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Pharmacokinetics of bestatin and oral activity for treatment of experimental metastases*

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Summary. Bestatin is a low molecular weight aminopeptidase inhibitor originally isolated from culture filtrates of *Streptomyces olivoreticuli*. The serum pharmacokinetics in mice are dependent on route of administration, with a short $t^1/_2$ (1.69 min $t^1/_{2\alpha}$ and 12.8 min $t^1/_{2\beta}$), but a high initial serum level following i.v. administration. When administered via the i.p., s.c., i.m., or p.o. routes of administration, bestatin had serum $t^1/_{2\beta}$s of 8.56, 16.91, 19.25, or 15.4 min, respectively. The maximum area under the curve (concentration × time) occurred following i.v. and i.m. administration, with a lower level following p.o. or i.p. administration. Bestatin had therapeutic activity for experimental metastases, not only following i.v., i.p., and i.m. routes of administration but also following oral administration. Because of its brief serum $t^1/_2$, bestatin's therapeutic activity depends on aggressive (either daily or twice daily injection, especially following p.o. administration) and high-dose administration. Thus, the rate-limiting aspect of bestatin's therapeutic activity appears to be associated with its pharmacokinetics.

Introduction

Bestatin is an aminopeptidase inhibitor isolated from the culture filtrate of *Streptomyces olivoreticuli* [34]. Bestatin has shown various benefivial immunological activities, including enhancement of immunological responses that have been weakened in the presence of cancer or in old age [1, 3, 7, 15, 37], activation of immune cells [5, 6, 10, 12, 16, 17, 23, 26, 27, 28, 29, 35], promotion of the host's antitumor activity [1, 2, 15, 21, 30, 33], carcinogenesis-suppressing activity [7, 11, 15], and resistance to infection [8, 13, 19]. It has been reported that bestatin binds to membrane receptors, including aminopeptidase β and leucine aminopeptidase, which are located on cells involved in the host defense mechanism [4, 18, 20, 24, 31]. Previously, Talmadge et al. [32] reported that bestatin produces a blastogenic effect in T cells, increased the mixed lymphocyte reaction, and augmented the adjuvant activity with tumor vaccines. It also activated macrophage cytotoxicity, but not killer cells in normal animals. Bestatin has also shown significant therapeutic activity against preexisting experimental and spontaneous metastases when administered at high doses for at least 4 weeks [32].

In this report, we discuss the therapeutic effect of bestatin on experimental metastases by oral administration, the pharmacokinetics of bestatin in mice, and the relationship of pharmacokinetics to the therapeutic protocol.

Materials and methods

Preparation of bestatin. Bestatin (*N*-[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine) was prepared by chemical synthesis by Nippon Kayaku Co. Ltd., Tokyo, Japan [25] and saline was used as the excipient. Radioactive bestatin, labeled on a portion of the 4-(2′-^{3}H) phenyl moiety (sp. act. 15 mCi/mg), was synthesized by the catalytic tritiation of *N*-[(2S,3R)-3-benzyloxycarbonylamino-2-hydroxy-4-(2′-bromo) phenylbutanoyl]-L-leucine benzyl-ester. Using thin-layer chromatography and HPLC, the material was determined to be greater than 99% pure.

Animals. Specific pathogen-free C57BL/6N mice, 4 weeks of age, were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (weight at use was 18–22 gm).

Therapy of established metastases. The metastatic melanoma variant B16-BL6 [14], selected in vitro from the B16 melanoma was used. At 6 to 8 weeks old syngeneic C57BL/6N mice were given i.v. injections of a single cell suspension of 4×10^5 in vitro-propagated B16-BL6 melanoma cells in calcium/magnesium-free Hanks' balanced salt solution. Bestatin was given p.o., i.m., i.p., or i.v. to C57BL/6 mice three times a week, daily, or twice a day for 3 or 4 weeks beginning 2 days after tumor cell injection. Necropsies were performed 24 h after the last administration; the lungs were fixed and the extent of pulmonary metastasis was determined by counting the metastases. The Mann-Whitney U test was used for statistical evaluation (N = 10). Polyinosinic acid-polycytidylic acid complexed with poly-L-lysine and carboxymethyl cellulose was used as the

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positive control for therapeutic activity throughout. Administration of bestatin had no effect on weight gain compared to control animals, i.e., it was nontoxic.

Pharmacokinetics studies. C57BL/6N mice were given bestatin at 1 mg/0.2 ml by the i.v., i.p., s.c., i.m. or p.o. routes. After bestatin administration, the mice were killed and blood was taken from the left axillary vein at the specified time. Pooled blood samples from 3 mice were allowed to clot in plastic tubes containing no anticoagulant at 4° C and centrifuged to obtain serum, which was stored at −80° C until use ($N = 3$).

Radioimmunoassay. The bestatin concentration in mouse serum was measured by radioimmunoassay (Koyama et al. in preparation). Briefly, antibestatin serum, which was obtained from a rabbit immunized wih bestatin bovine serum albumin conjugate, was diluted with 50 m*M* phosphate buffer, pH 7.3, containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3. The diluted antiserum (200 µl) was mixed with 100 µl of a test sample or a standard bestatin solution, 100 µl of ^3H bestatin (about 20,000 cpm), and 100 µl of phosphate buffer solution. The mixture was incubated at 5° C overnight. Following the incubation, 500 µl of 2% charcoal coated with 1% dextran T-70 was added to separate the antibody-bound fraction from the free fraction. After an additional incubation for 15 min, the mixture was centrifuged at 1600 *g* for 15 min. The supernatants were decanted and radioactivity was measured in an automatic liquid scintillation counter (Aloka LSC-75[3], Tokyo, Japan). The sensitivity of the radioimmunoassay using ^3H-labeled bestatin was 125 pg/0.1 ml of serum. There was no cross-reactivity against metabolites and stereoisomers of bestatin because the antiserum used in the radioimmunoassay recognized bestatin specifically.

Pharmacokinetic and statistical analyses. Data were subjected to 2 methods of analysis, backward stripping on log concentrations of sera and direct nonlinear estimation of parameters on untransformed concentrations [9]. Briefly, the method of backward stripping involved transforming the concentrations to natural or common logarithms, stripping initial observations to the plot of log-concentration versus time, and fitting the line of best fit using simple linear regression. Nonlinear regression required an iterative strategy which involved regressing the residuals or the partial derivatives of the model with respect to the parameters until the iterations converged. In this paper, results are presented in terms of estimates obtained through nonlinear estimation, as they were found to be more reliable. The median number of metastases as a measure of therapeutic activity was analyzed by the nonparametric Mann-Whitney U test ($N = 10$).

Results

The therapeutic activities of orally administered bestatin for experimental metastases from the B16 melanoma variant B16-BL6 are shown in Table 1, Exp. I. Oral therapy with bestatin significantly reduced the median number of preexisting experimental lung metastases when the agent was administered daily for 4 weeks. However, bestatin did not inhibit the growth of lung metastases when administered 3 times a week (i.e., reduce the number of metastases). These data suggested that bestatin was effective against lung metastases when administered orally and that daily consecutive administration may be more effective than intermittent bolus administration. To confirm and expand on the observation of therapeutic efficacy of bestatin by daily oral administration, bestatin was administered twice every day for 3 weeks as compared to once a day in a subsequent experiment. As shown in Table 1 (Exp. II), twice daily administration was also effective and was sig-

Table 1. Reduction in the number of B16-BL6 pulmonary colonies by oral bestatin administration[a]

Agent	Dose (mg/kg)	Schedule	Route	Median (range) number of metastases
Experiment I				
Saline	–	3x/week	i.v.	>300 (64–>300)
Poly(I,C)-LC[b]	0.5	3x/week	i.v.	18 (0–89)[c]
Bestatin	50	3x/week	p.o.	>300 (122–>300)
Bestatin	25	3x/week	p.o.	236 (37–>300)
Bestatin	50	Daily	p.o.	39 (2–>300)[c]
Bestatin	25	Daily	p.o.	>300 (54–>300)
Experiment II				
Saline	–	3x/week	i.v.	17 (7–27)
Poly(I,C)-LC[b]	0.5	3x/week	i.v.	4 (0–8)[c]
Bestatin	50	2x/day	p.o.	2 (0–14)[c]
Bestatin	25	2x/day	p.o.	3 (0–8)[c]
Bestatin	50	Daily	p.o.	6 (0–15)[c]
Bestatin	25	Daily	p.o.	10 (2–28)

[a] Syngeneic C57BL/6N mice ($N = 10$/group) received i.v. injections of 4×10^4 B16-BL6 tumor, and 2 days later, therapy was initiated with p.o. administration for 4 weeks (Exp. I), or 3 weeks (Exp. II). Necropsies were performed 24 h after the last administration

[b] Poly(I,C)-LC, polyinosinic-polycytidylic acid complexed with poly-L-lysine and carboxymethyl cellulose

[c] Significant reduction in the median number of pulmonary colonies ($P < 0.05$) according to the Mann-Whitney U test

Table 2. Reduction in the number of B16-BL6 pulmonary colonies by i.v. bestatin administration[a]

Agent	Dose (mg/kg)	Schedule	Median (range) number of metastases
Experiment I			
Saline	–	3x/week	17 (7–27)
Poly(I,C)-LC	0.5	3x/week	4 (0–8)[b]
Bestatin	50	3x/week	11 (4–35)
Bestatin	50	daily	2 (0–7)[b]
Experiment II			
Saline	–	3x/week	110 (8–>300)
Poly(I,C)-LC	0.5	3x/week	10 (0–92)[b]
Bestatin	50	2x/day	23 (0–>300)[b]
Bestatin	25	daily	26 (2–>300)

[a] Syngeneic C57BL/6N mice, aged 8 weeks (Exp. I) or 6 weeks (Exp. II) received i.v. injections of 4×10^4 B16-BL6 tumor. Then 2 days later, therapy was initiated with i.v. of administration of bestatin for 3 weeks

[b] Significant reduction in the median number of pulmonary metastases ($P < 0.05$) according to the Mann-Whitney U test

Table 3. Therapy of experimental metastases with bestatin[a]

Agent	Dose (mg/kg)	Schedule	Route	Median (range) number of metastases P^b
Experiment I				
Saline	–	–		65 (7–>300)
Poly(I,C)-LC	0.5	3x/week	i.v.	0 (0–3) 0.000
Bestatin	250	3x/week	i.v.	0.5 (0–4) 0.000
Bestatin	50	3x/week	i.v.	3 (0–50) 0.007
Bestatin	250	3x/week	i.m.	4 (0–59) 0.011
Bestatin	50	3x/week	i.m.	9 (0–39) 0.009
Bestatin	250	Daily	i.m.	1 (0–23) 0.003
Bestatin	50	Daily	i.m.	5 (0–93) 0.007
Experiment II				
Saline	–	–		243 (45–>300) –
Poly(I,C)-LC	0.5	3x/week	i.v.	31 (0–>300) 0.017
Bestatin	50	3x/week	i.v.	68 (12–>300) 0.041
Bestatin	25	3x/week	i.v.	130 (14–>300) 0.325
Bestatin	50	3x/week	i.p.	57 (1–>300) 0.038
Bestatin	25	3x/week	i.p.	186 (14–>300) 0.479
Bestatin	5	3x/week	i.p.	219 (67–>300) 0.289
Bestatin	5	Daily	i.p.	57 (3–>300) 0.032

[a] Syngeneic C57BL/6N mice, age 8 weeks (N = 10/group), received i.v. injections of 4×10^4 B16-BL6 tumor, and 2 days later, therapy was initiated with i.v., i.m., or i.p. administration for 4 weeks. Necropsies were performed 24 h after the last administration

[b] Significant reduction in the median number of pulmonary colonies ($P < 0.05$) according to the Mann-Whitney U test as compared to saline-treated animals

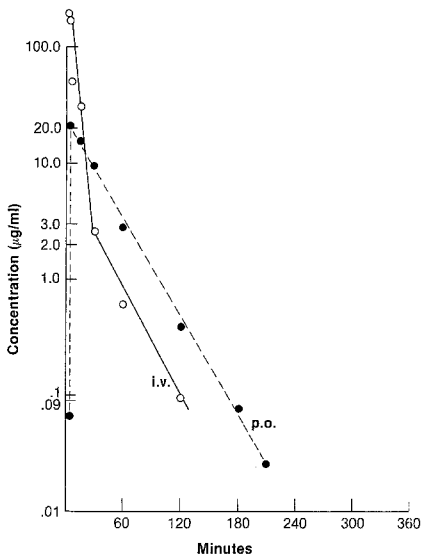

Fig. 1. Mean levels of bestatin in mouse serum after i.v. or p.o. administration. C57BL/6 mice were given bestatin at 50 mg/kg and were bled at the indicated times. Serum samples at 180 and 210 min following i.v. administration did not have analyzable levels of bestatin. Sera from 3 mice were pooled at each time interval and the level of bestatin determined by radioimmunoassay

nificantly more effective than daily administration at 25 mg/kg. Similar results were obtained with i.v. administration of bestatin (Table 2). Daily administration of bestatin produced significantly greater therapeutic activity than when the drug was administered 3 times a week. Therapy was administered for only 3 weeks because of the venous damage that occurs following such an aggressive injection schedule. However, the animals were necropsied 4 weeks after tumor challenge. Following i.m. administration, therapeutic activity was observed when bestatin was given either 3 times a week or daily (Table 3) but required higher doses than daily administration. However, significantly greater activity was apparent when 250 mg/kg of bestatin was administered daily rather than 3 times a week. Similar results were observed following the i.p. route of administration, such that daily administration at 5 mg/kg resulted in significantly greater activity than injections 3 times a week (P = 0.047, Table 3). These data suggested that bestatin was rapidly excreted or degraded, and it appeared that therapeutic activity depended on the maintenance of serum bestatin levels. Therefore, we further investigated the pharmacokinetics of bestatin following various routes of administration to provide additonal information on its optimal therapeutic protocol.

Bestatin was administered at 50 mg/kg via various routes and the serum levels were determined by a recently developed radioimmunoassay. Figure 1 shows the elimination of bestatin after i.v. or p.o. administration and Fig. 2 illustrates the agent's clearance after i.p., i.m., or s.c. administration. Although bestatin was eliminated rapidly after administration by any route, its rate of clearance was most rapid following i.v. or p.o. administration, when no peptide could be detected in the blood 4 h after the drug was given. On the basis of statistical analyses, data for the i.v. route fitted a 2-compartment open model [36], and those for the i.p., s.c., i.m., and p.o. routes of administration fitted a 1-compartment open model [36] with first order absorption kinetics. Table 4 shows serum elimination rate constants, serum $T\frac{1}{2}$s, area under the curve (concentration × time), and R-squared values for each route of administration. Bestatin had serum $T\frac{1}{2\beta}$s of 8.56, 12.84, 15.40, 16.91, and 19.25 min after i.p., i.v., p.o., s.c., and i.m. administration, respectively. The R-squared values were extremely high for the i.v., i.p., s.c., and i.m. routes

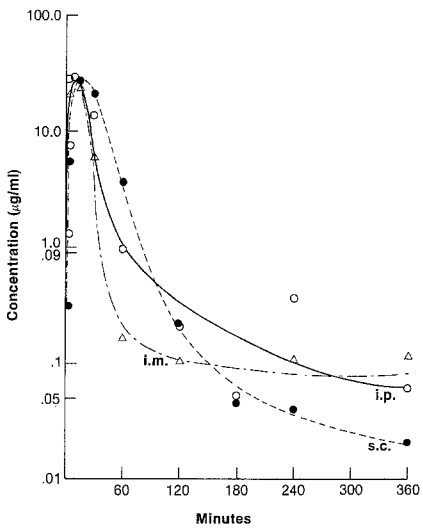

Fig. 2. Mean levels of bestatin in mouse serum after i.p., s.c., or i.m. administration. C57BL/6 mice were given bestatin at 50 mg/kg and were bled at the indicated times. Sera from 3 mice were pooled at each time interval and the level of bestatin determined by radioimmunoassay

Table 4. Pharmacokinetics of bestatin[a]

Route	Serum elimination rate[a] (l/min)	t 1/2[b] (min)	Range[c] lower bound) (upper bound)	CxT[d]	R^{2e}
i.v.	0.054 ± 0.062	12.84	(5.98 – 20)	1281.27	0.99
i.p.	0.081 ± 0.019	8.56	(6.93 – 11.18)	925.63	0.99
s.c.	0.041 ± 0.009	16.91	(13.86 – 21.66)	1171.39	0.98
i.m.	0.036 ± 0.007	19.25	(16.12 – 23.90)	1237.39	0.97
p.o.	0.045 ± 0.024	15.40	(10.04 – 30.00)	621.54	0.90

[a] Values represent parameter estimates obtained from nonlinear regression ± SE
[b] Bestatin following i.v. administration also had a $t\,1/2_{\alpha}$ of 1.69 min
[c] Confidence bounds correspond to deviations of 1 SE for serum rates
[d] Area under the curve (concentration × time) of bestatin obtained by analytic integration of estimated parameters
[e] R-squared values indicate the proportion of variance explained by the mathematical model to the total variation in the dependent measure

and moderately high for the p.o. route, indicating an excellent fit to the models selected for these data.

It should be noted that the greatest area under the curve [i,e., total serum levels (concentration × time)] and the highest serum bestatin levels were obtained following i.v. administration. Administration p.o. delivered a lower dose than any other route, which may have been due to either partial adsorption or degradation of the drug. This pharmacology is reflected in the observation that all routes of administration produced therapeutic activity following daily or twice daily administration at hight doses. Thus, maximal therapeutic activity appeared to depend on chronic administration with routes and schedules that produced and maintained high serum levels.

Discussion

In the present study, we demonstrated that oral administration of bestatin had therapeutic activity against preexisting experimental pulmonary metastases in mice and that therapeutic efficacy was increased when it was administered twice a day rather than daily or 3 times a week. In addition, bestatin had a greater effect on experimental lung metastases when it was administered i.v. every day rather than 3 times a week. Pharmacokinetic studies following high-dose administration demonstrated that this compound was well absorbed (> 50%), even when administered orally, but that it was also rapidly eliminated from the peripheral blood. The $t\frac{1}{2}{}_{\beta}$s of i.v. or oral doses were 12.8 and 15.4 min, respectively. However, bestatin was not detected 4 h after administration by either route. These findings are in agreement with the results of other investigations in humans and rats following administration at low doses [22, 36]. Rapid clearance from the blood might be one of the reasons that daily administration provides more effective antitumor activity than intermittent administration.

Previously, we reported that the optimal therapeutic dose of bestatin was about 5 mg/kg against transplantable murine tumors [1, 15] and that the optimal immunomodulating dose was also 5 mg/kg in mice, depending on which assay was used to measure immune functions. However, in this experiment, optimal doses appeared to be higher than those reported previously. These doses are not the same with both experimental therapy models. The therapeutic parameters for s.c. and i.p. tumors differ from those observed in systemic disease. Alternatively, different populations of effector cells may be responsible for antitumor activity against pulmonary versus s.c. disease, We conclude that bestatin is well absorbed following p.o. administration and has a short serum $t\frac{1}{2}$. Further, its ability to reduce pulmonary colonies of B16-BL6 tumors depends on its pharmacokinetics; the greatest therapeutic activity was observed following daily or twice daily i.v. or twice daily p.o. administration, these schedules and routes being the most effective for maintaining serum levels of bestatin. The ability of orally administered bestatin to have similar therapeutic activity compared to i.v. administration may be associated with the prolonged bioavailability resulting in greater effector cell augmentation.

We suggest that it may be more reasonable for bestatin to be administered by chronic infusion techniques such as osmotic pumps in preclinical studies or peristaltic pumps in the clinic. However, the demonstration of oral activity against preexisting metastatic disease suggests that this route is very attractive for chronic administration. In contrast to the clinical trials, undertaken with low-dose oral bestatin, frequent high doses of this nontoxic agent will be required for therapeutic activity.

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