Cytotoxicity of Compounds That Interfere with the Regulation of Intracellular pH: A Potential New Class of Anticancer Drugs

Daniela Rotini, Peter Wan, Sergio Grinstein, and Ian Tannock

Departments of Medicine and Medical Biophysics, Ontario Cancer Institute and University of Toronto, Toronto, Ontario M4X 1K9, Canada [D. R., P. W., I. T.], and Department of Cell Biology, The Hospital for Sick Children, Toronto, Ontario MSG 1X8, Canada [S. G.]

ABSTRACT

The extracellular pH (pH_e) in many solid tumors is often lower than in normal tissues. Cells may survive conditions of acid pH_e because antiports in their membrane exchange Na^+ for H^+, or HCO_3^- for Cl^-, and thus regulate the intracellular pH (pH_i). We have therefore assessed the effects of drugs which interfere with regulation of pH_i on survival of Chinese hamster ovary and human bladder cancer MGH-U1 cells in tissue culture. Nigericin, an ionophore which acidifies the cytoplasm when cells are placed in medium at low pH_e, was toxic at pH_i 6.5 or above but became very toxic as pH_i was reduced below this value. Amiloride and 4,4'-diisothiocyanostilbene 2,2-disulfonic acid, inhibitors of the Na^+/H^+ and HCO_3^-/Cl^- exchangers, respectively, decreased pH_i above but became very toxic as pH_i was reduced below this value. Chinese hamster ovary and human bladder cancer MGH-U1 cells in tissue culture. Nigericin, an ionophore which acidifies the cytoplasm when cells are placed in medium at low pH_e, was toxic at pH_i 6.5 or above but became very toxic as pH_i was reduced below this value. Amiloride and 4,4'-diisothiocyanostilbene 2,2-disulfonic acid, inhibitors of the Na^+/H^+ and HCO_3^-/Cl^- exchangers, respectively, decreased pH_i above but became very toxic as pH_i was reduced below this value. Cells may survive conditions of acid pH_e because antiports in their membrane exchange Na^+ for H^+, or HCO_3^- for Cl^-, and thus regulate the intracellular pH (pH_i). We have therefore assessed the effects of drugs which interfere with regulation of pH_i on survival of Chinese hamster ovary and human bladder cancer MGH-U1 cells in tissue culture. Nigericin, an ionophore which acidifies the cytoplasm when cells are placed in medium at low pH_e, was toxic at pH_i 6.5 or above but became very toxic as pH_i was reduced below this value. Amiloride and 4,4'-diisothiocyanostilbene 2,2-disulfonic acid, inhibitors of the Na^+/H^+ and HCO_3^-/Cl^- exchangers, respectively, decreased pH_i above but became very toxic as pH_i was reduced below this value. Chinese hamster ovary and human bladder cancer MGH-U1 cells in tissue culture. Nigericin, an ionophore which acidifies the cytoplasm when cells are placed in medium at low pH_e, was toxic at pH_i 6.5 or above but became very toxic as pH_i was reduced below this value. Amiloride and 4,4'-diisothiocyanostilbene 2,2-disulfonic acid, inhibitors of the Na^+/H^+ and HCO_3^-/Cl^- exchangers, respectively, decreased pH_i above but became very toxic as pH_i was reduced below this value. Cells may survive conditions of acid pH_e because antiports in their membrane exchange Na^+ for H^+, or HCO_3^- for Cl^-.

INTRODUCTION

Measurements of extracellular pH (pH_e) in solid tumors have shown considerable variation, but the average tumor pH_e appears to be about 0.5 pH unit less than in normal tissues (1-5). Typical ranges of pH_e are 6.5-6.9 in tumors and 7.0-7.5 in normal tissues, but pH_e values of 6.0 or lower have been detected in some tumors. In many tumors, cells located in regions distant from blood capillaries are hypoxic due to limited diffusion of oxygen. Hypoxia may contribute to the development of acidic conditions in tumors; under hypoxic conditions, cells must rely on anaerobic glycolysis to supply their energy requirements, although glycolysis may be limited by availability of glucose in poorly vascularized regions. Under conditions in which glucose supply is sufficient, hypoxia could lead to accumulation of lactic acid and hydrolysis of ATP (6), with consequent reduction of tumor pH_i. Thus the pH_i in hypoxic regions of tumors might be lower than mean values of pH determined by electrode measurements.

We have reported recently that the combination of hypoxia and low pH_i was toxic to cells in culture (7) and we have suggested that these conditions may contribute to cell death and necrosis found in many tumors. Since hypoxic cells are known to be resistant to radiation treatment and to some of the commonly used anticancer drugs, simulation or enhancement of conditions that lead to natural cell death in tumors might have chemotherapeutic potential.

Regulatory mechanisms allow the survival of cells in an acid environment by maintaining a higher intracellular pH (pH_i) than that predicted from the electrochemical equilibrium of H^+ and HCO_3^- ions across the cell membrane (8). The two known systems responsible for the regulation of pH_i in mammalian cells are (a) countertransport of external Na^+ for internal H^+, a process inhibited by the diuretic amiloride, and (b) countertransport of external HCO_3^- for internal Cl^- which is inhibited by stilbene derivatives such as DIDS (8). Inhibition of these regulatory mechanisms could influence cell viability. We have therefore investigated the cytotoxic effect of membrane-active compounds which might lead to a reduction of pH_i when cells are placed in an acidic environment. Two classes of compounds have been studied: (a) the ionophore nigericin which lowers pH_i by allowing exchange of intracellular K^+ for extracellular H^+ (9); and (b) the drugs amiloride and DIDS which inhibit the ion exchange agents.

MATERIALS AND METHODS

Cells. CHO cells and the human bladder carcinoma cell line MGH-U1 (kindly provided by Dr. G. Prout and colleagues, Urology Research Laboratory, Massachusetts General Hospital, Boston, MA) were maintained in complete α-medium supplemented with antibiotics and 10% FCS. Cultures, free of Mycoplasma, were reestablished from frozen stock at approximately 3-month intervals. MGH-U1 cells were grown routinely as monolayers in tissue culture flasks and were detached prior to experiments with 0.025% trypsin and 0.01% EDTA. CHO cells were transferred from culture flasks to spinners about 1 week prior to their use in experiments. All experiments were carried out with exponentially growing cells.

Reagents. Amiloride was a gift from Merck, Sharpe & Dohme (Quebec), and the tetraacetoxymethyl ester of BCECF was purchased from Molecular Probes (Eugene, OR). [14C]DMO (54 mCi/mmol), [3H]PEG (0.78 mCi/g), [3H]H_2O (5 mCi/ml) were obtained from New England Nuclear. Acetonitrile and KH_2PO_4 were obtained from Fisher (Fair Lawn, NJ), tetrabutylammonium phosphate from Waters Associates (Milford, Ontario, Canada), tri-chlorotrifluoroethane (Freon) from Matheson (Whitby, Ontario, Canada), and perchloric acid from J. T. Baker Chemical Co. (Phillipsburg, NJ). Nigericin, DIDS, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Survival Experiments. Ten ml of a suspension containing 10^6 cells/ml in α-medium plus 5% dialyzed FCS buffered to the required pH were added to small glass vials. The cells were stirred continuously at 37°C, and a humidified gas mixture of either air (plus 5% CO_2) or nitrogen (plus 5% CO_2, <10 ppm O_2) flowed through the vials, as described previously (10).

To achieve the desired pH, the appropriate amount of sodium bicarbonate was added to bicarbonate-free α-medium (plus 5% dialyzed

* The abbreviations used are: DIDS, 4,4'-diisothiocyanostilbene 2,2-disulfonic acid; CHO cells, Chinese hamster ovary cells; FCS, fetal calf serum; BCECF, 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein; DMO, 5,5-dimethyl-2,4-oxazolidinedione; PEG, polyethylene glycol; Na^+, K^+; intracellular Na^+ and K^+; Na^+, K^+, extracellular Na^+ and K^+.

Received 4/3/86; revised 10/16/86; accepted 12/1/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Supported by Grant CA 36913 from the NIH and by a grant from the National Cancer Institute of Canada.

2 Recipient of a research studentship from the National Cancer Institute of Canada.

3 To whom requests for reprints should be addressed, at Department of Medicine, Ontario Cancer Institute, 500 Sherbourne Street, Toronto M4X 1K9, Ontario, Canada.
COMPOUNDS INTERFERING WITH INTRACELLULAR pH REGULATION

FCS). Thus, bicarbonate was present in the media of all experiments. The stability of pH of CHO cells (1 × 10⁶ cells/ml) gassed with air or N₂ (each with 5% CO₂) at various initial pH values (i.e., pH of the medium before adding cells) was detailed previously (7). In brief, 1 h after initiation of gassing, pH decreased by 0.1–0.2 unit in air or in hypoxia at any initial pH, in the range of 6.0–7.2. pH varied minimally during the subsequent 4 h of gassing with only slight acidification (0.05–0.1 pH unit) of the cell suspension under hypoxic conditions. Nigericin (added 1 h after initiation of gassing) did not affect pH, for at least 4 h. In all figures (unless otherwise indicated) the indicated pH is the pH of the medium just before adding cells and gassing.

One h after initiation of gassing, appropriate concentrations of drug dissolved in 100 μl 50% ethanol were added to the vials. Each drug was added separately. The control vials received the same volume of 50% ethanol; maximum ethanol concentration in any experiment did not exceed 1.5%, a level that was not toxic to CHO or MGH-U1 cells. At selected times after adding drug or ethanol, 0.5-ml aliquots of the cell suspension were removed by passing a long needle attached to a syringe through the gas outlet tube. The cells were washed and resuspended in alpha-medium plus 10% FCS at pH 7.3, diluted, and plated in triplicate Petri dishes. Colonies were stained and counted 9–13 days later.

Measurements of Intracellular Na⁺ and K⁺ (Na⁺ and K⁺). Measurements were carried out essentially as described by Grinstein et al. (11). During the course of incubation of CHO cells with or without nigericin (1 μg/ml), 2.5 ml of a cell suspension containing about 2.5 × 10⁶ cells were removed and quickly washed twice with ice-cold choline buffer (free of Na⁺ and K⁺ containing 140 mM choline chloride, 10 mM glucose, 1 mM CaCl₂, 1 mM MgCl₂, and 20 mM Tris-2-(A'-morpholino)ethanesulfonic acid, pH 7.3). The buffer was aspirated and the pellet was stored at −70°C overnight. The pellet was then suspended in 1 ml Li⁺ standard solution (15 meq/liter; Instrumentation Laboratory Inc., Lexington, MA) and mixed vigorously to break the cells osmotically; after sedimentation of debris the Na⁺ and K⁺ content of the supernatant was measured by flame photometry (Photometer model 443; Instrumentation Laboratory). Concentrations of Na⁺ and K⁺ were calculated based on cell volume which was measured electronically (Coulter Counter ZM attached to Cl004 Coulter Channelizer).

To assess the amount of K⁺ and Na⁺ lost from cells during washing with Na⁺- and K⁺-free choline buffer, a parallel experiment was carried out in which cells were sedimented without washing. Briefly, after resuspension of 2.5 ml from the cell suspension, [3H]PEG (4 × 10⁵ cpm/ml final) was added to the cells, 1 ml (1 × 10⁶ cells) was sedimented through an oil-phthalate mixture, and an aliquot from the supernatant was counted by liquid scintillation (Packard Tri-Carb, C2425). The pellet was transferred to a separate tube containing Li⁺ buffer (15 meq/liter) and the cells were lysed by vigorous mixing. An aliquot of the pellet in Li⁺ was then counted by liquid scintillation. The fraction of H⁺ in the pellet indicated the amount of extracellular Na⁺ and K⁺ in the Li⁺ suspension and was used to correct values obtained by flame photometry.

Measurement of Intracellular pH. Intracellular pH was measured by two methods: (a) partition of the weak acid DMO, as described previously (12). Briefly, after 5 h exposure to medium containing nigericin or to the ethanol diluent (controls), CHO cells (2 × 10⁶ cells/ml) were equilibrated with media containing either [¹⁴C]DMO and H₂O or determination of DMO partition or with [¹³C]PEG (M, 4000) and H₂O for determination of intracellular volume and of trapped extracellular space. One ml of the cell suspension (2 × 10⁶ cells) was loaded on 300 μl oil-phthalate and sedimented through this mixture. The pellet (i.e., cells) and an aliquot of the supernatant (i.e., medium) were then counted by liquid scintillation; (b) intracellularly trapped fluorescent dye, as described elsewhere (11, 13, 14). The method is based on the penetration of the tetraacetoxyethyl ester of BCECF into cells, where it is cleaved by internal esterases, releasing the poorly permeant and highly fluorescent BCECF. BCECF serves as a cytoplasmic fluorescent indicator, with a pK₅₀ value close to 7.0 (13) and a linear relationship between fluorescence intensity and pH in the range of 6.0–7.5. For the experiments, cells were loaded with 2 μg/ml tetraacetoxyethyl ester-BCECF for 40 min at 37°C, sedimented, and resuspended in fresh α-medium (without serum) to yield 1.0–1.2 × 10⁶ cells/ml. Aliquots of either 4.8 × 10⁵ cells (MGH-U1) or 9.6 × 10⁵ cells (CHO) were then added to a cuvet containing Na⁺ buffer (containing 10 mM glucose, 1 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 140 mM NaCl) and pH₅ measurements were carried out in a Perkin Elmer LS3 fluorescence spectrophotometer. Excitation and emission wavelengths were 495 and 525 nm, respectively. Calibration of pH₅ versus fluorescence intensity was carried out on the same batch of cells for which pH₅ measurements were done, in K⁺ buffer (identical to the Na⁺ buffer only with isosmotic replacement of KCl for NaCl with nigericin. This ionophore sets H⁺/H⁺ = K⁺/K⁺ such that when cells are suspended in K⁺ buffer (containing approximately the same concentration of K⁺ as the cytoplasm) pH₅ follows pH₅ (9). In those experiments in which pH₅ measurements were carried out in α-medium (minus phenol red), a calibration curve was defined in the same medium, by using 5 μM monensin (a H⁺/Na⁺ ionophore) plus 2 μg/ml nigericin.

Measurements Relating to Cellular Energy Metabolism. The concentration of lactate and glucose in the medium were measured using a commercial kit (Sigma) as detailed previously (7). High performance liquid chromatography measurements of levels of AMP, ADP, and ATP were carried out as described in a previous publication (7). Energy charge was calculated as

\[
\frac{ATP + \frac{1}{2} ADP}{AMP + ADP + ATP}
\]

RESULTS

Effects of Nigericin. The cytotoxic effects of nigericin for CHO and MGH-U1 cells incubated under aerobic conditions at various pH₅ are shown in Fig. 1. Nigericin was toxic to cells at low but not at normal pH₅. The sensitivity of this cytotoxic effect to pH₅ and to concentration of nigericin is illustrated for CHO cells in Fig. 2. In the presence of nigericin cell survival began to decrease at pH₅ 6.5 and declined rapidly as pH₅ was reduced further (Fig. 2A). At pH₅ 6.3, an exponential decrease of plating efficiency was observed with increasing nigericin concentration between 0 and 0.5 μg/ml and a slower decrease of plating efficiency was observed at higher concentrations of the drug (Fig. 2B). Some variation in levels of survival was observed in multiple experiments, but results were always qualitatively similar. Fig. 2 indicates that small variations in pH₅ or in the concentration of nigericin may produce a large effect on cell survival.

The effect of nigericin on the concentration of Na⁺ and K⁺ is shown in Fig. 3. After 6 h incubation with the drug in air, K⁺ levels decreased (by 4-fold at pH₅ 7.0 and 30-fold at pH₅ 6.0–6.1) whereas Na⁺ levels increased (by approximately 3–4-fold) relative to untreated cells. The total concentration of Na⁺ plus K⁺ was greater in the controls than in cells treated with nigericin, especially at low pH₅. For example, at pH₅ 6.0–6.1, taking into account the differences in cell volume, the sum of Na⁺ plus K⁺ was 55% of the controls (Fig. 3). In the parallel experiment in which cells were not washed with Na⁺- and K⁺-free choline buffer (detailed in “Materials and Methods”), the corrected sum of Na⁺ and K⁺ at pH₅ 6.0–6.1 with nigericin was 79% of the controls (data not shown).

Nigericin caused equilibration of pH₅ with pH₅ in CHO cells incubated for 5 h at low but not at normal pH₅ (Table 1). Using the pH-sensitive fluorescent dye BCECF, nigericin was found to cause an immediate decrease of pH₅ in cells placed under acidic conditions (Fig. 4). Because nigericin is an uncoupler of oxidative phosphorylation, its effect on cellular energy metabolism was investigated. Measurements of lactate (Fig. 5) and glucose concentration (data not shown) in the medium revealed that following a 6-h incubation of CHO cells with nigericin, the
rate of glycolysis was increased slightly under aerobic conditions but decreased under hypoxic conditions relative to the controls. This effect was seen at both normal and low pHc. Cellular ATP pools and the energy charge of CHO cells treated with nigericin are shown in Fig. 6. ATP levels decreased at low pHc (Fig. 6A). They were lower under hypoxic than under aerobic conditions (see also Ref. 7) and they were also lower in the presence of nigericin than in untreated cells. The decrease of energy charge with decreasing pHc in cells treated with nigericin and in hypoxic cells was much slower than the decrease of ATP levels (Fig. 6B), with a decrease in energy charge below the normal values (0.85–0.95) seen only at pHc < ~6.5. Aerobic untreated cells exposed to pHc 5.9 had a normal energy charge despite an almost 40% decrease in ATP concentration.

Effect of Amiloride and DIDS on Cell Survival and on pHc.
We studied the cytotoxic effects of amiloride and DIDS either alone or in combination with nigericin at various pHc. At pHc 7.0, amiloride (0.1 mM) had no cytotoxic effect either alone or
化合物干扰细胞内pH调节

图3. 在选择性缓冲液中培养的CHO细胞的细胞内Na⁺和K⁺浓度。A: 无药物，1小时后改变pH。B: 6小时暴露于nigericin（或对照）。柱状图表示3次实验的平均值，误差线表示标准误差。

图4. 选择性缓冲液中培养的CHO细胞的细胞内pH（pHₗ）变化，检测方法见“材料和方法”部分。A: 超级敏感的CHO细胞；B: 中度敏感的MGH-U1细胞。柱状图表示3次实验的平均值，误差线表示标准误差。

图5. 选择性缓冲液中培养的CHO细胞的乳酸生产。柱状图表示3次实验的平均值，误差线表示标准误差。

表1. 不同pH值下CHO细胞的细胞内pH（pHₗ）。+：含有nigericin；-：无nigericin。

<table>
<thead>
<tr>
<th>pHₗ,</th>
<th>pHi,</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.24</td>
<td>6.93</td>
</tr>
<tr>
<td>(7.15-7.33)</td>
<td>(6.88-7.00)</td>
</tr>
<tr>
<td>6.74</td>
<td>6.88</td>
</tr>
<tr>
<td>6.64</td>
<td>6.75</td>
</tr>
<tr>
<td>(6.63-6.64)</td>
<td>(6.75-6.54)</td>
</tr>
<tr>
<td>6.25</td>
<td>6.42</td>
</tr>
<tr>
<td>6.18</td>
<td>6.17</td>
</tr>
</tbody>
</table>

a: pHₗ是通过DMO方法测定的。细胞浓度为2×10⁶个细胞/ml，nigericin浓度为1 μg/ml。

b: pH是5小时后添加药物时的值。细胞悬液在添加药物前1小时开始通气。

c: 为避免混淆，测量的pHi在括号内。

与nigericin共用的化合物显示了轻微的毒性或无毒性。

氨氯地平显示了轻微或无毒性，但暴露于nigericin后细胞的生存率下降了10⁻²倍。（图7A）。相似的结果被应用于DIDS（图7B）。这个化合物（0.1 mM）对CHO细胞本身在pHₗ 7.0或6.0下没有毒性，但在pHₗ 7.0下对nigericin表现出高度的细胞毒性。

图8B显示，细胞在空气中的存活率在6.5-7.0的pHi范围内变化，但没有观测到显著的pHi值变化。

图9显示，nigericin和DIDS（0.25 μg/ml）的组合在6.5-6.8的pHi范围内表现出毒性，但没有在pHi 7.0时观察到显著的毒性。

氨氯地平和DIDS（0.1 mM）的组合在pHₗ 6.9-7.1的范围内表现出毒性，但没有在pHi 7.0时表现出显著的毒性。

表2. 不同pH值下CHO细胞的细胞内pH（pHₗ）。+：含有nigericin；-：无nigericin。

<table>
<thead>
<tr>
<th>pHₗ,</th>
<th>pHi,</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.24</td>
<td>6.93</td>
</tr>
<tr>
<td>(7.15-7.33)</td>
<td>(6.88-7.00)</td>
</tr>
<tr>
<td>6.74</td>
<td>6.88</td>
</tr>
<tr>
<td>6.64</td>
<td>6.75</td>
</tr>
<tr>
<td>(6.63-6.64)</td>
<td>(6.75-6.54)</td>
</tr>
<tr>
<td>6.25</td>
<td>6.42</td>
</tr>
<tr>
<td>6.18</td>
<td>6.17</td>
</tr>
</tbody>
</table>

化合物与nigericin（0.25 μg/ml）的组合在pHₗ范围内表现出毒性，但在pHi 7.0时表现出轻微毒性。

氨氯地平和DIDS（0.1 mM）的组合在pHₗ 6.9-7.1的范围内表现出毒性，但没有在pHi 6.9-7.1时表现出显著的毒性。

图9显示，nigericin和DIDS（0.25 μg/ml）的组合在6.5-6.8的pHi范围内表现出毒性，但没有在pHi 7.0时表现出显著的毒性。
COMPOUNDS INTERFERING WITH INTRACELLULAR pH REGULATION

Fig. 6. Percentage of ATP (A) and energy charge (B) of CHO cells incubated without (controls) or with 1 µg/ml nigericin (Nig) for 4 h as a function of pH, (measured after 4 h incubation). ATP levels in (A) are expressed as percentage of the controls (pH 7.0, in air). Arrows in B, actual value less than the point shown which is the calculated value based on the detection limits of the high performance liquid chromatography. Energy charge is defined as

\[
\text{Energy charge} = \frac{\text{ATP} + \frac{1}{2}\text{ADP}}{\text{AMP} + \text{ADP} + \text{ATP}}
\]

Because nigericin was found to cause an immediate decrease of pH (Fig. 4), the effect of amiloride and DIDS on this nigericin-induced cytoplasmic acidification was studied. Fig. 4 shows that amiloride decreased further (by 0.2–0.3 unit) the reduction of pH caused by nigericin in both CHO cells (Fig. 4A) and MGH-U1 cells (Fig. 4B). To test the effect of DIDS, pH, measurements were carried out in α-medium (without phenol red, or serum, and with 3.85 mM NaHCO₃) using continuous gassing with 5% CO₂ in air. The results obtained were similar (albeit of smaller magnitude) to those obtained with amiloride; DIDS (0.1 mM) caused a further decrease of ~0.1 pH unit in pH, compared to that caused by nigericin alone. DIDS also added to the acidification caused by nigericin plus amiloride (data not shown). Neither amiloride nor DIDS (0.1 mM each) caused a decrease of pH when added alone in Na⁺ buffer or in α-medium (data not shown).

Effect of Hypoxia. We investigated the effects of nigericin, amiloride, and/or DIDS on survival of CHO cells incubated under hypoxic conditions. Exposure of cells to hypoxic conditions at low pH is toxic in the absence of drugs (Ref. 7; Fig. 10A). Fig. 10A shows that at pH 6.1, the addition of 0.25 µg/ml nigericin to cells incubated in hypoxia caused additional loss of survival by a factor of 10⁻³ after 2 h. Incubation of CHO cells (with or without nigericin) under hypoxic conditions caused fluctuation of Na⁺ and K⁺ levels; these fluctuations were qualitatively similar to those seen when aerobic cultures were exposed to nigericin (Fig. 3).

In contrast to nigericin (Fig. 10A), addition of amiloride and/or DIDS (0.1 mM each) did not increase cytotoxicity to CHO cells incubated under acidic (pH 6.3) and hypoxic conditions (data not shown). Addition of nigericin with amiloride and/or DIDS to cell cultures incubated under hypoxic conditions at pH 6.1 led to a much greater cell kill than that seen in the control (no drugs), in cells incubated with amiloride plus DIDS alone, or in cells incubated with nigericin alone (Fig. 10B). At the drug doses used (Fig. 10B), there was no detectable difference in the survival of cells incubated under aerobic or hypoxic conditions when all three drugs were added. In a preliminary study, using one-half the doses of the drugs (0.13 µg/ml nigericin plus 0.5 mM amiloride plus 0.05 mM DIDS) the surviving fraction after 4 h incubation was 0.68 in air and 0.17 in hypoxia (data not shown).
DISCUSSION

The present experiments show that (a) agents that interfere
with regulation of pH can increase cell death when cells are
placed in an acidic environment and that (b) the cytotoxic effect
of low pH, plus hypoxia can be increased by these agents.

Although the combination of hypoxia and pH, < 6.5 was
cytotoxic to at least two different cell lines in culture (7), the
average pH, values that have been measured in tumors range
between 6.5 and 6.9 (1–5). If acid pH is to be used to obtain
differential toxicity between tumors and normal tissues, it is
important to find agents that exert toxicity within the pH,
range of 6.5 and 6.9 but not at pH, 7.0 or above. In the present
study this was achieved by treating cells with nigericin, which
acidiﬁes the cytoplasm, plus amiloride and DIDS, which en-
hance this acidification, probably by inhibiting the ability of
cells to recover from a low pH.

Nigericin alone was toxic to CHO and MGH-U1 cells at pH,
< 6.5. Similar results were obtained by Haveman (15), who
-treated M8013S mammary carcinoma cells with the proton
ionophore carbonylcyanide 3-chlorophenylhydrazone. Nigeri-
cin is known to equilibrate pH, with pH, when extracellular
and intracellular K+ levels are equal (K+ = K+). Cells in our ex-
periments were exposed to medium with a low concentration of
K+ (5.5 mM) with the expectation that the outward K+ gradient
and the low pH, could cause movement of H+ ions into the cell,
and a fall in pH,. Indeed, CHO cells incubated with nigericin
showed a large decrease of K+, especially at low pH,. The
concomitant increase of Na+ suggests that nigericin probably
exchanged K+ for Na+ as well as for H+ and/or that cytoplasmic
acidification caused by nigericin may activate the Na+ /H+ ex-
changer resulting in an increase of Na+. In unpublished ex-
periments we have demonstrated the existence of an amiloride-
sensitive Na+/H+ exchanger in both CHO and MGH-U1 cells.
At low pH, nigericin caused a loss of total Na+ plus K+, with
a consequent reduction of cell volume. However, since both the
decrease of cell volume and loss of cations during washing with
Na+- and K+-free buffer could not account for the full reduction
of the sum of Na+ plus K+, it is possible that cell osmolarity
was maintained by other (unknown) metabolites or cations. The
large ﬂuctuations of Na+ and K+ probably do not cause cell
death, however, because they were detected at both pH, 7.0,
when nigericin was not toxic, and at pH, 6.0–6.1, when the
drug was very toxic (Figs. 1 and 3). However, we cannot exclude
the possibility that the difference in K+ levels between cells at
pH, 7.0 (28 mM) and at pH, 6.0 (2 mM) is crucial to cell
survival. Nevertheless, it seems likely that the major mechanism
whereby nigericin kills cells is through lowering of pH.

Lowering of pH, by nigericin probably leads to cell death in
an acidic environment by a combination of mechanisms, since
pH, has a marked effect on a variety of cellular processes,
including energy metabolism. The following mechanisms might
allow nigericin to contribute to cell death at low pH, by energy
depprivation: (a) Nigericin causes a slight increase in the rate of
glycolysis under aerobic conditions (Fig. 5), in agreement with
its known role as an uncoupler of oxidative phosphorylation.
Since glycolysis is inhibited at low pH (Fig. 5; see also Refs. 7
and 16), cells exposed to an acidic environment and to nigericin may then die of energy deprivation. The results showing decreased levels of ATP and energy charge (Fig. 6) in cells exposed to nigericin at low pH, are in support of this view. However, the uncoupling effect of nigericin could not solely account for cell death, because nigericin was found to add to the cytotoxicity of hypoxia plus low pH, in a situation in which respiration is already blocked. (b) The large fluctuations of K+ and Na+ caused by nigericin could lead to activation of Na+/K+-ATPase with the consequent loss of energy. This could explain the further reduction of ATP levels caused by nigericin under hypoxic conditions (Fig. 4). The consequent loss of energy. This could explain the further reduction of ATP levels caused by nigericin under hypoxic conditions (Fig. 4).

COMPOUNDS INTERFERING WITH INTRACELLULAR pH REGULATION

Fig. 10. Plating efficiency of CHO cells (A) exposed under aerobic and hypoxic conditions at normal or low initial pH, to nigericin (0.25 μg/ml) alone or (B) exposed under aerobic and hypoxic conditions at initial pH, 6.5 to various combinations of nigericin (Nig, 0.25 μg/ml), amiloride (Am, 0.1 mm), and DIDS (0.1 mm). Plating efficiency of cells exposed to amiloride and/or DIDS (0.1 mm each) at pH, 6.3 under hypoxia was the same as that of untreated cells. Data are plotted as mean (points) and range (bars) for triplicate plates.


D. Rotin, unpublished observations.


Cytotoxicity of Compounds That Interfere with the Regulation of Intracellular pH: A Potential New Class of Anticancer Drugs

Daniela Rotin, Peter Wan, Sergio Grinstein, et al.


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/6/1497

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.