Biochemical Pharmacology, Vol. 30, No. 24, pp. 3375–3377, 1981
Printed in Great Britain.

0006-2952/81/243375-03 $02.00/0

Bestatin, a stimulator of polysome assembly in T cell lymphoma (L 5178y)

(*Received* 8 *April* 1981; *accepted* 2 *July* 1981)

Bestatin, which has been isolated from culture filtrates of *Streptomyces olivoreticuli* by Umezawa *et al.* [1], was found to be a specific inhibitor of aminopeptidase B [1]. The molecular weight of bestatin is 195, and its chemical structure was determined as [(2S, 3R)-3-amino-2-hydroxy-4-phenyl-butanoyl]-L-leucine [2]. The enzyme aminopeptidase B appears both on the cell surface and intracellularly in monkey kidney cells, canine kidney cells, L cells and FM 3A cells [3, 4]. Bestatin enhances cell-mediated immunity (delayed-type hypersensitivity; immune resistance to cancer) [4, 3], probably by enhancement of the function of T lymphocytes [5]. This working hypothesis was supported by the finding [5], that bestatin enhances *in vitro* activation of T-cells, caused by concanavalin A [5]; the incorporation of [^{3}H]-dThd into DNA was chosen as parameter for determination of the amount of activation.

In the present report the effect of bestatin on both the cell proliferation and the polysome assembly was determined using L 5178y cells, a T cell lymphoma [6], as the test system.

Materials and methods

Compounds. The following materials were used: Heparin, bentonite and Triton X-100 from Serva, Heidelberg (West Germany); diethyl pyrocarbonate from EGA Chemie, Heidenheim (West Germany) and Nonidet NP 40 from Shell, Hamburg (West Germany).

Bestatin was a gift of Prof. H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan.

Cell culture. For the studies to determine polysome assembly L 5178y cells, a Thy-1,1-bearing, Fc receptor-positive T-lymphoma [6, 7] was used which was serially passaged in adult NMRI male mice. Lymphoma cells, harvested 12 days after intraperitoneal injection of 2×10^5 cells, were suspended in Fischer's medium and cultivated in suspension [8]. The lymphoma cell culture contained 2–5% macrophages, as judged by neutral red staining; variability was greater than 95% [9]. The cells were considered to be in the stationary phase of growth (during the incubation period used in the present study) by the following criteria: (a) there is no further increase in cell number and no mitotic figures are observed; (b) there is only little incorporation of dThd into DNA.

For the dose–response experiments L 5178y cells [7] were also used: the suspension cultures were serially passaged in Fischer's medium [8]. They were depleted of adherent and phagocytic cells (e.g. macrophages) and were used in the exponentially growing phase.

Incubation conditions. The dose–response experiments were performed in roller tubes, containing 5 ml of a suspension of 5×10^3 exponentially growing cells/ml. The cultures were incubated at 37° for 72 hr; the controls reached a cell concentration of about 3×10^5/ml (the cells needed 120 hr to reach the stationary phase). In some experiments different incubation conditions were used, as described in the text. The compound was added as described [8] and the cell concentration was determined with a Model B Coulter counter [8].

For the experiments to study the influence of bestatin on polysome formation, non-proliferating L 5178y cells were used. The cell density was adjusted to 5×10^6/ml and 50 ml assays were incubated for 2 hr in spinner cultures, using Fischer's medium [8].

Isolation of polysomes. After incubation the cells (2.5×10^8) were harvested by centrifugation (3000 *g*; 10 min; 2°), suspended in 2 ml Solution 1 (25 mM Tris–HCl, 25 mM NaCl, 5 mM $MgCl_2$, 140 mM sucrose, 2.5 μl diethyl pyrocarbonate/ml, 100 μg heparin/ml, pH 7.6) and homogenized by five strokes with a loose fitting pestle in a Dounce homogenizer. Then 2 ml of Solution 1, containing 2% Nonidet, and 2 ml of Solution 2 (5% sodium deoxycholate, 5% Triton X-100, pH 7.6) were added and homogenized again by six strokes. After centrifugation (15,000 *g*; 10 min.; 2°) the supernatant was first analyzed for DNA, RNA and protein. One ml of the supernatant contained 0.02 mg DNA, 2.4 mg RNA, and 7.3 mg protein. Subsequently an 0.5 ml aliquot of the supernatant was layered over a 0.4 to 1.5 M linear sucrose gradient containing Solution 1 and centrifuged at 38,000 rpm for 120 min at 2°C in a Spinco SW 50 rotor. The polysome profiles were analyzed from the top and the A_{254} profile was monitored through an ISCO flow monitor with a 2 mm path length and the absorbance measured was multiplied by 5.

All solutions used above were pretreated with bentonite to make them RNase-free [10].

Graphical integration of the optical density curves was used to estimate ribosomal distributions.

Analytical methods. DNA was determined by the method of Kissane *et al.* [11], RNA was determined by the orcinol reaction [12], and protein was determined according to Lowry *et al.* [13].

Results

Influence on cell proliferation. In the dose–response experiments, starting with 5,000 exponentially growing cells/ml and an incubation period of 72 hr, bestatin does not cause a reduction of cell proliferation. The highest compound concentration used was 100 μg/ml (=0.5 mM).

In a separate series of experiments, the same cell concentration was used as in the experiments, to determine the influence of bestatin on polysome formation. Starting with 5×10^6 exponentially growing cells/ml, a cell density of 8.1×10^6 was reached after an incubation period of 12 hr in the absence of bestatin. This increase of the cell density corresponds to 0.71 cell doublings [14]. In the presence of 1, 10 or 100 μg bestatin/ml, the cultures reached the same cell concentration ($8.0–8.2 \times 10^6$ cells/ml) as the controls.

After incubation of L 5178y cells (5×10^6 cells/ml) with 100 μg bestatin/ml for 12 hr no alteration of the cell volume was observed; controls: 1240 ± 160 μm^3.

Effect on polysome assembly. For these studies L 5178y cells, harvested 12 days after intraperitoneal injection from NMRI mice, were used. These cells were in the stationary phase during the period of time at which the following experiments were performed.

After *in vitro* incubation of the cells for 2 hr, polysomes were isolated by a linear sucrose gradient centrifugation. From the obtained polysome profiles, the amount of monomers (monosomal area) and that of oligo- and polymers (polysomal area) was determined by graphical integration as shown in Fig. 1. As a control, the extracted fraction was incubated with RNase as described earlier [8]; under these conditions the polyribosomes were disrupted and only monomers were traced after sucrose gradient centrifugation (data not shown).

In the absence of bestatin (Fig. 1A), the relative amount

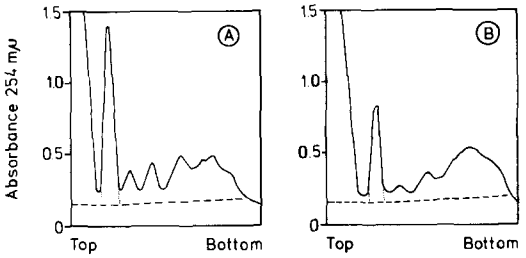

Fig. 1. Polysomal profiles observed after incubation of L 5178y cells with bestatin. (A) Incubation of the cells with 0 μg/ml; (B) incubation with 10 μg/ml bestatin. Total ribosome areas were calculated as follows; a broken line was extended down the left-hand side of the single ribosome peak to the base line, which was determined by measuring the optical density of blank gradients. The area bound by the single ribosome peak, the dotted lines and the broken line, was deemed the monosomal area; the polysome area was calculated by integration of the di-, tri- and polysomal peaks circumscribed by the dotted and the broken lines.

(O.D. units) of the monosomal area was 1.54 and the one of the polysomal area 1.75. In the presence of 10 μg bestatin/ml (Fig. 1B) the polysomal area (2.65) increases at the expense of the monosomal area (0.67). It should be noted, that the sum of the two areas is even, irrespective of the presence of bestatin (controls: 3.29; bestatin-treated: 3.32). This means that during the incubation period (2 hr) no *de novo* synthesis of ribosomes occurs. This finding is in accordance with previous results [15], indicating that within the first 12 hr polysomes are formed from pre-existing ribosomes in response to those hormones, which stimulate protein synthesis; only later do newly synthesized ribosomes enter the polysomes. Due to this fact it is reasonable to use the ratio between the polysomal area and the monosomal area as a criterion for the amount of polysome assembly. As shown in Fig. 2 the polysome assembly in L 5178y cells is strongly dependent upon bestatin treatment. After incubation with 10 μg bestatin/ml the ratio between the polysomal area and the monosomal area increases from 1.14 (in the absence of the compound) to 3.90. It is interesting that in the presence of higher concentrations of bestatin the ratios reach values identical with the controls.

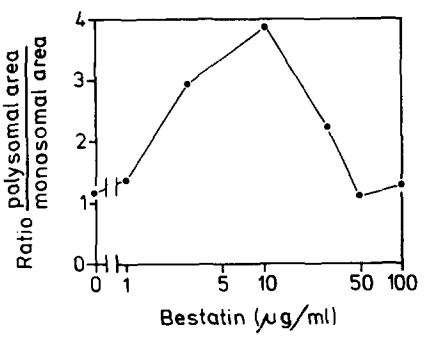

Fig. 2. Alteration of the ratio between the polysomal area and the monosomal area. L 5178y cells incubated in the presence of different concentrations of bestatin (abscissa); subsequently polysomes and monosomes were isolated by sucrose gradient centrifugation (Fig. 1). The polysomal area as well as the monosomal area was calculated (Fig. 1) and the ratio between their values was formed (ordinate).

Discussion

The present study shows that the aminopeptidase B inhibitor bestatin exerts no growth inhibitory activity on exponentially growing L 5178y cells, a T cell lymphoma, even at high concentrations (0.5 mM). In the presence of this compound, no increase of the average cellular volume is observed. This finding, that no "unbalanced growth" [16] occurs during incubation, is a strong hint that bestatin has no influence on DNA synthesis under our incubation conditions used. This assumption is supported by previous findings which showed that bestatin has no influence on dThd incorporation into DNA of normal lymphocytes [5].

Stimulated by the observation that bestatin enhances cell-mediated immunity [4], experiments were performed in the present study to determine the influence of bestatin on protein synthesis because a mRNA- and a subsequent protein synthesis is thought to be a prerequisite for the mobility of immunoglobulin receptors and other surface receptors of lymphocytes (e.g. ref. 17). As a criterion for an altered protein synthesis, the degree of organization of pre-existing ribosomes into polysomes may be used [15]. However such a type of experiment can only be performed in resting cells, in which neither *de novo* synthesis of ribosomes occurs nor interference with DNA synthesis-dependent mRNA formation exists. Therefore we used the same cell line, L 5178y, in the physiological state of non-proliferation. These cells were cultivated *in vivo* for 12 days intraperitoneally in mice before harvesting. After that period of growth, the L 5178y cells are in the G_0 phase and 50 per cent of the mice die of cancer at day 16. After harvesting, the cells were incubated in the presence of bestatin. Subsequently free as well as membrane-bound polysomes (treatment with deoxycholate [18]) were isolated and their relative amount was determined. Under the incubation conditions used, bestatin causes an increase of the polysome formation at concentrations within 1–50 μg/ml. Maximal increase of the relative polysome formation (6-fold over the controls) was observed after incubation with 10 μg/ml. Concentrations of bestatin higher than 50 μg/ml do not alter the amount of polysomes.

This concentration-dependent effect of bestatin is not yet understood, but has been already described in the case of concanavalin A-stimulated T cells [5]; also in this system bestatin causes an enhanced stimulation only in a limited concentration range.

Future studies must show whether the observed stimulation of polysome assembly in response to bestatin is restricted to lymphocytes alone. However based upon the studies already available and performed both on cellular [4] and on subcellular level ([5] and this report), it seems to be very likely that bestatin is involved in regulation of immune response.

In summary, bestatin, an aminopeptidase B-inhibitor of dipeptide nature, was tested in the L 5178y cell system for its effect on cell proliferation as well as for its influence on polysome assembly. The results showed, that bestatin influenced the extent of polysome assembly dose-dependently.

Acknowledgements—Supported by grants from the Landesversicherungsanstalt Rheinland-Pfalz, Speyer (West Germany) and from the Akademie der Wissenschaften und der Literatur, Mainz (West Germany).

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Biochemical Pharmacology, Vol. 30, No. 24, pp. 3377–3380, 1981.
Printed in Great Britain.

0006-2952/81/243377-04 $02.00/0
Pergamon Press Ltd.

Increases in cyclic AMP levels in rat brain regions *in vivo* following isoproterenol*

(*Received* 5 *March* 1981; *accepted* 12 *June* 1981)

Cyclic AMP may serve as a second messenger within the CNS [1, 2]. It is distributed throughout rat brain and responds to pharmacological and physiological manipulations [3–7]. The physiological effects of beta-adrenergic stimulation by cathecholamines in the periphery are thought to be mediated by adenyl cyclase activation [8]. Beta-adrenergic receptors have been described and measured by radioligand techniques throughout the mammalian CNS [9, 10]. Cyclic AMP increases in tissue from various brain regions after *in vitro* incubation with beta-adrenergic agonists such as isoproterenol suggesting that cyclic AMP may mediate the effect of beta-adrenergic stimulation in the CNS [11–13]. We were interested in determining whether *in vivo* administration of isoproterenol would alter cyclic AMP concentrations in selected regions of the brain.

The pineal has a beta-adrenergic receptor whose noradrenergic input from the superior cervical sympathetic ganglion varies greatly during the day versus the night [14, 15]. The sensitivity of the pineal beta-receptor also varies dramatically during the normal 24-hr period. The greatest sensitivity is seen during the light hours, and pineals of animals maintained in constant light show a supersensitive response *in vitro* to norepinephrine [16]. Therefore, in a separate experiment we studied cyclic AMP responsiveness to isoproterenol *in vivo* in animals maintained under constant light conditions.

Methods

Animals. In the first experiment, male Sprague–Dawley albino rats† (250–350 g) were obtained from Taconic Farms. In the second experiment, Wistar-derived rats from the Walter Reed Army Institute of Research colony were used. The animals had free access to food and water and were maintained in a 12 hr light–dark cycled room with lights on from 6.00 a.m. to 6.00 p.m. except for one group of rats in the second experiment which were maintained in constant light for 3 weeks. Experiments were performed between 8:30 a.m. and 12:30 p.m. to minimize circadian effects.

Solutions. DL-Isoproterenol HCl (Sigma) was dissolved in saline. The dose is expressed as the salt.

Habituation. The animals were habituated to the experimental procedure to minimize stress effects. Rats were injected with saline for a minimum of 3 days prior to the experimental day. Ten minutes after the injection, the rats were habituated to passing through a plastic open-ended cylinder similar to the plastic microwave applicator to be used on the experimental day. The animals were then returned to their home cage. Animals used in experiment 1 were habituated for 3 days, while rats in experiment 2 were habituated for 10 days. The longer habituation resulted in lower variability of cyclic AMP levels in saline-injected animals (see Discussion).

Experimental procedures. In the first experiment rats were alternately injected (i.p.) with either saline, 10 mg/kg isoproterenol, 20 mg/kg isoproterenol, or 30 mg/kg isoproterenol. Ten minutes later the rats were placed in a plastic

* This material has been reviewed by the Walter Reed Army Institute of Research, and there is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

† In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", a promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.