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ORIGINAL ARTICLE

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Low folate conditions may enhance the interaction of trifluorothymidine with antifolates in colon cancer cells

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Abstract *Purpose*: Trifluorothymidine (TFT) is a fluoropyrimidine that is part of the novel combination metabolite TAS-102, in which TFT is combined with a potent thymidine phosphorylase inhibitor (TPI). TAS-102 is currently tested as an orally chemotherapeutic agent in different schedules in a phase I study. In its monophosphate form, TFT can inhibit thymidylate synthase (TS) activity after binding to the TS-nucleotide binding site leading to dTTP depletion, and in its triphosphate form TFT is incorporated into DNA, eventually leading to DNA damage. In this in vitro study, we investigated whether TFT could potentiate cytotoxicity of the antifolate-based TS inhibitors AG337 (Nolatrexed), ZD1694 (Raltitrexed) and GW1843; and whether increased TS inhibition or DNA damage would be related to this result. *Methods*: The drug combinations were studied in colon cancer cell lines either grown at low or high folate conditions. Multiple drug effect analysis was performed after measuring growth inhibition when the drugs were combined (MTT Assay) and expressed as Combination Index (CI), where CI < 0.9 indicates synergism, CI = 0.9–1.1 indicates additivity and CI > 1.1 indicates antagonism. Drug target analysis was performed using the TS in situ inhibition assay and the FADU DNA-damage assay. Cells were exposed to either the drugs alone or in combination to determine the effect on TS activity and DNA damage induction, respectively. *Results*: Three experimental procedures were used to test the interaction of the drugs: either one of the drugs was kept at a constant concentration (IC_{25}) or two drugs were added in a 1:1 IC_{50}-based molar ratio. The combinations of TFT with one of the antifolates in which one of the drugs was kept at a constant concentration were synergistic for all antifolates in WiDr/F cells, which grow in low folate medium (CI = 0.6–0.8), but only additive to antagonistic for the cell lines growing in high folate medium: TFT-AG337: CI = 0.9–2.3; TFT-ZD1694: CI = 0.9–1.3; TFT-GW1843: CI = 0.8–1.7. The procedure in which the two drugs were added in a 1:1 IC_{50}-based molar ratio showed antagonism for all three combinations in all cell lines (CI > 2.7). TS inhibition (14.3%) and DNA damage (8%) were more pronounced than expected ($P < 0.05$) when TFT was combined with GW1843 in WiDr/F cells, in contrast to AG337 and ZD1694, which showed inhibiting effects as expected (additive). *Conclusions*: The combination of TFT with the antifolates AG337, ZD1694 and GW1843 is mainly additive when the drugs are given simultaneously and this is mediated by an additive TS inhibition and DNA damage. The drug interaction may partly be dependent on the folate homeostasis since WiDr/F cells growing at low folate conditions show pronounced synergism in growth inhibition, two-sided TS inhibition and DNA damage, especially when TFT is combined with the tight-binding TS inhibitor GW1843.

Keywords Antifolates · Colon cancer · DNA damage · Thymidylate synthase · Trifluorothymidine

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Introduction

In the treatment of colorectal cancer, most chemotherapy regimens used at present consist of combinations of drugs. Often 5-Fluorouracil (5FU) is combined with other cytostatic agents to improve the response rates [1–3]. In colorectal cancer, the expression of thymidylate

synthase (TS) [4] is usually high. TS is a rate-limiting enzyme in the pyrimidine *de novo* deoxynucleotide synthesis and therefore is an excellent target for chemotherapeutic strategies [5, 6]. TS catalyzes the methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP), where 5,10-methylene-tetrahydrofolate (CH_2-THF) serves as the methyl donor in the reaction [7]. TS can be inhibited by a variety of cytotoxic agents, which are active against colon cancer in clinical trials [8–11].

Besides 5-Fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), a metabolite derived from 5FU, TS can also be inhibited by the fluorinated dUMP analog 5-trifluoro-2′-deoxythymidine-5′-monophosphate (TF-TMP), the phosphorylated product from trifluorothymidine (TFT) [12,13]. TFT enters the cell by a nucleoside transporter, although the contribution of the concentrative and equilibrative nucleoside transporters is not known. Furthermore, TFT is part of the novel combination TAS-102 [14–16, 30], which is currently tested in a phase I study as an oral chemotherapeutic regimen given in different schedules [17]. In contrast to FdUMP, TF-TMP does not form a stable ternary complex and binds covalently to the active site of TS [12, 18].

Antifolates have been extensively studied as anticancer drugs [19]. Antifolates inhibit TS directly by binding to the CH_2-THF binding site of TS, such as AG337 (Nolatrexed or Thymitaq) [20, 21], ZD1694 (Raltitrexed or Tomudex) [22] and GW1843 (1843U89) [23, 24]. ZD1694 and GW1843 enter the cell via the reduced folate carrier (RFC) [25] and need folylpolyglutamate synthetase (FPGS) to inhibit TS effectively. AG337 enters the cell by passive diffusion and does not need to be polyglutamated to become more active. Antifolates have been studied in combination with 5FU in vitro and were shown to be mainly additive when given simultaneously depending on scheduling [26]. Caponigro et al. [27] summarized that combining ZD1694 and 5FU resulted in a well-tolerated schedule-dependent synergism in different clinical studies.

Since TFT and antifolates all inhibit TS, we hypothesized that they might enhance each other in TS inhibition. Inhibition of TS results in depletion of dTTP and an increase in dUTP in the cell, the so-called thymine-less state, thereby causing misincorporation of dUTP into DNA [28, 29]. In addition, the triphosphate form of TFT (TF-TTP) can also be incorporated into the DNA leading to DNA strand breaks [16, 30]. The imbalance in dTTP/dUTP and DNA damage induction probably results in an induction of downstream events leading to cell death [31].

In the present study, we aimed to determine whether sensitivity of colon cancer cells to antifolates could be increased by two-sided TS inhibition by addition of TFT. For this purpose, we used several colon cancer cell lines to investigate the effect of different combinations of TFT with the antifolates AG337, ZD1694 and GW1843 on inhibition of cellular growth, inhibition of TS and DNA damage induction.

Materials and methods

Chemicals

TFT was synthesized and kindly provided by Taiho Pharmaceuticals Co. (Tokushima, Japan). The antifolates AG337, ZD1694 and GW1843 were provided by Pfizer/Agouron Pharmaceuticals Inc. (La Jolla, CA, USA), AstraZeneca Pharmaceuticals Ltd. (Macclesfield, UK) and GlaxoSmithKline Inc. (Research Triangle Park, NC, USA), respectively. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma-Aldrich Chemicals (Zwijndrecht, The Netherlands). [5−^{3}H]-2′-deoxycytidine (specific activity 23 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA, USA). All other chemicals were of analytical grade and commercially available.

Cell cultures

Three colon cancer cell lines were used for the experiments including H630, WiDr and Colo320 [32, 33]. These cell lines were cultured in DMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL, Breda, The Netherlands) and 20 mM Hepes (Cambrex BioScience, Verviers, Belgium). Because this mixture contains 8.8 μM folic acid, which may influence the antiproliferative effects of TFT and antifolates, we used the WiDr subline WiDr/F, which was adapted to grow on low folate conditions [34]. WiDr/F was cultured in folate-free RPMI 1640 medium supplemented with 10% heat-inactivated and dialyzed FCS, 20 mM Hepes, 2 mM glutamine and 2.5 nM DL-leucovorin (LV; folinic acid). This did not affect the doubling time. Both DMEM and RPMI 1640 culture media were obtained from Cambrex BioScience (Verviers, Belgium). All four cell lines grew as adherent monolayers in a humidified atmosphere containing 5% CO_2 at 37°C and were maintained in exponential growth.

Growth inhibition studies

To assess cytotoxicities to TFT and the antifolates the MTT assay was used [35, 36]. In brief, a suspension of 5,000 cells/100 μl of medium was added to each well of Costar flat-bottomed 96-well plates (Corning Inc., NY, USA). After 24 h incubation, 100 μl drug containing medium was added to the wells in different concentrations and plates were incubated for another 72 h. Subsequently, the medium was removed from the wells and cells were incubated in 50 μl of MTT solution (end conc. 0.42 mg/ml medium) for 3 h at 37°C. The formazan crystals were dissolved in 150 μl of DMSO (Merck, Darmstadt, Germany) and absorbance was read at 540 nm using Spectra Fluor (Tecan, Salzburg, Austria). The IC_{25} and IC_{50} growth inhibition values were

expressed as the concentrations that corresponded to a reduction of cellular growth by 25% and 50%, respectively, when compared to values of untreated control cells.

Multiple drug effect analysis

All cell lines were exposed to TFT, the antifolates or a combination of TFT with an antifolate (simultaneously). Three combination variants were used to test the interaction of the drugs: (1) combination of various concentrations of an antifolate with a constant concentration of TFT (IC_{25}); (2) combination of various concentrations of TFT with a constant concentration of an antifolate (IC_{25}) or (3) various concentrations of the two drugs were added in a fixed 1:1 IC_{50}-based molar ratio. Using the MTT assay dose-effect curves were generated to obtain the IC_{25} and IC_{50} values and to perform multiple drug effect analysis using Calcusyn software (Biosoft, 1996). This program is based on the method of Chou and Talalay [37], which is distinct from other methods by the fact that both 'potency' and 'shape' of dose-effect curves of drugs and their combinations are taken into account [1]. This method has been used before in combination studies with anticancer agents [26, 38].

Each fraction affected (FA) was calculated by comparing the absorbance values of drug-treated wells to the absorbance of the control wells. A drug concentration that induces FA = 0.25 means a decrease in absorbance and growth of 25% (= IC_{25} concentration). Background absorbance was set at FA = 1. The program only allows using FA values in the range $0.01 < FA < 0.99$. From the median drug effect plots, the dose that reduced absorbance by 50% (D_x) and the slope (m) were calculated. The data were only applicable to this method of analysis when the linear correlation coefficient r of each obtained curve was >0.9. The program uses the formula $D_{1\text{-}FA} = D_x[FA/(1\text{-}FA)]^{1/m}$ to calculate the doses of the separate drugs and combination required to induce various levels of cytotoxicity. For each level of cytotoxicity a mutually non-exclusive combination index (CI) was calculated using the formula: $CI = [(D)_1/(D_{1\text{-}FA})_1] + [(D)_2/(D_{1\text{-}FA})_2] + [\alpha\ (D)_1\ (D)_2/(D_{1\text{-}FA})_1\ (D_{1\text{-}FA})_2]$. The parameters $(D)_1$ and $(D)_2$ represent the doses of the combination of drugs in a fixed ratio, whereas $(D_{1\text{-}FA})_1$ and $(D_{1\text{-}FA})_2$ are the doses of the individual drugs resulting in the effect 1−FA and $\alpha = 1$ for mutually non-exclusive drugs. Examples of Dose-FA and FA-CI plots are given in Figs. 1 and 2. In this method, according to the guidelines of the program, a $CI < 0.9$ indicates synergism whereas CI = 0.9–1.1 indicates additivity and $CI > 1.1$ indicates antagonism. A mean CI was calculated from datapoints with $FA > 0.5$ for the combination variants 1 and 2 and for the combination variant 3 a mean CI was calculated from the FA values 0.6, 0.75 and 0.9. We considered $FA < 0.5$ as not relevant growth inhibition [1].

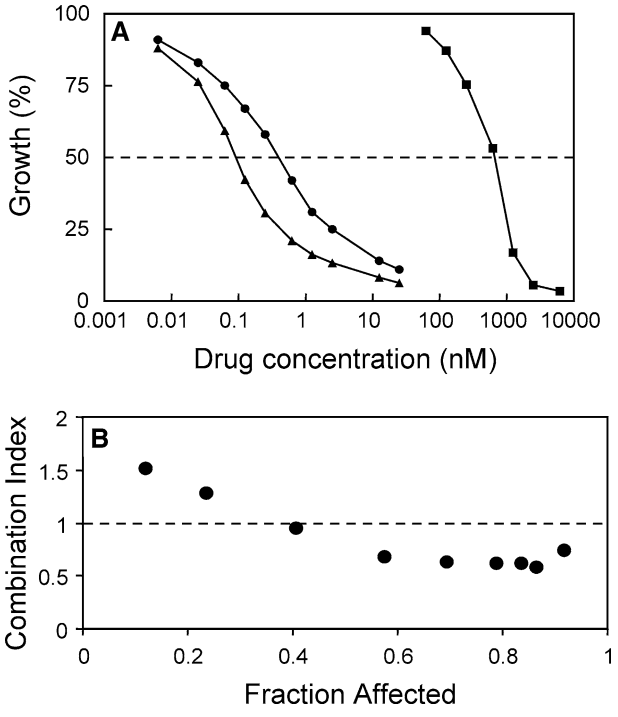


Fig. 1 Representative Dose-Effect (**A**) and FA-CI (**B**) plots for a combination of GW1843 with a fixed concentration of TFT in WiDr/F cells. GW1843 was added at various concentrations (GW1843v) and TFT was kept at a constant IC_{25}-concentration (TFTc). (*filled square*) = TFTv; (*filled circle*) = GW1843v; (*filled triangle*) = GW1843v + TFTc. An average CI value was calculated from datapoints with $FA > 0.5$, in this example $CI_{avg} = 0.64$. See also Materials and methods

TS in situ inhibition assay

Inhibition of TS in intact cells was determined by measuring the conversion of [5−^{3}H]-dUMP to dTMP and ^3H_2O catalyzed by TS, based on a previously described assay [39], and partly modified by our group [40]. Briefly, 2.5×10^5 cells/2 ml/well in Costar 6-well plates were incubated for 24 h with or without (controls) the drugs. [5−^{3}H]-2′-deoxycytidine (final concentration 1 μM) was added 2 h before the end of the incubation period. Blanks containing culture medium were also included. A 200 μl sample from the culture medium was taken and the reaction was stopped by addition of trichloroacetic acid (17.5% end concentration). The unconverted [5−^{3}H]-2′-deoxycytidine was removed by precipitation using activated charcoal. After centrifugation, the supernatants were transferred to liquid scintillation vials and counted for radioactivity. TS catalytic activity in cellular extracts was also measured using the tritium release assay [33].

For the combination studies, the concentrations of TFT and antifolates used were based on an expected inhibition of TS by each drug of 20–50% in order to evaluate the effects of the combinations. TFT was combined with the different antifolates simultaneously at these concentrations in order to determine whether inhibition of TS was changed. Representative TS

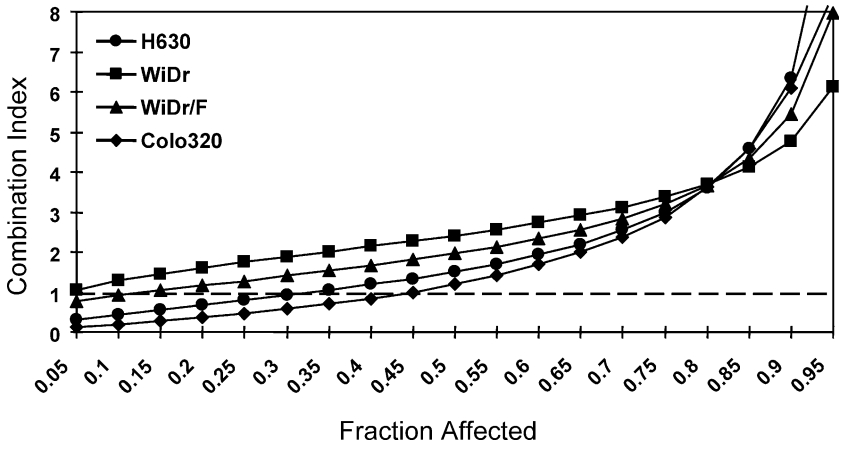


Fig. 2 Illustrative FA-CI plots for the combination TFT-GW1843 using a fixed ratio. TFT and GW1843 were combined in various concentrations in a ratio based on their IC_{50} values. An average CI value was calculated from datapoints with FA = 0.6, 0.75 and 0.9, in these examples all $CI_{avg} > 3.4$ (= antagonism). See also Materials and methods

inhibition curves induced by TFT for the colon cancer cell lines are depicted in Fig. 3. Fractional effect analysis was performed to predict the effect of two drugs by multiplying the relative effects of each single drug to compare it with the observed effect of the combination at the same concentrations of the single drugs [1]. The effect of the two drugs are synergistic when measured value < predicted value, additive when measured value = predicted value and antagonistic when measured value > predicted value.

FADU DNA damage assay

Differences in DNA damage between TFT, AG337, ZD1694, GW1843 or a combination of TFT with one of the antifolates was determined by the FADU assay (Fluorometric Analysis of DNA Unwinding) [26, 41]. The assay is based on the principle that the unwinding rate of double stranded DNA (dsDNA) in an alkaline environment is related to the extent of dsDNA breaks induced by the drugs (detected with ethidium bromide). DNA with high amount of strand breaks will unwind faster than DNA without strand breaks. DNA strand breaks were measured after 48 h exposure of WiDr and WiDr/F cells to the drugs alone or in combination. Concentrations of the single drugs were chosen that at least 60% of the dsDNA was still double stranded compared to the controls (the untreated cells). Cells were also exposed to 50 μM etoposide 2 h before harvesting as a positive control for DNA strand break formation. To predict the effect of a combination of drugs, fractional effect analysis was performed, as described for the TS in situ inhibition.

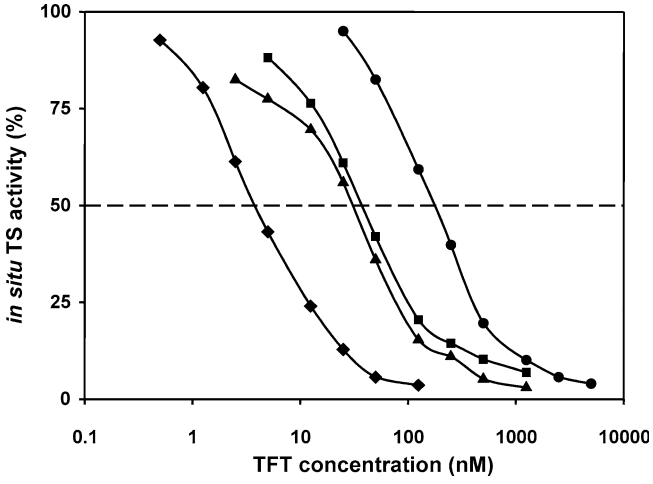


Fig. 3 Representative TS inhibition curves by TFT in H630 (*filled circle*), WiDr (*filled square*), WiDr/F (*filled diamond*) and Colo320 (*filled triangle*). The cell lines were exposed to a concentration range of TFT

Statistics

The Student's *t*-test for paired data was used for statistical analysis of data with two parameters. Changes were considered to be significant when $P < 0.05$.

Results

Evaluation of the combination TFT with the antifolates

The sensitivities of the cell lines for TFT and the antifolates AG337, ZD1694 and GW1843 are summarized in Table 1. The IC_{50} values were also used to calculate the drug ratios for the fixed ratio combinations. The cell lines were least sensitive to TFT and AG337 (all IC_{50} values > 450 nM) and most sensitive to ZD1694 and GW1843 (all IC_{50} values < 25 nM). GW1843 was the most potent inhibitor of cellular growth (all IC_{50} values, 2.2 nM or lower). The WiDr/F cell line growing on low folate conditions was more sensitive to all drugs compared to its parental cell line WiDr and also more sensitive to the antifolates compared to the other cell lines. H630 was most sensitive to TFT.

FA values were obtained after exposure of cells to a series of drug concentrations of TFT and/or antifolate, depending on the combination variant. The combinations of TFT with the antifolates were evaluated using

Table 1 Growth inhibition of the drugs and basal TS levels for the colon cancer cell lines

Cell line	Growth inhibition (IC_{50} values in nM)				Basal TS activity	
	TFT#	AG337#	ZD1694#	GW1843	at 1 μM dUMP# (pmol/h/10^6 cells)	at 10 μM dUMP# (pmol/h/10^6 cells)
H630	453 ± 114	3052 ± 506	21.3 ± 0.3	2.2 ± 0.7	201 ± 10	425 ± 24
WiDr	2025 ± 527	4840 ± 392	8.0 ± 1.3	1.5 ± 0.7	33 ± 2	131 ± 4
WiDr/F	900 ± 287	1733 ± 233	2.0 ± 0.9	0.6 ± 0.2	27 ± 2	148 ± 11
Colo320	533 ± 133	8750 ± 1750	7.4 ± 2.4	1.5 ± 0.2	105 ± 25	376 ± 50

Growth inhibition was determined as described in Materials and methods. Values are Means ± SEM of 3–5 experiments. # partly published: [4, 14, 42].

the FA-CI plots (see Figs. 1, 2). The mean CI values of all combination variants are given in Table 2. The combinations of TFT with one of the antifolates in which one of the drugs was kept at a constant concentration was not more than additive and was cell line dependent (TFT-AG337: CI = 0.9–2.3; TFT-ZD1694: CI = 0.9–1.3; TFT-GW1843: CI = 0.8–1.7), except for WiDr/F, where all three drug combinations showed CI = 0.6–0.8 and were clearly synergistic. Since we previously observed an aberrant binding pattern of GW1843 to TS [43], we also investigated preincubation with the antifolate. However, the CI did not change when WiDr/F cells were preincubated 24 h with GW1843 prior to TFT exposure. The combination variant in which the two drugs were added in a 1:1 IC_{50}-based molar ratio showed antagonism for all drug combinations in all cell lines (CI > 2.7).

In situ TS inhibition

To evaluate whether the interaction of the drugs was due to TS inhibition, we studied the effect of the drugs on in situ TS inhibition. Cells display a 3- to 4-fold difference in basal TS levels, but the TS levels were similar in WiDr and WiDr/F cells (Table 1). In order to evaluate in situ TS levels and inhibition, the colon cancer cell lines were exposed to concentrations inhibiting TS by at least 20%. These concentrations were based on the TS inhibition curves obtained after exposure of cells to a concentration series of the drugs (see Fig. 3). TS is inhibited most pronounced in WiDr/F cells and the highest TFT concentration to inhibit TS by 50% was required for H630 cells. The effect of each drug on the in situ TS activity in cells was used to predict the inhibition of the combinations. The expected and observed in situ TS inhibition is depicted in Fig. 4. In most cell lines the effects were additive, except in H630 cells, where a synergistic effect was induced for all three combinations ($P < 0.01$). GW1843 induced more TS inhibition with TFT in both WiDr (4.3%) and WiDr/F cells (14.3%; $P < 0.01$) than expected. In WiDr/F cells more TS inhibition was also seen for the combination TFT-ZD1694 (13.7%; $P < 0.05$) but not AG337 (additive).

DNA strand break formation

Since TFT can induce DNA damage next to and/or because of TS inhibition, we evaluated whether this effect was enhanced in the combinations. The FADU DNA damage assay demonstrated that more DNA strand breaks were seen in WiDr and WiDr/F cells for the combinations than for TFT alone ($P < 0.05$; Fig. 5). The most pronounced effects were seen for the combinations with GW1843, which were significantly better than expected in WiDr and WiDr/F (8%, $P = 0.05$). For the combinations of TFT with AG337 or ZD1694 only

Table 2 Combination Index values of TFT combined with the antifolates AG337, ZD1694 or GW1843 for the colon cancer cell lines

Cell line	TFT + AG337 combination			TFT + ZD1694 combination			TFT + GW1843 combination		
	TFTc	TFTv	Fixed ratio	TFTc	TFTv	Fixed ratio	TFTc	TFTv	Fixed ratio
H630	0.9 ± 0.2	2.3 ± 0.6	5.7 ± 0.9	1.1 ± 0.2	1.3 ± 0.3	3.3 ± 1.1	1.7 ± 0.3	1.1 ± 0.2	3.1 ± 0.5
WiDr	1.2 ± 0.2	1.0 ± 0.1	3.6 ± 0.8	1.0 ± 0.2	0.9 ± 0.1	4.7 ± 0.7	1.2 ± 0.1	1.2 ± 0.2	3.4 ± 0.5
WiDr/F	**0.7 ± 0.1**	**0.8 ± 0.1**	2.8 ± 1.4	**0.8 ± 0.2**	**0.6 ± 0.1**	4.3 ± 0.7	**0.6 ± 0.1**	**0.6 ± 0.2**	3.5 ± 0.9
Colo320	1.6 ± 0.1	1.5 ± 0.3	4.3 ± 0.5	0.9 ± 0.1	1.3 ± 0.3	4.0 ± 1.2	**0.8 ± 0.1**	1.7 ± 0.4	2.8 ± 1.3

Interpretation of CI values: CI < 0.9 means synergism; CI = 0.9–1.1 means additive; CI > 1.1 means antagonism. TFTc: combination of 2 drugs in which TFT was kept at a constant concentration (IC_{25}); TFTv: combination of 2 drugs in which an antifolate was kept at a constant concentration (IC_{25}); fixed ratio: the two drugs were added in a 1:1 IC_{50}-based molar ratio. For each experiment a mean CI was calculated from all datapoints with FA > 0.5 for all TFTc- and TFTv-combinations, and FA = 0.6, 0.75 and 0.9 for the fixed ratio-combinations. CI ≤ 0.8 are indicated in **bold**. Values (mean CI ± SEM) depicted here are based on 3–4 separate experiments.

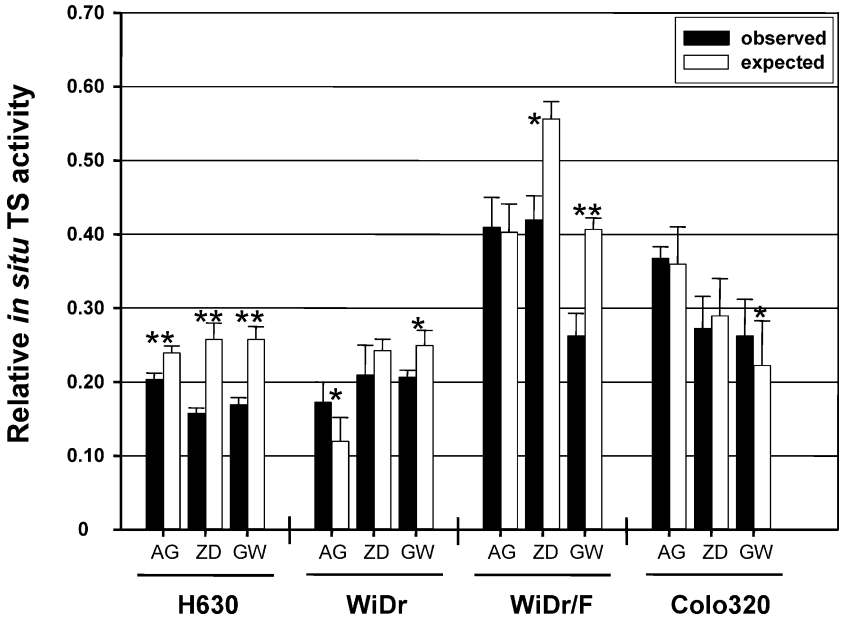


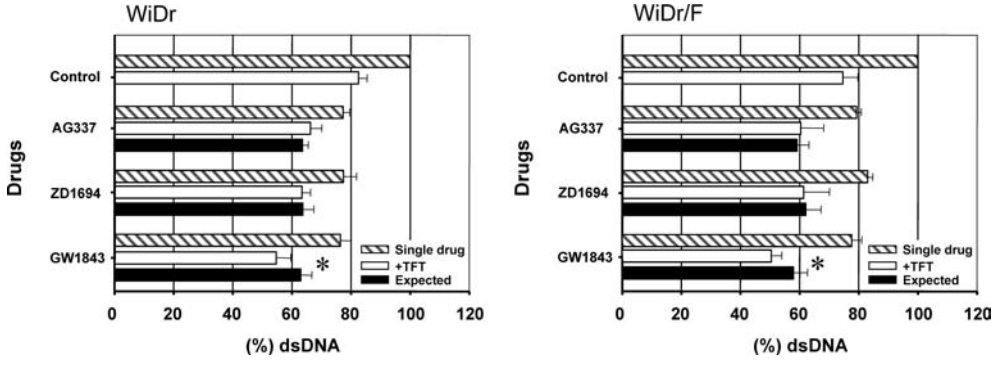
Fig. 4 In situ TS inhibition by TFT in combination with antifolates in the colon cancer cell lines. Cells were exposed for 24 h to TFT alone or in combination with antifolates simultaneously. TS in situ inhibition was measured by production of ^3H_2O. The single drug concentrations were: H630: 60 nM TFT, 422.5 nM AG337, 7.25 nM ZD1694, 1.1 nM GW1843; WiDr: 15 nM TFT, 102.5 nM AG337, 0.6 nM ZD1694, 0.27 nM GW1843; WiDr/F: 1.5 nM TFT, 80 nM AG337, 0.38 nM ZD1694, 0.34 nM GW1843; Colo320: 10 nM TFT, 250 nM AG337, 2 nM ZD1694, 1 nM GW1843. Values are Means ± SEM of 3–5 separate experiments. Significant differences between expected and observed values: $*P < 0.05$; $**P < 0.01$. TS activity$_{exp}$ = [rel. TS activity$_{TFT}$]×[rel. TS activity$_{antifolate}$]

additive effects were seen in both WiDr and WiDr/F cells; not more DNA strand breaks were seen than expected from the effects of the two single drugs.

Fig. 5 DNA damage induced by TFT and the antifolates in WiDr and WiDr/F. Cells were exposed for 48 h to TFT with or without antifolates and DNA damage was measured with the FADU assay. The single drug concentrations were: WiDr: 3 μM TFT, 6 μM AG337, 10 nM ZD1694, 2.5 nM GW1843; WiDr/F: 1.5 μM TFT, 2.5 μM AG337, 2.5 nM ZD1694, 1 nM GW1843. Values are Means ± SEM of 4 separate experiments. Significant differences between expected and observed values: $*P = 0.05$. (%) dsDNA$_{exp}$ = [((%)dsDNA$_{TFT}$/100)× ((%)dsDNA$_{antifolate}$/100)]×100%

Discussion

In the present study, we demonstrated the folate-status of cells may be an important determinant in the induction of a synergistic interaction with an antifolate. WiDr/F cells growing in low physiological folate conditions are more sensitive to two-sided inhibition of TS than WiDr. The combinations of TFT with the antifolates AG337, ZD1694 or GW1843 are mainly additive for the cell lines growing in high folate containing medium. The low folate status possibly increases the binding of antifolates to the TS co-factor binding site, resulting in a synergistic effect between TFT and antifolates. Although the antifolates are not all competitive inhibitors of TS, folate levels in medium and cells are an important determinant of the efficacy of the antifolates [25, 44], because culture conditions will reduce folate pools in colon cancer cells about 10-fold (unpublished data), thereby also reducing the availability of folates for other folate-requiring processes. This is clearly shown by the more potent TS in situ inhibition by TFT and antifolates in WiDr/F cells compared to WiDr cells despite comparable TS levels in the cells (Fig. 3; [45]). Therefore it can be concluded that low intracellular folate levels will enhance the efficiency of TS



inhibitors, and possibly also the interaction between two types of TS inhibitors, nucleotide- and folate-directed. A relative moderate increase to 50 nM LV is already very effective to abrogate antifolate effects [25], which is the reason why the experiments were performed at very low folate levels.

The interaction between TFT and antifolates may have some similarities to 5FU. FdUMP is a very potent TS inhibitor with a K_i of about 1 nM [46]. Our group previously showed that antifolates could enhance FdUMP-mediated TS inhibition in TS-overexpressing cells when exposed to 5FU. Especially at low antifolate concentrations (<0.3 μM) FdUMP binding to human TS can be facilitated, but is dependent on polyglutamate status [43]. We previously showed that TS inhibition by TF-TMP in cancer cells is rapid [47]. TF-TMP binds covalently to TS and does not form a stable ternary complex in contrast to 5FU, which forms a stable FdUMP-TS-CH_2THF complex. Thus after removal of TFT from medium TS activity restores more rapidly than after 5FU removal.

Because TFT induces dTTP depletion we expected to see an increased TS inhibition in combination with antifolates resulting in higher cytotoxicity, which might be related to an increase in TFTMP-TS binding. This was only seen in WiDr/F cells growing in low folate medium and most pronounced for the TFT-GW1843 combination. When these drugs were added simultaneously, synergistic effects were seen, both on the level of DNA damage induction and inhibition of TS. This was not shown for GW1843 preincubation, which can be explained by the high affinity of this compound for TS ([48]; $K_i = 0.09$ nM) and its unique way of binding to TS [43, 49]. In contrast to GW1843 and ZD1694, AG337 was less cytotoxic to the colon cancer cell lines. AG337 enters the cell by passive diffusion and does not need polyglutamylation to become more active and therefore migrates easier out of the cell. ZD1694 and GW1843 need polyglutamylation to be retained in the cell and bind to TS more easily. In addition, Longo et al. [50] previously showed that FdUMP mediated inhibition of TS can be increased by ZD1694 ([8]; $K_i = 1$ nM) when preincubated with the antifolate, eventually leading to higher cytotoxicity.

The combinations in which TFT and an antifolate were added to the medium in a fixed 1:1 IC_{50}-based molar ratio showed antagonistic effects, even for WiDr/F cells. In this combination variant dUMP levels are likely to be more increased at high antifolate levels, leading to an increased competition between dUMP and TF-TMP for binding to the substrate-binding site of TS, especially at FA > 0.5. Only at low TFT and antifolate concentrations synergism was observed for the 1:1 ratio combinations, possibly because dUMP levels would still be very low and two suboptimal inhibitory levels would add up. At high concentrations of both drugs, growth inhibition would be too high for each drug separately. In growth inhibition experiments with variable ratio, the ratio between antifolate and TFT concentrations will decrease, thereby diminishing the competition between dUMP and TF-TMP. Furthermore, the cooperativity of the antifolates in the binding of TF-TMP to TS may be negative at FA > 0.5 concentrations, as previously shown for the antifolates on FdUMP-binding to TS. Positive cooperation between the drugs was shown at concentrations in the low micromolar range (expressed as Hill coefficients) [43]. This would favor to combine TFT with a low and constant antifolate concentration, which would induce synergistic effects, since a similar enhancing effect would be seen at each TFT concentration. These data indicate that a fixed ratio is not always recommended to study synergistic effects between drugs properly, especially when the slopes of the growth inhibition curves are different, indicating different kinetics of inducing growth inhibition. Using one drug at a fixed concentration enables to compare the drugs more properly, since the effect of one drug would be similar while only the effect of the other drug changes. E.g. TFT in the formulation TAS-102 is given orally several times a day, leading to plasma concentrations in a small range; TS inhibition (Fig. 3) will be similar as well as DNA damage.

Alternatively, thymidine nucleotides produced via the TK salvage pathway might antagonize TS inhibition. This can be mediated by replenishing the TdR levels after depletion of nucleotide pools. Thymidine is not added to the culture medium and will not be able to replenish the thymidine nucleotide pools. Although some thymidine is present in fetal calf serum, this is very low and will be depleted very rapidly during culturing [40]. Therefore, differences in thymidine rescue are unlikely to play a role in the synergism in WiDr/F cells compared to WiDr cells.

The effect of TS inhibition may be dependent on folate status when cancer cells are exposed to antifolate based direct TS inhibitors. Maximum additive TS inhibition could be achieved when the antifolates were combined with TFT, but only at low folate conditions; at normal folate conditions the incorporation of dUTP is likely to be decreased. In contrast, the antitumor mechanism of TFT is primarily incorporation into the DNA resulting in the induction of DNA fragmentation (see also [16, 30]), which was enhanced when TFT was combined with GW1843. This antifolate is currently known as the liposomal formulation OSI-7904L [51]. Therefore, we can conclude that the antitumor effects of this formulation might be potentiated by TAS-102. In addition, TAS-102 and the novel multi-targeted drug Alimta (pemetrexed, LY231514) might be a more favorable combination. Alimta has recently been approved for use in the treatment of patients with malignant pleural mesothelioma [52].

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