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Original Paper

Azidothymidine in Combination with 5-Fluorouracil in Human Colorectal Cell Lines: *In Vitro* Synergistic Cytotoxicity and DNA-induced Strand-breaks

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The *in vitro* cytotoxicity of the combination of azidothymidine (AZT) and 5-fluorouracil (5-FU) against the human colorectal cancer cells SW-480, SW-620 and COLO-320DM was evaluated. The cytotoxic effects of 5-FU and AZT were determined by the assay using 2,3-bis(2-methoxy-4-nitro-5-sulfophenil)-2H-tetrazolium-5-carboxanilide inner salt (XXT), while drug-induced DNA strand-breaks were measured using a fluorometric analysis of DNA unwinding. After an exposure of 72 h, 5-FU and AZT induced a dose-dependent cytotoxicity against each cell line. The addition of 3, 10 and 30 μM AZT to various concentrations of 5-FU, as well as the addition of 0.5, 1 and 3 μm 5-FU to various concentrations of AZT, resulted in an enhanced cytotoxic effect. Isobologram analysis and the combination index (CI) method demonstrated that the interaction between 5-FU and AZT was clearly synergistic in each cell line, except for the 30% level of effect in SW-620, where borderline synergism was observed. The evaluation of DNA strand-breaks after an exposure of 16 h to 5-FU, AZT or 5-FU + AZT demonstrated that the 5-FU + AZT combination produced the greatest DNA damage, and that this interaction was synergistic in each cell line. In conclusion, our study supports the evidence that the potential antitumour activity of AZT can be modulated by combining it with agents which inhibit thymidylate (dTMP) formation, such as 5-FU, and that the increased cytotoxicity is related to enhanced DNA damage. These findings should encourage further experimental and clinical studies of the potential use of AZT in combination with inhibitors of *de novo* dTMP synthesis.

Key words: azidothymidine, 5-fluorouracil, colorectal cancer cell lines, synergistic cytotoxicity, DNA strand-breaks

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INTRODUCTION

AZIDOTHYMIDINE (AZT) is a thymidine analogue that is converted intracellularly to AZT triphosphate (AZTTP) by the sequential action of mammalian thymidine kinase (TK), thymidylate kinase and nucleoside diphosphate kinase, and is ultimately incorporated into DNA where it blocks chain elongation [1]. AZT was originally developed as an anticancer agent, but early studies did not find significant cytotoxic activity and its further development as an anticancer agent was therefore abandoned [2,3]. Instead, AZT has been found to show important *in vitro* activity against HIV and clinical utility in patients with AIDS and AIDS-related diseases [4,5]. AZT is, indeed, a more favourable substrate for HIV reverse transcriptase than for human DNA polymerase and is, therefore, preferentially incorporated into viral DNA [1].

More recently, experimental studies by Brunetti and colleagues [6] and Tosi and colleagues [7] have shown that the incorporation of AZT into cellular DNA of the human colorectal adenocarcinoma cell line, HCT-8, can be significantly enhanced when AZT is combined with agents that inhibit the *de novo* synthesis of thymidylate (dTMP), such as

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5-fluorouracil (5-FU) or methotrexate (MTX), thus resulting in enhanced AZT-induced DNA strand-breaks and cytotoxicity. The mechanism by which 5-FU and MTX enhance AZT cytotoxicity seems to be dependent on their ability to deplete intracellular thymidine triphosphate pools (dTTP) that consequently facilitate the utilisation of AZTTP in DNA synthesis [6–8]. Of interest, these studies have demonstrated that this effect is relatively specific for tumour cells, and this might reflect differences in thymidine salvage ability and transport between colorectal cancer cells and normal cells [6–8]. In fact, colorectal cancer cells usually have an elevated TK activity [9–12], and, therefore, a high capacity to activate AZT. In addition to this, cancer cells lack a Na^+-dependent concentrative nucleoside transport system that has been demonstrated in a variety of normal cells and which does not recognise AZT as a substrate [13–17]. These systems, by concentrating thymidine intracellularly, may protect normal cells from the action of AZTTP for competition between AZTTP and dTTP.

In this study, we extended our research on 5-FU and AZT to three human colorectal cancer cell lines to validate observations previously reported in HCT-8 cells and, in particular, to evaluate *in vitro* interactions between the two agents with regard to cytotoxicity and DNA damage.

MATERIALS AND METHODS

Drugs and chemicals

AZT was kindly provided by Burroughs Wellcome Co. (Beckenham, U.K.) and 5-FU was purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). The tetrazolium reagent 2,3-bis(2-methoxy-4-nitro-5-sulfophenil)-2H-tetrazolium-5-carboxanilide inner salt (XTT) was purchased from Polyscience (Warrington, Pennsylvania, U.S.A.) and prepared at a concentration of 1 mg/ml in prewarmed RPMI-1640 medium before use. Phenazine methosulphate (PMS) was purchased from Sigma and prepared at a concentration of 5 mM (1.53 mg/ml) in phosphate-buffered saline (PBS) and stored at 4°C for a maximum of 3 months. All other chemicals and solvents were obtained from Sigma Chemical Co.

Cell line and culture conditions

The human colorectal cancer cell lines SW-480, SW-620 and COLO-320DM were used in this study. These cell lines have been previously characterised [18–20] and were purchased from American Type Culture Collection (Rockville, Maryland, U.S.A.). Exponentially growing cells were grown in RPMI-1640 or Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine, and maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2.

In vitro *evaluation of cytotoxicity*

The XTT assay was used to determine the effect of 5-FU and AZT on colorectal cancer cell proliferation and was performed as previously described [21]. Briefly, single cell suspensions were prepared by trypsinisation, and approximately 2000 cells, suspended in 100 μl of complete medium, were seeded into each well of a 96-well microtitre plate, and incubated at 37°C with 5% CO_2. After 24 h, 100 μl of complete medium containing 5-FU and/or AZT was added to treat the cells for 72 h. Drug concentrations tested ranged between 0.5 and 100 μM for 5-FU and between 1 and 200 μM for AZT; in the combination studies, only one of the two agents was tested at three different concentrations that had achieved a percentage growth inhibition between 5 and 30%. Fifty microlitres of medium containing 50 μg of XTT and 0.38 μg of PMS was then added to each well and plates were incubated for an additional 3–4 h. The absorbance was measured at 450 nm with a microplate reader (Lab System Instruments, Helsinki, Finland). Cell growth inhibition was expressed as the percentage absorbance of untreated cells, and the 50% inhibitory concentration of cell growth (IC_{50}) was calculated by non-linear regression analysis.

Fluorometric detection of DNA strand-breaks

The fluorometric analysis of DNA unwinding was performed as previously reported [22]. In fact, when DNA is exposed to moderately alkaline solutions, the rate of DNA unwinding can be used as a sensitive measure of strand-breaks because the level of unwinding increases with increasing DNA strand-breaks. In this method, the fluorescent dye, ethidium bromide, that binds selectively to double-stranded DNA is used, and this procedure does not, therefore, require a physical separation of single-stranded DNA from double-stranded DNA as required in previous methods.

Briefly, $5–10 \times 10^6$ cells were plated in 100 mm sterile Petri dishes and exposed to 10 μM AZT or 1 μM 5-FU or their combination. After an exposure of 16 h to the drugs, cells were trypsinised, centrifuged and the pellet resuspended in 2 ml of solution A (0.25 M myo-inositol, 10 mM sodium phosphate, 1 mM $MgCl_2$). Aliquots of this suspension (0.2 ml) were distributed into 12 disposable glass tubes, designated T, P or B in groups of four. T tubes were used to estimate the total fluorescence (double-stranded DNA + contaminants), P tubes to estimate the unwinding rate of DNA, and B tubes (blank) to estimate the contribution of components other than double-stranded DNA to the fluorescence. For this purpose, 0.2 ml of solution B (9 M urea, 10 mM NaOH, 2.5 mM cyclohexanediaminetetraacetate, 0.1% sodium dodecylsulphate (SDS)) was added to each tube and incubated at 0°C for 10 min. After this time, during which cell lysis and chromatin disruption occurred, 0.1 ml of solution C (45% of solution B and 55% 0.2 N NaOH) and 0.1 ml of solution D (60% of solution B and 40% of 0.2 N NaOH) were gently added to the P and B tubes. After further incubation at 0°C for 30 min, during which the alkali diffused into the lysate to give a final pH of approximately 12.8, the contents of the B tubes were sonicated for 1–2 s to ensure rapid denaturation of the DNA in the alkaline solution. P and B tubes were further incubated at 15°C for 60 min and denaturation was then stopped by chilling the tubes to 0°C and adding 0.4 ml of solution E (1 M glucose, 14 mM β-mercaptoethanol) that lowered the pH to approximately 11.0. The lysates were briefly sonicated to render them homogeneous, diluted with 1.5 ml of solution F (ethidium bromide, 6.7 μg/ml, 13.3 mM NaOH) and their fluorescence was read at room temperature in a Perkin–Elmer spectrofluorometer (excitation wavelength 520 nm, emission 590 nm). The T tubes differed from the P tubes in that the neutralising solution E was added before the alkakine solutions C and D, so that the DNA was never exposed to a denaturing pH. The extent of DNA unwinding after a given time of exposure of cell extracts to alkali was calculated from the fluorescence values of the T, P and B samples. The percentage of double-stranded DNA was calculated using the formula $(P–B)/(T–B) \times 100$ where (P–B) provides an estimate of the amount of double-

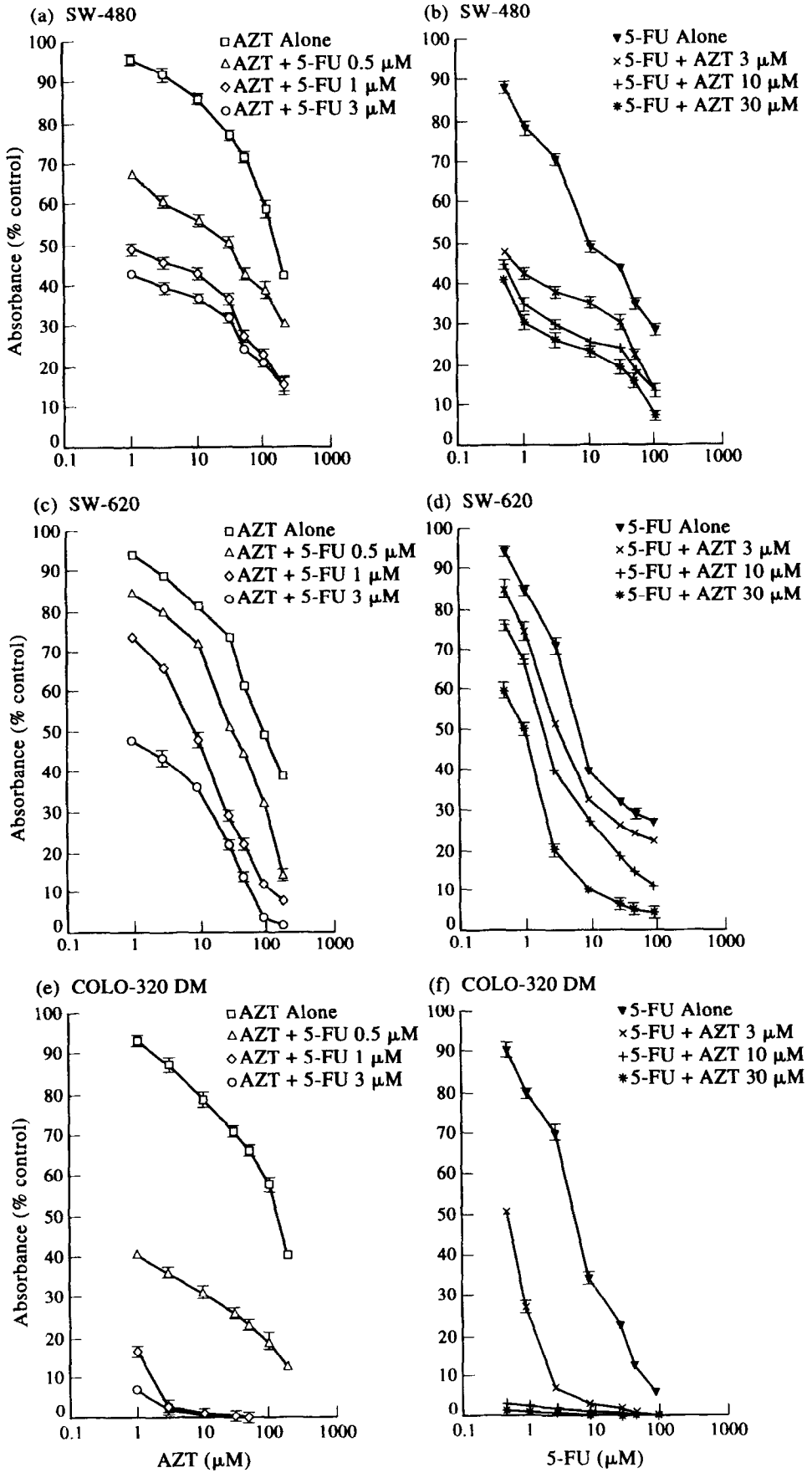

Figure 1. Cytotoxic effect of treatment for 72 h with azidothymidine (AZT), 5-fluorouracil (5-FU), AZT + 5-FU (0.5, 1 and 3 μM), and 5-FU + AZT (3, 10 and 30 μM) on cell survival of the human colorectal tumour cell lines SW-480, SW-620, and COLO-320DM as determined by percentage control absorbance in the 2,3-bis(2-methoxy-4-nitro-5-sulfophenil)-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay. Points are the mean of triplicate experiments each done in sextuplicate, with standard deviation shown by vertical bars.

stranded DNA remaining after exposure to alkali for 60 min, and (T–B) an estimate of the amount of double-stranded DNA in the cell extracts. Up to the point at which solution C was added, all steps were carried out under ordinary room illumination; after this point, manipulations were carried out in the dark and a covered bath was used for incubation. All solutions were kept at 0°C, except solutions B and F that were stored at room temperature.

Data analysis

An improved isobologram method that has been previously described [23,24] was used to determine the *in vitro* effects of combining AZT and 5-FU. In fact, for agents with a non-linear dose response curve, such as 5-FU and AZT, the expected additive response cannot be represented in the isobologram as a straight line, and it is necessary to determine an area called 'envelope of additivity' within which all responses are conceivably additive. Thus, when a data point lies within the envelope of additivity, an additive interaction is indicated; when a data point lies to the left side of the envelope, a supra-additive or synergistic interaction is indicated, and when it lies to the right side, a subadditive or antagonistic interaction is indicated. In order to determine the envelope of additivity, mode I, IIa and IIb lines were drawn. These lines were obtained by plotting the doses of AZT and 5-FU which give log survival values that add up to the chosen level of effect (30% and 50%): for mode I line, the increments in dose for both AZT and 5-FU starting from zero were taken; for mode IIa line, the increments in dose for AZT starting from zero and for 5-FU starting from where the effect of AZT had ended were taken; and for mode IIb line, the increments in dose for 5-FU starting from zero and for AZT starting from where the effect of 5-FU had ended were taken. The area surrounded by these three lines represents the envelope of additivity.

We also used the combination index (CI), as previously described [25], to compare the cytotoxic effects of AZT, 5-FU and AZT + 5-FU. The CI was defined as the sum of the relative concentrations (e.g. IC_{50} units) of each drug which yield an isoeffect (e.g. an inhibition of 0.5) when added together:

$$CI = (\text{concentration of AZT})/(\text{IC value of AZT}) + (\text{concentration of 5-FU})/(\text{IC value of 5-FU}).$$

With this method, $CI < 1$ indicates synergy, $CI > 1$ antagonism and $CI = 1$ additivity.

Interaction between 5-FU and AZT on DNA-induced strand-breaks was analysed using a multiplicative model as previously described [26,27]. Briefly, the multiplication of the fraction of double-stranded DNA (DS-DNA) remaining after exposure to either drug alone ($DS\text{-}DNA_{5\text{-}FU}$ or $DS\text{-}DNA_{AZT}$) is compared with the observed result. If $DS\text{-}DNA_{5\text{-}FU + AZT} < (DS\text{-}DNA_{5\text{-}FU} \times DS\text{-}DNA_{AZT})$, the interaction is synergistic; if $DS\text{-}DNA_{5\text{-}FU + AZT} = (DS\text{-}DNA_{5\text{-}FU} \times DS\text{-}DNA_{AZT})$, the interaction is additive; and if $DS\text{-}DNA_{5\text{-}FU + AZT} > (DS\text{-}DNA_{5\text{-}FU} \times DS\text{-}DNA_{AZT})$, it is antagonistic.

RESULTS

5-FU and AZT showed dose-dependent cytotoxicity against the cell lines SW-480, SW-620 and COLO-320DM; IC_{50} concentrations were, respectively, 10.25, 7.65 and 7.17 μM for 5-FU and 155.42, 94.76 and 149.80 μM for AZT after an exposure of 72 h (Figure 1, Table 1). The addition of AZT at 3, 10 and 30 μM to various concentrations of 5-FU, as well as the addition of 5-FU at 0.5, 1 and 3 μM to various concentrations of AZT, resulted in an enhanced cytotoxic effect (Figure 1, Table 1). Isobologram analysis of the IC_{50} and IC_{30} for the drug combinations (Figure 2) indicated that the interaction between the two agents was clearly synergistic in most cases, except for the 30% level of effect in SW-620, where only a borderline synergism was observed (Figure 2c). This observation was confirmed by comparing the cytotoxic effects of 5-FU, AZT and 5-FU + AZT with the CI method at different levels of effect (30% and 50%); in fact, the interaction was synergistic for each cell line, though synergism was more pronounced for SW-480 and COLO-320DM (Table 2).

As previous studies have suggested that 5-FU enhances AZT cytotoxicity by increasing the degree of AZTTP incorporation into DNA, thereby increasing AZT-induced DNA strand-breaks, the damage induced by 5-FU, AZT and 5-FU + AZT on DNA was measured. After an exposure of 16 h to 1 μM 5-FU, only modest amounts of DNA strand-breaks were detected in the cell lines (Figure 3). AZT, 10 μM, produced a significant increase in the extent of DNA strand-breakage, but the greatest effect was obtained by combining 5-FU and AZT (Figure 3). In fact, after an exposure of 16 h to the two agents, the mean values of double-stranded DNA

Table 1. *Cytotoxic effects of azidothymidine (AZT), 5-fluorouracil (5-FU) and AZT + 5-FU against human colorectal cell lines*

Cell line	Level of effect (%)	AZT concentration (μM)				5-FU concentration (μM)			
		AZT alone	AZT + 0.5 μM 5-FU	AZT + 1 μM 5-FU	AZT + 3 μM 5-FU	5-FU alone	5-FU + 3 μM AZT	5-FU + 10 μM AZT	5-FU + 30 μM AZT
SW-480	30	57.48	0.92	0.59	0.53	3.39	0.29	0.28	0.26
	50	155.42	33.00	0.99	0.88	10.25	0.48	0.46	0.43
	70	ND	ND	44.77	35.22	93.88	33.49	3.77	1.52
SW-620	30	35.30	11.90	1.85	0.57	3.26	1.40	0.87	0.37
	50	94.76	31.59	9.08	0.95	7.65	3.56	2.27	1.00
	70	ND	110.27	28.53	18.11	45.09	18.57	8.59	2.34
COLO-320DM	30	37.10	0.51	0.36	0.32	3.24	0.32	0.16	0.15
	50	149.80	0.85	0.60	0.54	7.17	0.56	0.26	0.25
	70	ND	16.53	0.84	0.75	19.33	0.96	0.36	0.36

ND, not determined.

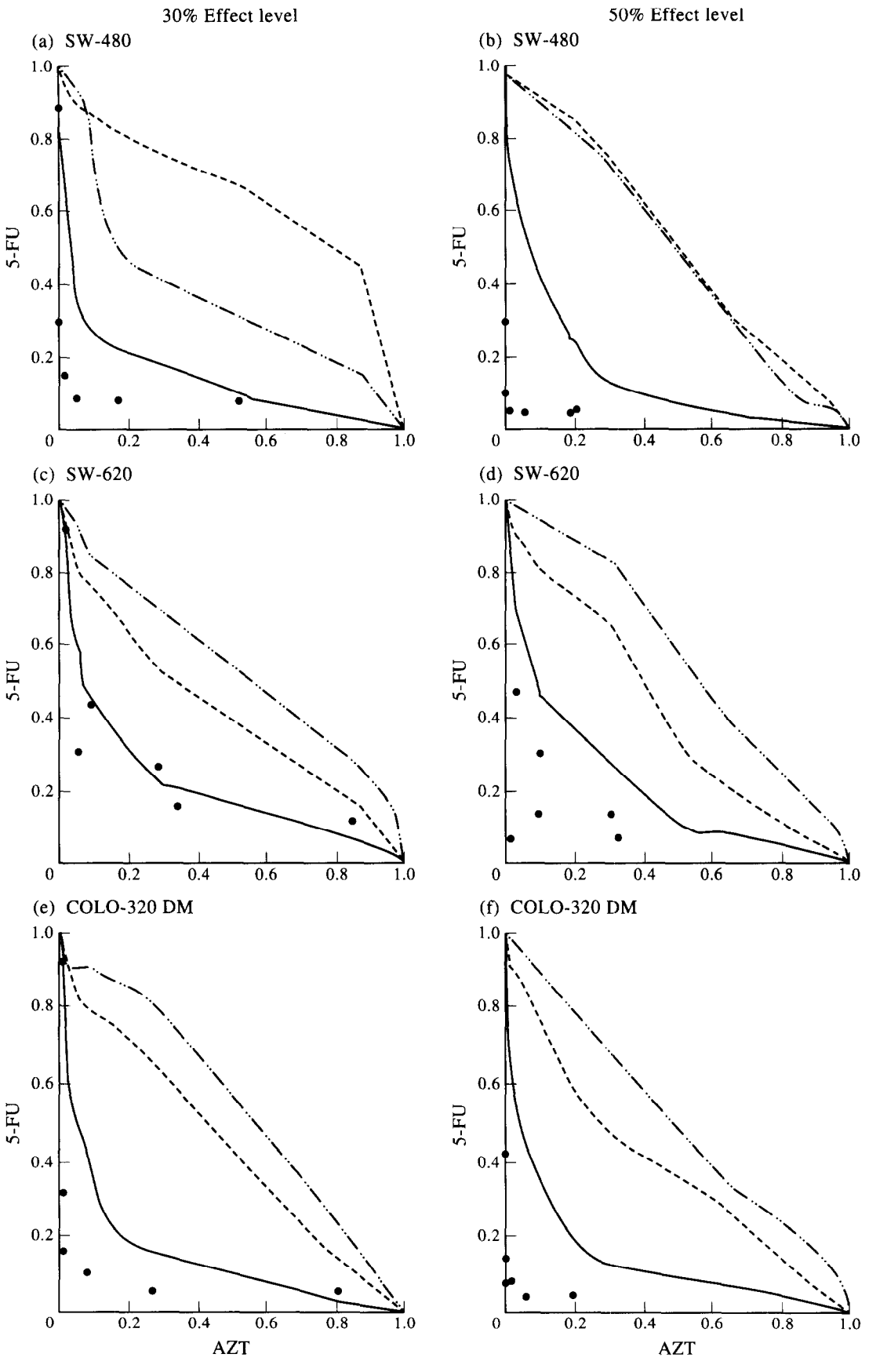

Figure 2. Isobologram analysis of the cytotoxic effects of the combination of azidothymidine (AZT) and 5-fluorouracil (5-FU) at 30% and 50% levels of effect in the human colorectal carcinoma cell lines SW-480, SW-620 and COLO-320DM. (●), Mean IC_{30} or IC_{50} values of three experiments each done in sextuplicate; (—), mode I line; (- - -), mode IIa line; (·—·—·), mode IIb line.

Table 2. Mean combination index (CI)

Cell line	Treatment	30% level of effect CI	30% level of effect Interaction	50% level of effect CI	50% level of effect Interaction
SW-480	AZT + 0.5 μM 5-FU	0.16	S	0.26	S
	AZT + 1 μM 5-FU	0.30	S	0.10	S
	AZT + 3 μM 5-FU	0.89	S	0.30	S
	5-FU + 3 μM AZT	0.14	S	0.06	S
	5-FU + 10 μM AZT	0.34	S	0.11	S
	5-FU + 30 μM AZT	0.60	S	0.43	S
SW-620	AZT + 0.5 μM 5-FU	0.49	S	0.40	S
	AZT + 1 μM 5-FU	0.36	S	0.23	S
	AZT + 3 μM 5-FU	0.94	S	0.40	S
	5-FU + 3 μM AZT	0.51	S	0.50	S
	5-FU + 10 μM AZT	0.55	S	0.40	S
	5-FU + 30 μM AZT	0.96	S	0.45	S
COLO-320DM	AZT + 0.5 μM 5-FU	0.17	S	0.07	S
	AZT + 1 μM 5-FU	0.32	S	0.14	S
	AZT + 3 μM 5-FU	0.93	S	0.42	S
	5-FU + 3 μM AZT	0.18	S	0.09	S
	5-FU + 10 μM AZT	0.32	S	0.10	S
	5-FU + 30 μM AZT	0.85	S	0.23	S

AZT, azidothymidine; 5-FU, 5-fluorouracil; S, synergistic.

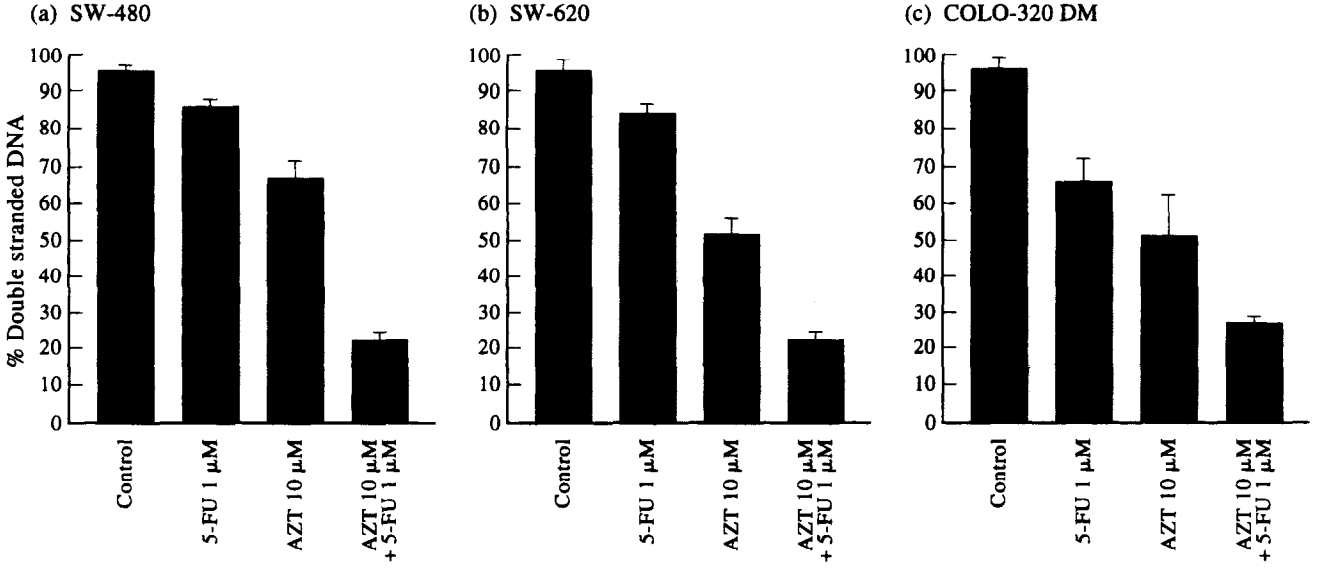

Figure 3. Effects of exposure for 16 h to 1 μM 5-fluorouracil (5-FU), 10 μM azidothymidine (AZT) or 1 μM 5-FU + 10 μM AZT on the amount of residual double-stranded DNA in SW-480, SW-620 and COLO-320DM colorectal carcinoma cell lines. Results are the mean of three experiments each done in duplicate, with standard deviation shown by vertical bars.

were 22, 21.8 and 27.4% in SW-480, SW-620 and COLO-320DM, respectively, in comparison to a mean control value of approximately 95%. The effect of combining 5-FU and AZT on DNA was analysed using the multiplicative model previously described, and it was found to be synergistic for each cell line.

DISCUSSION

Despite recent advances in medical oncology, the treatment of metastatic colorectal cancer remains unsatisfactory. Although 5-FU is considered to be the most effective drug, it induces remissions only in a minority of patients, with no significant impact on survival [28]. Numerous experimental studies have attempted to modulate 5-FU activity biochemically, but only modulation with leucovorin or MTX has produced some clinical improvement, leading to higher response rates and, in some cases, a modest survival advantage [29–31]. Recent studies have suggested that a new approach to enhance the cytotoxicity of 5-FU or other dTMP synthesis inhibitors, i.e. MTX, might be to combine them with a toxic thymidine analogue, such as AZT [6,7]. In fact, under conditions of *de novo* dTMP synthesis inhibition, incorporation of AZT into DNA is facilitated. Interestingly, *in vivo* studies have demonstrated that this effect is relatively specific

for tumour cells, and reflects differences in pyrimidine salvage capacity between tumour and normal cells [6,7].

Our study confirms, in SW-480, SW-620 and COLO-320DM human colorectal cancer cell lines, the previous *in vitro* observations for the cancer cell line HCT-8. Of interest, the interaction between 5-FU and AZT was clearly synergistic in at least two of the cell lines (SW-480 and COLO-320DM). In addition to this, our study suggests that the enhanced cytotoxicity induced by the drug combination is possibly related to an increase in DNA-induced strand-breaks that can be produced by enhanced AZT incorporation into DNA, as previously demonstrated.

Recently, other investigators have reported similar results by combining AZT with *de novo* dTMP synthesis inhibitors. Tosi and associates [32] showed that hydroxyurea, which inhibits ribonucleotide reductase activity and, therefore, reduces deoxyribonucleotide pools, increases the incorporation of AZT into DNA and synergistically enhances its cytotoxicity in two human chronic myeloid leukaemia cell lines [32]. Moreover, Presacco and Erlichman [33] reported that ICI D1694, a new folate-based thymidylate synthase inhibitor, also enhances AZT cytotoxicity and DNA-induced strand-breaks in the bladder MGH-U1 and colorectal HCT-8 tumour cell lines, but not in normal CFU-GM cells. Finally, a preliminary report by Szekeres and colleagues [34] has confirmed a synergistic cytotoxic effect of 5-FU and AZT in the human colon cancer cell lines HT-29, CCL-227, CCL-228 and Caco-2.

Clinical studies combining AZT with 5-FU or MTX have been initiated. Phase I studies have shown that the combination is clinically feasible and that AZT doses of up to 8–10 g/m^2 administered over 2–24 h do not significantly enhance 5-FU- or MTX-induced toxicities. Interestingly, the drug combination increases DNA strand-breaks dose-dependently in peripheral blood-nucleated cells [35,36]. Furthermore, preliminary phase II studies demonstrate promising results for 5-FU + leucovorin + AZT in metastatic colorectal cancer and for MTX + AZT in HIV-related high-grade non-Hodgkin's lymphomas [37,38].

In conclusion, our study supports the concept that the potential antitumour activity of AZT can be modulated by combining it with agents that inhibit dTMP formation and reduce intracellular dTTP pools, as in the case of 5-FU, and that the increased cytotoxicity is related to enhanced AZT-induced DNA damage. Because *in vitro* and *in vivo* studies indicate that this effect is relatively specific for tumour cells, the combination of AZT with agents that inhibit dTMP formation may be clinically useful and should be further evaluated.

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