Effects of agents which inhibit the regulation of intracellular pH on murine solid tumours

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Summary
Cell killing can be achieved in an acidic environment in tissue culture (medium pH < 7.0) by agents (nigericin, carbonylcyanide-3-chlorophenylhydrazone (CCCP)) which transport protons from the extracellular space into the cytoplasm. Cell killing is enhanced when these agents are used in combination with compounds (amiloride, 4,4'-disothiocyanostilbene-2,2'-disulfonic acid (DIDS)) which inhibit the membrane-based exchangers responsible for the regulation of intracellular pH (pHi). We describe experiments which assess the ability of these agents to kill tumour cells in spheroids and in vivo. Both nigericin and CCCP were observed to penetrate tissue based on their ability to kill tumour cells in spheroids. The mean extracellular pH (pHe) of the KHT fibrosarcoma and the EMT-6 sarcoma were observed to be 0.21 and 0.32 pH units more acidic than the mean pH in muscle tissue. Intrapertioneal (i.p.) administration of the vasodilator hydralazine (10 mg kg⁻¹) caused a reduction of the mean pHe of the KHT but not the EMT-6 tumour. Nigericin (2.5 mg kg⁻¹, i.p.) plus amiloride (10 mg kg⁻¹, i.p.) followed 30 min later by hydralazine (10 mg kg⁻¹, i.p.) reduced the surviving fraction of cells in the KHT and EMT-6 tumours, but had minimal effects on growth delay. When KHT tumours were treated with 15 Gy X-rays followed immediately by nigericin plus amiloride and hydralazine a reduced surviving fraction as well as an increase in tumour growth delay was observed compared to radiation alone. The administration of nigericin (2.5 mg kg⁻¹, i.p.) or the combination of nigericin (2.5 mg kg⁻¹, i.p.) followed by hydralazine (10 mg kg⁻¹, intravenous (i.v.)) resulted in reductions of tumour pH of 0.27 and 0.29 pH units respectively as determined by 31P magnetic resonance spectroscopy (MRS). Our results show that the combination of nigericin and hydralazine (with or without amiloride) can kill cells in rodent solid tumours and that cell killing is associated with a reduction in the mean pHi of tumour cells.

The pH of solid tumours has been determined by using pH-sensitive microelectrodes which measure predominantly pHf, and by using 31P-MRS which measures primarily pHi (Wike-Hooley et al., 1984; Vaupel et al., 1989). Values of pHf in solid tumours extend over a broad range (median 6.9 - 7.0) and are on average approximately 0.5 pH units more acidic than normal tissue pHf (median 7.4 - 7.5) (Wike-Hooley et al., 1984). Intracellular pH in solid tumours has been observed to have a wider range than that in normal tissue but median pHi (7.2) does not appear to be significantly lower in solid tumours as compared to normal tissue (Vaupel et al., 1989). Taken together these results indicate that cells in solid tumours are surrounded by an acidic extracellular fluid and that tumour cells are actively regulating their pHf to physiological levels. Since the above techniques have poor spatial resolution, it seems probable that microenvironments exist within solid tumours which are more acidic than the observed median value of pHf.

As solid tumours enlarge, deficient vascularisation as compared to normal tissue usually results in poor delivery of oxygen to many regions within them (Thomlinson & Gray, 1955; Tannock, 1968). Cells in a hypoxic microenvironment are dependent on anaerobic glycolysis for energy production and consequently produce large amounts of lactic acid. Glycolytic activity resulting in lactic acid production is thought to be a cause of tumour acidity (Wike-Hooley et al., 1984; Tannock & Rotin, 1989; Vaupel et al., 1989). However, in recent experiments from this laboratory tumours were generated from glycolysis-deficient variant cells which developed an acidic microenvironment in the absence of lactate production (Newell et al., unpublished observation). Regions of hypoxia probably coexist with regions of low pHf and possibly low pHi.

Hypoxic cells are known to be resistant to the effects of ionising radiation (Moulder & Rockwell, 1984; Vaupel et al., 1989). Cells at a distance from the vasculature may be resistant to chemotherapy due to limited penetration of agents to these cells, or the low proliferative rate of nutritionally-deprived cells may render them insensitive to the effects of drugs active against cycling cells (e.g. Tannock, 1982; Chaplin et al., 1985). Cells in a hypoxic/acidic microenvironment may therefore represent a subpopulation of tumour cells responsible for treatment failure. It might be possible to kill selectively cells in an acidic microenvironment in solid tumours by utilising the existing acidity to cause intracellular acidification.

Most cellular processes have pH optima at or near physiological pH (Trivedi & Danforth, 1966; Busa & Nuccitelli, 1984). The viability of cells in an acidic microenvironment depends therefore on the activity of membrane-based exchangers which regulate pHi (Rotin et al., 1989). When cells are exposed to a low pHe environment, the Na⁺/H⁺ antiporter (Grinstein et al., 1989) and the Na⁺ dependant HCO₃⁻/Cl⁻ exchanger (Cassel et al., 1988; Valburg-Reinertsen et al., 1988) are the two major membrane-based exchangers involved in the regulation of pHi. The Na⁺/H⁺ antiporter is inhibited by amiloride and the Na⁺ dependant H⁺/CO₃⁻/Cl⁻ exchanger is inhibited by stilbene derivatives such as DIDS.

Our laboratory has shown that it is possible to kill selectively cells in an acidic environment in vitro by using agents which cause intracellular acidification. Intracellular acidification leading to cell death was accomplished in vitro using agents which transport H⁺ from the extracellular space into the cytoplasm and this effect was enhanced by agents which inhibit the membrane-based mechanisms which regulate pHi. The ionophore nigericin (Rotin et al., 1987), which is capable of exchanging intracellular K⁺ for extracellular H⁺, or CCCP (Newell & Tannock, 1989), which is capable of transporting H⁺ equivalents into cells, as well as the weak acid succinate (Dobrowsky et al., 1991), were cytotoxic to tumour cells in vitro only at pH < 6.5. When nigericin or CCCP was combined with amiloride and/or the stilbene derivative DIDS, agents which inhibit the membrane-based exchangers responsible for the regulation of pHf, cytotoxicity was observed at pH < 7.0. In these in vitro studies cytotoxicity was...
associated with the ability to produce intracellular acidification.

The objective of the present study was to determine if agents which produce intracellular acidification and are selectively toxic at low pH in vitro have anti-tumour effects against two murine transplatable tumours.

Materials and methods

Cells

At the Ontario Cancer Institute, the KHT fibrosarcoma and the EMT-6 murine sarcoma cells were maintained in vitro in α-minimum essential medium (α-MEM) supplemented with 10% foetal bovine serum (FBS) and 0.1 mg ml⁻¹ kanamycin (complete medium). Both cell lines were maintained in culture for eight passages and then were passaged in syngeneic hosts. Routine tests for mycoplasma revealed that the cell lines were free from contamination. Tumour cells were discarded at 4–6 months intervals and re-initiated from frozen stock.

Spheroids

Spheroids were used in some experiments as a model of intermediate complexity between single cells and solid tumours to assess the ability of our agents to penetrate into tissue. Spheroids were grown from EMT-6 murine sarcoma cells. EMT-6 spheroids were initiated by seeding 10⁶ cells ml⁻¹ in 15 ml of FBS-containing α-MEM treated plastic dish. After 4 days cell aggregates were transferred into spinner flasks containing 200 ml of α-MEM minus bicarbonate supplemented with 25 mM hydroxyethylpiperazine-N₂-2-ethanesulfonic acid (Hepes) (pH 7.4), 10% FBS and 0.1 mg ml⁻¹ kanamycin (spheroid medium). Medium was changed every 48 h. Spheroid diameter was determined by measuring orthogonal diameters of a minimum of 30 spheroids and then determining a geometric mean diameter for the growing population. Spheroids were used for experiments when they had attained a mean diameter of approximately 800 μm.

To assess cell survival following exposure to potentially toxic agents, spheroids were washed once with α-MEM plus 10% FBS buffered with bicarbonate/[N-morpholino]-ethanesulfonic acid (Mes) (25 mM) to the desired pH and placed in 50 ml spinner flasks containing the same medium (Newell & Tannock, 1989). Spinner flasks were placed in a 37°C water bath and humidified 5% CO₂/95% air was continuously flowed over the suspensions (Whillans & Rauth, 1980). Medium pH drifted a maximum of 0.1 pH units during the exposure period. After 30 min equilibration, drugs were added. Samples were taken and spheroids were washed once with fresh complete medium and disaggregated by trypsinisation (0.05% trypsin in 0.53 mM ethylenediaminetetraacetic acid (Gitco; Grand Island, New York)) for 20 min at 37°C. The resulting single cell suspensions were counted electronically, diluted and plated in triplicate. After 11 days plates were stained and colonies containing greater than 50 cells were counted.

Tumours

At the Ontario Cancer Institute, the KHT fibrosarcoma and the EMT-6 sarcoma were propagated in 8–12 week old inbred female C3H/HeJ and BALB/c BYJ mice respectively. Mice were purchased from Jackson Laboratories (Bar Harbour, Maine). Tumours were initiated by intramuscular injection of 2.5–5.0 × 10⁵ cells into the left hind leg. Growth of tumours was monitored by passing the tumour-bearing leg through a strip of lucite with graded size holes. The diameter of the tumour-bearing leg was converted to an estimate of tumour weight using a previously defined calibration curve. Tumours were used for experiments when they had attained 0.3–0.5 g in weight, which required approximately 9 days for both tumour types.

The subline of the KHT fibrosarcoma that was used for studies of magnetic resonance spectroscopy (MRS) at the MRC Unit, UK was maintained by i.m. in vivo passage in C3H mice, with return to frozen stock after 15–20 passages. For MRS experiments tumours were generated by injecting a single cell suspension containing approximately 2 × 10⁶ cells intradermally on the back of the mouse. Tumours were studied after approximately 10 days growth, when they had a diameter of 6–8 mm.

Measurement of tumour pH

Mice were anaesthetised with tribromoethanol 0.50 mg kg⁻¹ mouse weight (Avertin). Measurements of pHe were made using a miniature glass electrode (model MI-408B, Microelectrodes Inc.) against a silver-silver chloride reference electrode (model MI-402, Microelectrodes Inc.) using a portable pH meter (model pH 103, Corning). The reference electrode was inserted subcutaneously on the back and the pH electrode was inserted directly into the tumour or muscle after incising the overlying skin. Measurements of tumour pHe were made at increments of 50–75 μm along a single track at a depth of 300–500 μm into the tumour by using a specially constructed micrometer to advance the electrode. A mean of 6 and a minimum of 4 pHe measurements were made per tumour.

Drugs

CCCP, nigericin, amiloride, hydralazine and all other chemicals were purchased from Sigma (St. Louis, Missouri). Mice were injected with various combinations of CCCP dissolved in 10% ethanol, nigericin dissolved in 10% ethanol, amiloride dissolved in sterile water, and hydralazine dissolved in sterile water. Nigericin was prepared and stored in glass vials due to its known ability to bind to plastic (Varnes et al., 1999). All drugs were delivered i.p. in a volume of 0.01 mg g⁻¹ body weight except for 31P-MRS experiments when hydralazine was administered i.v. Radiation was delivered to the tumour-bearing limb of unaesthetised mice restrained in a specially designed jig. The radiation source was a double-headed 100 kVp X-ray unit which has a dose rate of 10.2 Gy min⁻¹.

Excision assay

Tumours were excised 20–24 h after treatment, weighed and then minced with scissors in phosphate buffered saline (PBS). Single cell suspensions were prepared by enzymatic digestion with trypsin (Difco) and DNase I (Sigma) (Thomson & Rauth, 1974). Tumour cell suspensions were stained with trypan blue and dye-excluding cells were counted with a haemocytometer. Tumour cell suspensions were then diluted and plated in triplicate in complete medium. Plates were stained 11 days later and colonies containing greater than 50 cells were counted. Surviving fraction per tumour was calculated according to: SF/tumour = (plating efficiency treated/ plating efficiency control) × ((cells/gram treated)/(cells/gram control)).

Growth delay assay

Mice bearing tumours in the range of 0.3–0.5 g (8.5–9.5 mm leg diameter) were identified with ear tags and randomly distributed into treatment groups (minimum five mice per group). Mice were treated and leg diameters were recorded to the nearest 0.5 mm every 2–3 days by an observer who was unaware of the treatment history. Measurements were converted to estimates of tumour weight, and growth curves were compared for treated and control tumours. Mice were killed humanely when tumours attained a weight of approximately 1.5 g.
Tumour \(^{31}\)P-MRS measurements

Unanaesthetised mice bearing intradermal KHT tumours were restrained in a specially designed jig. MRS experiments were performed using a 4.7 Tesla 30 cm horizontal bore magnet (Oxford Instruments), interfaced with a SISCO 200 spectrometer. A 7 mm surface coil was placed over the tumour for RF transmission and signal collection. Acquisition parameters were set to minimise contamination from underlying tissue. Each spectrum consisted of 256 scans with a 2 s delay time, giving a total collection time of approximately 7 min per spectrum. Spectra from a single undistributed mouse were recorded at 15 min intervals up to 120 min after drug administration. Values of pH \(e\) were calculated by examining the change in the chemical shift of the inorganic phosphate peak relative to \(\alpha\)-ATP peaks. Since phosphocreatine was not present in the spectra of every tumour, this peak was not used to calculate pH.

Results

Spheroid toxicity

EMT-6 spheroids were exposed to concentrations of CCCP (15 \(\mu\)M) and nigericin (3.4 \(\mu\)M) which had been shown previously to be toxic to single cell suspensions (Rotin et al., 1987; Newell & Tannock, 1989). Cell killing was not observed for spheroids exposed to CCCP or nigericin at pH 7.4, or spheroids exposed to low pH (6.4) in the absence of ionophores (Figure 1). A time-dependent decrease in surviving fraction was observed for spheroids exposed to CCCP or nigericin at pH 6.4 (Figure 1), indicating that both drugs were able to penetrate to the interior cells of spheroids.

![Figure 1](image1.png)

Figure 1: Surviving fraction of cells from EMT-6 spheroids. Spheroids were exposed to the following conditions: pH 7.4, 6.4 (data not shown), pH 7.4, 6.4 plus 15 \(\mu\)M CCCP (\(\square\), ■), and pH 7.4, 6.4 plus 3.4 \(\mu\)M nigericin (\(\triangle\), ▲). No decrease in surviving fraction was observed for spheroids exposed to pH 7.4 or pH 6.4 in the absence of ionophores (Figure 1). Points represent mean and range from one experiment (range of values less than vertical extent of symbols). Qualitatively similar results have been obtained in a repeat experiment.

Tumour pH

The mean values of pH \(e\) in KHT (6.84) and EMT-6 (6.75) tumours were observed to be significantly more acidic than the mean pH \(e\) of muscle (7.06) (Table I). Since the in vitro cytotoxicity of the compounds to be tested increased sharply at lower values of pH \(e\), we attempted to decrease selectively tumour pH \(e\) by the i.p. administration of the vasodilator hydralazine, which has been shown to cause large transient increases in the hypoxic fraction of rodent solid tumours (Chaplin & Acker, 1987; Chaplin, 1989; Dunn et al., 1989). Forty-five minutes after hydralazine treatment there was a small increase in the pH differential between tumour and muscle tissue for both tumour types (Table I). For KHT tumours, tumour pH \(e\) was reduced and muscle pH \(e\) remained the same, whereas for EMT-6 tumours, tumour pH \(e\) remained the same and muscle pH \(e\) was increased.

Effect of nigericin and hydralazine on tumour pH

No reduction in pH \(e\) in KHT tumours was observed in control mice injected with PBS (Figure 2) or with hydralazine (10 mg kg\(^{-1}\), i.v.) (data not shown). Nigericin (2.5 mg kg\(^{-1}\), i.p.) caused a significant reduction in pH \(e\) with a maximum decrease of 0.27 pH units occurring 45 min after injection (Figure 2). The combination of nigericin followed by hydralazine was observed to cause a similar decrease in pH \(e\) (maximum 0.29 pH units) with a trend to a more prolonged period of acidification (Figure 2).

CCCP

The dose of CCCP which could be administered to mice was not limited by toxicity but by solubility of CCCP in sterile

![Figure 2](image2.png)

Figure 2: The effect of PBS (\(\bigcirc\)), nigericin (2.5 mg kg\(^{-1}\)) (\(\square\)), and nigericin (2.5 mg kg\(^{-1}\)) and hydralazine (10 mg kg\(^{-1}\)) (\(\triangle\)) on pH \(e\) in KHT tumours as determined by \(^{31}\)P-NMR. Points and error bars represent mean ± standard error from at least six tumours.

Table I: Effect of hydralazine on tumour pH

| Tissue   | pH \(e\) KHT | pH \(e\) EMT-6 | pH \(e\) 45 min after hydralazine (10 mg kg\(^{-1}\)) KHT | pH \(e\) 45 min after hydralazine (10 mg kg\(^{-1}\)) EMT-6 |
|----------|--------------|--------------|--------------------------------------------------|-----------------------------------------------------|
| Muscle   | 7.05 (0.08)\(^a\) | 7.07 (0.08) | 7.05 (0.08) | 7.21 (0.04) |
| Tumour   | 6.84 (0.06) | 6.75 (0.06) | 6.64 (0.11) | 6.82 (0.08) |
| \(\Delta\)pHe | 0.21 (0.10)\(^b\) | 0.32 (0.10) | 0.41 (0.14) | 0.39 (0.09) |

\(^a\)Values represent mean ± s.e.mean from at least six tumours.

\(^b\)\(\Delta\)pHe = (mean muscle pH\(e\)-mean tumour pH\(e\)).
water containing 10% ethanol. A dose of 1.0 g kg\(^{-1}\) CCCP was well tolerated alone or in combination with 10 mg kg\(^{-1}\) amiloride and/or 10 mg kg\(^{-1}\) hydralazine. Similar results were obtained when CCCP was dissolved in alternative solvents such as dimethylsulphoxide and N-N-dimethylacetamide.

Treatment of KHT tumours with CCCP plus amiloride followed 30 min later by hydralazine alone or in combination with 15 Gy X-rays did not lead to a decrease in surviving fraction per KHT tumour in excision assays (data not shown). No growth delay was observed for mice treated with CCCP plus amiloride followed by hydralazine either when these three agents were given alone, or in combination with 15 Gy X-rays (data not shown).

**Nigericin**

The maximum tolerated dose of nigericin dissolved in sterile water containing 10% ethanol was approximately 4.0 mg kg\(^{-1}\) alone and approximately 2.5 mg kg\(^{-1}\) in combination with amiloride (10 mg kg\(^{-1}\)) and/or hydralazine (10 mg kg\(^{-1}\)). Maximum tolerated dose was defined as the dose of drug which did not cause animal death within 30 days.

After excision of tumours the number of dye-excluding tumour cells per gram of tumour tissue was determined. It was observed that only nigericin followed by hydralazine or nigericin plus amiloride followed by hydralazine caused significant decreases in the number of dye-excluding cells per gram of tumour recovered (Table II). A similar reduction in cell recovery was observed when drugs were combined with 15 Gy X-rays (Table II). These conditions also led to a decrease in surviving fraction per tumour (Table II). The decrease in surviving fraction per tumour caused by the drug combination appeared to be additive to the decrease in surviving fraction per tumour caused by radiation. Similar effects were observed for the EMT-6 tumour (data not shown).

The decrease in surviving fraction per tumour for nigericin followed by hydralazine was observed to be dependent on the dose of nigericin (Figure 3a) and on the dose of hydralazine (Figure 3b). A decrease in surviving fraction per tumour was observed when hydralazine was administered 3 h prior to or 3 h after nigericin plus amiloride (Figure 4). Maximum reduction in surviving fraction per tumour was observed when hydralazine was administered from 1 h before to 1 h after nigericin.

The effects of nigericin plus amiloride followed by hydralazine on tumour growth delay are presented in Figure 5. When used alone these agents did not lead to a significant delay in tumour growth. When used with radiation, no additional growth delay was observed for EMT-6 tumours, whereas for the KHT tumour there was a trend to increased growth delay when drugs were used in combination with radiation (mean 12.3 days) as compared to radiation alone (mean 7.5 days) (Table III).

**Discussion**

The results from this study indicate that the ionophore nigericin, which produces pH-dependent cytotoxicity via intracellular acidification in vitro, is capable of killing cells in murine solid tumours.

Microelectrode measurements of pH within spheroids have shown that pH decreases as distance from the spheroid surface increases (Acker et al., 1987; Carlson & Acker, 1988). In medium at pH 7.4, minimum pH at a depth of 200 μm

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**Table II Number of dye excluding cells (× 10\(^{-7}\)) recovered per gram of tumour and surviving fraction per tumour for the KHT tumour**

| Treatment | Drugs alone | Drugs plus 15 Gy X-rays |
|-----------|-------------|------------------------|
|           | # cells per gram | S.F. per tumour | # cells per gram | S.F. per tumour |
| Control   | 7.7 (6.9–12.0) | 1.0 (0.85–1.0) | 4.8 (2.5–8.2) | 2.6 × 10\(^{-2}\) (2.1–3.0 × 10\(^{-2}\)) |
| Nigericin + Amiloride | 7.3 (4.0–10.1) | 0.73 (0.82–0.64) | 6.2 (2.0–10.4) | 2.7 × 10\(^{-2}\) (2.5–2.8 × 10\(^{-2}\)) |
| Hydralazine | 9.0 (7.9–10.1) | 0.70 (0.62–0.82) | 7.7 (6.4–9.0) | 1.2 × 10\(^{-2}\) (0.9–2.5 × 10\(^{-2}\)) |
| Nigericin + Hydralazine | 0.7 (0.5–1.0) | 1.3 × 10\(^{-2}\) (0.8–1.8 × 10\(^{-2}\)) | – | – |
| Nigericin + Amiloride | 1.5 (0.7–2.2) | 3.7 × 10\(^{-2}\) (3.5–3.8 × 10\(^{-2}\)) | 1.4 (0.9–1.9) | 3.4 × 10\(^{-4}\) (2.8–3.6 × 10\(^{-4}\)) |

Values represent mean (range) from at least two experiments.
from the surface of spheroids has been observed to be approximately 6.8. Cell killing was not observed when EMT-6 spheroids were exposed to CCCP or nigericin at pH 7.4. Since CCCP and nigericin have been shown previously to be selectively toxic at pH's less than 6.5, the failure to observe cell killing at pH 7.4 was probably due to the fact that at most a small proportion of cells within the spheroids had ambient pH within the range necessary for cytotoxicity of CCCP or nigericin. When spheroids were incubated in medium at low pH and exposed to CCCP or nigericin, time dependent cytotoxicity was observed with several orders of magnitude of cell killing (Figure 1). This result demonstrates that CCCP and nigericin are capable of penetrating into tissue and killing cells.

The mean pH in KHT (6.84) and EMT-6 (6.75) tumours growing in the legs of mice was observed to be 0.21 and 0.32 pH units more acidic than muscle pH (Table III). The observed values of pH for KHT and EMT-6 tumours are consistent with those reported for other rodent solid tumours (Wike-Hooley et al., 1984); however, mean values of pH within KHT and EMT-6 tumours were above the threshold (pH 6.5) for cytotoxicity by nigericin or CCCP alone (Rotin et al., 1987; Newell & Tannock, 1989). Nigericin and CCCP were therefore combined with the Na+/H+ exchange inhibitor amiloride which has been shown to allow killing of cells by CCCP and nigericin at values of pH below 7.0.

We could not demonstrate in vivo toxicity of CCCP when used in high concentration (1.0 g kg\(^{-1}\) body weight) either alone or in combination with other agents. The lack of cytotoxicity for CCCP may have been due to the limited solubility of CCCP in aqueous solutions which may prevent CCCP from reaching toxic concentrations within tumours. We have not performed pharmacological studies in an attempt to address directly this possibility.

Nigericin combined with amiloride was not cytotoxic to KHT or EMT-6 tumours in excision assays or in growth delay experiments. Failure to observe in vivo toxicity may have been due to pH values that were too high to allow cell killing at the doses achieved. We therefore used the vasodilator hydralazine in an attempt to reduce tumour pH. Hydralazine has been shown previously to produce a dose-dependent reduction in tumour blood flow (Chaplin, 1989; Lin & Song, 1990). This decrease in tumour blood flow may lead to a decrease in tumour pH because the rate of glycolysis may increase under induced anaerobic conditions, leading to accumulation of lactic acid, and because of reduced clearance of this and other metabolic acids. Hydralazine caused a decrease in pH of the KHT tumour with little change in pH of EMT-6 tumours. Our results with the EMT-6 tumour are consistent with a previous study which reported that hydralazine did not reduce pH in RIF-1 tumours (Tobari et al., 1988).

When nigericin plus amiloride was combined with hydralazine a decrease in surviving fraction was observed when the agents were given alone or in combination with 15 Gy radia-

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**Table III** Delay in growth of KHT and EMT-6 tumours following various treatments

| Treatment          | KHT Days to 1 g | Growth delay | EMT-6 Days to 1 g | Growth delay |
|--------------------|----------------|--------------|------------------|--------------|
| Control            | 4.7 (1.0)      | -            | 5.5 (0.9)        | -            |
| Nigericin + Amiloride + Hydralazine | 5.3 (0.5) | 0.6          | 7.6 (1.3)        | 2.1          |
| 15 Gy X-rays       | 12.2 (1.5)     | 7.5          | 16.5 (1.4)       | 11.0         |
| 15 Gy X-rays + Nigericin + Amiloride + Hydralazine | 17.0 (4.6) | 12.3         | 16.2 (1.3)       | 10.7         |

The values for days to 1 g represent mean ± s.e. from two growth delay experiments. Growth delay = [(days to 1 g)\(_{tumour}\) - (days to 1 g)\(_{control}\)].
tion (Table II). The toxic effects of nigericin plus amiloride followed by hydralazine appeared to be additive with those of radiation, and do not suggest selective toxicity towards nutritionally-deprived cells in hypoxic/acidic environments of solid tumours. However, this result might also be obtained if there was fluctuating hypoxia in the tumour (Minchinton et al., 1990) such that cells which had regained sensitivity to radiation had maintained an acidic microenvironment for a sufficiently long period to render them drug-sensitive. Nigericin plus amiloride followed by hydralazine alone or in combination (15 Gy X-rays) had produced a significant growth delay against the EMT-6 tumour (Table III). Nigericin plus amiloride followed by hydralazine produced a non-significant increase in the growth delay for the KHT tumour (approximately 4.8 days) only when combined with radiation (Table III). The apparent inconsistent effects when comparing excision assay results to growth delay results may be due to the modest decrease in surviving fraction per tumour caused by nigericin plus amiloride followed by hydralazine which may not be sufficient to produce a measurable growth delay; in addition, there may be smaller effects of the drugs to influence growth delay if they exert their effects selectively against a slowly-proliferating nutrient-deprived subpopulation of cells. In excision assays the decrease in surviving fraction per tumour was due partly to a loss in the number of cancer cells recovered which is due to rapid lysis of cells killed by the acidifying agents, but may be influenced by the trypsinisation procedure used to produce single cell suspensions. Disaggregation may have led to lysis of already damaged cells, since drug-damaged cells might be more susceptible to the effects of trypsin. If such drug-induced damage were repairable, the reduction in surviving fraction per tumour may be due in part to artifacts introduced by the trypsinisation procedure. Although this study does not indicate that nigericin plus amiloride are selectively toxic to nutritionally-deprived cells in solid tumours, it is encouraging that nigericin plus amiloride followed by hydralazine can produce tumour cell killing in vivo. Our results suggest that the approach of attempting to utilise agents which produce intracellular acidification may be useful if better tolerated and/or more potent analogues of nigericin and amiloride become available. In vivo studies with more potent analogues of amiloride are in progress in our laboratory.

Neither PBS or hydralazine influenced tumour pH (Figure 2) whereas nigericin or nigericin followed by hydralazine led to reductions in tumour pH of 0.27 and 0.29 pH units respectively. These observations suggest that nigericin enters tumour tissue and can produce a substantial decrease in tumour pH which may result in tumour cell cytotoxicity. The present study provides evidence which indicates that agents which cause intracellular acidification and cytotoxicity at low pH (<7.0) in vitro have small anti-tumour effects in vivo. The cytotoxic effect in vivo appears to be associated with the ability to produce intracellular acidification in vivo. Studies are in progress (using more potent analogues of amiloride) to determine if treatments are selectively toxic to specific subpopulations of cells by using the technique of Hoescht 33342 staining and fluorescence-activated cell sorting (Durand, 1982; Chaplin et al., 1985).

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