Determinants of trifluorothymidine sensitivity and metabolism in colon and lung cancer cells

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Trifluorothymidine (TFT) is a fluorinated thymidine analog that after conversion to its monophosphate derivative can inhibit thymidylate synthase (TS) and be incorporated into DNA. TFT is a good substrate for thymidine phosphorylase (TP), and the combination of TFT and a TP inhibitor (TPI), called TAS-102, has been developed to enhance the bioavailability of TFT *in vivo*, and is currently being studied in a phase I study. We aimed to determine the limiting factor(s) in the cytotoxicity of TFT with or without TPI to cancer cells. Colon cancer and lung cancer cell lines with either an overexpression or deficiency of one of the enzymes involved in TFT metabolism were used to study the effect of TPI on TFT sensitivity and the role of TS inhibition. The synthesis of radioactive TFT metabolites was studied using thin-layer chromatography together with the incorporation of TFT into DNA. We found that despite a high rate of TFT phosphorolysis, cells with high TP expression are not more resistant to TFT, while TPI did not increase TFT sensitivity. High TS-expressing cells were shown to be cross-resistant to a 72-h exposure to TFT compared to 5-fluorouracil (5-FU), although this was more pronounced at a 4-h exposure (3.4-fold or more for TFT and 1.4-fold or more for 5-FU). Despite a moderate inhibition of TS activity in cells expressing high TS, these cells were more sensitive to TFT than 5-FU (3.8-fold or more). Only in Colo320TP1 cells expressing high TP, inhibition of TFT phosphorolysis by TPI increased formation of active TFT metabolites 1.8-fold, although this was not related to an increase in TFT incorporation into DNA. These studies show that uptake of TFT and subsequent phosphorylation of TFT by cancer cells is very rapid. Despite a high rate of degradation, the activation pathways are still saturated and sufficient to inhibit TS and enable incorporation into DNA, although the contribution of each effect is exposure time dependent. *Anti-Cancer Drugs* 16:285–292 © 2005 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2005, **16**:285–292

Keywords: 5-fluorouracil, antifolate, DNA incorporation, thymidine phosphorylase, thymidylate synthase, time-dependent pharmacologic effect, trifluorothymidine

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This study was supported by Taiho Pharmaceutical Co., Ltd, Hanno, Japan.

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Received 6 July 2004 **Revised form accepted** 11 November 2004

Introduction

Thymidylate synthase (TS) is one of the rate-limiting enzymes in pyrimidine *de novo* deoxynucleotide synthesis. TS catalyzes the methylation reaction of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP) with 5,10-methylene-tetrahydrofolate (CH_2-THF) as the methyl donor [1]. Because TS plays a central role in DNA synthesis it continues to be a target for chemotherapeutic approaches [2,3]. TS can be inhibited by a variety of folate analogs, such as the TS-directed antifolates nolatrexed, raltitrexed and pemetrexed [4], and the fluorinated pyrimidine analogs 5-fluorouracil (5-FU) [5], 5-fluoro-2′-deoxyuridine (FdUrd) [6] and 5-trifluoro-2′-deoxythymidine (TFT; Fig. 1) [7]. FdUrd (derived from 5-FU) and TFT can be metabolized by thymidine kinase (TK) to their active phosphate derivatives FdUMP and TF-TMP, respectively. Therefore, TK and TS both have a potential role in resistance to fluorinated pyrimidines. FdUMP inhibits TS after formation of a ternary complex with CH_2-THF, which will enhance and prolong this inhibition [8]. In contrast to FdUMP, TF-TMP does not form a ternary complex and binds covalently to the active site of TS, thereby inhibiting its activity [9,10]. TF-TMP shares the same mechanism of cytotoxicity as FdUMP by inhibiting TS leading directly to depletion of dTMP and subsequently of dTTP (Fig. 2). Indirectly, lack of dTTP leads to an accumulation of dUMP, resulting in dUTP incorporation into the DNA [11]. In addition, the triphosphate forms of FdUrd [12] and TFT (FdUTP and TF-TTP, respectively) can be incorporated into the DNA causing cell death eventually because of DNA strand breaks.

Resistance to 5-FU is an important problem in cancer patients receiving 5-FU-based chemotherapy. A number of clinical evaluations demonstrated a relationship between TS expression in tumors and the clinical response and/or survival in these cancer patients

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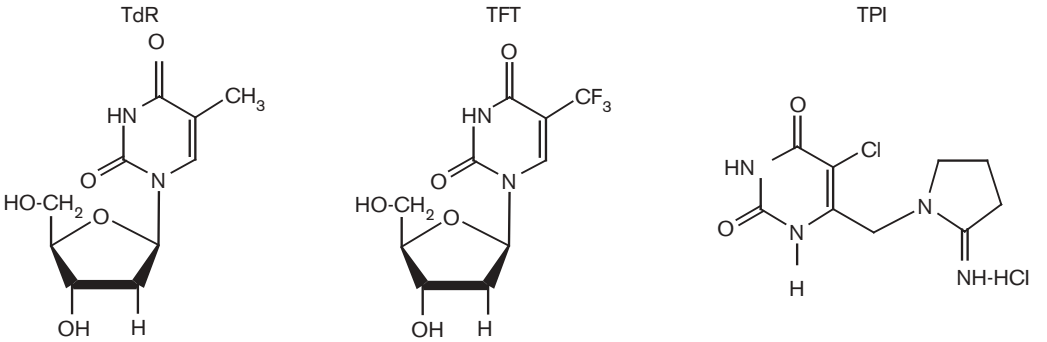
Fig. 1



Chemical structures of thymidine (TdR), trifluorothymidine (TFT) and thymidine phosphorylase inhibitor (TPI).

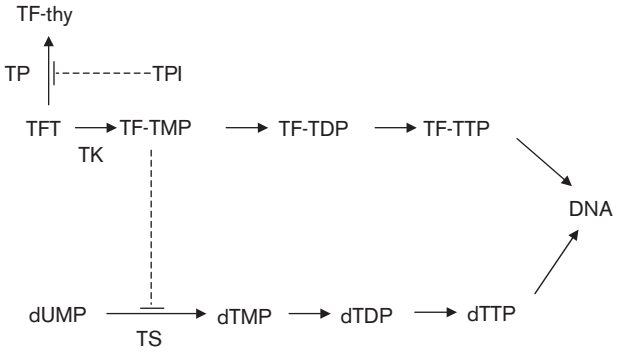
[13–15]. Poor response and/or survival rates are often associated with high TS levels in tumors. *In vitro* studies have shown that 5-FU resistance in human tumor cells is often the result of an increase in TS mRNA and/or protein expression [8,16]. Still, it has also been shown that 5-FU-resistant human tumor cells can have decreased 5-FU-activating enzymes, including orotate phosphoribosyl-transferase and uridine kinase [17,18]. Murakami *et al.* [19] showed that TFT may be more effective in colorectal cancer cells to overcome (acquired) 5-FU and/or FdUrd resistance caused by amplification and subsequent overexpression of TS.

TFT alone shows cytotoxicity against tumor cells, but this may be limited due to breakdown to TF-thymine (TF-thy) catalyzed by thymidine phosphorylase (TP). Previous studies showed that TP is often upregulated in tumor cells [20,21]. In contrast, 5-FU can be activated by TP, but under physiological conditions this pathway probably does not play an important role [22]. To avoid low bioavailability of TFT *in vivo*, degradation of TFT can be decreased by TP inhibition (Fig. 2). The very potent TP inhibitor 5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]-uracil hydrochloride (TPI; $K_i = 1.7 \times 10^{-8}$ M; Fig. 1) inhibits human TP, but not uridine phosphorylase [23].

TFT has been evaluated as an anticancer agent, but was not effective as a single agent, possibly because of its extensive degradation [24]. To overcome this problem, the combination of TFT and TPI has been developed in the molar ratio of 1:0.5 (called TAS-102) in which TPI should prevent degradation of TFT and thereby enhance the bioavailability of TFT to tumor cells [23,25]. TAS-102 is currently being tested in different schedules as an orally chemotherapeutic agent in a phase I study [26].

In this study we aimed to determine limiting factors in the *in vitro* cytotoxicity of TFT with or without TPI to cancer cells. For this purpose we focused on the metabolizing and target enzymes involved, and used cells with either an overexpression or deficiency (induced or intrinsic) of one of these enzymes and/or with a resistance to either 5-FU, FdUrd or antifolates. This was also related to synthesis of TFT metabolites and their incorporation into the DNA in cells.

Fig. 2



Mechanism of action of TFT and TPI.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium were obtained from Biowhittaker Europe (Cambrex, Verviers, Belgium). Fetal calf serum (FCS) was from Gibco/BRL (Life Technologies, Breda, The Netherlands). 5-FU was purchased from Sigma (St Louis, MO). TFT and TPI were provided by Taiho (Hanno, Japan) [23]. [5-^{3}H]2′-deoxycytidine (specific activity 28.5 Ci/mmol) and [6-^{3}H]5-trifluoromethyl-2′-deoxyuridine (specific activity 9.9 Ci/mmol) were purchased from Moravek (Brea, CA). All other chemicals

used in the experiments described below were of analytical grade and commercially available.

Cell cultures

Various cell lines originating from different tissues were used in this study. The human colorectal carcinoma cell lines H630, WiDr and their variants made resistant to either 5-FU [27] or an antifolate [pemetrexed (Pem) or multi-targeted antifolate (MTA)] [28] were used to investigate cross-resistance to TFT. WiDr-4Pem and WiDr-cPem are Pem-resistant variants from WiDr, which were developed by weekly exposure to Pem for 4 h at 50 μM and 72 h at 20 μM, respectively [28]. These cell lines have an overexpression of TS and were shown to be cross-resistant to other antifolates, e.g. ZD1694 and AG337. H630R1 and H630R10 are 5-FU-resistant variants of H630 with increased TS levels, which are grown continuously in medium containing 1 or 10 μM 5-FU [16,27]. Colo320TP1 is a Colo320 variant transfected with TP DNA and has a high TP expression compared to its parental colorectal carcinoma cell line Colo320 [22]. H460 is a human non-small cell lung cancer cell line with a naturally high TP expression [22]. WiDr/F is a WiDr variant adapted to grow at low folate conditions [29]. WiDr/F cells were cultured in folate-free RPMI 1640 medium supplemented with 10% dialyzed FCS, 2 mM glutamine and 2.5 nM DL-leucovorin (LV). H460 was cultured in RPMI 1640 medium only supplemented with 10% heat-inactivated FCS. WiDr, H630, Colo320 and their variants were cultured in DMEM supplemented with 10% heat-inactivated FCS. All cell lines were grown as monolayers at 37°C in a 5% CO_2 humidified atmosphere and grew exponentially during the course of all experiments.

Growth inhibition experiments

In order to determine cytotoxicity of the drugs we used the sulforhodamine B assay (SRB) for adhesive cell lines [30,31]. Cells were plated in 96-well plates, drugs were added after 24 h and cells were incubated for another 72 h. The SRB assay was performed before and after drug exposure. Briefly, cells were seeded in 100 μl of the cell-line-specific medium in triplicate in 96-well flat-bottom plates (Costar, Corning, NY). To ensure exponential growth, the cells were seeded in different densities depending on their growth rate (WiDr, Colo320 and H460 cell lines: 5000 cells/well; H630: 10000 cells/well). After 24 h, 100 μl of drug containing medium was added to the wells (in concentrations ranging from 10^{-3} to 10^{-8} M) with or without 10 μM TPI, a concentration that did not cause growth inhibition when added alone. When TPI was added to the medium, it was present during the complete incubation period of 96 h. Cells were either treated for 72 h with 5-FU, TFT with or without TPI (long-term exposure), or for 4 h with these drugs and incubated for another 68 h in fresh medium (short-term exposure). The Colo320, Colo320TP1 and H460 cells were also treated for 1 h with TFT or TFT + TPI and incubated for another 71 h in fresh medium. Colo320TP1 and H460 were used to determine the effect of TPI in high TP-expressing cells exposed to TFT. After the 72-h drug-exposure period the adhesive cells were fixed with 10% trichloroacetic acid (TCA) and stained with SRB protein dye. Based on the absorption values (measuring optical density at 540 nm) IC_{50} values could be calculated, defined as the drug concentration that corresponded to an inhibition in cellular growth by 50% compared to the values of untreated cells [32]. Resistance factors (RFs) were calculated by comparing the IC_{50} of the variant cell line with the IC_{50} of the parental cell line. Dose-modifying factors (DMFs) were calculated by comparing IC_{50} (+ TPI) with IC_{50} (–TPI).

TS *in situ* inhibition assay (TSIA)

This assay was used to determine whether TFT would affect intracellular TS activity in intact cells. Inhibition of TS was determined by measuring the TS catalyzed reaction of [5-^{3}H]dUMP to dTMP generating ^3H_2O [33]. In this assay [5-^{3}H]2′-deoxycytidine was added to the medium as precursor for intracellular [5-^{3}H]dUMP, the TS substrate. The H630 and WiDr cells (2.5×10^5 cells/well in Costar six-well plates in 2 ml medium) were incubated for 24 h at 37°C in a 5% CO_2 humidified atmosphere. Thereafter, the cells were exposed for 24 h to the drugs in a range from 10 nM to 4 μM. To each well [5-^{3}H]2′-deoxycytidine (final concentration 1 μM, 28.5 Ci/mmol) was added 1 h before the end of the total incubation period. A 200 μl sample from the culture medium was taken after the incubation period and was put in 2 ml Eppendorf tubes on ice. The reaction was stopped by putting the medium on ice and 200 μl of ice-cold 35% TCA was added together with 1 ml 10% (1 h pre-stirred) activated charcoal solution as described for the *in vitro* TS catalytic activity assay [34]. After vortexing, the samples were left on ice for 30 min and centrifuged (800 *g*, 30 min, 4°C) after which 450 μl of the aqueous supernatants (containing ^3H_2O) was transferred to liquid scintillation vials (J. T. Baker, Deventer, The Netherlands) and counted for radioactivity. Several drug concentrations were used to allow data to be expressed as TSI_{50}, the drug concentration needed to inhibit TS by 50% compared to the control TS activity.

TP activity

The TP activity was determined using an assay previously described [22,35]. In the present study both thymidine and TFT were used as a substrate for TP, and enzyme activity was calculated by their conversion to thymine and TF-thy, respectively, which were separated by HPLC analysis [35,36].

Formation of TFT metabolites and incorporation of TFT into DNA

In order to measure TFT metabolism subconfluent cells were trypsinized and resuspended in medium to a concentration of 5×10^6 cells/ml and 250 or 500 μl of this

suspension was put in 2.0-ml Eppendorf incubation vials, as previously described, to study 5-FU metabolism [37]. The cells were incubated in a shaking waterbath at 37°C and after 1 h pre-incubation 25 or 50 µl [6-^{3}H]5-trifluoromethyl-2′-deoxyuridine (9.9 Ci/mmol, final concentration 10 µM) was added, depending on the cell suspension volume, and incubation was continued for 3 h. The reaction was stopped by spinning down the cells (2 min, 3000 *g*). Blanks were obtained by adding labeled TFT to cell suspensions just before spinning down, thereafter these samples were treated identically to the other samples. The supernatants (containing extracellular TFT metabolites) were collected in separate vials to be analyzed later using thin-layer chromatography (TLC). Cells were resuspended/washed in 100 µl ice-cold saline solution. After centrifugation (1 min, 11 600 *g*) cells were resuspended in 45 µl ice-cold saline solution, and 5 µl 5 M $HClO_4$ was added to extract the cells to obtain the DNA and the intracellular TFT metabolites. After vortexing, the vials were left on ice for 20 min. The denatured material was spun down using a mini-centrifuge (5 min, 11 600 *g*) and the acid-soluble fraction was put into new vials to be neutralized by mixing with 100 µl trioctylamine-Freon solution (1:4). After centrifugation (2 min, 11 600 *g*) the supernatants (containing intracellular TFT metabolites) were collected in separate vials to be analyzed later using TLC. The acid-insoluble DNA pellets were washed at least 3 times in 100 µl cold saline solution (3 min, 11 600 *g*) until the solution was cleared from all free tritiated TFT metabolites. The pellets were dissolved in 200 µl 1 M NaOH and radioactivity was estimated.

To calculate the concentrations of TFT metabolites in cells and the medium, TLC was used, partially modified from a previously described method [37]. Non-radioactive tracer containing TFT, TF-thy, TF-TMP, TF-TDP and TF-TTP (5 µl, 2.5 mM each) was spotted onto PEI-cellulose sheets to permit localization of the radioactive compounds under UV light. Thereafter, 5 or 10 µl of the obtained supernatants was spotted onto the sheets. After development with distilled water-ethanol mixture (3:1, v/v) the spots were cut out. TFT and TF-thy had RF values of 0.91 and 0.80, respectively, and the phosphorylated TFT compounds remained at the origin. The spots were eluted with 1 ml 0.1 M HCl and 0.2 M KCl for 1 h (while shaking), and counted for activity after addition of 4 ml scintillation fluid.

Statistics

The experiments were analyzed by the Student's *t*-test. The calculated values were considered significant when $p < 0.05$.

Results

Growth inhibition

The growth inhibition experiments served several purposes: to determine whether a high TP activity or a deficient TP (intrinsic or by inhibition) would affect TFT sensitivity, to determine potential cross-resistance to 5-FU and antifolates, and to determine how TFT would exert its cytotoxicity. The role of TP was investigated by using high TP-expressing cells (Colo320TP1 and H460) and by inhibition of TP by TPI. In addition, we exposed cells for 72 h and for shorter periods (4 and 1 h) with or without TPI, since we observed that at long-term exposure to TFT its activation would be optimal and might hardly be affected by degradation. In contrast, at short-term exposure TFT would not be activated to a large extent and degradation would decrease the availability of TFT. In Table 1 the IC_{50} values of the cell lines exposed to TFT or 5-FU (± TPI) are shown, respectively. The IC_{50} values of TFT or 5-FU for the different cell lines varied considerably.

Colo320TP1 and H460 cells with high TP expression are not more sensitive to TFT in combination with TPI than TFT alone, both at long- and short-term exposure (DMF around 1.0). Comparable results were obtained when the cells were only exposed for 1 h to TFT followed by a 71-h incubation period in drug-free medium (data not shown). Colo320TP1 cells were shown to be less sensitive to TFT at short exposure compared to the parental cells (RF = 1.7, $p < 0.01$). De Bruin *et al.* [22] previously showed that in Colo320TP1 and high TP-expressing H460 non-small cell lung cancer cells, addition of TPI did reduce 5-FU sensitivity after 72-h exposure (DMF = 14.7 and DMF = 4.3; $p < 0.01$).

The 5-FU-resistant cell lines H630R1 and H630R10 were shown to be cross-resistant to TFT both at short- and long-term exposure (RF ≥ 10.4). The pemetrexed resistant cell lines WiDr-4Pem and WiDr-cPem were only cross-resistant to TFT at short-term exposure (RF = 3.4 and RF = 5.9, respectively, $p < 0.05$), although far less compared to the 5-FU-resistant cells. Remarkably, the pemetrexed-resistant cell lines were not cross-resistant to 5-FU both at short- and long-term exposure (RF ≤ 1.5). WiDr/F was more sensitive to TFT compared to WiDr ($p < 0.05$). TPI did not enhance TFT sensitivity in these cell lines significantly. The values for the ratio 4/72 h were higher for TFT than for 5-FU, showing that the growth-inhibiting effect was sustained longer for cells exposed to 5-FU compared to that of TFT.

TS inhibition

Since TS is a potential target for TFT we investigated whether and to what extent TFT can inhibit intracellular TS. To determine whether TFT would also be active in 5-FU- and antifolate-resistant cells we included these cells as well. The H630 and WiDr cells and their variants were incubated for 24 h with different concentrations of 5-FU and TFT. Table 2 shows that the *in situ* TS activity can be inhibited in the resistant variants, but to a far

Table 1 Growth inhibition by TFT or 5-FU (with or without TPI) for the cancer cell lines and their variants

Cell line	4 h drug + 68 h medium		72 h drug		DMF		RF		Ratio 4/72 h	
	TFT	TFT + TPI	TFT	TFT + TPI	4 h	72 h	4 h	72 h	TFT	TFT + TPI
H630	15.8 ± 2.2	15.3 ± 2.8	0.5 ± 0.1	0.5 ± 0.1	1.0	1.0			31.6	30.6
H630R1	273.3 ± 115.7	316.7 ± 92.8	4.7 ± 0.9	5.5 ± 1.0	1.2	1.2	17.3[a]	10.4[b]	58.1	57.6
H630R10	> 500	> 500	148.8 ± 8.5	155.0 ± 8.4	–	1.0	> 31.6[b]	330.6[b]	> 3.4	> 3.2
WiDr	29.8 ± 4.5	28.8 ± 1.8	2.0 ± 0.5	3.2 ± 0.8	1.0	1.6			14.9	9.0
WiDr-4PEM	101.7 ± 15.9	100.0 ± 20.8	3.7 ± 0.6	3.1 ± 0.7	1.0	0.8	3.4a	1.8	27.5	32.3
WiDr-cPEM	176.7 ± 31.8	186.7 ± 6.7	3.3 ± 0.4	2.7 ± 0.8	1.1	0.8	5.9a	1.6	53.5	69.1
WiDr/F	5.3 ± 1.4	7.7 ± 1.5	0.9 ± 0.3	0.9 ± 0.1	1.5	1.0	0.2[a]	0.4[a]	5.9	8.6
Colo320	15.0 ± 2.8	14.5 ± 1.0	0.5 ± 0.1	0.5 ± 0.1	1.0	1.0			30.0	29.0
Colo320TP1	25.7 ± 3.0	23.5 ± 2.4	0.6 ± 0.2	0.6 ± 0.1	0.9	1.0	1.7[b]	1.0	42.8	39.2
H460	11.2 ± 2.5	7.8 ± 1.0	0.6 ± 0.1[c]	0.6 ± 0.03[c]	0.7	1.0	–	–	18.7	13.0
	5-FU	5-FU + TPI	5-FU	5-FU + TPI					5-FU	5-FU + TPI
H630	24.0 ± 4.0	27.7 ± 1.5	2.4 ± 0.4	3.2 ± 0.8	1.2	1.3			10.0	8.7
H630R1	72.5 ± 2.0	92.5 ± 6.1	6.0 ± 1.0	6.5 ± 0.5	1.3	1.1	3.0[a]	2.5[a]	12.1	14.2
H630R10	> 1000	> 1000	175.0 ± 10.4	190.0 ± 15.3	–	1.1	> 41.7[b]	72.9[b]	> 5.7	> 5.3
WiDr	43.6 ± 6.4	41.6 ± 5.3	3.4 ± 0.4	3.5 ± 0.4	1.0	1.0			12.8	11.9
WiDr-4PEM	63.0 ± 6.2	52.0 ± 5.6	5.1 ± 1.8	6.0 ± 3.0	0.8[a]	1.2	1.4[a]	1.5	12.4	8.7
WiDr-cPEM	60.0 ± 8.9	54.4 ± 7.3	3.4 ± 0.2	3.8 ± 0.6	0.9	1.1	1.4	1.0	17.6	14.3

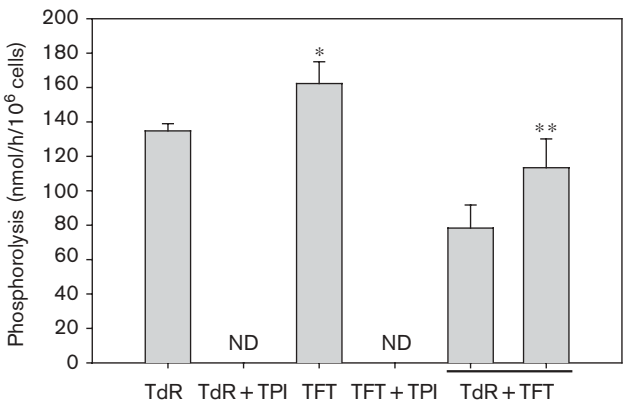
Growth inhibition was determined as described in Materials and methods. Results are given as mean IC_{50} values (μM) ± SEM of three to five experiments. TPI was added to a final concentration of 10 μM, 24 h before adding 5-FU/TFT. The DMF was calculated by IC_{50} (TFT + TPI)/IC_{50} (TFT alone). RF = resistance factor (compared to parentals). Significant differences are given as follows:
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] Previously published results [22].

Table 2 TSI_{50} values (nM) for H630 and WiDr cells and their resistant variants when exposed 24 h to 5-FU or TFT

Cell line	5-FU	TFT	RF	
			5-FU	TFT
H630	165 ± 56	43 ± 4		
H630R1	388 ± 66	100 ± 6	2.4[a]	2.3[b]
H630R10	> 4000	3500 ± 354	> 24.2[b]	81.4[b]
WiDr	127 ± 19	16 ± 3		
WilDr-4PEM	1867 ± 377	450 ± 81	14.7[b]	28.1[b]
WiDr-cPEM	3115 ± 176	> 4000	24.5[b]	> 250[b]

TSI_{50} means inhibition of TS activity by 50%. Results are means ± SEM from three separate experiments. TSI_{50} variants are compared with TSI_{50} parentals:
[a] $p < 0.05$.
[b] $p < 0.01$.

Fig. 3



Phosphorolysis of thymidine, TFT or in combination (in a 1:1 equimolar ratio) in Colo320TP1 cell lysates, measured as thymine or TF-thy. TPI was added to an end concentration of 10 μM. Indication significance when TFT is compared to thymidine: $*p < 0.05$, $**p < 0.01$. Error bars are SEM ($n = 4$). ND = nothing detected.

lesser extent than the parental cells. The TSI_{50} of the 5-FU- and Pem-resistant cells increased significantly when exposed to either 5-FU or TFT. H630R10 and WiDr-cPem are most resistant to TFT (RF = 81.4 and RF > 250, respectively), probably due to their highly upregulated TS levels [8,28].

TFT phosphorolysis

To determine thymidine phosphorolysis in Colo320TP1 cells the conversion of the two substrates thymidine and TFT to thymine and TF-thy was measured, respectively (see Fig. 3). When the substrates were given alone slightly more TFT was degraded compared to thymidine, indicating that TFT is a better substrate. When the substrates were given in equimolar concentrations about 45% more TFT was degraded than thymidine ($p < 0.01$). When TPI was added to the cell lysates no (TF-)thymine was detected.

TFT metabolism and incorporation into DNA

Using [^{3}H]TFT we determined TFT incorporation into DNA and measured the production of TFT metabolites in WiDr, H630, Colo320 (TP deficient) and the TP transfected Colo320TP1 cells (Table 3). The accumulation of TFT phosphate derivatives was about 4 pmol/3 h/10^6 cells in H630, WiDr and Colo320TP1, and 8.2 pmol/3 h/10^6 cells in Colo320. TPI increased the accumulation of TFT phosphate derivatives in Colo320TP1 cells from 3.9 to 7.2 pmol/3 h/10^6 cells ($p < 0.05$), comparable to the

Table 3 Metabolism of TFT and its incorporation into DNA

Cell line	TFT in DNA (pmol/3 h/10^6 cells)	TF-thy/TFT (in medium)	TFT metabolism (pmol/3 h/10^6 cells)	
			TFT-MP/DP/TP	TF-thy total
H630	2.3 ± 0.5	0.5 ± 0.1	4.2 ± 0.3	590.8 ± 54.6
H630 (+TPI)	2.7 ± 0.6	ND	4.3 ± 0.3	14.4 ± 8.4
WiDr	3.3 ± 0.7	0.6 ± 0.1	4.1 ± 0.4	648.7 ± 78.6
WiDr (+TPI)	4.6 ± 0.8	ND	4.7 ± 0.3	ND
Colo320	4.4 ± 0.5	ND	8.2 ± 1.0	ND
Colo320 (+TPI)	4.3 ± 0.5	ND	6.9 ± 0.4	ND
Colo320TP1	2.9 ± 0.5	6.3 ± 1.0	3.9 ± 0.3	1575.0 ± 54.0
Colo320TP1 (+TPI)	3.3 ± 0.2	ND	7.2 ± 0.7[a]	ND

Cells were incubated for 3 h in the presence of TFT (final concentration 10 μM). Results are given as means ± SEM of three or four experiments. TF-thy/TFT is the ratio of the metabolites found in the medium after the 3 h incubation period. Significant difference with/without TPI is given by [a]$p < 0.05$. ND = not detectable.

parental cell line. In the presence of TPI, the formation of TF-thy in cells containing TP was almost completely inhibited, while no TF-thy was formed in the Colo320 cells. TFT incorporation into DNA varied between 2.3 and 4.6 pmol/3 h/10^6 cells. TFT incorporation did not change significantly when 10 μM TPI was added to the medium.

Discussion

In this paper we demonstrate that cross-resistance to TFT was only observed in cells resistant to 5-FU and Pem with high TS levels, independent of the exposure times used. The TP inhibitor TPI completely inhibited TFT phosphorolysis, leading to increased formation of active TFT metabolites in cells with a very high TP. In contrast to TS inhibition, incorporation of TFT into DNA seems to be a more rate-limiting step for TFT-induced cytotoxicity.

Murakami *et al.* [19] showed that 5-FU-resistant DLD-1 colon cancer cells did not show any cross-resistance for both FdUrd and TFT at long-term exposure, suggesting that 5-FU resistance could be overcome by long-term exposure to TFT. Surprisingly, cells with no defect in 5-FU anabolism such as 5-FU-resistant H630 cells were cross-resistant to TFT, but antifolate-resistant cells were not. In contrast, after short exposure (4 h) both 5-FU- and Pem-resistant cells, which have increased levels of TS, showed cross-resistance to TFT, but the Pem-resistant cells were not cross-resistant to 5-FU, precluding a defect in 5-FU anabolism. These results can be explained due to the fact that, besides increased TS levels, reduced activity of 5-FU-anabolizing enzymes (e.g. decrease in orotate phosphoribosyltransferase or/and thymidine kinase activity) can cause resistance to 5-FU in the DLD-1 cells [17,19], but not in the H630 and WiDr cells.

Despite the presence of cross-resistance to TFT, the *in situ* TS activity was inhibited by TFT in these resistant cells with increased TS levels, leading to the conclusion that TFT is a more potent TS inhibitor than 5-FU. Although in WiDr cells TFT can inhibit the *in situ* TS activity, TS inhibition does not add to cytotoxicity in long-term exposure to TFT, therefore cytotoxicity should be related to TFT incorporation into DNA rather than TS inhibition. In the H630R10 cells TFT seems to be more TS directed, since both at short and long exposure these cells are cross-resistant to TFT, thereby blocking DNA synthesis stronger. There is a change in the mechanism of TFT cytotoxicity depending on exposure time, with incorporation into DNA being more important at longer exposure.

These data and previous data in FM3A cells [38] demonstrate that the conversion of TFT by TK to its active form is essential and because it only involves one activation step, TFT exerts cytotoxicity within a few hours, in contrast to 5-FU, which requires more conversion steps. Probably a low concentration of TFT is enough to induce dTTP depletion and is not influenced by degradation by TP [39]. It was shown that TS only remains inhibited when a constant influx of TFT is present; when TFT is removed TS activity recovers quickly [38].

The data in this report showed that TPI could block TP very effectively. We expected this would be associated with an increased cytotoxicity to TFT, especially because enzyme data suggested that TFT is even a better substrate for TP than thymidine. It has also been shown that thymidine levels may rescue the growth-inhibiting effect of TFT, because inhibition of both TFT and thymidine biotransformation by TPI can lead to an increase of intracellular thymidine [40]. Apparently, in cell culture, degradation occurs, but TFT activation to TF-TMP is very rapid and efficient, and can continue during continuous TFT exposure. This is due to the fact that TFT has a high affinity (low micromolar K_m) for TK, which in general has a low activity in cells; in addition the K_m for substrates of TP is much higher, but the activity of TP is also much higher. This means that the V_m/K_m ratio is more in favor for TK than TP. Thus, TFT will be maximally activated by TK so that it might not be

influenced by degradation by TP. It was expected that at short exposure TFT would not be activated to a large extent and degradation would decrease the availability of TFT. However, our data demonstrate that TPI only affects TFT cytotoxicity at very short exposure in cells with very high TP, Colo320TP1 and H460, but not in the other cell lines.

TFT alone is activated rapidly in tumor cell lines, possibly due to an efficient phosphorylation, since the affinity of some thymidine analogs for TK is higher than for TP [41,42]. It is also possible that TFT has more affinity for TK than thymidine. Higher TK affinity and/or conversion rate will cause a rapid activation of TFT to exert its cytotoxicity in cells. This high rate, as shown in the data obtained with intact cells, explains that TP inhibition does not have an *in vitro* effect anymore, both at short- and long-term exposure. Therefore, TFT incorporation into DNA did not increase significantly when TPI was added to the medium and the accumulation of TFT phosphate derivatives in the cells was only increased in the Colo320TP1 cells expressing high TP.

A high TP expression has been shown to correlate with a poor prognosis in colorectal cancer patients [36,43]. *In vivo* it has been shown that TPI enhanced the bioavailability of TFT due to prevention of degradation by liver cells (high TP) [23,25]. Thus, *in vivo*, TPI is required in the TAS-102 combination, since otherwise TFT does not exert any antitumor activity. This is in contrast to the *in vitro* data. Our *in vitro* data also show that the mode of cytotoxicity exerted by TFT is dependent on exposure time and that a longer exposure can bypass resistance due to increased TS expression. Therefore, this time-dependent pharmacologic effect of TFT should be translated in repeated administration of TAS-102 which will be the most optimal schedule, because it will lead to a prolonged exposure to TFT similar to in cell lines [44]. Since TFT acts both by inhibition of TS and incorporation into DNA, this opens various possibilities for potential combination studies.

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