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Effects of Disodium Etidronate in Murine Tumor Models*

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Abstract—*This study was designed to elucidate the effect of ethane-1-hydroxy-1,1-diphosphonate (EHDP) in experimental rodent tumors. EHDP had no antitumor activity against the L1210 leukemia implanted i.p. and against sarcoma 180, Lewis lung carcinoma (3LL) and Walker 256/B carcinoma injected i.p., s.c. or i.m. respectively. EHDP did not interfere with the antitumor activity of commonly used conventional chemotherapeutic agents (adriamycin, cyclophosphamide, 5-fluorouracil, bis-chloroethylnitrosourea) in the L1210 and 3LL models. EHDP reduced proportionally to the dose the hypercalcemia and hypercalciuria due to the Walker 256/B carcinoma growth. In an effort to evaluate whether EHDP-treated osseous tissues were more refractory to tumor growth, cells from sarcoma 180 and 3LL carcinoma were implanted intratibially (i.t.). Growth of 3LL cells was not consistently affected by EHDP, whereas a modest, but significant, growth inhibition was consistently observed with sarcoma 180 injected i.t. Growth of sarcoma 180 implanted i.p. or s.c. was not reduced by this drug, thus suggesting that inhibition of i.t. sarcoma 180 was in fact related to alterations of osseous tissues by EHDP. Inoculation of Walker 256/B carcinoma intra-aortically resulted in osteolytic bone lesions in the hind limbs. EHDP inhibited the formation of bone metastasis under these conditions.*

INTRODUCTION

CANCER is frequently associated with bone resorption and with hypercalcemia, as a consequence of localization of tumors to bones or of humoral mediators [1]. Diphosphonates have been shown to enhance the resistance of bones to osteolysis [2], including that induced by cancer cells *in vitro* [2–4]. Studies have been conducted in humans to evaluate the effect of diphosphonates in the control of hypercalcemia associated with neoplastic diseases and of the formation and progression of skeletal lesions [5–11]. Beneficial effects have been reported [4–11], though the role of these agents in the medical treatment of neoplasia remains to be firmly established and their mode of action to be fully elucidated.

In contrast to relatively numerous studies conducted in humans, little information is available on the effects of diphosphonate *in vivo* in experimental tumor models. The present study was designed to elucidate (a) whether ethane-1-hydroxy-1,1-diphosphonate (EHDP) had direct antitumor activity in experimental tumor models; (b) whether EHDP affected the antitumor activity of conventional chemotherapy; (c) whether this agent modified hypercalcemia in a rodent tumor model; and (d) whether growth of tumor cells directly implanted in bones and bone metastasis were affected by EHDP.

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MATERIALS AND METHODS

Animals

Male DBA/2, CD2F$_1$, C57Bl/6 and CD$_1$ mice (6–8 weeks old) and male CD-COBS rats (6 weeks old) were obtained from Charles River, Calco, Italy.

Tumors

Tumors and experimental procedures were as previously described [12–16] and will only be briefly mentioned here. The L1210 leukemia was maintained in ascitic form in syngeneic DBA/2 mice, and transplanted at a dose of 10^5 cells i.p. in compatible $CD2F_1$ mice. The Lewis lung carcinoma (3LL) was maintained in syngeneic C57BL/6 mice and 2×10^5 cells of the disaggregated tumor were inoculated i.m.

Sarcoma 180 (S180) was maintained by s.c. passage in CD_1 mice and transplanted (from 10^5 to 10^6 cells in different experiments) s.c. or i.p.

The Walker 256/B carcinoma [15, 16] was maintained s.c. in CD-COBS rats and 10^5 cells were transplanted i.m. in the same animal strain. Tumor cells from S180 and 3LL tumors (10^5 cells in 10 μl) were also inoculated in the tibia shafts (i.t.) of mice as previously described in detail [12].

Mice alive and disease-free beyond day 60 after L1210 leukemia transplantation were considered cured. Growth of solid tumors was measured 2–3 times/week with calipers. Animals transplanted with the 3LL and S180 tumors were also killed at specific times after transplantation and the weight of primary lesions and the number and weight of secondaries was measured as previously described [12–15]. Total calcium and inorganic phosphorus in plasma and urine were measured by colorimetric tests (Test Combination, Boehringer, F.R.G.). Both parameters were followed in each animal individually, throughout the whole period of observation (21 days).

Osteolytic bone metastases were obtained by injecting Walker 256/B carcinoma cells intra-aortically (i.a.) as described by Powles *et al.* [17]. Briefly, rats (250 g body weight) were anesthetized with chloralium (400 mg/kg i.p.) and the abdomen was open to expose the abdominal aorta. Cells (10^5) in 0.2 ml of sterile saline were injected i.a. By day 11 after tumor transplantation rats showed clinically obvious signs of hind limb paralysis, presumably because of pain related to osseous lesions. Rats were killed on day 14 and the hind limbs fixed in buffered formaline fixative and coded. X-rays were taken and examined by a clinical radiologist, unaware of treatment differences among groups. Coded samples were also examined independently by two of us. Samples were evaluated for the presence or absence of osteolytic bone lesions (see, for example, Fig. 2), which were scored as severe or borderline when their presence was doubtful.

Drugs

EHDP was obtained through the courtesy of Dr V. A. Uchtman, (Procter and Gamble, Cincinnati, OH). The compound was dissolved in saline (5–40 mg/ml).

Adriamycin (AM) was obtained from Farmitalia, Milano, Italy. Cyclophosphamide (Cy), bis-chloroethylnitrosourea (BCNU) and fluorouracil (5FU) were obtained from Dr H. J. Wood (Drug Research and Development Program, National Cancer Institute, Bethesda, MD).

Chemotherapeutic drugs were freshly dissolved in saline and given i.v. (AM), i.p. (Cy and BCNU) or s.c. (EHDP) in a volume of 0.1 ml/10 g body weight.

Statistical analysis

Eight to 15 mice and 4–9 rats per experimental group were used throughout and experiments were repeated at least twice.

The incidence of osteolytic bone lesions was analyzed by Fisher's exact test.

Calcium and phosphorus levels, monitored at different times after tumor inoculation, were analyzed by split-plot design and Tukey's test.

RESULTS

EHDP was given s.c. from day −7 to day 15 to mice transplanted with the L1210 leukemia (i.p.), 3LL carcinoma and S180 carcinoma (i.m. or s.c.). Doses of EHDP ranged from 5 to 40 mg/kg for the tumors in $CD2F_1$ (L1210) and CD_1 (S180) mice, and from 5 to 30 mg/kg for C57Bl/6 mice, the body weight of which was reduced by higher doses of this agent. EHDP under these conditions did not inhibit the growth and metastasis of these murine tumors (data not shown). Similarly, EHDP did not inhibit the growth of the Walker 256/B carcinoma: at day 21 the i.m. tumoral masses reached 32.3 ± 2.4 and 33.1 ± 2.2 g in untreated and EHDP (40 mg/kg)-treated rats respectively.

Diphosphonates have been (and presumably will be) given to tumor-bearing humans concomitantly with conventional chemotherapy. It was therefore of interest to evaluate whether EHDP interfered with the antitumor activity of commonly used cytotoxic agents such as AM, Cy, 5FU and BCNU. These experiments were conducted with the L1210 leukemia transplanted i.p. and 3LL carcinoma transplanted i.m. EHDP did not interfere with the antitumor activity of AM, Cy, BCNU and 5FU in these experimental models (data not shown). In one out of three experiments performed EHDP at a dose of 5 mg/kg significantly augmented the incidence of cures in L1210 bearing mice treated with Cy, but this was not confirmed in two subsequent experiments.

In an effort to evaluate whether EHDP affected the growth of tumor cells growing in bones, mice

were transplanted i.t. with S180 or 3LL carcinoma and treated with this agent. As shown in Table 1, the weight of S180 sarcoma at autopsy was significantly ($P<0.05$) lower in EHDP (40 mg/kg)-treated mice (6.08 ± 0.48 and 4.54 ± 0.4 g in the 2 experiments performed) compared to controls (7.31 ± 0.56 and 5.83 ± 0.55 g).

This finding was confirmed when tumor growth i.t. was measured by calipers (data not shown). S180 was only inhibited by EHDP, though marginally, when transplanted i.t., whereas growth s.c. or i.p. was not decreased (Table 2): if anything, treatment with 40 mg/kg significantly augmented growth of sarcoma 180 s.c. (Table 2). After i.p. inoculation (2×10^5 cells) the survival sime of EHDP-treated (40 mg/kg) mice inoculated with S180 cells was 16.8 ± 0.6 days, compared to 14.1 ± 0.7 for controls (Table 2). Unlike the S180 tumor, the 3LL carcinoma (Table 3) inoculated i.t. was not consistently inhibited by EHDP (20–30 mg/kg).

The effects of EHDP on tumor-induced hypercalcemia were studied in rats transplanted with the Walker 256/B carcinoma. This line was selected because, unlike a non-metastatic variant of the same tumor [15], it caused a significant and early hypercalcemia and hypercalciuria (Fig. 1).

In a first series of experiments the effects of EHDP in normal non-tumor-bearing rats were investigated. At the highest dose employed (40 mg/kg) EHDP caused some reduction of food intake and less increase of body weight (after 15 days of treatment the mean body weight value was 300 g for controls and 260 g for EHDP treated rats, data not shown). Treatment with this agent slightly increased plasma levels of calcium, with a value of 11.35 ± 0.19 (mg/100 ml) after 15 days of treatment (40 mg/lkg) compared to 10.36 ± 0.11 for controls. Calciuria was also augmented in EHDP-treated normal rats with values of 1.13 ± 0.08 and 7.59 ± 0.77 (mg/24 hr) for control and treated (40 mg/kg) rats respectively.

Table 1. Effect of EHDP on growth and metastasis of intratibially implanted sarcoma 180

	Treatment*	Dose (mg/kg, s.c.)	Mice with metastasis/total	Tumor weight (g ± S.E.)	Metastases (number ± S.E.)	Metastasis weight (mg ± S.E.)
Experiment 1	vehicle	-	16/16	5.82 ± 0.5	3.12 ± 0.3	346 ± 74.8
	EHDP	10	19/19	6.73 ± 0.5	3.37 ± 0.6	492 ± 86.1
	EHDP	40	16/17	4.54 ± 0.4†	3.25 ± 0.3	312 ± 93
Experiment 2	vehicle	-	13/13	7.31 ± 0.5	3.15 ± 0.2	468 ± 187
	EHDP	40	15/15	6.08 ± 0.4†	3.64 ± 0.4	501 ± 127

*Animals were treated from day -8 to day 15 and were killed on day 35.
†$P<0.05$.

Table 2. Effect of EHDP on sarcoms 180 implanted s.c. or i.p.

S180 implanted	Treatment*	Mice with tumor	MST†	Tumour weight g ± S.D.
i.p.	vehicle	10/10	16.8 ± 0.5	-
	EHDP, 20 mg/kg	10/10	14.1 ± 0.5	-
	EHDP, 40 mg/kg	10/10	14.1 ± 0.7	-
s.c.	vehicle	10/10	-	4.91 ± 1
	EHDP, 20 mg/kg	9/10	±	5.01 ± 0.2
	EHDP, 40 mg/kg	10/10	-	6.80 ± 0.98

*Animals were treated from day -8 to day 15.
†Mean survival time.

Table 3. Effect of EHDP on intratibial 3LL carcinoma

Treatment	Dose (mg/kg, s.c.)	Tumor weight (g ± S.E.)	Metastases No.	Metastases Weight (mg)
Vehicle	-	5.2 ± 0.4	9.1 ± 1.8	20 ± 8.7
EHDP	20	4.9 ± 0.7	5 ± 1.4	14.4 ± 5.6
EHDP	30	4.4 ± 0.3	6 ± 1.3	7.6 ± 3.4*

*Mean of 6 mice; the 7th had big metastases to kidney and lung metastases weighing 177 mg.

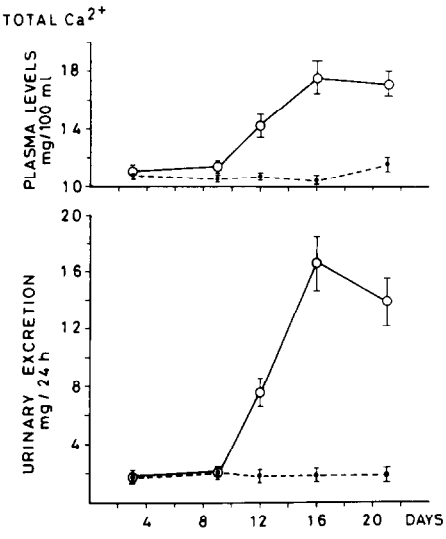

Fig. 1. Plasma levels (upper part) and urinary excretion (lower part) of total calcium in Walker 256/B carcinoma-bearing O——O *and normal* ●——● *rats.*

The effect of EHDP on Walker 256/B-implanted animals was then studied. The growth of the Walker 256/B tumor was not diminished by treatment with EHDP, with a tumor weight at the end of a typical experiment of 28.28 ± 2.35 and 31.63 ± 2.28 g for control and treated (40 mg/kg) rats respectively.

In general, onset of changes of biochemical and physiological parameters is characterized by a degree of variability among individuals bearing the Walker 256/B carcinoma. Nevertheless, this tumor, implanted intramuscularly, provoked a clearcut statistically significant increase in total calcium levels in plasma, starting from day 9 after tumor inoculation (Fig. 1, upper part).

As shown in Table 4, EHDP (20–40 mg/kg) caused a dose-dependent inhibition of tumor-induced hypercalcemia, with some effect at 10 mg/kg. Treatment with 40 mg/kg, instituted on day 1, kept plasma calcium close to controls.

The growth of W256/B carcinoma caused an appreciable increase of calciuria (Fig. 1, lower part) from day 12 onward. This increase was completely reversed by administration of EHDP 40 mg/kg given from day 1 or day 6 (Table 5) and partially reversed by all other doses.

When treatment with 30–40 mg/kg EHDP was instituted 1 day after tumor inoculation, urinary calcium excretion was initially (days 3–9) somewhat increased compared to untreated tumor-bearing rats (Table 5). Similar results were obtained when EHDP (40 mg/kg) was given to healthy controls.

Concomitantly with the W256/B induced hypercalcemia, the inorganic phosphorus in plasma decreased significantly and EHDP administration did not appreciably affect its levels (data not shown).

Upon i.a. inoculation of Walker 256/B carcinoma (Table 6, Fig. 2) osteolytic bone lesions were consistently observed in hind limbs, in agreement with a previous report [17]. Areas of tumor-induced osteolysis were particularly prominent in the distal femur and proximal tibia (Fig. 2). Treatment with EHDP (30 mg/kg on days −3 to +13 after tumor inoculation) protected rats from the development of osteolytic bone metastases. Only 1 of 7 treated animals showed minimal (doubtful) evidence of bone involvement, whereas 5 of 6 control vehicle-injected rats had diffuse areas of tumor-induced osteolysis and one

Table 4. Effect of EHDP on plasma calcium levels in Walker 256/B carcinoma-bearing rats

Rats	EHDP (mg/kg)	Calcium levels (mg/100 ml ± S.E.) on day: 3	9	12	16	21
Normal ($n = 4$)	-	10.79 ± 0.06	10.44 ± 0.48	10.66 ± 0.25	10.36 ± 0.11	11.56 ± 0.35
Tumor-bearing ($n = 7$)	-	11.09 ± 0.25	11.38 ± 0.48	14.79 ± 1.15‡	17.42 ± 1.08‡	17.13 ± 0.81‡
Tumor-bearing ($n = 7$)	10*	10.94 ± 0.20	11.28 ± 0.38	13.88 ± 0.66	16.01 ± 0.60	16.01 ± 0.52
Tumor-bearing ($n = 4$)	20*	11.18 ± 0.25	10.23 ± 0.11	13.35 ± 0.78	14.63 ± 0.24	14.33 ± 0.59
Tumor-bearing ($n = 4$)	30*	11.66 ± 0.25	11.23 ± 0.28	13.51 ± 0.22	13.37 ± 0.87	14.18 ± 0.69
Tumor-bearing ($n = 4$)	40*	11.44 ± 0.22	11.44 ± 0.64	12.97 ± 1.21§	10.89 ± 0.27§	12.06 ± 2.16§
Tumor-bearing ($n = 7$)	40†	10.60 ± 0.18	11.11 ± 0.59	12.92 ± 1.08§	11.78 ± 1.01	12.47 ± 1.29§

*EHDP was given on days 1–15 after tumor implantation.
†EHDP was given on days 6–15 after tumor implantation.
‡$P<0.01$ vs normal rats.
§$P<0.01$ vs control tumor-bearing rats.

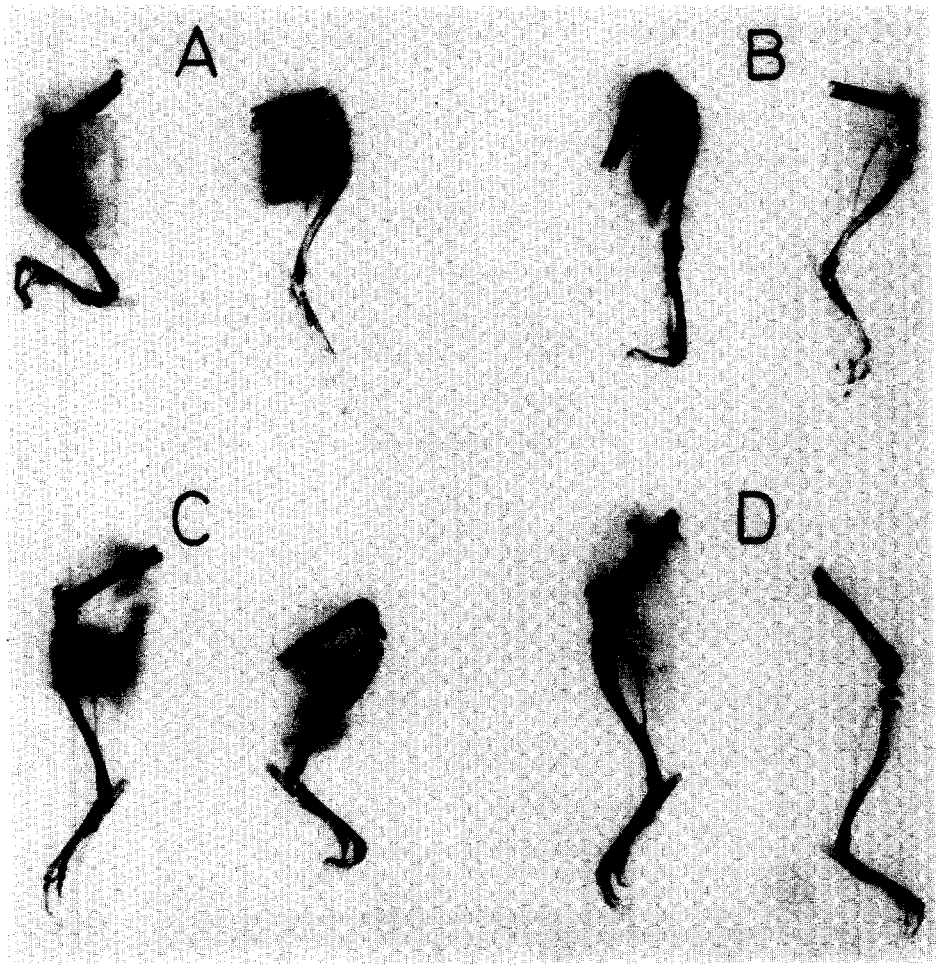

Fig. 2. Radiographs of hind limbs of 4 rats inoculated i.a. with Walker 256 carcinoma and treated with vehicle (A and B) or with EHDP (C and D) as detailed in Table 6.

Table 5 Effect of EHDP on calcium urinary excretion in Walker 256/B-bearing rats

Rats	EHDP (mg/kg)	Calcium urinary excretion (mg/24 hr ± S.E.) on day: 3	9	12	16	21
Normal ($n=4$)	-	1.74 ± 0.35	1.97 ± 0.18	1.84 ± 0.21	1.80 ± 0.26	1.84 ± 0.34
Tumor-bearing ($n=7$)	-	1.76 ± 0.30	1.95 ± 0.19	7.45 ± 0.95‡	16.63 ± 2.34‡	13.94 ± 1.58‡
Tumor-bearing ($n=4$)	10*	1.67 ± 0.50	3.16 ± 1.04	7.43 ± 1.49	9.77 ± 1.80§	8.76 ± 1.81
Tumor-bearing ($n=4$)	20*	1.71 ± 0.31	7.28 ± 1.16	7.69 ± 1.17	6.37 ± 1.57‖	2.25 ± 0.48‖
Tumor-bearing ($n=4$)	30*	2.89 ± 0.45	4.77 ± 1.30	6.42 ± 2.05	5.21 ± 1.63‖	2.94 ± 1.29‖
Tumor-bearing ($n=4$)	40*	4.72 ± 0.37	5.57 ± 0.34	2.10 ± 0.71	1.34 ± 0.60%	1.14 ± 0.60%
Tumor-bearing ($n=7$)	40†	1.66 ± 0.22	6.52 ± 1.02	3.40 ± 0.93	2.22 ± 0.71‖	2.43 ± 0.99%

*EHDP on days 1–15 after tumor implantation.
†EHDP on days 6–15 after tumor implantation.
‡$P<0.01$ vs normal rats.
§$P<0.05$ vs control tumor-bearing rats.
‖$P<0.01$ vs control tumor-bearing rats.

Table 6. Effect of EHDP on osteolytic bone metastases of i.a. inoculated Walker 256 carcinoma

Rats treated with:	Osteolytic lesions in hind limbs Borderline	Severe
Vehicle	1/6	5/6
EHDP	1/7	0/7

Rats were treated with EHDP (30 mg/kg/day) s.c. from day −3 to day +13 of tumor inoculations. Treatment was omitted on the day of tumor injection (day 0). The incidence of osteolytic lesions was significantly ($P<0.05$, Fisher's exact test) lower in EHDP-treated animals.

had minimal lesions (Table 6, Fig. 2). Moreover, also under these conditions EHDP prevented the hypercalcemia induced by Walker 256 carcinoma (data not shown).

DISCUSSION

Diphosphonates have been used in the treatment of human neoplastic disorders to reduce hypercalciuria and to inhibit the formation and progression of skeletal metastases [5–11]. So far studies in humans have been designed and conducted with little or no information on the effects of these agents in experimental tumors [3, 4], particularly as far as *in vivo* conditions are concerned. A first objective of this study was to assess whether EHDP had any direct *in vivo* antitumor activity *per se* and whether it affected the antineoplastic efficacy of commonly used conventional chemotherapeutic drugs. The latter point seemed worth investigating because diphosphonates have been given to subjects treated concomitantly with chemotherapy [10].

EHDP had no antitumor activity against the L1210 leukemia implanted i.p. and against the S180 sarcoma (i.p. or s.c.), 3LL carcinoma (i.m.) and Walker 256/B carcinoma (i.m.). It was reassuring to learn that this agent did not interfere with the antitumor activity against L1210 and 3LL tumor of AM, Cy, BCNU and 5FU, selected because deemed representative of classes of chemotherapeutic drugs of common use.

EHDP contrasted the hypercalcemia and hypercalciuria induced by the Walker 256/B carcinoma selected as a model of humoral hypercalcemia in malignancy. Preliminary evidence (data not shown) suggests that hypercalcemia in this model is related to generalized bone resorption, possibly due to humoral factors, in analogy with other experimental tumors [18] and some human cancers [1, 19]. These results suggest that EHDP rendered calcified tissues of Walker carcinoma-bearing rats more resistant *in vivo* to bone resorption. Further studies are required to elucidate further the mechanism(s) of action of EHDP in the Walker carcinoma model.

In an effort to evaluate whether EHDP rendered osseous tissues more refractory to tumor growth, cells of the S180 and 3LL tumor were implanted in the tibia shafts. In two experiments performed, EHDP caused a modest, but significant, inhibition of growth of i.t. implanted S180 cells, whereas the 3LL carcinoma was not consistently affected. As mentioned above, growth of S180 cells transplanted i.p. or s.c. was not inhibited by EHDP: if anything, s.c. S180 tumors grew faster in treated mice. These observations lend some support to the idea that the low, but significant,

inhibition of i.t. growing S180 tumor by EHDP is in fact related to a modification by this agent of osseous tissues.

Models of spontaneous bone metastasis are not available at present and therefore we induced bone lesions in hind limbs by injecting Walker 256/B carcinoma i.a. in rats. EHDP markedly reduced the appearance of osteolytic bone metastases following i.a. inoculation of 256 /B Walker carcinoma. The lack of antitumor activity of EHDP against i.m. Walker 256/B cells (see above) and the capacity of this compound to inhibit tumor-induced hypercalcemia following inoculation of carcinoma cells i.m. (Fig. 1) or i.a. (not shown) strongly suggests that inhibition of the formation of bone metastasis is in fact related to the ability of this compound to render osseous tissues more resistant to resorption and hence to tumor implantation and growth.

The major limitation of studies in experimental tumors with agents active on skeletal tissues is the present lack of syngeneic murine neoplasms spontaneously metastasizing to bones. Such models, if available, would be invaluable for analyzing the biology of implantation and growth of tumors in bones and drugs affecting skeletal metastasis. Efforts along this line are currently being made in this laboratory.

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