The chronic administration of drugs that inhibit the regulation of intracellular pH: in vitro and anti-tumour effects

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Summary Mean values of extracellular pH (pHe) in tumours tend to be about 0.5 pH units lower than in normal tissues, whereas values of intracellular pH (pHi) in tumours and normal tissues are similar. Previous studies have shown that drugs that acidify cells at lower pHe such as nigericin, used alone or with agents that inhibit the regulation of pHi, have toxicity to cultured cells at pHi < 6.5 in short-term exposure; these agents also lead to modest anti-tumour effects in mice when given acutely. To evaluate the long-term effects of these drugs at levels of pHi that might occur commonly in tumours, we exposed cells for up to 72 h at pHi 6.8 or 7.2 in vitro. Nigericin (0.033 μM) caused time-dependent cell killing of murine KHT and EMT-6 cells at pHi 6.8 (but not at pHi 7.2) with a surviving fraction approximately 5 x 10⁻³ after 72 h exposure. Cell killing was increased in the presence of 4,4-diisothiocyanostilbene 2,2-disulphonic acid (DIDS), an inhibitor of Na⁺⁺-dependent HCO₃⁻/Cl⁻ exchange, and to a lesser extent in the presence of 5-(Nethyl-N-isopropyl)amiloride (EIPA), an inhibitor of Na⁺⁺/H⁺ exchange. Cell killing was exquisitely sensitive to the level of pHi. Osmotic pumps were used to obtain a 72 h continuous infusion of nigericin in mice; this led to dose-dependent killing of cells in KHT tumours with surviving fraction of approximately 0.1 at maximum tolerated doses. Hydralazine, which may cause tumour hypoxia and lower pHi as well as pHe, caused cytotoxicity when given alone by chronic infusion, and enhanced the cytotoxicity due to nigericin. The addition of DIDS and/or EIPA (using two pumps) further enhanced anti-tumour toxicity, with a surviving fraction of approximately 0.002 at tolerated doses of the four drugs used to treat KHT tumours. The experiments demonstrate the activity of drugs that inhibit the regulation of pHi against murine tumours when delivered by chronic infusion.

Keywords: continuous infusion; inhibition of pH regulation; tumour acidification

Limited vascularisation of solid tumours often leads to inadequate delivery of oxygen and other nutrients to some tumour cells and to poor clearance of metabolic products as compared with normal tissues (Tannock, 1968). Tumour cells tend to metabolise glucose by glycolysis even under well-oxygenated conditions. Especially in hypoxic regions, tumour cells depend on anaerobic glycolysis as an energy source with consequent production of lactic acid, and cleavage of this and other acids produced by metabolism may lead to tumour acidity. Measurements of extracellular pH (pHe) using microelectrodes have shown that the pHe of tumours is on average about 0.5 pH units lower than that of normal tissues, with tumour pHe, typically in the range 6.6–7.0 and normal tissue pHe, between pHe 7.1 and 7.6 (Wike-Hooley et al., 1984). Although pHe in solid tumours tends to be acidic, intracellular pH (pHi) measured by ¹⁹F-nuclear magnetic resonance (NMR) spectroscopy is usually found to have similar values in solid tumours and normal tissues (Vaupel et al., 1989). Gillies et al. (1994) have measured pHe and pHi in tumours simultaneously using an extracellular pH marker and confirmed that tumour pHe was about 0.5 pH units lower than pHi in the same tumour. These results indicate that tumour cells are exposed frequently to an acidic environment and that the cells have active mechanisms that regulate their pHi to physiological levels.

The difference in pHi between tumours and normal tissues provides an opportunity for tumour-selective therapy through the development of drugs that have increased toxicity at low pH (Tannock and Rotin, 1989). Agents with this property include ionophores, such as nigericin, which abolishes the pH gradient across the cell membrane. The viability of cells in an acidic microenvironment also depends on the activity of membrane-based exchangers that regulate pH (Rotin et al., 1989). Two major exchangers known to be involved in the regulation of pHi, under acidic conditions are the Na⁺⁺/H⁺ antiport (Johnson and Epel, 1976; Aronson et al., 1982; Moolenaar et al., 1984) and the Na⁺⁺-dependent HCO₃⁻/Cl⁻ exchanger (Thomas, 1977; L'Allemain et al., 1985; Cassel et al., 1988). The former is inhibited by amiloride and its substituted analogues, and the latter is inhibited by stilbene derivatives such as DIDS (4,4-diisothiocyanostilbene 2,2-disulphonic acid).

Our previous studies have shown that the ionophore nigericin leads to intracellular acidification and is toxic to tumour cells under acidic conditions in vitro (Rotin et al., 1987). Nigericin also causes cellular acidification in a murine tumour, and when combined with hydralazine, which decreases tumour blood flow, leads to killing of tumour cells (Newell et al., 1992). In a previous study comparing three analogues of amiloride, 5-(N-ethyl-N-isopropyl)amiloride (EIPA) was found to be 200-fold more potent than amiloride in inhibiting Na⁺⁺/H⁺ antiport activity (Maidorn et al., 1993). Increased killing of tumour cells was found after injection of nigericin, EIPA and hydralazine into mice, but the surviving fraction was generally >10⁻³ (Hasuda et al., 1994). Others have shown that the combination of nigericin and DIDS augments the effect of hyperthermia on tumour growth when both drugs are given before heating an experimental tumour (Lyons et al., 1993).

Previous experiments have studied the effects of agents such as nigericin, EIPA or DIDS, given as short-term exposures of up to 6 h in vitro or by bolus injection in vivo. Major toxicity in cell culture has been observed at pHe < 6.5 but, as tumour pHe is generally higher than 6.5, it is not surprising that only limited cell kill has been observed in vivo. Long-term exposure to amiloride has been shown to cause the inhibition of proliferation of cells in mice treated repeatedly with amiloride. Agents that inhibit the regulation of pH, are dose- and time-dependent in their cytotoxic effects, so that long-term exposure might lead to increased cell killing at levels of pHe 6.8 found commonly in solid tumours. The aim of the present study was to determine if prolonged exposure to...
these agents was associated with therapeutic activity against tumour cells in culture and in experimental tumours.

**Materials and methods**

**Cell lines**

Exponentially growing KHT fibrosarcoma cells and EMT-6 murine sarcoma cells were used in the present experiments. The cells were maintained in vitro in alpha-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 0.1 mg ml⁻¹ kanamycin. The cells were re-established from frozen stock at 3 month intervals and were tested routinely for mycoplasma.

**Animals**

Inbred female C3H/HeJ and Balb/c mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were 8–12 weeks old when used in experiments.

**Reagents**

Nigericin, DIDS and EIPA were purchased from Sigma (St Louis, MO, USA). Nigericin was dissolved in 10% ethanol solution for assays in vitro and in 70% ethanol solution for osmotic pump infusion. DIDS and EIPA were dissolved in 10% dimethyl sulfoxide (DMSO) for in vitro survival assays and DIDS was dissolved in 70% DMSO for pump infusion. 2',7'-Bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes (Eugene, OR, USA).

**In vitro survival assays**

In most experiments, cell survival was assessed after varying duration of exposure to drugs at pH 6.8 and 7.2. Medium (α-MEM + 10% FBS) was buffered to pH 6.8 or 7.2 by adding MES (25 mM) and MOPS (25 mM) respectively, and was exposed to humidified 95% air/5% carbon dioxide. The pH of the medium was checked after 18 h preincubation, re-adjusted by adding hydrochloric acid or sodium hydroxide and sterilised by passing through a 0.22 μm filter. Exponentially growing tumour cells were detached from their flasks using 0.025% trypsin and 0.01% EDTA, and 2 × 10⁶ cells were seeded into four 250 mm dishes in each group. After 3 h incubation to allow attachment of cells, various drugs were added; control cultures were treated with diluents. The pH of the medium was checked periodically. After varying periods of exposure to drugs, cells in one dish from each group were trypsinised, resuspended and counted. Serial dilutions of cells were then plated in triplicate in α-MEM + 10% FBS. After 8–9 days, colonies were stained with methylene blue and counted. The surviving fraction (SF) was calculated according to:

\[
SF = \frac{\text{cell number in suspension treated}}{\text{cell number in suspension control}} \times \frac{\text{plating efficacy treated}}{\text{plating efficacy control}}
\]

Here the control conditions refer to untreated cells at pH 7.2. To evaluate the influence of small differences in pH, on cell survival, some experiments were also performed at pH 6.7 and 6.9.

**Measurement of the activity of Na⁺/H⁺ and Na⁺-dependent HCO₃⁻/Cl⁻ exchangers**

Intracellular pH (pHi) was measured as described previously (Boyer and Tannock, 1992). Briefly, cells grown as a monolayer on glass coverslips were loaded with BCECF-AM. The coverslip was then placed into a cuvette using a specially designed holder aligned at an angle of 30° to the excitation beam. Fluorescence was determined using an Aminco Bowman Series 2 luminescence spectrometer (SLM Instrument, NY, USA) with excitation and emission wavelengths set to 495 nm and 525 nm respectively. To determine the activities of the Na⁺/H⁺ antiport and the Na⁺-dependent HCO₃⁻/Cl⁻ exchanger, cells were first acidified to pH 6.5 or 6.8 by placing cells in NMG containing ammonium chloride for 30 min followed by replacement with NMG. The activity of the Na⁺/H⁺ antiport in the presence or absence of EIPA was quantified by adding sodium chloride to the cuvette. Activity of the Na⁺-dependent HCO₃⁻/Cl⁻ exchanger in the presence or absence of DIDS was quantified by adding sodium bicarbonate to the cuvette in the presence of EIPA. H⁺ efflux via each exchanger was calculated as described previously (Boyer and Tannock, 1992).

**Long-term exposure to drugs in vivo**

KHT or EMT-6 tumours were generated by intramuscular injection of 1 × 10⁶ cells into the left hind leg of syngeneic C3H/HeJ or Balb/c mice respectively. Growth of tumour was monitored by passing the tumour-bearing leg through a strip of lucite with graded size holes, and tumour weight was estimated from the diameter of the tumour-bearing leg using a previously defined calibration curve. Mice were used in experiments when their ID₅₀s had grown to about 0.5 g in weight, which took approximately 7 days.

Alzet micro osmotic pumps (model 1003D, Alza, CA, USA), with a capacity of 100 μl and which pumped the fluid at a steady rate of 1 μl h⁻¹ for 72 h, were implanted into mice. The pumps were loaded with various concentrations of nigericin with or without hydralazine. In some experiments, DIDS and/or EIPA were placed in a separate pump since nigericin, hydralazine and DIDS all form an insoluble precipitate if combined together at high concentration. The pumps containing nigericin were transplanted into the abdominal cavity of tumour-bearing mice and those containing DIDS were implanted into the subcutaneous region of the dorsum of the mice. Tumour size and body weight were measured daily.

Four days after implantation of the pump(s), i.e. 24 h after termination of the period of continuous infusion of drugs, each tumour was excised, weighed, and minced with scissors in phosphate-buffered saline (PBS). A single-cell suspension was obtained by enzymatic digestion with trypsin (Difco) and DNAase I (Sigma) and dye-excluding cells were counted with a haemocytometer. The suspensions were diluted and plated in triplicate in α-MEM + 10% FBS (Hasuda et al., 1994). After 8–10 days, cells were fixed and stained with methylene blue and colonies were counted. Surviving fraction per tumour was calculated according to:

\[
\text{SF/tumour} = \left( \frac{\text{cells/gram treated}}{\text{cells/gram control}} \right) \times \text{tumour weight treated} \times \text{tumour weight control}
\]

**Measurement of tissue pHe**

Mice bearing the KHT or EMT-6 tumours were anaesthetised with sodium pentobarbital given i.p. at a dose of 50 mg kg⁻¹. The pHe was measured using a miniature glass electrode in a 21 gauge needle (model MI-408B, Microelectrodes) against a silver–silver chloride reference electrode (model MI-402, Microelectrodes) using a pH-meter (model pH103, Corning). The reference electrode was inserted subcutaneously on the back and the pH electrode was inserted in the tumour or muscle after incising the overlying skin. Measurements of pHe were made at 50 to 75 μm increments along a single track at a depth of 3–5 mm into...
the tumour. A minimum of three pH measurements per tumour were recorded (Newell et al., 1993). The effect of continuous infusion of hydralazine on tumour pH was evaluated in comparison with bolus injection. The dose of hydralazine by bolus peritoneal injection was 10 mg kg$^{-1}$. For continuous infusion, the concentration of hydralazine in osmotic pumps that were implanted in the peritoneal cavity was 12.5 mg ml$^{-1}$.

Results

Assessment of exchangers

Table I shows rates of H$^+$ efflux in KHT and EMT-6 cells. When the cells were acidified to pH 6.5, the rate of H$^+$ efflux via the Na$^+$/H$^+$ exchanger was higher than that via the Na$^+$-dependent HCO$_3^-$/Cl$^-$ exchanger in both cell lines. As expected (Boyer and Tannock, 1992), the activity of both exchangers tended to be lower in the cell lines at pH 6.8 than at pH 6.5, but the Na$^+$-dependent HCO$_3^-$/Cl$^-$ exchanger was then quantitatively more important.

In vitro studies

During 72 h exposure, the pH of medium was relatively stable with maximum changes of <0.1 pH units from baseline at pH 7.2 and pH 6.8. Figure 1a shows cell growth during long-term exposure to nigericin (0.033 μM) with or without EIPA (0.5 μM), these doses are about one-tenth of the minimum doses that are cytotoxic following short-term (i.e. up to 6 h) exposure at pH 6.5. Minimal suppression of cell growth was observed with drug treatment at pH 7.2. Control cells at pH 6.8 grew slowly, and there was loss of cells exposed to nigericin+EIPA at pH 6.8. Surviving fractions under these conditions are shown in Figure 1b. Exposure of controls for 48–72 h to pH 6.8 led to a surviving fraction of 0.03–0.10 in multiple experiments. Nigericin (0.033 μM) caused time-dependent cell killing of KHT tumour cells at pH 6.8 with a survival fraction of 0.0017 at 72 h as compared with untreated cells at pH 7.2 (surviving fraction approximately 0.06 as compared with untreated cells at pH 6.8). Cell killing was increased minimally in the presence of EIPA.

The dose-dependent effects of EIPA and DIDS combined with nigericin (0.033 μM) on the survival of KHT cells are shown in Figure 2: Only minor effects were observed for EIPA at concentrations up to 5 μM (Figure 2a). A concentration of 0.4 mm DIDS was highly toxic to cells when used alone, and 0.1 mM DIDS enhanced cytotoxicity of nigericin with survival fraction reduced approximately 100-fold compared with nigericin alone (Figure 2b).

The dose-dependent effects of nigericin on surviving fraction of KHT and EMT-6 cells following 72 h exposure at pH 6.8 or 7.2 are shown in Figure 3a and 3b respectively. Cell killing by nigericin was enhanced in the presence of 0.05 mM DIDS, and by DIDS+EIPA (5 μM).

The effects of pH on survival of KHT and EMT-6 cells following 72 h exposure to nigericin with or without DIDS and EIPA are shown in Figure 4. Cell killing is very sensitive

| Table I Rate of H$^+$ efflux (in mm H$^+$ min$^{-1}$) in KHT and EMT-6 cells |
| Activity of exchangers | pH after acidification |
| | pH 6.5 | pH 6.8 |
| KHT | Na$^+$/H$^+$ exchanger | 7.0 ± 0.2 | 1.6 ± 0.2 |
| Na$^+$-dependent HCO$_3^-$/Cl$^-$ exchanger | 6.3 ± 0.3 | 4.1 ± 0.4 |
| Both combined | 13.9 ± 0.6 | 6.9 ± 0.6 |
| EMT-6 | Na$^+$/H$^+$ exchanger | 5.9 ± 0.2 | 1.8 ± 0.2 |
| Na$^+$-dependent HCO$_3^-$/Cl$^-$ exchanger | 3.7 ± 0.3 | 4.0 ± 0.4 |
| Both combined | 10.3 ± 0.5 | 8.0 ± 0.7 |

Results have been corrected for differences in buffering capacity.
to \( \text{pH}_6 \), with a decrease in survival of 10-fold or greater for a decrement of 0.1 \( \text{pH} \) unit. At \( \text{pH}_6 \) 6.7, survival of KHT cells exposed to nigericin + DIDS with or without EIPA was below the limit of detection.

**In vivo experiments**

Micro-osmotic pumps were loaded with 0.6, 2.0 or 6.0 mg ml\(^{-1}\) nigericin and implanted into mice. The estimated total release of nigericin over 72 h is about 2.2, 7.2 and 22 mg kg\(^{-1}\) body weight, and all of these doses were tolerated by mice. No severe early weight loss or late side-effects up to 2 weeks were observed. The maximum tolerated dose is close to 22 mg kg\(^{-1}\), as when DIDS (10 mg ml\(^{-1}\)) was added to the pumps death of the animals was observed; this compared with a maximum tolerated dose of 2.5 mg kg\(^{-1}\) nigericin by bolus injection (Hasuda et al., 1994). Neither weight loss nor abnormal behaviour was observed with administration up to 2 mg ml\(^{-1}\) nigericin and 10 mg ml\(^{-1}\) DIDS from the osmotic pumps.

Our unpublished data on pharmacokinetics of EIPA show a half-life of about 30 min in plasma of mice with slow conversion to the less potent amiloride, the concentration of which is still lower than that of EIPA up to 2 h later. Although EIPA administered from osmotic pumps containing a concentration of 3 mg ml\(^{-1}\) was not toxic to mice, the combination of nigericin + DIDS + hydralazine + EIPA caused death of Balb/c mice implanted with EMT-6 tumours.

**Figure 2** Effects of varying concentration of EIPA (a) or DIDS (b) alone or with nigericin (0.033 \( \mu \)M) on survival of KHT cells after 72 h exposure *in vitro*. Open symbols are for data at \( \text{pH}_6 \) 7.2 and closed symbols are for data at \( \text{pH}_6 \) 6.8. □, Without nigericin; ○, with nigericin. Points represent means ± standard deviation from three or more experiments.

**Figure 3** Effect of varying concentration of nigericin on survival of KHT (a) and EMT-6 (b) cells after 72 h exposure with or without DIDS (50 \( \mu \)M) or DIDS plus EIPA (5 \( \mu \)M). Open symbols are for data at \( \text{pH}_6 \) 7.2 and closed symbols are for data at \( \text{pH}_6 \) 6.8. □, Nigericin alone; ○, nigericin with DIDS; ○, nigericin with DIDS and EIPA. Points represent means ± standard deviation from three experiments.
The growth of KHT tumours during continuous 72 h exposure to various concentrations of nigericin with or without DIDS is shown in Figure 5a. Continuous administration of nigericin caused significant delay of tumour growth, which was enhanced in the presence of DIDS. The mean rate of regrowth was also slower than that of control tumours after these treatments. Figure 5b shows the surviving fraction per tumour: nigericin caused dose-dependent killing of KHT cells in mice when given by 72 h infusion, although the effect of nigericin at the highest tolerated dose was to reduce survival only to approximately $10^{-1}$. There was slight enhancement of cell killing in the presence of DIDS. The relationship between growth delay and surviving fraction is known to be complex owing to cell killing, environmental effects on potentially lethal damage, anti-proliferative effects and the proliferation and removal of damaged cells. The differences in survival fraction between each treated group and control shown in Figure 5b are consistent with or greater than the difference in tumour weight after 72 h treatment, which is shown in Figure 5a.

**Figure 4** Effect of pH on survival of KHT (a) or EMT-6 (b) cells exposed for 72 h to various agents. □, control (diluent only); ○, nigericin (0.033 μM); △, nigericin with DIDS and EIPA (5 μM). Each point represents mean ± standard deviation from three experiments.

**Figure 5** Growth of KHT tumours in mice during continuous infusion of agents from osmotic pumps. □, Diluents alone in pump; ○, 0.6 mg ml$^{-1}$ nigericin; △, 2.0 mg ml$^{-1}$ nigericin; △, 6.0 mg ml$^{-1}$ nigericin. Open and closed symbols represent nigericin alone and nigericin with 10 mg ml$^{-1}$ DIDS respectively. Each point represents mean ± standard deviation from four or more tumours. (b) Surviving fraction per KHT tumours after 72 h continuous infusion of varying concentration of nigericin with or without 10 mg ml$^{-1}$ DIDS using osmotic pumps. □, Nigericin alone; ●, nigericin and DIDS. Each point represents mean ± standard deviation from three or more tumours.

**Measurement of tumour pH and acidification by hydralazine**

Measurements of pH, in KHT tumours are indicated in Figure 6. Estimates of pH, after administration of hydralazine were significantly lower than estimates of pH, without treatment ($P<0.05$, paired $t$ test). The fall in tumour
The ionophore nigericin, which lowers pH, by allowing exchange of intracellular K⁺ for extracellular H⁺, has been shown to be cytotoxic to cultured cells at pH<6.5 (Rotin et al., 1987). The average values of pH in solid tumours, however, are usually about pH 6.9, which is only 0.4–0.5 pH units lower than those in normal tissues (Wike-Hooley et al., 1984). These values can be lowered slightly by vasodilator drugs (Newell et al., 1992) or by infusion of glucose with or without insulin (Hwang et al., 1991; Jährle et al., 1993). In the present study, pH 6.8 was selected as representative of the pHs that might be achieved in tumours, and long-term exposures of up to 72 h to low concentrations of nigericin were shown to kill tumour cells at pH 6.8 in vitro. This observation indicates the potential for continuous administration of nigericin to kill cells in solid tumours as compared with normal tissues based on differences in pH (6.8 Vs 7.2).

We attempted to enhance the cytotoxicity of nigericin with DIDS, which is an inhibitor of the Na⁺/H⁺ exchanger, as well as with EIPA, which is an inhibitor of the Na⁺/H⁺ exchanger, as both of the exchangers may have important roles in regulating pH, under acidic conditions (Cassel et al., 1988; Grinstein et al., 1989). In previous experiments, short-term exposure to EIPA or DIDS increased the killing of tumour cells by nigericin (Maidorn et al., 1993; Luo and Tannock, 1994) when used at pH 6.5. We measured the activity of both exchangers and confirmed our previous results, which indicate that the activity of the Na⁺/dependent HCO₃⁻/Cl⁻ exchanger is quantitatively more important in regulating pH, at values of pH, just below the physiological range (Boyer and Tannock, 1992). Consistent with this observation, we found that EIPA alone enhanced cell killing of KHT cells by nigericin only slightly at pH 6.8, whereas DIDS caused a greater enhancement of cell killing by nigericin. However, if EIPA was given in combination with nigericin and DIDS, there was increased cell killing at pH 6.8 in culture, so there may be therapeutic potential from pharmacological inhibition of both exchangers.

We demonstrated that small differences in pH between pH 6.7 and 6.9 greatly affected the degree of killing by nigericin with or without EIPA or DIDS in vitro. This exquisite sensitivity to pH suggests that there is considerable potential in combining the current approaches with treatments that lower pH in tumour tissue.

Our in vitro experiments have shown that chronic administration of nigericin can lead to a decrease in surviving fraction in both KHT and EMT-6 tumours, and that this effect is augmented by hydralazine, which inhibits tumour blood flow and lowers tumour pH, as well as pH, (Bhujwalla et al., 1990). The increase in cell killing caused by hydralazine could be due to acidification of tumour or to

**Table II**

| Treatment | Tumour weight (g) | No. of cells g⁻¹ (x 10⁷) | SF/tumour |
|-----------|------------------|--------------------------|-----------|
| **KHT**   |                  |                          |           |
| Control   | 1.17±0.45        | 1.68±0.52                | 1.0       |
| Nigericin 2.0 mg ml⁻¹ | 0.75±0.10 | 1.21±0.37 | 0.18±0.07 |
| Hydralazine 12.5 mg ml⁻¹ | 0.85±0.12 | 1.23±0.44 | 0.40±0.08 |
| Nigericin 2.0 mg ml⁻¹ + hydralazine 12.5 mg ml⁻¹ | 0.55±0.18 | 0.79±0.26 | 0.10±0.06 |
| Nigericin 2.0 mg ml⁻¹ + hydralazine 12.5 mg ml⁻¹ + DIDS 10 mg ml⁻¹ | 0.52±0.06 | 0.45±0.07 | 0.03±0.01 |
| Nigericin 2.0 mg ml⁻¹ + hydralazine 12.5 mg ml⁻¹ + DIDS 10 mg ml⁻¹ + EIPA 3 mg ml⁻¹ | 0.30±0.11 | 0.05±0.02 | 0.002±0.001 |
| **EMT-6** |                  |                          |           |
| Control   | 1.12±0.06        | 1.48±0.49                | 1.0       |
| Nigericin 2.0 mg ml⁻¹ + hydralazine 12.5 mg ml⁻¹ | 0.95±0.05 | 0.71±0.24 | 0.22±0.08 |
| Nigericin 2.0 mg ml⁻¹ + hydralazine 12.5 mg ml⁻¹ + DIDS 10 mg ml⁻¹ | 0.57±0.11 | 0.18±0.11 | 0.02±0.01 |

The drugs were given by 72 h infusion from micro-osmotic pumps and the concentration of each drug in the pump is indicated.

Values are mean±s.d. Each group comprised four or more tumours from three or more individual experiments.

**Figure 6** Extracellular pH (pHₑ) in KHT tumours with or without treatment with hydralazine by bolus injection (●) or by continuous infusion (○). □ Control. The dose of hydralazine by bolus injection was 10 mg kg⁻¹. The concentration of hydralazine in osmotic pumps was 12.5 mg ml⁻¹, leading to delivery of approximately 36 mg kg⁻¹ body weight over 72 h (i.e. 12.5 μg h⁻¹). Values are mean±s.d. Each point comprised four or more tumours. Values at 2 h after bolus injection, and at 24 h and 48 h after start of the infusion, are significantly different from control (P<0.05, paired t-test).

**Discussion**

The concentration of hydralazine in osmotic pumps was 12.5 mg ml⁻¹, leading to delivery of approximately 36 mg kg⁻¹ body weight over 72 h (i.e. 12.5 μg h⁻¹). Values are mean±s.d. Each point comprised four or more tumours. Values at 2 h after bolus injection, and at 24 h and 48 h after start of the infusion, are significantly different from control (P<0.05, paired t-test).
trapping of the drugs within the tumour as a result of decreased blood flow (Parks et al., 1994).

We found that DIDS enhanced cell killing in vitro by nigericin, although cell survival was reduced only to approximately 0.02 at tolerated doses in EMT-6 tumours in the presence of hydralazine. The effect of DIDS was greater than that in the absence of hydralazine. This observation is consistent with results obtained in vitro, which showed that DIDS enhanced cell killing by nigericin at lower pHs. Although we applied EIPA only to CHO/HeLa mice by continuous infusion, the greatest cell killing was observed when both exchangers were inhibited, consistent with results obtained in culture. This suggests the importance of inhibition of both the Na+/H+ antipor and the Na+-dependent HCO3-/Cl- exchanger in maximising this approach to tumour therapy.

There is evidence that the acute administration of agents that acidify cells may enhance the effects of hyperthermia against experimental tumours (Miyakoshi et al., 1986; Ruifrok et al., 1987; Kim et al., 1991; Song et al., 1993). Our current results using chronic administration of such agents might have relevance to studies of hyperthermia, although repeated or prolonged heat treatments within 72 h would only be useful if thermotolerance were inhibited.

The potential for using continuous infusion of drugs in patients is greater than in small animals, in which multiple infusions are technically difficult. Our experiments have shown: (i) the feasibility of selective cell killing in culture at pH 6.8, a value that may be representative for some regions of solid tumours, and (ii) the ability to obtain anti-tumour effects in an animal model. The exquisite sensitivity of cell survival to pH, at values close to 6.8 suggests the potential for enhancing therapeutic effects through mechanisms that lower tumour pH slightly and/or for using alternative measures to kill non-acidic tumour cells. Such experiments are in progress in our laboratory.

Abbreviations

EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; DIDS, 4,4-diisothio-cyanstilbene 2,2'-disulphonic acid; nMEM alpha-minimum essential medium; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5- and 6-carboxyfluorescein acetoxymethyl ester; FBS, fetal bovine serum; pHm, extracellular pH; pHi, intracellular pH.

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