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Nigericin enhances mafosfamide cytotoxicity at low extracellular pH*

Eckhard Jähde, Karl-Heinz Glüsenkamp, and Manfred F. Rajewsky

Institute of Cell Biology (Cancer Research), University of Essen Medical School, Hufeland-Strasse 55, D-4300 Essen 1, FRG

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Summary. The cytotoxicity of many alkylating anticancer drugs is increased at reduced intracellular pH (pH_i). The therapeutic index of such agents could therefore be improved by lowering pH_i in the target cells prior to their application. We have previously demonstrated that the formation of lactic acid can be selectively enhanced in malignant tissues via glucose-mediated stimulation of tumor cell glycolysis. However, the resulting reduction in pH_i is partly compensated by the extrusion of H^+ equivalents into the extracellular space, with pH_i remaining closer to the physiological value than extracellular pH (pH_e). For full exploitation of the proton-mediated increase in the cytotoxicity of alkylating agents, pH_i should therefore be equilibrated with pH_e in lactic acid-producing cells. In the present study we investigated the question as to whether nigericin, an H^+/K^+ antiporter enabling the entry into cells of H^+ ions at low pH_e, can be used to enhance the cytotoxic effect of mafosfamide (MAFO; a precursor of "activated" cyclophosphamide) on cultured M1R rat mammary carcinoma cells. At pH_e 7.4, the cytotoxic effect of combined treatment with MAFO and nigericin was not superior to treatment with MAFO alone. At acidic pH_e, however, MAFO cytotoxicity was potentiated by nigericin as indicated by the colony-forming capacity of M1R cells. For example, at pH_e 6.2 (corresponding to the approximate mean "aggregated pH" in actively glycolyzing tumors), the colony-forming fraction of cells treated with a combination of MAFO and nigericin was 3×10^{-5} that of controls, as compared with a value of 5×10^{-2} found for cells exposed to MAFO alone. These results suggest that agents counteracting cellular mechanisms that control pH_i may be candidate compounds for investigations aimed at the enhancement of alkylating drug cytotoxicity following glucose-mediated pH reduction in malignant tumors in vivo.

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Offprint requests to: Manfred F. Rajewsky

Introduction

The cytotoxic effects of a variety of alkylating anticancer drugs are sensitive to changes in intracellular pH (pH_I) [6]. We have previously demonstrated that glucose can be used to stimulate lactic acid formation *selectively* in malignant tumors [5]. Following intravenous administration of glucose to tumor-bearing hosts, the mean "aggregated pH" (for definition see [5]) decreased to ~6.2 (range, ~6–6.5) in various transplanted rat tumors as measured by pH microelectrodes [5]. This value corresponds to an increase in the intratumoral H^+ ion concentration by a factor of 15 as compared with levels in arterial blood. In contrast, pH distributions in normal tissues of tumor-bearing hosts remained unchanged (mean pH, 7.2) [5]. Similar results for both animal and human tumors have been reported by other investigators (for review see [6, 17]).

With the aim of improving the therapeutic index of alkylating anticancer agents, we are investigating the question as to whether the selective reduction of intratumoral pH can be exploited to improve target cell-directed drug cytotoxicity. We have recently shown that the cytotoxic effect of bis-chloroethylating agents, in particular that of oxazaphosphorines and nitrogen-mustard derivatives, on cultured malignant cells is potentiated when drug treatment is carried out in an acidic environment. Thus, the colony-forming capacity of M1R rat mammary carcinoma cells exposed to 4-hydroperoxycyclophosphamide (an analogue of "activated" cyclophosphamide, CP) at an extracellular pH (pH_e) of 6.2 was reduced by >100-fold as compared with that of control cells treated at pH_e 7.2 [6]. This effect is likely to be due to an increase at low pH in the alkylating activity of the respective terminal metabolites phosphoramide mustard and nornitrogen mustard [1]. The H^+ ion-mediated enhancement of the cytotoxic effects exerted by this class of agents is, therefore, critically dependent on changes in intracellular, particularly intranuclear, pH and only indirectly due to changes in pH_e [6].

Mammalian cells counteract an overload of H^+ ions, whether metabolically generated or of microenvironmental origin, by extrusion of H^+ ion equivalents so as to maintain

pH$_i$ within or near the physiological range [8]. For example, when the pH$_e$ of cultured M1R rat mammary carcinoma cells was lowered to 6.2, pH$_i$ decreased only to ~6.7 [6]. For full exploitation of a reduced "aggregated pH" in malignant tissues for tumor-selective potentiation of alkylating drug cytotoxicity, the transmembrane pH gradient should therefore be decreased so as to shift pH$_i$ (intranuclear pH) to values similar or equal to pH$_e$.

In the present study we determined whether an equilibration of pH$_i$ with a reduced pH$_e$ indeed enhances H^+ ion-mediated drug cytotoxicity. A frequently used method to facilitate the entry of H^+ ions into cultured cells involves the use of the antibiotic nigericin; an H^+ ionophore, this drug enables the electroneutral transport of extracellular H^+ ions in exchange for intracellular K^+ ions [13, 16]. The effects of nigericin on the ionic composition of the cytoplasm and on cellular energy metabolism have recently been described [9, 13]. We measured the colony-forming capacity of M1R rat mammary carcinoma cells following their exposure to mafosfamide (MAFO), a precursor of "activated" CP [10], either alone or in combination with nigericin, as a function of extracellular pH. The data show that the combination of MAFO and nigericin resulted in an increase in the cytotoxic effect by a factor of ~10^3.

Materials and methods

Drugs. MAFO [2-(bis-(2-chloroethyl))-amino-*cis*-4-((sulfoethyl)-thio)-tetrahydro-2H-1,3,2-oxazaphosphorine-*r*-2-oxide cyclohexylamine salt] was kindly provided by Dr. J. Pohl (Asta-Werke, Bielefeld, FRG) and was stored dry at –20° C. Immediately prior to use, solutions were prepared using phosphate-buffered saline (PBS, pH 5). Nigericin (Sigma, München, FRG) was dissolved and diluted in 50% (v/v) ethanol. The drug was added in volumes of 50 μl to dishes containing 5 ml culture medium. As shown in separate experiments, at a final concentration of 0.5%, ethanol was not cytotoxic in M1R cells (see below).

Cells and culture conditions. The M1R rat mammary carcinoma cell line (originally referred to as BICR-M1R$_{k-d}$) used in the present experiments was established from a clone of BICR-M1R$_{k-d}$ cells derived from a spontaneous mammary carcinoma in 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, penicillin, and streptomycin at 37° C in a humidified atmosphere containing 7% CO_2. Details of the conditions used for drug exposure and plating have been described elsewhere [6]. Briefly, drug treatment, to improve the buffer capacity of the culture medium at reduced pH, was performed in modified DMEM containing 20 mmol bis-TRIS/l [2-(bis(2-hydroxyethyl)imino)-2-(hydroxymethyl)-1,3-propanediol] and 15 mmol $NaHCO_3$/l. The pH of the culture medium was adjusted by the addition of 0.1 *N* HCl, providing for the pH shift after gassing with CO_2. Dishes whose pH differed from the appropriate value by >0.1 unit were discarded. Log-phase cultures of M1R cells were incubated with the drugs for 24 h unless otherwise stated. Following drug exposure, cells were rinsed with drug-free DMEM (pH 7.4), trypsinized and counted. Samples of 10^2, 10^3, 10^4, or 10^5 cells were seeded in triplicate onto 60-mm plastic dishes (Nunc, Wiesbaden, FRG) and incubated in DMEM (pH 7.4) for 12 days. Following fixation and staining with Loeffler's methylene blue, colonies measuring >1 mm in diameter were counted. The fractions of colony-forming cells (CFF) were calculated as previously described [6].

Results

Effects of nigericin on M1R cells as a function of pH$_e$

In the present study, three modalities were combined in the treatment of M1R cells: exposure to an acidic environment, to MAFO, and to nigericin. In aqueous solutions MAFO undergoes rapid spontaneous hydrolysis, resulting in the liberation of 4-hydroxycyclophosphamide ("activated" CP) [10]. The cytotoxic effects of MAFO and of an acidic pH$_e$ on M1R cells, either alone or in combination, have been described elsewhere [6]. The effect of nigericin per se on M1R cells as a function of drug concentration and pH$_e$ is shown in Fig. 1. At pH$_e$ 7.4, the drug was only slightly cytotoxic. Following 24 h exposure to the ionophore, the colony-forming fraction (CFF) of M1R cells decreased only to 0.65 and 0.4, respectively, of control values at the lowest (0.25 μg/ml) and highest (1 μg/ml) concentrations tested. In contrast, the CFF of M1R cells rapidly declined when the concentration of nigericin was raised above 0.25 μg/ml at pH$_e$ 6.2. For example, at 1 μg/ml the CFF was only 4×10^{-5} that of untreated controls.

Effects of nigericin on MAFO-induced cytotoxicity at acidic pH$_e$

To investigate whether a modification of the transmembrane H^+ ion gradient at acidic pH$_e$ could be exploited to enhance the H^+ ion-dependent cytotoxicity of MAFO, M1R cells were exposed to MAFO and nigericin simultaneously. To simulate the glucose-mediated reduction of intratumoral pH in vitro, the pH of the culture medium was adjusted to 6.2 or 6.5. In the concentration range tested (0.25–1 μg MAFO/ml), exposure to MAFO alone was only slightly cytotoxic at physiological pH$_e$ (7.4), this effect being hardly augmented by nigericin (Fig. 2). At pH$_e$

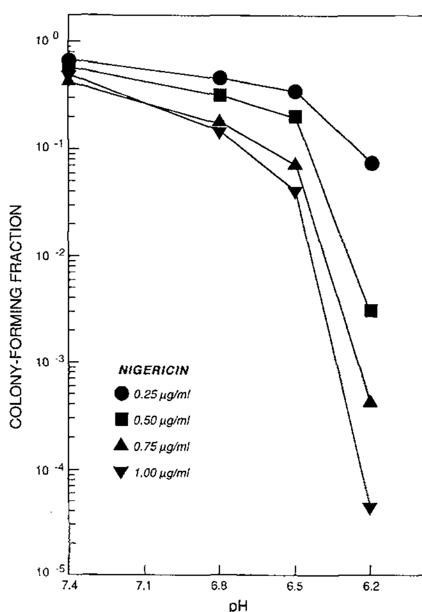

Fig. 1. Effect of nigericin on the colony-forming capacity of M1R cells as a function of pH$_e$

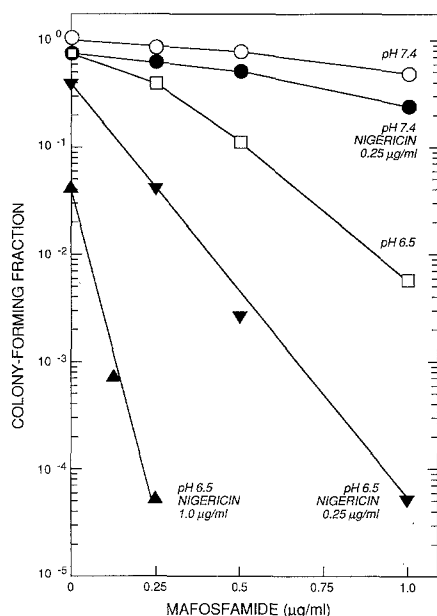

Fig. 2. Enhancement of MAFO cytotoxicity by nigericin on M1R cells at pH_e 6.5

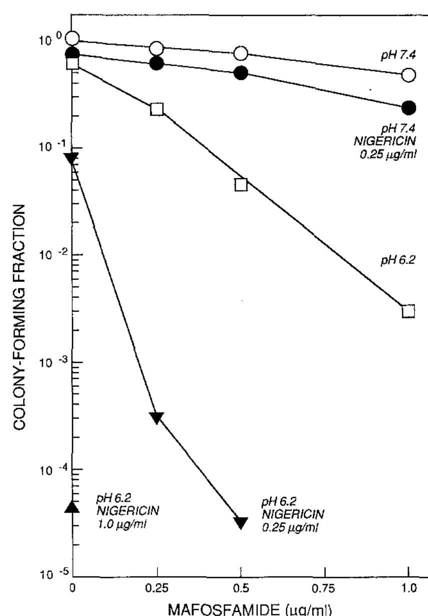

Fig. 3. Potentiation by nigericin of MAFO cytotoxicity to M1R cells at pH_e 6.2

6.5, however, both dose-dependent cytotoxicity for MAFO and an enhancement of MAFO cytotoxicity by nigericin were observed. When M1R cells were exposed to MAFO (0.25 µg/ml) at pH_e 6.5, the CFF decreased to 0.4 that of untreated controls (pH 7.4). Simultaneous exposure to nigericin at low concentration (0.25 µg/ml) resulted in a 10-fold enhancement of MAFO cytotoxicity (MAFO concentration, 0.25 µg/ml; CFF, 0.04). Under these conditions, nigericin exhibited a steep dose-response curve: When the concentration of the ionophore was raised by a factor of 4 (final nigericin concentration, 1 µg/ml), the cytotoxic effect of MAFO as measured by the colony-forming capacity of M1R cells was potentiated by a factor of 10^3 (CFF, 5×10^{-5}). The potentiation of MAFO cytotoxicity by nigericin could be further increased by lowering pH_e to 6.2. At this pH_e a nigericin concentration of 0.25 µg/ml was sufficient to reduce the CFF of M1R cells exposed to MAFO (0.5 µg/ml) to values $<10^{-4}$ that of untreated controls (Fig. 3). When applied at the highest concentration tested (1 µg/ml), nigericin at pH_e 6.2 was a potent cytotoxic agent per se (CFF, 4×10^{-5}).

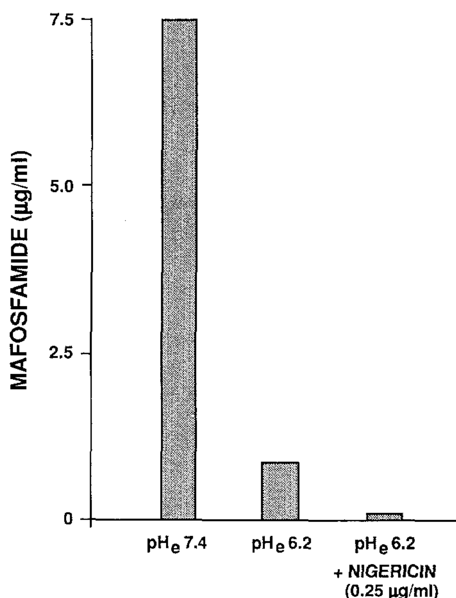

Fig. 4. Dose-sparing effect of nigericin on MAFO at acidic pH_e. *Bars* indicate the concentrations of MAFO required to reduce the colony-forming fraction of M1R cells to 0.01 of control values (IC_{99})

MAFO-sparing effect of nigericin at acidic pH_e

In acidic culture media, the MAFO concentration required to exert a defined level of cytotoxicity on M1R cells was lower than that required at physiological pH_e. This dose-sparing effect could be further enhanced by simultaneous treatment with nigericin. At pH_e 7.4, the CFF of M1R cells decreased to 0.01 that of untreated controls (IC_{99}) when the cells were exposed to MAFO at a concentration of 7.5 µg/ml (Fig. 4). The same effect was observed at pH_e 6.2 following MAFO treatment at ~1/10 of the former dose (0.8 µg/ml). Addition of nigericin to the medium resulted in a further 8-fold increase in MAFO potency, i.e. 0.1 µg MAFO/ml was sufficient to exert a 2-log reduction in CFF. Thus, by combining all three treatment modalities, the IC_{99} of MAFO could be reduced to <2% of the corresponding value for exposure to MAFO alone.

Kinetics of MAFO cytotoxicity in M1R cells simultaneously exposed to nigericin

The time-dependent changes in the CFF of M1R cells following exposure to MAFO (0.5 µg/ml) and nigericin

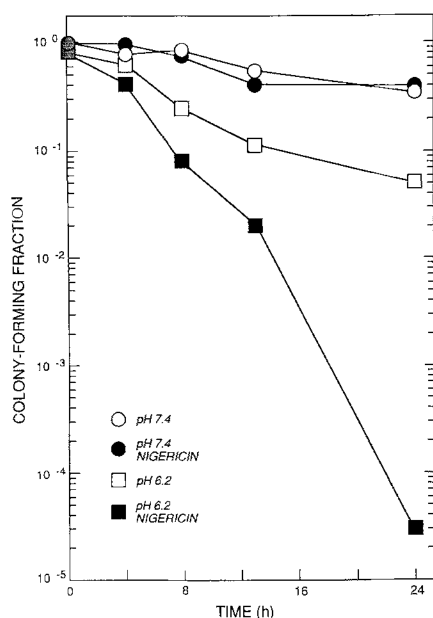

Fig. 5. Kinetics of the cytotoxic effect of MAFO (0.5 μg/ml) on M1R cells exposed to this agent either alone or in combination with nigericin (0.25 μg/ml) at pH_e 7.4 and 6.2, respectively

(0.25 μg/ml) are shown in Fig. 5. At pH_e 7.4, the curves for MAFO-treated cells leveled off at 13 h. This effect was independent of nigericin treatment. A similar result was obtained when the cells were exposed to MAFO alone at an acidic pH_e (6.2). However, when both drugs were applied in combination, the CFF of M1R cells continuously declined for 24 h; at that time, the colony-forming capacity of M1R cells was reduced to 3×10^{-5} that of controls as compared with 5×10^{-2} in the absence of nigericin.

Discussion

DNA monoadduct formation by bis-chloroethylating agents is thought to proceed via the formation of azeridinium intermediates; this process as well as the reactivity of the azeridine groups are pH-dependent [1, 7]. This is reflected by the potentiation of CP cytotoxicity to malignant cells in culture when drug exposure is performed at acidic pH_e [6]. However, the enhancement of drug cytotoxicity is lower than might be expected from the degree of pH_e reduction [6], due to the generation of a transmembrane pH gradient ($pH_i > pH_e$) when cells are exposed to an acidic environment [8]. pH_i homeostasis is crucial for a large variety of cellular functions, including the initiation of DNA replication [8, 15]. Cells therefore respond to an excess of H^+ ions by the extrusion of H^+ equivalents [8, 16]. Our previous observation that the cytotoxicity of melphalan to cultured malignant cells is primarily dependent on pH_i and is only indirectly influenced by changes in pH_e, is consistent with these findings [6].

To increase MAFO cytotoxicity to M1R cells above the level achieved by reduction of pH_e alone, we neutralized the membrane-based H^+ ion-transport mechanisms using the H^+ ionophore nigericin. Exposure of cultured cells to the ionophore at $\leqslant 1$ μg/ml results in an equilibration of pH_i with pH_e when the concentration of extracellular H^+ ions is raised above physiological values [13]. Intracellular acidification via the K^+ ion-driven import of H^+ ions occurs only at approx 10-fold higher concentrations of nigericin [9]. Over the concentration ranges tested, and regardless of whether applied alone or in combination, MAFO and nigericin were only slightly cytotoxic at physiological pH_e. Even at acidic pH_e, the cytotoxic effect increased only moderately when both agents were applied independently. However, when M1R cells were exposed to an acidic environment and to increasing concentrations of MAFO combined with nigericin, the CFF declined rapidly. Stimulation of lactic acid formation in malignant tumors by the administration of glucose in vivo is followed by a shift in the mean intratumoral "aggregated pH" from 6.9 to 6.3, with the upper limit of the pH distribution being ~6.5 [6]. At the latter pH_e, the cytotoxicity of MAFO was potentiated by factors of 10^2 and 10^4, respectively, when the cells were simultaneously exposed to the lowest and highest concentrations of nigericin tested. This effect was even more pronounced at pH_e 6.2, at which value very low concentrations of both agents, which were hardly cytotoxic at pH_e 7.4, decreased the CFF by 5 logs. As recently reported, even nigericin alone is a potent cytotoxic agent at pH_e 6.2 [13].

In the experiments described herein pH_e and, consecutively, pH_i [6] were modified by the exposure of cultured malignant cells to an increased extracellular concentration of H^+ ions. However, in malignant tumors lactic acid is produced intracellularly. This raises the question as to whether the present experimental procedure is a valid model of the conditions prevailing in vivo. We are aware of only one study of malignant tumors in which both pH_e and pH_i were measured simultaneously. Using the 5,5-dimethyloxazolidine-2,4-dione (DMO) method for the determination of pH_i and microelectrodes for measuring pH_e, Dickson and Calderwood [2] reported a reduction in pH_e in response to glucose, with pH_i remaining almost unchanged. However, these results are inconsistent with data reported by other investigators for pH_i alone [3]. As measured by NMR spectroscopy, in RIF-1 tumors, pH_i decreased by 0.45 units when the serum glucose concentration was elevated by a factor of 4 [3]. However, there are no data indicating that pH_i is lowered to the same extent as pH_e in actively glycolyzing tumor cells in vivo. In well-controlled tissue cultures (pH_e 7.4), the lactic acid produced by malignant cells is rapidly excreted and neutralized by an excess of extracellular buffer [16]. In malignant tumors, however, the drainage of acidic metabolites from the interstitial space is incomplete, particularly following glucose-mediated stimulation of aerobic glycolysis [5, 12, 17]. Accumulation of acidic metabolites in the interstitial fluid can partly compensate for cellular lactate export by the generation of an H^+ ion countergradient, which, in analogy to the situation in cultured cells, facilitates the entry of H^+ ions. Taken together, the present evidence thus suggests that pH_i declines in actively glycolyzing cells in vivo, although, a certain net H^+ gradient ($pH_i > pH_e$) is maintained due to pH_i regulatory mechanisms [4, 6]. This relation between pH_e and pH_i closely resembles the H^+ ion

distribution previously demonstrated in cultured malignant cells following acidification primarily of the extracellular fluid [6]. When M1R cells were incubated at a pH_e of 6.2, pH_i declined only to 6.7 [6]. We therefore believe that the experimental procedure used in the present study represents a valid model.

In combination chemotherapy of malignant disease, drugs are generally used whose principal effect is cytotoxicity. An alternative approach could be the use of combined modalities, cytotoxic as well as non-cytotoxic, that differ in their mode of action while acting synergistically to kill *defined* populations of target cells. In the approach outlined herein, the modality conferring tumor selectivity is the modulation of intratumoral pH. The agent used for the stimulation of intratumoral lactic acid formation is a non-toxic substrate, glucose [5]. When applied at acidic pH_e and in combination with nigericin, the concentration of the genuine cytotoxic modality used, MAFO, could be reduced to <2% of that required to exert an equal effect at physiological pH_e. At this reduced concentration, MAFO was hardly cytotoxic at pH_e 7.4. Thus, two of the three modalities used in this study, pH reduction and exposure to MAFO, comply with one of the main objectives of combination therapy: the reduction or absence of systemic (at pH_e 7.4) toxicity.

Nigericin has been widely used to counteract pH_i regulation in mammalian cells [9, 13, 16]. This compound was therefore chosen as a model agent for the modulation of pH_i in the present study, in spite of some data indicating that nigericin may not be suited for use in vivo [14]. For the potentiation of MAFO cytotoxicity by the reduction of pH_i, this argument is of only minor importance, particularly since the wide clinical use of calcium channel blockers would suggest that alternative drugs could be designed that interfere with ion fluxes in vivo without causing intolerable side effects. In summary, the results presented herein are consistent with the view that pharmacological modulation of pH_i may be used to enhance the H^+ ion-mediated potentiation of the cytotoxicity of alkylating drugs in malignant tumors in vivo.

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