resistant subline, PC-6/SN2-5H, which was selected from PC-6 cells by continuous exposure to SN-38 [205]. Therefore, it is strongly suggested that chemotherapeutic drugs may actively induce DNA methylation at the *MDR1* and the *ABCG2* promoters to mediate gene expression [189, 190, 194, 200, 206–210]. For example, the *MDR1* promoter has been found to be progressively demethylated during the course of chemotherapy and was correlated with overexpression of ABCB1 [190, 206, 209]. Similarly, analysis of patient samples at diagnosis and relapse has showed that tumor cells predominantly contained a hypomethylated *MDR1* promoter after chemotherapy, which results in the activation of *MDR1* expression [189, 194, 200, 207]. Furthermore, the chemotherapeutic drug, doxorubicin, has shown to induce the expression of *MDR1* by downregulating DNMT1 activity [208, 210].

Epigenetic process has been shown to be one of the mechanisms controlling ABC transporter expression [176, 188–190, 194, 200, 203, 211–222]. For example, several groups have demonstrated that basal expression of ABCB1 was mechanistically controlled at the chromatin level and that epigenetic modifications of DNA and histone have been shown to play a pivotal role in *ABCB1* gene expression in several tumor cell systems [188–190, 211–213]. In addition, the *MDR1* and the

ABCG2 promoters have been shown to contain a potential CpG island, which may be regulated by methylation [193, 200, 203, 214, 220, 223, 224]. CpG dinucleotides within the *MDR1* promoter region in both HeLa and KB cells were reported to be hypermethylated, and concomitantly, the expression level of ABCB1 was low [225]. In addition, hypermethylation of the CpG dinucleotides within *ABCB1* and *ABCG2* promoter regions has been reported to be reversed by treatment with a DNA methylation inhibitor, which induces re-expression and overexpression of ABCB1 [188–190, 194, 200, 203, 214, 219, 220, 222]. For example, demethylation of *MDR1* promoter following treatment with 5-aza-2′-deoxycytidine, a specific inhibitor of DNA methyltransferase, reportedly activates the gene, where the methylation pattern of the promoter in the cells resembled that of the drug-resistant cells and the expression of MDR1 mRNA can be induced [188, 226]. In human multiple myeloma cell lines NCI-H929 that do not express ABCG2, similarly, a CpG island in the *ABCG2* promoter was reported to be heavily methylated and demethylation of the promoter using 5-aza-2′-deoxycytidine could induce the expression of ABCG2 mRNA and protein [203]. In addition, melatonin also reportedly increased the methylation levels of the *ABCG2* promoter and its effects on ABCG2 expression and function were reportedly prevented by preincubation with 5-aza-2′-deoxycytidine [222]. Nakano et al. reported that 5-aza-2′-deoxycytidine dose-dependently re-expressed ABCG2 in non-ABCG2-expressing PC-6 cells at the mRNA and protein levels [219]. It has also been reported that human sporadic clear cell renal carcinoma cell lines UOK121 and UOK143, having a methylated *ABCG2* promoter, expressed a lower level of ABCG2 and were more sensitive to ABCG2 substrate drugs than the unmethylated cell line UOK181 [220]. Consistent with the role of DNA methylation in ABCG2 silencing, incubation of methylated cell lines UOK121 and UOK143 with 5-aza-2′-deoxycytidine resulted in upregulation of ABCG2 expression in a concentration-dependent manner [220]. These suggest that the methylation status of CpG islands at the *ABCB1* and the *ABCG2* promoter regions may be crucial for transcriptional regulation of these genes. On the other hand, the methyl-CpG-binding-protein-2 (MeCP2), a strong transcriptional repressor, was identified to localize to hypermethylated *MDR1* chromatin in CEM-CCRF cells and to be associated with methylation-dependent gene silencing, where the DNA demethylation reportedly caused the release of MeCP2, leading to histone acetylation and activation of the *MDR1* gene in several human cancers and cell lines [190].

The *MDR1* gene is reportedly transcribed from two promoters [227]. The majority of the transcripts originate from the downstream promoter which is used by most cell lines and tissues expressing the *MDR1* gene [228], which is well characterized; several cis-regulatory elements and binding factors acting in positive or negative regulation of *MDR1* have been found [212, 213, 229]. In contrast, the upstream promoter, located 112 kb upstream of the former, is active in some but not all multidrug-resistant cells whereas it is not active in cell lines or healthy human tissues [204, 227, 230–233]. For example, methylation of the −110 GC box within the *MDR1* downstream promoter appeared to activate *ABCB1* gene expression in colchicine-resistant KB-8-5 cells [194], which are known to produce transcript from the upstream promoter [230]. In contrast, demethylation of the −110 GC box within

the *MDR1* downstream promoter induced by 5-aza-2′-deoxycytidine treatment has been shown to decrease the expression of ABCB1 in adriamycin-resistant K562/ADM and docetaxel-resistant MCF-7 cells [191, 234].

MDR1 gene expression is also regulated by the modulation of histone acetylase and deacetylase activities, and the role of histone acetylation and deacetylation in the transcriptional regulation of *MDR1* has been established [200, 211, 221, 225, 235]. For example, the study using histone deacetylase inhibitors showed that *MDR1* gene transcription could be stimulated by the modulation of histone acetylase and deacetylase activities [211]. In addition, it has been reported that histones associated with the *MDR1* proximal promoter were acetylated in response to multiple inducers [200, 211]. This acetylation is reportedly dependent on the recruitment of p300/CREB-binding protein (CBP)-associated factor (PCAF), a histone acetyltransferase, to the *MDR1* enhanceosome which can form on an inverted CCAAT box binding NF-Y and recruiting PCAF [211]. Co-transfection of HATs, p300, CBP, or PCAF has been reported to co-activate the downstream *MDR1* promoter-luciferase construct, where binding of HATs to the *MDR1* promoter has been determined [211, 221, 225, 235]. On the other hand, ABCG2 has been reported to be transcribed by a TATA boxless promoter with multiple Sp1 sites and a CCAAT box [224, 236]. Characterization of the *ABCG2* gene promoter revealed that it is a TATA-less promoter with several Sp1, AP1, and AP2 sites and a CCAAT box downstream from a putative CpG island. In addition, it was also identified that HDAC1 and a corepressor, mSin3A, bind to the promoter region containing the CpG island, thereby suppressing ABCG2 transcription [220].

Elevated levels of histone acetylation in the chromatin of the *MDR1* promoter in drug-resistant cells have also been described so far [200, 218, 221]. For example, Baker et al. mapped both temporal and spatial changes in histone H3 and H4 acetylation levels on the *MDR1* 5′ region in response to chemotherapeutic drug treatment such as daunorubicin and etoposide [200]. Similarly, increased histone H3 and H4 acetylation in the chromatin of the *MDR1* promoter was found in drug-resistant cell lines expressing elevated levels of ABCB1 [221]. In addition, increased acetylation of histone H3 at the *MDR1* upstream promoter was also reported in drug-resistant human sarcoma (MES-SA) cells, where the upstream promoter was activated [204]. When compared to a T-cell leukemia line, two cell lines expressing increased levels of MDR1 mRNA have showed 3- to 30-fold levels of acetylated H3 in the *MDR1* promoter, first exon and transcribed region [200, 218, 221]. Furthermore, it has been reported that the *MDR1* locus in CEM-bcl2 and SW620 cells was enriched with H3 acetylation and MeH3K4 [200], consistent with other models of transcriptionally active genes or a transcriptionally permissive state of chromatin [237–240].

On the other hand, HDAC inhibitor treatment has been reported to increase ABCB1 and ABCG2 expressions through dynamic changes in chromatin structure and transcription factor association within the promoter region [103, 176, 200, 211, 215–218, 221, 225, 241–243]. For example, Huo et al. showed that trichostatin A induced H3K4me3 levels of the *MDR1* gene through its coding region [243]. In acute myeloid leukemia cells, trichostatin A treatment reportedly increased the expression not only of ABCB1 but also of genes encoding other drug transporters,

ABCG2 and ABCC11, thereby inducing a very broad drug resistance phenotype [242]. In addition, the histone deacetylase inhibitor phenylbutyrate was also shown to induce the expressions of ABCB1 and ABCG2 in acute myeloid leukemia KG-1a cells [242]. On the other hand, Kim et al. reported that HDAC inhibitor induced the expression of ABCB1 in HeLa, SiHa, and DLD-1 cells but not in A172, U87, or KB cells, indicating that HDAC inhibitor-induced ABCB1 expression is cell type selective [225]. In drug-sensitive cells, several studies have reported an increase in ABCB1 expression by HDAC inhibitors [176, 200, 211, 215–218, 221]. For instance, ABCB1 has been reported to be overexpressed in H69 human small-cell lung carcinoma, SW620 colon carcinoma, and CEM-Bcl2 cells exposed to trichostatin A, or KU812 and NB4 cells exposed to depsipeptide [200, 211, 216, 217, 221]. On the other hand, several researchers have reported that trichostatin A induced ABCB1 expression in a drug-sensitive cell line but decreased it in the drug-resistant derivative lines [215, 218, 221]. Similarly, Toth et al. reported that trichostatin A did not induce *MDR1* expression in drug-resistant MCF7-KCR cells, but in drug-sensitive MCF7 cells [176]. These data suggest that trichostatin A can be a therapeutic candidate for multidrug-resistant cancers or those with altered histone acetylation. Toth et al. also revealed that acetylation of H3K9 is elevated by two orders of magnitude in the promoter and first exon of the *MDR1* gene in a drug-resistant cell line, whereas H3K4, H3K14, H4K8, and H4K12 acetylations increased only mildly or not at all, compared to the drug-sensitive parental cell line [176]. Furthermore, other reports have showed that histone deacetylase inhibitors could induce specific increases in H3K4 methylation in many types of cells [103, 241]. Therefore these findings suggest that the extent and temporal kinetics of H4 and H3 acetylation at the *MDR1* promoter would be different among the cell lines.

It has been shown that trichostatin A treatment did not always induce *MDR1* transcription although it resulted in a significant increase in the level of acetylated histones at the *MDR1* promoter [190, 221], demonstrating that DNA methylation was a dominant MDR1-silencing mechanism. Robust MDR1 expression in CEM-CCRF cells was only obtained when demethylation induced by 5-azacytidine treatment was accompanied with trichostatin A treatment [190]. In addition, it was also reported that *MDR1* activation was accompanied by increased methylation on H3K4, and the mixed lineage leukemia 1 (MLL1) protein, a histone methyltransferase specific for H3K4, was required for *MDR1* promoter methylation, where knockdown of MLL1 resulted in a decrease in *MDR1* expression [243].

11.8 Conclusions and Perspectives

Drug resistance in the cancer cells during chemotherapy is an obstacle and needs to be overcome. Epigenetic modifications such as DNA methylation/demethylation and posttranslational modifications of histone proteins have been reported to underlie the phenomenon of ABC transporter-mediated drug resistance. As the induced epigenetic alterations are observed more frequently than genetic events in acquired drug

resistance, epigenetic modifications must be of importance in the development of a novel type of anticancer drugs. Taking into account the strong involvement of epigenetic mechanism in the determination of the MDR phenotype, in addition, it is not surprising that this phenotype can be induced or reversed by epidrug. On the other hand, the precise molecular mechanisms for epigenetic regulation have not been established. Therefore much more information from biochemical, structural, and in vivo biological characterizations of the known small-molecule inhibitors is needed before the mechanisms behind and the role of epigenetics for drug resistance by ABC transporters can be understood. We hope that this chapter can help you take new insights into the understanding of cancer cells to acquire drug resistance.

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ERRATUM TO

Chapter 8
ABC Transporter Modulatory Drugs from Marine Sources: A New Approach to Overcome Drug Resistance in Cancer

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