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# Clinical Multidrug Resistance in Cancer: A Multifactorial Problem

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## INTRODUCTION

RESISTANCE TO cytotoxic chemotherapy is a common problem in patients with cancer and a major obstacle to effective treatment of disseminated neoplasms. Resistance can be intrinsic or acquired. Tumours with intrinsic or *de novo* resistance fail to respond to the first chemotherapy given. In acquired resistance, tumours initially respond to chemotherapy but eventually progress in spite of treatment. In both scenarios, tumours are often found to be refractory to a variety of drugs with different structure and function. A similar experimental phenomenon has been termed multidrug resistance or MDR [1–4]. Various molecular mechanisms have been associated with MDR in experimental tumour models. These include enhanced efflux of drugs by transporter proteins such as P-glycoprotein (Pgp) or the human multidrug resistance-associated protein MRP [5–16], alterations in drug targets such as DNA topoisomerase II (topo II) [17–23], increased detoxification of compounds, e.g. by the glutathione (GSH) system [24–29], and overexpression of the human major vault protein LRP [30, 31], which might play a role in vesicular sequestration of drugs. Clinical studies have shown that any of these mechanisms can be deteced in human tumours, and some mechanisms have been associated with poor treatment outcome in particular cancers [32–55].

The ultimate goal of MDR research is to improve treatment outcome in patients with cancer by devising strategies that are able to prevent the emergence of MDR or to circumvent existing resistance. To achieve this goal, it is paramount to understand the mechanisms which render a patient with cancer—and not just the tumour cells in a patient's cancer—resistant to chemotherapy. In this special issue of the *European Journal of Cancer*, various molecular mechanisms known to be associated with experimental MDR and their potential role in clinical chemotherapy resistance are reviewed. However, clinical resistance to chemotherapy is likely to be multifactorial in most patients with cancer. What these factors are, how they might influence each other and treatment outcome, and what we know and do not know about their clinical relevance, are some of the issues discussed in this article.

Table 1 shows the terms used in this article to refer to particular MDR mechanisms and phenotypes. MDR itself is applied exclusively to refer to a phenotype of simultaneous resistance to multiple agents which differ in structure (and not necessarily function), without implying any particular mechanism. If applicable, these are specified by a prefix, as in Pgp-MDR or topo II-MDR. Furthermore, the terms apoptosis-MDR and clinical MDR are introduced. Alterations in apoptosis pathways have been shown to result in resistance to a variety of cytotoxic agents. Thus, it seems appropriate to refer to apoptosis-related chemotherapy resistance as a type of MDR. The same seems to apply to the phenomenon of clinical resistance to multiple cytotoxic agents. At a time of widespread interest in the phenomenon of MDR in cancer, from basic scientists to clinical oncologists and haematologists, it seems important to devise a terminology which is clear, unambiguous, and easy-to-comprehend for everyone with interest in the field. The terms used in this article are an effort in that direction.

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## THE IMPORTANCE OF DRUG CONCENTRATION—FROM DOSE TO TARGET

When patients with cancer are treated with cytotoxic agents, the pharmacological goal is to deliver as much active drug as possible to the molecular target in the cancer cells in order to cause sufficient molecular damage to lead to cell death. Cytotoxic agents can encounter various obstacles on that road to activity (Figure 1). These can be grouped into three categories: (a) factors upstream of the molecular target, which reduce the availability of active drug at target; (b) factors which reduce the availability of target molecules and thereby the ability of drugs to produce adequate molecular damage, and (c) factors downstream of the molecular target, i.e. from molecular damage to cell death, which reduce or abolish the ability of drug-induced molecular damage to lead to cell death.

Virtually all cytotoxic agents affected by one of the established molecular MDR mechanisms enter cancer cells via passive diffusion through the cell membrane, along the concentration gradient. Hence, extracellular concentration is the major determinant for how much drug can enter the cell. Various factors can reduce the amount of active drug reaching the cancer cells. These include low dose, metabolic inactivation, the presence of tumour cells in so-called pharmacological sanctuaries, and poor ability of drugs to diffuse through interstitial tumour tissue. Clinical resistance due to the latter

*Table 1. Terms used in this article for various types of MDR*

| Term | Mechanism | Characteristics |
|---|---|---|
| Pgp-MDR | Overexpression of *MDR1*/Pgp | Resistance to natural product drugs which differ in structure and function; reduced drug accumulation due to enhanced efflux<br>Can be reversed by chemosensitisers such as verapamil or cyclosporins |
| MRP-MDR | Overexpression of MRP | Phenotype similar to Pgp-MDR but little resistance to taxanes; changes in cellular pharmacology variable<br>Amphipathic cations need to be conjugated prior to transport<br>Low activity of typical Pgp-inhibitors |
| Topo II-MDR | Diminished content or activity of topo II α | Resistance to topo II drugs (i.e. drugs which differ in structure but not in function) |
| GSH-MDR | Increased content of GSH and/or increased activity of GSH S-transferases | Resistance to melphalan, cyclophosphamide, chlorambucil, BCNU, thiotepa (and possibly other drugs such as cisplatin and doxorubicin)<br>Increased phase II metabolism of drugs |
| Apoptosis-MDR | Blocked apoptosis; dysfunction of genes involved in apoptosis | Resistance to most (all?) cytotoxic agents |
| Clinical MDR | Can be multifactorial; extracellular mechanisms possible | Clinical resistance to multiple cytotoxic drugs which differ in structure (and possibly function) |

two mechanisms is often referred to as pharmacokinetic resistance. The relationship between dose and clinical activity of chemotherapy has been established in a number of tumours. However, we know from pharmacokinetic and pharmacodynamic studies that the same dose can result in plasma levels, areas under the plasma concentration–time curve (AUC) and adverse effects which vary greatly among patients. One reason for this variability are differences in hepatic drug metabolism, e.g. due to polymorphisms in drug-metabolising enzymes such as the families of cytochromes P450 or GSH S-transferases (GSTs) [56, 57]. Many cytotoxic agents are not able to pass the blood–brain barrier. Thus, the CNS is a typical pharmacological sanctuary where even highly drug-sensitive cancers such as acute leukaemias or malignant lymphomas do not usually respond to systemic chemotherapy. Recent data from studies in *MDR* knock-out mice have provided evidence that Pgp encoded by the mouse *Mdr1a* gene, one of the two functional homologues of the human *MDR1* gene, plays an important role in the protection of the CNS from xenotoxins, including cytotoxic drugs of natural origin such as vinblastine [58]. Particularly in solid tumours, various factors can limit the access of drugs to the cancer cells. After leaving the tumour capillaries, drugs need to get to the cancer cells by passive diffusion. Tumours can be poorly vascularised, resulting in long distances between capillaries and tumour cells. The interstitial tissue may be rich in solid structures such as collagen, e.g. in particular types of carcinomas or in scar tissues after radiotherapy. Cytotoxic agents have been found to differ in their capacity to diffuse through tissues [56–61]. For instance, 5-fluorouracil and cisplatin have been shown to penetrate more readily into tumour spheroids than do larger, amphipathic molecules such as anthracyclines or vinca alkaloids. A typical example of the negative effect the poor access of drugs to tumour cells can have on chemotherapy activity are the treatment results in head and neck cancer. If given prior to local therapy, chemotherapy is able to achieve response rates of up to 90%, with a significant number of complete remissions. By contrast, in patients with tumour relapse in areas of previous surgery or radiotherapy, i.e. in scar tissues, response rates with the same chemotherapy protocols are around 20–30% [62–64].

The first line of defence that agents can face upon entering tumour cells are membrane-bound efflux pumps, such as Pgp or MRP, which accept a number of cytotoxic agents as substrates for transport. The net-effect of a pump like Pgp, with respect to reduction of intracellular drug concentration, is determined mainly by two variables. One concerns Pgp itself, its density in the cell membrane and its activity, which is influenced by factors such as cellular ATP production and Pgp's phosphorylation status. The other variable is the number of drug molecules which need to be effluxed, a parameter determined mainly by the extracellular concentration of drug as the driving force for diffusion into the cell. We know from cell line studies that no matter how much Pgp is present in the cell membrane, beyond a certain extracellular concentration, enough drug accumulates in the cytoplasm to lead to cell death. In clinical tumours, the levels of Pgp overexpression are usually low relative to most experimental Pgp-positive cell lines. Hence, rather minor differences in extracellular drug concentration might decide whether Pgp is able to protect the cancer cells or not. This point appears important in the planning of clinical studies to overcome Pgp-MDR. In many studies conducted so far, little effort has been made to give maximum-tolerated doses of cytotoxic drugs when used in combination with chemosensitisers. Along the same lines, the increase in AUC observed for cytotoxic drugs when combined with chemosensitisers, such as cyclosporins or dexverapamil [65–68], may be an advantage rather than the problem it is often conceived to be. If a chemosensitiser does increase extracellular drug concentration and is able to block Pgp function in tumour cells it should increase the chance of reversing Pgp-MDR effectively. The problems in the interpretation of antitumour activity in such studies can be solved readily by adequate study design. Similarly, the potential increase in chemotherapy-induced toxicity does not seem to be a major problem, considering the many studies of high-dose chemotherapy being performed in a variety of cancer types. When we look at the results of chemosensitiser studies, it is not toxicity nor logistic issues but rather lack of activity which appears to be the prime concern and thus deserves the most attention.

Unlike dose of chemotherapy, the concept of prolonged drug exposure to overcome MDR has received a great deal of attention in recent years. This interest was stimulated by data

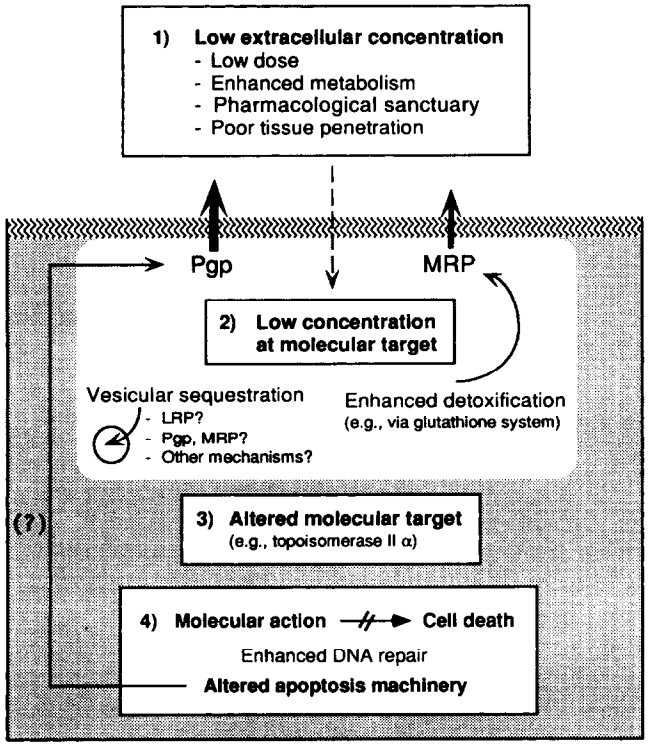


**Figure 1. Factors which can contribute to clinical MDR in patients with cancer. (1) Most cytotoxic agents enter cancer cells via passive diffusion through the cell membrane along the extra- to intracellular concentration gradient. The amount of drug molecules present outside the cancer cells is therefore the major determinant for cellular drug uptake. (2) Various molecular mechanisms can reduce the availability of active drug at the molecular target. These include efflux pumps such as Pgp or MRP, vesicular sequestration, and enhanced detoxification. As extracellular drug concentration is the driving force for drug uptake, it also affects the relative efficiency of these molecular mechanisms. A link seems to exist between phase II drug metabolism, e.g. conjugation, and MRP-mediated drug efflux (see text). (3) MDR can result from alterations in the amount, structure or activity of molecular targets of cytotoxic agents. (4) Adequate drug-induced molecular damage may not translate into cell death if cells are able to repair the damage effectively or are unable to die via apoptosis. Cytotoxic agents typically kill cancer cells via apoptosis and thus blocked apoptosis may result in broad chemotherapy resistance. Experimental data suggest a link between mutant *TP53*, i.e. a genetic alteration that usually leads to blocked apoptosis, and activation of *MDR1* transcription (see text).**

from two studies in patients with drug-refractory lymphomas, where continuous infusion chemotherapy proved capable of inducing remissions in a substantial number of patients [69, 70]. Furthermore, experimental studies have shown prolonged drug exposure diminishes resistance to doxorubicin in Pgp-positive colon carcinoma cell lines, while having no effect on Pgp-negative resistant cell lines [71]. However, *in vitro* studies in this laboratory have shown that effects of prolonged drug exposure on Pgp-associated resistance depend on the particular agent used [72]. Prolonged drug exposure had no effect on resistance to doxorubicin and docetaxel, even increased resistance to etoposide, and diminished only resistance to vinblastine. The results were similar when using cell lines which express high or low levels of Pgp, and in cell lines of epithelial or haematological origin. We and others have observed that extending the exposure time *in vitro* is able to increase the activity of chemosensitisers in reversing Pgp-mediated resistance [72–74], irrespective of the cytotoxic agent used. In most clinical studies, cytotoxic drugs are used in combination and the regimens are often comprised of various drugs pumped by Pgp. This is a scenario which is similar to the addition of a chemosensitiser to a single cytoxic agent. Thus, the continuous administration of such protocols over several days might, indeed, be able to diminish Pgp-mediated resistance.

Various cytoplasmic mechanisms have been identified which can reduce the amount of active drug available at target. One is sequestration of drugs in vesicles, and another is metabolic inactivation. In most MDR cell lines which express either Pgp or MRP, subcellular drug distribution has been found to be altered compared with the drug-sensitive counterparts [75–82]. The agents typically used in these studies are fluorescent compounds, such as anthracyclines or mitoxantrone. What can be seen in resistant cells is a shift of fluorescence from the nucleus to the cytoplasm, and there a punctate fluorescence pattern corresponding to cytoplasmic vesicles. The precise mechanism responsible for the accumulation of drugs in the vesicles is not yet known. A protein which might be involved in this process is the major human vault protein LRP [31]. Overexpression of LRP is often found in MRP-positive MDR cell lines [30], but has also been recently detected in a Pgp-positive cell line [83], and immunostaining of LRP in such cells typically shows a punctate cytoplasmic pattern. However, MRP or Pgp may also play a role in the process of vesicular sequestration of drugs [77, 82]. Typically, vesicular sequestration reduces the amount of drug available at the target without affecting the total concentration of drug accumulated in the cells. However, drugs sequestered in vesicles can also be extruded from the cells via exocytosis, a process which, of course, lowers cellular drug concentration [84].

In the cytoplasm, drugs are subjected to metabolism by various mechanisms, including conjugation with GSH by GSTs or glucuronidation. Overexpression of GSTs and increased cellular GSH content have been associated typically with resistance to the nitrogen mustard category of alkylating agents such as melphalan and cyclosphosphamide and to drugs such as BCNU and thiotepa [28, 29]. Various studies have also shown an association with resistance to other agents, e.g. anthracyclines or cisplatin [24–26]. Recent work has revealed a link between MRP-mediated transport and cellular phase II drug metabolism of cytotoxic agents. Unlike Pgp, MRP seems to require amphipathic agents which are cationic at physiological pH, i.e. the majority of cytotoxic drugs involved in the MRP-MDR phenotype, to be conjugated or glucuronidated to be a substrate for transport [85–90].

## FAILURE OF CANCER CELLS TO DIE DESPITE ADEQUATE DRUG CONCENTRATION AT TARGET

Even if cytotoxic agents reach their molecular target at adequate concentrations, they still may not be able to kill the cancer cell. One reason can be alterations in target molecules, e.g. decreased content or function. A typical example for such target-related drug resistance is topo II-MDR [17–23]. Expression of topo IIα, the 170 kDa isozyme which appears to be the main target for cytotoxic agents, is known to be proliferation- and cell-cycle-dependent [91–93]. In fact, topo IIα seems to be identical with the proliferation-associated

nuclear antigen Ki-S1 [94], which is frequently used in immunohistochemical studies to determine the rate of cell proliferation in tumour samples. As low proliferation and slow tumour growth are known to reduce the activity of most cytotoxic drugs [95], low expression of topo IIα in clinical tumour samples may just be an indicator for growth-related, i.e. kinetic, resistance. Accordingly, interpretation of results from clinical studies which show an association between low tumour content of topo IIα and poor treatment outcome seems difficult.

A mechanism which can lead to drug resistance in cancer cells despite adequate drug-induced damage at the molecular target is enhanced DNA repair, e.g. by enzymes such as $O^6$-alkylguanine-DNA-alkyltransferase or perhaps even excision repair mechanisms [96–101]. However, the most important mechanisms which can prevent cancer cells from dying despite appropriate drug action at the target seem to be processes that block apoptosis [102–106]. As most, if not all, cytotoxic agents appear to kill cancer cells via apoptosis, apoptosis-MDR could affect more drugs than any other molecular MDR mechanism. Altered function of genes involved in apoptosis, such as *TP53, RB, MYC, BCL-2* and various others has been shown to result in malignant transformation in experimental models and are known today to play an important role in tumorigenesis in man [107]. In fact, impaired ability to die via apoptosis seems to be a property of most human tumours and thus might be a significant factor in clinical MDR [108–111]. Importantly, if apoptosis-MDR is present, any resistance mechanism operating upstream of apoptosis may have little functional relevance. The same applies to circumvention of such mechanisms, e.g. of Pgp-MDR. Like any other form of MDR, apoptosis-MDR can be overcome in the laboratory by increasing the dose of cytotoxic drugs. Beyond a certain concentration, drugs kill cells by non-apoptotic mechanisms such as non-specific damage of cellular structures, e.g. biomembranes or mitochondria. However, the increase in drug concentration needed for this to occur seems well above the relative increase in cellular drug concentrations that can be expected in most clinical tumours from blocking Pgp function.

## OVEREXPRESSION OF MDR1/PGP IN CLINICAL TUMOURS—A MARKER FOR DRUG RESISTANCE OR TUMOUR AGGRESSIVENESS?

In various cancer types, such as acute myeloid leukaemia, various childhood tumours and locoregionally advanced breast cancer, overexpression of *MDR1*/Pgp has been found to correlate with poor outcome in patients treated with chemotherapy [33–40]. These data have been interpreted as an indication for Pgp-mediated drug resistance to be the cause of poor treatment outcome in Pgp-positive tumours. However, this kind of conclusion seems premature. Various clinical studies have suggested Pgp-positivity to be associated with more aggressive tumour behaviour. In colon cancer, Pgp was found to be expressed predominantly in the tumour cells at the invading edge of primary tumours, and Pgp-positivity in primary tumours was associated with a higher incidence of lymph node metastases [112]. In renal cell carcinoma, Pgp-positivity was found significantly more often in invasive than in non-invasive tumours [113], and in primary breast cancer, overexpression of *MDR1*/Pgp seems to be more common in advanced locoregional disease than it is in small tumours [114–116]. Recently, a significant correlation has been reported between Pgp-positivity and lower probability of event-free survival in patients with osteosarcoma treated with pre- and postoperative chemotherapy [40]. The extent of tumour necrosis after pre-operative chemotherapy, which has been found previously to be the most powerful predictor for long-term outcome in osteosarcoma, was also predictive for prognosis. However, multivariate analysis showed Pgp status to be a more powerful variable than was extent of tumour necrosis, and the two variables to be independent. Notably, no correlation was found between Pgp status and degree of tumour necrosis. Hence, Pgp-positivity was a strong predictor for poor treatment outcome in this study, but had no effect on tumour response to pre-operative chemotherapy as assessed by histological examination. These data seem to be the strongest evidence so far that Pgp-positivity indeed might be a marker for more aggressive tumour behaviour and thus poor treatment outcome, independent of its effect on chemosensitivity [116].

Experimental studies have suggested a relationship between the presence of mutant *TP53* in tumour cells and upregulation of *MDR1* transcription. In various independent studies, transfection of mutant but not of wild-type *TP53* was found to transactivate the *MDR1* promoter [117–120]. However, other experiments found wild-type but not mutant *TP53* to stimulate *MDR1* expression [121]. Furthermore, in a panel of drug-resistant human breast cancer cell lines, six cell lines showed overexpression of *MDR1* while containing wild-type *TP53* [122]. Perhaps most importantly, in clinical studies in B-cell chronic lymphocytic leukaemia, myelodysplastic syndromes and colon cancer, no correlation has been found between the presence of mutant *TP53* and overexpression of *MDR1*/Pgp [108, 123, 124].

*In vitro* transfection studies have shown the *MDR1* promoter is a target for the *HA-RAS* oncogene [117]. Furthermore, in two leukaemia cell lines established from a patient with acute multilineage leukaemia, one had high levels of *MDR1* expression and contained both mutant *TP53* and *N-RAS* whereas the other had low *MDR1* expression and contained only mutant *TP53* [125]. Mutations of *RAS* are believed to occur at later stages of tumorigenesis and to play a role in tumour aggressiveness. Thus, these data seem to be another piece of circumstantial evidence that *MDR1*/Pgp expression might be an indicator for aggressive tumour behaviour.

Currently it is unclear whether Pgp is a marker for tumour aggressiveness, for clinical chemotherapy resistance, or perhaps for both. This is an important question because it has profound implications for the effects we can expect from clinical reversal of Pgp-MDR. If mutant *TP53*, for instance, is indeed associated with upregulation of *MDR1*/Pgp expression, blocking of Pgp function in tumours which have impaired apoptosis is likely to have little impact on chemotherapy activity. Similarly, if Pgp is a marker for tumour aggressiveness, effective reversal of Pgp-MDR may have little impact on treatment outcome. Clearly, studies are needed which are able to answer these important questions conclusively.

## CLINICAL MDR AND RESISTANCE HETEROGENEITY

We know from experimental studies that various MDR mechanisms can be present in tumour cells simultaneously, e.g. MRP plus reduced amounts of topo IIα and overexpression of LRP [126, 127]. In clinical chemotherapy resistance, failure to achieve high enough extracellular drug concen-

tration, molecular mechanisms which reduce intracellular drug concentration, alterations in molecular targets and inability of cancer cells to die via apoptosis may all be operative at the same time, and in most patients with cancer, clinical MDR seems likely to be a multifactorial problem. As far as molecular MDR mechanisms are concerned, all clinical studies in which various mechanisms have been analysed in the same tumour samples have detected more than one such mechanism in a certain proportion of the tested specimens [42, 47, 50, 51, 128–133]. However, none of these studies has provided information on whether the various mechanisms were present in the same tumour cells or in differing subpopulations of cells.

Another phenomenon which may be common in clinical tumours is resistance heterogeneity. It is not unusual for clinical chemotherapy to produce mixed tumour responses, i.e. response of some tumour sites but progression of others. Experimental studies have shown the critical influence the microenvironment has on biological characteristics of tumours, including sensitivity to chemotherapy [134]. In murine tumour models, expression of *Mdr1*/Pgp has been found to vary from negative to strongly positive, dependent on the particular organ in which the tumour is growing [135]. Furthermore, access of drugs to tumour cells can vary in different tumour sites owing to differences in vascularisation or a proportion of tumour cells being present in a pharmacological sanctuary. Resistance heterogeneity may also exist within an individual tumour. For instance, Pgp expression in tumour biopsies is often heterogeneous, with some cancer cells lacking detectable Pgp whereas others have high levels of expression. In a large series of solid tumours, various biological parameters, including GSH content, differed widely when analysed in biopsies from different sites of the same tumour [136]. These observations led the authors to conclude that a minimum of three biopsies from different sites of an individual tumour is needed to assess biological parameters such as molecular resistance mechanisms with any accuracy. In clinical practice, this is impossible to do in the majority of patients. As most patients with solid tumours treated with chemotherapy have multiple metastases in different organ sites, the question arises whether we can expect to obtain meaningful information from analyses that are performed on a single biopsy taken from a single metastasis. Currently, this question is of particular importance in clinical studies which are evaluating the activity of chemosensitisers to overcome MDR. Analysis of *MDR1*/Pgp expression in tumour biopsies is viewed widely as being almost mandatory for the interpretation of clinical activity or inactivity of chemosensitisers. When we look at the available data, it seems likely that such analyses of *MDR1*/Pgp expression in single biopsies are not representative for the entire disease, and thus appear to have rather limited value in studies of chemosensitisers, particularly in solid tumours.

There are some tumour types, e.g. leukaemias and lymphomas, in which treatment response can be monitored readily and thus frequently. In such cancers, it is not uncommon to see a transient reduction in the number of circulating blasts or in the size of lymphomas after administration of chemotherapy, followed by rapid regrowth prior to the next treatment cycle. This type of resistance to chemotherapy has been termed regrowth resistance [137]. The cellular and molecular basis of regrowth resistance can differ, from a high proliferation rate to the presence of particular molecular mechanism in subpopulations of tumour cells. In solid tumours, close clinical monitoring of response is much more difficult and thus restricted to cancers treated with chemotherapy prior to local therapy, e.g. advanced breast cancer, head and neck cancer, and various childhood tumours. Similar to haematological neoplasms, it is seldom to see a rapid, early progression in such solid tumours. Usually, either an objective response is achieved or the tumour stops growing for at least some time. These clinical observations imply that in many cancers, a certain proportion of tumour cells tend to respond to chemotherapy even if the majority is resistant. This emphasises that resistance heterogeneity is a clinically relevant phenomenon.

## CONCLUSION

It seems reasonable to suggest that clinical resistance to chemotherapy is multifactorial and/or heterogeneous in most patients with cancer. Many of the mechanisms which can cause resistance are, to some extent, inter-related. Others are independent of each other, but may exist simultaneously in individual cancer cells, different subpopulations of cells in individual tumours, or in different metastases. The two dominant principles in clinical MDR appear to be inadequate drug concentration at the target and impaired apoptosis. At the moment, strategies which are capable of restoring normal function of genes involved in apoptosis are not available for clinical use. Thus, current clinical studies have to focus on efforts to enhance the concentration of active drug at the molecular target in cancer cells. Considering the potential complexity of clinical MDR, it seems difficult to improve chemotherapy efficacy by therapeutic measures which aim to overcome a single resistance mechanism. For this to happen, a particular mechanism needs to be by far the dominant factor for clinical MDR, a proposition which seems unlikely in most tumours. Nonetheless, this might turn out to be the case in certain tumour types, where such strategies could prove to have a significant impact on treatment outcome.

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