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Thymidylate synthase inhibition triggers apoptosis via caspases-8 and -9 in both wild-type and mutant p53 colon cancer cell lines

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Abstract

Thymidylate synthase (TS) is an important target for chemotherapy and increased levels are associated with resistance to colorectal cancer chemotherapy. TS can be inhibited by 5-fluorouracil (5-FU) and antifolates, ultimately resulting in apoptosis. We aimed to clarify whether activation of caspases and Fas signalling are crucial for the onset of apoptosis after specific inhibition of TS and whether p53 plays a role in activation of these downstream processes. For this purpose, wild-type (wt) and mutant (mt) p53 colon cancer cell lines, Lovo and WiDr, respectively, transfected with mt- and wt-p53, were treated with the specific TS inhibitor, AG337. Treatment with $10\times IC_{50}$ values of AG337 for 48 h resulted in S phase arrest in all Lovo and WiDr cells (up to 50% of cells being in S phase), irrespective of their p53 status. After 72 h, the induction of apoptosis was most pronounced in the AG337-sensitive cells. Approximately 30% apoptosis was detected in all of the WiDr cells, 20% in Lovo li (non-functional p53), 12–14% in Lovo 92 and B2 (wt p53) and only 7% in Lovo 175×2 cells (mt p53 transfected). The induction of apoptosis in Lovo cells, as determined using the classical sub-G1 peak after propidium iodide (PI) staining, was associated with an increase in the expression of Fas receptor. In addition, synergistic increases in apoptosis from approximately 10 to 35% after 48 h could be detected after simultaneous treatment of AG337 and the Fas activator antibody, CH11. Only additive effects were measurable in WiDr cells, without an increase in Fas receptor expression. Surprisingly, the Fas inhibitor, ZB4, could not decrease the amount of cell death in both cell lines after AG337 treatment. In contrast, simultaneous exposure of Lovo and WiDr cells to AG337 and inhibitors of caspases 8, 9 and 3 caused a decrease in the number of apoptotic cells compared with AG337 exposure alone. Inhibition of apoptosis by approximately 10–80% in Lovo and approximately 70–80% in WiDr cells could be detected. In conclusion, these results indicate that apoptosis induced after specific inhibition of TS is mediated via the caspases, but without clear involvement of Fas signalling. The status of p53 did not affect the onset of apoptosis by these caspases.

Keywords: Thymidylate synthase; p53; Fas; Caspases and AG337

1. Introduction

Thymidylate synthase (TS) catalyses the methylation of deoxyuridine-5′-monophosphate (dUMP) using 5,10-methylene-tetrahydrofolate (5,10-CH_2-THF) as the methyl donor to form deoxythymidine-5′-monophosphate (dTMP) [1]. Because TS is essential for the synthesis of dTMP, a precursor for DNA replication, it has been an important target for cancer chemotherapy for over 40 years [2].

TS can be inhibited by 5-fluorouracil (5-FU) and antifolates [2]. The active metabolite of 5-FU, 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) inhibits TS by forming a ternary complex with TS and CH_2-THF. 5-FU is one of the standard drugs for treatment of advanced colorectal cancer and is usually given in combination with leucovorin (LV) [3,4]. Antifolates (e.g. Raltitrexed [5], Thymitaq/AG337 [6] and pemetrexed/MTA [7]) are structural analogues of CH_2-THF and inhibit TS by binding to the CH_2-THF binding site of TS. All three antifolates are being evaluated in

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clinical trials, but, so far, only raltitrexed has shown a comparable activity as 5-FU/LV in some trials [8].

Inhibition of TS will result in single- and double-strand DNA breaks after imbalances in deoxynucleoside triphosphate (dNTP) pools and inhibition of DNA. We, and others, have shown that in both mutant (mt) and wild-type (wt) p53 colon cancer cell lines, cell cycle arrest and apoptosis were induced after TS inhibition [9–11].

Many anticancer drugs in current use such as 5-FU, have been shown to induce tumour cell death by apoptosis [12,13]. For the majority of these drugs, apoptosis appears to be initiated via mitochondria by activation of the cytochrome c/Apaf-1/caspase-9 pathway [14,15]. However, several studies, including those with 5-FU, suggested that the Fas system [16–18] might also play an important role in drug-induced apoptosis. Activation of the initiator caspase-8 by death receptors including Fas or caspase-9 by mitochondria can each activate the effector caspases 3, 6 and 7 resulting in the cleavage of structural proteins and, ultimately, in apoptosis [15,19].

p53 is important for the onset of apoptosis following DNA damage [16,20,21]. It can upregulate the expression of the pro-apoptotic protein bax [22] and Fas receptor [23], thereby facilitating the induction of apoptosis [21]. Although it has been shown that mt-p53 cells in the National Cancer Institute (NCI) cell line panel and our panel were less sensitive to 5-FU [24,25], it is not yet clear what the effect of p53 mutations is on the activation of caspases and Fas signalling.

Previously, we demonstrated that TS inhibition by antifolates induced apoptosis, which was associated with the induction of protein expression of p53 and bax [10]. In the present study, we aimed to clarify whether activation of caspases and Fas signalling are crucial for the onset of apoptosis after specific inhibition of TS and what effect mutations in p53 would have on the activation of these downstream processes. For this purpose, mt and wt p53 transfected colon cancer cell lines were treated with AG337, which is a specific TS inhibitor, in contrast to 5-FU with its additional capacity to be incorporated into RNA and DNA.

2. Materials and methods

2.1. Chemicals

AG337 (Thymitaq, Nolatrexed) was provided by Zarix, Limited (King of Prussia, PA, USA). The caspase inhibitors, z-IETD-fmk (FK-012), z-LEHD-fmk (FK-022), z-DEVD-fmk (FK-010) and z-VAD-fmk (FK-009) were obtained from Enzyme Systems Products (Livermore, CA, USA). The Fas receptor activator CH11 and the Fas receptor inhibitor, ZB4, were purchased from Kyamia Biomedical Company (Seattle, WA, USA).

Primary antibodies were obtained from several sources: TS (clone R30) from Dr. G.W. Aherne (Sutton, UK), p53 (Ab-2) and Fas receptor (clone DX2) from Oncogene Research products (Cambridge, MA, USA) and Fas ligand (clone 33) from Transduction Laboratories (Lexington, KY, USA). Biotinylated goat-anti-mouse immunoglobulins, p53 (DO-7), normal goat serum and streptavidin-biotin conjugated horse radish peroxidase (sABC-HRP) were obtained from DAKO (Glostrup, Denmark) and biotinylated goat-anti-rabbit immunoglobulins from Vector Laboratories (Burlinghame, CA, USA). Saponin, streptavidin, D-biotin, poly-L-lysine and di-amino-benzidine-HCL (DAB) were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). Unless otherwise specified, all other chemicals were of analytical grade and commercially available.

2.2. Cell lines

The human colon carcinoma cell lines, Lovo 92 and WiDr T, and their transfected variants were generously provided by Dr. Poupon [26] and Professor Takahashi [27]. Lovo 92 (wild-type (wt) p53) and WiDr T (mutant (mt)-p53; missense mutation at codon 273) are parental cell lines, and Lovo B2 and WiDr P are empty vector plasmid controls. WiDr B is a cell line derived from WiDr T transfected with wt-p53. Lovo 175X2 [mutation at position 175 (Arg→His)] and Lovo 273X17 [mutation at position 273 (Arg→His)] are Lovo 92 cells transfected with mt-p53. Lovo li is derived from Lovo 92 with functionally inactive p53, but without p53 mutations. Functional activity was determined previously as described by Flaman and colleagues in Ref. [28].

After transfer of the transfectants from Japan and France to our laboratory, identification of p53 mutations was checked by sequencing p53 exons 2–9 and exon 11. For that purpose, all exons were separately amplified using the polymerase chain reaction (PCR) (primers available on request) as previously described by Sidransky and colleagues in Ref. [29] and modified by Van Houten and colleagues in Refs. [30, 31]. Similar results to those earlier described were found for the transfectants [26,27].

In addition, the stable expression of p53 was determined by immunoblotting as reported earlier in Ref. [9]. As shown in Fig. 1, p53 expression was low in wt-p53

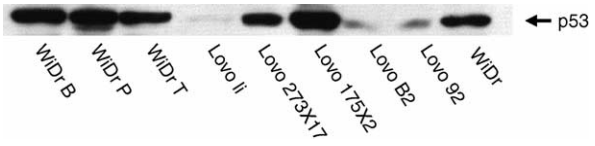

Fig. 1. Basal p53 expression in p53 transfected colon cancer cell lines. Western blotting analysis was performed as described in the Materials and methods. WiDr cells were used as a positive control. The experiments were performed at least three times with identical results.

cell lines, Lovo 92 and Lovo B2, while upregulation was found in the mt-p53 transfectants, Lovo 175X2 and Lovo 273X17. Hardly any p53 expression was detected in the p53 inactive cell line Lovo li. All WiDr variants expressed high levels of p53.

All cell lines were cultured at 37 °C in a 5% CO_2 humidified atmosphere in Roswell Park Memorial Institute (RPMI) (Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal calf serum (FCS) (GIBCO, Paisley, UK). Medium of the transfected cell lines was supplemented for selection with G418 (500 μg/ml). All cell lines were growing exponentially as monolayers during the course of all experiments.

2.3. Growth inhibition experiments

To evaluate the anti-proliferative effects of AG337, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test was used [32]. Previously, we have shown that this assay produces similar results as a clonogenic assay [33]. Lovo and WiDr cells (10,000 cells/well) were exposed to various concentrations of TS inhibitors ranging from 10^{-5} to 10^{-11} M for 72 h. Thereafter, medium was removed and cells were incubated for 3 h at 37 °C in 50 μl MTT (final concentration: 0.42 mg/ml). Formazan crystals were dissolved in 150 μl of dimethylsulphoxide and the optical density (OD) was measured at 540 nm. IC_{50} values were defined as the concentrations that correspond to a reduction of cellular growth by 50% when compared with values of untreated control cells [34].

2.4. Cell cycle analysis and induction of apoptosis

Cells were seeded in 10 cm^2 wells (2×10^5 cells/well) and after 24 h AG337 ($10\times IC_{50}$) was added. Changes in the cell cycle and induction of apoptosis after TS inhibition were evaluated after 48 and 72 h, respectively. These conditions were based on previous experiments evaluating time- and concentration-dependency [10]. To determine the involvement of caspases, the preferential specific caspase-8 inhibitor, z-IETD-fmk (30 μM), the preferential caspase-9 inhibitor, z-LEHD-fmk (40 μM), z-DEVD-fmk (an inhibitor of effector caspases; 50 μM) and the general caspase inhibitor, z-VAD-fmk (40 μM) were added together with AG337 for 72 h. The concentrations used were based on results from earlier studies performed in our department [33]. Inhibition of activation of the Fas receptor was obtained by preincubation with ZB4 (100 ng/ml) one day prior to the drug exposure. The effect of Fas receptor activation and TS inhibition was determined after simultaneous exposure of AG337 ($10\times IC_{50}$) and the Fas receptor activator, CH11 (0.5 μg/ml), for 48 h.

After drug treatment, adherent and floating cells were harvested and counted. After centrifugation (1200 rpm, 5 mm), the pellet was gently resuspended in 1 ml hypotonic propidium iodide (P1)-solution (50 μg/ml Pl, 0.1% sodium citrate, Triton X-100, 0.1 mg/ml ribonuclease A) to a concentration of 5×10^5–10^6 cells/ml in round-bottomed fluorescent activated cell sorting (FACS) tubes. Cell cycle distribution and induction of apoptosis was analysed by FACScan (Becton Dickinson, Mount View, CA) as previously described by Ferreira and colleagues in Ref. [35]. Initial experiments with Lovo-92 and WiDr-T cells demonstrated that determination of apoptosis by May Grünwald Giemsa [10] gave similar results.

2.5. Immunocytochemistry

Lovo and WiDr cells were seeded in 10 cm^2 wells (2×10^5 cells/well) and treated with AG337 ($10\times IC_{50}$). After 48 h, floating and adherent cells were harvested and cytospins containing 2×10^4 cells were prepared for detection of the protein expression of TS and of proteins involved in the process of apoptosis [36]. Cytospins were fixed for 10 min with 100% acetone. Slides were incubated for 1 h with p53 (1:500; DO-7), TS (1:250), Fas receptor (1:25) and Fas ligand (1:400) primary antibodies. Thereafter, TS primary antibody was detected with anti-rabbit biotinylated secondary antibodies (1:500) and p53, Fas receptor and Fas ligand primary antibodies with anti-mouse biotinylated secondary antibodies (1:500). After 30 min, staining was developed by incubation of sections with sABC-HRP (1:200; 1 h) and visualised by use of DAB (10 mg/ml) with 0.025% H_2O_2 for 3 min. For Fas ligand, an additional amplification step with a catalysed reporter deposition (CARD) enhancement kit (DAKO) was used [37]. Phosphate-buffered saline (PBS)/glucose (0.1%) was used for the washing procedures. All slides were counterstained with haematoxylin. Negative controls were performed for each sample by omitting the primary antibody.

Protein expression in untreated and treated Lovo and WiDr cells was determined by immunocytochemical scoring of the intensity (1, 2, 3 and 4) by two investigators without prior knowledge of the expected outcome. Changes in protein expression between untreated and treated cells were categorised into four groups: −, expression of treated cells decreased one or two intensity categories compared with the control cells; =, expression of treated and untreated cells was similar; +, expression of treated cells was one category higher compared with untreated cells; ++, expression of treated cells was two categories higher compared with the control cells.

2.6. Statistical analysis

Potential differences between the parental Lovo and WiDr cells and their transfectant variants for the

various parameters were evaluated using the two-tailed unpaired Student's *t*-test. Changes were considered significantly different when $P < 0.05$.

3. Results

3.1. Sensitivity to the specific TS inhibitor AG337

To investigate the role of caspases and the Fas system after specific inhibition of TS and the influence of p53 in these processes, we first determined the IC_{50} values of AG337 in the Lovo and WiDr p53 transfectants by performing growth inhibition experiments. The specific TS inhibitor AG337 was used for inhibition of TS to exclude the involvement of other mechanisms, which play a role in the sensitivity to 5-FU and other antifolates. As shown in Table 1, sensitivity to AG337 was comparable in the wt-p53 cell lines, Lovo 92 and B2, and the mt-p53 Lovo 273X17 cells, with IC_{50} values ranging from 4.6 to 6.3 μM. In contrast, Lovo 175X2 was approximately 3-fold before resistant to AG337 (19 μM; $P < 0.05$), whereas Lovo li was 3-fold more sensitive (2.1 μM; $P < 0.05$) compared with the parental cell line Lovo 92. No significant differences in sensitivity were observed for the WiDr panel.

3.2. S phase arrest and induction of apoptosis after TS inhibition

The potential effect of changes in the status of p53 on growth inhibition and cell death after TS inhibition was investigated. Therefore, we analysed cell cycle distribution and apoptosis using flow cytometry in all WiDr variants and in Lovo 92, Lovo 175X2 and Lovo li, which showed the most pronounced differences in sensitivity to TS inhibitors. Based on experiments with parental Lovo and WiDr cells previously performed in Ref. [10] we exposed cells to $10 \times IC_{50}$ concentrations of AG337 for 48 h. This resulted in an S phase arrest independent of the status of p53 in all variants, although there were quantitative differences (Fig. 2). In all untreated Lovo and WiDr variants 50–60% of the cells were in the G1 phase, 10–20% in S phase and approximately 30% in the G2/M phase. In Lovo 92 and Lovo 175X2 cells, AG337 exposure increased the proportion of cells in S phase to approximately 45–50% accompanied by a decrease in G1 cells to approximately 30% ($P < 0.05$; Fig. 2a and b). However, in Lovo li, the increase in S phase cells was significantly lower and there was a higher percentage of G_2/M phase cells ($P < 0.05$) compared with Lovo 92 cells (Fig. 2c). In WiDr cells, transfection of wt-p53 into mt-p53 cells did not significantly alter the cell cycle distributions after AG337 exposure (Fig. 2d and e). In both cell lines, an accumulation in S phase was observed.

Table 1
IC_{50} values of AG337 in p53 transfected cancer cell lines

	p53 status	AG337 (μM)
Lovo 92	wt-p53; parental	6.3±1.2
Lovo B2	Plasmid control	4.6±0.7
Lovo 175X2	Transfected with mt-p53	19.0±1.0*
Lovo 273X17	Transfected with mt-p53	4.8±0.2
Lovo li	Functional inactive p53	2.1±0.1*
WiDr T	mt-p53; parental	2.0±0.4
WiDr P	Plasmid control	1.4±0.3
WiDr B	Transfected with wt-p53	2.1±0.5

Growth inhibition was determined as described in the Materials and methods section. IC_{50} values are given as mean values (in μM) ±standard error of the mean (SEM) of at least three experiments. Significant differences ($P < 0.05$) between parental lines (Lovo 92 and WiDr T) and the transfected cell lines are indicated with *.

The induction of apoptosis was measured as the sub-G1 peak after PI staining using flow cytometry. As shown in Fig. 3, less than 7% apoptosis was observed in untreated Lovo and WiDr variant cells. AG337 treatment doubled the amount of apoptosis in both wt-p53 Lovo variants ($P < 0.05$). However, the induction of apoptosis was significantly lower in the mt-p53 transfected Lovo 175X2 (7%), but significantly higher in p53 functionally inactive Lovo li cells (20%). The highest amount of apoptosis was observed in all of the WiDr variants; irrespective of their p53 status rates ranged from 31 to 34%.

These results indicate that p53 does not affect the S phase arrest induced by the inhibition of TS, while apoptosis was more pronounced in cells that are sensitive to TS inhibition compared with the resistant variants.

3.3. The role of the Fas system in TS mediated apoptosis

Previously, we reported that 5-FU induced Fas receptor expression in the metastases of colorectal cancer patients [36]. In line with this, induction of Fas receptor expression was also found in all of the Lovo variants after AG337 treatment (Table 2). No induction, however, was observed in the WiDr variants. In the wild-type p53 cells, we observed a clear induction of p53 after treatment. The lack of induction of p53 in mutant p53 Lovo and WiDr cells, are completely in line with the phenotype of these cells. Fas ligand was elevated in all variants containing wt-p53 (Lovo 92, Lovo 175X2 and WiDr B), but not in cells with inactive p53 (Lovo li) or only mtp53 (WiDr T).

These data suggest that Fas signalling might play a role in the induction of apoptosis after TS inhibition. Therefore, we further investigated the involvement of Fas signalling by exposure of cells to the Fas receptor activator and blocker, CH11 (Fig. 4) and ZB4 (Fig. 5),

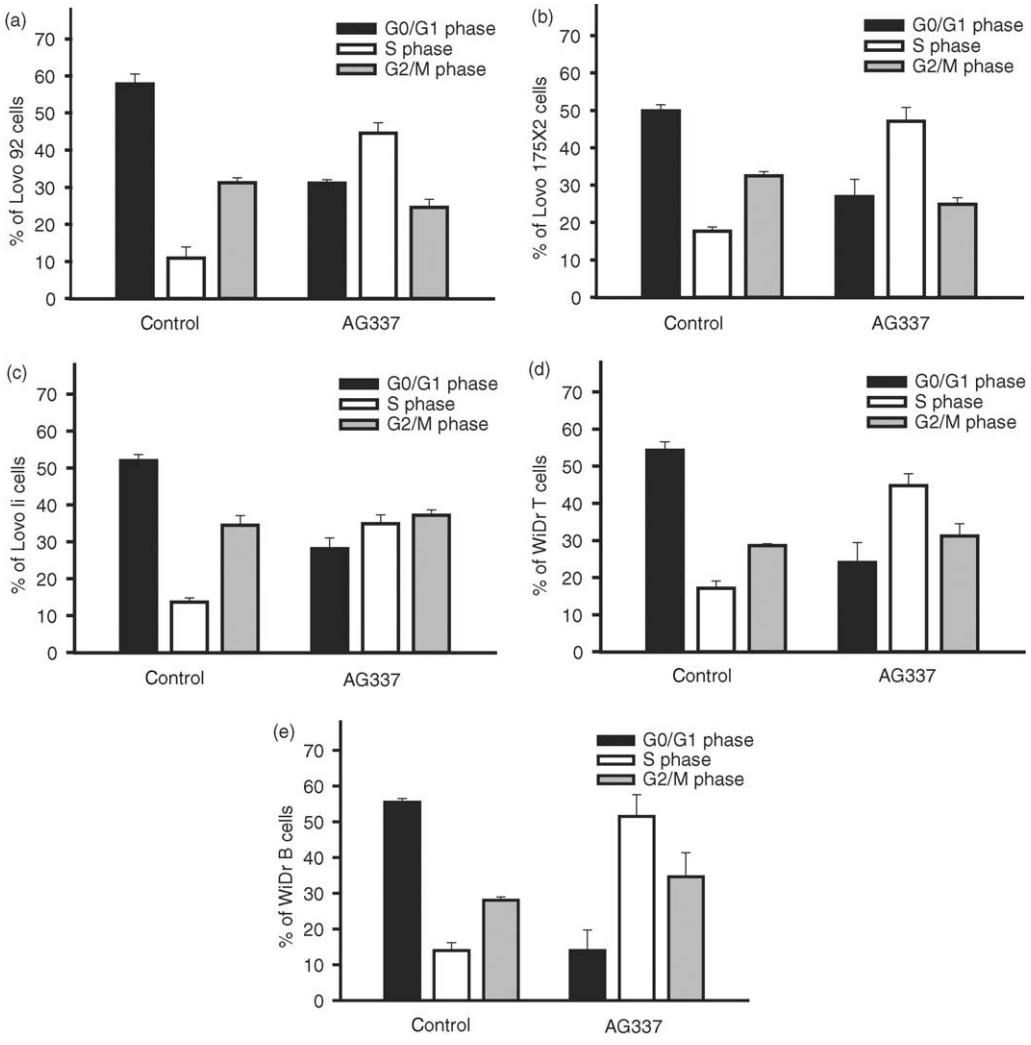

Fig. 2. Cell cycle distribution after TS inhibition. Lovo 92 (a), Lovo 175X2 (b), Lovo li (c), WiDr T (d) and WiDr B (e) were treated for 48 h with $10\times IC_{50}$ of AG337. Data were analysed by flow cytometry as described in Materials and methods and represent the mean values ± standard error of the mean (S.E.M.) of three separate experiments. Comparable results were found for the empty vector plasmid controls, Lovo B2 and WiDrP.

Table 2
Changes in the expression of p53, TS, Fas receptor and Fas ligand in p53 transfectants after inhibition of TS

	p53	TS	Fas-R	Fas-L
Lovo 92	++	+	++	++
Lovo 175X2	=	+	+	+
Lovo li	+	+	+	=
W1Dr T	=	+	=	=
WiDr B	=	+	=	+

Expression was determined after 48 h exposure to AG337 ($10\times IC_{50}$) using immunocytochemistry as described in the Material and methods. The basal expression of p53 in untreated Lovo and WiDr cells as measured using Western blotting is shown in Fig. 1. Changes in expression relative to the untreated cells were scored in four categories (−, expression of treated cells decreased one or two categories compared with the untreated control cells; =, expression similar to untreated cells; +, increased expression by one category and ++, strongly increased expression by two categories). All experiments were performed three times with similar results. No differences could be determined between the parental cell lines and the plasmid control cells.

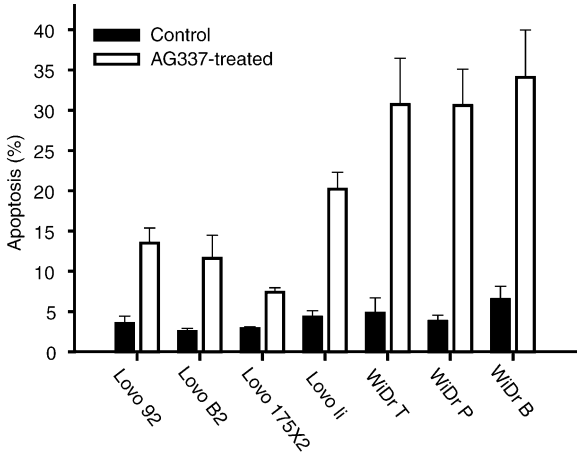

Fig. 3. Induction of apoptosis after TS inhibition. Cells were treated for 72 h with $10\times IC_{50}$ of AG337. Data were analysed by flow cytometry as described in Materials and Methods and represent the mean values ± S.E.M. of three separate experiments.

respectively. CH11 induced apoptosis in all of the Lovo variants (up to 11%; Fig. 4a) and WiDr variants (40–50%; Fig. 4b). The induction of apoptosis was slightly higher in WiDr B compared with WiDr T cells. Interestingly, the combined exposure to AG337 with CH11 resulted in a more than additive increase in apoptosis in the Lovo cells (up to 36%; Fig. 4a). In WiDr T and B cells, the effect of simultaneous exposure to AG337 and CH11 was additive (Fig. 4b). The general caspase inhibitor, zVAD-fmk, could block this effect in both the Lovo and WiDr variants pointing to the involvement of one or more caspases in this process.

Although all cells were sensitive to Fas activation, apoptosis induced by the inhibition of TS was not mediated via Fas signalling. Inhibition of the Fas receptor by ZB4 before and during exposure to AG337 did not reduce the induction of apoptosis (Fig. 5a and b). Similar results were obtained when another inhibitory Fas antibody (CLB-CD95/2) [35] was used (data not shown).

3.4. Both caspase-8 and-9 trigger apoptosis induced by the inhibition of TS

Although no role for the Fas system could be detected, the involvement of the caspases during the induction of apoptosis after TS inhibition was clearly demonstrated, independent of the status of p53. In Lovo 92 cells, apoptosis induced after AG337 exposure was inhibited by 50 and 90% by the general caspase inhibitors, z-DEVD-fmk and z-VAD-fmk, respectively (Fig. 5a). The inhibitory effects of the preferential caspase-8 inhibitor, z-IETD-fmk, and the preferential caspase-9 inhibitor, z-LEHD-fmk, were, however, less pronounced (47 and 12%, respectively). Similar results were found in Lovo 175X2 and Lovo li cells. Only in

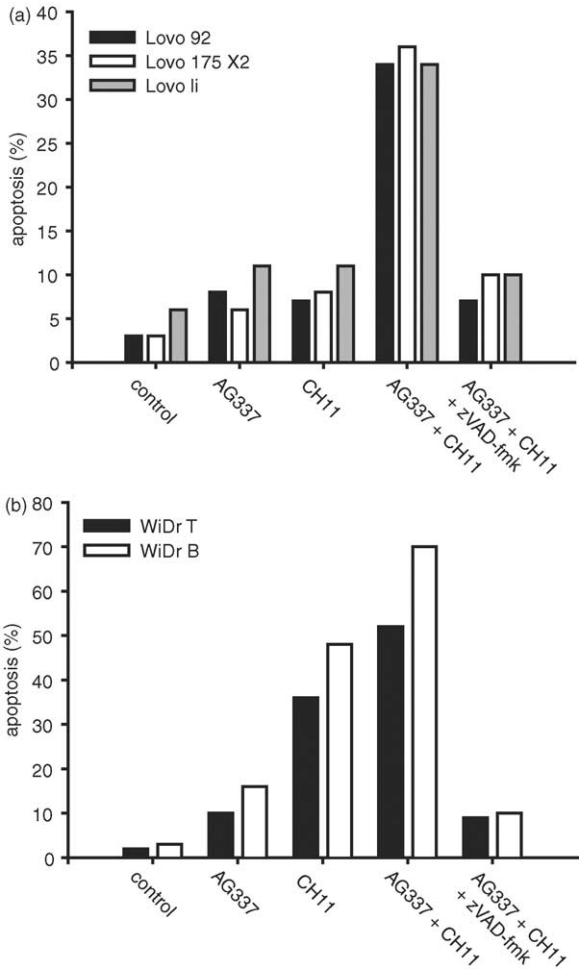

Fig. 4. Induction of apoptosis by AG337 and CH11 is inhibited by zVAD. (a) Lovo and (b) WiDr cells were treated for 48 h with AG337 ($10\times IC_{50}$)±CH11 (500 ng/ml)±zVAD-fmk (40 μM). Apoptosis was determined as a sub-G1 peak using PI staining on the flow cytometer. All experiments were performed three times with comparable results. No differences were found between the parental cell lines and the empty vector controls.

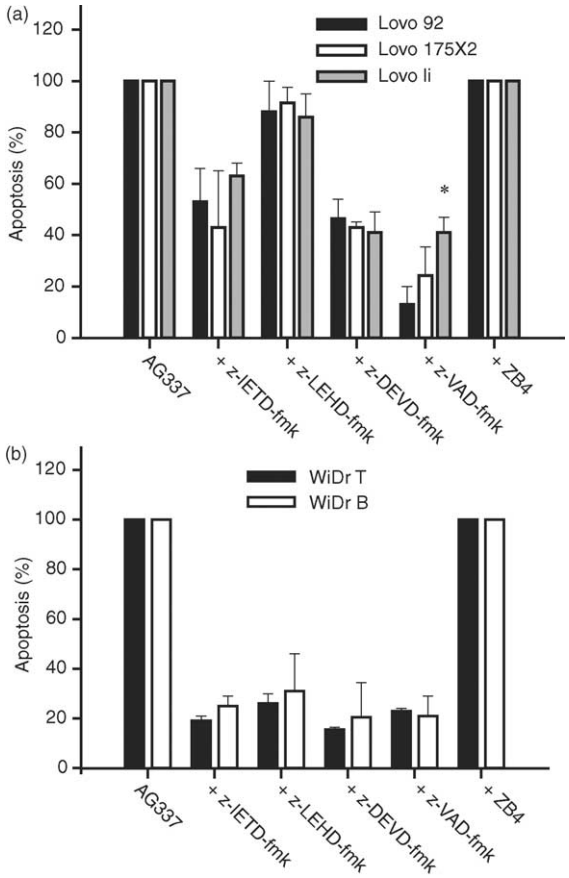

Fig. 5. Inhibition of AG337-induced apoptosls by caspase and Fas receptor inhibitors. (a) Lovo and (b) WiDr cells were treated for 72 h with AG337 ($10\times IC_{50}$)±z-IETD-fmk (30 μM), z-LEHD-fmk (40 μM), z-DEVD-fmk (50 μM), z-VAD-fmk (40 μM) or ZB4 (100 ng/ml). Apoptosis was determined as a sub-G1 peak using PI staining on the flow cytometer. Inhibition of apoptosis by caspase and Fas inhibitors was relative to the extent of apoptosis in the AG337-treated cells, which was set at 100%. Significant differences between transfected and parental cells are indicated with *. Data represent the mean±S.E.M. of three different experiments. No differences were found between the parental cell lines and the plasmid control cells.

Lovo li z-VAD-fmk did not block apoptosis as effectively as in Lovo 92 cells (59% inhibition). In WiDr T and B cells, apoptosis was blocked 70–80% by all four caspase inhibitors suggesting that both caspase-8 and-9 play an important role in drug-induced apoptosis in these cells (Fig. 5b); independent of their p53 status.

4. Discussion

This study demonstrates that inhibition of TS triggers apoptosis via activation of both caspases 8 and 9. In contrast to the non-specific TS inhibitor, 5-FU, specific TS inhibition after AG337 treatment does not seem to induce cell death via Fas signalling. In both Lovo and WiDr colon cancer cells, p53 is not involved in the regulation of caspase activation.

Previously, several groups have shown that TS inhibition will result in increased dUMP levels, inhibition of DNA synthesis after depletion of deoxythymidine triphosphate (dTTP) and, ultimately, in DNA damage [38] associated with cell cycle arrest [10] and apoptosis [39,40]. In the present study, we show that TS inhibition induced by the specific TS inhibitor, AG337, resulted in an S phase arrest independent of the status of p53. In line with these results, Matsui and colleagues [40] showed that ZD1694-induced cell cycle arrest in human colon carcinoma cells was not mediated via the induction of p53.

MTT results indicated that the status of p53 had no clear effect on the growth inhibition of Lovo and WiDr cells after AG337 treatment. Mt-p53 transfection decreased the sensitivity of Lovo 175X2 but did not affect Lovo 273X17 cells, whereas wt-p53 transfection in WiDr cells did not change the sensitivity to AG337. Although in the whole NCl cell line panel and in colon cancer cells it was shown that mt-p53 cells were less sensitive to 5-FU [24,25], our growth inhibition results are in line with that fact that, up to now, no clear survival benefit for wt-p53 could be observed for 5-FU-treated colorectal cancer patients when evaluating several immunohistochemical studies [41].

Induction of apoptosis in Lovo cells was most pronounced in cells that were sensitive to the inhibition of TS compared with the more resistant Lovo variants. However, as shown before [10], apoptosis could be induced independent of the p53 status. In addition, in both mt- and wt-p53 Lovo and WiDr cells, the Fas activator, CH11, could activate the Fas receptor and induce apoptosis, while apoptosis after CH11 exposure was more pronounced after transfection of wt-p53 into the mt-p53 cell line WiDr [27]. The upregulation of the Fas receptor by AG377 combined with CH11 in Lovo cells resulted in a more than additive increase in apoptosis compared with that observed with both agents alone. Similar results were obtained in HT29 cells treated with CH11 and interferon-γ (IFN-γ) [42], while in the WiDr cells only an additive effect of CH11 and AG337 was observed. Although the Fas system was functionally active, experiments with ZB4 show that apoptosis was not mediated via activation of the Fas receptor. Similar results were found after exposure to 5-FU [43]. In contrast, in other colon carcinoma cells, a Fas-dependent component was found in 5-FU-induced cytotoxicity [44]. These differences may be related to the use of different cell lines and different antibodies to block the Fas receptor.

Independent of the status of p53, caspases-3,-8 and-9 clearly mediate AG337-induced apoptosis, as well as 5-FU-induced apoptosis [43,45]. Caspase-8, which did not seem to be activated via the Fas system, might be activated by other death receptors, other caspases or might be mitochondria-dependent [33]. Furthermore, the induction of apoptosis seems to be regulated differently in Lovo and WiDr cells, with a minor involvement of initiator caspases 8 and 9 in Lovo cells. Other caspases or inhibitors of caspases might play a role in these cells.

In conclusion, these results indicate that apoptosis induced after specific inhibition of TS is mediated via caspases, but without clear involvement of the Fas signalling. Regulation of the downstream apoptosis processes is independent of the status of p53. The latter suggests that both mutant and wild-type p53 colorectal tumours can undergo apoptosis via caspases after TS inhibition. These results give a more detailed insight into the process of TS inhibition and its downstream events, which in the future can contribute to the development of better treatment strategies for patients with colorectal cancer.

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