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Effect of pH and moderate hyperthermia on doxorubicin, epirubicin and aclacinomycin A cytotoxicity for Chinese hamster ovary cells

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Abstract. The influence of extracellular pH on the cytotoxicity of the anthracyclines doxorubicin, epirubicin, and aclacinomycin A was examined at 37° C and 41° C in tissue culture. Chinese hamster ovary (CHO) cells were exposed for a total of 24 h to anthracyclines at doses ranging between 0.12 and 0.69 nmol/ml at pH 7.4, 6.7, and 6.4 and at 37° C. Temperature elevation to 41° C was carried out for 3 h after the initiation of the drug treatment. Doxorubicin and epirubicin were about equally cytotoxic in the pH range examined at both temperatures. Aclacinomycin A demonstrated a higher cytotoxicity at pH 7.4 and 37° C only at low doses. At low pH, however, aclacinomycin A was increasingly more effective with increasing dose as compared with doxorubicin and epirubicin. At 41° C and at higher doses aclacinomycin A was even less cytotoxic than doxorubicin or epirubicin. Doxorubicin and epirubicin were less effective at lower pH. However, aclacinomycin A at doses of greater than 0.25 nmol/ml was more cytotoxic at low pH. Moderate hyperthermia did not increase the cytotoxicity of the three drugs at low pH, except for aclacinomycin A at doses of less than 0.25 nmol/ml. At pH 7.4, aclacinomycin A was even less effective at the elevated temperature. At doses of greater than 0.25 nmol/ml, moderate hyperthermia decreased the cytotoxicity of aclacinomycin A at low pH.

Introduction

The extracellular pH in tumors tends to be lower in comparison with that in surrounding normal tissue by up to 0.5 units [26, 29]. Following hyperthermia, an additional drop in intralesional pH of up to 0.5 units has been reported in experimental tumors [10]. Although hyperthermia alone [5] or in combination with some chemotherapeutic agents [4] has been found to be more cytotoxic to cells at lower extracellular pH, the effect of hyperthermia and/or low pH on the transport and cytotoxicity of chemotherapeutic agents may be agent-specific. For instance, the cytotoxicity of the anthracyclines doxorubicin and epirubicin has been found to be reduced at subnormal pH at 37° C [1, 6]. Enhanced cytotoxicity of doxorubicin has been observed at 41°–43° C and a pH of 7.4 [2]. Furthermore, the cytotoxicity of non-anthracyclines has been increased by the combination of elevated temperature (41°–43° C) and low pH [8]. The main mechanism responsible for a decrease in doxorubicin cytotoxicity at low pH and 37° C is a decrease in drug uptake [1]. Hyperthermia at 40°–43° C enhances the doxorubicin uptake [4]. Therefore, it seems reasonable that hyperthermia might overcome the reduced cytotoxicity at lower pH. A temperature of 41° C can be readily maintained for whole-body hyperthermia. Modification of drug action for doxorubicin, epirubicin, and aclacinomycin A has not yet been examined at low pH and 41° C.

The anthracyclines as a group manifest heterogeneous drug characteristics [12, 13, 30, 31]. Doxorubicin and epirubicin interfere preferentially with DNA synthesis, whereas aclacinomycin A more markedly affects RNA synthesis [13, 31]. Since these effects are mediated at different cellular levels by different protein binding, transport factors, and enzyme activities, all of which are known to be pH- and temperature-dependent, the cytotoxicity of aclacinomycin A might differ in pH and temperature dependence in comparison with that of doxorubicin and epirubicin. There are conflicting data on the temperature dependence of aclacinomycin A cytotoxicity. It has been observed that hyperthermia of 41° C does not enhance the cell kill of malignant melanoma cells, whereas an additive effect results for Burkitt lymphoma cells [17]. However, augmented cytotoxicity at 42°/43° C has been obtained for aclacinomycin A without signs of thermotolerance [15].

The reported characteristics of anthracyclines suggest that the cytotoxicity of aclacinomycin A might not be re-

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duced at low pH as it is for doxorubicin and epirubicin, and it seemed reasonable that a reduction in cytotoxicity could be overcome by means of hyperthermia. Therefore, a comparative study of the anthracyclines doxorubicin, epirubicin, and aclacinomycin A was performed using a variation of the pH between 7.4 and 6.4 and at 37° and 41° C.

Materials and methods

Cell culture. Exponentially growing monolayer cultures of Chinese hamster ovary (CHO) cells were grown in McCoy's 5A medium supplemented with 17% fetal bovine serum at 37° C in humidified air containing 5% CO_2. The cell cultures were harvested twice weekly and replated in 10 ml of pH 7.4 medium at a cell density of 50,000 cells/flask (Falcon 3013). The doubling time ranged between 13 and 15 h.

Colony assay. Prior to treatment, multiplates (Lux 5215 4-well multiplate; well size, 24 × 67 mm) were filled with 3.0 ml of pH 7.4 medium per plate and preincubated for 24 h. Single-cell suspensions were obtained by trypsinization from 3- to 4-day-old cultures. Cell density was determined with a Coulter counter. In all, 150–10,000 cells were plated in each of the 4 multiplate wells by means of an automatic pipette (Eppendorf 4780).

Cells were incubated for 1.5–2.5 h to allow for attachment and to reduce the effect of multiplication. Prestudies had shown that this treatment did not interfere with normal morphological and growth characteristics. After cell attachment, the medium was replaced by 2.5 ml of fresh pH-adjusted medium with the specified dose of anthracycline. After 24 h of drug treatment, the medium was again replaced by fresh pH 7.4 medium and cells were reincubated for 7–9 days until colony formation was observed.

As maximal cytotoxicity would be expected with drug administration during hyperthermia in vitro [9], the heat treatment at 41° C for 3 h was initiated simultaneously with the 24-h drug treatment. Colony formation was evaluated after staining with crystal violet (1% crystal violet in 70% ethanol). Colonies containing more than 60 cells were scored as the endpoint of survival. Each survival rate calculated in one experiment was determined as the arithmetic mean value from four different wells of one multiplate.

Multiplicity was scored in separate plates at the initiation of treatment and was found in the range within 1.02–1.04 and accounted for in the calculation of the surviving fraction [23]. Plating efficiencies (PE) were determined from control plates to which no drug had been added (see Table 1). For the final evaluation, the results of three independent experiments were combined in a weighted regression analysis [24].

pH control. The pH of McCoy's media was adjusted to 7.4, 6.7, and 6.4 by adding 1 *N* hydrochloric acid. Measurements were performed with a combination glass electrode (Radiometer Copenhagen, type GK 1321C). The precision of the pH determination was greater than ±0.05 units. The pH was determined at the beginning and end of drug exposure and of temperature elevation in reference flasks. A stable pH within ±0.02 units had been verified by control experiments.

Temperature control. The multiplates were heated in an incubator with multiple stainless-steel compartments that were gassed with 5% CO_2 in air. Temperature adaptation from 37° to 41° C was achieved within 20 min. When 41° C had been reached, hyperthermia was maintained for 3 h; thereafter, the multiplates were removed to a 37° C incubator and were adjusted to 37° C within 16 min. Temperatures were measured by thermosensors based on a platinum resistor (PT 100 WK24, Ahlborn) compared with precision thermometers traced back to a national standard. The precision of temperature control was ± 0.4° C at 37° C (incubator) and ± 0.1° C at 41° C (hyperthermia chamber).

Drugs. Standard pharmaceutical preparations of doxorubicin hydrochloride (Adriblastin, Farmitalia Carlo Erba GmbH), 4-epirubicin hydrochloride (Farmorubicin, Farmitalia Carlo Erba GmbH), and aclarubicin hydrochloride (Aclaplastin, Behringwerke AG) were used. The powder preparations were stored at 4° C. At 3 h prior to the experiment they were exposed to room temperature. At 15 min before the drug treatment, a stock solution and further dilutions were produced as required using isotonic saline. In all, 10–30 µl of the drug dilution was added under shaking to 2.5 ml of pH-adapted McCoy's medium that had previously been filled into the multiplate wells.

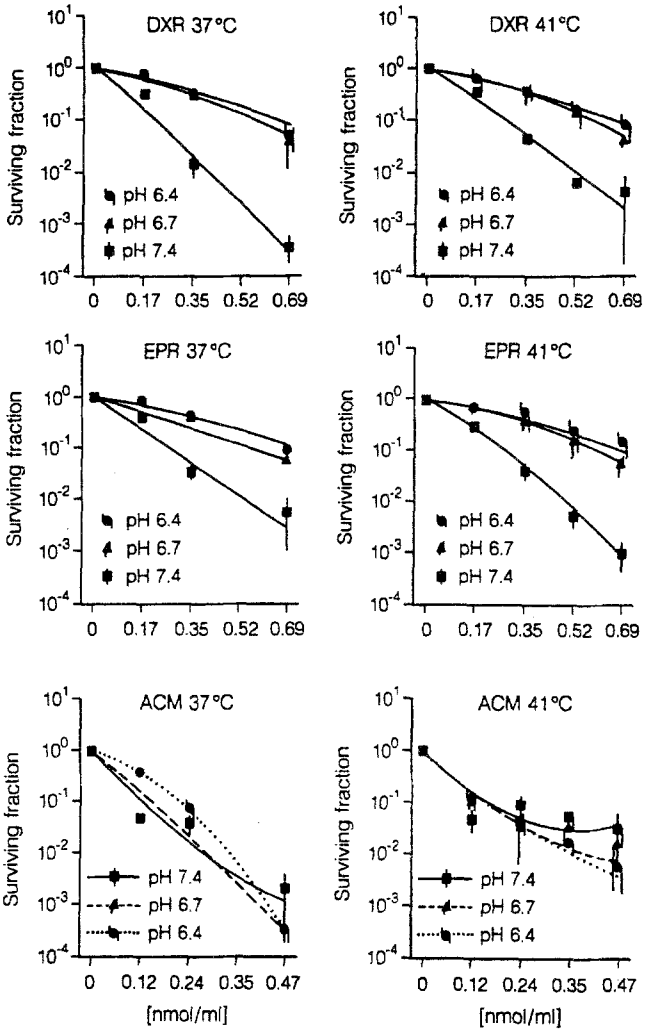

Fig. 1. Survival of CHO cells after treatment with doxorubicin (*DXR*), epirubicin (*EPR*), and aclacinomycin A (*ACM*) for 24 h. The surviving fractions plotted are normalized for the influence of pH and/or temperature (cf. Table 1). *Left column*, treatment at 37° C; *right column*, treatment with additional elevation of the temperature to 41° C for 3 h. The *symbols* represent the arithmetic mean value and the *vertical bars* indicate the standard error. The survival curves (*solid, broken, and dotted lines*, respectively) are fitted to linear-quadratic equations [24] by weighted regression analysis from three independent experiments performed simultaneously at 37° and 41° C

Results

Standard temperature

The survival of CHO cells after exposure to doxorubicin and epirubicin followed a linear-quadratic equation

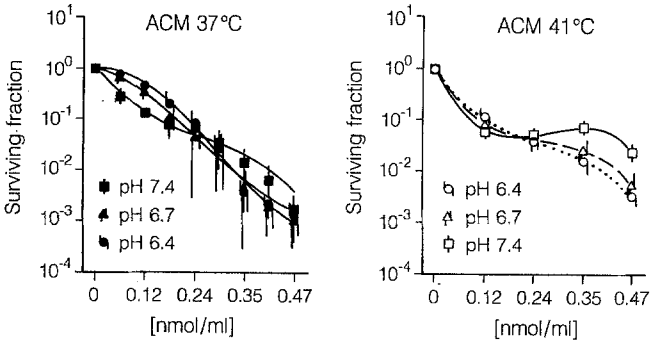

Fig. 2. Survival of CHO cells after treatment with aclacinomycin A (*ACM*) for 24 h at 37° C. The surviving fractions plotted are normalized for the influence of pH and/or temperature (cf. Table 1). *Left panel*, treatment at 37° C; *right panel*, treatment with additional elevation of the temperature to 41° C for 3 h. The *symbols* represent the weighted-regression mean value and the *vertical bars* indicate the 95% confidence range. The survival curves (*solid, broken, and dotted lines*, respectively) are fitted to linear-quadratic-cubic equations [24] by weighted regression analysis of three independent experiments performed simultaneously

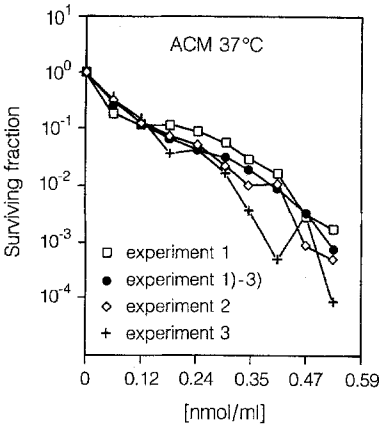

Fig. 3. Survival of CHO cells after treatment with aclacinomycin A (*ACM*) for 24 h at 37° C and at pH 7.4. The surviving fractions plotted are normalized for the influence of pH and/or temperature (cf. Table 1). The *open symbols* represent the arithmetic mean value from three observations and the *filled symbols* indicate the weighted-regression mean value fitted to linear-quadratic-cubic equations [24]

Table 1. Clonogenicity: mean ± SEM from three independent experiments

Temperature	pH	Clonogenicity
37° C	7.4	0.79 ± 0.06
	6.7	0.66 ± 0.04
	6.4	0.55 ± 0.09
41° C	7.4	0.72 ± 0.03
	6.7	0.65 ± 0.06
	6.4	0.45 ± 0.16

The value at pH 7.4 corresponds to the plating efficiency

Table 2. $D_{0.10}$ values, i.e., the dose necessary to obtain a 10% surviving fraction at different pH for the various drugs studied

Temperature	pH	Doxorubicin $D_{0.10}$	Epirubicin $D_{0.10}$	Aclacinomycin	
				$D_{0.10}$[a]	$D_{0.10}$[b]
37° C	7.4	0.21	0.28	0.13	0.14
	6.7	0.55	0.55	0.15	0.19
	6.4	0.64	0.71	0.22	0.23
41° C	7.4	0.28	0.28	0.16	0.08
	6.7	0.60	0.58	0.16	0.11
	6.4	0.69	0.65	0.16	0.13

All $D_{0.10}$ values are expressed in nmol/ml

[a] Values obtained from linear-quadratic fits [24] (Fig. 1)

[b] Values obtained from linear-quadratic-cubic fits [24] (Fig. 2)

(Fig. 1). At 37° C, doxorubicin and epirubicin exhibited decreased cytotoxicity with decreasing extracellular pH (Fig. 1). In contrast to the doxorubicin and epirubicin curves, the aclacinomycin A dose-effect curve shows a concave trend at pH 7.4, a nearly linear trend at pH 6.7, and a convex trend at pH 6.4 (Fig. 1). The crossing of the curves indicates lower cytotoxicity at an acidic pH at low drug doses and higher cytotoxicity at an acidic pH at higher drug doses relative to a normal pH. This effect was reproduced with additional data points (Fig. 2). The effect of pH and temperature on the cellular survival is demonstrated in Table 1. The dose values at surviving rates of 10% reflect the dependence of the effect on the dose at different pH and different temperatures (Table 2).

The dose-effect relation of aclacinomycin A included a cubic component, particularly at pH 7.4 (Fig. 3), which was reproduced in the different experiments. Therefore, the results were also evaluated in terms of a linear-quadratic-cubic fit.

For aclacinomycin A, the decrease in cytotoxicity with decreasing pH was limited to concentrations of less than 0.25 nmol/ml (Fig. 2). At an aclacinomycin A concentration of 0.25 nmol/ml, the surviving rate for pH 7.4 was independent of pH within the range examined. At higher aclacinomycin A concentrations (0.35–0.41 nmol/ml), the cytotoxicity for pH 7.4 was lower than that for acidic pH values. An interexperimental and fit-dependent variation should be considered in the interpretation of these dose ranges. The reproducibility of the general effect, however, was satisfactory.

The comparison of doxorubicin/epirubicin and aclacinomycin A cytotoxicity demonstrated that in the linear-quadratic-cubic fit at pH 7.4 and at doses of less than 0.25 nmol/ml, aclacinomycin A reached at least a 1.5-fold higher cytotoxicity than doxorubicin/epirubicin (Figs. 1, 2). With the linear-quadratic fit, the aclacinomycin A cytotoxicity at pH 7.4 increased with increasing dose up to a level 3.5-fold that of doxorubicin/epirubicin (Figs. 1, 2). In the dose range between 0.25 and 0.47 nmol/ml, the difference in cytotoxicity (linear-quadratic-cubic fit) was not significant. At pH 6.7 and 6.4, aclacinomycin A cytotoxicity increased about 100-fold at doses in the range between 0.12 and 0.47 nmol/ml. The corresponding increase in doxorubicin/epirubicin cytotoxicity was about 4-fold.

Elevated temperature

Elevation of the temperature to 41° C decreased doxorubicin cytotoxicity at pH 7.4 by factors of 4–8 at doses ranging between 0.52 and 0.69 nmol/ml (Fig. 1). Epirubicin exhibited no significant difference in cytotoxicity at 41° C as compared with 37° C in the entire pH range examined.

At 41° C and pH 7.4, aclacinomycin A was less cytotoxic than at 37° C at concentrations above 0.25 nmol/ml. At pH 6.7 and 6.4, however, the cytotoxicity of aclacinomycin A was increased at low concentrations and decreased at higher concentrations (Fig. 2). The increase at low doses was observed as a trend in the linear-quadratic fit and as being significant in the linear-quadratic-cubic fit. The decrease at high doses was significant at doses above 0.25–0.30 nmol/ml for both fit types (Figs. 1, 2).

The comparison of the cytotoxicities at 41° C demonstrated that at pH 7.4, aclacinomycin A was more cytotoxic than doxorubicin/epirubicin only at concentrations below 0.25 nmol/ml, whereas at pH 6.7 and 6.4, higher aclacinomycin A cytotoxicity with a factor of between 7.5 and 25 was observed throughout the concentration range of 0.12–0.47 nmol/ml.

Discussion

At 37° C and acidic pH, doxorubicin and epirubicin cytotoxicity were not found to differ significantly. The IC_{50} values (inhibitory concentration at which a surviving fraction of 50% is achieved) were used as a measure of cytotoxicity. A comparison of doxorubicin and epirubicin in terms of IC_{50} at pH 7.4 has resulted in IC_{50} ratios (doxorubicin/epirubicin) of between 0.83 and 1.08 for different Syrian hamster ovary and colon carcinoma cell lines [11, 13]. The results reported herein using CHO cells fall within this range with a ratio of 0.86 (Fig. 1).

For a comparison of doxorubicin and aclacinomycin A cytotoxicity at pH 7.4, IC_{50} ratios (doxorubicin/aclacinomycin A) in the range of 0.05 and 2.92 have been reported for lymphoma, leukemia, melanoma, Syrian hamster ovary, and colon carcinoma cell lines [13, 17, 18, 27]. The observed ratio of 2.21 lies within this range. At acidic pH, aclacinomycin A is more cytotoxic than doxorubicin or epirubicin.

At 41° C, doxorubicin and epirubicin exhibited no increase in cytotoxicity versus 37° C at normal and at low pH, which is in agreement with the observations reported for doxorubicin at pH 7.4 by Hahn et al. [7] and Morgan and Bleehen [16]. The comparable isoeffect doses of doxorubicin and epirubicin did not differ significantly at 41° C and all pH values studied (cf. Table 2). In contrast, aclacinomycin A was significantly more cytotoxic than doxorubicin and epirubicin at acidic pH.

Aclacinomycin A exhibited a negative momentum in the surviving curves, which resulted in a pH-dependent crossing or diverging. This effect can be discussed in terms of a linear-quadratic or a linear-quadratic-cubic fit. The cubic component of the dose response has been observed repeatedly following aclacinomycin A application [18, 21, 25]. The plateau formation has been explained by sublethal damage and partial repair [25] and by a concentration-dependent aclacinomycin A action on cell cycling with a blockage in relatively unsensitive phases [28]. At low pH the disappearance of the cubic component may therefore be a consequence of decreased repair.

At 41° C a complex heat-response pattern was observed for aclacinomycin A. This is consistent with a model of repair mechanisms and cellular drug uptake, both of which are augmented by hyperthermia but differ in pH and dose dependence. Previous observations [17] under different experimental conditions have shown no difference in cell survival for aclacinomycin and pH 7.4 at 37° and 42° C.

For a preferential cell kill at low pH, the concentration of aclacinomycin A should exceed about 0.25 nmol/ml at 37° and 41° C. A fit-dependent and interexperimental variation, however, should be considered in the interpretation of this critical value. As a consequence of the plateau formation in dose response, aclacinomycin A exhibits features distinctly different from those of doxorubicin and epirubicin. These complex response patterns are difficult to explain and emphasize the importance of preclinical studies that take into account a broad dose range and factors such as pH, temperature, and repair mechanisms.

A decrease in extracellular pH could result in an augmented cell kill with the application of growth-inhibiting modalities [29]. Conversely, a reduced effect of radiation and some cytotoxic drugs at subnormal extracellular pH has also been reported. Born and Eicholtz-Wirth [1], for instance, obtained reduced doxorubicin cytotoxicity in Chinese hamster cells at a lowered pH.

At pH 6.7 and 6.4, the reduced efficiency of the anthracycline antibiotics aclacinomycin A (at lower concentrations), doxorubicin, and 4-epirubicin may be based on pH-dependent variations in transport factors, enzyme activities, cell cycle, and drug metabolism [20, 22]. For doxorubicin a lower intracellular drug concentration has been reported with decreasing pH [1].

Assuming a passive permeation mechanism [22], the transport of anthracyclines is essentially influenced by the parameters pKa and polarity of the molecule. Polarity and lipophilicity can be estimated by the partition coefficient alcohol/buffer, which has been found to be 1.2 times higher for epirubicin and 44 times higher for aclacinomycin A in comparison with doxorubicin [19, 31]. At pH 7.4, the speed of uptake and release of doxorubicin and aclacinomycin correlate positively with their lipophilicity [31]. The intracellular drug concentration at steady state increases with increasing lipophilicity in the order doxorubicin, epirubicin, and aclacinomycin [19, 20].

In addition to lipophilicity, the degree of molecular ionization, which is dependent on pH and pKa, determines the transport action. Doxorubicin and epirubicin act as weak bases (pKa 8.34 and 8.08, respectively [33] and aclacinomycin A acts as a weak acid (pKa 7.30 [19]). Therefore, a decreasing extracellular pH leads to an increasing ionization of drug molecules and, hence, drug transport into cells is rendered more difficult [22].

Differences in cytotoxicity may be based on the interference with DNA synthesis by doxorubicin and epirubicin and on a preferential influence on RNA synthesis by aclacinomycin A [13]. This hypothesis is supported by the

absence of cross-resistance between aclacinomycin A and doxorubicin and epirubicin and the presence of complete cross-resistance between doxorubicin and epirubicin [31]. Furthermore, drug sensitivity depends on the cell cycle [11, 14, 27]. The slower proliferation at low pH may be an additional but unspecific factor [14, 29].

In conclusion, the present results demonstrate that pH modifies considerably the cytotoxicity of the anthracyclines studied and that 3 h of hyperthermia at 41° C do not lead to an enhancement, neither at pH 7.4 nor at low pH.

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