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Inhibition of p53 Protein Phosphorylation by 9-Hydroxyellipticine: A Possible Anticancer Mechanism

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Abnormality of p53, a tumor suppressor gene, is considered to be a potential cause of malignancy. We found that ellipticine and 9-hydroxyellipticine (9HE), antitumor alkaloids, caused selective inhibition of p53 protein phosphorylation in Lewis lung carcinoma and SW480 (human colon cancer cell line) in a concentration-dependent manner from 0.1 to 100 μM. 9HE suppressed cdk2 kinase activity concentration-dependently from 1 to 100 μM. By contrast, the inhibition of p53 protein phosphorylation by elliptinium and elliprabin (N2 substituted derivatives of 9HE) was very weak. A good correlation was observed between p53 phosphorylation inhibition and cytotoxic activity of these agents in terms of concentration-response relationships, suggesting that inhibition of p53 protein phosphorylation via kinase inhibition may be involved in the anticancer mechanism of these agents. In addition, this study demonstrated that brief exposure to 9HE caused apoptosis of cancer cells. It is suggested that accumulation of dephosphorylated mutant p53 may induce apoptosis.

Key words: Ellipticine — p53 — Phosphorylation inhibition — Apoptosis induction — Anticancer mechanism

Ellipticine (E), an alkaloid obtained from *Ochrosia acuminata* and from *Ochrosia elliptica*, and its analogues have anti-cancer activities in various tumor models and in man,[1] possibly as a result of DNA intercalation[2,3] or interaction with topoisomerase II-DNA complex,[4,5] although the mechanism remains to be proven.

During the course of screening of protein phosphorylation inhibitors, we found that E caused a specific inhibition of phosphorylation of p53 protein, the product of the p53 tumor-suppressor gene. Recently, changes in p53 gene have been noted as a potential cause of cancer, because loss of heterozygosity at polymorphic markers closely linked to p53 gene and point mutation of the remaining allele are frequently observed in a wide variety of human cancers.[6–9] It is now recognized that wild-type p53 gene has the properties of a tumor-suppressor gene.[10–14]

p53 protein is phosphorylated by p34 cdc2 kinase[15–18] and the phosphorylation occurs during the S and G2/M phase of the cell cycle.[16,19] p53 protein is also phosphorylated by casein kinase II[20,21] and DNA-activated protein kinase.[22–24] Recently it has been shown that overexpression of wild-type p53 induces apoptosis[25–29] and that G1 arrest induced by wild-type p53 is mediated through an increase in waf1/cip1 level associated with a decrease in cyclin-dependent kinase activity.[30] This occurs predominantly in the G1 phase.[26] In normal cells, however, only a little wild-type p53 is produced and apoptosis occurs when wild-type p53 is elevated by stimulation such as DNA damage.[31–33] In contrast, a large amount of mutant p53 protein is produced,[14,34–40] and highly phosphorylated in cancer cells.[41–43] However, mutant p53 has no ability to induce apoptosis.[44]

As reported herein, E and 9-hydroxyellipticine (9HE) cause selective inhibition of p53 protein phosphorylation through kinase inhibition in several human and animal tumor cell lines. We propose that certain ellipticines have a mechanism of antitumor action other than topoisomerase II inhibition. In addition, the significance of inhibition of mutant p53 phosphorylation in relation to tumor cell death or apoptosis will be discussed.

MATERIALS AND METHODS

Chemicals 9HE, elliptinium acetate (2-methyl-9-hydroxyellipticine, EA) and elliprabin (9-hydroxyellipticine arabinoside, ER) were synthesized in the Organic Chemistry Laboratory of Tanabe Seiyaku Co., Ltd. Etoposide and E were purchased from Sigma Chemical Co. (St. Louis, MO). Doxorubicin hydrochloride (adriamycin) was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo). Anti-p53 monoclonal antibodies, PAb 240 and 421, and anti-cdk2 kinase antibody, Ab-1, were purchased from Oncogene Science, Inc. (Uniondale, NY). Calf thymus double-stranded DNA was obtained from Sigma Chemical Co. Casein kinase II and histone H1 were purchased from Boehringer Mannheim Japan (Tokyo). A p13 *Suc*I expression vector was generously provided by Dr. P. Nurse (ICRF Cell Cycle Group, University of Oxford, UK).[45] CNBr-activated Sepharose 4B and protein A Sepharose beads were purchased from Pharmacia LKB (Uppsala, Sweden). [γ-^{32}P]ATP (>370

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GBq/mmol) and [^{32}P]H_3PO_4 (>314 TBq/mmol) were purchased from New England Nuclear (Boston, MA).

Cell lines and cultures SW480, a human colon carcinoma cell line in which p53 is mutated at codons 273 and 309,[6] was purchased from the American Type Culture Collection, and maintained in L15 containing 10% fetal calf serum. Mouse Lewis lung carcinoma (3LL), in which p53 is mutated at codons 32 and 334 (M. Gamanuma, unpublished results), was maintained by passage in mice and these cells were obtained from mice before use.

Cell fractionation 3LL, which was inoculated in the inguinal region of BDF1 mice (Shizuoka Laboratory Animal Center, Hamamatu) was resected and homogenized on ice with a homogenizer at 1200 rpm (10 strokes). The homogenate was centrifuged at 1,000*g* at 4°C for 10 min and the supernatant was further centrifuged at 30,000*g* at 4°C for 10 min. The microsomal fraction was pelleted by 1 h centrifugation at 100,000*g* and the precipitates were resuspended in 5 m*M* Tris-HCl (pH 7.0). Protein concentration of the resulting microsomal fraction was determined by Bradford's method.[46] The fractions were stored in liquid nitrogen until use.

Phosphorylation with ATP Fifty μl of the microsomal fraction (2 mg/ml) was incubated in an equal volume of the reaction medium containing 20 μM [γ-^{32}P]ATP, 20 mM $MgCl_2$, and 20 mM Na_3VO_4 for 5 min at 37°C. The reaction was terminated by addition of 100 μl of sodium dodecyl sulfate (SDS)-sample buffer and boiling in a water bath for 2 min. An aliquot of each sample was loaded on a 7.5% polyacrylamide gel in the presence of SDS and electrophoresed at 30 mA (SDS-PAGE). The gel was fixed, dried and placed in contact with an imaging-plate (Fuji Photofilm Co., Tokyo). The plate was subjected to analysis for phosphorylated proteins by using a bioimage analyzer, BAS 2000 (Fuji Photofilm Co.).

Phosphorylation with H_3PO_4 in whole cells Subconfluent monolayers of cells in a 96-well plate (4×10^4 cells/well) were washed with phosphate-buffered saline (PBS) and 80 μl of phosphate-free Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum was added. After addition of 10 μl of the drug or the medium (control), cells were radiolabeled with 10 μl of [^{32}P]-H_3PO_4 (74 MBq/ml) for 24 h at 37°C in a 5% CO_2 incubator. After the medium was removed, 100 μl of boiled SDS-sample buffer was added. The samples were loaded on a 7.5% SDS-polyacrylamide gel. After electrophoresis, the gel was fixed and dried. The dried gel was placed on an X ray film (DEF-5; Kodak) or on the imaging-plate. The films were developed for 5 min with Kodak GBX developer at 20°C. The plate was subjected to analysis by using the bioimage analyzer.

Immunoblotting Following SDS-PAGE, proteins were transferred electrophoretically to Immobilon transfer membrane (Millipore Corporation, Bedford, MA) for 45 min at 100 mA in the loading buffer containing 48 mM Tris, 39 mM glycine, 0.037% SDS and 20% methanol. The membrane was incubated with a 1:1000 diluted anti-p53 monoclonal antibody, PAb240 or PAb421 in TTBS buffer containing 100 mM Tris (pH 7.5), 0.9% NaCl, and 0.1% (v/v) Tween 20 for 20 min at room temperature. The membrane was washed with TTBS, and incubated with biotinylated horse anti-mouse IgG at 1 μg/ml in TTBS for 20 min. After further washing, a solution containing avidin-biotinylated complex (ABC reagent) was added. The membrane was rinsed well, and stained by addition of 0.02% of H_2O_2 and 0.1% diaminobenzidine.

Immunoprecipitation of p53 After phosphorylation with [γ-^{32}P]ATP, the labeled microsomal fraction from 3LL was lysed in 800 μl of the lysis buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.5% SDS, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Na_3VO_4 for 20 min on ice. The protein debris was removed by centrifugation and 200 μl of the supernatant was subjected to immunoprecipitation with PAb240 for 1 h. The immune complexes were collected by a subsequent 1 h incubation with 35 μl of a 50% suspension of Protein A-Sepharose beads. The beads were washed 5 times in RIPA (radio-immuno-protein assay) buffer containing 1% NP-40, 0.1% sodium deoxycholate, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM PMSF and 1 mM Na_3VO_4, boiled in SDS-sample buffer, and subjected to 7.5% SDS-PAGE. Immunoprecipitation of p53 from the cell lysates of SW480 was performed using PAb421 under the same conditions as described above.

Cyclin-dependent kinase assays The preparation of p13-Sepharose beads and the cdk2 kinase assay were carried out as described previously.[47,48] HeLaS3 cells (10^7 cells) were suspended in 1 ml of buffer A (50 mM Tris-Cl (pH 7.4), 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 50 mM NaF containing aprotinin and leupeptin, each at a concentration of 20 μg/ml, and 2 mM PMSF). After incubation on ice for 30 min, the lysate was centrifuged at 13,000*g* for 5 min at 4°C. To the supernatant, 90 μl of a 50% (v/v) suspension of p13-Sepharose beads was added. The mixture was incubated for 1 h at 4°C with gentle mixing. Then the mixture was centrifuged in a microcentrifuge for 30 s and washed with buffer A three times. After the last wash, the beads were resuspended in 500 μl of kinase buffer (50 mM Tris-Cl (pH 7.4), 10 mM $MgCl_2$, and 1 mM dithiothreitol). Fifteen μl of kinase buffer was added to the beads after removal of the supernatant. Fifty μM ATP, 1.2 μg of histone H1, an ellipticine derivative, and 187.5 kBq of [γ^{32}P]ATP were added, and the reaction mixture was incubated at 30°C for 2 min. The reaction was stopped by the addition of 30 μl of 2×SDS sample buffer. Twenty μl of the reaction

mixture was subjected to 10% SDS-PAGE. Following electrophoresis, phosphorylated histone H1 was visualized by autoradiography with BAS 2000.

Cytotoxic assay The chemosensitivity of 3LL was determined by a quantitative colorimetric assay using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide)[49] and that of SW480 cells was determined by a viable cell count after 24 h incubation with drugs. Cultures were adjusted to 1×10^5 cells/ml in a 96-well plate. Ten μl of a culture medium (control) or a drug solution with an appropriate concentration was added to each well of the plate. The plate was incubated in 5% CO_2 at 37°C for 24 h. MTT was added to each well to a final concentration of 500 μg/ml and the plate was incubated in 5% CO_2 at 37°C for 3 h. The supernatant was removed and 150 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve incorporated MTT. Absorbance at 595 nm was measured using a microplate reader, V_{max} (Molecular Devices, Menlo Park, CA). The number of viable cells was also determined by counting non-stained cells after treatment with trypan blue.

Analysis of DNA fragmentation Drug-treated and mock-treated cells were grown in 75 cm^2 culture bottles. Both attached and un-attached cells were harvested, washed with ice-cold PBS, resuspended in 0.5 ml of the lysis buffer (0.25% NP-40, 10 μg/ml RNase A, 1 m*M* EDTA and 5 m*M* Tris-boric acid, pH 8.0) and incubated for 30 min at 37°C. Proteinase K was added to a final concentration of 1 mg/ml and incubation was continued for another 30 min at 37°C. DNA was extracted with an equal volume of phenol/chloroform, and precipitated with 2.5 volumes of ethanol at −70°C for 10 min. DNA was pelleted at 13,000*g* for 10 min. The precipitates were dissolved in 0.5 ml of the buffer containing 1 m*M* EDTA and 10 m*M* Tris-HCl (pH 8.0). Samples were loaded onto 3% agarose gels, and DNA was visualized by ethidium bromide staining.

RESULTS

Inhibition of phosphorylation of 53 kDa protein by ellipticines in cell lysates and microsomal preparations During the course of screening of protein phosphorylation inhibitors, we found that E caused a selective inhibition of 53 kDa protein phosphorylation in the lysates prepared from mouse cancer cell lines, such as S180, L1210, P388, 3LL, colon 26 and B16 melanoma.[45] The phosphorylation of the 53 kDa protein was more marked in the 100,000*g* precipitates (microsomal fractions) than in the cell lysates, and was not detected in the mitochondrial fractions and cytosols (data not shown). Therefore, most of the experiments on phosphorylation with ATP were carried out using microsomal fractions. All phosphorylations were done in the presence of Na_3VO_4 (phosphatase inhibitor) to exclude the possibility of phosphatase activation by drugs. Fig. 1 shows autoradiograms demonstrating inhibition of phosphorylation with [γ-^{32}P]ATP by E, 9HE, EA and ER in the microsomal fractions of 3LL and B16 melanoma. 53 kDa protein was a major phosphorylated protein in the preparation. Phosphorylation of the 53 kDa protein was inhibited by E and 9HE in a concentration-dependent manner from 1 to 100 μM. The inhibition was highly selective for the 53 kDa protein, although phosphorylations of several other proteins were inhibited at 100 μM. By contrast, EA and ER showed almost no phosphorylation inhibition up to 10 μM. At 100 μM, EA caused a slight non-selective inhibition and ER caused strong non-selective inhibition.

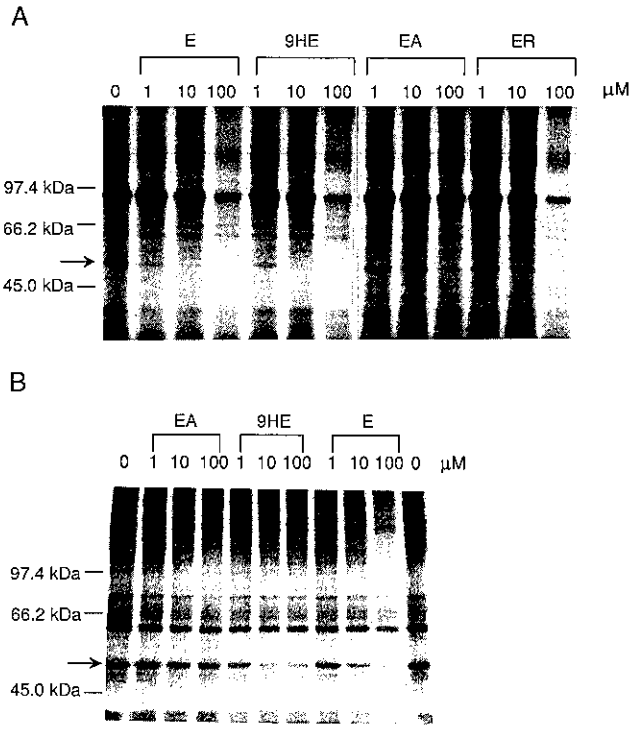

Fig. 1. Autoradiograms showing effects of ellipticines on protein phosphorylation in the microsomal fractions from 3LL (A) and B16 melanoma (B). The abbreviations for drugs are: E, ellipticine; 9HE, 9-hydroxyellipticine; EA, elliptinium acetate; ER, elliprabin. The numbers above each lane indicate drug concentrations (μM). The preparations were phosphorylated with [γ-^{32}P]ATP for 5 min in the presence or absence of drugs. After 7.5% SDS-PAGE, phosphorylated proteins were visualized by autoradiography using BAS 2000. Arrows indicate p53 bands. Bars indicate molecular markers of 97.4, 66.2 and 45.0 kDa.

Determination of p53 protein by immunoprecipitation The 53 kDa phosphoprotein described above was identified as p53 protein, a tumor suppressor gene product, by

immunoprecipitation. Fig. 2A shows an autoradiogram demonstrating inhibition of phosphorylation of the proteins immunoprecipitated with an anti-p53 monoclonal antibody, PAb 240. Two phosphorylated proteins (about 70 kDa and 40 kDa proteins) were co-precipitated with p53 protein, but only p53 was subject to phosphorylation inhibition by E and 9HE. The inhibition was concentration-dependent in a manner similar to that shown in Fig. 1. Another SDS slab gel simultaneously obtained in the same experiment was subjected to immunoblotting with PAb 240 (Fig. 2B). Three bands were seen. The uppermost band, located a little above the position of 53 kDa, was the heavy chain of IgG. The lowest band was the light chain of IgG. The middle band of 53 kDa was p53 protein. Since the intensity of the immunostained p53 bands was almost the same among the lanes, almost equal amounts of the samples had been applied to each lane in Fig. 2A.

Inhibition of p53 phosphorylation in whole cells Inhibition of p53 protein phosphorylation in whole cells was examined using SW480. In Fig. 3, autoradiographs show the phosphorylated proteins after incubation with $[^{32}P]H_3PO_4$ for 24 h in the presence or absence of drugs. Cell numbers decrease as a function of increasing drug concentration if the drug exhibits cytocidal effects. Therefore, total radioactivity in each lane of SDS-PAGE decreased as the drug concentration increased, as a consequence of a decrease in protein concentration when equal volumes of the reaction mixture were applied. In other words, the decrease in the total radioactivity in each lane reflects the cytocidal activity of the drug. The intensities of the 40, 51, 53 and 70 kDa bands relative to those of the other bands decreased markedly with increase in the concentration of 9HE (Fig. 3). The presence of p53 protein was proven in the lysates of SW480 by immunoprecipitation with anti-p53 monoclonal antibody, PAb421, after phosphorylation with $[\gamma\text{-}^{32}P]ATP$ for 5 min in the presence or absence of 9HE (Fig. 4). Two proteins of 51 and 53 kDa were precipitated with PAb421. The 53 kDa protein should be p53 and the 51 kDa protein may be a degradative fragment of p53.[50] 9HE inhibited phosphorylation of these proteins in a concentration-dependent manner from 0.1 to 10 μM, as shown in both Figs. 3 and 4. The similarity of the modes of the inhibition against p53, 51 kDa, 40 kDa and 70 kDa protein phosphorylations suggests that 9HE influences a common kinase active on all these proteins. In the case of ER, in contrast, decrease in the intensity of p53 protein phosphorylation paralleled decreases in the other bands (Fig. 3). This decrease in the apparent phosphorylation intensity was due to a decrease in the protein concentration in the samples applied to each lane. It is concluded, therefore, that ER did not cause selective inhibition of p53 phosphorylation.

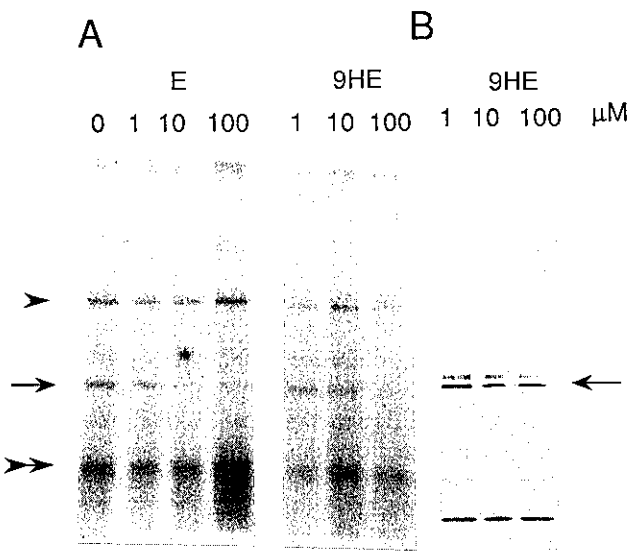

Fig. 2. A. Autoradiogram of the immunoprecipitates with anti-p53 monoclonal antibody PAb240 after phosphorylation of the microsomal fraction from 3LL with $[\gamma\text{-}^{32}P]ATP$ in the absence or presence of E, or 9HE for 5 min. The numbers above each lane indicate drug concentrations (μM). The arrow indicates p53 bands. The arrowheads indicate 70 and 40 kDa. B. Immunostaining with PAb240 after Western blotting of the gel shown in A. The arrow indicates p53 bands. The other bands are the heavy and light chains of IgG.

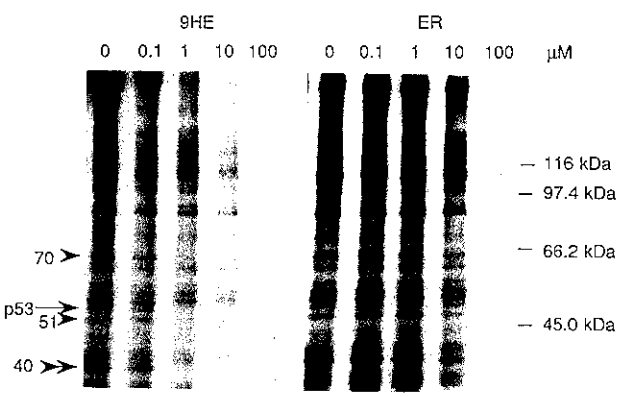

Fig. 3. Autoradiograms showing inhibition of protein phosphorylation by 9HE and ER in SW480 cells. SW480 cells underwent phosphorylation with H_3PO_4 for 24 h in the presence or absence of 9HE and ER. The numbers above each lane indicate drug concentrations (μM). An equal amount of the sample was applied to each lane and therefore the protein concentration decreases as the drug concentration increases because of cytocidal action of the drugs. Arrows indicate p53 bands. Bars indicate molecular markers of 116.0, 97.4, 66.2 and 45.0 kDa.

Effects of ellipticines on histone H1 phosphorylation by cdk2 kinase 9HE caused a concentration-dependent inhibition of histone H1 phosphorylation by cdk2 kinase from 1 to 100 μM, and inhibition by 9HE was much stronger than that by EA (Fig. 5). The inhibition was similar to that of p53 phosphorylation in terms of concentration range of the drugs and structure-activity relationship.

Relationship between inhibition of p53 phosphorylation and inhibition of tumor cell growth A good correlation was observed between inhibition of p53 phosphorylation and cytotoxic effects of Es (Figs. 6 and 7). The IC_{50}s of E for p53 phosphorylation inhibition and the cell growth inhibition of 3LL were 1.4 and 1.3 μM, respectively; those of 9HE were 1.7 and 1.3 μM, respectively (Fig. 6). On the other hand, ER and EA, in which N-2 is positively charged, showed weak cytotoxic effects and weak inhibition of p53 phosphorylation. The IC_{50}s of ER for inhibition of p53 phosphorylation and cell growth were 23 and 16 μM, respectively. EA showed little inhibition ($IC_{50} >$ 100 μM) of either phosphorylation or cell growth.

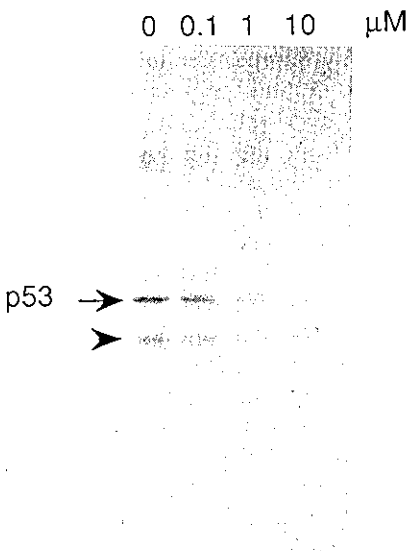

Fig. 4. Autoradiogram showing the result of 7.5% SDS-PAGE of the immunoprecipitates with anti-p53 monoclonal antibody PAb 421 after phosphorylation of the cell lysates from SW480 with [γ-^{32}P]ATP in the absence or presence of 9HE for 5 min. The same protein content was applied to each lane. The numbers above each lane indicate drug concentration (μM). The arrow indicates p53 bands and arrowheads indicate 51 kDa bands.

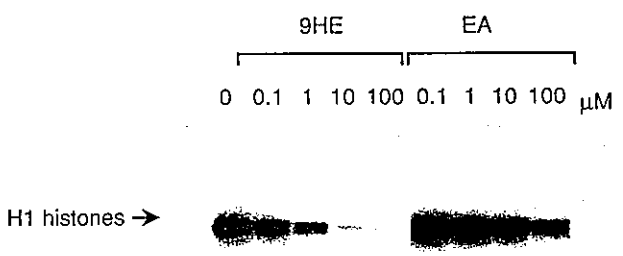

Fig. 5. Inhibition of cdk2 kinase activities by 9HE and EA. *In vitro* histone H1 kinase assays were performed for 2 min with the cdk2 kinase which was obtained by precipitation with p13-Sepharose beads. The concentrations (μM) of ellipticine derivatives are shown above the lanes. Phosphorylation of H1 histones was analyzed by autoradiography using BAS 2000 after 10% SDS-PAGE.

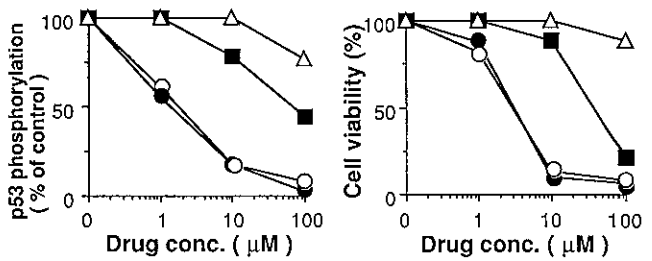

Fig. 6. Effects of E, 9HE, EA and ER on p53 phosphorylation (left) and cell growth (right) of 3LL. The microsomal preparations underwent phosphorylation with [^{32}P]ATP for 5 min in the presence or absence of the drug. Cell growth inhibition was determined by MTT assay after incubation with or without the drug for 24 h. ○, E; ●, 9HE; △, EA; ■, ER.

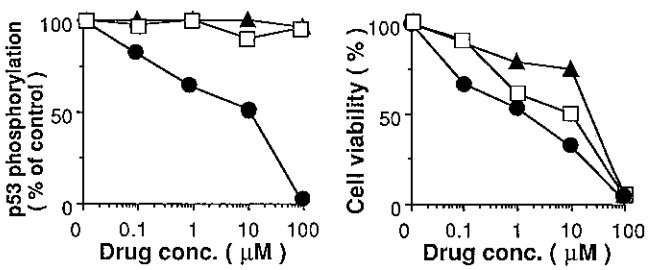

Fig. 7. Effects of 9HE, etoposide and adriamycin on p53 phosphorylation (left) and cell growth (right) of SW480. Phosphorylation was performed by incubation of SW480 cells with [^{32}P]H_3PO_4 in phosphate-free DMEM for 24 h. Cell growth inhibition was determined in terms of the number of viable cells after 24 h incubation. Although inhibition of phosphorylation could not be determined at 100 μM because of the small amount of the protein remaining due to the cytocidal effects of the drugs, 9HE completely inhibited p53 phosphorylation at 100 μM, but etoposide and adriamycin did not in the cell-free preparations. ●, 9HE; □, etoposide; ▲, adriamycin.

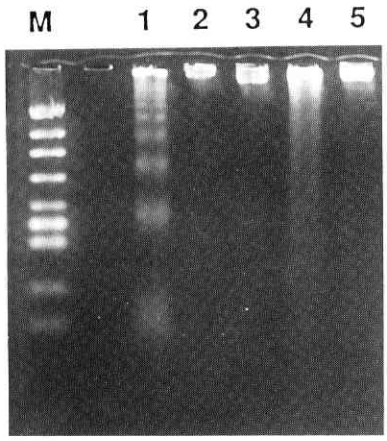

Fig. 8. DNA fragmentation in 3LL cells after treatment with 9HE at 37°C for 8 h (lane 1) and 10 μM adriamycin for 8 (lane 2), 24 (lane 3) and 48 h (lane 4). Lane 5 is the control (no drug). DNA samples obtained were loaded onto 3% agarose gels, and DNA was visualized by ethidium bromide staining. Lane M shows *Hinc*II-digested ϕX174 molecular weight markers.

SW480 cells were divided into 2 groups. One group was subjected to phosphorylation inhibition by 9HE, etoposide and adriamycin; the cells were incubated with $[^{32}P]H_3PO_4$ in the presence or absence of the drugs for 24 h. The other group was used to examine the antiproliferative effects of these drugs during incubation for 24 h. These experiments were carried out simultaneously. Fig. 7 shows the effects of 9HE, etoposide and adriamycin on p53 protein phosphorylation and cell growth. 9HE exhibited a good correlation between inhibition of p53 protein phosphorylation and inhibition of cell growth. In contrast, etoposide and adriamycin showed concentration-dependent cell growth inhibition, but these drugs did not inhibit phosphorylation of any of the proteins, including p53.

Induction of apoptosis 3LL cells exhibited DNA fragmentation characteristic of apoptosis after treatment with 9HE (10 μM) for 8 h (Fig. 8). Such apoptosis-type nucleosomal DNA ladder formation by this drug was also observed in SW480 and KB cells (data not shown). In contrast, adriamycin (10 μM) did not cause DNA fragmentation at 8 or 24 h after exposure, though vague DNA fragmentation appeared at 48 h (Fig. 8).

DISCUSSION

It was shown by immunoprecipitation and immunoblotting with anti-p53 monoclonal antibody that E and 9HE caused selective inhibition of the phosphorylation of p53 protein, a tumor suppressor gene product (Figs. 2 and 4). Inhibition was observed in the lysates or microsomal preparations of mouse cell lines (3LL and B16 melanoma in Fig. 1). It was also observed in P388, L1210, and colon 26 cell lines (data not shown). The inhibition was also seen in whole cells of SW480, a human colon cancer cell line (Fig. 3). By contrast, the inhibition was extremely weak when the preparations were treated with EA and ER, which have a strong positive charge on the quaternary ammonium ion at position 2 of their skeletons. Strong positive charge or substitution itself at that position may hamper the inhibitory effect on p53 phosphorylation. As far as phosphorylation inhibition is concerned, E and 9HE should be classified into a different group from the ellipticine derivatives having a quaternary ammonium moiety.

As for the mechanism of the anticancer action of Es, inhibition of topoisomerase-II has been proposed.[4, 5] However, neither a concentration-response nor a structure-activity relationship between topoisomerase-II inhibition and antitumor activity has been found with hydroxy ellipticines.[5] On the other hand, the following findings, reported in this study, suggest that inhibition of p53 phosphorylation may contribute at least in part to the anticancer actions of E and 9HE. The anti-proliferative effects of E and 9HE on 3LL and SW480 cells coincided well with the inhibition of p53 phosphorylation, in a concentration-dependent manner (Figs. 6 and 7). EA showed very weak antitumor effects in accordance with the extremely low inhibitory activities against p53 phosphorylation. Furthermore, we have reported that Saos-2 cells (a p53-deficient cell line) are resistant to 9HE but become sensitive after transfection with mutant p53, in which codon 143 mutates from Val to Ala,[51] showing the involvement of p53 in the anticancer mechanism of 9HE.

Mutation of the p53 gene is the most common event in human cancer[6–9] and p53 is frequently overexpressed in cancer cells.[14, 34–40] However, the significance of mutations of p53 with reference to tumorigenesis is not clearly understood. One hypothesis concerns loss of tumor-suppressing function of wild-type p53. Another concerns acquisition of tumor-generating ability by mutation. In any case, a large amount of mutant p53 protein is frequently found in cancer, whereas wild-type p53 protein is hardly detected in most normal tissues. It has been shown that phosphorylation of wild-type p53 is cell cycle-dependent; i.e., it is phosphorylated at the onset of S phase and dephosphorylated after mitosis.[16, 19] For mutant p53, the cell cycle dependency of phosphorylation has not been elucidated, but mutant p53 is present mostly in the phosphorylated state.[41–43] It was shown here that E and 9HE selectively inhibited p53 protein phosphorylation in living cells in parallel with their antiproliferative actions. Two alternative hypotheses are pos-

sible for the site of their actions. They may bind directly to p53 and inhibit its phosphorylation, or they may act on the kinases which regulate p53 functions.

The phosphorylation inhibition by 9HE is not specific for p53 protein, but is also observed with other proteins such as 40 and 70 kDa proteins in whole cells (Fig. 3). This suggests that the drug may act on common kinases related to p53, 40 and 70 kDa proteins; the 40 and 70 kDa proteins remain to be identified. 9HE inhibited cdk2 kinase but EA did not (Fig. 5). This is similar to the inhibition of p53 protein phosphorylation in terms of concentration range of the drugs and structure-activity relationship. This suggests that selective inhibition of p53 phosphorylation may be brought about through inhibition of a p53-related kinase or kinases. It has been reported that cdc2 kinase, casein kinase II and DNA-activated protein kinase can phosphorylate p53 *in vitro*, but their phosphorylating sites in p53 are different.[17, 21, 24] However, the kinase regulating p53 functions in living cells has not been identified as yet.

Growing evidence indicates that wild-type p53 suppresses tumor growth and overexpression of wild-type p53 induces apoptosis or programmed cell death.[25–29] The p53-induced apoptosis occurs after wild-type p53 protein levels are elevated by DNA damage in response to treatment with DNA-damaging agents or UV light.[28–33, 44] The p53-dependent DNA fragmentation appears within 8 h following treatment with anticancer agents.[44] However, such treatment does not cause the p53-induced apoptosis in cancer cells, since mutant p53 has no ability to induce apoptosis.[30, 32] Wild-type p53-deficient cancer cells are resistant to anticancer drugs, and undergo p53-independent cell death at high doses of drugs.[44] This study has demonstrated that 3LL cells undergo DNA fragmentation characteristic of apoptosis 8 h after exposure to 9HE (Fig. 8). In cancer cells, mutant p53 is frequently overexpressed and therefore, inhibition of p53 phosphorylation causes accumulation of a large amount of dephosphorylated mutant p53. In normal cells, induction of apoptosis by wild-type p53 occurs only when cells are in the G1 phase,[26] in which p53 is dephosphorylated.[16, 19] Interestingly, assays with p53-specific antibodies suggest that the wild-type p53 accumulated after DNA damage has a mutant-type conformation.[32] Taken together, it is suggested that dephosphorylated p53 having a mutant-type conformation induces apoptosis. By analogy with wild-type p53, mutant p53 could induce apoptosis in a dephosphorylated state. In cancer cells, most of the mutant p53 is phosphorylated, and may therefore lack the ability to induce apoptosis. Induction of p53-dependent apoptosis by DNA-damaging drugs occurs in normal cells but not in cancer cells, as described. This is unfavorable for cancer treatment from the point of view of selective toxicity. In contrast, the effects of inhibition of p53 phosphorylation on cell killing or apoptosis presented in this study might be more marked in cancer cells than in normal cells because of the presence of a large amount of mutant p53 in cancer cells and little wild-type p53 in normal cells. Recently, it has been reported that transforming growth factor-β1 inhibits cell growth by suppression of p53 phosphorylation.[52] Although E and 9HE themselves are not clinically used because of their renal and cardiovascular toxicities and low solubility,[1] inhibition of p53 phosphorylation or accumulation of dephosphorylated p53 may have the potential to be a promising anticancer mechanism from the viewpoint of selective toxicity in the formulation of new strategies for cancer treatment.

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