

# Effects of Amiloride on Tumor Growth and Intracellular Element Content of Tumor Cells *in Vivo*<sup>1</sup>

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## ABSTRACT

The effects of amiloride, a reported inhibitor of serum-stimulated sodium influx, were tested on tumor growth, tumor cell proliferation, and intracellular element content of cancer cells *in vivo*. We have shown previously that cancer cells have high intranuclear levels of sodium compared to those of their normal counterpart cells and have postulated that such a high level of sodium may be involved in the cancer state. We now report that amiloride, when given in a series of injections, inhibited both H6 hepatoma and DMA/J mammary adenocarcinoma growth *in vivo* in a dose-dependent fashion and that 3 injections of amiloride at a dose of 1.0  $\mu\text{g/g}$  body weight into mice bearing H6 hepatomas resulted in a significant decrease in the intranuclear content of sodium but not the content of magnesium, phosphorus, sulfur, chlorine, or potassium as measured by electron probe X-ray microanalysis in the H6 hepatoma cells. Amiloride at dosages as low as 1.0  $\mu\text{g/g}$  body weight per injection also inhibited tumor cell proliferation as measured by the tritiated thymidine autoradiography labeling index. Amiloride caused no changes in the mean profile diameters of metaphase or interphase H6 hepatoma or DMA/J mammary adenocarcinoma cells, suggesting that the action of amiloride on tumor growth was not due to cell volume changes. These data show that amiloride both inhibited tumor growth and decreased the proliferation of the tumor cells in the H6 hepatomas which was correlated with a decreased intranuclear sodium content.

## INTRODUCTION

Several reports have implicated that sodium plays a role in mitogenesis and/or oncogenesis (2-9, 11-13, 15, 16, 18, 20). It has been shown that rapidly proliferating normal cells have higher intracellular levels of sodium than do slowly proliferating normal cells and that transformed cells have even higher levels of sodium than do rapidly proliferating normal cells (4, 6, 7, 18, 24). Although the role of the high intracellular levels of sodium in transformed cells is not known, it has been speculated that the high level of sodium in transformed cells is mitogenic and/or oncogenic (4, 6, 18, 24).

This report deals with our efforts to determine the role and/or requirement for the high intracellular levels of sodium in transformed cells. We tested the effects of amiloride, which is reported to inhibit sodium influx and proliferation of normal cells (12, 20, 22, 26), on tumor growth, tumor cell proliferation,

and intranuclear element content of transformed cells *in vivo*. Briefly, amiloride reduced all 3 parameters.

## MATERIALS AND METHODS

Male A/J mice were used as tumor hosts and were obtained from The Jackson Laboratory (Bar Harbor, Maine). The DMA/J mammary adenocarcinoma (kindly supplied by David Morrison, Thorman Cancer Research Laboratory, Trinity University, San Antonio, Texas) and the H6 hepatoma were routinely propagated by s.c. injection of a 0.1- to 0.2-ml suspension of cells in lactated Ringer's solution. Amiloride was kindly supplied by Dr. E. Cragoe of Merck, Sharp, and Dohme (West Point, Pa.). Solutions of the drug were prepared fresh for each set of injections, and in the case of the tumor growth rate studies were given every 8 hr. The animals were killed 1 hr after the last injection. Tumor dimensions were determined daily by making caliper measurements of length and width. Tumor areas were then calculated as cross-sectional areas in sq cm using the formula for an ellipse. A linear regression program was used to determine the daily change in slope of tumor for each animal. The slope values from each group of mice were subjected to a one-way ANOVA.<sup>4</sup> This test was used to determine when amiloride significantly affected tumor growth. Each experiment was terminated when a significant inhibition of tumor growth was seen.

A separate experiment was run to determine how amiloride treatment influenced cell proliferation activity as well as the intranuclear content of sodium and several other elements. In this experiment, mice with an H6 hepatoma were randomly placed into 2 groups. The mice in one group were given 3 injections of amiloride made up in lactated Ringer's solution at a concentration of 1.0  $\mu\text{g/g}$  body weight, and each injection was spaced 8 hr apart. The other group of mice was given the same series of injections of lactated Ringer's but without amiloride. Both groups of mice were also given an injection of tritiated thymidine at a dose of 1  $\mu\text{Ci/g}$  (specific activity, 6.0 Ci/mmol) body weight 3 hr after the last injection of amiloride or Ringer's solution, and all mice were killed by decapitation 1 hr later.

Segments of the cortex of the tumors were then processed for either electron probe X-ray microanalysis or autoradiography.

In preparation for electron probe X-ray microanalysis, tissue was removed and attached to a cylindrical hollow brass tube 3 mm in diameter. The specimens were dissected and frozen by immersion in liquid propane cooled in a liquid nitrogen bath in <1 min. Each specimen was then transferred to a cryostat (Harris Manufacturing Co., North Billerica, Mass.) maintained at  $-40^\circ$  (19). Two- $\mu\text{m}$ -thick sections were cut with a Minot custom microtome (Damon/IEC Division, Damon Corp., Needham Heights, Mass.).

The 2- $\mu\text{m}$ -thick sections were positioned on a film of Formvar (0.9% in dioxane) spanning a 1.5-mm hole in a 3-mm carbon grid. To minimize curling or movement of the sections, a cover was laid over the sections. The cover consisted of a circular piece of heavy aluminum with a Formvar film stretched across a hole in the center. Thus, the section was sandwiched between 2 Formvar films. The sandwiched specimen was dried for 16 hr at  $-40^\circ$  in a custom-made cryoabsorption apparatus, warmed to room temperature, vented with nitrogen gas, and stored in a desiccator. At the time of analysis, the cover was teased

<sup>4</sup> The abbreviations used are: ANOVA, analysis of variance; PVP, polyvinyl pyrrolidone.

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away, leaving a flat dried section sandwiched between 2 layers of Formvar film. The sections were examined without coating at 25 kV in a JEOL JSM-35 scanning electron microscope equipped with an Si(Li) X-ray detector (Nuclear Semiconductor, Inc., Menlo Park, Calif.). The distance from specimen to detector was 1.5 cm, and the takeoff angle was 40°. The data were collected, stored, and processed by an NS-880 pulse-height analysis system (Tracor Northern, Middleton, Wis.). Morphology of the specimens was recorded by photographing the transmitted image from the recording cathode ray tube. For data collection, a rapid scan at a magnification of 30 to 75 × 10<sup>3</sup> was made for 100 seconds. The specimen area rastered was 0.39 to 2.44 sq μm. Specimen current as measured on the graphite planchet was 0.15 na. Our microanalysis quantitation was based upon the Hall mass fraction technique.

The nucleus within 25 μm of a blood vessel was analyzed for element content. X-ray pulse-height distribution was measured in the energy range of 0 to 10.22 keV with a resolution of 20 eV/channel. The Tracor Northern Super ML (multiple least squares) program was used to deconvolute the spectra and to calculate elemental peak:continuum ratios for each element in each spectrum. Continuum was arbitrarily designated as 4.50 to 5.00 keV, an energy interval in which no characteristic peaks were generated from the samples. For each of the detectable elements of biological relevance, the 2 groups of data were subjected to the ANOVA statistical test.

Quantification of data was performed with a series of standards prepared similarly to those of Cameron et al. (3). Known amounts of dried salts were added to aqueous solutions of PVP (special grade; Aldrich Chemical Co., Milwaukee, Wis.). The standards were prepared to a composition of approximately 20% dry solids, were frozen sectioned to 2 μm, and were freeze-dried in the usual procedure of this laboratory. By adjusting the relative amounts of the various salts, a series of multielement standards were prepared in which the total weight of electrolytes was kept nearly constant in all of the PVP standards. Microanalysis was done on PVP sections of 4 different concentrations for a given element and on 20% PVP without added salts. A least-squares linear regression analysis was used to obtain a constant of proportionality value for each element from the peak to continuum data. This constant of proportionality was used to convert from peak to continuum values, to content (mmol/kg dry weight) for each element.

For autoradiography and histological examination, pieces of tumor were removed at sacrifice and were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4 μm. The sections were then mounted on glass slides for autoradiography or histology.

To determine the effects of amiloride on proliferation of H6 cells, *in vivo* autoradiographs were prepared from deparaffinized mounted histological sections after they were washed in methanol:glacial acetic acid (3:1, v/v) followed by washes in distilled water. The slides were processed for autoradiography using NTB-2 emulsion (Kodak). Autoradiographic exposure time was 2 months, and then the slides were developed in Dektol (Kodak). The developed autoradiographs were stained through the emulsion with hematoxylin and eosin. The percentage of hepatoma cells with a labeled nucleus within 25 μm of a blood vessel were scored with the aid of a calibrated ocular grid. An average of 3 microscopic fields near the tumor cortex was scored for each mouse and each field averaged about 75 cells. To determine if the effect of amiloride on tumor growth was due to the actions of the drug which might affect cell volume, average metaphase and interphase cell diameters were measured in histological sections using a calibrated ocular micrometer.

**RESULTS**

The results of the initial experiment on H6 hepatoma growth *in vivo* are illustrated in Chart 1. After 14 injections, each spaced 8 hr apart, the percentage increases in tumor area for

the 3 groups were: (a) control (Ringer's injections), 298%; (b) amiloride (0.1 μg/g body weight), 293%; and (c) amiloride (1.0 μg/g body weight), 102%. The tumor areas of the control group and those of the amiloride (0.1 μg/g body weight) group had increased about 3 times as much as those of the group treated with amiloride (1.0 μg/g body weight). Tumor growth was also expressed as a rate by use of a linear regression program, and the slopes in sq cm/day from each animal in the treatment groups were subjected to a one-way ANOVA. The results of this analysis show that amiloride (1.0 μg/g body weight) (slope, 0.190 sq cm/day) significantly suppressed tumor growth compared to the control group (slope, 0.317; *p* < 0.001) and to the group treated with amiloride (0.1 μg/g body weight) (slope, 0.276; *p* < 0.01).

To determine if the influence of amiloride on tumor growth was due to an action which might cause a cell volume change, the diameters of metaphase and interphase hepatoma cells were measured. A one-way ANOVA showed that there were no significant differences within or among the groups when comparing metaphase and interphase cell diameters (Table 1).

The results of the effects of amiloride on DMA/J mammary adenocarcinoma growth *in vivo* are illustrated in Chart 2. After 17 injections, each spaced 8 hr apart, the percentage increases in tumor area for the 3 groups were: Group 1, control, 1022%; Group 2, amiloride (0.1 μg/g body weight), 226%; and Group 3, amiloride (1.0 μg/g body weight), 79%. Amiloride (1.0 μg/g body weight) (slope, 0.016) suppressed tumor

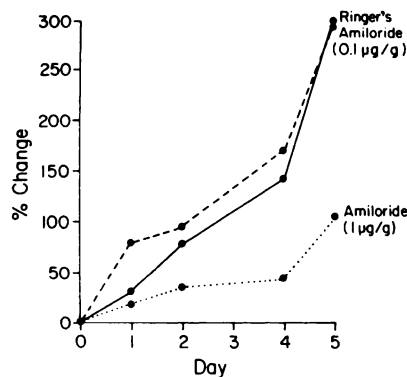


Chart 1. Cumulative percentage changes in H6 hepatoma tumor area relative to size at initiation of treatment. Tumors were measured daily using calipers. Each day, the mean tumor area for each group (≥4 animals/group) was determined and expressed as the cumulative percentage change in area from that at the initiation of treatment. Each animal received an injection every 8 hr for a total of 14 injections.

Table 1  
Effect of amiloride on mean profile diameters of interphase and metaphase H6 hepatoma cells and on DMA/J mammary adenocarcinoma cells *in vivo*

Treatment group	Cell cycle location	H6 cells		DMA/J cells	
		Cell diameters (μm)	n <sup>a</sup>	Cell diameter (μm)	n
Ringer's	Metaphase	11.00 ± 0.39 <sup>b</sup>	5	12.24 ± 0.54	6
	Interphase	11.40 ± 0.29	5	10.84 ± 0.16	6
Amiloride (0.1 μg/g)	Metaphase	10.25 ± 0.10	4	11.48 ± 0.33	5
	Interphase	10.64 ± 0.14	4	11.02 ± 0.17	5
Amiloride (1.0 μg/g)	Metaphase	10.55 ± 0.31	5	11.71 ± 0.29	7
	Interphase	11.47 ± 0.26	5	11.11 ± 0.21	7

<sup>a</sup> n, number of animals analyzed. Ten cells were measured per animal.  
<sup>b</sup> Mean ± S.E.; no significance was found between any of the means as determined by one-way analysis of variance.

growth compared to the control group (slope, 0.097;  $p < 0.05$ ). It can be seen in Table 1 that amiloride had no effect on the diameter of the metaphase or the interphase DMA/J mammary adenocarcinoma cells *in vivo*.

Further experiments to establish the dose response are summarized in Chart 3. Amiloride (5.0  $\mu\text{g/g}$  body weight) almost completely inhibited tumor growth during the 3-day period of the experiment. The total percentage increases in tumor area from the initiation of treatment until sacrifice were: control, 57%; amiloride (0.5  $\mu\text{g/g}$  body weight), 51%; amiloride (1.0  $\mu\text{g/g}$  body weight), 58%; and amiloride (5.0  $\mu\text{g/g}$  body weight), 5%. This corresponds to about a 10- or 11-fold difference between tumor area increase in the group treated with amiloride (5.0  $\mu\text{g/g}$  body weight) and that in the other 3 groups. The daily cumulative percentage changes in the control group and those in the groups treated with amiloride at 0.5  $\mu\text{g/g}$  body weight and 1.0  $\mu\text{g/g}$  body weight were virtually the same (Chart 3). As stated above, differences in tumor growth rate between groups were determined by one-way ANOVA of the slopes of tumor growth rate. Amiloride (5  $\mu\text{g/g}$  body weight), (slope, 0.092) significantly suppressed tumor growth when compared to the other 3 groups: control (slope, 0.316;  $p < 0.01$ ); amiloride (0.5  $\mu\text{g/g}$  body weight) (slope, 0.331;  $p < 0.01$ ); and amiloride (1.0  $\mu\text{g/g}$  body weight) (slope, 0.356;  $p < 0.01$ ).

Electron probe X-ray microanalysis of the nucleus on hepatoma cells of amiloride- and non-amiloride-treated mice revealed significant ( $p < 0.05$ ) changes in the content of sodium due to the amiloride treatment (Table 2). Of the other elements measured, chlorine showed a near significantly reduced concentration (content) in the amiloride-treated mice compared to that in the control group, but none of the other elements showed a significant content change.

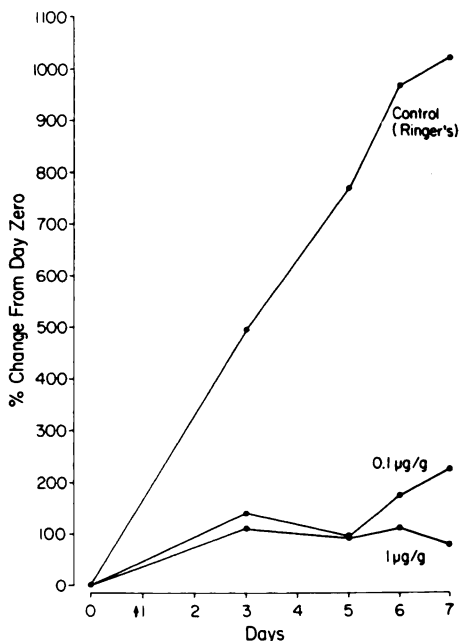


Chart 2. Effect of amiloride on DMA/J adenocarcinoma growth *in vivo*. Tumors were measured daily using calipers. The mean tumor area for each group ( $\geq 5$  animals/group) determined on the days indicated and expressed as percentage change in area from Day 0. Day 0 was designated as the day on which tumor measurements were made immediately prior to the initiation of amiloride treatment. Arrow, time at which amiloride treatment was initiated. Animals received injections every 8 hr for a total of 17 injections.

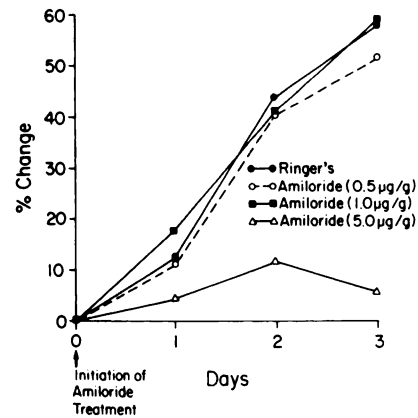


Chart 3. Effect of amiloride treatment on H6 hepatoma growth *in vivo*. Each day, the mean tumor area (in sq cm) for each group ( $\geq 10$  animals/group) was determined and expressed as the cumulative percentage of change in area from the initiation of treatment. Each animal received an injection at the indicated dosage every 8 hr for 65 hr.

Table 2

Effect of 3 injections of amiloride (1.0  $\mu\text{g/g}$  body weight each) given at 8-hr intervals on cell proliferation activity (autoradiographic labeling index) of H6 hepatoma cells *in vivo*

The mice were given injections of tritiated thymidine 1 hr before sacrifice.

Treatment group	No. of mice	% of labeled cells within 25 $\mu\text{m}$ of a blood vessel
Ringer's	5	44.3 $\pm$ 1.6 <sup>a</sup>
Amiloride in Ringer's	5	36.6 $\pm$ 1.9 <sup>b</sup>

<sup>a</sup> Mean  $\pm$  S.E.

<sup>b</sup>  $P < 0.025$ ; significantly lower than the Ringer's treatment.

The data in Table 2 show that a series of 3 injections of amiloride (1.0  $\mu\text{g/g}$  body weight) significantly suppressed the tritiated thymidine labeling index of the tumor ( $p < 0.025$ ).

## DISCUSSION

The present studies were undertaken to help determine the role of intracellular sodium in transformed cells. We tested the effects of amiloride on tumor growth, tritiated thymidine labeling index, and intracellular element content. Several recent studies give support to the idea that amiloride inhibits a passive sodium influx that is required for cell proliferation (12, 20, 22, 26). Therefore, the present studies were done in an effort to correlate the intracellular content of sodium to cellular proliferation and the inhibition of tumor growth.

Changes in ion transport and intracellular ion or element concentrations have been implicated in the control of mitogenesis and oncogenesis (4, 9-12, 21-23). Using the morpho-analytical technique of electron probe X-ray microanalysis, we have previously shown that the nucleus and the cytoplasm of H6 hepatoma cells have high levels of sodium compared to those of normal hepatocytes (24). Further studies comparing the intracellular element contents of normal and transformed cells *in vivo* have, in every case tested so far, shown that transformed cells have high sodium levels compared to those of their normal counterpart cells (4-7, 18, 24). Pool *et al.* (18) also showed that, after 5 days of treatment, the intracellular content of sodium in hepatocytes from animals exposed to a hepatocellular carcinogen was increasing towards the high levels found in overtly transformed hepatoma cells. It was concluded that not only is the elevation of sodium characteristic

Table 3

Effect of 3 injections of amiloride at 1.0 µg/g body weight each on intranuclear element content of H6 hepatoma cells *in vivo*

Values represent means from 3 animals in the amiloride group and 4 animals in the Ringer's group. Ten X-ray spectra were collected from each animal.

Element	Content (mmol/kg, dry wt)	
	Ringer's (4)	Amiloride (1.0 µg/g) (3)
Sodium	499 ± 60 <sup>a</sup>	275 ± 26 <sup>b</sup>
Phosphorus	635 ± 29	656 ± 99 <sup>c</sup>
Sulfur	156 ± 8	137 ± 14 <sup>c</sup>
Chlorine	328 ± 25	234 ± 26 <sup>d</sup>
Potassium	556 ± 51	664 ± 66 <sup>c</sup>
Magnesium	54 ± 4	67 ± 8 <sup>c</sup>

<sup>a</sup> Mean ± S.E.

<sup>b</sup>  $p < 0.05$ ; significantly different from Ringer's control values.

<sup>c</sup> Not significantly different from Ringer's control value.

<sup>d</sup>  $p < 0.10$ .

of transformed cells but also it may somehow be involved in the process of neoplastic transformation.

The results showed that, after 5 to 6 days of repeated injections of amiloride (1.0 µg/g body weight), a significant inhibition of the *in vivo* growth of both the H6 hepatoma and the DMA/J mammary adenocarcinoma was observed (Charts 1 and 2). The data also show that injections of amiloride (5 µg/g body weight) can inhibit H6 hepatoma growth (Chart 3) in a shorter period of time than that required for 1 µg/g body weight. Higher doses of amiloride, such as those Koch and Leffert (12) administered to the rat, could not be used in the A/J mouse, because preliminary studies using the A/J mouse showed that multiple injections of amiloride ( $\geq 10$  µg/g body weight) were lethal. Koch and Leffert (12) showed that a single injection of amiloride (50% inhibitory dose, ~25 µg/g body weight) inhibited rat hepatocyte DNA synthesis following 67% hepatectomy. Together, these data show that amiloride can inhibit the proliferation of normal and transformed cells *in vivo*. Further studies will be needed to determine if the lower doses of amiloride needed to inhibit tumor growth are due to the possible cumulative effect of doses given every 8 hr for several days. It is doubtful that any of the effects of amiloride on proliferation are due to its action on transport leading to change in cell volume as shown by the fact that amiloride had no effect on the cell diameters of H6 cells or DMA/J cells *in vivo* (Table 1).

Electron probe X-ray microanalysis was performed on control and amiloride-treated H6 hepatoma cells from the *in vivo* samples to determine if any changes in intranuclear element (ion) content could be correlated to changes in tumor growth. It was postulated that tumor cell proliferation might be inhibited by decreasing intranuclear sodium levels, since a correlation has been shown between intranuclear sodium content and the state of cell proliferation and cell transformation (1, 4, 5, 9, 18, 24, 25). As seen in Table 3, the intranuclear sodium content is significantly reduced in the amiloride-treated mice. No other element showed a significant change due to amiloride treatment. This demonstrated that a reduction in sodium content of transformed cells is correlated to inhibition of cell proliferation as measured by the tritiated thymidine labeling index. It therefore seems that amiloride, at a dosage which inhibited tumor growth, may have done so by inhibiting the sodium flux and/or sodium content increase that is associated with cell proliferation (12, 13, 15-17, 20, 22, 26). An alternate explanation for

the action of amiloride in inhibiting tumor growth may be that it directly inhibits protein synthesis (14).

In summary, amiloride suppressed H6 hepatoma growth and DMA/J mammary adenocarcinoma growth and caused a significant reduction in the intranuclear sodium content of H6 hepatoma cells. A direct correlation between intracellular sodium content, cell proliferation, and tumor growth after amiloride treatment has therefore been established in the H6 hepatoma.

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