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A Topoisomerase II Inhibitor, NK109, Induces DNA Single- and Double-strand Breaks and Apoptosis

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2,3-(Methylenedioxy)-5-methyl-7-hydroxy-8-methoxybenzo[*c*]phenanthridinium hydrogensulfate dihydrate, called NK109, is a benzo[*c*]phenanthridine derivative, which inhibits DNA topoisomerase II activity by stabilizing the DNA-enzyme-drug complex, and shows strong growth-inhibitory effects on several human cancer cells. In the present study, NK109 treatment induced DNA fragmentation and a rise in the level of cytoplasmic nucleosomes, which are markers of apoptosis, in human small-cell lung carcinoma SBC-3 cells. These effects were inhibited by zinc ions and enhanced by cycloheximide or actinomycin D. Dose-dependent single- and double-strand DNA breaks were observed, using alkaline and neutral elution assays, in SBC-3 cells treated with more than 0.2 μM NK109 for 4 h. Treatment with NK109 caused more DNA single- and double-strand breaks than treatment with an equimolar amount of VP-16. These results suggest that NK109 induces DNA strand breaks and apoptosis. In addition, it appears that this process does not require protein or RNA synthesis, but involves a specific endonuclease which is inhibited by zinc ions.

Key words: NK109 — Apoptosis — DNA strand break — Topoisomerase

Cell death occurs via either of two distinct mechanisms: necrosis and apoptosis.[1)] Whereas necrosis is a degenerative process caused by severe environmental disturbances, apoptosis is regarded as an active suicidal response to various physiological or pathological stimuli. Examples of apoptosis include glucocorticoid killing of thymocytes,[2)] selection of immature thymocytes,[3)] growth factor withdrawal,[4)] cell death after antibody binding to certain cell surface proteins,[5)] and cytotoxic T-cell killing.[6)] In addition, recent studies have shown that a wide variety of cytotoxic agents kill cells by apoptosis even though they damage different cellular targets.[7,8)] X-Ray irradiation and several chemotherapeutic drugs such as alkylating agents and topoisomerase inhibitors are known to trigger apoptosis.[9–11)] The genetic basis and signal transduction pathway of apoptosis are under intensive investigation.[12–18)]

DNA topoisomerases are enzymes that alter the topology of DNA by transiently breaking one or two strands of DNA, passing a single- or double-stranded DNA through the break, and finally resealing the break. These enzymes are involved in a number of crucial cellular processes, including replication, transcription and recombination, and they are considered to be important therapeutic targets in cancer chemotherapy. A novel antitumor agent, 2,3-(methylenedioxy)-5-methyl-7-hydroxy-8-methoxybenzo[*c*]phenanthridinium hydrogensulfate dihydrate, called NK109, has been developed from benzo[*c*]phenanthridine and inhibits DNA topoisomerase II activity by stabilizing the cleavable complex. The chemical structure of NK109 is shown in Fig. 1. NK109 strongly inhibits the growth of human cancer cell lines with an IC_{50} value of 0.2 μM.[19)]

In the present study, we demonstrated that NK109 induced single- and double-strand DNA breaks and DNA fragmentation, which is a marker for apoptosis, in a human small-cell lung cancer cell line. Furthermore, we showed that protein and RNA syntheses were not required for apoptosis.

MATERIALS AND METHODS

Drugs and chemicals NK109 was supplied by Nippon Kayaku Co., Ltd. (Tokyo). NK109 was dissolved in 0.9% NaCl solution and stored at 4°C. Etoposide was obtained from Bristol-Myers Squibb (Tokyo). RPMI-1640 medium and phosphate-buffered saline (PBS) were purchased from Nissui (Tokyo).

Cell line and culture The human small-cell lung cancer cell line, SBC-3, originally established at Okayama University School of Medicine, was donated by the Japanese Cancer Research Resources Bank (JCRB) Cell Division. SBC-3 cells were grown as attached cultures in RPMI-

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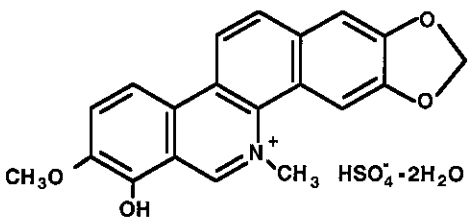

Fig. 1. Chemical structure of NK109

1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. The cells were routinely harvested by trypsinization and diluted with the medium to the appropriate concentrations. Cell numbers were measured using a Coulter Channalyzer C-256 system (Coulter Electronics, Hialeah, FL).

HL-60, a human promyelocytic leukemia cell line, was donated by the JCRB Cell Division.

Electrophoresis of DNA Inter-nucleosomal DNA fragmentation was analyzed by electrophoresis using the method of Smith *et al.*[20] with a slight modification. First 3×10^6 cells were washed twice with PBS, and the pellet was stored in an Eppendorf microcentrifuge tube at −70°C until electrophoretic analysis was carried out. Before electrophoresis, the cells were incubated at 50°C with 20 μl of 10 m*M* ethylenediaminetetraacetic acid (EDTA), 50 m*M* Tris-HCl (pH 8.0) containing 0.5% (w/v) sodium lauryl sarkosinate and 1 mg/ml proteinase K (Sigma) for 3 h. Then, each sample was treated with 8 μl of 10 mg/ml bovine pancreatic ribonuclease (RNase; Type III A, Sigma) at 50°C for a further 3 h. Samples were heated to 70°C for 5 min, mixed with 12 μl of 10 m*M* EDTA (pH 8.0) containing 1% (w/v) low-gelling-temperature agarose (FMC Corp., Rockland, MD), 0.025% (w/v) bromophenol blue and 40% (w/v) sucrose and loaded into the dry wells of a 2.5% (w/v) Nusieve 3:1 agarose gel (FMC Corp.). Electrophoresis was carried out in 40 m*M* Tris acetate, 1 m*M* EDTA at 2 V/cm until the marker dye had migrated about 8 cm. The gel was stained with 1 μg/ml ethidium bromide and washed with deionized water for 24 h, then the fluorescence of ethidium bromide-stained DNA was photographed. A 123-bp DNA ladder, suitable for use as ethidium bromide-stainable markers for gel electrophoresis, was purchased from Gibco BRL (Tokyo).

Filter elution assay Prior to filter elution analysis, exponentially growing SBC-3 cells were radiolabeled by incubation with [methyl-^{14}C]thymidine (0.02 μCi/ml) for 24 h. The cells were then washed free of radioactive medium, and incubated for at least 2 h before drug treatment and alkaline elution analysis.

For the determination of drug-induced DNA single-strand breaks or double-strand breaks in SBC-3 cells, the alkaline or neutral filter elution technique was performed as described previously.[21] Approximately 3×10^5 drug-treated cells were kept at ice temperature to prevent DNA repair. They were then diluted in cold PBS (137 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4) and gently deposited onto a polycarbonate filter. The cells were lysed on the filter for 1 h with 5 ml of a lysis solution containing 2% sodium dodecyl sulfate, 25 m*M* EDTA, 50 m*M* Tris, 50 m*M* glycine, and 0.5 mg/ml proteinase K, pH 9.6. The lysis solution was allowed to flow through the filter under gravity, and then the filter was rinsed three times with 3 ml of 20 m*M* disodium EDTA, pH 9.6, to remove most of the cell protein, membrane and RNA. The remaining DNA (more than 97% of that applied to the filter) was analyzed by elution with tetrapropylammonium-tetrahydroxy-EDTA, pH 12.1 for alkaline elution, or pH 9.6 for neutral elution, at a flow rate of 0.025 to 0.030 ml/min. Ten fractions of the eluate were collected directly into scintillation vials on a fraction collector at 1.5 h intervals for 15 h. The fractions were then mixed with five volumes of Clear-sol I (Nacalai Tesque Inc., Kyoto) containing 0.5% acetic acid, and the radioactivity was counted in an LS3801 liquid scintillation counter (Beckman).

Detection of nucleosomes in the cytoplasm Enrichment of nucleosomes in the cytoplasm of cells treated with drugs was detected by a Cell Death Detection ELISA (Boehringer Mannheim GmbH, Germany). In the first incubation step, the wells of a microtiter plate were coated with anti-histone antibodies by adsorption. Next, non-specific binding sites in the wells were saturated by treatment with a blocking solution. During the second incubation step, nucleosomes in the samples bound via their histone components to the immobilized anti-histone antibodies. In the third incubation step, peroxidase-conjugated anti-DNA antibodies reacted with the DNA of the nucleosomes. After removal of unbound peroxidase-conjugate by washing, the amount of peroxidase retained in the wells was determined photometrically, using ABTS (2,2′-azinodi[3-ethylbenzthiazoline sulfonate]),[22] as the substrate.

We calculated the degree of nucleosome enrichment in the cytoplasm by use of the following equation: Enrichment factor = [absorbance of the sample (drug-treated cells) − absorbance of blank]/[absorbance of the corresponding control (no drug treatment) − absorbance of blank]. Therefore, the enrichment factor of the control cells (exposed to drug-free medium) was 1.0.

Growth-inhibition assay We used the tetrazolium dye (MTT) assay described previously to evaluate the growth-inhibitory effects of the cytotoxic agents.[23]

RESULTS

DNA fragmentation, which is considered to be a marker for apoptosis, was detected at dose-dependent levels in extracts from SBC-3 cells treated with 2.0–20 μM NK109, by agarose-gel electrophoresis (Fig. 2A). DNA fragmentation was detected after more than 4 h of exposure of HL-60 cells to NK109, which induced internucleosomal DNA fragmentation in the same way as VP-16 (Fig. 2B).

Topoisomerase inhibitors are known to induce DNA strand breaks and apoptosis.[24] The DNA single- and double-strand breakage activities of NK109 were examined using pH 12.1 alkaline and pH 9.6 neutral elution assays, respectively, of SBC-3 cells. DNA single-strand breaks occurred dose-dependently in SBC-3 cells treated with more than 0.2 μM NK109 for 4 h, and DNA double-strand breaks also occurred dose-dependently in cells treated with more than 0.2 μM NK109 for 3 h (data not shown). We compared the DNA strand breakage activity of NK109 with that of VP-16. SBC-3 cells were exposed to 0.2, 2.0, or 20 μM NK109 or VP-16 for 5 h, and examined for single- and double-strand breaks by filter elution assays. NK109 treatment induced more of both DNA single- and double-strand breaks than the same concentration of VP-16 (Fig. 3). In particular, NK109 showed a stronger DNA double-strand break-inducing activity than VP-16 (Fig. 3B).

The presence of nucleosomes in the cytoplasmic fraction of cells, which is also considered as a marker for apoptosis, was examined in SBC-3 cells treated with or without NK109, as well as either cycloheximide, actinomycin D or ionic zinc for 6 h (Fig. 4). NK109 (2 μM) induced an increase of the enrichment factor to 1.8, suggesting the induction of apoptosis. The level of nucleosomes in the cytoplasmic fraction of cells treated with cycloheximide (1.0–100 μg/ml) or actinomycin D (0.5–2.0 μg/ml) increased dose-dependently, whereas it decreased in cells treated with zinc ions (0.5–2.0 mM). These changes were observed whether or not the cells were treated with NK109.

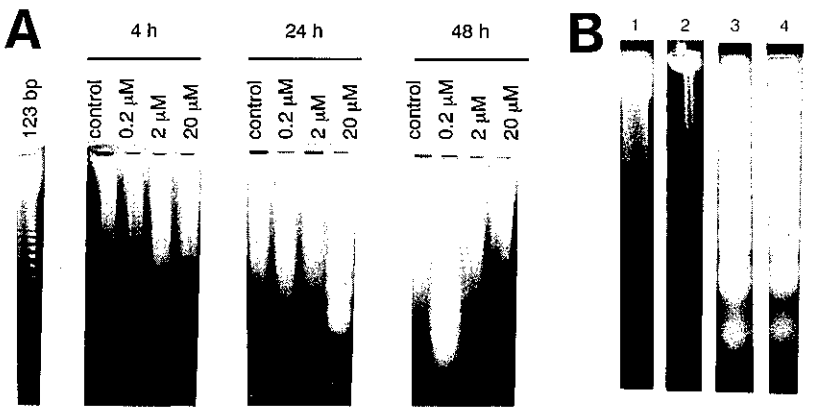

Fig. 2. Effect of NK109 on SBC-3 cells (A) and HL-60 cells (B) in agarose gel electrophoresis. A, SBC-3 cells were treated with NK109 for 4, 24 and 48 h, respectively. NK109 concentrations are shown in the figure. Control, free of drug. '123 bp' is the molecular weight marker, 123-bp DNA ladder. B, HL-60 cells were treated with NK109 or VP-16 for 20 h. Lane 1,123-bp DNA ladder; Lane 2, drug-free control; Lane 3, 2 μM NK109; Lane 4, 50 μM VP-16.

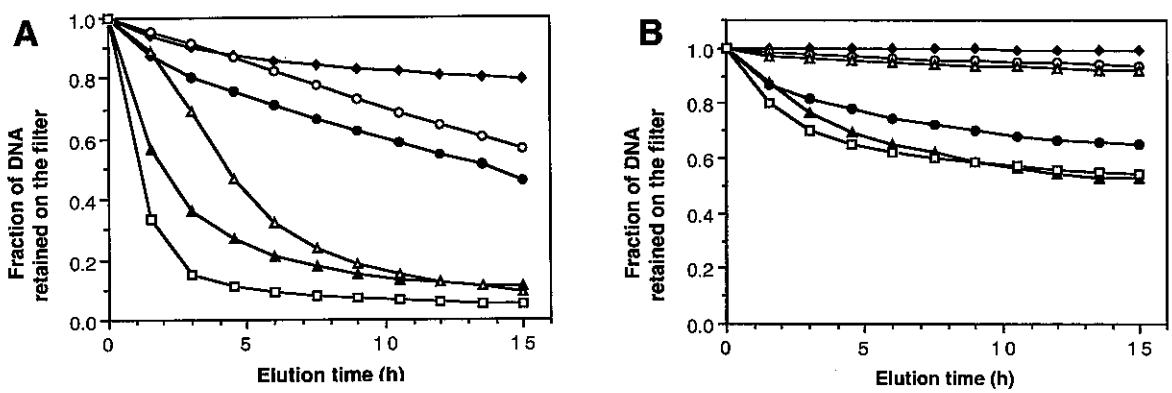

Fig. 3. DNA single- (A) and double- (B) strand breaks caused by 5 h exposure of SBC-3 cells to NK109 or VP-16. ◆, control; ●, 0.2 μM NK109; ▲, 2.0 μM NK109; ○, 0.2 μM VP-16; △, 2.0 μM VP-16; □, 20 μM VP-16.

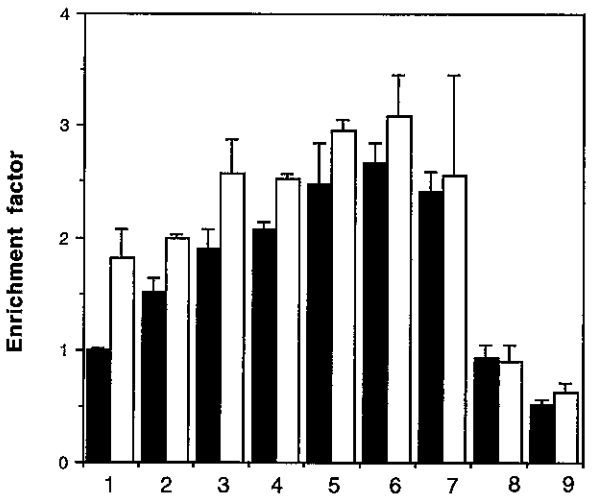

Fig. 4. The effects of NK109, cycloheximide, actinomycin D and zinc ions on the enrichment of nucleosomes in the cytoplasm of SBC-3 cells. SBC-3 cells were exposed to the drugs for 6 h. The enrichment factor of the control was 1.0. Lane 1, control; Lane 2, 1.0 μg/ml cycloheximide; Lane 3, 5.0 μg/ml cycloheximide; Lane 4, 20 μg/ml cycloheximide; Lane 5, 100 μg/ml cycloheximide; Lane 6, 0.5 μg/ml actinomycin D; Lane 7, 2.0 μg/ml actinomycin D; Lane 8, 0.5 m*M* zinc ions; Lane 9, 2.0 m*M* zinc ions; ■, without NK109; □, with 2 μ*M* NK109.

The growth-inhibitory activity of NK109 on SBC-3 cells was examined using the concentration which induced DNA strand breaks and apoptosis. The IC_{50} values of NK109 and VP-16 for SBC-3 cells, as determined by MTT assay, were 1.36 ± 0.17 μ*M* and 95.7 ± 33.2 n*M*, respectively. Ionic zinc (0.5-2.0 m*M*) did not have a cytotoxic effect on SBC-3 cells (data not shown).

DISCUSSION

Apoptosis is important in the homeostatic control of normal tissues, embryonic development, carcinogenesis, tumor development, and cancer therapy.[25,26] It is an active mechanism of cell death that is activated by exposure to a wide variety of stimuli. These observations suggest that apoptosis represents an underlying mechanism for cell death regardless of the initial stimuli and that the responses to divergent stimuli converge on a fundamental signal transduction pathway of cell death.

Apoptotic cell death is not only observed in continuously renewing tissues, but can be experimentally induced in cultured cells following incubation in serum-free medium or treatment with a variety of agents used for tumor therapy.[10,27-29] NK109, a benzo[*c*]phenanthridine derivative, has been demonstrated to inhibit strongly the growth of several human cancer cell lines including drug-resistant lines.[30] NK109 is a topoisomerase inhibitor and this function is considered to be its main mechanism of cytotoxicity.[19] In this study, we have demonstrated that cells treated with NK109 underwent DNA fragmentation (Fig. 2), and that the level of nucleosomes in the cytoplasmic fraction increased (Fig. 4). These findings suggest that NK109 induces apoptosis, and that the inhibition of topoisomerases is related to DNA cleavage and apoptosis.

It was difficult to detect DNA fragmentation or an increase in the level of cytoplasmic nucleosomes in the cells treated with 0.2 μ*M* NK109 (Fig. 2A), but 4 h treatment with more than 0.2 μ*M* NK109 induced both DNA single- and double-strand breaks (Fig. 3 and data not shown). Nelson and Kastan[24] demonstrated that DNA strand breaks induced by topoisomerase inhibitors induced p53 expression in cells with wild-type p53 alleles indicating the importance of strand breakage as a trigger of the p53-dependent DNA-damaging pathways. Although our data do not provide direct evidence for this relationship, they do indicate that an increase in the number of DNA strand breaks induced by NK109 triggers apoptosis. The p53 status in SBC-3 cells should be characterized.

NK109 induced more DNA strand breaks, especially DNA double-strand breaks, than the same concentration of VP-16 (Fig. 3). It is questionable whether the double-strand breaks induced by NK109 are non-protein-bound or not. Fig. 3 demonstrated that NK109 induced both single- and double-strand breaks. SDS/proteinase K treatment greatly increased the amount of VP-16-damaged DNA strand breaks detected by alkaline elution assay. The same treatment only slightly increased the amount of NK109-damaged DNA strand breaks detected by alkaline elution assay. Even at the washing step before the alkaline elution, most of the NK109-induced DNA strand breaks had already occurred.[19] This result suggests that NK109 induces DNA strand breaks independently of cleavable complex formation, although the possibility of cleavable complex formation by NK109 can not be ruled out.[19]

In addition, etoposide-resistant cells, with decreased topo II activity, exhibited as high sensitivity to NK109 as did their parent cells.[31] Apoptosis induced by NK109 was not inhibited by cyclohexide or actinomycin D (Fig. 4), whereas that induced by VP-16 was inhibited by them in SBC-3 cells (data not shown), as previously reported.[32] These results suggest that the mechanism of cytotoxicity of NK109 is different from that of etoposide.

In thymocytes, DNA fragmentation is induced by topoisomerase inhibitors and is inhibited by cycloheximide, a protein synthesis inhibitor, and actinomycin D, a transcription inhibitor.[33] Doxorubicin is reported to induce apoptosis that is inhibited by cycloheximide.[34] Induction of apoptosis by other topoisomerase II-tar-

geting drugs and inhibition of this process by cycloheximide and actinomycin D have also been demonstrated.[35] Although these reports suggest that protein or RNA synthesis is necessary for apoptosis induced by etoposide, our experiment showed that neither protein nor RNA synthesis is required to induce apoptosis. Therefore, there may be several pathways for the induction of apoptosis. Some studies have shown that Zn^{2+} blocks inter-nucleosomal DNA fragmentation and apoptotic cell death in a variety of cells *in vitro*,[36-40] and our data confirm those results. It has been demonstrated that the apoptotic death of cells is accompanied by cleavage of inter-nucleosomal DNA by a Ca^{2+}/Mg^{2+}-dependent endonuclease,[41,42] and that a physiological concentration of Zn^{2+} inhibits the Ca^{2+}/Mg^{2+}-dependent endonuclease activity and DNA fragmentation in intact splenocytes and cell extracts.[40] Apoptosis of SBC-3 cells induced by NK109 was shown to be related to a Ca^{2+}/Mg^{2+}-dependent endonuclease.

Recently it has been demonstrated that activations of interleukin 1β converting enzyme (ICE) and ICE-like protease are involved in VP-16-induced apoptosis.[43] We examined the effects of ICE inhibitors on the apoptosis induced by NK109. Apoptosis induced by NK109 was inhibited by 50 μg/ml ICE inhibitor (Ac-Tyr-Val-Ala-Asp-H, Peptide Res. Co., Osaka) or 25 μg/ml ICE/ICE-like protease inhibitor (Z-Asp-CH2-DCB, Peptide Res. Co.). These results suggest that ICE and ICE-like protease activation are involved in the pathway of apoptosis induced by NK109.

NK109, a novel topoisomerase II inhibitor, induced DNA single- and double-strand breaks and apoptosis. It appears that apoptosis induced by NK109 does not require protein or RNA synthesis, and that it involves a specific endonuclease, which is inhibited by ionic zinc.

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