We have studied the effect of 1-(5-isouquinolinesulfonyl)-2-methylpiperazine (H-7), a protein kinase inhibitor, on the regulation of apoptosis in the human neuroblastoma cell line, SH-SY5Y. H-7 (20–100 µM) induced apoptosis in these cells characterized by DNA fragmentation and chromatin condensation. Immunoblot analyses were performed with specific antibody against BCL-2, BCL-XL, BAX, JUNB, c-JUN, ICH-1, c-FOS, RB, CDK-2, and p53. H-7 treatment did not significantly alter the level of these proteins with the exception of p53. H-7, but not staurosporine, caused a dramatic nuclear accumulation of p53. The kinetics of nuclear accumulation of p53 correlates well with the kinetics of induction of apoptosis. The effect of H-7 was further assessed in a group of human cell lines. Only cells lines harboring the wild-type p53 gene were responsive to the stimulatory effect of H-7 on nuclear accumulation of p53. Furthermore, cell lines carrying a mutated p53 gene were resistant to the cytotoxic effect of H-7. The ability of H-7 in mediating apoptosis in the SH-SY5Y line expressing a dominant negative mutant of p53 was significantly diminished. Taken together, these data strongly suggest that a p53-dependent mechanism contributes to the cytotoxicity of H-7 in human neuroblastoma cells.

Apoptosis or programmed cell death was identified as a distinct process by virtue of the discrete series of morphological changes exhibited by cells undergoing programmed cell death. Apoptotic cells experience viability loss accompanied by cytoplasmic blebbing, chromatin condensation, and DNA fragmentation (1). The failure of cells to undergo apoptosis contributes to the origin and progression of human cancers.

The nuclear phosphoprotein p53 plays a key role in limiting the further expansion of cells containing damaged genomes. Loss of p53 functions in knock-out mice (2) results in tumor development early in life, whereas reconstitution of these functions in tumor cells usually confers growth arrest or apoptosis, depending on the cell type and circumstances (3–7). Over half of all human cancers are linked with loss of wild-type p53 function due to mutation of the p53 gene (8, 9). If wild-type p53 function can also be lost as a result of epigenetic changes, the impact of this natural tumor suppressor may have been grossly underestimated. An understanding of the p53 response and its regulation in tumor cell lines carrying the wild-type p53 gene are essential for evaluating this possibility.

The p53 protein can act as a transcription factor, and its role in growth arrest by modulation of specific target genes is well documented (4–6, 10, 11). For example, the induction of the gene encoding the Cdk inhibitor, p21/WAF1/CIP1, by p53 results in G1 arrest of cells (4–6). However, it is less certain how p53 regulates apoptosis. Some evidence indicates that induction of apoptosis by p53 may not require transcriptional activation of genes (12).

Neuroblastoma is one of the most common malignancies in childhood. It derives from the neural crest which gives rise to multiple cell lineages with neuronal, neurilemmal, or melanocytic phenotypes (13, 14). Unlike other tumor types, nearly all human neuroblastomas were found to carry wild-type p53 gene (15–18). Wild-type p53 protein is known to be present in many of the cell lines derived from neuroblastoma (19). These cell lines are therefore suitable model systems for investigating the regulation and functions of wild-type p53 in tumor cells.

Protein kinase inhibitors have been linked to apoptotic pathways in many cell types (reviewed in Ref. 20), but little is known regarding the role of protein kinases in regulating apoptosis in human neuroblastoma cells. In this study, we are interested in evaluating the roles of protein kinase inhibitors in apoptosis and p53 regulation in human neuroblastoma cells. We studied the effects of a series of known kinase inhibitors, including staurosporine and several isouquinoline analogues, in the human neuroblastoma cell line, SH-SY5Y.

EXPERIMENTAL PROCEDURES

Materials

1-(5-Isoquinolinesulfonfyl)-2-methylpiperazine (H-7),1 N-(2-guanidinoethyl)-5-isouquinoline sulfonamide (HA-1004), 5-isouquinolinesulfonylhomopiperazine (HA-1077), and staurosporine were purchased from RBI. 1-(5-Isoquinolinesulfonfyl)-3-methylpiperazine (iso-H-7) and hygromycin B were purchased from Calbiochem. All other chemicals were purchased from Sigma unless otherwise indicated.

Cell Cultures

21 human tumor cell lines were used for this study. The human neuroblastoma cell line, LA-N-5, was kindly provided by Dr. R. C. Seeger, Children's Hospital of Los Angeles, and the human neuroblastoma cell lines SH-SY5Y and SH-EP were kindly provided by Dr. W. C. Groshong, Children's Hospital of Los Angeles. The human neuroblastoma cell line, LA-N-5, was kindly provided by Dr. R. C. Seeger, Children's Hospital of Los Angeles.

1 The abbreviations used are: H-7, 1-(5-isouquinolinesulfonfyl)-2-methylpiperazine; HA-1004, N-(2-guanidinoethyl)-5-isouquinoline sulfonamide; HA-1077, (5-isouquinolinesulfonfyl)homopiperazine; Iso-H-7, 1-(5-isouquinolinesulfonfyl)-3-methylpiperazine; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; HA, influenza hemagglutinin; PK, protein kinase; PKA, protein kinase A; PKC, protein kinase C; TUNEL, terminal-deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; CA, carcinoma.
Sáde, Department of Pharmaceutical Chemistry, University of California, San Francisco. All other cell lines were obtained from the American Type Culture Collection. Cell lines were grown according to the directions provided by suppliers. All media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and antibiotics (100 mg of streptomycin/ml and 100 IU of penicillin/ml, GIBCO-BRL). Isotonic compounds were dissolved in H2O; staurosporine was dissolved in Me2SO/ethanol (1:1) and then diluted in PBS. When reagents containing Me2SO/ethanol were used, an equal volume of Me2SO/ethanol was added to the control cells.

**Plasmid and Constructs**

A CDNA fragment, isolated by polymerase chain reaction, encoding codons 302-393 of the human p53 was tagged at the amino terminus with a 9-amino acid epitope (YPYDVPDYA), derived from influenza virus hemagglutinin, by insertion into the PAS (Clontech, San Francisco, CA) containing the epitope sequence. The tagged fragment was subcloned into pCEP4 (Invitrogen Inc., San Diego, CA) expression vector. The absence of mutations in the final product was confirmed by sequencing. The resultant plasmid, named pCEPHA-90Cp53, was used to generate the permanent SH-SY5Y line expressing the HATagged 91-amino acid mini p53 protein.

**Northern Blot Analysis**

Total RNA from SH-SY5Y cells was extracted with RNAzol B solution (Biotechnix Inc., Houston, TX) according to the manufacturer’s instruction and electrophoresed in a denaturing 1.2% agarose-formaldehyde gel and transferred onto nitrocellulose membranes (P-80 Phast). Probes were generated using random primers (>8 x 10^6 cpm/µg). Blots were washed at 65 °C, 2 x SSC for 30 min. The blot was later stripped and rehybridized with human p21 and then the GAPDH probes. The various CDNA probes were the 891-base pair PstII-Sma fragment of human p53 cDNA, the 2.1-kilobase XhoI fragment of human p21, and the 316-base pair BamHI-SacI fragment of human GAPDH.

**Antibodies**

The following antibodies were purchased from Transduction Laboratories (Lexington, KY): mouse anti-p21 monoclonal antibody, mouse anti-p53 monoclonal antibody P21020 that recognizes the carboxyl terminus of the human p53 protein, mouse anti-RB monoclonal antibody, mouse anti-Cdk-2 monoclonal antibody, and mouse anti-ICH-1 monoclonal antibody. The mouse anti-Bcl-xL/100 monoclonal antibody, rabbit anti-Bcl-xl (S-19) polyclonal antibody, rabbit anti-Bax (N-20) polyclonal antibody, rabbit anti-c-Jun/AP-1 (N) X polyclonal antibody, rabbit anti-JunB (N-17) polyclonal antibody, and mouse anti-p53 (DO-1) monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-hemagglutinin (anti-HA) polyclonal antibody was from Babco (Richmond, CA), and the mouse anti-p21 monoclonal antibody was from Oncogene Science (Uniondale, NY). The rabbit anti-phospho-c-Jun polyclonal antibody was from New England Biolabs (Beverly, MA). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were from Amersham (Amersham Int., Buckinghamshire, UK).

**MTT Assay**

Drug-induced cytotoxicity was assayed by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay carried out in 96-well microtiter plates. The cells were treated with different drugs for 24 h. At 3 h before the end of drug exposure, MTT was added to each well to a final concentration of 1 mg/ml and incubated at 37 °C. The reaction was terminated by removing the supernatant, and the dye was dissolved by adding 100 µl of Me2SO, and 10 µl of Sorensen glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) followed by thorough mixing. The plates were read at 562 nm on a Microtiter Plate Reader Ceres 900 (Bio-tek Instruments Inc., Winooski, VT). Controls included untreated cells and medium alone. The assays were performed in replicate of four to six samples, and the mean and standard deviation were determined by standard methodology.

**DNA Ladder Assay**

Cells were grown on 5-cm tissue culture dishes (Nunc, Roskilde, Denmark). After drug treatment, the cells were harvested, washed once with ice-cold PBS, and resuspended in 250 ml of lysis buffer (10 mM Tris-HCl, pH 7.6, 20 mM EDTA, pH 8.0, 0.5% SDS, Triton X-100), and the samples were left at room temperature for 15 min. After centrifugation at 16,000 x g for 5 min, the supernatant was transferred to another Eppendorf tube, and sequential extractions were carried out. The samples were extracted once with an equal volume of phenol, once with phenol/chloroform (1:1), and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 0.1 volumes of 3 M sodium acetate, pH 4.8, and 2.5 volumes of ethanol at −20 °C overnight. The DNA was then pelleted at 16,000 x g for 30 min the next day. After washing once with 70% ethanol, the samples were digested with 0.1 µg of RNase A at 37 °C for 30 min before loading onto 1.5% agarose gels, and the DNA was visualized by ethidium bromide staining.

**TUNEL Assay**

Measurement of DNA fragmentation by the terminal-deoxynucleotidyltransferase (TnT)-mediated dUTP-biotin nick end labeling assay (TUNEL) was carried out using the Apop Tag detection kit (Oncor, Gaithersburg, MD) according to the supplier instructions. Briefly, nucleic acid digestion with exonuclease and deoxyribonuclease enzymes was terminated by removing the supernatant, and the DNA was digested by terminal deoxynucleotidyltransferase, and the anti-digoxigenin antibody peroxidase conjugate is used to stain the apoptotic DNA fragments.

**Nuclear Staining Assay**

Cells were seeded onto glass coverslips and treated with various reagents after which monolayers were washed twice with ice-cold PBS, pH 7.4. Thereafter, cells were fixed for 5 min at 4 °C with absolute methanol (−20 °C). The washing step with PBS was then repeated once. To stain the nuclei, the cells were incubated for 10 min with 10 µg/ml of Hoechst 33342 (Molecular Probes Inc., Eugene, OR) and then washed with PBS. The coverslips with the stained cells were mounted in 50% glycerol in PBS containing 1 mg/ml p-phenylenediamine, and examined with an Zeiss Axioplan microscope.

**Immunofluorescence Staining**

SH-SY5Y neuroblastoma cells and the dominant negative cell line, SY 5.6, expressing the p53 mini protein, were seeded onto glass coverslips and fixed as described above. The cells were blocked at 37 °C for 30 min with 2% bovine serum albumin, 5% fetal bovine serum, 5% normal goat serum (NGS) in phosphate-buffered saline (PBS) and then incubated at room temperature for 45 min with anti-p53 (Ab-2) antibody or anti-HA antibody (1:1000 dilution) in blocking buffer and washed with PBS. The cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR). After washing, the coverslips were mounted as described above.

**Preparation of Cell Extracts**

Whole Cell Lysates—Cells were grown in 9-cm dishes, cultured, and treated as above. To prepare the whole cell lysates, the medium was removed, and cells were washed twice with ice-cold Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris, pH 7.6) and lysed with 0.5 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 25 mM β-glycerophosphate, 1 mM PMSF, 2 mM benzamidine, 10 mM apoaptin, 10 mg/ml leupeptin, and 1 mM sodium orthovanadate) for 15 min. The lysed cells were transferred to 1.5-ml microtubes, centrifuged at 15,000 x g for 10 min at 4 °C, and the supernatants were collected.

**Nuclear Extracts**—As described previously (21), cells were washed with cold PBS twice and detached from plates by adding 1 ml of detachment buffer (150 mM NaCl, 1 mM EDTA, pH 8.0, 40 mM Tris, pH 7.6) for 5 min at room temperature. The cells were then transferred to Eppendorf tubes and centrifuged at 300 x g for 4 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 400 µl of cold buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, 2 mM benzamidine, 10 mM apoaptin, 10 mg/ml leupeptin, and 1 mM sodium orthovanadate) and incubated on ice for 15 min. 10 µl of 10% Nonidet P-40 was added, and the mixture was vortexed briefly. Nuclei were pelleted by centrifuging at 2800 x g for 4 min at 4 °C, and the supernatants were resuspended in 50 µl of ice-cold buffer B (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 2 mM benzamidine, 10 mg/ml apoaptin, 10 mg/ml leupeptin, and 1 mM sodium orthovanadate). The mixture was shaken vigorously for 15 min at 4 °C, centrifuged at 15,000 x g for 5 min, and the supernatant was collected. The protein concentration was determined by the Bradford assay (22).

**Immunoblotting**—Total cell lysates or nuclear extracts, volume adjusted according to the protein concentration, were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred to 0.2-mm pore size nitrocellulose membrane (Amersham Corp.). Filters were blocked with TBS containing 3% nonfat dry milk and 0.1% Tween 20 (TBST) for 3 h at room temperature and probed with primary antibodies diluted according to the supplier in...
surviving cells were re-plated, and the cells were maintained in media containing 0.1% Tween 20, probed with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (Amersham), washed again in TBST, and developed by using the enhanced chemiluminescence system (Amersham Corp.).

**Pulse-Chase Experiments**

Cells were grown until near confluence in 90-mm plates and labeled by incubation for 3 h in methionine-free medium (Life Technologies, Inc.) with dialyzed fetal bovine serum containing 25 μCi/ml [35S]methionine (DuPont NEN). After the labeling, the cells were washed three times with PBS before refeeding with fresh medium and then harvested at 2, 4, and 8 h later. The cells were lysed in lysis buffer (10 mM phosphate buffer, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, 1 mM PMSE, and 2 μg/ml aprotinin) and subjected to centrifugation at 140,000 × g at 4 °C for 20 min. Radioactivities of the supernatants collected were determined by scintillation counting, and protein concentrations were determined by Bio-Rad protein assay reagent (Bio-Rad). Immunoprecipitation of p53 protein was performed from samples containing equivalent amount of total cellular protein. The lysates were incubated with 1 μg of p53 monoclonal antibody, DO-1, at 4 °C for 1 h. The immune complexes were bound with protein A (4 °C, 1 h) followed by three washes with lysis buffer. Finally, samples were denatured by boiling in SDS-loading buffer (100 mM Tris, pH 6.8, 2% SDS, 0.1% bromphenol blue, 10% glycerol, 25 μM β-mercaptoethanol) and loaded onto a 10% SDS-polyacrylamide gel. Autoradiography was then performed on the dried gel. The intensities of the bands were quantitated with a PhosphoImager using ImageQuantTM (Molecular Dynamics). The half-life of the protein was estimated by an exponential equation with the relative level of p53 as a function of the chase period.

**Establishment of Permanent SH-SY5Y Line Expressing the Dominant Negative p53 Mini Protein**

SH-SY5Y cells were grown to 70% confluency on 9-cm plates, and the cells were transfected with either pCEP4HA or pCEP4HA-90Cp53 plasmid by Lipofectin (Life Technologies, Inc.). The transfected cells were selected by adding 400 μg/ml hygromycin B. After 2–3 weeks, the surviving cells were re-plated, and the cells were maintained in media containing 100 μg/ml hygromycin B.

**RESULTS**

**Induction of Apoptosis in the Human Neuroblastoma Cell Line, SH-SY5Y, by Protein Kinase Inhibitors—**We tested H-7, HA-1004, HA-1077, iso-H-7, and staurosorpin for their cytotoxic effect on the human neuroblastoma cells, SH-SY5Y. Upon treatment of cells with H-7 (50 μM) or staurosorpin (100 nM) for 24 h, but not other compounds, the cells became rounded and loosely attached to the plate, suggesting the cells were losing viability. Further analyses of the cell samples documented that the cells were undergoing apoptotic death. Most of the morphological hallmarks associated with apoptosis were detectable, including cell shrinkage, internucleosomal fragmentation, and chromatin condensation (Fig. 1A). DNA strand breaks, typical of the apoptotic process, were also demonstrated by TUNEL and DNA ladder assays (Fig. 1, A and B). The morphological changes resulting from H-7 and staurosporin treatment were indistinguishable (data not shown). Gross morphological changes associated with viability loss can be observed in 24 h, whereas signs of apoptosis, detectable by DNA ladder and TUNEL assays, were diminished in 10–12 h upon H-7 treatment. On the other hand, HA-1004, HA-1077, and iso-H-7, at concentrations up to 500 μM, did not induce noticeable morphological changes in these cells.

Staurosorpin, a PKC inhibitor, was found to induce apoptosis in neuroblastoma cells. This is not surprising as others have suggested that it is an universal inducer of apoptosis in all mammalian cell lines tested, regardless of the state of differentiation and cell cycle phase. The broad apoptotic activity of staurosorpin appears to be unique among other protein kinase inhibitors, raising the question whether a kinase mechanism is involved in the effect (25). Staurosorpin mediates its effect on death probably via a target proximal to the final death effector.

**Induction of Nuclear Accumulation of p53—**Two of the protein kinase inhibitors tested, H-7 and staurosorpin, showed apoptotic activity on SH-SY5Y cells. To facilitate our investigation into the mechanism underlying the apoptotic effect associated with H-7, we evaluated the effect of H-7 on a panel of proteins known to have a role in various apoptotic pathways. Many of these proteins are known activators or suppressors (e.g., BCL-2, BCL-XL, BAX, RB) of apoptosis and have already been shown to be expressed in the neuroblastoma cells (12, 26). The protein levels of these genes were evaluated by Western
analyses. The p53 was detectable in the whole cell lysate but not in the nuclear extract before treatment (data not shown). However, upon H-7 treatment, the levels of nuclear p53 and p21 were found to be enhanced. Interestingly, no changes were noted in the levels of ICH-1L, BCL-2, BCL-Xs/L, JUNB, c-JUN, c-FOS, BAX, RB, and CDK-2 (Fig. 2A), as a result of H-7 treatment. The protein level of the phosphorylated form of c-Jun and Rb also remained unchanged upon treatment with H-7 for the time interval of 5 min to 24 h (Fig. 2B).

HA-1004, HA-1077, iso-H-7, and staurosporine did not affect the level of nuclear p53 (Fig. 3A). The apoptotic effect of staurosporine on neuroblastoma cells is presumably p53-independent. Among the isoquinolines tested, there was a positive correlation between ability to induce apoptosis and ability to mediate nuclear accumulation of p53 (Fig. 3A). It is of interest to note that despite the close structural resemblance of iso-H-7 to H-7, iso-H-7 is devoid of any inductive effects on apoptosis and nuclear accumulation of p53 (Fig. 3B).

Kinetics of Induction of p53 and Apoptosis—The effect of H-7 on the nuclear accumulation of p53 appeared to be gradual and was not detectable within the first hour of exposure. It became detectable at about 2 h and reached peak levels at 5–6 h (Fig. 4A). In contrast to H-7, staurosporine (100 nM) was unable to induce nuclear accumulation of p53 over a period of 24 h (data not shown). The appearance of p53 in response to H-7 treatment coincided with the initiation of apoptosis as the DNA laddering pattern became detectable in 2 h (data not shown). Northern blot analysis revealed no significant changes in the p53 mRNA levels (Fig. 4B), indicating that the nuclear accumulation of p53 was not due to an increase in p53 transcripts. Consistent with the fact that the p53 induced in these cells was functional, the transcript for one of the p53 target genes, p21/WAF1, was significantly increased at the time that p53 level had just reached its peak (Fig. 4B).

The induction of p53 became detectable at 20 μM H-7 (Fig. 5A). The induction appeared abrupt which may suggest the presence of a cooperative mechanism. Similar observations were made in serum-free conditions suggesting that the source of the cooperative effect might not come from factors present in the serum (data not shown). As predicted from the Northern blot experiment, the steady-state p21 level had indeed increased significantly over the control, in parallel with the p53 protein. The concentration effect of H-7 on the induction of apoptosis, measured by DNA fragmentation assay and by nuclear accumulation of p53, correlated well with each other (Fig. 5B).

H-7 Increased the Half-life of p53—Because H-7 treatment did not seem to affect the transcriptional rate of the p53 gene, we then evaluated the effect of H-7 on the stability of p53 in SH-SY5Y cells. H-7 at 50 μM was able to prolong the half-life of the p53 protein in SH-SY5Y cells. The half-life of the wild-type p53 in the untreated SH-SY5Y cells was 2.5 h. Consistent with
previous reports of p53 in neuroblastoma cells, the protein was found to be more stable in SH-SY5Y than in the normal fibroblast in which the half-life of p53 was determined to be 20–30 min \( (19) \). Surprisingly, upon treatment with H-7, the p53 in SH-SY5Y cells was dramatically stabilized, and the half-life of the p53 was found to increase by approximately 10-fold, to 28 h (Fig. 6).

H-7 Selectively Induced Apoptosis in Tumor Cells with Wild-type p53—

We further investigated the relationship between the induction of functional p53 and the subsequent apoptosis mediated by H-7. If the effect of H-7 on apoptosis requires wild-type p53, one would assume that H-7 would not be cytotoxic to many tumor lines expressing a mutated p53 protein. A panel of human cell lines derived from different tumor origins with different p53 status was subjected to further analysis. Examples of these analyses concerning three cell lines with different p53 status are shown (Fig. 7). To facilitate quantitative analysis of the cytotoxic effect, we employed MTT assay, which serves as an index of cell viability by measuring the reduction of the tetrazolium salt to the blue formazan product \( (27) \).

SW 837, a human colon carcinoma line, is known to express a mutant form of p53 \( (28) \). The p53 protein is generally more stable in most cases when it is mutated \( (29, 30) \), and this is reflected by the presence of detectable amounts of p53 in the nucleus of SW 837 cells prior to H-7 treatment (Fig. 7). SAOS-2, a human osteosarcoma line, is a p53 null cell line \( (31) \). H-7 induced nuclear accumulation of p53 only in SH-SY5Y cells, which is carrying a wild-type p53 gene. Consistent with its role as a nonspecific inducer of apoptosis in mammalian cells, staurosporine induced apoptosis in all three cell lines with similar kinetics, whereas H-7 appeared only effective in killing SH-SY5Y cells (Fig. 7).

The results of our analysis of 21 human cell lines are summarized in Table I. Based on this limited analysis, it appears that H-7 had an effect on nuclear accumulation of p53 only in the cell lines carrying the wild-type p53 gene. H-7 induced cell cycle arrest rather than apoptosis in MCF-7 cells (data not shown), suggesting that the response to p53 in MCF-7 cells is different from neuroblastoma cells. Indeed, H-7 is cytotoxic to IMR-32 and LA-N-5, two other neuroblastoma lines tested. In accordance with the early stage of differentiation, neuroblastoma cells in tissue culture have been shown to undergo transdifferentiation between a neuroblast form and a non-neuronal (epithelial) phenotype \( (13, 14) \). SH-SY5Y is a neuroblast subclone isolated from the original parent line, SK-N-SH, whereas SH-EP is the epithelial subclone from this line \( (13, 14) \). Neuroblast- and epithelial-form of neuroblastoma cells display dramatic differences in morphological, growth, and functional characteristics \( (14, 39, 40) \). It has been reported that retinoic acid induced apoptosis selectively in epithelial-type cells in neuroblastoma but had no effect on the neuroblast form \( (41) \). It is therefore important to determine the effect of H-7 on both the SK-N-SH parent line and SH-EP epithelial subclone line. The data on nuclear accumulation of p53 and apoptosis for these two lines were identical to the SH-SY5Y line (Table I). Interestingly, the choriocarcinoma cell line Jeg-3, which carries only one wild-type p53 allele \( (36) \), remained sensitive to the inducive effect of H-7 on apoptosis and p53 accumulation (Table I).
Inhibition of the Apoptotic Effect by the Dominant Negative p53 Mutant—In order to provide direct evidence that the cytotoxic effect of H-7 is correlated with the availability of functional p53 in the cell, we created a permanent SH-SY5Y line expressing the dominant negative mutant of p53, tagged with a 9-amino acid peptide of influenza hemagglutinin (HA). This mini protein contains the carboxyl-terminal portion of p53 (amino acids 302–393). This carboxyl-terminal domain of p53, when expressed separately, exhibits strong transforming activity in cells which normally express low levels of endogenous p53 (42). This transforming activity is attributable, at least in part, to a negative dominant mechanism, involving the formation of non-functional hetero-oligomers between the carboxyl-terminal fragments and the intact endogenous wild-type molecule (42, 43). The dominant negative cell line, named SY 5.6, was found to express the p53 mini protein as detected by immunoblot analysis using the rabbit anti-HA polyclonal antibody (Fig. 8A). Treatment with H-7 also induced accumulation of this mini protein (Fig. 8A). In the vector control line, both p53 and p21 were faithfully induced upon H-7 treatment. In the dominant negative line, the basal level of p21 protein was slightly higher than in the control vector line, but treatment with H-7 actually decreased the level of p21. While the ability of staurosporine in mediating apoptosis was not affected in line SY 5.6 in comparison with vector control line, H-7-mediated cell killing had clearly become less effective in cells expressing the dominant negative mutant of p53 (Fig. 8B). Immunofluorescence staining experiments revealed that the level of the mini protein in SY 5.6 line did not appear to be homogeneous.

**Fig. 6.** H-7 increased the protein stability of p53. A, stability of the p53 protein in SH-SY5Y cells upon H-7 treatment. Cells were pulsed for 3–5 h in the presence of [35S]methionine and then chased with cold medium for the indicated times. H-7 was added to the media at the beginning of the pulse and throughout the chase period. The cell extracts were immunoprecipitated with anti-p53 antibody DO-1. The immune complexes bound to protein A were then fractionated on a 10% SDS-polyacrylamide gel followed by autoradiography. The experiment was repeated twice with similar results. B, graphic analysis of p53 protein stability in SH-SY5Y cells. Data from the pulse-chase experiments were quantitated with a PhosphoImager using ImageQuant™ program. Circles and triangles represent data from H-7-treated and control samples, respectively.

**Fig. 7.** The apoptotic effect of H-7 correlates with its ability to induce the nuclear accumulation of p53 protein. The effect of H-7 and staurosporine on the induction of apoptosis and nuclear accumulation of p53 in human colon carcinoma cell line SW837 (a), human neuroblastoma cell line SH-SY5Y (b), human osteosarcoma cell line, SAOS-2 (c). Cells were exposed to H-7 (open squares) or staurosporine (filled squares) for 24 h. The concentrations of H-7 and staurosporine are in μM and nM, respectively. The cells were plated onto a 96-well plate, and the viability of the cells was determined by MTT method. The 100% value of viable cells was defined by measurement obtained from the untreated cells. Values shown are means ± S.D. of triplicate samples. A duplicate experiment gave similar results. Immunoblot analyses of nuclear p53 were also performed to examine the effect of H-7 on p53 induction in these cells. Equal amounts of nuclear extracts from each indicated cell line before and after treatment with 50 μM of H-7 for 10–16 h were used for the analysis.
Regulation of p53-dependent Apoptosis by H-7

The viability of the cells was measured by MTT assay. The 100% value was derived from measurements obtained from untreated cells. The data are given as means ± S.D. of triplicate samples. The experiments were repeated 2–10 times and yielded similar results. p53 protein was examined by immunoblot analysis of the nuclear extracts isolated from each cell line before and after treatment with 50 μM H-7 for 10–16 h.

| Cell line | Tumor origin | p53 status | % Cell viability | Nuclear p53 protein detected | Induction of nuclear p53 |
|-----------|--------------|------------|----------------|----------------------------|-------------------------|
|           |              | 100 μM H-7 | 100 nM staurosporine |                           |                         |
| SW620     | Colorectal CA| R273H (32) | 84.46 ± 3.44 | 33.88 ± 5.15 | Yes | No |
| SW480     | Colorectal CA| R273H (28) | 74.28 ± 2.73 | 41.55 ± 4.84 | No | Yes |
| SW537     | Colorectal CA| R248W (28) | 84.09 ± 2.17 | 28.48 ± 4.06 | Yes | No |
| HT-29     | Colorectal CA| R273H (32) | 85.17 ± 1.12 | 12.82 ± 8.96 | No | Yes |
| MB231     | Breast CA    | R280K (33) | 78.05 ± 3.02 | 33.56 ± 8.58 | Yes | No |
| T47D      | Breast CA    | L194F (28) | 73.24 ± 2.00 | 14.26 ± 6.75 | Yes | No |
| MCF-7     | Breast CA    | Wild type (34) | 97.66 ± 1.57 | 22.93 ± 3.28 | No | Yes |
| SK-OV-3   | Ovarian CA   | Not expressed (35) | 74.50 ± 5.17 | 4.52 ± 8.08 | No | No |
| SAOS-2    | Osteosarcoma | Not expressed (31) | 79.71 ± 2.42 | 16.41 ± 3.39 | No | No |
| IMR-32    | Neuroblastoma| Wild type (19) | 35.65 ± 5.04 | 15.69 ± 10.6 | No | Yes |
| SK-N-SH   | Neuroblastoma| Wild type (19) | 32.56 ± 2.67 | 7.90 ± 4.44 | No | Yes |
| SH-SY5Y   | Neuroblastoma| Wild type (19) | 28.06 ± 3.05 | 16.16 ± 3.87 | No | Yes |
| SH-EP     | Neuroblastoma| Wild type (19) | 11.43 ± 4.62 | 4.66 ± 1.51 | No | Yes |
| LA-N-5    | Neuroblastoma| Wild type (19) | 35.11 ± 5.09* | 24.06 ± 8.92* | No | Yes |
| JEG-3     | Chorio CA    | Q167H      | 28.79 ± 7.12 | 6.41 ± 3.85 | No | Yes |
| M 103     | Lung CA      | ND         | 80.91 ± 5.36 | 28.63 ± 3.68 | Yes | No |
| SK-UT-L   | Leiomyosarcoma| R175H; R248Q (37) | 63.06 ± 5.49 | 18.04 ± 5.01 | No | Yes |
| HS 683    | Glioma       | ND         | 85.16 ± 9.37 | 6.85 ± 4.70 | No | Yes |
| HOS       | Osteosarcoma | R156P (38) | 69.39 ± 0.98 | 10.50 ± 6.79 | No | Yes |
| 5637      | Bladder CA   | ND         | 91.21 ± 2.03 | 13.09 ± 5.88 | Yes | No |
| RD        | Rhabdomysarcoma| R248W (37) | 90.48 ± 3.84 | 12.93 ± 2.93 | Yes | No |

* Concentration of H-7 = 50 μM.
+ Concentration of staurosporine = 50 nM.

The viability of the cellswas measured by MTT assay. The 100% value was derived from measurements obtained from untreated cells. The data are given as means ± S.D. of triplicate samples. The experiments were repeated 2–10 times and yielded similar results. p53 protein was examined by immunoblot analysis of the nuclear extracts isolated from each cell line before and after treatment with 50 μM H-7 for 10–16 h.

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Across the entire cell population. Approximately 30% of the cells seem to express low levels of the protein (data not shown). This finding may, in part, explain why H-7 was still able to achieve some killing effect in these cells at 100 μM concentration. However, one cannot rule out the possibility that a pathway other than p53 may also be involved in contributing to the cytotoxic effect of H-7.

**DISCUSSION**

The compound H-7 selectively induces apoptosis in human neuroblastoma cells. Several lines of evidence suggested that the apoptotic effect of H-7 in these cells was mediated, at least in part, through p53. 1) The kinetics of induction of apoptosis and p53 accumulation were similar. 2) Isoquinolone analogues that failed to induce apoptosis were also ineffective in p53 induction. 3) In p53-null cell line or cell lines expressing a mutant p53, H-7 failed to induce apoptosis. 4) Expression of a dominant negative form of p53 was sufficient to counteract the cytotoxic effect of H-7.

It is widely believed that restoring and/or enhancing wild-type p53 functions in tumor cells represents promising treatment strategies for human cancers (44). Unlike other tumors, neuroblastomas have extremely low rate of mutation in their p53 gene (15, 18). Recent evidence suggests that, despite the expression of a functional p53 protein in human neuroblastoma cells, these proteins appear sequestered in the cytoplasmic compartment and thereby prevent them from executing their normal functions in the nucleus (45, 46). Indeed, p53 protein of an undifferentiated neuroblastoma line such as SH-SY5Y was more readily detectable in the whole cell lysate (19). It would be of interest to determine the effect of H-7 on the trafficking of p53 between cytoplasmic and nuclear compartment in neuroblastoma cells. The effect of H-7 on affecting the phosphorylation pattern of p53 is currently also under investigation in our laboratory.

DNA damaging agents, such as etoposide and adriamycin, are known to induce p53 in cell lines harboring the wild-type gene. These agents are thought to enhance the level of p53 in cells by increasing its stability (47). Interestingly, pulse-chase experiments demonstrated an effect of H-7 treatment on enhancing the half-life of p53 in SH-SY5Y cells from 2.5 to 28 h. In agreement with the finding that H-7 stabilizes the p53 protein in SH-SY5Y cells, immunofluorescence examination of the cells upon H-7 treatment revealed that there was a strong elevation of p53 staining in the nucleus (data not shown). The kinetics of induction of p53 in SH-SY5Y cells by H-7 and etoposide were similar (data not shown). While it is clear that DNA damaging agents require functional p53 to exert its cytotoxic function in many cell types (48–50), the mechanism underlying its cytotoxicity can also be attributed to a p53-independent pathway (51).

At high concentrations (20–100 μM), H-7 is not known to be a selective kinase inhibitor (23). The reported activities of H-7 on kinase inhibition overlap with the other kinase inhibitors used in this study (24). Staurosporine, HA-1004, HA-1077, and iso-H-7 had no effect on p53 induction even at high concentrations, raising the question of whether the effect of H-7 can be assigned to any well established kinase pathway. Failure of staurosporine and HA-1077 to induce the p53 response at concentrations ranges up to ten times of their Ki values on PKC inhibition strongly suggests that the inhibition of PKC is not sufficient to trigger the inductive effect of p53 in these cells. It remains a formal possibility that the molecular target for H-7 in this case might not be a kinase.

This is the first report demonstrating an effect of H-7 on regulating the apoptotic function of wild-type p53 in human neuroblastoma cells. Several other structurally related isoquinolone analogues devoid of any apoptotic activities suggest that there is a unique and specific structural determinant,
Regulation of p53-dependent Apoptosis by H-7

Regulation of p53-dependent apoptosis by H-7 is a target gene for p53 (10). However, in SH-SY5Y cells, unlike p21, the level of BAX protein remained constant and did not subject to up-regulation by p53 (Fig. 2A). However, this result is consistent with the finding that H-7 induced differentiation in these cells was mediated by p53. It has been reported that BAX is a target gene for p53 (10). However, in SH-SY5Y cells, unlike p21, the level of BAX protein remained constant and did not subject to up-regulation by p53 (Fig. 2A). However, this result is consistent with the finding that in some cell types, BAX level appeared not to be induced by p53 (56).

H7 (50–100 μM) has previously been reported to induce apoptosis in HL-60 cells (57), MOLT-4 lymphoid leukemia cells, and normal lymphocytes (58). Conversely, H-7 has also been reported to prevent apoptotic death in murine thymocytes exposed to irradiation (59) or ethanol (60). HL-60 is a p53-null cell line (61), and presumably a p53-independent mechanism was involved in the H-7 effect. We found no changes in the steady-state level of ICH-1L, BCL-2, BCL-X/S/L, JUNB, c-FOS, RB, and CDK-2 upon treatment with H-7 (50 μM) for 24 h in SH-SY5Y cells. However, it is possible that H-7 can regulate some of these proteins in a cell-type-dependent manner. The list of proteins that seem to play important roles in various apoptotic pathways is growing rapidly. Whether H-7 can regulate apoptotic genes other than those that we have studied remains to be investigated.

H-7 induced p53 accumulation but failed to induce apoptosis in MCF-7 cells. While this work was in progress, a report confirmed our findings that H-7 induces p53 accumulation in MCF-7 cells (62). The inability of the induced p53 to elicit apoptosis in MCF-7 cells may be attributed to the cell-type-dependent response to wild-type p53. Alternatively, it may suggest the presence of an additional defect in one of the downstream steps in the apoptotic pathway in MCF-7 cells. To distinguish these two possibilities, more human cancer cell lines carrying the wild-type p53 genes need to be evaluated.

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