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International Journal of Pharmaceutics 116 (1995) 217–221

international journal of pharmaceutics

The effect of *p*-aminobenzoic acid on the uptake of thymidine and uracil by *Escherichia coli*

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Received 17 May 1994; accepted 30 August 1994

Abstract

Escherichia coli incubated with subinhibitory concentrations of *p*-aminobenzoic acid at pH 5.5 produced either larger or more opaque cells than the control culture. DNA synthesis was particularly sensitive to the presence of *p*-aminobenzoic acid. The antibacterial effect of *p*-aminobenzoic acid was reduced by the presence of folinic acid in the culture of *Pseudomonas aeruginosa*. These findings indicate that the effect of *p*-aminobenzoic acid on DNA synthesis may be via an action on the dihydrofolate reductase enzyme.

Keywords: Thymidine; Uracil uptake; Antibacterial effect

1. Introduction

p-Aminobenzoic acid is reported to inhibit growth of *Pseudomonas aeruginosa* and *Escherichia coli* and also to affect cell permeability, enhance antibacterial activity and increase uptakes of dibromopropamidine by *P. aeruginosa, Enterobacter cloacae*, *Proteus mirabilis* and *Staphylococcus aureus* (Eagon and McManus, 1989, 1990; Richards and Xing, 1992a,b) and *P. aeruginosa* cells grown in the presence of *p*-aminobenzoic acid were shown to be more susceptible to lysis by lysozyme and lysozyme plus disodium edetate. Electron micrographs indicated that cells of *P. aeruginosa*, *E. cloacae* and *S. aureus* cultured in the presence of *p*-aminobenzoic acid were larger than control cells (Richards and Xing, 1993). The molecular basis for this antibacterial action of *p*-aminobenzoic acid has not been fully established.

This investigation was to determine the effect of *p*-aminobenzoic acid on the uptake of thymidine and uracil and thus evaluate its effect on DNA and RNA synthesis in *E. coli*.

2. Materials and methods

2.1. Bacterial strains

E. coli ATCC 31389, *DAP, thy*, was obtained from the American Type Culture Collection, Rockville, MD, USA. *P. aeruginosa* NCTC 6750 was obtained from the National Collection of Type Cultures, Colindale, London, UK.

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SSDI 0378-5173(94)00303-3

2.2. Chemicals

Thymidine, uracil, *p*-aminobenzoic acid, sulphadiazine, folinic acid and 2,6-diaminopimelic acid were obtained from Sigma, Poole, UK. Unless otherwise stated, all the buffer salts and inorganic chemicals used in the minimal medium were commercial analytical grade and obtained from Fisons, Loughborough, Leicestershire, UK. Glucose was obtained from BDH Laboratory Supplies, Poole, UK. Yeast extract powder was obtained from London Analytical and Bacteriological Media Ltd, London, UK. Nutrient agar and Isosensitest broth were obtained from Oxoid, Basingstoke, UK. HPLC-grade methanol was obtained from Rathburn Chemicals Ltd, Walkerburn, UK and water for the capillary zone electrophoresis assay was glass distilled and then further purified by a Millipore Milli-Qsystem.

2.3. Bacteriological media

E. coli ATCC 31389 was incubated in supplemented minimal medium (SMM) (based on Eklund, 1980). This medium contained (g/l) KH_2PO_4, 3; K_2HPO_4, 7; sodium citrate, 0.5; $MgSO_4.7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; yeast extract, 1.0 and glucose, 5. Since *E. coli* ATCC 31389 is a thymidine- and diaminopimelic acid-dependent strain, thymidine and 2,6-diaminopimelic acid were added to all batches of SMM to give a final concentration of 40 μg/ml for thymidine and 100 μg/ml for diaminopimelic acid. Isosensitest broth was the medium used for *P. aeruginosa*.

2.4. Electropherograph equipment

The capillary zone electrophoresis (CZE) apparatus used was a Model 3850 electrograph from Isco (Nebraska, USA). Fused-silica capillaries of 50 μm i.d., total length 65 cm with a separation length of 45 cm, were employed. The sample was introduced into the column by hydrodynamic loading.

2.5. Turbidity measurement and viable counts

A 2 ml sample of 18 h *E. coli* culture in SMM (pH 7.0) was inoculated into a flask containing 98 ml of prewarmed SMM (pH 7.0) and incubated at 37°C in a water bath shaking at 100 oscillations per min until the turbidity reached 0.2 at 600 nm. Then 10-ml samples were inoculated separately into replicate flasks containing 90 ml SMM plus either *p*-aminobenzoic acid (1000 μg/ml) (pH 5.5) or SMM adjusted with hydrochloric acid to pH 5.5 as the control. The flasks were incubated as before and cell growth was determined at intervals by measuring culture turbidity at 600 nm and by determining colony-forming units (CFU) on overdried nutrient agar plates for both cultures.

2.6. Determination of uptake of thymidine and uracil by E. coli

Determination of the uptake of thymidine and uracil was based on the procedures described by Richards and Xing (1994). Briefly, 4 ml of 18 h culture was inoculated separately into three flasks containing 96 ml SMM prewarmed to 37°C and incubated in a shaking water bath for 4 h. Subsequently, 50 ml of the exponentially growing cells was transferred to 50 ml prewarmed SMM containing 500 μg/ml *p*-aminobenzoic acid (pH 6.0) and incubated with thymidine 40 μg/ml and uracil 40 μg/ml. SMM adjusted to pH 6.0 with hydrochloric acid containing no *p*-aminobenzoic acid was used as the control. All experiments were carried out in triplicate. Samples (5 ml) were removed at 30-min intervals and centrifuged for 10 min (13000 RPM) using an IFC Centra-4B centrifuge. Thymidine and uracil present in the supernatants were quantified by CZE. Sulphadiazine (200 μg/ml) was added as internal standard. The separation was carried out with running buffer 50 mM disodium tetraborate buffer, pH 9.1. The sample was loaded over a period of 10 s and the electrophoresis was performed at 24 kV. UV absorbance was detected at a wavelength of 270 nm. The ratio of the peak heights of these compounds was calculated with reference to the peak heights of the internal standards. The cell pellets were washed twice with 0.9% sodium chloride and assayed for protein using the assay of Lowry et al. (1951). Bovine serum albumin (from Sigma) was used as protein standard. The

uptake of the metabolites by the cells was represented as μg/mg of cell protein.

2.7. Determination of effect of folinic acid on the antibacterial activity of p-aminobenzoic acid

P. aeruginosa was used as the test organism for the study of the effect of folinic acid on the antibacterial activity of *p*-aminobenzoic acid since this organism had previously been shown to utilise free folinic acid (Richards et al., 1991). Samples (0.2 ml) of 18 h culture of *P. aeruginosa* in Isosensitest broth were inoculated separately into 9.8 ml Isosensitest broth containing either 1200 μg/ml *p*-aminobenzoic acid alone or plus 200 μg/ml folinic acid. Isosensitest broth containing 200 μg/ml folinic acid alone was used as control. All experiments were carried out in quadruplicate. The containers were incubated at 37°C in a water bath shaking at 100 oscillations per min. CFU for each culture at selected times were determined using overdried nutrient agar plates.

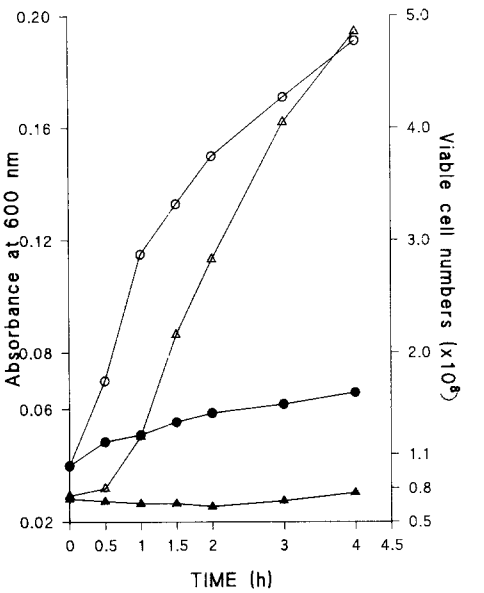

Fig. 1. Effect of *p*-aminobenzoic acid (1000 μg/ml) on the growth of *E. coli* at pH 5.5. At time zero, a culture was divided into two portions and *p*-aminobenzoic acid was added to one (filled symbols) whilst the other served as the control (open symbols). Culture turbidity (●,○) and CFU (▲,△) were determined at the times indicated.

3. Results and discussion

The effect of *p*-aminobenzoic acid on the growth of *E. coli* is shown in Fig. 1. Cell division occurred approx. 30 min later. Cell numbers increased approximately in parallel with the turbidity of the control cultures. The turbidity of the cultures treated with *p*-aminobenzoic acid (1000 μg/ml) slowly increased but no increase in CFU was observed during the same period. This indicated an increase in cell mass in the absence of cell division. It appears that during this non-replicating period the individual cells were larger and/or more opaque than those in the acid free control cultures. In a previous study (Richards and Xing, 1993) a comparison of cell diameters and lengths using electron micrographs indicated that cells of *P. aeruginosa*, *E. cloacae* and *S. aureus* cultured in the presence of *p*-aminobenzoic acid were larger than control culture cells. This indicated that subinhibitory concentrations of *p*-aminobenzoic acid could affect bacterial cell walls.

Fig. 2 and 3 show the uptake of thymidine and uracil which influence DNA and RNA synthesis, respectively, by *E. coli*. The choice of this organism was based on the knowledge that *E. coli* maintains its internal pH (pH_i) at 7.4–7.6 over an external pH (pH_o) range of 5–9 (Padan et al. 1981); *E. coli* ATCC 31389 is a thymidine-dependent strain which cannot utilise free folinic acid (unpublished results). Fig. 2 shows that the uptake of thymidine in the control cultures at pH 6.0 increased in a linear fashion over 2 h. In the presence of *p*-aminobenzoic acid, the uptake of thymidine remained at almost the same value as the control for the first 30 min, but after that the uptake of thymidine was reduced to about 50% of the control value. This indicates that DNA synthesis was inhibited by the presence of a subinhibitory concentration of *p*-aminobenzoic acid. Uptake of uracil was less sensitive than that of thymidine to the presence of *p*-aminobenzoic acid (Fig. 3). That is, the synthesis of RNA appeared to increase at only a slightly slower rate than in

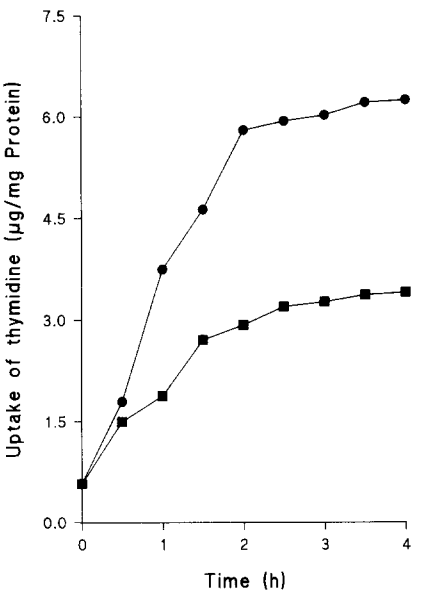

Fig. 2. Effect of *p*-aminobenzoic acid (500 µg/ml) on the uptake of thymidine by *E. coli* in supplemented minimal medium at pH 6.0. (●) Control; (■) *p*-aminobenzoic acid.

the control. This indicates that the DNA synthesis was much more sensitive than the RNA synthesis to the action of *p*-aminobenzoic acid (500 µg/ml). Similar results have been reported (Cherrington et al. 1990) for the effect of propionic acid and formic acid on the synthesis of DNA and RNA in *E. coli*. It was found that DNA synthesis was the most sensitive of the functions to propionic acid. The different susceptibilities of DNA and RNA synthesis to the acids could be a function of their individual sensitivity to pH, but it may also result from a more specific inhibition by the acid anion (Reynolds, 1975). Fig. 1 indicates that *p*-aminobenzoic acid caused inhibition of cell division for a period of at least 30 min after its addition to the culture. Since synthesis of molecules other than DNA may not be completely inhibited, bacterial mass increased as indicated by the absorbance measurements (Fig. 1). This observation that *p*-aminobenzoic acid treated cells were larger and/or more dense than cells from control cultures is consistent with preferential inhibition of DNA synthesis by the acid (Cherrington et al., 1990).

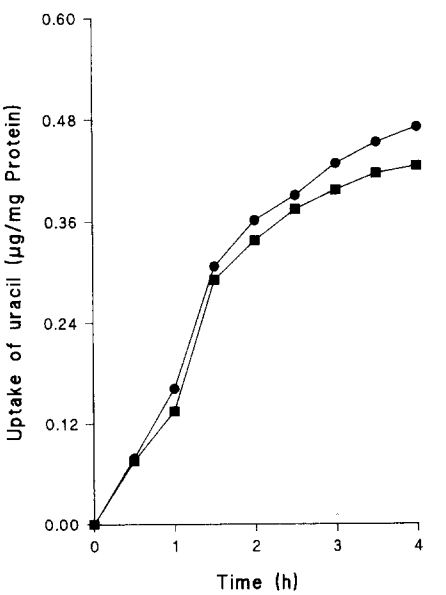

Fig. 3. Effect of *p*-aminobenzoic acid (500 µg/ml) on the uptake of uracil by *E. coli* in supplemented minimal medium at pH 6.0. (●) Control; (■) *p*-aminobenzoic acid.

The mechanism by which high concentrations of *p*-aminobenzoic acid inhibit DNA synthesis may be via an action on the dihydrofolate reductase enzyme as postulated by Then (1977) in a different context. Thus, low concentrations of *p*-aminobenzoic acid are involved in the normal bacterial synthetic processes (in combining with pteridine under the influence of dihydropteroate synthetase to produce dihydropteroic acid – a precursor of dihydrofolic acid); and high concentrations of the acid may be involved in the inhibition of tetrahydrofolic acid production and the subsequent synthesis of DNA and other metabolites. Fig. 4 shows that the influence of folinic acid on the activity of *p*-aminobenzoic acid on the cells growth of *P. aeruginosa*. It is known that *P. aeruginosa* is able to utilise free folinic acid (Richards et al., 1991). In this work, the growth inhibition caused by *p*-aminobenzoic acid was decreased in the presence of folinic acid (200 µg/ml) by 7–36% during the incubation period of 1–6 h. It had previously been noted that folinic acid (10 µg/ml) decreased by 87% the uptake of *p*-aminobenzoic acid by *P. aeruginosa* (Richards and Xing, 1992a) and it has been found that *p*-aminobenzoic acid inhibited the action of trimethoprim by checkerboard determinations

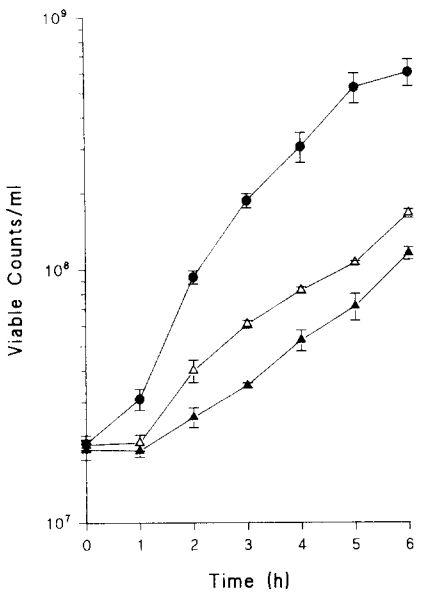

Fig. 4. Effects of *p*-aminobenzoic (1200 μg/ml) in the absence and presence of folinic acid (200 μg/ml) on the cell growth of *P. aeruginosa*. (●) Control; (▲) *p*-aminobenzoic acid; (△) *p*-aminobenzoic acid plus folinic acid.

(unpublished results). It could be postulated that high but relatively ineffective concentrations of *p*-aminobenzoic acid interfere with the action of lower but relatively more active concentrations of trimethoprim on the activity of the dihydrofolate reductase enzyme possibly by competing with trimethoprim for the pteridine portion of dihydrofolic acid and thus preventing the trimethoprim from having an antibacterial effect. The results shown in Fig. 1 and 2 could represent an effect of *p*-aminobenzoic acid on DNA synthesis via an effect on bacterial folate synthesis and the subsequent production of metabolites, via the thymidine → DNA pathways. The findings that growth of *P. aeruginosa*, *E. cloacae*, *Pr. mirabilis* and *S. aureus* in the presence of *p*-aminobenzoic acid affected cell wall structure similarly to but not identically to that reported for sulphonamides and trimethoprim also support this hypothesis (Richards et al., 1991; Richards and Xing, 1992a,b, 1993).

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