Novel regulators of endosome dynamics, MHCII antigen presentation and chemosensitivity

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Chapter 8:
Old drugs, novel ways out: drug resistance towards cytotoxic chemotherapeutics

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Drug Resistance Updates 28, 65-81

Abstract

Efficacy of chemotherapy in the treatment of malignancies is often hampered by drug resistance arising in the tumor. Understanding the molecular basis of resistance and translating this knowledge into personalized treatment decisions can enhance therapeutic and even curative outcome. Over the years, multiple drug resistance mechanisms have been identified that enable tumors to cope with the damage instigated by a specific drug or group of drugs. Here we provide an overview of the molecular pathways leading to resistance against conventional anti-cancer drugs, with emphasis on the utility of these pathways for rational selection of treatments for individual cancer patients. We further complement the review by discussing the pitfalls and difficulties in translating these findings into novel treatment strategies.

Introduction

Conventional chemotherapy is a major arm of anti-cancer therapy, but its effectiveness is often hampered by intrinsic, as well as acquired drug resistance (1-3). Understanding the resistance mechanisms for the different chemotherapeutic agents is crucial to develop better treatment strategies, ideally leading to a personalized drug regimen for better treatment responses, as well as preventing treatment with ineffective drugs (4, 5). Over the years ample evidence has delineated a multitude of drug resistance mechanisms, both general multi-drug resistance factors and factors specific for one (class of) drugs. Most of these mechanisms were initially discovered in tumor cell lines, and some have subsequently been validated in a clinical setting. Yet, few, if any, factors are actually used in the clinic as prognostic markers to select the appropriate individual cancer patient's treatment regimen for conventional chemotherapy.

On the other hand, personalized medicine is used successfully for targeted drugs. The evolution of genomic, proteomic and screening tools has yielded extensive knowledge about the molecular basis driving growth of individual tumors. Based on this information, drugs have been developed that specifically target a protein or pathway that is activated in the tumor. These are often activated kinases, like EGFR in lung cancer, BRAF in melanoma and FLT3 in AML patients (6, 7). Alternatively, targeted drugs instigate cytotoxic activity specifically in the context of a tumor-specific mutation, a concept known as synthetic lethality. This is exemplified by the usage of PARP-inhibitors for BRCA1/BRCA2 mutant tumors (8). These targeted drugs often present limited side effects compared to conventional chemotherapeutics and are effective during the initial responses. Unfortunately however, genomic instability and consequent tumor heterogeneity often allows cells to acquire drug resistance, by mutations in the drug target or activation of alternative signaling pathways, ultimately leading to ineffective treatment and poor outcome (9, 10).

Though conventional chemotherapeutic drugs also include antitumor agents with a molecularly defined target (like the antifolates) (3), most drugs target essential cellular systems that cannot be bypassed by the tumor. This does not imply that conventional chemotherapeutic drugs are spared from drug resistance, but merely that drug resistance is often multifactorial. Given the strong untoward side effects associated with most chemotherapeutics, drug resistance not only limits the therapeutic efficacy but also exposes patients to unnecessary toxicity in healthy tissues.

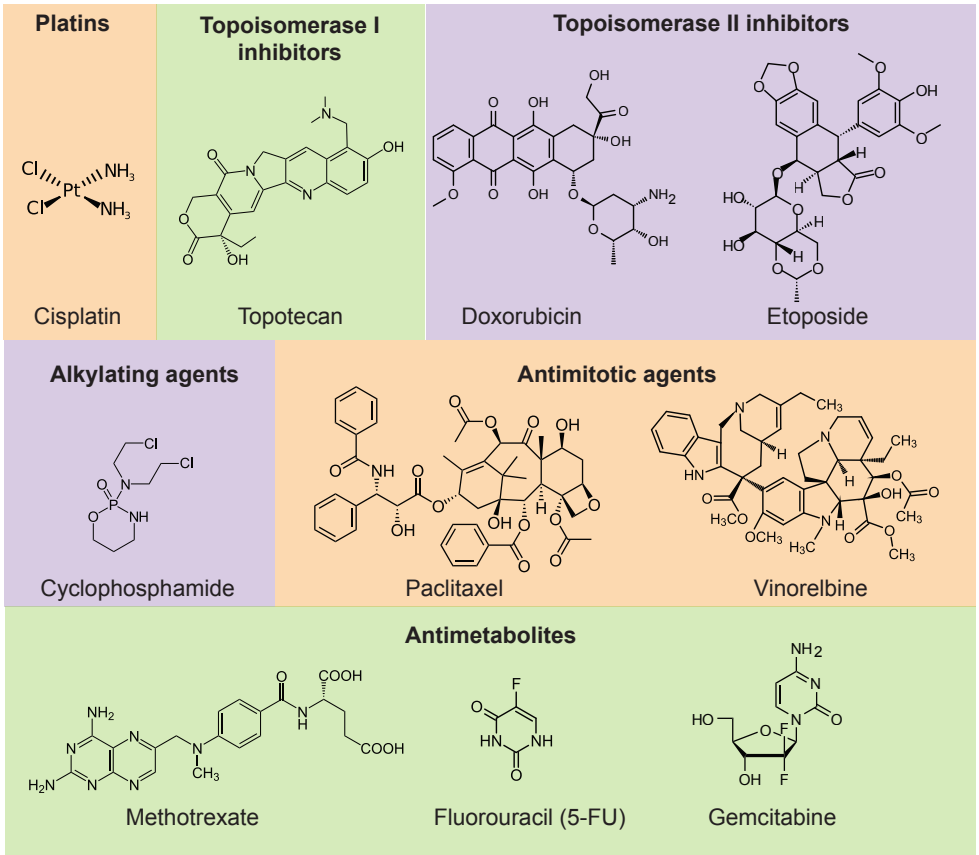

Figure 1: Chemical structures of members of the different classes of chemotherapeutics. *Six different chemotherapy classes are distinguished, some of which are divided into several subclasses. For every subclass, the structure of the most commonly used analogue is shown.*

Resistance mechanisms towards the different chemotherapeutic classes (Figure 1) have been extensively studied, both in tumor cell lines and in mouse models. Major mechanisms include enhanced drug efflux by multidrug transporters of the ABC superfamily, chemical modifications to convert drugs into non-effective metabolites, down-regulation of the major drug target, or bypassing the inhibited pathway (Figure 2). Only a few of these mechanisms have been shown to be operational outside the laboratory setting (i.e. in patients) and the translation of this fundamental knowledge into clinical practice has not been very successful. However, the recent sequencing of cancer genomes, as well as the discovery of novel molecular mechanisms of drug resistance, might spur the identification and validation of clinically relevant resistance mechanisms, yielding a better framework for implementing personalized treatment regimens.

In this review, we summarize the current challenges for personalized cancer medicine and provide an overview of the resistance modalities employed by tumors towards the different classes of anti-cancer drugs, with emphasis on novel, as well as clinically validated mechanisms.

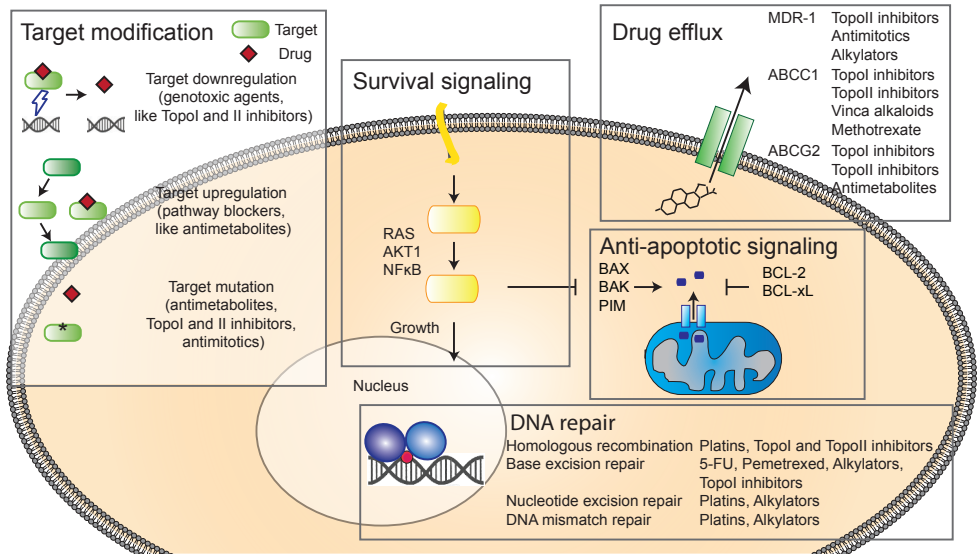

Figure 2: Common cell-intrinsic resistance mechanisms to chemotherapeutics. *Cancer cells can become generally resistant to chemotherapeutics by inhibiting the apoptotic pathway or constitutive activation of growth signaling pathways, which also inhibit apoptosis. Alternatively, the expression of drug transporters is upregulated, leading to resistance to a subset of drugs (as indicated). Cells also activate DNA damage repair pathways to cope with genotoxic stress induced by several chemotherapeutics. To avoid effective target inhibition, cells can mutate the drug target, upregulate its expression (in case of pathways blockers, as to prevent full inhibition of the total enzyme pool), or downregulate its expression (for genotoxic agents, to prevent harmful targeting by the drug).*

Challenges for predicting patient chemosensitivity

Applying personalized cancer treatment with conventional chemotherapeutics requires an accurate prediction of tumor sensitivity towards the different approved antitumor drugs. Given the varying responses observed in the clinic, molecular determinants that mediate chemoresistance should be at hand in order to allow selection of the appropriate individual drugs. Identifying and implementing these resistance factors have thus far been hampered by several issues, ranging from tumor heterogeneity to the difficulty of translating drug resistance mechanisms into reliable diagnostics and therapeutics. Overcoming or bypassing these impediments will provide a major headway towards personalized selection of the most efficacious drug(s) for optimal treatment as well as the lowest side effects.

The general types of chemoresistance

Generally speaking, drug resistance can be intrinsic (i.e. primary or inherent) or acquired during and/or following treatment. Intrinsic drug resistance arises when the tumor inherently contains traits that enable it to cope with the damage inflicted by the drug; for example, increased anti-apoptotic signaling or mutations in the DNA-damage response pathway. These characteristics are often instrumental in the establishment of the tumor and are intimately associated with its general survival. Since the therapeutic window of anti-cancer drugs is usually narrow, a relatively small resistance effect may render drugs therapeutically ineffective. Yet, decipher-

ing the molecular basis of such drug resistance mechanisms warrants tailored drug selection based on the characteristics of the tumor. This is more difficult in the case of acquired chemoresistance, where tumors acquire drug resistance during treatment, often by rewiring signaling pathways or epigenetic alterations that change the transcriptional landscape. Alternatively, resistance arises from the outgrowth of a preexisting subpopulation of tumor cells that already harbored mutations or altered expression of resistance genes, which is inherent to the heterogeneous nature of the tumor (11). These infrequent mutations can sometimes be detected using deep sequencing (10), while complex gene signatures may be able to predict acquired resistance (10, 12).
From a molecular perspective, drug resistance can be based on expression (quantitative alterations) and/or based on mutations (qualitative alterations). Mutation-based mechanisms of chemoresistance are often more stable, especially when these are cancer driver mutations essential for the growth of the tumor, or when they are somatic. Cells can also become drug resistant by up- or downregulating expression of relevant genes or pathways, expanding the inherent variation within the tumor and fueling resistance development (13). Given the heterogeneity of gene expression, selection on the basis of mutations seems most feasible as they are more stable and relatively homogeneous.

Multi-drug treatments

Whilst modeling of resistance towards a single drug seems feasible, most cancer patients are actually treated with drug combinations. In fact, combinatorial drug treatments constitute the treatment of choice in cancer therapy and yield better responses (14, 15). The concept of multi-drug treatments is that the tumor is simultaneously attacked from different angles for more effective eradication of the tumor and –in addition- preventing the occurrence of drug resistance phenomena. However, the contribution of each individual drug to the combined treatment effect is often unclear, which hampers linking drug resistance mechanisms observed in the clinic to a well-defined drug. Furthermore, in vivo validation of mechanisms identified in the lab is harder, since clinical effects can arise from drug-drug interactions in such a multi-drug chemotherapeutic cocktail. It is therefore often necessary to extrapolate the interactions between drugs and their individual resistance mechanisms based on data from mouse models or tumor cell lines to pin-point the mechanisms underlying resistance to drug-cocktails in cancer patients (16). This argues for more laboratory studies combining several anti-cancer drugs to model resistance to drug combinations that better reflect the genuine clinical treatment.

Tissue specific resistance

The efficacy of different chemotherapeutics varies markedly per tumor type. Whilst these differences can be partly attributed to different pharmacokinetics and pharmacodynamics (such as drug uptake by the tissue and tumor metabolism, reviewed in (17)), the genetic make-up of the tissue of origin affects many of the tumor's properties, among which is its drug sensitivity. As an extreme example of this, patients with hepatocellular carcinomas generally do not benefit from any type of chemotherapy, due to the high expression of multidrug efflux transporters, oxidative stress proteins

and metabolic enzymes, which is physiologically intrinsic to this tissue, the liver (18). Other tumors display more specific drug resistance or sensitivity profiles, for example testicular cancers are extremely sensitive to cisplatin, in part due to their wild-type p53 status (19).

While these are simple concepts, they cannot be employed by default as clinical oncologists have often observed excellent responses to such 'ineffective' drugs in a certain subset of patients. Hence, the inevitable question is; what are the molecular traits that define these subsets of patients or their tumors? Sometimes the underlying molecular basis can be well defined. For example, the EGFR inhibitor Cetuximab has only a small overall survival benefit for lung cancer patients, but it is effective on patients with lung tumors overexpressing EGFR (20). The effect on this small subgroup of patients is masked by the overall lung cancer population and would not have been noticed without phenotyping the individual patient. Retrospective studies with recently identified biomarkers on tumor tissues from responder versus non-responder patients could be useful in identifying subgroups of patients that might benefit from well-defined chemotherapeutics. One example is the use of platinum compounds in breast cancer therapy. Whereas cisplatin is hardly used in the clinic for the treatment of breast cancer due to a lack of activity, recent data have demonstrated that this may be different for breast tumors with mutations in BRCA1 or high levels of p63 (21, 22). This subgroup of breast cancer patients would thus favorably respond to a drug that is not generally used for this type of tumors. Similarly, it was recently shown in the laboratory setting that colon cancers harboring a BRAF mutant phenotype are very sensitive to vinorelbine, whereas overall, colon cancers are known to be resistant to this anti-microtubule agent (23). These examples illustrate that individual cancer patient profiling may facilitate stratification of patient subgroups that may respond to a well-defined anti-cancer drug, which has been omitted for therapy for the entire group of patients in that tumor type. Considering not only tissue types but also individual tumors may be a better and broader approach, yielding alternative treatment options for patients on the basis of traditional chemotherapy.

Clinical detection methods and validation of potential biomarkers

Most biomarkers identified in the lab for predicting chemotherapy response fail to be validated in the clinical setting. There are various reasons for this disparity. Firstly, tumor cell cultures markedly differ from the clinical in vivo setting, which includes the tumor microenvironment, drug bioavailability, pharmacokinetics and pharmacodynamics. While drug sensitivity screens are usually performed upon 48-72 hrs of continuous drug exposure on tissue cultured tumor cells, the pharmacokinetics of drugs in patients differ considerably (many compounds have half-lives of 4-8 hours). Secondly, it is difficult to establish and confirm a biomarker as being predictive of response to a given cytotoxic drug. This requires a randomized trial with patients treated with the single drug, as well as a control population that is untreated or treated with a different drug. Thirdly, potential biomarkers are often instrumental to the survival of the tumor, blurring the analysis. This is exemplified by βIII-tubulin, a subunit of the microtubules, which was initially discovered as a predictor for response to paclitaxel in NSCLC and ovarian cancer. Later findings indicated that βIII-tubulin expression actually acts as a prognostic factor independent of treatment, by promot-

ing cell survival in general (reviewed in (24)). These data indicate that when using a non-randomized trial, it is important to analyze the response to chemotherapy, rather than survival after treatment, to investigate the effect of a potential predictive biomarker for treatment outcome.
When it can be established that a well-defined genetic alteration allows the prediction of treatment responses to a defined drug, these can be used in a diagnostic setting as part of the treatment protocol for the patient. These should be robust methods of the detection with ideally a binary outcome (i.e. YES or NO predicted response), since decreased sensitivity is difficult to comprehend. Qualitative differences in a tumor, such as mutations, deletions and SNPs are mostly determined at the DNA level with little variation and allow for binary outcomes. Quantitative differences can be detected with immunohistochemistry, or at the RNA level using deep-sequencing, which requires a cut-off for expression to allow binary decisions. Given the complexity of anticancer drug resistance, it is likely that multiple resistance markers need to be combined in order to form a bona fide signature, hence arguing in favor of high-throughput measurements. The advantage of these drug resistance biomarker combinations is that the excess of obtained data could be correlated with the clinical responses of cancer patients to identify novel biomarkers predicting response. Understanding the relevant mechanisms may then de-convolute these data sets to define a condensed set of biomarkers for more easy prediction of treatment responses towards conventional anti-cancer drugs.

General drug resistance mechanisms

Influx and efflux drug transporters

The simplest way for a cancer cell to become resistant is by restricting intracellular drug accumulation. This can be achieved by either inhibiting import or by accelerating drug export. How drugs enter cells is not always clear. The general idea is that hydrophobic anticancer drugs mostly enter cells by passive diffusion across the membrane, but various transporters have been identified that can transport drugs into cells. Cisplatin can be imported by the VRAC transporter whose physiological function is to regulate anion transport (25), the anti-folates methotrexate and pemetrexed use specific influx transporters including the reduced folate carrier (RFC/SLC19A1) as well as the proton-coupled folate transporter (PCFT; SLC46A1) (26-28), and the DNA base analogue gemcitabine uses nucleoside transporters for cell entry (29). How anthracyclines and other DNA damaging drugs enter cells is not entirely clear at this point, though studies have shown a role for flip-flop based diffusion of anthracyclines as a mode of entry into cells (30, 31). Of note, anti-cancer drugs may compete with endogenous substrates to use these influx transporters, and such competition may also affect cell growth when essential cellular building blocks are not efficiently taken up by cells. This could be a secondary mechanism of action for some anti-cancer drugs.
Mechanisms of ATP-driven drug export have been extensively studied. Many drugs can be extruded from cells by members of the ATP-binding cassette (ABC) transporter superfamily (see Figure 2). These efficient drug efflux pumps display promiscuous substrate specificity and are hence termed multidrug resistance (MDR) proteins. Upregulation of ABCB1 (also named P-gp or MDR1), the best studied member of this

ABC superfamily, markedly decreases the intracellular accumulation of a multitude of drugs, including taxanes, anthracyclines epipodophylotoxins, Vinca alkaloids and actinomycin D, as well as lipophilic antifolates (1, 32, 33), leading to multidrug resistance (MDR). Other extensively studied members of the ABC transporter superfamily include ABCC1 (MRP1) and ABCG2 (BCRP), which have partially overlapping drug substrate specificities (reviewed in (34)). Transporters are highly expressed in the liver, the blood-brain barrier, the intestine and several endothelial tissues and are believed to be one of the reasons why hepatocellular carcinomas respond poorly to chemotherapy and why chemotherapy of brain tumors is often inefficient (35). Also, cancer cells can upregulate the expression of these ATP-driven drug exporters in response to drug exposure. Due to the collection of anti-cancer drugs that are sensitive to MDR1 expression, several transport inhibitors have been developed that specifically target MDR1 and other ABC transporter family members. While strongly linked to resistance in vitro and in mouse models, the clinical correlation between MDR1 expression and drug resistance is relatively poor, as is the clinical benefit from the use of MDR1 transport inhibitors (34, 36). This could be due to the redundancy of the different transporters, or because the drug dose during treatment is so high that ABC transporter activity contributes only marginally to intracellular drug accumulation.
While it is likely that ABC transporters and especially MDR1 contribute to MDR, expression of these drug pump proteins cannot be directly correlated to the clinical effect of drug resistance. Probably, the combination of impaired drug uptake and accelerated drug export will generate a more convincing clinical drug resistance case, but these data are yet unavailable.

Alterations in the anti-apoptotic pathway

The most common mechanism for cancer cells to undergo cell death in response to chemotherapy is via apoptosis. This programmed cell death (PCD) response is induced by an array of stimuli that eventually leads to activation of caspases that execute the PCD program (37). Many chemotherapeutic drugs instigate DNA damage that is sensed by p53, which subsequently provokes the BCL-2 family of pro-apoptotic and anti-apoptotic proteins. Induction of apoptosis results from an alteration in the balance towards pro-apoptotic factors, leading to mitochondrial membrane permeabilization and release of cytochrome c, hereby activating caspases that execute apoptosis (38). Due to their tumor promoting nature, genetic mutations or alterations that block apoptosis are frequently observed in various cancers (39), suggesting that these tumors are intrinsically more resistant to anti-cancer drugs. Indeed, cells overexpressing the anti-apoptotic BCL-2 protein are resistant to various chemotherapeutics (40). Especially in hematopoietic malignancies, which are exquisitely sensitive to apoptosis due to their immunological origin, a strong correlation between apoptotic capacity and response to chemotherapy has been observed (41). This has sparked strategies for patient selection on the basis of apoptotic protein expression, as well as the development of inhibitors for BCL-2 members, to be used as monotherapy or to increase the efficacy of chemotherapy (reviewed in (42)). Unfortunately, thus far, none of these inhibitors is clinically used due to severe side effects, but the development of more specific inhibitors might avoid this undesired toxicity (43). In solid

tumor types, some apoptotic factors correlate with response to chemotherapy as well. For example, in triple-negative breast cancer (TNBC), low expression of BCL-2 is associated with a better response to chemotherapy (44, 45). However, overall, the effects are less pronounced than in hematopoietic tumors. This could be explained by other BCL members that are functional in these tumors or because solid tumors have a different balance of apoptotic factors (46).
Cell death can also proceed via other pathways, like necroptosis, or via immunogenic cell death, or cells can become senescent, thereby not contributing to tumor growth (47, 48). The contribution of immunogenic cell death to treatment is of interest as chemotherapy responses are coupled to immune responses against tumors. As a result, the primed immune system can recognize tumor mutations and clear the tumor by the activity of the adaptive immune system (see Figure 3). For example, doxorubicin can trigger CD73 and A2A adenosine receptor signaling to activate the immune system (49), thereby stimulating a systemic response against the tumor, at least in mouse models. This type of cell death is not induced by all chemotherapeutics and the molecular basis is still being studied in more detail. Yet, the contribution of the immune system to tumor eradication following chemotherapy could be a great example for achieving two effects in a single drug maneuver.

Cell growth signaling pathways

In general, cancer is linked to systematic activation of growth signaling pathways and de-regulation of cell-cycle checkpoints (7, 39). These characteristics also enable tumor cells to counteract the cytotoxic effects inflicted by chemotherapeutics, by providing pro-survival signals and decreasing sensitivity to DNA damage. Combining classical chemotherapy with drugs that inhibit signaling pathways which promote tumor cell growth could alleviate the negative impact of sustained cell-signaling. Indeed, this concept is applied to a wide spectrum of tumors harboring activated growth receptors. For example, HER2-positive breast cancers and EGFR-mutated colon cancers are treated with a combination of receptor antagonists and conventional chemotherapeutics (50, 51). Clinical benefit is observed for many of these therapies combining novel drugs targeting signaling pathways with conventional chemotherapeutics. If drug resistance to the targeted therapy arises via signaling bypass, combinations of drugs that also target the signaling pathways bypass may prevent drug resistance, as illustrated for the addition of TGF-βR inhibitors to the EGFR inhibitor regimen (52).
Due to the robust nature of drug resistance provoked by these growth signals, activation of most signaling pathways acts as a general negative prognostic factor for response to chemotherapy. However, this picture is more complex, as signaling can also be required for optimal chemotherapy efficacy. This is perhaps best illustrated by NFκB, a master transcription factor for pro-survival genes and pro-inflammatory cytokines, that is activated in a variety of tumors and is actively linked to chemoresistance by inhibiting apoptosis (53). Although most patients with tumors containing active NFκB poorly respond to chemotherapy, recent studies demonstrated that under conditions where apoptosis is already inhibited, NFκB promotes senescence and is actually a positive prognostic factor for the response to the alkylating agent cyclophosphamide (54). Furthermore, NFκB is involved in activation of the immune

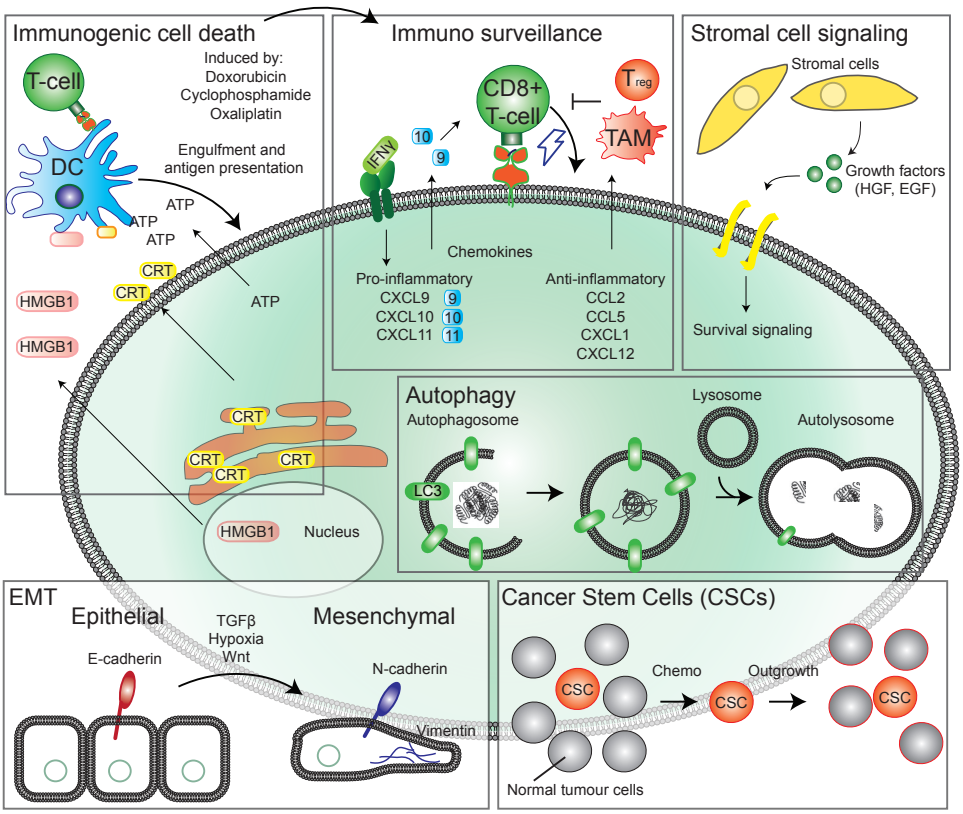

Figure 3: Overview of common cell-extrinsic or systemic mechanisms of drug resistance. *The tumor microenvironment can regulate resistance to chemotherapeutics via several mechanisms. Chemotherapeutics can induce immunogenic cell death, where dying cells secrete ATP and HMGB1, and present ER-resident protein calreticulin (CRT) at their cell surface. These signals are recognized by dendritic cells, which subsequently endocytose fragments of the dying cell and present their antigens by MHC-I molecules to CD8+ T-cells. This activates the T-cells, which can kill other tumor cells presenting the same antigens (immuno-surveillance). These T-cells can be recruited by IFNγ-induced chemokine expression and inhibited by tumor-associated macrophages (TAMs) or regulatory T-cells (Tregs), which can in turn be recruited by anti-inflammatory chemokine expression by the tumor. Alternatively, the tumor-microenvironment promotes drug resistance by secretion of growth factors that in turn activate survival signals in the tumor. Upregulation of autophagy by intrinsic or extrinsic factors also promotes survival of tumor cells, by sequestering damaged proteins and organelles. Drug sensitivity also depends on the differentiation status of the cell. Cells that have undergone epithelial-to-mesenchymal transition (EMT) or cancer stem cells (CSCs), are generally more resistant to chemotherapeutics. Some of these slow-cycling tumor cells can grow out to reform the tumor.*

system upon cell death (55), arguing that drugs that use an immune-system component following activation of the immunogenic cell death pathway are more effective in the presence of active NFκB. The balance of direct killing by chemotherapy versus indirect mechanisms utilizing the immune system is poorly understood, but can be important in the case of so-called immunogenic tumors like melanoma, lung and bladder cancer (56). A poor response to chemotherapy may then be compensated by an accelerated anti-tumor immune response that suffices to achieve the desired clinical effect, namely systemic eradication of the tumor.

Molecular resistance mechanisms to anticancer drugs

Platinum-containing drugs

One of the benchmark treatments for many malignancies are platinum-containing drugs, the most commonly used being cis-diaminedichloroplatinum(II) (CDDP or cisplatin, for structure see Figure 1). This important class of antitumor drugs, which also includes the analogs carboplatin and oxaliplatin, relies on a platinum at the core of the structure which, upon activation in the cytosol (via Cl- substitution for OH-, reviewed in (57)), reacts with several cellular structures, most notably guanine nucleobases, generating inter- and intra-strand DNA cross-links. These cross-links are believed to be the primary mode of cytotoxicity, leading to a block in cell growth, senescence and apoptosis (58). Tumor cells are more susceptible to cisplatin or its variants because they grow faster and can harbor mutations in the DNA repair machinery, rendering them less efficient in removing cross-links and thus inducing cell death.

Platinumdrug resistance

Although platinum drugs are effective in cancer therapy, resistance to these drugs is commonly observed (59-62). Most described resistance factors are related to DNA damage response (DDR; Table 1), supporting the notion that this is the main mode of action of cisplatin. However, several factors have been identified that act at other steps in the cisplatin action cycle and also contribute to drug resistance, as discussed below.

Mechanisms of DNA repair

Cisplatin induced inter- or intra-strand DNA crosslinks are mainly repaired by Nucleotide Excision Repair (NER), which is orchestrated by a multi-subunit complex of about 20 proteins, including Excision Repair Cross-complementation group 1 (ERCC1). Loss of NER increases the sensitivity of cells to cisplatin, but given the complex nature of this pathway, it is difficult to determine the limiting factor that effectively governs NER activity. In several tissues, ERCC1 is the limiting factor and its reduced expression is correlated with increased progression free survival (PFS) and overall survival (OS) after cisplatin treatment; however, a direct role of ERCC1 in the clinical response to cisplatin has not been demonstrated (reviewed in (63)). Inactivating or destabilizing mutations in any of the NER subunits also leads to increased sensitivity, as shown for mutations in ERCC2 in bladder cancer (64), XPG in NSCLC (65), ERCC1 in several tumor types (66, 67) and mutations in several NER proteins in a subset of epithelial ovarian cancers (68). Given the complexity of the NER pathway, other studies have measured DNA repair activity in peripheral blood cells as a surrogate for total NER activity and correlated that with the response of NSCLC to cisplatin (69). Collectively, these studies suggest an association between NER and the cellular sensitivity to cisplatin and its variants, but have not yielded a singular biomarker for selecting non-responding or responding patients.

Alternatively, DNA breaks are repaired by the Mismatch Repair machinery (MMR), which is also implicated in cisplatin resistance, albeit to a lesser extent. In vitro studies showed that cells deficient in MMR proteins, such as MSH2, are more resistant to cisplatin and carboplatin (but surprisingly not to oxaliplatin), likely because MMR members block efficient error-free repair of inter-strand crosslinks (70, 71). This was

Table 1: Summary of resistance mechanisms towards the different chemotherapeutic classes.

Drug class / gene	Mechanism	Mutation / expression	Effect	Occurrence	Impact	Prognostic factor[1]	Linked to response[2]	Predictive factor[3]
Platinum compounds								
ERCC1/2	Nucleotide Excision Repair	low expression / mutation	sensitizing	frequent	medium	yes	yes	yes, in NSCLC (Kamal et al., 2010)
BRCA1/2	Homologous Recombination	inactivating mutations	sensitizing	frequent	high	yes	yes	
MSH2	DNA mismatch repair	low expression	sensitizing	na	low	yes		yes, in NSCLC (Kamal et al., 2010)
LRRC8d	Imports cisplatin and carboplatin	low expression	resistance	na	low	yes		
Microtubule drugs								
Stathmin	Destabilizes microtubules	upregulation / activation	resistance	na	medium	yes	yes	
Syk	Destabilizes microtubules	upregulation	resistance	upregulated by treatment	medium		yes	
TEKT4	Functional part of microtubules	SNP	resistance	na	medium /low	yes		
CLIP-170	Stabilizes microtubules	low expression	resistance	na	low		yes	
Tau	Competititve binding with taxol	high expression	resistance	na	medium	yes	yes	
Methotrexate								
RCF	importer of MTX	low expression / mutation	resistance	na	high	yes	yes	
DHFR	Functional target	upregulation	resistance	na	high	yes	yes	
FPGS	polyglutamylates MTX for intracellular retention	downregulation / SNPs	resistance	na	low	yes		
Pemetrexed								
TS	Functional target	upregulation	resistance	na	high	yes	yes	yes, in mesothelioma (Righi et al., 2010)
FPGS	polyglutamylates PMX for intracellular retention	downregulation / SNPs	resistance	na	medium /low	yes		
dUTPase	Decreases dUTP pool to alleviate toxicity	upregulation	resistance	na	medium /low			
UDG	Removes misincorporated dUTP	low expression	sensitivity	na	medium /low			
Fluorouracil								
TS	Functional target	upregulation	resistance (most tumours, see text)	na	high	yes	yes	
dUTPase	Decreases dUTP pool to alleviate toxicity	upregulation	resistance	na	medium /low	yes		
DPD	Inactivation of 5-FU by enzymatic conversion	upregulation	resistance	na	low	yes		

Gemcitabine								
hENT1	Importer for gemcitabine	downregulation	resistance	na	high/ medium	yes	yes	yes, in pancreatic cancer (Greenhalf et al., 2014)
RRM1/2	Involved in formation of cytidine	upregulation	resistance	na	medium	yes		
dCK	Converts gemcitabine into active form	downregulation	resistance	na	medium	yes		
Topoisomerase I inhibitors								
Topoisomerase I	Functional target	low expression	resistance	na	medium	yes	yes	
Topoisomerase II inhibitors								
Topoisomerase II	Functional target	amplified	sensitizing	co-amplified with HER2 in breast tumours	low	yes	yes, in some cases	
SWI/SNF complex	Loading and activity of TopoII	Inactivating mutations	resistance	frequently deleted in many tumours	medium		yes	
BRCA1/2	Essential for Homologous Recombination	inactivating mutations	sensitizing	frequently mutated in multiple tumours	high	yes	yes	
Alkylating agents								
CYP2B6	enzymatic activation in the liver	SNP	Less drug exposure	frequent	medium	yes	yes	yes, in leukemia (Johnson et al., 2013)
MGMT	DNA de-alkylation	upregulation	resistance	na	low	yes		
XRCC1	Base excision repair	SNP	resistance	frequent	medium /low	yes		

Abbreviations: na. not applicable because the cohort was analyzed by division into high/low expression. [1] Factors linked to disease-free survival or progression free-survival after treatment with the respective drug. [2] Factors linked to the response towards the drug. [3] Factors for which randomized trials have demonstrated an effect specifically in the patient group treated with the indicated drug. References
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further corroborated in a large NSCLC cohort study demonstrating a correlation between low MSH2 levels and overall survival after cisplatin-based therapy (72). Why oxaliplatin deviated from its platinum family members is unclear, as this drug inflicts similar DNA inter- and intra-strand cross-links.

The third machinery to repair DNA crosslinks is Homologous Recombination (HR), which repairs double-strand breaks (DSBs) arising from inter-strand cross-links. Tumors deficient in HR are highly sensitive to platinum compounds and two HR genes, BRCA1 and BRCA2, are frequently inactivated in breast, ovarian and pancreatic

tumors, either somatic or during the course of treatment. These tumors are very sensitive to cisplatin but often restore HR via a secondary mutation in BRCA1/2 which re-activates the gene (9, 73). Recently, the DNA/RNA helicase SLFN11 was identified as a negative regulator of HR (74) and its loss is associated with cisplatin resistance, as well as topoisomerase (75) and PARP inhibitors (76). Together, these data indicate that patients with defective HR respond favorably to cisplatin, as well as to other drugs that rely on DSB repair. A defective HR is often observed in tumors and may be a useful biomarker for treatment stratification (77).

Alternative mechanisms of drug resistance

Platinum-based drugs must first enter tumor cells and accumulate in sufficient concentrations to target their macromolecular substrate (DNA). Reduced intracellular accumulation has been observed in several cisplatin-resistant cell lines and yielded several uptake and export receptors. Although a substantial part of cisplatin uptake appears to be via passive membrane diffusion (Ivy and Kaplan, 2013), uptake is also facilitated by the volume regulated anion channel (VRAC) subunits LRRC8a and LRRC8d (Planells-Cases et al., 2015), and their loss induces resistance to both cisplatin and carboplatin. Unlike many other anti-cancer drugs, export of cisplatin or its variants from cells is not mediated by P-gp, but probably by MRP2/ABCC2 (Borst et al., 2000), since MRP2 overexpression induces resistance to cisplatin. Other drug resistance determinants that reduce the amount of active platinum-based drugs that form DNA adducts are p22phox, which prevents translocation of cisplatin to the nucleus (Hung et al., 2015), and enzymes that produce nucleophilic species such as GSH, which convert hydroxylated cisplatin into an inactive glutathione-modified molecule (Galluzzi et al., 2012).

Other mechanisms contributing to cisplatin resistance that have recently been uncovered include downregulation of apoptotic factor FHIT (78), which is frequently mutated in tumors (79), upregulation of cell cycle regulator Dyrk1B (80), as well as activation of several signaling pathways, including p38MAPK and AKT1 (reviewed in (81)); though, due to their general nature, these factors are likely involved in resistance to other types of chemotherapeutics as well. While other pathways upstream or downstream of the DDR are also involved in cisplatin sensitivity, their relative contribution seems minor.

Topoisomerase I inhibitors

While platinum drugs directly target DNA, other chemotherapeutics inflict DNA damage by targeting enzymes that unwind DNA. These enzymes are topoisomerase I and topoisomerase II and inhibitors of both enzymes are major drug classes in cancer therapeutics. In normal physiology, topoisomerases are important to resolve topological problems that cells encounter during DNA replication, DNA repair, transcription and chromosomal segregation (Berger et al., 1996; Nitiss, 2009a). Topoisomerase I (Topo I) resolves DNA supercoiling by introducing single-strand breaks, allowing uncoiling followed by re-ligation of the introduced break (Stewart et al., 1998). Several drugs have been developed that target Topo I, with camptothecin and its analogues topotecan and irinotecan being best known in clinical practice (Hsiang et al., 1985; Pommier, 2013). These drugs bind and trap the cleavable complex of Topo I before re-ligation occurs, thereby leading to single-strand DNA breaks. At

lower doses, toxicity of Topo I inhibitors are replication dependent (Holm et al., 1989; Horwitz and Horwitz, 1973), and DNA double-strand breaks can be induced when replication forks collide with the drug-stabilized Topo I complex (Hsiang et al., 1989). In addition, DNA damage is generated during transcription, as Topo I is involved in transcription elongation, when DNA is in an uncoiled, open configuration (Wang, 2002). Since Topo I and Topo II are involved in similar cellular pathways during DNA replication and transcription, drugs targeting these different topoisomerases induce similar patterns of DNA damage at defined regions in the genome (Pang et al., 2015). Given the partial redundancy between both topoisomerases, sequential treatment with Topo I and Topo II inhibitors is currently tested as a strategy to prevent resistance by upregulation of the other topoisomerase (82).

Resistance to topoisomerase I inhibitors

One of the major mechanisms of resistance to Topo I drugs is drug efflux, predominantly via the multidrug efflux transporter ABCG2 (Schellens et al., 2000; Tiwari et al., 2013). Topo I is the only known target of camptothecin drugs, and most resistance mechanisms are centered on Topo I-dependent DNA break formation and repair. Multiple studies have shown that reduced expression of Topo I renders tumor cells resistant to treatment (Burgess et al., 2008; Horisberger et al., 2009; Ikeguchi et al., 2011). This suggests that determining Topo I expression levels could be a biomarker for predicting patients' drug response. In addition, Topo I mutations could confer resistance upon tumor cells to camptothecin (Pommier et al., 1999), though clinical correlations for this are still lacking. Modulators of Topo I activity also control sensitivity to camptothecins, as demonstrated for CK2. This kinase phosphorylates and activates Topo I and its loss desensitizes cells to camptothecin (Bandyopadhyay and Gjerset, 2011; Bandyopadhyay et al., 2012).

Alternatively, resistance can arise from alterations in the DDR pathway, leading to increased DNA repair or failure to induce apoptosis. The main pathway for cells to repair Topo I inhibitor-dependent single-stranded DNA damage is the DNA base repair pathway, involving PARP1, XRCC1, TDP1, among others (83). Indeed, overexpression of XRCC1 promotes resistance to camptothecin (84), whereas TDP1 depletion sensitizes cells (85). Furthermore, PARP1 inhibition was found to synergize with inhibition of Topo I (86). Repair of DNA DSBs induced by camptothecins is orchestrated by HR (87), as DSBs occur in S-phase during replication, and cells deficient in HR are more sensitive to Topo I inhibitors.

All mechanisms described herein await clinical validation, which is complicated by the fact that Topo I inhibitors are usually part of a multidrug regimen that also involves Topo II inhibitors or platinum drugs, which also rely on induction of DNA damage.

Topoisomerase II inhibitors

Like its counterpart, topoisomerase II inhibitors are an important class of chemotherapeutic agents. By introducing a DNA double-strand break, topoisomerase II unwinds the DNA to allow one DNA strand to pass another, followed by re-ligation of the broken strand. Topo II poisons block Topo II in its active form, when the transient DNA double-strand break is formed but before DNA ligation, yielding toxic DNA double-strand breaks. Since proliferating cells like cancer cells frequently have high levels of topoisomerase IIα (the most prevalent of the two isoforms) to adapt to more

active DNA replication and transcription (88, 89), cancer cells are supposedly more susceptible to inhibition by Topo II inhibitors than normal cells.
The best known class of Topo II inhibitors is the anthracycline family. Originally discovered as an antibiotic produced by Streptomyces bacteria, a striking anti-tumor activity was noticed for daunorubicin (Daun), which transformed its use into an anti-cancer drug. Subsequent mutagenesis of the daunorubicin-producing bacteria resulted in a superior analogue with minor chemical differences, named adriamycin (after the Adriatic Sea, only a few kilometers from where the producing strain was originally isolated) but later re-named doxorubicin (Doxo). Doxo is the most effective and broadly used member of the anthracycline family and it is used as a first line chemotherapeutic for a broad spectrum of cancers (90-93). Other structurally unrelated drugs have also been developed that target Topo II, among which Etoposide (Etop), a derivate of podophyllotoxin, is most frequently used in cancer chemotherapy. Interestingly, while Doxo and Etop share the same molecular target and induce DNA double-strand breaks in an almost identical manner, Doxo is more effective and has a broader anti-tumor spectrum (93). In fact, Doxo is used as monotherapy for some tumors (for instance breast cancer), whilst Etop is usually used in combination therapy. The difference between these drugs could be the consequence of a secondary effect of the anthracyclines; eviction of histones from the DNA (94, 95). Histone eviction by anthracyclines attenuates DNA repair by removal of histone H2AX, which is critical in the initiation of the DDR, thereby leading to prolonged DNA damage and enhanced p53 activation (96). Furthermore, histone eviction erases epigenetic marks based on histone modifications and alters the transcriptional landscape. Such effects disturb the normal cellular physiology and can also contribute to the cytotoxicity of this class of cytotoxic drugs. Different anthracycline drugs display selectivity for different histone modifications or chromatin structure, as Doxo and Daun prefer active chromatin regions marked by H3K4me3 and H3K36me3, whilst Acla (aclarubicin, an anthracycline that evicts histones but does not generate DNA breaks) also targets repressive chromatin regions decorated with H3K27me3 (96). Intercalation of Doxo into the DNA is sufficient to induce histone eviction and does not require active machinery, therefore identified resistance mechanisms against anthracyclines and etoposide are centered mostly around Topo II-mediated DNA break formation and repair (97).

Resistance to topoisomerase II poisons

The treatment of cancer patients with anthracyclines is limited by the cardiotoxic side effects that accumulate with increasing doses. This implies that many patients do not receive further treatment due to cardiotoxicity, whereas they still would have responded to the drug. Yet, patients can become resistant to Topo II poisons. The best studied resistance mechanism for both Doxo and Etop is drug efflux by the ABC transporter P-gp. Whereas in mice P-gp is certainly important in imparting Doxo resistance, its role in human cancer patients is less clear. Transport inhibitors of P-gp have not shown any clinical benefit for cancer patients treated with Doxo (34), suggesting that other mechanisms of resistance may be more prominent. Being a weak base that undergoes protonation in the highly acidic lumen of lysosomes, anthracyclines can also be sequestered in lysosomes (98-101), which effectively decreases nuclear drug exposure and efficacy. Lysosomal alkalinization achieved by treatment

with bafolimycin A1, which blocks vesicular ATPase, prevents this drug accumulation in lysosomes, restoring drug sensitivity (100).
Although Doxo is a Topo II inhibitor, the role of this enzyme in Doxo resistance is not unequivocal. Many reports have shown that in tissue cultured tumor cells or mouse models, decreased Topo II levels lead to Doxo resistance (102). Furthermore, clinical studies showed that HER2 amplified breast tumors with co-amplification of the neighboring Topo II gene are more sensitive to treatment with anthracyclines (103). However, in other tumor types, this correlation was not confirmed (104), suggesting that merely in the case of Topo II gene amplification there is a difference in sensitivity. In some cases, even tumor cells without detectable Topo IIα expression responded to Doxo (94). A potential explanation for this apparent discrepancy is the redundancy between Topo IIα and Topo IIβ, or alternatively additional regulators of Topo II activity are important determinants of Doxo sensitivity. The latter is supported by a recent genome-wide analysis of factors contributing to Doxo-resistance. This study identified the SWI/SNF complex, a chromatin remodeling complex that loads Topo II onto DNA and which is frequently mutated in cancer, as a factor mediating resistance to Doxo and Etop (97). Clinically, tumors with deletion or downregulation of this complex displayed an inferior response to Doxo-containing treatments, supporting the notion that factors controlling Topo II activity can influence sensitivity to Doxo. By using short incubation times with Doxo to recapitulate the clinical situation (mimicking the pharmacokinetics in patients), this screen also identified Keap1 and C9orf82/CAAP1 as factors involved in resistance to Topo II poisons. These respectively act by modulating Topo II poisoning and accelerating DNA repair. Other factors recently shown to be involved in resistance are regulators of homologous recombination (as for platinum-drugs and Topo I inhibitors), cell survival signaling, such as a pathway activating AKT1 that involves E2F7, Sphk1 and RacGAP1 (105, 106), DNA repair regulator FOXM1 (107) and inhibitor of apoptosis p57kip2 (108). Thus, resistance is analogous to other drug classes and centers around the control of DNA damage and subsequent repair.
Drug resistance to Topo II inhibitors could also be potentially averted by the use of different anthracycline analogues. For example, Acla does not induce DNA breaks and is not susceptible to at least some of the mechanisms contributing to Doxo resistance, including mutations in the SWI/SNF complex (97). This is confirmed in AML patients, where identical responses to an Acla-containing regimen were observed in patients refractory to Doxo/Daun-based chemotherapy versus chemo-naive patients, suggesting resistance to Doxo/Daun does not induce resistance to Acla (109). Furthermore, a fraction of diffuse large B-cell lymphomas harbors mutations in EZH2, which increases its H3K27me3 levels and renders the tumor cells more susceptible to Acla, which evicts those marked histones (96). It is compelling that in some instances deletion of the SWI/SNF complex increases H3K27me3 levels (110), suggesting that cells might actually become more sensitive to Acla. Elucidating the contribution of histone eviction to the overall anti-cancer effects of Doxo might thus yield novel treatment options to overcome resistance to anthracyclines.

Alkylating agents

Alkylating agents such as cyclophosphamide and ifosfamide are used mainly in com-

bination treatments for several malignancies. Synthesized as pro-drugs, they are bio-activated by the liver into their active nitrogen mustard moiety, which interacts with the DNA guanine base. The alkylated DNA can subsequently form intra-strand and inter-strand cross-links, leading to a DDR and subsequent cell death in a manner similar to platinum-based drugs (111).

Resistance to alkylating agents

Clinically, resistance to alkylating agents is poorly understood and few factors are correlated to response, possibly because cyclophosphamide is often administered in conjunction with other DNA damaging agents. The molecular determinants known to contribute to resistance center around its enzymatic activation and DNA repair following DNA alkylation. Enzymatic activation in the liver is orchestrated by the Cytochrome p450 family, and SNPs within this family, as well as liver expression levels of CYP2B6 have been linked to response (112, 113). On the other hand, CYP2B6 is also linked to the adverse effects observed from alkylating agents. Since higher p450 activity increases the conversion of the prodrug into the active form, CYP2B6 expression is better used as a biomarker for the dose that should be given to the patient, since higher p450 activity increases the total body exposure to the drug and hence untoward toxicity.

Upon hydroxylation, alkylating drugs diffuse into cells presumably via a flip-flop mechanism and can be inactivated by the action of aldehyde dehydrogenases, most notably ALDH1. Indeed, high ALDH1 levels correlate with a poor response to cyclophosphamide in breast cancer (114). However, ALDH1 is a marker for cancer stem cells and associated with the response to several chemotherapeutics, suggesting that ALDH1 is not a specific marker for response to these drugs (reviewed in (115)). Alkylating agents can also be modified by glutathione, but it is unclear what the clinical relevance of this pathway is (113). In addition, P-gp (ABCB1) or MRP2 (ABCC2) can be involved in clearing the drug from cells (113, 116).

Upon DNA alkylation, several repair mechanisms are involved in the restoration of DNA integrity. Direct de-alkylation of DNA is mediated by the enzyme MGMT, thus ablating the effect of alkylation and preventing cytotoxicity. Clinically, a correlation between MGMT expression and treatment outcome was found in some tumor types, which could not be confirmed in other tumors (117, 118). Cells can also repair DNA by removal of the alkylated guanine base, via the BER, NER or MMR pathways. Polymorphisms in XRCC1, involved in the BER pathway, are linked to survival after cyclophosphamide-containing treatment in several tumors (119, 120). Thus far, the NER and MMR pathways have not been associated with resistance to alkylating anti-cancer drugs. In summary, alkylating agents modify DNA and alterations in DNA repair appear to contribute to resistance to these drugs. MGMT and XRCC1 are the most promising markers, but convincing clinical validation is still lacking.

Antimetabolites

Another class of anti-cancer drugs, the antimetabolites, inhibits the formation of purine and pyrimidine nucleotides, effectively blocking DNA replication of rapidly proliferating cells. These drugs mimic endogenous reduced folates metabolites and act by either inhibiting enzymes essential in the biosynthesis of nucleotides, or by mimicking nucleotides to get incorporated into DNA or RNA, hereby frustrating repli-

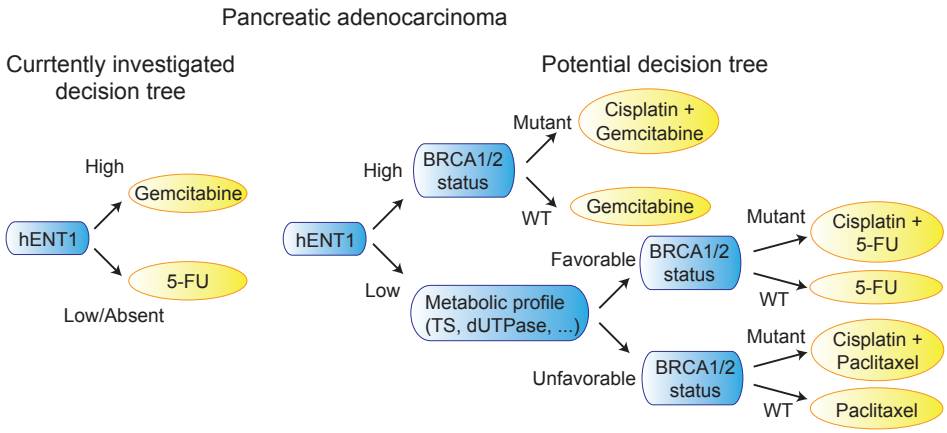

Figure 4: Potential stratification strategy for treatment decisions with conventional anti-cancer drugs. *A predictive factor for sensitivity to Gemcitabine in pancreatic cancer is the expression level of transporter hENT1. Tumors with low expression of hENT1 respond better to 5-FU treatment, while tumors with high expression respond favorably to Gemcitabine. BRCA1/2 mutations in pancreatic tumors and breast cancer can also be determined as they render tumors more susceptible to cisplatin treatment. The expression of Thymidylate Synthase, dUTPase or other factors potentially involved in resistance to 5-FU can be determined to select patients that will likely respond to 5-FU. Most mechanisms require validation in prospective studies.*

cation and inducing cell death (3). The first class is represented by the antifolate pioneer drug methotrexate (MTX), together with pemetrexed (PMX) and more recently pralatrexate, which inhibit the key enzymes in folate metabolism dihydrofolate reductase (DHFR) and thymidylate synthase (TS), both of which are important for purine and thymidine biosynthesis (26). Of the fluoropyrimidine nucleotide analogues, 5-fluorouracil (5-FU, and its pro-drug capecitabine) as well as gemcitabine are most commonly used. 5-FU is a thymidine analogue that blocks TS and gets incorporated into DNA, whereas gemcitabine is a cytidine analogue for which its incorporation into DNA is the main mode of antitumor activity.

Drug resistance mechanisms

Resistance to antimetabolites is frequently observed; the best documented resistance mechanism involves increased expression or mutation of the drug target. For 5-FU and PMX this means upregulation of TS, for which a prognostic role has been established in many different tumors. In the case of MTX, overexpression or mutations in its target DHFR affect drug sensitivity (reviewed in (3, 26)). However, in some settings and tissues high expression of TS can actually be beneficial for response to 5-FU, probably because cells with a higher expression of TS are metabolically more active and thus more vulnerable to inhibition of DNA building block synthesis (121).

Similar to other drugs, cancer cells can reduce import or increase export of the antimetabolites. 5-FU likely diffuses passively over the membrane, while MTX, PMX and pralatrexate are actively imported into cells, with MTX and pralatrexate predominantly transported by the reduced folate carrier (RFC) and PMX transported by both RFC and the proton-coupled folate transporter (PCFT), the latter one with higher affinity. RFC expression or polymorphisms are correlated to response to MTX in several tumor types (122-126), while for PMX a correlation between response

and mutation/expression of either RFC1 or PCFT has been less clear, probably due to the redundancy between the two transporters (3). Drug export can be mediated by several multidrug efflux transporters, including ABCC1-5, 11 and ABCG2, but no clinical relation has been established between any of these transporters and treatment outcome (26). Upon cellular entry, MTX, PMX and pralatrexate undergo polyglutamylation by folylpoly-gamma-glutamyl synthetase (FPGS), resulting in enhanced retention of these antifolate polyglutamates as they are no longer substrates for efflux transporters (127). As such, decreased FPGS expression, inactivating FPGS mutations as well as upregulation of γ-glutamyl hydrolase (GGH), which removes the polyglutamate tails, reduces sensitivity of cells to these drugs (128, 129). Clinically, high levels of polyglutamate-MTX are indeed correlated with enhanced drug responses (130, 131). Similar correlations with polymorphisms and increased expression of FPGS are also established (132, 133). For GGH, an effect on intracellular drug levels is observed but a clinical correlation to treatment outcome has only been established for a single polymorphism (134). This could be because GGH also modifies endogenous folates and its overexpression induces their export, reducing the production of DNA bases (3). Other factors influencing the response to antifolates are the levels of their natural reduced folate cofactor competitors. Increased reduced folate levels has been shown to induce resistance to MTX and PMX in vitro and in mouse models (135-138), however, a clinical correlation has not been established yet.

Mechanisms specific to TS inhibitors (5-FU and PMX) are related to dUTP metabolism, as blockade of TS leads to depletion of dTTP and accumulation of dUMP, which can be further phosphorylated to dUTP, shifting the nucleotide pool from dTTP to dUTP. This leads to the misincorporation of dUTP into DNA, which is subsequently repaired by the BER pathway. Loss of uracil–DNA glycosylase (UDG), responsible for the removal of dUTP incorporated into DNA, sensitizes cells in vitro to PMX, and to some extent to 5-FU (139, 140). High levels of dUTPase protect cells from 5-FU and PMX exposure by decreasing the concentration of dUTP, and this has been associated with poor treatment outcome in colorectal cancer (141-144).

5-FU resistance is also associated with decreased expression of genes involved in its conversion to the active metabolite FdUMP, namely thymidine kinase (TK), uridinemonophosphate kinase (UMPK) and orotate phosphorylase transferase (OPRT) (145), as well as overexpression of dihydropyrimidine dehydrogenase (DPD), which converts 5-FU into an inactive metabolite, and thymidine phosphorylase (TP), which opposes TK activity (26). The levels of TK, TP and DPD have been clinically correlated with treatment outcome, but results are conflicting and suggest a minor contribution for these enzymes in resistance against 5-FU (26, 146, 147).

Gemcitabine resistance relies on similar principles as the other antimetabolites. Cellular uptake of gemcitabine predominantly proceeds via the nucleoside transporters hENT1, hCNT1 and hCNT3 (148) of which low hENT1 has been correlated with poor overall survival of pancreatic patients after gemcitabine treatment (149-151). Similar results were obtained in other cancer types (152). No clinical correlation has been established for hCNT1, while a correlation between expression of hCNT3 and favorable responses in pancreatic cancer has been reported in one study (153). This suggests that hENT1 is likely the dominant influx transporter of gemcitabine.

Following uptake, the rate-limiting step for the conversion of gemcitabine into its active phosphorylated form is deoxycytidine kinase (dCK), whose expression has been linked to therapy response in several studies and tumor types (154). Before phosphorylation, gemcitabine can be converted by cytidine deaminase (CDA) into a metabolite that is secreted from cells, and high levels of CDA have been clinically linked to an unfavorable response. However, most CDA is produced in the liver and patients with low CDA levels generally also suffer from more side effects following gemcitabine treatment (155). Whether CDA can be used in individualized patient selection and/or whether the dose of gemcitabine should be adjusted to the patient's CDA levels remains unclear. Other enzymes involved in the conversion of gemcitabine are NMPK, NDPK and 5'-Nucleotidase, but no correlation between these enzymes and drug resistance has been documented in the clinic (29). Gemcitabin's active form dFdCTP competes with cytidine during incorporation into DNA. Cellular levels of cytidine are maintained by ribonucleotide reductase (RR). Upregulation of its two subunits RRM1 and RRM2 accelerates cytidine synthesis to compete with gemcitabine incorporation thus resulting in gemcitabine resistance, both under in vitro and in vivo conditions. Several studies have reported altered efficacy of gemcitabine in patients harboring SNPs or altered expression of RRM1, while other groups have failed to make these associations (156-158).

Out of all anticancer drug classes, the existence of predictive resistance markers has been most clearly demonstrated for the antimetabolites. A number of resistance factors either act at the level of intracellular accumulation or at the level of DNA base synthesis and incorporation. Currently, treatment of pancreatic patients on the basis of hENT1 expression is being evaluated in a clinical trial (159), rendering it the first stratification factor tested for conventional chemotherapy (Figure 4).

Anti-microtubule agents

This last class of conventional anti-cancer drugs targets another machinery essential for rapidly proliferating cells - the microtubules. Microtubules are critical for cytoskeletal structure, signaling and transport, as well as chromosome segregation during cell division. Dividing cells are very sensitive to cytoskeletal perturbations and hence multiple drugs have been developed that interfere with microtubule dynamics. These so-called spindle poisons bind β-tubulin and either stabilize microtubules (for example taxanes and epothilones), or destabilize microtubules (the Vinca alkaloids). Both drug classes act primarily by preventing proper spindle formation, thereby activating the spindle checkpoint, leading to a mitotic arrest. A prolonged arrest activates the apoptotic pathway or alternatively induces mitotic slippage, where cells exit the cell cycle without undergoing cell division (Kavallaris, 2010). Given their similar mechanism of action, many resistance mechanisms described are shared between both classes of spindle poisons (Dumontet and Jordan, 2010; van Vuuren et al., 2015).

Drug resistance mechanisms

Several mechanisms mediate resistance to tubulin-binding agents (TBAs), some of which are shared with other anti-cancer drug classes, like export via P-gp and upregulation of anti-apoptotic pathways, while others are more specific for the anti-microtubule agents, like mutations in β1-tubulin and overexpression of microtubule-associated proteins (MAPs).

β1-tubulin is a direct target for anti-microtubule drugs and multiple mutations have been identified that diminish drug sensitivity (Giannakakou et al., 1997; Hari et al., 2006; Yin et al., 2010), reviewed in (Kanakkanthara et al., 2013). Two types of mutations can be distinguished: mutations that alter drug binding, thus specific for a defined class of TBAs; and mutations that alter the general stability of microtubules. The latter destabilize microtubules to counteract the stabilization induced by taxanes (like paclitaxel), but sensitize cells to microtubule destabilizing drugs like the Vinca alkaloid vinblastine. These mutations are not germline mutations but acquired during treatment and thus difficult to use for patient stratification for treatment. Although resistance through β1-tubulin mutations is only tested in a tissue culture setting, several of these mutations are also found in tumors (Yin et al., 2010). However, the mutation frequency in β1-tubulin is low (<2%) (Gao et al., 2013), disfavoring the diagnostic value of such mutations.

An alternative escape route for cancer cells is to increase the expression of different β-tubulin isoforms, especially βIII-tubulin (Kavallaris, 2010). βIII-tubulin likely inhibits apoptosis via the PIM1-BCL2 mitochondrial pathway (160), contributing to a survival benefit for the tumor and a more general resistance to multiple types of chemotherapeutics (24, 161). Thus, βIII-tubulin acts as a general activator of anti-apoptotic pathways and not a specific factor that can predict responses to certain TBAs.

Apart from microtubules, microtubule associated proteins (MAPs) also influence microtubule dynamics and interfere with drug binding. For example, the Tau protein occupies the same binding site as taxanes (Kar et al., 2003), and de-sensitizes cells to paclitaxel (Rouzier et al., 2005). In vitro studies also demonstrated a role for mitotic centrosome associated kinase (MCAK) (Ganguly et al., 2011) and stathmin in sensitivity to paclitaxel (Alli et al., 2002), as well as FOXM1, a transcription factor regulating stathmin expression (Li et al., 2014a; Zhao et al., 2014). Clinically, the correlation between Tau and tumor sensitivity to paclitaxel has been confusing, with several studies validating an inverse correlation between response to paclitaxel and expression of Tau (Smoter et al., 2013; Werner et al., 2014), while others reported no significant impact of Tau (Bonneau et al., 2015). An inverse correlation between response to paclitaxel and (phosphorylated) stathmin expression has been demonstrated in several tumor types (Kuang et al., 2015; Werner et al., 2014), but it is unclear whether stathmin is a general prognostic factor or controls the actual response to paclitaxel under clinical conditions. Alternatively, cells can upregulate the expression of SYK kinase upon paclitaxel treatment to destabilize microtubules, likely by phosphorylating tubulin and several MAPS, which affects the effectivity of paclitaxel in recurrent ovarian carcinoma treatment (Yu et al., 2015). A different study has demonstrated that two germline variations in TEKT4 can destabilize microtubules and impair outcome (Jiang et al., 2014). Thus, several MAPs seem to be involved in regulating resistance to paclitaxel.

Translating microtubule deregulation to a cell death program is mediated by the spindle activation checkpoint (SAC), which in turn is activated by the chromosomal passenger complex (CPC) (Carmena et al., 2012). Downregulation of the SAC-members MAD2 and BubR1 (Furlong et al., 2012; Sudo et al., 2004), as well as overexpression of Aurora A, which overrides SAC activation (Anand et al., 2003), induces resistance to paclitaxel. Conversely, downregulation of CPC member Aurora B leads

to resistance, probably by defective targeting of MAD2 and BubR1 to the mitotic spindle (Ditchfield et al., 2003). In contrast to this, upregulation of survivin, also part of the CPC, leads to resistance to several TBAs. Survivin upregulation also leads to resistance to other chemotherapeutics such as doxorubicin and cisplatin, probably via its function as an anti-apoptotic factor (Zaffaroni et al., 2002). For several cancer types, such as breast cancer and NSCLC, high survivin levels are correlated with disease progression and poor response to chemotherapy (Huang et al., 2013; Li et al., 2014b). More mitotic factors have been recently linked to paclitaxel sensitivity, like PDCD4 (Xu et al., 2015), CASC1 and TRIM69 (Sinnott et al., 2014).
Since cells die by apoptosis in response to TBAs, deregulation of survival signaling pathways plays an important role in chemosensitivity. This has been demonstrated for the Keap1-Nrf2 oxidative stress signaling pathway (Leinonen et al., 2014), HER2 signaling (Knuefermann et al., 2003), Hippo signaling via TAZ (Lai et al., 2011), NFκb signaling (Kelly et al., 2006; Wee et al., 2015), and FAK1/YB-1 signaling (Kang et al., 2013). These pathways will not allow patient stratification, but inhibition of the mutated pathway enhances the efficacy of anti-microtubule agents, as demonstrated for the combination of HER2 inhibition (trastuzumab) and paclitaxel (Schramm et al., 2015).
Resistance to microtubule agents revolves around microtubule dynamics and translation of altered dynamics into a cell death program. Clinically, few factors have been unequivocally linked to clinical responses but the current evidence points towards the MAPs as the most likely prognostic factors for TBA resistance in patients.

Conclusions

Development of resistance is one of the major reasons for treatment failure of anti-cancer drugs. Over the years, a multitude of mechanisms have been identified that are employed by tumors to decrease their sensitivity to chemotherapy. Unfortunately, translation of these findings into personalized selection of patients for such drugs has proven difficult. Several reasons underlie this poor translation:

1. Most resistance mechanisms are initially identified in tissue culture systems and mouse models. Validation of these factors requires extensive prospective studies which take time and effort, delaying implementation. Large clinical datasets correlating response to gene expression can bridge the gap between the lab and clinic and used to filter out the most promising factors. Yet, a double blind prospective study remains essential for acceptance of prognostic markers for cancer therapy decisions.
2. Cancer treatment is often a multidrug treatment and resistance mechanisms to a combination therapeutic regimen are poorly understood.
3. Resistance mechanisms may not be present prior to treatment but acquired through selection under the pressure of chemotherapy. Only germline resistance mechanisms can be monitored for treatment decisions. Acquired resistance mechanisms may be avoided by searching for mechanisms of synthetic lethality and drugs (alone or in combination) that selectively act on the resistant tumor cells.
4. Tumors are heterogeneous and a small subpopulation of cells can harbor a mutation that renders them resistant. These cells will eventually become the recurrent tumor. Alternatively, cells that are intrinsically more resistant to chemotherapy,

such as cancer stem cells (162) and cells that underwent EMT (163, 164), grow out to re-form a tumor (Figure 3).

5. Tumor extrinsic factors such as the microenvironment (165) and even the microbiome (166, 167) can contribute to drug resistance, by producing growth factors or modulating the immune system. These factors are usually not included in studies focusing on drug resistance.

Despite these serious impediments, the first trial with personalized medicine for conventional chemotherapeutics is being set-up, likely followed by many others. The identification and validation of other resistance factors will yield leads that potentially influence the response to individual drugs, resulting in a multi-step decision process to select the optimal treatment of choice (see example in Figure 4). These biomarkers will hopefully aid the development of better and more optimized treatments for the individual cancer patient.

Acknowledgements

This work was supported by the Institute for Chemical Immunology, an NWO Gravitation project funded by the Ministry of Education, Culture and Science of the government of the Netherlands, a grant from the Dutch Cancer Society KWF, an NWO TOP grant and an ERC Advanced grant to J.N.

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