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DISULFIRAM-MEDIATED INHIBITION OF NF-κB ACTIVITY ENHANCES CYTOTOXICITY OF 5-FLUOROURACIL IN HUMAN COLORECTAL CANCER CELL LINES

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5-Fluorouracil (5-FU) is the major chemotherapeutic component for colorectal cancer (CRC) and other types of solid tumours. Resistance of cancer cells to 5-FU is considered the major obstacle for successful chemotherapy. NF-κB is a transcription factor. Cancer cells with high NF-κB nuclear activity demonstrate robust chemo- and radio-resistance. We demonstrated that nuclear NF-κB activity in CRC cell lines, DLD-1 and RKO_{WT}, was significantly induced by 5-FU in a concentration- and time-dependent manner. 5-FU induced IκBα degradation and promoted both NF-κB nuclear translocation and its DNA binding activity. 5-FU treatment did not influence the activities of AP-1, AP-2, Oct-1, SP-1, CRE-B and TFIID. Disulfiram (DS), a clinically used anti-alcoholism drug, strongly inhibited constitutive and 5-FU-induced NF-κB activity in a dose-dependent manner. DS inhibited both NF-κB nuclear translocation and DNA binding activity but had no effect on 5-FU-induced IκBα degradation. Used in combination, DS significantly enhanced the apoptotic effect of 5-FU on DLD-1 and RKO_{WT} cell lines and synergistically potentiated the cytotoxicity of 5-FU to both cell lines. DS also effectively abolished 5-FU chemoresistance in a 5-FU resistant cell line $H630_{5\text{-}FU}$ *in vitro*. As DS has extensive preclinical and clinical experience, translating its anticancer usage from *in vitro* study to clinical trials is relatively straightforward.
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Key words: *NF-κB; IκBα; 5-FU; disulfiram; colorectal cancer*

Nuclear factor-κB (NF-κB) is a transcription factor, which plays an important role in cell proliferation and malignant transformation.[1,2] Human NF-κB is composed of 5 subunits [p50/p105, p52/p100, p65 (RelA), RelB and c-Rel][3] that form homo- or heterodimer binding to DNA target sites (κB sites) to influence downstream gene expression. The most common dimer is a p50–p65 heterodimer. In most normal cells, NF-κB is retained in the cytoplasm as an inactive complex through the direct binding of the natural inhibitor of κB (IκB).[4] Upon various stimuli, IκB is phosphorylated, ubiquitinised and promptly degraded releasing NF-κB from the NF-κB-IκB complex. The liberated NF-κB dimers are translocated into the nucleus, bind to the promoter region of the relevant downstream genes, and trigger a series of transcriptional events.

Exposure of cancer cells to anticancer drugs, cytokines and radiation can induce the nuclear translocation and DNA binding activity of NF-κB.[5,6] Human cancer cells with induced NF-κB nuclear activity have demonstrated resistance to apoptosis induced by chemotherapy or radiotherapy.[2,7–9] The activated NF-κB can also antagonise the cytotoxicity of anticancer drugs by inducing the expression of anti-apoptotic genes (*e.g.*, *c-IAPs*, *IXAP*, *A1/Bfl-1* and *IEX-IL*).[10–12] Inhibition of NF-κB activity by IκBα can enhance the cytotoxicity of anticancer drugs *in vitro*[13] and *in vivo*.[14,15]

Although induction of tumour regression in nude mice could be achieved by combining use of anticancer drug and a gene therapy strategy that overexpressed mutant IκBα,[13,14] there are many obstacles to the clinical application of this approach. Therefore, a small molecule approach may be the most viable short-term strategy for inhibition of NF-κB. Disulfiram (DS) has been used in the treatment of alcoholism since the 1940s.[16] Several studies have demonstrated that DS and its metabolites can potentiate the effect of some anticancer drugs, such as cyclophosphamide[17] and nitrogen mustard.[18] The metabolites of DS can also protect normal tissues from radiotherapy and chemotherapy.[19–21] Recently, it was serendipitously found that DS could inhibit the maturation of P-glycoprotein (P-gp) and increase the sensitivity of P-gp-transfected cells to vinblastine and colchicine *in vitro*.[22] In addition, both DS and its metabolite, Diethyldithiocarbamate (DDC) inhibit oxidant-induced NF-κB activation.[23–25] DS downregulates the activity of NF-κB, AP-1 and bcl-2 and induces G1/S arrest and apoptosis in human hepatoma Hep G2 cells.[26] The precise anticancer mechanisms of DS are still largely unknown. We investigated DS-mediated changes in the NF-κB pathway after 5-fluorouracil (5-FU) treatment. The effect of DS on the cytotoxicity of 5-FU to drug sensitive and resistant colorectal cancer (CRC) cell lines was also assessed.

MATERIAL AND METHODS

Cell culture and cytotoxicity analysis

The 5-FU resistant CRC cell line $H630_{5\text{-}FU}$ and the relevant parent cell line $H630_{WT}$ and CRC cell lines DLD-1 and RKO_{WT} were chosen for our study (chemoresistant cell line was kindly provided by Prof. P.G. Johnston, The Queen's University of Belfast, Department of Oncology). The characteristics and culturing conditions have been described previously.[27–29] For *in vitro* cytotoxicity analysis, DLD-1 and RKO_{WT} cells (5,000/well), cultured overnight in 96-well flat-bottomed microtiter plates, were exposed to 5-FU (Sigma-Aldrich, St. Louis, MO), DS (Sigma-Aldrich) or a combination of both for 96 hr and then subjected to a standard 3-[4,5-dimethylthiazol-2-yl]diphenyltetrazolium bromide assay (MTT) as described previously.[27,30] The combined cytotoxic effect

Abbreviations: CI, combination index; CRC, colorectal cancer; DCC, diethyldithiocarbamate; DS, disulfiram; EMSA, electrophoretic mobility-shift assay; 5-FU, 5-fluorouracil; IKK, IκB kinase; RH, Rel homology; ROS, reactive oxygen species; TDX, Tomudex; TS, thymidylate synthase; TUNEL, deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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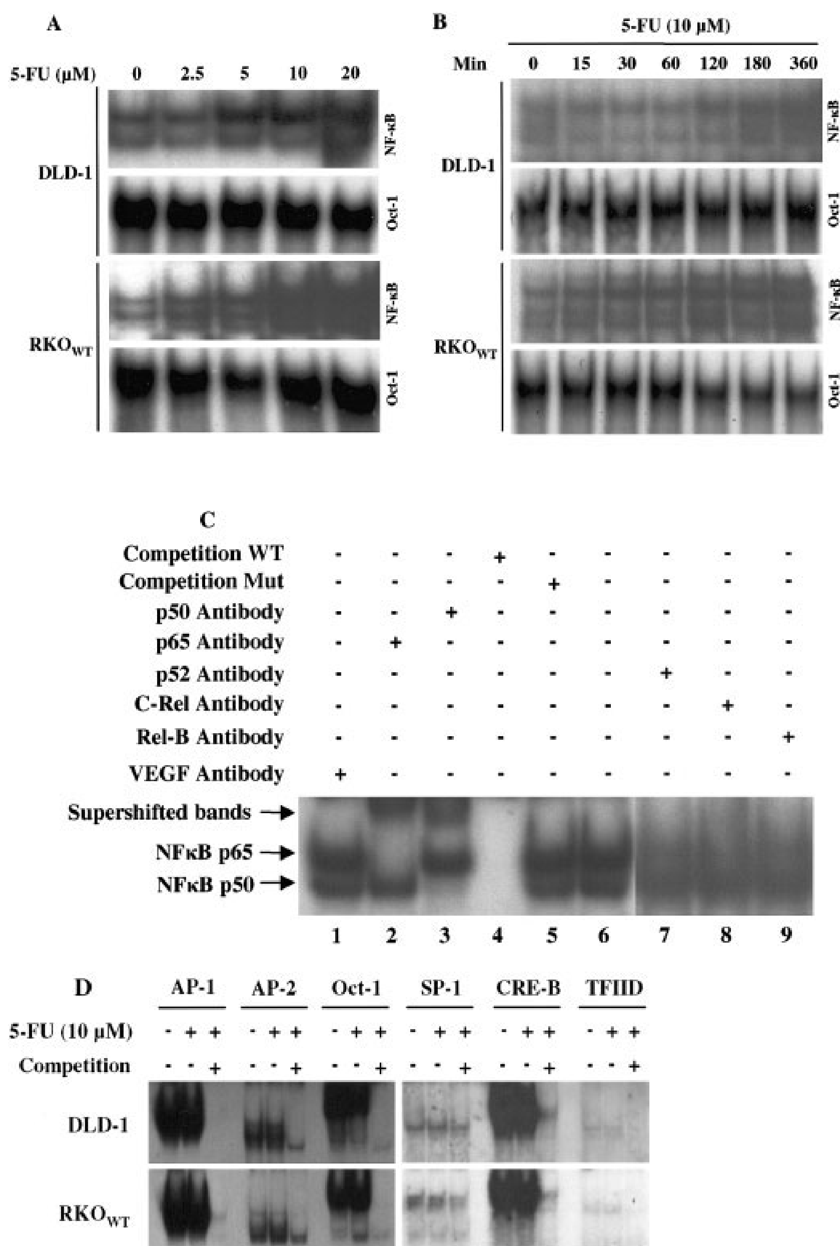

Figure 1 – NF-κB nuclear activity in DLD-1 and RKO$_{WT}$ cells was induced by 5-FU. *a*: The nuclear extract (5 μg/lane) from cells exposed to 0–20 μM 5-FU for 24 hr was analysed by EMSA. *b*: Nuclear extract (5 μg/lane) from cells exposed to 10 μM 5-FU for 0–360 min was subjected to EMSA. Oct-1 was used as loading control. *c*: Nuclear extract of RKO$_{WT}$ cells exposed to 5-FU (10 μM) for 24 hr was incubated with antibodies or unlabeled NF-κB probes and tested by EMSA. *d*: Influence of 5-FU on the DNA binding activity of AP-1, AP-2, Oct-1, SP-1, CRE-B and TFIID transcription factors. Nuclear extracts from cells treated with 5-FU (10 μM) for 24 hr were subjected to EMSA analysis using oligos containing consensus sequences for the above transcription factors.

of 5-FU with DS was determined by CI-isobologram using CalcuSyn software (Biosoft, Cambridge, UK).[31] Overnight cultured cells (5,000/well) were exposed to various concentrations of each drug or a combination of these 2 drugs at a constant ratio (5-FU: DS = 2:1) for 96 hr. The cells were then subjected to MTT analysis as mentioned above. Mutually exclusive equations were used to determine the combination index (CI).

Electrophoretic mobility-shift assay

The extraction of nuclear and cytoplasmic protein and electrophoretic mobility-shift assay (EMSA) analysis were carried out as described previously.[32] To test the effect of DS and 5-FU on the DNA binding activity of NF-κB, the cells were incubated with the compounds at indicated concentrations and time length. The nuclear protein concentration was determined using DC Protein Assay (Bio-Rad, Richmond, CA). Equal amount of the nuclear extract (5 μg) was incubated with 1 μg poly(dIdC) (Sigma-Aldrich) in binding buffer [50 mM Tris (pH 7.6), 250 mM KCl, 25 mM DTT, 5mM EDTA and 25% glycerol] for 10 min at room temperature. Approximately 20,000 cpm of ^{32}P-labeled NF-κB DNA probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was added and the reaction was incubated at room temperature for 20 min. The double-stranded oligonucleotides containing AP-1, AP-2, SP-1, Oct-1, CREB or TFIID consensus sequences were

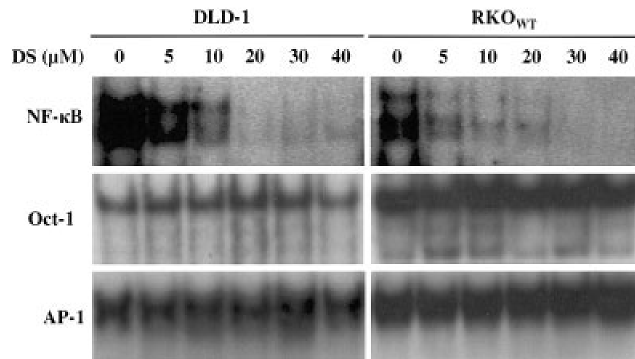

FIGURE 2 – Dose-dependent effect of DS on DNA binding activity of NF-κB, Oct-1 and AP-1. Cells were cultured in medium containing 10 μM 5-FU and 0–40 μM DS for 96 hr. Nuclear extracts (5 μg/lane) were analysed by EMSA.

purchased from Promega (Madison, WI). The EMSA for these transcription factors were carried out using the same conditions as those for NF-κB. For supershift assay and binding specificity determination, 5 μg of nuclear extract from RKO$_{WT}$ cells treated with 5-FU was incubated with 0.4 μg of antibody (p65, p50, p52, C-Rel, Rel-B or VEGF [Santa Cruz Biotechnology, Santa Cruz, CA]) or 20 × wild-type or mutant (5′-AGTTGATATTACTTT-TATAGGC-3′) unlabeled NF-κB probe for 30 min before EMSA analysis. The complexes were separated on a 6% polyacrylamide gel and exposed for autoradiography. The band intensity was analysed using Molecular Analyst software (Bio-Rad).

Western blot analysis

The nuclear or cytoplasmic protein (60 μg/lane) was electrophoresed through a 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, UK). The blots were stained with primary (NF-κB p50, 1:500; NF-κB p65 and IκBα, 1:100; Santa Cruz Biotechnology) and secondary (1:5,000; Amersham Pharmacia Biotech, NJ) antibodies respectively for 1 hr at room temperature. The loading quantity of protein was verified by staining the same membranes with anti-α-tubulin antibody for cytoplasmic protein (1:2,000, Sigma-Aldrich, Dorset, UK) or SimpleBlue SafeStain for nuclear protein (Invitrogen, Paisley, UK). The signal was detected using an ECL Western blotting detection kit (Amersham Pharmacia Biotech). The intensity of the bands was analysed using Molecular Analyst software (Bio-Rad).

Deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

The cells were grown on SuperCell culture slides (Erie Scientific Company, NH) until 70% confluent and exposed to variable concentrations of 5-FU, DS or 5-FU plus DS for 24 hr. After 1 hr fixation in 4% paraformaldehyde, the cells were stained according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). Briefly, the cells were fixed with a freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 1 hr at room temperature and then permeabilised with 0.1% Triton X-100/0.1% sodium citrate for 2 min on ice. After incubation with deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction mixture and Converter-AP, the apoptotic cells were detected using a chromogenic substrate (NBT/BCIP) (Sigma-Aldrich) and examined under light microscope (Zeiss, Welwyn Garden City, UK). Each experiment was carried out in duplicate.

Detection of internucleosomal DNA cleavage

DNA fragmentation was detected by a modified method described previously.[33] Seventy percent confluent cells in 25 cm^2 flasks were exposed to 5-FU, DS or 5-FU + DS for 24 hr and collected by trypsinisation. Genomic DNA samples (2 μg/lane) were electrophorised through a 1.5% agarose slab gel containing 0.5 μg/ml ethidium bromide and visualised under UV illumination.

Statistical analysis

All results were analysed by Student's *t*-test and ANOVA using SPSS 10.1.

RESULTS

5-FU induced NF-κB activity in DLD-1 and RKO$_{WT}$ cells

NF-κB activity in RKO$_{WT}$ and DLD-1 cells was induced by 5-FU at a concentration of 2.5 μM and 5 μM respectively (Fig. 1*a*). The NF-κB activity was further induced by increasing 5-FU concentrations. When cultured in 10 μM 5-FU for 0–360 min, the inductive effect of 5-FU on NF-κB activity was demonstrated in a time-dependent manner in both cell lines (Fig. 1*b*). To verify the binding specificity, 5 μg of nuclear extracts from 5-FU treated sensitive cells were pre-incubated with 20 times higher concentration of unlabeled wild-type or mutant NF-κB probe before EMSA. The retarded bands were removed by the unlabeled wild-type NF-κB probe but not by the mutant one (Fig. 1*c*, lanes 4 and 5). To determine which NF-κB subunits were involved, the nuclear extract was pre-incubated with different NF-κB antibodies (p65, p50, p52, c-Rel and RelB) for 30 min and examined by EMSA. Figure 1*c* illustrates that the supershifting bands were only produced by NF-κB p50 and p65 antibodies (lanes 2, 3) but not by p52, c-Rel or RelB (Fig. 1*c*, lanes 7–9). To verify the specific inductive effect of 5-FU on NF-κB DNA binding activity, the effect of 5-FU on other transcription factors was also tested. As shown in Figure 1*d*, the DNA binding activities of AP-1, AP-2, Oct-1, SP-1, CRE-B and TFIID were not affected by 24 hr exposure of DLD-1 and RKO$_{WT}$ cells to 10 μM of 5-FU.

DS inhibited 5-FU-dependent NF-κB activity

DS has been shown to inhibit the induction of NF-κB activity caused by reactive oxygen species (ROS).[24,25] To test the effect of DS on 5-FU-induced NF-κB activity, DLD-1 and RKO$_{WT}$ cells were cultured in medium containing 10 μM 5-FU and different concentrations of DS for 96 hr. EMSA results showed that DS inhibited 5-FU-induced NF-κB activity in a dose-dependent manner (Fig. 2). NF-κB activity was significantly inhibited by 5 μM DS (30% [DLD-1] and 10% [RKO$_{WT}$] of control, respectively) and the maximum inhibition occurred at 20 μM and 30 μM for DLD-1 and RKO$_{WT}$ cells, respectively. It is common that NF-κB and AP-1 are correlatively regulated by their stimuli and inhibitors.[34] We demonstrated that DS had differential effect on these 2 transcription factors. DS suppressed DNA binding activity of NF-κB but had no inhibiting effect on DNA binding activity of AP-1 in both DLD-1 and RKO$_{WT}$ cell lines (Fig. 2). Oct-1, which is constitutively expressed in many cell types, was used as a loading control for the EMSA analysis.

DS inhibited nuclear translocation and DNA binding affinity of NF-κB but not 5-FU-dependent degradation of IκBα

To test the effect of 5-FU and DS on the nuclear pool of the NF-κB p65 subunit, a major component of NF-κB activity,[35] cells were exposed to 5-FU or DS for 24 hr. Relatively high levels of nuclear NF-κB p65 protein were observed in both cell lines (Fig. 3*a*,*b*). 5-FU exposure increased the nuclear p65 pool to 130 and 140% of its background levels in RKO$_{WT}$ and DLD-1 cell lines respectively. DS reduced both constitutive and 5-FU induced nuclear pools of p65 protein in DLD-1 and RKO$_{WT}$ cell lines (Fig. 3*a*,*b*). Equivalent nuclear protein loading in each lane was verified by SimpleBlue SafeStain staining (Fig. 3*a*). To test the effect of DS on NF-κB DNA binding activity *in vitro*, the same amount of nuclear extract (5 μg) from 5-FU-treated (20 μM, 24 hr) cells was pre-incubated with indicated concentrations of DS for 30 min at 37°C and analysed by EMSA. Figure 3*c* demonstrates that the NF-κB DNA binding activity was inhibited by DS in a concentration-dependent manner. Inhibition was first observed at 0.1 μM and the binding activity was totally blocked by 10 and 50 μM DS

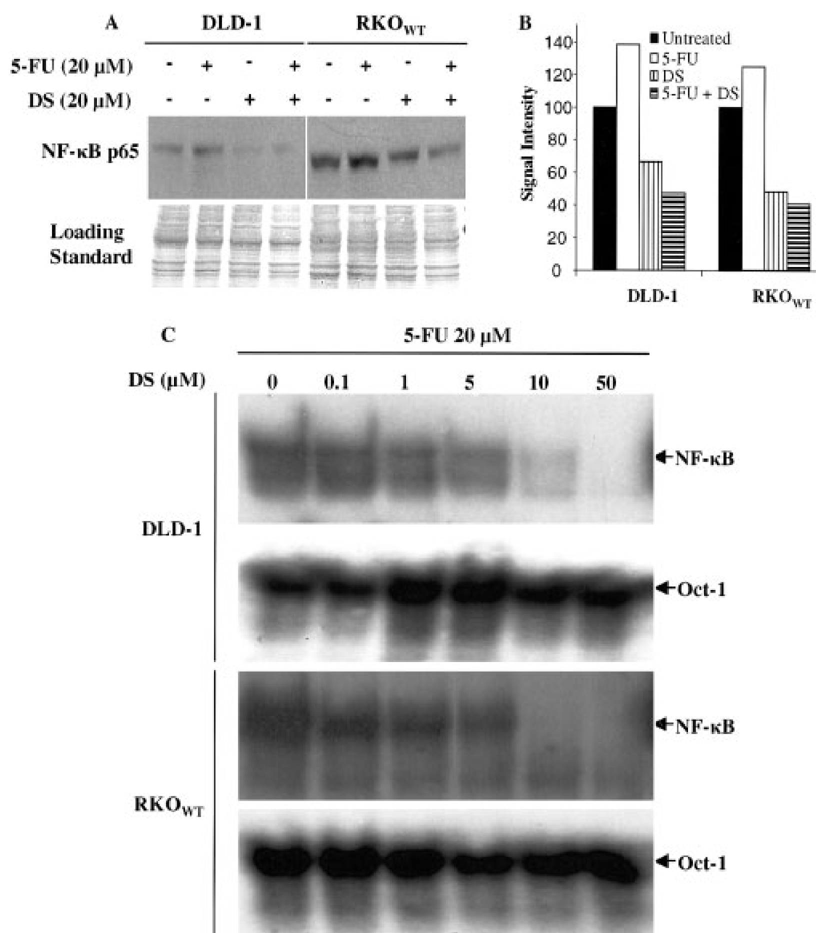

FIGURE 3 – The effect of 5-FU and DS on NF-κB nuclear translocation and *in vitro* DNA binding activity. *a*: Cells were treated with 5-FU or DS for 24 hr. The NF-κB/p65 protein pool in nuclear extracts was detected by Western blotting. The nuclear protein loading was standardized by SimpleBlue SafeStain staining (lower panel). *b*: Comparison of the band intensity in (*a*). *c*: DS inhibited NF-κB DNA binding activity *in vitro*. The nuclear extracts from 5-FU-treated cells were pre-incubated with indicated concentrations of DS at 37°C for 30 min and subjected to EMSA analysis. Oct-1 was used as loading control.

for RKO_{WT} and DLD-1 cells, respectively. To test if 5-FU and DS regulated NF-κB activity via the IκBα route, IκBα protein levels in the cytoplasm of drug-treated cell lines were measured by Western blotting. 5-FU influenced the cytoplasmic pool of IκBα in a time-dependent manner (Fig. 4*a*). IκBα protein degradation was observed within 15 min and reached its lowest level at 60 min after 5-FU treatment. DS did not protect IκBα from 5-FU-induced degradation (Fig. 4*a*). By comparison of the kinetics of NF-κB with IκBα after 5-FU treatment, our study indicated that IκBα degradation was mainly responsible for triggering NF-κB nuclear localisation at an early stage (within 120 min) instead of maintaining its activity at later stage. After 180–360 min exposure to 5-FU, cytoplasmic IκBα protein in RKO_{WT} and DLD-1 cells returned to baseline levels. NF-κB activity in both cell lines was constantly induced above control values (Fig. 1*b*, 4*b*).

DS enhanced 5-FU-mediated apoptosis

The upregulation of NF-κB activity by 5-FU might lead to autoantagonism of cytotoxicity. Inhibition of NF-κB activity with DS may therefore potentiate the anticancer effect. To test this hypothesis, the effect of DS on the cytotoxicity of 5-FU in DLD-1 and RKO_{WT} cell lines was studied using MTT analysis. DS was cytotoxic to the tested cell lines (Fig. 5*a*). The cytotoxicity of 5-FU in DLD-1 and RKO_{WT} cell lines was significantly increased by combining use of 5-FU and IC_{20} concentration of DS (Fig. 5*b*, Table I). To determine if the combining effect of DS and 5-FU is synergistic, the effect of these two drugs on DLD-1 and RKO_{WT} cells was subjected to CI-isobologram analysis.[31] The results (Table I) indicate a synergistic effect over a wide dosage range of IC_{50} to IC_{90}. The highest synergistic effect appeared at the IC_{75} dose of these 2 drugs in combination. DS also significantly enhanced the cytotoxicity of 5-FU to 5-FU resistant cell line $H630_{5\text{-}FU}$ and completely reversed the chemoresistance *in vitro* (Fig. 5*c*, Table I).

Either 5-FU or DS could induce apoptosis and the apoptotic effect was increased when the drugs were used in combination (Fig. 5*d*,*e*). Figure 5*d* shows the morphology of single and combination treatment with 5-FU (10 μm) and DS (10 μm) for 24 hr. More cells were detached from the flask surface after treatment with DS plus 5-FU and the floating cells demonstrated apoptotic morphology (nuclear condensation and fragmentation; inserts, Fig. 5*d*). Figure 5*e* shows *in situ* apoptotic status of DLD-1 and RKO_{WT} cells after drug treatment. The number of TUNEL stained nuclei is significantly higher in DS/5-FU-treated cells than in single-drug-treated cells (Fig. 5*f*, $p < 0.01$). DNA fragmentation was induced in both RKO_{WT} and DLD-1 cells after 24 hr exposure to 5-FU (10 μM) and DS (10 μM) (Fig. 5*g*, lanes 4, 8).

DISCUSSION

NF-κB is a transcription factor

NF-κB activity can be induced by exposing tumour cells to some anticancer drugs (*e.g.*, irinotecan and paclitaxel), cytokines (TNF-α) and irradiation.[1,7–9] In our study, we tested if 5-FU could also induce NF-κB activity. The CRC cell lines DLD-1 and RKO_{WT} had relatively high nuclear NF-κB baseline DNA binding activity and this activity was further induced by exposure to 5-FU. NF-κB p65 is the most transcriptionally active NF-κB subunits involved in tumourigenicity and blockage of its expression with antisense oligo nucleotides induces regression of colon and breast tumour xenografts in nude mice.[35] The supershift assay showed that NF-κB activity induced by 5-FU was composed of Rel A

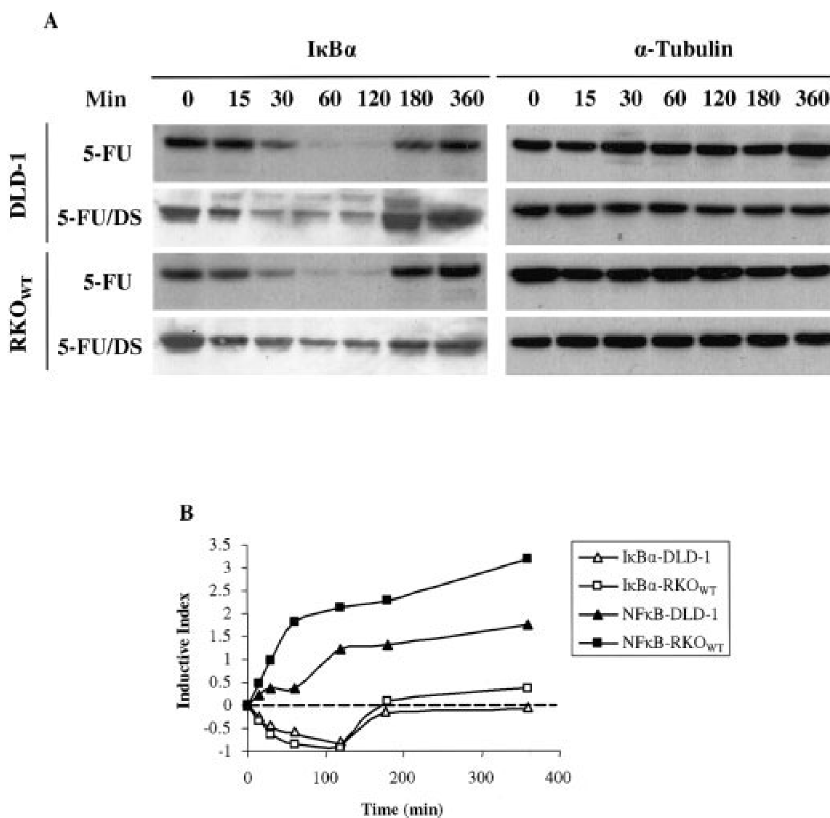

FIGURE 4 – The effect of 5-FU and DS on cytoplasmic pool of IκBα protein. *a*: Western blotting analysis of IκBα in cytoplasmic extract (60 μg/lane) from cells exposed to 5-FU (10 μM) or 5-FU (10 μM) plus DS (10 μM) for 0–360 min. Tubulin: loading standard. *b*: Kinetics of nuclear NF-κB binding activity (EMSA) and cytoplasmic IκBα protein pool (Western blotting analysis) after 5-FU (10 μM) treatment. Inductive index = band intensity of 5-FU-treated cells/band intensity of untreated cells.

(p65) and p50/p105. The p65/p50 is the most common NF-κB dimer and plays a major anti-apoptotic role in anticancer drug treated mammalian cells.[13,14,36] The enhanced NF-κB DNA binding affinity in tumour cells was partially due to 5-FU induced NF-κB p65 nuclear translocation (Figs. 1*a*,*b*, 3*a*,*b*).

IκBα is a predominant member of IκB family and its degradation is thought to be a major mechanism for induction and maintenance of high nuclear NF-κB activity.[4,37,38] 5-FU also induced IκBα degradation in DLD-1 and RKO_{WT} cells. It is well documented that high nuclear NF-κB can bind to the κB site on the promoter region of IκB and induce its re-synthesis.[38] The newly synthesized IκB will be translocated into nucleus, where it binds and transports NF-κB to cytoplasm.[39] The autoregulatory loop between NF-κB and IκB is responsible for maintenance of the homeostasis of NF-κB and IκB. In our study, IκBα was degraded in both cell lines as early as 15 min after 5-FU treatment. IκBα reached its lowest levels at 60 min and returned to pre-treatment levels by 180–360 min. The nuclear DNA binding activity of NF-κB, however, increased continuously during this period (Fig. 4). The high NF-κB activity was maintained for 48 hr after 5-FU treatment (data not shown). These results indicate that, at most, the 5-FU-induced IκBα degradation was mainly involved in triggering NF-κB activity at an early stage. Other mechanisms may be involved in the maintenance of 5-FU-induced NF-κB activity in CRC cell lines. It was reported that constitutive nuclear NF-κB activity in 2 chemoresistant lymphoma cell lines was caused by aberrant activation of IKKs and overexpression of defective IκBα.[40] NF-κB activity can also be modulated by IκB-independent NF-κB phosphorylations.[41] Further study is required to elucidate the mechanism maintaining 5-FU-induced NF-κB activity in CRC cell lines.

DS is already used in the clinic as an anti-alcoholism drug.[16] It has anti-oxidative activity and strongly inhibits ROS induced NF-κB activity in T cells.[24,25] Our results showed that DS significantly inhibited NF-κB activity in 5-FU treated cells in a dose-dependent manner (Fig. 2). In living cells, NF-κB activity is mainly influenced by its nuclear translocation and DNA binding activity.[3] DS strongly reduced constitutive and 5-FU-induced NF-κB nuclear translocation and inhibited NF-κB DNA binding activity in DLD-1 and RKO_{WT} cell lines *in vitro* (Figs. 2, 3). Structurally, all members of the NF-κB family contain an approximately 300 amino acid N-terminal Rel homology (RH) domain that plays a pivotal role in NF-κB dimerization, DNA binding and nuclear translocation. Some NF-κB inhibitors can modify free sulfhydryl groups and target key DNA binding amino acids in RH domain.[42,43] DS and its metabolite, DDC, can react with sulfhydryl groups on some enzymes and interfere with their biological functions.[44,45] Sulfhydryl group(s) in NF-κB protein may be a potential target for DS.

Similar to NF-κB, AP-1 is a eukaryotic transcription factor that is involved in an incredible diversity of molecular biological reactions. The activities of both NF-κB and AP-1 are correlated in response to some cytokines, anticancer drugs, oxidative stimuli and inhibiting small molecules.[26,34] In our study, these 2 transcription factors were regulated differentially. 5-FU and DS stimulated and suppressed NF-κB activity respectively but both had no effect on AP-1 activity. 5-FU specifically upregulated NF-κB activity in DLD-1 and RKO_{WT} cells, as the activity of other tested transcription factors (AP-1, AP-2, SP-1, Oct-1, TFIID and CREB) was not influenced by 5-FU treatment.

In most instances, anticancer drug-induced NF-κB activity confers chemoresistance.[10–12] Inhibition of NF-κB activity can enhance the cytotoxic effect of irinotecan and TNFα.[14,36] Liu *et al.*[26] reported that DS inhibits NF-κB activity, induces G1/S arrest and apoptosis in human hepatoma Hep G2 cells. Based on these facts, we tested the enhancing effect of DS on the cytotoxicity of 5-FU in sensitive DLD-1, RKO_{WT} and resistant $H630_{5\text{-}FU}$ cell lines. In combination with DS, the *in vitro* IC_{50} doses of 5-FU for RKO_{WT}, DLD-1 and $H630_{5\text{-}FU}$ cells were 4.5-, 9.1- and 83-fold lower than for 5-FU alone. 5-FU resistance in $H630_{5\text{-}FU}$ cells was totally

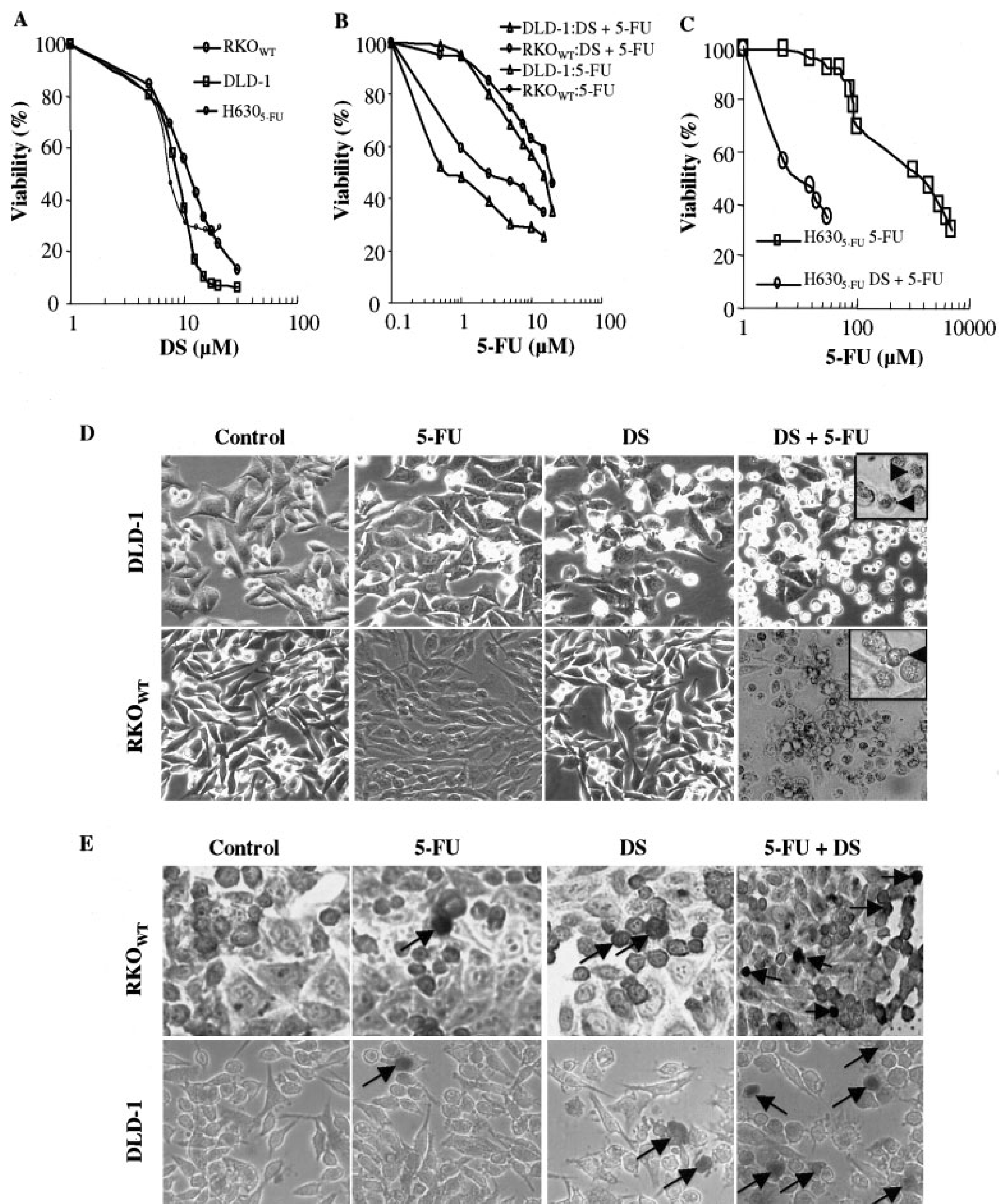

FIGURE 5 – DS-enhanced 5-FU-induced cytotoxicity and apoptosis to DLD-1, RKO$_{WT}$ and H630$_{5\text{-FU}}$ cells. *a*: Cytotoxicity of DS alone to DLD-1, RKO$_{WT}$ and H630$_{5\text{-FU}}$ cell lines. *b*,*c*: Cytotoxicity of 5-FU or 5-FU plus DS (5 μM for DLD-1, H630$_{5\text{-FU}}$ and 6.5 μM for RKO$_{WT}$ cells) to DLD-1, RKO$_{WT}$ (*b*) and H630$_{5\text{-FU}}$ (*c*). Cells were exposed to drugs for 96 hr and tested by MTT analysis as described. *d*: The micro-morphology of drug treated DLD-1 and RKO$_{WT}$ cells (×100 magnification). The arrowheads in the inserts: nuclear condensation and bulb (×200 magnification). *e*: Drug induced *in situ* apoptotic cells (arrows) were detected by TUNEL staining (×200 magnification). *f*: Quantification of TUNEL stained cells. The column and bar = mean and SD respectively. TUNEL-stained cells from 50 high power fields (HPF, ×200 magnification) were counted. *g*: Genomic DNA fragmentation in drug treated DLD-1 and RKO$_{WT}$ cells was analysed by 1.5% agarose gel electrophoresis.

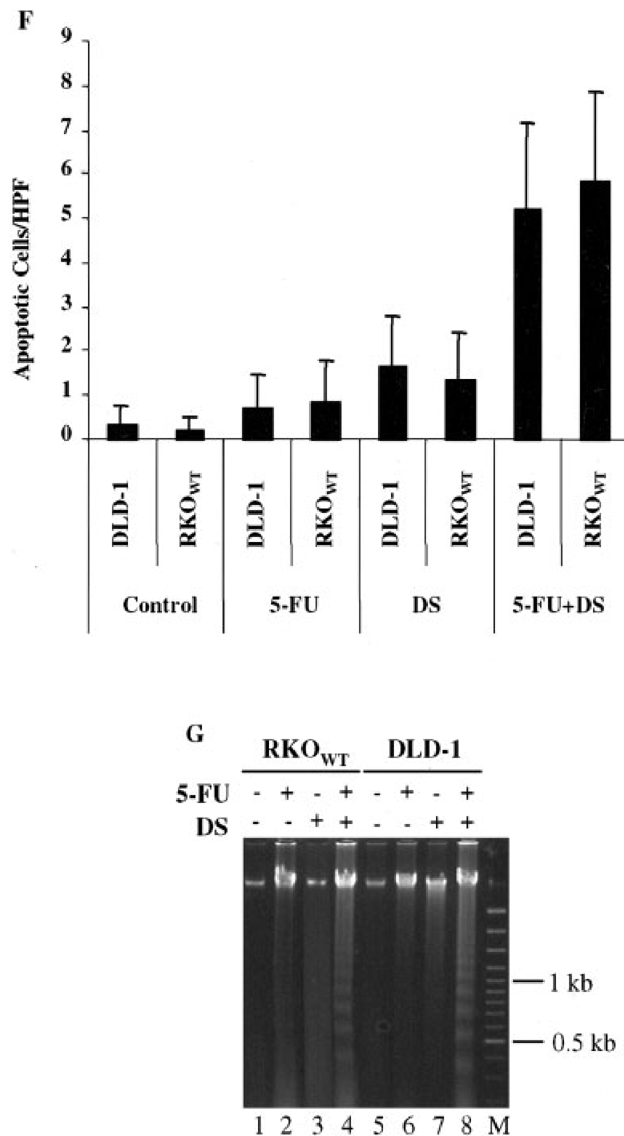

FIGURE 5 – CONTINUED

TABLE I – IC_{50} (μM) OF 5-FU USED ALONE OR IN COMBINATION WITH DS AND COMBINATION INDEX OF 5-FU + DS FOR DLD-1, RKO_{WT} AND $H630_{5\text{-}FU}$ CELL LINES

	DLD-1	RKO_{WT}	$H630_{5\text{-}FU}$	$H630_{WT}$
IC_{50} of 5-FU				
5-FU alone	13.6	17.1	1040	18.8
5-FU + DS[1]	1.5	3.8	12.5	ND
CI of 5-FU + DS				
IC50	0.600[2]	0.819	ND	ND
IC75	0.521	0.707	ND	ND
IC90	0.573	0.723	ND	ND

[1]DS concentrations: 5 μM for DLD-1, $H630_{5\text{-}FU}$ and 6.5 μM for RKO_{WT} cells.–[2]CI: 0.9–1.1 = additive effect; 0.8–0.9 = slight synergism; 0.6–0.8 = moderate synergism; 0.4–0.6 = synergism; 0.2–0.4 = strong synergism. ND, not determined.

reversed by the combining use of DS *in vitro* (Fig. 5, Table I). CI-isobologram results indicated a synergistic cytotoxic effect between DS and 5-FU (Table I). TUNEL and DNA fragmentation results confirmed that apoptosis was the mechanism of cell death. It remains unclear how DS enhanced the apoptotic effect of 5-FU on cancer cells. It may augment the cytotoxic effect of 5-FU by suppressing NF-κB activity and furthermore blocking the expression of NF-κB downstream anti-apoptotic proteins *e.g.*, the *IAP* proteins, *IEX-1L*, *TRAF1*, *TRAF2*, *Bcl-2* homologue *A1/Bfl-1* and caspase activity.[10–13] Chemoresistance is the bottleneck for successful cancer chemotherapy. Our unpublished data demonstrated that compared to parent cell lines, NF-κB activity was much higher in TS inhibitor (5-FU and TDX) resistant cell lines. Inhibition of NF-κB activity by DS may open a new avenue for treatment of TS inhibitor resistant cancers. Many advanced CRC tumours harbour p53 mutations that confer chemoresistance.[46] DS was able to enhance 5-FU cytotoxicity on both p53 mutant (DLD-1) and wild-type (RKO_{WT}) cell lines.

Intratumoural injection of a mutant IκBα can significantly augment the cytotoxic effect of irinotecan on human CRC cell derived xenografts.[14,37] Virus-based gene therapy approaches are not currently achieving success in clinical trials, however, and are also limited for use in widely disseminated metastases. DS may be able to overcome this dilemma. *in vivo*, DS is promptly reduced to its metabolite DDC that is also an NF-κB inhibitor.[47,48] DS blood concentrations after an oral dose of 500 mg in an adult patient are ~20 μM.[49] It is much higher than the DS concentrations needed to achieve *in vitro* cytotoxicity. DS can effectively protect normal cells in kidney, gut and bone marrow from the damage of cisplatin and radiation *in vivo* and increase the therapeutic index.[20,21] Thus, it should be relatively simple to translate its anticancer usage from animal study to clinical trials.

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