DIFFERENTIAL CYTOTOXICITY TO HUMAN LUNG NORMAL DIPLOID, VIRUS-TRANSFORMED AND CARCINOMA CELLS BY THE ANTITUMOR ANTIBIOTICS, AUROMOMYCIN AND MACROMOMYCIN, AND THEIR NON-PROTEIN CHROMOPHORES*

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(Received for publication August 18, 1982)

With the use of three human lung cultured cell lines: normal diploid fibroblasts (WI38), their SV40-transformants (VA13) and carcinoma cells (A549), whose doubling times were similar, the cytotoxicity of the protein antitumor antibiotics, auromomycin (AUR) and macromomycin (MCR), was studied by colony formation method. The susceptibilities of the three cell lines to these antibiotics were in the order: WI38<VA13<A549. This differentiality in the cytotoxic effect was similar between AUR and MCR. The differential cytotoxic effect did not depend on cell densities of each cell line at the time of the antibiotic-treatment. Alcohol-extracted chromophores of the antibiotics also showed a similar differential cytotoxic effect, while the apo-proteins of the antibiotics showed no cytotoxicity. It is concluded that the differential cytotoxic effect of AUR and MCR to normal, transformed and carcinoma cells is attributed to the chromophore moiety and the protein moiety is not involved in this effect.

Differential cytotoxicity toward normal and transformed or cancerous cells in culture may be useful to evaluate and screen antitumor agents. For this purpose, the cell lines should be derived from the same tissues and histological types, and have similar growth characteristics in culture. Normal cells may be characterized by the contact-inhibition of cell growth. Diploidy and limited life-span may fit rigorous criteria for normal cells.[1] Malignant cells are characterized by tumorigenicity,[2] though transformed cells do not necessarily induce tumors in recipient animals.[3] Several studies indicate that transformed or cancerous cells are more sensitive than corresponding normal cells to antitumor agents or other compounds,[4~8] while some other antitumor agents are more cytotoxic to normal cells than to corresponding transformed cells.[9]

Auromomycin (AUR) and macromomycin (MCR), protein antitumor antibiotics isolated from *Streptomyces macromomyceticus*,[10,11] are composed of polypeptides of similar amino acid compositions (molecular weight of 11,700) and non-protein chromophores.[11,12] AUR is converted to MCR on an Amberlite XAD-7 column by releasing the chromophore component.[11] Both the antibiotics induce cellular DNA strand scission as the primary action,[13~16] which is attributed to the chromophore moieties.[14,17~19] We have found that the chromophores of MCR are tightly bound to the protein moiety, while most of the chromophores of AUR are loosely bound.[20]

We have studied the cytotoxic effect of AUR and MCR using normal diploid, SV40-transformed and cancerous cells of human lung origin. In this paper, we report that both the antibiotics exhibit a

* This work was in part presented at the 102nd Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April 3, 1982.

Abbreviations: AUR, auromomycin; MCR, macromomycin; chr, chromophore; apo, apo-protein; D_{10}, 10% survival dose; PSS, physiological salt solution.

greater cytocidal activity against cancerous and transformed cells than normal cells, and that this differential effect is attributed to the chromophores of the antibiotics.

Materials and Methods

Auromomycin and Macromomycin

The antibiotics were generously provided by Kanegafuchi Chem. Ind. Co., Ltd., Takasago, Hyogo, Japan.

Cell Culture

Three monolayer cultured human lung cell lines were used: Normal cell WI38 is a diploid fibroblast originating from embryonic lung tissue[1]; VA13 is a SV40-transformed derivative of WI38 cell[21]; A549 is a malignant squamous epithelioid cell derived from pulmonary carcinoma tissue.[2] The three cell lines were maintained in Eagle minimum essential medium supplemented with 10% fetal calf serum (Flow Laboratories) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Medium I) in a humidified atmosphere of 5% CO_2 in air at 37°C. WI38 cells were used at population doubling levels of 25~32 where the plating efficiency did not change. A549 cells exhibit tumorigenicity in antithymocyte serum-treated mice.[2] The doubling times of WI38, VA13 and A549 cells were 22, 23 and 20 hours, respectively.

Determination of Cytotoxicity of AUR, MCR and Their Derivatives

The cytotoxic effect of the antibiotics was determined by the direct colony formation assay, where trypsinization of drug-treated cells for replating was avoided: Single-cell suspensions were prepared by treating the monolayer cultured cells in the logarithmic phase with 0.25% trypsin (Difco Lab.). The trypsinized cells were seeded on 60 mm-diameter plastic dishes containing 5 ml of Medium I at various cell densities of $4.9\times10^{0}\sim2.6\times10^{2}$, $1.5\times10^{0}\sim1.2\times10^{3}$ and $1.2\times10^{0}\sim1.3\times10^{4}$ cm^{-2} for WI38, VA13 and A549 cells, respectively. The dishes were incubated for 42 hours to allow the cells to attach, spread but not multiply. The medium was then substituted with 5 ml of fresh Medium I. In the dark, AUR and MCR in 10~100 μl of ice-cold PSS were added to the dishes. After incubation at 37°C for 2 hours, the drug-containing medium was aspirated. The cells were rinsed twice with 5 ml of Medium I and then developed for 12 days in fresh Medium I supplemented with 10% newborn calf serum (Flow Lab.). The colonies formed were fixed with 10% formalin and stained with 0.1% crystal violet. The plating efficiencies of WI38, VA13 and A549 cells were 11~19%, 43~78% and 65~93%, respectively.

Extraction of Chromophores from AUR and MCR

Lyophilized AUR and MCR (1 mg/ml) were suspended in methanol and ethanol precooled at −20°C, respectively. The suspensions were vigorously agitated with a vortex mixer for 5 minutes and left at −20°C for 15 minutes. This procedure was repeated three times in 1 hour. Then the suspensions were left for 2 hours at −20°C and centrifuged at 3,000 rpm for 30 minutes at 4°C. The upper three quarters of the supernatant were gently collected and used as the chromophore fractions (chr-AUR and chr-MCR). The sediments were washed twice with the alcohols, dissolved in PSS and used as the apo-protein fractions (apo-AUR and apo-MCR). The chromophore extraction was carried out in the dark and the extracts were used immediately. Amounts of the chromophores and apo-proteins were expressed as equivalent weights of intact AUR and MCR before the extraction.

UV Absorption Spectra

The spectra were read on a Hitachi 124 double beam-spectrophotometer and corrected for light scattering.[22] The molar absorption coefficients were calculated on the basis of the molecular weight of 12,500 for both AUR and MCR.[11]

Results

Differential Cytotoxicity of AUR and MCR to Normal, Transformed and Carcinoma Cells

The cytotoxic effect of AUR and MCR was greatest for carcinoma A549 cells (Fig. 1). The normal

Fig. 1. Cytotoxicity of AUR and MCR toward normal WI38, SV40-transformed VA13 and carcinoma A549 cells.

The cells were treated with the antibiotics at 37°C for 2 hours in the dark. The symbols represent the averages of four dishes in the experiments that were repeated six times. The bars represent the standard deviations.

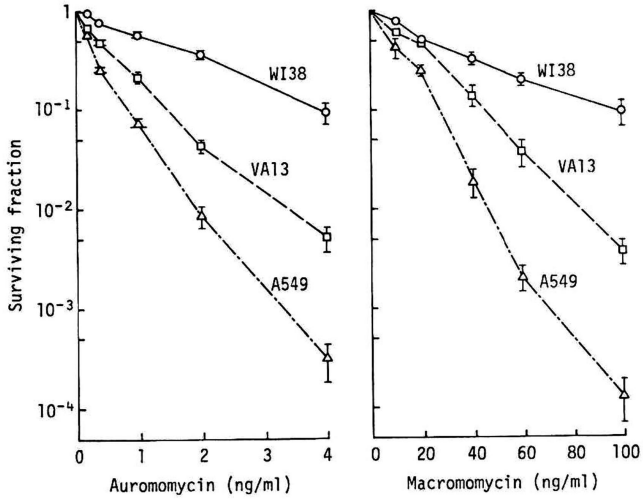
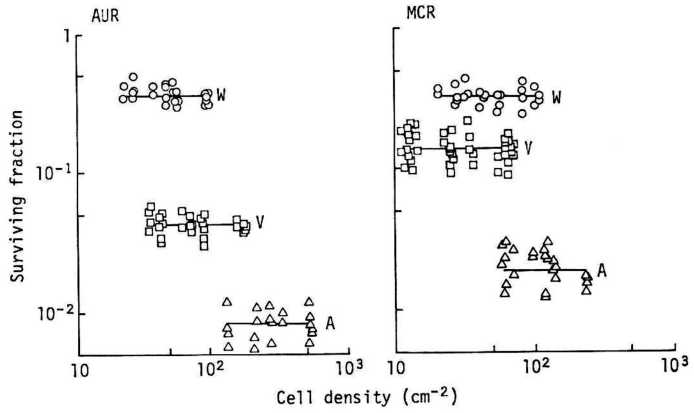


Fig. 2. Relationship between cell densities and cytotoxic effects of AUR and MCR.

The surviving fractions of WI38, VA13 and A549 cells treated with 2 ng AUR/ml and 40 ng MCR/ml for 2 hours were plotted against the cell densities used in each experiment. The bars represent the averages of surviving fractions in each cell line.

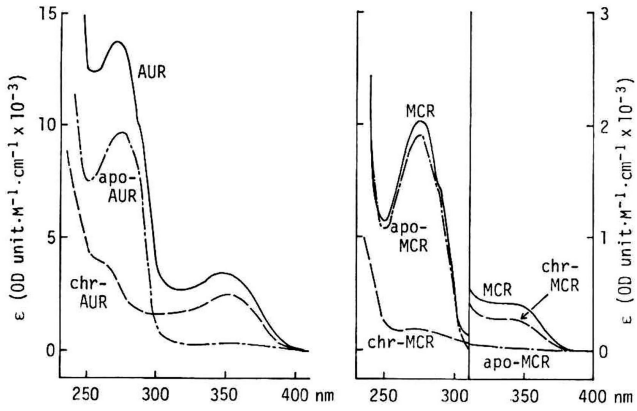


diploid WI38 cells were the least sensitive and the SV40-transformed VA13 cells were intermediately sensitive. A differential cytotoxic effect of AUR (2 ng/ml) and MCR (40 ng/ml) was observed at similar cell densities ($1.0 \sim 1.3 \times 10^2$ cm^{-2} and $0.6 \sim 0.7 \times 10^2$ cm^{-2}, respectively) of the three cell lines (Fig. 2). Furthermore, the cytotoxic effect of AUR and MCR did not depend on cell densities of each cell line at the time of treatment with the antibiotics.

UV Spectra and Purity of Chromophores and Apo-proteins of AUR and MCR

AUR and MCR were treated with alcohols to separate the chromophores and apo-proteins. Intact AUR and its chromophore had absorption maxima at 347 and 352 nm, respectively (Fig. 3). Intact MCR also showed a small maximum at 345 nm, and its molecular absorption coefficient was as low as

Fig. 3. UV absorption spectra of AUR, MCR, their chromophores and apo-proteins.



12% of that of AUR. The chromophore of MCR exhibited a shoulder-like absorption around 340 nm. The yield of the extracted chromophores could not be estimated from the absorption at this wavelength, since the absorbance of the intact antibiotics showed a bathochromic effect.[20] The chromophore fractions did not contain any proteins detectable by the method of LOWRY *et al.*[23] and all the proteins were recovered in the apo-protein fraction obtained by this alcohol extraction as previously reported by us[20] and others.[24] Moreover, the IR spectra of the chromophore fractions at amide I and II frequencies showed that they contained no peptides of the protein moiety.[20]

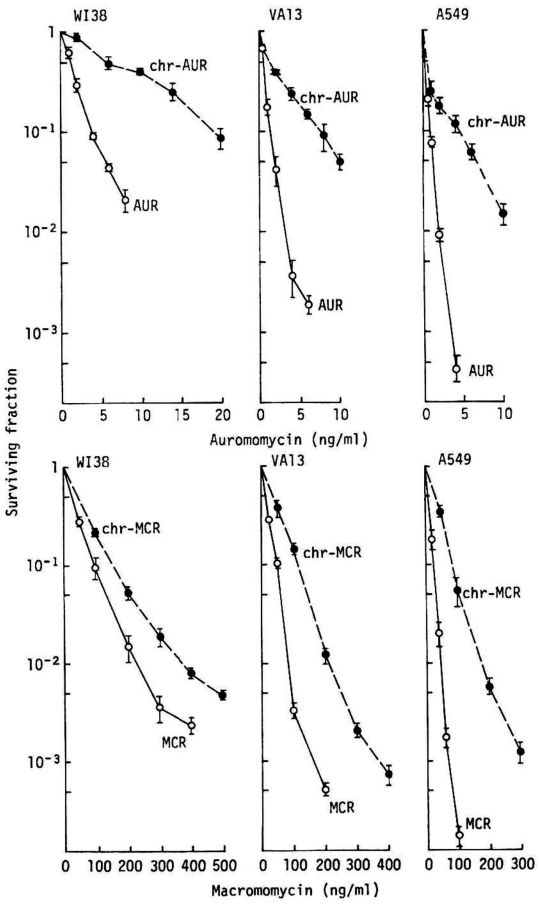
The apo-proteins of AUR and MCR had no absorption above 320 nm attributed to the chromophores (Fig. 3). AUR, MCR and their chromophores showed CD bands at the positive extrema of 371~392 nm, while their apo-proteins exhibited no CD above 320 nm.[20] These results indicated that the chromophores and apo-proteins were purely isolated from the intact antibiotics.

Differential Cytotoxicity of Chromophores of AUR and MCR

The chromophores of both the antibiotics also showed a differential effect on the three cell lines, though the cytotoxic effect was smaller than that of the intact antibiotics (Fig. 4). The reduction in cytotoxicity appeared to be more marked with the chromophore of AUR than with that of MCR. The apo-protein fractions showed

Fig. 4. Cytotoxicity of the chromophores of AUR and MCR.

Each point is the average of four dishes in the experiments that were repeated twice.



no cytotoxicity even at 100 times higher concentrations than the maximal doses of the antibiotics (data not shown). The alcohols contained in the chromophore fractions were not cytotoxic at the concentrations used (data not shown).

Discussion

The cytotoxic effect of AUR and MCR is greater for carcinoma or transformed cells than for normal diploid cells (Fig. 1). The doubling times of the three cell lines were similar. This differential cytotoxic effect is due to neither difference in cell densities at the time of antibiotic-treatment nor difference in the plating efficiencies of the three cell lines (Fig. 2).

This differential effect may be due to a greater plasma membrane permeability in the carcinoma and transformed cells to the antibiotics and/or due to a greater accessibility of the antibiotics to the target nuclear DNA.[13~16] Moreover, the differential effect may be attributed to a greater activation and/or lower inactivation of the antibiotics by intracellular cofactors in the more sensitive cells. This is inferred on the basis of the facts that the scission activity of AUR and MCR toward isolated DNA is potentiated by reductants such as $NaBH_4$ and dithiothreitol,[13,18,19] and inhibited by radical scavengers such as 2-propanol and α-tocopherol.[25]

The cytotoxic effect of the chromophores of AUR and MCR was smaller than that of the intact antibiotics (Fig. 4). This is not due to incomplete extraction of the chromophores with alcohols because the apo-proteins do not show cytotoxicity, UV absorption (Fig. 3) and CD[20] attributed to the chromophores. The decreased cytotoxic effect is due to the rapid inactivation of the free chromophores in the serum-containing medium (unpublished results).

The chromophores of AUR and MCR showed a similar differentiality in the cytotoxic effect when compared with that of the intact antibiotics with respect to the ratios of D_{10} for WI38 cells to D_{10} for VA13 or A549 cells (Table 1). The results indicate that the differential effect is solely attributed to the chromophore moieties of the antibiotics, and the protein moieties are not involved.

Table 1. Ten percent survival doses of AUR, MCR and their chromophores to three human lung cell lines by colony formation assay.

	D_{10} (ng/ml)			
	AUR	chr-AUR	MCR	chr-MCR
WI38	3.9	19.3	99	154
VA13	1.4	7.6	51	110
A549	0.9	4.5	25	84

The D_{10} values were obtained from Fig. 4.

Acknowledgments

We wish to express our thanks to Drs. KIYOSHI WATANABE and NORIYUKI NAOI, Kanegafuchi Chem. Ind. Co., Ltd., for generously supplying auromomycin and macromomycin and letting us read their manuscripts before publication. This work was partly supported by Special Coordinating Funds for Promoting Science and Technology from Science and Technology Agency, Japan.

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