Cancer Chemother Pharmacol (1994) 33: 371–377

Cancer Chemotherapy and Pharmacology

Laser flow cytometric studies on the intracellular accumulation of anthracyclines when combined with heat

Y. Sakaguchi[1], Y. Maehara[2], S. Inutsuka[1], I. Takahashi[1], M. Yoshida[1], Y. Emi[1], H. Baba[1], K. Sugimachi[1, 2]

[1] Cancer Center of Kyushu University Hospital, Kyushu University, Fukuoka, Japan
[2] Department of Surgery II, Faculty of Medicine, Kyushu University, Fukuoka, Japan

Received: 3 December 1992/Accepted 17 September 1993

Abstract. The effects of heat on intracellular accumulation of anthracyclines were investigated by laser flow cytometry analysis. Sarcoma-180 cells were exposed to Adriamycin (ADM), epirubicin (EPIR), daunomycin (DM), THP-Adriamycin (THP), ME-2303 (ME) and KRN-8602 (KRN) at 37° C and at higher temperatures. There was a dose-dependent increase in the fluorescence intensity of all drugs at 37° C, but heat varied the fluorescence intensity of each drug. At 43° C the cellular fluorescence of ADM and EPIR increased by approximately 200%, but for DM the increase was 110–130%. The cellular fluorescence of THP and ME was little affected by heat, and heat reduced that of KRN to 80–90%. Each drug showed was unique in the relationship between drug exposure time and the fluorescence intensity at 37° C and 43° C. Cytotoxicity determined by the MTT assay was enhanced with heat in the cases of ADM and EPIR, but not with DM, THP, ME, or KRN. Thus, ADM and EPIR are expected to show enhanced antitumor activities when given in combination with hyperthermia.

Introduction

Adriamycin (ADM), an anthracycline antibiotic, is one of the most effective drugs against a broad spectrum of tumors. ADM exerts cytotoxicity by various actions, which include intercalation into DNA, production of free radicals, binding to specific enzymes, e.g., topoisomerase II, and interaction with the cell membrane [1, 19, 29, 30]. As there is a direct relationship between the antitumor effects of ADM and the intracellular drug level, the potency of ADM can be predicted by measuring intracellular accumulation [10]. ADM has a characteristic fluorescence spectrum, and the intracellular accumulation of ADM in a cell population can be measured rapidly and easily by flow cytometry [11, 26]. A good correlation was found between the cytotoxic effect of ADM and mean cellular fluorescence [5]. New anthracyclines with a greater antitumor activity and lesser toxicity to normal tissues have been developed, and as most are also fluorescent, flow cytometry is useful for rapid detection and quantitation of fluorescence of these agents [12, 13].

Hyperthermia enhances the cytotoxicity of various antitumor drugs, including ADM. Hahn et al. [8, 9] reported that the combination of heat and ADM had a synergistic cytotoxicity against mouse mammary tumor cells, because a larger amount of ADM entered the cells at 43° C than at 37° C. Enhancement of the cytotoxicity of ADM by heat was the result of an increase in the intracellular accumulation of the drug [18, 23].

We report here findings of intracellular accumulation of six anthracyclines, with or without simultaneous heat exposure. The objective of this study was to compare the characteristics of these drugs and, by means of flow cytometry, to estimate the effects of heat on their cytotoxicity.

Materials and methods

Drugs. Structures of the six anthracyclines examined are shown in Fig. 1. Adriamycin (doxorubicin, ADM) and epirubicin (4′-epidoxorubicin, EPIR) were obtained from Kyowa Hakko Kogyo Co., Ltd. (Japan), daunomycin (daunorbicin, DM), THP-ADM (4′-*O*-tetrahydropyranyladriamycin, THP) [14, 32] and ME-2303 (14-hemipimelate-7-*O*-(2,6-dideoxy-2-fluoro-α-L-talopyranocyl) adriamycinone, ME) [31, 33] were obtained from Meiji Seika Kaisha (Japan), and KRN-8602 (3′-deamino-3′-morpholino-13-deoxy-10-hydroxycarminomycin, KRN) [34, 35] was obtained from Kirin Brewery Co. (Japan). Stock solutions of these drugs were prepared in Hanks' balanced salt solution. Fresh dilutions of the drugs were prepared in distilled water just before each experiment.

Tumor cells. Cells were grown in ddY mice that had been inoculated i.p. 7 days before with 1×10^6 sarcoma-180 ascites tumor cells resuspended in 0.5 ml of phosphate-buffered saline (PBS). The tumor cells were excised from the mice just before start of the experiments.

Correspondence to: Y. Maehara, Department of Surgery II, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

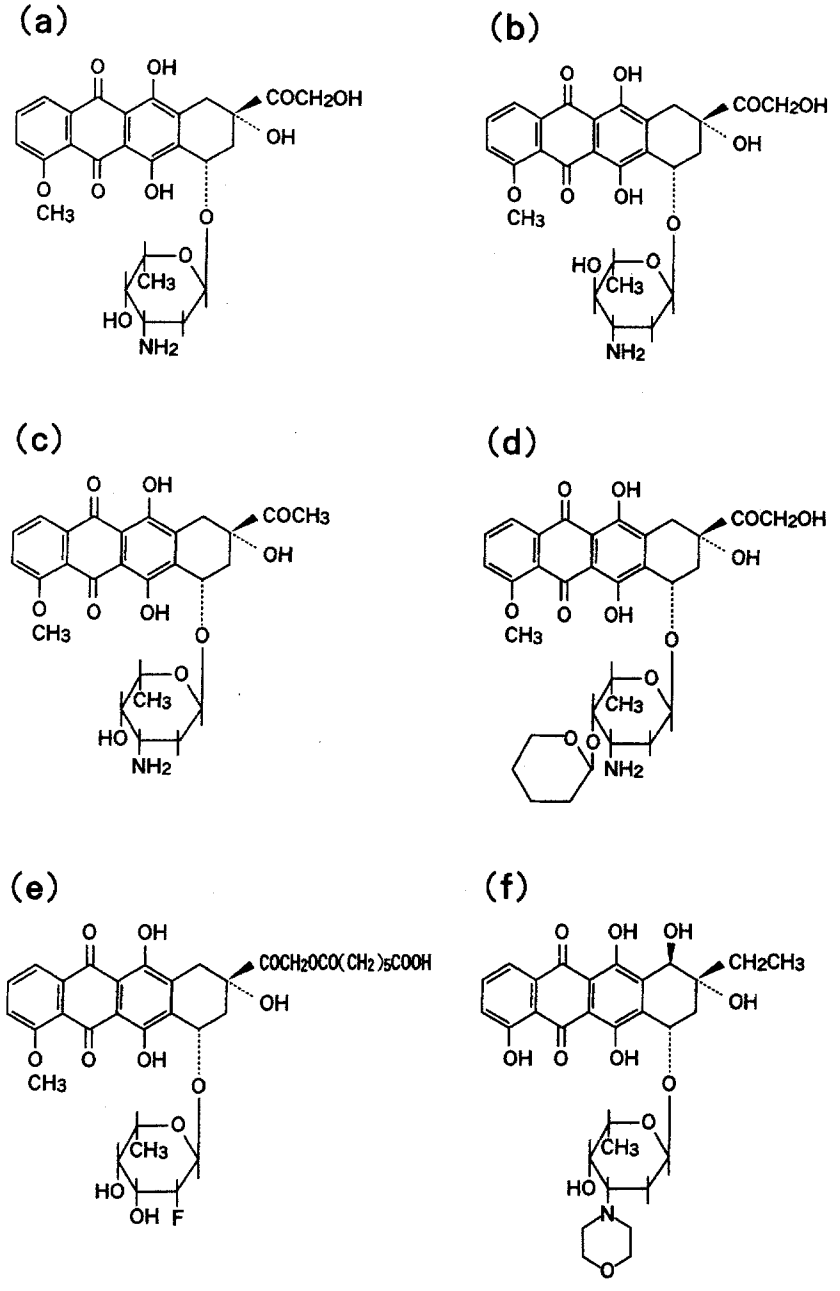

Fig. 1a–f. Structures of anthracyclines. **a** Adriamycin (ADM); **b** epirubicin (EPIR); **c** daunomycin (DM); **d** 4′-*O*-tetrahydropyranyl-ADM (THP); **e** ME-2303 (ME); **f** KRN-8602 (KRN)

The cells were retrieved by centrifugation and resuspended in PBS three times to give a final concentration of 2.5×10^5 cells/ml in Eagle's minimal essential medium (Nissui Pharmaceutical Co., Japan).

Measurement of cellular drug fluorescence. The cells were incubated with the drugs at the temperatures indicated in a model 7100 incubator (Napco Scientific Co., USA). The concentrations of the drugs used were 0.5–2.5 μM, and the duration of drug exposure ranged between 15 and 180 min.

Following drug exposure, the cells were washed twice with PBS, resuspended in PBS to a final concentration of $1-2\times10^5$ cells/ml and used immediately for experiments with the fluorescence-activated sorter. Cell size and cellular fluorescence intensity were determined with a FACScan/consort 30 (Becton Dickinson Co., USA). In each experiment, 1×10^4 cells were analyzed at a flow rate of 200–400 cells/s. To measure the fluorescence intensity, drug-exposed cells were analyzed with excitation at 488 nm (1 W power) with emission integrated above 530 nm, and were selected by gating the scattered light from the cell suspension. The data obtained were displayed in the form of a histogram of cell number versus fluorescence intensity. The mean cellular fluorescence was determined by calculating the mean of the fluorescence distribution and subtracting the mean spontaneous fluorescence of an untreated cell. Because the fluorescence intensity measured by flow cytometry is relative, the data in this report are shown as the fluorescence intensities relative to the fluorescence intensity of a cell treated with 1 μM ADM for 60 min. Each experiment was done at least in triplicate.

MTT assay. Cytotoxicity was determined by the MTT assay as described elsewhere [16, 17, 24]: 5×10^5 sarcoma-180 cells were incubated for 3 days during exposure to 1 μM of each drug at 37°C in a humidified atmosphere containing 5% CO_2. When the drug under scrutiny was combined with heat, the cells were maintained at 43°C for the first 60 min of incubation. The cells were then assayed for succinate dehydrogenase (SD) activity as a predictor of cell viability. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was used as the hydrogen acceptor for the SD activity [2, 3]. The formazan formed from MTT was extracted with acetone

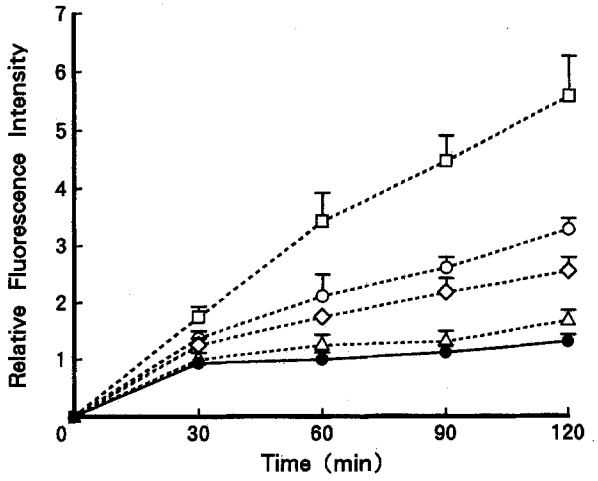

Fig. 2. Temperature dependence of cellular fluorescence of ADM. Sarcoma-180 cells were exposed to 1 μM ADM for 30–120 min at 37°C (●), 39°C (△), 41°C (◇), 43°C (○) and 45°C (□)

containing 0.5% trichloroacetic acid, and the absorption of formazan was measured at 565 nm. The SD activity was presented as the optical density. The cytotoxicity was evaluated by expressing the SD activity in the treated cells as a percentage of that in control cells. This assay was done in triplicate.

Statistical analysis. Significant differences between means were determined by Student's *t*-test. A *P*-value lower than 0.05 was considered significant.

Results

Cell size was clearly altered by heat. The fraction of cells with a larger volume was increased over that in the controls kept at 37°C. Exposure of the cells to 43°C for 60 min increased the mean volume of the treated cells by 115±6.4% (mean ± standard deviation) of that of untreated cells. Continued incubation up to a total of 180 min at 43°C did not further alter the cell size.

Figure 2 shows the temperature dependence of cellular ADM fluorescence in sarcoma-180 cells incubated with 1 μM of ADM for the times indicated at temperatures ran-

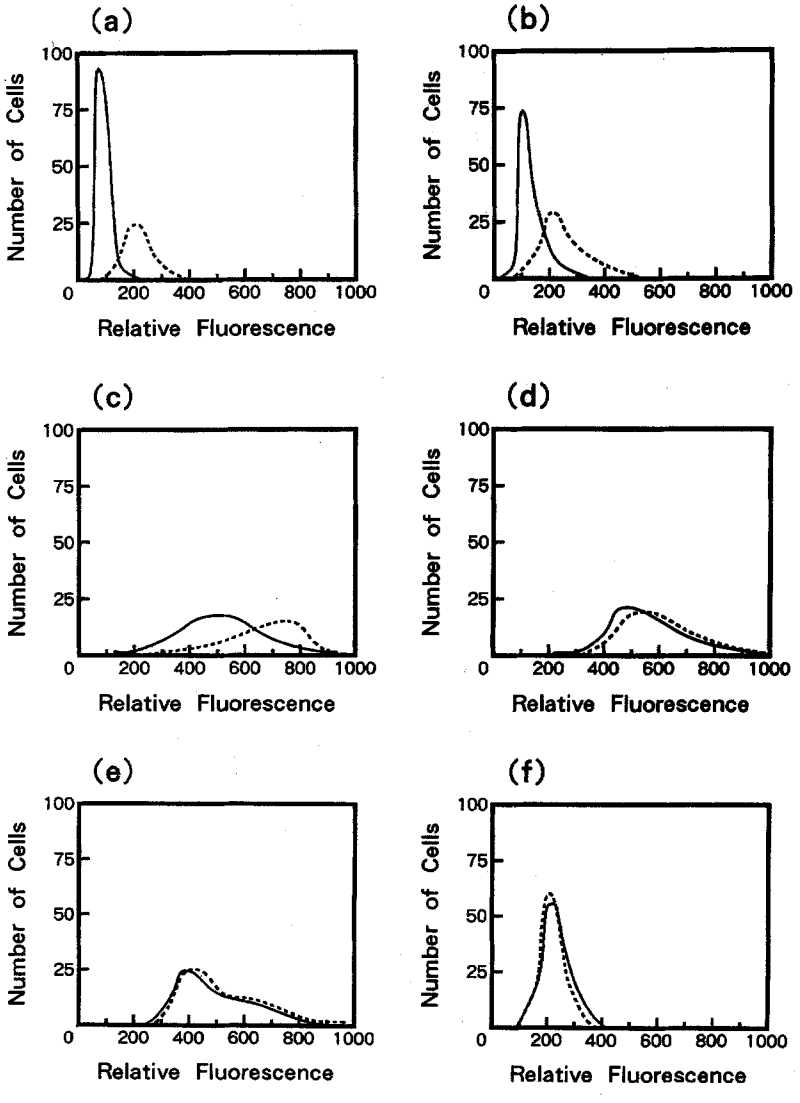

Fig. 3a–f. Distributions of fluorescence intensity of sarcoma-180 cells incubated with six anthracyclines. The cells were exposed to 1 μM of each drug for 60 min at 37°C (——) or 43°C (– – –). **a** ADM; **b** EPIR; **c** DM; **d** THP; **e** ME; **f** KRN

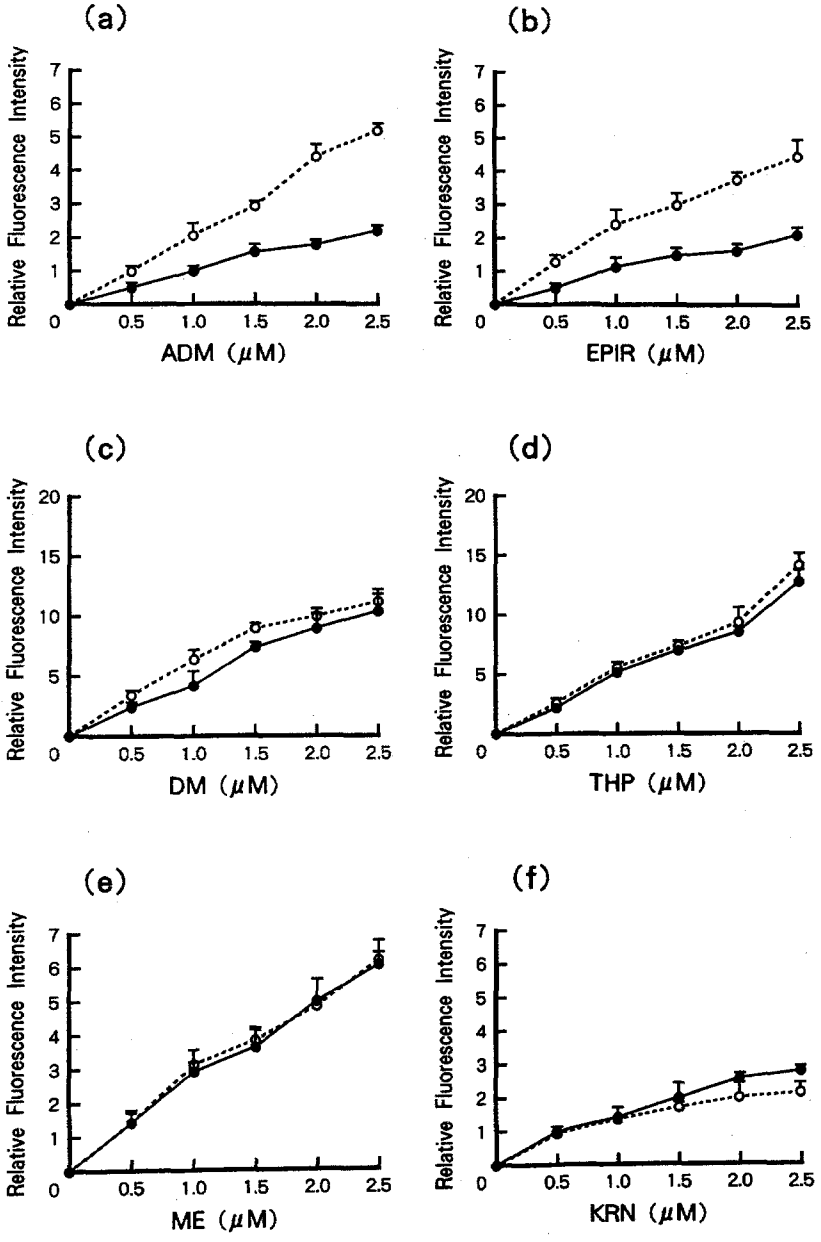

Fig. 4a–f. Relationship between drug concentration and mean fluorescence intensity of sarcoma-180 cells. The cells were exposed to the drugs at the concentrations indicated for 60 min at 37°C (●) or 43°C (○). **a** ADM; **b** EPIR; **c** DM; **d** THP; **e** ME; **f** KRN

ging from 37°C to 45°C. Fluorescence intensity increased in a temperature-dependent manner and did not reach a steady state during the study period. For the 120-min incubation, the cellular fluorescence of ADM at 45°C was 4.4 times greater that at 37°C, that at 43°C 2.6 times, that at 41°C, 2.0 times, greater and that at 39°C, 1.3 times the cellular ADM fluorescence at 37°C.

Differences in patterns of cellular fluorescence of the six anthracyclines were also investigated. Figure 3 shows the distribution of fluorescence intensity of sarcoma-180 cells exposed to 1 μM concentrations of the drugs for 60 min at 37°C or 43°C. ADM and EPIR had similar profiles of distribution at both 37°C and 43°C. The peaks of fluorescence shifted to the right and the mean fluorescence intensity was increased in the presence of heat. DM, THP, and ME had similar profiles of fluorescence distribution at 37°C, but fluorescence was greater and scatter was wider than with ADM or EPIR. The effects of heat on cellular fluorescence differed with these three drugs. Heat shifted the peak of fluorescence of DM to the right, but the change in distribution of THP and ME was slight. In contrast, KRN had a unique profile: the peak was shifted to the left by heat, with a decrease in fluorescence intensity.

The relationship between drug concentration and fluorescence intensity is shown in Fig. 4. Cellular fluorescence of each drug was measured after a 60-min incubation with 0.5–2.5 μM of each drug at 37°C or 43°C. For all six drugs, approximately linear relationships were obtained between the drug concentration and the fluorescence intensity at 37°C. Heat increased the cellular fluorescence of ADM and EPIR in a similar manner, by approximately 200% at temperatures between 37°C and 43°C. The fluorescence of DM was also increased by heat, but the difference was only 110–130% between 37°C and 43°C. With THP or ME, cellular fluorescence was little affected by heat. In contrast, heat reduced the cellular fluorescence of KRN to 80–90%.

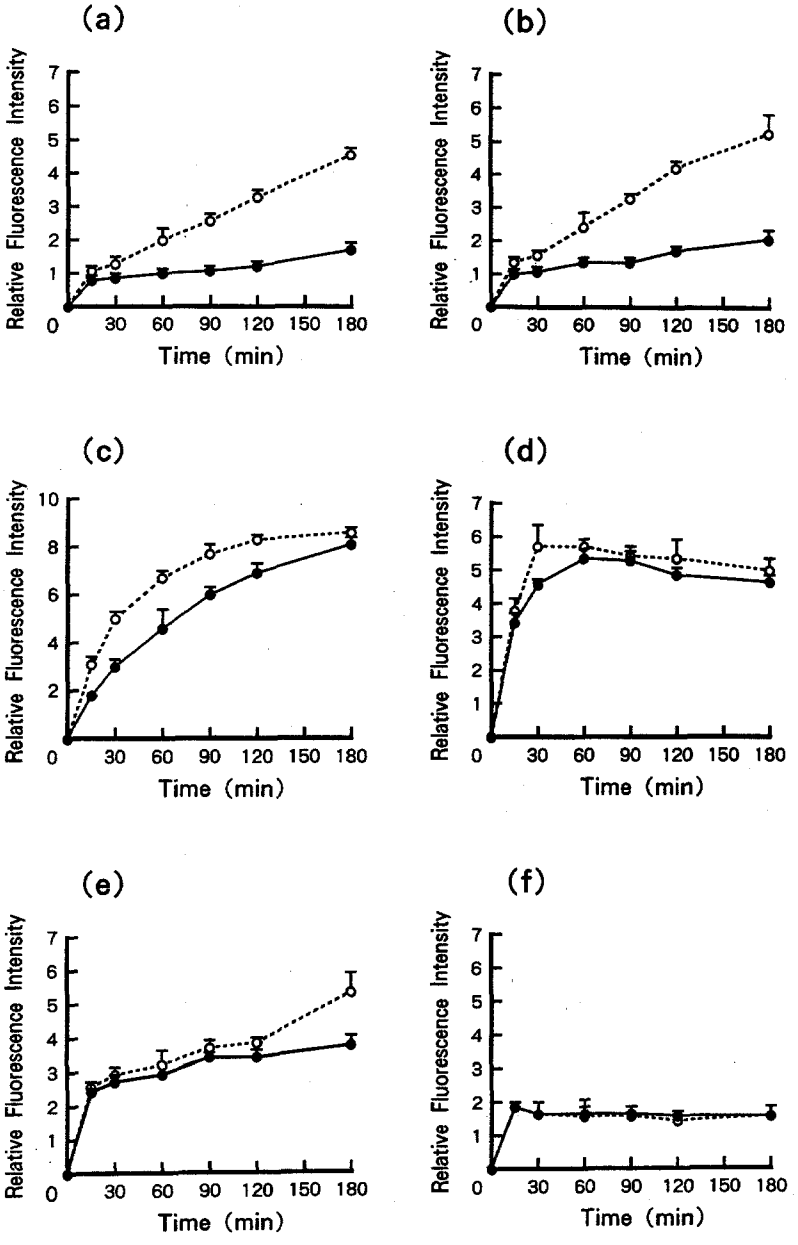

Fig. 5 a–f. Relationship between drug exposure time and mean fluorescence intensity of sarcoma-180 cells. The cells were exposed to 1 μM of each drug for the times indicated at 37°C (●) or 43°C (○). **a** ADM; **b** EPIR; **c** DM; **d** THP; **e** ME; **f** KRN

Additional experiments revealed relationships between drug exposure time and fluorescence intensity (Fig. 5). When cells were incubated with 1 μM of each drug for 15–180 min at 37°C or 43°C, the fluorescence of ADM increased rapidly for the first 15 min, and then slowly up to 180 min at 37°C. When the cells were incubated at 43°C, the fluorescence of ADM continued to increase for up to 180 min of exposure and at a higher rate than the fluorescence at 37°C. After 180 min, the fluorescence of ADM was 2.7 times greater at 43°C than at 37°C. EPIR showed similar characteristics. The cellular fluorescence of EPIR was increased 2.5 times by heat with a 180-min incubation. The fluorescence of DM continued to increase for up to 180 min at 37°C, but the rate decreased gradually. The effect of heat on the increase in the fluorescence of DM declined with continuation of the incubation time. The cellular fluorescence of THP increased rapidly with a peak at 60 min after exposure at 37°C and at 30 min at 43°C.

Table 1. Comparison of SD activities of sarcoma-180 cells following exposure to anthracyclines

Drug	SD activity (%)		*P*-value
	Heat (–)	Heat* (+)	
Adriamycin	60.8±6.6**	40.3±5.4	<0.05
Epirubicin	54.9±7.2	31.1±4.2	<0.05
Daunomycin	29.6±6.8	22.8±7.2	NS
THP[a]-Adriamycin	15.6±4.6	12.3±5.2	NS
ME-2303	16.3±5.3	13.7±4.2	NS
KRN-8602	21.5±4.5	17.4±5.8	NS

NS, not significantly different
* 43°C, 60 min
** Mean ± standard deviation

Fluorescence then decreased slowly during the steady state after passing the peak. Characteristics of ME at 37° C were similar to those of ADM. Although the effect of heat was slight with a 60-min incubation, the effects were obvious with a 180-min incubation. The cellular fluorescence of KRN reached a steady state within the first 15 min, which persisted throughout the study period at both 37° C and 43° C. Heat had no apparent effect on the accumulation of KRN.

The SD activity of sarcoma-180 cells exposed to the drugs was determined using the MTT assay, and the results are shown in Table 1, Exposure to 43° C for 60 min reduced the SD activity to 90% of that at 37° C. Decrease in SD activity by heat was obvious in cells exposed to ADM and EPIR ($P < 0.05$). Heat had little effect on cytotoxicities of DM, THP, ME, and KRN.

Discussion

Intracellular accumulation of anthracyclines involves a complex process: influx, distribution, binding to intracellular components and membrane, redistribution and efflux. Most of the drug is bound to intracellular organelles, mainly to the nucleus which has a strong binding site [7, 28]. The problem in measuring the intracellular fluorescence of anthracyclines is that quenching of drug fluorescence occurs when drugs are intercalated between the base pairs of DNA [6]. No quenching occurs when drugs are bound to intracellular components, including the nucleus. Drug fluorescence is quenched only by intercalation into DNA. Total cellular fluorescence thus indicates the level of anthracycline molecules that are not intercalated between the base pairs of DNA. Therefore, total cellular fluorescence does not actually reflect intracellular drug content [28]. The total cellular fluorescence has, however, been shown to correlate well with cellular toxicity and DNA damage caused by anthracyclines [5]. Thus, the cytotoxicity of anthracyclines can be estimated by quantitating the levels of drugs that can be measured as cellular fluorescence.

The efflux of anthracyclines is apparently due to active transport requiring energy [10]. This is a major cellular mechanism of resistance to these drugs. The influx of these agents takes place by passive diffusion, which is influenced by permeability of the cell membrane [22]. Uptake to intracellular components is also passive [28]. Since binding to the nucleus and intercalation into DNA are rapid, transport across the cell membrane is the rate-limiting step on the overall kinetics [7, 28]. Membrane permeability is important for anthracyclines to exert their activities. Therefore, if the cellular drug efflux does not change, an increase in the rate of drug influx will lead to a greater intracellular accumulation of the drugs and to enhancement of the cytotoxicity.

The passive diffusion of ADM is temperature-dependent [15, 21]. Heat seems to alter the membrane permeability to ADM and to increase the rate of ADM influx. Since heat does not affect the efflux of ADM [18, 25], the cytotoxicity of ADM is enhanced by heat as a result of high intracellular drug accumulation. Rice and Hahn [23] reported a good correlation between intracellular accumulation of ADM and cytotoxic effects when ADM was combined with heat. EPIR had a profile similar to that of ADM regarding cellular fluorescence, and heat increased the intracellular accumulation of EPIR in a dose- and time-dependent manner. The membrane permeability to EPIR seems to be increased by heat and enhancement of the cytotoxicity of EPIR by heat is of interest. Dahl [4] described the enhanced antitumor effect of EPIR combined with hyperthermia in vivo.

We found that with an incubation temperature of 43° C the cell volume increased by 115%, the result being a relative decrease in the intracellular concentration of drug. In the case of ADM and EPIR, however, the increase in content by heat was higher than that of the cell volume. We also observed an enhanced cytotoxicity of ADM and EPIR when these agents were combined with heat.

On the other hand, thermal enhancement of the cytotoxicity of DM was reported to be less than that of ADM [20], a result consistent with the findings in our present study. Drug accumulation might explain why the thermal enhancement is slight. It may be that heat does not increase membrane permeability to DM because cellular uptake of DM is rapid even at 37° C. THP is also rapidly taken up by cells, compared with ADM or EPIR. We observed that THP had a peak of cellular fluorescence 60 min after the exposure and that the peak appeared earlier with heat; however, there was little increase in the fluorescence of THP. Kunimoto et al. [14] suggested that different mechanisms might be involved in the transport of THP and ADM.

ME is a fluorine-containing anthracycline [31, 33], and KRN is a morpholino anthracycline [34, 35]. The manner of transport of these new anthracyclines into cells is not fully understood. We found that the uptake of these drugs was rapid at 37° C and that heat increased the fluorescence of ME only in the case of a long exposure. Heat slightly decreased the fluorescence of KRN. Although the efflux of ADM does not seem to be affected by heat [18, 25] and Tarasiuk and Garnier-Suillerot [27] reported that the binding of ADM to the nucleus is an exothermic process, our data do suggest that KRN may be effluxed by a heat-sensitive mechanism or that heat may increase the intercalation of KRN into DNA resulting in the quenching of fluorescence.

It is known that not all anthracyclines have enhanced effects when exposed to heat [20]. Based on our studies, of the six anthracyclines we tested only ADM and EPIR can be expected to have enhanced antitumor activities when combined with hyperthermia.

Acknowledgement. We thank M. Ohara for editorial assistance.

References

1. Bachur NR, Gordon S, Gee MV (1978) A general mechanism for microsomal activation of quinone anticancer agents to free radicals. Cancer Res 38: 1745
2. Carmichael J, Degraff WG, Gazdar AF, Minna JD, Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay. Assessment of chemosensitivity testing. Cancer Res 47: 936

3. Cole SPC (1986) Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. Cancer Chemother Pharmacol 17: 259
4. Dahl O (1983) Hyperthermic potentiation of doxorubicin and 4′-epi-doxorubicin in a transplantable neurogenic rat tumor (BT_4A) in BD IX rats. Int J Radiat Oncol Biol Phys 9: 203
5. Durand RE, Olive PL (1981) Flow cytometry studies of intracellular adriamycin in single cells in vitro. Cancer Res 41: 3489
6. Egorin MJ, Clawson RE, Ross LA, Bachur NR (1974) Cytofluorescence localization of Adriamycin and daunorubicin. Cancer Res 34: 2243
7. Frezard F, Garnier-Suillerot A (1991) DNA-containing liposomes as a model of the study of cell membrane permeation by anthracycline derivatives. Biochemistry 30: 5038
8. Hahn GM, Strande DP (1976) Cytotoxic effects of hyperthermia and Adriamycin on Chinese hamster cells. J Natl Cancer Inst 57: 1063
9. Hahn GM, Braun J, Har-Kedar I (1975) Thermochemotherapy: synergism between hyperthermia (42–43°) and Adriamycin (or bleomycin) in mammalian cell inactivation. Proc Natl Acad Sci USA 72: 937
10. Inaba M, Johnson RK (1978) Uptake and retention of Adriamycin and daunorubicin by sensitive and anthracycline-resistant sublines of P388 leukemia. Biochem Pharmacol 27: 2123
11. Krishan A, Ganapathi R (1979) Laser flow cytometry and cancer chemotherapy: Detection of intracellular anthracyclines by flow cytometry. J Histochem Cytochem 27: 1655
12. Krishan A, Ganapathi R (1980) Laser flow cytometric studies on the intracellular fluorescence of anthracyclines. Cancer Res 40: 3895
13. Krishan A, Sauerteig A, Gordon K, Swinkin C (1986) Flow cytometric monitoring of cellular anthracycline accumulation in murine leukemia cells. Cancer Res 46: 1768
14. Kunimoto S, Miura K, Takahashi Y, Takeuchi T, Umezawa H (1983) Rapid uptake by cultured tumor cells and intracellular behavior of 4′-*O*-tetrahydropyranyladriamycin. J Antibiot (Tokyo) 36: 312
15. Lane P, Vichi P, Bain DL, Tritton TR (1987) Temperature dependence studies of adriamycin uptake and cytotoxicity. Cancer Res 47: 4038
16. Maehara Y, Anai H, Kusumoto H, Sugimachi K (1987) Poorly differentiated human gastric carcinoma is more sensitive to antitumor drugs than is well-differentiated carcinoma. Eur J Surg Oncol 13: 203
17. Maehara Y, Anai H, Kusumoto H, Kusumoto T, Sugimachi K (1988) Colorectal carcinoma in vitro is more sensitive to 1-hexylcarbamoyl-5-fluorouracil compared with six other antitumor drugs: carboquone, Adriamycin, mitomycin C, aclacinomycin A, cisplatin, 5-fluorouracil. Dis Col Rect 31: 62
18. Nagaoka S, Kawasaki S, Sasaki K, Nakanishi T (1986) Intracellular uptake and retention and cytotoxic effect of adriamycin combined with hyperthermia in vitro. Jpn J Cancer Res 77: 205
19. Neidle S (1979) The molecular basis for the action of some DNA-binding drugs. Prog Med Chem 16: 151
20. Ohnoshi T, Ohnuma T, Beranek JT, Holland JF (1985) Combined cytotoxicity effect of hyperthermia and anthracycline antibiotics on human tumor cells. J Natl Cancer Inst 74: 275
21. Osborne EJ, Mackillop WJ (1987) The effect of exposure to elevated temperatures on membrane permeability to Adriamycin in Chinese hamster ovary cells in vitro. Cancer Lett 37: 213
22. Peterson C, Trouet A (1978) Transport and storage of daunorubicin and doxorubicin in cultured fibroblasts. Cancer Res 38: 4645
23. Rice GC, Hahn GM (1987) Modulation of Adriamycin transport by hyperthermia as measured by fluorescence-activated cell sorting. Cancer Chemother Pharmacol 20: 183
24. Saito A, Korenaga D, Maehara Y, Baba H, Okamura T, Sugimachi K (1992) In vitro succinate dehydrogenase chemosensitivity of gastric carcinoma – relationship to DNA content. Cancer Chemother Pharmacol 29: 185
25. Sakaguchi Y, Maehara Y, Emi Y, Kohnoe S, Sugimachi K (1993) Adriamycin combined with hyperthermia and dipyridamole is cytotoxic both in vitro and in vivo. Eur Surg Res (in press)
26. Tapiero H, Fourcade A, Vaigot P, Farhi JJ (1982) Comparative uptake of adriamycin an daunorubicin in sensitive and resistant Friend leukemia cells measured by flow cytometry. Cytometry 2: 298
27. Tarasiuk J, Garnier-Suillerot A (1992) Thermodynamics of the anthracycline–nuclei interactions in drug-resistant and drug-sensitive K562 cells. Biochem Pharmacol 43: 2575
28. Tarasiuk J, Frezard F, Garnier-Suillerot A, Gattegno L (1989) Anthracycline incorporation in human lymphocytes. Kinetics of uptake and nuclear concentration. Biochim Biophys Acta 1013: 109
29. Tewey KM, Rowe TC, Yand L, Halligan BD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II, Science 226: 466
30. Tritton TR, Yee G (1982) The anticancer agent Adriamycin can be actively cytotoxic without entering cells. Science 217: 248
31. Tsuchiya T, Takagi Y, Umezawa S, Takeuchi T, Komoro K, Nosaka C, Umezawa H, Fukatsu S, Yoneta T (1988) Synthesis and antitumor activities of 14-*O*-acyl derivatives of 7-*O*-(2,6-dideoxy-2-fluoro-α-L-talopyranosyl)adriamycinone. J Antibiot (Tokyo) 41: 988
32. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y (1982) 4′-*O*-Tetrahydropyranyladriamycin as a potential new anticancer agent. Cancer Res 42: 1462
33. Tsuruo T, Yusa K, Sudo Y, Takamori R, Sugimoto Y (1989) A fluorine-containing anthracycline (ME2303) as a new antitumor agent against murine and human tumors and their multidrug-resistant sublines. Cancer Res 49: 5537
34. Umezawa H, Nakajima S, Kawai H, Komeshima N, Yoshimoto H, Urata T, Odagawa A, Otsuki N, Tatsuya K, Ohtake N, Takeuchi T (1987) New morpholino anthracyclines, MX, MX2, and MY5. J Antibiot (Tokyo) 40: 1058
35. Watanabe M, Komeshima N, Nakajima S, Tsuruo T (1988) MX2, a morpholino anthracycline, as a new antitumor agent against drug-sensitive and multidrug-resistant human and murine tumor cells. Cancer Res 48: 6653