Mechanism of Hoechst 33342-Induced Apoptosis in BC3H-1 Myocytes*

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ABSTRACT

Hoechst 33342, a bisbenzimidazole dye, binds to adenine/thymine rich regions in the minor groove of deoxyribonucleic acid (DNA). This dye induces apoptosis in BC3H-1 myocytes. The mechanism of Hoechst 33342-induced apoptosis was investigated. Inhibitors of ribonucleic acid (RNA) synthesis, protein synthesis, and serine or cysteine proteases failed to prevent BC3H-1 myocyte death induced by Hoechst 33342. Apoptosis may be dependent on increased p53 expression. Hoechst 33342 had no effect on p53 expression in BC3H-1 myocytes. Lactate oxidation, a monitor of mitochondrial function, was altered by Hoechst 33342 in dose dependent manner. Also, nuclear extracts were used to assay endogenous topoisomerase I activity which was inhibited by Hoechst 33342 treatment of BC3H-1 myocytes. Therefore, Hoechst 33342 appears to initiate apoptosis in BC3H-1 myocytes by a pathway which is independent of de novo RNA and protein synthesis. However, the dye does initiate mitochondrial dysfunction and inhibition of nuclear topoisomerase I as two important steps in the apoptotic pathway.

Introduction

Apoptosis is a major form of cell death. Apoptosis is also a genetically-controlled process and often requires new protein synthesis. Apoptosis induced by etoposide, CPT-11 (a derivative of camptothecin), and γ-irradiation can be prevented by inhibitors of RNA and protein synthesis. Also, there are alternate apoptotic pathways that do not require de novo protein synthesis. Some gene products (e.g., p53, Fas, interleukin-1β converting enzyme) promote apoptotic processes. Since two kinds of apoptosis-related genes, ced-3 and ced-4, are required to execute the apoptotic program in the nematode Caenorhabditis elegans and since mammalian homologous of ced-3 gene product have been identified as an ICE cysteine protease, ICE-like proteases may play a key role in the induction of apoptosis in mammalian cells.

Further evidence supporting a critical role for ICE-like proteases in apoptosis comes from the ability of specific protease inhibitors to prevent the apoptotic process and the ability of overexpression of ICE to induce apoptosis. However, different insults trigger different apoptotic pathways, such as p53-dependent and p53-independent apoptotic pathways. The p53 gene encodes a transcription factor that can regulate cell proliferation and survival by modulating transcription of downstream target genes, resulting in G1

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arrest and/or apoptosis. The p53 is activated to promote G1 arrest or apoptosis by several stimuli. The most well characterized stimulus is DNA damage which initiates p53-dependent apoptosis. Therefore, the nucleus appears to play an important role in apoptosis since chromatin condensation and DNA degradation occur early in the process; there is an overexpression of death gene products and there is a requirement for new RNA and protein synthesis. However, anucleate cells (cytoblasts) can be induced to undergo apoptosis, suggesting that a cytoplasmic apoptotic pathway must function independently from the nucleus in some cases.

For example, mitochondrial dysfunction, as demonstrated by mitochondrial swelling, loss of mitochondrial inner transmembrane potential and release of protease and/or protease activators, may represent a rate-limiting, coordinating step to the irreversible pathway leading to apoptosis. Topoisomerase I and II inhibitors induce apoptosis in many cells. Topoisomerases are ubiquitous enzymes that modulate the topographic structure of DNA by transiently introducing breaks in the DNA backbone. Two classes of topoisomerases exist in eukaryotic cells: type I enzymes, which cause single-strand DNA breaks, and type II enzymes, which cause double-strand DNA breaks. The mechanism by which topoisomerase inhibitors, like camptothecin, induce apoptosis is unclear. Camptothecin initiates three different biochemical events at the level of DNA replication: irreversible arrest of the replication fork, generation of a double-strand break, and conversion of the reversible cleavable complex of topoisomerase I, DNA and camptothecin into an irreversible complex.

The formation of this irreversible complex appears to inhibit nuclear topoisomerase I activity (experimentally demonstrated in vitro) resulting in permanent double strand DNA cleavage and the initiation of apoptosis. Other potential antitumor drugs, including topotecan, BN80245, and nogalamycin, have also been demonstrated to inhibit topoisomerase I in vitro. Camptothecin-induced apoptosis is also dependent on the action of two protease families, a cysteine protease called caspase 3 (CPP32/Yama/Apopain) and serine proteases.

Bisbenzimidazole dyes (Hoechst 33342 or (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole), and Hoechst 33258 or (2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole)) are known to bind to the minor groove of DNA with adenine and thymine specificity and to cause widening of the minor grooves. In addition, bisbenzimidazoles have other effects on cells. Both Hoechst 33342 and Hoechst 33258 are potent inhibitors of topoisomerase I in vitro. Hoechst 33258, the parent compound of Hoechst 3342, also is an effective inhibitor of the formation of a complex composed on DNA and TATA box binding protein.

Recently, it was reported by us that Hoechst 33342 induced apoptosis in BC3H-1 myocytes in both a dose-dependent and a time-dependent manner. To investigate further the mechanisms by which Hoechst 33342 may induce apoptosis, the following questions were addressed: (1) Does Hoechst 33342 induce BC3H-1 myocyte apoptosis via a p53-dependent pathway? (2) Do RNA synthesis and protein synthesis inhibitors prevent BC3H-1 myocyte apoptosis induced by Hoechst 33342? (3) Does Hoechst 33342 induce mitochondrial dysfunction? (4) Does Hoechst 33342 inhibit endogenous topoisomerase I activity? (5) Do ICE-like protease or serine protease inhibitors inhibit Hoechst 33342-induced apoptosis in BC3H-1 myocytes?

**Materials and Methods**

**Cell Culture**

The murine muscle cell line (BC3H-1) was grown in Dulbecco's Modified Eagles medium (DMEM)* with 10 percent fetal bovine serum (FBS)† as previously described.

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Effect of Actinomycin D and Cycloheximide on Cell Viability

For these experiments, cells were plated at a density of \(1 \times 10^4\) cells/cm\(^2\) and were cultured for two days with 80 percent confluence prior to Hoechst 33342, actinomycin D, and/or cycloheximide treatment. Cells were rinsed twice in DMEM medium with 2 percent FBS. Hoechst 33342 and cycloheximide were dissolved in distilled water at 25 mg/mL and 50 mmol/L, respectively. Actinomycin D was dissolved in methanol at 5 mmol/L. The BC3H-1 myocytes were treated with 10 \(\mu g/mL\) Hoechst 33342, 5 \(\mu g/mL\) actinomycin D, 50 \(\mu mol/L\) cycloheximide, 5 \(\mu g/mL\) actinomycin D + 50 \(\mu mol/L\) cycloheximide, 10 \(\mu g/mL\) Hoechst 33342 + 5 \(\mu mol/L\) actinomycin D, 10 \(\mu g/mL\) Hoechst 33342 + 50 \(\mu mol/L\) cycloheximide, and 10 \(\mu g/mL\) Hoechst 33342 + 5 \(\mu g/mL\) actinomycin D + 50 \(\mu mol/L\) cycloheximide for 3 hours. Cell viability was determined by trypan blue exclusion. The number of trypan blue-negative cells was considered the number of viable cells. The effect of Hoechst 33342, actinomycin D, cycloheximide, and their combinations on BC3H-1 myocyte viability was determined in triplicate for each dose of Hoechst 33342, actinomycin D, cycloheximide, and their combinations.

Effect of Protease Inhibitors on Cell Viability

For these experiments, cells were plated at a density of \(1 \times 10^4\) cells/cm\(^2\) and were cultured for 2 days with 80 percent confluence prior to serine protease inhibitor Na-p-tosyl-L-lysine chloromethylketone (TLCK) and ICE inhibitor II (Ac-Try-Val-Ala-Asp-chloromethylketone, ATVAAcmk) treatment. Cells were rinsed twice in DMEM medium with 2 percent FBS. The TLCK was dissolved in distilled water at 10 mg/mL and ATVAAcmk dissolved in dimethyl sulfoxide at 100 mmol/L. The BC3H-1 myocytes were treated with 10 \(\mu g/mL\) Hoechst 33342, 40 \(\mu g/mL\) TLCK, 100 \(\mu mol/L\) ATVAAcmk, 10 \(\mu g/mL\) Hoechst 33342 + 40 \(\mu g/mL\) TLCK, 10 \(\mu g/mL\) Hoechst 33342 + 100 \(\mu mol/L\) ATVAAcmk for 3 hours. Cell viability was determined by trypan blue dye exclusion.

Effect of Hoechst 33342 on Lactate Oxidation

Lactate oxidation of BC3H-1 myocytes was measured as the amount of [1-\(^{14}\)C]-lactate oxidized in nmol of \(^{14}\)CO\(_2\) generated per mg of protein per minute, as described by Ofenstein et al. Briefly, BC3H-1 myocytes were rinsed twice in phosphate buffered saline (PBS) and treated with 0, 2.5, 5, 10, 15, 20, and 30 \(\mu g/mL\) Hoechst 33342 in RPMI-1640 with 1 mmol/L glucose for 3 hours. After the treatment, the assay was initiated by adding 10 \(\mu L\) of 5 \(\mumol/L\) D,L-lactic acid and 0.5 \(\muCi\) [D,L-\(^{14}\)C] lactate into each flask and the flasks slowly shaken at 37°C for 1 hour. Filter paper moistened with 0.2 mL of 1 mol/L benzethonium hydroxide used to collect released \(^{14}\)CO\(_2\) was suspended directly above the monolayer in each flask with an air-tight stopper. Each filter was transferred into scintillation vials and the amount of \(^{14}\)CO\(_2\) collected was determined by a liquid scintillation counter. The cells were then harvested for protein determination by bicinchoninic acid (BCA) protein assay reagent. Lactate oxidation was measured as the amount of lactate oxidized in nmol of \(^{14}\)CO\(_2\) generated/mg protein/min.

Immunoblot Analysis of p53 Gene Expression

The BC3H-1 myocytes treated with 20 \(\mu g/mL\) Hoechst 33342 for 0, 3, 6, 9, 12, and 24

\(\ddagger\) Sigma, St. Louis, MO.
\(\S\) Bachem, Torrance, CA.

\(\ddagger\) American Radiochemical Co., St. Louis, MO.
\(\S\) Pierce, Rockford, IL.
hours were harvested. Total cellular protein extracts were prepared by lysing untreated and treated cells with protein lysis buffer (910 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 percent triton X-100, 1 percent deoxycholate, 0.1 percent SDS, 0.5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin) and quantified by BCA protein assay reagent. p53 proteins levels in the cellular extracts were examined by p53 Western Blotting Kit.*

Preparation of Nuclear Extracts

Nuclear extracts from BC3H-1 myocytes were prepared as described by Tanizawa et al.[41] Untreated cells and BC3H-1 myocytes treated with 64 μmol/L Hoechst 33342 for 12 hours were rinsed with ice-cold nuclear buffer [150 mmol/L NaCl, 1 mmol/L KH₂PO₄, 5 mmol/L MgCl₂, 1 mmol/L ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 10 mmol/L 2-mercaptoethanol, 0.1 mmol/L phenylmethylsulfonyl fluoride, 10 percent (V/V) glycerol, pH 6.4], scraped, and centrifuged (14,000 × g). Cells were resuspended in 0.1 volume of ice-cold nuclear buffer, followed by 0.9 volume of cold nuclear buffer containing 0.3 percent Triton X-100. The mixture was gently rotated for 10 minutes at 4°C. The nuclei were pelleted (14,000 × g) and resuspended in Triton X-100-free nuclear buffer.

To obtain nuclear extracts, all procedures were carried out in the presence of 0.44 trypsin inhibitory unit/mL aprotinin, as well as phenylmethylsulfonyl fluoride. Isolated nuclei were washed once in Triton X-100-free nuclear buffer and resuspended in nuclear buffer containing 0.35 mol/L NaCl (final concentration). The salt extraction was performed for 30 minutes at 4°C with gentle rotation. The nuclei were pelleted (14,000 × g), and the supernatant was centrifuged again to remove any insoluble materials. Protein concentrations of the nuclear lysates were determined by BCA protein assay reagent. The supernatant (nuclear extract) was used for plasmid relaxation assay used to determine topoisomerase I activity.

Determination of Topoisomerase I Catalytic Activity in Nuclear Extracts

Endogenous topoisomerase I catalytic activity in the nuclear extracts from untreated and Hoechst 33342-treated BC3H-1 myocytes were examined by the plasmid relaxation assay described by Frydman et al.42 Briefly, reaction mixtures (20 μL each) containing 50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L dithiothreitol, 0.5 mmol/L EDTA, 30 μg/mL acetylated BSA, 1 μg of plasmid pClt and 5 μg total proteins of the nuclear extracts were incubated at 37°C for 15 minutes. One percent Sodium dodecyl sulfate (SDS) and 200 μg/mL proteinase K were added to terminate the reaction and incubation continued for 30 minutes at 60°C. The reaction mixtures were then analyzed by electrophoresis in an 0.8 percent agarose gel at room temperature.

Results

Effects of Actinomycin D and Cycloheximide on Hoechst 33342-induced BC3H-1 Myocyte Apoptosis

Cell viability was measured by trypan blue dye exclusion after BC3H-1 myocytes were incubated with 10 μg/mL Hoechst 33342, 5 μg/mL actinomycin D, 50 μmol/L cycloheximide, 5 μg/mL actinomycin D + 50 μmol/L cycloheximide, 10 μg/mL Hoechst 33342 + 5 μg/mL actinomycin D, 10 μg/mL Hoechst 33342 + 50 μmol/L cycloheximide, and 10 μg/mL Hoechst 33342 + 5 μg/mL actinomycin D + 50 μmol/L cycloheximide for 3 hours. As demonstrated in figure 1, Hoechst 33342 significantly induced BC3H-1 myocyte death.
Actinomycin D or cycloheximide or added in combination did not alter BC3H-1 myocyte viability compared to the control cells. However, both 5 \(\mu\text{g/mL}\) actinomycin D and 50 \(\mu\text{mol/L}\) cycloheximide alone, and the combination of 5 \(\mu\text{g/mL}\) actinomycin D and 50 \(\mu\text{mol/L}\) cycloheximide failed to prevent the death rate of BC3H-1 myocytes induced by 10 \(\mu\text{g/mL}\) Hoechst 33342, suggesting that Hoechst 33342-induced BC3H-1 myocyte death is not dependent upon protein or RNA synthesis.

**Effect of Protease Inhibitors on Hoechst 33342-induced BC3H-1 Myocyte Apoptosis**

To analyze the relationship between the serine and/or cysteine protease activation and Hoechst 33342-induced apoptosis two inhibitors were used: TLCK, a serine protease inhibitor and ATVAAcnk, an ICE or cysteine protease inhibitor. Our results showed that TLCK or ATVAAcnk alone or in combination failed to block the Hoechst 33342-induced reduction in BC3H-1 myocyte viability (figure 2).

**Effect of Hoechst 33342 on Lactate Oxidation of BC3H-1 Myocytes**

To assess mitochondrial function, lactate oxidation was measured after the treatment of BC3H-1 myocytes with 0, 2.5, 5, 10, 15, 20 and 30 \(\mu\text{g/mL}\) Hoechst 33342 for 3 hours in Rosewell Park Memorial Institute (RPMI) 1640. Twice the concentration of Hoechst 33342 is required to induce apoptosis in BC3H-1 myocytes when RPMI-1640 medium is used compared to DMEM (20 \(\mu\text{g/mL}\) compared to 10 \(\mu\text{g/mL}\)).\(^1\) The results indicated that lower doses of Hoechst 33342 (5 to 15 \(\mu\text{g/mL}\)) significantly increased lactate oxidation in BC3H-1 myocytes. In contrast, higher doses of Hoechst 33342 (20 to 30 \(\mu\text{g/mL}\)), which are known to induce apoptosis in BC3H-1 myocytes grown in RPMI-1640, significantly decreased lactate oxidation (figure 3). These results suggest that one step in the pathway by which Hoechst 33342 induces apoptosis in BC3H-1 myocytes is mitochondrial dysfunction.

**Effect of Hoechst 33342 on p53 Gene Expression**

Radiation and many known agents cause DNA damage, presumably leading to p53-
dependent apoptosis. Thus, identifying apoptotic agents that act independently or dependently of the p53 pathway is of fundamental importance. In this study, Western blot was used to detect the p53 protein level in BC3H-1 myocytes after treatment of Hoechst 33342. As indicated in figure 4, Hoechst 33342 failed to change p53 protein concentrations in BC3H-1 myocytes. This result showed that Hoechst 33342-induced BC3H-1 apoptosis is independent of the p53 pathway. However, low FBS (2 percent FBS) incubation for 24 hours increased p53 protein concentration in BC3H-1 myocytes (figure 4).

**Effect of Hoechst 33342 on Topoisomerase I Catalytic Activity**

Deoxyribonucleic acid minor groove drugs, including camptothecin and Hoechst 33342,
are effective inhibitors of DNA topoisomerase I in vitro. The topoisomerase I poison, camptothecin, induces apoptosis in different cell types. In this study, topoisomerase I catalytic activity assay was used to detect the effect of Hoechst 33342 on topoisomerase I activity in vivo. After BC3H-1 myocytes were treated with 64 \( \mu \text{mol/L} \) Hoechst 33342 for 12 hours, nuclear extracts were prepared from the untreated or Hoechst 33342-treated cells. The results indicate that Hoechst 33342 inhibited endogenous topoisomerase I enzyme activities after 12 hour treatment when compared with the control group (figure 5). This in vivo result suggests that Hoechst 33342 may induce apoptosis via inhibition of topoisomerase I activity.

**Discussion**

The following is concluded from this study: (1) Actinomycin D, an RNA synthesis inhibitor, and cycloheximide, a protein synthesis inhibitor, failed to protect BC3H-1 myocytes from cell death induced by Hoechst 33342; (2) Both TLCK, a serine protease inhibitor, and ATVAAcmk, an ICE protease inhibitor, did not prevent BC3H-1 myocytes from cell death induced by Hoechst 33342. (3) Hoechst 33342 treatment of BC3H-1 myocytes did not increase the cellular concentration of p53 protein; (4) BC3H-1 myocyte death induced by Hoechst 33342 was accompanied by mitochondrial dysfunction as measured by lactate oxidation; and (5) Hoechst 33342 inhibited endogenous topoisomerase I activity in nuclear extracts of BC3H-1 myocytes.

The p53 gene product is a critical mediator of G1 cell cycle arrest and apoptosis after DNA damage. Also, p53 has been reported to affect G2 cycle arrest, centrosome replication, mitotic spindle organization, and to regulate DNA repair. These responses may protect cells against replication of damaged DNA by permitting DNA repair or deleting the affected cell.\(^{43,44}\) It has become clear that cells have different responses to different apoptotic inducers. For example, \( \beta \)-lapachone- and camptothecin-mediated apoptosis follows a p53-independent pathway,\(^ {45,46,47}\) whereas DNA damage-mediated apoptosis usually utilizes a p53-dependent pathway.\(^ {48}\) The apop-
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Totic responses induced by Hoechst 33342 are not dependent upon p53, since Hoechst 33342 treatment of BC3H-1 myocytes did not result in an increase in nuclear p53 protein levels (figure 4).

Similar results have been observed with topoisomerase I inhibitors, camptothecin and α-lapachone. An apoptotic cell initiates a cascade of biochemical changes in response to the stimulus which may include the synthesis of macromolecules not related to p53. Protein synthesis and/or RNA synthesis inhibitors have been shown to prevent apoptosis induced by some compounds. To determine the effect of the inhibition of RNA and/or protein synthesis on BC3H-1 myocyte death induced by Hoechst 33342, trypan blue exclusion assay was used to evaluate the effect of actinomycin D and cycloheximide on cell death induced by 10 μg/mL Hoechst 33342 (figure 1).

Our results indicate that both actinomycin D and cycloheximide failed to prevent BC3H-1 myocyte cell death or apoptosis induced by Hoechst 33342, suggesting that Hoechst 33342-induced apoptosis does not require newly synthesized RNA or protein. These results were consistent with the previous results showing that Hoechst 33342-induced cell death or apoptosis failed to alter nuclear p53 protein levels in Hoechst 33342 treated BC3H-1 myocytes.

Protein degradation has also been implicated in apoptosis in both invertebrates and mammals. In the nematode, Caenorhabditis elegans, one ced-3 of the two genes essential for apoptosis, encodes a protein similar to the mammalian cysteine protease, caspase 3. Since overexpression of ced-3 and caspase 3 results in apoptosis and inhibitors of proteases can prevent apoptosis, these observations suggest that caspase 3 may play a critical role in the induction of apoptosis. Caspase 3 activates an endonuclease called caspase-activated Dnase which causes DNA degradation in nuclei. Caspase-activated DNA is bound to its inhibitor in an inactive complex until an apoptotic signal activates caspases which cleave the inhibitor protein from the active endonuclease. However, the role of ICE, caspase 3, and other cysteine proteins in apoptosis induced by different insults may be quite different. For example, TLCK, an inhibitor of serine protease, prevented camptothecin-induced apoptosis in Hep 3B cells. In contrast, ATVAAcmk, an inhibitor of ICE-like proteases, only minimally inhibited camptothecin-induced apoptosis. Shimizu and Pommier, using different serine and cysteine protease inhibitors, concluded that both caspase 3 (cysteine protease) and serine protease activation were essential for camptothecin-induced apoptosis in p53-null HL60 cells.

In the present study, our results demonstrate that both serine protease (TLCK) and cysteine protease (ATVAAcmk) inhibitors failed to prevent Hoechst 33342-induced apoptosis in BC3H-1 myocytes. However, further experiments with additional specific protease inhibitors may be required to determine which specific proteases may be activated during Hoechst 33342-induced apoptosis.

Since anucleate cells called cytoblasts can be induced to undergo programmed cell death, cytoplasmic structures including mitochondria have been shown to participate in the control of apoptotic nuclear disintegration. A variety of mitochondrial events have been reported to occur during the apoptotic process including mitochondrial swelling, hydrogen peroxide production, loss of mitochondrial inner transmembrane potential, and release of proteases and/or protease activators. To evaluate the effect of Hoechst 33342 on mitochondrial function in vivo, lactate oxidation assay was used. In this assay, [1-14C] lactate is converted by the myocytes to [1-14C] pyruvate by lactate dehydrogenase and finally to 14C CO2 by pyruvate dehydrogenase. This final reaction occurs within mitochondria. Hoechst 33342 at 5, 10, and 15 μg/mL significantly increased lactate oxidation levels above control in a biphasic response with maximum effect at 10 μg/mL (figure 3). Uncoupling of the mitochondrial electron chain with carbonyl cyanide chlorophenylhydrozone also increases lactate oxidation. Higher concentrations of
Hoechst 33342 (20 and 30 μg/mL), known to induce apoptosis when BC3H-1 myocytes when they are grown in RPMI-1640, significantly reduce lactate oxidation below control values. The complex I inhibitor, rotenone, decreases lactate oxidation in BC3H-1 myocytes, Chinese hamster ovary cells and fibroblasts.

Rotenone and a complex II inhibitor, thetynyltrifluoroacetone, but not the complex III inhibitor, antimycin A, block ceramide-induced mitochondrial hydrogen peroxide production as well as apoptosis. The blockade of ceramide-induced apoptosis by respiratory chain complex I and II inhibitors may be related to reduced mitochondrial hydrogen peroxide production which is an event that may not be important in the pathway leading to Hoechst 33342-induced apoptosis. These results suggest that Hoechst 33342 causes mitochondrial dysfunction during the apoptotic process potentially including initial uncoupling of the electron chain followed by inhibition of the mitochondrial respiratory chain. Further investigation is required to define further the role mitochondrial dysfunction plays in the apoptotic pathway initiated by Hoechst 33342.

Topoisomerases are enzymes that modify and regulate the topological state of DNA. These enzymes participate in many cellular metabolic processes, which are associated with separation of complementary DNA strands such as replication, transcription, recombination, and repair. Some studies have demonstrated that inhibitors of topoisomerase I and II (such as camptothecin, etoposide, and β-lapachone) can induce apoptosis in different cell types. The studies on bisbenzimidazoles showed that Hoechst 33342 and Hoechst 33258 both inhibited topoisomerase I in in vitro experiments.

In our present study, the results indicate that topoisomerase I activity in nuclear lysates was significantly inhibited after BC3H-1 myocyte treatment with 64 μmol/L Hoechst 33342 for 12 hours. These results suggest that Hoechst 33342-induced apoptosis has some relationship with the inhibition of endogenous topoisomerase I activity.

The precise mechanism by which Hoechst 33342 induce BC3H-1 myocyte apoptosis is unknown. Topoisomerase inhibitors (such as camptothecin), which induce apoptosis, are associated with three different biochemical events at DNA replication level: (1) irreversible arrest of the replication fork, (2) generation of a double-strand break, and (3) conversion of the reversible cleavable complex into an irreversible complex. An endogenous endonuclease is partially involved in camptothecin-induced apoptosis since apoptosis is partially blocked by aurintricarboxylic acid.

In addition, the parent compound of Hoechst 33342, Hoechst 33258, is an effective inhibitor of the formation of DNA/TATA box binding protein complex. The TATA box binding protein is a general transcription factor required for proper initiation of gene expression by RNA polymerase II. Based on these data, Hoechst 33342 disrupt DNA metabolism by inhibiting the formation of the DNA/TATA box binding protein complex and nuclear topoisomerase I activity which may be preceded or accompanied by mitochondrial dysfunction. Therefore, new protein and/or RNA synthesis is not a predominant requirement for this pathway. Future experiments need to be designed to determine the role of protease activation from mitochondria or cytoplasm and the role of the nucleus using anucleate cells in Hoechst 33342-induced apoptosis.

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