and ondansetron (10 μM) were routinely added to the Krebs solution. In addition, the 5-HT_2- and 5-HT_3-receptor selective agonists, α-methyl-5-HT and 2-methyl-5-HT exhibited little or no agonist activity under the conditions of the present experiments.

The observation that 5-CT was a full agonist, but more potent than 5-HT, is consistent with the contention that the response was mediated by 5-HT_1-like receptors (Bradley et al 1986), and this conclusion is corroborated by the observation that the responses to both 5-HT and 5-CT were blocked by methysergide and metergoline. The pK_B values calculated for these compounds against 5-HT as the agonist (8·0 and 7·3, respectively) do not match precisely with the published data for any 5-HT_1 binding site (Hoyer 1989) or with functional 5-HT_1-like receptors in dog saphenous vein (Apperley et al 1980; Humphrey et al 1988) and piglet vena cava (Sumner et al 1989). However, there are some similarities between the results in the present study and the findings of Kalkman et al (1986) in guinea-pig ileum. In the latter study Kalkman et al (1986) identified a 5-HT receptor-type mediating smooth muscle relaxation through a direct non-neuronal mechanism. 5-CT was a full agonist with an EPMR of 0·18 and metergoline and methysergide were antagonists with estimated affinity constants of 7·9 and approximately 8 respectively, values close to those obtained in the present study.

The relaxant responses to 5-methoxytryptamine and sumatriptan, with maximum responses significantly greater than those produced by 5-HT and 5-CT are difficult to interpret. Indeed, these responses were insensitive to high concentrations of methysergide (10 μM), suggesting a separate mechanism of action.

The antagonist data obtained in the present study does not agree well with published data (Costa & Furness 1979; Kojima & Shimo 1986). In the later studies methysergide (up to 2 μM) was without effect, whereas, in the present work, methysergide (0·1-1 μM) clearly antagonised the responses to 5-HT and 5-CT. The reason for this discrepancy is difficult to explain, although the different conditions used may provide some explanations; whereas in this study, relaxations were investigated using preparations with elevated tone, both of the other studies (see above) looked at relaxations of basal tone.

In summary, the present study has identified a neuronal receptor in guinea-pig proximal colon which mediates relaxation mediated by 5-HT, and which has been provisionally characterized as a 5-HT_1-like receptor. This receptor exhibits properties similar to that identified by Kalkman et al (1986) in guinea-pig ileum.

References

Apperley, E., Feniuk, W., Humphrey, P. P. A., Levy, G. P. (1980) Evidence for two types of excitatory receptor for 5-hydroxytryptamine in dog isolated vasculature. Br. J. Pharmacol. 68: 215-224
Bradley, P. B., Engel, G., Feniuk, W., Fozard, J. R., Humphrey, P. P. A., Middlemiss, D. N., Mylecharane, E. J., Richardson, B. P., Saxena, P. R. (1986) Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. Neuropharmacology 25: 563-576
Costa, M., Furness, J. B. (1979) The sites of action of 5-hydroxytryptamine in nerve-muscle preparations from the guinea-pig small intestine and colon. Br. J. Pharmacol. 65: 237-248
Hoyer, D. (1989) Receptor and effector coupling mechanisms. In: Fozard, J. R. (ed.) Peripheral Actions of 5-Hydroxytryptamine. Oxford University Press, UK, 72-99
Humphrey, P. P. A., Feniuk, W., Perren, M. J., Connor, H. E., Oxford, A. W., Coates, I. H., Butina, D. (1988) GR43175, a selective agonist for the 5-HT_1-like receptor in dog isolated saphenous vein. Br. J. Pharmacol. 94: 1123-1132
Kalkman, H. O., Engel, G., Hoyer, D. (1986) Inhibition of 5-carboxamidotryptamine-induced relaxation of guinea-pig ileum correlates with [^{125}I] LSD binding. Eur. J. Pharmacol. 129: 139-145
Kojima, S., Shimo, Y. (1986) The sites of action of 5-hydroxytryptamine in the longitudinal muscle of the guinea-pig proximal colon. Asia Pacific Pharmacol. 1: 111-116
Sumner, M. J., Feniuk, W., Humphrey, P. P. A. (1986) Further characterisation of the 5-HT receptor mediating vascular relaxation and elevation of cyclic AMP in porcine isolated vena cava. Br. J. Pharmacol. 97: 292-300

J. Pharm. Pharmacol. 1992, 44: 266-268
Communicated July 19, 1991

© 1992 J. Pharm. Pharmacol.

Increase in susceptibility to *Eco*RII restriction of bacteriophage λ produced by propagation on host cells growing in 5-azacytidine: a new in-vivo method for demonstration of DNA-methylation inhibition

L. RADNEDGE*, R. J. PINNEY, *Microbiology Section, Department of Pharmaceutics, The School of Pharmacy, University of London, Brunswick Square, London WC1N 1AX, UK*

Abstract—The efficiency of plating on *Eco*RII-restricting cells of bacteriophage λ_{vir} propagated on an *Escherichia coli* K-12 *dcm*$^+$ host decreased with increase in concentration of 5-azacytidine (5-azaC) in the propagating medium. This illustrates, in-vivo, the inhibition of DNA-cytosine methylation induced by 5-azaC and provides a simple system for the detection of DNA-methylation inhibitors.

* Present address: Laboratory of Chromosome Biology, NCI-Frederick Cancer Research and Development Center, PO Box B, Frederick, Maryland, MD 21702, USA.

Correspondence: R. J. Pinney, Microbiology Section, Department of Pharmaceutics, The School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, UK.

5-Azacytidine (5-azaC) is incorporated into nucleic acids of both eukaryotic and prokaryotic cells, producing defective rRNAs and tRNAs, and inhibiting protein synthesis (Cedar & Razin 1990). Uptake into DNA inhibits DNA synthesis and blocks DNA-cytosine methylation (Friedman 1981) by noncompetitive inhibition of DNA methyl transferases (Santi et al 1983, 1984). The chromosomal *dcm* gene of *Escherichia coli* codes for a DNA cytosine methylase that methylates, postreplicationally, the internal cytosine in the sequence 5′-CC(A/T)GG-3′ (May & Hattman 1975a, b). To date no phenotypic abnormality has been attributed to mutation within the *dcm* gene. However, 5-methylcytosine deaminates spontaneously to thymine, which upon replication introduces T. G mispairs into DNA. These are

corrected by very short patch (VSP) repair, but contrary to previous reports (Lieb 1987; Zell & Fritz 1987), *dcm* plays no role in VSP repair. Instead, another gene *vsr*, which is adjacent to *dcm* and transcribed from a common promoter, is involved (Sohail et al 1990). Thus, Vsr protein is made only when the Dcm methylase is produced, which is advantageous because VSP repair is required only when cellular DNA is methylated.

To initiate investigation of a possible role for cytosine methylation in DNA repair pathways other than VSP, we attempted to co-transduce a *dcm* allele with his^+ into various DNA repair-deficient strains of *E. coli* using the generalized transducing bacteriophage (phage) $P1_{vir}$. This was unsuccessful (Radnedge 1988), and we have therefore investigated whether 5-azaC-inhibition of cytosine-5-methyl transferase could be utilized to induce dcm^+ repair-deficient strains to become phenotypically Dcm^-.

The DNA sequence recognized and methylated by Dcm protein is identical with the recognition sequence of the *Eco*RII restriction-modification system (Schlagman & Hattman 1974) carried on N group bacterial plasmids (Birks & Pinney 1975). Phage propagated on dcm^+ hosts are therefore modified according to the *Eco*RII pattern and protected against *Eco*RII restriction. We now show that growing phage on a dcm^+ host in the presence of sub-inhibitory concentrations of 5-azaC inhibits methylation of DNA-cytosine, thereby increasing the susceptibility of the phage to *Eco*RII restriction by a factor of up to 100-fold.

We have previously described an in-vitro system for the investigation of inhibitors of DNA methyltransferases and suggested that such inhibition could form a basis for the design of new antibacterial agents (Crooks et al 1984). The system we now describe provides an in-vivo test for similar compounds.

Materials and methods

Bacterial strains, phages and plasmids. Bacterial strains were all derivatives of *Escherichia coli* K-12. Strains AB1157 *his* dcm^+ (Bachmann 1972) and LR68, a his^+ *dcm* derivative of AB1157 are both fully DNA repair-proficient. Strain LR68 was made by generalized transduction of AB1157 with a phage $P1_{vir}$ lysate of *E. coli* strain 1100 his^+ *dcm* (Hattman et al 1973), selecting for his^+ transductants and testing these for absence of cytosine methylase activity as described by Radnedge & Pinney (1991). Phages $P1_{vir}$ and λ_{vir} were from our laboratory collection. Plasmid RN3 *Eco*RII Res^+ Mod^+ and a Res^- Mod^+ derivative were as described by Schlagman & Hattman (1974).

Growth of E. coli *in 5-azaC.* Fully-supplemented Davis and Mingioli's medium (DM) (Davis & Mingioli 1950), 4·5 mL, was inoculated with a loopful of cells obtained by sweeping through the region of semi-confluent growth on a fresh fully-supplemented DM plate-culture of the organism and vortexed thoroughly. A sample of the resulting uniform suspension, 0·5 mL, was inoculated into 4 × 4·5 mL quantities of fully-supplemented DM medium, which were incubated overnight at 37°C with shaking. Each resulting 5 mL culture was then added to 95 mL fully-supplemented DM medium in a 250 mL conical flask at 37°C. Samples (3 mL) were immediately removed from each of the 4 flasks and their viable counts determined by diluting in Oxoid nutrient broth No. 2 (code CM67) and spread-plating on Oxoid Blood Agar Base (code CM55). The flasks were incubated at 37°C on an orbital shaker. Further samples were counted at hourly intervals. After 3 h, when cells were growing logarithmically, sufficient 5-azacytidine (Sigma) in aqueous solution was added to give the required final concentrations. Viable counts were continued at hourly intervals.

Propagation of λ_{vir} in the presence of 5-azaC. A 0·1 mL sample of a λ_{vir} phage preparation (5×10^4 plaque-forming units mL^{-1}) and 0·1 mL of an overnight culture of *E. coli* AB1157 dcm^+ (5×10^8 cells mL^{-1}), grown in DM were placed in a 3 mL molten DM base soft agar overlay held at 45°C. The overlay was mixed gently and poured onto a fully-supplemented DM agar plate. This procedure was repeated with 5-azaC in both overlays and plates at concentrations of 0·1, 1·0 or 10·0 mg L^{-1}. The plates were incubated at 37°C for 48 h and the phage harvested by scraping the overlays from the plates into 4·5 mL DM base. Phage were liberated from the agar by vortexing the suspension with 4 mm diameter glass beads, and the agar and bacteria separated by centrifugation at 4000 rev min^{-1} for 20 min. Any residential bacterial contamination in the supernatant was removed by membrane filtration.

Measurement of efficiency of plating of phage λ_{vir}. Plasmid RN3 codes for the *Eco*RII restriction endonuclease. Restriction was determined by titrating phage on strain LR68 *dcm* carrying the *Eco*RII Res^+ Mod^+ wild-type RN3 plasmid and on the same strain carrying a non-restricting Res^- Mod^+ derivative of RN3. The level of phage restriction was calculated by comparing the titres of the phage suspension on the two indicator strains, the ratio of the titres on the Res^+ and the Res^- strains giving the efficiency of plating (EOP) of the phage.

Results

Growth rate inhibition of E. coli *AB1157 produced by 5-azaC.* Addition of 0·1 mg L^{-1} 5-azaC to fully-supplemented DM medium caused a slight decrease in growth rate of *E. coli* AB1157 dcm^+ (Fig. 1), whereas 1·0 and 10 mg L^{-1} 5-azaC significantly reduced the rate of cell division but were not completely inhibitory (Fig. 1). Higher concentrations were lethal (results not shown). Since protein synthesis and cell growth are necessary for phage propagation, concentrations of 0·1, 1·0 and 10·0 mg L^{-1} were therefore suitable for the demonstration of the effect of 5-azaC on the plating efficiency of phage on *Eco*RII-restricting host cells.

Reduction in EOP of phage λ_{vir} after propagation in the presence of 5-azaC. Although both the *Eco*RII modification methylase and

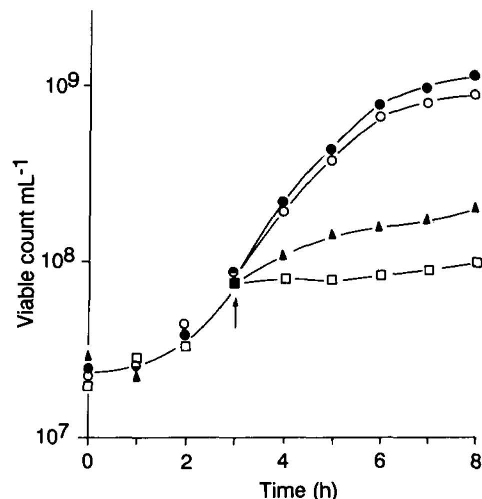

FIG. 1. Inhibition of growth in Davis and Mingioli's minimal medium of *E. coli* AB1157 dcm^+ by the addition of 5-azaC. Concentrations of 5-azaC: ● control, ○ 0·1, ▲ 1·0, □ 10·0 mg L^{-1}. Arrow indicates addition of 5-azaC.

the *dcm* gene product recognize and methylate the same DNA sequence, the Dcm enzyme is not as efficient as the plasmid-borne methylase at protecting against *Eco*RII restriction. Phage propagated on host cells carrying the RN3 *Eco*RII Mod^+ plasmid plate with an efficiency of unity on *Eco*RII-restricting bacteria (Hattman et al 1973), whereas the EOP of Dcm-methylated phage, propagated on a dcm^+ strain in the absence of plasmid RN3, is of the order of 10^{-1} (Fig. 2). However, increasing the concentration of 5-azaC in the DM medium used for phage propagation decreased the EOP of the phage from $1{\cdot}1 \times 10^{-1}$ in 5-azaC-free medium to $1{\cdot}8 \times 10^{-3}$ when the propagating host was grown in 10 mg L^{-1} 5-azaC (Fig. 2).

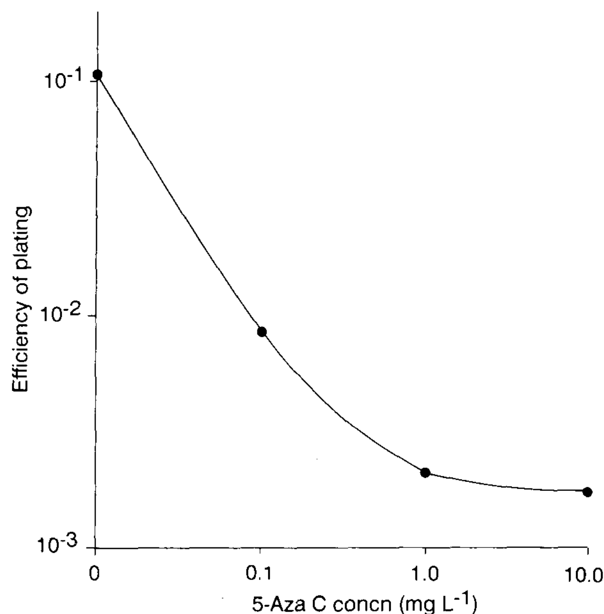

FIG. 2. Effect of propagation in the presence of 5-azaC on the plating efficiency of phage λ_{vir} on *Eco*RII-restricting and -non-restricting hosts. Phage crops propagated on *E. coli* strain AB1157 dcm^+ growing on Davis and Mingioli's medium in the presence of varying concentrations of 5-azaC were titrated on *E. coli* strains AB1157 *dcm* (RN3 *Eco*RII Res^+ Mod^+) and AB1157 *dcm* (RN3 *Eco*RII Res^- Mod^+).

Discussion

The in-vivo test we describe illustrates how phage can be used to demonstrate effective inhibition of DNA-cytosine methylation. Although the reduction in EOP produced by 5-azaC was not of the same order (10^{-5}) as that obtained by propagating phage on *dcm* strains such as LR68 (Radnedge & Pinney 1991), there is no doubt that specific inhibition of cytosine methylation is occurring.

E. coli DNA also contains methylated adenine residues. For example the sequence 5′-GAATTC-3′, which is recognized and cut by the plasmid-borne *Eco*RI restriction endonuclease, is protected from cleavage by methylation of the internal adenine by the associated modification methylase (Roberts 1978). It should therefore be possible to modify the method to detect compounds that inhibit DNA-adenine methylation.

References

Bachmann, B. J. (1972) Pedigree of some mutant strains of *Escherichia coli* K-12. Microbiol. Revs. 36: 525–557

Birks, J. H., Pinney, R. J. (1975) Correlation between thymineless elimination and absence of hspII (*Eco*RII) specificity in N group R factors. J. Bacteriol. 121: 1208–1210

Crooks, P. A., Tribé, M. J., Pinney, R. J. (1984) Inhibition of bacterial DNA cytosine-5-methyltransferase by *S*-adenosyl-L-homocysteine and some related compounds. J. Pharm. Pharmacol. 36: 85–89

Cedar, H., Razin, A. (1990) DNA methylation and development. Biochim. Biophys. Act 1049: 1–8

Davis, B. D., Mingioli, E. S. (1950) Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. 60: 17–28

Friedman, S. (1981) The inhibition of DNA(cytosine-5)methylases by 5-azacytidine: the effect of azacytosine-containing DNA. Mol. Pharmacol. 19: 314–320

Hattman, S., Schlagman, S., Cousens, L. (1973) Isolation of a mutant of *Escherichia coli* defective in cytosine-specific deoxyribonucleic acid methylase activity and in partial protection of bacteriophage λ against restriction by cells containing the N-3 drug resistance factor. J. Bacteriol. 115: 1103–1107

Lieb, M. (1987) Bacterial genes *mutL*, *mutS*, and *dcm* participate in repair of mismatches at 5-methylcytosine sites. Ibid. 169: 5241–5246

May, M. S., Hattman, S. (1975a) Deoxyribonucleic acid-cytosine methylation by host- and plasmid-controlled enzymes. Ibid. 122: 129–138

May, M. S., Hattman, S. (1975b) Analysis of bacteriophage deoxyribonucleic acid sequences methylated by host- and R-factor-controlled enzymes. Ibid. 123: 768–770

Radnedge, L. (1988) Cytosine Methylation and DNA Repair in *Escherichia coli*. Ph.D. Thesis, University of London

Radnedge, L., Pinney, R. J. (1991) Ultraviolet light induction of lambda from *dcm* host strains alleviates *Eco*RII restriction of phage. FEMS Microbiol. Letts. 79: 121–126

Roberts, R. J. (1978) Restriction and modification enzymes and their recognition sequences. Gene 4: 183–193

Santi, D. V., Garrett, C. E., Barr, P. J. (1983) On the mechanism of inhibition of DNA-cytosine methyltransferase by cytosine analogs. Cell 3: 9–10

Santi, D. V., Norment, A., Garrett, C. E. (1984) Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine. Proc. Natl. Acad Sci. USA 81: 6993–6997

Schlagman, S., Hattman, S. (1974) Mutants of the N-3 R-factor conditionally defective in *hsp* II modification and deoxyribonucleic acid-cytosine methylase activity. J. Bacteriol. 120: 234–239

Sohail, A., Lieb, M., Dar, M., Bhagwat, A. S. (1990) A gene required for very short patch repair in *Escherichia coli* is adjacent to the DNA cytosine methylase gene. J. Bacteriol. 172: 4214–4221

Zell, R., Fritz, H.-J. (1987) DNA mismatch-repair in *Escherichia coli* counteracting the hydrolytic deamination of 5-methyl-cytosine residues. EMBO J. 6: 1809–1815