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Enhancement of Bismuth Antibacterial Activity with Lipophilic Thiol Chelators

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The antibacterial properties of bismuth are greatly enhanced when bismuth is combined with certain lipophilic thiol compounds. Antibacterial activity was enhanced from 25- to 300-fold by the following seven different thiols, in order of decreasing synergy: 1,3-propanedithiol, dimercaprol (BAL), dithiothreitol, 3-mercapto-2-butanol, β-mercaptoethanol, 1-monothioglycerol, and mercaptoethylamine. The dithiols produced the greatest synergy with bismuth at optimum bismuth-thiol molar ratios of from 3:1 to 1:1. The monothiols were generally not as synergistic and required molar ratios of from 1:1 to 1:4 for optimum antibacterial activity. The most-active mono- or dithiols were also the most soluble in butanol. The intensity of the yellow formed by bismuth-thiol complexes reflected the degree of chelation and correlated with antibacterial potency at high molar ratios. The bismuth-BAL compound (BisBAL) was active against most bacteria, as assessed by broth dilution, agar diffusion, and agar dilution analyses. Staphylococci (MIC, 5 to 7 μM Bi^{3+}) and *Helicobacter pylori* (MIC, 2.2 μM) were among the most sensitive bacteria. Gram-negative bacteria were sensitive (MIC, <17 μM). Enterococci were relatively resistant (MIC, 63 μM Bi^{3+}). The MIC range for anaerobes was 15 to 100 μM Bi^{3+}, except for *Clostridium difficile* (MIC, 7.5 μM). Bactericidal activity averaged 29% above the MIC. Bactericidal activity increased with increasing pH and/or increasing temperature. Bismuth-thiol solubility, stability, and antibacterial activity depended on pH and the bismuth-thiol molar ratio. BisBAL was stable but ineffective against *Escherichia coli* at pH 4. Activity and instability (reactivity) increased with increasing alkalinity. BisBAL was acid soluble at a molar ratio of greater than 3:2 and alkaline soluble at a molar ratio of less than 2:3. In conclusion, certain lipophilic thiol compounds enhanced bismuth antibacterial activity against a broad spectrum of bacteria. The activity, solubility, and stability of BisBAL were strongly dependent on the pH, temperature, and molar ratio. Chelation of bismuth with certain thiol agents enhanced the solubility and lipophilicity of this cationic heavy metal, thereby significantly enhancing its potency and versatility as an antibacterial agent.

Bismuth compounds have been used in medicine for more than 2 centuries (2). Applications have been widespread, due to bismuth's antiseptic, astringent, protective, antacid, antisecretory, and local gastrointestinal properties (11). The range of clinical applications includes dyspepsia, diarrhea, syphilis, and warts. Bismuth compounds are also used in wound dressings, as an antiseptic for topical application on the skin, and for oral and upper-respiratory-tract infections (17). Special emphasis has been placed recently on the use of bismuth for duodenal ulcers, peptic diseases, and the eradication of *Helicobacter pylori* (11). Among the compounds used in treatment are bismuth subsalicylate (BSS), bismuth subnitrate, tripotassium dicitratobismuthate, bismuth subcitrate, and colloidal bismuth subcitrate. In addition, several organometallic bismuth compounds are used in bactericidal and fungicidal applications (12).

BSS (Pepto-Bismol), in combination with other agents, is often prescribed in the treatment of peptic ulcers and certain diarrheas (8, 10). Treatment with BSS decreased the duration of diarrhea in infants (9). Pepto-Bismol combined with ampicillin or tetracycline and metronidazole has been used to treat *H. pylori* peptic ulcers with reasonable success. Bismuth is an important component in the cure of ulcers, particularly with regard to recrudescence (10, 14). Triple therapy with a bismuth salt plus two antibiotics is one of the most-effective regimens in the eradication of *H. pylori* (5).

Despite its popular use, bismuth exhibits only modest antibacterial activity (4, 13). Therapy provides short-term effects, requiring administration of relatively large, frequent doses (8), which increase the chances of toxicity. Problems with toxicity have curtailed the use of bismuth compounds in wound dressings (19) and for gastrointestinal purposes (17, 18). The limited antibacterial effect is attributed to poor solubility of bismuth in water, since millimolar concentrations of bismuth are required to inhibit bacterial growth (13). However, solubility is dependent on the pH of the medium and the presence of certain compounds with hydroxy or sulfhydryl groups (17). Therefore, chelation of bismuth with thiol compounds would be expected to enhance bismuth solubility. This would reduce the dosage level necessary for effective treatment and would decrease toxicity.

While the literature is replete with studies of the toxicology of bismuth and the use of thiols in antidotal therapy (1, 17), virtually nothing has been reported on the microbiology of bismuth-thiol complexes. Several such complexes were generated in our efforts to improve bismuth activity and solubility. Some of these agents possessed broad-spectrum bactericidal activity at the low-micromolar level. The present study aims to characterize and optimize the activity, stability, and solubility

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of these new agents and to elucidate the mechanisms by which these novel compounds exert their antibacterial effects.

MATERIALS AND METHODS

Bacterial strains and cultivation. Bactrol reference cultures (Difco Laboratories, Detroit, Mich.) were used to test a broad spectrum of bacteria. Bactrol strains include *Enterobacter cloacae* ATCC 23355, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus vulgaris* ATCC 13315, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 8100, *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19615, *Enterococcus faecalis* ATCC 29212, and *Salmonella typhimurium* ATCC 14028. Other enteric pathogens used were enterotoxigenic *E. coli* ATCC 43896, enterohemorrhagic (verotoxin-producing) *E. coli* ATCC 35150, *Shigella flexneri* ATCC 12022, and *Yersinia enterocolitica* ATCC 27729. Vancomycin-resistant enterococci (VRE) and methicillin-resistant *S. aureus* (MRSA) were clinical strains. *Legionella pneumophila* 3-69, B2-5-D1, and ATCC 33152 and the Glover isolate were provided by Binax, Inc. Anaerobic bacteria included *Clostridium difficile* ATCC 9689, *Bacteroides fragilis* ATCC 23745, one *B. fragilis* clinical isolate, *Clostridium histolyticum* ATCC 19401, *Clostridium perfringens* ATCC 13124, *Bacteroides ovatus* ATCC 8483, and *Actinomyces odontolyticus* ATCC 17929. Another strain tested was *Enterococcus faecium* ATCC 2358. Anaerobic culture conditions were established by using the BBL GasPak Plus system. Anaerobic bacteria were cultured on Trypticase soy agar containing 5% sheep blood (BBL, Cockeysville, Md.). Six antibiotic-resistant *H. pylori* clinical strains were cultured on Mueller-Hinton blood agar in BBL Campy Pouch Microaerophilic Systems (Becton Dickinson, Cockeysville, Md.).

Susceptibility studies. Susceptibility studies were performed by several methods, including broth dilution, agar dilution, and agar diffusion. For broth dilution studies, starter cultures were grown to mid-log phase at 35°C for 4 h at 200 rpm and were used to prepare 0.5-McFarland standard suspensions, which were further diluted 1:100 (5×10^5 CFU/ml) in Mueller-Hinton broth medium (BBL). Cultures were incubated and monitored for turbidity in an Avantage Microbiology System (Abbott Laboratories, Irving, Tex.). Growth inhibition was determined by monitoring lag times before initiation of culture growth (6) and by standard plating of bacteria in triplicate at 24 h of growth. Culture lag times were obtained from computer-generated growth curves. The MIC was expressed as the lowest drug concentration that inhibited turbidity for 24 ± 2 h. Viable bacterial counts (in CFU per milliliter) and subcultures were performed by standard plating on nutrient agar (BBL). For agar diffusion studies, a sterile swab was used to spread a 0.5-McFarland standard onto nutrient agar. Absorbant paper disks were placed on the agar surface. The disks were impregnated with as much as 15 μl of solutions containing bismuth nitrate, dimercaprol (BAL), or both. The plates were incubated overnight at 36°C. The diameter of the zone of inhibition, including the 6-mm disk diameter, was measured with a vernier caliper. Agar diffusion studies were generally performed with Mueller-Hinton II agar. *Legionella* strains were cultured and tested on BCYE agar (BBL). For agar dilution studies (*H. pylori* and anaerobes), Mueller-Hinton blood agar containing the bismuth-BAL compound (BisBAL) was prepared. Molten Mueller-Hinton agar medium was cooled to 50°C, and defibrinated horse blood was added to a final concentration of 5%. BisBAL powder was added progressively while the agar plates were poured, producing agar medium with incremental concentrations of BisBAL. Suspensions equivalent to a no. 1 McFarland standard of *H. pylori* were spotted (10 μl) on the agar surface. The plates were incubated in Campy Pouches at 37°C for 5 days. The MIC was expressed as the lowest drug concentration that inhibited growth.

Bactericidal assays were performed in broth medium. Minimal bactericidal concentrations were recorded as the concentration of drug that reduced the initial viable bacterial count by 99.9% at 18 to 24 h of incubation. Viable bacterial counts were determined by standard plating on appropriate agar media. The influence of pH on bactericidal activity was determined by washing log-phase *E. coli* ATCC 25922 cells in saline and adjusting the viable count to 10^9 CFU/ml. Samples containing 10 mM of the following buffers at the indicated pHs were prepared: citrate, pH 4; 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5 and 6; 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7; and Tris, pH 8 and 9. BisBAL was added to the samples at 100 μM. Cultures were incubated at 36°C for 24 h and sampled repeatedly for colony counting.

Bactericidal activity of BisBAL against *E. coli* ATCC 25922 at several temperatures was assessed. A saline suspension of 10^9 late-log-phase bacteria per ml was incubated with BisBAL at a ratio of 75 μM bismuth to 37.5 μM BAL (75/37.5 μM BisBAL) at 25, 35, 42, and 50°C. Bacterial viability was determined by standard agar plating on nutrient agar (BBL).

Stability and solubility. BisBAL was prepared at molar ratios of between 2:1 and 1:2 by adding 2.5 to 10 μl of 10 M BAL (Sigma Chemical Co., St. Louis, Mo.) to 1 ml of 50 mM $Bi(NO_3)_3$ in propylene glycol. Samples were diluted in water or propylene glycol. The final concentration of propylene glycol was kept at ≤1% to avoid confounding antibacterial effects. The pH was adjusted by the addition of 10 N NaOH or concentrated HCl. Samples were tested weekly for stability against *E. coli* ATCC 25922 in broth cultures. Solubility testing involved mixing BisBAL components at various molar ratios and sedimenting precipitates by centrifuging for 2 min in an Eppendorf 5415 Microfuge. Sedimented BisBAL was lyophilized in preweighed tubes. Sediment weight was measured and divided by 20.8 (total weight in milligrams of components) to obtain percent solubility. Titration experiments involved the addition of NaOH to 5 ml of 100 mM BAL or 50/100 mM BisBAL in water and recording the pH increment after each addition of hydroxide.

The lipophilicity of bismuth-thiols was assessed by two-phase separation in 1-butanol. Bismuth-thiol solutions were prepared at 5/10 mM in purified water at pH 9 to 10 by the addition of 10 N NaOH. An equal volume of 1-butanol was added, and the tube was mixed vigorously for 30 s and pulse-centrifuged to separate liquid phases. A_{410} values of the bright-yellow solution in both phases in a Milton Roy Spectronic 601 UV/VIS spectrophotometer were recorded. The absorbance of BisBAL in butanol was greater than that in water and was adjusted to 0.8 of the raw absorbance data.

Biochemicals. Stock solutions of 50 mM $Bi(NO_3)_3$ (Sigma) were prepared in propylene glycol (Sigma). Thiol reagents and chelating agents obtained from Sigma were added at various ratios. The standard molar ratio in screening for synergy was 1:2 (bismuth to thiol). All solutions, except those used in stability studies, were prepared daily and kept at room temperature.

RESULTS

In an effort to enhance activity by increasing solubility, bismuth was combined with several potential chelating agents. The nonthiol compounds tested were D-penicillamine, 2,2′-dipyridyl, protocatechuate ethyl ester, 2,3-dihydroxybenzoate, desferrioxamine, 2,4-diaminobutyric acid, spermidine, *cis*-1,3-dichloropropene, EDDA, 2-bromo-2-nitro-1,3-propanediol, salicylhydroxamic acid, sodium bisulfite, and EDTA. The thiols that were tested included BAL, β-mercaptoethanol (βME), 2-mercaptoethylamine (MEN), dithiothreitol (DTT), dimercaptopropane-1-sulfonic acid (DMPS), dimercaptosuccinic acid (DMSA), 1-monothioglycerol (MTG), 1,3-propanedithiol, (PDT), 3-mercapto-2-butanol (MBO), 2-mercaptopyrimidine, 2-thiouracil, 1-thio-β-D-glucose, thiosalicylic acid, thimerosal, thiolactic acid, meso-1-1′-dimercaptoadipic acid, 2,3-dimercaptopropanol tributyrate, thioglycolic acid, thiostrepton, L-cysteine, reduced glutathione, *p*-thiocresol, thiodiglycol, 2-mercaptobenzothiazole, pyrithione, and thioanisole. The nonthiol compounds had no influence on bismuth antibacterial activity. However, the thiol chelators BAL, PDT, DTT, MBO, βME, MEN, and MTG enhanced bismuth antibacterial activity by 25- to 300-fold, as measured by inhibition of *E. coli* growth (Table 1). The MICs for bismuth nitrate or thiols separately were in the low-millimolar range (~3 mM) but could not be determined with precision due to their insolubility in growth media. None of the thiol acids (i.e., DMPS or DMSA) enhanced activity when combined with bismuth, although they exhibited similar properties in solution, such as yellow appearance and enhanced water solubility.

The seven active bismuth-thiol complexes showed optimum activity at different molar ratios. The relationship between molar ratio and MIC is illustrated in Fig. 1. The antibacterial activity of bismuth increased with increasing thiol concentrations but reached a plateau at which further addition of thiol had diminishing effects. The inhibitory concentration of bismuth at the optimum molar ratio (MIC_{opt}) was essentially the optimum antibacterial activity achieved with the least amount of thiol added. The MICs of the four most active bismuth-thiol compounds (BisPDT, BisBAL, BisDTT, and BisMBO) were comparably low when enough thiol was added. At their respective optimum ratios, the MICs were nearly 300-fold lower than that of $Bi(NO_3)_3$ (MIC, ~3,000 μM). However, only BisPDT and BisBAL were effective at a 3:1 ratio. The MIC_{opt}s for BisDTT and BisMBO occurred at ratios of 1:1. The MIC_{opt} for BisβME was twice that of BisBAL and occurred at a 1:2 molar ratio. Finally, the MIC_{opt}s for MEN and MTG occurred at molar ratios of 1:3 and 1:4, respectively, and were 8-fold higher than that of BisBAL but 25-fold lower than that of $Bi(NO_3)_3$.

A summary of the data for MICs against *E. coli*, both at the MIC_{opt} and at a 2:1 ratio, is given in Table 1. The dithiols produced the greatest synergy with bismuth at optimum bis-

TABLE 1. Relationship between pigment intensity, lipophilicity, optimum molar ratio, and bacteriostatic concentration for several bismuth-thiol compounds

Thiol	$MIC_{2:1}$[a]	MIC_{opt}[b]	Optimum ratio[c]	% Bismuth-thiol in butanol[d]	A_{410}[e]	Molecular formula
Dithiols						
PDT	8.6	12.0	3:1	58.5 ± 4.9	12.4 ± 0.88	$SHCH_2CH_2CH_2SH$
BAL	12.0	12.0	2:1	15.5 ± 1.7	6.2 ± 0.23	$OHCH_2CHSHCH_2SH$
DTT	20.9	10.8	1:1	2.7 ± 0.5	2.0 ± 0.04	$SHCH_2(CHOH)_2CH_2SH$
DMSA	3,000	3,000		0	1.9 ± 0.01	$CO_2H(CHSH)_2CO_2H$
Monothiols						
MBO	36.5	11.1	1:1	81.5 ± 0.5	1.0 ± 0.10	$CH_3CHSHCHOHCH_3$
βME	98.8	26.7	1:2	38.3 ± 0.1	1.5 ± 0.01	CH_2OHCH_2SH
MEN	291	154	1:3	8.1 ± 0.5	2.6 ± 0.05	$CH_2NH_2CH_2SH$
MTG	650	120	1:4	8.5 ± 0.5	2.1 ± 0.56	$CH_2OHCHOHCH_2SH$

[a] MICs are micromolar bismuth against *E. coli* in broth at a 2:1 molar ratio; means of three trials.
[b] MICs against *E. coli* at the optimum molar ratio; means of three trials.
[c] Molar ratio of bismuth to thiol producing optimum antibacterial activity.
[d] Percentages of yellow pigment from 5/10 mM bismuth-thiol solutions partitioned from H_2O into butanol.
[e] Yellow pigment intensity of a 5/10 mM bismuth-thiol solution.

muth-thiol molar ratios of 3:1 to 1:1. The monothiols were generally not as synergistic and required molar ratios of 1:1 to 1:4 for optimum antibacterial activity. There is a strong relationship between antibacterial activity and the lipophilicity of bismuth-thiol compounds, especially when monothiols and dithiols are separated. The most active bismuth-thiol in each category was also the most soluble in butanol (Table 1). BisPDT and BisBAL also produced the most yellow appearance in alkaline solution (Table 1), which is an indication of the amount of complex formed.

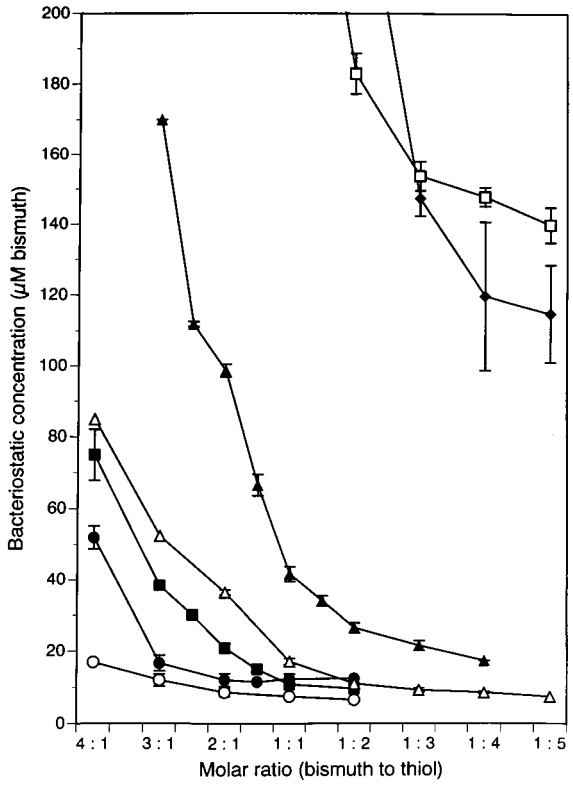

FIG. 1. Effect of molar ratio on bismuth-thiol chelator bacteriostatic activity. Bismuth nitrate was combined with PDT (○), BAL (●), DTT (■), MBO (△), βME (▲), MEN (□), and MTG (◆) at different molar ratios and added to broth medium. The susceptibility of *E. coli* was determined in triplicate at molar ratios of between 4:1 and 1:4. The bacteriostatic concentration was defined as the concentration of bismuth in combination with chelator that inhibited growth for 24 ± 2 h. Data are from trials performed in triplicate to obtain the means and standard deviations.

For comparison with bismuth, several trivalent metals were combined with thiol compounds and were tested for antibacterial activity. The metals tested included ferric iron, aluminum (III) chloride, chromium (III) chloride, gallium (III) oxide, ruthenium red, scandium (III) oxide, yttrium (III) nitrate, and ytterbium (III) oxide. No enhanced antibacterial activity against *E. coli* was noted with BAL combined with any of these metal ions at a 1:2 molar ratio. Enhancement by BAL of antimony or arsenic antibacterial activity was noted but was not of the same magnitude as that of bismuth (unpublished data).

Several bismuth salts were used in the preparation of BisBAL. Bismuth nitrate was the most convenient bismuth salt studied. Bismuth subgallate, bismuth citrate, bismuth oxide, and BSS were not as soluble as $Bi(NO_3)_3$ when they were combined with BAL at 2:1 molar ratios. Combinations of BAL with BSS (MIC, 12.4 μM Bi^{3+}) and bismuth citrate (MIC, 12.6 μM Bi^{3+}) were nearly as effective against *E. coli* as the combination of BAL with bismuth nitrate (MIC, 10.9 μM Bi^{3+}), but BAL plus bismuth subgallate (MIC, 27.3 μM Bi^{3+}) and bismuth oxide (MIC, 205.0 μM Bi^{3+}) were not as active.

The stability of concentrated BisBAL solutions was dependent on pH, temperature, and BAL concentration. BisBAL was stable in acidic but not in near-neutral or basic solutions. At pH 9, the half-life ($t_{1/2}$) of 500 μM Bi^{3+}–600 μM BAL at 25°C was approximately 3 weeks, while that for a 500–150 μM solution was <1 week. Thus, at alkaline pH, the stability depended on the BisBAL molar ratio. In contrast, 2:1 BisBAL solutions at pH 2 exhibited no noticeable loss of activity after 2 months at 25°C. BisBAL aqueous solutions (5 mM–2.5 mM) were stable indefinitely at pH <4 but gradually degraded at pH 6 to 7 ($t_{1/2}$, 1 to 2 weeks). BisBAL solutions prepared in propylene glycol showed no loss of stability at pH values of between 3 and 11.6, but the addition of 100 mM HCl destroyed activity. Autoclaving BisBAL solutions destroyed activity, but heating to 100°C for 30 min had no discernible effect on activity.

Bactericidal activity increased with increasing temperature. A 75/37.5 μM BisBAL solution reduced the viable *E. coli* count

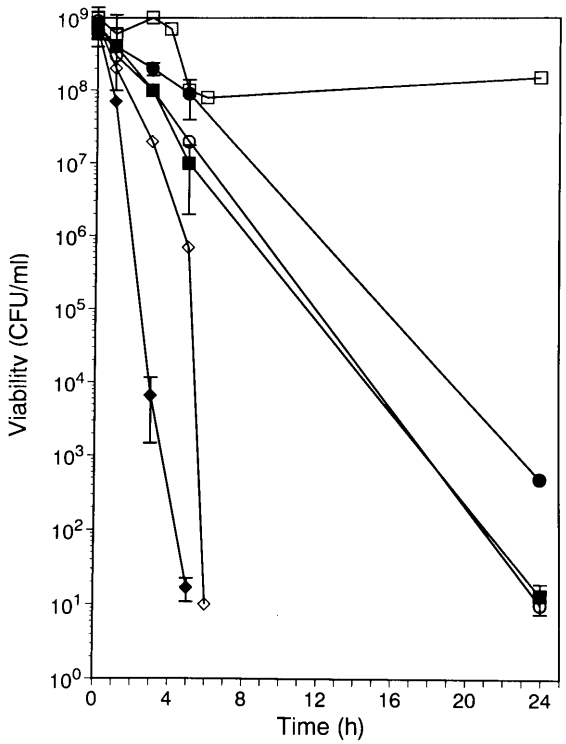

FIG. 2. Effect of pH on BisBAL bactericidal activity. *E. coli* cells were grown to mid-log phase in broth culture, washed once and resuspended in saline, and treated with 100/50 μM BisBAL in the presence of 10 mM buffer at pH 4 (□), pH 5 (■), pH 6 (○), pH 7 (●), pH 8 (◇), and pH 9 (◆). The cultures were sampled at 1, 3, 5, and 24 h. The reduction in viability over time was determined in triplicate by standard agar plating of appropriate dilutions to obtain the means and standard deviations.

at 24 h by 1 log unit at 25°C, by nearly 6 log units at 35°C, and by 9 log units at 42 or 50°C. Bacteria incubated at these temperatures without BisBAL present showed no decrease in viability at 25 or 35°C, 1 log unit decrease at 42°C, and a 3- to 4-log-unit decrease at 50°C.

Titrations of BAL and BisBAL (1:2 molar ratio) with NaOH were performed. The results of the BAL titration revealed two inflection regions of pH 9 to 10 and 10 to 11, corresponding to pK_a values of 8.5 and 10 for the two thiol groups on BAL. The solution was homogeneous and clear throughout the titration. The titration curve of BisBAL also revealed two inflection regions at pH 3.5 to 5.0 (pK_a 2.9) and pH 6.0 to 9.0 (pK_a 5.3). In contrast to the BAL titration, the solution was turbid, with a yellow precipitate, until pH 8.0, when the precipitate dissolved to form a clear yellow solution.

Another pH-dependent variable was antibacterial activity (Fig. 2). Bactericidal activity against 10^9 *E. coli* cells per ml with 100/50 μM BisBAL increased progressively from pH 4.5 to 9. This follows the titration curve of BisBAL with NaOH and the solubility behavior of the yellow BisBAL precipitate. At pH 8 and 9, bactericidal activity was relatively rapid, reducing viable bacteria to nearly 10^1 CFU/ml at 5 h of incubation. At pH 5 to 7, bactericidal activity was thorough at 24 h; however, only a 1- or 2-log-unit reduction was observed at 5 h of incubation. At pH 4, no bactericidal activity above that of control values was observed, indicating that BisBAL is largely inactive at this pH. Subjecting *E. coli* to pH 4 or 9 without BisBAL present had only marginal effects on viability.

The spectrum of activity for BisBAL proved to be rather broad. Inhibitory and bactericidal concentrations for BisBAL against several medically important bacteria are listed in Table 2. The MICs ranged from 5.9 to 63.0 μM Bi^{3+} for liquid and from 8.3 to 33.2 μg of powder per ml. *E. faecalis* was most resistant to BisBAL. In contrast, *S. aureus* and *S. pyogenes* were sixfold more sensitive to BisBAL. BisBAL also inhibited the enteric gram-negative pathogens, *S. typhimurium*, enterotoxigenic *E. coli*, enterohemorrhagic (verotoxin-producing) *E. coli*, *S. flexneri*, and *Y. enterocolitica*, as well as *E. cloacae*, *K. pneumoniae*, *P. vulgaris*, and *P. aeruginosa*. Inhibition by BisBAL of several pathogens was enhanced by >100-fold over that of bismuth alone. Bactericidal concentrations were 25 to 30% higher than the MIC. Overall, only *E. faecalis* was resistant to the bactericidal activity of BisBAL.

Several bacteria were also tested for susceptibility by agar diffusion. Generally, all bacteria tested were more sensitive to BisBAL than to bismuth nitrate or BAL alone, as demon-

TABLE 2. Susceptibility of bacteria to BisBAL in broth dilution[a]

Organism	BisBAL 2:1 liquid (μM Bi^{3+})		BisBAL powder (μg/ml)[b]	
	MIC	MBC	MIC	MBC
Gram-positive bacteria				
S. aureus ATCC 25923	10.7 ± 1.0	23.1 ± 1.3	8.3 ± 1.7	18.0 ± 2.0
S. pyogenes ATCC 19615	10.0 ± 0.9	36.3 ± 2.9	NP[c]	NP
E. faecalis ATCC 29212	63.0 ± 4.5	>350	33.2 ± 3.2	>200
Gram-negative bacteria				
Y. enterocolitica ATCC 27729	5.9 ± 0.7	7.5 ± 0.0	7.7 ± 0.6	9.3 ± 0.6
S. flexneri ATCC 12022	6.2 ± 0.8	7.0 ± 1.0	8.5 ± 0.7	10.3 ± 0.6
S. typhimurium ATCC 14028	8.0 ± 0.8	10.0 ± 0.4	11.0 ± 1.0	16.7 ± 1.5
E. cloacae ATCC 23355	9.7 ± 0.4	10.5 ± 0.0	18.4 ± 2.3	20.0 ± 1.8
E. coli ATCC 25922	10.9 ± 0.3	11.9 ± 0.9	16.6 ± 1.7	22.0 ± 2.3
K. pneumoniae ATCC 13883	11.0 ± 0.5	12.1 ± 0.3	18.7 ± 1.8	23.4 ± 1.1
E. coli ATCC 35150	13.0 ± 0.8	14.7 ± 1.4	17.3 ± 1.6	19.7 ± 1.5
E. coli ATCC 43896	14.7 ± 0.9	15.8 ± 0.2	19.8 ± 1.3	21.0 ± 1.0
P. aeruginosa ATCC 27853	15.5 ± 2.3	28.8 ± 12.0	25.0 ± 2.8	45.0 ± 1.0
P. vulgaris ATCC 13315	16.4 ± 4.5	23.5 ± 1.3	25.8 ± 0.5	28.7 ± 3.1

[a] MIC, drug concentration inhibiting bacterial growth for 24 ± 2 h; MCB, drug concentration reducing viability by 99.9%.
[b] Lyophilized precipitate from BisBAL at a molar ratio of 1.5:1 in basic aqueous solution.
[c] NP, not performed.

TABLE 3. Susceptibility of bacteria to BisBAL in agar diffusion

Bacterium (no. of isolates)	Zone of inhibition (mm) for:		
	$Bi(NO_3)_3$ (157 μg)	BAL (186 μg)	BisBAL (157/31 μg)
MRSA (47)	6.8	6.0	18.7 ± 3.1
VRE (18)	6.7	6.0	10.9 ± 1.6
E. coli (6)	7.3	6.0	12.5 ± 1.3
L. pneumophila (4)	6.0	6.0	15.6 ± 1.0
P. aeruginosa (3)	6.0	6.0	16.0 ± 1.1

strated by the zones of inhibition recorded in Table 3. Among gram-positive bacteria, BisBAL was very effective against staphylococci but less active against streptococci. Of 47 MRSA isolates and 18 VRE, the MRSA exhibited nearly twice the inhibition zone diameter that VRE did. Moreover, the MRSA showed a partial zone of inhibition extending to 27.9 ± 3.5 mm. No partial zones were seen for VRE. The gram-negative bacteria tested by agar diffusion included six *E. coli* strains, four *L. pneumophila* strains, and three *P. aeruginosa* isolates, all of which showed sensitivity to BisBAL but very little response to its component parts.

BisBAL was also effective against *H. pylori* and anaerobic bacteria. Table 4 summarizes agar dilution studies with various clinical isolates and reference strains. Several facultative bacteria were studied in tandem under anaerobic conditions. The MICs obtained varied from 2.2 to 100 μM Bi^{3+} in a 1:2 ratio with BAL. *Bacteroides* were generally more resistant than other bacteria, while *H. pylori*, *C. difficile*, and *S. aureus* were most sensitive. *H. pylori* results were the averages of six antibiotic-resistant isolates, including tetracycline-, ampicillin-, and metronidazole-resistant strains. Inhibition of *E. coli* by BisBAL also occurred under anaerobic conditions in agar dilution studies.

The solubility of BisBAL in water was also dependent on the pH and the molar ratio, as shown in Fig. 3. At pH 10, BisBAL (5 mM Bi^{3+}) became increasingly soluble as more thiol was added, to as much as a 1:1.8 molar ratio. BisBAL at ratios of >1.5:1 were least soluble in base, while ratios of <1:1.5 were most soluble. At pH 3, BisBAL (5 mM Bi^{3+}) became increasingly insoluble as more thiol was added, was completely soluble at a 1.2:1 molar ratio in acid, and was least soluble at molar ratios of <1:1.5. Very slight changes in the molar ratio higher than or less than 1:1 drastically changed the solubility in acid or base. In alkaline solutions, the 1:2 form of BisBAL increased Bi^{3+} solubility in H_2O to nearly 500 mM, while in acid solutions the 2:1 form increased Bi^{3+} solubility in propylene glycol to >50 mM Bi^{3+}. Other thiol compounds, such as DTT, βME, MTG, DMPS and DMSA, also significantly enhanced solubility in water in a similar fashion.

TABLE 4. Agar dilution sensitivities of anaerobically grown bacteria to BisBAL

Bacterial strain	MIC (Bi^{3+}/BAL [μM])
H. pylori (six clinical isolates)	2.2/4.4
C. difficile ATCC 9689	7.5/15
C. histolyticum ATCC 19401	22.5/45
C. perfringens ATCC 13124	50/100
B. fragilis ATCC 23745	15/30
B. ovatus ATCC 8483	100/200
B. fragilis clinical isolate	100/200
A. odontolyticus ATCC 17929	22.5/45
E. coli ATCC 25922	50/100
E. faecium ATCC 2358	50/100
S. aureus MRSA X22831	7.5/15

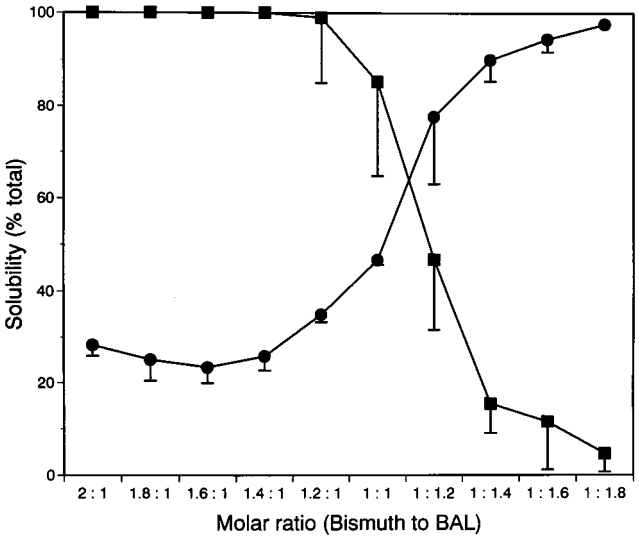

FIG. 3. Effect of molar ratio and pH on BisBAL solubility. Bismuth solutions of 5 mM in 10% propylene glycol were combined with BAL at various molar ratios, either at pH 3 (■) or at pH 10 (●). The precipitate formed was sedimented, lyophilized, and weighed. Percent solubility was determined by the weight of precipitate divided by the total weight of components added. Data are from trials performed in triplicate to obtain the means and standard deviations.

Some bismuth-thiol compounds were also soluble in 1-butanol, which was used as a measure of lipophilicity. The percentages of a 50/100 mM solution at pH 9 to 10 that partitioned in the butanol phase from water ranged from 2.7 to 81.5 (Table 1). The MIC correlated with lipophilicity, especially after separation of the dithiols from the monothiols. Complexes containing thiols and acidic groups, such as DMSA, DMPS, L-cysteine, dimercaptoadipic acid, dimercaptopropanol tributyrate, glutathione, or thiolactic acid, did not partition into butanol. These thiols also did not exhibit synergy with bismuth, while all complexes partitioning in butanol to some degree showed antibacterial synergy.

DISCUSSION

Several organic thiol compounds can enhance the antibacterial activity of bismuth. The active thiols are similar in chemical makeup with one to two sulfhydryl groups. All are amphipathic. Many of the active chelators are alcohols, but the presence of a hydroxyl group does not appear to enhance activity. Indeed, the most-active compound, BisPDT, contains no hydroxyl groups. However, the hydroxyl group on BAL seems to increase the stability of BisBAL without seriously hampering activity. The compounds BisβME and BisMEN differed by a hydroxyl or amino group in the same position. This difference amounted to a fivefold difference in activity and a different optimum molar ratio. Although DMSA is structurally similar to BAL, the presence of acidic groups completely abolished synergy with bismuth. Alcohol, amine, and especially acid group substitutions on thiols diminished activity, likely through their influence on lipophilicity. Numerous other thiol compounds tested were not synergistic with bismuth, indicating that certain chemical features on the thiol are necessary for activity. These include one to two thiol groups without a carboxylate on a small hydrocarbon backbone.

Dithiols were generally more active than monothiols, as they

are better chelators. Lower levels of dithiols than of monothiols were required to achieve optimum activity. Approximately three times as much MBO as BAL was required to achieve similar inhibitory activities. The compounds DMSA and DMPS were dithiols and excellent bismuth chelators but did not show antibacterial synergy, and they were not lipophilic. The lipophilicity of bismuth-thiol chelates is the best predictor of antibacterial activity. This model predicts that any hydrophobic monothiol or any dithiol with at least a modicum of hydrophobicity will be synergistic with bismuth.

The intensity of yellow in aqueous solution is predictive of enhanced activity at high molar ratios of bismuth to thiol. Most bismuth-thiols showed an absorbance at 410 nm with an absorption coefficient of 1.0 to 2.6, except for BisBAL and BisPDT, which had larger absorption coefficients (6.2 and 12.4, respectively). BisBAL and BisPDT were also the only agents that worked optimally at high bismuth-to-thiol ratios (3:1 to 2:1). The yellow appearance arises from ligand-to-metal charge-transfer bands (LMCT), which are common to metal ion complexes (7). The combination of the soft Bi^{3+} ion with the soft thiolate sulfur should favor LMCT. Therefore, the intensity of yellow can be used as a rough measure of the amount of bismuth-thiol complex formed and the extent of chelate formed. Yellow in alkaline bismuth-thiol solutions can then be used to screen for thiols that best chelate bismuth at low concentrations.

Bismuth-thiol combinations at a wide range of molar ratios were tested to determine optimal activity. However, for purposes of safety, cost, and practicality, the concentration of thiol was kept to a minimum. Although it was four- to sixfold less toxic when given intraperitoneally to mice than bismuth or BAL alone, BisBAL was most toxic at a 1:2 molar ratio (data not shown). High thiol content also proved malodorous and irritating to the skin. Addition of bismuth nitrate to the thiol solution eliminated the sulfur odor and the irritating effects of BAL at a ratio of 2:1 ratio, but not entirely at a ratio of 1:2 (personal observations). Bismuth-thiol compounds that achieve optimum activity only at higher thiol concentrations may not have much utility, due to these unfavorable effects. Thiols have also been shown to enhance bismuth absorption from the mouse gut (3). Nevertheless, BAL in excess has been used successfully as an antidote for rare cases of bismuth poisoning (15). The preliminary data suggest that each component of BisBAL mitigates the unfavorable characteristics of the other.

BisBAL was active against a broad range of bacteria. It was particularly effective against *H. pylori*, *S. aureus*, and *C. difficile* and was least effective against the enterococci and certain anaerobes. Of 47 MRSA tested, none showed any indication of resistance. Most bacteria are inhibited by BisBAL at a concentration of less than 17 μM Bi^{3+}. However, in agar dilution studies, the MIC against *E. coli* was threefold higher, suggesting neutralization of BisBAL activity by components in the agar medium. The data from agar diffusion studies correlate well with those seen in broth culture. The antibacterial activities of other bismuth-thiols have not been completely characterized yet. However, there is some indication that the different thiols are not interchangeable with regard to spectrum and that bismuth-thiol cocktails that strike all bacteria are possible.

The intent of combining bismuth with chelating agents was to increase water solubility. Although the lipophilicity of these agents is paramount for activity, water solubility is an obvious attribute. Solubility under a variety of conditions underscores the versatility and potential usefulness of bismuth-thiols. Many formulations and compositions with these agents are possible. Solubility in water was dependent on both pH and composition. Essentially, BisBAL was soluble in both acid and base, depending on the molar ratio. For example, with BisBAL, a powder was produced that retained most of the antibacterial activity. That BisBAL can be formulated to retain solubility in different environments adds to the versatility of this new class of compounds.

The influence of pH on so many aspects of BisBAL chemistry is a reflection of the ionization of the thiol groups of BAL. With BAL alone, the first thiol is fully deprotonated at pH 10 and the second is deprotonated at pH 11. This is dramatically altered when bismuth is added to the solution in a 1:2 molar ratio. Both deprotonations occur at much lower pH values, with the first thiol being complete at pH 5 and the second being complete at pH 9. A reasonable conclusion is that the coordination of BAL to the Bi^{3+} ion promotes ionization. It can be assumed that BAL remains coordinated to bismuth during this process. The change in solubility of BisBAL at pH 9 can be explained as follows. When the BisBAL complex is $Bi(BAL)_2$, then when the two thiols are deprotonated, the complex will be $Bi(BAL)_2^-$, which should be soluble, since it is ionic. However, basic conditions promote the autooxidation of the thiols to disulfides, which is an event that is accelerated by the presence of bismuth (16). This explains why both instability and antibacterial activity increase from pH 4.5 to 9.0. All of this activity can be enhanced further by increasing temperature and changing the molar ratio. With only one thiol ionized (pH 5 to 7), BisBAL exhibited less rapid activity. There was no activity and likely no complex formed at less than pH 4.5. Maximum activity and instability occur at the optimum molar ratio at high pH and temperature.

Seven bismuth-thiols with notable antibacterial activities have been discovered thus far. Although the ideal bismuth-thiol structure is not known, these data help predict what complexes might be maximally synergistic. Synergistic thiols possess a lipophilic hydrocarbon backbone with thiol groups. Multiple thiols per molecule facilitate chelation of metals and increase the amount of complex formed. High pH ionizes and activates sulfhydryl groups. The exact positioning of sulfhydryl groups, other substituents, and the number of carbon atoms per molecule will affect the degree of chelation and the lipophilicity. The lipid aspect of bismuth-thiols likely facilitates transport into and through lipid membranes in bacteria, with temperature affecting membrane fluidity. Bismuth is likely the antibacterial moiety, since it is known to exchange with thiols and inactivate membrane-bound enzymes involved in respiration, such as the F_1-ATPase of *H. pylori* (1). The enhanced antibacterial activity of bismuth-thiol complexes over simple bismuth salts likely involves a facilitated transport of bismuth into bacteria via lipophilic thiol carriers.

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