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In Vitro Antimicrobial Activity of Bismuth Subsalicylate and Other Bismuth Salts

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This report demonstrates that bismuth subsalicylate (BSS) effectively inhibits growth of a number of bacterial strains known to cause diarrhea, including *Escherichia coli*, *Salmonella*, *Shigella*, and *Campylobacter*. Other bismuth salts and sodium salicylate, a hydrolysis product of BSS in the gut, also were examined and were shown to have various degrees of activity. Growth of the organisms was monitored in vitro by inoculating culture fluid that contained one of the compounds to be tested and determining the concentration of viable organisms over a 24-hour period. Control cultures of each organism were grown in the absence of bismuth subsalicylate. BSS inhibited growth of all organisms examined in a dose-dependent fashion. Reductions of 2–6 logs, as compared with controls, were observed in cultures grown in the presence of 10–50 m*M* BSS. Other bismuth salts displayed various degrees of inhibition. These results suggest that the efficacy of BSS as an antidiarrheal agent may be related to an antimicrobial mechanism of action.

Few medicinal agents have a longer recorded history of use than do bismuth compounds. Since the early 1500s these compounds have been used for a number of applications, including systemic treatment of syphilis. Common applications in this century have focused on treatment of gastrointestinal ailments [1], and recent clinical trials have demonstrated that bismuth subsalicylate (BSS) is efficacious in both the prevention and treatment of travelers' diarrhea [2, 3], the treatment of *Campylobacter pylori* gastritis [4, 5], and the treatment of bacterial diarrhea in young children [6, 7]. Despite its widespread use, relatively little is known of the agent's mechanism of action. Because of its use in gastrointestinal ailments that involve an infectious component, we have been examining the ability of the bismuth salt to interfere with bacterial growth in vitro to begin to elucidate its potential mechanisms of action.

Methods

Cultivation. Because BSS and many other bismuth salts are insoluble, bacterial growth was evaluated in broth culture. In general, 30 mL of brain-heart infusion broth (BHI) (BBL, Cockeysville, Md.) containing various concentrations of BSS was inoculated with 100 μL of a late log-phase culture of a particular bacterial strain, yielding a bismuth-containing broth with an initial organism density of ~10^4 cells/mL. Control broths consisting of BHI without bismuth were inoculated in parallel. The flasks were incubated at 37°C on a rotary platform oscillating at ~250 rpm. Shaking was necessary to keep the bismuth salts evenly dispersed throughout the medium. For *Campylobacter* strains, brucella broth (BBL) was substituted for BHI and flasks were incubated under microaerophilic conditions using a Campybag (Scott Laboratories, Fiskville, R.I.).

At various times after inoculation, a sample was taken, diluted appropriately, and placed on trypticase soy agar (TSA) (Difco, Detroit) with use of a spiral plater (Spiral Systems, Bethesda, Md.). After overnight incubation at 37°C, colonies were read automatically with use of a laser counter (Spiral Systems). Each plate was read in triplicate, and a conversion program, calibrated to both the plater and counter, converted the value to cfu. In these experiments duplicate samples were taken from each broth culture and processed in parallel. Thus, data presented here represent the mean of duplicate samples. Further, each organism and bismuth salt combination was tested a minimum of three times to ensure reproducibility of results.

The following bismuth salts were evaluated in the scheme outlined above: bismuth subsalicylate (Procter & Gamble, Cincinnati); and bismuth sodium tartrate, bismuth citrate, bismuth sulfate, and bismuth oxychloride (Pfalz & Bauer, Waterbury, Conn.). In addition, acid-hydrolyzed BSS (produced as detailed below) and sodium salicylate (Sigma, St. Louis) were tested.

Acid hydrolysis of bismuth subsalicylate. A known amount of BSS was added to 50 mL of simulated gastric juice (USP formulation) and incubated

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with occasional stirring for 20 minutes at room temperature. Liberated salicylate was removed by extensive washing with distilled water, and the resulting bismuth salt was resuspended to a concentration of 100 m*M* in BHI. X-ray analysis of the resulting bismuth species revealed it to be identical to human gastric aspirates containing bismuth taken 1 hour after ingestion of BSS (W. B. Broering, personal communication).

Bacteria tested. The following organisms were used to measure the potential of BSS and other bismuth salts to inhibit growth: *Escherichia coli* H10407 (heat-labile toxin [LT]- and heat-stable toxin [ST]-positive) (provided by Dr. Dolores Evans, Baylor University, Houston), *Salmonella typhi* (ATCC no. 167), *Salmonella typhimurium* (ATCC no. 13311), *Shigella dysenteriae* (ATCC no. 11835), *Yersinia enterocolitica* (ATCC no. 27729), *Vibrio cholerae* Inaba (ATCC no. N16961), *V. cholerae* Ogawa (ATCC no. 395), *V. cholerae* El tor (ATCC no. 582), *Campylobacter fetus* subspecies *fetus* (ATCC no. 27374), and *C. fetus* subspecies *jejuni* (ATCC no. 29428).

In addition, a number of clinical isolates were obtained from Dr. Donna R. Morgan (University of Texas Medical School, Houston). These strains were isolated from American students residing in Mexico who presented with diarrhea. Table 1 details the identity and number of strains tested.

Results

Figure 1 illustrates the growth of *E. coli* strain H10407 (LT^+ and ST^+) in BSS at concentrations of 1–50 m*M*. As can be seen, control cultures grown in the absence of bismuth reached high densities after 24 hours. Addition of BSS resulted in a dose-dependent inhibition of growth. BSS at 5 m*M* (1.8 mg/mL) resulted in a 4-log decrease, as compared with the control, in viable counts after 24 hours. Bismuth at 50 m*M* (18.1 mg/mL) decreased viable counts below baseline values.

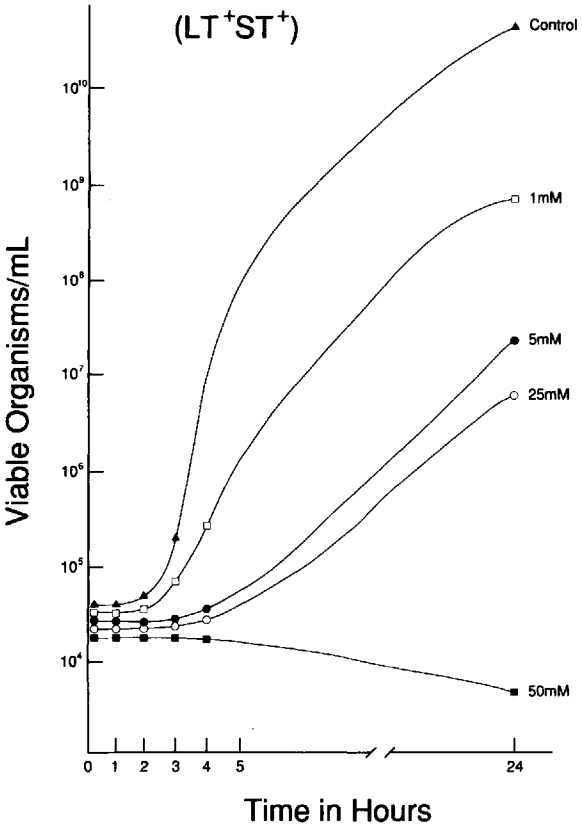

Figure 1. Dose-dependent inhibition of *Escherichia coli* growth by bismuth subsalicylate.

Similar results were seen when a number of different organisms (listed in Methods) were grown in BSS. In all cases addition of between 10 and 25 m*M* BSS resulted in at least a 2-log reduction, as compared with the control, in the number of viable organisms. Importantly, this effect was seen when human pathogens were examined.

Table 2 lists the log reductions in viable counts ([observed log cfu in bismuth-containing broth] – [observed log cfu in control broth]) after 24 hours' growth in 1, 10, and 50 m*M* of BSS for a number of clinical isolates obtained from American students with diarrhea during their stay in Mexico. While different strains showed some variation in sensitivity to bismuth, the presence of 10 m*M* BSS inhibited growth by 2–4 logs, as compared with control. A 50-m*M* dose of BSS resulted in an even greater reduction in viable organisms and, in some cases, sterilized the culture.

Table 1. Clinical isolates obtained from American students residing in Mexico.

Organism	No. of isolates tested
Toxigenic *E. coli* (LT^+, ST^+, or LT^+ST^+)	10
Plesiomonas species	5
Aeromonas species	1
Salmonella species	4
Shigella sonnei	11

Table 2. Inhibition of human clinical isolates by 1, 10, and 50 m*M* bismuth subsalicylate.

Organism (no. of strains)	Observed log reductions vs. control at indicated concentration		
	1 m*M*	10 m*M*	50 m*M*
E. coli (10)	<2	2–4	6–9*
Salmonella species (4)	<2	2.5–3	5–7
Shigella sonnei (9)	<2	2–5	5–8
Plesiomonas species (4)	<2	2	5–7
Aeromonas species (1)	<2	<2	4

* Three strains were below the detection limit.

In the gastrointestinal tract BSS is subject to acid hydrolysis, which results in the generation of free salicylate and numerous other bismuth species [1]. Thus, it was of interest to this investigator to evaluate the antimicrobial activity of the products of BSS acid hydrolysis. To this end, growth of these same organisms was examined in the presence of sodium salicylate or of the bismuth species resulting from acid hydrolysis of BSS.

Figure 2 presents counts of viable organisms obtained after 24 hours' growth in different concentrations of sodium salicylate (figure 2A) or acid-hydrolyzed BSS (figure 2B) for *E. coli* (H10407) and *S. typhi*. Both salicylate and the bismuth-containing insoluble fraction obtained from acid hydrolysis of BSS inhibited growth in a dose-dependent fashion. Like salicylate, 50 m*M* bismuth inhibited growth by ~5 logs as compared with control. Inhibition of growth by both acid-hydrolyzed BSS and sodium salicylate also was observed when *V. cholerae*, *S. typhimurium*, *Y. enterocolitica*, and *C. jejuni* were tested (data not shown).

Additional experiments showed that other bismuth salts also inhibited growth of the tested strains that were inhibited by BSS (data not shown). The potencies of these different compounds were directly compared by evaluating growth of one *E. coli* strain (H10407) in broths containing 50 m*M* bismuth from one of the bismuth salts.

Figure 3 presents the log reductions in counts of viable organisms obtained after 24 hours' growth. The values were obtained by subtracting the log of viable counts observed in the bismuth-containing broth from the log of viable counts in control broths grown in parallel. As can be seen, BSS and acid-hydrolyzed BSS were the most potent bismuth salts tested, as they provided the biggest reduction in viable counts (5.4 and 5.0 log reductions vs. control, respectively). Bismuth sodium tartrate was the third most potent salt (4.4 log reduction), followed by sodium salicylate (4.2 log reduction) and bismuth sulfate (4.1 log reduction), which had approximately equal potency. In these experiments, bismuth citrate (2.0 log reduction) was about half as potent as BSS.

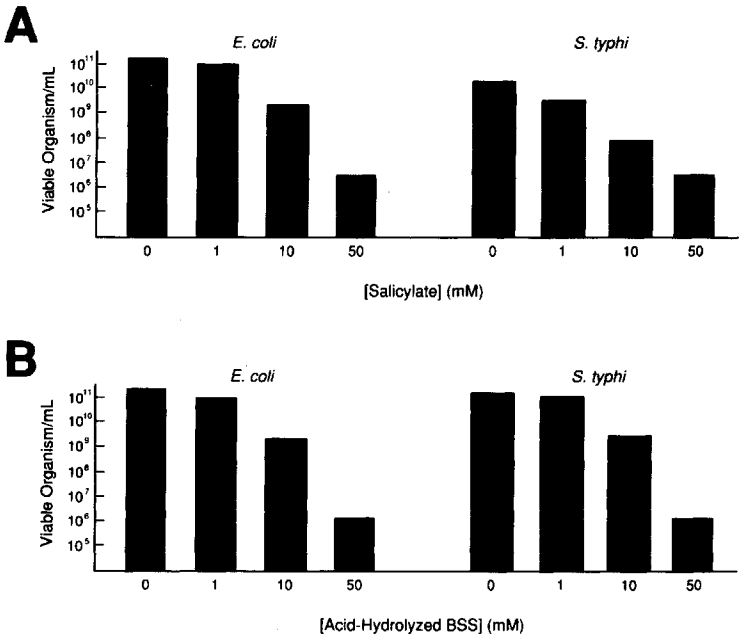

Figure 2. Inhibition of bacterial growth by the products of bismuth subsalicylate acid hydrolysis.

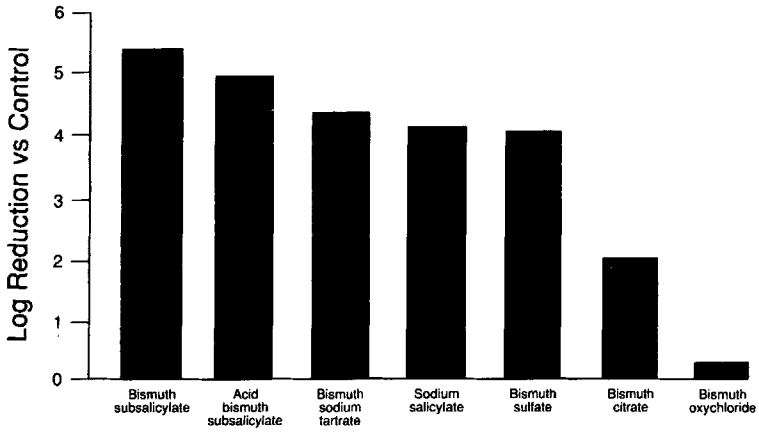

Figure 3. Inhibition of enterotoxigenic *Escherichia coli* growth by 50 m*M* bismuth salts.

Commercially obtained bismuth oxychloride did not show a significant antimicrobial effect (0.2 log reduction vs. control).

Discussion

These data demonstrate that BSS and other bismuth salts can inhibit the growth of a wide variety of potential human pathogens in vitro when present at concentrations between 1 and 50 m*M*. Graham et al. [8] and Cornick et al. [9] have also demonstrated that BSS has antimicrobial activity in vitro but used different methods than those employed here. The work presented here supports and extends these observations.

Direct comparison of the abilities of various bismuth salts to inhibit growth in vitro reveals marked differences among them. Of the salts tested here, BSS appears to be the most potent and commercially obtained bismuth oxychloride the least potent. Acid-hydrolyzed BSS (presumably bismuth oxychloride) was nearly as potent as BSS.

The apparent contradiction in results observed with commercially obtained bismuth oxychloride vs. the results obtained using acid-hydrolyzed BSS may be due to the different crystalline structures of these two species. X-ray analysis of the two bismuth forms revealed that acid-hydrolyzed BSS had an amorphous structure, with signals consistent with a mixed crystalline form, while commercially obtained bismuth oxychloride was highly crystalline in nature and uniform in structure (W. B. Broering, personal communication). Given the differences in antimicrobial potency between the two bismuth forms, it is tempting to speculate that crystalline structure may play a role in antimicrobial efficacy. Further work is needed to prove or disprove this hypothesis.

Sodium salicylate also demonstrates antimicrobial activity in the test system used here, raising the question of the relative contribution of bismuth and salicylate to any antimicrobial action in vivo. If BSS and the species formed upon the exposure of BSS to the human gut do demonstrate antimicrobial activity, it seems unlikely that salicylate would contribute significantly. Studies have shown that >90% of the salicylate that is theoretically available from BSS is absorbed after ingestion by either children [10] or adults [11]. It is possible that tissue salicylate could inhibit mucosa-associated organisms, but the work presented here suggests that concentrations >1 m*M* (138 μg/mL) would need to be maintained for salicylate to exert an antimicrobial effect in vivo. Thus, while salicylate demonstrates in vitro antimicrobial activity, one must question the relevance of this observation as it relates to the in vivo situation.

In general, the bismuth salts tested showed in vitro effects at or above concentrations of 10 m*M* (3,600 μg/mL), which are severalfold greater than what might be considered effective for antimicrobial agents. Thus, one must ask if after dosing the concentration of bismuth in the gastrointestinal tract is high enough to exert a clinically meaningful effect. Indirect evidence from human trials with BSS suggests that the answer is yes. Three studies of the efficacy of BSS in prevention or treatment of diarrhea demonstrate that a pathogen is isolated less frequently in patients treated with BSS than in patients treated with placebo. DuPont et al. [3] isolated pathogens from the stools of only four of 12 patients who were taking BSS but from 27 of a total of 38

placebo-treated patients. Similarly, Graham et al. [8] were able to detect enterotoxigenic *E. coli* (ETEC) in the stools of only two of 14 patients challenged with the organism who were given BSS prophylactically but from 13 of 15 patients given placebo before ETEC challenge. Finally, Steffen et al. [12], who examined the efficacy of a tablet formulation in prevention of diarrhea in travelers to Africa, isolated pathogens from none of six patients given BSS yet isolated pathogens from six of 12 stool specimens taken from patients receiving placebo. These data demonstrate that pathogens are consistently isolated less frequently from patients using BSS than from patients using placebo. This suggests that BSS has an antimicrobial effect on the human gut.

To examine in vivo antimicrobial effects more directly, we have recently begun to quantitate vibrios taken from ileal and jejunal segments of rabbits challenged 18 hours earlier with 10^8 *V. cholerae*. Although the preliminary results are subject to a rather large degree of experimental variation, indications are that dosing infected animals with 17.5 mg/kg of bismuth reduced counts of recoverable pathogens by 2–4 logs as compared with the control. This supports the hypothesis that bismuth has some direct antimicrobial effect in vivo.

In conclusion, the work presented here demonstrates that BSS inhibits growth of a broad spectrum of bacteria in vitro, including human diarrheal pathogens. Further, the products of BSS acid hydrolysis and other bismuth salts are also inhibitory. Finally, indirect evidence indicates that sufficient concentrations of bismuth are attained in the gut so as to exert an antimicrobial effect. For these reasons it is likely that the antidiarrheal efficacy of BSS is related at least in part to an antimicrobial mechanism of action.

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