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Biochemical Pharmacology, Vol. 30, No. 14, pp. 2026–2029, 1981.
Printed in Great Britain.

0006-2952/81/142026-04 $02.00/0

Inhibition of ribonucleic acid polymerase activity by ellipticine*

(*Received* 12 *August* 1980; *accepted* 13 *January* 1981)

Ellipticine (5,11-dimethyl-6H-pyrido-(4,3-b)-carbazole), isolated as a plant alkaloid from *Ochrosia* species [1], possesses antitumor activity in experimental animals [2]. Ellipticine binds to DNA by intercalation [3], and inhibits cellular DNA and RNA synthesis more efficiently than protein synthesis [4–6]. It kills cultured cells in all phases of the cell cycle [4], and causes chromosome damage of Chinese hamster ovary cells at a drug concentration of 1–4 μg/ml [4]. On interaction of ellipticine with poly d(A-T)·poly d(A-T), changes in the absorption spectra and the melting temperature have been observed in preliminary experiments [4]; with poly d(G-C)·poly d(G-C) no changes were observed, suggesting that there may be a preferential interaction of ellipticine with A:T base pairs of the nucleic acid [4]. This question has been explored in the present study by using an *in vitro* system of highly purified RNA polymerase.

Tritium-labeled nucleotides were obtained from Schwarz/Mann, Orangeburg, NY. Unlabeled nucleotides were purchased from the Worthington Biochemical Corp., Freehold, NJ, or from P-L Biochemicals, Milwaukee, WI. Calf thymus DNA was obtained from Worthington. Poly d(A-T)·poly d(A-T) and poly d(G-C)·poly d(G-C) were obtained from P-L Biochemicals. These templates were dissolved in 0.01 M Tris–HCl (pH 7.0) buffer containing 0.1 mM EDTA and 0.1 M NaCl. Twenty optical density units at 260 nm were taken to be equal to 1.0 mg template/ml. Creatine kinase and phosphocreatinine were obtained from Boehringer Biochemicals, Mannheim, Germany, and crystalline bovine serum albumin from Miles Laboratories, Elkhart, IN. Polymin P was a gift from the BASF-Wyandotte Corp., Parsippany, NJ. Frozen *Escherichia coli*, RNase-minus strain, MRE 600 cells, grown up to 3/4 log in enriched medium, were obtained from the Grain Processing Co., Muscatine, IA. DNA-cellulose was prepared according to the method of Alberts and Herrick [7]. Biogel A 1.5 m was obtained from Bio-Rad Laboratories, Richmond, CA. RNA polymerase activity was purified according to Burgess and Jendrisak [8]. The highly purified enzyme preparation was stored in small aliquots in 50% glycerol at −20°. The protein content of the enzyme preparation was determined by the method of Lowry *et al.* [9], using crystalline bovine serum albumin as a standard. Ellipticine (NSC 71795), daunomycin·HCl (NSC 82151), adriamycin·HCl (NSC 123127), and dactinomycin (NSC 3053) were obtained from Dr. John Douros, Developmental Therapeutics Program, Division of Cancer Treatment, NCI, Bethesda, MD. Stock solutions of these drugs were freshly made in 90% dimethylsulfoxide (DMSO) solution.

The highly purified RNA polymerase preparation had a 280:260 nm absorption ratio of 1.50. In a 7.5% polyacrylamide gel in 0.1% sodium dodecylsulfate, the enzyme preparation gave three major bands corresponding to $\beta\beta'$, σ and α subunits of the enzyme [8]. One unit of enzyme

* Supported by a Center grant (CA-12197) from the National Cancer Institute, Department of Health, Education and Welfare, and the Forsyth Cancer Society. A portion of this work has been reported previously in abstract form [V. S. Sethi, *Proc. Am. Ass. Cancer Res.* **20**, 77 (1979)].

activity was defined as that which incorporated 1 pmole of [^{3}H]UMP or [^{3}H]GMP into RNA at 37° in 10 min. The V_{max} values of the RNA polymerase preparation for calf thymus DNA, poly d(A-T)·poly d(A-T), and poly d(G-C)·poly d(G-C) were, respectively, 5.5×10^5 units, 8.7×10^5 units and 2.85×10^4 units per mg of enzyme protein. In the presence of 4.5% DMSO, the enzyme activity with poly d(A-T)·poly d(A-T) was inhibited slightly (4–8 per cent), and with poly d(G-C)·poly d(G-C) it was stimulated slightly (5–10 per cent). With calf thymus DNA the enzyme activity was not affected significantly with 4.5% DMSO. These results are in agreement with the effects of DMSO on RNA synthesis [10–12].

Inhibition of RNA polymerase activity at increasing concentrations of ellipticine, with poly d(A-T)·poly d(A-T) as template, when expressed according to Dixon [13] gave linear equations at increasing template concentrations (Fig. 1A). The intersection points of these lines gave $\bar{x} = -38.43 \pm 3.00$ and $\bar{y} = +0.05 \pm 0.04$. These data were consistent with a noncompetitive type of inhibition with an apparent K_i of 38.4 μM ellipticine. When poly d(G-C)·poly d(G-C) was used as template, linear regression lines at increasing template concentrations (3.3 to 16.5 μM) were obtained (Fig. 1B). Intersection of these lines with the axes did not meet around a point but had a much wider scatter with $\bar{x} = 53.99 \pm 39.41$ and $\bar{y} = +6.0 \pm 4.80$. In this plot, concave inhibition curves were noticed especially at 6.6 μM and higher template concentrations. Interpretation and significance of these results are not clear. With calf thymus DNA, linear regression lines at increasing template concentrations (3.3 to 16.5 μM) were obtained (Fig. 1C). The average intersection point of these lines was $\bar{x} = -77.02 \pm 24.93$ and $\bar{y} = 2.49 \pm 1.48$, suggesting an apparent K_i of 77.0 ± 24.9 μM. In some of the inhibition curves in this plot, slight concave curvature could also be detected which could be due to the contribution of the G-C component in the DNA.

Inhibition of RNA polymerase activity also occurred with dactinomycin, adriamycin and daunomycin. Concentrations of the drugs needed to inhibit enzyme activity fifty per cent (IC_{50}) were determined, and the molecular ratios of the drug to the nucleotide base pairs at IC_{50} were calculated (Table 1). Dactinomycin did not show inhibition with poly d(A-T)·poly d(A-T) but it was most inhibitory with poly d(G-C)·poly d(G-C). These results indicated a much higher affinity of the drug for the latter template, and they were consistent with published reports [14–16]. Adriamycin and daunomycin showed an equal inhibition for the synthetic polynucleotides. With calf thymus DNA, ratios of the drug to the nucleotide base pairs at 50% RNA polymerase inhibition level were 1:128, 1:5 and 6:1, respectively, for dactinomycin, adriamycin and ellipticine, suggesting that ellipticine is about 30-fold weaker as an inhibitor than adriamycin, and 768-fold weaker as an inhibitor than dactinomycin. Data from Table 1 further suggest that ellipticine showed inhibition of the enzyme activity that was higher for the A-T template than for the G-C.

DNA reactive antitumor agents have been classified into two groups [17], namely those that cause single-strand breaks in DNA, such as bleomycin and neocarzinostatin, and those that bind to DNA by ionic bonds and/or by intercalation. To the latter group belong dactinomycin, adriamycin, daunomycin, anthramycin, acridinylaniside, and ellipticine. All these agents ultimately interfere with DNA and RNA synthesis [17, 18]. It is not known at what stage of RNA synthesis [19]—namely binding of the enzyme to the template or initiation or elongation of the RNA chain—these intercalators interfere. Some DNA intercalators, such as adriamycin, dactinomycin and ellipticine, have also been shown recently to induce single-strand breaks in DNA [20]. In a comparative study of the effects of antitumor agents on RNA synthesis of L1210 murine leukemic cells, it was noted that ellipticine selectively inhibited nucleolar RNA synthesis and its processing [5, 21]. Under our experimental conditions of pH 8.0 and 200 mM salt, where ionic interactions are presumably minimal, it seems that ellipticine has a higher affinity for the A-T base pair template than for the C-G base pair polynucleotide. The apparent drug inhibition constant for poly d(A-T)·poly d(A-T) was 38.4 μM. Ellipticine concentrations needed to inhibit 50 per cent enzyme activity were 3- to 4-fold lower for the alternating A-T template than the G-C template. It is interesting to note that the ellipticine concentrations (1–4 μg/ml) needed for murine leukemia cell kill activity [4], inhibition of RNA or DNA synthesis in these cultured cells [4], or cell detachment from a glass surface at 16.6 μg/ml [4], are in the same drug concentration range (7–12 μg/ml) for the inhibition of RNA synthesis with poly d(A-T)·poly d(A-T) and calf thymus DNA. Furthermore, inhibition of adenine methylation in tRNA by methylases with ellipticine takes place at a base pair:drug ratio of 1:3–5 [22] which is quite comparable to our data. Whether these *in vitro* properties of ellipticine are related to the antitumor and other biological properties [4, 5, 23] needs further exploration.

Table 1. Concentrations of drugs (μM) needed for fifty per cent inhibition of RNA polymerase activity (IC_{50}) with different templates, and the molecular ratios of drug:nucleotide base pair at IC_{50}*

Drugs	IC_{50} (μM)		
	Calf thymus DNA	Poly d(A-T)·poly d(A-T)	Poly d(G-C)·poly d(G-C)
Ellipticine	50 (6:1)†	35 (4:1)†	120 (14:1)†
Dactinomycin	0.13 (1:128)	‡	0.58 (1:28)
Adriamycin	3.0 (1:5)	6.0 (1:3)	6.0 (1:3)
Daunomycin	4.0 (1:4)	8.0 (1:2)	9.0 (1:2)

* Enzyme reaction conditions were the same as described in the legend of Fig. 1. For dactinomycin, adriamycin and daunomycin, 1.0 μg template per assay was used. The control values (100 per cent) for calf thymus DNA, poly d(A-T)·poly d(A-T), and poly d(G-C)·poly d(G-C) were 705, 3875, and 219 pmoles [^{3}H]XMP incorporated per assay respectively. The inhibition data were plotted on a log-logit scale and the IC_{50} values were determined.

† Values in parentheses are the molecular ratios of drug:nucleotide base pairs at IC_{50}. In these calculations, average molecular weights of the nucleotide base-pair for calf thymus DNA, poly d(A-T)·poly d(A-T), and poly d(G-C)·poly d(G-C) were taken to be 598, 589, and 606 respectively. The molecular weights for dactinomycin, adriamycin, daunomycin, and ellipticine are 1255, 543, 527, and 246 respectively.

‡ No inhibition of the enzyme activity was obtained.

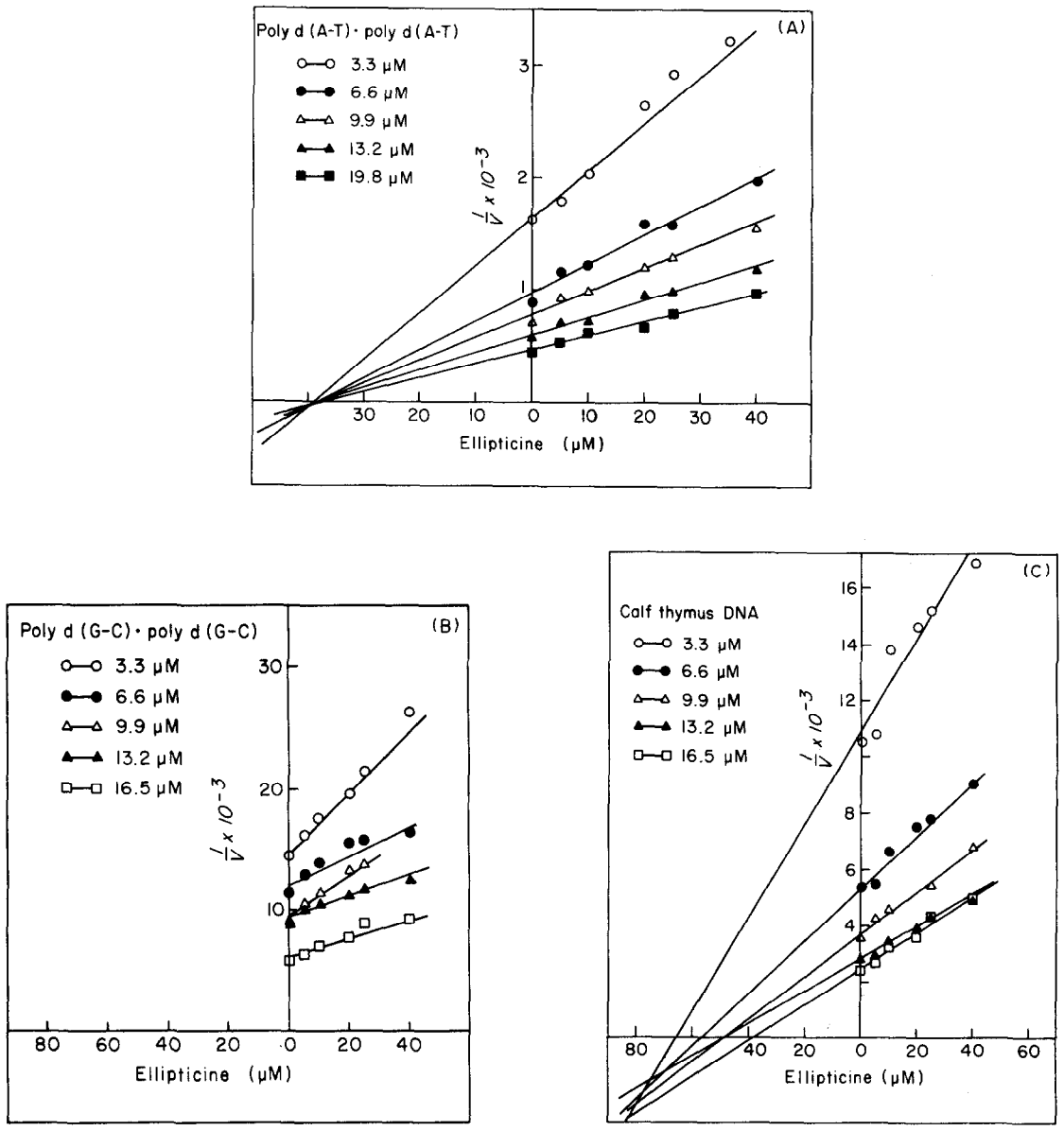

Fig. 1. Dixon plots of inhibition of RNA polymerase activity by ellipticine at increasing concentrations of poly d(A-T)·poly d(A-T) (A), poly d(G-C)·poly d(G-C) (B), and calf thymus DNA (C). The enzyme assay mixtures of 100 μl contained 5 μmoles Tris–HCl (pH 8.0), 0.2 μmole $MnCl_2$, 0.1 μmole $MgCl_2$, 0.4 μmole phosphocreatinine, 5 μg phosphocreatin kinase, 0.1 μmole NaF, 20 μmoles KCl, 0.4 μmole dithiothreitol, 5% (v/v) glycerol, 5 μg RNA polymerase, 25 nmoles each of unlabeled nucleotides, the appropriate amount of labeled nucleotide in increasing amounts of 3.3 μM (○—○), 6.6 μM (●—●), 9.9 μM (△—△), 13.2 μM (▲—▲), 16.5 μM (□—□) or 19.8 μM (■—■) template, and various amounts of ellipticine (0–40 μM). All experiments including control (without ellipticine) had 4.5% DMSO). For calf thymus DNA, ATP, CTP, GTP, and 23.5 nmoles [^{3}H]UTP (10 cpm/pmole) were used in the assay mixtures; for poly (d(A-T)·poly d(A-T), 25 nmoles ATP and 23.5 nmoles [^{3}H]UTP were used; and for poly d(G-C)·poly d(G-C), 25 nmoles CTP and 8 nmoles [^{3}H]GTP (47 cpm/pmole) were used. The enzyme assays were incubated for 10 min at 37°. The reactions were stopped by chilling in ice-water, and 25 μl of 0.1 M EDTA-disodium salt, pH 7.0, was added to each tube and mixed well on a Vortex mixer. Seventy-five microliters of each reaction mixture was spotted uniformly onto a 2.5 cm circular DE-81 (Whatman) filter disc, kept at room temperature for 15 min, and washed batchwise by swirling four times in 10 ml of a 5% (w/v) solution of $Na_2HPO_4 \cdot 7H_2O$ per filter, followed by two washings each of water and 95% ethanol. The filters were dried under a heat lamp for 15 min, and the radioactivity on each filter was counted in 6 ml of toluene–1,4-bis-[2-(4-methyl-5-phenyl-oxazolyl)]benzene (POPOP)–2,5-diphenyloxazole (PPO) scintillation fluid. Plots of ellipticine concentration versus the reciprocal of the enzyme activity $1/v$, were drawn. With the help of a computer program (Texas Instruments, Ti 58C), linear regression analysis of each set of data points was performed, and the points of intersection of the lines with the axes were determined.

In conclusion, ellipticine inhibited RNA polymerase activity by interacting preferentially with the A : T base pair template as compared to the G : C template. Ellipticine was considerably less effective than adriamycin, and far less so than dactinomycin, in the inhibition of RNA synthesis.

Acknowledgements—I am most grateful to Dr. Charles L. Spurr for his encouragement and continued support. The excellent technical assistance of Janice Whitaker is greatly appreciated.

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