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Influence of Both Salvage and DNA Damage Response Pathways on Resistance to Chemotherapeutic Antimetabolites

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ABSTRACT. The resistance of 3 human embryo fibroblast cell lines to the antimetabolites methotrexate (MTX), N-phosphonacetyl-L-aspartate (PALA) and 5-fluorouracil (5-FU) has been studied. The cell lines were of common genetic origin, all originating from the normal KMS parental cell line, which was irradiated with ^{60}Co to produce the immortalised derivative KMST which, in turn, was transfected with an activated N-*ras* oncogene to produce the tumourigenic KN-NM cell line. Previous work from this group, using dialysed versus nondialysed serum, has provided evidence for the involvement of salvage pathways of purine and pyrimidine biosynthesis in the increased resistance to antimetabolites of those cell lines (KMST and KN-NM) tending towards increased tumourigenicity. The present study has extended this work by using the nucleoside and nucleobase transport inhibitor dipyridamole, to further assess the contribution of the salvage pathways to the increased cellular resistance to the three antimetabolites. The salvage pathways were found to contribute to the resistance of cell lines to PALA and MTX, but had no effect on the resistance to 5-FU. The addition of excess uridine in the case of PALA, and hypoxanthine plus thymidine in the case of MTX, could be used to "rescue" cells from the effects of dipyridamole-induced salvage pathway inhibition. The data will be discussed in relation to 1. the effect of limited substrate availability, 2. the induction of DNA damage and DNA damage-response pathways, and 3. DNA-damage protection by the salvage pathways of purine and pyrimidine biosynthesis. BIOCHEM PHARMACOL 52;3:425–431, 1996.

KEY WORDS. drug resistance; antimetabolites; salvage pathways; dipyridamole

Resistance of tumour cells to chemotherapeutic agents is a major problem in the treatment of human malignancy. Differences in the metabolic and structural properties of cells may lead to drug-resistant phenotypes, and many of these mechanisms of resistance have been extensively studied and reviewed [1] (and refs. therein). The antimetabolite group of drugs block the *de novo* synthesis of the purines and pyrimidines required for DNA synthesis [1]. The resistance of tumour cells to certain of these antimetabolites has been attributed to either gene amplification [2] (and refs. therein) or to an increase in the activity of the salvage pathways for purine and pyrimidine biosynthesis [3]. Resistance to MTX† has been linked to the amplification of its target gene, dihydrofolate reductase (DHFR) [4] and increased resistance to PALA has been attributed to amplification of the multifunctional CAD gene [5]. Despite these data, gene amplification mediating drug resistance has been documented in very few human malignancies [2, 6]. Resistance has also been reported to be due to the increased activities of the enzymes in the *de novo* pathways or of the enzymes in alternative pathways for purine and pyrimidine biosynthesis, the salvage pathways. A marked rise in the activities of the enzymes of both pathways has been observed in cancer cells in logarithmic growth, and in hepatomas of differing growth rates [3]. Previous work from this group has demonstrated differences in the sensitivity of a series of human embryo fibroblast cell lines, of common genetic origin, to the antimetabolites MTX and PALA [6]. The data obtained, using MTX and PALA in the presence of either nondialysed or dialysed serum, showed increasing resistance to both drugs to parallel increasing tumourigenicity as a consequence of salvage pathway involvement.

Mammalian cells possess, in their plasma membranes, specific transport elements that mediate the entry and exit of purine and pyrimidine nucleosides and bases by facilitated diffusion [7–9]. By blocking these transport processes, the availability of nucleosides and bases as substrates for the salvage pathways is greatly reduced. Dipyridamole is one of

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† *Abbreviations:* MTX, methotrexate; 5-FU, 5-fluorouracil; PALA, N-(phosphonacetyl)-L-aspartate; FdUMP, fluorodeoxyuridine monophosphate; TS, thymidylate synthetase; TTP, thymidine-tri-phosphate; CTP, cytidine-tri-phosphate; dUMP, deoxy-uridine-mono-phosphate; dTMP, deoxy-thymidine-mono-phosphate; GTP, guanine-tri-phosphate; ATP, adenine-tri-phosphate.

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many compounds known to block nucleoside and nucleobase transport [7]. It has been shown to inhibit nucleoside uptake in the heart [10], erythrocytes [11], rat hepatoma cells [12, 13], chick fibroblasts [14] and the HCT 116 colorectal tumour cell line [15]. The mechanism of this "blockade" on nucleoside and nucleobase transport is non-specific, as demonstrated in studies using murine leukaemia cells [16].

Thus, the previous studies from this group have been extended to include a third antimetabolite, 5-FU, and the effect of dipyridamole on the resistance of the same 3 human fibroblast cell lines to all 3 drugs, MTX, PALA, and 5-FU. The results will be discussed in relation to current theories that drug resistance is determined not only by the nature of the initial drug-target interaction and the extent of DNA damage produced, but on how the cell responds to the DNA damage present [17, 18]. This theory suggests that the phenotype of the cell determines the nature of the DNA damage response and changes in the genotype may create differing cellular responses to DNA damage. Thus, the intrinsic resistance pattern of a particular cell may become altered. The concept of the salvage pathways providing DNA damage protection will also be discussed.

METHODS

Cell Lines

The cell lines used in the study were the normal KMS-6 human embryo fibroblast cell line, its ^{60}Co- immortalised derivative KMST-6 [19] and the KN-NM cell line established from a fibrosarcoma that resulted from activated N-ras infection of the immortalised KMST-6 cell line [20]. Previous immunocytochemical and cell cycle analysis by this group has provided strong evidence to suggest that the normal KMS cell line has wild type p53 functionality, in contrast to both the immortal KMST and the tumourigenic KN-NM cell lines that appear to have lost p53 function [21]. All cell lines were routinely maintained in Dulbecco's modified Eagle's medium (Life Technologies Ltd., Paisley, Scotland) supplemented with 10% foetal bovine serum (ICN Biomedicals, Irvine, Scotland) and 200 mM glutamine at 37°C in a humid atmosphere of 5% CO_2/95% air. The same serum batch was used for the entire series of experiments. Cells were routinely plated at a density of 1×10^5 per 25 cm^2 tissue culture flask in 5 mL of medium and passaged approximately every 4 to 5 days by trypsinisation.

Measurement of Intrinsic Drug Sensitivities

To assay for drug resistance (sensitivity) 500 test cells were plated on to a feeder layer of 5×10^4 gamma irradiated (5000 rads.) EJ human bladder carcinoma cells in a 10 cm tissue culture plate (5 plates/point) and allowed to attach for 4 hr. The feeder cell line was necessary only for the successful cloning of the normal fibroblast cell line, but was included for all cell lines to eliminate any complications that might arise from feeder cell metabolites with regard to the supply of "salvage pathway" substrates. After 4 hr, the medium was removed and replaced with medium containing MTX (1×10^{-9} M–1×10^{-5} M) (Sigma, Poole, U.K.), 5-FU (1×10^{-7} M–1×10^{-3} M) (Sigma) or PALA (1×10^{-6} M–1×10^{-3} M) (Drug Synthesis and Chemistry Branch, NIH, Bethesda, MD, U.S.A.). The medium was replaced with drug-free medium at the end of weeks 1 and 2. The plates were incubated for a total of 3 weeks to permit colony formation. After 3 weeks, any colonies were fixed, stained, and counted, as described previously [6]. The percent cell survival was calculated from the number of colonies on treated plates expressed as a percentage of the colonies on the control drug-free plates. Identical experiments were performed using dipyridamole (5 μM) to block nucleoside transport and, where appropriate, hypoxanthine (100 μM), thymidine (100 μM), and uridine (200 and 500 μM) purchased from Sigma Chemical Co., (Poole, Dorset, England) were added. All experiments were repeated at least 3 times.

Assessment of Cell Growth

To assess the effect of the three antimetabolites on cell growth, cultures were monitored over a 3-week period. Cells were plated at a density of 5×10^5 cells onto a 75 cm^2 flask. The cells were allowed enough time to attach before the medium was aspirated, and replaced by drug-containing medium at the relevant drug concentrations. The cells were exposed to 5-FU (1×10^{-5} and 1×10^{-4} M), MTX (1×10^{-7}, 1×10^{-6}, and 1×10^{-5} M) and PALA (1×10^{-5}, 1×10^{-4}, and 1×10^{-3} M). Every 48 hr thereafter, for the first 8 days, the cell counts were obtained at each of the different drug concentrations. At 8 days, the drug-containing medium on the remaining flasks was replaced with fresh drug-free medium and cell counts were, again, obtained on day 14. Following a further medium change of the remaining flasks, the final cell count for each drug concentration was obtained on day 21. The experiment was repeated on 3 separate occasions and in the presence and absence of dipyridamole (5 μM).

RESULTS

Intrinsic Cell Line Sensitivities to the Three Antimetabolites in the Presence and Absence of Dipyridamole

Differences in the sensitivities of the 3 cell lines could be observed when tested for resistance to all 3 antimetabolites. In the case of PALA, the normal embryonic KMS fibroblast cell line demonstrated increased sensitivity with increasing concentration and a 13.6 ± 4.5% cell survival at 1×10^{-3} M (Fig. 1). The experiment was repeated on 2 other occasions and the cell survival was over the range of 9.6 to 21% at 1×10^{-3} M. The chromosomally abnormal, immortalised derivative of KMS, KMST, showed increased resistance in comparison to KMS, with a 68% cell survival at 1×10^{-3} M

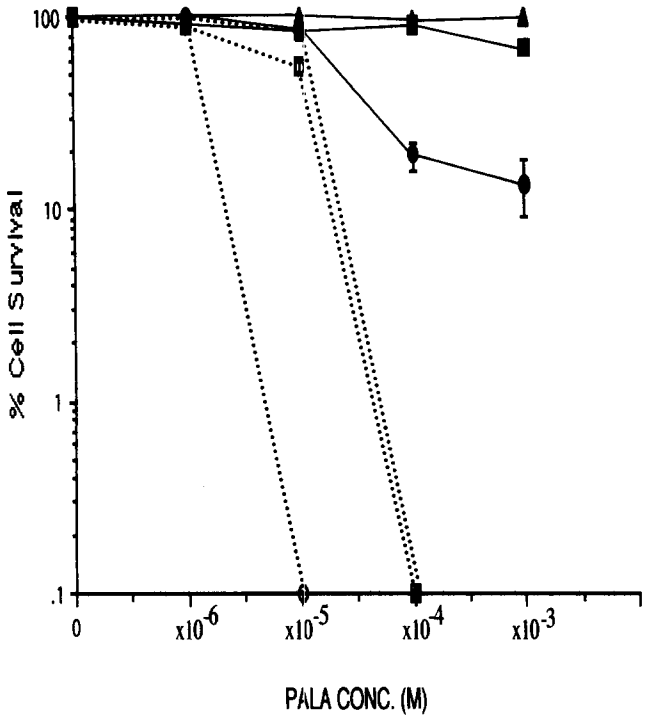

FIG. 1. Intrinsic PALA sensitivities for the cell lines in the presence and absence of dipyridamole (5 μM). —●—, KMS normal fibroblasts; —■—, KMST immortalised fibroblasts; —▲—, KN-NM tumourigenic fibroblasts; --○--, KMS normal fibroblasts + dipyridamole; ··□···, KMST immortalised fibroblasts + dipyridamole; and ··△··· KN-NM tumourigenic fibroblasts + dipyridamole. Representative data from a single experiment (5 plates point).

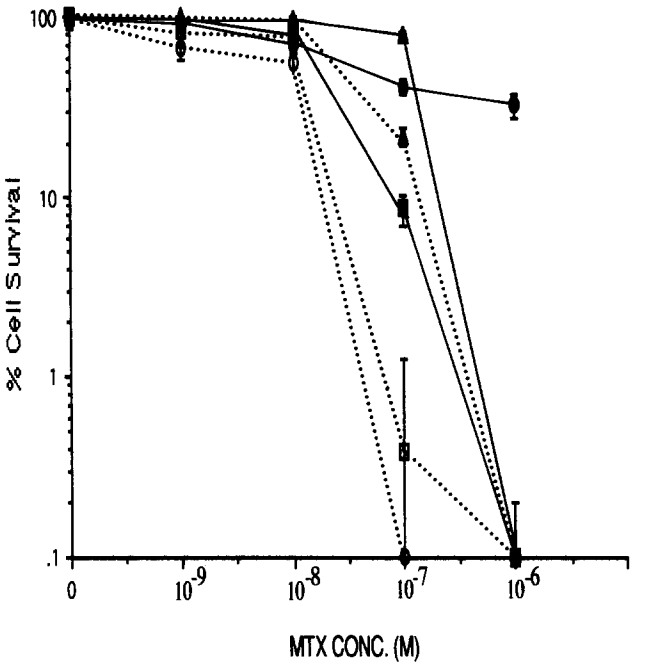

FIG. 2. Intrinsic MTX sensitivities for the cell lines in the presence and absence of dipyridamole (5 μM). —●—, KMS normal fibroblasts; —■—, KMST immortalised fibroblasts; —▲—, KN-NM tumourigenic fibroblasts; ···○··, KMS normal fibroblasts + dipyridamole; ··□···, KMST immortalised fibroblasts + dipyridamole; and ···△··· KN-NM tumourigenic fibroblasts + dipyridamole. Representative from a single experiment (5 plates point).

PALA. Resistance was increased (to 100% cell survival) in the N-*ras* infected tumourigenic KN-NM cell line at the highest PALA concentration of 1×10^{-3} M. The cell survivals at this concentration were 59.6 and 72.3% for the KMST and 91.1 and 100.6% for KN-NM on 2 other occasions. When the experiments were repeated in the presence of dipyridamole, the sensitivities of all the cell lines were greatly increased (Fig. 1). The sensitivities of the KMST and the KN-NM cell lines were very similar and no cell survival was observed above a drug concentration of 1×10^{-4} M for either cell line. In contrast, the normal KMS cell line was more sensitive than both the KMST or KN-NM cell lines, with no cell survival apparent above a drug concentration of 1×10^{-5} M. This phenomenon was partly reversed by the addition of the salvage pathway substrate uridine to the drug-containing medium, which resulted in increased resistance of all 3 cell lines (data not shown). Uridine, at a concentration of 200 μM, when added to the KN-NM cell line, resulted in a cell survival of 21% at 1×10^{-4} M PALA and, at a concentration of 500 μM uridine, 59% and 4% of cells survived at PALA concentrations of 1×10^{-4} M and 1×10^{-3} M, respectively.

In the case of MTX, the differences in sensitivity were not as marked as those seen with PALA (Fig. 2). The KMS cell line was more resistant than expected, with a 33% survival at 1×10^{-6} M MTX and was, in fact, more resistant than either the KMST or KN-NM cell line. A similar pattern was observed when the experiments were repeated on 2 other occasions with 55.2 and 43.4% survival for the KMS cell line at 1×10^{-6} M MTX. The KN-NM cell line was more resistant than the KMST cell line. However, no cell survival was apparent at 1×10^{-6} M MTX for either cell line (Fig. 2) in any of 3 experiments. When the experiments were repeated in the presence of dipyridamole, the sensitivities of all 3 cell lines were increased and the KMS cell line became more sensitive than either the KN-NM or KMST cell lines, as one might have originally predicted. This increase in sensitivity in the presence of dipyridamole could be reversed by the simultaneous addition of hypoxanthine (100 μM) and thymidine (100 μM) to the culture medium. No "rescue" effect was observed when the two nucleosides were added separately (data not shown).

A pattern of resistance, similar to that observed with PALA alone, was observed following exposure to 5-FU in that the KMS cell line was more sensitive than either the KMST or KN-NM cell lines (Fig. 3). No cell survival was apparent at 1×10^{-5} M 5-FU for the KMS cell line. This was in contrast to the KMST and KN-NM cell lines, which did not demonstrate zero cell survival until a drug concentration of 1×10^{-4} M. In turn, the KMST and KN-NM cell lines showed very little difference in resistance compared with one another. Surprisingly, the addition of dipyrid-

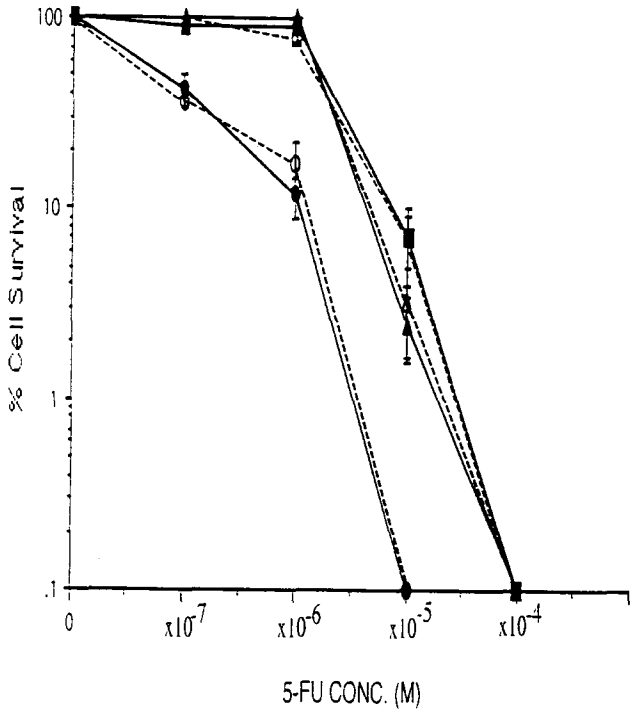

FIG. 3. Intrinsic 5-FU sensitivities for the cell lines in the presence and absence of dipyridamole (5 μM). —●— KMS normal fibroblasts; —■—, KMST immortalised fibroblasts; —▲—, KN-NM tumourigenic fibroblasts; --○--, KMS normal fibroblasts + dipyridamole; --□--, KMST immortalised fibroblasts + dipyridamole; and --△-- KN-NM tumourigenic fibroblasts + dipyridamole. Representative data from a single experiment (5 plates point).

amole did not alter the sensitivity of any of the cell lines to 5-FU (Fig. 3).

Permanent Arrest of the Normal Fibroblast Cell Line in the Presence of 5-FU But Not MTX and PALA

Previous work by this group has established that the normal KMS cell line responds to presumed 5-FU-induced DNA damage by arresting its cell cycle in G1 [21]. To test the duration of this G1 arrest, monolayer cultures were set up to establish the effects of 5-FU, MTX, and PALA on cell number over a 3-week period. The cell counts obtained during the initial 8-day period of exposure to 5-FU and over the following 2 weeks in fresh drug-free medium, Fig. 4a, show that the cell number increased in control drug-free medium from 2.9×10^5 at time zero to 2.42×10^6 on day 8. The cells began to encounter contact inhibition and reach confluence between days 6 and 8, but still managed to increase their cell number to 2.72×10^6 on day 14 and 2.83×10^6 on day 21. This was in contrast to the situation with 1×10^{-5} and 1×10^{-4} M 5-FU, where the cell number never increased above the plating cell density of 2.9×10^5 and stayed static over the entire 3-week period. The KMST and KN-NM cell lines continued to proliferate at 1×10^{-5} M FU even in the presence of dipyridamole (5 μM) [21].

In the case of MTX and PALA, Fig. 4b and c, respectively, the cell numbers for the KMS cell line increased steadily in the presence of both MTX and PALA over both the first 8 days and the following 2 weeks in fresh drug-free medium. Round, detached cells could be observed under the microscope even in the slower growing cultures at high drug concentration, which were consistent with cell populations that were still proliferating. In the presence of dipyridamole, the KMS cells went into permanent growth arrest at a PALA concentration of 1×10^{-5} M and a MTX concentration of 1×10^{-7} M (data not shown).

DISCUSSION

The importance of the role of the salvage pathways for purine and pyrimidine biosynthesis in increasing the resistance of tumour cells to the chemotherapeutic antimetabolites has been reported previously [1, 3, 6]. The aim of the present study was to extend the studies of Kinsella and Haran [6] to investigate further the contribution of the salvage pathways to the resistance to the antimetabolites observed in a series of human fibroblast cell lines. To this end, resistance to the three antimetabolites MTX, PALA, and 5-FU, in the presence and absence of the nucleoside transport inhibitor dipyridamole, has been studied using clonogenic assays.

Intrinsic Cellular Sensitivity to MTX, PALA and 5-FU

A slight increase in resistance to PALA, paralleling increasing tumourigenicity, was observed but essentially all 3 cell lines were very resistant to the PALA-induced *de novo* block (Fig. 1). This high level of resistance may be explained by virtue of the fact that PALA blocks a very early step in the *de novo* pathway of pyrimidine synthesis [3, 22]. Thus, all substrates distal to the block will have to be utilised before any PALA-induced toxicity resulting from diminishing pyrimidine pools is observed. The increased sensitivity of all 3 cell lines to PALA in the presence of dipyridamole, Fig. 1, also suggests that high levels of nucleosides and bases in the surrounding environment are being taken up by the salvage pathways to replenish the depleted pools. The normal KMS cell line remained the most sensitive of all 3 cell lines, and the immortalised KMST and tumourigenic KN-NM cell lines exhibited identical sensitivities (Fig. 1). With the addition of dipyridamole and the resultant inhibition of nucleoside and nucleobase transport, one can speculate that the availability of uridine and thymidine as substrates for the salvage pathways was greatly decreased. Thus, in turn, the supply of CTP and TTP for DNA synthesis and repair would be severely depleted, causing the increased sensitivity to PALA. Uridine is the key nucleoside for PALA rescue, in that both CTP and TTP can be produced from it. This was confirmed in the present study when the addition of exogenous uridine (200 μM) was seen to prevent toxicity in the

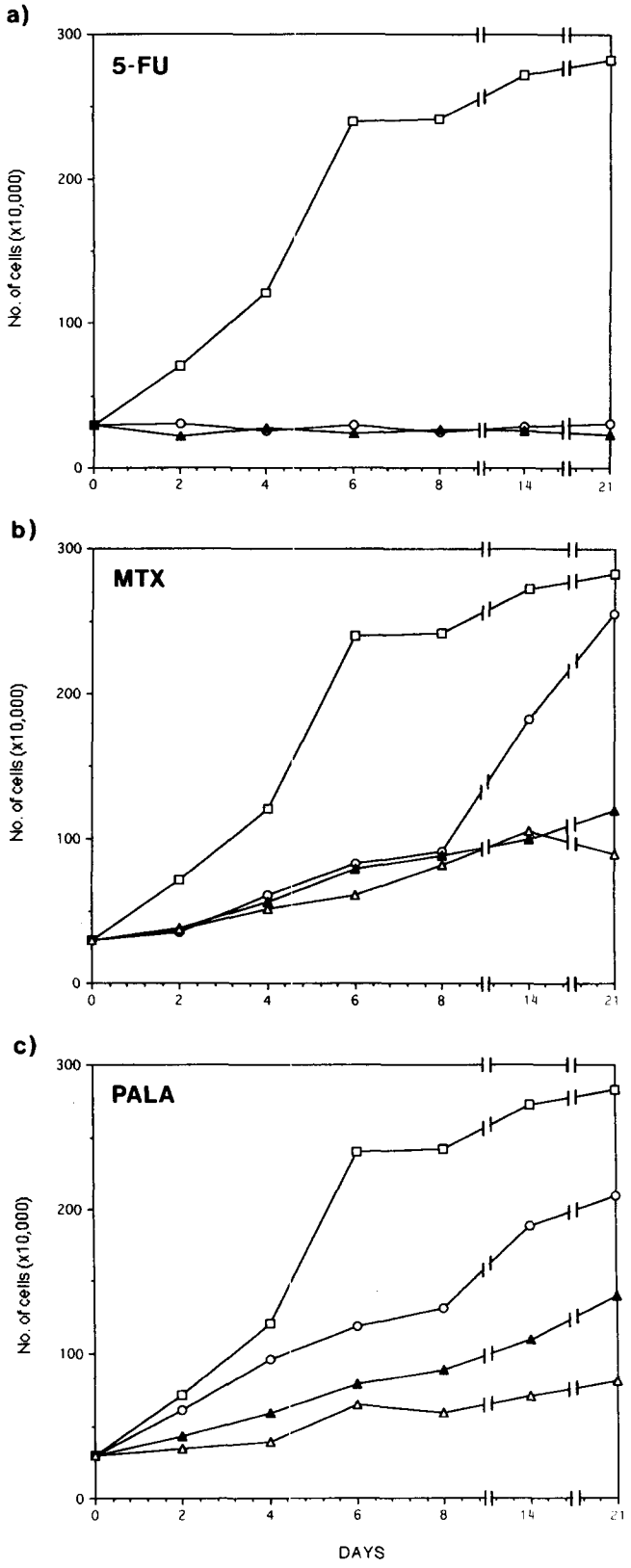

FIG. 4. Growth of the KMS cell line over a 3-week period during and following exposure to drug-free medium (□) and (a) 5-FU at concentrations of 1×10^{-5} (○) and 1×10^{-4} (▲) M; (b) MTX at concentrations of 1×10^{-7} (○), 1×10^{-6} (▲), and 1×10^{-5} (△) M; and (c) PALA at concentrations of 1×10^{-5} (○), 1×10^{-4} (▲), and 1×10^{-3} (△) M. Fresh drug-free medium was applied to the cells on day 8 and day 14. The data are from one representative experiment.

KN-NM cell line treated with PALA plus dipyridamole (data not shown). Thymidine (100 μM) failed to prevent toxicity at the same PALA and dipyridamole concentrations (data not shown), presumably due to a lack of source nucleosides and nucleobases for CTP production.

The sensitivities of the same 3 cell lines to the antimetabolite MTX, in the presence and absence of dipyridamole, are shown in Fig. 2. Methotrexate is a folate analogue and blocks the regeneration of tetrahydofolates [23]. Two specific sites in the *de novo* pathway for purine synthesis are blocked, as well as inhibition of thymidylate synthetase in the pyrimidine biosynthesis pathway [3]. Thus, MTX depletes 3 nucleotide pools, namely GTP, ATP and TTP. Contrary to expectation, the normal KMS cell line was more resistant to MTX administered alone than either the immortal KMST or the tumourigenic KN-NM cell line (Fig. 2). However, in the presence of dipyridamole, the situation changed. The sensitivities of all 3 cell lines increased and the normal KMS cell line became more sensitive than either the KMST or the KN-NM cell line (Fig. 2). From these data, it was clear that the salvage pathways were functioning particularly well, especially in the case of the KMS cell line, to protect the cells from MTX-induced cytotoxicity. This was confirmed in the present study by the "rescue" from methotrexate- and dipyridamole-induced cytotoxicity of the KN-NM cell line on adding the nucleosides hypoxanthine (100 μM) and thymidine (100 μM) in combination (data not shown), both substrates of the salvage pathway enzymes.

Finally, in the case of 5-FU, the normal KMS cell line was more sensitive than either the immortalised KMST or tumourigenic KN-NM cell lines (Fig. 3). The main cause of 5-FU cytotoxicity remains unclear, but it is thought to operate *via* 3 mechanisms. First, the metabolites of 5-FU can inhibit thymidylate synthetase (TS), blocking dTMP formation [24, 25]. Second, they can be incorporated into DNA and cause strand breaks and fragmentation [26, 27] and, third, 5-FU can be directly incorporated into both nuclear and cytoplasmic RNA altering processing and function [28–30]. Previous studies have reported that the growth inhibitory effects of 5-FU inhibition of TS in mouse sarcoma and mouse L cells can be reversed by the addition of exogenous thymidine [31, 32]. Low concentrations of 5-FU have also been reported to be growth-limiting as a result of TS inhibition in colon carcinoma cell lines [33, 34]. However, in the present study, the absence of any effect of dipyridamole (Fig. 3) and exogenous nucleotides (data not shown) on the resistance of the 3 fibroblast cell lines to 5-FU suggests that salvage pathways are not contributing to the increased resistance of the KMST and KN-NM cell lines to 5-FU. Thus, it would appear that the inhibition of TS and the depletion of the nucleotide pools are not the important actions of 5-FU in these cell lines. These data suggest that the direct DNA-damaging action of 5-FU on DNA [26, 27] may actually be the most important cytotoxic action of 5-FU in these cell lines. They are also

consistent with the observations made in clinical trials that levels of drug-activating enzymes and TS levels do not correlate with 5-FU response in patients [35].

A previous study by the authors on the resistance of the same series of human fibroblast cell lines to 5-FU provided evidence to suggest that differences in the cell cycle response, not apoptosis, were responsible for the differences in resistance [21]. These cell-cycle data coupled with immunohistochemical analysis suggested that there was, in fact, a difference in p53 functionality between the cell lines. The normal KMS cell line was found to undergo G1 arrest in response to increasing concentrations of 5-FU, in contrast to both the KMST and KN-NM cell lines, which continued to cycle in the presence of the DNA insult [21].

The p53-dependent DNA damage response described by Kastan *et al.* [36] was a transient G1 arrest, presumably allowing the cell to repair the DNA damage before continuation of the cell cycle [36]. Closer inspection of the nature of the G1 arrest of the normal KMS cell line in response to presumed 5-FU-induced DNA damage in this study revealed that the arrest was, in fact, permanent, lasting at least 3 weeks, even with the addition of drug-free, fresh medium (Fig. 4a). This finding was similar to the permanent arrest described recently in fibroblasts following ionising radiation [37]. Thus, one can speculate that permanent G1 growth arrest may be a third type of p53-dependent DNA damage response, in addition to the already well-characterised transient cell cycle arrest [36, 38, 39] and apoptosis [40, 41, 42]. It is possible that this permanent cell cycle arrest may, in fact, be senescence [39, 43]. Recent studies have shown that senescence in human diploid fibroblasts (HDFs) is p53-dependent, but WAF 1 p21/Cip1-independent [39, 43]. Thus, the sensitivity of the KMS cell line to 5-FU can be explained on the basis that after DNA damage and strand breaks have been created, the cells respond by permanently arresting/senescing, allowing no further proliferation to take place.

The permanent arrest/senescence observed in the KMS cell line in response to 5-FU did not occur in response to either MTX or PALA. The cell number increased in the presence of both drugs over the first 8 days and continued throughout weeks 2 and 3 (Fig. 4b and c). One can speculate that the salvage pathways were operating to repair and synthesise DNA to such a degree that the strand breaks expected to occur, in response to MTX and PALA insult, failed to materialise. Only at high drug concentrations did the substrates for the salvage pathways become rate-limiting and DNA strand breaks begin to appear in some cells. The cells with strand breaks would, presumably, respond by permanently arresting in either G1 or G2/M, but there would be enough cells that were able to continue through the cell cycle and divide. In the presence of dipyridamole, the supply of purines and pyrimidines *via* the salvage pathways would decrease. As a consequence, strand breaks would become a more common occurrence and many more cells would respond by permanently arresting their cell cycle. Thus, the cell population would fail to increase in number and become more sensitive over the same range of concentrations of PALA and MTX (Figs. 1 and 2). In the case of the KMST and KN-NM cell lines, which have lost the ability to arrest their cell cycles in response to DNA damage, the only limitation on continued DNA synthesis, division, and increasing population size would appear to be the supply of nucleotides. Thus, in the presence of dipyridamole and inhibition of nucleotide supply *via* the salvage pathways, the sensitivities of these two cell lines to MTX and PALA were also increased.

In conclusion, it would appear that the resistance of the fibroblast cell lines was dependent on their ability to respond to DNA damage. In the case of those antimetabolites that do not induce direct DNA strand breaks (e.g. MTX and PALA), the salvage pathways of purine and pyrimidine biosynthesis maintain the nucleotide pools and delay the onset of the DNA damage response in normal cells, protecting them from antimetabolite-induced cytotoxicity. In the case of 5-FU, its ability to induce direct DNA damage elicits an immediate DNA damage response and permanent growth arrest in the normal cell population, which is totally independent of salvage pathway involvement. The immortalised KMST and tumourigenic KN-N-M cell lines will always appear more resistant by virtue of the fact that they continue to proliferate in the presence of either direct or indirect DNA damage. P53 may be important in controlling this response and, thus, may play a role, either alone or in combination with the salvage pathways, in modulating drug resistance.

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