Mechanisms of resistance to alkylating agents

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Abstract

Alkylating agents are the most widely used anticancer drugs whose main target is the DNA, although how exactly the DNA lesions cause cell death is still not clear. The emergence of resistance to this class of drugs as well as to other antitumor agents is one of the major causes of failure of cancer treatment. This paper reviews some of the best characterized mechanisms of resistance to alkylating agents. Pre- and post-target mechanisms are recognized, the former able to limit the formation of lethal DNA adducts, and the latter enabling the cell to repair or tolerate the damage. The role in the pre-target mechanisms of reduced drug accumulation and the increased detoxification or activation systems (such as DT-diaphorase, metallothionein, GST/GSH system, etc...) are discussed. In the post-target mechanisms the different DNA repair pathways, tolerance to alkylation damage and the 'downstream' effects (cell cycle arrest and/or apoptosis) are examined.

Introduction

Alkylating agents (AA) are the most widely used anticancer drugs, being major components of combination chemotherapy regimens for disseminated solid tumors and for high-dose/stem cell support treatment regimens (De Vita *et al.*, 1997; Armitage *et al.*, 1995). This review mainly focuses on the mechanisms of AA resistance, the major reason for failure of cancer treatment.

Mode of Action

AA currently used in cancer chemotherapy are highly reactive small molecules that bind covalently to electron-rich nucleophilic moieties. This binding to cellular DNA is believed to explain these drugs' cytotoxic effects. The most frequent site of alkylation of DNA is the N7 position of guanine (Povirk *et al.*, 1994; Hemminki, 1994; Hemminki *et al.*, 1986; Buble *et al.*, 1994) though the O6 and N1 positions of guanine, the N7, N3 and N1 positions of adenine, the N3 position of cytosine and the O4 position of thymine

are also alkylation sites. Depending on the chemical structures and the charges of the non-reactive part of the molecule, there may be selective binding to specific base sequences in the DNA, so that not all the N7's of guanine are equally likely to be targets for alkylation. Most of the DNA lesions occur in the major groove, but recently a new class of AA has been identified having higher affinity for the minor groove of DNA (mainly alkylating the N3 position of adenine) and is currently under clinical investigation (D'Incalci *et al.*, 1997; Hurley *et al.*, 1984).

As summarized in Table I, various classes of AA can be recognized with different potency, toxicity and disease selectivity. Bifunctional agents, such as melphalan, chlorambucil and busulfan, form monoadducts and, in a second reaction, bi-adducts, mainly interstrand DNA adducts. Nitrosoureas decompose in aqueous solution to produce chloroethyl carbonium ion and an isocyanate group; the former reacts with DNA and creates a mono and then a bifunctional DNA crosslink. Cisplatin (DDP) covalent binding to DNA involves the formation of monofunctional adducts, particularly at the N7 position of adenine and guanine. These monoadducts react with another purine base or

Table 1. Classification of alkylating agents

Mustards:	Meclotretamine Cyclophosphamide Melfhalan Clorambucil Isophosphamide Busulfan
Nitrosureas:	MNU Carmustine (BCNU) Lemustine (CCNU) Semustine (MeCCNU) Fotemustine Streptozotocin
Tetrazine:	Dacarbazine Mitozolomide Temozolomide
Aziridine:	Thiotepa Mytomicine AZQ
Cisplatin and analogs	
Non-classical alkylating agents:	Procarbazine Hexamethylmelamine

nucleophilic center of a protein to form bifunctional adducts (Zwelling *et al.*, 1979; Strandberg *et al.*, 1982; Eastman, 1983).

How exactly these adducts cause cell death is not clear. It is likely that attempts by the cell to replicate or repair the damaged DNA cause the formation of double-strand breaks or single-strand gaps in the DNA and these DNA lesions can lead to chromosomal aberrations and breakage during mitosis. Either DNA adducts themselves or the subsequent DNA damage caused by the lesions can trigger apoptosis or other mechanisms of cell death.

The major limit to the successful treatment of malignancies with AA is the emergence of drug-resistant tumor cells. Cellular resistance to these drugs is multifactorial, as depicted in Table II, there being mechanisms that limit the formation of lethal DNA adducts and mechanisms that enable the cell to repair or tolerate the damage once it has occurred.

Pre-Target Mechanisms of resistance

Pre-target mechanisms of resistance limit the formation of cytotoxic DNA lesions in response to AA and DDP.

Reduced drug accumulation

Transport-mediated resistance has been well characterized for some anti neoplastic agents acting through the drug efflux pump P-glycoprotein, but little is known about the mechanisms of uptake, accumulation and efflux of AA. However reduced DDP and melfhalan accumulation has been reported in cancer cell lines resistant to these drugs (Moscow *et al.*, 1993; Johnson *et al.*, 1996). It remains to be established whether the low intracellular drug concentration is because the resistant cell is more able to pump the drug out or DDP is less able to cross the plasma membrane.

Activation and detoxification mechanisms of resistance

Some cellular factors have been reported to be involved in the activation or detoxification of AA, with consequent changes in their cytotoxic effects.

Reductases

Mitomycin C requires metabolic activation by a reductase (DT-diaphorase) to be cytotoxic. A recent study reported a correlation between DT-diaphorase activity and Mitomycin C sensitivity (Fitzsimmons *et al.*, 1996).

Bleomycin hydrolase

The gene encoding for a specific bleomycin-detoxifying enzyme (bleomycin hydrolase) has been recently cloned and its expression levels were higher in head and neck carcinomas than in adjacent normal tissue and very low or undetectable in lymphomas (Ferrando *et al.*, 1996), suggesting this enzyme might serve as a marker to identify patients potentially refractory to bleomycin therapy.

Aldehyde dehydrogenase (ALDH) isoenzymes

ALDH isoenzymes catalyze the oxidation of aldehydes (one of the active intermediates of cyclophosphamide) to the inactive carboxyphosphamide. Overexpression of a cytosol form of ALDH (class 1) is associated with resistance to cyclophosphamide in L1210 (Hilton, 1984), while a class 3

Table 2. Mechanisms of resistance to alkylating agents

Pre-target mechanisms: (mechanisms that limit the formation of lethal DNA adducts)	Reduced drug accumulation Increased detoxification or activation systems Reductase, hydrolase, aldehyde dehydrogenase Metallothionein, GSH/GST system
Post-target mechanisms: (mechanisms that enable a cell to repair or tolerate DNA damage)	DNA repair mechanisms Tolerance to the damage Cell cycle arrest and/or apoptotic response

ALDH has been reported to be overexpressed by about 100-fold in human adenocarcinoma cells showing a 30-fold oxazaphosphorine-specific resistance (Sreerama *et al.*, 1993). Resistance to cyclophosphamide was reversed by treatment with ALDH inhibitors such as diethylaminobenzaldehyde (Sladek *et al.*, 1985) and resistance was acquired by transfecting MCF-7 cells with a cDNA encoding a rat class 3 ALDH isoenzyme (Bunting *et al.*, 1993).

Metallothioneins (MTs)

MTs are low-molecular-weight intracellular proteins with an unusually high cysteine content (30%), able to bind cytotoxic agents such as platinum compounds and AA. As intracytoplasm binding to MT stops the active molecules reaching their target (cellular DNA), increased levels might influence the AA's cytotoxic action. Elevated MT levels have been observed in some malignant cells with acquired resistance to AA and DDP (Kelley *et al.*, 1988; Lohrer *et al.*, 1989). Very low or undetectable expression of MT has been reported in seminomas, and quite high levels in advanced nonseminomatous germ cell tumors, suggesting a role of MT in the DDP-resistance of germ cell tumors (Chin *et al.*, 1993).

GSH/GST system

The GSH/GST system is an important cellular drug metabolizing system involved in resistance to anticancer agents. The glutathione S-transferases are a family of enzymes able to detoxify a broad class of electrophiles catalyzing their conjugation to the tripeptide glutathione (GSH, the most abundant cellular thiol) with the formation of compounds that are generally less reactive. The role of GST in the formation of GSH conjugates has been demonstrated for several AA: melphalan, chlorambucil, and cyclophosphamide metabolites (Ciaccio *et al.*, 1991; Bolton *et al.*, 1993; Dirven *et al.*, 1994). An increased concentration of

GST should in principle be linked to enhanced detoxification of AA, and therefore be responsible for the development of resistance (Waxman, 1990; Tsuchida *et al.*, 1992). In fact, many acquired AA and DDP resistant tumor cells have a high GSH concentration and/or an increase in the activity of enzymes such as GST (Tew, 1994). Depletion of GSH by agents such as buthionine sulfoximide or inhibition of GST activity by ethacrynic acid restores sensitivity to AA (Tew *et al.*, 1988). However recently it has been reported that, in contrast to expectation, higher GST activity levels were associated with a better chance of response to chemotherapy and more favorable clinical outcome in cancer patients (Ferrandina *et al.*, 1997; Buser *et al.*, 1997).

Post-Target Mechanisms of Resistance

Post-target mechanisms enable a cell to repair or to tolerate the cytotoxic damage induced by antitumor agents and either to survive or else 'to opt' to die.

DNA repair systems

As DNA is the main target of AA, increased repair of DNA damage is one possible way of becoming resistant to the cytotoxic effect of AA.

MGMT (O6-alkylguanine DNA alkyltransferase)

MGMT is an enzyme that transfers an alkyl group from a DNA base to a specific cysteine residue in the active center of the protein itself, through a suicide mechanism. Its best substrate is an O6-methyl guanine, though other alkyl groups from O6 guanine and from O4 thymine can be removed, with varying efficiency. Cells with high levels of this enzyme were called MER+ and were more resistant to nitrosoureas than cells with lower levels (MER- cells) (Wu *et al.*,

1992; Yarosh *et al.*, 1986). Nitrosoureas alkylate the O6 position of guanine which, if left unrepaired, leads to a lethal interstrand crosslink between the N3 position of guanine and the N1 position of cytosine. In human tumors and human cancer cell lines, the levels of endogenous MGMT vary considerably (Egyhazi *et al.*, 1991; Hotta *et al.*, 1994) and the interstrand crosslinks and sensitivity to chloroethylnitrosoureas (CNU) are inversely correlated to MGMT activity (Egyhazi *et al.*, 1991; Hotta *et al.*, 1994; Bodell *et al.*, 1988). A recent multicenter study suggested that changes in MGMT activity affected the therapeutic response of human brain tumors *in vivo* (Belanich *et al.*, 1996). However, some tumors show no correlation between expression of MGMT and resistance to nitrosoureas, suggesting that other mechanisms contribute to CNU resistance (Beith *et al.*, 1997).

Methyladenine DNA glycosylase (APNG)

This glycosylase recognizes a wide variety of alkylated bases including N3-methyladenine, N3-methylguanine, N7-methylguanine and ethylated bases. Whereas introduction of the human APNG gene into *E. coli* deficient in 3-methyladenine DNA glycosylase II activity resulted in resistance to chloroethylnitrosoureas, the lack of APNG steeply increases the toxicity of 1,3-bis-nitrosourea-chloroethyl (BCNU) and mitomycin C in mouse embryonic stem (ES) cells created by homologous recombination (Engelward *et al.*, 1996). However, over-expression of APNG in human or murine cancer cell lines did not alter the sensitivity to AA (Bramson *et al.*, 1995) and no correlation was found between APNG activity and sensitivity to AA in a panel of human cancer cell lines (Damia *et al.*, 1996).

Nucleotide excision repair (NER) pathway

The removal of DDP-DNA adduct is believed to depend on nucleotide excision repair (NER) and it is likely that this repair requires some recombinational events. Increased repair of platinum DNA adducts is associated with DDP resistance in human ovarian cancer and murine leukemia cell lines, investigated using different methods (unscheduled DNA synthesis, host cell reactivation of DDP damaged plasmid DNA and atomic absorption spectrometry) (Chaney *et al.*, 1996). While many DDP-resistant cell lines present enhanced repair activity and this enhanced activity is very likely caused by an increase in NER, it is hard to demonstrate a specific increase in NER activity, because of the inherent variability of the *in vitro* excision

repair assay (Nichols *et al.*, 1992). Increases in the disappearance of interstrand cross-links and in host cell reactivation of melphalan-treated DNA have been reported in some nitrogen-mustard resistant cell lines and chronic lymphocytic leukemia cells resistant to nitrogen mustard therapy (Panasci *et al.*, 1988; Torres Garcia *et al.*, 1989).

Little is known about the expression of the different NER genes in human tumors or cancer cell lines and their correlation with the response to AA chemotherapy, and that little is controversial. While some authors found that Xeroderma Pigmentosum group A (XPA) and Excision Repair Cross Complementing group 1 (ERCC1), two proteins involved in the recognition-incision step of the NER expression, correlated with the response to DDP-based therapy in ovarian cancer, others reported that ERCC1 levels were not increased in some DDP-resistant tumors (Dabholkar *et al.*, 1994; Yu *et al.*, 1996; Codegoni *et al.*, 1997). There is as yet no clear evidence that over-expression of the NER genes in cell lines or tumors is associated with DDP resistance, but studies comparing the expression of these genes with therapeutic responses are accumulating and should provide important additional information.

Damage recognition proteins

There is a group of proteins containing the HMG ('high mobility group') domain, a recently identified DNA-binding motif (Bruhn *et al.*, 1992; Brown *et al.*, 1993), that bind to DDP DNA intrastrand crosslinks. These proteins may affect the antitumor activity of DDP, though their exact mechanisms of action have yet to be determined. Two possible mechanisms have been proposed. As these proteins are also known to be transcriptional factors, their binding to DDP adducts might triter them away from their normal sites, interfering with the transcription of specific genes (McA'Nulty *et al.*, 1996). It has also been suggested that they interfere in the repair of such lesions, by preventing the excision complex from recognizing DDP-DNA adducts. In fact HMG-proteins can selectively inhibit the human nucleotide excision repair complex *in vitro* (Huang *et al.*, 1994) and inactivation of an HMG-protein (IXR1) in yeast doubled the resistance to DDP (Huang *et al.*, 1994). The precise significance of these findings is still unclear, as no difference in the steady-state levels of HMG-domain proteins has been reported in DDP-resistant cell lines (Hill *et al.*, 1994).

Another class of protein UV-damage recognition protein (UV-DRP – also known as DDB-damaged

DNA binding protein or XPE-binding factor) is present at higher levels in DDP-resistant cells with increased repair activity (Chu *et al.*, 1990; Chao *et al.*, 1991). However *in vitro* the XPE protein is not essential for the NER system (Mu *et al.*, 1995) and another study found no correlation between the steady-state levels of UV-DRP in cell lines sensitive and resistant to DDP (Vaisman *et al.*, 1995).

A novel DNA binding protein able to bind to minor groove AA damaged DNA has now been identified and its role in the cellular response to minor groove AA is currently under investigation (Colella *et al.*, 1996).

Tolerance to the damage

Post replicative repair

Post replicative repair, also known as replicative bypass, refers to a cell's ability to synthesize DNA past a site of DNA damage. This process, bypassing DNA damage during S phase, allows a cell to get to G2 phase where it can potentially arrest the cell cycle and repair the damage before proceeding into mitosis (Kaufmann, 1989). In murine leukemia and human ovarian cancer cell lines replicative bypass of DDP-DNA adducts was enhanced (Gibbons *et al.*, 1991), the increase in activity being proportional to the increased tolerance of platinum DNA adducts. This could in theory explain a significant part of the increased tolerance (Mamanta *et al.*, 1994).

Mismatch repair (MMR)

Defective MMR has been implicated in acquired resistance to some cytotoxic drugs (Modrich *et al.*, 1996; Karran *et al.*, 1994). Tolerance to AA such as MNNG has been observed in cell lines (Kat *et al.*, 1993; Branch *et al.*, 1993) also deficient in MMR, that showed microsatellite instability (Branch *et al.*, 1995). Mismatch proteins have also been reported to bind to DDP intrastrand crosslinks and MMR deficient cell lines have shown resistance to DDP (Anthoney *et al.*, 1996; Drummond *et al.*, 1996). The development of DDP resistance was accompanied by loss of DNA mismatch.

These results might have important implications for acquired resistance to the drug. In fact DDP-induced loss of DNA MMR can directly produce resistance to methylating and possibly to other alkylating agents. Secondly, loss of MMR dramatically increases the mutation rate of the affected cells, potentially increasing the rate of development of resistance to many other drugs.

Cell cycle arrest or apoptotic response

After a cytotoxic insult, a cell has two choices. If the injury is mild, it can arrest the cell cycle (G1 and G2 blocks of the cell cycle) and repair the damage. If the injury is severe, causing irreversible damage, the cell undergoes irreversible apoptosis. The role of p53 in genetic stability is a consequence of its ability to prevent the entry of damaged cells into the S phase of the cell cycle and thus prevents the replication of damaged DNA (Gottlieb *et al.*, 1996). After DNA damage, p53 protein levels rise, leading to either a G1 block or apoptosis. The G1 arrest pathway probably involves activation of p21 which inhibits G1 cyclin-cdc complex and inhibition of phosphorylation of RB protein, preventing the cell entering the S phase (Bodell *et al.*, 1988). Recent studies have shown that p21 ^{-/-} cells are defective in repair of the *in vitro* DDP damaged β -galactosidase reporter gene (El-Deiry, 1996; McDonald *et al.*, 1996; Waldman *et al.*, 1996).

The ability of cells to engage an apoptotic response after DNA damage is an important mechanism that could explain resistance to different anticancer agents, including AA and DDP (Hannun, 1997). The genetic regulation of apoptosis involves a complex interplay between p53, bcl-2 and bcl-2 related proteins (Cotter *et al.*, 1997); p53 functions as an inducer of cell death, especially in response to DNA damaging events (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Ryan *et al.*, 1993). Mutations of p53 are very common in human cancers and loss of p53 function contributes to tumorigenesis, genomic instability and drug resistance (Greenblatt *et al.*, 1994). Results are contrasting on the role of wild type or mutated p53 in DDP sensitivity or resistance in different tumor cell types (Fan *et al.*, 1995; De Feudis *et al.*, 1997; Perego *et al.*, 1996).

Bcl-2 is a 26 kD protein that inhibits programmed cell death by different pathways including c-myc, p53 and DNA damaging agents (Hockenbery *et al.*, 1990). Bcl-2 was over-expressed in DDP-resistant ovarian cancer cells and this correlated with DDP resistance in neuroblastoma cells (Eliopoulos *et al.*, 1995; Dole *et al.*, 1994; Lasorella *et al.*, 1995).

Bcl-2 related genes (bcl-x and bax) are involved in the positive and negative regulation of apoptosis. There are two alternative spliced bcl-x mRNA, bcl-xl and bcl-xs, the former being an inhibitor and the latter an inducer of apoptosis (Boise *et al.*, 1993; Sumantran *et al.*, 1995). Over-expression of bcl-xl reduces cytotoxicity induced by DDP (Minn *et al.*, 1995). The bax gene product heterodimerises with and inactivates

bcl-2 and it has been suggested that the ratio of bax to bcl-2 governs survival or death after an apoptotic signal.

Other factors have been described (over- or under-expression of certain genes) that can influence AA and DDP resistance in tumor cells, but the mechanisms are not yet understood. For example, DDP resistance has been correlated both with *in vitro* over-expression of c-myc (Sklar *et al.*, 1991; Niimi *et al.*, 1991) and with MDM-2 over-expression in human glioblastoma cells (Kondo *et al.*, 1995).

The relation between the expression of oncogenes, tumor suppressor genes and proteins involved in the pathways activated by these proteins and sensitivity or resistance to AA now requires research to establish whether there are consistent patterns in different tumors. These studies require simultaneous assessment of the expression and possibly of the function of hundreds of genes in the same cells. Recent new technologies make this kind of evaluation realistic in the near future.

Conclusions

Most of the knowledge of the best-characterized mechanisms of resistance to alkylating agents described in this paper was obtained in cellular systems and only in few cases have there been attempts to verify these findings *in vivo*, in tumors growing in rodents or *ex vivo* in human tumor biopsies.

For this reason, our knowledge of the mechanisms of resistance to anticancer drugs must be viewed with caution as regards their clinical application. The lack of success of clinical attempts to reverse the mechanisms of resistance are probably mainly due to the complexity of these mechanisms in human tumors, compared to cancer cell lines growing *in vitro*. The huge variety of mechanisms of resistance to drugs, coexisting in the same tumor, is a daunting obstacle. However, in the last decade a number of compounds have been discovered and investigated which inhibit enzymes involved in specific mechanisms of resistance to AA. For example, potent inhibitors of the enzymes involved in DNA repair mechanisms (e.g. O⁶-alkylguanine DNA alkyltransferase) or in drug detoxification (e.g. GSH transferase) have been identified. Although some clinical investigations with these inhibitors are still in progress, the idea of reversing resistance by using specific inhibitors does not seem promising on account of the multiple mecha-

nisms of resistance simultaneously present in the same tumor. In addition, as briefly outlined in this paper, the downstream mechanisms of sensitivity or resistance (e.g. cell cycle effects, apoptosis) are vital to the therapeutic outcome, and we cannot yet influence these complex biological pathways, still only partially elucidated. However many new agents which act at several steps in the signal transduction pathways are under preclinical and clinical investigation. It is conceivable that in the near future the effectiveness of AA will be increased by the combined use of these new agents which could render tumor cells more vulnerable to AA-induced damage, ideally in a selective manner. Although preclinical data on this kind of combination is already promising, only appropriately designed clinical trials will tell us whether this approach significantly improves the efficacy of AA.

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