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Neutral Proteinase Inhibitors and Antimetastatic Effects in Mice*

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Abstract—*The effects of a series of neutral proteinase inhibitors have been examined in mice bearing Lewis lung carcinoma. The substances tested are three non-steroidal anti-inflammatory agents (chloroquine, indomethacin and phenylbutazone), a broad spectrum non-specific enzyme inhibitor (aurintricarboxylic acid), and two intracellular inhibitors prepared from bovine spleen (SNPIs). All of these substances significantly reduced the formation of spontaneous pulmonary metastases. Primary tumor growth was unrelated with depression of metastasis formation, and a significant inhibition was caused by chloroquine and indomethacin only. These findings are consistent with previously reported inhibitory effects of non-steroidal anti-inflammatory agents on tumor development in animals, and indicate that, in addition to the suggested mechanisms, proteinase inhibition might be involved. They also support our previous observations on the antimetastatic effects caused in mice by neutral proteinase inhibitors.*

INTRODUCTION

THE MECHANISM of the process by which malignant tumors metastasize is complex, and not yet fully elucidated. However, evidence exists that enzymes capable of degrading extracellular substance participate to early phases of hematogenous dissemination, namely tumor cell entrance into the blood stream. Collagenolytic activity has been detected either in human [1–5] or in animal transplantable neoplasms [6–9]. Similarly, cathepsin B has been found present in malignant tumor tissues at levels higher than those observed for normal tissues or benign tumors [10, 11]. The activity of these enzymes is of particular interest, since they are capable of degrading collagen and proteoglycans [12, 13], the two major constituents of extracellular substance. At the same time, they cause cellular detachment [14, 15], and might also be responsible for vascular invasion by the tumor [16, 17].

The antimetastatic effects caused in mice bearing Lewis lung carcinoma by collagenase and neutral proteinase inhibitors are in accord with the above reported findings. Aprotinin, a broad spectrum proteinase inhibitor, and LNPI, inhibitor of elastase and chymotrypsin-like neutral proteinase [18, 19], resulted to be active in preventing pulmonary metastasis formation [20, 21]. Similarly active were *N*-diazoacetylglycinamide [22, 23] and *p*-carboxamidophenyl-3,3-dimethyltriazene [24]; these substances have been tested *in vitro* against elastase and chymotrypsin-like neutral proteinase, which they were found to inhibit ([19, 25] and unpublished results).

Consequently, we thought it worthwhile to test a further series of inhibitors of natural and synthetic origin. This series consists of SNPI-1, inhibitor of chymotrypsin-like neutral proteinase and of the thiol proteinases cathepsin B and H, and SNPI-2, active on elastase and chymotrypsin-like neutral proteinases [26, 27]. In addition to SNPIs, chloroquine, indomethacin and phenylbutazone have been included in this investigation. These substances are clinically used as antirheumatic drugs. Though their mechanism of action is complex, it appears that they may act in preventing extracellular substance breakdown by inhibiting collagenase, which they were found to inhibit *in vitro* at *in vivo* attainable concentrations [28–31]. Furthermore, chloroquine has been also reported to inhibit *in*

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Abbreviations used: ATA, aurintricarboxylic acid; SNPI, spleen neutral proteinase inhibitor; LNPI, leucocyte neutral proteinase inhibitor.

vitro cathepsin B [32]. Also the effects of ATA, a broad spectrum nonspecific enzyme inhibitor [33] were examined. The effects observed testing these substances in mice bearing Lewis lung carcinoma on primary tumor growth and spontaneous lung metastasis formation are hereafter reported.

MATERIALS AND METHODS

Enzyme inhibitors

Chloroquine, indomethacin, phenylbutazone and ATA were obtained from Sigma Chemical Co. Proteinase inhibitors, sample SNPI-1 (mol. wt of about 15,000) and SNPI-2 (mol. wt of about 40,000) were isolated from the postmicrosomal fraction of disrupted spleen cells, by procedures described previously [26].

In vivo *experimental system*

The Lewis lung carcinoma was transplanted by aseptically implanting with a trocar tumor fragments in the axillary region of C57BL mice weighing 18–20 g. Donors were similarly inoculated 2 weeks before [34].

The treatment was performed daily for 14 days following tumor implantation by i.p. administration of freshly prepared drug solutions. The solvent used was water for ATA and chloroquine, 0.1 N $NaHCO_3$ for indomethacin and phenylbutazone, and 50 mM Tris–HCl buffer pH 7.5 containing 50 mM NaCl for SNPIs.

Primary tumor weight was determined on day 15 by caliper measurements, assuming tumor density to be equal to 1, as the volume of the rotation elipsoid having the long and the short axes equal to a and b respectively:

$$\text{Tumor weight} = \pi/6 \times a^2 \times b \qquad (1)$$

The number of lung metastases was determined at sacrifice on day 21 using a dissection magnifying lens. The mass of metastases was estimated as the sum of their individual weight, determined using equation (1).

RESULTS

Data reported in Table 1 show the effects obtained testing the four synthetic compounds. The dose used for each compound is a maximum tolerated dose, and is equal to half of the LD_{50} obtained in normal mice with the same treatment schedule, using the method of Litchfield and Wilcoxon [35]. A significant reduction of primary tumor growth was caused by chloroquine and indomethacin. On the other hand, ATA caused the more pronounced effect on metastases, consisting of the significant reduction of the number of large metastases, and of their total number and weight; 50% of the treated animals were also found free of large metastases at sacrifice. The effects of indomethacin consisted of a significant reduction of the number of large metastases and metastasis weight. Chloroquine and phenylbutazone significantly reduced the number of large metastases and metastasis weight respectively.

The results obtained examining the effects of SNPIs are reported in Table 2. None of the inhibitors tested affected appreciably primary tumor growth. On the contrary, SNPI-1 significantly reduced the number of large metastases, and their total number and weight. A significant reduction was caused by SNPI-2 on the number of large metastases and metastasis weight.

DISCUSSION

The presently reported results were obtained testing the synthetic compounds at equitoxic dosages, and thus represent the potency of each substance. Consequently, ATA results to be the most effective compound in reducing the formation of lung metastases, whereas it is ineffective on primary tumor growth. On the other hand, chloroquine and indomethacin cause significant inhibition of primary tumor growth, while being less effective than ATA on metastases in terms of reduction of large metastasis formation. Phenylbutazone significantly reduces the weight of metastases only. The lack of correlation between effects on primary tumor and lung metastasis formation, indicates that the effects on metastases are not caused exclusively by cytotoxic effects of the drugs on tumor cells. In fact, purely cytotoxic agents such as cyclophosphamide, cause in the same experimental system strictly related inhibition of subcutaneous tumor growth and pulmonary metastasis formation [36, 37].

The above reported findings indicate that three non-steroidal anti-inflammatory agents (indomethacin, phenylbutazone, chloroquine) and ATA, reduce metastasis formation in mice bearing Lewis lung carcinoma. These data are in agreement with the inhibitory effects observed for indomethacin and aspirin on the growth of some rodent transplantable

Table 1. Effects of proteinase inhibitors on primary tumor growth and lung metastasis formation in mice bearing Lewis lung carcinoma

Compound	Dose (μmole/kg/day)	Primary tumor weight (mg)	Effects on metastases: Number small*	Number large	Number total	Weight (mg)	Animals free of large metastases
Controls	—	2129±312	27.1±3.0	7.3±1.2	34.4±3.8	119±25	0/9
ATA	57	1706±219	23.5±3.8	1.7±0.7†	24.4±4†	26± 8†	4/8
Chloroquine	68	1274±167†	35.0±3.6	4.1±1.0†	38.5±4	70±15	1/8
Controls	—	1180±146	37.3±3.3	9.7±1.9	47.0±4.6	130±31	0/8
Indomethacin	7	590± 47.2†	39.0±4.9	2.5±0.6†	41.5±5	40± 8†	0/8
Phenylbutazone	325	1015±118	27.0±1.9	5.0±1.4	32.0±2.5	60±14†	0/8

*Diameter smaller than 2 mm.
†Means significantly different from controls ($P<0.05$).
Groups of 8 mice were treated daily from day 1 to 14 after tumor implantation: each value is expressed as the average (±S.E.) for each group. The statistical analysis performed is the Student–Neuman–Keul test [45].

Table 2. Effects of SNPIs on primary tumor growth and lung metastasis formation in mice bearing Lewis lung carcinoma

Compound	Dose (μmole/kg/day)	Primary tumor weight (mg)	Effects on metastases: Number small*	Number large	Number total	Weight (mg)
Controls	—	880±114	17.2±1.3	4.8±0.9	20.9±2.0	37.4±9.6
SNPI-1	0.08	1021±202	13.2±2.2	2.2±0.6†	14.4±2.1†	10.5±3.4†
SNPI-2	0.3	854±158	16.7±3.6	1.5±0.5†	17.8±3.5	11.6±3.7†

*Diameter smaller than 2 mm.
†Means significantly different from controls ($P<0.05$).
Groups of 8 mice were treated daily from day 1 to 14 after tumor implantation: each value is expressed as the average (±S.E.) for each group. The statistical analysis performed is the Student–Neuman–Keul test [45].

tumors [38–40], and also with a previous report on the antimetastatic effects of aspirin in mice [41]. Several mechanisms have been suggested to be responsible for the effects caused by indomethacin and aspirin. Cytotoxicity for tumor cells was excluded for indomethacin because of its lack of effects on *in vitro* cultures of tumor cells [39]. Other mechanisms proposed for indomethacin and aspirin were direct effects on tumor cells, such as reduction of the abnormally high level of prostaglandins, or effects on the host, such as restoration of the depressed immune function or influence on blood coagulation [39, 42]. No definite conclusion was drawn by these authors; their data, as well as those here reported, are consistent with the hypothesis that the effects observed are caused, at least partially, by tumor proteinase inhibition. This might be operative for aspirin also, since this drug has been shown to inhibit *in vitro* collagenase at *in vivo* attainable concentration [29].

In the case of the inhibitors of natural origin, it has to be noted that the dosages used have been chosen on the basis of the amount available. At the same time, SNPIs caused no body wt reduction or any other detectable toxic effect at the dosage used. Doses higher than those used in this investigation might thus be tolerated and cause more pronounced effects. This is further indicated by the fact that a dose of SNPI-1 of 1.7 μmole/kg was required to cause significant inhibition of neutral proteinases (active at pH 7.5 on fibrinogen, casein and hystones) in tumor homogenates of *in vivo* treated animals (T. Giraldi *et al.*, to be published). For LNPI

and SNPIs it is interesting to note that, in addition to their direct inhibitory effects on proteinases, they might alter also the activity of other enzymes indirectly. Indeed, LNPI and SNPI-1, by inhibiting cathepsin B, may reduce the conversion of latent collagenases into their active forms [43]. At the same time it appears that no *in vivo* inactivation, and consequent reduction of their pharmacological effects, occurs for SNPIs. No formation of antibodies was detected by immunodiffusion in the sera of normal and tumor bearing mice treated with SNPI-1, and SNPI-1 is resistant to possible occurrence of inactivation by tissue proteinases such as cathepsin D [44].

In conclusion, these data support our previous observations on the antimetastatic effects of neutral proteinase inhibitors in mice bearing Lewis lung carcinoma [20–24]. Further research is needed, and is in progress to determine the actual inhibition of tumor proteinases after *in vivo* administration of inhibitors, the nature of the enzyme(s) whose inhibition is related with antimetastatic effects, as well as the examination of the effects caused by higher dosages of SNPIs.

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