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Review Article

Molecular mechanisms of drug resistance

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Abstract

Resistance to chemotherapy limits the effectiveness of anti-cancer drug treatment. Tumours may be intrinsically drug-resistant or develop resistance to chemotherapy during treatment. Acquired resistance is a particular problem, as tumours not only become resistant to the drugs originally used to treat them, but may also become cross-resistant to other drugs with different mechanisms of action. Resistance to chemotherapy is believed to cause treatment failure in over 90% of patients with metastatic cancer, and resistant micrometastic tumour cells may also reduce the effectiveness of chemotherapy in the adjuvant setting. Clearly, if drug resistance could be overcome, the impact on survival would be highly significant. This review focuses on molecular mechanisms of drug resistance that operate to reduce drug sensitivity in cancer cells. Drug resistance can occur at many levels, including increased drug efflux, drug inactivation, alterations in drug target, processing of drug-induced damage, and evasion of apoptosis. Advances in DNA microarray and proteomic technology, and the ongoing development of new targeted therapies have opened up new opportunities to combat drug resistance. We are now able to characterize the signalling pathways involved in regulating tumour cell response to chemotherapy more completely than ever before. This will facilitate the future development of rational combined chemotherapy regimens, in which the newer targeted therapies are used in combination with cytotoxic drugs to enhance chemotherapy activity. The ability to predict response to chemotherapy and to modulate this response with targeted therapies will permit selection of the best treatment for individual patients.

Keywords: chemotherapy; cancer; drug resistance

Introduction

Drug resistance is a major problem that limits the effectiveness of chemotherapies used to treat cancer. Tumours may be intrinsically resistant to chemotherapy prior to treatment. However, drug resistance can also be acquired during treatment by tumours that are initially sensitive to chemotherapy. A frustrating property of such acquired resistance is that the tumours not only become resistant to the drugs originally used to treat them, but may also become cross-resistant to other drugs with different mechanisms of action. Drug resistance, whether intrinsic or acquired, is believed to cause treatment failure in over 90% of patients with metastatic cancer, and resistant micrometastic tumour cells may also reduce the effectiveness of chemotherapy in the adjuvant setting. Clearly, if drug resistance could be overcome, the impact on survival would be highly significant.

There are many factors that affect drug sensitivity. These include mechanisms such as those that limit the amount of drug reaching the tumour and those affecting the tumour micro-environment. Here we will provide an overview of cancer cell-specific mechanisms of drug resistance and highlight examples that have clinical relevance. Cancer cell resistance to chemotherapy can occur at many levels, including increased drug efflux and decreased drug influx; drug inactivation; alterations in drug target; processing of drug-induced damage; and evasion of apoptosis (Figure 1).

Drug influx and efflux

The mechanism by which many chemotherapeutic drugs are taken up by cells is unknown. However, it has been well established that folate pathway inhibitors (anti-folate drugs) such as the dihydrofolate reductase (DHFR) inhibitor methotrexate (MTX) and the thymidyate synthase (TS) inhibitor tomudex (TDX) enter the cell predominantly via the reduced folate carrier (RFC) [1,2]. Decreased expression of the RFC and inactivating mutations are documented mechanisms of MTX resistance [3,4]. A recent study has indicated that the cellular uptake of MTX by the RFC may play an important role in determining response to this agent [5]. Children with chronic myeloid leukaemia (CML) with a particular *RFC* genotype (80AA) had significantly higher plasma MTX levels, suggesting decreased drug uptake, and had a significantly shorter event-free survival compared with those

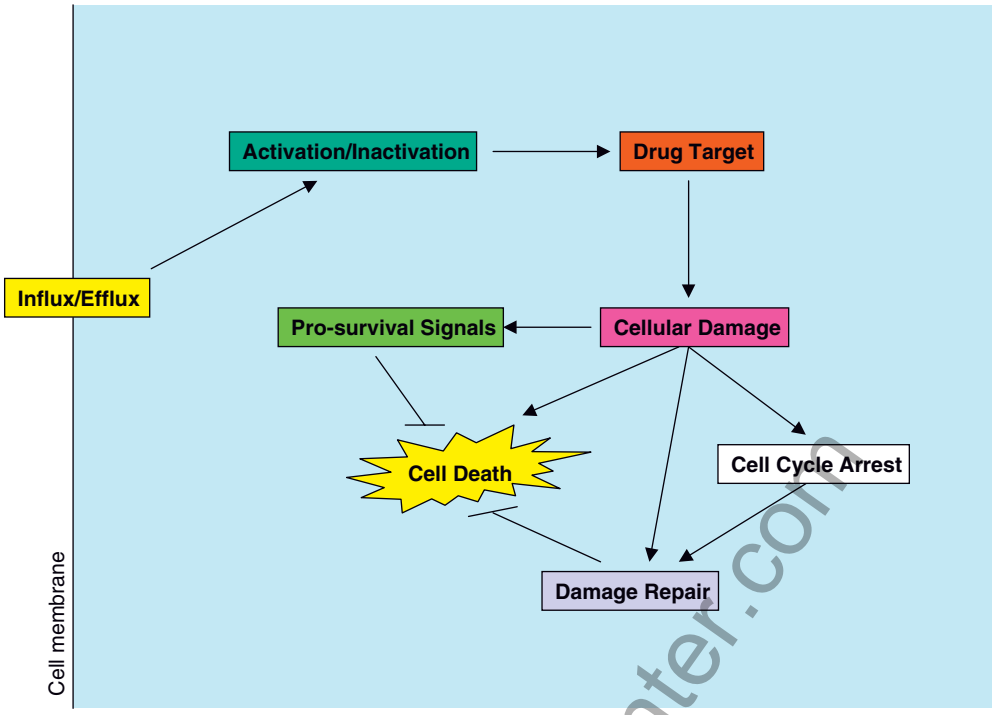

Figure 1. Overview of cancer-cell-specific mechanisms of drug resistance

with a different *RFC* genotype (80GG). Impaired uptake of TS-targeted anti-folates by the RFC has also been demonstrated to be a resistance mechanism to this agent in cell line models [6], suggesting that impaired uptake by the RFC may constitute an important general resistance mechanism to anti-folates.

Alterations in drug efflux due to ABC transporter proteins such as P-glycoprotein (P-gp) and multi-drug resistance protein (MRP) have been demonstrated *in vitro* [7,8]. These proteins can actively transport drugs out of cells and particularly target natural hydrophobic drugs such as taxanes, anthracyclines, and vinca alkaloids [9,10]. Furthermore, expression of these proteins has been reported to correlate with resistance to chemotherapy *in vitro*. The DNA topoisomerase I (topo-I) inhibitor irinotecan (CPT-11) and its active metabolite SN-38 are also targets for ABC transporter proteins, as are cisplatin and methotrexate [9]. Multi-drug resistance has most often been linked to overexpression of P-gp, which is overexpressed in many drug-resistant cell lines and in a number of leukaemias and solid tumours [9,11]. This has led to the development of P-gp inhibitors. The first generation of P-gp inhibitors such as cyclosporin were limited by unacceptable toxicity, while second-generation agents such as vaslapodar (PSC-833) had unpredictable pharmacokinetic interactions and also targeted other transporter proteins [9,11]. However, the third generation of P-gp inhibitors such as tariquidar and zosuquidar have high potency and specificity for P-gp, and are being tested in phase III clinical trials in conjunction with chemotherapy to determine whether P-gp inhibition can restore, enhance or prolong drug sensitivity [12,13].

Drug inactivation

Mechanisms that inactivate drugs can diminish the amount of free drug available to bind to its intracellular target. More than 80% of the anti-metabolite 5-fluorouracil (5-FU) is normally catabolized by dihydropyrimidine dehydrogenase (DPD), primarily in the liver [14]. *In vitro* studies have demonstrated that DPD overexpression in cancer cell lines confers resistance to 5-FU [15]. Furthermore, high levels of DPD mRNA expression in colorectal tumours have been shown to correlate with resistance to 5-FU [16], presumably reflecting greater DPD-mediated degradation of 5-FU in these tumours.

The formation of conjugates between the thiol glutathione (GSH) and platinum drugs such as cisplatin, carboplatin, and oxaliplatin is a key step in the inactivation of these drugs [17]. GSH is a powerful antioxidant, which inhibits oxidative stress that can damage DNA and RNA. Platinum drugs become covalently linked to GSH and the resulting complex is a substrate for ABC transporter proteins, promoting drug efflux [18]. High levels of GSH have been found in tumour cells resistant to platinum drugs [19]. The process of GSH conjugation is catalysed by the glutathione-S-transferase (GST) family of enzymes, and high levels of the GST-π sub-type have been reported to correlate with resistance to cisplatin in ovarian cancer cells [20,21] and tumours [22]. Furthermore, a low level of GST-π has been correlated with improved overall survival following cisplatin treatment of head and neck cancer [23]. Other enzymes involved in GSH synthesis such as γ-glutamylcysteine synthetase (γ-GCS) and γ-glutamyltransferase (γ-GT) may also play a role

in regulating platinum drug resistance [24]. Inactivation of platinum drugs may also occur through binding to the small, sulphur-rich thiol metallothionein (MT) proteins. Some *in vitro* studies have demonstrated a correlation between MT expression and cisplatin sensitivity [25,26]; however, other studies failed to find a correlation [27]. Furthermore, Murphy *et al* found no correlation between MT levels and response to platinum-based chemotherapy in ovarian tumours [28], so the contribution of MT to clinical platinum drug resistance is not clear. In an attempt to bypass inactivation by GSH and MT, a platinum analogue, ZD0473, has been developed that avoids binding to these cytoplasmic thiols [29].

CPT-11 can be inactivated by cytochrome P450 enzymes and its active metabolite SN-38 is a target for glucuronidation by uridine diphosphogluronysl transferase 1A1 (UGT1A1) [30]. An *in vitro* study by Cummings *et al* concluded that increased drug clearance regulated by glucuronidation may contribute to CPT-11 resistance [31], and polymorphisms in the *UGT1A1* gene have been associated with CPT-11 toxicity [32], suggesting an important clinical role for SN-38 glucuronidation.

Decreased drug activation can also play an important role in drug resistance. Drugs such as 5-FU and CPT-11 must be converted to their active metabolites to exert their anti-cancer effects. CPT-11 is converted to SN-38 by carboxylesterase (CE) [30], and several *in vitro* studies have indicated that the level of CE activity in cancer cells is an important determinant of CPT-11 sensitivity [33,34]. The activation of 5-FU to its active metabolites is complex, and the levels of 5-FU activating enzymes such as thymidine phosphorylase (TP), uridine phosphorylase (UP), and orotate phosphoribosyl transferase (OPRT) have been associated with 5-FU sensitivity [35–37]. Anti-folate drugs such as MTX and TDX are polyglutamated by folylpolyglutamate synthase (FPGS), which increases their cellular retention and substrate-binding affinity [38]. Decreased polyglutamation has been observed as a resistance mechanism to anti-folates [6,39].

Drug targets

Alterations in expression levels or mutation of a chemotherapeutic drug target can have a major impact on drug resistance (Figure 2). The 5-FU metabolite fluorodeoxyuridine monophosphate (FdUMP) is a potent inhibitor of TS, and it is the inhibition of TS that is believed to be the primary anti-cancer activity of 5-FU [40]. Numerous pre-clinical studies have demonstrated that TS expression is a key determinant of 5-FU sensitivity [41]. Furthermore, immunohistochemical and reverse transcriptase-PCR studies have shown improved response rates to 5-FU-based chemotherapy in patients with low tumour TS expression [42,43]. More recently, genotyping studies have found that patients homozygous for a particular polymorphism in the *TS* promoter (TSER3/TSER3) that increases TS expression are less likely to respond to 5-FU-based chemotherapy than patients who are heterozygous (TSER2/TSER3), or homozygous for the alternative polymorphism (TSER2/TSER2) [44]. Collectively, these studies indicate that high TS expression correlates with increased 5-FU resistance. Treatment with 5-FU has been shown to acutely induce TS expression in both cell lines and tumours [45,46]. This induction of TS seems to be due to inhibition of a negative feedback mechanism in which ligand-free TS binds to its own mRNA and inhibits its own translation [47]. When stably bound by FdUMP, TS can no longer bind its own mRNA and suppress translation, resulting in increased protein expression. This constitutes a potentially important resistance mechanism, as acute increases in TS would facilitate recovery of enzyme activity. *In vitro* studies have demonstrated that acute increases in TS expression induce resistance to TS inhibitors such as 5-FU, TDX, and the multi-targeted anti-folate (MTA, Alimta) [48,49].

The CPT-11 active metabolite SN-38 is a potent inhibitor of DNA topoisomerase-I (topo-I) [30]. Topo-I relaxes super-coiled double-stranded DNA in front of replication forks by inserting a nick in one strand of the DNA double helix, passing the other strand through the cleavage site and re-ligating the cleaved strand. SN-38 stabilizes the complex between topo-I and the cleaved DNA, thereby inhibiting the re-ligation of the single-strand break. It has been hypothesized that collision of the DNA replication fork with the stabilized topo-I/DNA complex converts these single-strand breaks into more cytotoxic double-strand breaks [50]. We found that topo-I mRNA was highly down-regulated in a CPT-11-resistant colon cancer cell line [33]. Similarly, Jansen *et al* found that DNA topo-I activity correlated with CPT-11/SN-38 sensitivity in a panel of human colon cancer cell lines [51]. In addition, topo-I mutations have been reported to affect CPT-11 sensitivity, with the mutant topo-I enzymes having reduced affinity for SN-38 [52]. In a series of colorectal cancer xenografts, the levels of topo-I/DNA complexes were found to be predictive of response to CPT-11 [53]. However, the clinical relevance of topo-I expression or mutant topo-I enzymes for CPT-11 resistance remains to be established. DNA topoisomerase-II (topo-II) is also vital during DNA replication. Topo-II is a target for anthracyclines such as doxorubicin and epipodophyllotoxins such as etoposide. Similar to CPT-11, alterations in topo-II enzyme activity through decreased levels of topo-II protein or topo-II mutations have been found in cell lines resistant to topo-II-targeted drugs [54,55].

Microtubules are highly dynamic cytoskeletal fibres composed of tubulin subunits (most commonly α- and β-tubulin) that have crucial roles in maintaining cell shape, in cell signalling, in cell division and mitosis, and in the transport of vesicles and mitochondria and other cellular components throughout the cell [56]. The rapid polymerization/de-polymerization

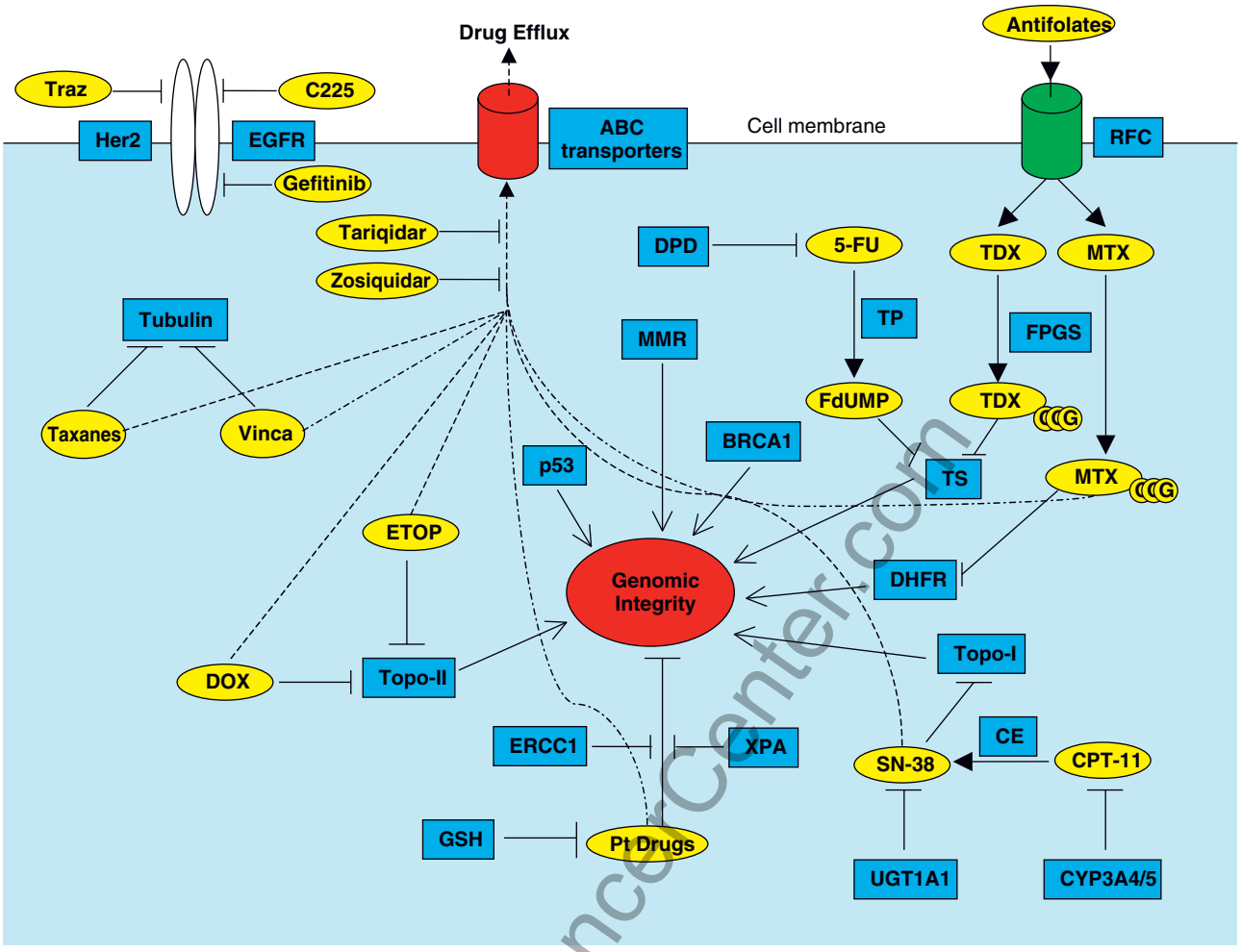

Figure 2. Mechanisms of chemotherapeutic drug activity, highlighting potentially important determinants of drug resistance. Abbreviations not in the text: C225, cetuximab; CE, carboxylesterase; CYP3A4/5, cytochrome P450 enzymes; DOX, doxorubicin/adriamycin; ETOP, etoposide; G, glutamate; Pt drugs, platinum drugs; Traz, herceptin/trastuzamab; Vinca, vinca alkaloids

dynamics of microtubules are critical for proper spindle function and accurate chromosome segregation during mitosis. Taxanes such as paclitaxel and docetaxel, and vinca alkaloids such as vinblastine and vincristine suppress microtubule polymerization dynamics, which results in the slowing or blocking of mitosis at the metaphase–anaphase boundary [57,58]. Mitotically blocked or slowed cells eventually die by apoptosis [57]. Microtubule polymer levels and dynamics are regulated by many factors, including expression of different tubulin isotypes [56]. Changes in microtubule dynamics and levels of tubulin isotypes have been correlated with resistance to paclitaxel and vinca alkaloids [59–61]. In addition, a reduction in total intracellular tubulin levels has been observed in paclitaxel-resistant cells. Therefore, the development of resistance to anti-microtubule agents may involve changes in the microtubule polymer mass and expression of different tubulin isotypes. Furthermore, specific β-tubulin mutations that alter sensitivity to paclitaxel have been reported *in vitro* [61,62]. The clinical relevance of these mechanisms of resistance to anti-microtubule agents remains to be determined.

DNA damage repair

The capacity for a cancer cell to repair DNA can determine resistance to chemotherapeutic drugs that induce DNA damage either directly (for example, platinum drugs) or indirectly (for example, 5-FU and topoisomerase poisons). The response to DNA damage is either repair or cell death, and therefore has a profound effect on tumour chemosensitivity and chemoresistance. Signalling pathways have evolved to arrest the cell cycle following DNA damage to allow time for DNA repair. Only when repair is incomplete, for example when the DNA damage is too extensive, will cells undergo apoptosis. However, the relationship between cell cycle arrest and apoptosis following chemotherapy is complex and dependent on both the chemotherapy used and the molecular phenotype of the tumour cell.

Nucleotide excision repair (NER) is the major pathway for platinum–DNA adduct removal and repair of platinum drug-induced DNA damage. Both oxaliplatin- and cisplatin-induced DNA adducts are removed with similar efficiency by NER [63]. The

importance of NER is highlighted by the finding that defects in this pathway result in hypersensitivity to cisplatin, and that restoration of NER activity reduces sensitivity to more normal levels [64,65]. NER is a complex process involving at least 17 different proteins; however, up-regulation of only a few rate-limiting components of the NER system is necessary to increase a cell's capacity for NER [66]. One of these important rate-limiting factors is the excision repair cross-complementing 1 protein (ERCC1), which several pre-clinical studies have demonstrated plays an important role in determining cisplatin sensitivity, with increased expression of ERCC1 associated with cisplatin resistance [67–69]. Furthermore, ovarian cancer cells in which ERCC1 expression was down-regulated using an antisense expression construct were found to be more sensitive to cisplatin than control cells in cell line and xenograft models [70]. Clinically, high levels of ERCC1 have been reported to correlate with poor response to platinum-based chemotherapy in ovarian, gastric, and non-small cell lung cancers (NSCLC) [71–73]. In addition, high mRNA expression of ERCC1 and TS has been shown to be predictive of poor response to combined oxaliplatin/5-FU treatment of advanced colorectal cancer [74]. Another central component of the NER machinery, *xeroderma pigmentosum* group A (XPA), has been found to be overexpressed in cisplatin resistance [71]. Collectively, these studies suggest an important role for NER in mediating resistance to platinum-based chemotherapies.

TS inhibition mediated by the 5-FU metabolite FdUMP or TS-targeted anti-folates results in nucleotide pool imbalances that severely disrupt DNA synthesis and repair [75]. TS inhibition can result in the accumulation of deoxyuridine triphosphate (dUTP), as conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) is blocked [76]. Both dUTP and another 5-FU metabolite, fluorodeoxyuridine triphosphate (FdUTP), can be misincorporated into DNA. Repair of uracil- and 5-FU-containing DNA is mediated by the base excision repair enzyme uracil-DNA-glycosylase (UDG) [77]. However, this repair mechanism is futile in the presence of high (F)dUTP/dTTP ratios and only results in further false nucleotide incorporation. These futile cycles of misincorporation, excision, and repair eventually lead to DNA strand breaks. DNA damage due to dUTP misincorporation is highly dependent on the levels of the pyrophosphate dUTPase, which limits intracellular accumulation of dUTP [78,79]. Increased dUTPase expression has been associated with resistance to TS inhibitors [78–81]. dTMP can be salvaged from thymidine by thymidine kinase (TK), alleviating the adverse effects of TS inhibition on nucleotide levels and DNA damage [82]. This salvage pathway represents a potential mechanism of resistance to TS-targeted therapies.

Inherited defects in the DNA mismatch repair (MMR) genes, especially *hMLH1* and *hMSH2*, are common in certain familial forms of cancer such as hereditary non-polyposis colon cancer (HNPCC) and are also observed in a variety of sporadic tumours, including colorectal, breast, and ovarian [83–86]. The main function of the MMR system is to scan newly synthesized DNA and remove single nucleotide mismatches that arise during replication [87]. DNA MMR deficiency gives rise to the microsatellite instability (MSI) phenotype, which is detected as variations in lengths of DNA repeat sequences present in the genome [88].

Experimental data implicate MMR deficiency in the development of resistance to a wide range of DNA-damaging agents, including platinum drugs [87]. The exact mechanism by which MMR-deficient cells become resistant to cisplatin is unclear. It is possible that MMR recognition of DNA damage may trigger an apoptotic pathway, or that futile cycles of DNA damage and repair mediated by the mismatch repair machinery generate lethal DNA strand breaks. *In vitro* selection for cisplatin resistance has been demonstrated to result in cell lines deficient in hMLH1 or hMSH2 in a high number of cases [89]. A common feature of cisplatin resistance is hypermethylation of the *hMLH1* promoter [90]. One clinical study found that ovarian tumours that were initially microsatellite-stable before cisplatin-based chemotherapy exhibited MSI in the residual tumours after chemotherapy, and this was most often due to loss of hMLH1 expression [91]. So, clinical and pre-clinical studies strongly implicate loss of MMR, in particular loss of hMLH1, in resistance to cisplatin. Reversal of *hMLH1* promoter methylation by 2′-deoxy-5-azacytidine (DAC), which inhibits DNA methyl transferase activity, has been shown to re-sensitize cells to a range of anti-cancer agents, including platinum drugs [90]. DAC has now entered clinical trials in combination with carboplatin in ovarian cancer. Importantly, MMR deficiency does not affect resistance to the newer platinum drug oxaliplatin, which forms DNA adducts that are not recognized by the MMR machinery [92]. Furthermore, enhanced ability to synthesize DNA past the site of DNA damage (replicative bypass) has been demonstrated in cisplatin-resistant, but not oxaliplatin-resistant ovarian carcinoma cell lines [93]. This additional mechanism of resistance to cisplatin has been linked to the MMR system [94].

Meyers *et al* found that restoration of hMLH1 in MMR-deficient HCT116 colon cancer cells renders them more sensitive to 5-FU, suggesting that MMR-deficient cells are more resistant to 5-FU [95]. However, the MSI phenotype has been associated with excellent survival in colorectal cancer patients receiving adjuvant 5-FU-based chemotherapy [96]. These apparently contradictory findings may be due to intrinsic biological differences between MSI-positive and MSI-negative colorectal tumours, with MSI-positive (MMR-deficient) tumours being less aggressive. For example, it has been suggested that MSI may lead to an increased anti-tumour immune response [97]. In

addition, it has been demonstrated that the majority of MSI-positive tumours express wild-type p53 [98], which is an important determinant of 5-FU sensitivity (see below). This highlights the complexities involved when different molecular mechanisms of drug resistance overlap.

Several *in vitro* studies have demonstrated that loss of DNA MMR activity results in increased resistance to topo-II poisons [99–101]. In one of these studies, Fedier *et al* demonstrated that loss of either hMLH1 or hMSH2 resulted in resistance to the topo-II inhibitors doxorubicin, epirubicin, and mitoxantrone [99]. Perhaps not surprisingly, this study found that MMR deficiency had no effect on sensitivity to the non-DNA-targeted anti-cancer agents paclitaxel and docetaxel. Furthermore, this study also found that loss of hMLH1, but not hMSH2, was associated with a low level of resistance to topo-I inhibitors such as camptothecin. In contrast, Jacob *et al* found that MSI-positive colorectal cancer cells were more responsive to the topo-I inhibitor camptothecin and the topo-II inhibitor etoposide [102], while Magrini *et al* found that lack of hMLH1 enhanced CPT-11-induced apoptosis in HCT116 colon cancer cells [103]. Another study found that MSI-positive human tumour xenografts were moderately more sensitive to CPT-11, although only a limited number of tumours ($n = 5$) were analysed [104]. Most importantly, a recent clinical study found that MMR deficiency was a predictive factor for tumour response to CPT-11 in advanced colorectal cancer [105]. Collectively, these studies suggest that topo-I poisons may have greater activity against MMR-deficient (MSI-positive) colorectal tumour cells.

Cell cycle arrest versus apoptosis

As already mentioned, there is a critical balance between cell cycle arrest (promoting DNA repair and survival) and cell death following chemotherapy. The tumour suppressor protein p53 plays a central role in the regulation of cell cycle arrest and cell death [106]. The gene encoding p53, *TP53*, is the most frequently mutated gene in human cancers, with ~50% of all tumours estimated to carry a mutation [107]. Furthermore, in p53 wild-type tumours, the activity of p53 may be compromised by inactivation of positive regulators of p53 activity (such as $p14^{ARF}$ [108]) or overactivation of negative regulators of p53 activity (such as Akt [109]). DNA damage results in the activation of upstream kinases such as ATM (ataxia–telangiectasia mutated), ATR (ATM and Rad-3 related), and DNA-PK (DNA-dependent protein kinase), which can directly or indirectly activate p53 [110]. Phosphorylation of p53 by upstream kinases inhibits its negative regulation by MDM-2, which targets p53 for ubiquitin-mediated degradation [110]. The role of p53 in determining cell fate following DNA damage has been attributed to its role as a transcription factor. p53 transcriptionally up-regulates genes such as those encoding $p21^{WAF-1/CIP-1}$ and GADD45, which induce cell cycle arrest in response to DNA damage [111,112]. However, depending on the cellular context, p53 can trigger elimination of the damaged cells by promoting apoptosis through the up-regulation of pro-apoptotic genes such as *Bax, NOXA, TRAIL-R2* (*DR5*), and *Fas* (*CD95/Apo-1*) [113–116]. Both p53-induced cell cycle arrest and apoptosis act to maintain genomic integrity and prevent damaged DNA being passed on to daughter cells.

Several models have been proposed to explain how cells choose between p53-mediated cell cycle arrest and apoptosis. One model suggests that more profound DNA damage induces higher and prolonged activation of p53, which increases the chances of apoptosis over arrest [117]. Another model suggests that different cell types might keep different p53-regulated genes in regions of active chromatin, which determines the cassette of genes that are transcriptionally up-regulated [118]. A third model proposes that the availability of transcriptional co-factors determines the ability of p53 to activate different subsets of genes [119,120].

A number of experimental reports have indicated that lack of functional p53 contributes to drug resistance, and this has been attributed to an inability to undergo p53-mediated apoptosis. For example, *in vitro* studies have reported that loss of p53 function reduces chemosensitivity to 5-FU [49,121]. Furthermore, a number of clinical studies have found that p53 overexpression (a surrogate marker for p53 mutation) correlated with resistance to 5-FU-based chemotherapy [96,122,123], although other studies have found no such correlation [124]. Such conflicting findings may be due at least in part to the fact that p53 overexpression does not actually reflect *TP53* mutation in as many as 30–40% of cases [125]. In addition, gain-of-function p53 mutations have been described that actively contribute to transformation and drug resistance [126]. Indeed, a recent study has suggested that certain p53 mutants may increase dUTPase expression, resulting in 5-FU resistance [80]. So, 5-FU chemosensitivity may be dependent on the particular *TP53* genotype.

A number of *in vitro* studies have demonstrated decreased cisplatin-induced apoptosis in p53 mutant tumour cells [127–129]; however, some studies have reported that disruption of p53 function actually enhances sensitivity to cisplatin [130,131]. This enhanced chemosensitivity was attributed to defects in p53-mediated cell cycle arrest, which reduced time for DNA repair. Similarly, O'Connor *et al* found a wide range of overlapping responses to cisplatin in p53 wild-type and mutant models from the NCI cell line panel [132]. A number of clinical studies have demonstrated that ovarian tumours with mutant p53 have a worse clinical outcome following platinum-based chemotherapy [133–136], although whether the p53 status of these tumours predicts a poor response to platinum drugs, or an inherently more aggressive

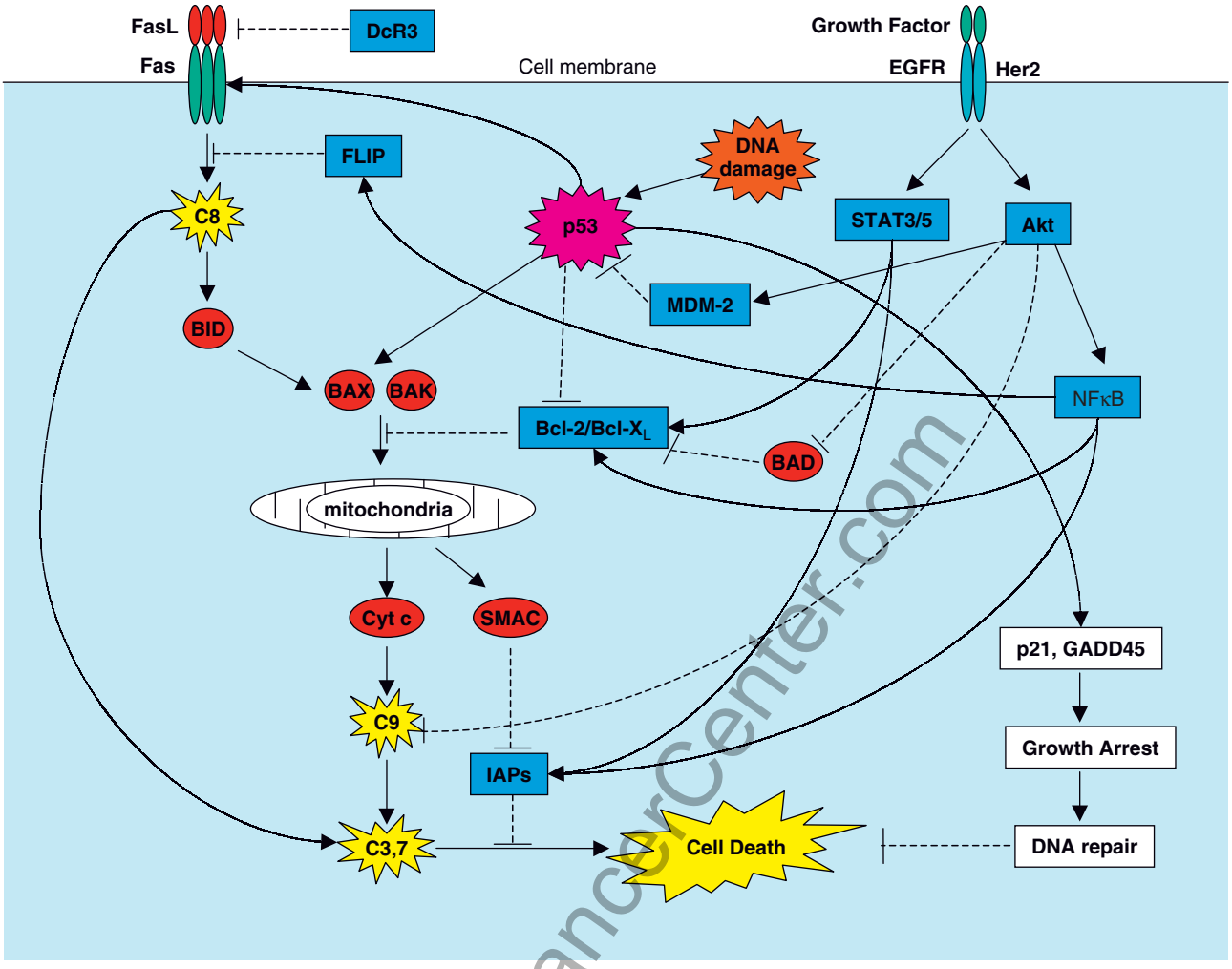

Figure 3. Interactions between pro-apoptotic and pro-survival signal transduction pathways that may play important roles in determining response to chemotherapy. Abbreviations not in the text: C3, caspase 3; C7, caspase 7; C8, caspase 8; C9, caspase 9; cyt c, cytochrome c

tumour phenotype is unclear. *In vitro* data from our laboratory and others suggest that oxaliplatin-induced apoptosis in colon cancer cells is highly dependent on the presence of wild-type p53 [33,137]; however, the clinical relevance of p53 status for oxaliplatin sensitivity remains to be determined.

An *in vivo* study found that doxorubicin was more active against p53 wild-type tumour xenografts than p53 mutant and null cells [138]. In this study, 5-FU activity was also enhanced in the presence of wild-type p53, whereas no difference in cisplatin sensitivity was observed between p53 wild-type and mutant tumours. Furthermore, sensitivity to doxorubicin in acute lymphoblastic leukaemia cell lines was found to be dependent on the presence of wild-type p53 [139]. Clinically, p53 mutations have been correlated with lack of response to doxorubicin in patients with locally advanced breast cancer [140]. These data indicate that doxorubicin is more active against p53 wild-type tumours.

There have been conflicting studies regarding the role of p53 in regulating the anti-tumour activity of CPT-11. Fichtner *et al* found no correlation between p53 status and sensitivity to CPT-11 in a panel of 15 xenotransplanted colorectal tumours [141]. Similarly, Jacob *et al* found that p53 status did not correlate with CPT-11 chemosensitivity in a panel of colorectal cancer cell lines [102]. In contrast, another study found that the presence of mutant p53 correlated with poor response to CPT-11, although only a small number of tumours ($n = 5$) were examined [104]. We have found that CPT-11 induces apoptosis to a similar extent in isogenic p53 wild-type and null colon cancer cells [33]. Pavillard *et al* found no correlation between p53 status and response to CPT-11 in a small series ($n = 9$) of colorectal cancers [142]. Clearly a much larger trial will be necessary to establish the clinical relationship between CPT-11 sensitivity and p53 status.

A number of *in vitro* studies have found that p53 status does not affect tumour sensitivity to paclitaxel, or that loss of p53 correlates with increased paclitaxel chemosensitivity [143–145]. Furthermore, a study by Lavarino *et al* reported that paclitaxel in combination with cisplatin was more effective against p53 mutant ovarian tumours than p53 wild-type tumours [146], while Gadducci *et al* found that p53 status was not

predictive of response to paclitaxel-based chemotherapy in advanced ovarian cancer [147]. These studies suggest that p53 mutant tumours may be equally sensitive, or possibly more sensitive, to paclitaxel than p53 wild-type tumours. However, conclusive proof of this will require correlation of p53 status with response to paclitaxel as a single agent.

BRCA1 is another tumour suppressor gene that regulates cellular responses to DNA damage. Germline mutations of the *BRCA1* gene account for 5–10% of breast and ovarian cancer cases, and lower than normal BRCA1 expression may be an important contributing factor in sporadic cancers [148]. BRCA1 plays a major role in various DNA repair processes, and has recently been found to reside in a large DNA repair complex that includes various mismatch repair proteins including hMLH1 and hMSH2 [149]. BRCA1 also plays a role in the activation of cell cycle checkpoints in response to different types of cellular stress including DNA damage and disruption of microtubule dynamics [150–152]. In addition, BRCA1 has been implicated in the regulation of apoptosis [153]. It appears that, similar to p53, BRCA1 may function as a sensor of cell stress by relaying signals to either the cell-cycle checkpoint machinery or cell death machinery. A recent study by Quinn *et al* found that BRCA1 acts as a differential modulator of chemotherapy-induced apoptosis in breast cancer cells [154]. They found that BRCA1 enhanced sensitivity to apoptosis induced by anti-microtubule agents such as paclitaxel and vinorelbine, but inhibited apoptosis induced by DNA-damaging agents such as cisplatin and etoposide. These results may be clinically relevant for the treatment of breast and ovarian cancers and suggest that BRCA1 may be a useful predictive marker of response to different chemotherapies used to treat these cancers.

Induction of apoptosis

The ultimate goal of cytotoxic chemotherapies is to induce cell death in tumour cells. The onset of apoptosis is regulated by multiple intra- and extra-cellular signals, amplification of these signals by second messengers, and activation of the effectors of apoptosis, the caspases. There are two main pathways for the activation of caspases: the intrinsic pathway regulated by Bcl-2 proteins, and the extrinsic pathway regulated by members of the tumour necrosis factor (TNF) receptor superfamily [155] (Figure 3). Activation of proximal caspases in these pathways leads to activation of downstream effector caspases, most importantly caspases 3 and 7. These executioner caspases cleave a cassette of cellular substrates to bring about the morphological and biochemical changes that characterize apoptosis, including chromatin condensation and nuclear fragmentation, membrane blebbing, and cell shrinkage [155]. Eventually, the cell breaks into small membrane-bound fragments (apoptotic bodies) that are cleared by phagocytosis without causing an inflammatory response.

Activation of the intrinsic apoptotic pathway is regulated by the Bcl-2 family of proteins (Figure 3). Although these proteins share some homology, some Bcl-2 family members such as Bax, Bak, and Bad promote apoptosis, whereas others such as Bcl-X_L, Mcl-1, and Bcl-2 itself are anti-apoptotic [155,156]. Pro-apoptotic Bcl-2 proteins promote the release of cytochrome c from mitochondria. In the cytosol, cytochrome c forms a complex known as the apoptosome with caspase 9 and APAF-1 (apoptosis protease activating factor-1) [155]. Formation of the apoptosome activates caspase 9, leading to cleavage and activation of effector caspases and apoptosis. Anti-apoptotic Bcl-2 proteins oppose the action of the pro-apoptotic proteins by binding to them and inhibiting the changes in mitochondrial membrane potential that promote cytochrome c release.

Not surprisingly, the role of Bcl-2 family members in regulating response to chemotherapy has been extensively studied. For example, *in vitro* studies have demonstrated that Bcl-2 overexpression inhibits cell death induced by a number of chemotherapies [157,158], whereas overexpression of Bax sensitizes cancer cells to chemotherapy-induced death [159]. It has also been demonstrated that down-regulating Bcl-2 and Bcl-X_L using antisense techniques sensitizes cells to chemotherapy [137,158,160], whereas loss of Bax expression has been found to decrease chemosensitivity [161]. Clinically, the picture is less straightforward. Several studies have demonstrated that high Bcl-2 expression in cancers (particularly lymphomas) correlates with poor response to chemotherapy [118,162–164]. However, several studies have shown no correlation between Bcl-2 expression and drug response [165–167], while others have found that Bcl-2 expression is actually associated with a good prognosis [167–169]. These findings may be explained by the association between Bcl-2 expression and favourable clinicopathological parameters such as oestrogen and progesterone receptor positivity and low tumour grade in breast cancer, which are indicative of a less aggressive and possibly more chemosensitive tumour phenotype [158,167,170]. Some clinical studies have demonstrated a correlation between Bax expression and response to chemotherapy [171,172]; however, other studies have found no correlation [167,173]. So, although Bcl-2 family members undoubtedly play important roles during chemotherapy-induced apoptosis, their usefulness as molecular predictive markers of response may be limited. It may be that analysing only one, two, or even four [167] Bcl-2 family members is insufficient, and that more complete analysis of this family of proteins is needed to predict chemotherapy response. Oblimersen (G3139, Genasense) is an antisense oligonucleotide designed to specifically down-regulate Bcl-2 that has been shown to synergize with cytotoxic drugs in pre-clinical models [174]. Various

clinical trials are currently evaluating the anti-tumour potential of combining oblimersen with chemotherapy in haematological cancers and solid tumours.

The extrinsic apoptotic pathway is regulated by cell surface 'death receptors' of the TNF-receptor family, such as Fas (CD95/APO-1), DR4 (TNF-related apoptosis-inducing ligand receptor 1, TRAIL-R1), and DR5 (TRAIL-R2). The best studied of these receptors is Fas, which when bound by Fas ligand (FasL) recruits caspase 8 via the adapter molecule FADD (Fas-associated death domain) to form a death-inducing signalling complex (DISC) [175]. At the DISC, caspase 8 molecules become activated and can subsequently activate the caspase cascade, leading to apoptosis (Figure 3). If there are sufficient levels of caspase 8 activation (type I cells), DISC formation can lead directly to activation of the caspase cascade and apoptosis [175]. However, in some cells (type II cells), amplification of the DISC-mediated apoptotic signal through the mitochondrial pathway is required. This is mediated by the Bcl-2 family member BID, which when cleaved by caspase 8 translocates to the mitochondrial membrane and promotes cytochrome c release [175]. *In vitro* studies have shown that targeting death receptors with recombinant death ligands or agonistic antibodies can induce apoptosis and/or enhance chemotherapy-induced apoptosis [114,176–178]. Efficient induction of Fas-mediated apoptosis in response to agonistic Fas antibodies and recombinant FasL has been demonstrated *in vivo*; however, systemic treatment with Fas-targeted agents has been shown to cause severe liver damage [179]. Most pre-clinical studies are now focused on local administration of rFasL, or the use of FasL-expressing vectors as gene therapy [179]. However, Ichikawa *et al* have successfully developed a non-hepatotoxic agonistic Fas antibody [180], suggesting that it is possible to develop less toxic Fas antibodies. A more promising clinical approach may be to target the TRAIL receptors, as TRAIL has been shown to exert marked anti-cancer activity without systemic toxicity in mice [181]. Clinical trials are currently examining TRAIL as an anti-cancer agent.

A clinical study by Backus *et al* found that 5-FU treatment up-regulated Fas expression in colorectal tumours [182], suggesting that Fas is a clinically important mediator of response to chemotherapy. In several cancers, down-regulation of Fas and up-regulation of FasL have been demonstrated to occur during disease progression [183–186]. It has been postulated that tumour FasL induces apoptosis of Fas-sensitive immune effector cells, thereby inhibiting the anti-tumour immune response [187]. A recent study found that Fas-negative/FasL-positive breast cancer tumours had significantly shorter disease-free survival (DFS) and overall survival (OS) following adjuvant chemotherapy [188]. However, a number of clinical studies have failed to show any relationship between Fas/FasL expression and response to chemotherapy [167,189,190].

Death receptor-mediated apoptosis can be inhibited by decoy receptors: decoy receptor 3 (DcR3) in the case of Fas, and DcR1 and DcR2 in the case of the TRAIL receptors [181]. These decoy receptors bind to FasL/TRAIL, but lack the intracellular domains necessary for DISC formation and therefore inhibit death receptor-mediated apoptosis. A recent study in stage II and III colorectal cancer found that the *DcR3* gene was amplified in 63% of cases and that the DcR3 protein was overexpressed in 73% of cases [191]. Although adjuvant chemotherapy was found to be significantly more beneficial in patients with normal *DcR3* gene copy number in this study, DcR3 protein expression was not associated with the effectiveness of adjuvant 5-FU-based chemotherapy. The clinical significance of decoy receptor expression for drug resistance has yet to be demonstrated, but may prove to be an important clinical determinant of sensitivity to TRAIL. Apoptosis mediated by both Fas and DR4/DR5 can also be inhibited by cytoplasmic factors, most notably c-FLIP (FADD-like interleukin-1β-converting enzyme-inhibitory protein), which binds to the DISC and inhibits caspase 8 activation [192]. c-FLIP overexpression has been found to inhibit death receptor-mediated apoptosis in a number of *in vitro* studies [193–195]. Recently, we have found that RNAi targeting of c-FLIP dramatically sensitizes a panel of colon cancer cell lines to 5-FU, oxaliplatin, and CPT-11 (manuscript submitted), suggesting an important role for c-FLIP in regulating colon cancer cell chemosensitivity. Interestingly, c-FLIP has been found to be overexpressed in a high percentage of colonic and gastric carcinomas [196,197]; however, the significance of c-FLIP overexpression for clinical drug resistance has not yet been studied.

Another important level at which apoptosis is regulated is through inhibition of caspase activity by members of the IAP (inhibitors of apoptosis) family, which include c-IAP1, c-IAP2, X-IAP, and survivin [198]. IAPs can bind directly to caspases, such as caspases 3, 7, and 9, and inhibit their activity (Figure 3). IAPs themselves are negatively regulated by Smac/DIABLO, which is released from mitochondria along with cytochrome c in response to apoptotic stimuli [198]. Overexpression of survivin has been shown to inhibit chemotherapy-induced apoptosis *in vitro* [199]. Furthermore, expression of a dominant negative mutant of survivin and RNAi targeting of survivin have been shown to enhance chemotherapy-induced apoptosis in cancer cells [160,200]. Survivin is overexpressed in a wide range of human cancers *in vivo* and was identified as being among the most common transcripts up-regulated in cancer compared with normal tissues [201]. Clinically, low levels of survivin have been correlated with better response to chemotherapy and improved prognosis in a range of cancers [200,202,203], suggesting that it may be a useful clinical marker. High X-IAP and survivin expression has been reported to be a negative prognostic factor in AML [204]. However, a study by

Ferreira *et al* found that expression of c-IAP1, c-IAP2, and X-IAP did not predict response to chemotherapy in patients with advanced non-small cell lung cancer [205].

Pro-survival signalling

Protein tyrosine kinases (PTKs) can have an important impact on drug resistance through their regulation of anti-apoptotic signal transduction pathways [206]. Overexpression and oncogenic mutations of many PTKs have been described in human cancers [206]. Some of the best-characterized PTKs are the epidermal growth factor receptor (EGFR) family, which comprise EGFR (ErbB1, Her1), Her2 (ErbB2, Neu), Her3 (ErbB3), and Her4 (ErbB4) [207,208]. Binding of growth factors such as epidermal growth factor (EGF), transforming growth factor α (TGF-α), and heregulins results in homo- and hetero-dimerization of EGFR, Her3, and Her4, with the preferred binding partner being Her2, for which there is no known ligand [208]. Receptor dimerization leads to cross auto-phosphorylation of key tyrosine residues in the receptor cytoplasmic domain, which creates docking sites for downstream signal transducers. Anti-apoptotic downstream signals activated by these receptor tyrosine kinases include the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B, PKB) pathway and the STAT (signal transducers and activators of transcription) pathway, specifically STAT3 and STAT5 (see below) [206,207].

Numerous reports have described EGFR overexpression or mutation in various human cancers, indicating an important role for this receptor in cancer [206,209]. Furthermore, both EGFR and Her2 have been strongly implicated in tumour progression, where receptor overexpression is associated with advanced disease and poor prognosis [208]. *In vitro* overexpression of EGFR and Her2 has been shown to increase resistance to chemotherapeutic drugs [210–212]. EGFR and Her2 have long been recognized as potential drug targets, and specific inhibitors of these receptors have entered the clinical arena [209,213]. The Her2 inhibitor herceptin (trastuzamab) is used to treat the 20–40% of breast cancers that are Her2-positive [214]. The EGFR inhibitor gefitinib (Iressa, ZD1839) is currently being tested in clinical trials in a number of solid tumours and is approved for use in the treatment of advanced non-small cell lung cancer [213]. Another EGFR inhibitor, cetuximab (C225), is in phase III trials in combination with chemotherapy in advanced colorectal cancer [213,215]. Other growth factor receptors and protein tyrosine kinase may also prove to be effective therapeutic drug targets. The modulation of response to cytotoxic chemotherapy using targeted inhibitors or antisense techniques is one of the most exciting areas of contemporary anti-cancer research. *In vitro* and xenograft studies have found that gefitinib enhances the cytotoxic effects of a variety of chemotherapies [216–219]. However, in two large phase III trials in chemonaïve patients with NSCLC (INTACT-1 and -2), the addition of gefitinib to standard chemotherapy did not improve survival, time to disease progression, or response rate [220,221].

One of the downstream pathways activated by PTKs is mediated by PI3K. Activation of PI3K generates phosphatidylinositol 3,4,5-trisphosphate (PIP_3), a lipid second messenger essential for the translocation of Akt to the plasma membrane, where it is phosphorylated and activated by 3-phosphoinositide-dependent kinase-1 (PDK-1) [222,223]. Akt phosphorylates and regulates the function of many cellular proteins, including several key regulators of apoptosis such as Bad (Figure 3). Phosphorylation of Bad inhibits its negative regulation of anti-apoptotic Bcl-X_L [224]. Bad can also be inactivated by the Ras–Raf–MEK–ERK signalling cascade, another downstream pathway activated by PTKs [224]. Other anti-apoptotic functions of Bad include inhibition of caspase 9 and activation of the transcription factor nuclear factor-κB (NF-κB) (see below) [222,223]. Akt can also affect p53 activity as it promotes phosphorylation of MDM-2 and its translocation to the nucleus, where it down-regulates p53 expression [109]. Akt is frequently constitutively activated in human cancers due to gene amplification or mutations in signalling pathways that regulate Akt activity, such as overexpression of EGFR or mutation of PTEN, which is one of the most frequently mutated tumour suppressor genes [206,225]. PTEN dephosphorylates PIP_3, thereby inhibiting Akt activation; therefore its loss through mutation promotes constitutive activation of Akt. Activation of the PI3K pathway has been shown to increase chemoresistance in cell lines [226]. Furthermore, *in vitro* studies have demonstrated that inhibiting the PI3K/Akt pathway enhances the cytotoxic effects of a variety of chemotherapeutic agents [227,228]. Inhibition of Akt signalling, either directly or by interfering with its upstream regulators, may represent an effective anti-cancer strategy. A recent clinical study found that NSCLC patients with phospho-Akt-positive tumours had a better response rate and time to disease progression following gefitinib treatment than patients with phospho-Akt-negative tumours [229], suggesting that Akt activity could be a good predictive marker of response to EGFR-targeted therapies.

STAT proteins transmit cytoplasmic signals from cytokine and growth factor receptors to the nucleus, where they activate transcription of a diverse set of target genes [230]. Persistent activation of STATs, in particular STAT3 and STAT5, has been demonstrated in a large number of cancers, and this often occurs due to constitutive activation of an upstream tyrosine kinase [230]. Activation of STAT proteins involves their recruitment to the activated receptor and phosphorylation, which is usually mediated by a receptor-associated tyrosine kinase of the Janus kinase (JAK)

family [230]. Phosphorylated STAT proteins form activated dimers that translocate to the nucleus and up-regulate target gene transcription. STAT3 and STAT5 have been shown to regulate Bcl-X_L expression and apoptosis in wide range of tumour cells [231–236]. Mcl-1 is another anti-apoptotic Bcl-2 family member that is a target for STAT3 and STAT5 [232,237]. Furthermore, a study by Real *et al* demonstrated that STAT3-dependent overexpression of Bcl-2 inhibited chemotherapy-induced apoptosis in breast cancer cells [238], while Masuda *et al* demonstrated that inhibition of STAT3 enhanced the sensitivity of head and neck cancer cells to 5-FU [239]. In addition, STAT3 has also been shown to induce survivin expression [240,241]. Given its anti-apoptotic activity and the frequency of its activation in cancer, STAT3 is a particularly attractive therapeutic target.

The transcription factor NF-κB is a key regulator of oncogenesis through its promotion of proliferation and inhibition of apoptosis [242]. NF-κB exerts its anti-apoptotic effects by up-regulating a number of anti-apoptotic proteins, including IAPs, TNF-receptor associated factors (TRAFs), c-FLIP, Bcl-2, Bfl-1 (A1), and Bcl-X_L [242]. NF-κB is negatively regulated by IκB, which binds to NF-κB and sequesters it in the cytoplasm. Phosphorylation of IκB by the IκB kinase (IKK) complex targets it for ubiquitin-mediated proteasomal degradation and allows NF-κB to translocate to the nucleus [242]. As mentioned above, NF-κB is also activated by Akt [242]. NF-κB has been connected with multiple pathways involved in oncogenesis, including cell cycle regulation and apoptosis [243,244]. Chuang *et al* demonstrated that a wide range of cytotoxic drugs (5-FU, doxorubicin, paclitaxel, and cisplatin) activated NF-κB in a panel of cancer cell lines [245]. This suggests that NF-κB activation is a general feature of cancer cell response to chemotherapy. Whether constitutively or inducibly activated, NF-κB appears to be a critical determinant of drug resistance, with NFκB activation blunting the ability of chemotherapy to induce cell death [244]. A number of *in vitro* studies support this notion, having demonstrated that inhibition of NF-κB sensitizes cancer cells to chemotherapy-induced apoptosis [246–248]. Furthermore, increased NF-κB activity in patients with oesophageal cancer has been correlated with reduced response to neoadjuvant chemotherapy and radiation therapy [249]. NF-κB is believed to be a major target for proteasome inhibitors, as proteasome inhibition prevents degradation of IκB, blocking NF-κB nuclear translocation [250,251]. Clinical trials with proteasome inhibitors such as bortezomib are underway. Inhibiting NF-κB signalling may prove to be an effective strategy to enhance drug-induced apoptosis in a range of cancers.

Future perspectives

Molecular drug resistance is complex and multifactorial and would appear to be an almost intractable problem. However, the advent of DNA microarray technology, advances in proteomic technology, and the ongoing development of new targeted therapies have opened up new opportunities to combat drug resistance. Furthermore, clinical studies have shown that combining information from more than one molecular biomarker increases our ability to predict tumour drug response [16]. This predictive ability will be further enhanced by increasing the number of biomarkers analysed, which may be achieved by analysing the expression of subsets of marker genes using DNA microarray and proteomic profiling. Indeed, the validity of this approach has already been demonstrated *in vitro* [252]. Ultimately, these technologies could be used to prospectively identify tumours most likely to respond to a given chemotherapy. As well as analysing the molecular phenotype of the tumour, pharmacogenomic and pharmacogenetic profiling of normal tissue may also be useful in predicting systemic toxicity. Indeed, a number of genotypes that predict enhanced toxicity to certain drugs have already been identified [253]. Individualization of therapy according to the molecular phenotype of tumour and patient should dramatically increase the effectiveness of chemotherapy.

The development of these new technologies now enables us to characterize the upstream and downstream signalling pathways involved in regulating tumour cell response to chemotherapy more completely than ever before. This will facilitate the future development of rational combined chemotherapy regimens designed to maximize drug activity. The newer targeted therapies, including antisense approaches, are likely to be used increasingly in combination with cytotoxic drugs to enhance chemotherapy activity. In the future, the ability to predict tumour and patient response to cytotoxic drugs and to modulate this response with targeted therapies will permit selection of the best combined treatment for an individual patient.

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