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Resistance mechanisms to cancer chemotherapy

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1. ABSTRACT

Resistance to chemotherapy ('drug resistance') is a fundamental problem that limits the effectiveness of many chemotherapies currently used to treat cancer. Drug resistance can occur due to a variety of mechanisms, such as increased drug inactivation, drug efflux from cancer cells, enhanced repair of chemotherapy-induced damage, activation of pro-survival pathways and inactivation of cell death pathways. In this article, we review some of the major mechanisms of drug resistance and discuss how new molecularly-targeted therapies are being increasingly used to overcome these resistance mechanisms.

2. INTRODUCTION

It is generally accepted that cancer results from a series of genetic mutations that lead to an accumulation of genetic disarray which results in uncontrolled growth (1). A number of genetic mutations must occur within one cell before it becomes malignant, such as gain-of-function mutations of oncogenes and loss-of-function mutations in tumour suppressor genes. Many of the mutations affect genes which control cell cycle and apoptosis. It has been suggested that cells must gain six types of alterations to become malignant: self sufficiency in growth signals; insensitivity to anti-growth signals; evasion of apoptosis;

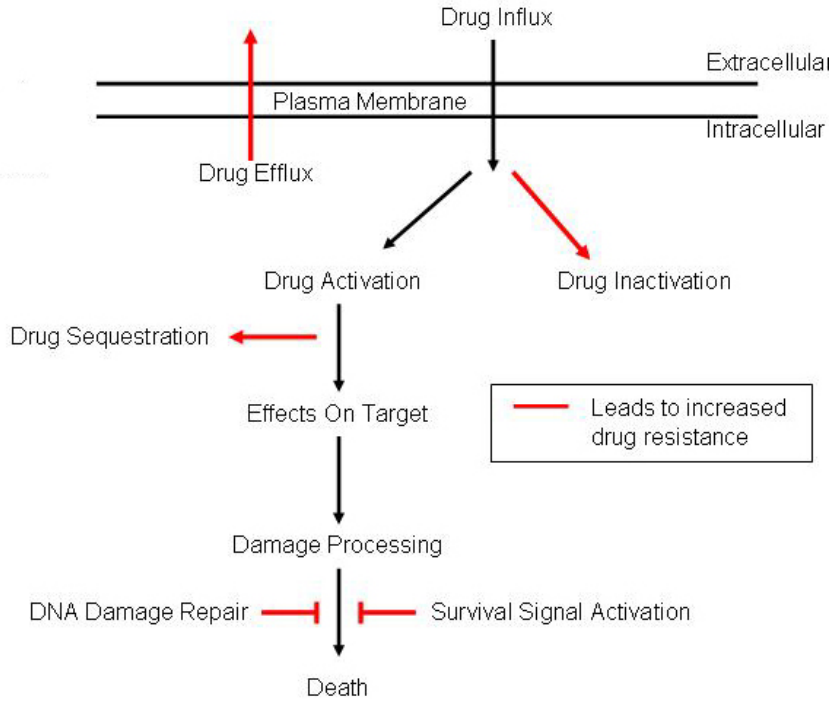

Figure 1. Overview of mechanisms of drug resistance. See text for details.

limitless replicative potential; angiogenesis; and the ability to invade distant tissues and metastasise (2). The resulting malignant cell displays uncontrolled proliferation and may fail to undergo programmed cell death (apoptosis) when cellular damage, such as DNA damage, occurs.

Ideally, chemotherapeutic agents should function to target only highly metabolic, malignant cells and produce cytotoxic effects that lead to tumour regression or increase overall survival. However, one of the main problems limiting the effectiveness of chemotherapies is the presence or development of drug resistance. There are two forms of drug resistance – intrinsic and acquired resistance. Intrinsic drug resistance is present at the time of diagnosis (3). Acquired drug resistance occurs after chemotherapy treatment, and may enable the tumour to become cross-resistant to a range of chemotherapies with differing modes of action.

There are four main steps in the cytotoxic action of a chemotherapeutic drug on a cell:

- The drug enters or is actively taken up by the cell.
- The drug is activated (or its activity is preserved) within the cell.
- The drug exerts its effects on its target(s) within the cell.
- If the damage caused is irreparable, cell death may be induced (4).

Each of these four steps can be the target of drug resistance mechanisms (Figure 1). In this article, we will discuss and give examples of resistance mechanisms that can operate at each of these stages.

3. DRUG INFLUX/EFFLUX

The intracellular concentration of active chemotherapeutic agents must be sufficiently high to produce an anti-proliferative effect. The intracellular drug concentration is determined by the balance between drug influx and efflux, and alterations in either of these processes can lead to drug resistance.

3.1. Drug influx

Influx may be affected by the position of the tumour within the body, for example, if it is sequestered behind the blood-brain barrier (3). Influx may also be reduced by poor vascularisation of the tumour, or by extensive necrosis of the tumour which can lead to reduced blood flow. In addition, as drug influx can occur by passive diffusion, alterations in the composition of the plasma membrane may reduce the amount of drug which enters the cells (4, 5). The intracellular pH also determines the amount of drug which can accumulate in cells. Tumour cells tend to have a slightly acidic pH due to increased aerobic glycolysis (6). Thus, cytotoxic drugs that are weak bases tend to accumulate in the cytosol of these cells. A shift of pH to more alkaline conditions leads to a decrease in the amount of these drugs that enters the cells, which may lead to, or contribute to drug resistance (7). Some drugs, such as methotrexate and other antifolates, rely on specific transport systems for their take-up. The major transporter for antifolates is the reduced folate carrier (RFC), an 85kDa membrane glycoprotein which mediates

the transport of folates and hydrophilic antifolates across the plasma membrane. Decreases in RFC expression have been shown to lead to increased methotrexate resistance in osteosarcoma (8) and breast cancer cell lines (9), and has been shown to lead to a poor response to methotrexate in osteosarcoma (10) and leukaemia patients (11).

3.2. Drug efflux

Even if sufficient quantities of the chemotherapeutic agents are able to enter the malignant cells, drug resistance may still occur if mechanisms of drug efflux are upregulated. Enhanced drug efflux may prevent the intracellular drug concentration reaching a therapeutically effective level. Drug efflux is mediated by the activity of transporter proteins such as members of the ABC transporter family and P-glycoprotein (P-gp), a 170kDa transmembrane protein which contains two ATP binding sites. This receptor functions to actively pump certain drugs out of cells. Increased expression of P-gp leads to increased drug efflux, and therefore increased resistance to drugs such as doxorubicin, vincristine, actinomycin D, paclitaxel and etoposide (12). As this receptor functions to reduce the intracellular concentrations of several different types of drugs, its expression causes multi-drug resistance (resistance to drugs with different mechanisms of action). A number of tumour types have been shown to overexpress the gene for P-gp (*MDR-1*), including neuroblastoma, myeloma, non-Hodgkins lymphoma, colon carcinoma and breast carcinoma (3), and it has been shown that overexpression of P-gp correlates with poor prognosis and poor response to chemotherapy in various cancer types such as ovarian carcinoma (13), colon carcinoma (14) and leukaemia (15).

Multidrug resistance-related proteins (MRP1 and MRP2) have also been shown to be involved in drug efflux from cells and are involved in the export of organic anions such as drugs conjugated to glutathione, glucuronate or sulphate (MRP1) and platinum-based drugs (MRP2) (16). In addition, the expression of lung resistance-related protein (LRP) has also been shown to correlate with resistance to chemotherapies such as doxorubicin, vincristine, cisplatin, carboplatin and melphalan in several human cancer cell lines (17).

The intracellular drug concentration may also be reduced by compartmentalisation/sequestration within organelles. As many cytotoxic drugs are weak bases, they can accumulate in acidic membrane compartments, such as the *trans*-Golgi network, the recycling endosomes and lysosomes, thereby decreasing the amount of free drug available to bind to intracellular targets. Alternatively, the drug compartments can be transported to the cell surface and removed from the cells by exocytosis (18).

4. DRUG ACTIVATION AND INACTIVATION

Once a significant concentration of drug has accumulated in the cell, it may require modification or processing before it becomes fully active. Furthermore, the active drug must have a sufficient intracellular half-life for it to exert its cytotoxic effects. Thus, drug activation and inactivation are two further levels at which resistance can occur.

4.1. Drug activation

A number of chemotherapeutic drugs need to be activated before they can bind to their targets; two examples are 5-fluorouracil (5-FU) and irinotecan (CPT-11). 5-FU is a fluoropyrimidine antimetabolite which is converted to its active form in a stepwise manner. It is first converted to flouordeoxyuridine by thymidine phosphorylase (TP), and this is then converted to its active metabolite fluorodeoxyuridine monophosphate (FdUMP). FdUMP binds to and inhibits thymidylate synthase (TS), the nucleotide synthetic enzyme which provides the sole *de novo* source of thymidylate in cells and is therefore required for DNA synthesis and repair (19). Enhanced TP activity has been shown to correlate with increased 5-FU sensitivity in colon carcinoma cells (20). CPT-11 is administered as the inactive pro-drug which is converted to its active metabolite SN-38 by carboxylesterase (CE). SN-38 functions to stabilise the bonds between DNA topoisomerase-I (topo-I) and nicked DNA at replication forks, preventing the re-ligation of the DNA and ultimately leading to DNA double strand breaks. Decreased CE expression has been correlated with irinotecan resistance in colorectal cancer (21) and non-small cell lung carcinoma (NSCLC) cell lines, and furthermore it has been shown that gene transfer of human CE cDNA can overcome this resistance in NSCLC cells (22).

4.2. Drug inactivation

Drugs can also be metabolised and inactivated. For example, more than 80% of 5-FU administered is metabolised by dihydropyrimidine dehydrogenase (DPD), which is mainly found in the liver. This reduces the bioavailability of 5-FU and reduces its effectiveness (19). If DPD is overexpressed in malignant cells, the 5-FU reaching the cells can also be inactivated by this mechanism, resulting in increased resistance. Increased expression of DPD has been shown to lead to increased resistance to 5-FU treatment in lung cancer cell lines (23), and DPD expression has also been shown to correlate with response to 5-FU treatment in colorectal tumours (24). Platinum drugs can be inactivated by cytoplasmic interactions with thiol-containing molecules such as glutathione (GSH) and metallothioneins (MT). These interactions inactivate the drugs and target them for transport out of the cells by the ABC proteins. Levels of GSH have been reported to correlate with the resistance of ovarian cancer cell lines to cisplatin treatment, and bladder and oesophageal cancer cell lines which express high levels of MT have been shown to be more resistant to cisplatin treatment (25).

5. DNA DAMAGE REPAIR

Resistance to chemotherapy can be caused by alterations in the drug target, for example, the 5-FU target enzyme TS is acutely up-regulated by 5-FU treatment (19). This constitutes a potentially important resistance mechanism, as acute increases in TS expression in response to 5-FU therapy would facilitate recovery of enzyme

activity. Many chemotherapeutic agents cause damage to DNA either directly (for example, platinum-based drugs) or indirectly (for example, TS inhibitors and topisomerase poisons), and this is the primary cause of their cytotoxic activity. Cells possess various mechanisms by which they can repair damage to DNA and can use these mechanisms to repair damage caused by chemotherapy agents. Ideally, chemotherapy treatment should lead to cellular damage which is severe enough to induce cancer cell death. If the cells are able to repair the DNA damage caused by chemotherapy treatment, they will not undergo cell death and will therefore be more resistant to treatment. There are five main pathways which cells can use to repair DNA damage – homologous recombination (HR), non-homologous end joining (NHEJ), nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR) (Reviewed by (26)).

5.1. DNA double strand break repair

HR and NHEJ are both used to repair double strand breaks in DNA. The process of homologous recombination involves the exchange of genetic material with a homologous chromosome, which is used as a template for the repair of damage to ensure that the repair is error-free, while non-homologous end joining repairs the breaks by joining the ends together and can lead to mutations and deletions. HR involves the binding of proteins such as RAD52, BRCA1, p53, ATM and RAD51 to the site of the DNA break, which initiates the synthesis of new DNA strands. NHEJ is initiated by the binding of the heterodimer Ku70-Ku80 at both ends of the DNA break. This leads to the recruitment of DNA-PK, which aligns the ends of the DNA and allows them to be joined by DNA ligase. It has been reported that an increase in the activity of the NHEJ pathway contributes to increased resistance of human chronic lymphocitic leukaemia B cells to chemotherapy treatment (27), while increased activity of the HR pathway through overexpression of RAD51 leads to increased resistance of small cell lung cancer cell lines to etoposide (28).

5.2. Nucleotide excision repair

Nucleotide excision repair is the process by which damaged DNA is removed and replaced with new DNA using the complementary strand as a template for its synthesis. In this way, DNA damage such as cross-links induced by treatment with platinum-based agents and UV-induced photoproducts can be repaired. The NER pathway involves over 30 proteins, which are involved in damage recognition, incision/excision of the damaged DNA, repair and ligation (29). There are two different sub-pathways in NER: transcription-coupled repair; and global genome repair. Different sets of proteins recognise the DNA damage in these pathways. In transcription-coupled repair, DNA damage is recognised by the elongating RNA polymerase II complex and by CS-A and CS-B (Cockayne Syndrome A and B). In global genome repair, the damage is recognised by xeroderma pigmentosum (XP) proteins, in particular XPC, which is specific to global genome-nucleotide excision repair. Both pathways then proceed through recruitment of ERCC1 (excision repair cross-complementing protein 1) and further XP proteins (XPA, B, D, E, F and G) to the site of DNA damage, leading to DNA unwinding, excision of the damaged section and *de novo* synthesis and ligation. It has been reported that deficiencies in NER leads to an enhanced response to cisplatin treatment in testicular cancer (30, 31). Gene silencing of *ERCC1* has been shown to sensitise ovarian cancer cell lines to cisplatin-induced apoptosis (32), and silencing of XPA has been shown to sensitise lung cancer cell lines to cisplatin-induced cell death (33). Conversely, overexpression of the proteins involved in the NER pathway has been shown to correlate with resistance to cisplatin. For example, Ferry *et al.* demonstrated that cisplatin-resistant ovarian cancer cell lines expressed elevated levels of ERCC1, XPA, XPB, XPC and XPG mRNA compared to a control cisplatin-sensitive cell line (34). Also, siRNA studies targeting *ERCC1* in prostate cancer cells found that downregulation of ERCC1 sensitised these cells to cisplatin- and mitomycin C-induced apoptosis (35). Moreover, high levels of ERCC1 mRNA have been shown to correlate with poor response to platinum agents in gastric (36) and non-small cell lung carcinomas (37), and ERCC1 protein expression has been reported to correlate with poor response to platinum-based chemotherapy in non-small cell lung cancer patients (38).

5.3. DNA mismatch repair

The DNA mismatch repair system functions to identify and excise DNA replication errors such as base/base mismatches and insertion/deletion loops which have escaped proof-reading polymerases during DNA replication. Failure to repair mismatches during DNA replication, for example as a consequence of mutations of mismatch repair genes, leads to increased microsatellite instability within tumours. Inheritance of mutations in MMR genes such as *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2* causes HNPCC (hereditary non-polyposis colon cancer), which accounts for approximately 5% of colorectal cancer cases (39). HNPCC patients develop early onset colon tumours, and also have a predisposition to develop tumours of the endometrium, stomach, ovaries and urinary and biliary tracts (40).

There have been conflicting reports on the role of mismatch repair in chemotherapy resistance. It has been reported that loss of DNA mismatch repair contributes to resistance of several cell lines to chemotherapy treatment, for example the resistance of ovarian, colorectal and endometrial cancer cell lines to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), 6-Thioguanine and cisplatin treatment (41). Fink *et al.* demonstrated that defects in MMR pathways lead to increased cisplatin resistance in colorectal cancer both *in vitro* and *in vivo* (42), and Carethers *et al.* reported that colorectal cancer cells show a better response to 5-FU treatment when the MMR pathway is intact (43). The mechanism by which MMR deficient cells can become more drug resistant is unclear, but it has been suggested that recognition of DNA damage by the MMR complex may lead to the initiation of apoptosis, or that the DNA repair carried out by the MMR machinery leads to the formation of lethal DNA strand breaks (44). Despite the reports demonstrating that loss of MMR leads to increased resistance of cell lines to chemotherapy

Table 1. Summary of clinical reports on the effects of p53 status on response to chemotherapy.

Cancer	Correlation	Refs
Breast	No correlation between p53 overexpression and response to paclitaxel	151
	Negative p53 expression corresponds to increased response to paclitaxel and doxorubicin	152
NSCLC	No correlation between p53 expression and response to platinum-based therapies	153, 154
Colorectal	Correlation between p53 overexpression and poor response to 5-FU-based regimens	155-157
	No correlation between p53 overexpression and response to 5-FU-based regimens	158
Pancreatic	p53 overexpression correlates with a poor prognosis following a 5-FU/cisplatin-based adjuvant therapy	159
Ovarian	p53 mutations correlate with a worse prognosis following platinum-based therapies	160-163

treatment, clinical studies have reported that the presence of microsatellite instability, which occurs when the MMR pathway is defective within tumour cells, is associated with a more favourable outcome. Black *et al.* reported that the presence of MSI in endometrial carcinoma is associated with a better outcome (45), and it has been reported that loss of hMLH1 in advanced NSCLC is associated with a statistically significant improvement in prognosis (46). Moreover, germline mutations of *MLH1* have been reported to lead to a significantly higher survival rate in patients with HNPCC (47). Significantly, it has been shown that MSI-positive colorectal tumours very often express wild type p53 (48), which may (at least in part) explain why tumours with MSI have an improved prognosis (see below).

5.4. Base excision repair

Base excision repair is used to repair distinct base errors, such as removal of non-bulky adducts or alkylated bases, which have not distorted the DNA helix. Initially, glycosylases recognise the damage and excise the affected base, creating an apurinic/apyrimidinic (AP) intermediate site. This site is recognised by an AP endonuclease, APE1, which creates a strand break and allows the DNA damage to be repaired by DNA polymerases (49). The expression of AP endonucleases has been shown to be directly correlated with resistance to chemoradiotherapy and poor survival in squamous cell head-and-neck cancer patients treated with platinum-based therapies (50) and resistance of pancreatic cancer cells to gemcitabine treatment (51).

6. p53

The processing of chemotherapy-induced damage is a critical step in a cancer cell's response to chemotherapy and is therefore a key determinant of drug sensitivity/resistance. The tumour suppressor p53 plays a key role in regulating a cell's response to a toxic insult (such as chemotherapy treatment) through its regulation of apoptosis and cell cycle arrest (52). The gene encoding p53, *TP53*, is mutated in approximately 50% of all human cancers, making it the most frequently mutated gene in this disease (53), and in many of the remaining cases, p53 activity may be compromised by alternative mechanisms (54). DNA damage results in the activation of upstream kinases such as ataxia-telangiectasia mutated (ATM), ATM- and Rad3-related (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) that can directly or indirectly activate p53. Phosphorylation of p53 by these upstream kinases results in enhanced p53 stability and activity by inhibiting its interaction with HDM-2 (55). The role of p53 in determining cell fate following DNA damage is attributed to its role as a transcription factor. p53 transcriptionally up-regulates genes that can induce cell cycle arrest, such as $p21^{WAF-1/CIP-1}$ and *GADD45*, thus allowing the cell time to repair the DNA damage (56, 57). Alternatively, p53 can induce apoptosis if the DNA damage is irreparable (54). p53 can regulate apoptosis through both the extrinsic and the intrinsic pathways. p53 transcriptionally up-regulates the *Fas* (58) and *DR5* (59) genes and has been implicated in trafficking of the Fas receptor from the Golgi to the cell membrane (60). p53-responsive elements have been found in the promoters of genes of the BH3-only subset of the Bcl-2 protein family, namely *PUMA* (61) and *Noxa* (62). p53 also induces expression of APAF-1, an integral part of the caspase-9 apoptosome, through a p53-responsive element within the *APAF-1* promoter (63). p53 can also abrogate pro-survival signals; for example, p53 transcriptionally up-regulates the *PTEN* tumour suppressor gene, a phosphatase that negatively regulates the PI3K/Akt pro-survival pathway (64). p53 also regulates apoptosis by directly binding to and inhibiting the anti-apoptotic activities of Bcl-2 and Bcl-X_L (65).

The role of p53 in regulating chemotherapy-induced cell death appears to depend on the type of chemotherapy used. In bladder cancer cell lines, p53 wild-type and heterozygous $p53^{+/-}$ cells underwent apoptosis following chemotherapy treatment, however p53 null cell lines showed little or no increase in apoptosis (66). In our laboratory, we found that HCT116 p53 wild-type and null colorectal cancer cells were equally sensitive to irinotecan (CPT-11), whereas the p53 null cell line was significantly less sensitive to both 5-fluorouracil and oxaliplatin (67). Similarly, Fichtner *et al.* found no correlation between p53 status and response to irinotecan in xenotransplanted colorectal tumours (68). To assess the role of p53 mutations and response to chemotherapy, Koike *et al.* generated twenty-one human tumour xenografts, ten of which were p53 mutant. Of the nine chemotherapy drugs tested, resistance was only observed in p53 mutant xenografts treated with mitomycin C (69). Clinically, studies attempting to correlate p53 mutation and response to chemotherapy have produced mixed and sometimes conflicting results. Table 1 summarizes some of those studies. p53 overexpression is often used as a surrogate marker for p53 mutations, however it has been reported that p53 overexpression does not actually reflect mutations of the *TP53* gene in as many as 40% of cases (70). This may be the cause of some of the conflicting reports on the role of p53 in drug resistance.

As p53 is a key regulator of cellular response to cytotoxic drugs and is mutated in approximately 50% of cancers, restoration of normal p53 signaling in cancer cells is a major therapeutic strategy currently in development.

One such strategy is to inhibit the binding of p53 to HDM-2, and subsequently prevent its proteasomal degradation. Such molecules include a family of imidizole compounds known as nutlins (71). *In vitro* studies have demonstrated that nutlins can sensitize cancer cell lines to radiotherapy (72) and chemotherapy (73, 74). *In vivo* studies demonstrated that nutlin treatment resulted in 90% growth inhibition of established osteosarcoma xenografts in nude mice (75). Another HDM-2 antagonist RITA was discovered by screening the NCI library for compounds that inhibited the growth of p53 wild-type HCT116 cells, but not p53 null HCT116 cells (76). Treatment of SCID mice carrying HCT116 p53 wild-type and null xenografts with RITA significantly suppressed the growth of p53 wild-type, but not p53 null xenografts. Genetic reintroduction of wild-type p53 has also been studied extensively as a therapeutic strategy. Pre-clinical studies have indicated that re-introduction of wild-type p53 can sensitize cancer cells to treatment with radiation *in vitro* (77) and *in vivo* (78). An adenovirus expressing wild-type p53 (Ad-p53) called Advexin has been developed by Introgen Therapeutics. Phase I and II trials have shown that Advexin can be safely administered by intratumoural injection into patients with advanced esophageal cancer (79) and intravenously in patients with advanced malignancies (80, 81). Advexin is currently undergoing a phase III trial in patients with head and neck cancer (www.introgen.com). Other p53 expressing adenovirus therapies being tested clinically include ONYX-015 (Onyx Pharmaceuticals), a genetically engineered virus that only replicates in and lyses p53 deficient/mutant cells. ONYX-015 in combination with numerous chemotherapies has been tested in a phase II/III clinical trials of patients with head and neck cancer, with no observed treatment-related toxicity (82, 83). Another p53 expressing adenovirus, SCH58500 (Schering-Plough), has undergone successful phase I and II trials for hepatic arterial infusion to patients with colorectal cancer and interperitoneal administration to patients with ovarian cancer (84, 85).

7. CELL DEATH

The ultimate goal of most cancer therapies is the induction of cell death, which can occur in two main ways – necrosis and apoptosis. Necrosis is a pathological response to cellular injury, which results in lysis of the plasma membrane and spillage of the cellular contents, leading to the triggering of a general inflammatory response. In contrast, apoptosis is a controlled method of cell death that involves chromatin condensation, DNA laddering, cell shrinking and plasma membrane blebbing. The cell contents are then packaged into membrane-bound fragments, released and subsequently phagocytosed. As a result, an inflammatory response is avoided. Apoptosis can be induced in two ways: via the intrinsic (mitochondrial) pathway; and the extrinsic (death-receptor mediated) pathway (Figure 2). Mutations of the genes or alterations in the expression of proteins that regulate apoptosis have been shown to occur in many cancers. Dysregulation of apoptosis is believed to make a significant contribution to drug resistance.

7.1. Intrinsic apoptotic pathway

The intrinsic apoptotic pathway can be initiated in response to a number of different types of stimuli, including UV-induced DNA damage, chemotherapy treatment and growth factor withdrawal. In response to these stimuli, the mitochondrial outer membrane becomes permeabilised and pro-apoptotic molecules such as cytochrome c, Smac (second mitochondria-derived activator of caspases)/DIABLO (direct IAP binding protein with low Pi) and AIF (apoptosis inducing factor) are released. Cytochrome c can bind to ATP, APAF-1 (apoptotic protease activating factor) and procaspase-9 in the cytoplasm to form the apoptosome, which leads to caspase-9 activation. Active caspase-9 can then activate the downstream executioner caspases, caspases-3 and -7 (86). Smac functions to bind to and inactivate the inhibitor of apoptosis (IAP) family of proteins, and AIF functions to induce chromatin condensation and DNA fragmentation, which can lead to apoptosis in the absence of caspase activity.

The intrinsic apoptotic pathway is regulated by the Bcl-2 family of proteins. This family of proteins contains both pro-apoptotic proteins such as Bax, Bak, Bad and Bid, which function to promote the release of cytochrome c from the mitochondria, and anti-apoptotic proteins such as Bcl-2 and Bcl-X_L, which interact with and inhibit the pro-apoptotic Bcl-2 proteins. It is the relative balance between the pro- and anti-apoptotic Bcl-2 family members within cells that is believed to determine whether they undergo apoptosis (Figure 2).

It has been reported that the overexpression of anti-apoptotic Bcl-X_L leads to poor prognosis in NSCLC patients treated with chemotherapy (87), and increased Bcl-X_L protein expression corresponds to increased resistance of ovarian cancer cells to cisplatin, paclitaxel, topotecan and gemcitabine treatment *in vitro* and *in vivo* (88). Bcl-2 overexpression has been correlated with increased cisplatin resistance *in vitro*, and knock-down of Bcl-2 using siRNA leads to increased sensitivity to cisplatin in bladder cancer cells (89) and increased sensitivity of pancreatic cancer cells to gemcitabine treatment (90). Downregulation of Bcl-2 has also been shown to increase the effectiveness of cisplatin in renal cell cancer (91). Mutations in genes encoding Bid and Smac/DIABLO have been shown to be related to resistance to the tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and to chemotherapy in glioma cells (92). Furthermore, Bcl-2 protein levels have been inversely correlated with response to chemotherapy treatment in neuroblastoma patients, whereas Bax protein levels have been directly correlated with response (93). Bcl-2 inhibitors such as the antisense oligonucleotide Oblimersen have been developed to overcome the resistance caused by overexpression of Bcl-2, and have been shown to be successful in the clinical setting. The addition of Oblimersen to fludarabine treatment in a recent Phase III clinical trial in chronic lymphocitic leukemia patients has been reported to significantly improve response rates and response duration compared to chemotherapy treatment alone (94). Furthermore, use of the small molecule inhibitors ABT-737

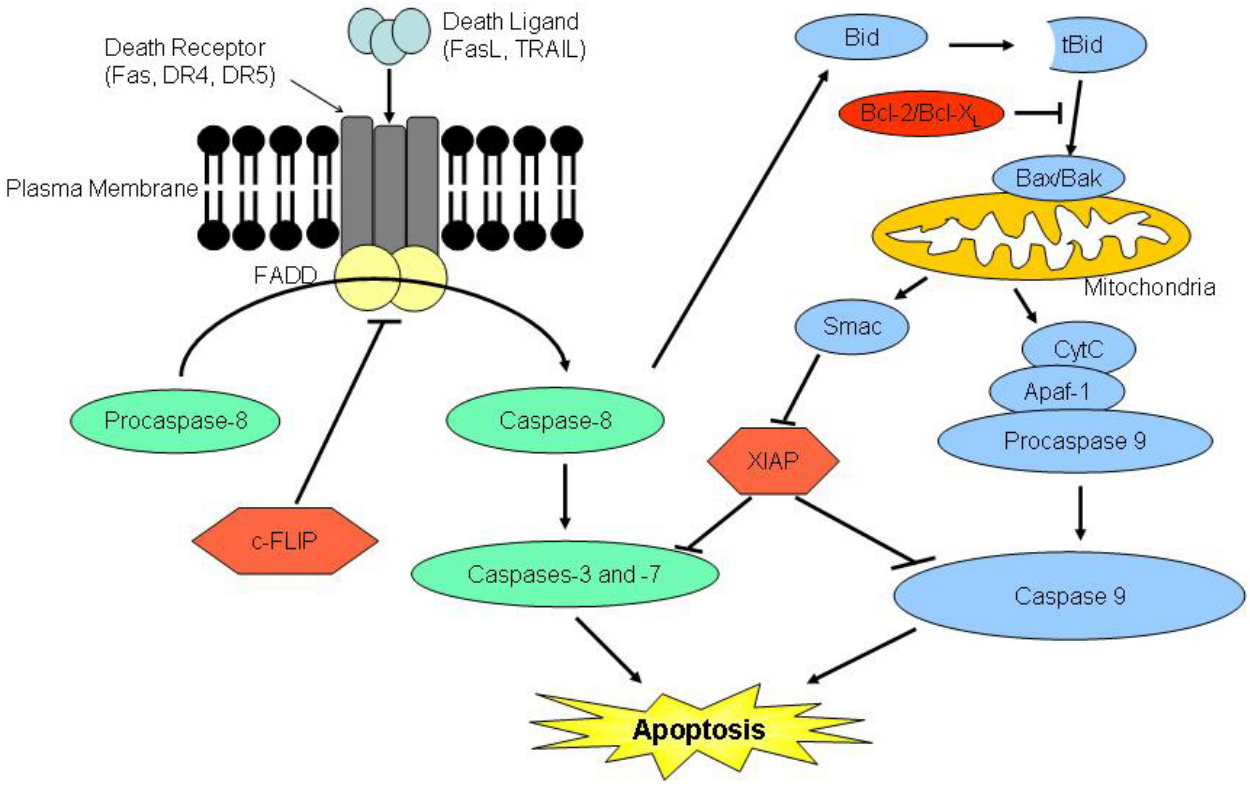

Figure 2. Overview of the death receptor pathway. Following binding of their ligands, the TRAIL death receptors (DR4 and DR5) and the Fas death receptor recruit the adapter molecule FADD, which in turn recruits procaspase 8 to form the death-inducing signaling complex (DISC). At the DISC, procaspase 8 is cleaved and activated. Active caspase 8 can then dissociate from the DISC and activate the downstream executioner caspases, caspase 3 and caspase 7, which then cleave a cassette of protein substrates to induce apoptosis. Caspase 8 activation at the DISC is inhibited by c-FLIP. Caspase 8 can also cleave and activate the Bcl-2 family member BID, which translocates to the mitochondria and promotes Bax- and Bak-mediated release of pro-apoptotic molecules such as cytochrome c and Smac from the mitochondria. This process is inhibited by pro-survival Bcl-2 family members such as Bcl-2 itself and Bcl-X_L. Cytochrome c forms a complex with APAF-1 and procaspase 9 known as the apoptosome, in which caspase 9 is activated and can then cleave and activate the downstream executioner caspases. Smac inhibits the action of inhibitor of apoptosis proteins (IAPs) such as XIAP, which acts to inhibit caspase 3, 7 and 9 activity.

(Abbott Laboratories) and GX15-070 (Geminex), which target the anti-apoptotic Bcl-2 family members, have been shown to enhance the effectiveness of chemotherapy. ABT-737 has been shown to increase the effectiveness of vincristine, dexamethasone and L-asparaginase both *in vitro* and in *in vivo* mouse models derived from patient samples with acute lymphoblastic leukemia (95), and GX15-070 treatment has been reported to significantly enhance cisplatin-induced apoptosis in NSCLC cell lines (96).

7.2. Extrinsic apoptotic pathway

Death receptors are members of the TNF (tumour necrosis factor) receptor superfamily and include TNFR-1, Fas and TRAIL (TNF-related apoptosis inducing ligand) -R1 (death receptor 4 or DR4) and TRAIL-R2 (death receptor 5 or DR5). The binding of a death ligand to its corresponding receptor leads to the recruitment of an adaptor molecule FADD (Fas associated death domain), either directly or indirectly via the adaptor molecule TRADD. FADD can then recruit two molecules of procaspase-8 or -10 to form the DISC (death inducing signalling complex), in which caspase-8 and -10 become activated and subsequently activate the downstream executioner caspases-3, -6 and -7 (97). In addition, active caspase-8 can activate the Bcl-2 family member Bid, which translocates to the mitochondria to induce Bax/Bak-mediated release of cytochrome c and other pro-apoptotic proteins (86) (Figure 2).

Alterations in the proteins involved in the extrinsic apoptotic pathway can contribute to drug resistance. Decoy receptors present on the cell surface such as DcR1 and DcR2, which bind TRAIL, and DcR3, which binds FasL, bind to the death ligands and thereby inhibit their binding to DR4/5 and Fas. (98). Overexpression of DcR1 contributes to increased resistance of fibrosarcoma cells to cyclohexamide (99), and expression of DcR2 has been reported to correlate with response to TRAIL treatment in MCF7 breast cancer cells (100). A direct correlation has been reported between the expression of Fas and DR5 and response to adriamycin and etoposide

treatment in acute lymphoblastic leukaemia cell lines (101). Furthermore, it has been demonstrated that mutations in the genes encoding DR4, DR5, caspase-8 and FADD are related to resistance to TRAIL and chemotherapy treatment in glioma cells (92), and low FADD protein expression has been correlated with chemoresistance and a worse clinical outcome in patients with acute myeloid leukaemia (102).

Death receptors have been investigated as novel targets for cancer therapy. We and others have shown efficient induction of Fas-mediated apoptosis in response to agonistic Fas antibodies *in vitro* (103, 104), however, systemic treatment with Fas-targeted antibodies has been shown to cause severe liver damage *in vivo* (105). Targeting the TRAIL receptors appears to be a more promising strategy (106). Pre-clinical studies have shown that rhTRAIL exhibits very little toxicity in normal cell lines of various lineages, but is capable of inducing apoptosis in a range of cancer cell lines (107, 108). Furthermore, high concentrations of rhTRAIL do not cause any toxicity in non-human primates such as chimpanzees or cynomolgus monkeys (109). rhTRAIL has also shown tumoricidal activity *in vivo* against xenografts of various cancers either alone or in combination with chemotherapy (108, 110, 111). Several clinical trials are currently evaluating the effectiveness of various TRAIL agonists.

7.3. Inhibition of apoptosis

The IAP family of proteins, of which six members have been identified (cIAP1, cIAP2, XIAP, NIAP, survivin and Bruce), can bind to and inhibit caspases-3, -7 and -9, preventing apoptosis. In addition to this, cIAP1, cIAP2 and XIAP can promote degradation of caspases (112). It has been shown that increased cIAP1 and survivin levels lead to increased resistance of thyroid cancer cells to chemotherapy, and RNAi-mediated silencing of these two proteins leads to increased sensitivity to doxorubicin or cisplatin treatment in these cells (113). cIAP1 and cIAP2 expression has also been correlated with resistance of pancreatic cancer cells to 5-FU, cisplatin, doxorubicin and paclitaxel treatment, and downregulation of cIAP2 and XIAP enhances sensitivity to chemotherapy treatment (114). Resistance of hepatoma cell lines to chemotherapy has been correlated with increased survivin expression (115), and IAP expression has been reported to be an important determinant of drug resistance in leukaemia cell lines (116). Furthermore, knock down of XIAP has been shown to sensitise Hodgkins lymphoma cells to a number of cytotoxic agents (117) and prostate cancer cells to cisplatin treatment (118). Use of XIAP small molecule inhibitors have also been shown to sensitise pancreatic cancer cells to gemcitabine treatment (119), and treatment of NSCLC cells with a small molecule Smac mimetic enhances sensitivity to cisplatin (120). In addition, the XIAP-targeted antisense oligonucleotide AEG35156 has been developed which leads to a significantly enhanced response to docetaxel treatment in lung and pancreatic cancer xenografts (121) and is currently being evaluated in clinical trials.

Death receptor-mediated apoptosis is inhibited by the caspase-8 inhibitor c-FLIP. c-FLIP protein expression has been shown to be an important determinant of response to chemotherapy in a number of different cancer cell line models, including colorectal (122, 123), ovarian (124), prostate (125), cervical (126) and lung cancer (127). Interestingly, c-FLIP over-expression has been linked to a poor clinical outcome and increased drug resistance in Burkitt's lymphoma (128), and to poor prognosis in colorectal cancer patients (129).

8. PRO-SURVIVAL SIGNALING

Cells may be inherently resistant to apoptosis induced by chemotherapeutic agents due to the constitutive activation of pro-survival signalling pathways that is a hallmark of many cancers. Furthermore, these pathways may be activated by chemotherapy treatment and thereby induce acute resistance. Pro-survival pathways can be activated by cytoplasmic and receptor tyrosine kinases such as c-Src and the epidermal growth factor receptor (EGFR) family, of which there are four members, namely EGFR (HER-1, ErbB1), HER-2 (ErbB2), HER-3 (ErbB3) and HER-4 (ErbB4). Binding of ligands to these receptors leads to homo- or heterodimerisation, which in turn leads to the activation of downstream pro-survival pathways such as those mediated by phosphatidylinositol-3 kinases (PI3Ks), Ras/Raf and STAT3/5 (signal transducers and activators of transcription).

8.1. EGFR signalling

The expression of the EGFR family of receptors has been implicated in tumour progression and as sign of poor prognosis. Overexpression of EGFR family members occurs in many different cancer types and has been reported to increase resistance to chemotherapy, for example HER2/*neu* overexpression in the breast cancer cell line MCF-7 increases resistance to the platinum analogue CBDCA and 5-FU treatment (130) and to paclitaxel treatment (131). The use of EGFR inhibitors has been shown to enhance response to chemotherapy in a number of different cancer cell lines. The EGFR-targeted monoclonal antibody Cetuximab (Erbitux) inhibited growth and improved response to chemotherapy in both *in vitro* and *in vivo* pancreatic cancer models (132). Cetuximab has been reported to show considerable activity in the treatment of metastatic colorectal cancer both as a monotherapy and in combination with chemotherapy (133). Furthermore, Gefitinib treatment has been demonstrated to increase sensitivity to the FOLFOX-4 chemotherapy regime in patients with previously treated advanced colorectal cancer in a Phase II clinical trial (134). Gefitinib has also been reported to sensitise a number of non-small cell lung carcinoma (135) and colorectal cancer cell lines (136) to chemotherapy treatment; interestingly, the interations between gefitinib and chemotherapy were found to be dependent on the activation of EGFR in response to chemotherapy treatment in these cell lines, suggesting that these cancer cells activate EGFR as an acute survival response to chemotherapy treatment.

8.2. PI3K/PTEN/Akt pathway

Phosphatidylinositol-3 kinases (PI3Ks) are a family of lipid kinases which are activated by growth factor

receptors. Activation of PI3Ks leads to the downstream activation of the serine/threonine kinase Akt, which is involved in various processes including cell survival, cell cycle progression and cell growth. Inhibition of Akt increases the efficacy of paclitaxel treatment in ovarian (137), lung and oesophageal cancer cells (138), and enhances apoptosis induced by etoposide in small cell lung carcinoma cells (139). The activation of Akt is inhibited by the tumour suppressor PTEN, the overexpression of which has been shown to reduce drug resistance in ovarian cancer cell lines (140) and prostate cancer cells (141). Furthermore, overexpression of pAkt and loss of PTEN are correlated with poor prognosis in non-small cell lung carcinoma, as shown by significantly worse five year survival rates and median survival time (142).

8.3. NFκB

The NFκB transcription factor is a heterodimer consisting of p50 (NFκB1) and p65 (RelA) subunits. It normally remains sequestered in the cytoplasm bound by the inhibitory IκB protein. Activation of NFκB involves the phosphorylation of IκB and its subsequent degradation by the proteasome pathway. Active NFκB translocates to the nucleus where it binds to promoter regions of target genes to induce their expression and is therefore a key regulator of a number of cellular responses. NFκB has been reported to be activated in response to chemotherapy, resulting in inducible chemoresistance in human gastric cancer cell lines. Consistent with this, inhibition of NFκB enhances chemotherapy effectiveness in this cancer type (143). NFκB inhibition also leads to increased sensitivity of myeloma cells to apoptosis (144), and increased sensitivity of pancreatic cancer cells to gemcitabine (145). A proteosome inhibitor, Bortezomib (Velcade), has been developed, which has been shown to induce apoptosis in chronic lymphocitic leukaemia cells (146) and human multiple myeloma cancer cell lines, and also to sensitise these cells to chemotherapy (147). The pro-apoptotic activity of Bortezomib is thought to be due (at least in part) to inhibition of IκB degradation, which results in NFκB signalling being blocked. This agent is currently in clinical trials, where it has shown considerable single-agent activity in T-cell lymphoma (148) and multiple myeloma (149), and it has also been reported to lead to a sensitisation to doxorubicin treatment in multiple myeloma patients (150).

9. DISCUSSION

The presence or development of drug resistance is known to be a major limiting factor in the effectiveness of chemotherapy treatment of cancer. Drug resistance can develop via numerous mechanisms, some of which have been outlined in this review. Recent advances in the understanding of these processes have led to the development of a number of different strategies to overcome the effects of drug resistance. These strategies include antagonists of Bcl-2 family members (Oblimersen, ABT-737, GX15-070), EGFR/Her2 inhibitors (Cetuximab, Herceptin, Lapatanib), and the use of death-inducing ligands such as TRAIL agonists which function to directly activate the apoptotic pathway. Alongside these developments, DNA microarray technology is starting to identify gene expression patterns that predict tumour response to chemotherapy. This may enable the identification of patients who are more likely to respond to a particular therapy and (just as importantly) identify patients who are unlikely to benefit. In the future, the combined use of molecular-targeted therapies and tumour/patient profiling could lead to an era in which patient treatment for cancer is individualized, with chemotherapy being combined with targeted therapies according to the genomic profile of the tumour and patient.

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Abbreviations: RFC: reduced folate carrier, MRP: multidrug resistance-related protein, 5-FU: 5-fluorouracil, CPT-11: irinotecan, TP: thymidine phosphorylase, CE: carboxylesterase, NSCLC: non-small cell lung carcinoma, DPD: dihydropyrimidine dehydrogenase, HR: homologous recombination, NHEJ: non-homologous end joining, NER: nucleotide excision repair, BER: base excision repair, MMR: mismatch repair, CS: cockayne syndrome, XP: xeroderma pigmentosum, HNPCC: hereditary non-polyposis colorectal cancer, MSI: microsatellite instability, AIF: apoptosis inducing factor, APAF: apoptotic protease activating factor, IAP: inhibitor of apoptosis, TRAIL: tumour necrosis factor-related apoptosis inducing ligand, FADD: Fas-associated death domain, DISC: death inducing signaling complex.

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