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PHASEOLINONE, A NEW MYCOTOXIN, INHIBITS RNA POLYMERASE(S) OTHER THAN RNA POLYMERASE II

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DNA-dependent RNA synthesis in an isolated rat liver nuclei in vitro assay was inhibited by 41 and 61% in the presence of α-amanitin, the inhibitor specific for RNA polymerase II and phaseolinone, a new mycotoxin, respectively. This inhibition reached 97% when these two inhibitors were present together. The RNA polymerase activity present in a nucleolar fraction was inhibited by greater than 80% in presence of phaseolinone, but phaseolinone and DNA showed no interaction as judged from CD spectra or density gradient centrifugation of toxin-treated DNA. This evidence suggested that phaseolinone inhibited RNA polymerase activity other than RNA polymerase II.

Differential sensitivity of α-amanitin, a mycotoxin produced by Amanita phalliods (1), to the inhibition of DNA-dependent RNA polymerases proved extremely useful in characterisation of the multiple forms of this enzyme and in nucleic acid enzymology (2-4). Extensive studies on RNA polymerase I were reported possible when other forms in the presence of this toxin were kept to an inhibited state (5-7). However, studies on RNA polymerase in which RNA pol I was subjected to inhibition were not possible due to the lack of an inhibitor compound that inhibits RNA polymerase I only.

Phaseolinone (Fig.1), a new mycotoxin, appears to be such an inhibitor for the RNA polymerase (s) other than polymerase II. The toxin

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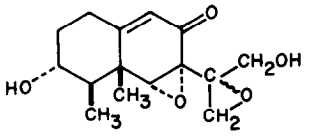


Fig 1 : Structure of phaseolinone.

produced by Macrophomina phaseolina, a fungal pathogen of plant, was shown to be a strong inhibitor of seed germination (black gram seeds) (8,9). In experimental animals such as weaning mice it resulted in weight loss (10). In bacterial systems it inhibited microbial growth and showed reversion of excession-repair-deficient bacterial strains, such as Escherichia coli WP-2 and Salmonella typhimurium TA-100, indicating its mutagenic action (11). The latter observation suggested a possible interaction between phaseolinone and bacterial DNA as the basis of mutagenic action. Durine one of our studies on RNA polymerases using isolated rat liver nuclei in vitro assay (12), the toxin was included and was shown to have no effect at the DNA level (unpublished results). The present study suggests that the toxin phaseolinone did not interact with DNA but inhibits DNA-dependent RNA synthesis, probably interacting with RNA polymerase I.

MATERIALS AND METHODS

Preparation of phaseolinone

Phaseolinone was purified to a crystalline form, using 7-day-old unshaken culture filtrate of M. phaseolina Goid (9).

Preparation of rat liver nuclei

Rat liver nuclei were isolated from fresh liver (13). The purity and integrity of the preparation were checked microscopically. DNA content of the nuclei was determined by the diphenyl method of Burton (14) using calf thymus as the standard.

Preparation of nuclear and nucleoplasmic RNA polymerase

Nucleolar and nucleoplasmic fractions having RNA polymerase activity were prepared according to the methods of Sugden and Sambrook (15) using the isolated liver nuclei. Briefly, nuclear pellet isolated from 24 g liver was suspended in a solution of 0.01 M $(NH_4)_2SO_4$, 0.05 M Tris-HCl, pH 7.9, 0.006 M $MgCl_2$, 0.001 M EDTA, 0.005 M β-mercaptoethanol, 30% glycerol, and 0.5% Triton X for 60 min at 4°C. After the nuclei were pelleted again, the supernatant was taken as a nucleolar fraction of RNA polymerase. The pellet was resuspended in 0.05 M Tris-HCl, pH 7.9, 0.006 M $MgCl_2$. 0.001 M EDTA, 0.00005 M dithiothreitol, and 15% glycerol solution to a minimum volume of 2 ml. To this, $(NH_4)_2SO_4$ solution (5.3 M at pH 7.9) was added to 0.3 M. The viscous material thus obtained was sonicated (4 x 15 sec), diluted three folds and centrifuged at 40,000g for an hour. The supernatant was collected as nucleoplasmic fraction.

Assay of RNA polymerase

(i) Assay with isolated nuclei: Isolated rat liver nuclei served as the source of RNA polymerase as well as functional DNA template (endogenous). The assay was based on Goldberg et. al. (4), as modified by Lazarus and Warnick (16) and carried out as earlier (12). Briefly 0.6 ml assay volume included 30 µmol Tris-HCl pH 7.9, 3 µmol NaF, 1 µmol $MnCl_2$ 0.6 µmol dithiothreitol 24 µmol $(NH_4)_2SO_4$, 0.3 µmol of ATP, GTP and CTP, 0.1 µmol UTP and 1 x 10^{-3} µmol ^3H UTP (25000 µci/µmol) and 0.1 ml of nuclear suspension having total DNA-content 60-50 µg. Except UTP, all other ingredients were incubated for 5 min at 37°C with or without the inclusion of toxin. Then UTP was added to initiate the reaction. After 15 mins the reaction was terminated and TCA insoluble material was collected on Whatman GF/C filter disc. This was then washed with TCA containing 0.01 M $Na_2P_4O_7$, dried and counted for radioactivity. The blank was carried out by assaying the synthesis in absence of trinucleotides but with the inclusion of radioactive UTP. The synthesis of RNA was then estimated by subtracting the blank value from total incorporation.

(ii) Assay with nucleolar and nucleoplasmic fractions : RNA polymerase activity was determined as described by the authors, reporting the preparation of these fractions from Hela cells (15). 200 µl assay mixture was made up 0.5 mM of ATP, CTP and GTP, 50 µg per ml or otherwise of deactivated calf thymus DNA, 50 mM $(NH_4)_2SO_4$, 1.5 mM $MnCl_2$, 0.125 mM EDTA, 0.1 mM dithiothreitol, 75 mM Tris-HCl pH 7.9 containing 4 µci per ml ^3H UTP [25000 µci/µmol] and 50 µl of enzyme. The toxins for inhibition study were included in the assay mixture. Rest of the assay was similar to nuclei assay except the incubation was for 30 min.

Binding of phaseolinone to DNA

(i) Circular Dichroism Study : Different concentrations of phaseolinone were used to mix with DNA solution (1 mg in 10 ml of 0.02 M phosphate buffer pH 7.2) and circular dichroism of these solutions were recorded in JASCO spectropolarimeter model J-20 with expansion 10 nm per cm, time constant 16 and scan speed 20 nm per min.

(ii) Density gradient centrifugation : This was carried out essentially by the method of Abelson and Thomas (1966) (17) using ^3H-thymidine labelled E. coli DNA prepared by us. Both control and toxin treated DNA solution was layered on 4.5 ml of 5-20% (w/v) sucrose gradient (adjusted to pH 11.8 with NaOH) and run for 2 h at 28000 r.p.m. in a Beckman SW 50.1 rotar at 20°C. Fractions were collected as drops and radioactivity was estimated for both the control and toxin treated samples.

RESULTS AND DISCUSSION

Figure 2 represents the dose response curve of phaseolinone on the inhibition of RNA synthesis in an isolated rat liver nuclei assay system. The curve appeared slightly sigmoidal and the inhibitory response apparently levelled off at a dose 20-25 µg per ml, phaseolinone showing nearly 60% inhibition of RNA synthesis. The toxin might have more than one interacting sites in this enzyme system. And one of the sites affected by a dose below 2.5 µg per ml is possibly least effective in modulating enzyme activity considerably. But at doses 2.5 to 10 µg per ml, the

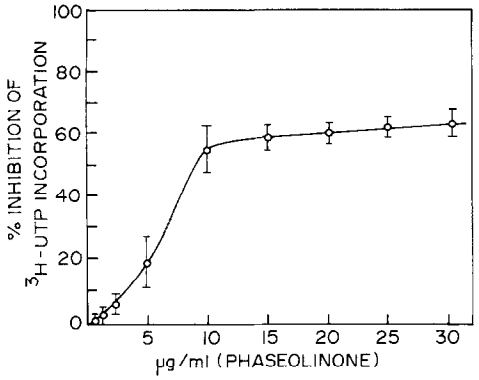


Fig 2 : Inhibition of RNA synthesis in presence of phaseolinone. Each data point presents mean ± S.D of five experiments and each experiment done in triplicate.

toxin interacts with the critical sites responsible for enzymatic activity. The difference in inhibition for doses 10 to 30 μg per ml was within the limit of experimental variation. And phaseolinone at largest dose (30 μg per ml) was used throughout the study.

Table 1 demonstrated the inhibition of RNA synthesis in an isolated rat liver nuclei in presence of α-amanitin and phaseolinone. Phaseolinone 30 μg and α-amanitin 3 μg per ml if added together the inhibition was nearly the sum of their individual inhibitions (Table 1). This result implied that the sites of actions of these two toxins are distinctly sepa-

Table 1 : Inhibition of RNA synthesis by phaseolinone and α-amanitin in isolated whole rat liver nuclei

Inhibitors	Cpm incorporation per μg of DNA	% of inhibition
None	17727 ± 1369	0
α-amanitin (3 μg/ml)	10443 ± 624	41.20
α-amanitin (6 μg/ml)	9683 ± 309	45.00
Phaseolinone (30 μg/ml)	6994 ± 654	61.00
Phaseolinone (30 μg/ml) + α-amanitin (3 μg/ml)	539 ± 92	97.00

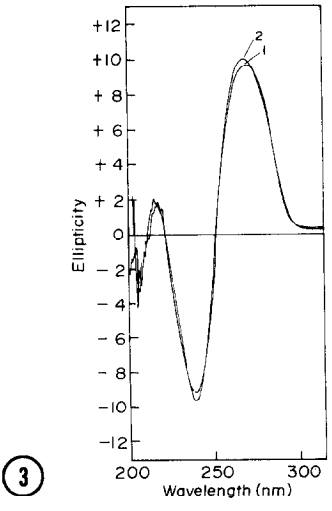
Mean ± S.D. for five sets of experiments and triplicate in each set.

Table 2 : Effect of phaseolinone and α-amanitin on RNA synthesis by nucleolar and nucleoplasmic fractions

Treatment	Nucleolar fraction (I)				Nucleoplasmic (II)			
	UMP incorporation (Cpm) for DNA dose (μg/ml)		Inhibition % for DNA dose (μg/ml)		UMP incorporation (Cpm) for DNA dose (μg/ml)		Inhibition % for DNA dose (μg/ml)	
	50	25	50	25	50	25	50	25
None	5026 ± 1204	2161 ± 114	0	0	3963 ± 765	1562 ± 121	0	0
α-amanitin (3 μg/ml)	5054 ± 472	1819 ± 144	8	8	1093 ± 308	472 ± 69	73	70
Phaseolinone (30 μg/ml)	906 ± 207	431 ± 50	82	80	3421 ± 318	1287 ± 117	14	18

[a] Mean ± S.D. for five sets with duplicate in each set.
[b] Inhibition was calculated on the values of means only.

rated and each of them acts independently. Since α-amanitin specifically inhibits RNA polymerase II (4) and does not interact with DNA, phaseo linone would possibly interact with the component (s) of RNA polymerase other than polymerase II. But its interaction with DNA template is not ruled out in these experiments.



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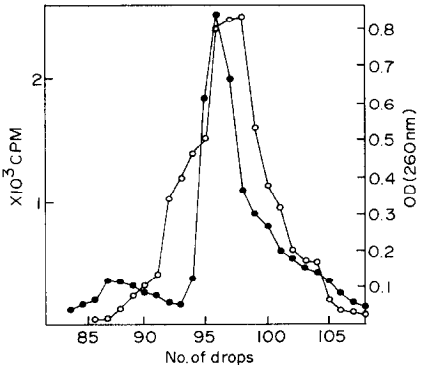

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Fig 3 : Ellipticity of calf thymus DNA in presence (curve 2) and absence (curve 1) of phaseolinone. [Since 1-2 μg/ml phaseolinone did not show any strong CD bands hence 1 μg/ml phaseolinone was used].

Fig 4 : Alkaline sucrose density gradient centrifugation of DNA, with (-●-) and without (-O-) phaseolinone treatment. To 0.25 ml of DNA (5 μg) solution 0.1 ml of water or phaseolinone (100 μg) solution was mixed and kept for centrifugation.

RNA polymerase activity in nucleoplasmic fraction is primarily due to RNA polymerase II (15) and is inhibited by greater than 73% in presence of ∝-amanitin (Table 2) while nucleolar fraction having RNA polymerase I as the major component is resistant to it (only 8% inhibition of RNA synthesis is observed in presence of ∝-amanitin, Table 2). But phaseolinone inhibits greater than 80% of the RNA polymerase activity present in the nucleolar fraction. A small portion of RNA polymerase activity in nucleoplasmic fraction shows sensitive to phaseolinone possibly due to cross-contamination with RNA polymerase II. In a similar way sensitivity of ∝-amanitin to a minor portion of the polymerase activity in nucleolar fraction could be argued. Both these two nuclear fractions in these in vitro systems were assayed with an equal amount of identically treated exogeneous DNA template. Hence if there be any interaction between the DNA template and phaseolinone, it would result an equal inhibition of RNA synthesis. Furthermore, the results of C.D. spectra of DNA and phaseolinone treated DNA shown in Fig 3 indicated the lack of interaction between these two since there was no difference in their spectral patterns. Similarly sucrose density gradient centrifugation of DNA and DNA-phaseolinone samples (Fig.4) clearly showed that there was no change in their molecular sizes. Therefore, it further strengthened the support that phaseolinone is not involved in any interaction with DNA. The dose of ∝-amanitin as used in this assay was not sufficient for inhibition of RNA polymerase II and the contents of RNA polymerase III and I in the isolated rat liver nuclei were reported to be 11% and 71% of the total RNA polymerase activity respectively (4). In mouse the fraction of RNA polymerase activity resistant to 3 µg per ml ∝-amanitin is due to a mixture of about 90% RNA polymerase I and 10% RNA polymerase III (16). Therefore the proportion of RNA polymerase III in the nucleolar fraction would be expected to be very low and 80% inhibition in presence of phaseolinone would account mostly for the RNA polymerase(s) which is not at all sensitive to ∝-amanitin. So the inhibition of RNA synthesis in presence of phaseolinone is primary due to the inhibition of RNA polymerase I.

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