

Induction of Biphasic DNA Double Strand Breaks and Activation of Multiple Repair Protein Complexes by DNA Topoisomerase I Drug 7-Ethyl-10-hydroxy-camptothecin

JIAXI WU, MING-BIAO YIN, GUNNAR HAPKE, KÁROLY TÓTH, and YUCEF M. RUSTUM

Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York

Received October 1, 2001; accepted January 2, 2002

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Camptothecins demonstrate a broad spectrum of antitumor activity. Although they are known to trap DNA topoisomerase I on DNA, form cleavable complexes, and generate DNA breaks upon collision with DNA or RNA polymerases, the precise mechanisms predictive for antitumor activity remain to be identified. Recent studies using panels of colorectal and breast cancer cell lines indicate that events downstream of cleavable complexes are more relevant. In this study, we chose SN-38, an active metabolite of irinotecan, to characterize DNA double strand breaks and repair mechanisms induced by this type of drugs using a human head and neck squamous cell carcinoma cell line A253. The results showed that 2-h exposure of cells to an IC₅₀ concentration of SN-38 induces biphasic DNA double-strand break (DSBs): an immediate phase, which was greatly reduced within 8 h, and a lagging phase, culminating 24 h after

drug removal. Three DNA double-strand break repair protein complexes were activated: DNA-dependent protein kinase (DNA-PK), NBS1-MRE11-RAD50, and BRCA1. Aphidicolin, a DNA polymerase inhibitor, abolished both phase I DSBs and the activation of repair protein complexes, suggesting that they resulted from the collision between the cleavable complex and DNA polymerase of S-phase cells. This is in contrast to ionizing radiation-induced activation of DNA-PK and NBS1-MRE11-RAD50 complexes that occur predominantly among non-S-phase cells. The trigger for phase II DSBs cannot be abolished by aphidicolin. The data also indicate that DNA fragments in the size of 50 to 200 kilobases were detected in the lagging phase. This suggests that the late DNA DSBs were associated with apoptotic cell death.

TOP1 inhibitors are a group of novel and promising agents showing broad antitumor activity. They trap TOP1 on DNA, generating reversible cleavable complexes that are not cytotoxic per se. The cleavable complexes are converted into DNA breaks through collision with either DNA or RNA polymerase. Collision with DNA polymerases generates DSBs on the leading strand and TOP1-linked single-strand breaks on the lagging strand (Hsiang et al., 1989). On the other hand, collision with RNA polymerases produces predominantly TOP1-linked single-strand breaks, provided the cleavable complexes are located on the transcribed strand (Wu and Liu, 1997). Collision with RNA polymerases can also lead to DSBs when two cleavable complexes are seated closely on the opposite strands of the DNA duplex (Wu and Liu, 1997). Although the mechanisms of action of TOP1 drugs are well defined, the basis for their selective antitumor activity is not

completely understood. Recent studies using panels of colorectal and breast cancer cell lines have suggested that processing of cleavable complexes and repair of the drug-induced DNA damage are relevant to differential sensitivity among various cell lines (Goldwasser et al., 1995; Davis et al., 1998).

A number of chemical or physical agents, including CPT, induce DSBs, which have been proposed to be responsible for cell death (Frankenberg-Schwager and Frankenberg, 1990; Bennett et al., 1993; Lee et al., 1998). In mammalian cells, three major mechanisms involve DNA DSB repair, including single-strand annealing, nonhomologous end rejoining, and homologous recombination. Although more proteins involved in DSB repair are found and more interactions between these proteins are discovered (Hendrickson, 1997; Karran, 2000; Khanna and Jackson, 2001; van Gent et al., 2001), several research groups have suggested that four protein complexes have thus far been identified to play important roles in the execution of these three repair mechanisms. They are DNA-dependent protein kinases NBS1-MRE11-RAD50, RAD51-

This work is supported by project grant CA65761 and Cancer Center Support Grant CA16056 from the National Cancer Institute. G.H. is supported by grant HA3116/1-1 from the Deutsche Forschungsgemeinschaft, Germany.

ABBREVIATIONS: TOP1, topoisomerase I; CPT, camptothecin; DSB, double-strand break; dThyd, thymidine; SN-38, 7-ethyl-10-hydroxy-camptothecin; DNA-PK, DNA-dependent protein kinase; HBSS, Hanks' balanced salt solution; CFGE, constant-field gel electrophoresis; kb, kilobase; PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; IR, ionizing radiation; ATM, ataxia telangiectasia mutated; SRB, sulforhodamine B.

RAD52-RAD54-RAD55-RAD57, and probably BRCA1/2 (Hendrickson, 1997; Khanna and Jackson, 2001; Karan, 2000; van Gent et al., 2001).

In this study, we used SN-38, an active metabolite of irinotecan, to characterize the DSBs and the repair complexes in a head and neck squamous cell carcinoma line A253. The results showed that SN-38 induced biphasic DSBs. Although phase I DSBs could be inhibited by the DNA polymerase inhibitor aphidicolin the majority of phase II DSBs was not. This suggests the latter were independent of DNA replication. On the other hand, the repair complexes, including DNA-PK, NBS1- MRE11-RAD50, and BRCA1, were activated by SN-38 and could be abolished by aphidicolin.

Materials and Methods

Drugs and Chemicals. SN-38 was provided by Pharmacia (Peapack, NJ). Aphidicolin, proteinase K, RNase A, and alkaline phosphatase were obtained from Roche Applied Science (Indianapolis, IN) and/or Sigma (St. Louis, MO). SN-38 and aphidicolin were dissolved in dimethyl sulfoxide to concentrations of 5 and 1 mM, respectively. [¹⁴C]dThyd (specific activity, 56 mCi/mmol) and [³H]dThyd (specific activity, 20 Ci/mmol) were purchased from Moravsek Biochemicals (Brea, CA) and PerkinElmer Life Sciences (Boston, MA), respectively.

Cell Line and Growth Inhibition Assay. The A253 cell line was purchased from American Type Culture Collection (Manassas, VA) and cultured according to its instructions. All treatments were carried out using exponentially growing cell cultures. The test for detection of mycoplasma in cultured cells was performed using the GEN-PROBE mycoplasma tissue culture rapid detection system every two months (GEN-PROBE Inc., San Diego, CA). In vitro cell growth inhibition was determined using the total protein SRB assay described previously (Yin et al., 1992). Briefly, 600 cells were seeded in 96-well plates. After 24 h, exponentially growing cells were treated with drug for 2 h. At four doubling times after drug exposure, cells were fixed with 10% trichloroacetic acid and further processed according to the published SRB procedure. Absorbance was measured at 570 nm using an automated Bio Kinetics reader (Model EL 340; Bio-Tek Instruments, Winooski, VT). Growth inhibition data from single drug exposures were modeled with the Hill equation $E = E_{con} / [C^m / (C^m + IC_{50}^m)]$, as described previously (Greco et al., 1990). In this equation, E is the measured effect, E_{con} is the control response at zero drug concentration, IC_{50} is the concentration of drug inducing a 50% decrease in the maximal effect when given alone, m is the slope of the concentration-effect curve, and C is the drug concentration.

Preparation of Agarose Plugs. DNA plugs were prepared as described previously (Yin et al., 2000). Approximately 5×10^6 cells were washed using HBSS and resuspended in HBSS. An equal volume of 2% low melting point agarose prepared in HBSS was added at 50°C, and the mixture was immediately poured into molds. Each plug contained approximately 1×10^6 A253 cells. Cells embedded in agarose were digested in 20 volumes of lysis buffer containing 0.5 M EDTA, pH 8.0, 10 mM Tris, 1% Sarkosyl, and 1 mg/ml of proteinase K for 24 h at 50°C. After washing in Tris/EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), the DNA plugs were then incubated for 1 h in Tris/EDTA buffer containing 0.2 mg/ml RNase A. The agarose plugs, which contain purified DNA, were kept in a storage buffer (10 mM Tris-HCl and 50 mM EDTA, pH 8.0) at 4°C and used for electrophoresis.

Constant-Field Gel Electrophoresis. DNA plugs were inserted into wells of a 1.8% agarose gel, which was prepared in 1× Tris/acetate/EDTA buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3). A conventional horizontal submerged unit was used for electrophoresis in Tris/acetate/EDTA buffer at room temperature. After electrophoresis, gels were stained with ethidium bromide and photo-

graphed on a UV-transilluminator. Drug-induced DNA DSBs were quantified by prelabeling with [¹⁴C]dThyd for 24 h. ¹⁴C-labeled DNA was electrophoresed. After CFGE, the bands were cut out, digested with 1 M HCl at 70°C for 1 h, and the quantity of ¹⁴C-labeled DNA fragments was determined by scintillation counting. The counts were normalized as an expression of the ratio of [count of band]/[total DNA count].

Pulsed-Field Gel Electrophoresis. Agarose gels were prepared in 0.5× Tris/borate/EDTA buffer (75 mM Tris, 25 mM boric acid, and 0.1 mM EDTA, pH 8.9) as described previously (Panadero et al., 1995). Agarose plugs containing purified DNA were inserted in to the well of a 0.8% agarose gel, and sealed with a small amount of melted agarose. Electrophoresis was carried out using Hex-A-Field horizontal gel electrophoresis apparatus (Invitrogen, Carlsbad, CA), which contains a hexagonal array of electrodes having a reorientation angle of 120°. The electrophoresis was performed in Tris/borate/EDTA buffer at 14°C with buffer circulation. After electrophoresis, gels were stained with ethidium bromide and then photographed on a UV-transilluminator.

Immunoblot and Immunoprecipitation Analyses. Analysis of protein expression was performed by Western blot. Cells were washed twice with cold HBSS and the cell pellet was lysed in radio-immunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% SDS, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 trypsin inhibitor units of aprotinin). The protein content was determined by the DC protein assay (Bio-Rad, Hercules, CA). Fifty micrograms of total protein were subjected to Western blotting with anti-RPA2 monoclonal antibody (NA-18; Dr. Terry A. Beerman, Roswell Park Cancer Institute, Buffalo, NY); anti-BRCA1 monoclonal antibody (MS110; Oncogene Research Products; Boston, MA), anti-RAD51 antibody (Ab-1; Oncogene Research Products); anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, Lake Placid, NY); or anti-NBS1 antibody (Novus Biologicals, Littleton, CO). Bands were visualized using Renaissance Western blotting kit from PerkinElmer Life Sciences. Immunoprecipitation and phosphatase treatment were performed as described elsewhere (Nagy et al., 1997; Zhao et al., 2000).

Morphological Detection of Apoptosis. Exponentially growing cells were exposed for 2 h to SN-38, washed once, and then maintained in drug-free medium for 24 h and 48 h. Cells were harvested at the indicated time points. Approximately 5×10^4 cells were used for cytopins using the Cytospin 3 from Shandon (Pittsburgh, PA). Apoptosis was evaluated by morphology after conventional hematoxylin and eosin staining. Nuclear condensation and fragmentation identified apoptotic cells. Apoptotic index was calculated as the percentage of apoptotic cells among 300 cells in randomly selected areas of the cytopins. The presence of apoptotic cells were confirmed by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL assay), according to the manufacturer's instructions (Apoptag in situ Apoptosis Detection kit; Intergen, Purchase, NY).

Results

Growth Inhibition of A253 Cell by SN-38. Growth inhibition of A253 cells by 2-h exposure to SN-38 was measured by both SRB and clonogenic assays, and the mean value of IC_{50} from at least three independent experiments was determined to be 0.35 μ M (Fig. 1), which was used for the following study.

Biphasic Profile of DSBs Induced by IC_{50} Concentration of SN-38. The kinetics of DSBs induced by 0.35 μ M SN-38 were quantitated using CFGE. [¹⁴C]dThyd pre-labeled control and drug-treated cells were harvested and the fragmented DNA or DSBs were resolved by CFGE (Fig. 2). A biphasic profile of DNA DSBs was detected. Significant amounts of DNA fragmentation (3.7%) were produced at the

end of the 2-h drug exposure and subsequently reduced to 1.1% at 8 h after the removal of the drug. However, the second phase of DNA fragmentation appeared progressively afterward and accounted for 8.3 and 6.4% of the total amount of DNA at 24 and 48 h, respectively.

Effects of Aphidicolin on SN-38-Induced DNA Fragmentation. To assess whether the induction of DSBs relies on DNA replication, cells were pre- and cotreated with aphidicolin. The data in Fig. 3 shows that the addition of aphidicolin almost completely blocked the generation of phase I DSBs caused by SN-38, whereas only a small proportion of the second phase disappeared, suggesting that the first phase of DSBs were dependent on DNA replication and the second phase was not. No significant alterations of DSBs were observed when cells were treated with aphidicolin alone (data not shown).

Activation of DSB Repair Pathways by IC_{50} of SN-38. We then investigated the four DNA DSB repair complexes, namely NBS1-MRE11-RAD50, RAD51-RAD52-RAD54-RAD55-Rad57, BRCA1/BRCA2, and DNA-PK, which have been suggested to play major roles in the repair of DNA DSBs in mammalian cells. Previous studies using ionizing radiation demonstrated that activation of the first three pathways can be represented by the phosphorylation of NBS1, RAD51, and BRCA1 (Yuan et al., 1998; Chen et al., 1999; Cortez et al., 1999; Lee et al., 2000; Wu et al., 2000; Zhao et al., 2000). In addition, CPT-induced phosphorylation of RPA2 has been reported to be solely dependent on the activation of DNA-PK (Shao et al., 1999). In this study, the activation of DSB repair complexes by SN-38 was evaluated by examining the phosphorylation of these proteins. Figure 4A shows that the mobility of a fraction of NBS1, BRCA1, and RPA2 was retarded after 2-h treatment with SN-38 and the retardation persisted for up to 24 h. To confirm that the retarded bands were the phosphorylated products, the samples were treated with alkaline phosphatase before Western blot analysis. As illustrated in Fig. 4B, the mobility of the retarded bands was

restored to the original position, suggesting that the changes in the mobility were caused by phosphorylation.

Because the dephosphorylated and phosphorylated RAD51s cannot be separated by SDS-polyacrylamide gel electrophoresis, the latter has been detected by anti-phosphotyrosine antibodies after immunoprecipitation with RAD51 antibodies (Yuan et al., 1998; Chen et al., 1999). As shown in Fig. 5A, no significant amount of phosphorylated RAD51 (about 38 kDa) was detected by the anti-phosphotyrosine antibody 4G10, which has been used successfully for studying IR-induced RAD51 phosphorylation (Yuan et al., 1998; Chen et al., 1999). The nitrocellulose membrane was stripped and reprobed with RAD51 antibody and the results showed that RAD51 was not lost during immunoprecipitation (Fig. 5B). To exclude the possibilities that immunoprecipitation procedures might interfere with the phosphorylation of RAD51, cell extracts were subjected to Western blot without immunoprecipitation. RAD51 phosphorylation was not observed (Fig. 5). The alterations of phosphorylated RAD51 was not detected when A253 cells were treated with SN-38 at doses ranging from IC_5 to more than IC_{95} ; A253 cells were X-ray-irradiated with 30 Gy; A253 grown to confluence from 50 to 100% were treated with IC_{50} and IC_{95} concentrations of

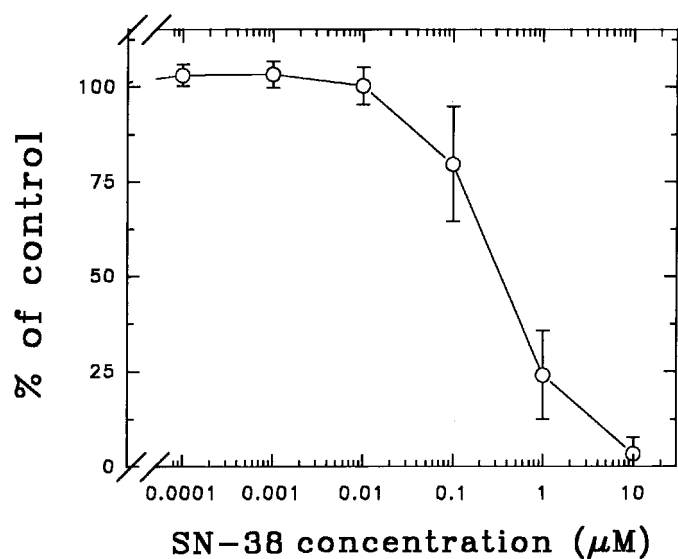


Fig. 1. Growth inhibition of A253 cells. A253 cells were treated with SN-38 for 2 h and growth inhibition was determined after four doubling times by SRB assay as described under *Materials and Methods*. Data are from one representative experiment. Similar results were obtained from at least three independent experiments.

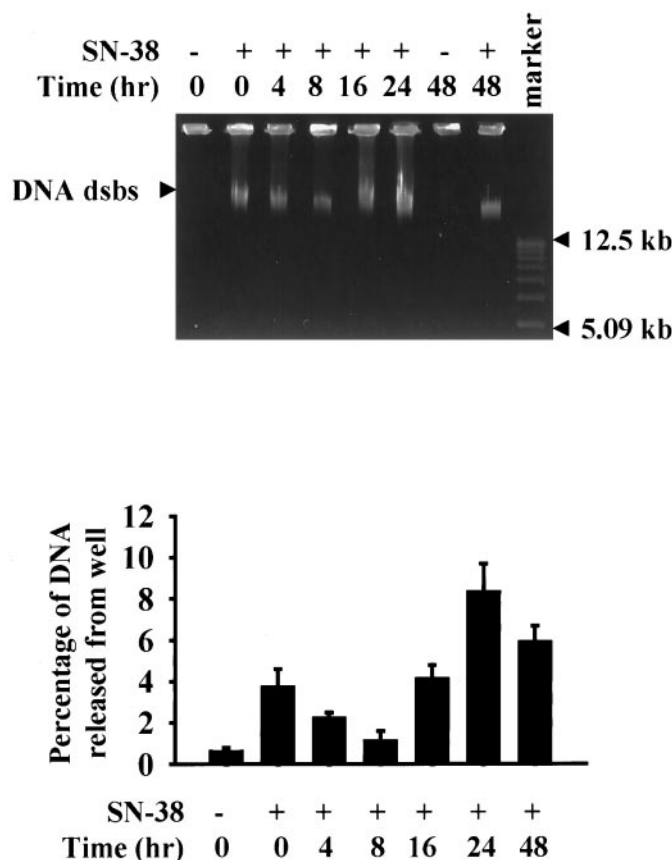


Fig. 2. Kinetics of DNA double-strand breaks induced by SN-38 at IC_{50} . Exponentially growing cells were prelabeled for 20 h with 0.250 μ Ci/ml of [14 C]dThyd, exposed to 0.35 μ M SN-38 for 2 h, washed twice with medium, reincubated in drug-free medium, and collected at the indicated time points. Determination of DNA fragmentation was performed using CFGE with the agarose plugs as described under *Materials and Methods*. The total amount of fragmented DNA or DSBs was quantified as the percentage of DNA released from the well. The upper shows the agarose gels stained by ethidium bromide and the lower are the quantitative plots measured by 14 C incorporation into the DNA.

SN-38; or T24 cell line (which was confirmed to have an elevated level of phosphorylated RAD51 after ionizing radiation) and a normal human fibroblast cell line GM00637 (the phosphorylated RAD51 of which was detected by the 4G10 antibody) were treated with 0.35 and 10 μ M SN-38 (data not shown). However, the phosphorylation of NBS1 in A253 cells was observed under these same four conditions, except when the concentration of SN-38 was below IC_{20} (data not shown). In addition, compared with the control, no evident accumulation of nuclear foci was found when A253 cells were treated with SN-38 at IC_{50} or IC_{95} (data not shown).

Phosphorylation of Repair Proteins Is Abolished by DNA Polymerase Inhibitor Aphidicolin. The effect of aphidicolin on the phosphorylation of repair proteins was examined. As presented in Fig. 6, the retarded bands disappeared not only at the end of drug treatment but also at time points after drug treatment, up to at least 24 h when phase II DSBs were predominant (Fig. 2). These results indicate that the phosphorylation was related to DNA replication and probably induced by first- rather than second-phase DSBs.

PFGE Analysis for the Size of SN-38-Induced DNA Fragments. To assess the patterns of DNA fragmentation induced by SN-38, the size of DNA fragments was further analyzed by PFGE. The results show that the first wave of drug-induced DNA damage was primarily associated with megabase DNA fragmentation. The secondary wave of DNA damage is associated with the induction of 50- to 200-kb DNA fragmentation in addition to the megabase DNA damage (Fig. 7). This is consistent with previous reports suggesting

that the appearance of higher molecular weight DNA fragmentation ranging between 30 and 300 kb was typically associated with apoptotic cell death (Oberhammer et al., 1993; Collins et al., 1997).

Induction of Apoptosis by IC_{50} Concentration of SN-38. To further investigate the downstream effect of the in-

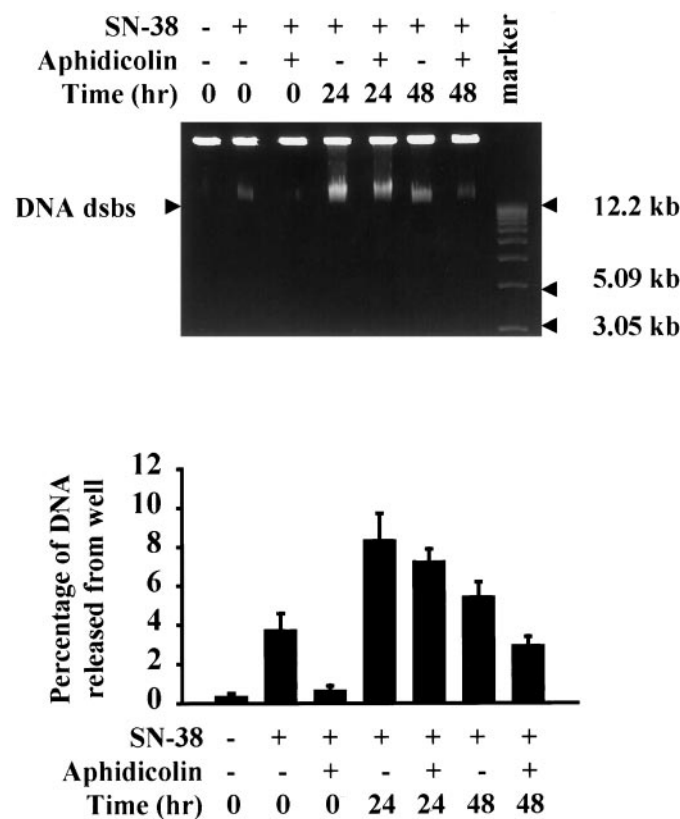


Fig. 3. Effects of aphidicolin on SN-38-induced DNA fragmentation. Cells were exposed to IC_{50} SN-38 in the presence (including a 2-h preincubation) or absence of 1 μ M aphidicolin. DNA damage is quantified as described in Fig. 2.

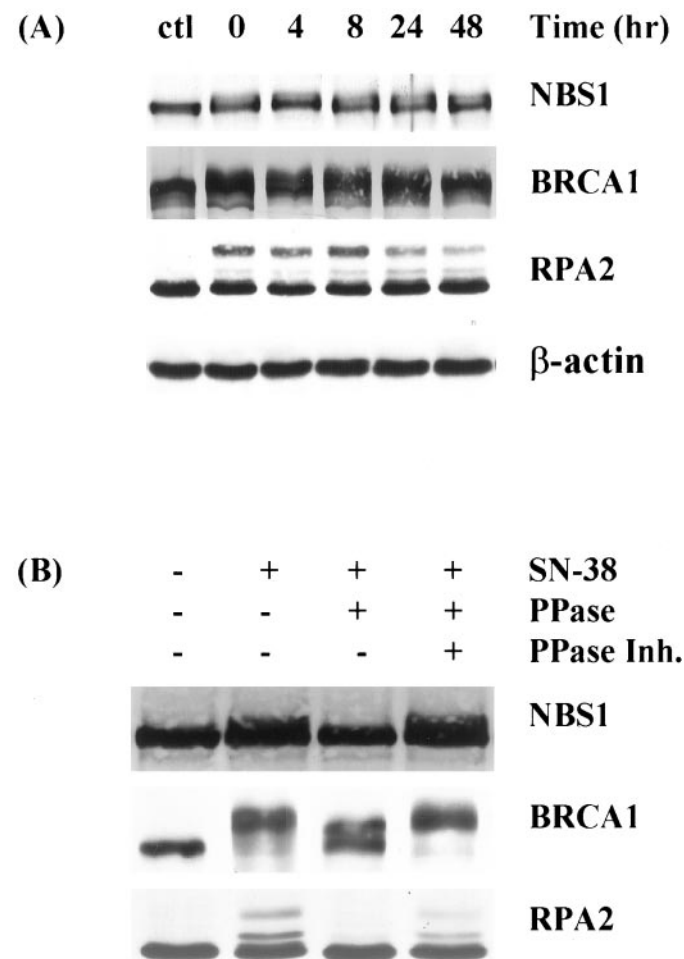


Fig. 4. Phosphorylation of NBS1, BRCA1, and RPA2. Cells were treated with SN-38 at IC_{50} and proteins were extracted. Protein (50 μ g), treated with or without alkaline phosphatase, was resolved by SDS-polyacrylamide gel electrophoresis and blotted with antibodies. Upper, phosphorylation of NBS1, BRCA1, and RPA2 and loading amount control β -actin; lower, alkaline phosphatase treatment for confirmation of phosphorylation.

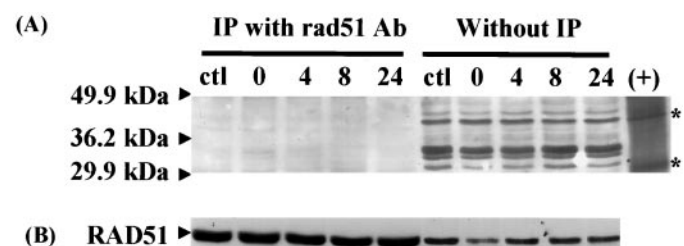


Fig. 5. Absence of RAD51 phosphorylation. Cells were treated with IC_{50} SN-38. Cell extracts were either immunoprecipitated with RAD51 antibody or not before Western blots using anti-phosphotyrosine antibodies. +, lane represents the positive control of anti-phosphotyrosine antibody, which detected the phosphotyrosine containing proteins (indicated by *) extracted from EGF-stimulated A431 cell lysates provided by the Upstate Biotechnology. A, the nitrocellulose membrane was probed by anti-phosphotyrosine antibodies. B, the membrane was stripped and reprobed with RAD51 antibodies.

duction of the two phases of DNA DSBs, the percentage of apoptotic cells were detected in cytopins using the TUNEL assay (Fig. 8, A and B). Beginning 24 h after drug treatment, an increased percentage (control, $0.5 \pm 0.58\%$; 24 h, $1.5 \pm 0.5\%$; 48 h, $8.5 \pm 2.3\%$) of apoptotic cells could be detected. Coherent with this finding cleaved poly(ADP-ribose) polymerase (PARP), a substrate for caspase 3 could be detected beginning at 24 h and increased at further 48 h as shown in Fig. 8C. No increased numbers of apoptotic cells or induction of PARP cleavage could be detected during the early time-points.

Discussion

We have demonstrated that SN-38 at IC_{50} causes two phases of DSBs. Phase I DSBs appear quickly after drug exposure and are gradually reduced, and presumably repaired. These DSBs can be abolished by a DNA replication inhibitor, aphidicolin, suggesting that they result from the

collision between DNA polymerase and cleavable complexes. Phase II DSBs, on the other hand, are not abolished by aphidicolin. The origin of these breaks is not clear. PFGE analysis used to size DNA fragments shows that the first wave of drug-induced DNA damage was primarily associated with megabase DNA fragmentation. The secondary wave of DNA damage is associated with the induction of 50- to 200-kb DNA fragmentation in addition to the megabase DNA damage (Fig. 7). Previous reports suggested that the appearance of molecular weight DNA fragments ranging between 30 and 300 kb was typically associated with apoptotic cell death (Oberhammer et al., 1993; Collins et al., 1997). To support the suggestion, the percentage of apoptotic cells were detected in cytopins using the TUNEL assay. Beginning 24 h after drug treatment, an increased percentage (control, $0.5 \pm 0.58\%$; 48 h, $8.5 \pm 2.3\%$) of apoptotic cells could be detected. Coherent with this finding, cleaved PARP, a substrate for caspase 3, could be detected beginning at 24 h and increased further at 48 h (Fig. 8). This suggests that the late DNA DSBs are at least partially associated with apoptotic cell death.

In addition, it was reported that the collision between cleavable complexes and RNA polymerase produces DSBs (Wu and Liu, 1997). DNA damage during transcription is also known to directly lead to apoptosis. Whether transcription plays a role in the generation of the second-phase DSBs remains to be confirmed. However, investigation for the connections between CPT-induced, transcription-dependent DNA damages and drug sensitivity is complicated and, to our knowledge, unsuccessful thus far, probably because of the lack of specific inhibitors for RNA polymerase I, which transcribes the ribosomal DNA in the nucleolus where the majority of TOP1 is localized under physiological conditions (Buckwalter et al., 1996; Danks et al., 1996). Whatever causes phase II DSBs, it seems that these DSBs are not responsible for the phosphorylation of NBS1, RPA2, and BRCA1.

We show that NBS1, RPA2, and BRCA1 are phosphorylated in response to SN-38, indicating that they or their complexes are activated as indicated by IR studies. IR- and SN-38-activated DSB repair complexes share several common features, including activation of multiple complexes and the persistence of the phosphorylated status of repair proteins hours after the removal of the damaging agents. However, unlike those induced by IR, SN-38 does not induce the phosphorylation of RAD51 (Fig. 5). Moreover, the phosphorylation of the other three complexes is inhibited by the DNA replication inhibitor aphidicolin (Fig. 6), suggesting that these complexes are activated during S- rather than the G₁- or G₂/M-phase of the cell cycle, as in the case of IR (Hendrickson, 1997). The discrepancy between the IR- and SN-38-induced activation of DSB repair complexes probably reflects the complexity of the repair network. The absence of RAD51 phosphorylation is unexpected because the hamster cell line *irs1*, which is defective for XRCC2 (one of the five paralogs of RAD51), is hypersensitive to CPT. On the other hand, overexpression of RAD51 did not change the rate of nonhomologous recombination induced by CPT (Arnaudeau et al., 2001). Therefore, it is possible that the activation of RAD51 could be cell type- or species-specific (Karran, 2000).

The kinases that phosphorylate the proteins are not defined in this study. However, IR studies showed that BRCA1

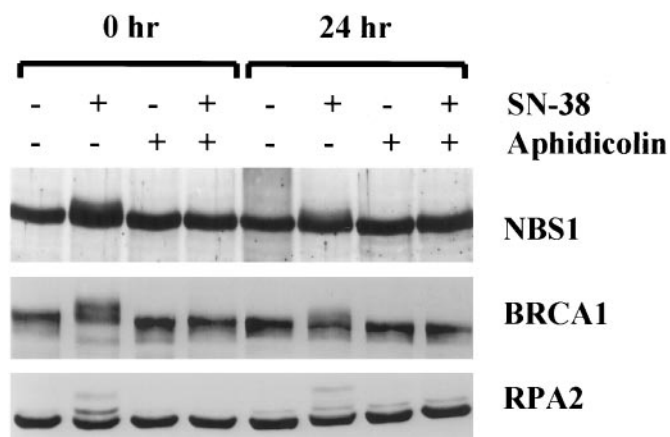


Fig. 6. Effects of aphidicolin on phosphorylation of NBS1, BRCA1, and RPA2. Cells were treated with SN-38 in the presence (including 2 h preincubation) or absence of aphidicolin. Proteins were extracted at indicated time points and then subjected to Western blot.

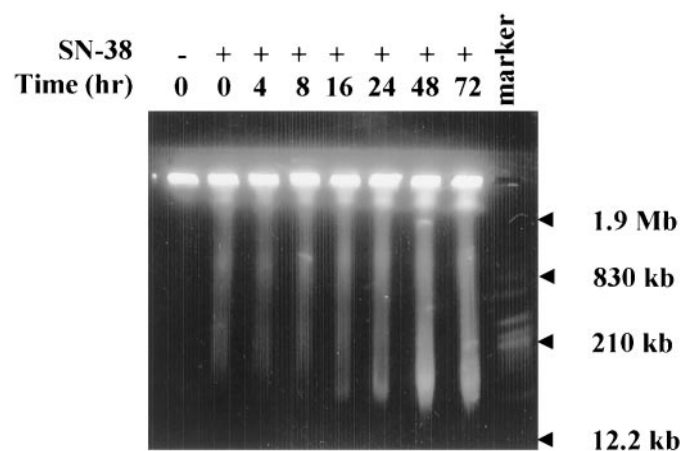


Fig. 7. PFGE analysis for patterns of SN-38-induced DNA fragmentation. Exponentially growing cells were exposed to $0.35 \mu\text{M}$ SN-38 for 2 h and then incubated in drug-free medium for 0, 4, 8, 16, 24, 48, and 72 h. Agarose plugs containing DNA were processed as described under *Materials and Methods*. Determination of DNA damage by pulsed-field gel electrophoresis was carried out in two phases. Phase I consisted of a 20-min pulse time and a 48-h running time, and phase II consisted of a 6-min pulse time and a 24-h running time, at 14°C in 0.8% agarose. Megabase I DNA standards are suitable for sizing double strand DNA from 0.1 to 1.9 megabases.

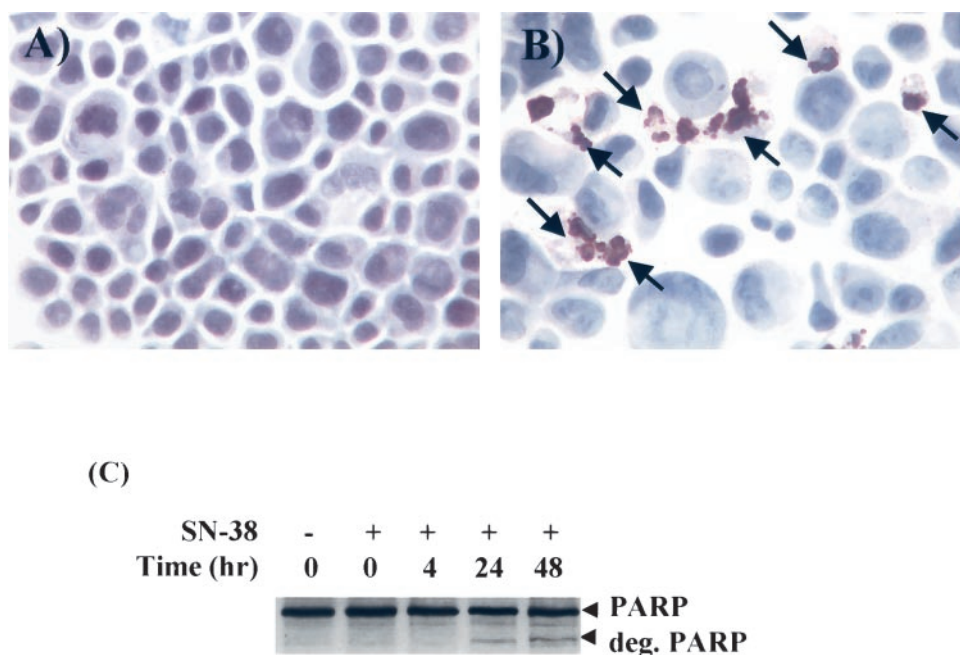


Fig. 8. Detection of apoptosis after treatment with IC_{50} SN-38. Apoptotic cells were detected in cytopins using the TUNEL assay. Shown are two representative examples for untreated control (A) and 48 h after (B) drug treatment. C, detection of PARP degradation in cell extracts by Western blot at indicated time points.

can be phosphorylated by ATM and its related kinases or hCds1/chk2, whereas NBS1 can be phosphorylated by ATM (Cortez et al., 1999; Lee et al., 2000; Wu et al., 2000; Zhao et al., 2000). Although it remains to be confirmed, ATM could be a candidate kinase in the case of TOP1 drugs because cell lines derived from ataxia telangiectasia patients are hypersensitive to CPT (Falk and Smith 1992). As for DNA-PK, it is autophosphorylated in the presence of free DNA ends. Such types of DNA damage are induced by TOP1 drugs (Hsiang et al., 1985; Wu and Liu, 1997).

The molecular events in relation to the repair roles of individual protein/protein complexes are not yet clear, probably because of the complicated damaged DNA products by IR. Although the precise roles of the NBS1-MRE11-RAD50 complex are not yet completely understood, its possession of exonuclease and helicase activities suggests that it might process the free DNA ends (Paull and Gellert, 1999). BRCA1 can bind to the branched DNA structure and inhibits the nucleolytic activities of the NBS1-MRE11-RAD50 in an in vitro study (Paull et al., 2001). TOP1 drugs produce both free DNA ends and branched DNA. Therefore, the TOP1 drug may provide a unique type of agent to address the complicated DNA repair network because, unlike the damaged DNA products induced by IR, the structures of TOP1 drug-induced DNA damage products are well defined (Hsiang et al., 1985, 1989; Wu and Liu, 1997).

In addition to the four major DSB protein complexes as suggested by several research groups, recent studies have shown that protein from one complex can also interact with proteins from other complexes. BRCA1 can form complexes with different proteins under various conditions (Chiba and Parvin, 2001). Ku70, which forms a complex with DNA-PK, also assists in targeting MRE11, a component of NBS1-MRE11-RAD51, to subnuclear foci upon IR exposure (Goedecke et al., 1999). RAD51, the involvement of which in homologous recombination is generally accepted, enhances the frequency of spontaneous nonhomologous recombination when overexpressed (Arnaudeau et al., 2001). The modification and regulation of these

repair proteins by their kinases make the situation more complicated. ATM activates *ABL*, which subsequently inactivates DNA-PK (Shangary et al., 2000). On the other hand, *ABL* also is well known for its role in activating RAD51. Therefore, it seems the activation of specific repair complexes and their interactions are explicitly regulated, dependent on the damaged DNA structure, the cell cycle and the specific cell types.

The activation of DNA double-strand break complexes in response to SN-38 indicates that they are probably required to repair TOP1-drug-induced DNA damage. Cells defective of these repair complexes should be hypersensitive to TOP1 drugs. In fact, it has been reported that cells deficient of DNA-PK and cells derived from Nijmegen syndrome patients whose NBS1 is mutated, are hypersensitive to TOP1 drugs (Pommier et al., 1999). Cancer cells usually have defective DNA repair mechanisms and/or DNA damage checkpoint pathways. Moreover, patients with DNA-repair deficient syndromes are often susceptible to cancer (van Gent et al., 2001). Therefore, it will be interesting to examine whether the antitumor activity of TOP1 drugs can be caused by the defective repair capacity of cancer cells.

Acknowledgments

We thank Cheryl Frank and Carol Wrzosek for their technical help, Dr. Harry K. Slocum for his invaluable comments, and Pharmacia (Peapack, NJ) for providing us with SN-38.

References

- Arnaudeau C, Rozier L, Cazaux C, Defais M, Jenissen D, and Helleday T (2001) RAD51 supports spontaneous non-homologous recombination in mammalian cells, but not the corresponding process induced by topoisomerase inhibitors. *Nucleic Acids Res* **29**:662–667.
- Bennett CB, Lewis AL, Baldwin KK, and Resnick MA (1993) Lethality induced by a single site-specific double-strand break in a dispensable yeast plasmid. *Proc Natl Acad Sci USA* **90**:5613–5617.
- Buckwalter CA, Lin AH, Tanizawa A, Pommier YG, Cheng YC, and Kaufmann SH (1996) RNA synthesis inhibitors alter the subnuclear distribution of DNA topoisomerase I. *Cancer Res* **56**:1674–1681.
- Chen G, Yuan SS, Liu W, Xu Y, Trujillo K, Song B, Cong F, Goff SP, Wu Y, Arlinghaus R, et al. (1999) Radiation-induced assembly of Rad51 and Rad52 recombination complex requires ATM and c-Abl. *J Biol Chem* **274**:12748–12752.

- Chiba N and Parvin JD (2001) Redistribution of BRCA1 among four different protein complexes after replication blockage. *J Biol Chem* **276**:38549–38554
- Collins JA, Schandi CA, Young KK, Vesely J, and Willingham MC (1997) Major DNA fragmentation is a late event in apoptosis. *J Histochem Cytochem* **45**:923–934.
- Cortez D, Wang Y, Qin J, and Elledge SJ (1999) Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science (Wash DC)* **286**:1162–1166.
- Danks MK, Garrett KE, Marion RC, and Whipple DO (1996) Subcellular redistribution of DNA topoisomerase I in anaplastic astrocytoma cells treated with topotecan. *Cancer Res* **56**:1664–1673.
- Davis PL, Shaiu WL, Scott GL, Iglehart JD, Hsieh TS, and Marks JR (1998) Complex response of breast epithelial cell lines to topoisomerase inhibitors. *Anticancer Res* **18**:2919–2932.
- Falk SJ and Smith PJ (1992) DNA damaging and cell cycle effects of the topoisomerase I poison camptothecin in irradiated human cells. *Int J Radiat Biol* **61**:749–757
- Frankenberg-Schwager M and Frankenberg D (1990) DNA double-strand breaks: their repair and relationship to cell killing in yeast. *Int J Radiat Biol* **58**:569–575.
- Goedecke W, Eijpe M, Offenberg HH, van Aalderen M, and Heyting C (1999) Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis. *Nat Genet* **23**:194–198.
- Goldwasser F, Bae I, Valenti M, Torres K, and Pommier Y (1995) Topoisomerase I-related parameters and camptothecin activity in the colon carcinoma cell lines from the National Cancer Institute anticancer screen. *Cancer Res* **55**:2116–2121.
- Greco WR, Park HS, and Rustum YM (1990) Application of a new approach for the quantitation of drug synergism to the combination of cis-diamminedichloroplatinum and 1-beta-D-arabinofuranosylcytosine. *Cancer Res* **50**:5318–5327.
- Hendrickson EA (1997) Cell-cycle regulation of mammalian DNA double-strand-break repair. *Am J Hum Genet* **61**:795–800.
- Hsiang YH, Hertzberg R, Hecht S, and Liu LF (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* **260**:14873–14878.
- Hsiang YH, Lihou MG, and Liu LF (1989) Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res* **49**:5077–5082.
- Karran P (2000) DNA double strand break repair in mammalian cells. *Curr Opin Genet Dev* **10**:144–150.
- Khanna KK and Jackson SP (2001) DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet* **27**:247–254.
- Lee JS, Collins KM, Brown AL, Lee CH, and Chung JH (2000) hCds1-mediated phosphorylation of BRCA1 regulates the DNA damage response. *Nature (Lond)* **404**:201–204.
- Lee SE, Moore JK, Holmes A, Umez K, Kolodner RD, and Haber JE (1998) Saccharomyces Ku70, mre11/rad50 and RPA proteins regulate adaptation to G₂M arrest after DNA damage. *Cell* **94**:399–409.
- Nagy JI, Li WE, Roy C, Doble BW, Gilchrist JS, Kardami E, and Hertzberg EL (1997) Selective monoclonal antibody recognition and cellular localization of an unphosphorylated form of connexin43. *Exp Cell Res* **236**:127–136.
- Paull TT, Cortez D, Bowers B, Elledge SJ, and Gellert M (2001) From the Cover: Direct DNA binding by Brca1. *Proc Natl Acad Sci USA* **98**:6086–6091
- Paull TT and Gellert M (1999) Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex. *Genes Dev* **13**:1276–1288
- Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, and Sikorska M (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO (Eur Mol Biol Organ) J* **12**:3679–3684.
- Panadero A, Yin MB, Voigt W, and Rustum YM (1995) Contrasting patterns of DNA fragmentation induced by thymidylate synthase inhibitors, ZD1694 and AG-331. *Oncol Res* **7**:73–81.
- Pommier Y, Pourquier P, Urasaki Y, Wu J, and Laco GS (1999) Topoisomerase I inhibitors: selectivity and cellular resistance. *Drug Resist Updat* **2**:307–318.
- Shangary S, Brown KD, Adamson AW, Edmonson S, Ng B, Pandita TK, Yalowich J, Taccioli GE, and Baskaran R (2000) Regulation of DNA-dependent protein kinase activity by ionizing radiation-activated abl kinase is an ATM-dependent process. *J Biol Chem* **275**:30163–30168
- Shao RG, Cao CX, Zhang H, Kohn KW, Wold MS, and Pommier Y (1999) Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA dependent protein kinase and dissociates RPA:DNA-PK complexes. *EMBO (Eur Mol Biol Organ) J* **18**:1397–1406.
- van Gent DC, Hoeijmakers JH, and Kanaar R (2001) Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet* **2**:196–206.
- Wu J and Liu LF (1997) Processing of topoisomerase I cleavable complexes into DNA damage by transcription. *Nucleic Acids Res* **25**:4181–4186.
- Wu X, Ranganathan V, Weisman DS, Heine WF, Ciccone DN, O'Neill TB, Crick KE, Pierce KA, Lane WS, Rathbun G, et al. (2000) ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response. *Nature (Lond)* **405**:477–482.
- Yin MB, Guimaraes MA, Zhang ZG, Arredondo MA, and Rustum YM (1992) Time dependence of DNA lesions and growth inhibition by ICI D1694, a new quinazoline antifolate thymidylate synthase inhibitor. *Cancer Res* **52**:5900–5905.
- Yin MB, Guo B, Vanhoefer U, Azrak RG, Minderman H, Frank C, Wrzosek C, Slocum HK, and Rustum YM (2000) Characterization of protein kinase chk1 essential for the cell cycle checkpoint after exposure of human head and neck carcinoma A253 cells to a novel topoisomerase I inhibitor BNP1350. *Mol Pharmacol* **57**:453–459.
- Yuan ZM, Huang Y, Ishiko T, Nakada S, Utsugisawa T, Kharbanda S, Wang R, Sung P, Shinohara A, Weichselbaum R, et al. (1998) Regulation of Rad51 function by c-Abl in response to DNA damage. *J Biol Chem* **273**:3799–3802.
- Zhao S, Weng YC, Yuan SS, Lin YT, Hsu HC, Lin SC, Gerbino E, Song MH, Zdzienicka MZ, Gatti RA, et al. (2000) Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. *Nature (Lond)* **405**:473–477.

Address correspondence to: Dr. Youcef M. Rustum, Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. E-mail: youcef.rustum@roswellpark.org