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Protection of Cultured Malignant Cells from Mitoxantrone Cytotoxicity by Low Extracellular pH: A Possible Mechanism for Chemoresistance *In Vivo**†

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Abstract—*In malignant tumors the distribution of pH values is shifted to lower values (range, pH* 5.8–7.4) *as compared to normal tissues (range, pH* 6.9–7.4) *or peripheral blood (pH* 7.35–7.45). *We have investigated whether the cytotoxic effect of the anthracenedione anti-cancer drug mitoxantrone (MX) on malignant cells in culture is dependent on changes of extracellular pH. The clonogenic fraction of M1R rat mammary carcinoma cells was measured after exposure to MX at an extracellular pH (pH_e) of* 6.5–7.4. *At pH_e* 6.8 *(approximately the average pH measured in a number of malignant tumors* in vivo*) the clonogenic fraction of M1R cells exposed to MX* (0.1 μg/ml) *only decreased to* 1×10^{-1} *as compared to* 2.5×10^{-4} *at pH_e* 7.4, *corresponding to a* 400-*fold inhibition of MX cytotoxicity at reduced environmental pH. The H^+ ion-mediated resistance of M1R cells to MX could be partially reversed by verapamil, suggesting that a reduced microenvironmental pH possibly interferes with intracellular MX accumulation. Therefore, drugs like MX may not be effective in the elimination of cells in acidic tumor areas. Moreover, investigations on anti-cancer drug activity* in vitro *at what is frequently referred to as 'physiological pH' may be irrelevant in terms of the cytotoxic effects of the respective agents at the pH values prevailing in malignant tissues* in vivo.

INTRODUCTION

THE CYTOTOXIC EFFECT of anti-cancer agents in malignant—and normal—cells is not only a function of the dose administered. It is, in addition, modulated by a complex interplay between a variety of determinants of drug activity, including physical parameters (e.g. accessibility of cells to the drug) and the chemical composition of the extracellular fluid (e.g. oxygen concentration [1]). One of the major differences between the interstitial fluid (IF) in tumors and normal tissues is the distinctly higher concentration of lactic acid in malignant tissues [2]. The accumulation of acidic metabolites in the extravascular space of malignant tumors exceeds the buffer capacity of the IF, resulting in a reduced intratumoral pH. This was shown by several investigators using pH microelectrodes, 5,5-dimethyloxazolidine-2,4-dione (DMO), and NMR spectroscopy for the determination of local pH distributions in malignant tissues *in vivo* (Table 1). In a transplanted mouse mammary carcinoma, for example, minimum local pH values as low as 5.8 have been reported [3], corresponding to a more than 25-fold increase in H^+ ion concentration as compared to normal tissues (range, pH 6.9–7.4; mean, ~7.2 [4]).

The investigational screening of new drugs for cytotoxicity and, in particular, the primary evaluation of potential anti-cancer drugs, is generally performed in malignant cells maintained in tissue culture. Routinely, cells are exposed to the drugs at an extracellular pH (pH_e) adjusted to the average pH of rodent or human blood (7.35–7.45 [5]). However, as stated above, this range of pH values may differ considerably from the pH in the microenvironment of malignant cells *in vivo*. As demonstrated by several investigators [6, 7], some of the determinants of anti-cancer drug activity, e.g. intracellular drug accumulation and drug reactivity with DNA, may be highly sensitive to alterations of pH. Results of studies evaluating the cytotoxic effects of

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drugs at what is considered to be a 'physiological pH' may thus be misleading with regard to the conditions prevailing in malignant tissues *in vivo*. In the present study we demonstrate that the cytotoxic effect of mitoxantrone (MX; 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]-ethyl]amino]-9,10-anthracenedione dihydrochloride), a recently developed anti-cancer agent, is critically dependent on microenvironmental pH. MX is an anthracenedione derivative exhibiting structural similarities to anthracyclines [8]. MX has been shown to possess substantial antitumor activity in mammary cancer, acute leukemia, and malignant lymphoma [9, 10].

MATERIALS AND METHODS

Drugs

Mitoxantrone and verapamil were obtained as pharmaceutical preparations from Cyanamid (Wolfratshausen, Germany) and Knoll (Ludwigshafen, Germany), respectively. Dilutions were prepared immediately prior to use in phosphate-buffered saline (PBS), pH 7.4.

Cells and cell culture conditions

BICR-M1R_{k-d} rat mammary carcinoma cells (termed M1R cells in the present report) were used in all experiments. This cell line originated from a spontaneous mammary carcinoma of a Marshall rat [11]. For routine use, cells were grown as monolayers in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% newborn calf serum and antibiotics. Cells were incubated at 37°C under a humidified atmosphere containing 7% CO_2. Details of the procedures used for drug exposure and cell plating have been published elsewhere [4]. Briefly, drug exposure was performed in a modification of DMEM containing 2-[bis(2-hydroxyethyl)imino]-2-(hydroxymethyl)-1,3-propanediol (BIS-TRIS; 20 mmol) and $NaHCO_3$ (15 mmol). The pH of the culture medium was adjusted by adding 0.1 N HCl, providing for the pH shift after gassing with CO_2. Log-phase M1R cells were incubated with the drugs for 24 h. Thereafter, cells were rinsed, trypsinized and counted.

Four different dilutions in DMEM (10^2, 10^3, 10^4, and 10^5 cells per 60 mm dish) were plated in triplicate at pH 7.4. After incubation for 12 days colonies were stained with Loeffler's methylene blue and counted. The colony-forming frequency (fraction of clonogenic cells) was calculated as the ratio of the number of colonies formed to the number of cells inoculated. The calculated value was normalized to the colony-forming frequency of untreated M1R control cells (60 ± 5%) and corrections were made for the fraction of cells lyzed during drug exposure.

RESULTS

The cytotoxic effect of MX on M1R cells at pH_e 7.4 and in more acidic culture media (pH 6.8 and pH 6.5, respectively) is shown in Fig. 1. At 0.1 μg of MX/ml the clonogenic fraction was 2.5×10^{-4} of untreated control cells. In contrast, the cytotoxic effect of MX was inhibited to a large extent when the pH of the culture medium was lowered to 6.8, i.e. approximately to the average pH reported for various malignant tumors *in vivo* (Table 1). At this pH_e, the clonogenic fraction of M1R cells was 0.1 (0.1 μg of MX/ml). This value corresponds to a more than 400-fold inhibition of MX cytotoxicity at pH_e 6.8 as compared to pH_e 7.4. Further acidification of the culture medium had only a small additional protective effect: the clonogenic fraction at pH_e 6.5 was 0.2 (0.1 μg of MX/ml).

The inhibition of MX cytotoxicity at reduced pH_e was not due to an acid catalyzed decomposition of the drug to inactive metabolites. This was shown by incubation of MX in cell-free culture medium at pH 6.8 for 24 h (37°C). Following pH adjustment to 7.4 by addition of 0.1 N NaOH, the drug-containing medium was then added to untreated cultures of M1R cells. Cells were plated 24 h later. The results obtained in these experiments were identical to those shown in Fig. 1 (pH_e 7.4).

The protective pH effect also could not be ascribed to a 'kinetic resistance' resulting from an inhibition of cell proliferation in the more acidic culture medium. At pH_e 6.8, the population doub-

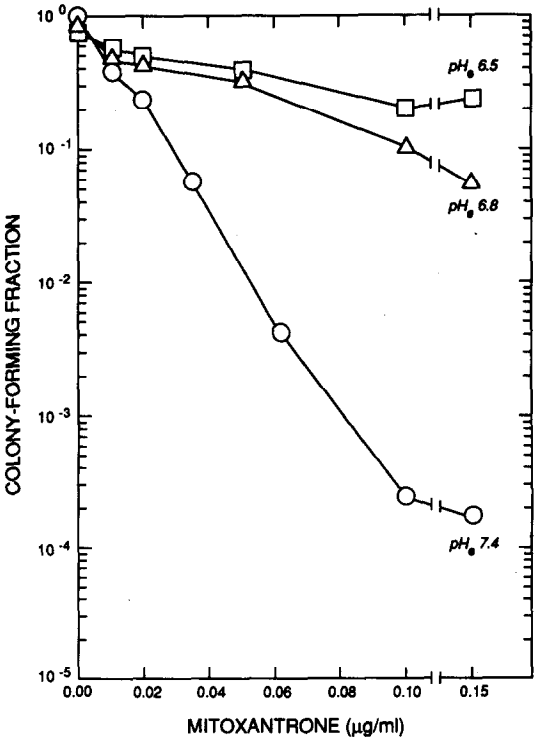

Fig. 1. Cytotoxicity of mitoxantrone on M1R cells at different pHs, as a function of drug concentration.

ling time of M1R cells was not significantly different from that of M1R cells incubated at pH_e 7.4 [12].

The kinetics of MX-induced cytotoxicity in terms of the inhibition of the colony-forming capacity of M1R cells are shown in Fig. 2. At pH_e 7.4, the fraction of clonogenic cells decreased sharply for up to ~16 h after the beginning of drug treatment. The curve then levelled off gradually and maximum cytotoxicity was observed after 24 h. At pH_e 6.5, the shape of the curve was similar to that at pH_e 7.4; however, the inhibition of colony formation capacity was much less pronounced than at pH_e 7.4. Again, maximum cytotoxicity was seen after 24 h of exposure to MX (0.06 μg/ml).

As a first approach to investigate possible mechanisms underlying the H^+ ion-mediated inhibition of MX activity, we varied the time sequence between drug exposure and pH shift. Inhibition of MX cytotoxicity at reduced pH_e was only observed when the cells were exposed to the drug and to an acidic environment simultaneously (Fig. 3C). Preincubation of M1R cells at pH_e 6.5 for 24 h prior to the beginning of a 24 h-period of drug treatment (0.06 μg/ml MX) at pH_e 7.4 did not result in decreased cytotoxicity (Fig. 3A). Similarly, no decrease in cytotoxicity was seen when cells were exposed to pH_e 6.5 for a 24 h-period immediately following removal of the drug after MX-treatment for 24 h at pH_e 7.4 (Fig. 3E). In contrast, cell kill was inhibited when both treatment modalities were applied simultaneously (Fig. 3C), or (as expected from the kinetics of the cytotoxic effect of MX) to a lesser extent when both treatment modalities overlapped during a 12 h-period (Fig. 3B and D, respectively).

Calcium channel blockers such as verapamil have been shown to increase the intracellular accumulation of anthracycline antibiotics in cultured malignant cells, resulting in an enhancement of drug toxicity [13]. In order to investigate whether a putative inhibition of MX efflux by verapamil would counteract the H^+ ion-mediated protection of M1R cells from MX cytotoxicity, we analyzed the effect of verapamil on MX cytotoxicity at reduced pH_e. As shown in Fig. 4, verapamil enhances the cytotoxicity of MX in both alkaline and acidic culture media. This effect was dose-dependent, but somewhat more evident at pH_e 7.4 than at pH_e 6.5. For example, the clonogenic fraction of cells exposed to MX (0.06 μg/ml) at pH_e 7.4 in combination with verapamil (50 μg/ml) was

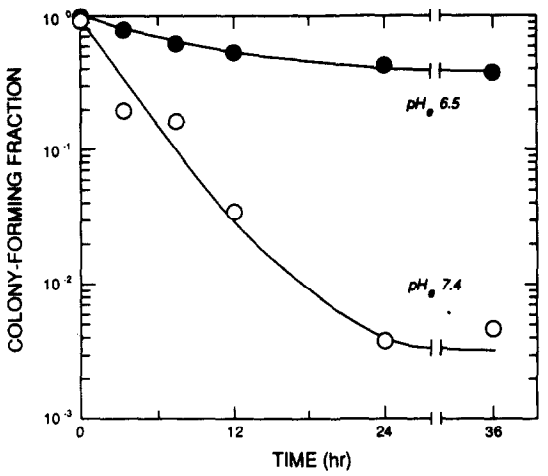

Fig. 2. Cytotoxic effect of mitoxantrone (0.06 μg/ml) on M1R cells at pH_e 7.4 and 6.5, respectively, as a function of time of drug exposure.

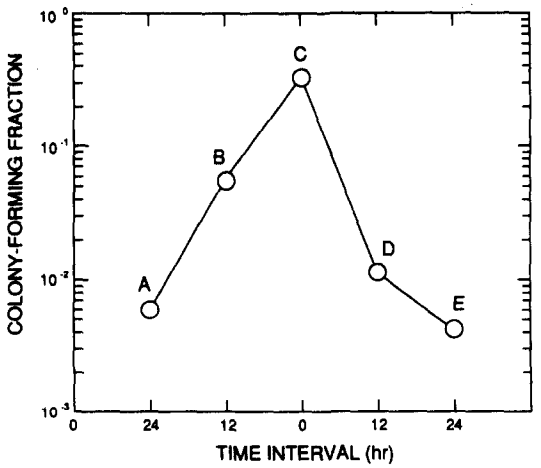

Fig. 3. Protection of M1R cells from mitoxantrone cytotoxicity by low pH_e, as a function of the time interval between the beginning of MX exposure and the shift to an acidic environment. (For details, see Results.)

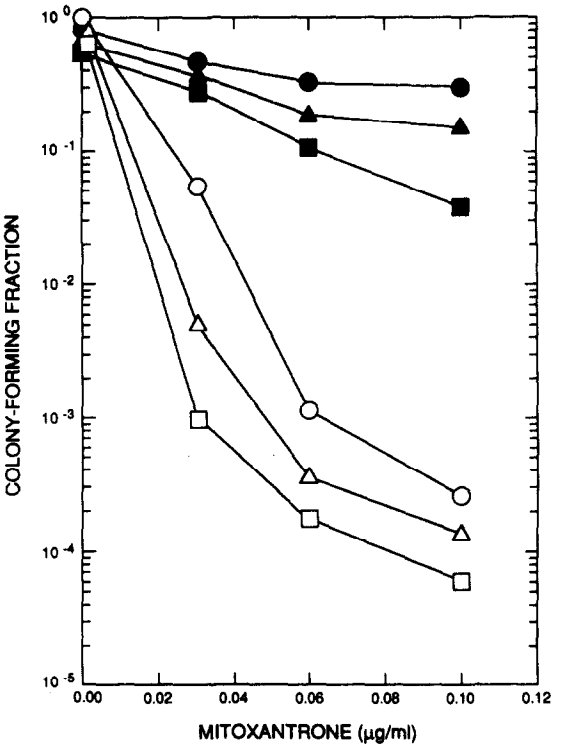

Fig. 4. Partial reversal of H^+ ion-mediated inhibition of mitoxantrone cytotoxicity in M1R cells by verapamil. Cells were incubated with MX at pH_e 6.5 (closed symbols) or 7.4 (open symbols) either in the absence (○/●) or in the presence of verapamil (△/▲, 10 μg/ml; □/■, 50 μg/ml).

reduced by a factor of 6 as compared to treatment with MX alone. For comparison, the clonogenic fraction of M1R cells subjected to identical combinations of drug treatment at pH_e 6.5 was only reduced by a factor of 3.

The H^+ ion-mediated protection of malignant cells from MX cytotoxicity can also be overcome by increasing the dose of the drug. The modifying factor of pH, i.e. the ratio of the MX concentration required to decrease the clonogenic fraction of M1R cells to 0.1 at pH_e 6.8 to the concentration of MX exerting the same level of cytotoxicity at pH_e 7.4, was calculated from the curves presented in Fig. 1. In order to reduce the fraction of clonogenic M1R cells by the same factor, the MX concentration at pH_e 6.8 must be increased to 4-fold the concentration of MX at pH_e 7.4.

DISCUSSION

The present experiments show that the cytotoxic effect of MX, a recently introduced anti-cancer drug, on M1R rat mammary carcinoma cells in culture is highly sensitive to variations of microenvironmental pH (pH_e). Shifting pH_e from 7.4 to 6.8 (i.e. approximately to the average pH of malignant tumors *in vivo*; see Table 1) was sufficient to reduce the fraction of clonogenic M1R cells by a factor of 400. We cannot offer a conclusive explanation for this finding. However, the need for simultaneous exposure of M1R cells to both treatment modalities, the drug and reduced pH_e, would seem to suggest that pertubations of energy metabolism or DNA repair are unlikely to represent major mechanisms contributing to this effect. To our knowledge, only a few studies have thus far addressed effects of shifts in microenvironmental pH on drug–target cell interactions. Two H^+ ion-sensitive mechanisms have been identified in the sequence of events leading from drug uptake to macromolecular cell damage. In EMT6 tumor cells incubated at reduced pH_e, DNA cross-link formation by mitomycin C was enhanced [7]. Skovsgaard [6] has presented evidence that the intracellular accumulation of Adriamycin® is inhibited at low pH_e. Whether similar mechanisms prevail with regard to MX awaits further investigation. Nevertheless, it should not be overlooked that the molecular structure of MX differs from that of Adriamycin® only by the absence of the amino sugar moiety at C9. In particular, the ring structure is common to both molecules. From studies of pleiotropic drug resistance it is known that different heterocyclic drugs may share common transport systems [14]. In analogy to the anthracyclines, inhibition of MX cytotoxicity at reduced pH_e could, therefore, be due to changes of the ratio between cellular drug uptake and efflux. Our finding that verapamil, an agent known to interfere with the transport of heterocyclic drugs, is able to partially revert the H^+ ion-mediated inhibition of MX cytotoxicity, is consistent with the hypothesis that the net uptake of MX into cells is decreased at reduced pH_e.

All known anti-cancer drugs are only effective against a limited number of tumors of different histological types. The molecular, cellular, and host-mediated mechanisms responsible for this differential effectiveness are poorly understood. There is evidence that cells and cell lines derived from human cancers may be sensitive to cytotoxic agents *in vitro* whereas the respective primary tumors, either individually or as histological entities, are not [15, 16]. Clearly, these differences in drug sensitivity are based on microenvironmental factors and tumor cell kinetics, as well as on biochemical and

Table 1. pH of malignant tumors

Tumor	Minimum pH	Mean pH	Maximum pH	Authors and reference No.
Malignant melanoma*	6.63	6.81	7.00	Ashby [19]
DS carcinosarcoma	6.78	6.94	7.10	Rauen *et al.* [20]
Primary rat and murine tumors	6.80	6.97	7.14	Von Ardenne and Reitnauer [21]
Yoshida sarcoma	7.06	7.19	7.32	Dickson and Calderwood [22]
Mammary carcinoma	6.20	6.75	7.00	Bicher *et al.* [23]
Mammary carcinoma	5.80	6.73	7.10	Vaupel *et al.* [24]
DS carcinosarcoma	6.10	6.60	7.20	Müller-Klieser *et al.* [25]
TV1A neurinoma	6.70	6.90	7.10	Jähde and Rajewsky [17]
RIF-1 fibrosarcoma	6.81	6.93	7.05	Evelhoch *et al.* [26]
Walker 256 carcinoma	6.50	6.70	6.90	Jain *et al.* [27]
Different histologies*	6.50	7.25	7.70	Wike-Hooley *et al.* [28]
Brain tumors	7.17	7.26	7.35	Hossmann *et al.* [29]
Guerin carcinoma	6.85	6.88	6.92	Osinsky *et al.* [30]
Different histologies*	6.80	7.14	7.40	Thistlethwaite *et al.* [31]
RIF-1 fibrosarcoma	6.75	6.80	6.85	Tobari *et al.* [32]
Mammary carcinoma*	5.78	6.81	7.59	Kallinowski *et al.* [33]
Mean	6.58	6.92	7.17	

*Primary human tumors or human tumor xenografts.

pharmacokinetic mechanisms. As outlined in Table 1, the pH values reported for tumors of different origin may, in their extremes, vary from 7.6 to 5.8, corresponding to variations in the microenvironmental H^+ ion concentration by a factor of 60. The results presented here are, therefore, consistent with the assumption that the concentration of acidic metabolites in tumor tissues may be an important determinant for the activity of anti-cancer drugs *in vivo*. In malignant tissues an inhibition of drug activity by reduced pH_e may be of particular relevance in poorly vascularized areas which are in general drained inefficiently of lactic acid [17]. Such areas are known to contain high proportions of non-proliferative, 'kinetically resistant' clonogenic cells which are also less accessible for anti-cancer drugs. After the completion of chemotherapy, proliferation-competent cells located in these 'sanctuaries' may repopulate the tumor by re-entry into the cell cycle [18]. They represent, therefore, subpopulations of cells that are critically relevant for successful therapy. To eliminate these cells, drugs would be required whose cytotoxic activity is *enhanced* by an acidic cellular microenvironment. More information regarding the pH dependence of anti-cancer drug activity is therefore needed, and the evaluation of cytotoxic drug effects on malignant cells in culture should not be restricted to a 'physiological' microenvironmental pH but rather take into account the range of pH values present in malignant tissues *in vivo*.

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