Evaluation of BW942C, a Novel Antidiarrheal Agent, Against Enterotoxins of *Escherichia coli* and *Vibrio cholerae*

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BW942C, an enkephalin-like pentapeptide with anti-diarrheal activity, was tested against crude toxins of *Escherichia coli* and *Vibrio cholerae* in the Y-1 adrenal cell assay, rabbit ileal loop assay, and suckling mouse assay. The effects of BW942C on in vitro ion transport were measured in rabbit ileum mounted in Ussing chambers. In vitro, BW942C decreased basal short-circuit current (2.26 and 3.15 μeq cm^{-2} h^{-1} in experimental samples and controls, respectively; $n = 7$, $P < 0.05$) and increased basal net Cl absorption (1.59 and 0.50 μeq cm^{-2} h^{-1} in experimental samples and controls, respectively; $P < 0.025$). Net Na absorption was also increased, but not significantly. BW942C did not block the secretory response to a maximal dose of purified heat-stable toxin. BW942C directly enhanced intestinal fluid absorption. In the Y-1 adrenal cell assay, 5 mg of BW942C per ml inhibited the cytopathic effect caused by cholera toxin or heat-labile enterotoxin of *E. coli*. In the rabbit ileal loop assay, *E. coli* heat-stable toxin, *E. coli* heat-labile enterotoxin, and cholera toxin were inhibited 35 to 70% by administration of BW942C. With the suckling mouse model, the fluid accumulation caused by *E. coli* heat-stable toxin was ablated by prior treatment with BW942C. The drug is currently being evaluated in patients with acute secretory diarrhea to determine its effect on clinical symptoms.

In most regions of the developing world, acute diarrhea represents a major if not the major cause of infant mortality. In the United States, one in every six illnesses of adults involves the gastrointestinal tract; one of the most common complaints is diarrhea (2). In addition, nearly 50% of the travelers from the United States to developing countries experience infectious diarrhea (12, 22, 40, 47). Regardless of etiology, diarrhea is inconvenient to the patient. Availability of a drug which would reduce intestinal fluid losses and improve clinical symptoms in patients with diarrhea would be met with enthusiasm.

Several drugs are now available for the symptomatic relief of diarrhea. Kaolin-pectin, bismuth subsalicylate, codeine phosphate, paregoric, and diphenoxylate with atropine have been available for many years. Loperamide hydrochloride has been introduced more recently.

The potential for abuse, the presence of undesirable side effects, and the lack of gastrointestinal specificity associated with certain currently available antidiarrheal agents have impelled research efforts to discover antidiarrheal agents which act directly on the intestine to control diarrhea and which do not have significant effects on the central nervous system.

BW942C is a chemically novel enkephalin-like pentapeptide [Tyr-D-Met-(O)-Gly-pNO$_2$-Phe-Pro-NH$_2$ acetate]. The compound has limited constipating effects, low abuse potential, limited effects on the central nervous system, and a high therapeutic index. Recently, methionine enkephalins have been shown to augment the immune system (36). In this study, we evaluated the ability of BW942C to inhibit the activity of enterotoxins by using in vitro and in vivo models.

MATERIALS AND METHODS

Toxin preparation. Crude toxins were prepared from human *Escherichia coli* isolates H10407 and TX-1 and *Vibrio cholerae* ATCC 25870 as previously described (13, 37, 38, 49). Purified heat-stable toxin (ST) of *E. coli* (kindly provided by D. C. Robertson, University of Kansas, Lawrence) were used in the Ussing chambers.

Electrical and ion flux measurements. New Zealand White male rabbits, each weighing 2 to 3 kg, were fed standard rabbit chow and water ad libitum. Rabbits were killed by cervical dislocation; the distal ileum was removed rapidly from each rabbit, opened along its mesenteric border, and rinsed clean of its luminal contents with cold Ringer solution containing (in millimoles per liter): NaCl, 114; KCl, 5; Na$_2$HPO$_4$, 1.65; NaH$_2$PO$_4$, 0.30; CaCl$_2$, 1.25; MgCl$_2$, 1.1; and NaHCO$_3$, 25. The serosa and two major layers were removed by placing a 10-cm sheet of ileum (serosa up) on a Plexiglas plate moistened with Ringer solution, making a transverse incision through the muscle layers with a razor blade, and peeling the layers off longitudinally with fine, curved forceps.

Transepithelial electrical potential difference, total conductance, and short-circuit current (I_{sc}) were measured as described previously (45). Four to six pieces of intestinal mucosa were mounted in Ussing chambers (exposed surface area, 1.12 cm^2) and bathed with 10 ml of Ringer solution on each side. Solutions were gassed with 95% O$_2$–5% CO$_2$, circulated by gas, and maintained at 37°C in reservoirs with water jackets. Glucose (10 μmol/ml) was added to the serosal medium. An equimolar amount of mannitol was added to the mucosal medium.

Ion fluxes were measured over two successive periods. After the tissues had been mounted for 45 min, ^{22}Na and ^{36}Cl (New England Nuclear Corp., Boston, Mass.) were added to either the mucosal or the serosal reservoirs, and the tissues

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were short-circuited by an automatic voltage clamp (Control Instruments, Houston, Tex.). Tissues were paired by matching resistances. If at any time during flux measurements the resistance of paired tissues differed by more than 30%, the experiment was rejected.

Two successive flux measurements were made in pair-matched sets of tissues. The initial flux (period 1) lasted 30 min and measured either the basal transport rate of tissue (no agents added) or the ion movement in the presence of 10^{-6} M BW942C (added serosally). After the first flux period, 2×10^{-10} mg of ST was added to the mucosal reservoir. After a 20-min equilibration period, a second flux measurement was made over a 40-min period (period 2).

Unidirectional mucosal-to-serosal and serosal-to-mucosal fluxes (J_{m-s} and J_{s-m}) and net fluxes ($J_{net} = J_{m-s} - J_{s-m}$) of Na and Cl were calculated from samples taken at the beginning and end of each flux period. Unidirectional ion fluxes were calculated by dividing the steady-state rates of radioisotope transfer by the specific activity of the initially labeled side and by the surface area of exposed tissue. The net flux is calculated as the difference between oppositely directed unidirectional fluxes of tissue pairs. From these measurements, the residual ion flux [$J^R = I_{sc} - (J_{net}^{Na} + J_{net}^{Cl})$] which represents that part of the I_{sc} not attributed to the movement of Na or Cl (7), was calculated. Duplicate paired determinations were made for each animal and averaged to provide one set of results for each animal.

YAC assay. Y-1 adrenal cell (YAC) monolayers were prepared and challenged with serial twofold dilutions (1:2 to 1:512) of crude heat-labile toxin (LT) of *E. coli* and cholera toxin (CT), as previously described (10, 13, 37, 38, 49). BW942C (Burroughs Wellcome Co., Research Triangle Park, N.C.) was dissolved in minimal essential medium supplemented with serum and antimicrobial agents. Three dilutions of BW942C (1.25, 2.5, and 5.0 mg/ml), which were not cytotoxic, were added to cell monolayers simultaneously with crude toxin. After 15 min of incubation at 37°C in 5% CO_2, the drug-containing medium was removed and replaced with fresh complete medium without BW942C. After overnight incubation at 37°C in 5% CO_2, the monolayers were read for percent cytopathic effect (CPE). Greater than 20% CPE was considered positive (37, 49).

Suckling mouse assay. Crude ST was injected intragastrically into mice, each weighing 2 g, as previously described (6, 13, 20, 25, 38, 49). BW942C was dissolved in phosphate-buffered saline (pH 7.4). Three doses of BW942C (10, 100, and 1,000 μg per mouse) were injected intraperitoneally into each mouse 90 min before toxin administration. Fluid accumulation (FA) ratios > 0.090, measured 120 min after challenge with crude ST, were considered positive (25).

Ligated ileal loop model. Crude ST, LT, and CT FA ratios were measured in the adult rabbit ligated ileal loop assay (17, 43). BW942C was dissolved in phosphate-buffered saline (pH 7.4). Doses of BW942C (ranging from 1 to 20 mg per animal) were administered orally or intramuscularly to rabbits, each weighing 2 kg, 4 h before surgery and at 4 h intervals until the animals were sacrificed (6 h for ST and 18 h for LT and CT). In addition, doses of BW942C (5 and 10 mg per loop) were administered directly into the ligated ileal loop 5 min before toxin injection. FA ratios of >0.5 for ST and >1.0 for LT and CT were considered positive.

Statistical analysis. Significant reduction in FA ratios was determined by the two-tailed, paired Student t test and chi square test. The paired Student t test was used for comparing control and BW942C-treated tissues in periods 1 and 2 of Ussing chamber experiments.

RESULTS

Effect of BW942C on transport across isolated ileum. BW942C significantly altered basal transport parameters (Fig. 1). I_{sc} was reduced to 70% of control values, from 3.15 to 2.26 μeq cm^{-2} h^{-1} ($P < 0.05$). J_{net}^{Cl} was increased by approximately 2 μeq cm^{-2} h^{-1}; this was due primarily to a decrease in the unidirectional flux J_{s-m}^{Cl} from 9.3 ± 0.7 to 6.5 ± 1.0 μeq cm^{-2} h^{-1} ($P < 0.05$). Although there was a trend toward increased Na absorption (from 0.9 to 2.3 μeq cm^{-2} h^{-1}), this was not statistically significant. BW942C did not have an effect on either conductance or residual ion flux.

ST has previously been shown to cause active ion secretion, mediated by an increase in intracellular cyclic GMP (cGMP) (26). We initially performed dose response studies which indicated that 2×10^{-10} mg of ST per ml elicited a maximal increase in I_{sc}. This concentration was used in subsequent studies. The toxin effect was characterized by an increase in I_{sc} and both Na and Cl secretion with a decreased conductance; the changes were similar in control and BW942C-treated ilea (Fig. 2).

Effect of BW942C on CPE in YAC monolayers. BW942C was not toxic to YAC monolayers at doses of 5 mg/ml. When added to cells (in two experiments performed with paired, duplicate wells) with crude toxins, BW942C inhibited the CPE. In the absence of the drug, a 1:256 dilution of LT was sufficient to cause CPE. In the presence of 5 mg of BW942C per ml, the highest dilution which exhibited CPE was 1:32. Similarly, without drug a 1:128 dilution of CT was cytopathic, whereas only a 1:8 dilution of CT in the presence of 5 mg of BW942C per ml caused CPE. Lower dilutions of BW942C did not consistently alter the CPE (data not shown).

Effect of BW942C on FA in suckling mice. BW942C was administered intraperitoneally to mice, each weighing 2 g, in 10-, 100-, and 1,000-g amounts 90 min before intragastric challenge with crude ST (Table 1). All three doses reduced FA in this model ($P < 0.001$). No toxicity was noticed in the animals.
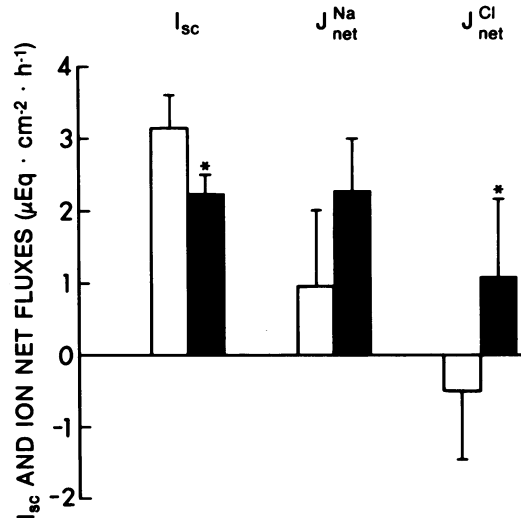

FIG. 1. Effect of BW942C on basal electrolyte transport in rabbit ileum. J_{net}^{Na}, J_{net}^{Cl}, and I_{sc} were measured in rabbit ileum mounted in Ussing chambers. The effects of 10^{-6} M BW942C (black bars) on ion fluxes and short-circuit currents are compared with untreated pair-matched controls (white bars). Results are expressed as mean ± standard error of the mean ($n = 7$). *, $P = 0.05$.

Effect of BW942C on FA in adult rabbits. BW942C was toxic to adult rabbits. Fatality rapidly occurred when 5-mg intramuscular doses (total of 25 mg) or 20-mg oral doses (total of 80 mg) were administered. Death was rapid, with no signs of apparent toxicity. Intra-ileal administration (total dosage, 10 mg) of BW942C was ineffective against LT and CT in an 18-h assay (Table 2). Oral (total of 60 mg) and intramuscular (total of 15 mg) dosing did inhibit LT- and CT-induced FA in rabbit ileal loops ($P < 0.05$). Similarly, oral (40 mg) and intramuscular (2 mg) BW942C inhibited ST-induced FA in a 6-h assay ($P < 0.05$). Intra-ileal administration (10 mg) of BW942C failed to interfere with FA.

DISCUSSION

Opiates are one of the most effective classes of anti-diarrheal agents. Although their anti-motility effects have long been appreciated, it has become increasingly apparent that they may have an important role in epithelial ion transport. The recognition of endogenous (enkephalin, endorphin) in addition to exogenous opiates, as well as different classes of opiate receptors, attests to the complexity of the opiate response.

Although the exogenous opiates such as codeine and morphine (8, 35) alter ion transport, their major effect may be on intestinal motility (44). Enkephalins, presumably through receptors, increase Na and Cl absorption in the
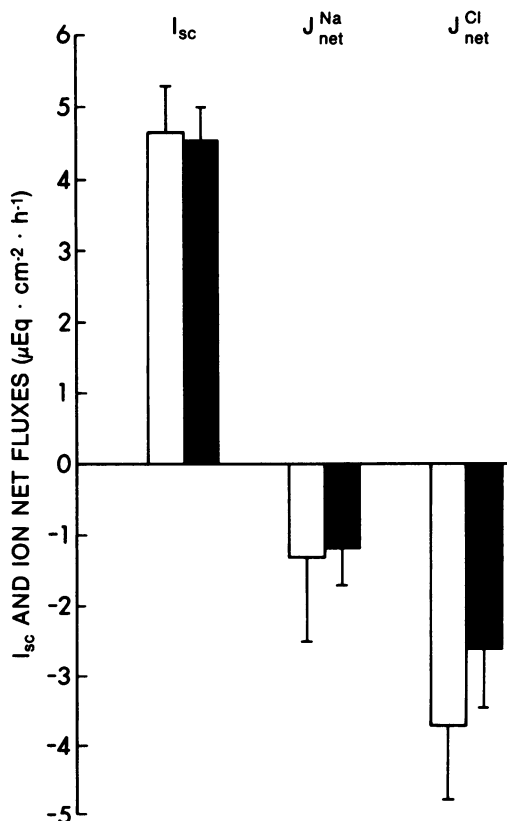

FIG. 2. Effect of BW942C on *E. coli* ST-induced secretion. J_{net}^{Na}, J_{net}^{Cl}, and I_{sc} were measured in rabbit ileum mounted in Ussing chambers. J_{net}^{Na}, J_{net}^{Cl}, and I_{sc} after exposure to *E. coli* ST are compared in BW942C-treated (black bars) and control (white bars) tissues. Results are expressed as mean ± standard error of the mean ($n = 6$).

TABLE 1. Effect of BW942C on enterotoxin-induced FA in the suckling mouse model

No. of mice	Dose (μg)[a]	FA[b]
14	0	0.140
12	10	0.090
9	100	0.087
11	1,000	0.085

[a] BW942C was administered to suckling mice intraperitoneally 90 min before intragastric challenge with crude ST.

[b] FA was measured 120 min after challenge with crude ST; $P < 0.05$ in all three drug-treated groups when compared with the controls.

small bowel and colon (8, 19, 30, 31). BW942C is a chemically unique enkephalin-like compound with potential therapeutic value in decreasing the symptoms of gastroenteritis. BW942C induces similar changes in ion transport as have been previously reported for methionine-enkephalin: a reduction in I_{sc}, with increases in J_{net}^{Na} and J_{net}^{Cl}. Additonally, like methionine-enkephalin, it does not alter the change in I_{sc} or ion fluxes induced by secretagogues. Therefore, the effects on in vitro transport of this chemically novel pentapeptide parallel those of the endogenous enkephalin.

Agents effecting epithelial transport may have an anti-diarrheal effect in two different manners: by specifically blocking the effect of a secretory stimulus or, alternatively, by stimulating an independent absorptive mechanism. For example, indomethacin may block the effect of a variety of secretagogues without altering basal rates of ion absorption (46). Alternatively, glucocorticoids increase Na and Cl absorption but do not alter the magnitude of response to CT or 8-Br-cAMP (4, 45). Both types of effects result in decreased net ion secretion, which clinically can be translated into an anti-diarrheal response.

The anti-secretory capability of BW942C was studied in vitro and in vivo. In vitro transport studies demonstrated that BW942C directly enhanced intestinal fluid absorption. The compound failed to block the secretory response to a maximum enterotoxin dose, however. Further in vitro studies indicated that BW942C was able to interfere with toxin-induced CPE in cultured mammalian cells. In vivo animal models demonstrated that BW942C was able to inhibit, and in some cases ablate, FA induced by the enterotoxins of *E.*

TABLE 2. Effect of BW942C on enterotoxin-induced FA in the ligated ileal loop model in rabbits

Crude toxin	No. of loops challenged	Dose (mg)[a]	Route of BW942C administration	FA[b]
LT	6	10	Intra-ileal	1.23
	6	60	Oral	0.44
	6	15	Intramuscular	0.71
	18	0		1.43
CT	6	10	Intra-ileal	1.17
	6	60	Oral	0.88
	6	15	Intramuscular	1.03
	18	0		1.77
ST	6	10	Intra-ileal	0.77
	6	40	Oral	0.32
	6	2	Intramuscular	0.31
	18	0		0.71

[a] BW942C was administered to animals 4 h before surgery and at 4-h intervals until sacrifice.

[b] FA measured 18 h (LT, CT) or 6 h (ST) after challenge with crude toxin.

coli and *V. cholerae*. The drug was well tolerated in mice but less so in rabbits.

Many pharmacological agents have been demonstrated to inhibit the activity of ST (1, 24, 25, 32, 34), LT (16, 28, 33), and CT (9, 16, 23, 28, 29, 33). Several compounds are effective against all these toxins, despite the differences in the mode of enterotoxin activity, as well as pharmacological activity differences. ST is known to cause fluid secretion via stimulation of guanylate cyclase and elevation of intracellular cGMP (11, 27, 39). LT and CT, in contrast, are ADP ribosylating toxins which bind to monosialoganglioside residues and cause activation of adenyl cyclase and accumulation of intracellular cAMP (5, 18, 21). Recent studies have shown that prostaglandin synthesis and calcium regulation may be involved in both mechanisms of secretory responsiveness (3, 11, 29, 48). Therefore, it follows that anti-inflammatory agents, prostaglandin antagonists, and calcium channel blockers might be effective antisecretory agents.

Few agents which have effective anti-secretory activity in vitro as well as in in vivo animal models have demonstrated in vivo effectiveness in field tests. Ericsson et al. (16) demonstrated the effectiveness of bismuth subsalicylate in laboratory models and followed with effective clinical trial data on bismuth subsalicylate in the treatment and prevention of travelers' diarrhea (14, 15). Recent data from our laboratories have demonstrated that loperamide is effective against ST and LT in vitro and in vivo (manuscript in preparation); clinical trials have shown this agent to be effective in treating chronic diarrhea (41, 42).

BW942C was effective in vitro and in vivo against the enterotoxins of *E. coli* and *V. cholerae*. The drug may prove useful in rapid relief of diarrheal symptoms. Perhaps symptomatic treatment of diarrhea in conjunction with an antibiotic in selected cases could provide prompt and permanent relief of symptoms. Clinical trials are under way to determine the effectiveness of BW942C with and without an antimicrobial agent in the treatment of acute travelers' diarrhea.

ACKNOWLEDGMENTS

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