Signaling for apoptosis induced by topoisomerase poisons

Roles of NF-κB and 26S Proteasome in Apoptotic Cell Death Induced by Topoisomerase I and II Poisons in Human Non-Small Cell Lung Carcinoma

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SUMMARY

Activation of signaling pathways following DNA damage induced by topoisomerase poisons can lead to cell death by apoptosis. Treatment of human non-small cell lung carcinoma (NSCLC-3 or NSCLC-5) cells with the topo I poison SN-38 or the topo II poison etoposide (VP-16) leads to activation of NF-κB prior to induction of apoptosis. Inhibiting the degradation of IκBα by pre-treatment with the proteasome inhibitor MG-132, significantly inhibited NFκB activation and apoptosis, but not DNA damage, induced by SN-38 or VP-16. Transfection of NSCLC-3 or NSCLC-5 cells with dominant negative mutant IκBα (mIκBα) inhibited SN-38 or VP-16 induced transcription and DNA binding activity of NFκB, without altering drug-induced apoptosis. Regulation of apoptosis by mitochondrial release of cytochrome c and activation of pro-caspase 9 followed by cleavage of poly-ADP-ribose polymerase by effector caspases-3 and -7 was similar in neo and mIκBα cells treated with SN-38 or VP-16. In contrast to pre-treatment with MG-132, exposure to MG-132, following SN-38 or VP-16 treatment of neo or mIκBα cells, decreased cell cycle arrest in the S/G2 + M fraction and enhanced apoptosis compared to drug alone. In summary, apoptosis induced by topoisomerase poisons in NSCLC cells is not mediated by NFκB but can be manipulated by proteasome inhibitors.
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INTRODUCTION

Human non-small cell lung carcinoma (NSCLC) is clinically responsive to chemotherapy with topoisomerase (topo) poisons (1). Etoposide (VP-16) which poisons the nuclear enzyme topoisomerase II (topo II) is currently used in a number of therapeutic protocols. More recently, the topoisomerase I (topo I) poison e.g. irinotecan, has also shown activity in the clinical management of NSCLC (1). The chemotherapeutic efficacy of topoisomerase I and II poisons is presumed to be due to stabilization of a topoisomerase -DNA cleavable complex leading to protein-linked DNA breaks and cell death by apoptosis (2-4). Tumor cell resistance to apoptosis induced by topoisomerase poisons has primarily focused on the membrane efflux pumps affecting cellular drug pharmacokinetics. A major impact of these studies has been the demonstration that reduced cellular drug levels due to overexpression of P-glycoprotein encoded by mdr 1 gene leads to reduced DNA damage, apoptosis and cell death. (5). Both experimental and clinical studies have suggested that overexpression of P-glycoprotein is frequently associated with resistance to topoisomerase II poisons, and occasionally with resistance to topoisomerase I poisons (5). However, unlike the vinca-alkaloids, the magnitude of alterations in cellular drug levels per se, mediated by P-glycoprotein does not correlate with DNA damage or apoptosis in topoisomerase II poison-treated cells (6). Alternatively, reduced formation of drug stabilized topoisomerase -DNA cleavable complex can be due to decreased topoisomerase I/II protein levels or mutations in the enzyme which can directly impact on sensitivity to drug induced apoptosis. (2-4). Although a cause and effect relationship of DNA damage leading to apoptosis appears relatively straightforward, determining signaling events and linking it to DNA damage and apoptosis could potentially lead to understanding the mechanistic basis governing
topoisomerase poison induced apoptotic cell death.

NF-κB is an inducible transcription factor involved in the regulation of genes during inflammatory, acute phase and immune responses (7,8). The inappropriate regulation of NFκB has been implicated in a variety of diseases including cancers (9). Specifically, the activation of NFκB by tumor necrosis factor and the subsequent induction of apoptosis would suggest that these events are linked. However, it has been recently suggested that the activation of NFκB can indeed be anti-apoptotic in response to tumor necrosis factor or topoisomerase poisons (10,11). In addition to tumor necrosis factor, topoisomerase poisons also induce activation of NFκB (11). Since apoptotic cell death is frequently observed in topoisomerase I/II poison treated cells (12), establishing a functional link between NF-κB and drug-induced apoptosis has been pursued (11,13,14).

In the present study using pharmacological inhibitors of proteasome function and the molecular strategy of transfecting a dominant negative IκBα to manipulate the activation of NF-κB in topoisomerase I and II poison treated human NSCLC cells, we determined the signaling pathways contributing to apoptotic cell death. Our data in two independent model systems of human NSCLC, suggest that inhibiting the activation of NFκB by the proteasome inhibitor or transfection of a dominant negative IkBα result in markedly different responses to apoptosis induced by topoisomerase I and II poisons. While the dominant negative IkBα and proteasome inhibitor MG-132 do not affect DNA damage induced by topoisomerase I and II poisons, pre- or post-treatment with proteasome inhibitor MG-132, inhibits or enhances apoptosis respectively. In addition, following DNA damage induced by topoisomerase I or II poisons, neither activation of transcription or the DNA binding
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activity of NF-κB affects drug-induced apoptosis based on the regulation of mitochondrial release of cytochrome c and activation of caspase 9, followed by cleavage of poly-ADP ribose polymerase by effector caspases - 3 and -7.

EXPERIMENTAL PROCEDURES

Materials

The topo I poison SN-38 (active metabolite of irinotecan) and the topo II poison VP-16 were obtained from Pharmacia & Upjohn Co. and Sigma Chemical Co. respectively. Stock solutions of these drugs were prepared in dimethylsulfoxide (Sigma Chemical Co.) and stored frozen at -20°C. The dominant negative IκB (S32A/S36A) cDNA cloned into pUSEamp(+) expression vector and the empty control pUSEamp(+) expression vector were obtained from Upstate Biotechnology, Lake Placid, New York. The PathDetect cis-reporting system pNF-κB-Luc reporter plasmid and pFC-MEKK positive control plasmid were obtained from Stratagene, LaJolla, California. Antibodies to: caspase 8 were purchased from Santacruz Biotechnology Inc., Santa Cruz, Califronia; caspase 9 and cytochrome c were obtained from BD Pharmingen, San Diego, California; kBα were obtained from Upstate Biotechnology Inc., Lake Placid, New York; PARP were purchased from Enzyme Systems Inc. Livermore, California. The fluorogenic substrate LEHD-AFC for determining caspase 9 activity was obtained from BioVision Inc. Palo Alto, California. Cell culture medium and fetal bovine serum were obtained from BioWhittaker Inc., Gaithersburg, Maryland. All other chemicals of analytical grade were obtained from commercial sources.

Cell Lines and Transfection

The human non-small cell lung carcinoma model systems, NSCLC-3 and NSCLC-5 were
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established in culture in the laboratory using specimens obtained from patients during surgical resection of the tumor (15). The parental wild-type NSCLC-3/wt and NSCLC-5/wt cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine and maintained at 37°C in a humidified 5% CO2 plus 95% air atmosphere. Doubling time in vitro for the NSCLC-3/wt and NSCLC-5/wt cells was ~ 35 h.

Transfection of parental wild-type NSCLC-3 or NSCLC-5 cells with a dominant negative IkBα (S32A/S36A) in pUSEamp(+) expression vector under control of the CMV promoter or the empty pUSEamp(+) expression vector was carried out using 4 µg DNA/2 x 10⁶ cells/1.2 ml containing 6 µl of DMRIE-C (GIBCO/BRL, Gaithersburg, Maryland). Stable transfectants were selected by culturing in 1 mg/ml G418.

Measurement of Apoptosis, Drug Stabilized DNA-topo Cleavable Complex Formation and Drug Cytotoxicity

The cells in all experiments were either pre- or post-treated for 30 min with the proteasome inhibitor MG-132 (20 µM). Treatment with the desired concentration of SN-38 or VP-16 for 60 min either followed or preceded treatment with the proteasome inhibitor MG-132. After required drug treatment, control and treated cells were washed in drug-free medium and re-incubated in drug-free medium to determine: (a) target protein levels and/or their activity; and (b) potential signaling events of apoptosis. DNA-topoisomerase cleavable complex formation induced by SN-38 or VP-16 was determined by a modification of the SDS-KCl method (16) using the NSCLC-3 or NSCLC-5 cells labeled overnight with [³⁴C]-thymidine. Apoptosis in drug treated cells was determined using the technique of Muscarella et al (17). Briefly, 2 x 10⁶ control or treated cells were re-suspended in 100
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\(7\) \(\mu l\) staining solution (70\(\mu g/ml\) Hoechst 33342 and 100\(\mu g/ml\) propidium iodide in phosphate buffered saline) and incubated at 37\(^\circ\)C for 15 min. The stained cells were viewed in a fluorescence microscope with the appropriate filters, so as to visualize simultaneously the blue fluorescence from Hoechst 33342 and the red fluorescence from propidium iodide. Normal viable cells fluoresce blue within the nucleus and the apoptotic cells show condensation of chromatin and formation of small masses of varying sizes. Necrotic cells stain pink, but these cells are swollen and the chromatin is not condensed and fragmented as in apoptotic cells. Flow cytometry for cell cycle traverse perturbations was carried out following staining with propidium iodide as described earlier (18). Cytotoxicity induced by SN-38 or VP-16 was determined by a soft agar colony forming assay. Cells were treated with a range of drug concentrations for 60 min at 37\(^\circ\)C in a humidified 5% CQ plus 95 % air atmosphere. Following treatment cells were washed and 3 \(\times\) 10\(^4\) cells plated in triplicate in 35 x 10-mm Petri dishes using RPMI 1640 supplemented with 2 mM L-glutamine and 20 % fetal bovine serum. Colonies were counted following incubation of the Petri dishes for 10-12 days in a humidified 5% CQ plus 95 % air atmosphere.

**Electromobility Shift Assays**

Nuclear extracts from control and treated cells were prepared as described by Dignam et al (19). Electromobility shift assays (EMSA) were carried out using nuclear extracts containing equivalent amounts of protein (10 \(\mu g\)) which were incubated with \(^{32}\)P labeled oligonucleotide containing the consensus sequence (5'-GGGACTTTCC-3'), corresponding to the \(\kappa\)-light chain enhancer motif (20). Assays for supershift were carried out by incubation of nuclear extracts with antibodies to the p65 subunit and the labeled oligonucleotide prior to analysis by EMSA. Cells
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transfected with PathDetect pNF-κB-Luc plasmid cis-reporting system were used to test for the effect of drug treatment on transcriptional activation of NF-κB. Transfection with the pFC-MEKK plasmid was used as the positive control.

Cell Lysis and Western Blotting

Cell lysates prepared in 50mM Tris, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, protease inhibitors (1µg/ml each of aprotinin, leupeptin, pepstatin), and phosphatase inhibitors (1mM NaVO₄, 1mM NaF) were used for detection of IκBα protein levels in western blots. Cell lysates (50 µg protein) were resolved by 10% SDS-PAGE, electroblotted onto nitrocellulose (0.45 µm) and blocked by incubation in 3% non-fat dry milk in PBS for 3 h at room temperature. The membranes were probed with antibody to IκBα (1 µg/ml) overnight at 4°C followed by incubation with secondary antibody for 1 h at room temperature for signal detection by chemiluminescence.

Cytosolic extracts were prepared in extraction buffer (21) containing 220mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol and protease inhibitors to determine cytochrome c protein levels. Control or treated cells were incubated on ice in extraction buffer for 30 min, followed by disruption with a “B” pestle in a glass Dounce homogenizer. After centrifugation of the cell homogenates at 14,000x g, the supernatant containing 50 µg of cytosolic protein was resolved on a 15% SDS-polyacrylamide gel. After electrophoresis, the gels were electroblotted onto PVDF membrane (0.2 µm) and blocked by incubation in 3% BSA, 3% non-fat milk, 0.1% Tween 20 in phosphate buffered saline (PBS) for 3
h at room temperature. The PVDF membrane was probed by incubating with antibodies to cytochrome c (dilution 1: 500) overnight at 4°C followed by horseradish coupled secondary antibodies for 1 h at room temperature for signal detection by chemiluminescence. Lysates prepared from aliquots of control and treated cells were tested for caspase 9 activity using the fluorogenic substrate LEHD-AFC, peptide substrate leucine-glutamic acid-histidine-aspartic acid (LEHD) coupled to 7-amino-4-trifluoromethylcoumarin (AFC).

Cell lysates prepared in 62.5 mM Tris, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, 5%\(\beta\)-mercaptoethanol were used for detection of caspase 9 and PARP cleavage. The lysate samples from control and treated cells were resolved on 10% SDS-polyacrylamide gel, electroblotted onto nitrocellulose, blocked in 5% non-fat dry powdered milk in PBS, and probed with antibody to caspase 9 or antibody C2.10 for PARP, followed by horseradish coupled secondary antibody for detection by chemiluminescence. The antibody to caspase 9 detects both pro- and cleaved active forms of caspase 9 and C2.10 antibody detects the 116 kDa monomeric PARP and cleaved 89 kDa PARP.

**RESULTS**

**Differential effect on DNA binding activity of NF-κB in cells treated with SN-38, VP-16, cis-platinum or paclitaxel**

Results in Figure 1A demonstrate that following treatment of NSCLC-3/wt cells with the topo I poison SN-38 or the topo II poison VP-16, there is a significant increase in the DNA binding activity of NF-κB compared to the untreated control. The activation of NF-κB by SN-38 and VP-16 is dose dependent and specific for topoisomerase poisons, since treatment with the microtubule poison.
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paclitaxel (PCT) or the DNA damaging agent cis-platinum (CDDP) produced no measurable increase in the DNA binding activity of NF-κB. In the next series of experiments we determined the time course governing the enhanced DNA binding of NF-κB in topo poison treated cells and the results in Figure 1B demonstrate that maximal enhancement of DNA binding activity of NF-κB in topo poison treated cells occurs at 2-3 h. The results in Figure 1C indicate that with the various drugs at concentrations tested for activation of NF-κB there is significant apoptosis in the NSCLC-3/wt cells by 24 - 48 h and the topo poisons SN-38 and VP-16 induce apoptosis much more rapidly than cis-platinum or paclitaxel. As shown in Figure 2, treatment of NSCLC-5/wt cells with SN-38 or VP-16 but not cis-platinum or paclitaxel, significantly enhanced the DNA binding activity of NF-κB. The data in Figures 1 and 2 suggest that in NSCLC-3/wt or NSCLC-5/wt cells treated with a topo I or topo II poison, activation of NF-κB precedes the subsequent induction of apoptosis.

The Proteasome inhibitor MG-132 inhibitstopo poison induced NF-κB DNA binding activity and apoptosis

It is well recognized that degradation of phosphorylated κB by the 26S proteasome, regulates the DNA binding of NF-κB subunits (22). Thus, using MG-132 as a pharmacological inhibitor of proteasome activity, we determined the effect of pre-treatment for 30 min of NSCLC-3/wt cells with MG-132 on the DNA binding activity of NFκB in cells treated with SN-38 or VP-16. The results in Figure 3A indicate that pre-treatment with 20 µM MG-132 for 30 min, significantly inhibits the DNA binding activity of NFκB in SN-38 or VP-16 treated cells. The attenuated activation of NF-κB in cells pre-treated with MG-132 is consistent with the data in Figure 3B,
Signaling for apoptosis induced by topoisomerase poisons demonstrating diminished degradation of IκBα protein. In subsequent experiments, we analyzed drug induced apoptosis in an attempt to correlate the apoptotic response with the DNA binding activity of NF-κB in cells pre-treated with MG-132. The results in Table 1 indicate that pre-treatment with MG-132, which resulted in the inhibition of NFκB DNA binding activity, also inhibited SN-38 or VP-16 induced apoptosis.

Differential activation of NFκB and degradation of IκBα in topo poison treated neo or mIκBα transfected cells

The data with MG-132 pre-treatment (Figure 3) suggest that in NSCLC-3/wt cells treated with SN-38 or VP16, reduced activation of NF-κB and apoptosis is correlated. To establish a functional link between NFκB activation and apoptotic signaling pathways in SN-38 or VP-16 treated cells, experiments were carried out in NSCLC-3/wt or NSCLC-5/wt cells stably transfected with vector control (NSCLC-3.neo, NSCLC-5.neo) or mutant IκBα (NSCLC-3/mIκBα or NSCLC-5/mIκBα). The results in Figure 4A demonstrate that following treatment with SN-38 or VP-16, the significant increase in the DNA binding activity of NFκB observed in the parental or vector control (neo) cells, is absent in cells transfected with dominant negative mutant IκBα (mIκBα). Consistent with this differential response to the DNA binding activity of NFκB in neo versus mIκBα cells treated with SN-38 or VP-16, the transcriptional activation of NF-κB is also inhibited in themIκBα, but not in neo cells treated with VP-16 (Figure 4B). Treatment with VP-16 results in the rapid degradation of IκBα protein in the neo but not mIκBα cells (Figure 4C). Similar to the data obtained in NSCLC-3 cells, a differential response to SN-38 or VP-16 stimulated DNA binding activity of NF-κB is seen in NSCLC-5 cells.
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κB, transcriptional activity of NF-κB and degradation of IκBα protein is also observed between the neo and mIκB transfected NSCLC-5 cells (Figure 5A - 5C).

Signaling events and apoptosis induced by SN-38 or VP-16 are similar in neo or mIκB transfected cells

Since drug-induced NF-κB activation in the neo and mIκBα transfected cells was different, the temporal regulation of events which lead to the induction of apoptosis in NSCLC-3/neo or NSCLC-3/mIκBα and NSCLC-5/neo or NSCLC-5/mIκBα cells treated with SN-38 or VP-16 was determined. Preliminary studies revealed that SN-38 or VP-16 induced apoptosis in the neo or mIκBα transfectants was not mediated by Fas or FasL and the parental and transfected cells were caspase 8 deficient (data not shown).

Immunoblotting results in Figure 6A indicate that following treatment with either SN-38 or VP-16 a detectable increase in cytosolic cytochrome c was apparent at 4 h. This was followed by conversion of caspase 9 from the pro- to the active form at 6 h with maximal levels being detectable at 24 h (Figure 6A). Consistent with the immunoblot results on activation of caspase 9, experiments on caspase 9 activity using the specific substrate LEHD-AFC also revealed detectable activity at 6 h (Figure 6A). Cleavage of PARP by the effector caspase -3 and -7 following activation of caspase 9 was detected as early as 6 h and maximal levels of cleaved PARP product occurred at 24 h (Figure 6A). The morphological determination of apoptosis by fluorescence microscopy indicated that drug induced apoptosis was dose dependent and similar between the neo and mIκBα transfected NSCLC-3 (Figure 6B). Confirming the data on topo poison induced apoptosis (Figure 6B), the results in Table 2 indicated that the clonogenic cell survival of VP-16 or SN-38 treated NSCLC-3/neo and NSCLC-
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3/mIκBα is similar in soft agar colony forming assay. The data in Figure 7 show results on mitochondrial release of cytochrome c and activation of caspases which lead to SN-38 or VP-16 induced apoptosis in NSCLC-5.neo and NSCLC-5/mIκBα cells. Overall, following treatment with VP-16 or SN-38, results on mitochondrial release of cytochrome c, the conversion of unprocessed pro-caspase 9 (46-48 kDa) to active caspase 9 (35 kDa, 37 kDa), caspase 9 activity and the cleavage of PARP by effector caspases -3 and –7, suggest no apparent differences in the temporal regulation of apoptotic pathways between the neo and mIκBα transfected NSCLC-3 or NSCLC-5 cells.

Post-treatment with the proteasome inhibitor MG-132 potentiates topo poison induced apoptosis and is independent of NF-κB activity

Earlier experiments (Figure 3) on pre-treatment with the 26S proteasome inhibitor MG-132 indicated that the DNA binding activity of NFκB, degradation of IκBα and apoptosis induced by SN-38 or VP-16 were inhibited. However, comparative studies with the neo and mIκB transfectants of NSCLC-3 (Figure 6) or NSCLC-5 (Figure 7) cells demonstrated that SN-38 or VP-16 induced activation of NF-κB may not be required for apoptosis. To further evaluate the mechanisms contributing to these discrepant results we determined the effect of pre- or post-treatment with MG-132 on SN-38 and VP-16 induced cell cycle traverse perturbations and apoptosis in NSCLC-3/wt or the neo and mIκB transfectants of NSCLC-3 or NSCLC-5 cells. Results in Figure 8A demonstrate that in SN-38 or VP-16 treated NSCLC-3 cells: (a) pre-treatment with MG132 significantly inhibits apoptosis, and (b) post-treatment for 30 min with MG-132 results in a significant (>3-fold) increase in apoptosis. As shown in Figure 8B, an analysis of cell cycle traverse perturbations demonstrated that treatment with VP-16 alone or the pre-treatment with MG-132 followed by VP-16, resulted in the
accumulation of cells in the S + G₂/M boundary at 24 hr and a measurable increase in the apoptotic
(sub-G₁) cell population. In contrast, post-treatment for 30 min with MG-132 after VP-16 exposure,
resulted in no remarkable accumulation of cells at the S-G₂ +M boundary but produced a marked
increase in the apoptotic (sub-G₁) population suggesting either apoptosis in this phase or transit
through cycle and mitotic catastrophe. The differential effect of pre- or post-treatment with MG-132
on SN-38 or VP-16 induced apoptosis is not unique to NSCLC-3/wt cells or dependent on NF-κB
activation, since post-treatment with MG-132 also enhanced apoptosis in the neo or mIκB transfected
NSCLC-3 or NSCLC-5 cells (Figure 9 and Figure 10).

**DISCUSSION**

Drugs that poison the enzymes topo I or topo II stabilize topo - DNA cleavable complex
formation, which leads to protein linked DNA strand breaks and cell death (2-4). Although, it is
generally accepted that topoisomerase I or topoisomerase II poisons produce DNA damage and
induce cell death by apoptosis, it remains to be addressed whether the signaling pathways that
regulate the initiation of apoptosis induced by topo poisons are dependent on the DNA damage. Since
it is well established that the activation of NFκB is an inducible stress response and topo poisons are
effective in stimulating this pathway (20) we examined the functional role for NFκB activation in
apoptosis induced by SN-38 or VP-16. Our results indicate that NF-κB (based on an increase in DNA
binding activity) is indeed activated following treatment with the topo I poison SN-38 or the topo II
poison VP-16, but not with cis-platinum or taxol, although all of these agents are potent inducers of
apoptosis. The anti-apoptotic role of NF-κB has been suggested as a mechanism of resistance to
chemotherapy, since attenuation of NF-κB activity in topoisomerase poison treated cells leads to
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stimulation of an apoptotic response (11). The present data demonstrating reduced apoptosis following inhibition of NF-κB activity by pre-treatment with the proteasome inhibitor MG-132 suggests that activation of NF-κB mediates apoptosis induced by SN-38 or VP-16. However, these results contradict data obtained with the neo and mIκBα transfected NSCLC-3 or NSCLC-5 cells, wherein the differential activation of NFκB did not alter apoptosis or clonogenic cell survival (NSCLC-3/neo and NSCLC-3/mIκBα) in a soft agar colony assay following treatment with SN-38 or VP-16.

A proposed sequence of events regulating chemically induced apoptosis involves release of cytochrome c followed by activation of initiator caspases 8 and/or 9 and the effector caspases -3 and -7 (21). In the present study we were able to detect changes in the protein levels and activity of initiators and effectors of apoptosis in a temporal manner after treatment of NSCLC-3 or NSCLC-5 cells with SN-38 and VP-16. Measurable increases in the mitochondrial release of cytochrome c, an initiator of chemically induced apoptosis, were observed 2 - 4 h following treatment with SN-38 and VP-16, and maximal levels were apparent between 24 and 48 h following drug treatment. In these caspase 8 deficient NSCLC-3 and NSCLC-5 cells, the mitochondrial release of cytochrome c was followed by the detection of unprocessed inactive pro-form and the active proteolysed forms of caspase 9 (in western blots) as well as changes in caspase 9 activity. The subsequent cleavage of PARP by effector caspases -3 and -7 was maximal at 24 - 48 h. Signaling pathways regulating SN-38 or VP-16 induced apoptosis in neo and mIκB transfectants of NSCLC-3 and NSCLC-5 cell were also similar, since no differences in the levels or activity of proteins initiating or effecting apoptosis were apparent on a temporal basis. Thus, these results suggest that in human non-small cell lung
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carcinoma cells, NF-κB may not be functionally involved in affecting the initiation or execution of
topoisomerase I/ II poison induced apoptosis.

The differential apoptotic response with the proteasome inhibitor MG-132, whether the
treatment precedes or follows exposure to SN-38 or VP-16, is unique, and indeed suggests that the
downstream apoptotic response can be manipulated without any apparent change in the magnitude
of the DNA damage induced by topoisomerase poisons. The apoptotic response which is decreased
with pre-treatment and increased by post-treatment with MG-132 is not due to altered degradation
of either topo I (23) or topo II in the SN-38 and VP-16 treated cells respectively (data not presented).
The data on reduction in apoptosis with MG-132 pre-treatment suggest that either a delay in
mitochondrial release of cytochrome c or the activation of the pro-caspases may be involved.
However, the increased apoptosis at 24 h with MG-132 post-treatment suggests that an alternate
mechanism based on cell cycle traverse perturbations may exist. Data on cell cycle traverse in VP-16
treated cells indicate that pre-treatment with MG-132 does not affect cell cycle arrest in the S + G₂
/ M fraction. However, post-treatment with the proteasome inhibitor which does not result in
sustained arrest in the S + G₂ / M fraction, leads to enhanced apoptosis possibly due to continued cell
cycle transit. Although a precise mechanism for this response is not readily apparent, the data strongly
support the possibility that interference with proteasome function following DNA damage induced
by topo poisons can affect cell cycle arrest in the late S + G₂ / M fraction. In both pre- or post-
treatment with MG-132, activation of NF-κB induced by SN-38 or VP-16 is inhibited. However, the
role of NF-κB mediating this enhanced apoptotic response is countered by data demonstrating that
post-treatment with the proteasome inhibitor (which inhibits NFκB activity in neo cells) results in
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enhancement of SN-38 or VP-16 induced apoptosis in the neo or mIκBα transfected NSCLC-3 or NSCLC-5 cells.

In summary, the present results demonstrate that in human NSCLC cells treated with topo I poison e.g. SN-38 or a topo II poison e.g VP-16, the apoptosis downstream of drug-induced DNA damage is initiated by the mitochondrial release of cytochrome c, followed by the processing of caspase 9, and the subsequent cleavage of PARP by the effector caspases -3 and -7. These apoptotic pathways are not regulated by activation of NF-κB induced by topoisomerase poisons. In contrast, while inhibitors of the 26S proteasome do not affect topoisomerase poison induced DNA damage, the use of a proteasome inhibitor following treatment with SN-38 or VP-16 remarkably affects the course of drug induced cell cycle traverse perturbations, and significantly enhances the apoptotic response independent of NF-κB activation. Future studies on the role of proteasome function in cellular response to chemically induced DNA damage could provide additional information on the signaling pathways of apoptosis that may be useful in improving the therapeutic benefit of topoisomerase poisons in cancer chemotherapy.

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FIGURE LEGENDS

Figure 1: (A) DNA binding activity of NF-κB determined by EMSA in NSCLC-3/wt cells treated with etoposide (VP-16), active metabolite of irinotecan (SN-38), paclitaxel (PCT) or cis-platinum (CDDP) for 1 h; (B) Time course of DNA binding activity of NF-κB detected in NSCLC-3/wt cells. Cells were treated for 1 h with 100 µM VP-16,
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washed, re-incubated in drug-free medium and nuclear extracts from aliquots of cells retrieved at 0 - 4 h were analyzed for NF-κB DNA binding activity by EMSA; (C) Apoptosis induced by VP-16, SN-38, PCT or CDDP in NSCLC-3/wt cells treated for 1 hr. Following treatment cells were re-incubated in drug-free medium and apoptosis analyzed by fluorescent microscopy at 4 hr, 24 hr and 48 h.

Figure 2: DNA binding activity of NFκB determined by EMSA in NSCLC-5/wt cells treated with etoposide (VP-16), active metabolite of irinotecan (SN-38), paclitaxel (PCT) or cis-platinum (CDDP) for 1 h.

Figure 3: (A) Effect of pre-treatment for 30 min with 20 µM MG-132 followed by 100 µM VP-16 or 0.1 µM SN-38 for 60 min on DNA binding activity of NFκB detected by EMSA in nuclear extracts from NSCLC-3/wt cells; (B) Effect of pre-treatment for 30 min with 20 µM MG-132 followed by 100 µM VP-16 or 0.1 µM SN-38 for 60 min on degradation of IκBα protein in NSCLC-3/wt cells.

Figure 4: (A) Effect of treatment with VP-16 or SN-38 for 60 min on DNA binding activity of NF-κB detected by EMSA in nuclear extracts from neo (NSCLC-3/neo) and mIκBα transfected NSCLC-3/wt cells. Control, Lanes 1, 4, 7, 10, 12 and 14; 40 µM VP-16, lanes 2, 5 and 8; 100 µM VP-16, lanes 3, 6 and 9; 0.1 µM SN-38, lanes 11, 13 and 15. The NF-κB signal and the non-specific (N.S.) binding are identified by arrows; (B) Transcriptional activation of NF-κB in NSCLC-3/neo and NSCLC-3/mIκBα cells transfected with the pNF-κB Luc plasmid cis-reporting system and treated with VP-
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16 for 60 min; (C) Degradation of IkBα protein in NSCLC-3/neo and NSCLC-3/mIkBα cells treated with VP-16 for 60 min.

Figure 5: (A) Effect of treatment with VP-16 (100 µM) or SN-38 (0.1 µM) for 60 min on DNA binding activity of NF-κB detected by EMSA in nuclear extracts from neo (NSCLC-5/neo) and mIkBα (NSCLC-5/mIkBα) transfected NSCLC-5 cells; (B) Transcriptional activation of NF-κB in NSCLC-5/neo and NSCLC-5/mIkBα cells transfected with pNF-κB Luc plasmid cis-reporting system and treated with VP-16 for 60 min; (C) Degradation of IkBα protein in NSCLC-5/neo and NSCLC-5/mIkBα treated with VP-16 for 60 min.

Figure 6: (A) Effect of treatment with VP-16 or SN-38 for 60 min on levels of cytochrome c, caspase 9 levels/activity, and cleavage of PARP in NSCLC-3/neo and NSCLC-3/mIkB cells; (B) Dose dependent effects of VP-16 and SN-38 on induction of apoptosis at 4hr and 24 hr in NSCLC-3/neo and NSCLC-3/mIkBα cells.

Figure 7: Effect of treatment with VP-16 or SN-38 for 60 min on levels of cytochrome c, caspase 9 levels/activity, and cleavage of PARP in NSCLC-5/neo and NSCLC-5/mIkBα cells. Apoptosis at 48 h in NSCLC-5/neo or NSCLC-5/mIkBα treated with 100 µM VP-16 and 0.1 µM SN-38 was ~50% and 27% respectively.

Figure 8: (A) Effect of pre- or post-treatment for 30 min with 20 µM MG-132 on VP-16 or SN-38 induced apoptosis at 24 h in NSCLC-3/wt cells. Apoptosis in NSCLC-3/wt cells treated with VP-16 followed by MG-132 was significantly higher p< 0.007 than
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treatment with VP-16 alone; apoptosis in NSCLC-3/wt cells treated with MG-132 followed by SN-38 was significantly lower p< 0.03 than treatment with SN-38 alone; apoptosis in NSCLC-3/wt cells treated with SN-38 followed by MG-132 was significantly higher p< 0.03 than treatment with SN-38 alone; (B) Effect of pre- or post-treatment with MG-132 on VP-16 induced cell cycle traverse perturbations and apoptosis (% cells in sub G₁ fraction) in NSCLC-3/wt cells.

Figure 9: (A) Effect of pre- or post-treatment with for 30 min with 20 µM MG-132 on VP-16 (100 µM) induced apoptosis at 4 h and 24 h in NSCLC-3/neo and NSCLC-3/mIκBα cells. Apoptosis at 4 hr and 24 hr in NSCLC-3/neo or NSCLC-3/mIκBα cells treated with MG-132 followed by VP-16 was significantly lower p< 0.03 than treatment with VP-16 alone; apoptosis at 4 h in NSCLC-3/neo or NSCLC-3/mIκBα cells treated with VP-16 followed by MG-132 was significantly lower p< 0.01 than treatment with VP-16 alone; apoptosis at 24 h in NSCLC-3/neo or NSCLC-3/mIκBα cells treated with VP-16 followed by MG-132 was significantly higher p< 0.003 than treatment with VP-16 alone; (B) Effect of pre- or post-treatment with for 30 min with 20 µM MG-132 on SN-38 (0.1 µM) induced apoptosis at 4 hr and 24 hr in NSCLC-3/neo and NSCLC-3/mIκBα cells. Apoptosis at 4 h and 24 h in NSCLC-3/neo or NSCLC-3/mIκBα cells treated with MG-132 followed by SN-38 was significantly lower p< 0.04 than treatment with SN-38 alone; apoptosis at 4 h in NSCLC-3/neo or NSCLC-3/mIκBα cells treated with SN-38 followed by MG-132 was significantly lower p< 0.01 than treatment with SN-38 alone; apoptosis at 24 h in NSCLC-3/neo or NSCLC-
Signaling for apoptosis induced by topoisomerase poisons

3/mIκBα cells treated with SN-38 followed by MG-132 was significantly higher p< 0.003 than treatment with SN-38 alone.

Figure 10: (A) Effect of pre- or post-treatment with 20 μM MG-132 on VP-16 (100 μM) induced apoptosis at 48 h in NSCLC-5/neo and NSCLC-5/mIκBα cells. Apoptosis in NSCLC-5/neo or NSCLC-5/mIκBα cells treated with MG-132 followed by VP-16 was significantly lower p< 0.008 than treatment with VP-16 alone; apoptosis in NSCLC-5/neo or NSCLC-5/mIκBα cells treated with VP-16 followed by MG-132 was significantly higher p< 0.03 than treatment with VP-16 alone. (B) Effect of pre- or post-treatment with MG-132 on SN-38 (0.1 μM) induced apoptosis at 48 h in NSCLC-5/neo and NSCLC-5/mIκBα cells. Apoptosis in NSCLC-5/neo or NSCLC-5/mIκBα cells treated with MG-132 followed by SN-38 was significantly lower p< 0.001 than treatment with SN-38 alone; apoptosis in NSCLC-5/neo or NSCLC-5/mIκBα cells treated with SN-38 followed by MG-132 was significantly higher p< 0.002 than treatment with SN-38 alone.
Table 1

Effect of Pre-Treatment with 20 μM MG-132 for 30 min on Apoptosis Induced in NSCLC-3/wt Cells Treated for 60 min with 100μM VP-16 or 0.1 μM SN-38

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptosis (%)</th>
<th>4 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>2.9 ± 0.9</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>20 μM MG-132</td>
<td></td>
<td>3.9 ± 0.9</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>100 μM VP-16</td>
<td></td>
<td>25.1 ± 2.6</td>
<td>28.7 ± 3.9</td>
</tr>
<tr>
<td>20 μM MG-132→100 μM VP-16</td>
<td></td>
<td>16.3 ± 1.8&lt;sup&gt;2&lt;/sup&gt;</td>
<td>22.5 ± 4.1</td>
</tr>
<tr>
<td>0.1 μM SN-38</td>
<td></td>
<td>20.6 ± 1.4</td>
<td>27.2 ± 1.8</td>
</tr>
<tr>
<td>20 μM MG-132→0.1 μM SN-38</td>
<td></td>
<td>13.3 ± 1.3&lt;sup&gt;3&lt;/sup&gt;</td>
<td>22.3 ± 2.0&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> NSCLC-3/wt cells were treated for 60 min with indicated concentrations of SN-38 or VP-16 without or with pre-treatment for 30 min with 20 μM MG-132. Following treatment cells were washed, re-incubated in drug-free medium and apoptosis determined by fluorescence microscopy. Results are the mean± S.D. from triplicate experiments.

<sup>2</sup> Significantly different from treatment with VP-16 alone p=0.001.

<sup>3</sup> Significantly different from treatment with SN-38 alone p=0.003.

<sup>4</sup> Significantly different from treatment with SN-38 alone p=0.035.
Signaling for apoptosis induced by topoisomerase poisons

Table 2

Colony Formation in Soft-Agar of NSCLC-/neo and NSCLC-3/\(\kappa B\)α Cells

Treated with VP-16 or SN-38

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival (% of Control) (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NSCLC-3/neo (^2)</td>
</tr>
<tr>
<td>VP-16</td>
<td></td>
</tr>
<tr>
<td>2.5 μM</td>
<td>41.4 ± 2.8 (^3)</td>
</tr>
<tr>
<td>10 μM</td>
<td>13.9 ± 2.6</td>
</tr>
<tr>
<td>40 μM</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>100 μM</td>
<td>0.3</td>
</tr>
<tr>
<td>SN-38</td>
<td></td>
</tr>
<tr>
<td>0.0025 μM</td>
<td>50.9 ± 1.4 (^3)</td>
</tr>
<tr>
<td>0.01 μM</td>
<td>44.4 ± 2.7</td>
</tr>
<tr>
<td>0.04 μM</td>
<td>40.9</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>30.3 ± 4.1</td>
</tr>
</tbody>
</table>

\(^1\) NSCLC-3/neo and NSCLC-3/\(\kappa B\)α cells were treated with indicated concentrations of SN-38 and VP-16 for 60 min.

\(^2\) Colony forming efficiency of NSCLC-3/neo and NSCLC-3/\(\kappa B\)α was 11.1% and 8.9% respectively.

\(^3\) Data are the mean ± standard deviation using triplicate Petri dishes from at least duplicate experiments.
Figure 1

A

<table>
<thead>
<tr>
<th></th>
<th>VP-16 (µM)</th>
<th>SN-38 (µM)</th>
<th>PCT (µM)</th>
<th>CDDP (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40</td>
<td>0.01</td>
<td>0.5</td>
<td>10</td>
</tr>
<tr>
<td>100 µM</td>
<td>100</td>
<td>0.1</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

B

C

Control 100µM VP-16 0.1µM SN-38 100µM CDDP 1µM PCT

4hr

Apoptosis 1.9 ± 0.7% 28.4 ± 7.0% 20.1 ± 5.4% 2.0 ± 0.5% 2.0 ± 1.3%

24hr

Apoptosis 3.3 ± 2.2% 33.3 ± 2.2% 38.7 ± 1.8% 10.5 ± 2.3% 5.1 ± 0.9%

48hr

Apoptosis 6.8 ± 0.7% 43.1 ± 8.1% 60.9 ± 9.0% 44.4 ± 5.0% 39.3 ± 6.6%
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
VP-16 (100 µM)  

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
<th>C</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
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</thead>
<tbody>
<tr>
<td>NSCLC-5/neo</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NSCLC-5/mIκBα</td>
<td>1.59</td>
<td>3.30</td>
<td>6.76</td>
<td>1.16</td>
<td>2.39</td>
<td>4.16</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

SN-38 (0.1 µM)  

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
<th>C</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
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</thead>
<tbody>
<tr>
<td>NSCLC-5/neo</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSCLC-5/mIκBα</td>
<td>2.46</td>
<td>5.32</td>
<td>10.06</td>
<td>2.06</td>
<td>3.76</td>
<td>8.73</td>
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</tbody>
</table>

Cytochrome c  

Proform Caspase 9  

p37/p35 Caspase 9 activity (fold-increase)  

PARP  

Figure 7
Figure 8
Figure 9
Figure 10
Roles of NF-κB and 26S proteasome in apoptotic cell death induced by Topoisomerase I and II poisons in human non-small cell lung carcinoma
Masahiro Tabata, Rika Tabata, Dale R. Grabowski, Ronald M. Bukowski, Mahrukh K. Ganapathi and Ram Ganapathi

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