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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 (pH_e) in tumours tend to be about 0.5 pH units lower than in normal tissues, whereas values of intracellular pH (pH_i) in tumours and normal tissues are similar. Previous studies have shown that drugs that acidify cells at lower pH_e such as nigericin, used alone or with agents that inhibit the regulation of pH_i, have toxicity to cultured cells at $pH_e < 6.5$ 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 pH_e that might occur commonly in tumours, we exposed cells for up to 72 h at pH_e 6.8 or 7.2 *in vitro*. Nigericin (0.033 μM) caused time-dependent cell killing of murine KHT and EMT-6 cells at pH_e 6.8 (but not at pH_e 7.2) with a surviving fraction approximately 5×10^{-3} after 72 h exposure. Cell killing was increased in the presence of 4,4-diisothiocyanstilbene 2,2-disulphonic acid (DIDS), an inhibitor of Na^+-dependent HCO_3^-/Cl^- exchange, and to a lesser extent in the presence of 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), an inhibitor of Na^+/H^+ exchange. Cell killing was exquisitely sensitive to the level of pH_e. 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 pH_i as well as pH_e, caused cytotoxity 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 pH_i 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 clearance of this and other acids produced by metabolism may lead to tumour acidity. Measurements of extracellular pH (pH_e) using microelectrodes have shown that the pH_e of tumours is on average about 0.5 pH units lower than that of normal tissues, with tumour pH_e typically in the range 6.6-7.0 and normal tissue pH_e between pH_e 7.1 and 7.6 (Wike-Hooley *et al.*, 1984). Although pH_e in solid tumours tends to be acidic, intracellular pH (pH_i) measured by ^{31}P-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 pH_i and pH_e in tumours simultaneously using an extracellular pH marker and confirmed that tumour pH_e was about 0.5 pH units lower than pH_i 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 pH_i to physiological levels.

The difference in pH_e between tumours and normal tissues provides an opportunity for tumour-selective therapy through the development of drugs that have increased toxicity at low pH_e (Tannock and Rotin, 1989). Agents with this property include ionophores, such as nigericin, which abolish 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_i (Rotin *et al.*, 1989). Two major exchangers known to be involved in the regulation of pH_i under acidic conditions are the Na^+/H^+ antiport (Johnson and Epel, 1976; Aronson *et al.*, 1982; Moolenaar *et al.*, 1984) and the Na^+-dependent HCO_3^-/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-diisothiocyanstilbene 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 $\geq 10^{-2}$ (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 $pH_e < 6.5$ but, as tumour pH_e 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 culture (Szolgay-Daniel *et al.*, 1991). Sparks *et al.* (1983) have demonstrated suppression of growth of the H6 hepatoma and DMA/J mammary carcinoma in mice treated repeatedly with amiloride. Agents that inhibit the regulation of pH_i are dose- and time-dependent in their cytotoxic effects, so that long-term exposure might lead to increased cell killing at levels of pH_e found commonly in solid tumours. The aim of the present study was to determine if prolonged exposure to

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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 sulphoxide (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_e 6.8 and 7.2. Medium (α-MEM + 10% FBS) was buffered to pH_e 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_e of the medium was checked after 18 h preincubation, readjusted 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 2×10^5 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:

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

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

Measurement of the activity of Na^+/H^+ and Na^+-dependent HCO_3^-/CL^- exchangers

Intracellular pH (pH_i) 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 HCO_3^-/Cl^- exchanger, cells were first acidified to pH_i approximately 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 HCO_3^-/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 1×10^6 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 tumours 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 formed 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:

$$\text{SF/tumour} = \frac{(\text{cells/gram } \textit{treated}) \times \text{ tumour weight } \textit{treated}}{\text{cells/gram } \textit{control}) \times \text{ tumour weight } \textit{control}} \times \frac{\text{plating efficacy } \textit{treated}}{\text{plating efficacy } \textit{control}}$$

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 pH_e 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 pH_e 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_e measurements per tumour were recorded (Newell *et al.*, 1993). The effect of continuous infusion of hydralazine on tumour pH_e 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_i 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_i 6.8 than at pH_i 6.5, but the Na^+-dependent HCO_3^-/Cl^- exchanger was then quantitatively more important.

In vitro *studies*

During 72 h exposure, the pH_e of medium was relatively stable with maximum changes of <0.1 pH units from baseline at pH_e 7.2 and pH_e 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_e 6.5. Minimal suppression of cell growth was observed with drug treatment at pH_e 7.2. Control cells at pH_e 6.8 grew slowly, and there was loss of cells exposed to nigericin $\pm$ EIPA at pH_e 6.8. Surviving fractions under these conditions are shown in Figure 1b. Exposure of controls for 48–72 h to pH_e 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_e 6.8 with a survival fraction of 0.0017 at 72 h as compared with untreated cells at pH_e 7.2 (surviving fraction approximately 0.06 as compared with untreated cells at pH_e 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_e 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_e 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

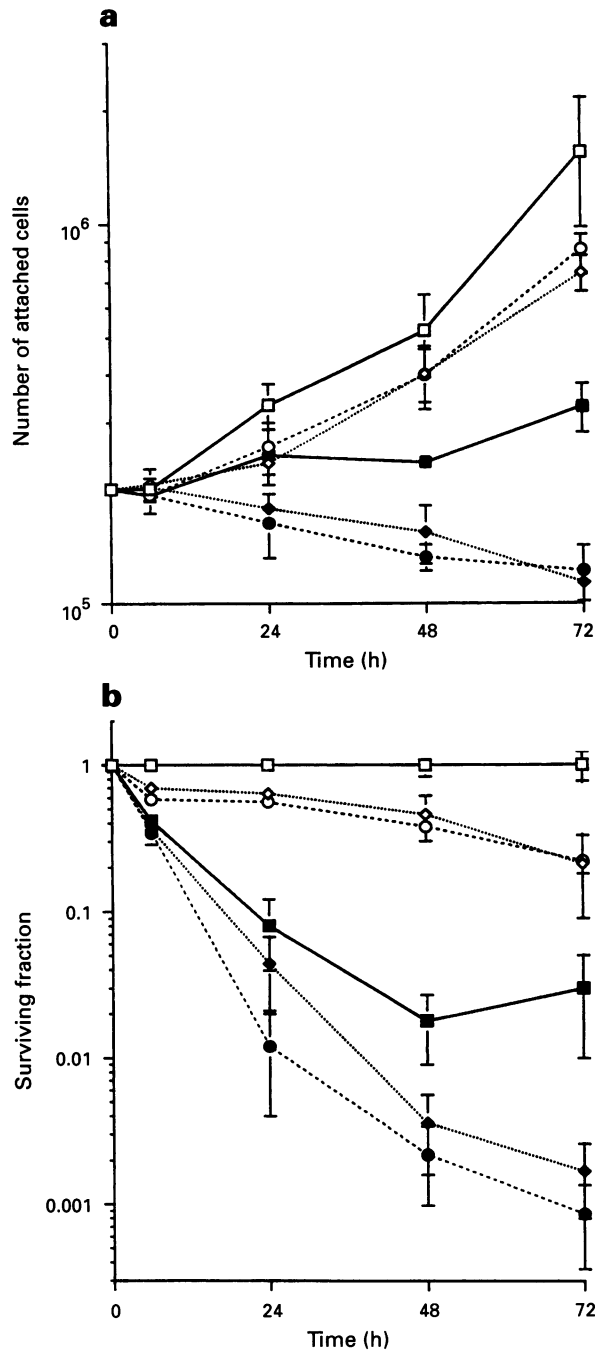

Figure 1 Growth (**a**) and survival (**b**) of KHT cells during 72 h exposure to nigericin 0.033 μM with or without EIPA 0.5 μM at pH_e 7.2 or 6.8 *in vitro*. Open symbols are for data at pH_e 7.2 and closed symbols are for data at pH_e 6.8. □, Control (diluent only); ◇, nigericin alone; ○, nigericin and EIPA. Each point represents the mean $\pm$ standard deviation from three experiments.

Table I Rate of H^+ efflux (in mM H^+ min^{-1}) in KHT and EMT-6 cells

Activity of exchangers	*pH_i after acidification* pH_i 6.5	pH_i 6.8
KHT		
Na^+/H^+ exchanger	7.0 $\pm$ 0.2	1.6 $\pm$ 0.2
Na^+-dependent HCO_3^-/Cl^- exchanger	6.3 $\pm$ 0.3	4.1 $\pm$ 0.4
Both combined	13.9 $\pm$ 0.6	6.9 $\pm$ 0.6
EMT-6		
Na^+/H^+ exchanger	5.9 $\pm$ 0.2	1.8 $\pm$ 0.2
Na^+-dependent HCO_3^-/Cl^- exchanger	3.7 $\pm$ 0.3	4.0 $\pm$ 0.4
Both combined	10.3 $\pm$ 0.5	8.0 $\pm$ 0.7

Results have been corrected for differences in buffering capacity.

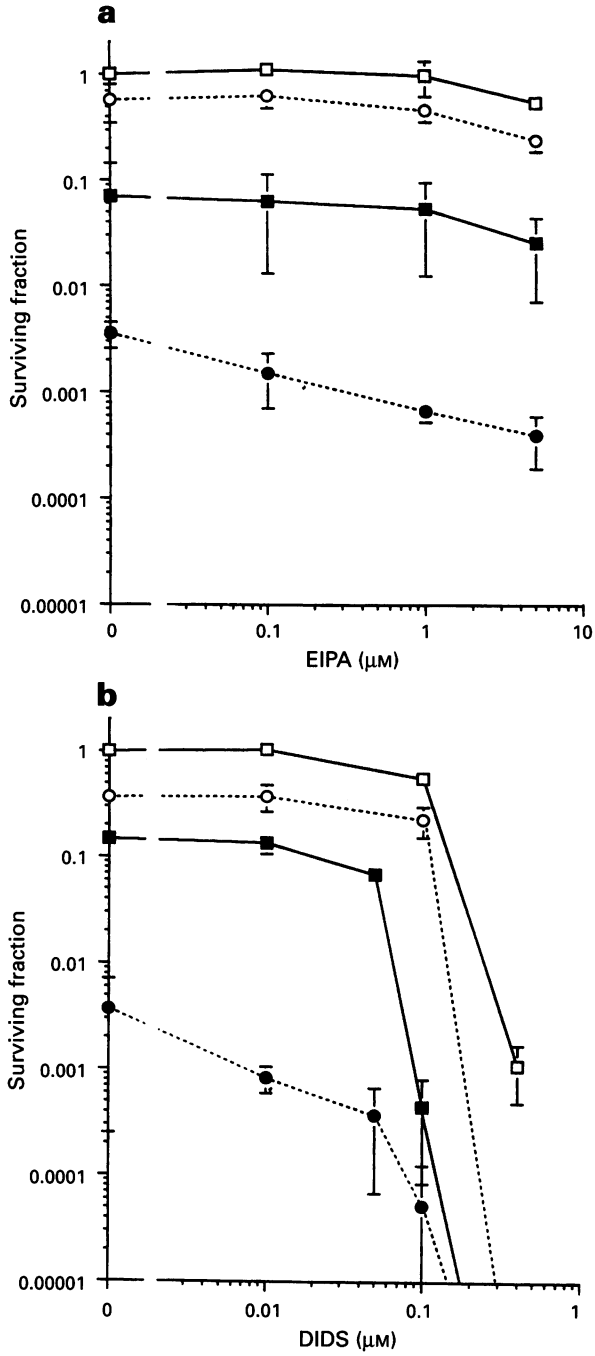

Figure 2 Effects of varying concentration of EIPA (**a**) or DIDS (**b**) alone or with nigericin (0.033 μM) on survival of KHT cells after 72 h exposure *in vitro*. Open symbols are for data at pH_e 7.2 and closed symbols are for data at pH_e 6.8. □, Without nigericin; ○, with nigericin. Points represent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

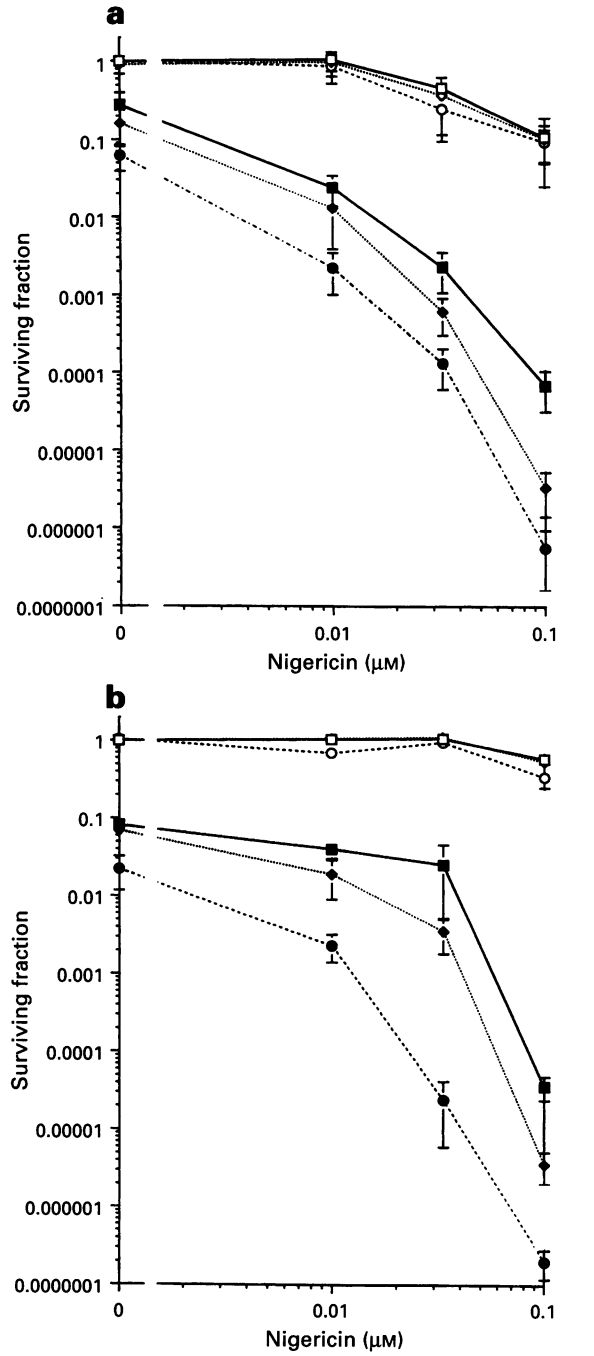

Figure 3 Effect of varying concentration of nigericin on survival of KHT (**a**) and EMT-6 (**b**) cells after 72 h exposure with or without DIDS (50 μM) or DIDS plus EIPA (5 μM). Open symbols are for data at pH_e 7.2 and closed symbols are for data at pH_e 6.8. □, Nicericin alone; ◇, nigericin with DIDS; ○, nigericin with DIDS and EIPA. Points represent means ± standard deviation from three experiments.

to pH_e, with a decrease in survival of 10-fold or greater for a decrement of 0.1 pH unit. At pH_e 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.

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 compex 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.

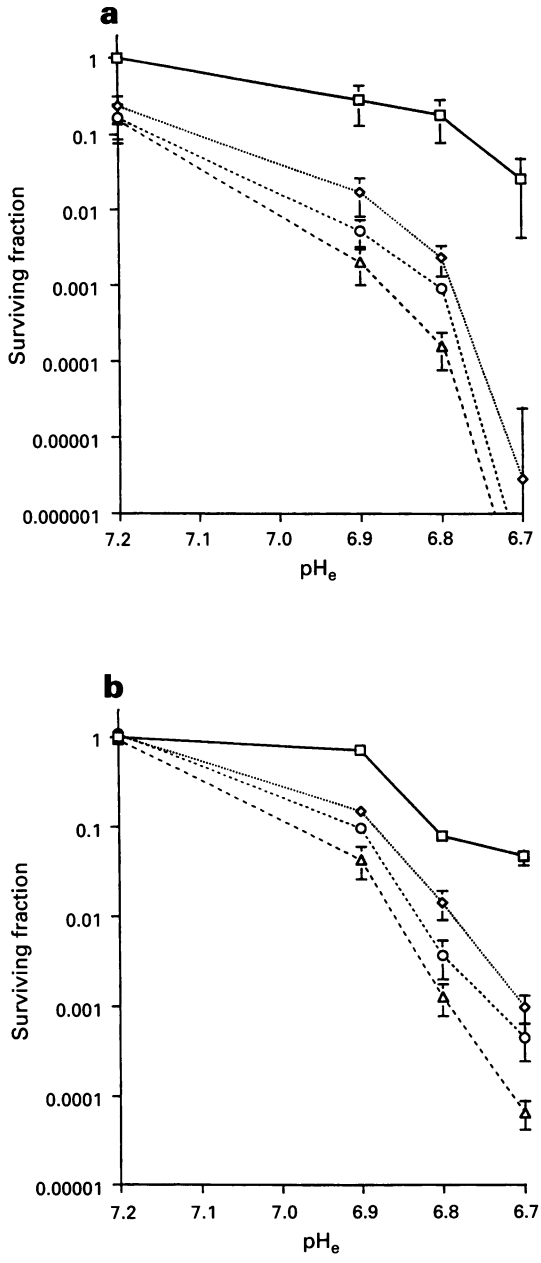

Figure 4 Effect of pH_e on survival of KHT (**a**) or EMT-6 (**b**) cells exposed for 72 h to various agents. □, control (diluent only); ◇, nigericin (0.033 μM); ○, nigericin with DIDS (50 μM); △, nigericin with DIDS and EIPA (5 μM). Each point represents mean ± standard deviation from three experiments.

Measurement of tumour pH_e and acidification by hydralazine

Measurements of pH_e in KHT tumours are indicated in Figure 6. Estimates of pH_e after administration of hydralazine were significantly lower than estimates of pH_e without treatment ($P<0.05$, paired t test). The fall in tumour

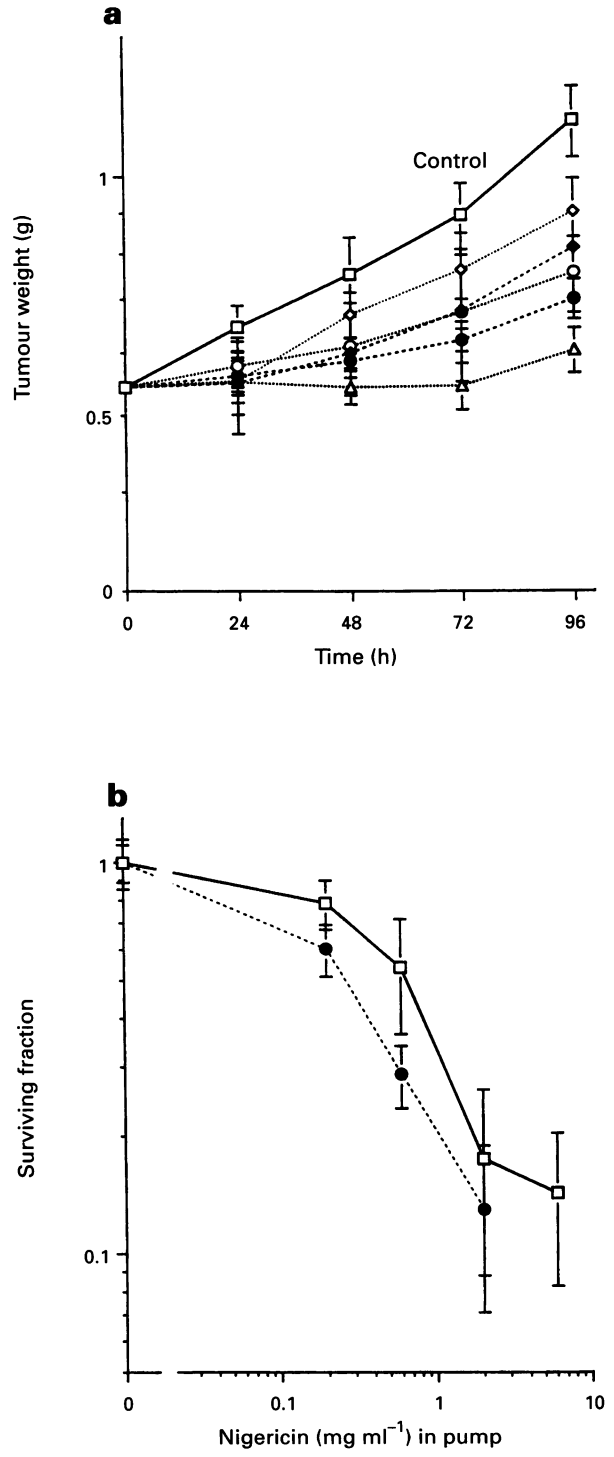

Figure 5 Growth of KHT tumours in mice during continuous infusion of agents from osmotic pumps. □, Diluents alone in pump; ◇, 0.6 mg ml^{-1} nigericin; ○, 2.0 mg ml^{-1} nigericin; △, 6.0 mg ml^{-1} nigericin. Open and closed symbols represent nigericin alone and nigericin with 10 mg ml^{-1} DIDS respectively. 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.

pH_e after bolus injection was observed only up to 4 h with a maximal decrease at 2 h after injection, whereas that obtained by continuous infusion lasted throughout the period of infusion.

Table II indicates surviving fractions of cells in KHT and EMT-6 tumours of mice treated with continuous 72 h infusions of nigericin, hydralazine and DIDS via two infusion pumps. Mice were able to tolerate this combination of drugs and there were no animal deaths up to 2 weeks. The combination of nigericin and hydralazine caused an approximately 2-fold increase in cell killing as compared with nigericin alone. If DIDS was given with this combination of agents, the surviving fraction per tumour was reduced to about 0.02–0.03.

When EIPA was added to the second osmotic pump together with DIDS, this led to death of Balb/c mice implanted with EMT-6 tumours. C3H mice bearing KHT tumours were able to tolerate this treatment. EIPA caused a marked augmentation in cell killing by the combination of nigericin, DIDS and hydralazine with a surviving fraction per tumour reduced approximately 10-fold compared with the combination of the drugs without EIPA.

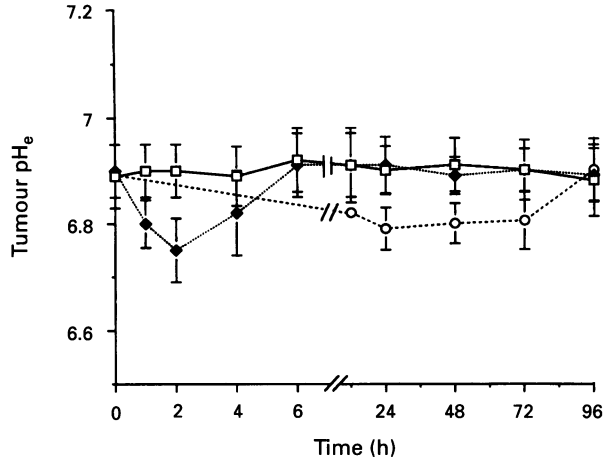

Figure 6 Extracellular pH (pH_e) in KHT tumours with or without treatment with hydralazine by bolus injection (◆) or by continuous infusion (○). □, Control. The dose of hydralazine by bolus injection was $10\,mg\,kg^{-1}$. The concentration of hydralazine in osmotic pumps was $12.5\,mg\,ml^{-1}$, leading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

Discussion

The ionophore nigericin, which lowers pH_i by allowing exchange of intracellular K^+ for extracellular H^+, has been shown to be cytotoxic to cultured cells at $pH_e \leqslant 6.5$ (Rotin *et al.*, 1987). The average values of pH_e in solid tumours, however, are usually about pH_e 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; Jähde *et al.*, 1992). In the present study, pH_e 6.8 was selected as representative of the pH_e 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_e 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_e (6.8 *vs* 7.2).

We attempted to enhance the cytotoxicity of nigericin with DIDS, which is an inhibitor of the Na^+-dependent HCO_3^-/Cl^- exchanger, and with EIPA, which is an inhibitor of the Na^+/H^+ exchanger, as both of the exchangers may have important roles in regulating pH_i 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_e 6.5. We measured the activity of both exchangers and confirmed our previous results, which indicate that the activity of the Na^+-dependent HCO_3^-/Cl^- exchanger is quantitatively more important in regulating pH_i at values of pH_e 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_e 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_e 6.8 in culture, so there may be therapeutic potential from pharmacological inhibition of both exchangers.

We demonstrated that small differences in pH_e 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_e suggests that there is considerable potential in combining the current approaches with treatments that lower pH_e in tumour tissue.

Our *in vivo* 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_i as well as pH_e (Bhujwalla *et al.*, 1990). The increase in cell killing caused by hydralazine could be due to acidification of tumour or to

Table II Surviving fraction per tumour for KHT and EMT-6 tumours following various treatments

Treatment	*Tumour weight (g)*	*No. of cells g^{-1} (× 10^7)*	*SF/tumour*
KHT			
Contol	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 72h 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.

trapping of the drugs within the tumour as a result of decreased blood flow (Parkins *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 pH_e. Although we could apply EIPA only to C3H/HeJ 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^+ antiport and the Na^+-dependent HCO_3^-/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_e 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_e at values close to 6.8 suggests the potential for enhancing therapeutic effects through mechanisms that lower tumour pHe 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-diisothiocyanstilbene 2,2-disulphonic acid; αMEM alpha-minimum essential medium; BCECF-AM 2′,7′-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxymethyl ester; FBS, fetal bovine serum; pH_e, extracellular pH; pH_i, intracellular pH.

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References

ARONSON PS, NEE J AND SUHM MA. (1982). Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Nature*, **299**, 161–163.

BHUJWALLA ZM, TOZER GM, FIELD SB, MAXWELL RJ AND GRIFFITHS JR. (1990). The energy metabolism of RIF-1 tumours following hydralazine. *Radiother. Oncol.*, **19**, 281–291.

BOYER MJ AND TANNOCK IF. (1992). Regulation of intracellular pH in tumour cell lines: influence of microenvironmental conditions. *Cancer Res.*, **52**, 4441–4447.

CASSEL D, SCHARF O, ROTMAN M, CRAGOE JR EJ AND KATZ M. (1988). Characterization of Na^+-linked and Na^+-independent Cl^-/HCO_3^- exchange systems in Chinese hamster lung fibroblastoma. *J. Biol. Chem.*, **263**, 6122–6127.

GILLIES RJ, LIU Z AND BHUJWALLA Z. (1994). ^{31}P-MRS measurements of extracelluar pH of tumors using 3-aminopropylphosphonate. *Am. J. Physiol.*, **267**, C195–203.

GRINSTEIN S, ROTIN D AND MASON MJ. (1989). Na^+/H^+ exchanger and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim Biophys. Acta*, **988**, 73–97.

HASUDA K, LEE C AND TANNOCK IF. (1994). Anti-tumor activity of nigericin and 5-(*N*-ethyl-*N*-isopropyl) amiloride: an approach to therapy based on cellular acidification and the inhibition of regulation of intracellular pH. *Oncol. Res.*, **6**, 259–268.

HWANG YC, KIM S-G, EVELHOCK JL, SEYEDSADR M AND ACKERMANN JH. (1991). Modulation of murine radiation-induced fibrosarcoma-1 tumor metabolism and blood flow *in situ* via glucose and mannitol administration monitored by ^{31}P and ^{2}H nuclear magnetic resonance spectroscopy. *Cancer Res.*, **51**, 3108–3118.

JÄHDE E, VOLK T, ATEMA A, SMETS LA, GLÜSENKAMP K-H AND RAJEWSKY MF. (1992). pH in human tumor xenografts and transplanted rat tumors: effect of insulin, inorganic phosphate, and m-iodobenzylguanidine. *Cancer Res.*, **52**, 6209–6215.

JOHNSON JD AND EPEL D. (1976). Intracellular pH and activation of sea urchin eggs after fertilizaton. *Nature*, **304**, 371–374.

KIM GE, LYONS JC AND SONG CW. (1991). Effects of amiloride on intracellular pH and thermosensitivity. *Int. J. Radiat. Oncol. Biol. Phys.*, **20**, 541–549.

L'ALLEMAIN G, PARIS S AND POUYSSÉGUR J. (1985). Role of a Na^+-dependent Cl^-/HCO_3^- exchanger in regulation of intracellular pH in fibroblasts. *J. Biol. Chem.*, **260**, 4877–4883.

LUO J AND TANNOCK IF. (1994). Inhibition of the regulation of intracellular pH: potential of 5-(*N,N*-hexamethylene) amiloride in tumour selective therapy. *Br. J. Cancer*, **70**, 617–624.

LYONS JC, ROSS BD AND SONG CW. (1993). Enhancement of hyperthermia effect in vivo by amiloride and DIDS. *Int. J. Radiat. Oncol. Biol. Phys.*, **25**, 95–103.

MAIDORN RP, CRAGOE JR EJ AND TANNOCK IF. (1993). Therapeutic potential of analogues of amiloride: inhibition of the regulation of intracellular pH as a possible mechanism of tumour selective therapy. *Br. J. Cancer*, **67**, 297–303.

MIYAKOSHI J, ODA W, HIRATA M, FUKUHORI N AND INAGAKI C. (1986). Effects of amiloride on thermosensitivity of Chinese hamster cells under neutral and acidic pH. *Cancer Res.*, **46**, 1840–1843.

MOOLENAAR WH, TERTOOLEN LGJ AND DE LAAT SW. (1984). The regulation of cytoplasmic pH in human fibroblasts. *J. Biol. Chem.*, **259**, 7563–7569.

NEWELL K, WOOD P, STRATFORD I AND TANNOCK I. (1992). Effects of agents which inhibit the regulation of intracellular pH on murine solid tumours. *Br. J. Cancer*, **66**, 311–317.

NEWELL K, FRANCHI A, POUYSSÉGUR J AND TANNOCK I. (1993). Studies with glycolysis-deficient cells suggest that production of lactic acid is not the only cause of tumor acidity. *Proc. Natl Acad. Sci. USA*, **90**, 1127–1131.

PARKINS C, CHADWICK JA AND CHAPLIN DJ. (1994). Enhancement of chlorambucil cytotoxity by combination with flavone acetic acid in a murine tumour. *Anticancer Res.*, **14**, 1603–1608.

RUIFROK ACC AND KONINGS AWT. (1987). Effects of amiloride on hyperthermic cell killing of normal and thermotolerant mouse fibroblast LM cells. *Int. J. Radiat. Biol.*, **52**, 385–392.

ROTIN D, WAN P, GRINSTEIN S AND TANNOCK I. (1987). Cytotoxity of compounds that interfere with the regulation of intracellular pH: a potential new class of anticancer drugs. *Cancer Res.*, **47**, 1497–1504.

ROTIN D, STEELE-NORWOOD D, GRINSTEIN S AND TANNOCK I. (1989). Requirement of the Na^+/H^+ exchanger for tumor growth. *Cancer Res.*, **49**, 205–210.

SONG WC, LYONS JC, GRIFFIN RJ, MAKEPEACE CM AND CRAGOE JR EJ. (1993). Increase in thermosensitivity of tumor cells by lowering intracellular pH. *Cancer Res.*, **53**, 1599–1601.

SPARKS RL, POOL TB, SMIKTH NKR AND CANERON IL. (1983). Effects of amiloride on tumor growth and intracellular element contact of tumor cells *in vitro*. *Cancer Res.* **43**, 73–77.

SZOLGAY-DANIEL E, CARLSSON J, ZIEROLD K, HOLTERMANN G, DUFAU E AND ACKER H. (1991). Effects of amiloride treatment on U-118 MG and U-251 MG human glioma and HT-29 human colon carcinoma cells. *Cancer Res.*, **51**, 1039–1044.

TANNOCK IF. (1968). The relation between cell proliferation and vascular system in a transplanted mouse mammary tumour. *Br. J. Cancer*, **22**, 258–273.

TANNOCK IF AND ROTIN D. (1989). Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res.*, **49**, 4373–4384.

THOMAS RC. (1977). The role of bicarbonate, chloride and sodium ions in the regulation of intracellular pH in snail neurones. *J. Physiol.*, **273**, 317–338.

VAUPEL P, KALLINOWSKY F AND OKUNIEFF P. (1989). Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res.*, **49**, 6449–6465.

WIKE-HOOLEY JL, HAVEMAN J AND REINHOLD JS. (1984). The relevance of tumour pH to the treatment of malignant disease. *Radiother. Oncol.*, **2**, 343–366.