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Inhibition of Nucleolar Function and Morphological Change by Adriamycin Associated with Heat Shock Protein 70 Accumulation

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Adriamycin (ADR) has been considered to target mainly DNA metabolism in the nucleus. Recently, we observed the nuclear translocation of heat shock protein 70 (HSP70) after ADR treatment. We examined which intranuclear changes might be related to this alteration of HSP70 localization. We found considerable alternations in the nucleolar morphology and function in ADR-treated tumor cells, i.e., a ring-shaped segregation of granular components of almost all nucleoli and a dramatic reduction of nucleolar 45S ribosomal precursor RNA biosynthesis in HeLa cells exposed to 100 μM ADR for 2 h. Concomitantly with these changes, HSP70 was concentrated into the nucleoli, as in the case of heat shock treatment. These results indicate a novel anticancer effect of ADR via the suppression of cellular protein biosynthesis, in addition to its effect on DNA.

Key words: Adriamycin — HSP70 — Nucleolus — DNA damage — HeLa cells

Adriamycin (ADR) is an anthracycline chemotherapeutic agent against a wide range of human malignant neoplasms, including acute leukemia, breast cancer, ovarian cancer and others.[1] ADR directly intercalates into double-stranded DNA and prevents DNA replication.[2] There are, however, other mechanisms by which ADR kills tumor cells. In the presence of DNA, ADR is complexed with iron and then generates reactive oxygen species (ROS) such as hydroxyl radical, which damage DNA.[3–5] An alternative pathway is to attack DNA topoisomerase II (topo II).[6,7] In ADR-treated cells, topo II and ADR have been shown to exist as a complex associated with DNA strand breaks, probably due to interference with the religation activity of topo II by ADR.[8] The formation of these inhibitory intermediate-complexes plays a crucial role in the anticancer action of ADR.[8]

We have previously reported that heat shock protein 70 (HSP70) transiently translocated from the cytoplasm to the nucleus after the exposure of human amniotic cultured cells to hydrogen peroxide, and suggested that HSP70 might participate in the protection of DNA and recovery from DNA damage induced by oxidative stress.[9] Several reports have indicated that HSP70 proteins can be induced by ROS and that they protect cells against the cytotoxicity of free radicals.[10–12] It is likely that both the protection against the initial damage in the nucleus and the efficient recovery from both nuclear DNA damage and protein degradation/aggregation are important for cell survival.[13]

The major aim of this study was to examine whether intranuclear change other than DNA damage can induce nuclear accumulation of HSP70 after ADR treatment. We observed that HSP70 translocated into the nucleus, particularly into the nucleolus, in HeLa cells after addition of ADR into the medium, and this was associated with drastic changes in the nucleolar morphology and function. It is likely that the decrease of ribosome biosynthesis also contributes to the suppression of tumor growth in addition to the effect of ADR on the DNA.

MATERIALS AND METHODS

Cell line and chemicals HeLa cells were maintained in a MEM (Eagle) medium supplemented with 10% fetal calf serum (GIBCO BRL, Life Technologies, Inc., Grand Island, NY) and 2 mM glutamine at 37°C in an atmosphere of 95% air and 5% CO_2. For experiments, cells (5×10^6) were grown in a 15 cm-diameter culture dish (FALCON 3025, FALCON®, NJ) and used after 3 days. Adriamycin (ADRIACIN) was a gift of Kyowa Hakko Kogyo Co., Ltd., Tokyo. Bacterial alkaline phosphatase type III, and nuclease P1 were purchased from Sigma Chemical Co., St. Louis, MO. Other chemicals were of the highest purity commercially available.

Cell viability HeLa cells were seeded at 8×10^3/100 μl in 96-microwell plates. After 2 days, serial dilutions of ADR were added. After incubation for 2 h, cells were washed twice with phosphate buffered saline (PBS), and their viability was determined by measuring the conversion of MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to

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formazan by dehydrogenase enzymes found in metabolically active cells, using the MTS assay kit (CellTiter 96 AQ Assay: Promega Co., Madison, MI).

Determination of 8-hydroxydeoxyguanosine (8-OHdG) After treatment of HeLa cells with 100 μM ADR, DNA was isolated using the DNA Extractor WB Kit (Wako Pure Chemical Industries, Ltd., Tokyo). The extracted DNA was digested to deoxynucleosides by treating it with nuclease P1 and acid phosphatase.[14] 8-OHdG in the digested DNA was assayed by an electrochemical detector, and deoxyguanosine was simultaneously assayed by a UV monitor coupled to a high-pressure liquid chromatograph.[15]

Immunofluorescence studies HeLa cells grown on Cellmatrix type I (Iwaki Glass Co., Tokyo) coated slide glasses were treated variously as described in the text and washed with PBS three times, after which they were fixed with methanol at $-20°C$ for 10 min. The cells were treated with PBS containing 5% normal rabbit serum for 20 min at room temperature in a humidified chamber to block non-specific binding of IgG. Samples were incubated with mouse anti-human HSP72 monoclonal antibody (IgG1) (RPN1197, Amersham) for 60 min. This antibody was diluted 1/200 in 1% bovine serum albumin (BSA) in PBS (BSA/PBS). After incubation, samples were washed and then labeled with fluorescein (DTAF)-conjugated rabbit anti-mouse IgG (CHEMICON, CA) at a dilution of 1: 50 with BSA/PBS for 60 min in a dark chamber. The slides were washed extensively with PBS, coverslips were placed on them and the cells were examined using a Zeiss AXIOPHOT microscope or a Zeiss laser scanning confocal microscope (LSM410). For the negative control, mouse monoclonal antibody ($\times$931, IgG1, DAKO, Copenhagen) was used as a primary antibody.

Electron microscopy HeLa cells were seeded on collagen membrane (CELLGEN, CM-24: KOKEN, Tokyo) in a culture dish. After various treatments, specimens were fixed in a mixture of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h at 4°C, postfixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections were made on an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a JEOL 1200 EX electron microscope.

Isolation of nuclei Nuclei were prepared according to Muramatsu *et al.*[16] In brief, HeLa cells were harvested by trypsin treatment and collected by centrifugation at 500g for 5 min. The resulting pellets were suspended in 20 vol (of the cell pellet) of hypotonic buffer (reticulocyte standard buffer, RSB: 1 mM $MgCl_2$, 10 mM KCl, 10 mM Tris-HCl, pH 7.6) and stood in an ice bath for 5 min, then a solution of 10% Nonidet P40 (Sanyo Chemical Industries, Ltd., Kyoto) was added to give a final concentration of 0.2–0.3%. The mixture was homogenized in a tightly fitted Dounce homogenizer and centrifuged at 1200g for 5 min to sediment crude nuclei. The pellet was homogenized in 10 vol of 0.34 M sucrose containing 3 mM $MgCl_2$, layered over an equal volume of 0.88 M sucrose, and centrifuged at 1200g for 10 min. The pellet was designated as purified nuclei and used for extraction of nuclear RNA.

Northern blot analysis Total nuclear RNA (20 μg) prepared by the guanidinium thiocyanate procedure[17] was electrophoresed in 1% agarose-formaldehyde gels. To improve the transfer of high-molecular-weight RNA, gels were washed with distilled water for 5 min to remove formaldehyde, soaked at room temperature in a solution (50 mM NaOH and 10 mM NaCl) for 30 min, and then neutralized in 0.1 M Tris-HCl (pH 7.5) for 30 min and in 20$\times$SSC for 1 h. Subsequently, partially digested RNA was transferred to a nylon membrane (Hybond-N, Amersham, UK). The probe used was 5.8 kb human ribosomal DNA (5′-portion-specific *Eco*R I fragment of pHr21Ab plasmid).[18] The fragment was labeled with [α-^{32}P]dCTP using a Nick Translation Kit (Takara Shuzo Co., Kyoto). Hybridization was carried out as described previously.[19] The hybridized membranes were exposed to Bio-imaging plates (FUJIX, Tokyo), and analyzed using a Bio-imaging analyzer BAS 2000 (FUJIX).

RESULTS

Increase of oxidative DNA damage by ADR To examine the general biological effects of ADR on HeLa cells, we

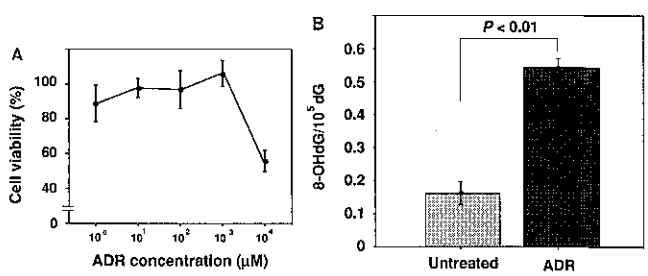

Fig. 1. Determination of cytotoxic effects of ADR in HeLa cells. A, after incubation with indicated concentrations of ADR for 2 h, cell viability was determined by MTS assay as described in "Materials and Methods." Cell viability at each concentration was plotted as a percentage of that of the untreated control. Data represent the means±SD of five experiments. B, cells were exposed to 100 μM ADR for 1 h, and the formation of 8-OHdG was measured by HPLC as described in "Materials and Methods." Data represent the means±SD of four experiments. The significance of differences was determined by using the t test.

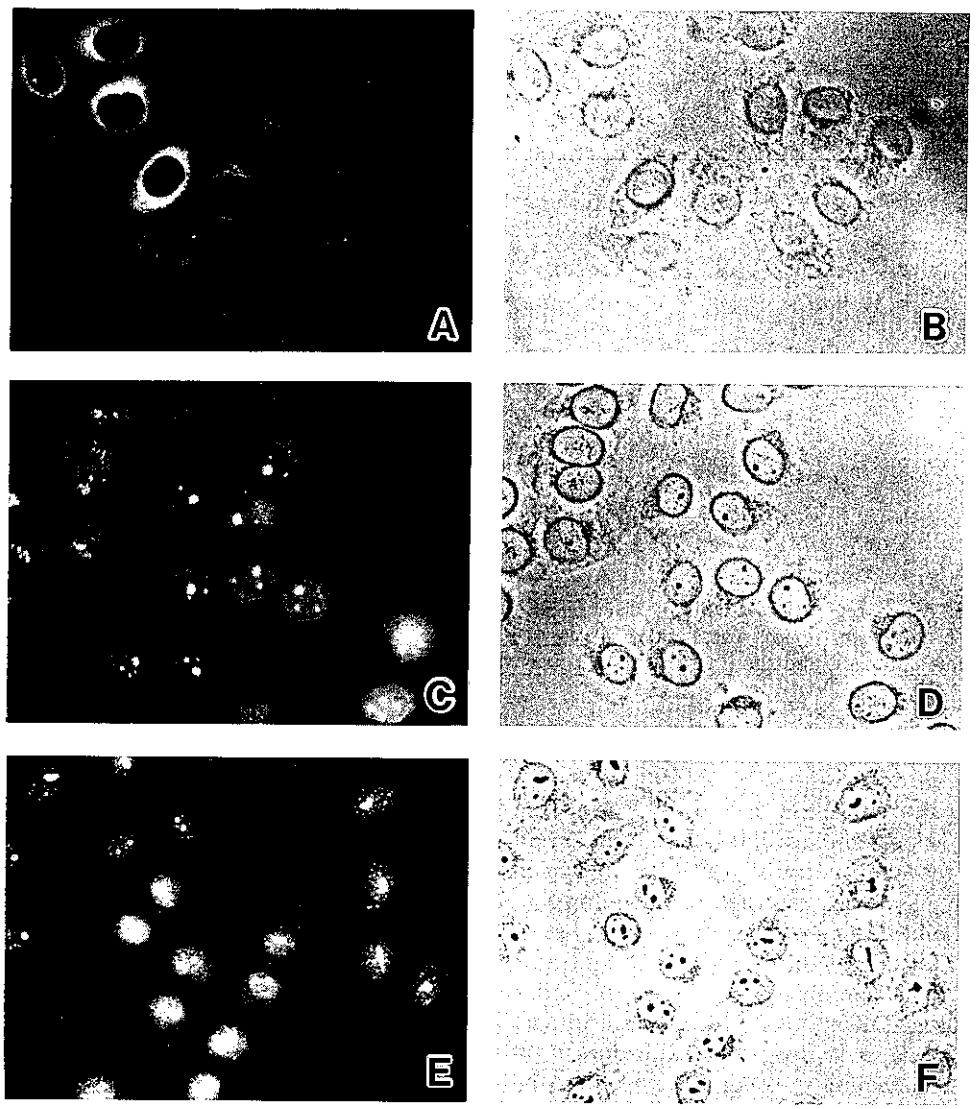

Fig. 2. Intracellular distribution of HSP72 protein in HeLa cells after ADR treatment. A, C, and E, fluorescence micrographs; B, D, and F, the corresponding phase-contrast micrographs. (A, B) untreated cells, (C, D) 100 μM ADR for 2 h, and (E, F) heat shock (43°C, 30 min).

determined their viability and the extent of DNA damage under the following conditions. The levels of cell viability were not altered significantly by ADR in the range of less than 1 mM for 2 h, at least with the MTS assay, which depends on metabolically active cells (Fig. 1A). Since ADR is known to produce ROS, we measured the formation of 8-OHdG in the DNA of HeLa cells treated with ADR, as a measure of the extent of oxidative damage.[14] Upon incubation of HeLa cells with 100 μM ADR for 1 h, the content of 8-OHdG increased to 4 times that of the untreated control (Fig. 1B). These results suggested that there is some DNA damage in HeLa cells treated with 100 μM ADR, but the general effect of ADR at this dose was not so severe as to kill the cells during the period of this experiment.

HSP70 accumulation in the nucleolus after ADR treatment An isoform of HSP70 (HSP72) is constitutively expressed in unstressed HeLa cells (Fig. 2A and B) and is also heat-inducible.[20] The translocation of HSP72 into the nucleolus frequently occurred in heat-treated cells (Fig. 2E and F). Previously, we have shown the nuclear translocation of HSP70 protein after the exposure of

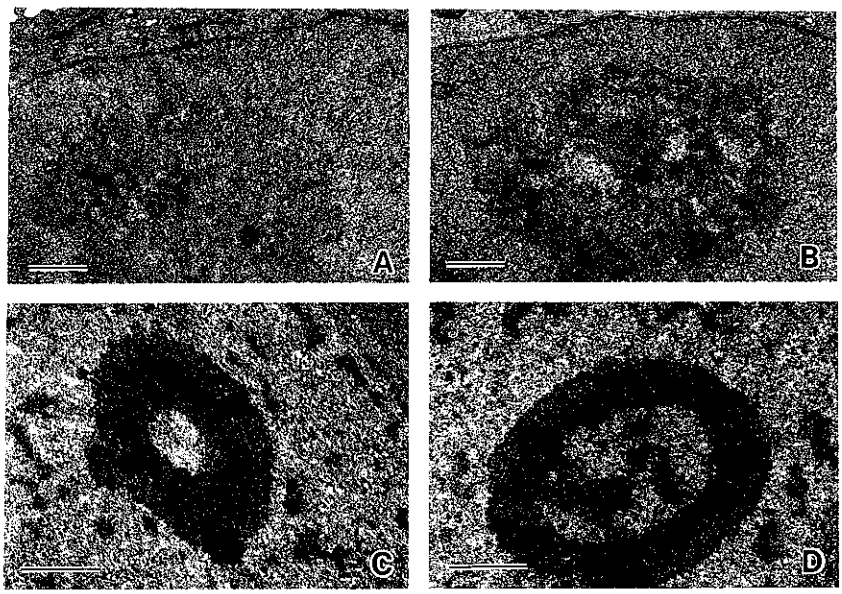

Fig. 3. Analysis of ADR-treated HeLa cells by electron microscopy. A, nucleolus in an untreated cell. B, heat-shocked (43°C, 30 min) cells. The high-density region was slightly increased in the nucleolus. C, D, cells incubated with 100 μM ADR for 2 h. The nucleolus became compact, and the low density area was surrounded by segregated high density components. Bar=0.5 μm.

human amniotic cultured WISH cells to hydrogen peroxide.[9] The HSP70 protein was diffusely distributed all over the area of the nucleus transiently after hydrogen peroxide treatment, and then returned to the cytoplasm within 1 h.[9] At the beginning of the present study using human tumor cells, we thought that the nuclear translocation of HSP70 protein would occur via a similar mechanism, because ADR attacks DNA at least in part via ROS in the same way as hydrogen peroxide does.[3, 9, 21] Immunofluorescence staining showed, however, that the HSP72 was translocated from the cytoplasm to the nucleus and accumulated in the nucleolus after treatment with 100 μM ADR and remained there even after 2 h (Fig. 2C and D).

Morphological changes of nucleolus in ADR-treated cells

We further examined the ultrastructure of the nucleolus in HeLa cells treated as above, using electron microscopy. After heat treatment at 43°C for 30 min, a slight increase in the distinction between high and low density areas in the nucleolus was seen, although the size and fine structure of their boundary from the nucleoplasm were not significantly altered during this grade of heat treatment (Fig. 3B). On the other hand, in the nucleus of the ADR-treated cell (100 μM for 2 h), the nucleolar size decreased and the contour became rounded (Fig. 3C and D). The internucleonemal spaces[22] were also lost and the nucleolus became compact. The area containing the granular components was segregated sharply toward the peripheral zone of the nucleolus and a low density area remained at the center of the nucleolus. These characteristics of the ring-shaped nucleolus were frequently observed and may be related to the distribution of HSP72 protein observed by laser scanning confocal fluorescence microscopy, as shown in Fig. 4.

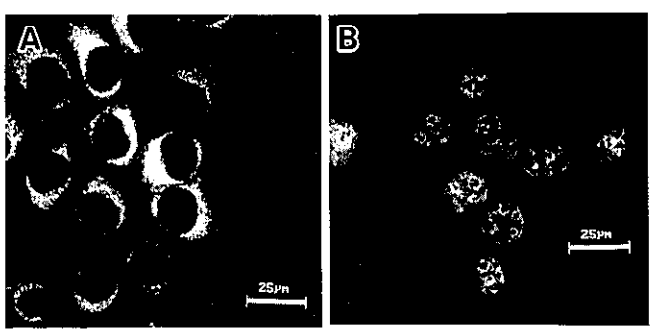

Fig. 4. Laser scanning confocal fluorescence micrographs showing HSP72 protein distribution in HeLa cells. A, untreated cells. B, cells treated with 100 μM ADR for 2 h.

The inhibition of nucleolar function in ADR-treated cells

Since we observed drastic morphological changes in the nucleolar fine structure, the effects of ADR on the nucleolar function were examined using a probe that detects 45*S*, 35*S*, and part of 18*S* RNA.[18] The synthesis

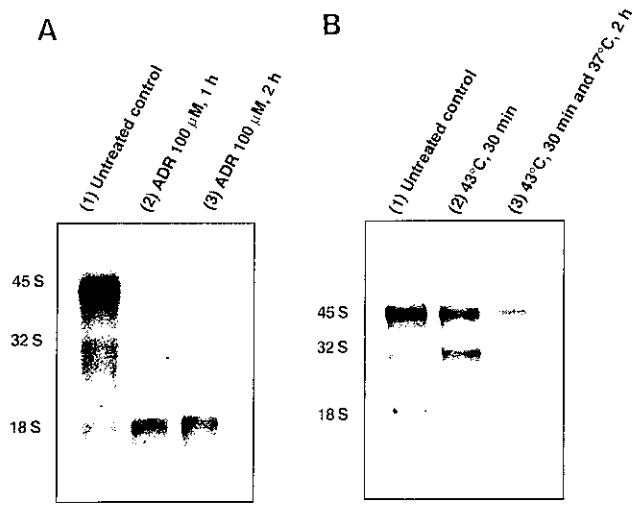

Fig. 5. Northern blot analysis for 45S ribosomal RNA synthesis in nuclei isolated from HeLa cells. Following treatment with either 100 μM ADR (A) or heat shock (43°C, 30 min) (B), nuclear RNA was prepared and analyzed as described in "Materials and Methods." The probe used was the 5′-portion (5.8 kb) of human ribosomal DNA involving the external terminal sequence (ETS) and a part of 18S RNA.[18]

of 45*S* RNA was somewhat decreased after heat shock (43°C, 30 min), to 80% of control (Fig. 5B). On the other hand, the synthesis of 45*S* RNA was significantly inhibited in the nucleus of ADR-treated HeLa cells (Fig. 5A). These results are compatible with the drastic changes of nucleolar morphology after treatment of HeLa cells under the same conditions.

DISCUSSION

ADR can produce ROS both *in vivo* and *in vitro* in organisms.[23–25] Due to its reactivity, the ROS reacts instantaneously with cellular components such as DNA and proteins. 8-OHdG is one of the markers of oxidative DNA damage.[21, 26] We observed an approximately 4-fold increase of 8-OHdG content in the DNA of HeLa cells exposed to 100 μM ADR (Fig. 1B). Previously, we suggested a possible relationship between the DNA damage induced by hydrogen peroxide and the transient translocation of HSP70 protein from the cytoplasm to the nucleus.[9] It has also been reported that HSP70 may contribute to either the defense of nuclear components or the better recovery of physiological nuclear functions.[13, 27, 28] Recently Karlseder *et al.*[29] showed that HSP70 overexpression is sufficient to provide protection against the cytotoxicity of doxorubicin (ADR). p53 is also induced and accumulated in the nucleus after treatment of cells with various DNA damaging agents.[30, 31] However, it is still not clear what really triggers the translocation of HSP70 protein.

In the present study using immunofluorescence staining, HSP70 protein apparently accumulated in the nucleolus when HeLa cells were exposed to ADR (Fig. 2). This differed from the situation of WISH cells treated with hydrogen peroxide, in which predominant nucleolar accumulation of HSP70 was not observed.[9] These changes of HSP70 distribution were reminiscent of the nucleolar accumulation of HSP70 after heat shock.[20, 32, 33] Concerning the nucleolus, the translocation of HSP70 to the nucleolus was commonly associated with visible changes in the nucleolar structures.[31, 34] In other words, agents which do not cause nucleolar damage, but can induce heat shock response, do not lead to the nucleolar accumulation of HSP70.[32, 34] Daskal *et al.*[35] reported the nucleolar ultrastructural aberration of Novikoff hepatoma cells after treatment with ADR, although they did not note the typical ring-shaped segregation of granular components as in the present study and did not mention 45*S* RNA or HSP70. In the present study, we clearly demonstrated that most of, if not all, nucleoli showed ring-shaped segregation and a decrease of nucleolar 45*S* RNA biosynthesis in HeLa cells treated with ADR.

The question arises, what is the difference between nuclear translocation and nucleolar accumulation of HSP70 protein? The nucleolar HSP70 is generally bound tightly and not removed by treatment with nonionic detergents, whereas most of the nucleoplasmic HSP is extracted under the same conditions.[36, 37] Previous electron microscopic fine localizations using microautoradiographic and immunological techniques showed that HSP70 is almost exclusively found in the region with granular preribosomal materials.[34, 38] The fibrillar centers of the nucleolus are largely free from HSP70. ATP was required to remove unwanted protein aggregates from the nucleolus.[36]

It is well known that ADR inhibits topo II activity.[8] Regarding the mechanism of nucleolar inhibition by ADR, we speculate that topo IIβ may be involved, although we did not examine its localization in the nucleolus of HeLa cells due to the lack of a specific antibody to topo IIβ. Topo II has two isozymes, that is, topo IIα and topo IIβ.[39] These two isozymes differ in their intracellular location; topo IIα is mainly localized in the nucleoplasm while topo IIβ is in the nucleolus.[40] Our findings regarding the effects of ADR on the structure and function of the nucleolus suggest that topo IIβ might be a target of this anticancer drug. Besides topoisomerase I (topo I), which is well known to be involved in the transcription of ribosomal genes by RNA polymerase I,[41] topo IIβ may play some roles in the nucleolus.

Interestingly, the association of HSP70 and topo I in the nucleolus after heat shock has been reported by Ciavarra *et al.*[42] Heat shock proteins may contribute to maintain or reactivate various enzymes in the nucleolus and also to remove aberrant ribosomal precursors from it.

In conclusion, we observed the translocation of HSP70 into the nucleolus after ADR treatment and the effects of ADR on both the structure and function of the nucleolus in HeLa cells. We also confirmed an increase of oxidative DNA damage after ADR treatment. Therefore, ADR prevents cell growth at the DNA level by direct intercalation, oxidative damage and inhibition of DNA replication, and also suppresses protein synthesis indirectly by inducing dysfunction of the nucleolus. We feel that our present study offers some useful clues to the development or design of new anticancer drugs.

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