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The multi-herbal drug STW 5 (Iberogast®) has prosecretory action in the human intestine

D. KRUEGER,* L. GRUBER,* S. BUHNER,* F. ZELLER,† R. LANGER,‡ S. SEIDL,‡ K. MICHEL* & M. SCHEMANN*

*Department of Human Biology, Technische Universität München, München, Germany
†Department of Surgery, Clinical Centre Freising, Freising, Germany
‡Department of Pathology, Technische Universität München, München, Germany

Abstract *There is growing evidence that STW 5 (Iberogast®, fixed combination of hydroethanolic herbal extracts), besides being effective in functional dyspepsia, also improves symptoms in irritable bowel syndrome (IBS). Clinical data indicate that modulation of mucosal secretion is a promising approach to treat intestinal disorders associated with IBS. We therefore explored the effect of STW 5 on secretion in the human intestine and the mechanisms by which it acts. The Ussing chamber technique was used to measure mucosal secretion in human intestinal mucosa/submucosa preparations and in human epithelial cell line T84. In addition, we recorded STW 5 effects on human enteric neurons with voltage sensitive dye imaging. In human tissue and T84 cells STW 5 induced a dose-dependent increase in ion secretion that was significantly reduced by the Na–K–Cl cotransporter blocker bumetanide, the adenylate cyclase inhibitor MDL-12 330, the non-specific and selective cystic fibrosis transmembrane conductance regulator (CFTR) inhibitors glibenclamide and $CFTR_{inh}$-172, respectively, and the blocker of calcium dependent Cl^- channels (ClCa) SITS (4-acetamido-4-isothiocyanatostilbene-2,2-disulphonic acid). It was unaffected by amiloride, a blocker of epithelial Na^+ channels. In human tissue, the nerve blocker tetrodotoxin significantly suppressed the STW 5 response. STW 5 evoked an increased spike discharge in 51% of human submucous neurons. Results suggest that STW 5 is a secretogogue in the human intestine by direct epithelial actions and through activation of enteric neurons. The prosecretory effect is due to increased epithelial Cl^- fluxes via CFTR and Ca-dependent ClCa channels. STW 5 may be a novel option to treat secretory disorders associated with IBS and constipation.*

Keywords *chloride secretion, cystic fibrosis transmembrane conductance regulator, enteric nervous system, human intestine, Iberogast.*

INTRODUCTION

STW 5 (Iberogast®) is a fixed combination of a fresh plant extract from bitter candytuft and drug extracts from greater celandine herb, angelica root, lemon balm leaves, peppermint leaves, caraway fruit, liquorice root, chamomile flower and milk thistle fruit. Several studies reported that all extract components contribute in a synergistic manner to the overall efficacy of the drug (for review).[1] STW 5 is best known for its beneficial effect in functional dyspepsia.[2,3] The inhibitory and excitatory action of STW 5 on motility of the proximal and distal stomach, respectively, may contribute to symptom relief in functional dyspepsia.[4–6]

Studies in experimental models demonstrated a broad action profile of STW 5 including modulation of muscle activity in the stomach,[4] radical scavenging,[7] anti-inflammatory[8] and antioxidative properties.[9] These actions very likely depend on local and systemic effects of STW 5 supported by the good bioavailability of lead substances characteristic for each of the extracts.[10] As for other multi-herbal drug preparations, the concept of multitargeting may improve treatment options for gut diseases that are associated with multifactorial disorders.[11] This is supported by clinical studies, which demonstrated that STW 5 improved the overall symptom score in irritable bowel (IBS) patients.[12,13] However, there are no data attempting to explore the mode of action of STW 5 in the intestine

Address for correspondence
Professor Michael Schemann, Human Biology, Technische Universität München, Hochfeldweg 2, D-85350 Freising-Weihenstephan, Germany.
Tel: 49 8161 71 5483; fax: 49 8161 71 5785;
e-mail: schemann@wzw.tum.de
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except for its motility modulating effects in rat and guinea-pig ileum *in vitro*.[14] STW 5 exhibits spasmolytic effects in precontracted but, at higher concentrations, it increases smooth muscle tone in relaxed muscle preparations. Sensori-motor dysfunctions in IBS include altered epithelial secretion very likely involving disturbed enteric network activity.[15] Recent studies demonstrated that linaclotide and lubiprostone, both of which increase fluid secretion, improve symptoms in constipation predominant IBS[16,17] and chronic constipation.[18,19] They also emphasized the potential of increasing fluid secretion for a novel approach to IBS treatment.[16,17]

We therefore hypothesized that STW 5 may modulate intestinal functions in humans and wanted to characterize its effect on secretion in the human intestine and in the human epithelial cell line T84. Furthermore, it was our aim to provide mechanistic insights into its mode of epithelial action.

MATERIAL AND METHODS

Tissue samples

Human tissue samples of small and large bowel were obtained from 79 patients (age, mean ± SD: 68.8 ± 12.6; 37 male, 42 female) undergoing surgery at the Medical Clinics in Freising and Munich. Diagnoses that led to the surgery were as follows: carcinoma (61 patients), diverticulitis of the sigmoid colon (10 patients), colon polyps (five patients), ileus (two patients) and unspecified bleeding (one patient). Samples were taken from macroscopically unaffected areas as determined by visual inspection of the pathologist (RL). Experiments were performed in 317 tissue preparations (275 colon, 11 rectum, 24 ileum and seven duodenum).

After removal, specimens were placed in cold, oxygenated, sterile Krebs solution and immediately transferred to the laboratory. All procedures were approved by the ethics committee of the Technische Universität München (project approval 1746/07). Segments were carefully dissected in ice-cold Krebs solution to obtain mucosa/submucosa preparations containing the submucous plexus.

Human epithelial cell line T84

T84 cells (ECACC, Salisbury, UK) were seeded on Millipore filters (Bedford, MA, USA) with 0.45 μm pore size, and incubated at 37 °C and 95% O_2 and 5% CO_2 (Carbogen) in Dulbecco's modified Eagle medium/Ham's Nutrient Mixture F-12, supplemented with 10% heat-inactivated fetal calf serum, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2.75 μg mL^{-1} amphotericin B (all from Sigma-Aldrich, Schnelldorf, Germany) to attain a monolayer. The medium was replaced daily. After 12–14 days, filters were mounted in Ussing chambers for ion-transport studies.

Ussing chamber experiments

To test the effects of STW 5 on ion transport in intact human mucosa/submucosa preparations and T84 cells, we used the Ussing voltage clamp technique (Easy Mount chambers, Physiologic Instruments, San Diego, CA, USA).[20] The tissue specimens were mounted into plexiglas Ussing chambers. The exposed tissue area was 0.5 cm^2. Mucosal and serosal sides were bathed separately in 5 mL Carbogen-bubbled Krebs solution maintained at 37 °C. The experiments were performed employing the 4-electrode Ussing technique as previously described in detail.[20] The set-up allowed simultaneous measurements of up to eight mucosa/submucosa preparations dissected from one specimen. Parameters were the transepithelial, active electrogenic transport (short-circuit current, I_{sc}) expressed in μA cm^{-2}; the values were corrected for bath resistance. Positive I_{sc} indicated a net anion current from the serosa to the lumen. The tissue was electrically stimulated by silver electrodes placed on either side of the tissue and connected to a constant voltage stimulator (Grass SD-9; Astro-Med Inc., West Warwick, RI, USA). Neural stimulation of the tissue was achieved by delivering a train of pulses with supramaximal stimulus parameters: pulse amplitude, 20 V; pulse frequency 10 Hz; pulse duration 1 ms; train duration 10 s [electrical field stimulation (EFS)]. STW 5 and other drugs were either added to the serosal bathing solution (basolateral application) or the mucosal bathing solution (apical application). Before starting the actual measurements, the tissues were allowed to equilibrate for at least 30 min. No change of the pH (7.4) in the Krebs solution was observed with any of the drugs used in this study.

Neuroimaging technique

The multisite optical recording technique (MSORT) is a fast imaging technique using a potentiometric dye that allows us to record action potential discharge in the human submucous plexus and has been previously described in detail.[21] Samples from the specimens also used for Ussing experiments were dissected to obtain preparations of the inner submucous plexus (5 × 10 mm final size), then placed in a recording chamber and continuously perfused with Carbogen-gassed

37 °C Krebs solution (pH 7.4). Individual ganglia were stained with the fluorescent voltage sensitive dye Di-8-ANEPPS (1-(3-sulphonatopropyl)-4-[beta[2-(di-*n*-octylamino)-6-naphthyl]vinyl]pyridinium betaine (Molecular Probes Mobitec, Göttingen, Germany). Controlled illumination of the preparation did not exceed 5 s to avoid dye bleaching. Signals were acquired with a frequency of 1.6 kHz and processed by an array of 464 photodiodes (RedShirt Imaging, Decatour, GA, USA). The MSORT setup measures relative changes in fluorescence ($\Delta F/F$) which are linearly related to changes in membrane potential.[22] The photodiode system is an AC-coupled system which allowed the recordings of action potential with the compromise that slowly developing, small amplitude changes in membrane potential are not detected. Dye staining of the nerve cells does not change their electrophysiological properties.[22]

STW 5 was applied to single submucous ganglia by pressure ejection from micropipettes (20 psi, 200 ms duration, approximately 200 μm distance to the ganglion). According to previously published calibration curves,[21] the pipettes were filled with 5120 μg mL^{-1} STW 5 to achieve a concentration of about 512 μg mL^{-1} at the ganglion level.

Drugs and solutions

STW 5, the lyophilized liquid of Iberogast®, was provided by Steigerwald Arzneimittelwerk GmbH Darmstadt, Germany. It was dissolved in Krebs buffer and added to the mucosal or serosal bathing solution yielding final concentrations ranging from 128 to 1024 μg mL^{-1}. The concentrations are well below the amount of 51.3 mg of STW 5 provided by one therapeutic serving of 20 drops (equivalent to 1 mL). The Krebs solution contained (in mmol L^{-1}): 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$ and 11 glucose (all from Sigma-Aldrich, Steinheim, Germany). Stock solutions of amiloride, 4-acetamido-4-isothiocyanatostilbene 2 (SITS), bumetanide, forskolin, glibenclamide, MDL-12 330 (all from Sigma-Aldrich, Schnelldorf, Germany) and $CFTR_{inh}$-172 (Calbiochem Merck, Darmstadt, Germany) were prepared in 100% dimethyl sulphoxide. Tetrodotoxin (TTX; Tocris Cookson, Bristol, UK) was dissolved in water. At the final concentrations used none of the solvents had any effect on the transport properties of the tissues.

Data analysis and statistics

Di-8-ANEPPS incorporates into the outer membrane revealing the outline of individual cell bodies. The overlay of signals and ganglion image allowed us to analyse the response of individual cells.[20] The total number of neurones for each ganglion was determined by visual inspection of images from the Di-8-ANEPPS stained ganglion.

For Ussing chamber experiments, the statistical values are based on the number of patients (for experiments with human tissue) or filters (for experiments with T84). For the dose-dependent effects, we calculated the maximum increase in I_{sc} (ΔI_{sc}) which was tested with Student's paired *t*-test (SIGMASTAT 3.1; Systat Software Inc, Erkrath, Germany). Each dose was tested in a different tissue rather than cumulative applications in one tissue. The same test was used to test for differences in spike discharge of individual neurons. The data are illustrated as mean ± standard error of means (SEM). The median effective concentrations (EC_{50}) were calculated with SIGMASTAT Software using Hill equation curve fit function.

Blockers were added to the mucosa/submucosa preparations 20 min before STW 5 application. For each blocker, a separate tissue from a directly adjacent region served as a control for STW 5 effects in untreated preparations. STW 5 was applied at the same time to the control tissue and the pretreated tissue. The non-normally distributed data are expressed as their median together with the 10th, 25th, 75th and 90th percentiles and tested with the Mann–Witney Rank Sum test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Effect of STW 5 on intestinal secretion and activity of submucous neurons

Basolateral application of STW 5 to human intestinal tissues led to dose-dependent increase in I_{sc} with an EC_{50} of 250 μg mL^{-1} (Fig. 1). For 256, 512 and 1024 μg mL^{-1} STW 5, this increase was significant. In the same tissues, we recorded the response to EFS, which evoked nerve-mediated secretion. We found that the increase in I_{sc} evoked by 256, 512 and 1024 μg mL^{-1} STW 5 was 146%, 205% and 224%, respectively, greater than the EFS-evoked increase. The STW 5 response peaked after 9.9 ± 0.7 min (n = 57) independent of the concentration used and slowly declined to pre-STW 5 levels with mean durations of 20.4 ± 2.7 min at 256 μg mL^{-1} (n = 10), 25.8 ± 3.3 min at 512 μg mL^{-1} (n = 14) and 29.6 ± 4.1 min at 1024 μg mL^{-1} (n = 10). Apical application of STW 5 also evoked an increase in I_{sc} with an EC_{50} of

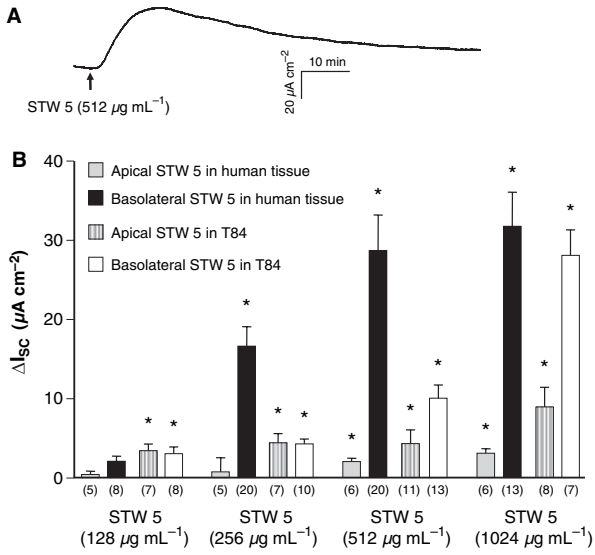

Figure 1 STW 5 evokes an increase in ion secretion in human tissue and in the epithelial cell line T84. Panel A shows the long lasting increase in short circuit current (I_{sc}) after basolateral application (addition to the serosal bath) of STW 5 in human tissue; time of application is marked by the arrow. Panel B shows the dose-dependent increase in I_{sc} after basolateral and apical (addition to the mucosal bath) application in human tissue and in T84 cells. Numbers in parenthesis below the bars indicate the number of tissues (equal to number of patients) studied. Asterisks mark significant changes.

555 μg mL^{-1} (Fig. 1). However, the effect was smaller and less robust than after basolateral STW 5 application.

The EFS evoked increase in I_{sc} was not changed by any of the STW 5 concentrations (21.3 ± 2.8 μA cm^{-2} *vs* 23.6 ± 3.2 μA cm^{-2} measured after 1 h STW 5 incubation; $P = 0.678$; $n = 41$). However, two findings revealed a strong nerve-mediated component of the STW 5 response. Firstly, tetrodotoxin (1 μmol L^{-1}) significantly suppressed the STW 5-evoked increase in I_{sc} by 75 ± 11% at 256 μg mL^{-1}, 68 ± 12% at 512 μg mL^{-1} and 76 ± 12% at 1024 μg mL^{-1} (Fig. 2A). Secondly, STW 5 had an excitatory action on human submucous neurons. Direct application of STW 5 onto colonic submucous ganglia via the ejection pipette elicited action potential discharge (Fig. 2B) in 27 of 46 neurons with 51 ± 18% STW 5-responsive neurons per ganglion (four ganglia, three tissues and three patients). The spike frequency significantly increased from zero (all responsive neurons were quiescent) to 2.4 ± 0.2 Hz.

The results so far suggested that STW 5 had a prosecretory action by direct effects on the epithelium and by activation of enteric neurons.

We further confirmed the direct epithelial effects by experiments in T84 cells. Basolateral application of STW 5 led to a dose-dependent significant increase in I_{sc} with an EC_{50} of 1000 μg mL^{-1} (Fig. 1). The STW 5 response peaked after 9.4 ± 0.6 min ($n = 36$) independent of the concentration used and slowly declined to pre-STW 5 levels with mean durations of 25.9 ± 7.3 min at 128 μg mL^{-1} ($n = 3$), 25.2 ± 5.0 min at 256 μg mL^{-1} ($n = 7$), 33.6 ± 4.4 min at 512 μg mL^{-1} ($n = 9$) and 31.7 ± 3.4 min at 1024 μg mL^{-1} ($n = 6$). Apical application also caused a significant but smaller increase in I_{sc} with an EC_{50} of 4241 μg mL^{-1} (Fig. 1).

Pharmacology of the STW 5 induced increases in I_{SC}

To further characterize the mode of action of basolateral application of STW 5, we applied specific blockers of ion channels and transporters in human mucosa/submucosa preparations and T84 cells. Due to the limited availability of human tissue, we performed these experiments with 512 μg mL^{-1} STW 5 only, a concentration which produced robust responses in both models. The small response to apical application prevented a detailed evaluation of its pharmacology.

We first wanted to identify the ion flux responsible for the increase in I_{sc}. There are basically two possibilities: increase in Cl^- secretion or enhanced Na^+ absorption. In human tissue and T84 cells blockade of the basolateral Na–K–2Cl cotransporter with bumetanide reduced the response to STW 5 by 70% ($P = 0.015$) and 85% ($P = 0.001$), respectively (Fig. 3). Pre treatment of human tissue and T84 cells with apically applied amiloride, a blocker of epithelial Na channels (ENaC), had no influence on the STW 5 response ($P = 0.878$ and 0.635; Fig. 3). As ENaC is differentially expressed along the gut, the experiments with amiloride were all performed in colonic tissue. The efficacy of amiloride was confirmed by the expected decrease in baseline I_{sc} by 38 ± 19 μA cm^{-2} ($n = 8$). These results indicated that the STW 5 induced increase in I_{sc} was due to an increased Cl^- secretion and not to an enhanced Na^+ absorption.

The two most prominent candidates responsible for Cl^- secretion are the cAMP-dependent cystic fibrosis transmembrane conductance regulator (CFTR) and the Ca^{++}-dependent Cl^- channels ClCa. Involvement of CFTR was supported by the observation that apical application of the unspecific CFTR blocker glibenclamide reduced the response to STW 5 by 42% ($P = 0.038$) and 68% ($P = 0.001$) in human tissue and T84 cells, respectively (Fig. 3). Strong cAMP dependency was also evident because basolateral application of the adenylate cyclase inhibitor MDL-12 330 suppressed the STW 5 response by 70% ($P = 0.001$) and

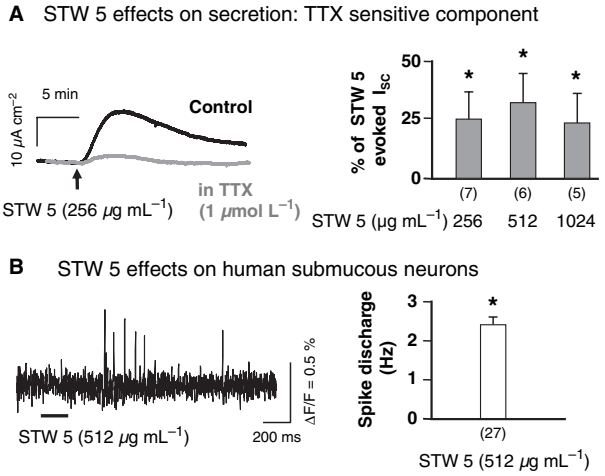

Figure 2 STW 5 evoked increase in ion secretion in human tissue is partly mediated by activation of enteric nerves. Panel A shows on the left side a control STW 5 evoked increase in I_{sc}. In a different tissue from the same patient the nerve blocker tetrodotoxin (TTX) suppressed this response. As shown on the right side TTX decreased the response at all concentrations. Panel B shows that STW 5 directly activates human submucous neurons. The bar below the trace indicates the 200 ms application of STW 5. Shortly after application the neuron fired action potential discharge (sharp upward deflections). This effect was significant (see right side). Asterisks mark significant differences and the numbers in parenthesis indicate the number of tissues (equal to number of patients) studied.

51% ($P = 0.023$) in human tissue and T84 cells, respectively (Fig. 3). To further confirm the involvement of CFTR channels, we used the highly specific CFTR channel blocker $CFTR_{inh}$-172.[23] In native tissue, the potency of $CFTR_{inh}$-172 is limited by the relatively low negative membrane potential, which reduces cytoplasmatic accumulation of $CFTR_{inh}$-172.[23] Therefore, we tested the efficacy of $CFTR_{inh}$-172 by applying the cAMP activator forskolin (10 μmol L^{-1}) at the end of the experiments. In eight of 12 experiments, $CFTR_{inh}$-172 significantly reduced the forskolin-induced increase in I_{sc} by 43% ($P = 0.031$). In these tissues, $CFTR_{inh}$-172 also reduced the STW 5 evoked response by 32% ($P = 0.022$; Fig. 3). $CFTR_{inh}$-172 suppressed the forskolin response in all 12 experiments with T84 cells by 64% ($P = 0.009$) and at the same time reduced the STW 5-induced increase in I_{sc} by 59% ($P = 0.003$, Fig. 3).

Both in human tissue and in T84 none of the CFTR inhibitors fully blocked the response. To test the involvement of ClCa channels, we used apical application of SITS which reduced the response to STW 5 by 42% ($P = 0.03$) and 43% ($P = 0.001$) in human tissue and T84 cells, respectively (Fig. 3).

The conclusion from the above experiments was that the STW 5 increased Cl^- secretion was due to an activation of both CFTR and ClCa channels.

DISCUSSION

Our study revealed that STW 5 acts as a secretagogue in the human intestine through an epithelial and a nerve-mediated action. The increased secretion is caused by activation of cAMP and calcium-dependent Cl^- channels. This action profile may help to understand its beneficial effect in IBS patients[12] and should stimulate further clinical studies with STW 5 in gut disorders that are associated with, or caused by, impaired secretion.

This is the first study demonstrating effects of STW 5 on intestinal epithelial transport functions. We did not attempt to identify which of the individual extracts in STW 5 or which of their phytochemical components are responsible for the prosecretory effect for two main reasons. Firstly, it was the mother compound (STW 5) that was effective in patients.[12,13] Secondly, we have previously shown that the effects of STW 5 on gastric motility are a result of the combined action of several components because each individual extract had an effect on motility.[5] This was also reported for the effects of STW 5 and its components on electrical smooth muscle activity in the intestine.[23] However, we cannot discount the possibility that only few extracts or even a single component is involved in the prosecretory action of STW 5. Therefore, the effects of individual extracts on mucosal secretion need to be investigated in future studies.

The prosecretory effect of STW 5 is due to multiple modes of action including stimulation of transporters and channels on epithelial cells and activation of enteric neurons. The direct epithelial effects are supported by the STW 5 responses in T84 cells and by the occurrence of a TTX-insensitive secretory component in human tissue. The bumetanide sensitivity suggests that STW 5 strongly activates basolateral Cl^- influx which represents the main driving force for luminal Cl^- secretion through apical CFTR and ClCa channels. Consequently, STW 5 evoked secretion was also suppressed by blockers of the CFTR and ClCa channels. The involvement of both Cl^- channels may prove to be of clinical relevance as STW 5 would increase secretion even under conditions where one of the channels may be down-regulated. Whether STW 5 can directly activate the apical Cl^- channels remains to be shown but its prosecretory action upon apical application would be in agreement with such a mode of action.

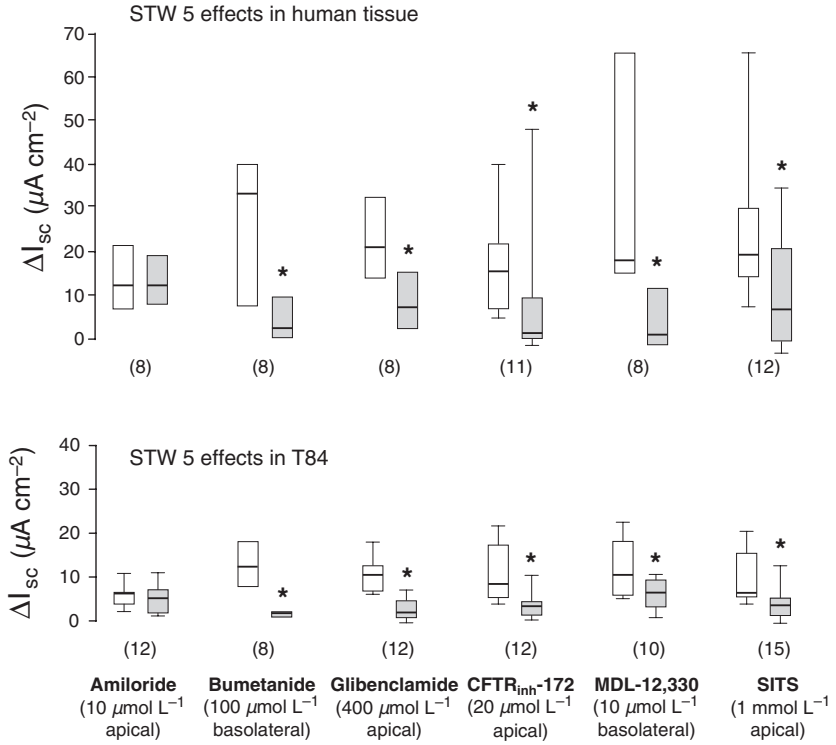

Figure 3 Both in human tissue and in T84 cells, STW 5 evoked increase in I_{sc} is due to increased Cl^- secretion involving the basolateral Na–K–2Cl transporter (bumenatide sensitivity), the cAMP-dependent CFTR channel (sensitivity to glibenclamide, MDL-12 330 and $CFTR_{inh}$-172) and the Ca^{++} dependent CaCl channel (SITS sensitivity). The epithelial Na channel ENaC is not involved (amiloride insensitivity). For the action of all inhibitors (grey bars) separate tissues from the same patient served as control (white bars). Asterisks mark significant differences between control STW 5 responses and STW 5 responses in pretreated tissues. The numbers in parenthesis indicate the number of tissues (equal to number of patients) or wells studied.

The strong TTX sensitivity of the STW 5 evoked secretion and the finding that STW 5 activated human submucous neurons highlight the prominent role of enteric nerves. The activation of enteric nerves will lead to enhanced release of transmitters which are known to activate Cl^- secretion via CFTR and ClCa channels.[24,25] The most likely reason for the finding that the EFS was not potentiated by STW 5, despite its activation of enteric neurons, is that we used stimulus parameters which already produced supramaximal stimulation.

Although the response to a single STW 5 application was long lasting, we did observe a slow decline with time. There are two possible explanations: firstly, biochemical and/or metabolic inactivation of STW 5 or secondy, run-down of signalling cascades. The latter is an often encountered phenomenon under *in vitro* conditions. For example, even carbachol and forskolin would evoke a transient increase in chloride secretion (own unpublished data).

While we found that acute application of STW 5 caused an excitatory action in neurons of the submucous plexus others described a decreased mechano- and chemosensitivity of visceral afferents after animals have been pretreated with STW 5 for 2 h.[26] Whether the differences are due to the different application protocols (acute in our study *vs* 2 h pretreatment in the study on visceral afferents) or rather suggest differential actions of STW 5 on visceral afferents *vs* enteric neurons remains unknown. It is tempting to speculate that the improved IBS symptom and abdominal pain score after STW 5 treatment[12] may be explained by the differential actions which would lead to decreased pain threshold by inhibiting visceral afferents and at the same time normalized intestinal functions by activating enteric nerves.

Although speculative at this stage the prosecretory effect of STW 5 may provide some mechanistic insights into its favourable effect in IBS patients.[12] It is obvious that enhanced secretion may improve symptoms in constipation predominant IBS. This has also been shown in trials with linaclotide and lubiprostone, both of which improve gastrointestinal symptoms in IBS constipation and are known to increase intestinal secretion.[16,17] It is noteworthy that the clinical study with STW 5 reported

an improved IBS symptom and abdominal pain score in all IBS subgroups – constipation, diarrhoea and alternating IBS.[12] It is conceivable that a secretion enhancing drug may dilute luminal threats, thus help to maintain and restore intestinal barrier functions which altogether may contribute to symptom relieve. Above all, it is likely that other STW 5 actions, such as modulation of muscle activity,[14,27] anti-inflammatory properties[7] and inhibition of nociceptive pathways[26] contribute to its clinical efficacy.

In the IBS trial, patients took 20 drops Iberogast® (corresponding to 51.3 mg mL^{-1} STW 5) three times daily.[12] It is difficult to assess how the concentrations used in our study can be extrapolated to the clinical situation as colonic concentrations of STW 5 or its components after oral intake have not been determined. We believe that our result have functional relevance as the smallest effective concentration was 200th of one therapeutic serving. Moreover, besides local actions of STW 5 in the colon an additional systemic effect appears likely.[10] Preliminary reports demonstrating efficacy of STW 5 in treatment of experimental colitis would support a systemic mode of action.[28,29] Based on our results in the Ussing chamber, topical and systemic actions are conceivable as apical and basolateral application were effective. Nevertheless, the results of our study would favour a predominant action of STW 5 on the basolateral epithelium and enteric nerves.

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Part of this work has been published in abstract form.[30]

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