Biochemical Pharmacology, Vol. 32, No. 3, pp. 449–453, 1983.
Printed in Great Britain.

0006-2952/83/030449-05 $03.00/0

ANTIMICROBIAL ACTION OF COMPOUND 48/80—II

MECHANISM OF ACTION*

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(*Received* 29 *June* 1981; *accepted* 28 *June* 1982)

Abstract—The mixture of compounds called compound 48/80 had been shown to have antimicrobial activity against a wide variety of microorganisms. In this paper it is shown that its primary site of attack appears to be on the membrane of the cell. In its presence, *Tetrahymena* became much more sensitive to osmotic stress, and α-methylglucose was rapidly released from preloaded *Escherichia coli* cells. The drug also had effects on cell viability, respiration, cell division, and the release of material absorbing at 260 nm. In general, its effects paralleled those of polymyxin B, although its structure is quite different except for the presence of amino groups and hydrophobic regions in both molecules. The activity of 48/80 was not due to detergent-like, surface-active properties and was antagonized by magnesium and other cations and by phosphatidylserine. Purification of the active principle might provide a relatively simple and readily modifiable probe of membrane function and possibly a new family of useful antimicrobial compounds.

Compound 48/80 is a family of polymers of *p*-methoxyphenethylmethylamine which produces marked depression of blood pressure when injected into mammals [1]. Paton [2] discovered that the hypotensive effect of 48/80 was due to the release of histamine, and since then it has been used extensively to study mast cell degranulation and histamine release. Previous studies of the antimicrobial effects of 48/80 indicated that it has activity against a wide spectrum of microorganisms, including protozoa, fungi, and bacteria [3, 4]. These studies also suggested that the polymers found in 48/80 might be purified or modified to separate the antimicrobial and hypotensive activities. Because of the continuing need for drugs that are effective against fungal and protozoal parasites or that can supplement existing antibacterial compounds, further studies on the mode of action of 48/80 were undertaken.

MATERIALS AND METHODS

Growth of microorganisms. Tetrahymena pyriformis strain GL and *Escherichia coli* strain AB 1157 were grown in peptone broth containing 1% proteose peptone (Difco, Detroit, MI), 0.1% K_2HPO_4, 0.1% glucose, and 0.1% sodium acetate. *T. pyriformis* and *E. coli* stock cultures were maintained in cotton-stoppered upright test tubes (18 × 150 mm) containing 7 ml of medium at room temperature. Cultures for experiments were prepared by inoculating 200 ml volumes of peptone broth with 2 ml of a 3 to 4-day-old stock culture and incubating this overnight at 28–29° with rotation on a New Brunswick (New Brunswick, NJ) incubator-shaker. Growth of organisms was measured by turbidity in a spectrophotometer (Spectronic 20, Bausch & Lomb, Rochester, NY) or by cell counts. Viability of *E. coli* was determined by standard plate count methods.

Compound 48/80. Initially, compound 48/80 was obtained from Burroughs Wellcome (Research Triangle Park, NC, courtesy of Dr. R. Baltzly), but later we synthesized it ourselves according to the procedure of Baltzly *et al.* [1]. The composition of the different lots from Burroughs Wellcome varied somewhat with regard to polymer distribution, as did our products, but all behaved qualitatively the same in the microbial assays.

Surface activity. The surface activities of solutions containing 48/80, benzalkonium chloride (Winthrop Laboratories, New York, NY), and Triton X-100 (Sigma Chemical Co., St. Louis, MO) were compared with that of distilled water by measuring their drop size. The volume/1000 drops was determined from the volume remaining in a burette after 1000 drops had fallen.

Release of cellular constituents. A modification of the procedure of De Lumen and Tappel [5] was used to assay for cathespin release. *T. pyriformis* was incubated in Ryley's buffer [6] at 28–29° in an incubator-shaker with either 48/80 or Triton X-100. A 0.2-ml sample of each supernatant fraction (5 min at 300 *g*) was mixed with 0.4 ml of cathespin activating solution (15 mM EDTA and 30 mM dithiothreitol, Sigma Chemical Co.) and incubated for 10 min at room temperature. Aliquots (0.2 ml) were added to tubes containing 3.1 ml of 0.05 M *N*-ethylmorpholine (pH 7.8) (Aldrich Chemical Co., Mil-

* Portions of this work were supported by grants from the National Institutes of Health, the Leahi Foundation, and the Hawaii Thoracic Society.

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Table 1. Effects of various compounds on reversal of the inhibition of *Tetrahymena pyriformis* cell division in peptone broth by 50 μg 48/80/ml*

Compound	Cell numbers (%)	P
48/80 Alone	49 ± 2	
1.7 mM Arg	95 ± 1	0.001
1.7 mM His	86 ± 6	0.02
1.7 mM Lys	92 ± 6	0.01
1.7 mM 48/80 Monomer	91 ± 7	0.02
48/80 Alone	48 ± 3	
1.0% Maltose	85 ± 8	0.02
0.5% Glucose	95 ± 2	0.006
0.5% Fructose	101 ± 5	0.01
0.5% Galactose	100 ± 3	0.01
48/80 Alone	40 ± 6	
5 mM Magnesium	82 ± 4	0.002
50 μg/ml Phosphatidylserine	59 ± 3	0.02

* Experiments were done in triplicate with approximately twenty-one fields (ranging from 21 to 450 cells per field) counted per replicate. The values represent the average percent increase (± S.E.) in cell number of treated cultures relative to control cultures (not shown).

waukee, WI) and 0.05 ml of 0.017 M α-*N*-benzoyl-DL-arginine-β-napthylamine HCl (BANA, the cathepsin substrate, Sigma Chemical Co.) in dimethyl sulfoxide (Sigma Chemical Co.). After incubation at 30° for 30 min, the flourescence of each solution was measured on a Aminco–Bowman spectrophotometer set for excitation at 340 nm and for emission at 404 nm.

Release of material absorbing light at 260 nm was measured by harvesting cells of *E. coli*, resuspending them in 1% NaCl with the indicated concentrations of drug, and sampling after 6 hr. The samples were harvested by centrifugation at 10,000 *g* for 10 min, and the supernatant fraction was read at 260 nm in a Beckman DU spectrophotometer. Parallel cultures containing no drug were used as controls.

Respiration. Oxygen consumption was measured with an oxygen meter (model 54, Yellow Springs Instrument Co., Yellow Springs, OH) which was calibrated against aerated distilled water using standard temperature corrections.

α-Methylglucose uptake. E. coli used in the α-methylglucose (α-MG) uptake and release experiments was grown overnight in peptone broth in which 10 g/l glycerol replaced the glucose. Cells were harvested by centrifugation (10 min at 12,000 *g*) and resuspended in the original volume of distilled water containing 0–50 μg 48/80/ml and 10 μl of α-MG (23,000 cpm/10 μl, uniformly labeled ^{14}C, 300 mCi/mmole, New England Nuclear Corp., Boston, MA). After incubation at 37° for 15 min, 0.1 ml was removed from each tube and filtered through a 0.45 μm cellulose nitrate filter (Millipore, Bedford, MA), washed twice with distilled water, dried in air overnight, and counted in a liquid scintillation counter [7]. A similar procedure was followed in experiments on the release of α-MG from preloaded cells, except that the mixture of cells and α-MG was incubated at 37° for 10 min before the drug was added. The time–course studies utilized a single, 1.5 ml incubation mixture to which 30 μl of α-MG was added. This was sampled immediately and at intervals before and after compound 48/80 or polymyxin B (Sigma Chemical Co.) was added.

Osmotic stress experiments. Cultures of *T. pyriformis* 18–24 hr old were collected by aspiration of

Table 2. Release of 260 nm absorbing material from *E. coli* in the presence of 50 μg 48/80/ml or 30 μg polymyxin B/ml and possible antagonists of their actions*

Treatment	Solutions in which bacteria were suspended					
	NaCl (1%)	Lys (1.7 mM)	His (1.7 mM)	Arg (1.7 mM)	$MgSO_4$ (0.01 M)	Glucose (0.5%)
No drug	100	106	115	98	65†	87
	†	†		†		†
48/80	142	158	149	149	71†	140
	†	†	†	†		†
Polymyxin B	200	184	201	192	84†	186

* Release is relative to that of control cultures in 1% NaCl (taken as "100", which represents a reading of about 0.300 at 260 nm) to which no drug was added. Each datum represents the average of five experiments.

† $P < 0.05$. For comparisons made vertically (i.e. between the "No drug" and "48/80" or "Polymyxin B" rows), the dagger (†) appears above the number; for comparisons made horizontally (i.e. between 1% saline and the amino acid, $MgSO_4$ or glucose solutions), the dagger appears to the right of the number.

the top 90% of the supernatant fraction after centrifugation (5 min at 300 *g*). A 0.1-ml sample of this stock suspension was diluted into 0.9 ml of distilled water or other test solutions.

Statistics. Probabilities for Tables 1 and 2 were calculated using Student's *t*-test for paired samples.

RESULTS

Surface activity of 48/80. Solutions containing 500 μg 48/80/ml yielded the same volume per 1000 drops as distilled water, while the same concentrations of the detergents benzalkonium chloride and Triton X-100 gave volumes of 58 and 45% of the control respectively. Thus, 48/80 at concentrations more than ten times that necessary for inhibition of the growth of microorganisms did not alter the surface tension of water. *T. pyriformis* cultures treated with up to 10 mg 48/80/ml and analyzed for the presence of the lysosomal enzyme cathepsin B did not release this enzyme. However, 1 mg Triton X-100/ml released all of the enzyme present, as determined by sonication.

Effects of medium composition. Higher concentrations of 48/80 were required to inhibit cell division by *T. pyriformis* in peptone-liver medium than in peptone broth, so the broth was supplemented with various constituents of the richer medium in order to identify the protective compounds. It was found that 1.7 mM of any one of the basic amino acids (arginine, histidine, or lysine) or *p*-methoxyphenethylmethylamine (48/80 monomer) could diminish the effects of 48/80 during one division cycle (Table 1). Increasing the magnesium concentration to 5 mM or the glucose concentration to 0.5%, or substituting equivalent concentrations of a number of other sugars, had similar effects. Phosphatidylserine also antagonized the antimicrobial action of 48/80 as it does the histamine-releasing activity of 48/80 [8], but water-soluble vitamins or non-basic amino acids had no effect.

Response to osmotic stress. T. pyriformis observed at 100-fold magnification in the absence of 48/80 remained normal in form and motility. When suspended in 20 μg 48/80/ml or more, virtually all cells were immobile after 10 min. Some had lysed, and most were round and swollen with prominent vacuoles. When dilution was made into Ryley's buffer rather than into distilled water, most cells remained motile and retained their usual form even in the presence of 50 μg 48/80/ml. When magnesium was omitted from Ryley's buffer the medium still protected the protozoa for at least 10 min, but $MgSO_4$ in the concentration found in Ryley's buffer (0.001 M) did not. However, the cells were almost completely protected against 50 and 100 μg 48/80/ml by 0.01 M $MgSO_4$. Sucrose (0.14 M) reduced the swelling and lysis seen in distilled water but did not prevent the drug from immobilizing the protozoa. Sodium chloride at concentrations of 0.01 M did not protect the cells, but at 0.1 M it was effective in maintaining normal morphology and motility in most of the *T. pyriformis*. The basic amino acids (Lys, His, or Arg, 2 mM) had no protective effect in these experiments.

After 10 min in distilled water containing 15 μg polymyxin B (PMB)/ml, many cells appeared normal while cells in a somewhat smaller fraction were swollen and immobile. Similar results were observed when the drug concentration was doubled. Although PMB is not usually considered to be effective against eukaryotic microbes, the high concentrations used in these experiments did seem to damage a significant fraction of the protozoa. Ryley's buffer lacking magnesium appeared to protect the organisms against PMB also, as virtually all cells suspended in this medium retained normal activity after 10 min in 30 μg PMB/ml.

Release of material absorbing at 260 nm. Because PMB causes material absorbing light at 260 nm to be released from Gram-negative organisms [9, 10], the effects of PMB and 48/80 on *E. coli* were compared (Table 2). Ultraviolet-absorbing material was released from cells suspended in distilled water or 1% NaCl during a 6-hr incubation period, but this release was increased by both 48/80 and PMB. Release from *E. coli* was slow, and both control and drug-treated cultures were still liberating material at a linear rate at the end of 6 hr. Glucose and the basic amino acids had no effect on such release, but 0.01 M $MgSO_4$ appeared to reduce the amount of 260 nm absorbing material liberated by untreated cultures or cultures treated with either drug. No release from *T. pyriformis* was observed with either drug.

Viability studies. PMB rapidly kills *Salmonella typhimurium* [11]. Similarly, *E. coli* exposed to 50 or 100 μg 48/80/ml for 30 min and then diluted and plated exhibited 3- and 20-fold decreases in the number of viable cells. It appeared that 48/80 killed some of the bacteria, but many cells survived rather large doses of this drug.

Respiration. Oxygen utilization by both *T. pyriformis* and *E. coli* suspended in distilled water or 0.14 M sucrose was rapidly inhibited by 50 μg 48/80/ml or 30 μg PMB/ml (Fig. 1). The presence of peptone broth permitted normal respiration, apparently due to the peptone itself, because this constituent had the effect by itself, while other components of the medium did not. Glucose (1%) and the basic amino acids (His, Arg, and Lys, 2 mM each) did not protect the cells against 48/80, but 0.01 M $MgSO_4$, $MgCl_2$ or $MnCl_2$ did permit normal respiration to continue in the presence of the drug. The inhibition of respiration by the two drugs occurred in parallel in *E. coli*, but in *Tetrahymena* 48/80 appeared to inhibit respiration more rapidly than PMB (Fig. 1).

Uptake and release of α-methylglucose. α-MG is taken up by bacterial cells and may be phosphorylated, but it is not further metabolized [12]. *T. pyriformis* did not appear to take up this compound, but *E. coli*, suspended in distilled water, took it up rapidly, reaching steady-state intracellular concentrations in about 15 min, and retained it for an hour or more. Addition of 50 μg 48/80/ml or 30 μg PMB/ml led to rapid loss of most of the label from the cells (Fig. 2). Low levels of 48/80 seemed to stimulate concentration of α-MG by the cells, but concentrations above 15 or 20 μg/ml caused rapid efflux of the label. In peptone broth, efflux of α-MG was slower and prolonged.

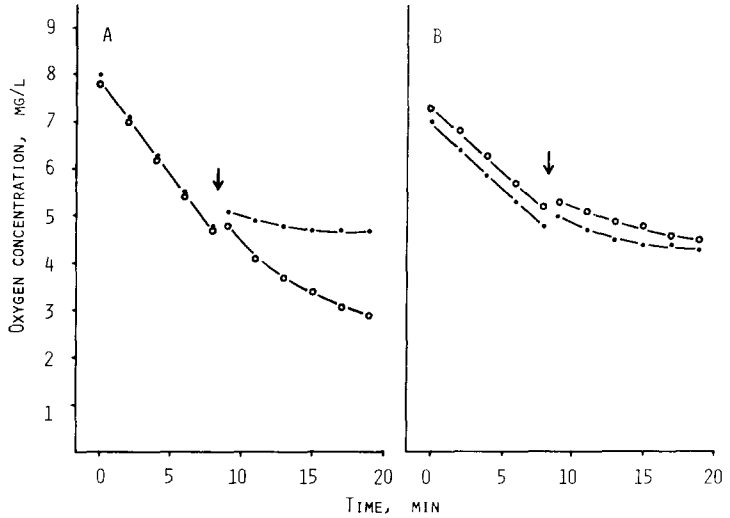

Fig. 1. Representative experiments of utilization of oxygen by (A) *T. pyriformis* and (B) *E. coli* in the presence of 50 μg 48/80/ml (●) or 30 μg PMB/ml (○). The arrows indicate the time at which each drug was added.

As has been reported for polymyxin B [11], treatment of preloaded cells with 48/80 did not lead to complete excretion of α-MG. This is consistent with the observation that many of the cells remained viable in the presence of the drug concentrations used and indicates that the permeability of a portion of the cells may not have been affected by the drug, of that the breakdown of the permeability barrier was not complete.

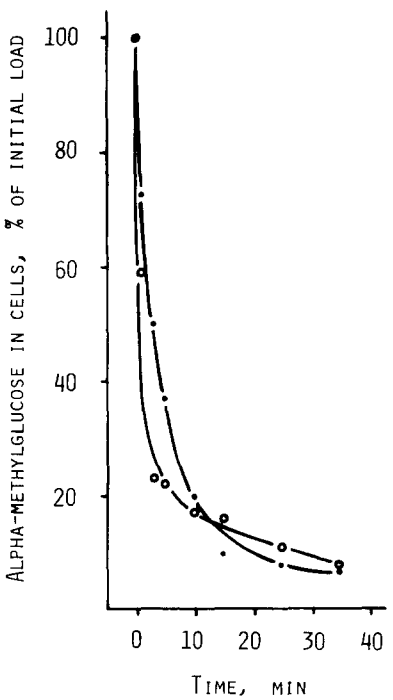

Fig. 2. Representative experiments of release of [^{14}C]-α-MG from pre-loaded cells of *E. coli* by 50 μg 48/80/ml (●) or 30 μg PMB/ml (○). Each drug was added at time zero. The amount of α-MG in the cells is plotted as a percent of the initial load (3500 cpm/sample) measured just before drug was added.

DISCUSSION

Compound 48/80 contains both hydrophobic and hydrophilic regions and, therefore, could have been acting as a detergent in inhibiting the division of the cultures. However, studies on its surface-active properties indicated that it had essentially no surfactant activity. Unlike the detergent Triton X-100, it failed to release the cathepsin B from cells of *T. pyriformis*.

PMB, like 48/80, stimulates the release of histamine from mast cells [13]. When it was observed that magnesium inhibited the effect of 48/80 on the growth of *T. pyriformis*, just as it inhibits release of 260 nm absorbing material from *E. coli* treated with PMB [10], it seemed possible that the antimicrobial activities of the two drugs involved similar mechanisms. Therefore, these two drugs were compared for their effects on osmotic sensitivity, membrane permeability, and respiration.

The rapid efflux of α-MG from preloaded cells in the presence of 48/80 suggests that the primary action of this material was on the membrane of the cell and affected its permeability. This conclusion is supported by the increased sensitivity to osmotic stress seen in *T. pyriformis* in the presence of 48/80. PMB had similar effects. The release of 260 nm absorbing material from *E. coli* by both drugs was much slower than that of α-MG, suggesting that it may have resulted from the degradation of nucleic acids in injured or dying cells, or to a progressive increase in the permeability of the membrane to large molecules. It seems probable that the effects of 48/80 on respiration were also secondary to its action on membrane permeability. The more rapid inhibition of respiration in *Tetrahymena* by 48/80 than by PBM is consistent with the selectivity of the latter compound for prokaryote membranes. The data suggest that 48/80 is much less selective in this respect.

It is not clear why low levels of 48/80 stimulate uptake of α-MG. However, this phenomenon sup-

ports the hypothesis that 48/80 acts on the plasma membrane of the cell, because it seems unlikely that an effect on other possible targets of the drug, such as a metabolic pathway or the synthesis of a macromolecule, would lead to increased uptake of the unmetabolizable α-MG.

The ability of magnesium and other cations to antagonize the action of 48/80 can be explained if there is a negatively charged site in the membrane to which the drug binds, and for which these other cations can compete. Although the structures of 48/80 and polymyxin B must be very different in detail, they are similar in having multiple amino groups which are positively charged at physiological pH, and in containing aromatic, hydrophobic residues. Membrane phospholipids have been implicated as the probable binding site for polymyxin B [11, 14], and so similar sites may be involved in the activity of 48/80. This hypothesis is supported by the observation that phosphatidylserine antagonizes both its histamine-releasing and antimicrobial actions, and that it is able to form an inactive complex with it [8]. Further evidence bearing on the question of membrane binding was obtained in the experiments on osmotic stress. Ryley's phosphate buffer, with or without magnesium, protected *T. pyriformis* against 50 μg 48/80/ml, while approximately equal osmolar sucrose prevented swelling but not immobilization of the cells. Magnesium (0.01 M) or NaCl (0.1 M) also protected the protozoa, indicating that the effect was not specific to one cation. It seems plausible that the cations in these salt solutions compete successfully with 48/80 for the membrane binding site and, thus, prevent the increased permeability to water and other membrane effects. The protective effects of peptone broth can probably be explained by a similar mechanism, with cationic peptides competing with 48/80 for the membrane binding sites. Sucrose can alleviate the osmotic stress induced by increased membrane permeability but not the effects due to leakage of cell contents or alterations of the membrane structure. The alternative explanation, that the salt solutions provide ions to replace those lost from the cells when the membrane becomes more permeable, seems unlikely because of the variety of cations that provide protection, including sodium, which is not found in the cytoplasm in high concentration.

Sugars and the basic amino acids permitted division of *T. pyriformis* inhibited by 48/80, but did not protect *T. pyriformis* from osmotic stress, reduce the loss of A_{260} absorbing material from *E. coli*, or protect either organism from inhibition of respiration. Possibly these compounds provide the extra nutrients necessary to permit survival of the organisms in the partially protected environment of the peptone broth and do not compete as effectively for the 48/80 binding site to protect the cells under more stressful test conditions.

These data suggest that 48/80 contains some potent antimicrobial compound(s). Since the material is a mixture of many components, it is not possible to determine just how effective the active principle is against test organisms. It seems likely, however, that only a fraction of the total mixture has significant antimicrobial activity, and it is possible that different components possess different activities. If 48/80 is to be considered a candidate for therapeutic use, a problem will be its powerful histamine-releasing activity. There is some evidence that the histamine-releasing and antimicrobial activities reside in different fractions of the mixture [4]. If it is possible to separate these two activities, the antimicrobial compound could prove to be a valuable therapeutic agent, particularly against such microorganisms as fungi and protozoa, which are difficult to control with current drugs. Its relatively simple structure should permit numerous modifications, so perhaps the histamine-releasing activity could be removed by structural alterations. Even if 48/80 proves to be unsuitable for medical use, however, it might become a useful tool for exploring membrane structure and function.

Acknowledgements—We thank Mrs. Charlotte Oda for the synthesis of the 48/80 used in this work and Dr. E. F. Kiefer for supplying the materials for its preparation. We are also indebted to Dr. S-C. Chou for giving us the *Tetrahymena* strain used and to Mr. D. Ichinotsubo for the E. coli.

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