Cancer Chemother Pharmacol (2008) 61:189–201
DOI 10.1007/s00280-007-0461-4

ORIGINAL ARTICLE

Cell death in response to antimetabolites directed at thymidylate synthase

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Received: 1 December 2006 / Accepted: 6 March 2007 / Published online: 30 March 2007

Abstract
Purpose Thymidylate synthase (TS) is an indispensable enzyme in the de novo biosynthesis of TMP during DNA replication and cell growth, and has, therefore, been an important target for several classes of antimetabolites used in cancer chemotherapy. While most investigations of the action of TS-directed agents have focused on apoptosis as the primary means of cell death, little is known regarding the role, if any, of non-apoptotic mechanisms. In the present study, we have examined the mode of cell death induced by several TS inhibitors.
Methods Apoptosis and necrosis in response to TS inhibitors was assessed. The roles of caspases and the transcriptional regulator nuclear factor kappa B (NFκB) in drug-induced cell death were analyzed. Finally, drug-mediated changes in expression of several proteins involved in regulation of apoptosis were analyzed.
Results Though human colon tumor cells exposed to TS inhibitors undergo classical apoptosis, it is not the predominant mechanism of response; rather, a necrosis-like mechanism prevails. The apoptotic response to TS inhibitors is caspase-dependent, and is promoted by NFκB. In contrast, the necrosis-like response is independent of both caspases and NFκB. Exposure to TS inhibitors induces PARP cleavage, but does not alter expression of the pro or activated forms of caspases-3 or caspases-8, Fas, or FasL. Treatment with the death-inducing cytokine TNFα, like TS inhibitors, results in a limited extent of apoptosis that is both caspase- and NFκB-dependent; however, unlike TS inhibitors, the cytokine does not induce necrosis.
Conclusion Classical apoptosis occurs to a limited extent in human colon tumor cells exposed to TS inhibitors, with caspase-independent necrosis being the prinicipal mechanism of cell death. We suggest that the role of necrosis and necrosis-like mechanisms should be considered in future studies of the action of TS-directed antimetabolites, as well as other chemotherapeutic agents.

Abbreviations

$CH_2H_4PteGlu$	N^5, N^{10}-methylenetetrahydrofolate
dThd	Deoxythymidine
dTMP	Deoxythymidine monophosphate
FdUrd	5-Fluoro-2′-deoxyuridine
FUra	5-Fluorouracil
RTX	Raltitrexed
NFκB	Nuclear factor kappa B
TNFα	Tumor necrosis factor-α
TS	Thymidylate synthase

Introduction

Thymidylate synthase (TS) (EC.3.2.1.1.4) catalyzes the reductive transfer of a methyl group from N^5,N^{10}-methylenetetrahydrofolate ($CH_2H_4PteGlu$) to dUMP, generating TMP and dihydrofolic acid [1, 2]. In the absence of an exogenous source of thymidine, TS is indispensable for DNA synthesis and cell growth. For quite some time, TS has been an important target at which anti-neoplastic agents are directed. Inhibition of the enzyme leads to TMP deficiency, followed by increases in dUTP pools, misincorporation of uracil into DNA, genome damage, and cell death [3]. The fluoropyrimidine analogs 5-fluorouracil (FUra) and

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5-fluoro-2′-deoxyuridine (FdUrd), which are cytotoxic by virtue of their being converted to the powerful TS inhibitor 5-fluoro-2′-deoxyuridylic acid (FdUMP), have been useful in the treatment of carcinomas of the ovary, breast, and gastrointestinal tract [2, 4]. FdUMP, being an analog of the substrate dUMP, can form a catalytic ternary complex with the enzyme and its co-substrate CH_2H_4PteGlu; however, the presence of the fluorine atom precludes subsequent steps in the reaction, so that the ternary complex accumulates [2, 4]. This complex, which is termed the inhibitory ternary complex (ITC), is held together by the same two covalent linkages that occur in the catalytic complex. Enzyme inhibition by FdUMP is, therefore, a stoichiometric "titrating-out" of active sites. Dissociation of the ITC, which is stabilized by folates, is enzyme-catalyzed.

Folate-based inhibitors of TS have been designed on the basis of information from the structures of the natural substrates and the enzyme's active site cleft [2, 5, 6]. Indeed, these inhibitors were among the first to be developed using such rationally-based criteria. Several, such as raltitrexed (RTX), LY231514, ZD9331 and GW1843U89, have advanced to clinical trials, and have demonstrated significant activity against a variety of cancers [5–7]. Structure-based design of new TS inhibitors continues to be a major effort in drug development.

In established tumor cell lines, resistance to TS inhibitors can occur by any of a number of mechanisms, depending upon the particular cell line and the conditions under which it is maintained. Overproduction of TS via amplification of its structural gene is one major mechanism [8, 9]. TS structure is also important, as indicated by the existence of amino acid substitutions in the TS polypeptide that alter binding of inhibitory ligands and that modify drug sensitivity [10, 11]. Human colon tumor cell line HCT116 is intrinsically resistant to FdUrd due to its expression of a variant TS molecule containing a Tyr → His substitution at residue 33 [10, 12]. A mutant TS molecule has been identified in FdUrd-resistant derivatives of cell line HCT15 [13]. All of the mutants studied so far exhibit altered patterns of FdUMP and/or CH_2H_4PteGlu binding.

Numerous studies have focused on caspase-dependent apoptosis as the primary mechanism of cell death following thymidylate deprivation in cells treated with TS inhibitors. The Fas signaling pathway, followed by caspase activation, has been shown to play a pivotal role in initiating the apoptotic response [14–18]. Additional gene products, including the death receptor KILLER/DR5 [19], the transcription factor NFκB [18], the thymine glycosylase MBD-4 [20–22], and the cyclin-dependent kinase inhibitor $p21^{Cip1}$ [23] have been identified as modulators of apoptosis in cells treated with TS inhibitors. Apoptosis is clearly an important mode of cell death following TS inhibition and subsequent thymidylate deprivation; however, it may not be the only mode. As pointed out by several authors, cell death can occur by any of several signaling pathways that are caspase-independent and that are distinct from classical apoptosis [24–28]. Indeed, necrotic forms of cell death have been observed in cells exposed to certain chemotherapeutic agents, including TS inhibitors [29–34].

In the current study, we find that apoptosis occurs in human colon tumor cells treated with TS-directed antimetabolites, but is not the major pathway of cell death; rather, non-apoptotic mechanisms, closely resembling necrosis, prevail. In contrast to apoptosis, the necrosis-like response to TS inhibition is caspase-independent, and is resistant to modulation by nuclear factor-κB (NFκB), a transcription factor that regulates a large body of target genes involved in cell proliferation, innate and adaptive immunity, and tumorigenesis [35, 36]. These findings add to the increasing awareness of the importance of non-apoptotic cell death pathways in mediating response to cytotoxic agents such as TS inhibitors.

Materials and methods

Reagents

Antibodies recognizing caspases-3 and caspases-8 were purchased from Alexis Biochemicals (San Diego, CA, USA), those recognizing IκBα, Fas, FasL, and PARP were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and that recognizing actin was from Sigma-Aldrich Co. (St. Louis, MO, USA). Secondary antibodies were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The broad-spectrum caspase inhibitor zVAD-fmk, the caspase-3 inhibitor zDEVD-fmk, the caspase-8 inhibitor zIETD-fmk, and the caspase-9 inhibitor zLEHD-fmk were obtained from R&D Systems (Minneapolis, MN, USA). Oligonucleotides were purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA). TNFα, FdUrd, FUra, and dThd were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). RTX was provided by AstraZeneca (Macclesfield, Cheshire, UK).

Cell lines and IC_{50} determinations

Human colon tumor cell line HCT116 (originally obtained from Dr. Michael G. Brattain) was maintained in RPMI 1640 medium (Cellgro) containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals) at 37°C in a humidified 5% CO_2 atmosphere. To produce a line deficient in nuclear NFκB expression, HCT116 cells were stably transfected with a plasmid that expresses a dominant-negative mutant form of IκB-α under control of a cytomegalovirus promoter [37]; the plasmid was provided by Dr. Vincent

Bours (University of Liege, Belgium). Transfection was carried out with FuGENE-6 (Roche Applied Science), according to the manufacturer's instructions. Cells were selected in 4 mg/ml G418 (FisherBiotech), and a clonal population was isolated to generate cell line HCT116/dnIκB. Passaging this line in non-selective media for 4 months resulted in a G418-sensitive revertent population of cells, one clone of which was isolated and used as the source of cell line HCT116/dnIκB-R.

To determine growth response to drugs, cells were plated in six-well plates (100,000 cells/well), and allowed to adhere overnight; various concentrations of drug (FUra, FdUrd, or RTX) were then added. After 5 days, the number of surviving cells was assessed by staining with Trypan blue and direct counting with a hemocytometer. The concentration of drug required to inhibit growth by 50% (IC_{50}) was determined.

Measurement of apoptotic and necrotic cell death

Cells were grown for the indicated times in the presence or absence of TS inhibitors (FUra, FdUrd, or RTX); where indicated, caspase inhibitors were dissolved in DMSO and diluted in media to a final concentration of 10 μM. Apoptotic indices were determined by TUNEL assays, performed using the In Situ Cell Death Detection Kit, POD (Roche Applied Science, Indianapolis, IN, USA). Cells were stained according to the manufacturer's instructions, counterstained with hematoxylin, and viewed under a light microscope at 400× magnification. Apoptotic nuclei were counted manually, based on staining and morphology, and the apoptotic index was calculated as the ratio of apoptotic/total cells. A total of 500 cells (both attached and non-attached) were included in each determination.

Necrosis was monitored by measuring cellular swelling. Cells were photographed at 400× under the light microscope, and relative cell areas were determined by pixel counting of the images, using Scion Image software. A total of 100–200 cells were included in each determination.

Transmission electron microscopy

Cells were pelleted at 2,000*g* for 5 min, washed, fixed overnight at 4°C in PBS containing 2.4% glutaraldehyde, and rinsed in PBS. The cells were post-fixed for 1 h in 2% aqueous osmium tetroxide, rinsed, dehydrated through a graded EtOH series, and embedded in PolyBed 812 (Polysciences Inc., Warrington, PA, USA). Sections (100 nm) were stained with uranyl acetate and lead acetate, and imaged in a JEOL 200CX transmission electron microscope equipped with a XR40 4-megapixel CCD camera (Advanced Microscopy Techniques Corp., Danvers, MA, USA).

Western blot analysis

Cells were washed with ice-cold PBS and resuspended in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM dithiothreitol, 5 mM phenylmethylsulfonyl fluoride, 200 μg/ml aprotinin, 50 μg/ml leupeptin, and 100 μg/ml pepstatin A). After brief sonication, the crude lysates were cleared by centrifugation at 15,000*g* for 1 h at 4°C, and the protein was quantified using the Bio-Rad assay reagent, with BSA as a standard. Equivalent amounts of protein were resolved by SDS-PAGE gel electrophoresis through either 4–15% SDS-PAGE gradient gels, or 12% SDS-PAGE gels, depending upon the target protein. The fractionated proteins were transferred to nitrocellulose membranes (Amersham, Piscataway, NJ, USA). After blocking in PBS containing 0.5% Tween-20 and 5% non-fat milk for 1 h at room temperature, the membranes were incubated overnight with primary antibodies, followed by incubation with horseradish peroxidase-linked IgG secondary antibodies for 1 h; immunoreactive bands were visualized by chemiluminescence using the ECL detection system (Amersham, Piscataway, NJ, USA). To control for equal loading, blots were stripped and re-probed with antibodies against actin.

Electrophoretic mobility shift assays

To prepare nuclear extracts, cells were collected in PBS by scraping, and were centrifuged at 3,000*g* for 5 min at 4°C; the cell pellet was gently resuspended in buffer (10 mM HEPES, pH 8.0, containing 10 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2.5 mM dithiothreitol, 5 mM phenylmethylsulfonyl fluoride, 200 μg/ml aprotinin, 50 μg/ml leupeptin, and 100 μg/ml pepstatin A), and incubated on ice for 15 min. Nonident P-40 was added to 0.3% and the mixture was vortexed vigorously. Nuclei were pelleted by centrifugation at 14,400*g* for 1 min, and buffer (20 mM HEPES, pH 8.0, containing 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2.5 mM DDT, 5 mM phenylmethylsulfonyl fluoride, 200 μg/ml aprotinin, 50 μg/ml leupeptin, and 100 μg/ml pepstatin A-l) was added to the pellet and incubated on ice for 15 min with occasional vortexing. The lysate was centrifuged at 11,000*g* for 5 min at 4°C, and the supernatant was collected. Protein was quantified using the Bio-Rad assay reagent, with BSA as a standard.

For the electrophoretic mobility shift assays, 3 μg of nuclear protein were incubated for 30 min at 37°C in 10 μl reaction mixtures containing 0.2 M HEPES, pH 7.9, 4 mM EDTA, 4 mM dithiothreitol, 50% glycerol, 1 mg/ml poly[dI-dC], and 100,000 cpm ^{32}P-end-labeled oligonucleotide probe. The probe was a 21-bp double-stranded oligonucleotide corresponding to a consensus binding sequence for NFκB (5′-AGTGAGGGGACTTTCCCAGG

C-3′). DNA-protein complexes were resolved by electrophoresis through 6% polyacrylamide gels, and the gels were dried and visualized by autoradiography.

Results

Patterns of death in HCT116 cells treated with TS inhibitors

Human colon tumor cell line HCT116 has been used extensively in our laboratory to study mechanisms of response to TS inhibitors [10, 12, 13, 38]. The IC_{50}s for growth inhibition of HCT116 cells are 1.9 ± 0.1 μM for FUra, 13.4 ± 1.9 nM for FdUrd, and 4.9 ± 0.7 nM for RTX. We determined extents of apoptosis following exposure to each of the TS inhibitors. Cells were treated with various concentrations of FUra, FdUrd, or RTX for 48 h, and apoptotic indices were determined by TUNEL assay. For each drug, a concentration-dependent increase in the apoptotic index was observed (Fig. 1a). A similar result was obtained for cells treated with the pro-death cytokine TNFα (Fig. 1a). Apoptosis in response to both the TS inhibitors and to TNFα was inhibited almost completely by zVAD-fmk (Fig. 1b), indicating it to be caspase-dependent.

Interestingly, the apoptotic indices never exceeded 10–25%. Such low extents of apoptosis were observed over a wide range of drug concentrations (10 nM–10 mM FUra, 10 nM–10 mM FdUrd, and 1 nM–100 μM RTX, 5–400 ng/ml TNFα), indicating they are not due to non-specific effects of excessive drug. In all further experiments, we utilized concentrations of TS inhibitors that resulted in measurable and reproducible levels of apoptosis, i.e., 10 μM FUra, 100 μM FdUrd, 10 nM RTX, and 50 ng/ml TNF).

Using light microscopy, we monitored the gross morphology of cells treated with each of the cytotoxic agents. During the first 24 h of treatment with TS inhibitors, most of the cells began to swell, with a small percentage undergoing shrinkage and becoming refractile. The swelling continued over the subsequent 24–48 h (shown for FUra in Fig. 2a), after which time the cells detached from the plate, and lysed. By 96 h, nearly all of the cells were dead, with the few remaining on the plate being severely bloated. This pattern of response to TS inhibitors was not affected by exposure to the broad-spectrum caspase inhibitor zVAD-fmk (Fig. 2a), suggesting that it is caspase-independent. No cellular swelling was observed following treatment with TNFα (Fig. 2a).

To quantitate the swelling phenomenon, direct measurement of relative cell areas under the light microscope were carried out. Figure 2b shows that each of the TS inhibitors

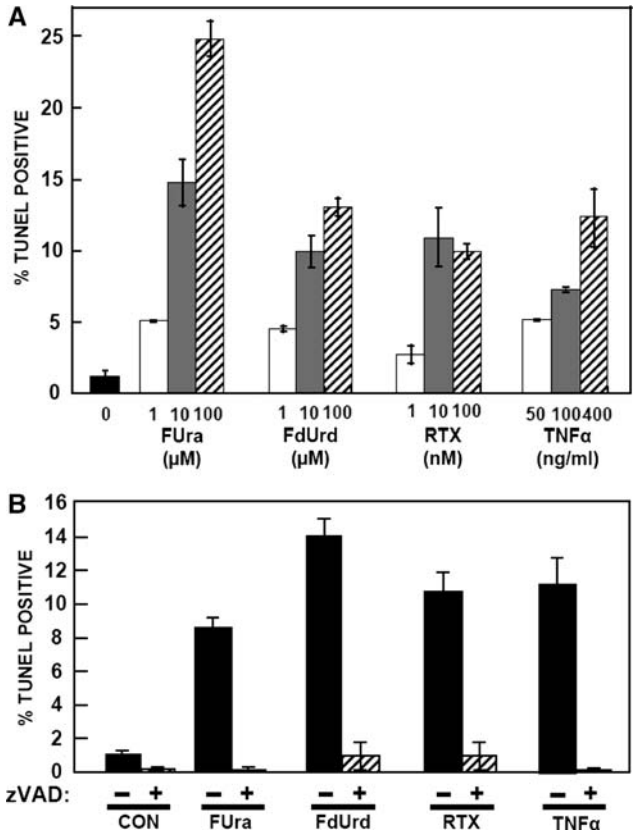

Fig. 1 Apoptosis in response to TS inhibitors. **a** HCT116 cells were grown for 48 h in various concentrations of FUra (1, 10, or 100 μM), FdUrd (1, 10, or 100 μM), RTX (1, 10, or 100 nM), or TNFα (50, 100, or 400 ng/ml), and extents of apoptosis were determined by TUNEL assay. *Bars* represent the percentage of cells that were TUNEL-positive (±SE). **b** HCT116 cells were grown for 48 h in FUra (10 μM), FdUrd (100 μM), RTX (10 nM), or TNFα (50 ng/ml) in the absence (−) or presence (+) of zVAD-fmk. Extents of apoptosis were determined by TUNEL assay. *Bars* represent the percentage of cells that were TUNEL-positive (±SE). *CON* control cells not treated with TS inhibitor

elicited a fourfold to fivefold increase in average cell area; no increase was observed in cells treated with TNFα. The swelling response was maintained in the presence of zVAD-fmk (Fig. 2b), verifying its caspase-independent nature.

Similar results to those described above were obtained with other human colon tumor cell lines, including SW837, HCT15, and SW480 (data not shown). Thus, the observed phenotypes are not a particular characteristic of HCT116. Overall, the experiments indicate that colon tumor cells are generally refractory to apoptotic cell death following treatment with TS inhibitors or TNFα; caspase-independent cellular swelling, followed by lysis, is the major mode of response to TS-directed metabolites, suggesting a necrosis-like form of cell death [24, 26, 28].

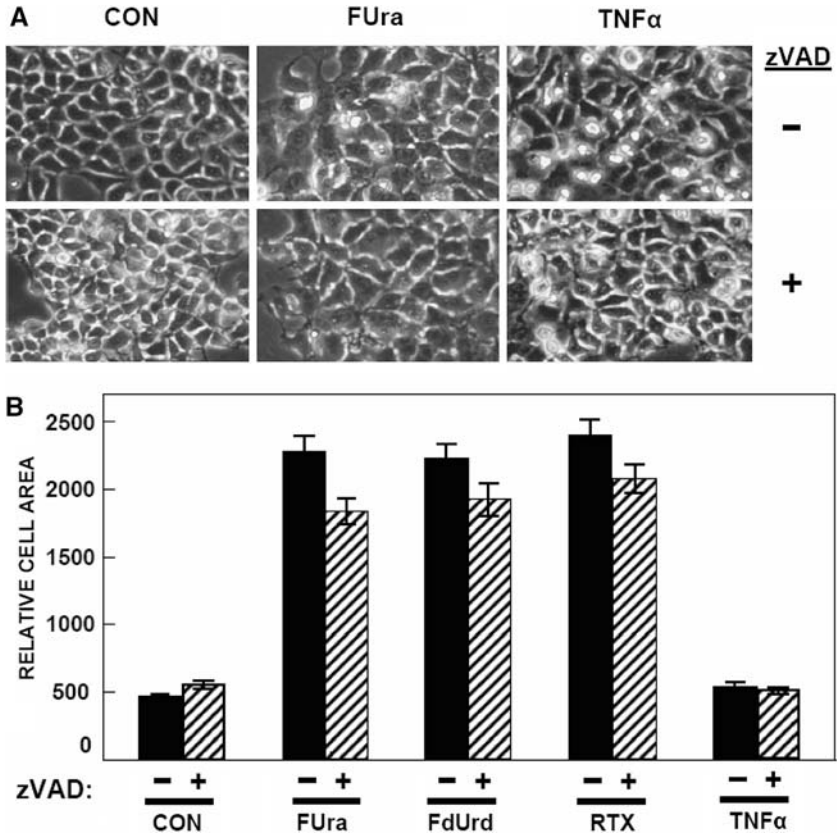

Fig. 2 Cellular swelling in response to TS inhibitors. **a** HCT116 cells were grown for 48 h in FUra (10 μM) or TNFα (50 ng/ml), in the absence (−) or presence (+) of 10 μM zVAD-fmk, and photographed at 400×. *CON* control cells not treated with FUra. **b** HCT116 cells were grown for 48 h in FUra (10 μM), FdUrd (100 μM), RTX (10 nM), or TNFα (50 ng/ml), in the absence (−) or presence (+) of 10 μM zVAD-fmk. Cells were photographed, and the images were used to assess the relative cellular area (see Materials and methods). *Bars* represent the relative areas (±SE), presented in arbitrary units. *CON* control cells not treated with TS inhibitor

Rescue of drug-induced cell death by dThd

To verify that cell death induced by the TS inhibitors is due to TS inhibition followed by dTMP deprivation, we tested the ability of dThd to reverse the toxicity. As shown in Fig. 3, dThd reduced both the swelling (Fig. 3a) and the apoptotic (Fig. 3b) responses to FUra, FdUrd, and RTX. Thus, each drug is indeed targeting TS.

Occurrence of both apoptosis and necrosis in drug-treated cells

Using transmission electron microscopy, we examined the morphology of drug-treated cells in greater detail. Nuclei of control HCT116 cells exhibited relatively smooth membrane contours, and contained aggregates of condensed chromatin (Fig. 4a). The cytoplasms had a uniform granular appearance, with few vacuoles or lysosomes (Fig. 4a). Upon exposure to FUra (Fig. 4c, d), FdUrd (Fig. 4e, f), or RTX (Fig. 4g), some cells (arrowheads in Fig. 4) were shrunken, and exhibited condensation of both the cytoplasm and nucleus. The cytoplasms of these cells were electron-dense and often contained numerous vacuoles, while the nuclei showed evidence of fragmentation. These features defined the cells as undergoing apoptosis. In contrast, a more abundant population of cells (arrows in Fig. 4) displayed pronounced swelling of the nucleus, cytoplasm, and mitochondria, and exhibited disruption of cell membranes. In most, the cytoplasms were decondensed and non-granular, while nuclei were intact and electron-light. These characteristics are typical of cells undergoing necrosis. Only normal and apoptotic cells were observed in cultures treated with TNFα; no necrosis-like morphologies were detected (Fig. 4b).

Thus, as deduced from Figs. 1 and 2, above, TS inhibitors promote cell death via both apoptotic and necrotic mechanisms, with the latter occurring in the majority of cells. This contrasts with TNFα, which elicits a low degree of apoptosis, but no necrosis.

Effects of caspase inhibitors

The data in Fig. 1b indicated that apoptosis in response to TS inhibitors and to TNFα is sensitive to the broad-spectrum caspase inhibitor zVAD-fmk. We determined the effects of several other caspase inhibitors that target particular members of this protease family. As shown in Fig. 5, the caspase-3 inhibitor zDEVD-fmk reduced apoptosis in response to each of the TS inhibitors by about 50%; zIETD-fmk and zLEHD-fmk, which are specific to caspases-8 and caspases -9, respectively, exerted rather mild 30–50% reductions in apoptosis in cells treated with FdUrd and

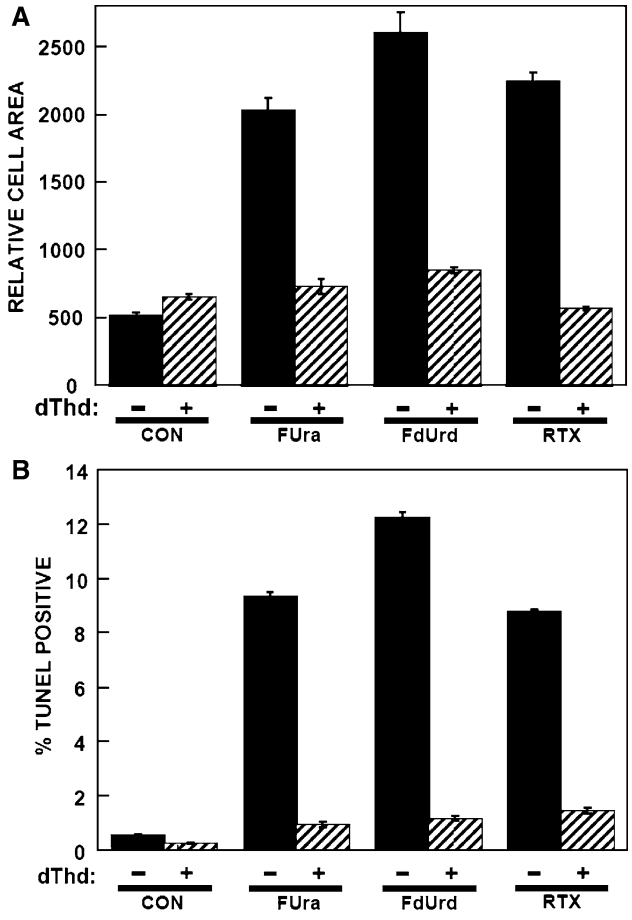

Fig. 3 dThd rescue of cellular swelling and apoptosis. HCT116 cells were grown for 48 h in FUra (10 μM), FdUrd (100 μM), RTX (10 nM), or TNFα (50 ng/ml), in the absence (−) or presence (+) of 100 μM dThd. **a** Cellular swelling was determined as described in Materials and methods (see Fig. 1b). **b** Apoptosis was determined as described in Materials and methods (see Fig. 1)

RTX, with little, if any, effect on that in cells exposed to FUra. All three caspase inhibitors caused >90% reduction in apoptosis in TNFα-treated cells (Fig. 5).

In all, the data indicate that while apoptosis induced by TS inhibitors is caspase-dependent (Fig. 2b), it is only modestly sensitive to specific inhibitors of caspases-3, caspases-8, and caspases-9. Thus, each of the individual caspase family members contribute to, but do not fully account for, the apoptotic response to TS inhibitors.

Role of NFκB in apoptotic and necrotic responses to TS inhibitors

It has been shown that transcription factor NFκB promotes Fas-mediated apoptosis during thymidylate deprivation [18]. NFκB has been extensively studied for its role in cell growth, apoptosis, and tumorigenesis, and is generally considered to exert an anti-apoptotic function through the induction of genes that inhibit apoptotic signaling pathways [35, 36]. In normal cells, NFκB is sequestered in the cytoplasm via binding to the so-called Inhibitor of kappa B-α (IκBα). Activation of NFκB involves phosphorylation of IκBα by IκB kinase-β (IKKβ), which results in targeting of the IκBα for degradation by the 26S proteasome. This leads to translocation of NFκB to the nucleus, and modification of the transcription of a large number of target genes involved in several aspects of cell function, including apoptosis [39].

To test the role of NFκB in both the apoptotic and necrotic response to TS inhibitors, we utilized HCT116 cells in which activation of the transcription factor was inhibited by expression of a dominant-negative mutant form of IκBα. This mutant contains Ser → Ala replacements at residues 32 and 36 of the IκB polypeptide, rendering these sites resistant to phosphorylation by IKKβ, and preventing down-regulation of the polypeptide; this results in constitutive sequestration of NFκB in the cytoplasm [40, 41]. In effect, expression of the mutant IκBα leads to reduced levels of activated NFκB in the nucleus. A cell line expressing a dominant-negative IκBα protein was generated and denoted HCT116/dnIκB (see Materials and methods). The mutant protein is readily detected in HCT116/dnIκB cells, and is resistant to down-regulation by TNFα (Fig. 6a). Furthermore, the cells contain barely detectable levels of NFκB in the nucleus, even after treatment with TNFα (Fig. 6b). To assure that any observed phenotypic changes are indeed due to attenuated NFκB activation, we also isolated a revertent of HCT116/dnIκB (designated HCT116/dnIκB-R; see Materials and methods). This line contains reduced levels of the dominant-negative IκBα protein and rescues NFκB activation (Fig. 6b).

Growth inhibition experiments (data not shown) indicated that the IC_{50} values for FUra (1.9, 2.6, and 2.0 μM in HCT116, HCT116/dnIκB, and HCT116/dnIκB-R, respectively) and for RTX (4.9, 5.0, and 6.0 nM in HCT116, HCT116/dnIκB, and HCT116/dnIκB-R, respectively) were similar among the three cell lines. This was not the case for FdUrd (IC_{50} values of 13, 36, and 35 nM in HCT116, HCT116/dnIκB, and HCT116/dnIκB-R, respectively), where resistance was observed in both HCT116/dnIκB and HCT116/dnIκB-R. Since the latter two cell lines exhibited the same level of resistance, it is unlikely that such resistance is caused by changes in NFκB expression. Overall, we conclude that there are no significant effects of altered NFκB activation on growth response to TS inhibitors.

We compared extents of apoptosis in the three lines, both in the presence and absence of TS inhibitors. Each line was exposed for 48 h to various concentrations of FUra (1–100 μM), FdUrd (1–100 μM, or RTX (1–100 nM), and the apoptotic indices were determined. Figure 7a shows that for each inhibitor, HCT116/dnIκB exhibited a consistent reduction in the apoptotic index relative to the

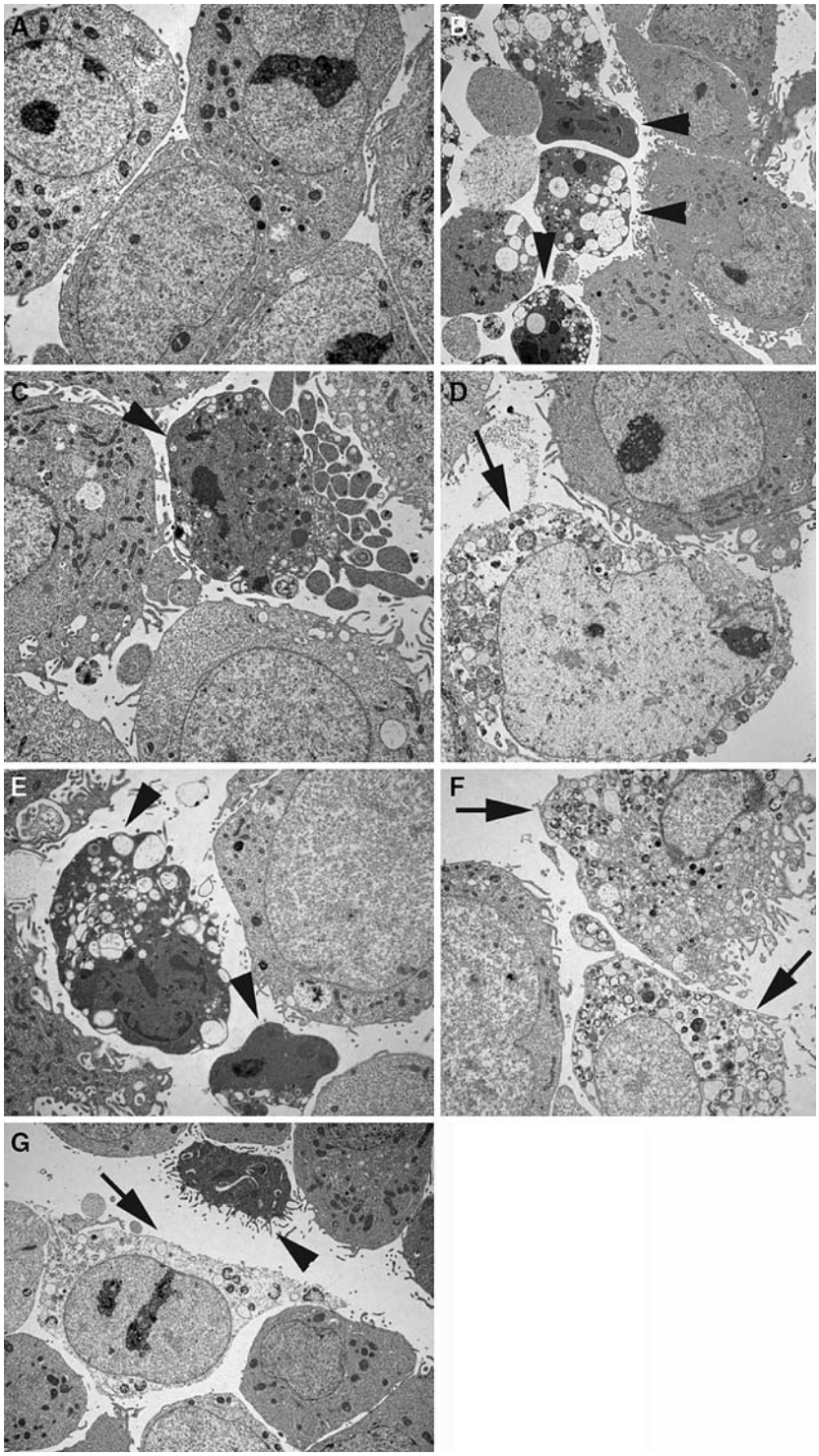

Fig. 4 Electron microscopic analysis of cellular morphology. HCT116 cells were grown for 48 h in FUra (10 μM), FdUrd (100 μM), RTX (10 nM), or TNFα (50 ng/ml), collected, and processed for visualization under the electron microscope (see Materials and methods). Untreated controls are shown in (**a**), TNFα in (**b**), FUra in (**c**) and (**d**), FdUrd in (**e**) and (**f**), and RTX in (**g**). *Arrowheads* indicate cells with apopototic morphology; *arrows* indicate cells with necrotic morphology

parental line HCT116; furthermore, this reduction was partially or completely rescued in HCT116/dnIκB-R. Thus, reduced basal NFκB levels resulted in decreased apoptosis in response to TS inhibitors, indicating that NFκB plays a pro-apoptotic role in response to these drugs. This was somewhat surprising in view of the fact that the transcription factor is generally considered to function as an anti-apoptotic protein [36].

Apoptosis in response to TNFα showed a similar pattern among the three cell lines. The apoptotic index was reduced in HCT116/dnIκB relative to HCT116, and was rescued in HCT116/dnIκB-R (Fig. 7a). Similar results were obtained in cells treated with TRAIL, another death-inducing ligand (C. Voelkel-Johnson, unpublished).

We assessed the impact of altered NFκB levels on the necrosis-like response to TS inhibitors by measuring the

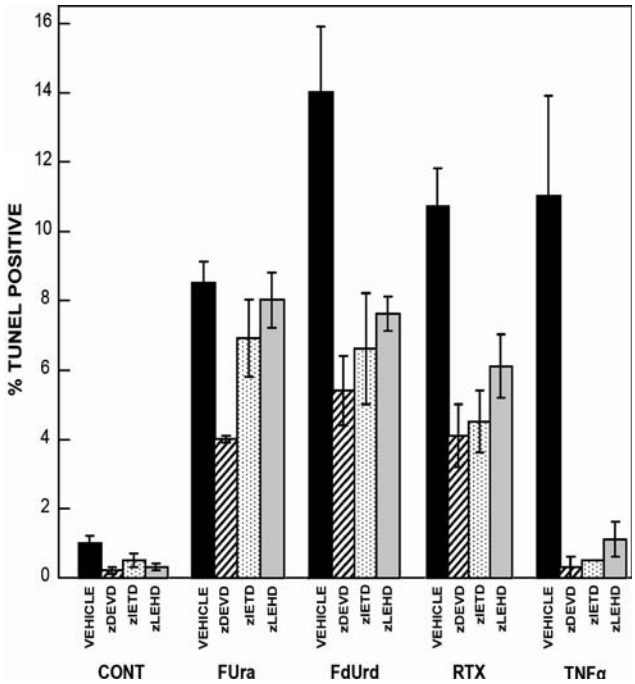

Fig. 5 Effects of caspase inhibitors on apoptotic response to TS inhibitors. HCT116 cells were grown for 48 h in FUra (10 μM), FdUrd (100 μM), RTX (10 nM), or TNFα (100 ng/ml) in the absence or presence of the indicated caspase inhibitors (10 μM each). Extents of apoptosis were determined by TUNEL assay. *Bars* represent the percentage of cells that were TUNEL-positive (±SE). *CONT* control cells not treated with TS inhibitor; *VEHICLE* cells not treated with caspase inhibitor

degree of cellular swelling following drug treatment. Similar to parental HCT116 cells, both HCT116/dnIκB and HCT116/dnIκB-R cells underwent threefold to fivefold increases in relative cell area following drug exposure (Fig. 7b). Thus, alterations in the nuclear concentrations of NFκB have little or no impact on the necrotic response to TS-direct agents.

Expression of apoptosis mediators

The effects of TS inhibitors on expression of several additional proteins known to be involved in the apoptotic process were measured. These proteins included procaspases-3 and procaspases-8, their activated cleavage products, Fas, FasL, and PARP. Expression of Fas and FasL were of particular interest, since these proteins have been shown to be induced in cells treated with TS inhibitors [17, 42], as well as in TS-deficient cells subjected to thymidylate deprivation [18]. HCT116 cells were exposed to FUra, FdUrd, or RTX at concentrations that induce cell death, and expression of each of the various proteins was measured by Western blotting. As shown in Fig. 8, none of the inhibitors affected expression of procaspases, activated caspases, Fas, or FasL. PARP cleavage, on the other hand, was significantly increased by all three drugs. Similar results were observed with cell lines HCT116/dnIκB and HCT116/dnIκB-R (Fig. 8). Thus, cell death in response to TS inhibitors

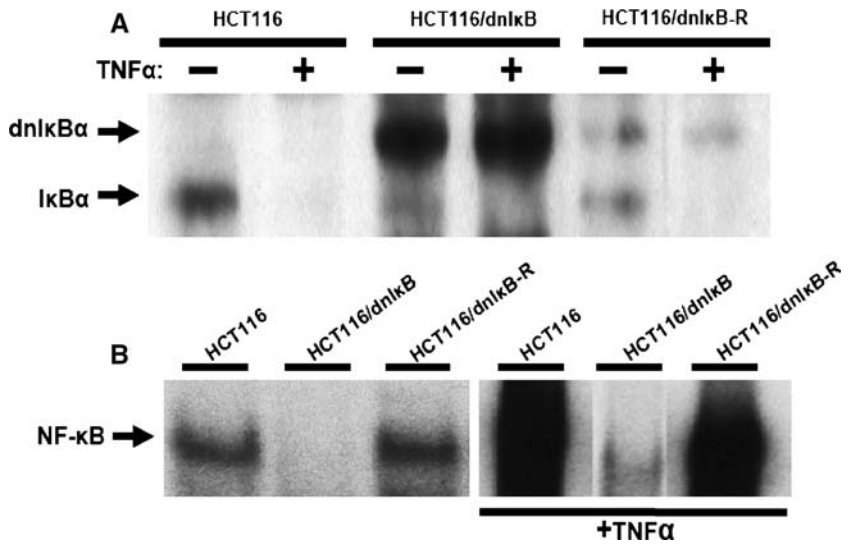

Fig. 6 Characterization of HCT116-derived cell lines with altered NFκB activation. Cell lines HCT116, HCT116/dnIκB (deficient in NFκB activation due to expression of the dominant-negative IκB-α mutant protein; see Materials and methods), and HCT116/dnIκB-R (a revertent line with normal activation of NFκB; see Materials and methods) were compared. **a** IκBα expression was analyzed by Western blotting probed with an anti-human IκBα antibody. Samples from control cells or TNFα-treated cells are denoted by (−) or (+), respectively. *Arrows* indicate the endogenous and the dominant-negative IκBα proteins, which are derived from human and mouse, respectively, and have different mobilities. **b** NFκB expression was assessed by electrophoretic mobility shift assay of nuclear extracts. Only the region of the gel containing the DNA-protein complex is shown. Samples from cells treated with TNFα are indicated

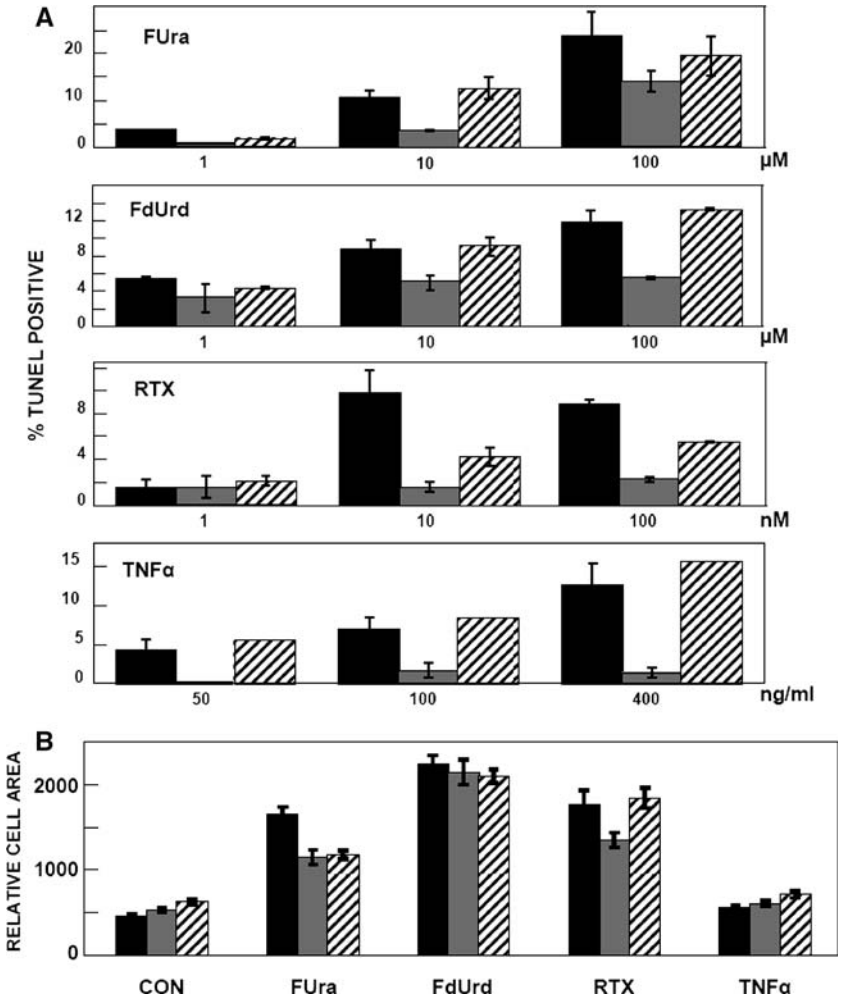

Fig. 7 Effects of altered NFκB activation on apoptotic and necrotic cell death following exposure to TS inhibitors. **a** Cells were grown for 48 h in various concentrations of FUra (1, 10, or 100 μM), FdUrd (1, 10, or 100 μM), RTX (1, 10, or 100 nM), or TNFα (50, 100, or 400 ng/ml), and extents of apoptosis were determined by TUNEL assay. *Bars* represent the percentage of cells that were TUNEL-positive (±SE). HCT116 is represented by the *filled-in bar*, HCT116/dnIκB by the *gray bar*, and HCT116/dnIκB-R by the *cross-hatched bar*. **b** Cells were grown for 48 h in FUra (10 μM), FdUrd (100 μM), RTX (10 nM), or TNFα (50 ng/ml). They were photographed, and the images were used to assess relative cellular area (see Materials and methods). *Bars* represent the relative areas (±SE), presented in arbitrary units. HCT116 is represented by the filled-in bar, HCT116/dnIκB by the gray bar, and HCT116/dnIκB-R by the *cross-hatched bar*

is accompanied by induction of PARP cleavage, but is not associated with activation of procaspase-3, procaspase-8, Fas, or FasL. Furthermore, there is no effect of altered NFκB activation on expression of these proteins, or on their patterns of response to TS inhibitors. The fact that only a fraction of the cell population undergoes apoptosis following drug-treatment may underlie the inability to detect changes in these mediators.

TNFα induced PARP activation, but had no effect on expression of procaspases-3 or procaspases-8, (or their cleaved derivatives), Fas, or FasL (Fig. 8). PARP activation by the cytokine was abrogated in HCT116/dnIκB cells, and rescued in HCT116/dnIκB-R cells (Fig. 8), indicating it to be NFκB-dependent. Interestingly, we observed that expression of procaspases-3 and procaspases-8 (as well as their activated cleaved forms) were repressed by TNFα in HCT116/dnIκB cells, but not in HCT116/dnIκB-R (Fig. 8). This was examined in more detail in Fig. 9, which shows a TNFα-mediated, dose-dependent repression of both procaspases in cell line HCT116/dnIκB, but not in cell line HCT116 or HCT116/dnIκB-R. These observations suggest that attenuation of NFκB activation promotes TNFα-mediated repression of procaspase-3 and procaspases-8 expression. This effect of NFκB may be a contributing factor in its ability to promote apoptotic response to the death-promoting cytokine.

Discussion

We have shown that exposure of HCT116 cells to TS inhibitors causes both apoptotic and necrotic modes of cell death, with the latter mode being far more prevalent. The apoptotic response is caspase-dependent, and is regulated by transcription factor NFκB. In contrast, the necrotic response is caspase-independent, and is unaffected by NFκB. Treatment with the pro-death cytokine TNFα, similar to TS inhibitors, results in induction of apoptosis that occurs in only a fraction of the cell population; however, the cytokine does not elicit a necrosis-like mode of cell death. Thus, colon tumor cells, under the conditions of our experiments, were generally resistant to induction of apoptosis by TS inhibitors and by TNFα, with the former, but not the latter, promoting a necrosis-like cell death response.

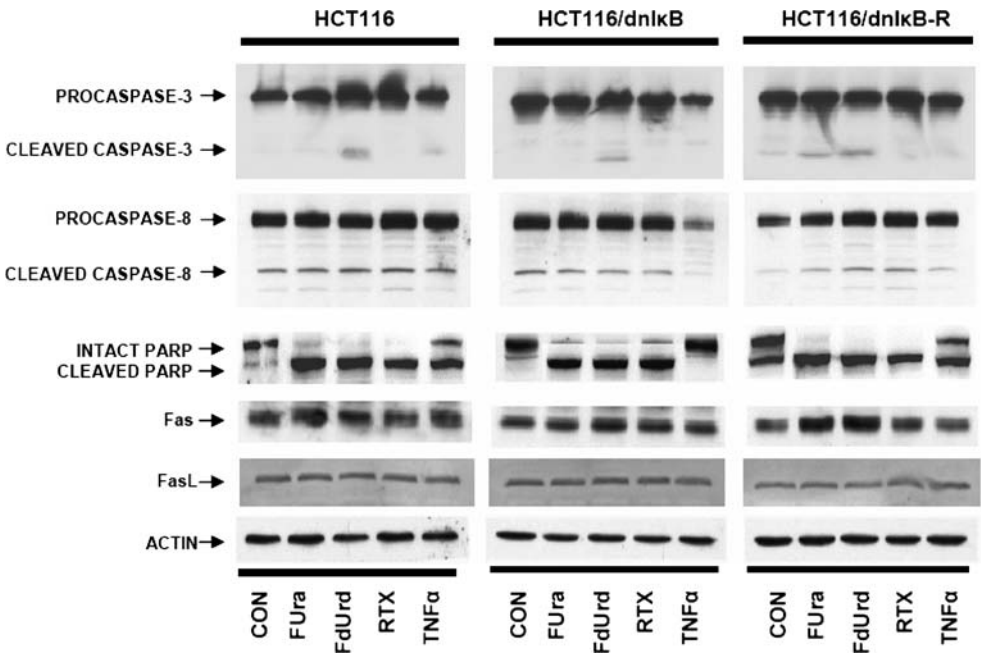

Fig. 8 Expression of signaling proteins involved in cell death. HCT116, HCT116/dnIκB, and HCT116/dnIκB-R cells were grown for 48 h in FUra (10 μM), FdUrd (100 μM), RTX (10 nM), or TNFα (100 ng/ml), and subjected to Western blot analysis, using antibodies to the indicated proteins as probes. Procaspases and their activated forms, as well as intact and cleaved forms of PARP are indicated by *arrows*. Actin was included as a control for loading. *CON* control cells not treated with TS inhibitor

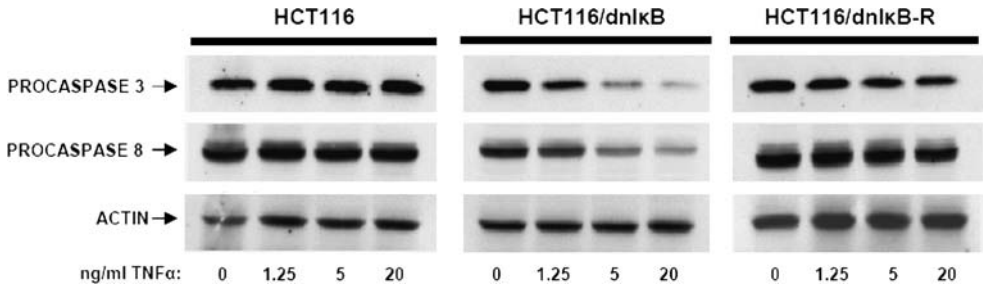

Fig. 9 TNFα-mediated repression of caspase-3 and caspase-8. HCT116, HCT116/dnIκB, and HCT116/dnIκB-R cells were grown for 48 h in various concentrations of TNFα (0, 1.25, 5, or 20 ng/ml), and procaspases-3 and procaspases-8 expression were measured by Western blotting. Actin was included as a control for loading

No drug-mediated alterations in expression or activation of procaspase-3 or procaspases-8 were detected in HCT116 cells. This may indicate that only a fraction of the cell population (i.e., the apoptotic fraction) undergoes caspase activation. Alternatively, there may be a generalized disruption of caspase expression and/or activation in these cells, leaving them with constitutively low levels of active enzymes, and, as a consequence, low apoptotic indices following drug treatment. PARP cleavage did occur in cells treated with TS inhibitors, and was maintained when NFκB levels were reduced (and the apoptotic index was attenuated) by introduction of a dominant-negative IκBα mutant. The role, if any, of PARP cleavage in either the apoptotic or necrotic mode of cell death in HCT116 cells is not known.

In earlier studies, we demonstrated that HCT116 cells express a variant form of TS that causes relative resistance to TS inhibitors [10, 38]. It might be suggested that this variation is responsible for the necrosis-like responses measured in the current study. This is unlikely, however, since other human colon tumor cell lines that we tested (e.g., SW837, HCT15, and SW480) respond to TS-directed agents similarly to HCT116, i.e., exhibiting low levels of apoptosis and marked necrosis. Thus, the phenotypes we have observed are not unique to HCT116.

Apoptosis has been considered to be the major mechanism of cell death following exposure to anti-cancer drugs, and the vast majority of studies have focused on its mechanism and regulation. This has been the case for TS inhibitors as well [17, 18, 23, 42]. However, evidence has been accumulating to indicate that non-apoptotic modes of cell death do occur following exposure to cytotoxic agents, and may in certain circumstances be the predominant mechanism. For example, human leukemic cells undergo caspase-independent, necrosis-like cell death in response to the BCR-ABL inhibitor imatinib [29]. In mouse embryo fibroblasts, alkylating agents such as nitrogen mustard and MNNG induce PARP-dependent necrosis that occurs independently of caspases, p53, and Bax/Bak [30]. The marine-derived antitumor agent kahalalide F exerts its cytotoxic effects via a necrotic-like mechanism in breast and prostate cancer cells

[31]. Finally, two sublines of mouse mammary tumor FM3A cells that exhibit different modes of cell death following FdUrd treatment have been isolated: one subline undergoes apoptosis in response to drug, while the other undergoes necrosis [32]. Collectively, these studies show that while apoptosis is clearly an important means of cell death induced by toxic drugs, necrosis or necrosis-like mechanisms also play a role, and may predominate in some cases.

Several factors are likely to have contributed to the notion that apoptosis, as opposed to other forms of cell death, is the prevailing response to cytotoxic agents. Typically, standard assays such as TUNEL and flow cytometry are utilized to measure the apoptotic index in drug-exposed cells. It may be that the non-apoptotic fraction, which can be 50% or more of the cell population, has simply been ignored or discounted during the course of experiments. In addition, a great deal of knowledge exists on molecular mediators of apoptosis, and how they function in various signaling pathways. This provides a rather large foundation on which to base ongoing experimental studies. Finally, it is quite likely that the particular mode of cell death is context dependent, i.e., the physiological state of cultured cell lines may predispose to one or another cell death mechanism. Apoptosis may be the predominant mechanism under the conditions of many studies, while necrosis may prevail in others. Several authors have described a regulatory "switch" between necrotic and apoptotic modes of cell death that is controlled by factors such as genetic background [43], ATP levels [44, 45], and redox status [33]. Subtle alterations in such contextual factors among cell lines or among laboratories using the same cell line could result in the operation of different cell death pathways upon exposure to cytotoxic agents.

The molecular mechanisms underlying apoptotic and necrotic cell death induced by TS inhibitors are not fully understood. The formation of reactive oxygen species in the mitochondria has been invoked as a key component of necrosis in some situations [24]. Along these lines, recent studies have shown that FUra induces ferredoxin reductase [46, 47] and spermidine/spermine *N*-acetyltransferase [48, 49], two enzymes whose activities lead to generation of hydrogen peroxide and/or reactive oxygen species as by-products. These by-products have the potential to cause oxidative stress leading to cell death. It will be of interest to determine the role, if any, of redox status in both the apoptotic and necrotic response to TS inhibitors.

The current study identified a pro-apoptotic role for NFκB in cells responding to TS inhibitors. A number of investigations have shown that decreased NFκB levels sensitize cells to the action of TS inhibitors [50–53]. Such observations are consistent with the broadly accepted function of NFκB as an anti-apoptotic factor [36]. Our current results, which are in direct opposition to this view, are consistent with the findings of Houghton and colleagues, who showed that apoptosis during thymidylate deprivation in human colon carcinoma cells is promoted by NFκB [18]. A pro-apoptotic role for NFκB has also been observed in other studies [54–56], making it apparent that the factor has dual, context-dependent functions in the control of apoptosis. Indeed, this has prompted some authors to urge caution in the use of drugs targeted at NFκB for control of cancer.

Several studies have reported activation of NFκB following treatment with TS inhibitors [18, 50–53]. We have found no observable effects of FUra, FdUrd, or RTX on NFκB concentrations in the nucleus of HCT116 cells (K. Barbour, unpublished), indicating that under the conditions of our experiments, TS inhibitors do not on their own promote NFκB activation. Thus, basal levels of the transcription factor are adequate for regulating the response to TS-directed agents.

In conclusion, it is clear that necrosis may be a prevailing mechanism of cell death in response to TS inhibitors under certain conditions. The detailed signaling pathways that regulate necrosis are unknown. It will be critical to identify such pathways in future work, and to determine how the decision to undergo cell death via an apoptotic versus a necrotic mechanism is made. Increased recognition of necrosis as a means of cell death will likely be important in efforts aimed at enhancing the efficacy of TS-directed, as well as other, chemotherapeutic agents.

Acknowledgments This research was supported by a grant (CA 44013) from the National Cancer Institute. We thank AstraZeneca for raltitrexed, Dr. Robert Price of USC's Histology and Imaging Core Facility for carrying out electron microscopy, and members of the Berger lab for help and support during the course of these studies.

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