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# Antimetastatic Effects and Tumor Proteinase Inhibition by Spleen Intracellular Inhibitors of Neutral Proteinases*

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**Abstract**—*The mechanism of the antimetastatic effects of spleen intracellular inhibitors of neutral poteinases (SNPI's) has been examined in mice bearing subcutaneous Lewis lung carcinoma. SNPI-1 and SNPI-2 selectively depress the formation of spontaneous lung metastasis. A dose-dependent inhibition of the proteolytic activity of subcutaneous primary tumor homogenate at neutral pH was observed after i.p. administration of SNPI-1; its selective antimetastatic effects were evident only at the lower dosage used. This finding indicates that the administration of SNPI's, in spite of causing a dose-dependent depression of tumor proteinases, which presumably participate in the early phases of the process of metastasis formation, may also exert other actions, reducing their antimetastatic effects.*

## INTRODUCTION

SEVERAL different steps are involved in the process of metastasis formation, during which single tumor cells or tumor cell clumps [1] detach from the primary neoplastic lesion, finally lodging in the target organ where secondary tumor will outgrow. Tumor proteinases appear to be involved in the early phases of this process. Indeed, a high level of proteinases, such as collagenase, cathepsin B and plasminogen activator, has been detected in solid malignant tumors, as compared with benign tumors of the same tissue as well as with control normal tissue (for a summary of the relevant literature, see reference [2]). These enzymes share the property of degrading the main components of extracellular matrices, namely collagen, glycoproteins and proteoglycans [3]. After vascularization of the primary neoplasm [4], the action of these enzymes causes two major events leading to tumor cell entrance into the blood stream, i.e., vascular invasion by the tumor [5] and tumor cell detachment from tumor parenchyma [6–8]. Several compounds have been found capable of selectively inhibiting the formation of spontaneous metastases in mice. Among them, *N*-diazoacetylglicinamide, dimethyltriazenes and ICRF 159, in addition to their selective antimetastatic effects [9–14], share the property of inhibiting *in vitro*, at *in vivo* attainable concentrations, neutral proteinases [9–15]. *In vitro* inhibition of neutral proteinases and antimetastatic effects in mice are also caused by phenylbutazone, indomethacin and chloroquine [16], by the nonspecific enzyme inhibitor aurintricarboxylic acid [16] and by the physiological tissue inhibitors, aprotinin [17], leucocyte neutral proteinase inhibitor [18] and spleen neutral proteinase inhibitors [16] (for a summary of the data on the antimetastatic effects and proteinase inhibition, see also reference [2]).

The spleen neutral proteinase inhibitors used consist of a protein having a molecular weight of about 15,000 dalton, inhibiting *in vitro* chymotrypsin-like neutral proteinase and

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the thiol proteinases, cathepsin B and H (SNPI-1), and a protein with a molecular weight of about 40,000 dalton, inhibiting chymotrypsin-like neutral proteinase and elastase (SNPI-2) [19, 20].

In the previous experiments with SNPI's [16] the dosages used were limited by the total amount of each inhibitor available. The aim of the present investigation was, therefore, that of examining the effects of higher dosages of SNPI-1 on the formation of spontaneous pulmonary metastases in mice bearing Lewis lung carcinoma. The mechanism of action of these inhibitors has also been studied by examining the effects of different treatment schedules against spontaneous metastases, as well as their effects on artificial metastases. Finally, the effects of the *in vivo* administration to tumor-bearing mice of therapeutically active dosages of SNPI-1 on the *in vitro* proteolytic activity of primary tumor homogenate has been determined.

## MATERIALS AND METHODS

*Preparation of SNPI's*

SNPI's have been prepared as already described in detail elsewhere [19].

*Determination of the effects on tumor growth and on metastases*

Female BDF1 mice, weighing approximately 18–20 g, purchased from Charles River, Calco, Como, Italy, were used. A single cell suspension of Lewis lung carcinoma was prepared using tumors obtained from donors inoculated subcutaneously two weeks before. The tumor, freed of capsule and necrotic parts, was gently forced through a 40 mesh metal sieve and suspended in 20 volumes of Dulbecco phosphate-buffered saline (DPBS) [21]. The cell suspension was filtered through a double layer of gauze and washed with DPBS by centrifugation at 500 *g* for 10 min. Cell viability, as determined by trypan blue exclusion, was about 33–40%. For the experiments on primary tumor growth and on the formation of spontaneous metastases, $10^6$ viable cells were inoculated s.c. in the axillary region of BDF1 mice; in the experiments on artificial metastases an i.v. inoculum of $3 \times 10^5$ viable cells was used. Primary tumor growth and metastasis formation were evaluated as previously described [10]. The treatment was performed i.p., as indicated, by administering the inhibitors in 0.1 ml per 10 *g* of animal weight of 50 mM Tris–HCl buffer, pH 7.5, containing 50 mM NaCl; the control animals were treated with the same amount of this solution.

*Proteolytic activity assay*

Mice bearing two-week-old subcutaneous tumors were treated i.p. with SNPI-1 and killed 4 hr later. The subcutaneous tumor was removed and homogenized, using a Potter–Elvehjem tissue grinder, in 4 volumes of 50 mM Tris–HCl buffer, pH 7.4. Proteolytic activity of homogenate was determined by the method of Anson [22], using the following protein solutions as substrate: 2% hemoglobin in 0.1 M acetate buffer, pH 3.5, and 1% casein, histones and fibrinogen with plasminogen or plasminogen-free (Kabi or Miles, USA) in 0.2 M phosphate buffer, pH 7.5. Histones were prepared using the modified [23] method of Zubay and Dodty [24]. Two ml of substrate solution were added to 0.4 ml of tumor homogenate and incubated at 37°C (30 min–18 hr). TCA-soluble products of hydrolysis were measured spectrophotometrically using the Folin–Ciocalteau reagent. Blanks having TCA added before the beginning of incubation were run; enzyme activity is expressed as ΔOD at 750 nm per mg protein per hr. Protein concentration was determined by the method of Lowry *et al.* [25], using crystalline bovine serum albumine as standard.

## RESULTS AND DISCUSSION

Data reported in Table 1 show that SNPI-2 (0.5 μmol/kg) significantly reduced the number and weight of spontaneous pulmonary metastases when the treatment was performed on days 15–21. A lesser degree of reduction, restricted to the number of large metastases and metastasis weight, was caused respectively by the treatment with SNPI-2 (0.25 μ mol/kg) on days 1–14, and by SNPI-1 on days 1–14 (0.15 μ mol/kg) and 15–21 (0.30 μ mol/kg). The effects of any other treatment schedule on spontaneous metastasis formation were not statistically significant. At the same time, no appreciable alteration of primary tumor growth, determined at the end of treatment, was observed. It thus appears that SNPI's do not affect tumor cell replication in subcutaneous primary tumors, while they selectively reduce the formation of spontaneous pulmonary metastases. The choice of the dosages used for the different treatment schedules employed was such that the total amount of inhibitor administered was the same, with the exception of the treatment on days 1–14 where also a 10-fold greater dose of SNPI-1 was used. From these data it appears that a significant inhibition of metastasis formation is observed for an early treatment, performed during the first two weeks following tumor implantation,

*Table 1. Effects of SNPI's on the number and weight of spontaneous pulmonary metastases after s.c. implantation of Lewis lung carcinoma*

| Inhibitor | Daily dose μmol/kg | Inclusive days of treatment | Effects on metastases (% of controls) number Small[a] | Large | Total | Weight (mg) |
|---|---|---|---|---|---|---|
| | | | 100 ± 18 (27 ± 4.9)[b] | 100 ± 17 (7.5 ± 1.3)[b] | 100 ± 18 (33 ± 6.0)[b] | 100 ± 22 (101 ± 22)[b] |
| SNPI-1 | 0.10 | 1–21 | 74 ± 9 | 122 ± 39 | 75 ± 9 | 85 ± 28 |
| | 0.15 | 1–14 | 94 ± 10 | 64 ± 13 | 90 ± 8 | 57 ± 12* |
| | 1.40 | 1–14 | 74 ± 13 | 82 ± 24 | 78 ± 11 | 76 ± 19 |
| | 0.30 | 1–7 | 98 ± 8 | 93 ± 33 | 97 ± 12 | 66 ± 24 |
| | 0.30 | 8–14 | 125 ± 9 | 121 ± 22 | 128 ± 10 | 115 ± 24 |
| | 0.30 | 15–21 | 104 ± 7 | 43 ± 7* | 87 ± 4 | 54 ± 12* |
| SNPI-2 | 0.17 | 1–21 | 65 ± 9 | 194 ± 17 | 75 ± 9 | 131 ± 39 |
| | 0.25 | 1–14 | 129 ± 13 | 62 ± 11* | 118 ± 12 | 69 ± 11 |
| | 0.50 | 1–7 | 83 ± 14 | 83 ± 17 | 81 ± 9 | 62 ± 19 |
| | 0.50 | 8–14 | 89 ± 16 | 94 ± 28 | 87 ± 39 | 93 ± 26 |
| | 0.50 | 15–21 | 58 ± 10* | 55 ± 0* | 57 ± 9* | 49 ± 12* |

[a]: Diameter smaller than 2 mm.

[b]:Actual finding obtained in the control groups.

The values represent mean percent ratios (treated over controls) ± S.E. obtained using groups of 8 mice. The data have been obtained in 3 separate experiments, and the values of control groups have been pooled.

The statistical analysis performed is the Student–Newman–Keuls test [45]; *means significantly different from controls, $P < 0.05$.

and for a late treatment during the last week before death.

The formation of artificial metastases appears to be significantly reduced by SNPI-2 only and a lesser reduction, not statistically significant, is caused by SNPI-1 (Table 2). The daily doses used are the same as the lower one used for the treatment on days 1–14 of the experiments reported in Table 1, which were found to be active. Pulmonary tumor development thus appear to be inhibited, at least by SNPI-2. A possible inhibitory effect of SNPI's on the lodgement of tumor cells in the lungs can be ruled out since the treatment in the case of artificial metastases was started 24 hr after injection of tumor cells,when the process of lodgement was presumably completed. For SNPI-2 in particular, the effectiveness of the late treatment, together with that on artificial metastases, appears to indicate that tumor cell growth in the lungs is significantly and selectively inhibited.

The effects of the *in vivo* administration of a single dose of SNPI-1 on the *in vitro* proteolytic activity of tumor homogenates have been examined. The doses used are 0.15 μmol/kg, which were found to be active against the formation of spontaneous metastases when given daily on days 1–14, and 1.4 μmol/kg. A high variability of the data in control animals has been already observed and reported [26]; correspondingly, a high variability is also found for the inhibitory effects of SNPI-1 (Table 3). For this reason the inhibition caused by the

*Table 2. Effects of SNPI's on the number and weight of pulmonary tumors after i.v. transplantation of Lewis lung carcinoma*

| Inhibitor | Daily dose μmol/kg | Inclusive days of treatment | Effects on metastases (% of controls) Number Small[a] | Large | Weight Total | (mg) |
|---|---|---|---|---|---|---|
| | | | 100 ± 32 (12 ± 4.0)[b] | 100 ± 20 (7.9 ± 1.6)[b] | 100 ± 18 (20 ± 3.1)[b] | 100 ± 18 (125 ± 23)[b] |
| SNPI-1 | 0.15 | 1–8 | 64 ± 8 | 91 ± 24 | 85 ± 21 | 75 ± 19 |
| SNPI-2 | 0.25 | 1–8 | 84 ± 28 | 63 ± 21 | 86 ± 14 | 43 ± 18* |

[a]:Diameter smaller than 2 mm.

[b]:Actual finding obtained in the control group.

The values represent mean percent ratios (treated over controls) ± S.E. obtained using groups of 8 mice. The statistical analysis performed is the Student–Newman–Keuls test [45]; *means significantly different from controls, $P < 0.05$.

*Table 3. Effects of* in vivo *treatment of mice bearing subcutaneous Lewis lung carcinoma with SNPI-1 on the* in vitro *proteolytic activity of primary tumor homogenates*

| Inhibitor | Dose μmol/kg | Substrate: Hemoglobin | Casein | Histones | Fibrinogen without plasminogen | Fibrinogen with plasminogen |
|---|---|---|---|---|---|---|
| SNPI-1 | 0.15 | 1.43 ± 9.84 (−22.8; 24.6) | 23.8 ± 8.24 (−6.2; 37.5) | 35.5 ± 2.91* (29.0; 42.0) | 30.7 ± 5.22* (13.4; 44.9) | 23.0 ± 8.56 (−1.6; 51.9) |
| SNPI-1 | 1.40 | −38.2 ± 10.8 (−54.5; −17.8) | 10.2 ± 20.0 (−26.2; 41.7) | 44.2 ± 20.6 (2.91; 66.7) | 97.5 ± 1.61* (94.5; 100) | 59.4 ± 14.9* (38.9; 88.4) |

Individual measurements were performed using 4 different treated and untreated tumor bearing mice. Each value is the mean percent inhibition ± S.E. (the range of the measured inhibition is in parentheses) of the treated groups as compared with untreated controls.

*Values significantly different from that of controls, Students '*t*' test with paired data [46], $P < 0.05$.

treatment as compared with the relevant controls has been determined in individual experiments and the average inhibition observed, as well as its range has been reported in Table 3. It is evident, however, that the proteolytic activity at neutral pH of tumor homogenates is inhibited by SNPI-1 in a dose-dependent way on histones and fibrinogen, particularly in the case of fibrinogen free of plasminogen. The inhibitory effects on casein and those on hemoglobin at acidic pH are not significant. It is possible that the inhibition caused by SNPI-1 on tumor proteinases after repeated administrations, as used for the experiments on metastasis formation, may be significantly more pronounced. These data indicate that, in spite of the fact that SNPI-1 is a protein of molecular weight of about 15,000 dalton, it is appreciably absorbed after i.p. injections and reaches, in the subcutaneous tumor, concentrations high enough to inhibit tumor proteinases. On the other hand, 1.4 μ mol/kg of SNPI-1 on days 1–14 were inactive against spontaneous metastases, whereas 0.15 μ mol/kg given in the same period were significantly active. This discrepancy does not appear to be caused by immunological inactivation of SNPI-1 after repeated administrations. In fact, the examination of the blood of the treated animals after death by immunoelectrophoresis, as well as by immunodiffusion, did not reveal the presence of antibodies against the inhibitor. Possible explanations might take into account biological activities of SNPI-1 different from those exerted on primary tumor proteinases, which should lead to an increase of spontaneous metastasis formation. In other words, small doses of SNPI-1 could cause a decreased release of tumor cells into the blood stream of the host, whereas at a higher dosage, SNPI-1 should cause an increase in the efficiency of lung colony formation large enough to overcome the reduction of circulating cancer cells. Indeed, SNPI's might reduce the host's immunological response against Lewis lung carcinoma, thus increasing lung metastasis formation [27], since proteinase inhibitors have been reported to depress several functions of phagocytes and lymphocytes, such as chemotaxis [28], phagocytosis [29], exocytosis [30], mitogenic activity [31], antibody-dependent cell-mediated cytotoxicity [32] and direct cytotoxicity mediated by proteinases [33].

As far as the inhibition of tumor proteolytic activity by SNPI-1 is concerned, the nature of the tumor proteinases inhibited has not yet been identified. It has to be noted, however, that a proteinase inhibitor may also affect the activity of proteolytic enzyme(s) indirectly. In fact, tumors have been shown to also contain latent proteinases [34–38], as well as proteinase inhibitors [39–41]. At the same time, latent proteinases may be activated by other proteinases [34–38, 42], which may also inactivate spleen proteinase inhibitors [43]. This complex equilibrium [40] might thus be influenced by the treatment, leading to the observed effects. No collagenase activity has been detected using Lewis lung carcinoma homogenates [26] and, consequently, the activity of this enzyme has not yet been examined. It is noteworthy that the action of collagenase can be detected using intact Lewis lung carcinoma [36; unpublished results], that it appears to be relevant in the process of metastasis formation [35] and that several neutral proteinases, including plasminogen activator, are capable of activating latent collagenases [40, 44].

Further research, which is partially in progress in our laboratory, is needed in order to better evaluate for antimetastatic agents the relationships between tumor proteinase inhibition and antimetastatic effects.

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