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Investigation of the antibacterial activity of *p*-aminobenzoic acid against *P. aeruginosa* and *E. cloacae*

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Summary

p-Aminobenzoic acid was found to have a bactericidal action against *Pseudomonas aeruginosa* and *Enterobacter cloacae*. This action was not the result of a reduction in pH. An open porin strain of *P. aeruginosa* was more sensitive to *p*-aminobenzoic acid than the mother strain, indicating uptake via the outer membrane porin protein. Subinhibitory concentrations of a combination of *p*-aminobenzoic acid with dibromopropamidine isethionate were shown to have synergistic antibacterial activity against *P. aeruginosa*. Bacterial uptakes of the antibacterials determined by an HPLC assay method combined with dry cell weight determinations indicated that enhancement of activity of the combination was related to the fact that both compounds increase the bacterial uptake of the other. *P. aeruginosa* cells grown in the presence of subinhibitory concentrations of *p*-aminobenzoic acid sustained damage which made the cells more sensitive to the action of both benzalkonium chloride and EDTA and to lysis by lysozyme plus EDTA. These cells also exhibited lysis when treated with lysozyme alone indicating that *p*-aminobenzoic acid causes damage to the outer membrane. Folinic acid was found to block the antibacterial activity of *p*-aminobenzoic acid and to increase bacterial resistance to benzalkonium chloride and EDTA.

Introduction

It has been reported that the high concentrations of *p*-aminobenzoic acid required to inactivate sulphadiazine in cream formulations possessed marked antibacterial activity (Richards, et al., 1991) The antibacterial activity of *p*-aminobenzoic acid against *Enterobacter cloacae* was greater than that of sulphadiazine (unpublished results). Therefore, it was decided to investigate the activity of the potentially less toxic *p*-aminobenzoic acid against *Pseudomonas aeruginosa* and *E. cloacae*, particularly in combination with dibromopropamidine isethionate, which had been shown to have synergistic activity with sulphadiazine and several other antibacterial agents (Richards and Xing, 1991; Richards, et al., 1991).

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Materials and Methods

P. aeruginosa NCTC 6750 and *E. cloacae* NCTC 10005 were obtained from the National

Collection of Type Cultures, Colindale, London, U.K. *P. aeruginosa* 799 (ATCC 12055) was obtained from the American Type Culture Collection. *P. aeruginosa* K 799/61 (ATCC 35151) was a gift from Dr W. Zimmermann, CIBA-GEIGY Ltd, Basel, Switzerland. *P. aeruginosa* K 799/61 is a mutant open porin strain and 799 (ATCC 12055) is the mother strain. Isosensitest broth, isosensitest agar and nutrient broth were all obtained from Oxoid, Basingstoke, U.K. Inactivating recovery medium was made with nutrient broth, Tween 80, 3.0% w/v and lecithin 0.5% w/v.

p-Aminobenzoic acid, folinic acid, folic acid, lysozyme, EDTA and benzalkonium chloride were all obtained from Sigma, Poole, U.K. Dibromopropamidine isethionate was a gift from May and Baker, Dagenham, U.K. Tris and lecithin were obtained from BDH, Poole, U.K. Tween 80 was purchased from ICI, Leatherhead, U.K. Other chemicals were all obtained from BDH, Poole, U.K.

Measurement of pH

The pH of bacterial cultures was determined using a Kent EIL 7020 pH meter at 20–25°C.

Minimum inhibitory concentrations (MICs)

These were determined by two separate methods:

(i) MICs were determined in duplicate using 9.9 ml volumes of isosensitest broth containing a series of dilutions of *p*-aminobenzoic acid alone or plus folinic acid either 10 or 500 μg/ml, by inoculating with either 0.1 ml of an 18 h culture, or of a 1:100 dilution of an 18 h culture, of one of the four test organisms to give either approx. 5×10^5 or 5×10^3 cells/ml. These tubes were incubated for 24 h at 37°C and the lowest concentrations showing no growth in both tubes were recorded as the MICs.

(ii) MICs were also determined in duplicate by the agar plate test method (Gould and Path, 1975). Known concentrations of *p*-aminobenzoic acid were incorporated in plates of isosensitest agar and over-dried at 37°C for 2 h. The different bacterial suspensions were inoculated onto the surface of each plate to give an inoculum of either approx. 5×10^3 or 5×10^5 cfu/ml. The plates were then incubated for 24 h at 37°C. The lowest concentration of *p*-aminobenzoic acid that entirely prevented the growth of the bacteria on the plate was recorded as the MIC. Plates showing no growth has 0.001 m^2 removed and aseptically transferred to 9.9 ml nutrient broth which was then incubated for 7 days at 37°C to determine the MBC. The pH of broth plus 0.001 m^2 samples of agar containing the highest concentration of *p*-aminobenzoic acid (2700 μg/ml) was 6.95 and supported growth of low inocula of both *P. aeruginosa* and *E. cloacae*.

Effect of p-aminobenzoic acid on the viable count

P. aeruginosa and *E. cloacae* cells were grown in isosensitest broth for 18 h and then centrifuged ($6000 \times g$, 15 min, 4°C). The cell pellets were washed in 0.9% w/v sodium chloride, recentrifuged and the cell concentration adjusted to approx. 5×10^7 cells/ml for both *P. aeruginosa* and *E. cloacae*. Volumes of 0.1 ml of the *P. aeruginosa* cell suspension were added separately to duplicate tubes containing 9.9 ml volumes of a range of concentrations of *p*-aminobenzoic acid buffered at pH 5.0 in phosphate buffer and equilibrated at 37°C. The *E. cloacae* cells were added to unbuffered aqueous solutions of *p*-aminobenzoic acid. Samples (0.5 ml) were removed from each tube at predetermined intervals and transferred to 9.5 ml volumes of inactivating recovery medium which after appropriate dilution in 0.9% w/v sodium chloride were then inoculated onto the surface of four replicate plates. Viable counts were determined after incubation for 24 h at 37°C. Control counts were evaluated on suspensions adjusted to pH 5 with phosphate buffer for *P. aeruginosa* and to pH 4 with hydrochloric acid for *E. cloacae*. This latter pH value was equivalent to that of the strongest *p*-aminobenzoic acid solution.

High-performance liquid chromatography (HPLC)

Determinations of bacterial cell uptake were based on the HPLC assay previously described (Taylor et al., 1990; Richards et al., 1991). The method involved pretreatment of the broth by solid-phase extraction using CH-bonded silica fol-

lowed by preconcentration and ion-pairing reverse-phase chromatography using a 100 × 4.6 mm i.d. ODS Hypersil column and UV detection at 254 nm.

Determination of uptake of p-aminobenzoic acid and dibromopropamidine isethionate

Samples (1 ml) of 18 h culture in isosensitest broth were inoculated separately into replicate flasks containing 99 ml isosensitest broth plus the selected concentrations of the chemicals and incubated for 18 h. The cultures were centrifuged (6000 × g, 15 min, 4°C) and the supernatants removed; the pellets were washed by resuspension in distilled water and recentrifugation. The washed pellets were used for dry cell weight determinations and the concentration of chemical in the supernatants and in the washings determined using the HPLC method, the resultant concentrations being combined. The levels of bacterial cell uptake were calculated from the differences between the original and the resultant concentrations.

Lysis

Samples (1 ml) of 18 h culture were inoculated into 99 ml isosensitest broth containing either selected concentrations of *p*-aminobenzoic acid and folinic acid used alone, or *p*-aminobenzoic acid in combination with folinic acid, and incubated for 18 h at 37°C. The cultures were centrifuged (6000 × g, 15 min, 4°C) and the pellets resuspended in 0.9% w/v sodium chloride, recentrifuged and the pellets resuspended in Tris buffer (pH 7.4). The cell concentrations were adjusted to approx. 5×10^8 cells/ml. Readings of the absorbance of the test suspension at 600 nm were made using a Unicam SP 1800 Ultraviolet Spectrophotometer at room temperature before the addition of either lysozyme alone or lysozyme plus EDTA to give a reading at zero time. The reference cuvette in the spectrophotometer contained Tris buffer. Either lysozyme or EDTA solution was added to give a final concentration of 33 μg/ml for lysozyme and 25 μg/ml for EDTA. The subsequent absorbance at 600 nm was recorded over a period of 10 min. Three individual sets of readings were determined for each sample and standard deviations calculated. For the purpose of this investigation the percentage reduction in absorbance was taken to be equivalent to the percentage lysis of the cells.

Determination of killing times

Estimates of the comparative resistance of cells grown in isosensitest broth alone and in isosensitest broth plus either *p*-aminobenzoic acid 250 μg/ml or *p*-aminobenzoic acid 250 μg/ml in the presence of folinic acid 10 μg/ml were determined as previously described (Richards and McBride, 1973; Richards and Mizrahi, 1978). After 18 h incubation at 37°C, the bacterial cultures were centrifuged and washed as for the above lysis experiments except that the cell pellets were resuspended in 0.9% w/v sodium chloride. Duplicate tubes containing 9.9 ml of the antibacterial solutions under test were equilibrated in a water bath at 37°C and inoculated with 0.1 ml of cell suspension to give approx. 5×10^6 cells/ml. At intervals of 15, 30, 45 60, 90, 120, 150, 180, 240 and 300 min after inoculation, 0.5 ml samples were aseptically transferred to 9.5 ml of inactivating recovery medium and incubated at 37°C for 72 h. The test antibacterial solutions were benzalkonium chloride 25 μg/ml and EDTA 600 μg/ml both of which are known to have marked membrane activity against *P. aeruginosa* cells. Positive controls were prepared by adding 5×10^3 cells from the cell suspension above to duplicate tubes of inactivator medium plus either 100 μg/ml of benzalkonium or 800 μg/ml of EDTA. This was to demonstrate the effectiveness of the inactivators.

Checkerboard MIC determination

Checkerboard MIC estimates with two chemical combinations were based on the method of Sabbath (1968). *P. aeruginosa* was the test organism. A 10 × 10 checkerboard of test-tubes was prepared in duplicate using isosensitest broth. Each test-tube was inoculated to give 5×10^3 cells/ml. MICs were determined after 24 h incubation at 37°C and isobolograms plotted.

Results and Discussion

This investigation demonstrates that *p*-aminobenzoic acid has a bactericidal action against *P. aeruginosa* and *E. cloacae*. No *P. aeruginosa* cells from an inoculum of 5×10^5 cells/ml were detectable 30 min after contact with *p*-aminobenzoic acid 3000 μg/ml (Fig. 1) and a concentration of *p*-aminobenzoic acid of 3000 μg/ml produced a 99% kill against 10^6 *E. cloacae* cells/ml within 3 h (Fig. 2). All *p*-aminobenzoic acid solutions used were either of equivalent pH to the control (*P. aeruginosa*) or of a higher pH than the control (*E. cloacae*). Therefore, this activity is not a pH effect. The action is, however, related to the -COOH group since sodium *p*-aminobenzoate was shown to be much less active than *p*-aminobenzoic acid (unpublished results).

P. aeruginosa mutant 799/61 was more sensitive to *p*-aminobenzoic acid than the wild-type strain (Table 2) and this provides further evidence that mutant 799/61 is more permeable to hydrophilic antibacterials than the wild-type strain K799 (Angus et al., 1982). This also indicates that *p*-aminobenzoic acid may enter the cells of *P. aeruginosa* through outer membrane porin protein.

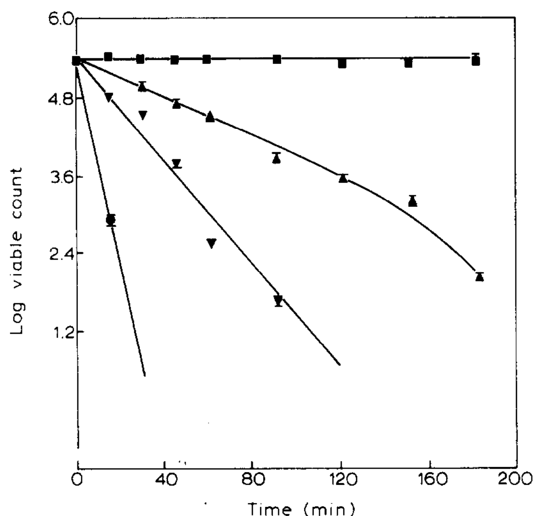

Fig. 1. Effect of *p*-aminobenzoic acid in phosphate buffer at 37°C on the viable count of *P. aeruginosa* (mean with SD). (■) Control (pH 5); (▲) PABA 1000 μg/ml (pH 5); (▼) PABA 2000 μg/ml (pH 5); (●) PABA 3000 μg/ml (pH 5).

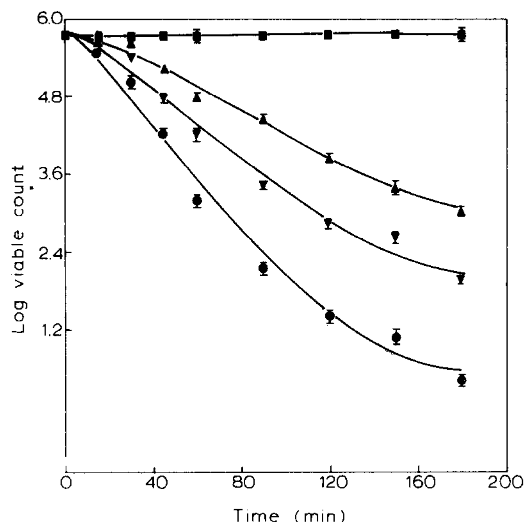

Fig. 2. Effect of *p*-aminobenzoic acid in distilled water at 37°C on the viable count of *E. cloacae* (mean with SD). (■) HCl control (pH 4.0); (▲) PABA 2400 μg/ml (pH 4.3); (▼) PABA 2700 μg/ml (pH 4.2); (●) PABA 3000 μg/ml (pH 4.0).

The MIC for *p*-aminobenzoic acid alone against *P. aeruginosa* NCTC 6750 was 1340 μg/ml but this increased to 1355 and 1436 μg/ml in the presence of folinic acid 10 and 500 μg/ml, respectively.

Addition of either lysozyme or lysozyme plus EDTA to suspensions of *P. aeruginosa* cells, which had been grown for 18 h in the presence of *p*-aminobenzoic acid 500 μg/ml caused approx. 10 and 50% lysis of cells (Tables 3 and 4). This indicates that growth in the presence of *p*-aminobenzoic acid has an effect on the cell envelope permeability which makes the cells permeable to lysozyme. Therefore, it can be concluded

TABLE 1

MIC values for p-aminobenzoic acid related to the effect of pH on the growth of P. aeruginosa and E. cloacae

Test organism	MIC (μg/ml)	pH of broth at MIC value	Highest pH adjusted with HCl giving no growth
P. aeruginosa	1500	4.45	4.1
E. cloacae	1200	4.60	< 4.0

TABLE 2

MIC and MBC determinations for p-aminobenzoic acid in isosensitest agar and MIC determinations in isosensitest broth against P. aeruginosa and E. cloacae

Organism	Agar		Broth	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
P. aeruginosa (NCTC 6750)				
5×10^3	1400	2400	1345	2100
5×10^5	1600	2700	1500	2400
E. cloacae (NCTC 10005)				
5×10^3	800	2100	1050	2000
5×10^5	1000	2400	1200	2100
P. aeruginosa (K 799/61 ATCC 35151)				
5×10^5	1050	...	...	...
P. aeruginosa (12055 ATCC 799)				
5×10^5	1200	...	...	...

that *p*-aminobenzoic acid causes damage to the outer membrane. Previously, *p*-aminobenzoic acid has been reported to enhance the antimicrobial activity of phosphanilic acid (Eagon and McManus, 1989). The present investigation showed that the combination of subinhibitory concentrations of *p*-aminobenzoic acid and dibromopropamidine exerts a synergistic action against *P. aeruginosa* (Fig. 3). This synergistic activity can be explained by the fact that both compounds increase the uptake of the other. It occurred despite the reported reduction of dibromopropamidine activity at lower pH values (Woodside, 1973). Dibromopropamidine at subinhibitory concentrations has been shown to have an effect on the cell envelope permeability which results in the increased uptake of a second antibacterial present in the medium (Richards et al., 1991). It would appear that *p*-aminobenzoic acid also has an effect on the cell permeability which produces a

TABLE 3

Lysis of P. aeruginosa cells grown for 18 h in isosensitest broth alone or plus either p-aminobenzoic acid (PABA) or PABA plus folinic acid (FA) and then treated with lysozyme (33 μg / ml)

Source of bacterial cells [broth or broth + chemicals (μg/ml)]	Lysis (%) (±SD) (mean of 3 determinations)
Control	2.6 ± 0.20
PABA (250)	9.1 ± 0.15
PABA (250) + FA (10)	6.5 ± 0.15
PABA (500)	9.6 ± 0.11
PABA (500) + FA (10)	7.1 ± 0.30

TABLE 4

Effect of EDTA (25 μg / ml) plus lysozyme (33 μg / ml) on the lysis of P. aeruginosa (NCTC 6750) grown for 18 h in isosensitest broth alone or plus either PABA or folinic acid or PABA plus folinic acid

Source of bacterial cells [broth or broth + chemicals (μg/ml)]	Lysis (%) (±SD) (mean of 3 determinations)		Percentage change in lysis
Control	25.7 (mean of 5)	0.88	0
FA (10)	16.9	0.61	−34.2
PABA (250)	41.4	0.92	+61.1
PABA (500)	48.0	0.74	+86.7
PABA (250) + FA (10)	18.9	1.02	−26.5
PABA (500) + FA (10)	25.8	1.20	no change

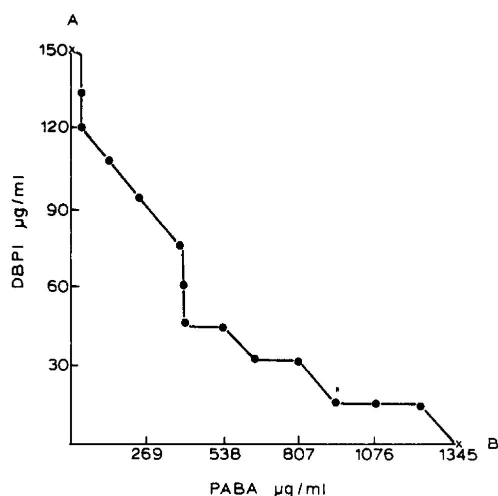

Fig. 3. Isobologram constructed from checkerboard MIC data showing combinations of DBPI with PABA against *P. aeruginosa* in isosensitest broth. MIC of DBPI 150 µg/ml, MIC of PABA, 1345 µg/ml.

more than doubling of the uptake of dibromopropamidine (Table 5). Folinic acid 10 µg/ml decreased by 87% the uptake of *p*-aminobenzoic acid 200 µg/ml by *P. aeruginosa* cells (Table 5). *p*-Aminobenzoic acid has been shown to be effective in increasing the uptake of Mn^{2+} (Morariu et al., 1987) and this could also be the result of an effect on cell permeability.

The results of the killing time determinations indicated that cells grown in the presence of

TABLE 5

Bacterial uptake of dibromopropamidine isethionate (DBPI) and p-aminobenzoic acid (PABA) alone or in combination.

PABA (µg/ml culture)	DBPI (µg/ml culture)	FA (µg/ml culture)	Uptake (µg/mg dry cell weight) (mean of 2 determinations)	
			PABA	DBPI
200	...	...	0.075	...
...	100	...	...	10.866
200	100	...	0.172	27.795
200	...	10	0.010	...

P. aeruginosa was cultured for 18 h in isosensitest broth and in broth plus the antibacterials or plus PABA and folinic acid (FA).

TABLE 6

Killing times for benzalkonium chloride (Bzk) and ethylenediaminetetra-acetic acid (EDTA) solutions against washed suspensions of approx. 5 × 10^6 P. aeruginosa cells grown in isosensitest broth alone or plus either p-aminobenzoic acid (PABA) or folinic acid (FA) or plus a combination of PABA and FA

Source of inoculum [broth or broth + chemicals (µg/ml)]	Killing times (min), at 37°C (mean of 2 determinations)	
	Bzk (25 µg/ml)	EDTA (600 µg/ml)
Control	180	300
FA (10)	240	> 300
PABA (250)	60	180
PABA (250) + FA (10)	150	300

p-aminobenzoic acid were more sensitive to the action of both benzalkonium chloride and EDTA (Table 6). This bacterial cell sensitising effect is thought to result from damage to the permeability control of the cells grown in the presence of *p*-aminobenzoic acid. A similar effect has been observed previously with cells grown in the presence of disodium edetate and phenylethanol and subsequently tested against another antibacterial (Richards et al., 1969). Both disodium edetate and phenylethanol are known to affect the *P. aeruginosa* cell envelope permeability and to enhance access of a second antibacterial agent present in the medium (Brown and Richards., 1965; Richards et al. 1969; Richards and McBride, 1973; Richards, 1975).

Cells grown in the presence of *p*-aminobenzoic acid plus folinic acid 10 µg/ml were less sensitive to lysis by either lysozyme alone or lysozyme plus EDTA than the cells grown in the presence of *p*-aminobenzoic acid alone (Tables 3 and 4). This could indicate that p-aminobenzoic acid has an inhibitory effect on bacterial folate synthesis. With sulphadiazine an action on folate synthesis affected the permeability properties of the cell and it was shown that this type of action was reversed by folinic acid (Richards et al., 1991). The results with *p*-aminobenzoic acid might also indicate that folinic acid facilitated more effective synthesis and repair of those cell envelope components damaged by some other

action of *p*-aminobenzoic acid. The finding that cells grown in the presence of 10 μg/ml folinic acid were more resistant to the antibacterial action of EDTA and benzalkonium chloride (Table 6) would support the latter hypothesis.

If *p*-aminobenzoic acid had an effect on the folate synthesis pathway then it might be via the excess production of folic acid. However, folic acid 50 μg/ml added to the MIC test system did not inhibit the cells in any detectable way nor did it enhance or block the effect of *p*-aminobenzoic acid.

It may be concluded that *p*-aminobenzoic acid has a bactericidal action against the test organisms and appears to enter *P. aeruginosa* cells via outer membrane porin protein. At subinhibitory concentrations it affects the permeability properties of the cell envelope of *P. aeruginosa* which results in increased bacterial uptake of dibromopropamidine isethionate. Folinic acid reverses the effect of *p*-aminobenzoic acid on the permeability properties of *P. aeruginosa* cells.

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