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Review

Resistance to chemotherapeutic antimetabolites: a function of salvage pathway involvement and cellular response to DNA damage

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Summary The inherent or acquired (induced) resistance of certain tumours to cytotoxic drug therapy is a major clinical problem. There are many categories of cytotoxic agent: the antimetabolites, e.g. methotrexate (MTX), *N*-phosphonacetyl-L-aspartate (PALA), 5-fluorouracil (5-FU), 6-mercaptopurine (6-TG), hydroxyurea (HU) and 1-β-D- arabinofuranosylcytosine (AraC); the alkylating agents, e.g. the nitrogen mustards and nitrosoureas; the antibiotics, e.g. doxorubicin and mitomycin C; the plant alkaloids, e.g. vincristine and vinblastine; and miscellaneous compounds, such as cisplatin. There are also many mechanisms of drug resistance elucidated principally from in vitro studies. These include mutation of target genes, amplification of target and mutated genes, differences in repair capacity, altered drug transport and differences in nucleoside and nucleobase salvage pathways (Fox et al, 1991). The aim of the present review is to evaluate in detail the mechanisms of response of both normal and tumour cells to three chemotherapeutic antimetabolites, MTX, PALA and 5-FU, which are routinely used in the clinic either alone or in combination to treat some of the commonest solid tumours, e.g. breast, colon, gastric and head and neck. The normal and tumour cell response to these agents will be discussed in relation to the operation of the known alternative 'salvage pathways' of DNA synthesis and current theories of DNA damage response.

Keywords: methotrexate; 5-fluorouracil; *N*-phosphonacetyl-L aspartate; p53; salvage pathways

BACKGROUND

The majority of anticancer drugs mediate their cytotoxicity either by inhibiting DNA synthesis or by damaging the DNA template (Fox et al, 1991). The chemotherapeutic antimetabolites are used to target 'key' enzymes in the pathways of de novo purine and pyrimidine biosynthesis (Weber, 1983). The original hypothesis was that the rapidly proliferating tumour cells might be more sensitive to their cytotoxic action than the normal cells. Paradoxically, however, both acquired resistance and inherent resistance to cytotoxic drugs, and chemotherapeutic antimetabolites in particular, are now accepted to be more common features of tumour cells than normal cells (Wright et al, 1990; Kinsella and Haran, 1991; Lucke-Huhle, 1994). The evidence for this comes from the in vitro observations that tumour cells more readily amplify their DNA than normal cells in response to chemotherapeutic agents (Wright et al, 1990 and refs therein). In vitro studies have also shown that tumour cells are inherently more resistant to the cytotoxic effects of the chemotherapeutic antimetabolites because of progression- linked changes in their pathways of purine and pyrimidine biosynthesis (Weber, 1983; Kinsella and Haran, 1991; Weber and Prajda, 1994).

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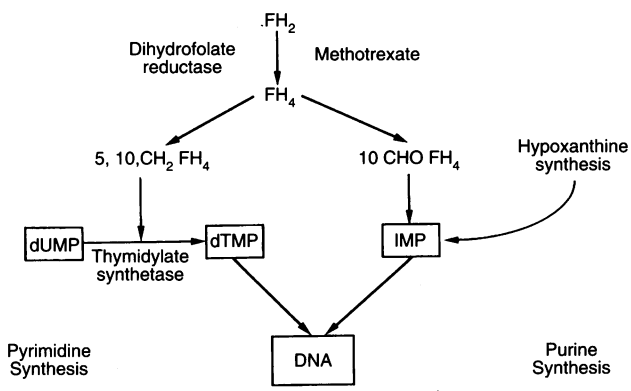

Figure 1 Mechanism of action of MTX by depletion of reduced folate pools

MECHANISMS OF ACTION OF MTX, PALA AND 5-FU

All three of the chemotherapeutic antimetabolites MTX, PALA and 5-FU inhibit key steps in the pathways for the generation of the purine (dATP, dGTP) and pyrimidine (dTTP, dCTP) nucleotides, which are the precursors of DNA synthesis. In simple terms, inhibition of these pathways leads to a shortage of the building blocks for DNA, inhibition of DNA synthesis and,

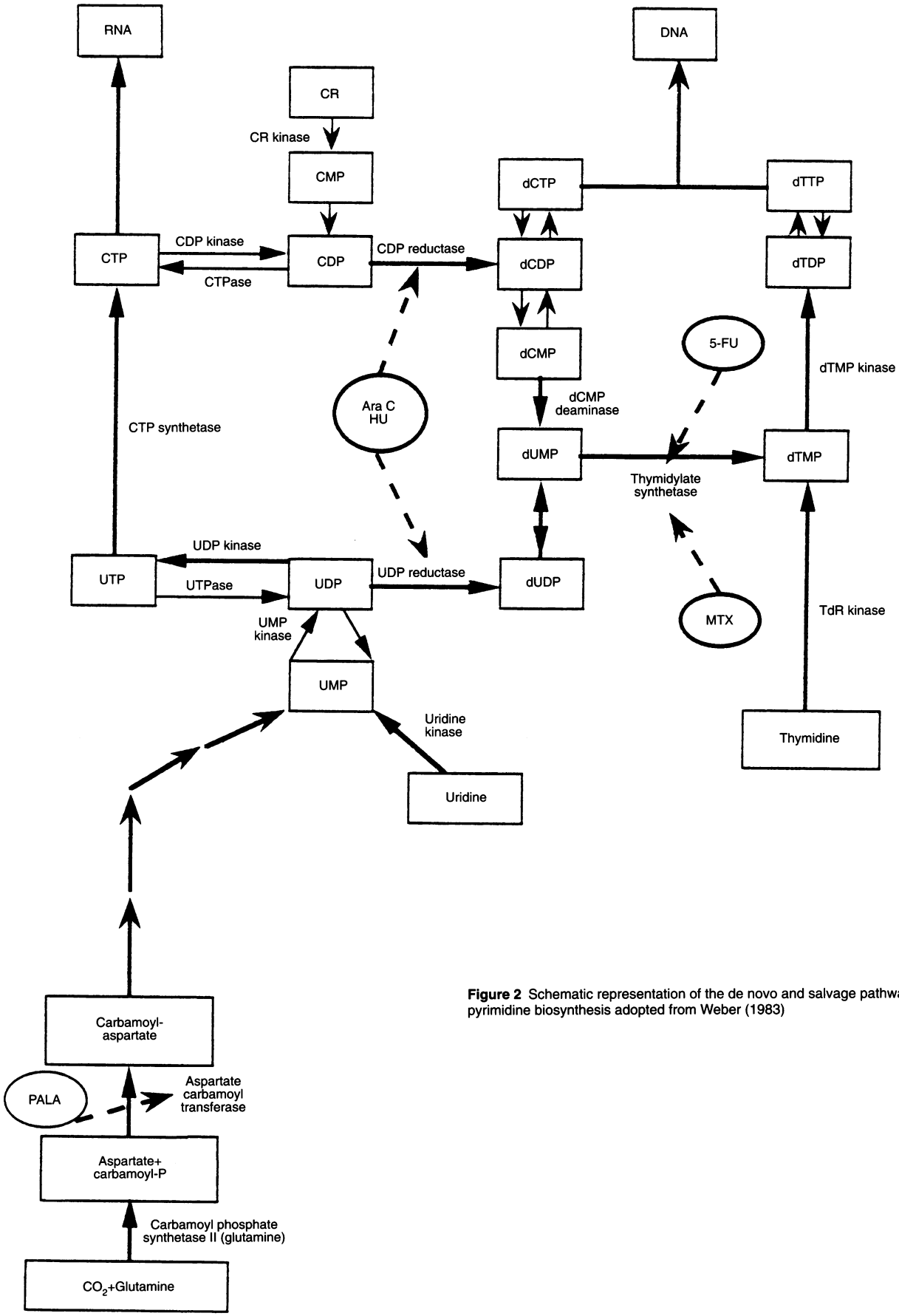

Figure 2 Schematic representation of the de novo and salvage pathways of pyrimidine biosynthesis adopted from Weber (1983)

depending on the drug and the cell type, the rapid or eventual induction of DNA strand breaks. The detailed mechanisms of their action are outlined below.

Methotrexate

Methotrexate is an antifolate and a specific inhibitor of the enzyme dihydrofolate reductase (DHFR), which plays a critical role in intracellular folate metabolism (Chabner and Collins, 1990). It exerts its antineoplastic effects by limiting the synthesis of the reduced folates that act as co-factors for several 'key' enzyme reactions of purine and pyrimidine nucleotide synthesis (Figure 1). Reduced folates are essential for firstly the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), a key step in pyrimidine nucleotide synthesis (Figure 2) which is catalysed by the enzyme thymidylate synthase (TS), and secondly for purine synthesis (Figure 1) (Erlichman, 1992). Thus, MTX depletes three nucleotide pools, namely guanine triphosphate (GTP), adenine triphosphate (ATP) and thymidine triphosphate (TTP). In addition, it is thought that the formation of MTX and dihydrofolate polyglutamates results in further inhibition of the same folate-dependent enzymes (Allegra et al, 1986, 1987; Allegra, 1990). This reduction in the pools of available pyrimidine and purine nucleotides and the impairment of the ability to repair sites of DNA damage eventually results in MTX-induced single- and double-strand breaks (Lorico et al, 1988; Borchers et al, 1990). In addition, the intracellular build-up of dUMP because of TS inhibition and negative feedback inhibition by dCMP deaminase results in the incorporation of dUTP into DNA (Figure 2), resulting in chain elongation and inhibition of DNA synthesis. Excision repair of the DNA containing these dUTP moieties may lead to further DNA strand breaks and fragmentation (Borchers et al, 1990).

Resistance to MTX is conferred on cells by a variety of mechanisms which include: alteration of MTX transport, resulting in a non-inhibitory cell concentration; mutation of the DHFR gene, resulting in a protein product with a reduced binding affinity for MTX; and overproduction of the DHFR gene product as a consequence of gene amplification (Schimke, 1984*a*). Treatment with anti-cancer drugs themselves can enhance the emergence of drug resistance. In the case of MTX, pretreatment of mouse cells with hydroxyurea (Brown et al, 1983) or UV irradiation (Tlsty et al, 1984) or in hamster cells transient hypoxia (Rice et al, 1986) or pretreatment with MTX itself (Tlsty et al, 1982) or AraC (Goz et al, 1989) enhanced the appearance of MTX-resistant clones in clonogenic assays. Molecular analysis of the basis for these changes showed all three mechanisms (altered transport, altered affinity and gene amplification) to be increased (Flintoff et al, 1976; Brown et al, 1983; Tlsty et al, 1984). Administration of the tumour promoter TPA was shown to enhance the emergence of MTX resistance in mouse but not in hamster cells in the absence of gene amplification (Bojan et al, 1983). This was later attributed to the influence of the tumour promoter TPA on the cell cycle (Szallasi et al, 1988). While in a series of human fibroblast cell lines differences in inherent resistance, in the absence of gene amplification, have been attributed to differences in salvage pathway involvement (Kinsella and Haran, 1991). Salvage pathways circumvent the normal de novo pathways of nucleotide biosynthesis by using nucleosides in the surrounding cellular environment as substrates for their enzymes in the process of nucleotide synthesis.

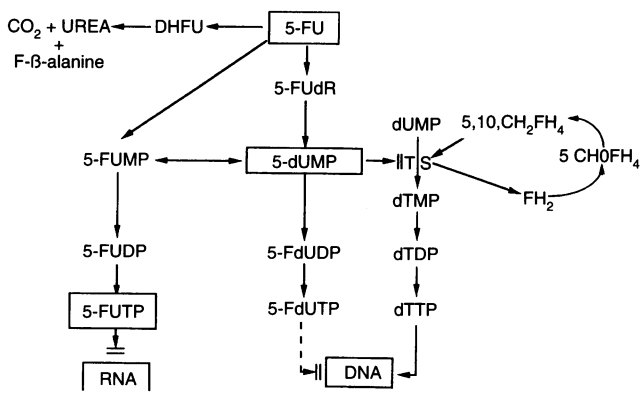

Figure 3 The metabolic activation of 5-fluorouracil produces the metabolites 5-FUTP and 5-FdUMP which can incorporate into RNA or DNA or block thymidylate synthetase activity

PALA

The chemotherapeutic antimetabolite PALA was specifically synthesized to be a stable inhibitor of the enzyme aspartate transcarbamylase, which catalyses the second step of de novo pyrimidine biosynthesis (Figure 2) (Collins and Stark, 1971). It is a potent inhibitor of de novo pyrimidine nucleotide synthesis (Swyryd et al, 1974; Yoshia et al, 1974; Martin et al, 1983) and specifically causes decreases in the UTP and CTP pools (Wahl et al, 1979; Moyer et al, 1982). Inhibition of DNA synthesis and the secondary induction of DNA strand breaks is the primary limitation on replication for cells grown in low concentrations of PALA. However, inhibition of RNA synthesis may become an important factor during prolonged exposure to high concentrations (Moyer et al, 1982). The molecular basis for resistance to PALA is considered almost exclusively to be amplification of the multifunctional *CAD* gene, the products of which catalyse the first three reactions of de novo pyrimidine synthesis (Wahl et al, 1979; Stark and Wahl, 1984; Goz et al, 1989). However, the enhanced inherent resistance of premalignant and malignant human fibroblasts to a single exposure of PALA has been shown to be a consequence of the efficacy of the salvage pathways of pyrimidine biosynthesis in the absence of any amplification of the target gene (Kinsella and Haran, 1991).

5-FU

5-FU was specifically synthesized for the clinic (Heidelberger et al, 1957; 1983) to resemble the pyrimidine bases uracil and thymine. The drug is rapidly taken up by cells and is rapidly metabolized by a number of enzymes along several pathways (Sotos et al, 1994 and refs therein) (Figure 3) to produce two active metabolites, i.e. 5-FUTP, which may be incorporated directly into RNA, and 5-dFUMP. 5-dFUMP in the presence of reduced folates inhibits TS activity and depletes dTTP, a necessary precursor of DNA synthesis (Mandel, 1969; Sommer and Santi, 1974).

Alternatively, it may be phosphorylated to the triphosphate and 5-FdUTP incorporated directly into DNA, inhibiting chain elongation and altering DNA stability, resulting in the production of single-strand breaks and DNA fragmentation (Cheng and Nakayama, 1983). The fluoropyrimidines may also induce DNA strand breaks without being incorporated into DNA, possibly through the inhibition of DNA repair as a consequence of dTTP depletion (Yoshioka et al, 1987). The relative contribution of each of these mechanisms remains unclear and may depend on the specific patterns of intracellular 5-FU metabolism associated with different normal and tumour cell types.

Resistance to 5-FU is achieved through a variety of mechanisms, including deletion of the key enzyme required for its activation, the increased activity of a catabolic enzyme, a lack of reduced folate substrate and an alteration in TS activity through gene amplification, over-expression or mutation (Jastreboff et al, 1983; Berger et al, 1985; Barbour et al, 1990; Chu et al, 1991). There is also evidence of salvage pathway involvement (Grem and Fischer, 1989).

ACQUIRED RESISTANCE

Resistance to antineoplastic drugs can develop through a variety of mechanisms as cited at the beginning of this review and, more specifically, above for the chemotherapeutic antimetabolites. One of the mechanisms of acquired drug resistance commonly associated with the chemotherapeutic antimetabolites MTX, PALA and 5-FU is the amplification of the genes coding for the specific target enzymes of purine and pyrimidine biosynthesis.

Gene amplification, the process which gives rise to multiple copies of a single gene within a single cell, is the result of stepwise drug selection and results in overproduction of a normal protein and eventually high levels of resistance (Schimke, 1984*b*). It is now recognized that the propensity for gene amplification correlates for the most part but not exclusively with the transformed phenotype (Cillo et al, 1989; Lucke-Huhle, 1989; Otto et al, 1989; Perry et al, 1992). Recently, permissivity for MTX-induced gene amplification was shown to correlate with the metastatic phenotype (Lucke-Huhle, 1994). Oncogenes, e.g. myc and ras are frequently amplified in tumour cell lines (Schwab and Amler, 1990; Brennan et al, 1991), and immortalized and tumour cell lines readily develop resistance to MTX, PALA and another antimetabolite hydroxyurea (HU) (Figure 2) by amplification of the corresponding target genes (Stark and Wahl, 1984; Stark, 1986). Mutant cell lines with high rates of amplification and which are doubly resistant to both MTX and PALA have been selected from rodent cells (Giulotto et al, 1987). These cell lines were said to have an 'amplificator phenotype,' i.e. to be permissive for amplification. What is not certain is whether gene amplification is a manifestation of genetic instability or the driving force for genetic instability (Stark et al, 1989; Windle and Wahl, 1992; Stark, 1993; Ishizaka et al, 1995) and this remains difficult to assess because the drugs themselves, e.g. MTX, PALA and 5-FU, generate DNA damage and genetic instability.

However, we do know that normal rodent and human cells in vitro fail to develop resistance to drugs like MTX, PALA and 5-FU and fail to amplify the genes normally targeted by these drugs. Support for this comes from the fact that amplification has not been reported in the normal cells of patients undergoing prolonged cytotoxic drug therapy (Wright et al, 1990). Normal cells do not exhibit the genetic instability exhibited by the immortalized or tumour cells. Recent mechanistic studies suggest that the tumour-suppressor gene p53 is required to maintain the non-permissive state with respect to gene amplification (Livingstone et al, 1992; Yin et al, 1992; Lucke-Huhle, 1994). As DNA damage is likely to be the first step in the process leading to gene amplification, it is understandable why cells with an intact wild-type (WT) p53 pathway do not produce resistant subclones at experimentally measurable rates (Tlsty et al, 1989; Tlsty, 1990; Wright et al, 1990). In contrast loss of WT p53 function allows immortalized non-tumorigenic and primary fibroblasts to cycle in the presence of chromosome breaks and undergo gene amplification at experimentally measurable rates (Kastan et al, 1991; Livingstone et al, 1992; Yin et al, 1992). Titration experiments indicate that the p53-dependent arrest mechanism in normal human fibroblasts can be activated by very few double-strand breaks and that just one may be sufficient (Huang et al, 1996).

Thus the process is complicated. It is not simply a case of gene amplification being facilitated by a more aggressively transformed phenotype. We now know that pretreatment with other drugs facilitates increased resistance and may be a feature of the ability of these agents to inhibit DNA synthesis or induce DNA damage (Stark et al, 1989). One can speculate that repeated exposure to chemotherapeutic antimetabolites either alone or in combination creates the conditions for amplification of the target genes in tumour cells that are perhaps already primed for amplification by virtue of their genetic instability (Schimke et al, 1986; Stark et al, 1989). This may in turn reflect the genetic status of the target cell. Thus, tumour cells, unlike their normal counterparts, have properties that facilitate the acquisition of resistance to repeated exposures of chemotherapeutic antimetabolites.

INHERENT RESISTANCE

Acquired resistance after prolonged and repeated exposure to a chemotherapeutic agent is only one mechanism of resistance. Many tumours seem to be inherently resistant to the chemotherapeutic agent used. An example of this is 5-FU, which is the mainstay of therapy for advanced colorectal and gastric malignancies (Sotos et al, 1994). The efficacy of 5-FU is limited by the lack of response in a substantial percentage of the patients who receive the drug. It only achieves a response rate of 10–20 per cent when used as a single agent (Grem and Fischer, 1989; Sotos et al, 1994). There have been many attempts at biomodulation of 5-FU in an effort to improve its efficacy. These have included the use of MTX, PALA, cisplatin, alpha interferon and leucovorin (folinic acid) (Sotos et al, 1994). Of these, only leucovorin has proved to be clinically useful. The rationale for the combination of 5-FU and folinic acid was based on biochemical and cell culture studies using a number of cell lines (Ullman et al, 1978; Waxman et al, 1978; Evans et al, 1981; Houghton et al, 1981). These studies demonstrated that an excess of intracellular reduced folates was necessary for optimal inhibition of thymidylate synthase and for an increased cytotoxic effect of fluorinated pyrimidines. This observation led to the first clinical trial of 5-FU and folinic acid by Manchover et al (1982). Subsequently several randomized trials of 5-FU + folinic acid vs 5-FU alone were performed in advanced colorectal carcinoma patients. In 1992, such patients were subjected to meta-analysis which showed a statistically significant advantage in terms of response rate, i.e. 23% vs 11% (for 5-FU + folinic acid vs 5-FU alone) (Advanced Colorectal Meta-analysis Project). At present, 5-FU modulated by folinic acid remains the

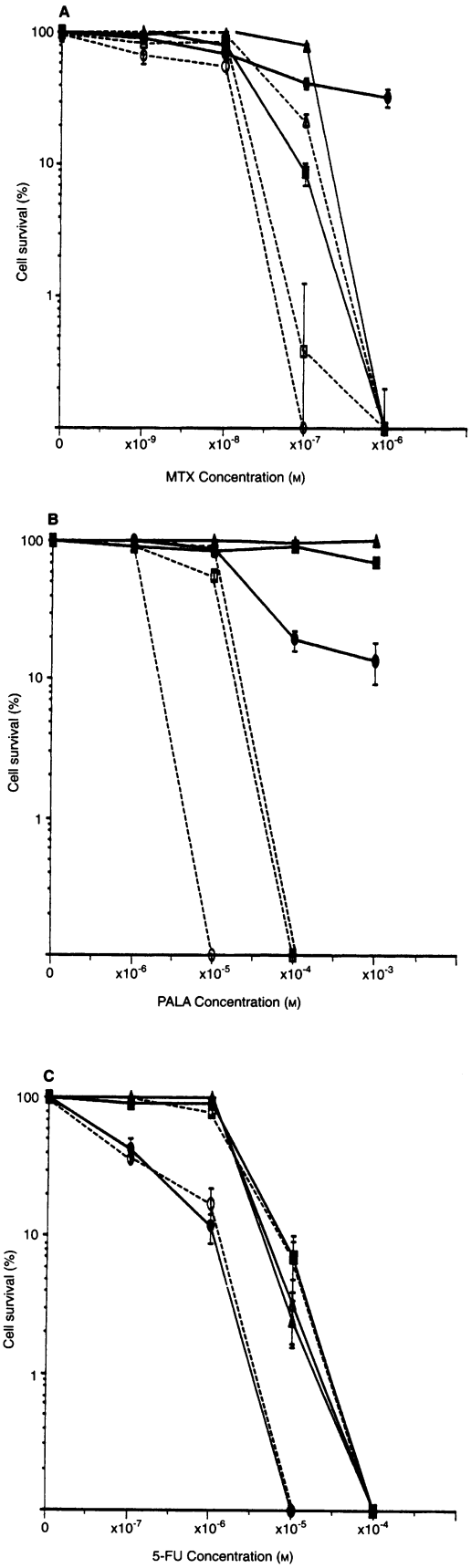

Figure 4 The intrinsic sensitivities of the normal KMS, immortalized KMST and tumorigenic KN-NM cell lines to (**A**) MTX, (**B**) PALA and (**C**) 5-FU in the presence and absence of the nucleoside transport inhibitor dipyridamole

most effective available treatment for advanced colorectal cancer. However, it seems that certain colorectal malignancies have an inherent resistance to this agent.

Over the years, it has become increasingly obvious that drugs do not only need to be targeted against the key enzymes of the de novo pathways of purine and pyrimidine biosynthesis but also against the activities of the 'salvage pathways' of purine and pyrimidine synthesis, which can circumvent the inhibition of the pathways of de novo synthesis (Weber, 1983; Kinsella and Haran, 1991; Fox et al, 1991; Weber and Prajda, 1994). It has long been recognized from in vitro studies that inhibition of de novo synthesis leads to the increased accumulation of substrates of the salvage pathways (Plagemann et al, 1978; Cadman and Benz, 1980). Studies by Weber and co-workers have demonstrated over a number of years that cancer cells in the logarithmic phase of growth and hepatomas of different growth rates show a marked rise in the activities of both the de novo and salvage enzymes of purine and pyrimidine synthesis. More recently, studies in rat hepatoma, rat sarcoma and human colorectal cancer have shown the activities of the enzymes of the salvage pathways to be higher than the rate-limiting enzymes of de novo synthesis (Natsumeda et al, 1989). Camici et al (1990) reported higher levels of the purine salvage pathway enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) in tumours than in peritumour tissues. This highlighted the role that salvage pathways might play in circumventing the action of the antimetabolites in a therapeutic setting.

Certainly there are a spectrum of reports in vitro of augmentation of the effects of MTX by inhibition of thymidine salvage using the nucleoside transport inhibitor dipyridamole (Marz et al, 1977; Cabral et al, 1984; Nelson and Drake, 1984; van Mouwerik et al, 1987). Dipyridamole is a reversible competitive inhibitor of nucleoside transport. The ability of dipyridamole to prevent nucleoside salvage in general has been exploited in human cell lines in in vitro studies of both MTX and PALA cytotoxicity (Chan and Howell, 1985; Kennedy et al, 1986). Moreover, AraC enhancement of MTX and PALA resistance was blocked by the nucleoside transport inhibitor dipyridamole (Goz and Jeffs, 1994). Previous work by the authors (Kinsella and Haran, 1991) showed increasing resistance to the chemotherapeutic antimetabolites MTX and PALA paralleling progression towards tumorigenicity in a series of isogenic human fibroblast cell lines. An increase in the sensitivity of all the cell lines to both MTX and PALA was observed when the experiments were performed in dialysed serum from which essentially all the salvage pathway substrates had been eliminated. This suggested an important role for the salvage pathways of purine and pyrimidine biosynthesis and their substrates in the original resistance of these cell lines. This was confirmed by the restoration of the resistance of the KN-NM tumorigenic cell line to PALA by the addition of uridine to the medium. Uridine is the key nucleoside for PALA 'rescue' (Figure 2) in that both CTP and TTP can be produced from it. This was the first comparative study in isogenic cell lines and showed not only that salvage pathways were involved in resistance but also that there were very clear differences in the levels of the salvage pathway activity between normal and tumour cells.

Extension of these initial in vitro studies to include the nucleoside transport inhibitor dipyridamole and another chemotherapeutic antimetabolite, 5-FU, provided an unexpected insight into the possible operative mechanisms. As expected, from our previous observations with MTX and PALA (Kinsella and Haran, 1991), the tumorigenic human fibroblast cell line KN-NM

was more resistant than the immortalized cell line KMST from which it was derived, which in turn was more resistant than the normal cell line KMS to a single exposure of 5-FU in clonogenic assays. Addition of dipyridamole increased the intrinsic sensitivities of all three cell lines to MTX and PALA (Figure 4A and B), which was consistent with the earlier observations in dialysed serum (Kinsella and Haran, 1991). The effects of dipyridamole on PALA resistance were reversed by the addition of the salvage pathway substrate uridine and on MTX resistance by the addition of hypoxanthine and thymidine in combination (Pickard et al, 1995). However, addition of dipyridamole had no effect on the resistance of any of the three cell lines to 5-FU (Figure 4C). This suggested that, contrary to expectation, there was no salvage pathway involvement in the increased resistance of the immortalized (KMST) and tumorigenic (KN-NM) cell lines to 5-FU (Figure 4C).

So, what is going on in these cells in response to 5-FU? Early reports in the literature have shown 5-FU inhibition of TS to be growth limiting in mouse sarcoma and mouse L cells and have shown that this can be reversed by the addition of exogenous thymidine (Madoc- Jones and Bruce, 1968; Evans et al, 1980). This suggests that dipyridamole may be able to enhance 5-FU cytotoxicity by inhibiting thymidine salvage in cells in which the DNA-directed effects of 5-FU predominate. This has been confirmed in human colon carcinoma cell lines in which inhibition of TS has been shown to be growth limiting at relatively low concentrations of 5-FU (Miller et al, 1987; Schwartz et al, 1987). Obviously, in our study, no such effect of dipyridamole is operative. Dipyridamole has previously been shown to enhance 5-FU induced cytotoxicity in a human colon carcinoma cell line (Grem and Fischer, 1985), but this was shown not to be as a result of depletion of the TTP pools and was unrelated to the availability of exogenous thymidine. In fact, dUMP was shown to be the key effector of cytotoxicity (Grem and Fischer, 1986). This lack of a role for salvage pathway involvement is reasonably consistent with the evidence from clinical studies.

Whether or not a thymidine salvage pathway contributes to clinical resistance to 5-FU is difficult to determine with absolute certainty. Several phase I/II trials have been performed using dipyridamole in combination with 5-FU-based chemotherapy in an attempt to address this question and in general the results suggest that little if any benefit accrues from the addition of dipyridamole (Tsavaris et al, 1990; Grem, 1992). This is in keeping with the clinical observation that folinic acid potentiates the cytotoxicity of 5-FU, suggesting that dUMP interaction with thymidylate synthase is the major effector of 5-FU cytotoxicity, which would be consistent with the early in vitro observations of Grem and Fischer (1986). However, it is probable that none of the studies investigating 5-FU + dipyridamole achieved concentrations of dipyridamole in the range known to biomodulate 5-FU in vitro in cell culture systems. In one trial, dipyridamole and 5-FU were administered as a continuous intravenous infusion over 72 h (Grem, 1992). At the maximum-tolerated dose of dipyridamole, the steady state concentration was of the order of 25 nM, some 20-fold lower than that known to produce optimal biomodulation of 5-FU in the HCT116 colorectal carcinoma cell line. In addition, in this trial, dipyridamole caused an increase in the clearance of 5-FU, resulting in a reduced steady-state plasma concentration and possibly reducing the effectiveness of the drug. A further piece of evidence is an early study in which thymidine was actually combined with 5-FU (Vogel et al, 1979). This was based on evidence that thymidine could enhance the incorporation of 5-FU into RNA and that thymidine could delay the breakdown of 5-FU by the hepatic enzyme dihydrouracil dehydrogenase and thus prolong the 5-FU plasma half-life. In this trial, thymidine did not alter the effect of 5-FU. It appears therefore that at clinically relevant doses neither dipyridamole nor thymidine affect resistance to 5-FU.

RESISTANCE TO 5-FU A FUNCTION OF DNA DAMAGE RESPONSE

Recently, it has been postulated that the outcome of drug therapy is determined by the response of a cell according to its phenotype rather than by the nature of the primary drug target interaction (Dive and Hickman, 1991). The hypothesis was that tumour cells were resistant to the genetic programme for cell death and therefore the tumour cell population continued to expand and appeared to be resistant to cytotoxic drugs. It is now known that the p53 gene product in its role as 'guardian of the genome' (Lane, 1992; Levine et al, 1993), plays an important role in determining the cellular response to DNA damage. Expression and stability of the p53 gene product is induced in cells following exposure to DNA damaging agents (Kastan et al, 1991) and leads either to cell cycle arrest, which may facilitate DNA repair (McIlwrath et al, 1994; Nelson and Kastan, 1994), or cell death by apoptosis (Clarke et al, 1993; Lowe et al, 1993*a*, *b*), dependent on cell type.

In human fibroblast studies undertaken by the authors, immunohistochemical analysis (Hall and Lane, 1994) suggested that the normal p53 function had been disrupted in the immortalized and tumorigenic cell lines (Pickard et al, 1995). This led us to postulate that the differences in the sensitivities of the cell lines to 5-FU (Figure 4C) might be a consequence of differing cellular responses to drug-induced damage. Measurement of 5-FU-induced apoptosis in the three cell lines showed the normal KMS cell line with WTp53 to apoptose at a lower level than its more resistant immortalized and tumorigenic derivatives (Pickard et al, 1995). Thus, the differences in resistance could not be explained on the basis of differences in apoptosis between the cell lines. Detailed cell cycle analysis and proliferation studies, however, showed that the normal human fibroblast cell line ceased to proliferate in response to increasing concentrations of 5-FU and entered a permanent growth arrest at a 5-FU concentration as low as 1×10^{-5}M. This was in contrast to the immortal and tumorigenic cell lines that continued to proliferate at a 5-FU concentration of 1×10^{-5}M, apparently regardless of the drug insult. This suggested that the G_1 checkpoint had been lost in these cell lines. The loss of p53 function may be an important factor in determining the increased resistance of the immortalized and tumorigenic cell lines to 5-FU.

IS THERE A ROLE FOR DNA DAMAGE RESPONSE IN RESISTANCE TO MTX AND PALA?

Recent studies have identified increased levels of p53 protein that coincide with the appearance of strand breaks induced by all three of the antimetabolites MTX, PALA (Nelson and Kastan, 1994) and 5-FU (Fritsche et al, 1993). However, the evidence of a strong salvage pathway involvement in the resistance to MTX and PALA allows one to speculate that the salvage pathways may be operating to repair and synthesize DNA to such a degree that the strand breaks expected to occur in response to antimetabolite insult fail to materialize. For example, in the case of the resistance of the human fibroblast cell

lines to PALA (Figure 4B) (Pickard and Kinsella 1996), all the substrates distal to the block (Figure 2) will have to be used. Moreover, the nucleosides and bases in the surrounding environment can be used by the salvage pathways to regenerate any depleted pools. Any available thymidine will be converted to TTP and any available uridine will be converted to CTP and TTP. Thus, the nucleotides required for DNA synthesis and repair continue to be provided, and so no strand breaks occur and no DNA damage response pathway is initiated. With the addition of dipyridamole and the inhibition of nucleoside and nucleobase transport, the levels of available uridine and thymidine for the salvage pathways are greatly decreased and the availability of CTP and TTP is diminished. As a result, DNA synthesis and repair are inhibited and DNA strand breaks occur, eliciting a delayed DNA damage response in the presence of normal p53 in the KMS cell line and allowing the continued proliferation of the KMST and KN-NM cell lines with their dysfunctional p53. If we look in detail at the effect of dipyridamole (5 uM) on the resistance of the three cell lines to PALA (Figure 4B), we see that dipyridamole increases the intrinsic sensitivities of both the immortalized (KMST) and the tumorigenic (KN-NM) cell lines so that they are equally sensitive and increases the sensitivity of the already sensitive normal (KMS) cell line. However, even in the presence of dipyridamole, there is still a difference in resistance to PALA between the normal and the more tumorigenic cell lines (Figure 4B). Thus, the differing abilities of the cell lines to elicit a DNA damage response explains the enhanced resistance of the KMST and KN-NM cell lines to PALA compared with the KMS cell line, even in the presence of dipyridamole (Figure 4b). There are therefore two clear components to the response of these cells to PALA.

The situation with MTX (Figure 4A) is more complicated but one can speculate that the same principles apply (Pickard and Kinsella, 1996). We know from the 5-FU data that the normal KMS cell line responds to DNA damage. Thus, the differences in the resistance of these particular human fibroblast cell lines to both MTX and PALA are clearly not only a function of differences in the salvage pathways but also of differences in the abilities of the cells to elicit a p53-dependent DNA damage response. In the case of 5-FU (Figure 4C), one has to assume in the absence of an effect of dipyridamole that the drug is mediating its effects via its direct action on DNA (Parker and Cheng, 1990) and that, as stated previously, only the DNA damage response is important. No growth arrest was observed in any of the cell lines at concentrations of PALA up to and including 10 mM, except in the case of the normal cell line in the presence of dipyridamole.

Recently, however, in a different human fibroblast cell line (Linke et al, 1996), UTP, GTP and CTP depletion following treatment with PALA (or pyrazofurin) resulted in reversible antimetabolite-induced growth arrest in the G_1 phase of the cell cycle, which correlated with p53 induction in the absence of apparent DNA damage. In the same fibroblast cell line, MTX and 5-FU treatment resulted in an early S phase arrest that could be prevented by co-treatment with the salvage pathway substrate thymidine. Thus, Linke et al (1996) speculated that p53 might play a role in the induction of a quiescent state in response to metabolite depletion and senescence or permanent arrest, such as that reported by the authors in human fibroblasts in response to 5-FU (Pickard et al, 1995) and by Di Leonardo et al (1994) in response to irradiation in the presence of DNA damage. It is known that p21, a downstream effector of p53, increases reversibly in quiescent cells and irreversibly in senescent cells (Noda et al, 1994). These studies and those of Li et al (1995) also provide evidence of a link between p53 and the product of retinoblastoma tumour-suppressor gene (RB) in the resistance of certain cells to the chemotherapeutic antimetabolites (Almasan et al, 1995; Li et al, 1995). RB codes for a nuclear protein implicated in the transition from the G_1 to S phase of the cell cycle. The G_1 cyclin-dependent kinase (cdk) complexes, which are inhibited by p21 a downstream effector of p53, phosphorylate the RB protein (Harper et al, 1993; Dulic et al, 1994), allowing the release of the transcriptional activator E2F and the activation of S-phase genes, such as DHFR (Blake et al, 1989; Slansky et al, 1993) and TS (Johnson, 1994). Consistent with this, growth inhibition by MTX and 5-FU was increased, and DHFR and TS activities and expression were correspondingly decreased in SaOS-2 human sarcoma cells containing a truncated RB protein that had been stably transfected with an RBcDNA (Li et al, 1995).

CLEAR EVIDENCE FOR A ROLE FOR THE P53 DAMAGE RESPONSE IN ACQUIRED AND INHERENT DRUG RESISTANCE

The evidence for p53 being involved in both acquired and inherent resistance is consistent with what we know about the role of p53 (Lane, 1992; Levine et al, 1994). p53 plays an important role in genetic stability as a consequence of its ability to prevent the entry of damaged cells into the S phase of the cell cycle and thus preventing the replication of damaged DNA. It is known that many environmental insults and cancer therapies, including administration of the chemotherapeutic antimetabolites MTX, PALA and 5-FU, increase the levels of p53 protein expression, leading to either arrest in the G_1 phase of the cell cycle or apoptosis (Kastan et al, 1991; Clarke et al, 1992; Kuerbitz et al, 1992; Livingstone et al, 1992; Yin et al, 1992; Lowe et al, 1993*a,b*; Di Leonardo et al, 1994; Pickard et al, 1995). As cited above, the G_1 arrest pathway probably involves activation of p21, which inhibits G_1 cyclin–cdk complexes and which in turn inhibits phosphorylation of the RB protein and progression into the S phase of the cell cycle (Harper et al, 1993; Xiong et al, 1993; Dulic et al, 1994). It is also likely that other factors downstream of p53 are involved in this arrest response. Mutations in the *p53* gene eliminate these p53-dependent responses. Mutations in the *p53* gene are the commonest genetic change in human tumours and occur in 60% of tumours overall and 75% of colorectal malignancies (Hollstein et al, 1991; Levine et al, 1991). The presence of a mutated or dysfunctional *p53* gene product will permit the continued replication of damaged DNA, producing the conditions that facilitate gene amplification. This is consistent with the observation that cells with no p53 protein (null mutation) amplify their DNA at least a million times more readily than cells containing the normal wild-type *p53* gene product (Livingstone et al, 1992; Yin et al, 1992). Similarly, in situations of inherent resistance to single doses of chemotherapeutic antimetabolites, facilitated by the 'salvage pathways' of purine and pyrimidine biosynthesis, mutant or dysfunctional p53 allows the cells that eventually accumulate DNA damage (because of restricted substrate availability) to continue to proliferate and therefore manifest their resistance. Loss of the p53 damage response therefore contributes to both acquired and inherent drug resistance.

CLINICAL IMPLICATIONS

Support for the circumvention of the de novo pathways of purine and pyrimidine biosynthesis by the salvage pathways comes from the observation that there are sufficient levels of nucleotides and

nucleosides in human plasma to overcome the inhibitory and cytotoxic effects of the drugs (Gordon, 1985; Sinkeler et al, 1994). The plasma nucleotide concentrations represent a balance between the release of nucleotides and their degradation by extracellular nucleotidases (Boyle et al, 1989). In addition, the cytotoxic therapy itself leads to destruction of tumour cells, which is thought to provide high local concentrations of nucleosides and nucleotides in tumour patients (Fox et al, 1991).

However, few studies have dealt with the genetic factors that might control the resistance (inherent and acquired) to DNA-damaging agents and chemotherapeutic antimetabolites in vivo. The recent evidence of a role for the tumour-suppressor gene *p53* in mediating the response of cells to chemotherapeutic agents in vitro (Lowe et al, 1993*b*; Nelson and Kastan, 1994; Pickard et al, 1995) suggests that p53 dysfunction assessed quite simply by immunohistochemistry (Hall and Lane, 1994) might provide us with a preliminary predictor of the likelihood of a response to chemotherapy for individual tumours. On theoretical grounds, one can predict that the loss of normal p53 function will be associated with more rapidly advancing disease because of the fact that the replication of damaged DNA will be associated with the induction of additional point mutations and loss of heterozygosity (Harris and Hollstein, 1993; Greenblatt et al, 1994). Indeed, p53 protein expression has already been implicated as an independent prognostic indicator in carcinomas of the colon, stomach, breast, bladder and lung (NSCLC) (Dowell and Hall, 1994). The availability of immunohistochemical and polymerase chain reaction (PCR)-based diagnostic techniques means that studies can be performed on archival material, even from completed clinical trials. This will provide clinicians with the opportunity both to identify the more aggressive tumours and assess the significance of p53 and its related pathways and pathway molecules, e.g. p21, Gadd45, bcl2, bax, mdm2, p16 and RB (Di Leonardo et al, 1994; Hartwell and Kastan, 1994; Miyashita et al, 1994; Reed, 1994) in predicting chemotherapeutic and other therapeutic responses. Such studies can address the specific and general hypotheses associated with the progression to malignancy. For example, certain tumours with p53 mutations might be more resistant to drug-induced apoptosis, while others may be more sensitive to amplification of genes which might influence therapy. In the clinical situation, p53 mutations have been associated with resistance to chemotherapy in haematological malignancies (Fan et al, 1994; Hecker et al, 1994; Wattell et al, 1994) and in a study by the authors on 59 advanced colorectal carcinomas (Brett et al, 1996). Conversely, in testicular tumours elevated levels of Wtp53 may contribute to their sensitivity to DNA-damaging chemotherapeutic agents, which might explain their high cure rate (Chresta et al, 1996; Chresta and Hickman, 1996; Lutzker and Levine, 1996). Moreover, tumours lacking RB or having functional abnormalities of this protein might be more resistant to treatment with drugs that target DHFR or TS. For example, cell lines from RB patients and small-cell lung carcinomas lacking a functional RB protein are intrinsically resistant to MTX. Cancer cell survival after chemotherapy will depend on the specific cell cycle checkpoints or repair functions that have been lost. Greater susceptibility to these agents will be observed when the repair function is most important and greater resistance when it is not and the cells continue to cycle or resist apoptosis. We can predict a time when it will be possible to characterize tumours for these functions and thereby predict their responses to specific therapies in the clinic. Assessing the p53 and RB status of tumours would seem an important and worthwhile first step in the process of identifying subsets of patients with favourable and unfavourable prognoses in response to standard treatment protocols.

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