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Modification of thermosensitivity by amrubicin or amrubicinol in human lung adenocarcinoma A549 cells and the kinetics of apoptosis and necrosis induction

SACHIKO HAYASHI[1], MASANORI HATASHITA[3], HIDEKI MATSUMOTO[1], ZHAO-HUI JIN[1], HIROKI SHIOURA[2] and EIICHI KANO[1]

Departments of [1]Experimental Radiology and Health Physics, [2]Radiology, Faculty of Medical Science, Fukui University, Matsuoka, Fukui 910-1193; [3]The Wakasawan Energy Research Center, Research and Development Department, Tsuruga 914-0192, Japan

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Abstract. The effects of amrubicin (AMR) and its active metabolite, amrubicinol (AMROH), on the sensitivity of human lung adenocarcinoma A549 cell line to hyperthermia at 44°C were investigated. The cell phase response as well as the kinetics of apoptosis and necrosis induction were also analyzed. The cytocidal effects of 44°C hyperthermia on A549 cells exhibited low thermosensitivity with a T_0 value of 12 min. The slope of the survival curve in the exponential phase, described semilogarithmically, in 44°C hyperthermia combined treatment with AMROH (0.02 μg/ml) was approximately parallel to 44°C hyperthermia alone. The initial shoulder shape portion of the survival curve from 44°C hyperthermia alone, indicating the repair of sublethal thermal damage (SLTDR), was reduced with the sequential combined treatment of AMR or AMROH. Sequential treatments with AMR or AMROH prior to 44°C hyperthermia resulted in additive thermo-enhancement effect by reducing not only survival but was shoulder wide. Furthermore, like AMR and AMROH, adriamycin (ADM) and etoposide (VP-16) are DNA topoisomerase II inhibitors, and the effects of these 4 agents on 44°C hyperthermia were compared. All 4 agents exhibited comparable thermo-enhancement effects. Using synchronized A549 cells, AMR or AMROH did not elicit cell phase responses, irrespective of the concentration. The induction of apoptosis was investigated at 48 and 72 h after AMROH treatment, 44°C hyperthermia or the combined treatment, in which apoptosis was not significantly induced after any treatment. Furthermore, the incidence of necrosis was examined as well as apoptosis. The incidence of necrosis at 48 and 72 h after AMROH was 2.4 and 4.3%, respectively; after 44°C hyperthermia was 3.3 and 4.0%, respectively; and after the combined treatment it was 10.7 and 8.7%, respectively. The necrosis induced after the combined treatment was circa 3 times higher than that in either of the single treatments.

Correspondence to: Dr Sachiko Hayashi, Department of Experimental Radiology, Faculty of Medical Science, Fukui University, Matsuoka, Fukui 910-1193, Japan
E-mail: hayashis@fmsrsa.fukui-med.ac.jp

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Introduction

Amrubicin hydrochloride (AMR) is the first totally-chemically synthesized anthracycline derivatives (1,2), and it exhibits anti-tumor activity against human non-small cell lung, small cell lung and superficial bladder cancer (3-5). AMR exerts its antitumor effects by inhibiting topoisomerase II activity and suppressing DNA synthesis (6,7). AMR is metabolized to AMROH *in vivo* (2), and the results of our *in vitro* study using human lung cancer A549 cells evidenced that the cytocidal effects of AMROH were about 10 times more potent than those of AMR. The survival curves of A549 cells treated with isotoxic doses of AMR and AMROH showed the same profile. When cancer cells are exposed to hyperthermia, the cells usually acquire thermo-tolerance during heating or afterwards, which has been one of the problems associated with thermotherapy (8-10). Thermo- and chemo-therapy, a combined treatment with hyperthermia and chemical agents, has been adopted as an interdisciplinary treatment strategy to overcome the problems of thermotolerance.

We previously reported that the lethal sensitivity in the simultaneous combined treatment with 42°C hyperthermia and adriamycin (ADM) for the initial short time was markedly higher but followed by reduced lethal sensitivity, the sequential treatment showed the tolerant lethal sensitivity (11). In this study, the modification of thermo-sensitivity by AMR or AMROH was investigated using A549 cells *in vitro*. A549 cell line originating from human lung adenocarcinoma was established by Giard *et al* (12,13). Several studies have been conducted on the sensitivity of A549 cells to radiation and hyperthermia (14-17).

It has been clarified at the molecular level that A549 cells have a mutated K-ras gene (18) and the wild-type p53 gene (19). When DNA is damaged, wild-type p53 protein is

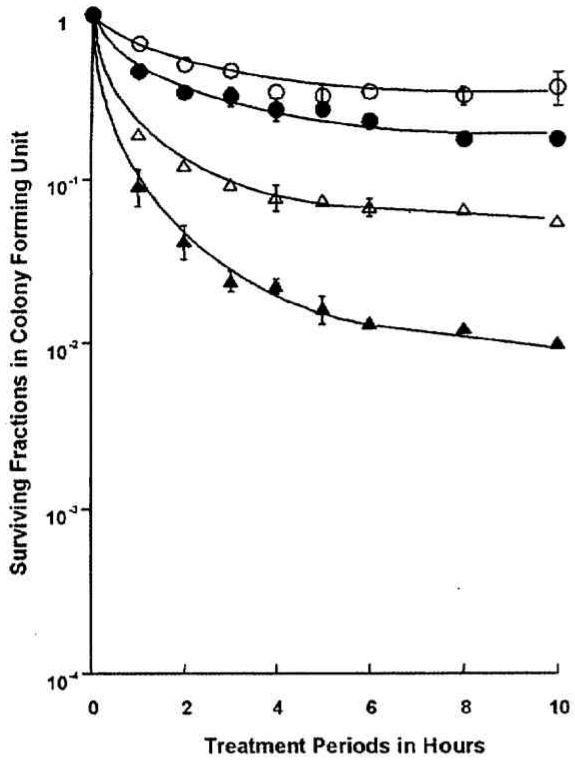

Figure 1. Cytocidal effect of AMROH in graded concentration on survival of A549 cells. Ordinate: log surviving fractions (SFs) in colony forming unit. Abscissa: treatment periods of AMROH in hours. Symbols of the survival curve represent the concentration of AMROH in open circles (0.01 μg/ml), closed circles (0.02 μg/ml), open triangles (0.1 μg/ml) and closed triangles (0.2 μg/ml), respectively. Symbols with vertical bars represent means with standard errors obtained from three independent SFs. Symbols without a bar represent the standard error within the symbols.

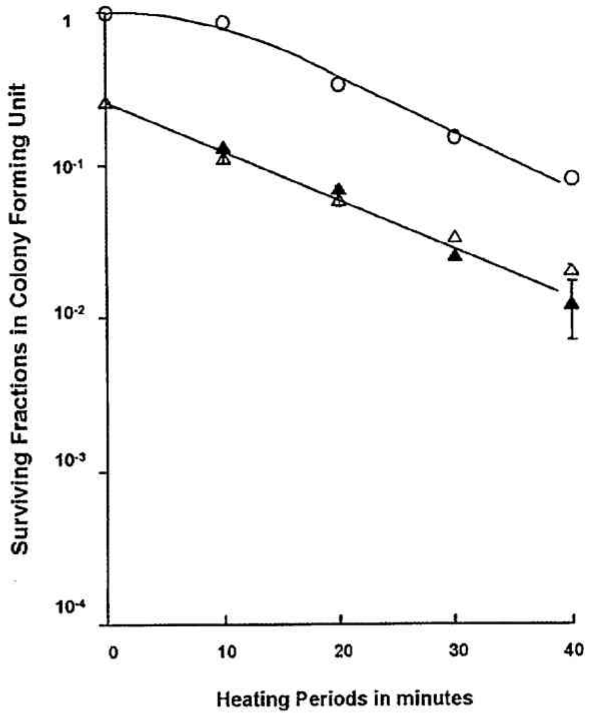

Figure 2. Effect of thermo-enhancement of AMR (0.2 μg/ml) in A549 cells. Ordinate: log surviving fractions (SFs) in colony forming unit. Abscissa: heating periods at 44°C in minutes. Open circles represent SFs of cells from 44°C hyperthermia alone. Open triangles represent SFs of cells treated with hyperthermia at 44°C for graded periods followed by AMR for 3 h. Closed triangles represent SFs of cells from hyperthermia at 44°C for graded periods after pre-treatment with AMR for 3 h. Symbols with vertical bars represent means with standard errors obtained from three independent SFs. Symbols without a bar represent the standard error within the symbols.

activated by phosphorylation on signaling pathway, and the activated p53 exhibits antitumor effects by inducing either apoptosis or G_1 arrest (20-25). The *ras* gene was one of the first oncogenes discovered, and it is involved in cellular proliferation and differentiation (26-28), but there have not been any studies investigating the relationship between the *ras* gene and thermosensitivity.

In the present study, we analyzed the effects of AMR and AMROH on the thermosensitivity of A549 cells with the above-mentioned genetic backgrounds. We examined the thermo-enhancement effects of isotoxic doses of AMR and AMROH on the sensitivity of A549 cells to 44°C hyperthermia and compared the effects of AMR and AMROH with those of two other DNA topoisomerase II inhibitors (ADM and VP-16). With regard to cell phase responses to hyperthermia, the survival curve for mild hyperthermia at 40°C was flat and responses were not appreciable for any phase (16), but the responses for experimental hyperthermia at 44°C showed marked cytotoxicity in the S phase (30,31).

We conducted a study using bleomycin, a DNA synthesis inhibitor, and confirmed that the survival curve for synchronized cells was flat regardless of drug concentration, and that cell phase responses were not appreciable during cell phase progression (30). The cell phase responses to AMR and AMROH have not been clarified. Therefore, we investigated the cell phase responses of A549 cells synchronized with hydroxyurea to AMR and AMROH. In murine L cells having wild-type p53 that show high thermosensitivity, hyperthermia effectively induced apoptosis (8).

Here, in A549 cells that have wild-type p53 gene and are not thermosensitive, the induction of apoptosis (32,33) and necrosis (34,35) was investigated following AMROH treatment combined sequentially with 44°C hyperthermia. The effects of AMR and its active metabolite, AMROH, on the sensitivity of A549 cells to 44°C hyperthermia were analyzed at the kinetic and molecular levels.

Materials and methods

Cells and culture medium. A549, a human lung adenocarcinoma cell line, cultured in Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) containing NCTC-135 (Gibco Laboratories, Grand Island, NY, USA), lactalbumin hydrolysate solution (Difco Laboratories, Detroit, USA) and 15% newborn calf serum (Gibco) (hereinafter referred to as MLN-15), was used (12,13).

Chemicals, hyperthermia and treatments. AMR or AMROH (Sumitomo Pharmaceuticals Co., Ltd. Osaka, Japan) was dissolved in the culture medium to an appropriate final concentration for the treatment. The adhered cells in the flasks were treated with AMR or AMROH by replacement with 6 ml of the AMR or AMROH solution in MLN-15 for graded

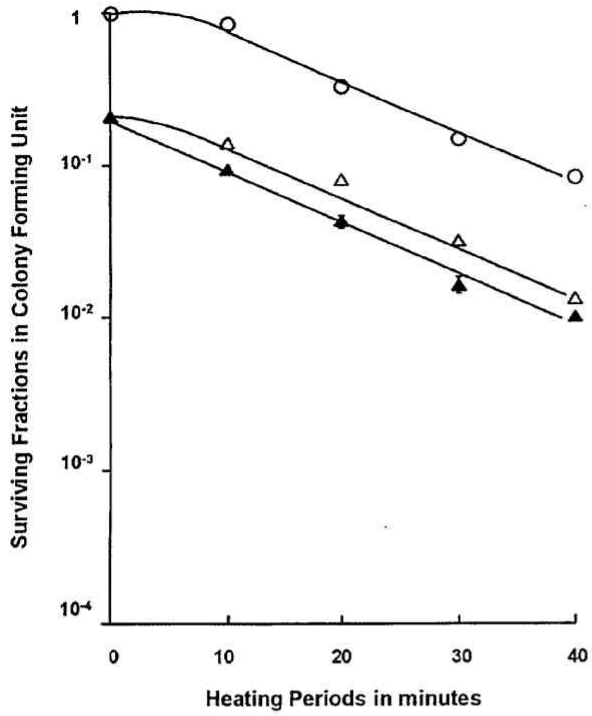

Figure 3. Effect of thermo-enhancement of AMROH (0.02 μg/ml) in A549 cells. Ordinate: log surviving fractions (SFs) in colony forming unit. Abscissa: heating periods at 44°C in minutes. Open circles represent SFs of cells from 44°C hyperthermia alone. Open triangles represent SFs of cells treated with hyperthermia at 44°C for graded periods followed by AMROH for 3 h. Closed triangles represent SFs of cells from hyperthermia at 44°C for graded periods after pre-treatment with AMROH for 3 h. Symbols with vertical bars represent means with standard errors obtained from three independent SFs. Symbols without a bar represent the standard error within the symbols.

periods. After the exposure to AMR or AMROH, the adhered cells were chased, gently rinsed twice and re-fed with 6 ml of the culture medium, MLN-15. Thus, treated flasks were incubated stationarily for visible colony formation.

Hyperthermia was carried out by immersion of culture flasks of the tightened screw tops in a temperature-regulated water bath (Model EPS-47, Tokyo Seisakusho Co., Tokyo, Japan) pre-set at 44°C. The temperature was maintained within the error of ±0.05°C as measured with a thermistor (Model D116-1251, Takara Thermistor Instruments Co., Yokohama, Japan).

For combined treatment with chemotherapy and thermotherapy were carried out sequentially. Cells treated with AMR or AMROH for 3 h were rinsed twice with culture medium containing 3% serum, placed in MLN-15 and followed by 44°C hyperthermia. Kinetic assessment of the sensitivity of A549 cells to agents and hyperthermia was carried out by the colony formation method and corrected based on the plating efficiency of the control (i.e., 80-90%). The average colony multiplicity was <1.1. The T_0 value, adopted as the criterion of cellular thermo- or chemo-sensitivity, represented the treatment period required to reduce survivals by 1/e in the exponentially regressing portion of the survival curves, i.e., the linear portions of the treatment period in survival curves semilogarithmically.

For cell phase responses, A549 cells were treated with 4 mM hydroxyurea (HU) (Sigma Chemical, St. Louis, MO, USA) (36,37) for 5 h to synchronize them at the G_1-S border, and after an interval of 1-9 h, cells were treated with AMR or AMROH for 4 h. Cell phase responses were also analyzed by the colony formation methods.

Analysis of apoptosis and necrosis. Induction of apoptosis was analyzed at 48 and 72 h after treatment with 0.02 μg/ml AMROH for 4 h, 44°C hyperthermia for 35 min, or the combined treatment. Trypsinized cells were placed in MLN-15 to stop trypsinization, rinsed with PBS(-), fixed overnight in 1% glutaraldehyde (Nacalai Tesque Inc., Kyoto, Japan), and placed in PBS(-). For observation, cells were stained with 0.2 mM Höchst 33342; Bisbenzimide H33342 Fluorochrome, Trihydrochloride (Calbiochem, Merck KGoA, Dormstodt, Germany) and were analyzed under a fluorescence microscope (Nikon Eclipse E600, Nikon Co. Ltd, Tokyo, Japan). A total of 1,000 cells were counted, and the percentage of typical apoptotic cells was calculated. The induction of necrosis was also determined after the above-described treatments. After double-staining the cells using acridine orange (Sigma-Aldrich Co. St. Louis, USA) and ethidium bromide (Nacalai Tesque Inc., Kyoto, Japan) (AO/EB) (38), cells were observed in real-time under a fluorescence microscope, and the percentage of typical necrotic cells was calculated.

Results

Cytotoxic effects of AMROH. A549 cells were treated with graded concentrations of AMROH (0.01, 0.02, 0.1 or 0.2 μg/ml) for 1-10 h in order to determine its cytotoxic effects as shown in Fig. 1. For each concentration, the surviving fraction of cells were plotted against treatment time on a semilogarithmic chart. All curves showed steeply a slope during the initial stages of treatment. At concentrations of 0.01 and 0.02 μg/ml, the curves gradually reached a plateau after 4 to 6 h, and the plateau was maintained ≤10 h at all concentrations. AMROH was markedly cytotoxic during the early stages of treatment, but no marked changes in sensitivity were seen beyond 4 h.

Heating period (44°C) - survival relationships of A549 cells. With regard to the thermosensitivity of A549 cells, the T_0 value (dose required to reduce the survival by 1/e), which is the reciprocal of the slope of the survival curve in the exponential phase, was 12 min for 44°C hyperthermia. As is the case with the Elkind recovery associated with low-dose radiotherapy, the repair of sublethal thermal damage (SLTDR), which is seen as a curve shoulder, was identified during the early stages of hyperthermia. The Tq value, a sort of cellular capacity for SLTDR, was 6.5 min as shown in Fig. 2.

The effects of AMROH on hyperthermia. Using A549 cells, the thermo-enhancement effects of AMR and AMROH were investigated based on the survival curves drawn after 44°C hyperthermia and AMROH combination therapy in Figs. 2 and 3. The survival curve of the cells performed with 44°C hyperthermia alone elicited a shoulder, indicating the repair of SLTD, whereas the curve of sequentially combined

Table I. Comparison of thermo-enhancement by four topoisomerase II inhibitors.

	44°C alone T_0	Chemical - 44°C T_0	Enhancement ratio
AMR	1.40	1.05	1.33
AMROH	1.40	1.00	1.40
ADM	1.40	1.05	1.33
VP-16	1.40	0.95	1.47

The thermo-enhancement effects to 44°C hyperthermia of 4 agents in DNA topoisomerase II inhibitor, AMR, AMROH, ADM and VP-16, were compared by T_0 value. The A549 cells were treated with agent prepared at isotoxic dose, AMR (2.5 μg/ml), AMROH (0.02 μg/ml), ADM (0.02 μg/ml) or VP-16 (2.0 μg/ml) for 3 h respectively and followed by hyperthermia at 44°C for 30 min in the combined treatment.

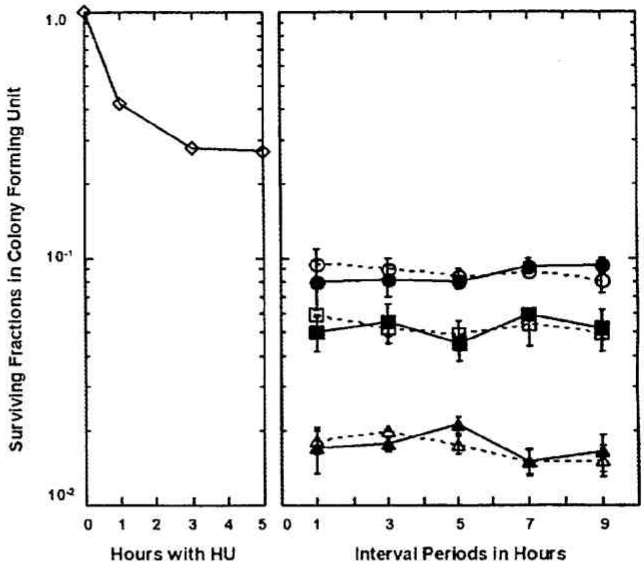

Figure 4. Left panel shows the HU treatment period-survival relationship of cells. Right panel shows the cell phase responses to AMR or AMROH in gradational concentration for A549 cells synchronized by the exposure to HU for 5 h. Ordinates: log surviving fractions (SFs) in colony forming unit. Abscissa in left panel: treatment periods with 4 mM HU in hours. Abscissa in right panel: incubation periods at 37°C between the pre-treatment with HU for 5 h and the post-treatment with AMR or AMROH for 4 h. Open circles, open squares and open triangles represent SFs from post-treatment with AMR in 0.1, 0.5 and 2.5 μg/ml, respectively. Closed circles, closed squares and closed triangles represent SFs from post-treatment with AMROH in 0.004, 0.02 and 0.1 μg/ml, respectively. Symbols with vertical bars represent means with standard errors obtained from three independent SFs. Symbols without a bar represent the standard error within the symbols.

treatment with AMR or AMROH was reduced. The SLTDR associated with short-term hyperthermia was blocked by AMR or AMROH. The slope of the survival curve in the exponential phase for 44°C hyperthermia and the combination therapy with AMR or AMROH were parallel, i.e. administration of AMR or AMROH prior to hyperthermia showed additive thermo-enhancement effects. The thermo-enhancement effects of 4 topoisomerase II inhibitors, ADM, VP-16, AMR and AMROH, on 44°C hyperthermia for 30 min were compared as shown in Table I. The effects of 4 agents in isotoxic doses were comparable, markedly enhancing the effects of hyperthermia.

Cell phase responses to AMR and AMROH. A549 cells synchronized at the G_1-S border by treatment with 4 mM HU for 5 h were incubated for graded periods of time, and were further exposed to AMR and AMROH in order to analyze the cell phase responses as shown in Fig. 4. As a control, synchronized cells were incubated for graded periods of time and were treated with 4 mM HU for 2 h, not shown in the figure. Cell survival was plotted in order to compare the results with the standard cell-cycle phase. After a specified interval, synchronized A549 cells were treated with 3 graded concentrations of AMR or AMROH for 4 h respectively, and the survival curve was largely flat for all cell-cycle phases at all concentrations. AMR and AMROH did not elicit cell phase responses, irrespective of the concentration.

Effects on apoptosis induction. The thermo-enhancement effects of AMROH on A549 cells were investigated based on the induction of apoptosis as shown in Fig. 5. After AMROH for 4 h, 44°C hyperthermia for 35 min or combination therapy, cells were harvested at 48 and 72 h after incubation and were fixed with glutaraldehyde. Fixed cells were stained with Höchst 33342 solution, and cellular morphology was observed under a fluorescence microscope in order to determine the percentage of apoptotic cells. At 48 and 72 h after treatment with AMROH alone, the incidence of apoptosis was 0.9% and 1%, respectively; circa 1.3% (at both time points) for 44°C hyperthermia alone; and 2.4 and 1.7% for combination therapy, respectively.

Effects on necrosis induction. As was the case with apoptosis, the thermo-enhancement effects of AMROH on A549 cells were investigated in the induction of necrosis. The incidence of necrotic cells following AMROH treatment, 44°C hyperthermia or the combination therapy was investigated by AO/EB double staining at intervals of 48 and 72 h after treatment. The incidence of necrosis at 48 and 72 h after treatment with AMROH was 2.4 and 4.3%, respectively, after 44°C hyperthermia was 3.3 and 4.0%, respectively, and after combined treatment it was 10.7 and 8.7%, respectively as shown in Fig. 6.

Discussion

In the present study, the modification of AMR and AMROH on the thermosensitivity of A549 cells was investigated.

Cytotoxicity of AMROH. In our study, AMROH exhibited marked cytotoxic activity during the early stages. AMR and its active metabolite, AMROH, are DNA topoisomerase II inhibitors (3,4). DNA topoisomerase II is reported to be vital for essential biological reactions such as replication, transcription and genetic recombination (39-41). In 1984, Liu *et al* discovered that mAMSA [4'-(9-acridinylamino) methanesulfon-m-anisidide], an antitumor agent, inhibited topoisomerase II

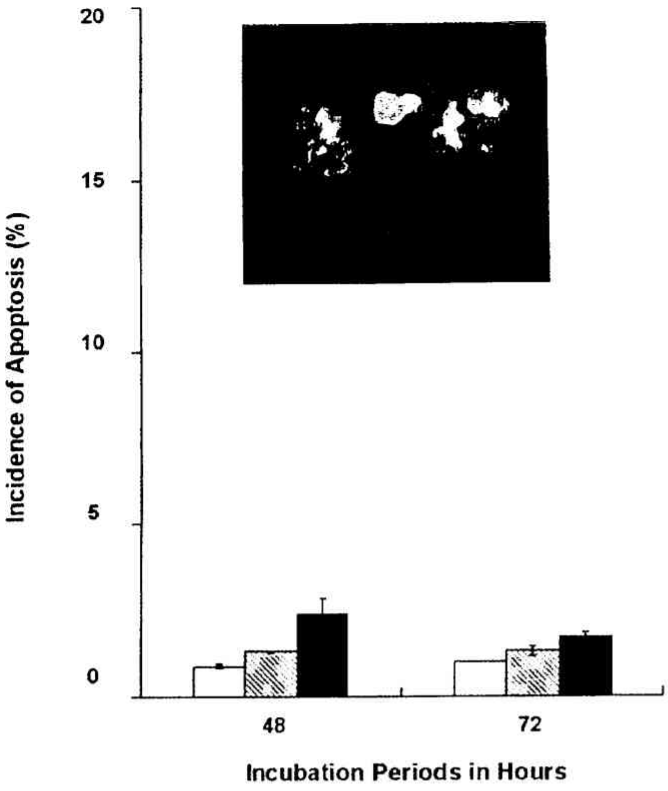

Figure 5. Kinetics of apoptosis induction at 48 or 72 h of incubation periods after AMROH, 44°C hyperthermia or the combined treatment. At indicated time, A549 cells stained with Höchst 33342 were observed under a fluorescent microscope. The cells of DNA fragmented nuclei with typical morphological features of apoptosis were counted as apoptotic cells. Ordinate: percentages of apoptosis in the total cells. Abscissa: 37°C incubation periods in hours after treatment. Columns represent AMROH (0.02 µg/ml) for 4 h, 44°C hyperthermia for 35 min and the combined treatments in order from the left, respectively. Incidence of apoptosis after treatment with AMROH or hyperthermia alone were slightly induced in interval periods at 48 and 72 h. In the combined treatment, no frequency of apoptosis increased significantly.

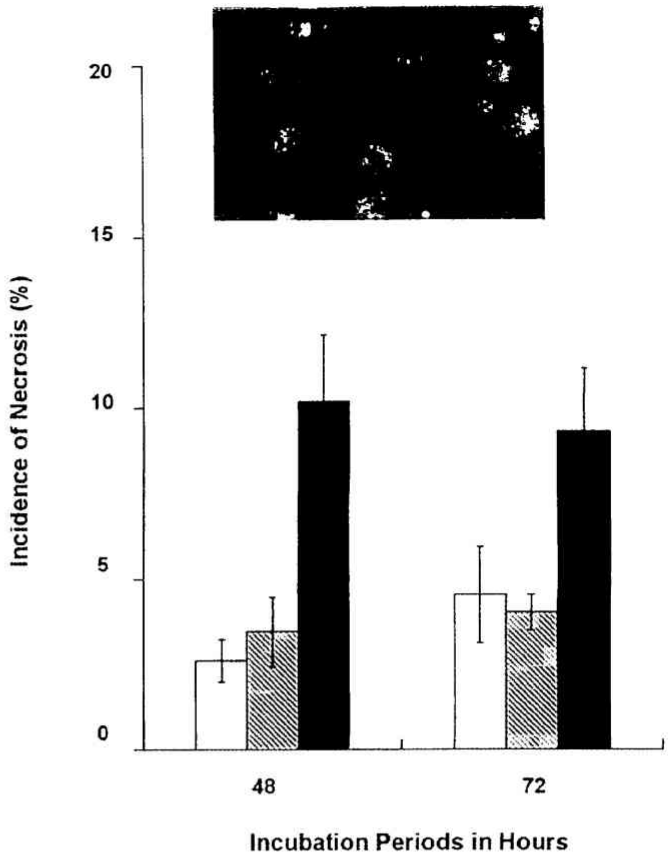

Figure 6. Kinetics of necrosis induction at 48 or 72 h of incubation periods after AMROH, 44°C hyperthermia or the combined treatment. At indicated time, A549 cells by double staining with AO/EB were observed under a fluorescent microscope. The rubious cells were counted as necrotic cells. Ordinate: percentages of necrosis in the total cells. Abscissa: 37°C incubation periods in hours after treatment. Columns represent AMROH (0.02 µg/ml) for 4 h, 44°C hyperthermia for 35 min and the combined treatments in order from the left, respectively. Incidences of necrosis after AMROH or hyperthermia alone were both induced slightly in interval periods at 48 and 72 h. In these combined treatment, the frequency of necrosis increased significantly.

in mammals (42). Therefore, topoisomerase II inhibitors are effective anticancer agents because topoisomerase II inactivation and inhibition severely affect cell survival (43,44).

Effects on hyperthermia. When A549 cells were treated with AMR or AMROH prior to 44°C heating *in vitro*, the SLTDR seen during the early stages of hyperthermia was inhibited. AMR and AMROH additively enhanced the effects of 44°C hyperthermia. In thermotherapy for cancer, the time required to achieve 10% survival (0.1 on a survival curve) for hyperthermia alone was 30 min, but that for the combination therapy was 10 min. Comparable results were obtained in about one-third of the time by combining hyperthermia with AMR or AMROH. The fact that combining AMR or AMROH cuts the duration of hyperthermia to one-third is beneficial in multidisciplinary anticancer therapy. We have investigated the ability of various chemicals to modify the effects of hyperthermia using different cells at the kinetic and molecular levels (11,30,45-47). The effects of AMR, AMROH, ADM (48) and VP-16 (49), which target DNA metabolism, on the thermosensitivity of A549 cells were compared. When A549 cells were treated with isotoxic doses of AMR, AMROH, ADM or VP-16 for 3 h and then sequentially subjected to 44°C hyperthermia for 30 min, comparable degrees of enhancement were noted. These findings clarify that all topoisomerase II inhibitors increase the thermosensitivity of A549 cells.

Cell phase responses. In order to clarify the mechanisms of thermal enhancement by AMR or AMROH, we investigated the survival curves through cell phase progression, the cell phase response of A549 cells to 0.1, 0.5 and 2.5 µg/ml of AMR or 0.004, 0.02 and 0.1 µg/ml of AMROH were analyzed. All survival curves were flat, i.e., cell phase responses were not seen regardless of the concentrations of AMR or AMROH. We reported that bleomycin (29), which inhibits DNA synthesis, was cell cycle non-specific regardless of concentration (30). When exposed to more than moderate hyperthermia or radiation, cells are most sensitive to hyperthermia during the G_1-S phase and to radiation during the G_2-M phase (50,51). There was no cell phase response to mild hyperthermia that could be assessed uniformly in all phases for thermosensitivity (30), but with 44°C experimental

hyperthermia, sensitivity was high during the G_1-S phase (31). While various studies have been conducted to determine the target of hyperthermia, no conclusive evidence has been obtained. The reason that chemicals such as AMR in Fig. 4 and BLM lack cell phase responses may be due to the fact that they target different types of DNA damage (52-54). Hence, more effective synergism may be achieved by combined treatment with hyperthermia and drug administration, as the mirror contrast on each cell phase response, in order to enhance and complement antitumor effects.

Induction of apoptosis and necrosis. The induction of apoptosis following 44˚C hyperthermia or 0.02 μg/ml AMROH increased slightly with time, but there was no significant increase following sequential combination therapy. However, the induction of necrosis following 44˚C hyperthermia increased with time, and there was a significant increase in the induction of necrosis after 4 h of AMROH exposure followed by 44˚C hyperthermia. p53-mediated apoptosis is thought to be cell cycle dependent (55,56). We reported that apoptosis was induced effectively by 44˚C hyperthermia in thermosensitive murine L cells with wild-type p53 (8). The results of the present study were unexpected in that the cytotoxicity of AMROH against A549 cells having wild-type p53 in hyperthermia was due to increased necrosis, not increased apoptosis. In addition, with A549 cells, cell phase responses were not seen. This suggests that AMR and AMROH directly act on DNA topoisomerase II without affecting the p53 cascade. AMR and AMROH were shown to dynamically enhance the thermosensitivity of A549 cells. Thus, in multidisciplinary anticancer therapy, the antitumor effects of hyperthermia can be improved by administering AMR or its metabolite, AMROH (57).

Acknowledgements

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