THE ROLE OF MESSENGER RNA IN TYROSINE AMINOTRANSFERASE SUPERINDUCTION: EFFECTS OF CAMPTOTHECIN ON HEPATOMA CELLS IN CULTURE.

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Summary: Camptothecin inhibited the hydrocortisone but not the insulin induction of tyrosine aminotransferase activity in hepatoma cells in culture. However, camptothecin did not cause "superinduction" of tyrosine aminotransferase activity even though it reportedly inhibits messenger RNA synthesis. In hydrocortisone pre-induced cultures, camptothecin treatment caused a rapid decline in tyrosine aminotransferase activity suggesting it did not block degradation of the enzyme. A comparison of actinomycin D with camptothecin indicated that some of the effects of actinomycin D on tyrosine aminotransferase activity may not be mediated through inhibition of messenger RNA synthesis.

Actinomycin D has been extensively utilized in studies on the role of messenger RNA synthesis in regulation of protein synthesis (1-4). However, there is evidence that not all of the effects of this inhibitor on protein synthesis are a direct result of turnover of intracellular messenger RNA (2-5). Thus, comparison of actinomycin D with other inhibitors of mRNA synthesis is necessary in formulation of models for gene regulation of enzyme synthesis.

Recently, we reported that cordycepin, an inhibitor of mRNA processing, blocked the cortisol induction of tyrosine aminotransferase activity in Reuber (H35) cells in culture (3). This finding supported actinomycin D experiments implicating an mRNA synthesis requirement for the cortisol induction of this enzyme (1). Cordycepin, like actinomycin D, inhibited neither the insulin nor the cyclic AMP induction of tyrosine aminotransferase (3). These inductions apparently result from translational stimulation of enzyme synthesis (6). However, the cordycepin effects differed from those of actinomycin D in that cordycepin did not cause "superinduction" of tyrosine aminotransferase activity (3). The term "superinduction" has been coined to describe increases in enzyme levels resulting from treatment either with actinomycin D or with

another RNA inhibitor or analog (1). Tomkins et. al. (1) postulated that actinomycin D "superinduction" of tyrosine aminotransferase activity was due to inhibition of messenger RNA synthesis. However, results with cordycepin failed to confirm this hypothesis (3).

It has been recently reported that camptothecin, a plant alkaloid with antitumor activity, almost completely inhibited labelling of mRNA in HeLa cells (7). The effects of this inhibitor have also been reported to be reversible (7). The present report shows that camptothecin experiments support the earlier findings with cordycepin (3).

Methods and Materials

Reuber (H35) cells were grown as monolayers at 37°C on 60 mm plastic plates in 4 ml of Swim 77 (S-77) medium (8) which was modified to contain 4 mM glutamine and supplemented with 20% horse serum and 5% fetal calf serum. The gas phase was 5% CO_2 and 95% air. The cells were grown until each plate contained about 3×10^6 nuclei and 1.2 mg protein. At this point, cells were transferred to S-77 medium containing glutamine but lacking serum. Experiments were conducted 18 hours after the medium change. Inducing agents were dissolved in S-77 medium while inhibitors were dissolved in dimethyl sulfoxide. Each was added in a volume not exceeding 2% of total volume. Cells were harvested by aspirating he medium and adding 2.0 ml of a solution containing 200 mM potassium phosphate pH 7.3, 10 mM α-ketoglutarate and 0.040 mM pyridoxal phosphate to each plate. Cells were frozen and thawed three times and then cells were removed from the plates with the aid of a rubber policeman. The resulting suspensions were transferred to 12 ml centrifuge tubes and centrifuged at 1000 xg for 10 minutes. The supernatants were assayed for tyrosine aminotransferase activity according to the method of Diamondstone (3,9). One unit of activity was defined as equal to formation of 1 μmole of p-hydroxyphenylpyruvic acid per hour at 37°C. Protein determinations done according to the method of Lowry (10) indicated that within a given experiment there was less than 5% difference in supernatant protein per

Table 1. Effect of camptothecin on hydrocortisone induction of tyrosine aminotransferase in H-35 cells

Treatment	Concentration	Units tyrosine aminotransferase/ plate
none		0.22 ± 0.02[a]
hydrocortisone	2×10^{-6} M	2.80 ± 0.04
hydrocortisone + camptothecin[b]	2×10^{-6} M 4 µg/ml	0.30 ± 0.03
hydrocortisone + camptothecin	2×10^{-6} M 8 µg/ml	0.12 ± 0.01
hydrocortisone + camptothecin	2×10^{-6} M 20 µg/ml	0.02 ± 0.01

[a]Values are average of duplicate plates with the range indicated.

[b]Camptothecin was added 30 min before hydrocortisone and the cells were harvested 10 hours after addition of hydrocortisone

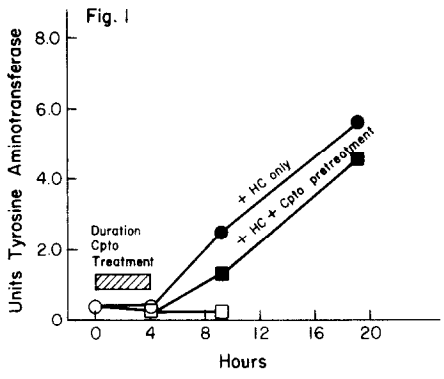
plate. Therefore, the tyrosine aminotransferase activities were expressed as enzyme units per plate. Camptothecin was generously supplied by H. B. Woods of the Drug Research and Development Branch, Division of Cancer Treatment, National Cancer Institute.

Results

Table 1 shows that camptothecin at doses between 4 µg/ml and 20 µg/ml was very effective in inhibiting the hydrocortisone induction of tyrosine aminotransferase activity. Cordycepin and actinomycin D also inhibited the hydrocortisone induction of tyrosine aminotransferase activity (3).

Figure 1 illustrates that the inhibitory effect of camptothecin on the inducibility of tyrosine aminotransferase activity by hydrocortisone was largely reversible even at a high dose of this inhibitor. Its inhibition of RNA synthesis in HeLa cells was also reported as reversible (7).

The data in fig. 2 illustrate that camptothecin did not block the trans-



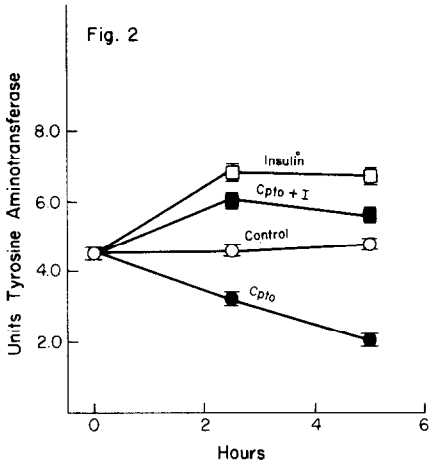


Fig. 1.

The reversibility of the effect of camptothecin (20 µg/ml) on hydrocortisone (2x10-6M) inducibility of tyrosine aminotransferase activity. Cells were treated with 20 µg/ml camptothecin for 4 hours. The camptothecin containing media was aspirated and plates washed once with 4 ml of S-77 media. 4 ml of fresh S-77 media was then added to each plate. Control plates were treated similarly with the exception of camptothecin treatment. O, no camptothecin pretreatment; □, camptothecin pretreatment. The values are the average of duplicate plates with the range indicated by the brackets. HC, hydrocortisone; cpto, camptothecin.

Fig. 2

Effect of camptothecin (20 µg/ml) on induction of tyrosine aminotransferase activity in H-35 cells by insulin (0.5 µg/ml). Hydrocortisone (2x10-6M, final concentration) was added to the plates 18 hours prior to the addition of camptothecin. Insulin was added 30 min later. Values are average of duplicate plates within the range indicated. O, control; ●, camptothecin. I, insulin; cpto, camptothecin.

lational induction of tyrosine aminotransferase activity by insulin in hydrocortisone pre-induced cells. However, in the absence of insulin camptothecin caused a rapid decrease in tyrosine aminotransferase activity. In fact, its activity in camptothecin-treated cells declined as if hydrocortisone had been removed from media (3). In another experiment, we found that increasing hydrocortisone to 5×10^{-5}M and decreasing camptothecin to 4 µg/ml (data not shown) did not alter camptothecin's effectiveness in blocking hydrocortisone induction. Thus, the camptothecin effect was not due to competition with hydrocortisone.

Table 2. Comparison of the actinomycin D effect with the camptothecin effect on tyrosine aminotransferase levels in H-35 cells preinduced with hydrocortisone

Treatment	Concentration	Units tyrosine aminotransferase/plate
none[a]		4.51 ± 0.16[b]
Camptothecin	20 μg/ml	2.80 ± 0.04
Actinomycin D	5 μg/ml	6.91 ± 0.11
Actinomycin D + camptothecin	5 μg/ml + 20 μg/ml	6.84 ± 0.29

[a]Cells were pre-induced 18 hours with hydrocortisone (2×10^{-6}M). Cells were then harvested 6 hours after addition of inhibitors.

[b]Values are average of duplicate plates with range indicated.

Table 2 compares effects of camptothecin and actinomycin D on hydrocortisone pre-induced cells. As above, camptothecin treatment resulted in a rapid decline in tyrosine aminotransferase activity. Actinomycin D, however, brought about an increase in tyrosine aminotransferase activity. When both drugs were added together, the actinomycin D "superinduction" effect predominated.

Discussion

Actinomycin D binds to guanine regions in DNA (11) resulting in blockage of transcription of all classes of RNA. In eukaryotes, enzyme inductions dependent on RNA synthesis are inhibited by actinomycin (1). However, it is not known whether increases in enzyme levels caused by actinomycin D treatment result from its transcriptional effects (3). A comparison of actinomycin with other RNA inhibitors is needed to resolve this problem.

Camptothecin, a plant alkaloid with antitumor activity, inhibits RNA but not protein synthesis in L1210 (12) and HeLa cells (7). While its mechanism of action is unknown, it altered HeLa cellular DNA such that strand breaks could be detected under alkaline but not neutral conditions (13). Presumably, transcriptional inhibition could result from its effects

on DNA. In HeLa cells, labeling of heterogeneous nucleoplasmic RNA was inhibited while almost no label appeared in cytoplasmic mRNA (7). Camptothecir also blocked labeling of ribosomal but not transfer RNA (7,12). In the present study, it was found that camptothecin blocked the hydrocortisone induction of tyrosine aminotransferase in H35 cells, a process which is considered to require transcriptional production of an mRNA for tyrosine aminotransferase (1,3). In contrast to actinomycin D "superinduction" of tyrosine aminotransferase activity in hydrocortisone pre-induced cells, camptothecin treatment resulted in rapid decline in TAT activity.

Previously, Tomkins postulated that actinomycin D "superinduction" of tyrosine aminotransferase resulted from its inhibition of mRNA synthesis (1). A critical discussion of his model was presented elsewhere (3). The present results with camptothecin suggest that inhibition of mRNA synthesis may play no role in actinomycin D mediated increases in tyrosine aminotransferase activity. Recent evidence indicates that actinomycin D inhibits initiation of protein synthesis (2) and selectively alters translation of existing mRNA (4).

The data reported here serve to confirm earlier results obtained with cordycepin (3). Both inhibitors blocked the hydrocortisone but not the insulin induction of tyrosine aminotransferase activity. Addition of either drug to hydrocortisone pre-induced cultures resulted in rapid decrease in tyrosine aminotransferase activity. Neither inhibitor, however, blocked actinomycin D "superinduction" of tyrosine aminotransferase activity. Cordycepin (3-deoxyadenosine) is phosphorylated to 3'-deoxy ATP which inhibits poly A formation thus presumably accounting for its inhibition of cytoplasmic mRNA labelling (14). Therefore, camptothecin and cordycepin appear to inhibit cytoplasmic mRNA labelling by quite different mechanisms. While cordycepin appears to inhibit synthesis of only mRNA containing poly A (14), there is no evidence that camptothecin selectively inhibits mRNA synthesis (7).

Recently, multiple forms of tyrosine aminotransferase activity in rat liver have been separated by hydroxyapatite (15) and CM-sephadex chromatography (16). At least 2 of the multiple forms probably arise due to post-transcriptiona enzyme modification (16). Studies on the effects of RNA inhibitors on enzyme modification may aid in understanding TAT superinduction.

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