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Original Articles

Increased Uptake of Actinomycin D in Ehrlich Ascites Tumour Cells Induced by Daunorubicin

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Summary. *The influence of anthracyclines on the uptake of actinomycin D in Ehrlich ascites tumour cells was studied in vitro. The anthracycline daunorubicin significantly increased the [^{3}H]actinomycin D uptake both in whole tumour cells and in isolated nuclei of tumour cells. On the other hand, actinomycin D increased daunorubicin uptake only to a very small degree.*

Experiments with other intercalating drugs did not influence [^{3}H]actinomycin D binding, suggesting a specific interaction between actinomycin D and the anthracyclines. The mechanism behind this interference is discussed.

The increased cellular uptake of actinomycin D in the presence of daunorubicin may have implications for antineoplastic combination chemotherapy.

Introduction

Actinomycin D and the anthracyclines are among the most important cytostatic antibiotics. The biological activity of both groups probably depends mainly on the ability to bind with double-helical DNA. It has been shown by circular dichroism that daunorubicin and adriamycin facilitate the actinomycin D binding to synthetic polynucleotides, possibly by changing the helical geometry [4].

This paper is concerned with the interaction between actinomycin D and the anthracyclines in vitro at the nuclear and the cellular level. Daunorubicin was chosen as representative of the anthracyclines.

Materials and Methods

Drugs and Isotopes. Daunorubicin was obtained as hydrochloride from Farmitalia. [^{3}H]Daunorubicin (0.5 mCi/mg) was kindly supplied by Rhone-Poulenc, Research Laboratory, Paris, France. Actinomycin D and acridine orange were obtained from Merch & Co., West Point, USA. [^{3}H]Actinomycin D was obtained from the Radiochemical Centre, Amersham, England (radiochemical purity 98%; specific activity 21 C/mmol). Proflavin was obtained from May & Baker, England. Ethidium bromide was obtained from BDH Chemicals, England. *N*-Acetyl-daunorubicin was synthesized as previously described [9]. Daunomycinone was obtained by hydrolysing daunorubicin in 0.1 *N* HCl at 100° C for 5 min, followed by washing in water three times. Nonidet P-40 was obtained from Shell, Carrington, England.

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Tumour Cells. All experiments were carried out on a hypotetraploid Ehrlich ascites tumour obtained from mice 6–8 days after transplantation. The origin and maintenance of the tumour have already been described [7]. Unless otherwise stated, the cell preparations were made as previously described [8]. Suspensions of cells with a final density of 0.5% v/v were prepared and preincubated for 10 min at 37° C before exposure to drugs. Cell viability was estimated by the ability of the cells to exclude 0.1% nigrosin. The medium was composed of 62.0 m*M* NaCl, 5.0 m*M* KCl, 1.3 m*M* $MgSO_4$, 60.0 m*M* sodium phosphate, 5% calf serum, and 10 m*M* glucose; pH was adjusted to 7.45.

Preparation of Isolated Nuclei. Washed tumour cells were added to a medium composed of sucrose 250 m*M*, $CaCl_2$ 5.0 m*M*, and Tris-HCl 25 m*M*, pH 7.45 at 37° C, final density of the suspension 0.5% v/v as for intact tumour cells. The detergent Nonidet P-40 0.1% v/v was added, instantly lysing the cells. A control specimen was stained with Giemsa and observed under the microscope. All cells were completely lysed, but nuclei remained intact.

Thus the concentration of nuclei was equal to the concentration of cells in the suspension of intact cells.

Incubation and Sampling Procedure. Media containing either the suspensions of cells or of nuclei were incubated at 37° C. At time zero drugs were added to the mixture. Serial samples of 2,000 µl were withdrawn at varying time intervals to conical centrifuge tubes, and placed on ice. Ice-cold Ringer solution (8 ml) was aded to terminate the uptake process. Intact cells were centrifuged at 5,000 *g* for 1.75 min and the pellet was washed twice in ice-cold Ringer solution. The supernatant was discarded and the wash fluid drained from the pellet as much as possible [8]. Isolated nuclei were centrifuged at 5,000 *g* for 2 min. The washing procedure was omitted for isolated nuclei, and correction for trapped medium was made as previously described [8].

Determination of Cellular Drug Content. [^{3}H]Actinomycin D was extracted from the drained pellet with 900 µl 0.5 *N* KOH at 80° C for 10 min, followed by centrifugation at 5,000 *g* for 10 min. [^{3}H]Daunorubicin was extracted from the drained pellet for 24 h with 1,500 µl 0.3 *N* HCl–50% ethanol solution according to Bachur et al. [1]. Then 100 µl of the supernatant was added to 10 ml scintillation fluid (Insta-Gel), and radioactivity was counted in a Beckman LS 250 liquidscintillation spectrometer.

All the drugs were tested individually for quenching, but no quenching was observed. Neither was any chemo- or photoluminescence observed.

Results

Figure 1 shows the time course of net uptake of [^{3}H]actinomycin in Ehrlich ascites tumour cells with increasing concentrations (1–50 μ*M*) of the anthracycline daunorubicin in the incubation medium. When daunorubicin was present in the medium, the uptake of [^{3}H]actinomycin D was significantly increased. It appears from the figure that uptake of [^{3}H]actinomycin D approaches steady state at 180 min in the control and in experiments with a low concentration of daunorubicin, whereas the uptake of the drug in combination with daunorubicin at high concentrations still shows a significant increase at 180 min. In the presence of 1 μ*M* daunorubicin the increase in uptake at 180 min was 12% and in the presence of 50 μ*M* daunorubicin it was 62%.

To study whether this increase in uptake was due to an influence on membrane permeability or to interference with the nuclear binding, experiments were carried out on isolated nuclei obtained by lysis of the tumour cells. As was the case for intact cells, [^{3}H]actinomycin D was added at a concentration of 1 μ*M* in the incubation medium, and daunorubicin was added in increasing concentrations from 1 to 50 μ*M*. It appears (Fig. 2) that the presence of daunorubicin results in a significant increase in binding of actinomycin D to isolated nuclei. This ranges from 64% in the presence of 1 μ*M* daunorubicin to 214% in the presence of 50 μ*M* daunorubicin at steady state, which was obtained after 30–60 min.

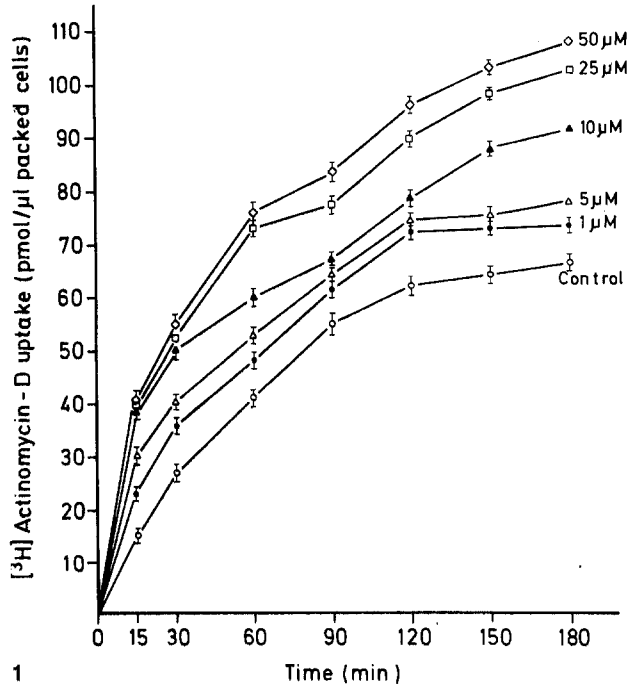
This effect of daunorubicin on nuclear binding of [^{3}H]actinomycin D did not depend on the order in which the two drugs were added to the suspension, as appears from data in Table 1.

To investigate whether a corresponding enhancement of the binding of daunorubicin might be induced by actinomycin D, the time course of binding of [^{3}H]daunorubicin to isolated nuclei was studied in the presence of increasing actinomycin D concentrations in the medium. [^{3}H]Daunorubicin was added at time zero at a concentration of 10 μ*M*, and actinomycin D at the concentration of 10, 25, and 50 μ*M*. Data are presented in

Table 1. Binding of [^{3}H]actinomycin D to isolated nuclei of tumour cells 60 min after addition of [^{3}H]actinomycin D

Time of daunorubicin addition[a]	Binding of [^{3}H]actinomycin D after incubation for 60 min with 10 μ*M* [^{3}H]actinomycin D (pmol/μl packed cells)	
	Mean	± SD
−15 min	119.0	0.4
0	118.9	0.2
+15 min	118.8	0.5

[a] Added at a concentration of 10 μ*M*



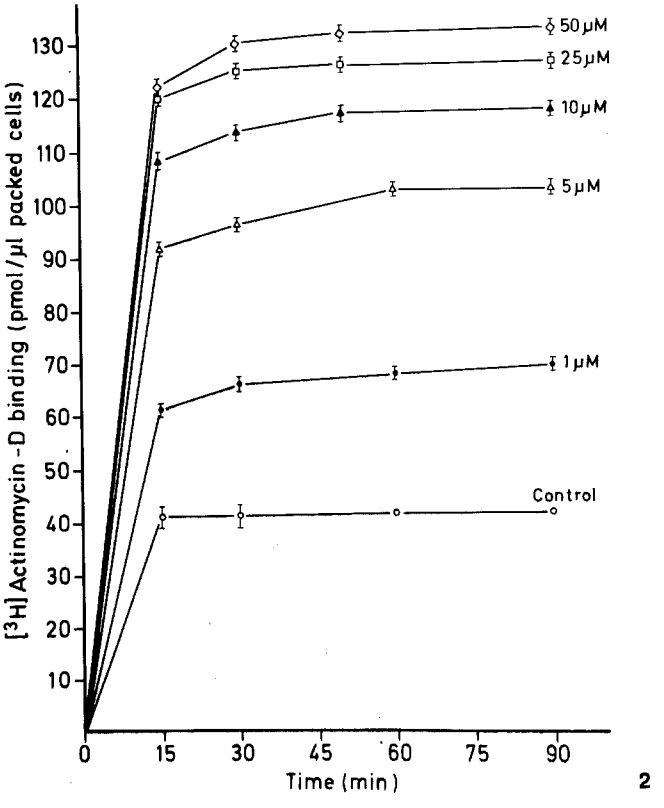


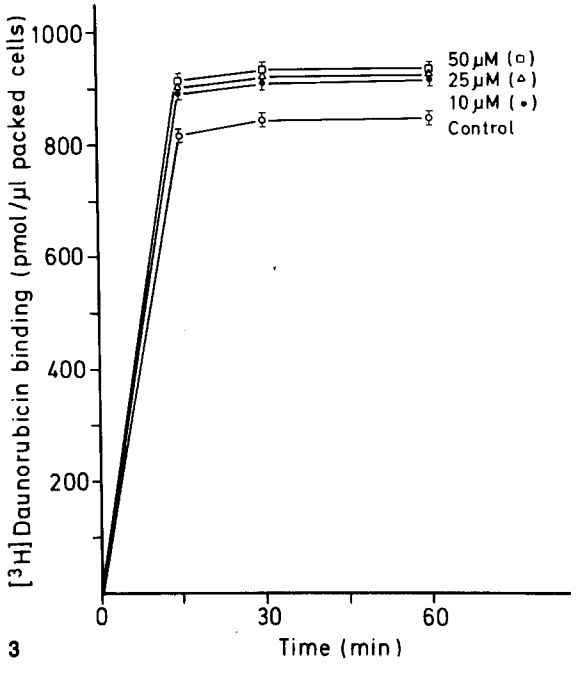
Fig. 1. [^{3}H]Actinomycin D uptake in Ehrlich ascites tumour cells. Daunorubicin was added to the incubation medium in increasing concentrations, as indicated in the figure. Concentration of [^{3}H]actinomycin D was 1 μ*M* in all experiments. [^{3}H]Actinomycin D was added immediately after daunorubicin. *Bars* indicate standard deviation over three determinations

Fig. 2. Binding of [^{3}H]actinomycin D to isolated nuclei from Ehrlich ascites tumour cells in the presence of increasing daunorubicin concentrations. [^{3}H]Actinomycin D was added at a concentration of 1 μ*M* immediately after the addition of daunorubicin. *Bars* indicate standard deviation over three determinations

Fig. 3. The level at steady state obtained after 30 min was increased by 10% in the presence of 10 μ*M* actinomycin D, but no further increase in binding was observed following addition of 25 or 50 μ*M* actinomycin D to the media.

The specificity of the interaction between anthracyclines and [^{3}H]actinomycin D with respect to nuclear binding was investigated by comparing the effect of four other intercalating drugs. Figure 4 shows [^{3}H]actinomycin D binding to isolated nuclei at steady state in the presence of increasing concentrations of the drugs daunorubicin, *N*-acetyl-daunorubicin, proflavin, ethidium bromide, and acridine orange in the incubation medium. [^{3}H]Actinomycin D was added at time zero at a concentration of 1 μ*M*. The marked effect on [^{3}H]actinomycin D binding was only observed for daunorubicin; *N*-acetyl-daunorubicin exerted a weak effect, whereas the other drugs had no statistically significant influence on binding. It appears from the figure that the effect of daunorubicin on the [^{3}H]actinomycin D binding levels off with increasing concentrations. As shown in Table 2, in the same range of concentrations, the anthracycline analogue adriamycin had a nearly identical effect on the [^{3}H]actinomycin D binding to isolated nuclei. However, addition of the aglycone of daunomycin, daunomycinone, did not influence the binding of [^{3}H]actinomycin D.

Figure 5 shows the effect of daunorubicin at a concentration of 25 μ*M* on nuclear binding of [^{3}H]actinomycin D, plotted



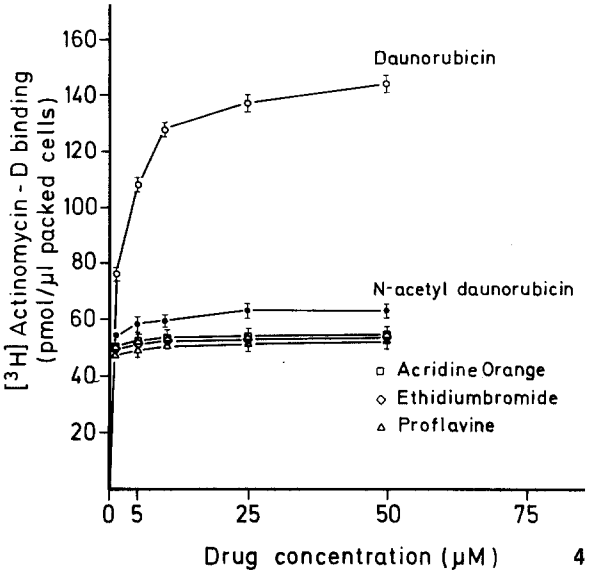


Fig. 3. Binding of [^{3}H]daunorubicin to isolated nuclei from Ehrlich ascites tumour cells. Actinomycin D was added to the incubation medium in increasing concentrations (10–50 μ*M*), whereas the concentration of [^{3}H]daunorubicin was 10 μ*M* in all experiments. *Bars* indicate standard deviation over three determinations

Fig. 4. [^{3}H]Actinomycin D binding to isolated nuclei after 60 min of incubation. Daunorubicin and four other intercalating drugs were added in increasing concentrations to the incubation medium immediately before the addition of [^{3}H]actinomycin D at time zero. [^{3}H]Actinomycin D was added at a concentration of 1 μ*M*. *Bars* indicate standard deviation over three determinations

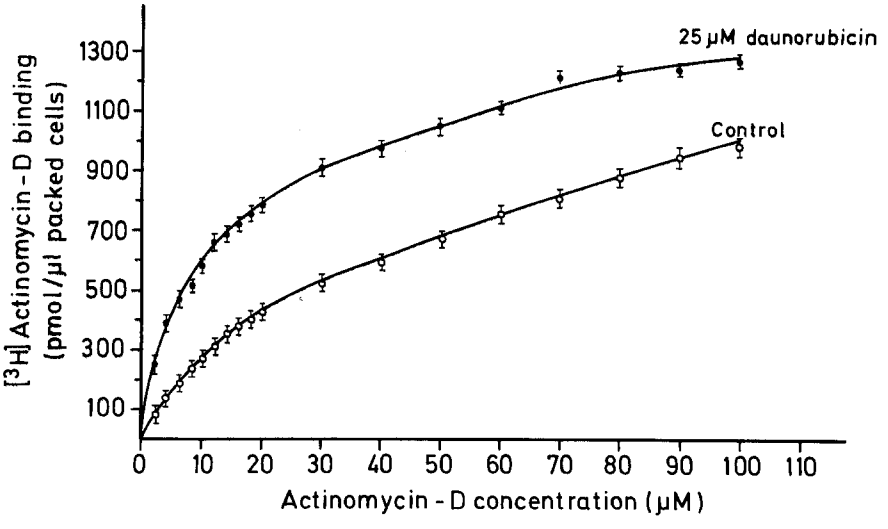


Fig. 5. Influence of daunorubicin on binding of [^{3}H]actinomycin D to isolated nuclei as a function of increasing concentrations of actinomycin D in the incubation medium. Binding pattern was studied after 60 min. *Bars* indicate standard deviation over three determinations

Table 2. [^{3}H]Actinomycin D binding[a] to isolated nuclei (pmol/μl packed cells) in the presence of adriamycin, daunorubicin, and daunomycinone

Time	10 μ*M* Adriamycin	10 μ*M* Daunorubicin	10 μ*M* Daunomycinone	Control
30 min	102.8 ± 0.9	103.0 ± 0.5	40.79 ± 0.2	41.4 ± 0.3
60 min	105.3 ± 0.8	105.4 ± 0.6	41.07 ± 0.3	41.5 ± 0.3
Time	50 μ*M* Adriamycin	50 μ*M* Daunorubicin	50 μ*M* Daunomycinone	Control
30 min	130.0 ± 0.4	130.5 ± 0.3	41.07 ± 0.1	41.4 ± 0.3
60 min	131.4 ± 0.4	131.1 ± 0.2	41.66 ± 0.3	41.9 ± 0.1

[a] Mean and standard deviation

as a function of [^{3}H]actinomycin D in the medium. The effect of daunorubicin on [^{3}H]actinomycin D binding increases with increasing concentrations of [^{3}H]actinomycin D in the medium until a maximum is obtained at 20 μ*M*. If [^{3}H]actinomycin D in the medium exceeds this concentration, no further increment in binding is observed.

This biphasic course could be the result of two binding processes: one a specific binding showing a saturation phenomenon around 20 μ*M* and the other a linear non-specific adsorption to the nuclei.

Discussion

The present data show that daunorubicin induces a significant increment in uptake of [^{3}H]actinomycin D in Ehrlich ascites tumour cells. A similar but much more pronounced effect was observed for isolated nuclei, indicating that daunorubicin exerts its effect at the nuclear level and not on the membrane transport. As both actinomycin D and daunorubicin bind mainly to DNA, the change in binding characteristics of nuclei can most probably be ascribed to an influence of daunorubicin on the mechanism of the binding of actinomycin D to DNA. This interpretation agrees well with the finding that daunorubicin facilitates the binding of actinomycin D to poly · (dA−dT) poly · (dA−dT) [4].

As actinomycin D does not bind to double-stranded poly · (dA−dT) poly · (dA−dT) (in contrast to the anthracyclines), the experiments performed by Krugh and Young indicate that the intercalation of daunorubicin is accompanied by changes in the conformation of the polynucleotide, resulting in the formation of new stable binding sites for actinomycin D. The authors proposed that the effect could be an expression of an opening of new binding sites in consequence of unwinding of double-helical DNA, which is a well-documented phenomenon accompanying intercalation [5].

Our studies on isolated nuclei and the results obtained on synthetic DNA by Krugh and Young demonstrate that another intercalating drug, ethidium bromide, is unable to facilitate the binding of actinomycin D. We observed a similar lack of effect for the acridines proflavin and acridine orange, which also intercalate in double-helical DNA. These findings indicate that the phenomenon is characteristic only for the interaction between actinomycin D and the anthracyclines. The finding is probably not a question of base specificity, as anthracyclines [3, 6], ethidium bromide [10], and proflavine [11] all fail to show base specificity.

The effect of the anthracyclines on nuclear binding of actinomycin D could also result from an interaction between anthracyclines and DNA apart from intercalation. However, a stable intercalation seems to be a prerequisite for the facilitating effect on actinomycin D binding, as the aglycone daunomycinon, which is unable to form a stable binding to DNA [2], did not affect actinomycin D binding. Furthermore, the anthracycline analogue *N*-acetyl-daunorubicin, which had a considerably lower affinity to DNA [12], only exerted a weak effect on the binding of actinomycin D.

The data shown in Fig. 5, demonstrating that the absolute increment obtained in nuclear binding of [^{3}H]actinomycin D by adding daunorubicin is constant over a wide range of [^{3}H]actinomycin D concentrations, are consistent with the formation of a constant number of new binding sites.

According to this hypothesis, the effect of daunorubicin may be ascribed to an unwinding and subsequent opening of binding sites adjacent to adenine-thymine base pairs in DNA for binding of actinomycin D in addition to the well-known sites adjacent to the guanine−cytosine bases.

As several other intercalating compounds are unable to facilitate actinomycin D binding, the unwinding angle may be a very critical characteristic for the formation of new binding sites for actinomycin D. Another explanation could be that the ion binding formed between the amino sugar of the anthracyclines and the phosphate backbone of DNA is a prerequisite for the phenomenon.

The interaction in vitro may result in a synergistic cytotoxic effect of the two antibiotics in vivo, which could be of importance for the use of these drugs in clinical combination chemotherapy.

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