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HU-331, a novel cannabinoid-based anticancer topoisomerase II inhibitor

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Abstract

Anthracyclines, a large group of quinonoid compounds, are used to treat some forms of cancer. Although highly effective in cancer therapy, the mechanism of action of these compounds is not specific; they act on cancer and other cells by numerous mechanisms. A new anticancer quinone (HU-331) was synthesized from cannabidiol. It shows significant high efficacy against human cancer cell lines *in vitro* and against *in vivo* tumor grafts in nude mice. In this study, we investigated its mode of action and present evidence on its unique mechanism. HU-331 does not cause cancer cell cycle arrest, cell apoptosis, or caspase activation. HU-331 – caused cell death of human cancer cell lines is not mediated by reactive oxygen intermediates/species, as exposure to HU-331 failed to elicit the generation of reactive oxygen species. HU-331 inhibits DNA topoisomerase II even at nanomolar concentrations but has only a slight nonsignificant effect on DNA topoisomerase I action. The cannabinoid quinone HU-331 is a highly specific inhibitor of topoisomerase II, compared with most known anticancer quinones. It might represent a new potent anticancer drug. [Mol Cancer Ther 2007;6(1):173–83]

Introduction

Anthracyclines, a large group of quinonoid compounds produced by different strains of streptomyces, exert

antibiotic and antineoplastic effects and are used to treat some forms of cancer (1). The best known members of this family are daunorubicin and doxorubicin, the first identified anthracyclines (2). Other quinones are also used as anticancer drugs. Mitomycin C and streptonigrin produced by streptomyces and the synthetic epirubicin and mitoxantron are well-known examples (3). These compounds act on cells by numerous mechanisms, such as apoptosis, abrogation of the cell cycle, activation of caspases, stimulation of the production of reactive oxygen species (ROS), inhibition of topoisomerases I and II, activation of intracellular second messengers, etc. (4, 5). Anthracyclines may also cause severe side effects in tumor-bearing hosts, among them cumulative cardiotoxicity (6, 7). The development of quinonoid compounds that display antineoplastic activity, but are more specific in their actions and are less toxic, is a major therapeutic goal.

A new anticancer quinone (HU-331) was synthesized from cannabidiol. It showed very high efficacy against human cancer cell lines *in vitro* and against *in vivo* grafts of human tumors in nude mice (8).

We investigated the mechanism by which HU-331 kills cancer cells. Treatment of human cancer cells with HU-331 did not lead to apoptosis, cell cycle arrest, activation of caspases, and topoisomerase I inhibition and did not cause ROS production. HU-331 was shown to be a highly specific inhibitor of topoisomerase II.

Materials and Methods

Cell Culture

Jurkat, Raji, and HT-29 cells were chosen for the assays, as these were the most sensitive to HU-331-mediated death cells. Jurkat and Raji cells (a generous gift from Prof. H. Ben-Bassat, The Hebrew University, Jerusalem, Israel) were suspended in RPMI 1640 supplemented with 12.5% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 0.01 mg/mL streptomycin at 37°C in a 5% CO₂ humidified atmosphere. HT-29 and BE cell lines (a generous gift from Dr. D. Ross, University of Colorado, Denver, CO) were suspended in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 0.01 mg/mL streptomycin at 37°C in a 5% CO₂ humidified atmosphere.

Apoptotic Cell Death

The population of cells undergoing apoptosis was evaluated by the binding of Annexin V. Jurkat, Raji, or HT-29 cells were incubated for 1.5 h with HU-331 at a 0.3 mmol/L concentration (dissolved in ethanol, so that the final ethanol concentration was 0.5%) and for 48 h with HU-331 at a 4.75 and 1.2 μmol/L concentrations or 0.5% ethanol (as a negative control). The concentration of 4.75 μmol/L approximately parallels the concentration of

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2.5 mg/kg used *in vivo*. To 1×10^6 of cells suspended in 100 μ L binding buffer, 1 μ L of Annexin V B-FITC (25 μ g/mL, IQProducts, Groningen, the Netherlands) was added. The mixture was incubated for 20 min at 4°C, and 10 μ L propidium iodide (IQProducts) was added for 10 min at 4°C, then the cells were analyzed by flow cytometry. Assays with every cell line were carried out in three repeated experiments.

Caspase-3 Staining

Jurkat or Raji cells were incubated for 1.5 h with HU-331 at a 0.3 mmol/L concentration (dissolved in ethanol, at a final concentration of 0.5%), 0.5% ethanol (as a negative control), or 2 μ L/mL anti-Fas (as a positive control). For intracytoplasmic immunofluorescent staining, washed pellets of 10^7 cells were resuspended in 250 μ L buffered formaldehyde acetone (pH 6.8) for 2 h at room temperature (9). The cells were washed twice in chilled PBS containing 10% FCS, 0.1% bovine serum albumin, 0.1% NaN₃ (PBFN) solution and incubated in 2 mL PBFN solution for 1 h at 4°C. Thereafter, 0.5 to 1×10^6 cells were resuspended in 50 to 100 μ L PBFN and incubated with FITC-conjugated anti-caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 4°C in the dark. For each test, 5,000 to 10,000 cells were collected and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Assays with every cell line were carried out in three repeated experiments.

Cell Cycle Analyses

Jurkat or Raji cells were incubated with HU-331 (dissolved in ethanol, 0.5% final ethanol concentration) at a 0.3 mmol/L concentration for 3 h or at a 4.75 μ mol/L concentration for 48 h or with 0.5% ethanol (as a negative control). Washed pellets of 1×10^7 cells were resuspended in 250 μ L buffered formaldehyde acetone (pH 6.8) for 2 h at 0°C. The cells were washed twice in chilled PBS containing 10% FCS, 0.1% bovine serum albumin, 0.1% NaN₃ (PBFN) solution and incubated with 10 μ g RNase for 30 min at 37°C. Then they were stained with 50 μ g/mL propidium iodide in 0.1% sodium citrate. Cells were analyzed after a minimum 30-min incubation in the dark at room temperature with a FACScan flow cytometer. The assay was carried out in three repeated experiments.

Cell Viability Test

Cancer cells were dispensed at 200 μ L volumes into wells of 96-well tissue culture plates at a density of 0.02×10^6 per well. Various concentrations of HU-331 were added into the wells, alone or together with various compounds, and their efficacy was tested, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures mitochondrial dehydrogenase activity (i.e., presumably viable cell number). The principle of this assay is that cells that survived following exposure to various compounds can reduce MTT to a dark-colored formazan, whereas dead cells are incapable of doing so. The assay was done as described previously (10–12). In each MTT assay, every concentration of the cytotoxic substance was tested in five replicates in microplate wells. Assays were carried out in two to three repeated experiments.

Receptor Involvement

Cannabinoid receptor antagonists SR141716A (CB1R antagonist, 1 μ mol/L) and SR144528 (CB2R antagonist, 1 μ mol/L) in 0.5 volume % of DMSO were added together with HU-331 (in various concentrations) to Jurkat cells for a 3-day incubation period, and their efficacy in preventing HU-331-mediated cell death was assayed by MTT test as described above. (+)-HU-331 was synthesized as previously described for (–)-HU-331 (8), and its ability to mediate Jurkat cell death was assayed by MTT test as described above. The assay was carried out in two repeated experiments.

DT-Diaphorase Involvement

To assess the extent to which DT-diaphorase (DTD) contributes to the cytotoxicity of HU-331, MTT test was conducted in HT-29 and BE human colon carcinoma cell lines as described above. HT-29 cells have a high level of DTD activity, whereas the BE cell line has a C-to-T point mutation in the gene encoding for DTD that results in production of a mutant enzyme with no measurable activity (13). The assay was carried out in two repeated experiments.

ROS Involvement

To assess the involvement of ROS in HU-331-caused cell death, we explored the ability of various compounds to modulate the cell-damaging effect of HU-331 by MTT as described above. The following compounds were added 15 min before HU-331: superoxide dismutase mimic [1 mmol/L (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl)]; an iron chelator (5 mmol/L deferoxamine mesylate); diffusible and cell permeable iron chelators (5 mmol/L zinc desferrioxamine and 15 mmol/L acetylhydroxamic acid); a precursor of reduced glutathione and intracellular ROS scavenger (5 mmol/L *N*-acetyl cysteine); the hydroxyl radical scavengers (0.5 volume % DMSO and 10 mmol/L dimethylthiourea); a rapidly diffusible scavenger of H₂O₂, O₂^{•-}, and OH [5 mmol/L *N*-(2-mercaptopropionyl) glycine (MPG)]; and antioxidants (10 μ mol/L α -tocopherol and 1 mmol/L sodium salicylate). Assays were carried out in two to three repeated experiments.

Quantification of Hydroxyl Radicals by Interaction with Salicylate

Raji or Jurkat cells were treated with 1 mmol/L of sodium salicylate and, 15 min after, with different concentrations of HU-331 (0.6, 0.3, and 0.15 mmol/L), H₂O₂ (20, 8, and 4 mmol/L) for a positive control, or 0.5% ethanol for a negative control. Three hours after the compound addition, the cells were centrifuged, and the supernatants were frozen at –70°C until use. The cells were washed with PBS, and the cell pellets were lysed by the addition of double-distilled water and three cycles of freezing and thawing and then frozen at –70°C until use. The samples were prepared by taking 100 μ L of the liquid (the supernatant or cell lysate) and adding 10 μ L of 30% trichloroacetic acid to precipitate the proteins. We quantified the level of \cdot OH-mediated conversion of salicylate to its dihydroxybenzoate derivatives (DHBA), and levels were measured by high-performance liquid chromatography coupled with electrochemical detection using a Varian 5000 liquid chromatograph (Varian Medical Systems,

Palo Alto, CA), equipped with a Rheodyne 7125 sample injector (20 μ L loop; Rheodyne LLC, Rohnert Park, CA). The column used for separation of salicylate and DHBA was a 25 cm \times 4 mm Li Chrospher 100 RP-18, 5 μ m (E-Merck, Darmstadt, Germany). The mobile phase contained 0.03 mol/L citric acid, 0.03 mol/L acetic acid, 0.2 g/L sodium azide, and 2% methanol. The mobile phase was titrated with solid NaOH to pH 3 followed by titration with CH₃COONa to a final pH of 3.6. The flow rate was 1 mL/min; retention times for 2,5-DHBA, 2,3-DHBA, and catechol were 8.9, 10.1, and 11.5 min, respectively. The system was equipped with two detectors in series. Salicylate was identified and measured fluorometrically using a FD-300 model fluorescence detector (Spectrovision, Chelmsford, MA) using excitation and emission wavelengths of 300 and 412 nm, respectively. DHBA derivatives and catechol were quantified using an electrochemical amperometric detector (Model 4A; Bioanalytical Systems, West Lafayette, IN), with a plastic cell equipped with a glass carbon electrode operated at +0.80 V, 100 mA, using an Ag-AgCl reference electrode. The signals from the detector were acquired on an EZChrom data acquisition and handling system (EZChrom Elite, Scientific Software, Inc., San Ramon, CA) and subsequently processed. Assays with every cell line were carried out in three repeated experiments.

Topoisomerase I Assay

Purified calf thymus topoisomerase I (5 units) was added to a reaction mixture containing, at a final volume of 25 μ L, 20 mmol/L Tris-HCl (pH 8.1), 1 mmol/L DTT, 20 mmol/L KCl, 10 mmol/L MgCl₂, 1 mmol/L EDTA, 30 μ g/mL bovine serum albumin, and 225 ng pUC19 supercoiled DNA plasmid (MBI Fermentas, Hanover, MD). Different concentrations (1 μ mol/L to 10 mmol/L) of HU-331 were added. After incubation at 37°C for 30 min, the reaction was terminated by adding 5 μ L of stopping buffer [final concentration; 1% SDS, 15% glycerol, 0.5% bromophenol blue, and 50 mmol/L EDTA (pH 8)]. The reaction products were analyzed by electrophoresis on 1% agarose gel using a Tris-borate/EDTA buffer (89 mmol/L Tris-HCl, 89 mmol/L boric acid, and 62 mmol/L EDTA) at 1 V/cm, stained by ethidium bromide (1 μ g/mL), and photographed using a short-wavelength UV lamp (ChemImager 5500; Alpha Innotech, San Leandro, CA).

Topoisomerase II Assay

Purified human topoisomerase II α (2 units) was added to a reaction mixture containing, at a final volume of 25 μ L, 50 mmol/L Tris-HCl (pH 8.1), 0.5 mmol/L DTT, 120 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L EDTA, 30 μ g/mL bovine serum albumin, 1 mmol/L ATP, and 225 ng pUC19 supercoiled DNA plasmid (MBI Fermentas). Different concentrations (100 nmol/L to 10 mmol/L) of HU-331 were added. After incubation at 37°C for 30 min, the reaction was terminated by adding 5 μ L of stopping buffer (see above). The reaction products were analyzed by electrophoresis on 1% agarose gel using a Tris-borate/EDTA buffer at 1 V/cm, stained by ethidium bromide, and photographed using a short-wavelength UV lamp (ChemImager 5500; Alpha Innotech).

Topoisomerase II-DNA Complexes Assay

The examination of topoisomerase II-DNA complexes was done as previously described (14). Briefly, 5 units of purified topoisomerase II were added to a reaction mixture containing 10 ng (6×10^5 cpm) of [α -³²P]-labeled pUC19 DNA. Where indicated, either etoposide (VP-16) or HU-331 was added to the reaction before the addition of the enzyme. Following incubation at 37°C for 30 min, 5 μ L of stopping buffer was added (1% SDS, 15% glycerol, 0.5% bromophenol blue, and 50 mmol/L EDTA). The reaction products were analyzed on 0.7% agarose gel electrophoresis, and the gel was exposed to autoradiography.

Protein Extraction and Western Blotting

Jurkat cells were treated with either vehicle or HU-331 (100 μ g/mL for 45 min or 30 μ g/mL for 2 h). The washed cell pellets were resuspended in lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 2 mmol/L EDTA, 6 mmol/L 6-mercaptoethanol, 1% Triton X-100, 0.1% SDS, and 10 mmol/L NaF, plus the protease inhibitors leupeptin 10 μ g/mL, aprotinin 10 μ g/mL, and 0.1 mmol/L phenylmethylsulfonyl fluoride]. The protein concentration of each sample was estimated using the Bradford assay (15). For Western blotting, samples containing 25 μ g of total cell lysate were loaded onto a SDS-polyacrylamide gel (10% for phosphorylated *c-jun*, phosphorylated extracellular signal-regulated kinase, and phosphorylated p38 mitogen-activated protein kinase or 15% for phosphorylated H2AX) and subjected to electrophoresis. Proteins were transferred to nitrocellulose membranes in transfer buffer (25 mmol/L Tris, 190 mmol/L glycine, 20% methanol) at 300 mA for 1 h at room temperature. The membranes were blocked with blocking buffer (PBS/0.05% Tween 20/5% bovine serum albumin) for 1 h at room temperature and washed thrice for 5 min in washing buffer (PBS/0.05% Tween 20). The membranes were incubated with the primary antibodies overnight at 4°C. The membranes were washed as described above and incubated with secondary antibodies (1:20,000) for 1 h at room temperature. The immune detection was done using the Enhanced Chemiluminescence Western blotting detection system (Amersham, Piscataway, NJ). Intensities of the different proteins were quantified by densitometric scanning. All experiments were repeated at least thrice and yielded similar results.

Genomic DNA Preparation and PCR Methods

Jurkat cells were exposed to vehicle, HU-331 (0.1 mmol/L for 2 h, 2 or 1 μ mol/L for 24 h), VP-16 (20 μ mol/L for 2 h), or a combination of VP-16 and HU-331. Then, the cells were washed twice in PBS. Cell pellets were resuspended in 400 μ L lysis buffer [50 mmol/L Tris (pH 8), 100 mmol/L EDTA, 0.5% SDS, 7.5 μ L of 10 mg/mL RNase, and 10 μ L of a 10 mg/mL solution of proteinase K] and incubated overnight at 55°C on a rocking platform. The aqueous phase was extracted with phenol (pH 8) and chloroform. The DNA was precipitated by adding of 70 μ L of 3 mol/L sodium acetate (pH 6) and 400 μ L absolute ethanol, washed with 70% ethanol, dried, and

dissolved in 100 μ L of double-distilled water (pH 8) with 1 mmol/L EDTA. Each PCR reaction contained \sim 50 ng of total genomic DNA. The assay was carried out in three repeated experiments.

DNA Lesion Determination

Lesion frequencies per strand are usually calculated by Poisson's equation (16–18). Briefly, amplification of treated samples is normalized to controls to generate an amplification ratio. Assuming a random distribution of lesions along the target amplification and employing the Poisson expression $f(x) = e^{-\lambda} \lambda^x / x!$, where λ is equal to average lesion frequency, lesion frequencies of treated samples are calculated. To use this expression to calculate lesion frequencies, the control samples are assumed to have no lesions. Thus, the control samples are set to $x = 0$,

reducing the Poisson expression to $f(0) = e^{-\lambda}$. The lesion frequency per amplicon is then calculated as $-\ln(A_D/A_C)$, where A_D represents the amount of amplification of the damaged template, and A_C represents the amplification of the control template. However, as was aforementioned, using the Poisson equation, we assume that in the control DNA, there are no strand breaks, which is incorrect, as DNA is naturally broken by topoisomerases for replication, repair, transcription, etc. Thus, cells exposed to compounds that inhibit topoisomerase at the stage of its catalytic cycle where DNA is unbroken will have less strand breaks than the control cells and thus will have negative values of number of breaks per 10 kb using the Poisson equation. We have used the simple comparison of the long (2,700 bp) band amplification (normalized by

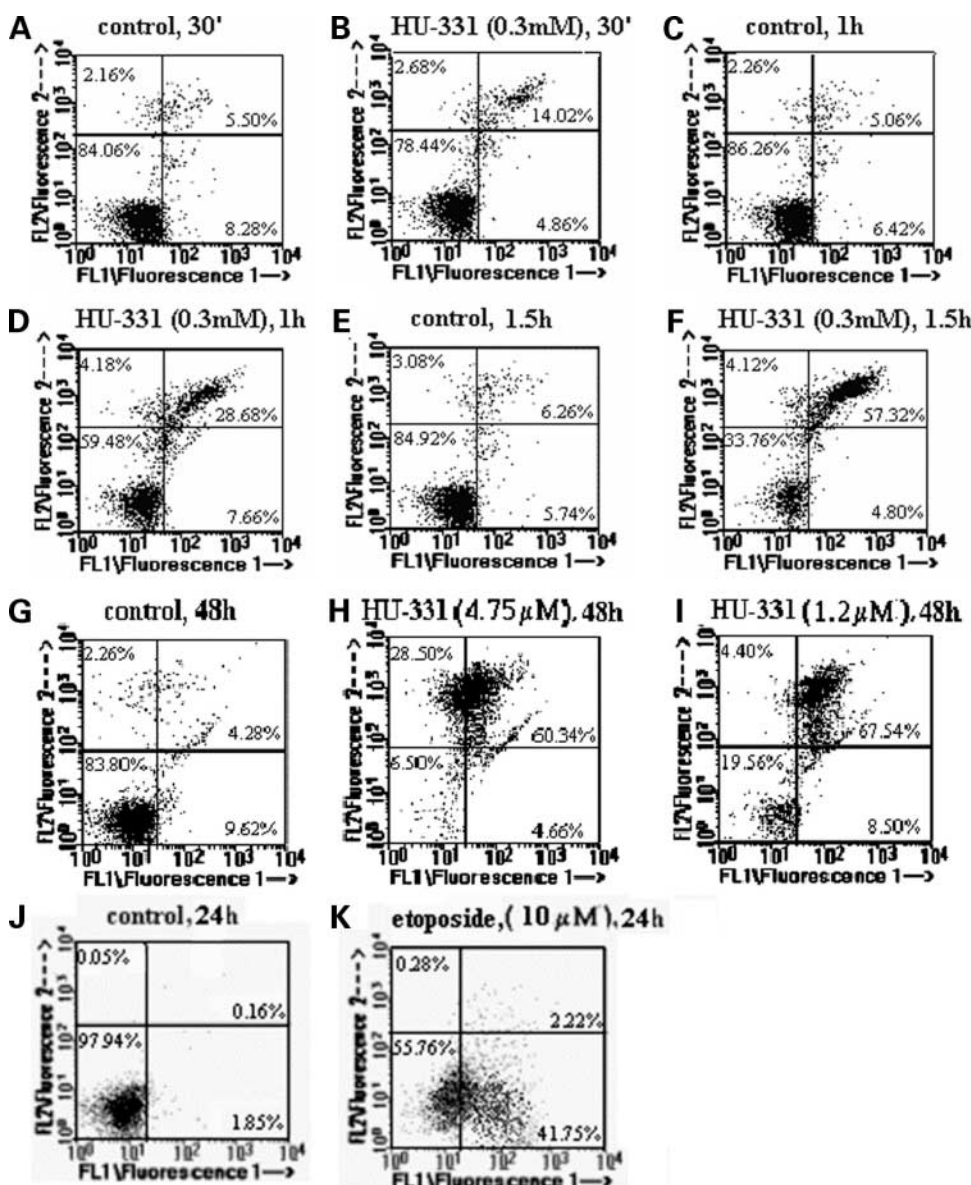


Figure 1. HU-331-mediated Jurkat cell death is not apoptotic. FL1 = Annexin V-FITC, FL2 = propidium iodide. Representative of three experiments. **A**, negative control cells (0.5 volume % ethanol), 30 min. **B**, cells exposed to 0.3 mmol/L HU-331, 30 min. **C**, negative control cells, 1 h. **D**, cells exposed to 0.3 mmol/L HU-331, 1 h. **E**, negative control cells, 1.5 h. **F**, cells exposed to 0.3 mmol/L HU-331, 1.5 h. **G**, negative control cells, 48 h. **H**, cells exposed to 4.75 μ M HU-331, 48 h. **I**, cells exposed to 1.2 μ M HU-331, 48 h. **J**, negative control cells, 24 h. **K**, positive control for apoptotic cells: cells exposed to 10 μ M/L VP-16, 24 h.

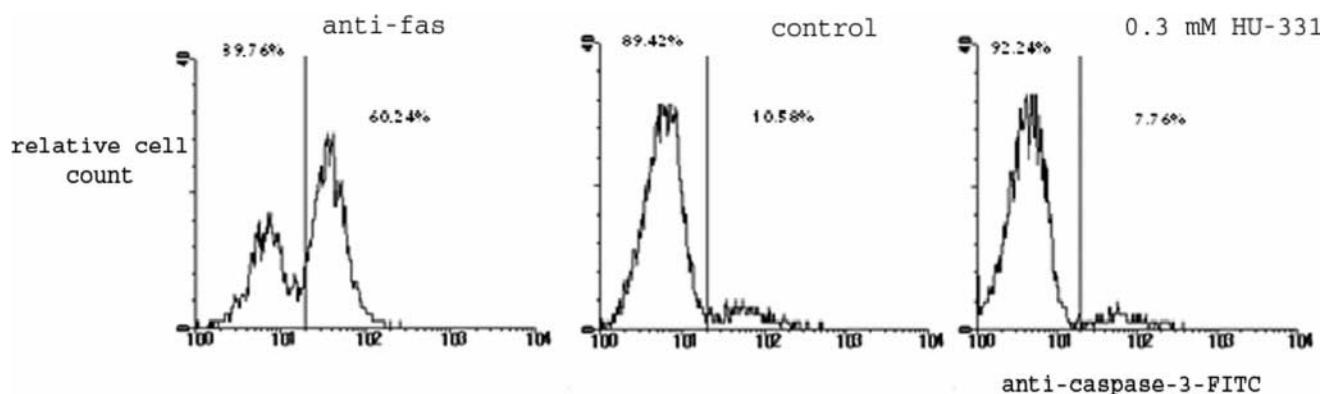


Figure 2. Caspase-3 is not involved in HU-331-mediated Jurkat cell death. Representative caspase staining. Jurkat cells treated with HU-331 (*right*), 0.5 volume % ethanol (a negative control, *middle*), or anti-Fas antibody (a positive control, *left*) were stained with FITC-conjugated anti-caspase-3 antibody.

taking into account the short 226-bp band amplification, as in such a short sequence, it is statistically unlikely for a strand break to fail) to answer whether HU-331 causes DNA strand breaks or not.

Results

HU-331-Mediated Jurkat, Raji, and HT-29 Cell Death Is Not Apoptotic

The population of cells undergoing apoptosis in HU-331-treated Jurkat, Raji, or HT-29 cells was determined by comparing the binding of Annexin V-FITC to the outer cell membrane (which is a marker of apoptosis) with intracytoplasmic staining with propidium iodide (a marker of cells with damaged plasma membrane: dead cells), which stained DNA in cytoplasm derived from dead nuclei. In negative control cells (Fig. 1), the cells do not bind Annexin and are not stained by propidium iodide; these cells are alive and are not undergoing apoptosis. In cells exposed to 0.3 mmol/L HU-331 for 30 to 90 min (a concentration that kills 20–60% cells during this time period, based on a preliminary MTT test), both Annexin V and propidium iodide stained cells to a similar extent (Fig. 1). During that period, the proportion of dead cells, stained by both Annexin V and propidium iodide, increased from 14% to 57%. Parallel results were observed in cells treated with 4.75 or 1.2 $\mu\text{mol/L}$ HU-331 for 48 h (concentrations that kill 50–70% cells during this time period, based on a preliminary MTT test), where the portion of double-stained cells increased from 4.28% to 60.34% and 67.54%. No evidence was found for an early phase of staining for Annexin V without propidium iodide staining that is a characteristic for apoptosis, leading to the conclusion that the HU-331-mediated cell death of Jurkat, Raji, and HT-29 cells is apparently not apoptotic. In contrast, in the positive control cells, exposed to 10 $\mu\text{mol/L}$ of VP-16 for 24 h, early apoptosis can be detected ($\sim 50\%$ of the cells were stained only with Annexin V and not with propidium iodide).

Caspase 3 Is Not Involved in HU-331-Mediated Jurkat and Raji Cell Death

To evaluate the involvement of caspases cascade in HU-331-mediated cell death, Jurkat or Raji cells were incubated with HU-331, 0.5% ethanol (as a negative control), or 2 mg/mL anti-Fas (as a positive control for caspase activation) and then stained with FITC-conjugated anti-caspase-3 antibody. In the negative control cells (Fig. 2, *middle*), there was no caspase-3 activation. Treatment of Jurkat cells with anti-Fas elicited the activation of caspase-3 (Fig. 2, *left*; the left peak is of cells with no activated caspase and the right peak is of cells with activated caspase). In contrast, exposure to HU-331 for 1.5 h failed to activate caspase-3 (Fig. 2, *right*), whereas under these assay conditions, there are 44.46% dead cells (as can be seen from Fig. 1), suggesting that caspases cascade is not involved in HU-331-mediated death of Jurkat or Raji cells.

HU-331-Mediated Jurkat and Raji Cell Death Is Not Due to Cell Cycle Arrest

In cell cycle analysis, the control untreated cells showed the normal distribution of cells in the G_1 , S, and M phases of the cell cycle (Fig. 3A and C). There were very few cells in the sub- G_1 fraction (apoptotic cells). Treatment of Jurkat or Raji cells with 4.75 $\mu\text{mol/L}$ HU-331 for 48 h (Fig. 3B) or with 0.3 mmol/L HU-331 for 1.5 h (Fig. 3D) increased the amounts of cells in the G_1 phase compared with untreated cells (78.8% versus 68.6% for 48 h, 71.0% versus 61.0% for 3 h), whereas at the aforementioned time intervals, $>50\%$ of cells were dead (as described above). The population of HU-331-treated cells contained a lower amount of cells (15–25%) in the S and M phase compared with untreated cells, suggesting a slight inhibition of the cell cycle that is not crucial for the HU-331-mediated cell death. HU-331 treatment did not induce an increase in the proportion of cells in the sub- G_1 phase, an indicator for apoptotic cells, suggesting that HU-331-induced cell death is not an apoptotic process.

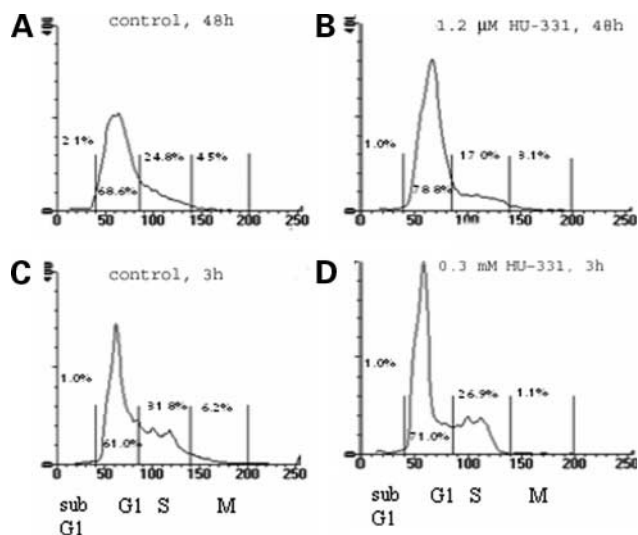


Figure 3. HU-331-mediated Jurkat cell death is not due to cell cycle arrest. Representative cell cycle profiles. **A**, negative control cells (0.5 volume % ethanol), 48 h. **B**, cells exposed to 1.2 $\mu\text{mol/L}$ HU-331, 48 h. **C**, negative control cells (0.5 volume % ethanol), 3 h. **D**, cells exposed to 0.3 mmol/L HU-331, 3 h.

HU-331-Mediated Cell Death Is Not Mediated by Cannabinoid Receptors

As HU-331 is a cannabinoid derivative, and as in previous articles, some cannabinoids were shown to kill various cancer cells through cannabinoid receptors (19–21), we evaluated the involvement of these receptors in HU-331-mediated Jurkat cell death. Antagonists of cannabinoid receptors were added simultaneously with HU-331 to Jurkat cells and incubated for 3 days. The antagonists of cannabinoid receptors did not inhibit HU-331-mediated cell death (Fig. 4A). No significant differences in the level of cell death in the presence of CB1 antagonist, CB2 antagonist, both antagonists, or no antagonists were observed.

(+)-HU-331 and (-)-HU-331 Have Comparable Cytotoxic Effects

(+)-HU-331 was synthesized from (+)-CBD by the same method as HU-331 from (-)-CBD (8). The level of cell death of (+)-HU-331 did not significantly differ from that observed with HU-331 (Fig. 4B), which suggests that HU-331 presumably does not act through receptor binding but probably enters the cell due to its lipophilicity and then acts through its quinone moiety.

HU-331 Is Not Activated by DTD

DTD is a cytosolic FAD-containing enzyme that catalyzes an obligatory two-electron reduction of a variety of quinones using NAD(P)H as cofactor (22). Although the physiologic role of this enzyme is not known, it is believed to involve the detoxification of quinones by inhibiting formation of highly reactive radical anion intermediates (23, 24). In some cases, this enzyme was shown to activate quinones and render them more toxic to cancer cells (4).

To assess the extent to which DTD contributes to the cytotoxicity of HU-331, MTT tests were conducted on two lines of human colon carcinoma cells: HT-29, which has a high level of DTD activity versus BE cells containing a mutated inactive enzyme (C-to-T point mutation in the gene encoding for DTD). No difference in the sensitivity of these two lines to the cytotoxic effect of HU-331 was observed (Fig. 4C).

Intracellular Pathway Involvement

The Western blot analyses of proteins from Jurkat cells exposed to 0.3 mmol/L HU-331 for 10 min to 3 h or to 1.2 to 4.75 $\mu\text{mol/L}$ HU-331 for 24 or 48 h did not show changes in any pathway checked. The levels of phosphorylated *c-jun*, phosphorylated extracellular signal-regulated kinase, and phosphorylated p38 mitogen-activated protein kinase remained unchanged, whereas in the cells exposed to UV irradiation, used as positive control for all the pathways, all of these proteins were elevated (data not shown). When pifitrin- α , a p53 inhibitor, was added to the cells simultaneously with HU-331, it did not interfere with HU-331-caused cell death (data not shown).

Cell Death by HU-331 Is Not Mediated by ROS

To assess the involvement of free radicals in HU-331-mediated cell death, 0.3 mmol/L HU-331 was added to Jurkat cell culture in the presence of radical scavengers/iron chelators, and their ability to interfere with cell death was monitored. HU-331-mediated cell death was partially inhibited by *N*-acetyl cysteine and MPG and, to a very small extent, by α -tocopherol but not by the superoxide dismutase mimic 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, the iron chelator desferrioxamine mesylate, diffusible and cell permeable iron chelators zinc desferrioxamine and acetylhydroxamic acid, and the antioxidants dimethylthiourea, DMSO, and sodium salicylate (Fig. 4D). The inhibition of HU-331-mediated cell death by MPG, the most potent of the antioxidants used, was assayed also with smaller doses of HU-331 during larger time intervals (the concentrations of 1.2 $\mu\text{mol/L}$ to 0.3 mmol/L, for 24 or 48 h). The results paralleled those obtained with 0.3 mmol/L HU-331 for 3 h.

The ability of MPG to inhibit HU-331-mediated cell death was tested on HT-29 cell line as well (at concentrations of 1.2 $\mu\text{mol/L}$ to 0.3 mmol/L, for 24, 48, or 72 h or 0.3 mmol/L for 3 h) and also showed the same results (data not shown). However, the ability of MPG and *N*-acetyl cysteine to inhibit HU-331-mediated cell death is probably not due to their antioxidant activity but rather reflects mechanisms other than scavenging of free radicals, as none of the other antioxidants and iron chelators were able to inhibit HU-331-mediated cell death.

Sodium salicylate is a highly effective hydroxyl free radical scavenger, which, upon scavenging $\cdot\text{OH}$, forms 2,3-DHBA and catechol by hydroxylation. We quantified the level of $\cdot\text{OH}$ -mediated conversion of salicylate to its 2,3-DHBA and catechol (2,3-DHBA decarboxylation product) by high-performance liquid chromatography coupled with electrochemical detection. As 2,5 DHBA can be a product not only of $\cdot\text{OH}$ addition to salicylate, but also of salicylate

oxidation by cytochrome P450, its levels were not quantified. The results (depicted in Fig. 4E), show that there is no significant difference between the levels of 2,3 DHBA + catechol in HU-331-exposed supernatants and control supernatants of Raji cells. Even at doses of HU-331 that kill ~50% of cells, the level of these ·OH indicators remains similar to that of the control cells. In contrast, in the positive controls, exposed to H₂O₂, the levels of 2,3 DHBA + catechol are elevated even at concentrations in which all the cells are still alive. Comparing the 0.6 mmol/L concentration of HU-331 with the 20 mmol/L concentration of H₂O₂ (both of which kill ~50% of Raji cells during 3 h of treatment), the level of 2,3 DHBA + catechol is nearly 5-folds higher in supernatants of H₂O₂-exposed Raji cells. The same results are obtained for Raji cell lysates and for Jurkat cell supernatants and cell lysates (data not shown). Altogether, these results suggest that the killing of Raji and Jurkat cells by HU-331 is not mediated by ROS.

HU-331 at Nanomolar Concentrations Inhibits the DNA Relaxation Activity of Human Topoisomerase II α

HU-331 at various doses was added to a specific reaction mixture containing purified human topoisomerase II α enzyme and supercoiled DNA plasmid as a substrate. Under the assay conditions, topoisomerase II converted all the supercoiled DNA molecules (Fig. 5A, lane 1) to their partially and fully relaxed forms (Fig. 5A, lane 2). The

addition of HU-331 to the reaction mixture significantly inhibited the DNA relaxation activity of topoisomerase II α even at low doses of the drug (100 nmol/L; compare lane 3 with lane 2). The inhibitory effect of HU-331 on the DNA relaxation activity of topoisomerase II α is dose dependent (see lanes 3–8), and ~100% inhibition is observed at 10 μ mol/L of HU-331 (lane 5).

To determine the effect of this drug on DNA topoisomerase I, various concentrations of HU-331 were added to a specific reaction mixture containing purified topoisomerase I and supercoiled DNA plasmid as the substrate. The results depicted in Fig. 5B show that DNA topoisomerase I converted the supercoiled DNA molecules (Fig. 5B, lane 1) to their relaxed and partially relaxed forms (lane 2), and no effect on the topoisomerase I DNA relaxation activity was observed when 1 μ mol/L to 1 mmol/L of HU-331 were added to the reaction mixture (lanes 3–6). A slight inhibitory effect on the topoisomerase I DNA relaxation activity was observed only at a high dose (10 mmol/L) of HU-331 (lane 7).

To examine the possibility that HU-331 inhibits topoisomerase II activity by its interaction with the enzyme protein, a classic biochemical competition-based assay was done. Topoisomerase II activity was measured in the presence of a constant amount of DNA (225 ng), a constant amount of HU-331 (1 μ mol/L), and increasing amounts of

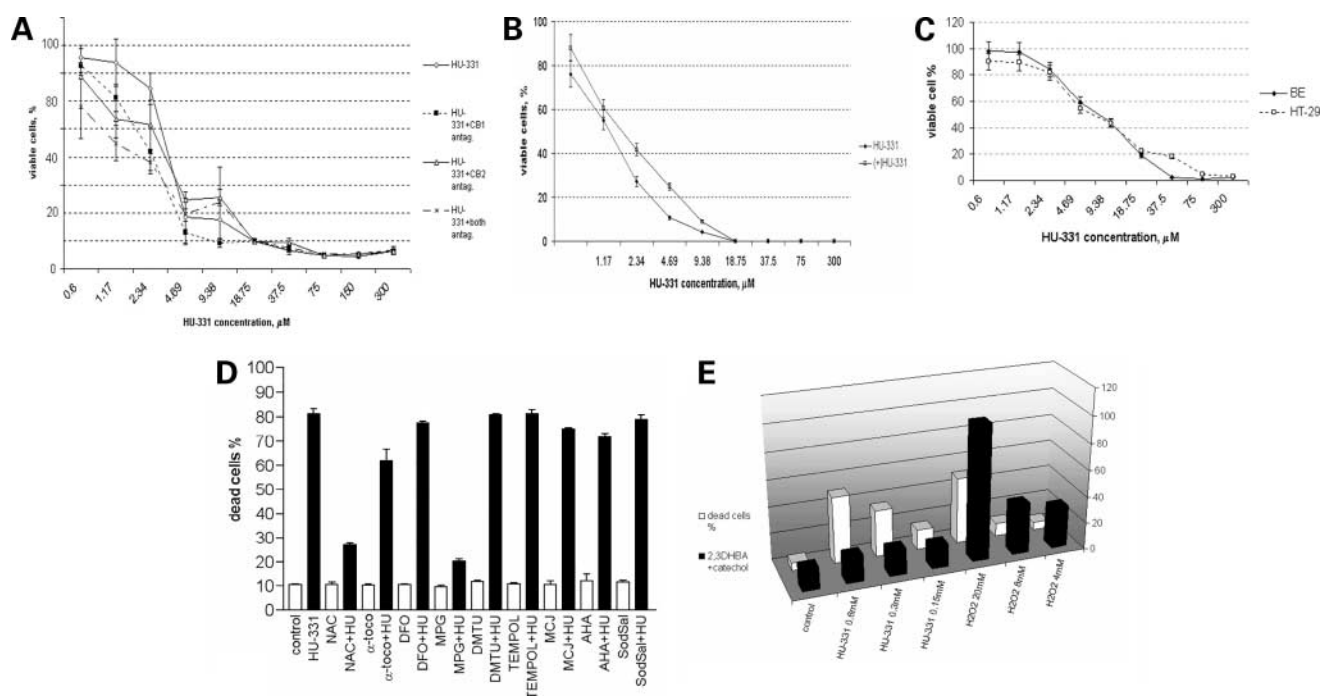


Figure 4. **A**, influence of cannabinoid receptors antagonists on HU-331-induced Jurkat cell death. **B**, (-)-HU-331 and (+)-HU-331 inhibition of the growth of Jurkat cells. **C**, BE versus HT-29 cell death caused by HU-331. **D**, interference of free radicals scavengers with HU-331-caused cell death. Ethanol = 0.5% ethanol, the negative control for cell death. HU-331 = 0.3 mmol/L HU-331 in 0.5% ethanol, the positive control. **E**, induction of free radicals by HU-331 (expressed by the sum of 2,3-DHBA and catechol) versus cell death. NAC, N-acetyl cysteine; HU, HU-331; DFO, deferoxamine mesylate; α -toco, α -tocopherol; DMTU, dimethylthiourea; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; MCJ, zinc desferrioxamine; AHA, acetylhydroxamic acid; SAL, sodium salicylate.

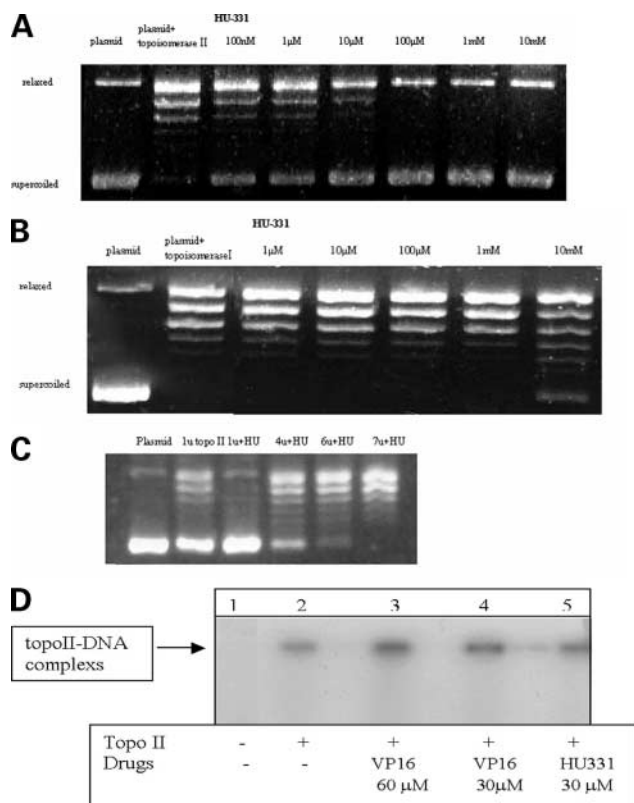


Figure 5. HU-331 at nanomolar concentrations inhibits the DNA relaxation activity of human topoisomerase II α . HU-331 specifically inhibits the DNA relaxation activity of human topoisomerase II α (*topo II α*). Purified human topoisomerase II (A and C) or calf thymus topoisomerase I (B) were added to specific reaction mixtures containing DNA supercoiled plasmid (lane 1 in A–C) as the substrate. Various HU-331 doses (A and B) were added to the reaction mixtures as described in Materials and Methods, and the reaction products were analyzed by agarose gel electrophoresis. C, increasing amounts of topoisomerase II enzyme were added to a reaction mixture containing supercoiled DNA plasmid and 1 μ mol/L of HU-331 (lanes 3–6). Lane 2, 1 unit of topoisomerase II without HU-331. D, purified topoisomerase II (5 units) were added to [32 P]-labeled fragments of pUC 19 DNA in the absence (lane 2) or presence of VP-16 (lanes 3 and 4) or HU-331 (lane 5). The reaction products were analyzed by agarose gel electrophoresis followed by autoradiography.

topoisomerase II enzyme (1–7 units). The results in Fig. 5C show that by increasing the amount of topoisomerase II enzyme, it is possible to overcome the inhibitory effect of HU-331 (compare lanes 4–6 with lane 3). These results suggest that HU-331 is a potent inhibitor of topoisomerase II that exerts its inhibitory effect probably by its interaction with the topoisomerase II protein.

HU-331 Slightly Stabilizes DNA-Topoisomerase II Complexes

To elucidate the mode of action by which HU-331 inhibited topoisomerase II activity, we compared the enzyme-DNA complexes formed in the presence of HU-331 with those formed in the presence of VP-16. A significant increase in topoisomerase II-DNA complexes was observed in the presence of VP-16 (Fig. 5D, compare

lanes 3 and 4 with lane 2) and only a slight increase in the presence of HU-331 (compare lane 5 with lane 2). VP-16 is a topoisomerase II inhibitor that stabilizes topoisomerase II-DNA cleavable complexes, prevents the DNA ligation step, and thus causes double-strand DNA breaks. Therefore, in the presence of this drug, an increase in the enzyme-DNA complexes is observed. The slight insignificant increase in these complexes in the presence of HU-331 suggests that this drug action as topoisomerase II inhibitor differs from that of VP-16, which points to the possibility that HU-331 might act as a catalytic inhibitor of topoisomerase II and not as a topoisomerase II poison like VP-16.

HU-331 Does Not Cause DNA Strand Breaks

Phosphorylation of histone H2AX on Ser¹³⁹, also referred to as γ H2AX induction, is an established marker of DNA double-strand breaks in cells (25, 26). As can be seen from Fig. 6A, the amount of phosphorylated H2AX in HU-331-treated cells is not higher but even lower than in control cells. Actually, no phosphorylated H2AX can be seen in HU-331-treated cells. These data suggest that HU-331 does not cause DNA strand breaks and thus differs from the commonly known DNA topoisomerase II inhibitors (e.g., VP-16; refs. 27, 28). To prove in one more way that HU-331 does not cause DNA damage and, moreover, protects DNA from damage (although killing the cell meanwhile by a different pathway), quantitative PCR was done. Examination of the presence of double-strand breaks by

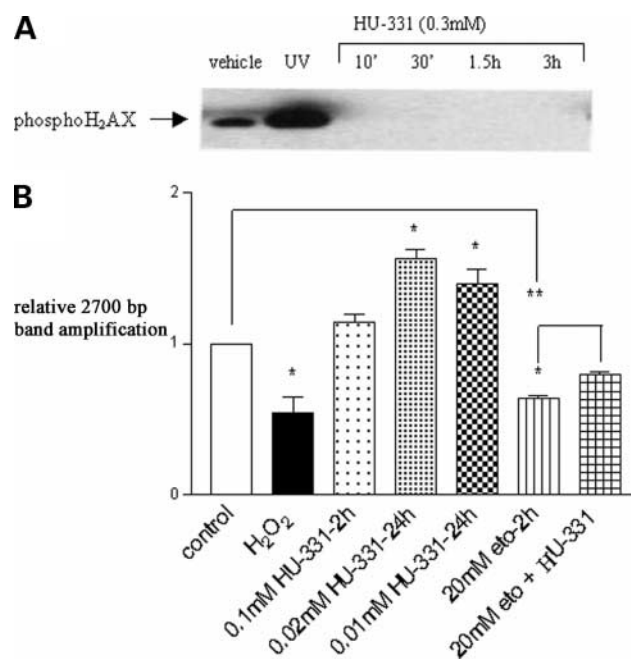


Figure 6. HU-331 does not cause DNA strand breaks. A, Western blot of phosphorylated H2AX (*phosphoH₂AX*) in HU-331-exposed cells comparing with vehicle-treated cells (negative control) and UV-treated cells (positive control for DNA damage). Vehicle = 0.5 volume % ethanol. B, quantitative PCR results for DNA damage: 2,700-bp band amplification (normalized by taking into account 226-bp band amplification). Control = ethanol 0.5 volume %. eto, etoposide. VP-16 and H₂O₂ are the positive controls for DNA breaks. *, $P < 0.05$; **, $P < 0.01$.

quantitative PCR assay is based on reports that DNA lesions can affect the Taq polymerase and thereby result in a decrease in the amplification of a damaged DNA segment compared with the amplification of the same undamaged DNA segment (16, 17). A 226-bp fragment within the 2,700-bp amplified fragment was used as the internal standard of DNA extraction and PCR reaction preparation, as it is unlikely for DNA strand damage to fall within such a short sequence. Figure 6B shows a comparison of vehicle-treated and HU-331-treated cells; after 2 h of exposure to 0.1 mmol/L HU-331, when 60% of the cells are already dead (counted by trypan blue dye exclusion; data not shown), there is still more 2,700-bp band amplification than in the control cells. This difference is much more prominent and statistically significant when the cells are exposed to 2 μ mol/L HU-331 for 24 h (30% dead cells by trypan blue exclusion) or 1 μ mol/L HU-331 for 24 h (nearly all cells are still viable). In the positive control cells, which were exposed to hydrogen peroxide, there is a significant reduction in the 2,700-bp band amplification compared with the control cells. These results show that in HU-331-exposed cells, there is less DNA damage than in control cells. Hence, HU-331 seems to be a catalytic inhibitor of topoisomerase II, rather than a topoisomerase II poison. Catalytic topoisomerase II inhibitors are known to protect the cell from DNA damage caused by topoisomerase poisons. To investigate this possibility, Jurkat cells were exposed to vehicle, VP-16 (20 μ mol/L), a known topoisomerase poison, or a combination of VP-16 (20 μ mol/L) + HU-331 (0.1 mmol/L) for 2 h; the genomic DNA was prepared and analyzed by PCR. The results depicted in Fig. 6B show that VP-16 causes DNA strand breaks, as in VP-16-exposed cells, a reduction in the amplification of the 2,700-bp band (normalized by taking into account the 226-bp band amplification) is observed compared with control untreated cells (65% of control amplification). However, when a combined treatment with VP-16 and with HU-331 was done, the 2,700-bp band amplification is significantly higher than with VP-16 alone (80% of control amplification, 120% of VP-16 amplification). Thus, HU-331 protects the cells from VP-16-mediated DNA damage, which is typical for a catalytic topoisomerase II inhibitor.

Discussion

Chemotherapeutic drugs exert their cytotoxic effect on target cells through numerous nonexclusive mechanisms. Doxorubicin (Adriamycin) and other anticancer quinones have been in use for the chemotherapy of human cancer for many years; yet, the mechanism of action of these drugs remains the subject of considerable controversy (5). Doxorubicin was shown to damage DNA by intercalation, by generation of free radicals and by inhibition of DNA topoisomerases I and II. It induces single and double DNA strand breaks. The protein associated with these breaks was shown to be topoisomerase II, and the damage to DNA was shown to be catalyzed by this enzyme (29).

Cannabinoids can act as anticancer compounds (30) causing death of various cancer cells following direct

interaction with cannabinoid receptors. Thus, glioma growth *in vivo* was inhibited by selective activation of CB2 cannabinoid receptors (15), and the endogenous cannabinoid anandamide inhibited human breast cancer cell proliferation (19, 20). The antitumor effect of HU-331 does not seem, however, to be mediated by known cannabinoid receptors because antagonists of cannabinoid receptors failed to inhibit HU-331-mediated cell death.

In a previous study, HU-331 was shown to exert an antiangiogenic effect accompanied by apoptosis of endothelial cells (31). In contrast, in the present study, HU-331 did not cause death of human cancer cells by eliciting apoptosis. The conclusion that HU-331 did not elicit apoptosis in cancer cells is based on the finding that treatment with the drug did not increase the proportion of cells with sub-G₁ DNA content and failed to elicit the expression of caspase-3 in cancer cells. This notion is supported by the fact that in cancer cells exposed to HU-331, staining with anti-Annexin V was not detected before their staining with propidium iodide. In addition to apoptosis, a "cell suicide" program that is targeted by many anticancer drugs, some nonapoptotic types of cell death exist, such as necrosis, autophagy, senescence, paraptosis, and mitotic catastrophe (32, 33). It is unlikely that HU-331-mediated cancer cell death is caused by either senescence (which is an irreversible cell cycle arrest) or mitotic catastrophe (during which cells proceed to mitosis, although their DNA is damaged, due to G₂ checkpoint defect), as in both of these cases, there should be serious alterations in the cell cycle. Although the treatment of Jurkat cancer cells with HU-331 increased the amounts of cells in the G₁ phase compared with untreated cells, there were only 10% more cells in G₁, whereas at the aforementioned time intervals, >50% of cells were already dead. The population of HU-331-treated cells contained a somewhat lower amount of cells (15–25%) in the S and M phase compared with untreated cells, suggesting a slight inhibition of the cell cycle. However, these slight cell cycle alterations do not seem to be crucial for the HU-331-mediated cell death. As there is no pronounced cell cycle arrest, HU-331-mediated cell death is most probably not due to senescence, and as there are fewer cells in S + M phases and no tetraploid cells are found, mitotic catastrophe can also be ruled out as the mode of HU-331-mediated cancer cell death. Paraptosis, a cell death mode on which there are only a few reports until now, seems to act through mitogen-activated protein kinases (34). As HU-331 does not activate extracellular signal-regulated kinase and c-Jun NH₂-terminal kinase pathways (the intracellular pathways that were found to be activated during paraptosis), it is unlikely that it causes cancer cell death by this mode. Autophagy (or, as it is called, type 2 programmed cell death, whereas apoptosis is called type 1 programmed cell death) is a catabolic process where the cytoplasmic content of a cell is sequestered within double-membrane vacuoles, called autophagosomes, and delivered to the lysosome for degradation. Although autophagy can function as a survival mechanism in starving cells, extensive autophagy

is commonly observed in dying cells, leading to its classification as an alternative form of programmed cell death (35). It is yet unclear if HU-331 is able to activate this type of cell death. Necrosis was for decades considered a chaotic, unregulated mode of cell dying. However, recently, some cases of regulated necrosis were discovered (36–38). Nowadays, it seems that this process is even more complicated: some “apoptosis-like” and “necrosis-like” kinds of programmed cell death were found (39). HU-331 might kill cancer cells through this kind of death mode, which is supported by the observation that the cells swell a little before dying (data not shown). Other quinones, such as β -lapachone, were found to cause necrotic-like cancer cell death (40). Previously, most of cancer research was focused on triggering apoptosis in tumor cells. However, noting the fact that many cancers have defective apoptosis machinery, it is reasonable to consider whether activating other death pathways, such as necrosis, may be an effective rationale for cancer therapy. Thus, the inability to cause apoptosis in cancer cells, while working through some different mode of cell death, might be rather the strength than the disadvantage of HU-331.

The quinone structure of doxorubicin and daunorubicin permits these compounds to act as electron acceptors of free electrons, which converts the quinones into semi-quinone free radicals that may kill cancer cells. It has been argued that although chemotherapy-induced free radicals may be formed under aerobic conditions in tissue culture, this mechanism may be negligible under the anaerobic conditions of tumor growth (5). The observation that some free radical scavengers (such as MPG and *N*-acetyl cysteine) blocked the cytotoxicity of HU-331, while all others (such as zinc desferrioxamine, acetylhydroxamic acid, deferoxamine mesylate, dimethylthiourea, α -tocopherol, and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) had no such inhibitory activity, raised the possibility that the active compounds may antagonize the toxicity of HU-331 through mechanisms other than scavenging of free radicals. The present study showed that indeed HU-331 did not elicit the production of ROS; therefore, the anticancer effect of HU-331 is not mediated by ROS.

Inhibition of topoisomerase II was previously reported for other chemotherapeutic agents such as doxorubicin. HU-331 was found to specifically inhibit the activity of topoisomerase II *in vitro*, while lacking an inhibitory effect on topoisomerase I. Topoisomerase II participates in most DNA transactions such as replication, transcription, chromosomal segregation, and nucleosomal assembly.

Topoisomerase II DNA relaxation activity is reduced by HU-331 even at a low dose of 100 nmol/L, and a significant to full inhibition is observed at 10 to 30 μ mol/L. For comparison, reduction of topoisomerase II activity by VP-16 is seen at a concentration of 20 μ mol/L, and a full inhibition is observed with 50 to 100 μ mol/L (41, 42).⁵

⁵ Unpublished observations.

Topoisomerase inhibitors are divided into two groups: topoisomerase poisons, which stabilize the topoisomerase-DNA cleavable complex and thus introduce DNA strand breaks leading to apoptosis, and topoisomerase catalytic inhibitors, which hamper the activity of these enzymes without introducing DNA strand breaks. HU-331 seems to be a catalytic inhibitor of topoisomerase II probably through binding to the enzyme protein. Indeed, we show that this drug does not cause DNA damage and protects the cells from natural damage, or damage induced by other topoisomerase II inhibitors that act as topoisomerase poisons. Even when 60% of the target cells are killed by HU-331 treatment, their DNA content remains undamaged, with less strand breaks than in the control DNA. H2AX Western blots revealed more phosphorylated H2AX in control cells than in HU-331-exposed cells. The fact that in the presence of HU-331, only a slight increase in cleavable complex formation was observed, whereas with VP-16, a much more prominent increase in the enzyme-DNA complexes was observed, also supports the conclusion that HU-331 is a catalytic topoisomerase inhibitor rather than a topoisomerase poison. Thus, whereas doxorubicin and other anthraquinones act by numerous mechanisms, such as apoptosis, abrogation of the cell cycle, activation of caspases, generation of ROS, inhibition of both topoisomerases, activation of intracellular second messengers, etc., HU-331 has a highly specific activity, which gives it a high potential to develop into a new anticancer drug.

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