

Effect of echinomycin on DNA methylation

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Although echinomycin is reported to intercalate and to bind to DNA at CG dinucleotides, the effects of the drug on DNA methylation in vitro and in vivo are much less apparent than are the effects on DNA synthesis and cell growth.

DNA methylation; DNA methylase; Echinomycin

1. INTRODUCTION

Echinomycin is a member of the quinoxaline group of antibiotics which has been shown to bind tightly to DNA, especially in GC-rich regions [1]. Detailed kinetic and DNase footprinting studies suggest that the antibiotic acts as a bifunctional intercalating agent showing high specificity for the symmetrical CG dinucleotide [2,3]. The intercalation reaction involves an altered helix conformation and is inhibited by monovalent cation concentrations which interfere with the breathing of native DNA [2,4,5]. No interaction is observed with single-stranded DNA [5].

The above characteristics are reminiscent of the conditions required for interaction of eukaryotic DNA methylase with native DNA [6,7] and we therefore have investigated whether the reported effects of echinomycin on the growth of tumours might be related to an inhibition of DNA methylation by the drug.

2. MATERIALS AND METHODS

DNA methylase purified from Krebs II ascites tumour cells [8] was incubated with a limiting

amount of native *M. luteus* DNA in a low salt buffer. The DNA was preincubated with echinomycin in the methylase assay buffer containing 35% methanol and the assays were performed in the presence of 20% methanol. The echinomycin was a generous gift of Drs Peter and Scheibi of Ciba-Geigy AG, Basel.

DNA synthesis and methylation in vivo were assayed by incubating mouse L929 cells with [6-³H]uridine (10 μ Ci/ml) for 6 h. The DNA was then purified and the bases separated on a column of Aminex A6 (BioRad) [9]. The radioactivity in DNA cytosine and methylcytosine is a measure of the rate of DNA synthesis (similar results were obtained following incubation with tritiated thymidine) and the level of DNA methylation is expressed as cpm in methylcytosine $\times$ 100/cpm in cytosine and methylcytosine.

3. RESULTS

Low et al. [2] report that protection of DNA from DNase I action was obtained with echinomycin concentrations of between 2 μ M and 8 μ M and DNA becomes saturated with echinomycin when there is one drug molecule for every five base pairs of DNA [5] though this must be dependent on DNA base composition.

We, therefore, preincubated 1 μ g *Micrococcus luteus* or other DNA with the drug at various con-

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centrations up to $3.5 \mu\text{M}$ which would supply 1.8 echinomycin molecules for every CG dinucleotide pair, i.e. twice the number required to saturate the DNA. The DNA was then used as a substrate in a DNA methylase assay. At DNA/drug ratios of 20–40 bp/drug there is a 35% stimulation of methylase activity perhaps resulting from a loosening of the tight double helical structure of the DNA, but as this ratio falls to 6 the enzyme reaction is inhibited to about 40% of the maximum value (fig.1).

Could such an inhibition of DNA methylation account for the reported effect of the drug on cell growth? Fig.2 shows the effect of different drug concentrations on the growth of mouse L929 cells and on DNA synthesis in these cells. 50% inhibition is achieved with 10–25 nM echinomycin, i.e. 60 pmol per dish. The control dishes contained 10^6 cells, i.e. about $10 \mu\text{g}$ DNA which, because of the low GC content and the CG dinucleotide deficiency [10] would contain about 120 pmol CG

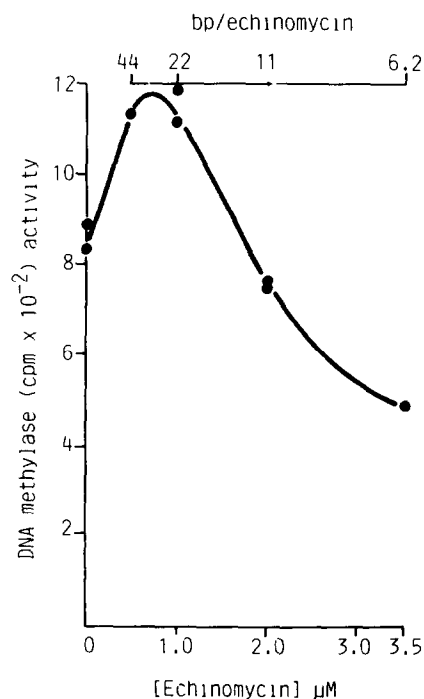


Fig.1. Effect of echinomycin on DNA methylase activity. $1 \mu\text{g}$ native *M. luteus* DNA was incubated with echinomycin at 37°C for 20 min in $40 \mu\text{l}$ and then used as a substrate for a partially purified DNA methylase. The concentration of echinomycin is that present in the assay (volume $70 \mu\text{l}$) in which the DNA is limiting.

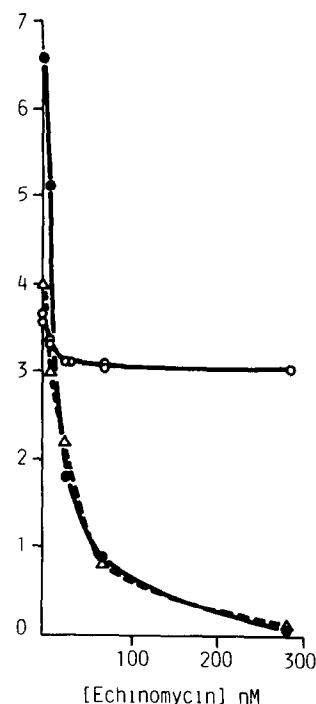


Fig.2. Effect of echinomycin on DNA synthesis and methylation in vivo and on cell growth. Mouse L929 cells were cultured in the presence of the indicated concentrations of echinomycin. DNA synthesis and methylation were assayed as described in section 2 after incubation with $[6\text{-}^3\text{H}]\text{uridine}$ from 16–22 h after drug addition. The level of DNA methylation (○) is expressed as cpm in methylcytosine $\times 100/\text{cpm}$ in cytosine + methylcytosine. The rate of DNA synthesis (●) is the cpm incorporated into cytosine plus methylcytosine $\times 10^{-3}$. (Similar results for inhibition of DNA synthesis were obtained by following incubation with $[^3\text{H}]\text{thymidine}$.) Cell number was measured 48 h after drug addition and the results are expressed as fold increase in cell number (Δ).

dinucleotide pairs. Thus 50% inhibition of DNA synthesis and cell growth is obtained at a CG pair/drug ratio of about 2 compared to 0.8 which is required to inhibit in vitro DNA methylation to a similar extent. The drug was always administered in a constant volume of 35% methanol ($56 \mu\text{l}$ per ml culture medium) and this by itself inhibits DNA synthesis by 50%. All controls were therefore treated with methanol. Investigation of the effect of echinomycin on in vivo methylation of DNA shows that the DNA made 16–22 h after drug administration is only slightly less methylated than

control (fig.2). Thus while 280 nM echinomycin inhibits incorporation of [6-³H]uridine into DNA-cytosine + methylcytosine by 99%, the DNA which is produced has 3.0% of its cytosine residues methylated compared with 3.6% for control DNA.

4. DISCUSSION

In an assay for DNA methylase activity in which the rate limiting DNA was preincubated with echinomycin, 50% inhibition of maximal activity was obtained at a drug concentration of 2.5 μ M. This represents a base pair per drug ratio of 8.3 which, because of the high CG dinucleotide content of the *M. luteus* DNA used represents 8.3 base pairs or about 0.8 CG pairs per drug molecule. In this de novo methylase reaction both cytosines in the CG dinucleotide pair are available for methylation yet when these are fully complexed with echinomycin only 50% inhibition of methylation occurs.

In vivo 12 nM echinomycin causes 50% inhibition of DNA synthesis and growth of cultured mouse L929 cells. This represents 250 base pairs per drug molecule but because of the CG dinucleotide deficiency of mouse DNA, this represents 2 CG pairs per drug molecule. Thus the two DNA strands may be cross linked only every 250 base pairs but every other CG dinucleotide pair is associated with a molecule of echinomycin. Despite this the effect on in vivo methylation is very slight. In this case the methylation occurring in mouse L929 cells is maintenance methylation but it shows even less sensitivity than the de novo methylase activity assayed in vitro.

It is possible that the DNA methylation observed in drug-treated cells is occurring in a small fraction of cells not affected by the drug. However, autoradiography of [³H]thymidine-labelled cells shows that the percentage of cells making DNA only falls from $32.1 \pm 4.7\%$ in the absence of echinomycin to $17.2 \pm 3.0\%$ in the presence of 70 nM echinomycin. In the control, 62% of the labelled cells had over 100 grains per cell whereas this figure was only 22% in cells treated with 70 nM echinomycin. No labelled cells were detected in samples treated with 280 nM echinomycin. This shows that no resistant cells are present and that the rate of DNA synthesis is reduced to a similar extent in all cells.

The failure of echinomycin to inhibit DNA methylation while strongly inhibiting DNA and RNA synthesis may imply that methylation does not involve the transient separation of the base pairs. The bifunctional intercalating agent has two quinoxaline rings held rigidly parallel and 1 nm apart by the perpendicular peptide disc [1]. When the quinoxaline rings intercalate into native DNA they sandwich 2 base pairs and extend the DNA chain by about 0.7 nm per drug molecule bound. The peptide cross bridge gives specificity by intercalating with base pair substituents in the narrow groove of the helix, e.g. the 2-amino group of guanine [1]. In this respect the drug may not block access to the major groove and may not seriously affect the conformation of a sandwiched CG dinucleotide pair.

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REFERENCES

- [1] Gale, E.F., Cundliffe, E., Reynolds, P.E., Richmond, M.H. and Waring, M.J. (1981) in: *The Molecular Basis of Antibiotic Action*, 2nd edn, pp.333–337, John Wiley, London.
- [2] Low, C.M.L., Drew, H.R. and Waring, M.J. (1984) *Nucleic Acids Res.* 12, 4865–4879.
- [3] Fox, K.R. and Waring, M.J. (1984) *Nucleic Acids Res.* 12, 9271–9285.
- [4] Fox, K.R., Wakelin, L.P.G. and Waring, M.J. (1981) *Biochemistry* 20, 5768–5779.
- [5] Wakelin, L.P.G. and Waring, M.J. (1976) *Biochem. J.* 157, 721–740.
- [6] Turnbull, J.F. and Adams, R.L.P. (1976) *Nucleic Acids Res.* 3, 677–695.
- [7] Adams, R.L.P., McKay, E.L., Craig, L.M. and Burdon, R.H. (1979) *Biochim. Biophys. Acta* 561, 345–357.
- [8] Adams, R.L.P., Gardiner, K., Rinaldi, A., Bryans, M., McGarvey, M. and Burdon, R.H. (1986) *Biochim. Biophys. Acta* 868, 9–16.
- [9] Adams, R.L.P., McKay, E.L., Craig, L.M. and Burdon, R.H. (1979) *Biochim. Biophys. Acta* 565, 72–81.
- [10] Swartz, M.N., Trautner, T.A. and Kornberg, A. (1962) *J. Biol. Chem.* 237, 1961–1967.