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MODE OF ACTION OF ANTITUMOUR ANTIBIOTICS-III : MODULATION OF PERMEABILITY OF NUCLEAR MEMBRANE IN THE PRESENCE OF THE ANTIBIOTICS*

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Summary:

The binding characteristics of the antibiotics to nuclei and their effect on the permeability of nuclear membrane with respect to histones and ribonucleic acids have been investigated. The binding constant for chromomycin A_3 was found to be $1.4 \times 10^{4} M^{-1}$ and number of binding sites was equal to $3.48 \pm 1.08 \times 10^{12}$ molecules/nuclei. The antibiotic chromomycin A_3 enhanced the uptake of lysine-rich histone, actinomycin D decreased the uptake and ethidium bromide had no effect. Chromomycin A_3 also enhanced the release of acid insoluble fraction containing RNA from the nuclei, actinomycin D and ethidium bromide inhibited the release of acid insoluble fraction containing RNA. The relevance of this finding to the role of nuclear envelope in understanding the mechanism of action of the antibiotic has been discussed.

Introduction:

Chromomycin A_3 and actinomycin D exhibit antitumour properties against various experimental tumours and have been clinically used against certain cancers (1). The locus of action of these antibiotics appears to be the nucleus, where they inhibit the DNA dependent RNA-polymerase reaction by interacting with double helical DNA. Presence of a divalent cation is an obligatory for chromomycin A_3 to interact (2). Although, a detailed study on the interaction of various antibiotics to DNA and their effects on the inhibition of transcription has been investigated (3-6) it has not been

*Part I and II - see references (5) and (6)

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possible to establish a relationship between the dose and the response. For example, the binding constant for chromomycin A_3 is 1000 times lower than that of actinomycin D, but it inhibits transcription by 50% at concentrations comparable to actinomycin D (5). This finding supports the assumption that antibiotics have other modes of action in addition to their interaction with DNA. It has been shown in case of actinomycin D that it inhibits the nucleo-cytoplasmic migration of RNA due to the decrease in the pore size (7). Furthermore, Roth and Hans have shown that in Hela cell nuclei ribosomal proteins are taken up more rapidly than cytoplasmic proteins, thus, showing the selectivity of the nuclear membrane (8). Therefore, it remains to be established whether uptake of antibiotics are different in each case and the permeability of the nuclear membrane to biologically important molecules is altered in their presence. Thus to explore further the mechanism of action, a systematic study on the binding of various antibiotics to the nuclei and their effects on the permeability of the nuclear membrane to histones and ribonucleic acid in an *invitro* system was undertaken. Here we wish to report some of the preliminary results.

Materials and methods:

Actinomycin D and Ethidium bromide were obtained from Sigma Chemical Company (St. Louis) and Chromomycin A_3 from Cal Biochem (Switzerland). The concentration of the antibiotics was determined spectrophotometrically. The solution of Chromomycin A_3 was always stored in dark and the concentration was determined at 405 nm using molar extinction coefficient of 8.8×10^3.

Male rats (Indian Institute of Science strain) weighing about 150 gms were used for the isolation of nuclei.

Isolation of nuclei:

Nuclei were isolated according to the method of Blobel and Potter (9). The nuclei prepared was stored in 25% glycerol 0.01M tris-HCl, 0.001M $MgCl_2$ pH 7.5 until use. The concentration of nuclei was determined by counting them in an hemacytometer.

The purity and integrity was checked by chemical composition and phase contrast microscopy.

Labelling of histones:

The calf thymus lysine-rich histones (Sigma Chemical Company, St. Louis) were acetylated with [^{14}C]-acetyl phosphate according to the method of Ramponi et.al (10). After the reaction, the histones were precipitated with 18% trichloroacetic acid, centrifuged and washed with acetone and dried in vacuo.

The total histones were isolated from rat liver according to the method of Bonner et.al (11). Histones were labelled as mentioned above. The purity of histones was checked by polyacrylamide gel electrophoresis according to Panyim and Chalkley (12).

Labelling of ribonucleic acid (RNA):

This was labelled in vivo by injecting 500 µCi of [^{32}p] phosphate one hour before sacrifice of the animal.

Binding of antibiotic to nuclei:

Nuclei suspended in 0.25M sucrose in tKM buffer (0.05M tris HCl, 0.025M KCl, 0.005M $MgCl_2$ pH 7.5) was incubated for 10 minutes with the antibiotic at 25°C. At the end of the period nuclei were pelleted by centrifugation at 800 g for 10 minutes. The supernatant was used to measure the concentration of free drug spectrophotometrically.

Effect of the antibiotic on the nucleo-cytoplasmic transport of histones and acid insoluble fractions:

The suspension of nuclei containing 8.3×10^7 counts/ml were incubated with the drug (5×10^{-5}M) in 0.25M sucrose in tKM buffer at 25°C for 10 minutes prior to the addition of histones. The total volume of the mixture was 0.5 ml. At the end of 20 minutes, the nuclei were pelleted as before. The pellet was solubilized with 10% Sodium dodecyl sulphate by agitation. The radioactivity in both pellet and supernatant were counted in a Beckmann LS-100 counter using 0.5% ppo in toluene-triton X-100 in the ratio 2:1 as the cocktail.

For the release experiments, the [^{32}p] labelled nuclei (8.3×10^7 counts/ml) were incubated with the antibiotic (5×10^{-5}M) for 10 minutes and the nuclei were pelleted as before. RNA in both pellet and supernatant were precipitated with 0.3M perchloric acid and centrifuged for 10 minutes at 800 g. The precipitate was washed thrice with 0.3N perchloric acid and was digested with 0.3N potassium hydroxide overnight and centrifuged. The supernatant was neutralized with perchloric acid centrifuged and aliquot of the supernatant was counted.

Both uptake and release experiments were carried out about an hour after preparation of nuclei. Light microscopic examination of nuclei showed their intactness.

Evaluation of binding constant (K) and number of binding sites (n):

The values for K and n were calculated from the equation

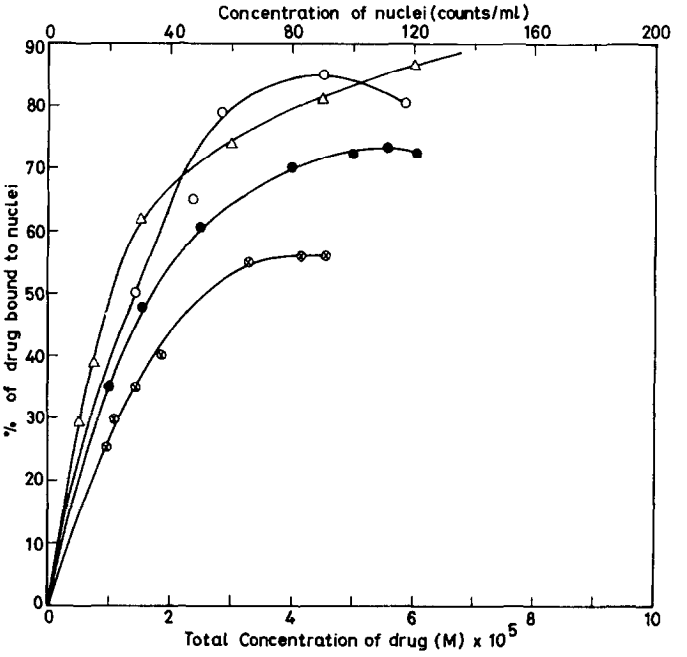

Figure 1. Uptake of the antibiotic by the nuclei at 25°C, at fixed concentration of nuclei (counts/ml) (i) (⊛)26x10^6; (ii) (●)40 x 10^6; (iii) (○) 50x10^6; (iv) (△)at fixed concentrations of antibiotic (5x10^{-5}M).

$1/r = \frac{1}{Kn} x \frac{1}{m} + \frac{1}{n}$ according to Steck (13), where r = amount of drug bound to nuclei, m = amount of free drug.

Results and Discussion:

Fig. (1) shows the percentage of chromomycin A_3 bound to nuclei when varying concentrations of the antibiotic are added to a constant amount of nuclei and vice versa. It indicates significant binding in the range of concentration of nuclei (5-200 x 10^6/ml). Further, the concentration of nuclei required for 50% saturation of the antibiotic is 1.5 times higher for ethidium bromide as compared to chromomycin A_3 (data not shown). This is in accordance with the higher value of binding constant (5). The noteworthy feature here is, the concentration of chromomycin A_3 is much higher than that calculated from the DNA-drug binding data (5).

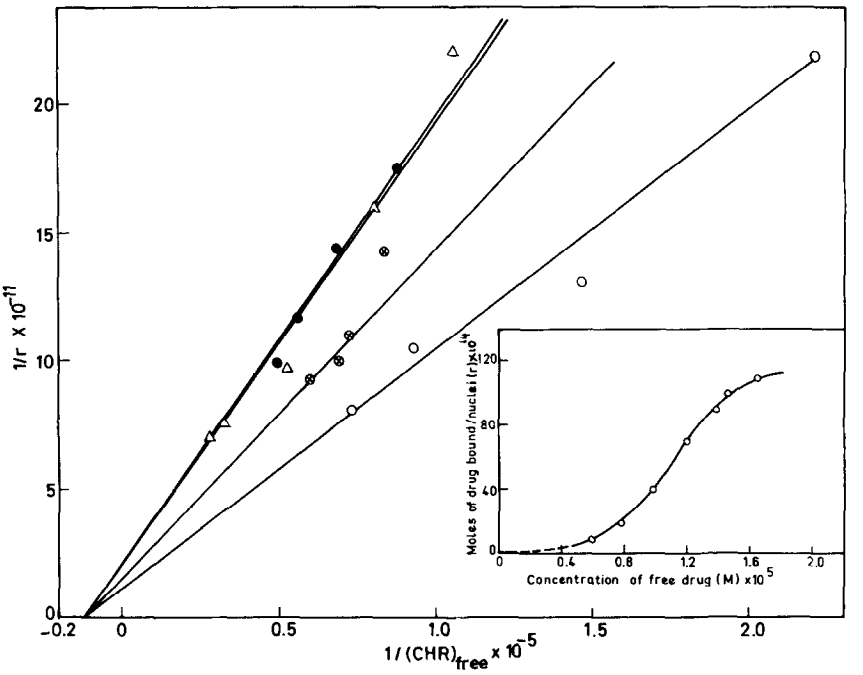

Figure 2. Plot of 1/r against 1/m for binding of Chromomycin A_3 to nuclei. The concentration of nuclei (counts/ml) (i) (●) 26×10^6; (ii) (⊗) 40×10^6; (iii) (○) 50×10^6 and (iv) (△) at fixed concentrations of the antibiotic (5×10^{-5}M).

The inset shows the plot of amount of the antibiotic bound/nuclei against the amount of free antibiotic. The concentration of nuclei 40×10^6 counts/ml.

This suggests that it binds to sites, presumably, nuclear membrane in addition to DNA. These data were further analysed to calculate the binding constant and number of binding sites as shown in Fig.(2). The values for K and n were $1.4 \times 10^4 M^{-1}$ and $3.48 \pm 1.08 \times 10^{12}$ molecules/nuclei. This discrepency in the value of n is probably due to the use of different batches of nuclei.

The results of the uptake of histones by the nuclei and the release of the acid insoluble fraction in the supernatant are summarised in (tables I and II). It is seen that uptake of lysine-rich histones by the nuclei depends on the nature of the antibiotic.

Table I. Effect of antibiotics on uptake of [^{14}C]-acetylated histones by the nuclei.

Concentration of histones (M) x 10^7	-Antibiotic	Counts in the nuclei (cpm) +Chromomycin A_3 ($5x10^{-5}$M)	+Ethidium bromide ($5x10^{-5}$M)	+Actinomycin D (5×10^{-5}M
Total histones				
8.6 (10,000)	500	700	-	-
21.5 (28,000)	2520	3150	-	-
45.0 (52,000)	10400	11400	-	-
Lysine rich histones				
8.6 (8,000)	810	1200	820	775
18.5(16,000)	2540	3700	2565	2260
39.0(38,000)	9500	12500	9510	4550
75.0(69,000)	18610	27600	18540	9318

The numbers in the parenthesis denote the total counts (cpm).

Chromomycin A_3 stimulates the uptake of lysine rich histones whereas the difference in the uptake of total histones was not significant, presumably, because of the aggregation behaviour of histones under these experimental conditions (14).

Since the antibiotic is transported by passive diffusion under these experimental conditions, it is logical to assume that it might affect the transport of other molecules by same process. This is evident from the fact that both the uptake of histones and the release of acid insoluble and alkali hydrolysable fraction (RNA) are concomitantly stimulated in case of Chromomycin A_3. In contrast Actinomycin D brought about a decrease in both the uptake of histone and the release of acid insoluble fractions. The observed differences with respect to 3 different drugs suggest that interaction of drug with nuclear envelope leading to subtle changes in organisation of the membrane are different in each case. It remains still to be established how such changes are brought about and the mechanism of uptake of histones and the nature of the released components. Such studies are in progress. Nevertheless, present data do indicate,

Table II. Effect of antibiotics on the release of $[^{32}p]$ labelled fractions*

Type of the antibiotic	Concentration of the antibiotic (M) X 10^5	counts (cpm) in Nuclei	counts (cpm) in total acid insoluble fractions	counts (cpm) in released fraction	counts (cpm) in acid insoluble released fraction	% count of total insoluble fractions released into supernatant
Chromomycin A_3	0 (batch I)	30017	21011	9200	4200	20.0
	0 (batch II)	38888	5938	12300	2500	42.0
	1.5	30017	21011	9350	6130	29.0
		38888	5938	13600	2930	49.5
	3.0	30017	21011	9400	7550	35.8
		38888	5938	12600	3330	56.0
	5.0	30017	21011	9500	9400	44.5
		38888	5938	13600	3500	59.0
	8.3	38888	5938	13800	3580	60.5
	12.0	38888	5938	14100	3500	59.0
Ethidium bromide	1.5	30017	21011	7900	3820	18.1
	3.0	30017	21011	8120	3420	16.2
	5.0	30017	21011	8200	2410	11.4
Actinomycin D	1.5	30017	21011	7980	3140	14.8
	3.0	30017	21011	8850	2940	13.9
	5.0	30017	21011	9500	2940	13.9

*It may be noted that the total invivo incorporation of $[^{32}p]$ label into acid insoluble fraction differs significantly from batch to batch and the extent of release of acid insoluble fraction is also higher as compared to the value reported for RNA (15). This difference is not due to the lysis of the nuclei as judged by the phase contrast microscopic examination, but in all probability due to the presence of $[^{32}p]$ labelled acid insoluble fractions other than RNA and due to the difference in the composition of the incubation medium.

for the first time, that in determining the therapeutic potential of an antibiotic both the affinity constant and effect of the antibiotic on the permeability of nuclear envelope to other functional molecules in transcription must be taken into consideration.

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