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ORIGINAL ARTICLE

Mechanisms of acquired chemoresistance to 5-fluorouracil and tomudex: thymidylate synthase dependent and independent networks

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Abstract
Purpose Thymidylate synthase (TS) over-expression is widely accepted as a major molecular mechanism responsible for 5-fluorouracil (5-FU) and tomudex (TDX) resistance. In this study, the importance of TS in 5-FU and TDX resistance was evaluated.
Methods The sensitivity of TS-over-expressing 5-FU (3) and TDX (3) resistant cell lines to 5-FU and TDX was analysed. The cross-resistance between 5-FU and TDX resistant cell lines was determined. The relationship between p53 and NF-κB status and the sensitivity to 5-FU and TDX was evaluated.
Results Compared to relevant parental sensitive cell lines, the 5-FU resistant cell lines were highly cross-resistant to TDX (over 20,000-fold). In contrast, over-expression of TS did not significantly confer 5-FU resistance on the TDX resistant cell lines (0.8- to 1.3-fold). Thymidine (20 μM) rescue induced TDX resistance in TDX sensitive cell lines (over 10,000-fold) but only moderately influenced 5-FU sensitivity in 5-FU sensitive cell lines (1.1- to 2.4-fold). Uridine moderately protected one cancer cell line (RKO) from 5-FU-induced, but not TDX-induced, cytotoxicity. NF-κB transfected MCF-7 and p53 knockout HCT116 cells were resistant to 5-FU (4.4- and 2.4-fold, respectively) but not to TDX. TS protein expression in NF-κB transfected and p53 knockout cell lines was comparable to the relevant parental cell lines.
Conclusion In some cancer cell lines, TS-independent molecular events may play a key role in 5-FU resistance. Loss of p53 function and NF-κB over-expression may be involved in TS-independent 5-FU chemoresistance in some cancer cell lines.

Keywords 5-Fluorouracil · Tomudex · Thymidylate synthase · Cross-resistance · NF-kappaB

Abbreviations

5-FU	5-Fluorouracil
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
dUTP	Deoxyuridine triphosphate
FdUMP	5-Fluorodeoxyuridine monophosphate
MTT	Tetrazolium-based semiautomated colorimetric
NF-κB	Nuclear factor-kappa B
TdR	Thymidine
TDX	Tomudex
TS	Thymidylate synthase
Urd	Uridine

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Introduction

5-Fluorouracil (5-FU) has been effectively used in the treatment of a broad-spectrum of solid tumours e.g. colorectal, pancreas, breast, head and neck, gastric and ovarian cancers [17]. The 5-FU derivative, 5-fluorodeoxyuridine monophosphate (FdUMP), can compete with deoxyuridine monophosphate (dUMP) to inhibit thymidylate synthase (TS, EC 2.1.1.45) activity. TS catalyses the methylation of dUMP to deoxythymidine monophosphate (dTMP). dTMP is then metabolized intracellularly to deoxythymidine triphosphate (dTTP), an essential precursor for DNA biosynthesis. Inhibition of TS activity will deprive the de novo dTTP supply and inhibit DNA synthesis [3, 23]. 5-FU can also induce DNA and RNA strand breaks and apoptosis by direct incorporation of fluorinated nucleotides into DNA and RNA. As with other anticancer drugs, inherent and acquired resistance is still one of the major obstacles for the success of 5-FU based chemotherapy. 5-FU sensitivity is inversely related to the level of TS protein and enzyme activity in cancer cells and 5-FU resistant tumours commonly express high levels of TS protein [5, 9, 31]. High expression of TS protein and/or RNA in cancer tissue is also a strong indicator for poor prognosis of 5-FU-based chemotherapy for colorectal cancer patients [19]. It has been widely thought that TS is the main molecular mechanism governing 5-FU sensitivity and targeting TS is a major strategy for reversing 5-FU chemoresistance.

Tomudex (TDX) is a specific TS inhibitor [23] and effectively used in the treatment of human tumours such as colorectal, breast and lung cancers [24, 27]. Although TS over-expression has been widely accepted as a major mechanism of chemoresistance to TDX and 5-FU, the relationship between TS levels and 5-FU sensitivity is controversial [15, 26]. It has been reported that TS inhibitors (TDX and Pemetrexed) resistant cancer cell lines which over-express TS are not resistant to 5-FU [7, 25]. High TS expression does not account for all non-responding tumours in colorectal cancer patients treated with 5-FU [8, 21, 22]. 5-FU sensitivity is also influenced by expression levels of dihydropyrimidine dehydrogenase (DPD) [6, 21], the genetic status of p53 [1, 2], NF-κB [29, 32], DNA mismatch-repair genes [14] and cell cycle disturbance [11, 16]. Therefore, the anticancer and resistant mechanisms of 5-FU may be multi-factorial.

Both 5-FU and TDX resistant cancer cells express high levels of TS protein. If TS over-expression plays a major role in resistance to both, the specific and direct TS inhibitor, TDX, and the multi-modal indirect TS inhibitor, 5-FU, there should be a strong cross-resistance between 5-FU and TDX. In this study, we analysed TS protein expression levels in a panel of 6 5-FU or TDX resistant cancer cell lines. The role of TS in the sensitivities of these cell lines to 5-FU and TDX was evaluated to elucidate the relative importance of TS gene over-expression and the de novo thymidylate pathway in chemoresistance to 5-FU or TDX. Since p53 loss of function and high NF-κB activity in cancer cells are involved in 5-FU resistance, we also tested the role of p53 knockout and high NF-κB activity in 5-FU and TDX resistance.

Materials and methods

Cell culture

The 5-FU resistant $H630_{R10}$ and TDX resistant $H630_{TDX}$, RKO_{TDX}, $MCF\text{-}7_{TDX}$ and relevant parental cell lines were generously provided by Prof. PG Johnston (The Queen's University of Belfast, Department of Oncology). The 5-FU resistant breast cancer cell lines ($MCF\text{-}7_{FU10}$ and $T47D_{FU2.5}$) were generated in our lab by continuously culturing the drug sensitive parental cell lines ($MCF\text{-}7_{WT}$ and $T47D_{WT}$) in medium containing increasing concentrations of 5-FU in a stepwise procedure over 2 years. The 5-FU and TDX resistant and relevant parental cell lines, NF-κB p50 + p65 transfected MCF-7 and empty vector transfected cell lines ($MCF\text{-}7_{p50+p65}$ and $MCF\text{-}7_{pcDNA3.1}$, generated in our lab [29]), and p53 knockout and parental HCT116 cell lines ($HCT116_{p53-/-}$ and $HCT116_{WT}$; generously provided by Dr. L. Scott, Beatson Institute for Cancer Research, Glasgow, UK) were cultured in RPMI 1640 medium or DMEM (HCT116) supplemented with 10% foetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin and appropriate concentrations of the selection drugs (TDX: $H630_{TDX}$, 1 μM; RKO_{TDX} and $MCF\text{-}7_{TDX}$, 2 μM; 5-FU: $H630_{R10}$ and $MCF\text{-}7_{FU10}$: 10 μM, $T47D_{FU2.5}$: 2.5 μM; $MCF\text{-}7_{pcDNA3.1}$, and $HCT116_{p53-/-}$: G418 500 μg/ml; $MCF\text{-}7_{p50+p65}$: G418 500 μg/ml + hygromycin 150 μg/ml). The drug resistant cells were cultured in drug free medium for 2 weeks before harvesting for protein extraction and western blotting analysis.

MTT assay

Cytotoxicity of 5-FU and TDX was determined in all cell lines using the MTT assay. Cells were cultured in 96-well plates (5×10^3/well) overnight and then treated with 5-FU or TDX (consistently cultured in

drug-containing medium for 96 h). After drug treatment, the cells were subjected to MTT analysis as described previously [18]. The drug exposure and MTT analysis were carried out in RPMI 1640 or DMEM (HCT116 cells) medium supplemented with 10% dialysed FCS. To determine the importance of TS or RNA damage in 5-FU or TDX induced cytotoxicity, the MTT assay was also carried out in the same medium supplemented with 20 μM thymidine or 1 mM uridine.

Western blot analysis

Cells (80% confluence) grown in 75 cm^2 flasks were washed in ice-cold PBS and lysed in 500 μl RIPA buffer. The lysate was centrifuged for 5 min in a microfuge and the supernatants retained. The protein (200 μg/cell line) was electrophoresed through a 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, Watford, UK) using an electrophoretic transfer chamber (Millipore, Watford, UK). The blots were blocked for nonspecific binding by incubating the membranes for 1 h in TBS-T with 5% non-fat milk, which was also used to dilute primary (TS, monoclonal, Calbiochem, Nottingham, UK, 1:250; NF-κB p50 and p65, rabbit polyclonal, Santa Cruz, CA, USA, 1:250; p53, Pharmingen, CA, USA, 1:250) and HRP-conjugated monoclonal secondary (Amersham, Little Chalfont, UK, 1:5,000) antibodies. The signal was detected using ECL Western blotting detection kit (Amersham, Little Chalfont, UK).

Results

TS protein over-expression and resistance to TS inhibitors

Compared to the sensitive parental cells, the drug-induced 5-FU and TDX resistant cell lines were 36- to 115-fold and 8,276- to >29,411-fold resistant to 5-FU or TDX, respectively (Table 1). TS protein expression was detected by western blotting analysis. High expression of TS protein was detected in all drug resistant cancer cell lines, with low or undetectable TS protein in the sensitive parental lines (Fig. 1).

To investigate the relative importance of TS over-expression in the resistance of cancer cells to 5-FU and TDX, the TDX- and 5-FU-induced resistant cancer cell lines were also treated with 5-FU or TDX, respectively. The resistance of 5-FU-induced resistant cell lines to TDX was comparable to that of TDX resistant cell lines (>20,000-fold, Table 1). In contrast,

Table 1 The cytotoxicity of 5-FU and TDX in cancer cell lines

	$MCF\text{-}7_{WT}$	$MCF\text{-}7_{FU10}$	$MCF\text{-}7_{TDX}$	$H630_{WT}$	$H630_{R10}$	$H630_{TDX}$	$T47D_{WT}$	$T47D_{FU2.5}$	RKO_{WT}	RKO_{TDX}
5-FU										
IC_{50} (μM)	3.33 ± 0.17	149.98 ± 22.19	4.44 ± 0.16	3.80 ± 0.24	438.72 ± 43.3	2.98 ± 1.40	2.67 ± 0.57	97.23 ± 23.19	2.77 ± 1.29	2.33 ± 0.33
Resistant fold	–	45	1.3	–	115.5	0.8	–	36.4	–	0.8
TDX										
IC_{50} (μM)	0.0024 ± 0.0002	>50	>50	0.0017 ± 0.0005	>50	>50	0.002 ± 0.0003	>50	0.0034 ± 0.0004	28.14 ± 2.94
Resistant fold	–	>20833.3	>20833.3	–	>29411.7	>29411.7	–	>25000.0	–	8276.5

The IC_{50} figure is mean from three replicates ± SD

Resistant fold the IC_{50} ratio of resistant cell line/sensitive cell line, *TdR* thymidine, *Urd* uridine

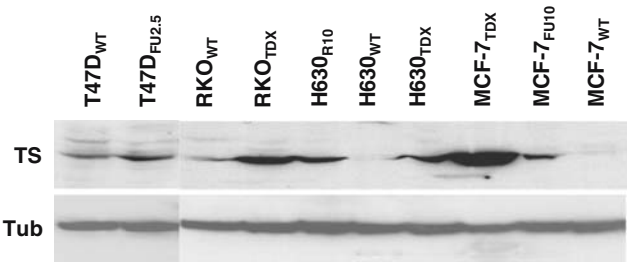

Fig. 1 5-FU and TDX resistant cancer cell lines express high levels of TS protein. TS protein expression was analysed by western blot in drug (5-FU and TDX) sensitive and resistant cell lines. *Tub* tubulin, used as a loading control

there was no significant difference between the chemo-sensitivity of TDX-induced TDX resistant and the paired sensitive cell lines to 5-FU (0.6- to 1.3-fold, Table 1).

Thymidine protects cancer cells from TDX-induced but not from 5-FU-induced cytotoxicity

To further determine the importance of TS in 5-FU and TDX chemoresistance, we tested the protective effect of thymidine on 5-FU- and TDX-induced cytotoxicity in the drug sensitive cell lines. Supplementation of the cultured cells with exogenous thymidine allows TS-independent synthesis of thymidylate for DNA synthesis and repair, thereby bypassing the cytotoxic effects of TS inhibition. As shown in Table 2, supplementation of cultured sensitive parental cells with 20 μM thymidine completely prevents TDX-induced cytotoxicity (14,705- to 31,250-fold resistance), with IC_{50} values comparable to that observed in TDX resistant lines. In contrast, thymidine only conferred at the most a very moderate rescuing effect (1.1- to 2.4-fold) from 5-FU-induced cell death (Table 2).

Uridine rescued 5-FU-induced cytotoxicity in some cancer cell lines but not TDX-induced cytotoxicity

The 5-FU derivative, FUTP, can incorporate into and damage RNA, thereby inhibiting pre-mRNA splicing, polyadenylation of mRNA, processing of pre-rRNA, post-transcriptional modification of tRNA and assembly and activity of snRNA/protein complexes. To determine the importance of this mechanism in 5-FU-induced cytotoxicity, analysis was carried out with supplementation of an excess concentration (1 mM) of exogenous uridine in the culture medium. Uridine supplementation conferred a relatively high rescuing effect on 5-FU-induced cytotoxicity in RKO$_{WT}$ cells (7.9-fold) but only offered moderate or no protection in the other cell lines (0.8- to 1.9-fold, Table 2). Uridine had no significant rescuing effect on TDX-induced cytotoxicity (0.9- to 1.6-fold, Table 2).

NF-κB over-expression and p53 mediate 5-FU, but not TDX, resistance

It has been reported that p53 mutations and NF-κB over-expression can confer 5-FU resistance in human cancer cells [2, 32]. In this study, we tested 5-FU and TDX cytotoxicity in p53 knockout HCT116 colon cancer and NF-κB p50 + p65 transfected MCF-7 breast cancer cell lines. The NF-κB transfected cells expressed high levels of NF-κB p50 and p65 proteins (Fig. 2a), and had high NF-κB DNA binding and transcriptional activity (data not shown). No p53 protein was detected in the p53 knockout cells (Fig. 2b). Compared with relevant parental cells, NF-κB transfection and p53 knockout induced 4.4- and 2.4-fold resistance to 5-FU, respectively but neither molecular alteration induced resistance to TDX (Table 3). TS protein levels were comparable between the parental and NF-κB transfected or p53 knockout lines (Fig. 2c). These

Table 2 The rescuing effect of thymidine or uridine on the cytotoxicity of 5-FU and TDX in sensitive cell lines

	MCF-7$_{WT}$	T47D$_{WT}$	H630$_{WT}$	RKO$_{WT}$	HCT116$_{WT}$
IC_{50}					
5-FU + TdR	3.86 ± 0.007	6.31 ± 1.04	4.36 ± 0.56	5.77 ± 1.23	1.94 ± 0.14
5-FU + Urd	4.05 ± 0.75	2.76 ± 0.12	7.37 ± 1.85	21.78 ± 2.35	2.73 ± 0.12
TDX + TdR	>50	>50	>50	>50	>50
TDX + Urd	0.0034 ± 0.0002	0.0018 ± 0.0008	0.0025 ± 0.0001	0.0056 ± 0.0006	0.0018 ± 0.0008
Rescue fold					
5-FU + TdR	1.2	2.3	1.1	2.4	0.6
5-FU + Urd	1.2	1.0	1.9	7.9	0.8
TDX + TdR	>20833.3	>25000	>29411.7	>14705.9	>31250.0
TDX + Urd	1.4	0.9	1.5	1.6	1.1

The drug sensitive cell lines were treated with 5-FU or TDX and rescued with thimidine (TdR, 20 μM) or uridine (Urd, 1 mM). The IC_{50} value is the mean from three replicates ± SD. Rescue fold: the IC_{50} of drug in combination with thymidine or uridine/drug without thymidine or uridine

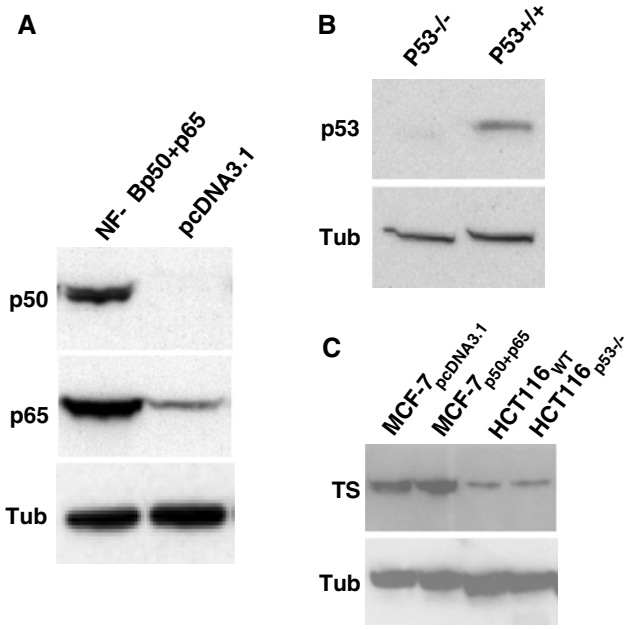

Fig. 2 Western blotting analysis of the expression status of NF-κB p50 and p65 (**a**), p53 (**b**) and TS (**c**) proteins in NF-κB transfected and p53 knockout cell lines. *Tub* tubulin, used as a loading control

results indicate that in some cancer cell lines, 5-FU resistance conferred by NF-κB over-expression or loss of p53 protein is TS-independent.

Discussion

Both 5-FU and TDX are TS inhibitors. TS is over-expressed in 5-FU and TDX resistant cancer cell lines. To determine the relative importance of TS in acquired 5-FU and TDX resistance, we tested the cross-resistance of six 5-FU or TDX resistant cell lines to each TS inhibitor. 5-FU resistant cells were highly cross-resistant to TDX. The level of resistance to TDX in the cells whose resistance was induced by 5-FU exposure was comparable to that observed in the cell lines with resistance induced by TDX exposure. In contrast, although both TDX and 5-FU resistant cell lines expressed high levels of TS protein, the TDX resistant cell lines were not significantly resistant to 5-FU (Table 1).

To further evaluate the role of TS in TDX and 5-FU resistance, we added exogenous thymidine into the culture medium. As shown in Table 2, the drug sensitive cancer cell lines became highly resistant to TDX in the presence of thymidine. In contrast, thymidine did not introduce a significant rescuing effect on 5-FU cytotoxicity in these cell lines. These data are in agreement with a previous study demonstrating MCF-7 and RKO cells are not rescued from 5-FU cytotoxicity by thymidine [7]. Our data indicate that high TS protein over-expression and maintenance of cellular dTMP levels play an important, if not exclusive, role in acquired TDX resistance. In contrast, acquired 5-FU resistance appears to be multi-factorial, with TS-dependent maintenance of cellular dTMP levels playing at the most, a relatively minor role in these human cancer cells.

In a meta-analysis derived from over 3,000 colorectal cancer patients received a variety of adjuvant or advanced disease treatments, it was shown that overall survival of colorectal cancer patients with tumours expressing high levels of TS protein is significantly poorer, although the association was moderate [19]. Therefore, the expression levels of TS in tumour tissue may be a prognostic biomarker for patients receiving 5-FU based chemotherapy. However, gene expression profiling data [13] and the data in this report indicate that predictive markers such as TS, may not necessarily represent molecules critical to the acquisition or maintenance of the phenotype under investigation. TS protein over-expression may be a predictive marker for response to 5-FU without playing a significant biological role in acquired 5-FU resistance.

Uridine inhibits incorporation of 5-FU metabolites into RNA and rescues colorectal cells from 5-FU-induced cytotoxicity [7, 20]. RNA mediated cytotoxic effects of 5-FU is p53-dependent in colon epithelia [20] and it may play a role in acquired 5-FU resistance [10, 12]. It has also been reported that uridine can prevent apoptosis in response to 5-FU in normal but not tumour tissues [4, 20]. Our data demonstrated that only RKO cells were protected from 5-FU induced cytotoxicity by the addition of uridine, indicating that 5-FU kills RKO cells, at least partially, via an RNA-mediated mechanism. However, uridine conferred no significant protection on breast cancer (MCF-7, T47D) or

Table 3 IC_{50} of 5-FU and TDX to NF-κB transfected MCF-7, p53 knockout HCT116 and relevant parental cell lines

	$MCF\text{-}7_{pcDNA3.1}$	$MCF\text{-}7_{p50+p65}$	$HCT116_{WT}$	$HCT116_{p53-/-}$
5-FU (μM)	5.12 ± 0.32	22.37 ± 5.35	3.47 ± 0.16	8.22 ± 0.2
TDX (μM)	0.0029 ± 0.0009	0.0018 ± 0.0004	0.0016 ± 0.0001	0.0017 ± 0.0001

Data represent the mean IC_{50} from three replicates ± SD

TdR thymidine, *Urd* uridine

the other colorectal cancer (H630 and HCT116) cell lines. Therefore, the RNA-mediated 5-FU induced cell death may not be the major mechanism of action of 5-FU in human cancer cell lines.

p53 is a tumour suppressor gene whose genetic status plays a key role in resistance of cancer cells to some anticancer drugs including 5-FU [2]. Over-expression and high DNA binding activity of the transcription factor NF-κB has been identified in TS inhibitor (5-FU and TDX) resistant cell lines [28] and blocking the NF-κB pathway sensitizes cancer cells to 5-FU in vitro [30, 32]. NF-κB transfection induces 5-FU resistance in cancer cells (Table 3, [29]). Both p53 knockout and NF-κB transfected cells expressed comparable levels of TS protein to their control parental lines. In vitro cytotoxicity analysis demonstrated 5-FU resistance was induced by p53 knockout (2.4-fold) and NF-κB transfection (4.4-fold). In contrast, neither p53 knockout nor NF-κB transfection induced resistance to TDX. These data indicate that in these cell lines p53 mutation and NF-κB over-expression are TS-independent molecular events involved in acquired 5-FU resistance. Although NF-κB is involved in 5-FU resistance, the precise molecular mechanisms and the down stream molecular pathways are still not fully elucidated. The resistant levels induced by either of p53 knockout (2.4-fold) or NF-κB over-expression (4.4-fold) were much lower than the acquired resistance induced by 5-FU exposure (36- to 115-fold). This suggests that p53 and NF-κB independent mechanisms may be primarily responsible for acquired 5-FU resistance in the resistant cancer cell lines evaluated in this study.

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References

1. Boyer J, McLean EG, Aroori S, Wilson P, McCulla A, Carey PD, Longley DB, Johnston PG (2004) Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin, and irinotecan. Clin Cancer Res 10:2158–2167
2. Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Williams J, Lengauer C, Kinzler KW, Vogelstein B (1999) Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J Clin Invest 104:263–269
3. Chu E, Callender MA, Farrell MP, Schmitz JC (2003) Thymidylate synthase inhibitors as anticancer agents: from bench to bedside. Cancer Chemother Pharmacol 52(Suppl 1):S80–S89
4. Codacci-Pisanelli G, Kralovanszky J, van der Wilt CL, Noordhuis P, Colofiore JR, Martin DS, Franchi F, Peters GJ (1997) Modulation of 5-fluorouracil in mice using uridine diphosphoglucose. Clin Cancer Res 3:309–315
5. Copur S, Aiba K, Drake JC, Allegra CJ, Chu E (1995) Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. Biochem Pharmacol 49:1419–1426
6. Ishibiki Y, Kitajima M, Sakamoto K, Tomiki Y, Sakamoto S, Kamano T (2003) Intratumoural thymidylate synthase and dihydropyrimidine dehydrogenase activities are good predictors of 5-fluorouracil sensitivity in colorectal cancer. J Int Med Res 31:181–187
7. Johnston PG, Behan KA, Allegra CJ, Drake JC (1995) Fluorouracil: active in ZD1694 (tomudex)-resistant cell lines with markedly elevated thymidylate synthase levels. J Natl Cancer Inst 87:1558–1559
8. Johnston PG, Benson AB, Catalano P, Rao MS, O'Dwyer PJ, Allegra CJ (2003) Thymidylate synthase protein expression in primary colorectal cancer: lack of correlation with outcome and response to fluorouracil in metastatic disease sites. J Clin Oncol 21:815–819
9. Johnston PG, Lenz HJ, Leichman CG, Danenberg KD, Allegra CJ, Danenberg PV, Leichman L (1995) Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. Cancer Res 55:1407–1412
10. Kufe DW, Major PP (1981) 5-Fluorouracil incorporation into human breast carcinoma RNA correlates with cytotoxicity. J Biol Chem 256:9802–9805
11. Longley DB, Harkin DP, Johnston PG (2003) 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 3:330–338
12. Lonn U, Lonn S (1986) DNA lesions in human neoplastic cells and cytotoxicity of 5-fluoropyrimidines. Cancer Res 46:3866–3870
13. Maxwell PJ, Longley DB, Latif T, Boyer J, Allen W, Lynch M, McDermott U, Harkin DP, Allegra CJ, Johnston PG (2003) Identification of 5-fluorouracil-inducible target genes using cDNA microarray profiling. Cancer Res 63:4602–4606
14. Meyers M, Wagner MW, Hwang HS, Kinsella TJ, Boothman DA (2001) Role of the hMLH1 DNA mismatch repair protein in fluoropyrimidine-mediated cell death and cell cycle responses. Cancer Res 61:5193–5201
15. Mirjolet JF, Barberi-Heyob M, Merlin JL, Marchal S, Etienne MC, Milano G, Bey P (1998) Thymidylate synthase expression and activity: relation to S-phase parameters and 5-fluorouracil sensitivity. Br J Cancer 78:62–68
16. Mirjolet JF, Didelot C, Barberi-Heyob M, Merlin JL (2002) G(1)/S but not G(0)/G(1)cell fraction is related to 5-fluorouracil cytotoxicity. Cytometry 48:6–13
17. Pinedo HM, Peters GF (1988) Fluorouracil: biochemistry and pharmacology. J Clin Oncol 6:1653–1664
18. Plumb JA, Milroy R, Kaye SB (1989) Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. Cancer Res 49:4435–4440
19. Popat S, Matakidou A, Houlston RS (2004) Thymidylate synthase expression and prognosis in colorectal cancer: a systematic review and meta-analysis. J Clin Oncol 22:529–536
20. Pritchard DM, Watson AJ, Potten CS, Jackman AL, Hickman JA (1997) Inhibition by uridine but not thymidine of p53-dependent intestinal apoptosis initiated by 5-fluorouracil: evidence for the involvement of RNA perturbation. Proc Natl Acad Sci USA 94:1795–1799
21. Salonga D, Danenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, Lenz HJ, Leichman CG, Leichman L, Diasio RB, Danenberg PV (2000) Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. Clin Cancer Res 6:1322–1327

22. Sobrero A, Kerr D, Glimelius B, Van Cutsem E, Milano G, Pritchard DM, Rougier P, Aapro M (2000) New directions in the treatment of colorectal cancer: a look to the future. Eur J Cancer 36:559–566
23. Touroutoglou N, Pazdur R (1996) Thymidylate synthase inhibitors. Clin Cancer Res 2:227–243
24. Van Cutsem E (1999) Raltitrexed (Tomudex) in combination treatment for colorectal cancer: new perspectives. Eur J Cancer 35(Suppl 1):S1–S2
25. van der Wilt CL, Backus HH, Smid K, Comijn L, Veerman G, Wouters D, Voorn DA, Priest DG, Bunni MA, Mitchell F, Jackman AL, Jansen G, Peters GJ (2001) Modulation of both endogenous folates and thymidine enhance the therapeutic efficacy of thymidylate synthase inhibitors. Cancer Res 61:3675–3681
26. van Triest B, Pinedo HM, van Hensbergen Y, Smid K, Telleman F, Schoenmakers PS, van der Wilt CL, van Laar JA, Noordhuis P, Jansen G, Peters GJ (1999) Thymidylate synthase level as the main predictive parameter for sensitivity to 5-fluorouracil, but not for folate-based thymidylate synthase inhibitors, in 13 nonselected colon cancer cell lines. Clin Cancer Res 5:643–654
27. Vokes EE, Goh BC, Bertucci D, Vogelzang NJ, Mani S, Ratain MJ (1999) A phase I study of raltitrexed and paclitaxel given every 3 weeks to patients with solid tumors. Cancer 86:528–532
28. Wang W, Cassidy J (2003) Constitutive nuclear factor-kappa B mRNA, protein overexpression and enhanced DNA-binding activity in thymidylate synthase inhibitor-resistant tumour cells. Br J Cancer 88:624–629
29. Wang W, Cassidy J, O'Brien V, Ryan KM, Collie-Duguid E (2004) Mechanistic and predictive profiling of 5-fluorouracil resistance in human cancer cells. Cancer Res 64:8167–8176
30. Wang W, Collie-Duguid E, Cassidy J (2002) Cerivastatin enhances the cytotoxicity of 5-fluorouracil on chemosensitive and resistant colorectal cancer cell lines. FEBS Lett 531:415–420
31. Wang W, Marsh S, Cassidy J, McLeod HL (2001) Pharmacogenomic dissection of resistance to thymidylate synthase inhibitors. Cancer Res 61:5505–5510
32. Wang W, McLeod HL, Cassidy J (2003) Disulfiram-mediated inhibition of NF-kappaB activity enhances cytotoxicity of 5-fluorouracil in human colorectal cancer cell lines. Int J Cancer 104:504–511