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● *Hyperthermia Original Contribution*

ENHANCEMENT OF HYPERTHERMIA EFFECT *IN VIVO* BY AMILORIDE AND DIDS

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Purpose: Intracellular pH is regulated mainly by Na^+/H^+ antiport and Cl^-/HCO_3^- exchange through the cell membrane. Amiloride (3,5-diamino-6-chloro-N-(diaminomethylene)pyrazine carboxamide) is a diuretic drug that blocks Na^+/H^+ antiport and DIDS (4,4-diisothiocyanatostilbene-2,2'-disulfonic acid) is an inhibitor of Cl^-/HCO_3^- exchange. We investigated the potency of these drugs to lower pH_i and increase the thermosensitivity of tumors *in vivo*.

Materials and Methods: The cytocidal effect of heat in combination with drug effect in vivo was studied using the in vivo-in vitro clonogenic assay method and the tumor growth delay method with SCK tumors, a mammary adenocarcinoma, on the hind limbs of A/J mice. The effects of amiloride and DIDS on tumor pH_i and high energy phosphate levels were investigated using ^{31}P-NMR.

Results: We observed that amiloride or DIDS alone increased the effect of hyperthermia at 42.5°C or 43.5°C to suppress tumor growth. The thermosensitization was greater when the two drugs were combined. For example, hyperthermia at 43.5°C alone resulted in a tumor growth delay of about 4 days. When 10 mg/kg amiloride or 25 mg/kg DIDS was injected prior to heating, the growth delay increased to about 6 days. When both drugs were injected prior to heating, a total growth delay of 8 days was obtained. *In vivo-in vitro* excision assays for cell survival demonstrated that these drugs enhanced the heat-induced tumor cell death. An i.p. injection of 10 mg/kg amiloride plus 25 mg/kg DIDS did not lower the tumor pHi over a 120 min interval. Heating the tumors at 42.5°C for 1 hr significantly lowered the pHi and when the tumor-bearing mice were injected i.p with amiloride and DIDS, and the tumors were heated 1 hr later, the drop in pH_i was greater relative to that by heating alone. Heating alone significantly lowered the tumor energy levels as indicated by PCr/P_i and β-ATP/P_i ratios and an i.p. injection of 25 mg/kg amiloride prior to heating further reduced the energy status in the tumors.

Conclusion: Amiloride or its analogs and DIDS may be useful in increasing the therapeutic efficacy of hyperthermia treatments by enhancing the reduction in tumor pH_i.

Intracellular pH, Thermosensitization, Amilorade, DIDS.

INTRODUCTION

The interstitial pH in most tumors is known to be acidic relative to normal tissues (16, 20, 22). This tumor acidity is one of the few physiological characteristics which may be exploited in the treatment of tumors. For example, it has been suggested that certain drugs which alter the intracellular pH and which are toxic only under acidic conditions may have therapeutic potential due to the high degree of tumor acidity (10, 13, 19).

Hyperthermia in combination with radiotherapy and chemotherapy is being used for the control of human tumors. The low pH associated with tumors is believed to be one of the reasons why hyperthermia may be preferentially cytotoxic to tumors relative to surrounding normal tissues. It has been demonstrated that the intracellular pH (pH_i) and not the extracellular pH (pH_e) is the critical factor which influences the cellular thermosensitivity (1, 2, 7). Mammalian cells have a great degree of control over their pH_i and can maintain a relatively neutral pH_i even when the external pH is quite acidic (7). For example, SCK tumor cells, cultured *in vitro* at pH 7.4 or 7.2 maintain their pH_i near those values but when placed into acidic culture media at pH 6.6 they maintain pH_i values 0.3 to 0.4 pH units higher than the surrounding environment (7). It is reasonable to suspect then that drugs which interfere with the mechanisms which control pH_i may be able to sensitize the cells to heat in an acidic environment.

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We indeed observed that amiloride (3,5-diamino-6-chloro-N-(diaminomethyulene)pyrazine carboxamide) alone or with DIDS (4,4-diisothiocyanatostilbene-2,2′-disulfonic acid) lowered the pHi of SCK cells in culture and that these drugs markedly enhanced the thermal killing of tumor cells *in vitro*, particularly in an acidic extracellular environment (7). In the present study, the capability of amiloride and DIDS to sensitize *in vivo* tumors was investigated. In addition, we studied the effects of these drugs on pH_i and the metabolic status in heated and unheated SCK tumors using ^{31}P-NMR.

METHODS AND MATERIALS

Drugs

Both amiloride and DIDS were purchased from a commercial source*. Amiloride has an LD_{50} of 50 mg/kg in A/J mice with animal deaths beginning after 30 mg/kg and reaching 100% mortality at 70–80 mg/kg. DIDS is markedly less toxic than amiloride with no deaths occurring even at 100 mg/kg. The LD_{50} for DIDS was found to be 150 mg/kg in A/J mice. The standard dose for DIDS used in this study was 25 mg/kg. The combined injection of 25 mg/kg DIDS with 25 mg/kg amiloride, the highest amiloride dose used in this study, produced about 20% mortality. Therefore, 10 mg/kg was chosen as the highest amiloride dose to be used with DIDS in the tumor growth studies. No animal deaths were ever observed due to the injection of 10 mg/kg amiloride with 25 mg/kg DIDS. Amiloride and DIDS were dissolved separately at the appropriate concentrations in saline and were administered to the mice in a 0.2 ml i.p. injection one hr before the hyperthermia treatments. When both amiloride and DIDS were injected into the same mice, amiloride was injected first and DIDS was injected 20–30 min later. The drugs were administered separately in two i.p. injections because amiloride and DIDS form an insoluble precipitate if combined together at high concentrations. DIDS is readily soluble in saline, but amiloride at these high concentrations requires a brief heating in a microwave oven for it to completely dissolve.

Tumor growth delay

Throughout this study, SCK tumors of A/J† mice were used. About 2×10^5 SCK cells in 0.05 ml RPMI media without calf serum were injected under the superficial layer of the muscle in the right thigh of female A/J mice. About 7 days later, when the tumors had grown to approximately 7 by 9 mm in diameter (about 250 mm^3), the mice were separated randomly into groups of 8–10 animals per cage. Mice were fitted onto plastic jigs by a thread secured around one or two toes on the right foot and were fastened in place on the jigs by adhesive tape.

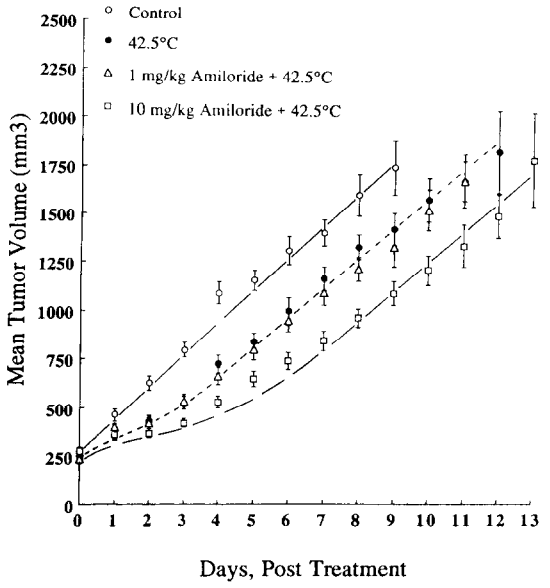

Fig. 1. Effect of heating at 42.5°C for 1 hr alone or heating after an injection of 1 mg/kg or 10 mg/kg amiloride on the growth of SCK tumors is shown.

The tumor-bearing right legs were submerged into a water bath preheated at 42.5°C or 43.5°C. After heating the tumors for 60 min, the mice were released from the jigs and returned to their cages. Control mice were left untreated. The long and short tumor diameters were measured daily with a caliper until the death of the animal. Tumor volume was calculated using the standard formula $V = a^2b/2$, where a is the short tumor diameter and b is the long tumor diameter. The growth curve data presented in Figures 1–4 are from 3–5 combined experiments totalling 30–40 mice for each experimental condition. The Student's t-test was used to test for statistical significance between mean values for different treatments.

Clonogenic assay of tumors in vivo

The survival of cells in the heated tumors was determined by use of the *in vivo-in vitro* excision assay. The mice and tumors were handled identically to that described above for the tumor growth delay study. Upon completion of the hyperthermia treatments, the mice were removed from the jigs, killed by cervical dislocation, and the tumors were excised from the animals. The tumors were trimmed of any muscle tissue, weighed, minced into small fragments with a pair of scissors, and dispersed to single cells by gentle agitation in RPMI media containing 25% trypsin and 15 units/ml DNAse for 20 min at room temperature. The cell suspension was strained through sterile gauze to remove any debris and the trypsin was neutralized by the addition of calf serum. The cell suspension was centrifuged and the cell pellet was rinsed once,

* Sigma Chemical Co., St. Louis, MO.
† Jackson Laboratories, Bar Harbor, ME.

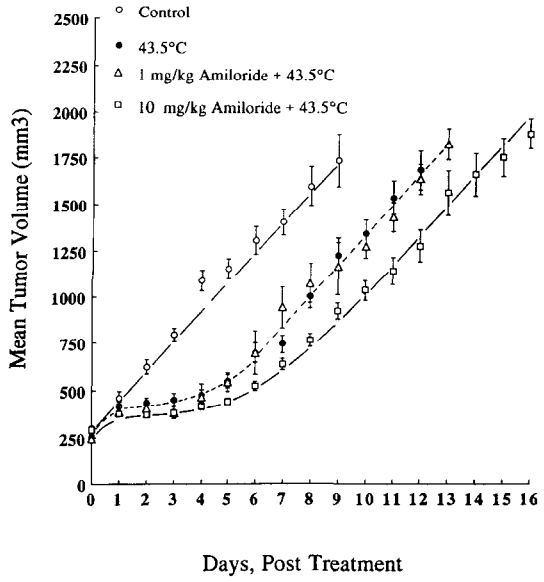

Fig. 2. Effect of heating at 43.5°C for 1 hr alone or heating after an injection of 1 mg/kg or 10 mg/kg amiloride on the growth of SCK tumors is shown.

recentrifuged and resuspended in fresh culture media. Appropriate dilutions of the cell suspension were made, the number of cells was counted, and the number of cells obtained per gram of tumor was calculated. Appropriate numbers of cells were then plated into five replicate culture flasks. After 7 days incubation at 37°C, the resulting SCK colonies were counted and the plating efficiency of the cells was determined. From the number of cells obtained per gram and the plating efficiency of the cells, the number of clonegenic cells per gram tumor was calculated.

Combined effects of drug(s) and heat on hypoxic cells

The thermosensitizing effect of amiloride and DIDS under aerated and hypoxic conditions *in vitro* was studied. Appropriate numbers of SCK cells in exponential growth phase were plated into glass tissue culture flasks and incubated for 16 hr at 37°C. The culture media was replaced with new media with or without amiloride and/or DIDS. The flasks were sealed with tight-fitting rubber caps and two 22-gauge hyperdermic needles were pierced through the rubber caps for gassing. To create acidic and hypoxic conditions simultaneously, the flasks were gassed through one of the needles with a mixture of 33% CO_2 and 67% nitrogen for 45 min at room temperature. The other needle served as the gas outlet. The pH of the culture media under these conditions dropped to 6.57 ± 0.02. Upon completion of the nitrogen gassing, the needles were removed from the rubber cap leaving the flasks completely sealed and the flasks were immersed in a 43°C preheated water bath. For hyperthermia treatments under oxygenated conditions, 25 ml CO_2 gas was added to each flask before it was tightly capped. Media pH under these conditions was 6.63 ± 0.02. When the heating was finished, the rubber caps were removed and the media in the flasks

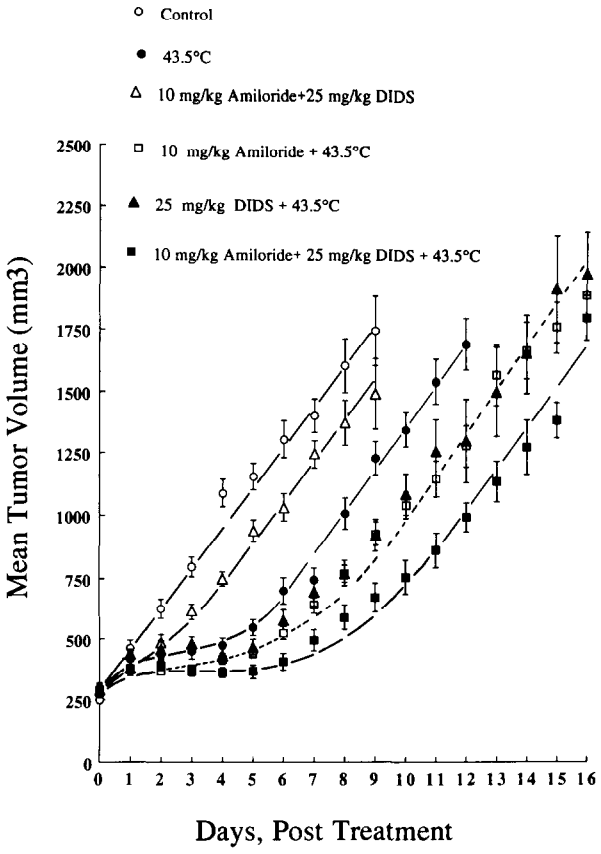

Fig. 3. Effect of heating at 43.5°C for 1 hr alone, 10 mg/kg amiloride + 25 mg/kg DIDS, heating after an injection of 10 mg/kg amiloride or 25 mg/kg DIDS alone and combined on SCK tumor growth is illustrated.

was removed by aspiration. The flasks were rinsed with culture media twice to remove any remaining drugs, 8 ml new culture media was added to the flasks and the cells were incubated in a 5% CO_2 incubator at 37°C for 7 days to allow for colony formation.

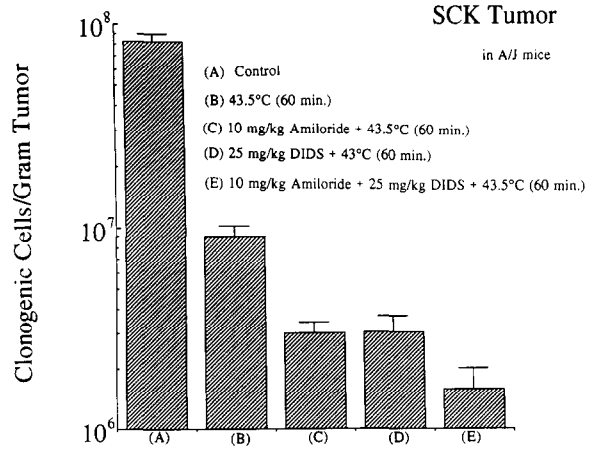

Fig. 4. Effect of hyperthermia treatment in combination with 10 mg/kg of amiloride and/or 25 mg/kg of DIDS on the number of clonogenic cells/gram tumor is shown.

^{31}P-NMR measurements of pH_i and energy status

^{31}P-NMR experiments were performed at 121.4 MHz on a Spectroscopy Imaging Systems Corporation (SISCO) NMR system equipped with an 18.3 cm horizontal bore 7.0 Tesla magnet. ^{31}P-NMR spectra were obtained using an 8 mm single turn surface coil which was also switchable to the ^{1}H frequency (299.994 MHz) for shimming purposes. Other parameters were: 4K data points per FID, spectral width of 8000 Hz, repetition time of 2.3 sec, 400 signal averages per spectrum (15 min total acquisition time), and a pulse length of 14 μs. ^{31}P-NMR spectra were apodized with a 50 Hz exponential line broadening function prior to Fourier transformation. Spectral baselines were corrected using a cubic spline method. Quantification of ^{31}P resonance areas was accomplished using a spectral deconvolution routine provided with the SISCO NMR system. The pH_{NMR} was calculated from the chemical shift of P_i relative to PCr. When the PCr was absent from the tumor spectra, the H_2O resonance from the proton spectrum obtained during shimming procedures was used to calculate the position of the PCr which then allowed the chemical shift of P_i, and hence pH_{NMR} to be determined, as previously described (8). The absolute accuracy of pH measurements with ^{31}P-NMR has been reported to be 0.1 pH units, and pH changes can be measured within 0.05 pH units (3). The tissue pH measured using microelectrodes is believed to be predominantly interstitial/extracellular with a small component of intracellular pH due to cells ruptured by the insertion of the electrode. On the other hand, the tissue pH measured by ^{31}P-NMR is believed to be predominantly intracellular as almost all of the phosphate is intracellular with a small contribution from extracellular phosphate which may have leaked from adjacent cells. Consequently, the pH value measured using NMR in this study will be referred to as pH_{NMR} with the understanding that it is assumed to reflect tumor pHi.

For NMR observation, unanesthetized mice were secured to a plastic jig which restricted the movement of the tumor-bearing legs. This arrangement allowed positioning the tumor next to the surface coil probe. Two types of NMR studies were conducted. The first involved the continuous measurement of tumor pH_{NMR} following drug injections. For each tumor, a pretreatment ^{31}P spectra was acquired, the mouse was taken out of the magnet and given an i.p. injection of 10 mg/kg amiloride followed by 25 mg/kg DIDS. The mouse was repositioned in the magnet, the magnetic field was optimized by shimming and acquisition of ^{31}P spectra was resumed approximately 10 min following DIDS administration. Spectra were collected at 15 min intervals for up to 120 min following drug administration and pH_{NMR} was determined at each of these 15 min timepoints.

In an alternative method of studying the drug effect on the ^{31}P spectra *in vivo*, ^{31}P spectra were obtained before heating and in the same tumor immediately following a 60 min heating in a 42.5°C water bath. To ensure that the NMR surface coil was repositioned as closely as possible to the original measurement site, a dot was marked on the skin over the tumor as a guide to center the surface coil over the tumor. Four groups of mice were used. The first was sham heated in a water bath at 37°C for one hr. The second group was heated in the water bath at 42.5°C for one hr. The third group of mice received 25 mg/kg amiloride 1 hr prior to heating, and the fourth group received 25 mg/kg amiloride plus 25 mg/kg DIDS prior to heating. Any tumor which exhibited low PCr and ATP peaks in the initial spectra was deemed to be in poor condition and was discarded.

RESULTS

Drug effect on tumor growth control

One hr heating at 42.5°C or 43.5°C delayed tumor growth by 1.8 and 3.6 days, respectively (Table 1, Figs. 1 and 2). A single injection of 25 mg/kg amiloride resulted in a growth delay of 1.1 days (Table 1) and an injection of 10 mg/kg amiloride plus 25 mg/kg DIDS resulted in a growth delay of 1.7 days (Table 1 and Fig. 3). The combination of heat and amiloride at 1 mg/kg, the lowest drug dosage used in this study, did not increase the tumor growth delay beyond that observed with heat alone (Table 1, Figs. 1 and 2). An injection of amiloride at 5, 10 and 25 mg/kg increased the tumor growth delay by about 2.5 days at 42.5°C and by about 2 days at 43.5°C compared to the tumor growth delay observed with heat alone. There was no significant difference in drug effect between these three amiloride dosages. An injection of DIDS alone at 25 mg/kg was effective in increasing the tumor response to heat as shown by a 1.6 day increase in growth delay beyond that due to heat alone at 43.5°C (Table 1, Fig. 3). When 10 mg/kg amiloride and 25 mg/kg DIDS were combined with 43.5°C, an additional growth delay of 3.9 days beyond that observed with heat alone was achieved (Table 1, Fig. 3).

As may be expected, the drug effect in enhancing tumor growth delay was reflected in an increase in animal survival (Table 1). Untreated animals died 7.6 $\pm$ 0.3 days after the tumors had grown to about 250 mm^3. A single injection of 25 mg/kg amiloride or an injection of 10 mg/kg amiloride plus 25 mg/kg DIDS increased the survival of the tumor-bearing mice by about 1.1 days. Hyperthermia treatments alone at 42.5°C and 43.5°C increased mouse survival by 2.3 and 4.7 days, respectively. The injection of 25 mg/kg amiloride prior to heating the tumors at 42.5°C or 43.5°C resulted in an increase in animal survival about 2.0 days longer than that observed with heat alone at both temperatures. When 10 mg/kg amiloride and 25 mg/kg DIDS were injected before hyperthermia treatments at 43.5°C, the animal survival was prolonged by about 2.5 days beyond that resulting from the same hyperthermia treatment without drugs.

Clonogenic cells within SCK tumors

The clonogenic cell number in the untreated tumors was about 8.29×10^7 cells/g (Fig. 4). A hyperthermia

Table 1. Study results

Treatment	Days for tumor to increase to 4× initial volume		Total days growth delay	Days growth delay due to drug(s)	Days animal survival		Days increase in survival due to treatment	Days enhanced survival post-treatment due to drug(s)
Drug alone								
Control (untreated animals)	4.1 ± 0.2				7.6 ± 0.3			
25 mg/kg Amiloride	5.2 ± 0.4	$p < .05$	1.1	1.1	8.6 ± 0.7	$p = .09$, BS	1.1	1.1
10 mg/kg Amiloride + 25 mg/kg DIDS	5.8 ± 0.5	$p < .001$	1.7	1.7	8.5 ± 0.5	$p = .06$, BS	1.0	1.0
42.5°C	5.9 ± 0.2		1.8		9.9 ± 0.4		2.3	
1 mg/kg Amiloride + 42.5°C	5.8 ± 0.4	NS	1.6	−0.2	8.8 ± 0.8	NS	1.2	
5 mg/kg Amiloride + 42.5°C	8.4 ± 0.3	$p < .001$	4.2	2.4	10.1 ± 0.3	NS	2.5	0.2
10 mg/kg Amiloride + 42.5°C	8.4 ± 0.5	$p < .001$	4.3	3.2	11.1 ± 0.5	$p = .08$, BS	3.5	1.2
25 mg/kg Amiloride + 42.5°C	8.6 ± 0.6	$p < .001$	4.4	3.3	11.7 ± 0.6	$p < .05$	4.1	1.8
43.5°C	7.7 ± 0.4		3.6		12.3 ± 0.4		4.8	
1 mg/kg Amiloride + 43.5°C	7.9 ± 0.6	NS	3.8	0.2	11.8 ± 1.2	NS	4.2	−0.6
5 mg/kg Amiloride + 43.5°C	9.7 ± 0.5	$p < .001$	5.6	2.0	11.8 ± 0.5	NS	4.3	−0.5
10 mg/kg Amiloride + 43.5°C	9.6 ± 0.4	$p < .001$	5.5	1.9	13.8 ± 0.5	$p < .05$	6.2	1.5
25 mg/kg Amiloride + 43.5°C	9.8 ± 0.3	$p < .001$	5.6	2.0	14.3 ± 0.5	$p < .001$	6.7	2.0
25 mg/kg DIDS +k 43.5°C	9.3 ± 0.3	$p < .05$	5.2	1.6	13.2 ± 0.5	NS	5.6	0.9
10 mg/kg Amiloride + 25 mg/kg DIDS + 43.5°C	11.6 ± 0.6	$p < .001$	7.5	3.9	15.0 ± 0.6	$p < .001$	7.4	2.6

NS = Non-significant ($p > .1$); BS = Barely significant ($.1 < p > .05$).

treatment of 43.5°C for 60 min reduced this value to about 9.03×10^6, which was 10.9% of the control value. When 10 mg/kg amiloride was given 1 hr prior to the hyperthermia treatment, the number of clonogenic cells declined further to 2.99×10^6, which was 3.6% of the control value. When 25 mg/kg DIDS was injected prior to heating, 3.05×10^6 clonogenic cells were obtained, which was 3.7% of that in the untreated tumors. Finally, when both amiloride and DIDS were administered prior to the hyperthermia treatment, the number of clonogenic cells was only 1.58×10^6, 1.9% of the number in control tumors.

Drug effect under hypoxic conditions in vitro

Amiloride has been reported to increase thermal killing of cells *in vitro* under acidic conditions (4, 7, 9, 14), whereas DIDS did not sensitize SCK cells to heat *in vitro* under either alkaline or acidic conditions (7). However, the present study shows that both amiloride and DIDS enhanced the heat-induced growth delay of tumors *in vivo*. This result suggested that the response of the tumor cells to the drug may have been modified by some condition *in vivo* that is not present under *in vitro* conditions. Therefore, the effect of the drug was examined under both oxygenated and hypoxic conditions.

Figure 5 illustrates the effect of amiloride alone or combined with DIDS on the heat sensitivity of SCK cells *in vitro* under oxygenated or hypoxic conditions. There was only a slight difference in the cell survival after heating at 43°C for 90 min under aerated and hypoxic conditions when drugs were not present. However, in the presence of 0.5 mM amiloride, the reduction of cell survival by heat was much greater under hypoxic conditions than under oxygenated conditions. Specifically, when the cells with 0.5 mM amiloride under aerated and hypoxic conditions were heated at 43°C for 90 min, the cell survival decreased to 45% and 10%, respectively, to that observed without the drug under aerated and hypoxic conditions. DIDS at 0.1 mM had almost no effect on thermosensitivity in the presence of oxygen, but under hypoxic conditions, DIDS reduced the cell survival to 50% of that observed after applying hyperthermia alone. Finally, when amiloride and DIDS were combined, the thermosensitizing effect of the drugs was profoundly greater under hypoxic conditions than in the presence of oxygen.

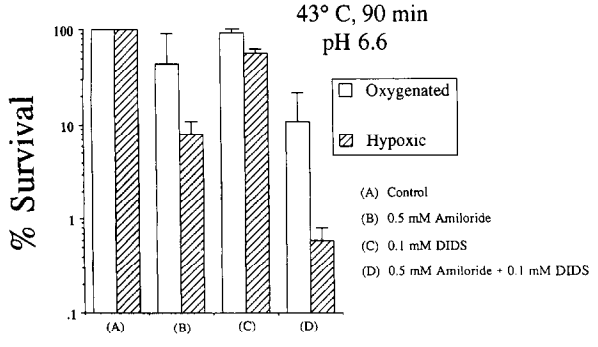

Fig. 5. Effect of amiloride and/or DIDS combined with hyperthermia at 43°C for 90 min on SCK cells *in vitro* under oxygenated and hypoxic conditions is illustrated. All cell survival data was normalized using the cell survival under oxygenated or hypoxic conditions without drugs as 100%.

Drug effect in vivo as measured by ^{31}P-NMR spectroscopy

The pre-treatment pH_{NMR} for all SCK tumors ranged from 6.61 to 7.08 with a mean value of 6.86 ± 0.02 (SE), 0.12 (SD), with a total sample size of 33 tumors. When the pH_{NMR} was continuously monitored at ambient temperature following the i.p. injection of 10 mg/kg amiloride and 25 mg/kg DIDS, the baseline tumor pH_{NMR} was essentially unchanged during the 120 min period that the tumors were monitored (Fig. 6). Hyperthermia alone at 42.5°C for 60 min reduced tumor pH_{NMR} from 6.85 ± 0.04 to 6.65 ± 0.06, a drop of 0.2 pH units (Fig. 7). When 25 mg/kg amiloride was injected prior to heating, the pH_{NMR} dropped from 6.80 ± 0.06 to 6.45 ± 0.09, a decline of 0.35 pH units. When both amiloride and DIDS were injected prior to the heat treatment, the pH_{NMR} dropped from 6.81 ± 0.04 to 6.20 ± 0.16, a drop of 0.6 pH units.

Amiloride was also found to significantly affect the heat-induced changes in cellular energy status when injected before the hyperthermia treatment. Hyperthermia treatments were found to reduce tumor energy status as defined by the β-ATP/Pi and PCr/Pi ratios. The tumor β-ATP/Pi ratios were reduced to 35% of the pre-treatment values by the 42.5°C heating for 60 min (Fig. 8). The tumors in mice which had received an amiloride injection prior to hyperthermia treatment exhibited more marked reductions in ATP levels, which were visually apparent in the majority of the spectra obtained. The β-ATP/Pi and PCr/Pi ratios following hyperthermia with 25 mg/kg amiloride injected prior to heating were 12% of the pre-treatment values. When 25 mg/kg of DIDS was injected into the mice in addition to 25 mg/kg of amiloride prior to the hyperthermia treatments, the changes in PCr/Pi and β-ATP/Pi ratios were indistinguishable from those observed in animals which had received only amiloride prior to the hyperthermia treatment (data not shown in Fig. 8). That is, DIDS did not add to the degree of high energy phosphate depletion observed with amiloride in combination with hyperthermia. Reduction of tumor ATP and PCr levels following amiloride and DIDS injections was not observed during the continuous study of pH_{NMR} performed at room temperature.

DISCUSSION

We observed in the present study that the thermal damage in SCK tumors of A/J mice, as measured using the tumor grow delay method and *in vivo-in vitro* excision assay, could be enhanced by amiloride or DIDS which interfere with the cellular regulation of pH_i. A combination of amiloride and DIDS was more potent than either drug alone in enhancing the thermal damage in the tumors *in vivo*. A comparison of NMR spectra of the tumors before and after heating showed that the heat-induced reductions in the pH_i and ATP content in the tumor were enhanced when the drugs were given to the host mice prior to heating the tumors. It thus appeared that the thermosensitization by the drugs was at least in part mediated by the decline in the pHi and reduction in the energy status in the tumors.

It has been reported that the interstitial pH (pH_e) in the SCK tumor was 7.05 ± 0.14 (SD) (6) or 6.96 ± 0.19 (SD) (11), and that the pH_e in these tumors decreased by about 0.2 pH units when the tumors were heated at 43.5°C

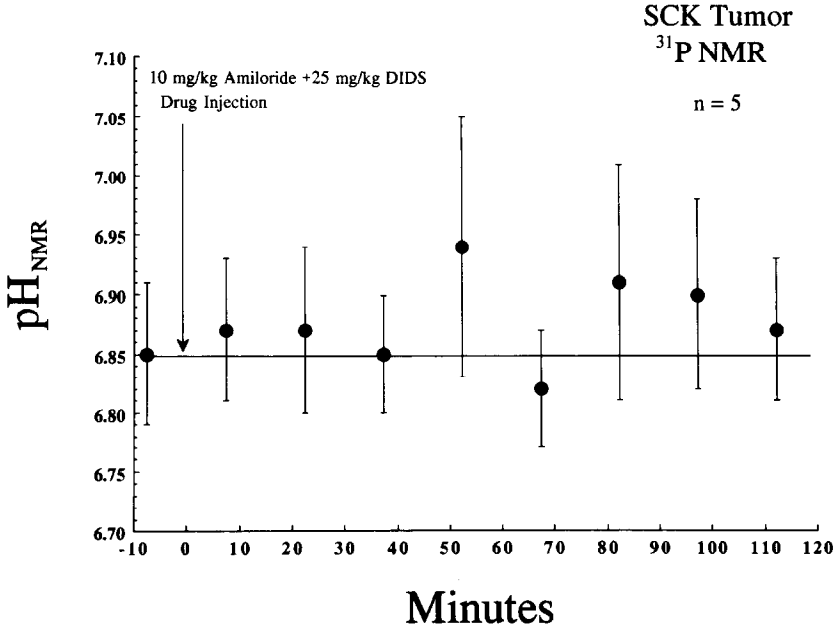

Fig. 6. The pH_{NMR} of SCK tumors following i.p. injection of 10 mg/kg amiloride and 25 mg/kg DIDS as measured continuously for 120 min using ^{31}P-NMR spectroscopy. Results shown are the mean values from five tumors. Error bars indicate S.E.

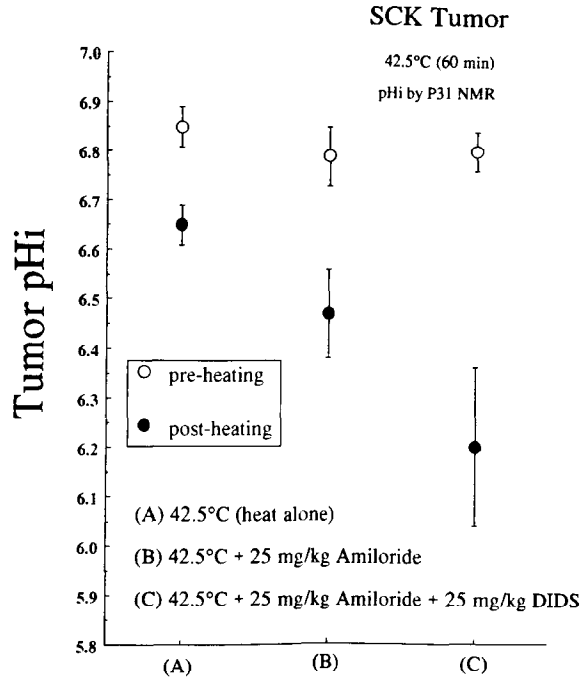

Fig. 7. The pH_{NMR} of SCK tumors before and after hyperthermia treatments with or without amiloride and DIDS. Results shown are the mean values of 6 to 8 tumors. Error bars indicate S.E.

for 30–60 min (6, 11). The mean pH_{NMR} in untreated SCK tumors obtained in the present study was 6.86 ± 0.12 (SD). Because the measurement of pH_e using the microelectrode method and the measurement of pH_i with the NMR method could not be done for the same tumors, it was not possible to make an exact comparison between pH_i and pH_e in this tumor line. Nevertheless, based on the aforementioned data, it would be reasonable to assume that the resting pH_i is approximately equal to the pH_e in the untreated SCK tumor. The lack of change in the pH_{NMR} after injection of 10 mg/kg of amiloride plus 25 mg/kg of DIDS in unheated tumors in the present study (Fig. 7), may then be attributed to the state of equilibrium having been reached between the pH_i and pH_e before the drug treatment. It is likely that under these conditions, both Na^+/H^+ antiport and Cl^-/HCO_3^- exchange may be operating at very low levels of activity. Consequently, the effect of amiloride and DIDS would be negligible.

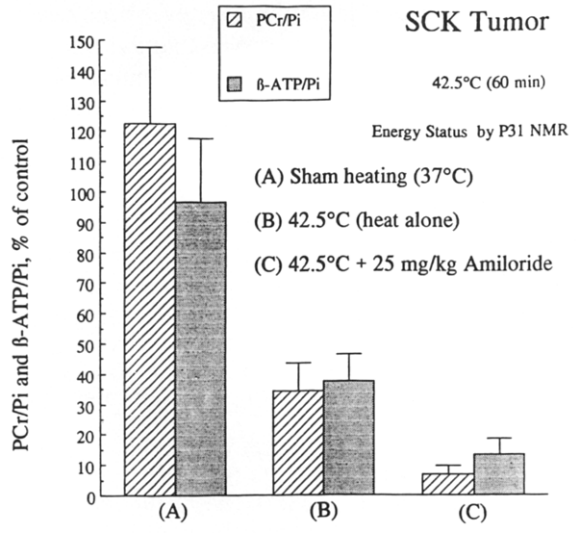

Fig. 8. Effect of hyperthermia treatments on tumor energy status as indicated by PCr/Pi and β-ATP/Pi ratios. Sham-heated animals were treated the same as the animals that were heated without drug injections except the water bath temperature was 37°C. Group A are the results from three mice, Group B are the results from 13 mice, and Group C are the results from 15 mice.

Hyperthermia at 42.5°C for 1 hr reduced the pH_{NMR} of SCK tumors about 0.2 pH units (Fig. 7), which was in general agreement with previous NMR studies of heated tumors (5, 15, 21) and our previous observations that heating reduced the pH_i in SCK tumor cells *in vitro* (4, 7). The mechanisms for such heat-induced declines in pH_i in cultured cells are unknown, but they have been attributed to hydrolysis of ATP and increased production of acidic metabolites such as lactic acid (16, 20). Our finding that amiloride and DIDS did not lower the pH_{NMR} of unheated SCK tumors but did reduce the pH_{NMR} in heated tumors may be partially due to the fact that the declining pH_e in heated tumors exerts a downward influence on the tumor cell pH_i. It would then be reasonable to expect that under conditions of heat stress, the activity of regulatory mechanisms for pH_i, such Na^+/H^+ antiport and Cl^-/HCO_3^- exchange are of greater importance in maintaining neutral pH_i. Therefore, any reduction in the pH_i regulating capacity by the drugs may contribute to more marked pH_i decreases. The reduction of pH_i or prevention of recovery of pH_i by these drugs then would enhance the cytocidal effects of heat to tumor cells *in vivo*.

In agreement with previous reports (5, 15, 21), we observed a significant reduction in high energy phosphate levels in the SCK tumors (Fig. 8) after heating. An injection of 25 mg/kg of amiloride to the tumor-bearing mice prior to heating the tumors resulted in a further reduction of ATP and PCr levels compared with that detected after heating alone (Fig. 8). It is unclear whether heating enhanced the effect of amiloride to reduce the ATP content or conversely, amiloride potentiated the effect of heat to reduce ATP content in the tumor cells. It is also not known whether the increased depletion of ATP observed is the result of direct drug interaction within the cell or whether it may just be a reflection of the greater degree of cell damage and death in the heated tumor in the presence of the drug.

In agreement with the observation that amiloride and/or DIDS enhanced the heat-induced tumor growth delay, these drugs also enhanced the effect of hyperthermia treatments to reduce the number of clonogenic cells in the tumors (Fig. 4). It has been reported that additional cell death occurs in SCK tumors after heating due to the heat-induced noxious environment (12, 17). The data presented in Figure 4 shows the drug effect on the clonogenic cell number in heated SCK tumors measured im-

mediately after heating. Whether the drugs would increase the additional cell death that occurs after heating remains to be determined.

Interestingly, an i.p. injection of 25 mg/kg of DIDS increased the thermal damage to tumor cells *in vivo* (Table 1, Figs. 3 and 4), whereas DIDS failed to thermosensitize tumor cells *in vitro* (7). The cause for this discrepancy between the effects of DIDS *in vitro* and *in vivo* is obscure. It is generally believed that the relative role of Na^+/H^+ antiport in pH_i regulation in most cells is greater than that of Cl^-/HCO_3^- exchange. It is conceivable that the role of Cl^-/HCO_3^- exchange in regulating pH_i depends on various conditions and that its role in SCK tumors *in vivo*, particularly after heating, may be more important than under the optimal culture conditions *in vitro*. The enhancement of thermal damage by the injection of amiloride plus DIDS was greater than that by either drug alone (Figs. 3 and 4). This result is in agreement with our previous observation with the SCK tumor cells *in vitro* (7) and may be attributed to a greater reduction in pH_i by the combination of these two drugs than by amiloride alone (Fig. 7).

Our observation that the thermosensitization by amiloride alone or combined with DIDS was greater under hypoxic conditions than in the presence of oxygen is of great interest (Fig. 5). It has been well known that tumors tend to be hypoxic compared to normal tissues and that hyperthermia at 43°–44°C reduces blood flow and creates extremely hypoxic conditions in rodent tumors. Therefore, the observation that amiloride and DIDS exerted a more pronounced thermosensitizing effect *in vitro* under hypoxic conditions is relevant to the *in vivo* situation. In a previous study (7), amiloride under aerated and alkaline culture conditions *in vitro* exhibited a very modest sensitizing ability on SCK cells as shown by the reduction of Do from 32.6 to 30.2 min, a Drug Enhancement Ratio (D.E.R.) of only 1.08. However, under conditions of acidity (pH 6.6) and hypoxia in the present study, 0.5 mM amiloride reduced the Do from 20.5 min to 12.5 min (data not shown), a D.E.R. of 1.64. These results suggest amiloride should have significant thermosensitizing ability under acidic and hypoxic tumor conditions and would not significantly affect heat sensitivities of oxygenated, and alkaline normal tissues. The reason for the increased effect of amiloride and DIDS observed under hypoxic conditions is not known. However, a likely and possible explanation is the increased intracellular lactic acid level due to the high rates of anaerobic glycolysis under hypoxic conditions. Decreased cellular energy levels under hypoxic conditions may also be involved. The antiport proteins do not require ATP directly for their operation, but their function may be diminished under conditions of energy deprivation. The thermosensitization of tumors *in vivo* by amiloride and DIDS, in particular by a combination of these two drugs, may then be attributed to several mechanisms, namely reduction in pHi, reduction in ATP content and the effect of heat-induced hypoxia in the tumors.

It has been reported that an injection of 1 mg/kg or 5 mg/kg of amiloride every 8 hr could inhibit growth of the DMA adenocarcinoma and H6 hepatoma (18). In the present study, our finding that amiloride alone could modestly delay tumor growth is in basic agreement with the above mentioned study. Amiloride is used clinically as a diuretic drug. The commonly used dosage of this drug in humans is 5–20 mg/day. The LD_{50} of amiloride for A/J mice was found to be 50 mg/kg. The dose of amiloride which could increase the thermoresponse of SCK tumors in A/J mice in the present study was 5 mg/kg. The dose of amiloride needed to increase the thermoresponse of human tumors remains to be determined. There are a number of amiloride analogues which are much more potent than amiloride in inhibiting the Na^+/H^+ antiport. The potential usefulness of these analogues as thermosensitizers is being investigated in our laboratory. To our knowledge, DIDS has never been tested for animal or human use. We observed that the LD_{50} of this drug for A/J mice is about 150 mg/kg. Clinical usefulness of this drug as a thermosensitizer also remains to be investigated.

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