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PLEIOTROPIC EFFECT OF ANTICAPSIN ON HeLa S_3 CELLS**

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Anticapsin, the terminal epoxyaminoacid moiety of tetaine, inhibits irreversibly growth of HeLa S_3 cells.

The antibiotic decreases to a similar extent incorporation of ^{3}H-labelled precursors into nucleic acids and protein in intact cells: inhibition of protein synthesis prevails on prolonged incubation. Also incorporation of [^{3}H]dTTP and [^{3}H]UTP is inhibited in the presence of anticapsin into permeabilized cells.

These effects, however, are not due to the interference with DNA or RNA polymerases since anticapsin only slightly suppresses RNA polymerase activity and has no effect on DNA polymerase in the cell-free systems.

The results indicate that the mechanism of antiproliferative action of anticapsin in HeLa S_3 cells differs from that of tetaine and imply that inhibition of protein synthesis might be the primary effect of anticapsin.

Anticapsin, L-β-(2,3-epoxycyclohexyl-4-on)alanine, is produced by a strain of *Streptomyces griseoplanus* [1]. Also it constitutes the C-terminal epoxyaminoacid moiety of the peptide antibiotic tetaine [2] (synonyms: bacilysin [3, 4], bacillin [5].

It has been found that tetaine transported by peptide permease [6] is enzymatically cleaved within microbial cells to release anticapsin which, in turn, inhibits cellular glucosamine-6-phosphate synthetase (2-amino-2-deoxy-D-glucose-6-phosphate keto-isomerase, aminotransferring, EC 5.3.1.19). This leads to inhibition of microbial growth [7]. The released anticapsin acts apparently as a glutamine analogue inactivating irreversibly the enzyme,

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probably by covalent binding of its epoxide grouping to the sulphydryl group(s) in the enzyme molecule [8]. Recently, we have also found that tetaine inhibits growth of cultured mammalian tumour cells, presumably by impairing DNA and RNA synthesis [9].

In contrast to tetaine, anticapsin itself is devoid of antibacterial activity since it is not transported into prokaryota. However, some, though only marginal, inhibitory activity of this compound was observed against the fungus *Candida albicans* [10]. Hence, there is a possibility that other types of eukaryota, such as mammalian cells, might also take up anticapsin and are suceptible to the inhibitory action of this agent.

In the present studies we have examined the growth inhibitory properties of anticapsin against cultured HeLa S_3 cells, and its effect on biosynthesis of nucleic acids and protein in several systems.

MATERIALS AND METHODS

Anticapsin was a gift from Dr. N. Neuss from Eli Lilly Co. (Indianapolis, IN, U.S.A.).

L-[4,5-^{3}H]Leucine (53 - 60 Ci/nmol) and D-[1-^{3}H]glucosamine (3 Ci/nmol) were obtained from the Radiochemical Centre (Amersham, Bucks., England), while [*methyl*-^{3}H]thymidine (22 - 25 Ci/nmol), [5-^{3}H]uridine (29 Ci/nmol), [*methyl*-^{3}H]thymidine-5'-triphosphate (23 - 27 Ci/nmol) and [5-^{3}H]uridine-5'-triphosphate (26 Ci/nmol) were purchased from UVVVR (Prague, Czechoslovakia).

HeLa S_3 cells were grown in monolayer or in suspension culture as described by Witkowski *et al.* [11].

The growth inhibitory action of anticapsin in HeLa S_3 cells (ED_{50} value) was estimated according to Konopa *et al.* [12] after 72 h incubation with the antibiotic.

The incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine was determined as described previously [11] and reffered to the cell number.

HeLa S_3 cells were permeabilized by incubation with lysolecithin (L-α-lysophosphatidylcholine, type I, from egg yolk, Sigma Chemical Co., U.S.A.) at a concentration of 100 μg/ml for 2 min at 0 - 4°C. Synthesis of DNA and RNA was measured in the permeabilized cells by the method of Castellot as described previously by Witkowski *et al.* [11].

Partial purification and the assay of DNA polymerase activity in crude cell-free extracts were performed according to Noy & Weissbach [13].

Calf thymus DNA (type II, Sigma Chemical Co., U.S.A., was activated after Fansler & Loeb [14].

RNA polymerases were solubilized and partially purified according to Schwartz *et al.* [15] and their activity was assayed as described by Weil & Blatti [16].

Incorporation of [^{3}H]glucosamine into protein was determined according to Kessel [17].

The protein content was determined by the method of Schacterle & Pollack [18] with crystalline bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Incubation of HeLa S_3 cells with anticapsin for 72 h at 37°C resulted in the dose-dependent inhibition of cellular growth (Fig. 1). Half-maximal inhibitory concentration (ED_{50}) was found to be 10 μM (2 μg/ml). Thus,

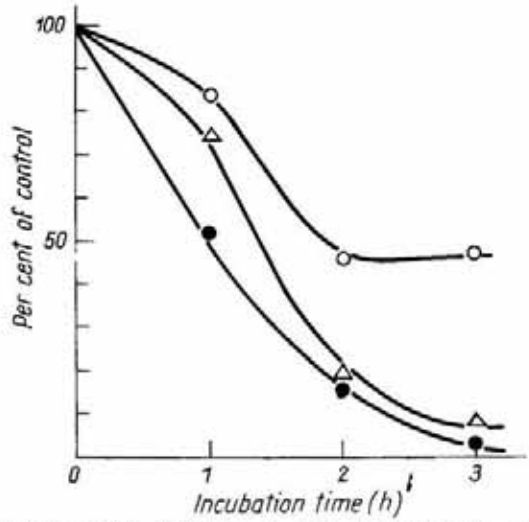

Fig. 1. The inhibition of growth of HeLa S_3 cells by anticapsin concentrations of 0.01 mM (○), 0.03 mM (△) and 0.1 mM (●). The cells were plated 16 - 19 h prior to the experiment to yield about 2×10^4 cells per Leighton tube. The samples were taken out at the intervals indicated and the cells were counted

not only did anticapsin inhibit growth of mammalian cells but also its antiproliferative potency was somewhat higher than that of tetaine which has the ED_{50} value of 35 μM against HeLa S_3 cells [9]. This observation points to a major difference between the effects of tetaine and anticapsin in mammalian and bacterial cells, since anticapsin is essentially inactive in the latter cells.

In order to obtain information on the possible target(s) of anticapsin in HeLa S_3 cells, we assayed its effect on biosynthesis of nucleic acids and protein. Incubation of HeLa S_3 cells with anticapsin for 2 h at 37°C resulted in a strong dose-dependent inhibition of the incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine into macromolecules (Fig. 2A). The extent of inhibition was similar for all three [^{3}H]-labelled precursors.

Inhibition of incorporation of all precursors by 0.1 mM anticapsin was gradually enhanced during 6 h incubation with the antibiotic (Fig. 2B). The rates of incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine (±S.E.M.) in control cells were: 1.25±0.3, 1.05±0.3 and 2.3±0.2 dpm/10^5

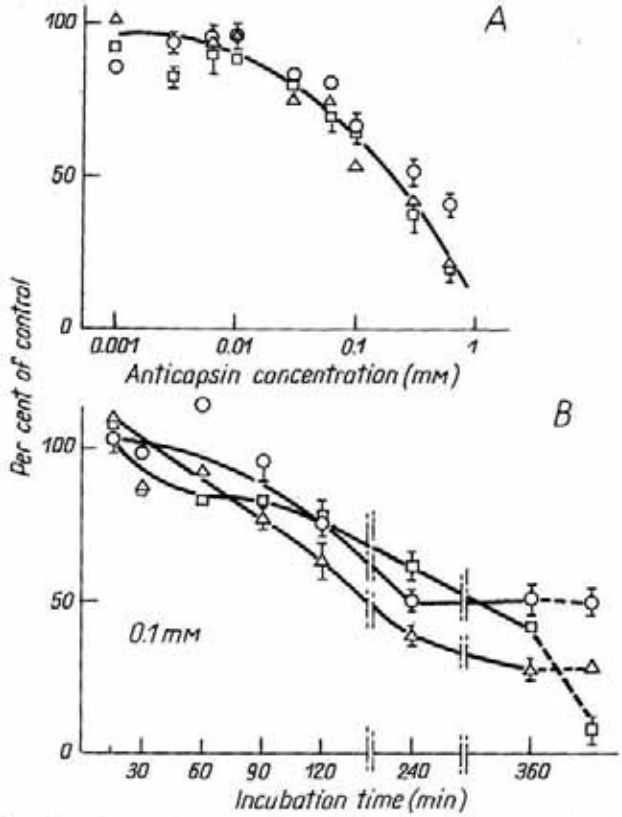

Fig. 2. Effect of anticapsin on incorporation of [^{3}H]thymidine (○), [^{3}H]uridine (△) and [^{3}H]leucine (□) into HeLa S_3 cells. A, The cells were incubated with anticapsin for 2 h at concentrations indicated. The labelled precursors were added after one hour of incubation. B, The cells were incubated with 0.1 mM anticapsin for the time indicated. [^{3}H]-labelled precursors were added for the last 30 min of incubation

cells per hour, respectively as averaged from 12 determinations. Incorporation of [^{3}H]leucine was completely inhibited on prolonged incubation (up to 24 h) but no further decrease of [^{3}H]thymidine and [^{3}H]uridine incorporation was observed (Fig. 2B).

The suppressed incorporation of [^{3}H]thymidine or [^{3}H]uridine might reflect the effect on nucleoside uptake or phosphorylation apart from the inhibition of nucleic acid biosynthesis [19]. Therefore, in attempt to distinguish the effect of anticapsin on DNA and RNA biosynthesis we used the

immediate precursors, [^{3}H]dTTP and [^{3}H]UTP, respectively, in the permeabilized cells capable of incorporating nucleoside triphosphates.

Anticapsin inhibited [^{3}H]dTTP and [^{3}H]UTP incorporation in this system both when preincubated with the cells prior to permeabilization and when applied directly to the permeabilized cells together with the nucleosides (Fig. 3A, 3B). These findings confirm the ability of anticapsin to inhibit

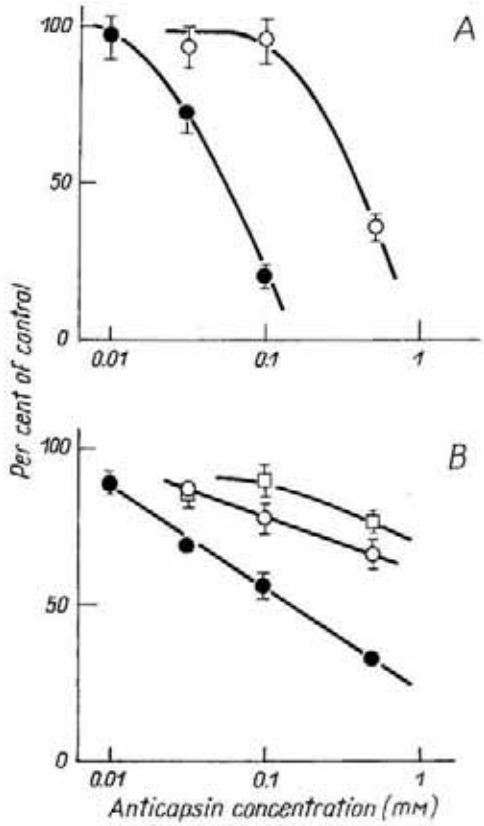

Fig. 3. The effect of anticapsin on [^{3}H]dTTP (A) and [^{3}H]UTP (B) incorporation into the permeabilized HeLa S_3 cells. The cells (0.5×10^6 cells/ml) were permeabilized with lysolecitin (100 μg/ml, 0 - 4°C, 2 min) without preincubation with the antibiotic (○) or after 0.5 h (□) and 2 h (●) preincubation period with anticapsin

nucleic acids biosynthesis in HeLa S_3 cells. The mean values of [^{3}H]dTMP and [^{3}H]UMP incorporated into control cells ($\pm$S.E.M.) were 54.9 ± 1.6 and 10.9 ± 1.1 pmol/10^6 cells per hour, respectively (n 10 or 12). At anticapsin concentration of 0.1 mM the incorporations of [^{3}H]dTTP and [^{3}H]UTP were about 20% and 35% of the control, respectively (Fig. 3A).

Interference of anticapsin with nucleic acid biosynthesis suggested that the antibiotic might, like tetaine, inhibit DNA- and RNA-polymerases *in vitro*

in the HeLa S_3 cell extracts. However, it was found that anticapsin even at concentration as high as 1 mM failed to inhibit DNA polymerase and only slightly suppressed RNA polymerase activity (Fig. 4). Thus, the inhibition of cellular DNA and RNA biosynthesis does not seem to be due to the interference with the respective polymerases. In contrast, inactivation of the polymerases is apparently the cause of selective inhibition of DNA and RNA synthesis in HeLa S_3 cells by tetaine [9].

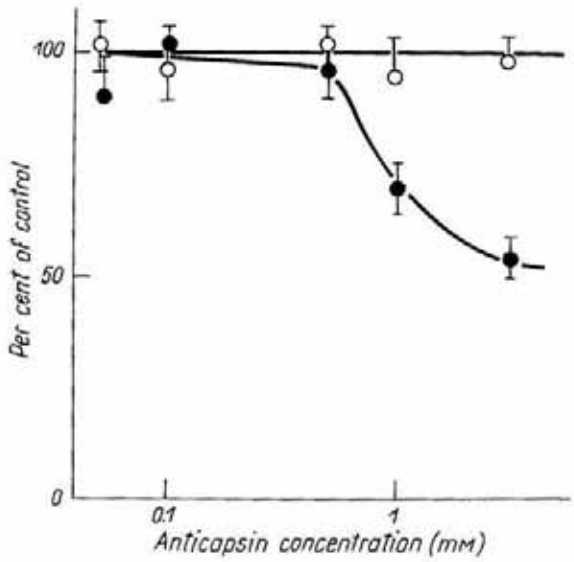

Fig. 4. The inhibition of DNA (○) and RNA (●) polymerases *in vitro*. The DNA polymerase assay was performed in 0.1 ml reaction mixture with the crude cell-extract as a source of the enzyme (2.4 mg of protein/ml). The crude cell-extract for the RNA polymerase assay contained 4.0 - 4.5 mg of protein/ml and the activity was measured in 50 μl reaction mixtures. The samples were incubated for 0.5 h at 37°C, then the reactions were stopped and radioactivities were measured

The growth inhibitory effect of tetaine in bacterial and yeast cells could be prevented by addition of glucosamine or *N*-acetylglucosamine [20] since anticapsin generated inside the cells acts as a glutamine analogue and inhibits glucosamine synthetase, and, in consequence, biosynthesis of glycoproteins and other glucosamine containing macromolecules [21]. Hence, we wondered whether inhibition of HeLa S_3 cell growth by anticapsin could also be prevented by the product of glucosamine synthetase. It appeared, however, that addition of *N*-acetylglucosamine failed to protect HeLa S_3 cells against the growth inhibitory action of anticapsin and it did not release inhibition of [^{3}H]thymidine incorporation. Furthermore, although anticapsin did inhibit incorporation of [^{3}H]glucosamine into glycoprotein, the extent of this inhibition coincided with that of protein

Table 1

The inhibition of incorporation of [^{3}H]glucosamine into HeLa S_3 cells after 2 h incubation with anticapsin

Anticapsin [mM]	% inhibition of incorporation of	
	[^{3}H]glucosamine	[^{3}H]leucine
0.03	23 ± 8	20 ± 2
0.1	47 ± 1	40 ± 3

synthesis (Table 1) suggesting that the latter might be the direct cause of the former. These findings indicate that in mammalian cells in contrast to microbial cells, glucosamine synthetase is not the main target of the antibiotic.

It seems that inhibition of protein synthesis is the key inhibitory effect of anticapsin in HeLa S_3 cells, at least after prolonged incubation. Besides, the inhibitors of protein synthesis are known to inhibit DNA synthesis after brief incubation [22]. Thus, it is conceivable that inhibition of protein synthesis could be the primary effect of anticapsin, whereas inhibition of nucleic acid biosynthesis a secondary one. One can not exclude, however, that effect of anticapsin in HeLa S_3 cells could result from the interference with another still unknown target(s). It is also quite likely that the drug might cause no single "primary" event, but would exhibit pleiotropic effects which are typical of other glutamine analogues in mammalian cells [23].

Although the precise mode of action of anticapsin in mammalian cells remains to be elucidated, the results reported demonstrate that it is clearly different from the mechanism of tetaine action both in mammalian and microbial cells.

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