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The type 2 CCK/gastrin receptor antagonist YF476 acutely prevents NSAID-induced gastric ulceration while increasing iNOS expression --Manuscript Draft--

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Abstract:	Purpose: YF476 differs from the PPI esomeprazole in mode of action by antagonizing the type 2 receptor of cholecystokinin/gastrin (CCK-2R). YF476 protection against diclofenac-induced gastric ulcers was compared to esomeprazole and correlated with plasma levels of hormones related to gastric pH (gastrin, ghrelin, somatostatin), gastric gene expression of these hormones, their receptors, and inducible nitric oxide synthase (iNOS). Methods: YF476 or esomeprazole pretreatments were followed by diclofenac. Four hours later, gastric tissue was excised and analyzed for ulcer index. An intragastrically implanted Bravo capsule measured pH for five days during YF476 plus pentagastrin treatment. Changes in gene expression were assayed for gastrin, ghrelin and somatostatin, their receptors and iNOS. Results: YF476 acutely (within 4 h) protected against diclofenac-induced gastric ulcers equivalent to esomeprazole. Gastric pH recorded during five days in presence of pentagastrin was 1.83 (± 0.06). YF476 raised pH to 3.67 (± 0.09) and plasma ghrelin, gastrin and somatostatin increased. YF476 increased expression of gastrin and SstR2 genes, while ghrelin receptor decreased. Expression of ghrelin, somatostatin and CckbR receptor remained unchanged. In presence of diclofenac, esomeprazole increased expression of all these transcripts and NOS2 gene (iNOS), while YF476 yielded only decreased CckbR and increased NOS2. Conclusions: YF476 is a potential new preventative treatment for patients at risk of

	NSAID-induced ulceration. Gastric gene expressions of ghrelin, gastrin and somatostatin and their receptors differ between esomeprazole and YF476. Despite these differences and different modes of action to raise gastric pH, both drugs acutely increase iNOS, suggesting iNOS expression parallels pH.
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The type 2 CCK/gastrin receptor antagonist YF476 acutely prevents NSAID-induced gastric ulceration while increasing iNOS expression

Short title: YF476 prevents diclofenac-induced ulcers

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Abstract

Purpose: YF476 differs from the PPI esomeprazole in mode of action by antagonizing the type 2 receptor of cholecystokinin/gastrin (CCK-2R). YF476 protection against diclofenac-induced gastric ulcers was compared to esomeprazole and correlated with plasma levels of hormones related to gastric pH (gastrin, ghrelin, somatostatin), gastric gene expression of these hormones, their receptors, and inducible nitric oxide synthase (iNOS).

Methods: YF476 or esomeprazole pretreatments were followed by diclofenac. Four hours later, gastric tissue was excised and analyzed for ulcer index. An intragastrically-implanted Bravo capsule measured pH for five days during YF476 plus pentagastrin treatment. Changes in gene expression were assayed for gastrin, ghrelin and somatostatin, their receptors and iNOS.

Results: YF476 acutely (within 4 h) protected against diclofenac-induced gastric ulcers equivalent to esomeprazole. Gastric pH recorded during five days in presence of pentagastrin was 1.83 (± 0.06). YF476 raised pH to 3.67 (± 0.09) and plasma ghrelin, gastrin and somatostatin increased. YF476 increased expression of gastrin and SstR2 genes, while ghrelin receptor decreased. Expression of ghrelin, somatostatin and CckbR receptor remained unchanged. In presence of diclofenac, esomeprazole increased expression of all these transcripts and NOS2 gene (iNOS), while YF476 yielded only decreased CckbR and increased NOS2.

Conclusions: YF476 is a potential new preventative treatment for patients at risk of NSAID-induced ulceration. Gastric gene expressions of ghrelin, gastrin and somatostatin and their receptors differ between esomeprazole and YF476. Despite these differences and different modes of action to raise gastric pH, both drugs acutely increase iNOS, suggesting iNOS expression parallels pH.

Key words: Diclofenac, gastric pH, gastrin, ghrelin, somatostatin, nitric oxide synthase.

Abbreviations

CCK-2R, type 2 receptor of cholecystokinin/gastrin; CckbR, rat gene coding type 2 receptor of cholecystokinin; CV, intra-assay coefficient of variation; Gast, rat gastrin gene; Ghrl, rat ghrelin gene; GhsR, rat ghrelin receptor; H2RA, histamine H2 receptor antagonist; iNOS, inducible nitric oxide synthase (i.e., expressed protein possessing enzymatic activity); NOS2, gene coding iNOS; IV, intravenous; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate buffered saline; PPI, proton pump inhibitor; qPCR, quantitative polymerase reaction; SstR2, rat gene coding type 2 receptor of somatostatin; Sst, rat somatostatin gene; SC, subcutaneous; YF476, (R)-1-[2,3-dihydro-2-oxo-1-pivaloylmethyl-5-(2-pyridyl)-1H-1,4-benzo-diazepin-3-yl]-3-(3-methylaminophenyl)urea.

Introduction

NSAIDs have the unwanted property of inducing gastric lesions that progress to gastric ulcers. Histamine H₂-receptor antagonists (H₂RAs) and proton pump inhibitors (PPIs) prevent this (Leontiadis et al. 2007). However, chronic suppression of acid secretion by H₂RAs or PPIs causes hypergastrinaemia. In the case of H₂RAs, hypergastrinaemia is associated with acid suppression tolerance (Wilder-Smith et al. 1990). PPIs such as esomeprazole are currently the preferred treatment for acid related conditions (Frazzoni et al. 2006). In some cases, rebound hyperacidity and dyspepsia occur after withdrawal of prolonged treatment with H₂RAs (Nwokolo et al. 1991) or PPIs (Reimer et al. 2009). Consequently, even PPIs effectively promote the conditions they are intended to treat (McColl et al. 2009). New drugs that escape acid suppression tolerance and rebound hyperacidity while still protecting against NSAID- induced gastric ulcers are desired.

YF476 is an orally active, selective antagonist of the gastrin receptor CCK-2R (Semple et al. 1997). YF476 is more potent than H₂RA or PPI at inhibiting gastric acid secretion in the rat and dog (Takinami et al. 1997; Takemoto et al. 1998). As with H₂RAs and PPIs, acid suppression by YF476 results in hypergastrinaemia (Ding et al. 1997; Chen et al. 2000). However, in the rat, this does not progress to tolerance to gastric acid inhibition or rebound hyperacidity after stopping YF476 (Kitano et al. 2000; Chen et al. 2000). Two characteristic advantages of gastrin antagonists are that they prevent, 1) PPI induced ECL-cell hyperplasia (Chen et al. 2000) and 2) rebound hyperacidity (Nishida et al. 1995). Thus, YF476 has advantages over H₂RAs and PPIs for treatment of acid related conditions (Tari 1997; Cui and Waldum 2007).

In healthy human volunteers, single doses of YF476 cause dose dependent increases in basal and food stimulated gastric pH (Boyce et al. 2000). Consistent with this, single doses of YF476 cause dose-dependent inhibition of pentagastrin-stimulated titratable acid in gastric

aspirate, and this activity is not lost after repeated doses of YF476 (Boyce et al. 2004). Recent studies in rats further demonstrated continued acid inhibition over time (Barrett et al. 2012). Thus, YF476 causes prolonged blockade of CCK-2R in rats and humans under more hyperacidic conditions, but it is not currently known how YF476 affects the various hormones/pathways regulating gastric pH.

Targeted gene disruption experiments in mice have demonstrated at least 3 pathways controlling acid secretion (Chen et al. 2005): the gastrin–histamine pathway, the CCK–somatostatin pathway and a neural pathway. Somatostatin inhibits gastric acid secretion (Kapuscinski and Shulkes 1995; Schubert 2008), and ghrelin (neural pathway) stimulates it, and these have been described as being mediated via both the vagus nerve and histamine (Yakabi et al. 2006; Schubert 2008; Yakabi et al. 2008). Crosstalk between the 3 pathways therefore seems plausible. Similar to somatostatin, ghrelin has also been shown to decrease pentagastrin-stimulated acid secretion (Levin et al. 2005). It was therefore essential in this study to investigate how YF476 influences gastrin, somatostatin and ghrelin. It was further reasoned that receptor signaling could also be altered through changes in receptor expression.

The primary aims of this study were therefore to compare the effect of YF476 with the PPI esomeprazole on NSAID-induced gastric ulceration in rats and to investigate the effect of YF476 on pentagastrin-stimulated gastric pH. Other aims were to assess the effect of YF476 on circulating levels of the pH regulatory peptides gastrin, ghrelin and somatostatin, and gene expression of these hormones and their receptors in the gastric mucosa. Finally, YF476 was studied in two conditions under which acid suppression would be expected, if anything, to protect against tissue damage leading to inflammation and tissue repair using NOS2 mRNA expression (gene encoding iNOS) as marker; during acute diclofenac co-treatment with YF476 and after 5 days of chronic YF476 treatment.

Material and methods

Animals

Male Sprague-Dawley rats (300-350 g, Scanbur B&K AB, Sollentuna, Sweden) were acclimated in wire meshed cages at 24 °C with constant humidity and 12 h light-dark cycles for at least 7 days before experiments. They were fed *ad libitum* with commercial rat chow (LABFOR, Lactamin R36, Kimstad, Sweden) and tap water. During recovery after surgery, rats were trained daily to accept experimental conditions. Experiments were conducted in conscious animals after fasting for maximum 15 h in wire bottomed cages with access to water. Experiments were approved by the Animal Ethics Committee in Stockholm (permits/Dnr: 157/05, 100/07, 191/08, 353/09) according to the “ARRIVE” guidelines (Animals in Research: Reporting *In Vivo* Experiments).

Surgical procedures

Surgery was performed with a mixture of midazolam (5 mg·mL⁻¹, midazolam Aktavis AB, Stockholm, Sweden) and Hypnorm (fentanyl citrate, 0.315 mg·mL⁻¹ plus fluanisone, 10 mg·mL⁻¹; Janssen-Cilag, Sollentuna, Sweden) given subcutaneously (SC) at a dose of 1.5-2 mL·kg⁻¹ body weight. Buprenorfin (Temgesic® 0.3 mg·mL⁻¹, Schering-Plough, Stockholm, Sweden) was given at 0.05 mg·kg⁻¹ body weight SC after surgery to relieve postoperative pain.

A Bravo capsule (electronic pH sensor encapsulated in PVC plastic, 25x5x5 mm; Synmed Medicinteknik AB, Spånga, Sweden) was surgically implanted inside the stomach, as previously described (Rudholm et al. 2008). A midline incision was made, creating an opening in the proximal greater curvature, and gastric contents were evacuated. The capsule was sutured to the

stomach mucosa with pH sensor pointing distally. An indwelling silastic catheter (Dow Corning Co., Midland, MI, USA) pretreated with heparin was inserted into the external jugular vein for administration of saline and pentagastrin, and blood sampling. Finally, a small flange catheter (Dow Corning Co.) was anchored in the fundus of the stomach near the lesser curvature. The catheters were tunneled at the back of the animal's neck, and the opening soldered.

Chemicals

YF476 was supplied by Trio Medicines Ltd, London, UK. Esomeprazole was from AstraZeneca (Nexium Södertälje, Sweden). Pentagastrin was obtained from Cambridge Laboratories (Wallsend, UK). Diclofenac was from Novartis (Voltaren, Basel, Switzerland). Saline solution ($\text{NaCl } 9 \text{ g}\cdot\text{L}^{-1}$) was from Fresenius Kabi (Halden, Norway). Other chemicals were from Sigma-Aldrich Sweden AB (Stockholm, Sweden).

NSAID-induced gastric ulcers

In total, 32 rats were deprived of food but had free access to water 18-20 h before the start of experiments. They were separated into 4 groups of 8 rats ($n=8$), and given a single pretreatment of saline (vehicle), esomeprazole ($100 \text{ mg}\cdot\text{kg}^{-1}$) or YF476 ($100 \text{ mg}\cdot\text{kg}^{-1}$). This dosage was chosen to ensure that hypergastrinaemia was achieved similarly by both drugs (Takeuchi et al. 2003 and Martinsen et al. 2003). After one hour, diclofenac was administered ($30 \text{ mg}\cdot\text{kg}^{-1}$), except for one saline pretreatment group that received a second dose of saline instead of diclofenac (negative control). All administrations were by gavage. Four h later, rats were sedated with CO_2 and euthanized by cervical dislocation. Stomachs were excised, washed in PBS and photographed. Ulcer index was quantified according to Jansson et al. 2007 and is expressed in mm, reflecting total length of gastric lesions per stomach, judged by three researchers blinded to

the treatments. Tissue was then processed for qPCR measurement of mRNA. In 2 cases, mRNA was excluded because of poor mRNA content, resulting in n=6 for related data.

Intragastric pH, gastric regulatory peptides and gene expression

Experiments were performed in conscious rats (n=8) two days after surgically implanting the Bravo capsule, one experiment in each rat. The animals gained weight (mean 8.0 ± 1.2 g), exhibiting normal behavior and feeding. There were no postmortem mucosal lesions, pyloric obstructions or gastric distensions. Bolus doses of YF476 were administered at $100 \text{ mg} \cdot \text{kg}^{-1}$ through a gastric catheter twice daily (8 am and 5 pm) for a total of 5 days. At the same time pentagastrin ($90 \text{ pmol kg}^{-1} \cdot \text{min}^{-1}$, IV) was continuously administered on all 5 days. The pH recorded by the Bravo capsule was transmitted with 6 Hz sampling frequency to a receiver outside the cage. Recordings were made continuously over a total of 120 h (i.e., 5 days), with exception of two short, obligatory intermissions every 48 h to download data and replace batteries. From these 8 rats/group, 2 rats/group were excluded due to poor mRNA content. Plasma from associated rats was also excluded from peptide measurements, resulting in n=6 for the two experiments. Plasma peptide measurements are therefore matched to gene expressions in the same animals.

Samples of 200 μL blood were drawn 1.5 h after the morning administration of YF476 on the fifth (last) day, centrifuged at 2500 RCF, 10 min, 4 °C and ~100 μL of plasma collected and stored at -20 °C for assays of regulatory peptides. Assays used competitive enzyme-linked immunosorbent assay kits from Phoenix Pharmaceuticals, Burlingame, CA, USA. Samples were measured in duplicate. CV values were 5-7%, n=6 rats. Ghrelin (total) was measured using rat EK-031-31. Gastrin was measured using a human gastrin I kit (EK-027-04) that cross-reacts with rat gastrin. Somatostatin was measured using a rat kit (EK-060-03), which cross-reacts 100%

with somatostatin-25 and -28 and [des-Ala¹]-somatostatin, but not pro-somatostatin. Matched gastric tissue samples were processed for corresponding mRNA and NOS2 (rat iNOS) as described below.

qPCR

At the end of above experiments involving pH or diclofenac, animals were sedated with CO₂ and euthanized by cervical dislocation. Tissue segments (20-30 mg) from the corpus of the stomach were collected from six animals in each group, quickly placed in RNA stabilizing reagent (RNAlater, Qiagen, Hilden, Germany), stored at 4 °C for 24 h, and stored at -20 °C until analysis by quantitative polymerase chain reaction (qPCR). For RNA extraction, tissue samples were placed in RTL cell lysis buffer (Qiagen, Hilden, Germany) and homogenized by a rotor-stator knife (Ultra-Turrax T8, IKA®-Werke, Staufen, Germany). Total RNA was then extracted using the RNeasy Mini Kit according to the manufacturer's instruction (Qiagen). Finally, a DNase digestion step (DNase I; Promega, Madison, WI, USA) with incubation at 37 °C for 30 minutes was done to remove traces of chromosomal DNA. Samples with A260/A280 ratio ≥ 1.8 were used for qPCR. Complementary DNA (cDNA) synthesis was performed on 1–5 µg of total RNA using oligo dT primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

mRNA expression for rat ghrelin (Ghrl), gastrin (Gast), somatostatin (Sst), their respective receptors GhsR, Cck2R and SstR2 and also inducible nitric oxide synthase (NOS2) were analyzed using qPCR with an ABI 7300 PCR System (Applied Biosystems, Foster City, CA) or a CFX96 Real-Time System (Bio-Rad Labs, Hercules, CA) using ready-made gene-specific primers and TaqMan probe mixes (gene expression assays no. Rn01644838, Rn00561967, Rn00572319, Rn00561646, Rn00565867, Rn00583419, Rn00571116; all from Applied

Biosystems). Each reaction was normalized against the expression of hypoxanthine phosphoribosyl transferase 1 (Hprt1; gene expression assay no. Rn01527840, Applied Biosystems) as internal control. See Table 1 for details. Complementary DNA subjected to qPCR was performed in 25 μ L reaction volumes with 20 x TaqMan universal PCR master mix (Applied Biosystems). Amplification was carried out at 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 sec, and 60 °C for 1 min. All samples were analyzed in triplicate for each transcript. Serial dilutions of a mix of all cDNAs was amplified to generate standard curves for the threshold cycles (C_t) of each mRNA transcript, as well as to determine the levels of expression in the samples by comparison. Raw C_t values were recalculated with REST software (Pfaffl et al. 2002) to obtain the relative expression of mRNA in each treatment group relative to the appropriate control. Data are presented as fold-changes in arbitrary units relative to the mean of the control samples.

Data analysis and statistical analysis

Bravo capsule pH data was analyzed with POLYGRAM NET ^{TM} (pH Testing Application software, Synmed Medicinteknik, Spånga, Sweden), calculating changes in pH relative baseline (defined as 0.5 h before onset). Ulcer indexes were compared by Kruskal-Wallis test. Plasma hormone concentrations were compared using Mann-Whitney test.

Results

YF476 gastroprotection from diclofenac-induced ulcers

Figure 1 shows representative photographs depicting gastroprotection by YF476. Figures 1C and 1D show that YF476 and esomeprazole similarly protected the gastric mucosa from

diclofenac-induced ulceration compared to control (Figure 1A). Ulcer index was quantified from these photographs (figure 1E, n=8). Compared with controls (79 ± 14 mm), YF476 (4.8 ± 2.4 mm; $P < 0.001$) and esomeprazole (5.4 ± 1.1 mm; $P < 0.01$) dramatically reduced the ulcer index.

Intragastric pH elevated by YF476 in presence of pentagastrin

Figure 2 illustrates pH during 120 h (5 days) of pentagastrin IV. In controls, mean 24 h pH across the 5 days (n=5 days) was 1.83 ± 0.06 . In the YF476 treated group, mean 24 h pH was significantly higher at 3.67 ± 0.09 ($p < 0.001$). This effect was seen on all 5 days, with daytime peak pH in the YF476 treated group reaching between pH 4 and 5; that is, 2-3 logs above controls. There were no significant differences in diurnal pH (9 am to 3 pm vs. 3 pm to 9 am) when YF476 was given, except that pH was maintained at a higher level.

YF476 effects on hormone concentrations and gene expressions in presence of pentagastrin

Data in figures 3 and 4 are matched results for same rats in presence of pentagastrin. Figure 3 shows YF476 treatment increased plasma ghrelin 2.6-fold ($P < 0.05$), gastrin 9.1-fold ($P < 0.01$) and somatostatin 6.2-fold ($P < 0.01$). Following YF476 treatment (figure 4), mRNA expression of Gast gene increased 40.9-fold ($P < 0.05$) and SstR2 gene increased 9.9-fold ($P < 0.01$), while GhsR decreased (20.3-fold ($P < 0.05$)). Expression of Ghrl, Sst and CckbR in stomach did not change after YF476.

Gene expression with YF476 and diclofenac

Results are shown in figure 5 for acute (4 h) conditions. Relative to untreated controls, diclofenac did not change gene expression of any of the hormones or their receptors. Esomeprazole increased expression of all transcripts measured ($P < 0.05$ or $P < 0.01$ as indicated),

while YF476 only yielded decreased expression of CckbR (81.7-fold, $P<0.01$) compared to controls. When compared to the diclofenac group without additional treatment, added esomeprazole induced expression of CckbR, SstR2, ghrelin and iNOS, while YF476 showed decreased expression of CckbR 34-fold ($P<0.05$) and increased expression of NOS2 35-fold ($P<0.01$). Hence, in the acute setting, both esomeprazole and YF476 induced over-expression of NOS2.

Discussion

YF476 prevented diclofenac-induced lesions associated with ulceration of the gastric mucosa equal to esomeprazole. Directly blocking gastrin through CCK-2R antagonism may offer clinically relevant advantages such as preventing hypergastrinaemia induced ECL-cell hyperplasia and potentially adverse downstream paracrine signaling cascades (Dimaline and Varro 2007).

The Bravo capsule continuously measures gastric pH in free roaming animals for long periods (Rudholm et al. 2008). The disadvantage of measuring pH is the logarithmic scale. Gastric pH may change little despite a large change in hydrogen secretion (Johnston and Wormsley 1993). However, YF476 induced pH changes covering as many as three logs during the daytime peaks. While gastric pH does not quantify changes in volume, the magnitude of pH changes seen in this study cannot be explained as mere changes in volume. Further, while the alternative of total hydrogen collected over time is a more exact readout of acid suppression, it is not applicable to continuous monitoring in free roaming animals over extended periods.

As expected, YF476 caused a significant increase in gastric pH in rats stimulated with intravenous pentagastrin, consistent with inhibition of gastric acid secretion by parietal cells. Onset was rapid and persisted throughout five days. These results are compatible with those of

others showing that the effect of YF476 and another CCK-2R antagonist, YM022, persist even after chronic dosing up to 8 weeks in the rat (Chen et al. 2000; Kitano et al. 2000). The CCK-2R antagonist JNJ-26070109 also prevents PPI (omeprazole)-induced acid rebound in the rat (Barrett et al. 2012). Hence, persistent acid inhibition with repeated dosing over considerable time is a general and reproducible property of CCK-2R antagonists.

The increased plasma gastrin following YF476 treatment seen in this study confirms several studies (e.g., Ding et al. 1997; Chen et al. 2000; Kitano et al. 2000; Cui et al. 2006). Such increases in gastrin are understood to be secondary to acid suppression. This study extends on this knowledge by revealing that components of the other two pathways regulating gastric pH (i.e., somatostatin and ghrelin) parallel gastrin.

The mechanism by which YF476 affects gene expression has yet to be studied, but from the present data, it can be concluded that since gene expression differed so markedly between esomeprazole and YF476, pH alone is insufficient to explain all the changes. The differences must be accounted for through selective intracellular signaling through the respective receptors.

The NOS2 results, reflecting iNOS synthesis and NO production, were surprising to us in the case of the acute setting. To our knowledge, diclofenac does not increase gastric pH (Alioth et al. 1993) and diclofenac on its own was also not seen to acutely increase NOS2 expression despite clearly evident lesions. Yet, both esomeprazole and YF476, which predictably raise pH, albeit by different signaling mechanisms, induced over-expression of NOS2 where gastric lesions had never evolved. This implies that elevated gastric pH increases NOS2 expression in the absence of tissue injury that would otherwise be associated with inflammation. Our data is in agreement with previous studies indicating a pH-dependent NO production as shown in hepatocellular damage in the rat (Shu et al. 1997). This offers important mechanistic insights into how these drugs prevent NSAID-induced ulcers.

Gastric pH and NO are connected through non-NOS-dependent mechanisms in the lumen; hydrogen in the gastric lumen is required for non-enzymatic reduction of ingested nitrate to NO. It is noteworthy that in this study, iNOS over-expression in response to acute YF476 and esomeprazole, favoring NO production in tissue, increased with increased gastric pH, a condition under which non-NOS-dependent mechanisms in the gastric lumen disfavor NO production. There may exist a direct antagonistic coupling between gastric pH, iNOS expression and NO production between the non-NOS-dependent and NOS-dependent pathways. The present results speak in favor of an acute acid dependent NO regulatory mechanism in the stomach that enforces NO levels. Evidence for such a tight coupling can be seen in data published by others (e.g., Tatemichi et al. 2003; Takeuchi K et al. 1999). It is noteworthy that this effect, while seen consistently in acute (4 h) experiments was not sustained in most cases after 5 days of YF476 treatment, suggestive of a desensitizing mechanism or isoform switching of NOS, which is known to occur (Yoshizumi et al. 1993; Vo et al. 2005), although the physiological meaning of this is not understood. The other NOS isoforms were not studied here because they are not generally regarded to be tightly coupled to inflammation, which was of more immediate interest in the context of preventive action against NSAID-mediated gastric lesions.

With the exception of Ghrl, the mRNA expression of the three regulatory peptides and their receptors differed markedly between acute and chronic YF476 treatment. It is clear YF476 had a very similar effect on pH at 4 h and 5 days. Diclofenac did not acutely affect mRNA expression of any of the genes in this study. It seems YF476 treatment results in different mRNA expressions contingent upon duration of treatment.

As regards the use of a CCK-2R antagonist in acid-related disorders it would be of interest to investigate whether the putative mucosal anti-trophic actions of receptor antagonism (Håkanson et al. 1994, Koh and Chen 2000) would outweigh the acid suppression aspect in

healing gastric ulcers. In our experiments in the rat this was not the case as YF476 and omeprazole had the same preventative effect on NSAID-induced gastric ulcerations. Diclofenac did not acutely affect mRNA expression of any of the genes in this study. Chronic YF476 treatment apparently results in a transition in mRNA expressions relative to what is obtained during acute treatment. Similarly, whereas NOS2 up-regulation was consistent with acute treatment of either esomeprazole or YF476, following 5 days of YF476 treatment, NOS2 expression varied between individuals indicating a successive adaptation of the mucosal injury and inflammatory response.

In the organ bath system, gastrin has been shown to induce contractions in about one third of muscle strips (Bennett et al. 1967). It has been seen in this lab that YF476 gives relaxation in one third of gastrin pre-treated strips. The presence/absence of response differed by patient with strong reproducibility between strips from the same preparation (Halim et al, to be published). In examining the distribution of changes in gastric gene expression in response to acute YF476, cluster analysis revealed two separable sets of responders with this same frequency distribution. For instance, the strongest expressions for each of the genes in figure 4 were from the same rat. Similarly, the weakest expressions were traced to one other rat. YF476 is now being applied to explore heterogeneity in changes in gene activation and responses in the organ bath system.

In conclusion, YF476 prevents NSAID-induced gastric ulceration as effectively as esomeprazole and inhibits pentagastrin-stimulated gastric acid secretion. Expression of NOS2, and by extension iNOS, parallels acid suppression regardless of which pathway acted upon to raise pH. YF476 is a potential new preventative treatment for patients at risk of NSAID-induced ulceration and is now being applied to in situ research.

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References

Alioth C, Blum RA, D'Andrea DT et al (1993) Application of dual radiotelemetric technique in studying drug-drug interaction between diclofenac sodium and ranitidine HCl in volunteers. Pharm Res 10:1688-1692

Barrett TD, Lagaud G, Wagaman P et al (2012) The cholecystokinin CCK2 receptor antagonist, JNJ-26070109, inhibits gastric acid secretion and prevents omeprazole-induced acid rebound in the rat. Br J Pharmacol 166:1684-1693

Bennett A, Misiewicz JJ, Waller SL (1967) Analysis of the motor effects of gastrin and pentagastrin on the human alimentary tract in vitro. Gut 8:470-474

Boyce M, David O, Darwin K, Mitchell T, Johnston A, Warrington S (2012) Single oral doses of netazepide (YF476), a gastrin receptor antagonist, cause dose-dependent, sustained increases in gastric pH compared with placebo and ranitidine in healthy subjects. Aliment Pharmacol Ther. 36:181-189

Boyce M, Warrington S, Johnston A, Harris A (2000) Effect on gastric pH of single doses of YF476, a new gastrin antagonist, compared with ranitidine and placebo. Br J Clin Pharmacol 49:493P-494P

Boyce M, Warrington S, Nentwich H, Norris V, Hull R, Black J (2004) Effect of repeated doses of YF476, a gastrin antagonist, on pentagastrin-induced changes in volume, hydrogen content and

pH of gastric aspirate in healthy subjects. Br J Clin Pharmacol 57:684P.

Chen D, Friis-Hansen L, Håkanson R, Zhao C-M (2005) Genetic dissection of the signaling pathways that control gastric acid secretion. Inflammopharmacol 13:201-207

Chen D, Zhao C-M, Norlén P et al (2000) Effect of CCK-2 receptor blockade on rat stomach ECL cells. Cell Tissue Res 299:81-95

Cui G, Takaishi S, Ai W et al (2006) Gastrin-induced apoptosis contributes to carcinogenesis in the stomach. Lab Invest 86:1037-1051

Cui G, Waldum HL (2007) Physiological and clinical significance of enterochromaffin-like cell activation in the regulation of gastric acid secretion. World J Gastroenterol 28:493-496

Dimaline R, Varro A (2007) Attack and defence in the gastric epithelium – a delicate balance. Exp Physiol 92:591-601

Ding X-Q, Lindström E, Håkanson R (1997) Evaluation of three novel cholecystokinin-B/gastrin receptor antagonists: A study of their effects on rat stomach enterochromaffin-like cell activity. Pharmacol Toxicol 81:232-237

Frazzoni M, Manno M, De Micheli E, Savarino V (2006) Intra-oesophageal acid suppression in complicated gastro-oesophageal reflux disease: esomeprazole versus lansoprazole. Dig Liver Dis 38:85-90

Håkanson R, Chen D, Andersson K et al (1994) The biology and physiology of the ECL cell. *Yale J Biol Med* 67:123-134

Jansson EA, Petersson J, Reinders C et al (2007) Protection from nonsteroidal anti-inflammatory drug (NSAID)-induced gastric ulcers by dietary nitrate. *Free Radic Biol Med* 15:510-518

Johnston D, Wormsley K (1993) Problems with the interpretation of pH measurements. *Clin Investig* 72:12-17.

Kapuscinski M, Shulkes A (1995) Secretory and biosynthetic responses of gastrin and somatostatin to acute changes in gastric acidity. *J Gastroenterol Hepatol* 10:405-412

Kitano M, Norlén P, Ding XQ, Nakamura S, Håkanson R (2000) Long-lasting cholecystokinin(2) receptor blockade after a single subcutaneous injection of YF476 or YM022. *Br J Pharmacol* 130:699-705

Leontiadis GI, Sreedharan A, Dorward S et al (2007) Systematic reviews of the clinical effectiveness and cost-effectiveness of proton pump inhibitors in acute upper gastrointestinal bleeding. *Health Technology Assessment* 11:1-164

Martinsen TC, Kawase S, Håkanson R et al (2003) Spontaneous ECL cell carcinomas in cotton rats: natural course and prevention by a gastrin receptor antagonist. *Carcinogenesis* 24:1887-1896

McColl KE, Gillen D (2009) Evidence that proton-pump inhibitor therapy induces the symptoms it is used to treat. *Gastroenterology* 137:20-22

Nishida A, Kobayashi-Uchida A, Akuzawa S et al (1995) Gastrin receptor antagonist YM022 prevents hypersecretion after long term acid suppression. *Am J Physiol* 269:G699-705

Nwokolo C, Smith J, Sawyerr A, Pounder R (1991) Rebound intragastric hyperacidity after abrupt withdrawal of histamine H2 receptor blockade. *Gut* 32:1455-1460.

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30:e36

Reimer C, Søndergaard B, Hilsted L, Bytzer P (2009) Proton-pump inhibitor therapy induces acid-related symptoms in healthy volunteers after withdrawal of therapy. *Gastroenterology* 137:80-87

Rudholm T, Hellström PM, Theodorsson E, Campbell CA, McLean PG, Näslund E (2008) Bravo capsule system optimizes intragastric pH monitoring over prolonged time: effects of ghrelin on gastric acid and hormone secretion in the rat. *World J Gastroenterol* 28:6180-187

Shu Z, Jung M, Beger HG et al (1997) pH-dependent changes of nitric oxide, peroxynitrite, and reactive oxygen species in hepatocellular damage. *Am J Physiol.* 273:G1118-1126

Schubert ML (2008) Gastric secretion. *Curr Opin Gastroenterol* 24:659-664

Semple G, Ryder H, Rooker D et al (1997) (3R)-N-(1-(tert-butylcarbonylmethyl)-2,3-dihydro-2-oxo-5-(2-pyridyl)-1H-1,4-benzodiazepin-3-yl)-N'-(3-(methylamino)phenyl)urea (YF476): a potent and orally active gastrin/CCK-B antagonist. *J Med Chem* 31:40:331-341

Takemoto Y, Yuki H, Nishida A et al (1998) Effects of YF476, a potent and selective gastrin/CCK-B receptor antagonist, on gastric acid secretion in beagle dogs with gastric fistula. *Arzneim Forsch* 48:403-407

Takeuchi K, Kato S, Konaka A, Sugawa Y (1999) Luminal calcium in regulation of nitric oxide release and acid secretion in rat stomachs after damage. *Dig Dis Sci* 44:515-522

Takeuchi Y, Kitano S, Bando T, Matsumoto T, Baatar D, Kai S (2003) Acceleration of gastric ulcer healing by omeprazole in portal hypertensive rats. Is its action mediated by gastrin release and the stimulation of epithelial proliferation? *Eur Surg Res* 35:75-80

Takinami Y, Yuki H, Nishida A et al (1997) YF476 is a new potent and selective gastrin/cholecystokinin-B receptor antagonist in vitro and in vivo. *Aliment Pharmacol Ther* 11:113-120

Tatemichi M, Ogura T, Sakurazawa N, Nagata H, Sugita M, Esumi H (2003) Inducible nitric oxide synthase activity induced by sodium chloride solution prolongs luminal pH elevation in rat and mouse stomachs. *J Gastroenterol Hepatol* 18:1039-1046

Tari A, Kamiyasu T, Yonei Y et al (1997) Role of gastrin/CCK-B receptor in the regulation of gastric acid secretion in rat. *Dig Dis Sci* 42:1901-1907

Vo PA, Lad B, Tomlinson JA, Francis S, Ahluwalia A (2005) Autoregulatory role of endothelium-derived nitric oxide (NO) on lipopolysaccharide-induced vascular inducible NO synthase expression and function. *J Biol Chem* 280:7236-7243

Waldum HL, Kleveland PM, Sandvik AK et al (2002) The cellular localization of the cholecystokinin 2 (gastrin) receptor in the stomach. *Pharmacol Toxicol* 91:359-362.

Wilder-Smith C, Halter F, Ernst T et al (1990) Loss of acid suppression during dosing with H₂-receptor antagonists. *Aliment Pharmacol Ther* 4(Suppl 1):15-27.

Yakabi K, Kawashima J, Kato S (2008) Ghrelin and gastric acid secretion. *World J Gastroenterol* 7:6334-6338

Yakabi K, Ro S, Onouhi T *et al* (2006) Histamine mediates the stimulatory action of ghrelin on acid secretion in rat stomach. *Dig Dis Sci* 51:1313-1321

Yoshizumi M, perella MA, Burnett JC, Lee ME (1993) Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ Res* 73:205-209

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Table 1. Characteristics of gene-specific primers.

mRNA	Assay No.	Ref Seq	Exon	Probe sequence	Amplicon length
			Boundary		
HPRT1	Rn01527840	NM_012583.2	8-9	TTCAGGGATTTGAATCATGTTTGTG	64
CCKB-R	Rn00565867	NM_013165.2	1-2	AACCGGGACCAGAGAATTGGAGATG	109
Gastrin	Rn00561967	NM_012849.1	1-2	CCAGCTCTGCAGACAAGATGCCTCG	60
Ghrelin	Rn01644838	NM_021669.2	3-4	GAAGAGGTCAAAGAGGCGCCAGCTA	82
SSTR2	Rn00583419	NM_019348.1	1-2	TCGTGGAAAAGCAAGATGTCACGAT	91
GhsR	Rn00561646	U94321.1	1-1	TCCCCAGCGGCCTCTATTTCTGTTT	78
SST	Rn00572319	NM_012659.1	1-2	GCCACCGGGAAACAGGAACTGGCCA	117
NOS2	Rn00571116	NM_012611.3	2-3	CTATTCCCAGCCCAACAACACAGGA	77

Legends to Figures

Fig. 1A-E. Gastroprotective effect of YF476 pretreatment against diclofenac-induced ulcers.

Representative photographs of excised rat stomachs 4 h after diclofenac treatment. (A) Positive control given saline pretreatment 1 h prior diclofenac ($30 \text{ mg}\cdot\text{kg}^{-1}$). (B) Negative control in which animals received only saline pretreatment and saline instead of diclofenac. Protective effects of (C) esomeprazole ($100 \text{ mg}\cdot\text{kg}^{-1}$) and (D) YF476 ($100 \text{ mg}\cdot\text{kg}^{-1}$). All treatments given by gavage. (E) Quantification of ulcer index indicating statistically significant protective effect of YF476 of comparable magnitude as esomeprazole. Bars represent means (** $P<0.01$, *** $P<0.001$, $n=8$).

Figure 2. Changes in pH $\pm$ SEM during *ad libitum* conditions after bolus dose of YF476 ($100 \text{ mg}\cdot\text{kg}^{-1}$ intragastrically) under continuous pentagastrin stimulation ($90 \text{ pmol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, IV) for a total of 120 h (5 days). Data presented as mean $\pm$ SEM. $P<0.001$ for all times points following YF476 treatment (all data points $n=8$).

Fig. 3. Regulatory peptide concentrations in plasma after YF476 bolus administration ($100 \text{ mg}\cdot\text{kg}^{-1}$, intragastrically) during continuous pentagastrin stimulation ($90 \text{ pmol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, IV) compared to controls (* $P<0.05$, ** $P<0.01$, $n=6$). Data expressed as mean $\pm$ SEM.

Fig. 4. Chronic effects (5 days) of intragastric YF476 bolus ($100 \text{ mg}\cdot\text{kg}^{-1}$ IV) and pentagastrin IV ($90 \text{ pmol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) on mRNA expression of different gut peptides and their receptors. Values are expressed relative to mean of the respective controls. Bars indicate mean value (* $P<0.05$, ** $p<0.01$, $n=6$).

Figure 5. Acute (4 h) effects of YF476 on mRNA expression of gut peptides, their receptors, and NOS2 (iNOS). A pretreatment bolus of YF476 (100 mg·kg⁻¹), esomeprazole (100 mg·kg⁻¹) or vehicle (NaCl, positive control) was administered. Diclofenac (30 mg·kg⁻¹) was administered 1 h later. An additional vehicle pretreatment group received a second administration of vehicle (NaCl + NaCl, negative control). All treatments were given by gavage. After 4 h, animals were sacrificed. The values are relative to mean of respective controls. Rats are same as in figure 1 where ulcer index was quantified. Bar indicates mean value (* P<0.05, ** P<0.01, n=6).

Figure 1
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Figure 2

Figure 3

Figure 4

● saline + pentagastrin

□ YF476 + pentagastrin

Figure 5