Ghent University – Faculty of Medicine and Health Sciences
Department of Basic and Applied Medical Sciences
Heymans Institute of Pharmacology

APPLICATION OF THE HEME OXYGENASE-1/CARBON MONOXIDE PATHWAY AND THE HYDROGEN SULFIDE PATHWAY AS NEW APPROACH TO PREVENT POSTOPERATIVE ILEUS

Jonas Van Dingenen

Promotors: Prof. Dr. Lindsey Devisscher & Prof. Dr. Romain Lefebvre

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Dissertation submitted to Ghent University in fulfilment of the requirements for the degree of Doctor in Health Sciences - 2022

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Promotors

Prof. Dr. Lindsey Devisscher
Department of Basic and Applied Medical Sciences
Faculty of Medicine and Health Sciences
Ghent University, Belgium

Prof. Dr. Romain Lefebvre
Department of Basic and Applied Medical Sciences
Faculty of Medicine and Health Sciences
Ghent University, Belgium

Examination committee

Prof. Dr. Johan Vande Walle (chair)
Department of Internal Medicine and Pediatrics
Faculty of Medicine and Health Sciences
Ghent University, Belgium

Prof. Dr. Wim Ceelen
Department of Human Structure and Repair
Faculty of Medicine and Health Sciences
Ghent University, Belgium

Prof. Dr. Danny De Looze
Department of Internal Medicine and Pediatrics
Faculty of Medicine and Health Sciences
Ghent University, Belgium

Prof. Dr. Guy Joos
Department of Internal Medicine and Pediatrics
Faculty of Medicine and Health Sciences
Ghent University, Belgium

Prof. Dr. Benedicte De Winter
Department of Gastroenterology and Hepatology
Laboratory of Experimental Medicine and Pediatrics (LEMP)
Antwerp University, Belgium

Prof. Dr. Gianluca Matteoli
Department of Chronic Diseases and Metabolism
Translational Research in GastroIntestinal Disorders
Leuven University, Belgium

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LIST OF ABBREVIATIONS

3-MST	3-mercapto-sulfurtransferase
5-HT$_4$	5-hydroxytryptamine-4
ACh	acetylcholine
ADHP	10-acetyl-3,7-dihydroxy-phenoxazine
ANOVA	one-way analysis of variance
ARE	antioxidant response element
ATP	adenosine triphosphate
Bach1	BTB domain and CNC homolog 1
CAT	cytoplasmic aspartate aminotransferase
CBS	cystathionine-β-synthase
CGRP	calcitonin gene-related peptide
CLP	cecal ligation and puncture
CNS	central nervous system
CO	carbon monoxide
COHb	carbon monoxide hemoglobin
CoPP	cobalt protoporphyrin IX
CORM	carbon monoxide-releasing molecule
COX	cyclo-oxygenase
CRF	corticotrophin-releasing factor
CrMP	chromium mesoporphyrin
CSE	cystathionine-γ-lyase
DAMPs	damage-associated molecular patterns
DMF	dimethyl fumarate
DMSO	dimethyl sulfoxide
DSS	dextran sulfate sodium
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
eNOS	endothelial NO synthase
ENS	enteric nervous system
E-OCORS	esterase-triggered oral carbon monoxide release system
ERAS	enhanced recovery after surgery
ERK	extracellular signal–regulated kinases
FD70	fluorescein-labeled dextran (70kDa)
FDA	Food and Drug Administration

Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
GALT	gut-associated lymphoid tissue
GC	geometric center
GSH	glutathione
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulfide
HCAR2	hydroxycarboxylic acid receptor 2
HETAB	hexadecyl-trimethylammonium bromide
HO	heme oxygenase
HYCO	hybrid carbon monoxide-releasing molecule
i-CORM	carbon monoxide-depleted CORM
i.g.	intragastric
i.p.	intraperitoneal
I/R	ischemia/reperfusion
i.v.	intravenous
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
IKB	inhibitor of IKB
IKK	IκB kinase
IL	interleukin
IM	intestinal manipulation
iNOS	inducible NO synthase
JNK	c-Jun N-terminal kinase
K_{ATP} channels	adenosine triphosphate-dependent K-channels
Keap1	Kelch-like ECH-associated protein
LES	lower esophageal sphincter
LHM	laparoscopic Heller myotomy
LPS	lipopolysaccharides
MAPK	mitogen-activated protein kinase
MCP-1	monocyte-chemoattractant protein-1
MMC	migrating motor complex
MMF	monomethyl fumarate
MPO	myeloperoxidase
MS	multiple sclerosis
Na_2S	sodium sulfide

nAChRs	nicotinic ACh receptors
NADPH	dihydronicotinamide-adenine dinucleotide phosphate
NaHS	sodium hydrosulfide
NANC	non-adrenergic non-cholinergic
NEC	necrotizing enterocolitis
NEMO	nuclear factor kB essential modulator
NF-kB	nuclear factor kB
NH_3	ammonia
NO	nitric oxide
NOSE	natural orifice specimen extraction
NOTES	natural orifice transluminal endoscopic surgery
Nrf2	nuclear factor (erythroid-derived 2)-like 2
NSAIDs	non-steroidal anti-inflammatory drugs
O_2	oxygen
O_2^-	superoxide anion radicals
OCORS	oral carbon monoxide release system
$ONOO^-$	peroxynitrite anion
PAG	propargylglycine
PAMPs	pathogen-associated molecular patterns
PI3K/Akt	phosphatidylinositol 3-kinase/protein kinase B
PLP	pyridoxyl 5'-phosphate
POEM	peroral endoscopic myotomy
POI	postoperative ileus
PTKs	protein tyrosine kinases
RAGE	receptors for advanced glycation end-products
ROS	reactive oxygen species
sGC	soluble guanylate cyclase
StRE	stress-responsive elements
T_H1	T helper type 1
TLR	Toll-like receptors
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF-α	tumor necrosis factor-α
UES	upper esophageal sphincter
VIP	vasoactive intestinal peptide

CHAPTER I

LITERATURE SURVEY

CHAPTER I

Literature survey

I.1. Gastrointestinal motility

I.1.1. Anatomy and histology of the gastrointestinal tract

The human gastrointestinal tract is a canal passing through the body from mouth to anus with an in vivo average length of 5 m; it is associated with the accessory digestive organs (salivary glands, liver, gallbladder and exocrine pancreas) which empty their content into the canal (Helander & Fändriks, 2014). The canal itself consists of the mouth, the upper esophageal sphincter (UES), the esophagus, the lower esophageal sphincter (LES), the stomach (subdivided in the fundus, corpus and antrum), the pyloric sphincter, the small intestine (subdivided in the duodenum, jejunum and ileum), the ileocecal sphincter, the large intestine (subdivided in the caecum, colon and rectum) and the anus, enclosed by the internal and external sphincter (Figure I.1; Silverthorn, 2009).

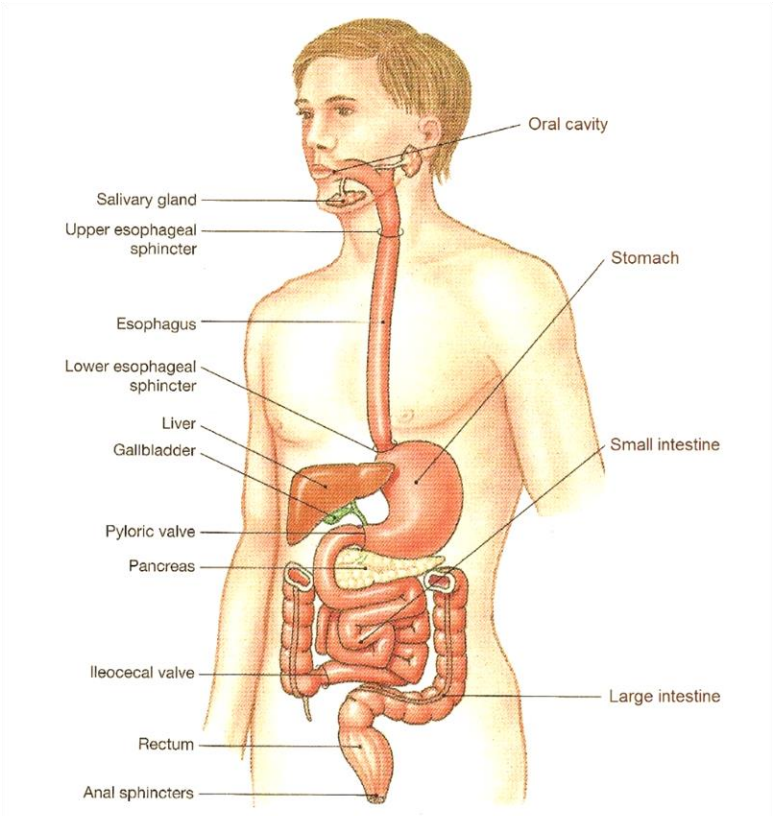


Figure I.1 Representation of the human gastrointestinal tract (Adapted from Silverthorn, 2009).

The basic histological structure of the gastrointestinal wall is quite similar throughout the gastrointestinal tract and consists of four layers (Figure I.2):

The inner **mucosa** is facing the lumen and consists of (1) a single layer of epithelial cells, (2) subepithelial connective tissue, named the lamina propria and (3) a thin layer of smooth muscle, named the muscularis mucosae. Several structural adjustments within the mucosa increase the total mucosal surface area in particular in the small intestine. First, the entire wall is crumbled into folds, the valvulae conniventes. Fingerlike extensions, called villi, project into the lumen and tubular invaginations, called crypts, extend down into the connective tissue to create additional surface area, which is further increased by the brush border of the epithelial cells created from numerous microvilli on the apical surface.

The **submucosa**, composed out of connective tissue, holds larger blood and lymph vessels. It also contains the submucosal nerve plexus that innervates the cells in the epithelial layer as well as the smooth muscle cells of the muscularis mucosae.

The **muscularis externa** primarily consists of two layers of smooth muscle tissue, an inner circular layer and an outer longitudinal layer. The stomach has an incomplete third muscular layer between the circular muscle and the submucosa, called the oblique layer. Between the circular and longitudinal muscular layer lies the myenteric nerve plexus, which controls the motor activity of the muscularis externa.

The outer **adventitia** is a thin layer of connective tissue surrounding the gastrointestinal tract. Where the gut lies within the abdominal cavity, the adventitia is covered by the visceral peritoneum and referred to as the serosa.

I.1.2. Physiological functions of the gastrointestinal tract

The primary function of the gastrointestinal tract is to take in food, digest it to extract energy and nutrients, and expel the remaining waste. To accomplish this, four main processes are involved:

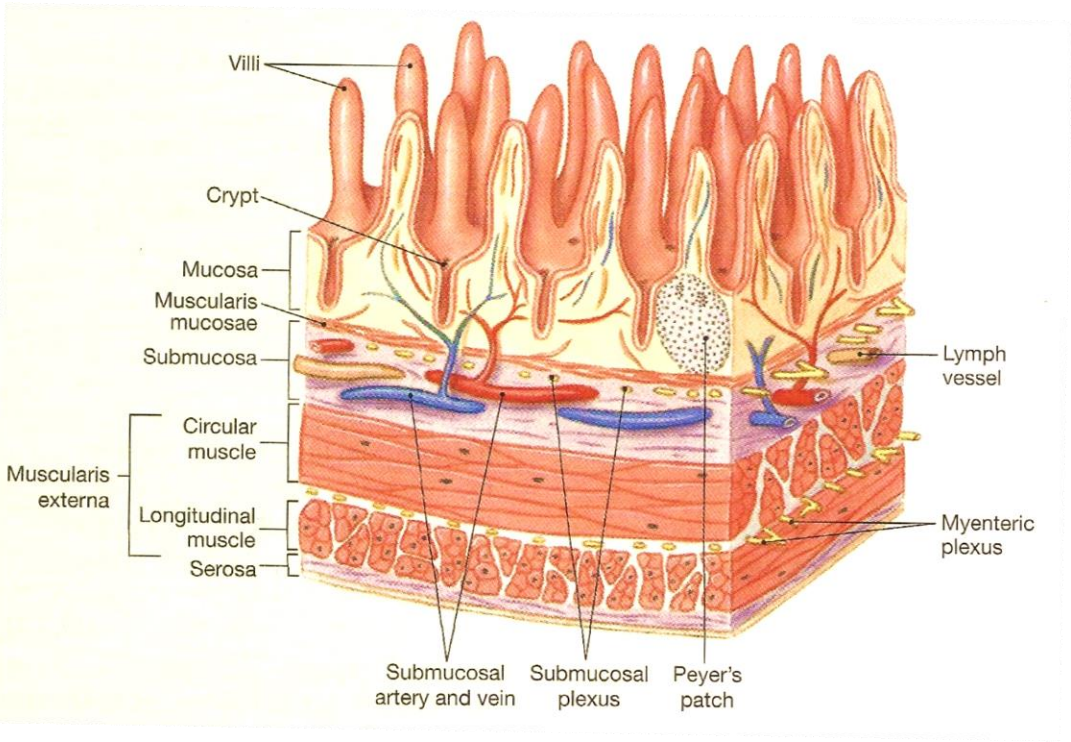


Figure I.2 Sectional view of the intestinal wall (Adapted from Silverthorn, 2009).

Digestion is the combination of the mechanical and chemical breakdown of food into absorbable components. The mechanical digestion starts by chewing in the oral cavity, creating a softened, moistened bolus that can be easily swallowed; it continues with grinding by the muscular activity of the stomach and small intestine, creating a larger surface area exposed to digestive enzymes. These enzymes take care of the chemical breakdown and function best at a certain pH; enzymes that act in the stomach prefer an acidic pH, whereas enzymes in the small intestine work better in an alkaline environment. This chemical digestion is already initiated in the oral cavity by enzymes such as lingual lipase and α-amylase present within the saliva; α-amylase will start the breakdown of starch in the oral cavity (pH optimum of 7.4), whereas lingual lipase will be mostly active after it is swallowed and reaches the acidic environment of the stomach (pH optimum of 4.0).

Absorption is the migration of nutrients (by passive diffusion or active transport) from the digestive tract to adjacent blood and lymphatic vessels. Most absorption takes place in the small intestine, with additional absorption of water and ions in the large intestine.

Motility results in the movement of food from mouth to the anus; it is determined by the properties of the smooth muscle within the gastrointestinal wall and regulated by the chemical input from nerves, hormones and paracrine signals.

Secretion of digestive enzymes, water and other substances such as pancreatic bicarbonate, which is important in buffering of acid chyme entering the duodenum, into the lumen of the gastrointestinal tract is needed for the chemical breakdown of food.

The gut microbiota, containing a wide variety of bacteria and fungi, was shown to play a role in immunological, metabolic and neurological processes of the human body; they even influence structural aspects of the gastrointestinal tractus as they influence crypt and villus development and play a role in tight junction regulation (Adak & Khan, 2019). However, the intestinal epithelium of the gastrointestinal tract is also continuously exposed to disease-causing pathogens such as bacteria and viruses and potential toxic substances. Therefore, the gut is equipped with the largest peripheral lymphoid tissue in the body named the gut-associated lymphoid tissue (GALT). The GALT is composed out of immune cells of both the innate and adaptive immune system, such as macrophages, dendritic cells, mast cells, and T and B lymphocytes, scattered throughout the mucosa and clusters of immune cells in Peyer's patches. Specialized cells, called M cells, reside in the region of the epithelium covering the Peyer's patches and take up intestinal microbial antigens to deliver them to the GALT for efficient mucosal as well as systemic immune responses. But also other, non-immunological mechanisms contribute to the protection against pathogens, e.g. enzymes in the saliva and the highly acidic environment of the stomach (Wershil & Furuta, 2008; Silverthorn, 2009; Ohno, 2016).

I.1.3. Regulation of gastrointestinal motility

The motility pattern in the gastrointestinal tract differs between the interdigestive state and the digestive state upon food intake. The interdigestive pattern is characterized by the migrating motor complex (MMC); this has a "housekeeping" function sweeping undigested food

remnants, basal secretion and lost epithelial cells through the gastrointestinal tract. The MMC starts between the upper and middle third of the stomach and moves then aborally towards the end of the small intestine in 80–110 min; it is repeated untill food is taken. At a given location, four phases can be differentiated in the MMC. Phase I is a quiescent period with only rare contractions. During phase II, contractions progressively increase in frequency and during phase III the region undergoes intense rhythmic contractions for a time period of 5 – 15 min. It is followed by a period of less intense activity (phase IV) which functions as a short period of transition between phase III and phase I. The MMC of the interdigestive state disappears soon after a meal is taken and is replaced by the fed pattern of activity, which consists of ongoing phasic contractile activity. These contractions are highly irregular, making it difficult to define their frequency, which is approximately 12–15 contractions per min.

The fed pattern of activity both propels and mixes the contents via respectively peristalsis and segmentation. Peristalsis consists of progressive waves of contractions that move from one section of the gastrointestinal tract to the next as circular muscles contract just behind the bolus of food. This results in movement of the bolus forward into a receiving segment where the circular muscles are relaxed; this receiving segment then contracts, continuing the forward movement (Figure I.3 A). During segmentation, non-adjacent segments of the intestine alternately contract and relax. Because the active segments are separated by inactive regions, segmentation moves the food forward and then backward, mixing the food with intestinal secretions rather than propelling it forward (Figure I.3 B) (Kunze & Furness, 1999; Silverthorn, 2009; Takahashi, 2013).

The different functions of the gastrointestinal tract, including motility, are controlled by two types of mechanisms: hormonal and neuronal, allowing for a continuous optimal adjustment in response to a meal. This thesis will only focus on the neuronal control of gastrointestinal motility, which is mediated via both intrinsic and extrinsic pathways. The intrinsic pathway, also called the enteric nervous system (ENS), is located within the wall of the gastrointestinal tract and includes the myenteric plexus and the submucosal plexus. The extrinsic pathway, that

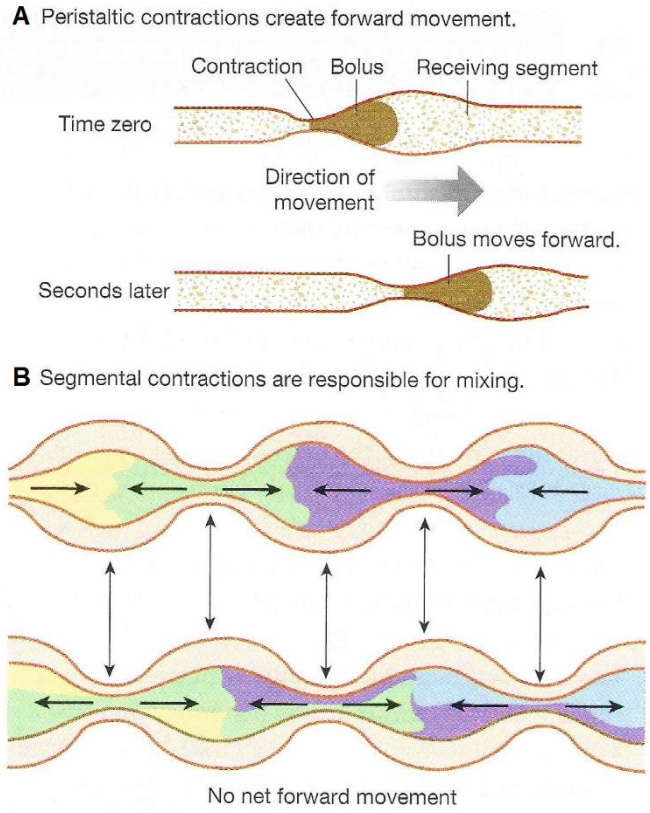


Figure 1.3 (A) In peristalsis, the segment of the alimentary canal proximally from the bolus contracts while that distally relaxes, moving food distally along the gastrointestinal tract. (B) In segmentation, non-adjacent segments, separated by inactive regions, of the intestine alternately contract and relax, mixing the food rather than propelling it forward (Silverthorn, 2009).

receives input from the central nervous system (CNS), can be subdivided into a parasympathetic and sympathetic branch (Silverthorn, 2009).

I.1.3.1. Intrinsic innervation

Large numbers of neurons, about 200-600 million in humans, are contained in the ENS and are organized in thousands of small ganglia. Two major sets of ganglia are found: the myenteric plexus and the submucosal plexus. The myenteric plexus lies between the outer longitudinal and inner circular muscle layers, and extends along the full length of the digestive tract from the esophagus to the rectum; whereas the submucosal plexus is well developed in the small and large intestines, but only contains few ganglia in the stomach. Within these ganglia, approximately 20 types of enteric neurons can be defined which can be divided into three different classes: sensory neurons, interneurons and motor neurons. Sensory neurons detect the physical (for example tension) and chemical effects of the luminal content on the gastrointestinal tract and send this information, via the interneurons, to the motor neurons. The

motor neurons will react to these signals by initiating appropriate reflex control of motility, secretion and blood flow. The motor neurons, which regulate motility, consist of both excitatory and inhibitory neurons that innervate the longitudinal and circular smooth muscle layers, as well as the mucosal muscle layer. Excitatory motor neurons primarily release acetylcholine (ACh), which binds on muscarinic receptors of smooth muscle cells, but also contain excitatory non-adrenergic non-cholinergic (NANC) neurotransmitters such as tachykinins (e.g. substance P); inhibitory motor neurons release inhibitory NANC neurotransmitters such as nitric oxide (NO) and vasoactive intestinal peptide (VIP). Although the ENS can autonomically control smooth muscle activity, it is subjected to modification by signals emanating from the extrinsic innervation (Kunze & Furness, 1999; Furness, 2000).

I.1.3.2. Extrinsic innervation

The parasympathetic innervation of the gastrointestinal tract consists of the vagus nerve and the pelvic nerves. The innervation to the esophagus, stomach, small intestine and proximal part of the colon is supplied by the vagus nerve, while the pelvic nerves innervate the distal part of the colon, the rectum and the anal canal. The information from sensory nerves in the gut, which can be triggered by a wide range of both chemical and mechanical stimuli, is transmitted to the CNS via parasympathetic afferent fibers. For the vagus nerve, the cell bodies of these fibers lie within the nodose ganglia and their central terminals enter the brainstem via the tractus solitarius; whereas for the pelvic nerves, the cell bodies are located in the dorsal root ganglia. In the CNS, the sensory information will result in an appropriate efferent response through the dorsal motor nucleus in the brainstem (vagus nerve) or the lumbosacral spinal cord (pelvic nerves). The preganglionic efferent nerve fibers enter the gut wall via the vagus or pelvic nerves, where they synapse with excitatory cholinergic neurons or inhibitory NANC neurons in the ganglia of the ENS (Figure I.4 A). As the parasympathetic nervous system can regulate both excitatory and inhibitory effects on gastric and intestinal tone and motility, it has a finely tuned and complex influence on gastrointestinal activity.

The cell bodies of the sensory neurons in the sympathetic nervous system lie within the dorsal root ganglia. The majority of preganglionic sympathetic efferent neurons innervating the stomach and small intestine arise from spinal thoracic levels, while neurons innervating the colon arise predominantly from spinal lumbar levels. These nerve fibers are cholinergic and activate postganglionic neurons, innervating the gastrointestinal tract, that are contained in well-defined prevertebral ganglia; the connections between the paravertebral sympathetic column and the prevertebral ganglia are the splanchnic nerves. Postganglionic sympathetic neurons innervating the stomach and lower esophageal sphincter are contained within the celiac ganglion, while neurons innervating the intestine reside in the superior mesenteric ganglion (small intestine and proximal part of the colon) and the inferior mesenteric ganglion (distal part of the colon). The sympathetic innervation of the proximal esophagus on the other hand is derived primarily from cervical and upper thoracic paravertebral ganglia (Figure I.4 B). The postganglionic sympathetic neurons regulate motility indirectly via inhibition of the release of excitatory neurotransmitters from preganglionic parasympathetic neurons or ganglionic enteric neurons; except for sphincters in which they induce constriction via a direct action on the smooth muscle cells. The sympathetic nervous system thus exerts a predominantly inhibitory effect on gastrointestinal smooth muscle. It also has an inhibitory influence on mucosal secretion and regulates gastrointestinal blood flow via neurally mediated vasoconstriction (Cunningham & Sawchenko, 1990; Browning & Travagli, 2014).

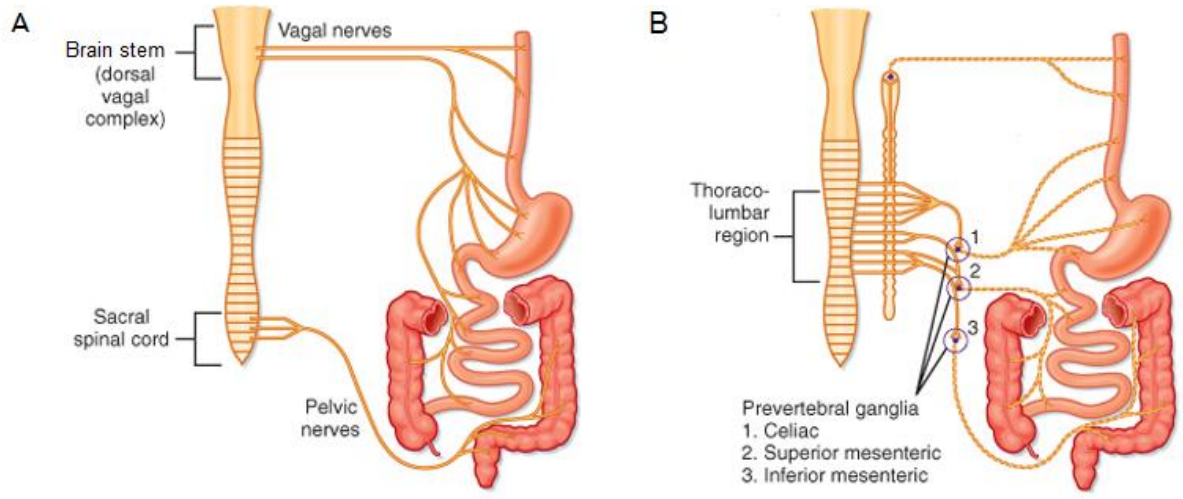


Figure I.4 Schematic representation of the extrinsic innervation of the gastrointestinal tract. (A) The parasympathetic nervous system. (B) The sympathetic nervous system (Koeppen & Stanton, 2008).

I.2. Postoperative ileus

I.2.1. Definition and symptoms

Postoperative ileus (POI) is defined as transient cessation of the coordinated bowel motility after surgical intervention, which prevents effective transit of intestinal contents or tolerance of oral intake. This inhibition of gastrointestinal motility involves the entire gastrointestinal tract; however not all segments are equally affected. Small intestinal motility is on average disturbed for approximately 24 h and gastric motility between 24 and 48 h, whereas colonic motility is impaired between 48 and 72 h. Patients undergoing major abdominal surgery are at highest risk for developing POI, but also other surgical procedures, such as cardiac or orthopedic surgery, may be associated with POI (Luckey et al., 2003; Augestad, 2010). A certain degree of POI might be considered as a normal response of the intestine to a traumatic event, but POI clearly has a significant impact on patient morbidity, with symptoms such as delayed passage of gas and stool, abdominal distention, lack of bowel sounds, and accumulation of gas and fluid in the bowel (Boeckxstaens & de Jonge, 2009). The observation that POI can prolong hospital stay, slow patient recovery, increase postoperative morbidity and increase healthcare costs, has risen the interest in this condition. This is further illustrated by the annual socio-economic impact of prolonged POI in the United States alone, which is estimated up to 1.46 billion US$ (Goldstein et al., 2007).

In a systematic review, Wolthuis et al. (2016) investigated the incidence of prolonged POI across 54 studies. The principal finding of this meta-analysis was that the overall incidence of prolonged POI after colorectal surgery was around 10% both in non-randomized and randomized clinical trials. However, this incidence varied according to the definition used. Most studies defined prolonged POI as the need to reinsert the nasogastric tube, whilst other referred to the absence of bowel function on either postoperative day 3, postoperative day 5 or postoperative day 7; in the 17 randomized clinical trials considered, the incidence ranged from 2 to 61 %. To overcome this heterogeneity, Vather et al. (2013) proposed definitions for normal POI, prolonged POI and recurrent POI depending on the severity and duration. Normal

POI is described as the interval from surgery until passage of flatus or stool and the tolerance of an oral diet, occurring before day 4 postoperatively. Prolonged POI is defined if two of the following five criteria are met on or after day 4 postoperatively: nausea or vomiting, inability to tolerate an oral diet over the last 24 h, absence of flatus over the last 24 h, abdominal distension or radiologic confirmation of POI by computed tomography. When after an apparent resolution of POI, two or more of the latter mentioned criteria reoccur, it is classified as recurrent POI (Delaney, 2006; Vather et al., 2013). Venara et al. (2020) conducted a prospective study over a total of 786 patients scheduled for colorectal surgery to determine the incidence rate of POI based on the definition of Vather et al. (2013) and concluded that 15.4% of all patients were diagnosed with POI.

I.2.2. Pathogenesis of POI

The pathophysiology of POI is complex involving three main mechanisms: neurogenic inhibitory reflexes, inflammatory responses and pharmacological factors. Before further discussing these mechanisms, it is important to emphasize that the current insight is mainly based on rodent models using standardized manipulation of the small intestine and that these data may not directly translate to the human situation. These animal studies have shown that abdominal surgery triggers two phases: an early neurogenic phase and a prolonged and clinically more important inflammatory phase, starting 3-4 h after surgery. Although there is a slight overlap between these two phases, the importance of each mechanism varies in time as shown in Figure I.5 (Boeckxstaens & de Jonge, 2009).

I.2.2.1. Neurogenic phase

During abdominal surgery, different neural pathways are activated depending on the intensity of the nociceptive stimulus applied (Figure I.6). Skin incision and laparotomy only briefly result in a reduction of gastrointestinal activity, primarily via activation of an adrenergic inhibitory pathway. Laparotomy activates a low-threshold spinal loop in which afferent splanchnic nerves synapse in the spinal cord, leading to the activation of an inhibitory pathway involving prevertebral adrenergic efferent neurons, inhibiting the motility of the entire gastrointestinal

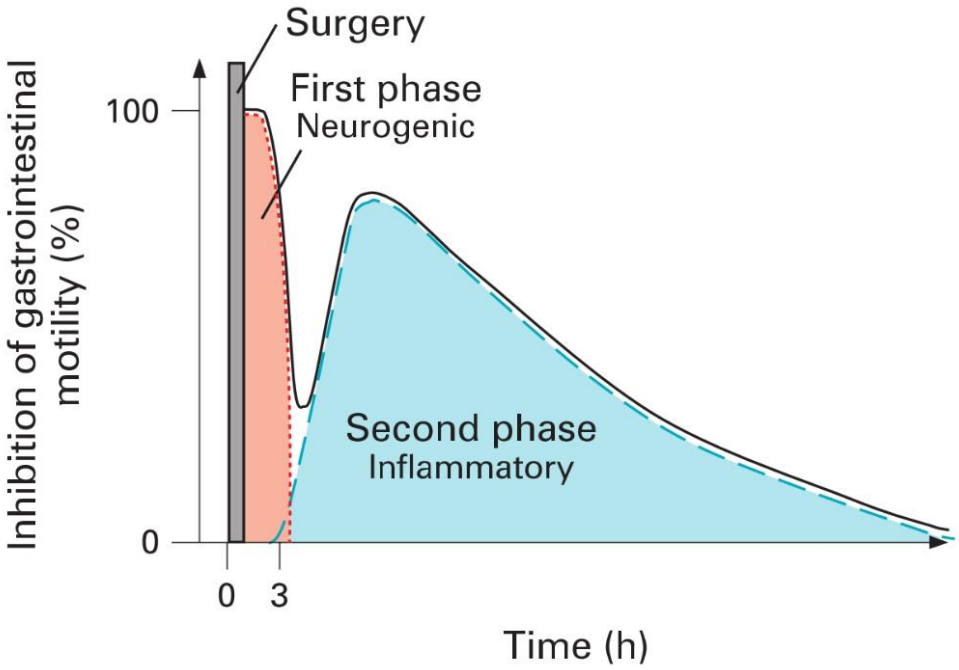


Figure I.5 Representation of the two phases involved in postoperative ileus. The first phase starts during surgery and ends soon after, whereas the inflammatory phase starts 3-4 h after surgery and lasts much longer (Boeckxstaens & de Jonge, 2009).

tract. In contrast, the nociceptive stimuli during intestinal manipulation (IM) are more intense and activate high-threshold supraspinal pathways (Boeckxstaens et al., 1999). It is believed that within this pathway, release of corticotrophin-releasing factor (CRF), triggered by afferent signals transmitted to the brainstem, plays a central role as it leads to stimulation of neurons in the hypothalamus. From the hypothalamus, projections are sent to the spinal cord, including the intermediolateral column of the thoracic cord, where they will activate sympathetic preganglionic neurons which synapse to prevertebral adrenergic nerves. Activation of these adrenergic nerves will subsequently lead to the prolonged inhibition of gastrointestinal motility observed with IM (Taché et al., 1993; Luckey et al., 2003). This prolonged inhibition can only be partially blocked by adrenergic antagonists as IM also results in additional activation of vagally mediated NANC inhibitory pathways (Boeckxstaens et al., 1999). Upon activation, the motor nucleus of the vagus nerve will synapse to inhibitory neurons, releasing NO and VIP (De Winter et al.,1997; De Winter et al., 1998).

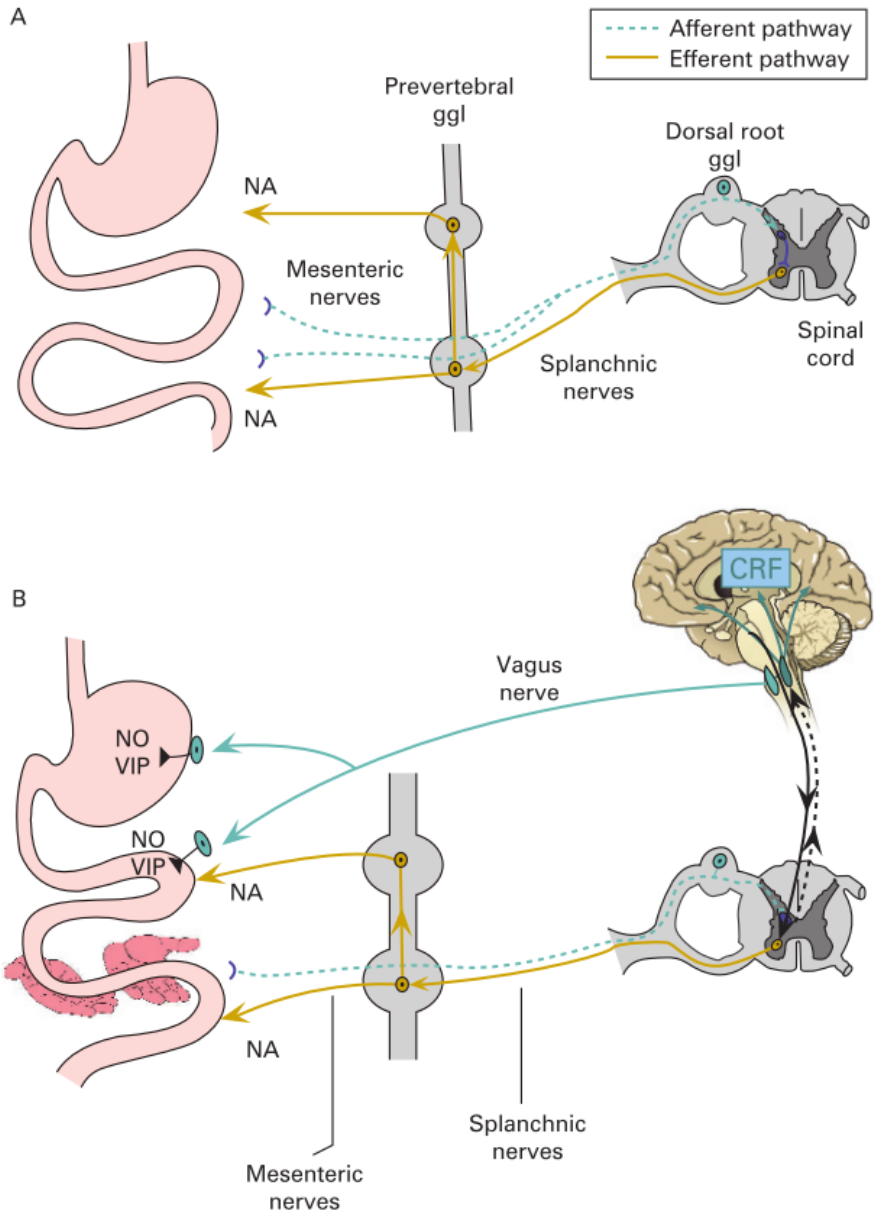


Figure I.6 Schematic representation of the neural pathways involved in the inhibition of gastrointestinal motility induced by laparotomy (A) and more intense intestinal manipulation (B). Laparotomy activates spinal afferents which will synapse in the spinal cord where they activate an inhibitory pathway involving prevertebral adrenergic neurons. The release of noradrenaline (NA) will abolish the motility of the entire gastrointestinal tract (A). During intestinal manipulation, additional pathways mediated by the brainstem come into play. The more intense stimuli will transmit afferent signals to the brainstem where they trigger the release of corticotrophin-releasing factor (CRF). In response to CRF, the hypothalamus sends projections to the spinal cord where spinal efferents will synapse to sympathetic preganglionic neurons releasing NA. In addition, the motor nucleus of the vagus nerve is activated, synapsing to inhibitory nitrergic (NO) and vipergic (VIP) neurons and further contributing to the inhibition of the gastrointestinal motility. ggl, ganglion (Boeckxstaens & de Jonge, 2009).

I.2.2.2. Inflammatory phase

The activation of the neural pathways caused by mechanical stimuli, diminishes once the abdomen is closed. This was first discovered by Bueno et al. (1978) as they observed an initial complete inhibition of electrical spiking activity in the small intestine which ceased at the end

of surgery in dogs and sheep. More interestingly was the observation of a secondary period of inhibition lasting from 6 to 72 h after surgery, of which the exact origin remained unclear. Twenty years after this first description of biphasic gastrointestinal inhibition during POI, Kalff et al. (1998) demonstrated the association between this secondary, long-lasting phase of POI and the inflammation of the intestinal muscularis as they demonstrated the activation of normally quiescent macrophages present within the muscular layer upon surgical manipulation of the small bowel in rats. Activation of these macrophages results in the activation of intracellular signaling pathways such as the mitogen-activated protein kinases (MAPK), including p38, extracellular signal–regulated kinases (ERK) 1/2 and c-Jun N-terminal kinase (JNK), and pro-inflammatory transcription factors such as nuclear factor kB (NF-kB). Subsequently, this will lead to an increased release of pro-inflammatory cytokines such as interleukin (IL)-6, IL-1β and tumor necrosis factor-α (TNF-α) and chemokines such as monocyte-chemoattractant protein-1 (MCP-1), as well as to increased expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1). As a result, additional circulatory leukocytes, mainly neutrophils and monocytes, will enter the muscularis, further contributing to the intestinal inflammation. The inhibitory effect of these inflammatory cells on intestinal smooth muscle cells is mediated by the enhanced release of NO and prostaglandins, through upregulation of inducible NO synthase (iNOS) and cyclo-oxygenase (COX)-2 respectively. Both NO and prostaglandins exert potent inhibitory effects on the gastrointestinal tract, leading to ileus (Kalff et al., 1999a; Kalff et al., 1999b; Kalff et al., 2000; Schwarz et al., 2001; Turler et al., 2006; Boeckxstaens & de Jonge, 2009). The group of Guy Boeckxstaens proposed to further subdivide this inflammatory phase into an early phase (lasting until 6 h post IM), mediated by innate cytokines released by resident macrophages, and a second phase of smooth muscle inhibition (starting approximately 6 h post IM), which is temporarily associated with the influx of circulatory inflammatory cells peaking at 24 h after IM (Farro et al., 2016).

The mechanisms leading to the activation of these resident macrophages remain unclear. One potential mechanism may be through the release of damage-associated molecular patterns

(DAMPs), such as adenosine triphosphate (ATP), into the extracellular space by cells that are damaged or stressed during IM. Besides intracellular molecules, degradation of the extracellular matrix has also been demonstrated to contribute to the inflammation in a murine model of POI (Moore et al., 2011). As the intestinal permeability is transiently increased following IM, also pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) from translocated bacteria could trigger the innate immune system. Both DAMPs and PAMPs are recognized by pattern recognition receptors such as Toll-like receptors (TLR) and receptors for advanced glycation end-products (RAGE), which upon activation stimulate macrophages to secrete pro-inflammatory cytokines and chemokines (Figure I.7) (Schwarz et al., 2002; Boeckxstaens & de Jonge, 2009). However, translocated bacteria and their products only start to appear in the muscularis externa at 6 h after IM (Schwarz et al., 2002), whereas an increase in ICAM-1 mRNA can already be observed 15 min post IM (Kalff et al., 1999b), indicating that PAMPs do not initiate the activation of resident macrophages, but more likely strengthen the inflammatory response. Moreover, TLR2 and TLR4 knockout mice are not protected against POI, questioning the involvement of bacterial recognition via the TLRs in the activation of the innate immune response (Stoffels et al., 2014).

Another possible mechanism involves the release of pro-inflammatory neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) during the intense activation of afferent nerve fibers in the neural phase, implicating that the initial neural phase could be the trigger for the inflammatory phase. Studies in the 1990s already pointed out the importance of CGRP in motility disturbances within the acute phase of POI (Plourde et al., 1993; Zittel et al., 1994). Recent evidence also suggests a pro-inflammatory role for CGRP as both CGRP depletion via capsaicin and the CGRP-receptor antagonist BIBN4096BS reduced the expression of IL-1β and IL-6 mRNA in the muscularis externa at 3 h after surgery in mice. Furthermore, CGRP protein within muscular nerve fibers was undetectable by immunofluorescence 3 h after IM, indicating IM-induced release and depletion of pre-stored CGRP from neuronal endings within the myenteric plexus. The same investigators

demonstrated that CGRP does not influence mast cell activation and CGRP-receptor antagonism fails to prevent IM-induced mast cell degranulation (Glowka et al., 2015). However, other neuropeptides like substance P are believed to activate mast cells, present in mesentery, lamina propria, submucosa and serosa, resulting in an increase of mast cell mediators such as histamine and proteases in peritoneal fluid and gut tissue. These mediators will increase the mucosal permeability leading to translocation of intraluminal bacteria and activation of resident macrophages (Figure I.7) (Bueno et al., 1997; De Winter et al., 2012).

The involvement of mast cells in POI has been described in pre-clinical murine models of POI; it was demonstrated that IM elicited a significant increase in mast cell protease-I levels in peritoneal fluid and that treatment with mast cell stabilizers like ketotifen or doxantrazole prevented both IM-induced inflammation and gastroparesis, whereas mast cell-deficient mice were resistant to IM-induced delay in gastrointestinal transit and muscular inflammation (de Jonge et al., 2004; Snoek et al., 2012). Moreover, Snoek et al. (2012) showed that IM in mice leads to an impaired barrier function via mast cell activity, which leads to postoperative

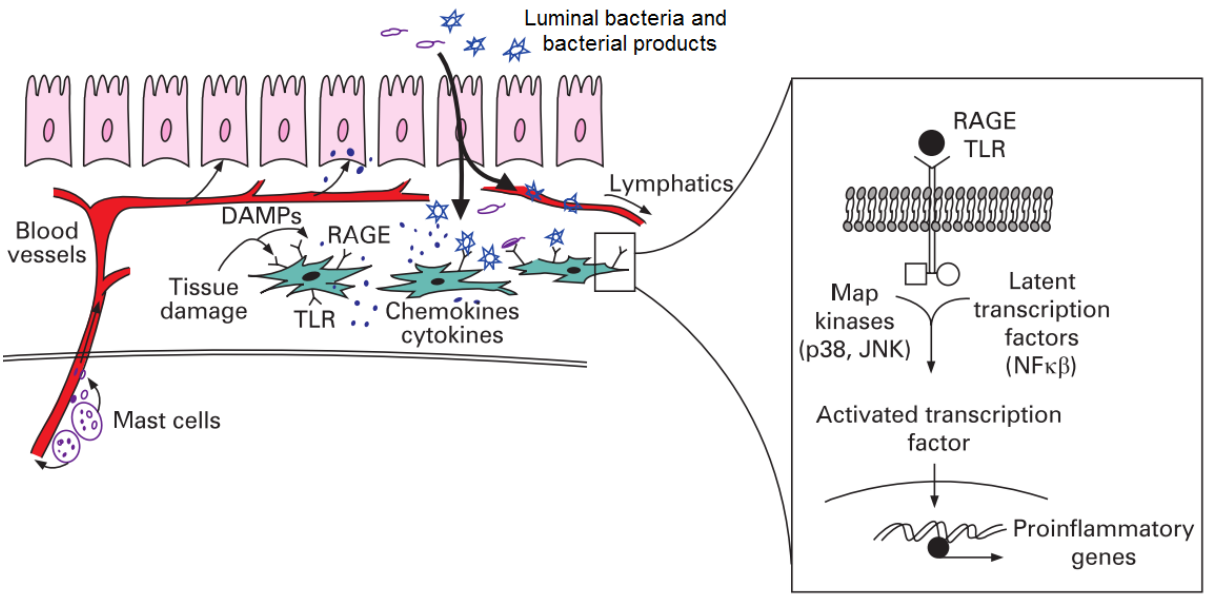


Figure I.7 Schematic representation of the possible involvement of mast cells and damage-associated molecular patterns (DAMPs) in triggering the inflammatory cascade upon intestinal manipulation. Intestinal handling will activate mast cells residing around mesenteric vessels, resulting in the release of mast cell mediators which will diffuse into the blood vessels and increase the mucosal permeability. Subsequently, entrance of luminal bacteria and bacterial products such as lipopolysaccharides can interact with Toll-like receptors (TLRs) on resident macrophages. Also DAMPs released by damaged tissue might interact with TLRs and receptors for advanced glycation end-products (RAGE). Binding with TLRs or RAGE on resident macrophages will result in activation of intracellular pathways such as MAP kinases p38 and c-Jun N-terminal kinase (JNK) and transcription factors such as nuclear factor-κB (NF-κB), leading to the transcription of proinflammatory genes and the production of chemokines and cytokines (Adapted from Boeckxstaens & de Jonge, 2009).

bacterial translocation towards draining mesenteric lymph nodes. A more recent study of Gomez-Pinilla et al. (2014a) in mice, showed that mast cells play no role in the pathophysiology of POI; whereas previous studies used mast cell-deficient mouse models with abnormal Kit signaling, which also lack interstitial cells of Cajal, resulting in aberrant motility even prior to surgery, Gomez-Pinilla et al. (2014a) used a specific mast cell-deficient mouse model (Cpa3-Cre) with intact Kit signaling. Data on the role of mast cells in rodent models of POI should thus be interpreted with caution (Peters et al., 2015). Also in humans there is evidence that mast cells might play a role in the pathophysiology of POI. Treatment with ketotifen, known as a mast cell stabilizer, significantly improved gastric emptying after abdominal surgery; however the exact underlying mechanism of ketotifen remains unclear and may not be related to mast cell stabilization (see further I.2.4. New targets for management of POI – I.2.4.1 Mast cell stabilization) (The et al., 2008; The et al., 2009). Still, studies measuring the peritoneal concentration of mast cell mediators support the role of mast cells in human POI. Tryptase release, which is an indicator for mast cell activity, was significantly increased in peritoneal fluid of patients undergoing abdominal surgery with palpation of the small intestine (The et al., 2008). A more recent study also found increased mast cell tryptase and chymase in the peritoneal fluid of patients with POI (Berdún et al., 2015).

A last potential mechanism involves the activation of intestinal CD103+CD11b+ dendritic cells, present in the lamina propria, by luminal antigens. These dendritic cells will release IL-12, promoting memory T helper type 1 (T_H1) cells to secrete interferon (IFN)-γ leading to activation of resident macrophages. Moreover, IL-12 release will stimulate the migration of T_H1 cells from the surgically manipulated site through the bloodstream to non-manipulated areas, contributing to impairment of motility of the entire gastrointestinal tract (Engel et al., 2010; Pohl et al., 2017).

I.2.2.3. Pharmacological mechanisms

Besides the neurogenic and inflammatory phase, also pharmacological treatment to induce anesthesia or to reduce pain might play a role in POI. However, new intravenous (i.v.) anesthetics (such as propofol) and gaseous anesthetic agents (such as sevoflurane and

desflurane) can difficultly contribute to prolonged POI as they have a relatively short half-life. The low incidence and magnitude of POI in patients who undergo non-abdominal procedures under general anesthesia further supports the notion that the overall contribution of actual general anesthesia to POI is small. Nowadays patients usually recover from general anesthesia relatively quickly and the adverse effects of anesthesia do not last more than a few hours (Person & Wexner, 2006).

Opioids such as morphine are very effective analgesics and are often used following various types of surgery to reduce postoperative pain. But it is also well known that they have a major impact on gastrointestinal function and can complicate and prolong POI. There are three major classes of opioid receptors that mediate their effects: μ, δ and κ receptors. Activation of these receptors will hyperpolarize target cells, making them less responsive to depolarizing pulses and reducing the release of neurotransmitters at synapses and neuromuscular junctions. In the gastrointestinal tract, activation of opioid receptors on enteric neurons results in inhibition of gut motility via inhibition of neurotransmitter release from enteric excitatory neurons. The receptor primarily involved in the adverse effects of opioids on gastrointestinal motility is the μ-receptor. Importantly, opioid receptor agonists can also interrupt inhibitory neural inputs to gastrointestinal muscle, resulting in depression of NO release from inhibitory motor neurons and disinhibition of gastrointestinal muscle activity causing non-propulsive motility patterns or spasm. As the opioid receptors in the central nervous system mediate the analgesic actions of opioids, selectively blocking the peripheral opioid receptors in the gut is a logical therapeutic target for managing opioid-induced bowel dysfunction (Holzer, 2009; Camilleri et al., 2017) (see further *I.2.3.2. Pharmacological strategies – I.2.3.2.2 Alvimopan*).

I.2.3. Current clinical management of POI

As the causes of POI are multifactorial and include inflammation, inhibitory neural reflexes and exogenous opioids used to manage pain, the management of POI requires a multimodal approach using both non-pharmacological and pharmacological strategies. Therefore, enhanced recovery after surgery (ERAS) protocols or fast-track programs aim to shorten POI

by a series of general measures, such as perioperative fluid management, forced patient mobilization, early oral feeding and optimal analgesia (Kehlet, 1997; Holte & Kehlet, 2002; Kehlet, 2011). Multiple studies have shown the beneficial impact of fast-track care in patients undergoing abdominal surgery, mainly colorectal surgery, resulting in a rapid recovery and early discharge from hospital (Wind et al., 2006). Still ERAS protocols do not fully prevent prolonged POI (Grass et al., 2017). Treatment of POI remains mainly supportive and no single standard treatment is currently available.

I.2.3.1. Non-pharmacological strategies

I.2.3.1.1. Laparoscopy

In a study in mice, Gomez-Pinilla et al. (2014b) demonstrated that animals undergoing laparotomy and IM, but not those undergoing laparoscopy and IM, developed a significant delay in gastrointestinal transit compared to laparotomy or laparoscopy alone. In addition, only IM via laparotomy resulted in significant intestinal inflammation. Similarly, they demonstrated in patients undergoing segmental colectomy, that the levels of pro-inflammatory cytokines detected in the peritoneal lavage fluid were significantly lower under laparoscopic conditions, a finding that was associated with a shorter POI compared to patients undergoing open surgery as judged from the days till tolerance of solid food and hospital discharge. Numerous other clinical studies have shown that laparoscopy compared to open surgery has a beneficial effect on the length of ileus, postoperative morbidity and hospital stay after abdominal surgery (Xu & Chi, 2012; Qingbin et al., 2017; Wang et al., 2018; Xubing et al., 2018). The use of minimally invasive techniques produces less physical tissue trauma compared with open surgery, whereby the exposure of the intestine to the hyperoxic and dry air environment after laparotomy might enhance the tissue trauma. This leads to reduced postoperative pain, less need for analgesia and weaker immune response with lower levels of pro-inflammatory cytokines; whether the reduction of the length of POI is a result of the reduced immune and pain response or a combination of additional factors, is yet to be determined (Person & Wexner, 2006).

Although laparoscopy only requires small incisions in the abdomen, usually 0.5 – 1.5 cm, new surgical techniques have been developed over the last decades to minimalize the invasiveness of surgery even more. Natural Orifice Transluminal Endoscopic Surgery (NOTES) or Natural Orifice Specimen Extraction (NOSE) are a fusion of therapeutic endoscopy and laparoscopy surgery; they access the site of surgery via one of the bodies' natural orifices, for example anus, mouth or vagina, instead of via incisions made in the abdomen (Wang & Meng, 2012). Sometimes a laparoscopic procedure is combined with the NOTES/NOSE technique in so called hybrid NOTES/NOSE. It is expected that these techniques reduce the trauma required to perform the surgery and thus also reduce pain scores and the incidence of POI. This is supported by a meta-analysis of Steinneman et al. (2017), which included 27 trials, comparing hybrid NOTES and standard laparoscopy; hybrid NOTES resulted in a lower pain scale on postoperative days 1 and 2 and reduced the need for analgesia; they did not include the incidence of POI as outcome parameter in their study. The occurrence of POI following NOTES can not be excluded as it was reported as a postoperative complication in multiple studies on NOTES including transanal rectal resection (de Lacy et al., 2013); transanal total mesorectal excision (Velthuis et al., 2013), transanal hybrid colon resection (Fuchs et al., 2013) and endoscopic mucosal resection (Khan et al., 2020). However, Kumbhari et al. (2015) showed that with peroral endoscopic myotomy (POEM), which represents the NOTES approach to laparoscopic Heller myotomy (LHM), not one out of 49 patients developed POI whereas LHM resulted in POI in 3 out of 26 patients. Evidence indicates that POI is also less common with NOSE as a quicker return of bowel function, measured by time to first passage of flatus following colorectal surgery, has been described (Xingmao et al., 2014; Ma et al., 2015).

I.2.3.1.2. Perioperative fluid management

Surgery will lead to salt and water retention as a result of increased anti-diuretic hormone, cortisol and aldosterone levels. Liberal perioperative fluid administration can therefore lead to intestinal edema, which might contribute to the development of POI (Bragg et al., 2015). It was shown that bowel edema secondary to volume overload is associated with delayed recovery

of gastrointestinal function and extended hospital stay (Lobo et al., 2002). Conversely, intraoperative fluid restriction in patients undergoing intra-abdominal surgery has been shown to reduce the time to recovery of gastrointestinal function (Nisanevich et al., 2005). Balanced administration of fluid within a restrictive regime should therefore be utilized during abdominal surgery.

I.2.3.1.3. Nasogastric tubes

Routine use of nasogastric tubes at the end of abdominal surgery is intended to hasten the recovery of bowel function, prevent pulmonary complications, increase patient comfort and shorten hospital stay. However, clinical studies have shown that prophylactic insertion of nasogastric tube did not deliver the intended beneficial effects; nasogastric tube usage was even associated with increased upper airway inflammation and lower airway infections. Therefore, nasogastric tubes are only recommended for the management of POI when vomiting and abdominal distension predominate (Verma & Nelson, 2007; Rao et al., 2011).

I.2.3.1.4. Early enteral nutrition

Fasting after gastrointestinal surgery was believed to prevent nausea, vomiting and abdominal distension and to allow healing time for surgical anastomoses. However, several clinical studies have shown that early oral feeding is safe and well tolerated by over 70% of patients with absence of nausea, vomiting or abdominal distension. The effect on the duration of hospital stay and POI is less clear and probably moderate as only a few studies found decreased length of hospital stay and time to restoration of bowel function. Moreover, other factors in multimodal care may have influenced these beneficial outcomes as only one study investigated the effect of early feeding outside of ERAS programs and demonstrated hastened gut function (Gianotti et al., 2011). Nevertheless, since early feeding did not show any detrimental effects and might even enhance anastomotic and wound healing, early postoperative enteral feeding should be included as routine in perioperative care (Lewis et al., 2001; Ng & Neill, 2006; Person & Wexner, 2006).

I.2.3.1.5. Sham feeding

Sham feeding, accomplished by gum chewing, is thought to stimulate gastrointestinal motility via cephalic vagal stimulation, which results in increased salivary and pancreatic secretions. Although this effect is also accomplished with early enteral feeding, it might offer a safe and costless solution for those patients who do not tolerate an oral diet. A recent meta-analysis, including twelve randomized controlled trials, suggested that the degree of improvement is small and of limited clinical significance (Su'a et al., 2015). Furthermore, both a small and large scale study have shown that chewing gum does not further enhance bowel recovery following abdominal surgery in an ERAS environment, which included early postoperative feeding (Lim et al., 2013; de Leede et al., 2018).

I.2.3.1.6. Early postoperative mobilization

It is recommended that patients with POI ambulate regularly, although there is no evidence that ambulation has a prokinetic effect on gastrointestinal motility. It was even shown that ambulation following laparotomy did not confer any significant differences in myoelectric activity in the stomach, small bowel or colon (Waldhausen & Schirmer, 1990). However, it is believed that mobilization reduces the risk of developing postoperative and thrombotic complications, leading to its inclusion in multimodal postoperative care programs (Kehlet, 1997).

I.2.3.2. Pharmacological strategies

I.2.3.2.1. Epidural anesthesia and analgesia

As activation of neurogenic inhibitory reflexes originates from incision and gastrointestinal manipulation is proposed to play a role in POI (see *I.2.2. Pathogenesis of POI – I.2.2.1 Neurogenic phase*), blockade of afferent and efferent inhibitory reflexes by epidural anesthesia and analgesia will result in a beneficial effect on POI. Furthermore, it may reduce the need for systemic opioids. Numerous clinical studies have shown hastened recovery of bowel function with epidural local anesthetics and analgesia compared to administration of systemic opioids. It has to be noted that the location of epidural placement might play a role in its efficacy; Scott

et al. have shown that thoraric epidural analgesia has distinct advantages over lumbar epidural analgesia and some studies using lumbar or low-thoracic epidural anesthetics have failed to demonstrate the positive effects on ileus due to insufficient visceral afferent/efferent blockade (Liu et al., 1995; Scott et al., 1996; Holte & Kehlet, 2000; Leslie et al., 2011).

I.2.3.2.2. Alvimopan

As mentioned earlier, opioids adversely affect intestinal motility as they also activate μ-opioid receptors in the gastrointestinal tract. Peripherally acting μ-opioid receptor antagonists which do not cross the blood brain barrier, such as alvimopan, can be used to counteract this side effect without affecting pain reduction. Various clinical studies have shown a beneficial effect of alvimopan on bowel recovery after abdominal surgery in patients receiving opioids, with reduced time to tolerance of solid food and first defecation or flatus (Wolff et al., 2004; Viscusi et al., 2006; Ludwig et al., 2008). However, routine use of alvimopan is not advised as its beneficial effects in an ERAS environment are marginal and may not outweigh the additional cost (Keller et al., 2016; Nair, 2016).

I.2.3.2.3. NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit algesic COX pathways and their postoperative use is advocated as part of a multimodal postoperative analgesic strategy to reduce opioid consumption and their inhibiting effect on intestinal motility. Because COX-2 activation and production of prostaglandins play an important role in the pathophysiology of POI, they may also be useful to accelerate gastrointestinal function postoperatively. Clinical trials indeed show that inhibition of COX-2 by use of the classic NSAID diclofenac or the COX-2 selective NSAID celecoxib, may reduce the development of prolonged POI following abdominal surgery; it is worth mentioning that in both studies a first dose was given one or two hours before surgery, indicating that preoperative administration of COX-2 inhibiting NSAIDs may increase their efficacy to reduce the development of POI (Wattchow et al., 2009; Raju et al., 2015). Furthermore, caution may be needed with prescribing non-selective NSAIDs to patients with pre-existing risk factors for leak, as emerging data suggest that usage of these

NSAIDs may be associated with a higher risk of anastomotic leakage (Gorissen et al., 2012; Bhangu et al., 2014; Huang et al., 2018).

I.2.3.2.4. Prokinetic agents

The use of prokinetic agents to improve gastrointestinal motility after abdominal surgery seems obvious. In a study in which transit in rats was measured at 1 h 20 min after abdominal surgery, known as the neurogenic phase of POI, De Winter et al. (1999) show that prokinetics may be effective. However, results were diverse as only cisapride was able to significantly improve the delay in transit seen after IM; other prokinetics such as prucalopride, metoclopramide and erythromycin had no significant effect. Also in a clinical setting classic prokinetics are generally not effective. Traut et al. (2008) analyzed 39 randomized control trials, concerning 15 different prokinetic agents including cisapride, metoclopramide and domperidone, to evaluate their effect in POI. Most agents showed no positive effect, or the evidence was not sufficiently conclusive to attribute a beneficial effect as trials were too small or of poor methodological quality. Only i.v. lidocaine and neostigmine might exert beneficial effects, but more evidence on clinically relevant outcomes is needed. However, in the past decade there has been emerging evidence that selective 5-hydroxytryptamine-4 (5-HT_4) receptor agonists, such as mosapride and prucalopride, do provide protection against POI. These agents have prokinetic actions as a direct result of increased ACh release from cholinergic myenteric nerve endings innervating smooth muscle, via presynaptic 5-HT_4 receptors on these nerve endings. Clinical trials studying the effect of mosapride after colectomy demonstrated a reduction of time to first flatus and bowel movement and shortened hospital stay (Narita et al., 2008; Toyomasu et al., 2011); also recent clinical studies with prucalopride showed improved postoperative recovery in humans (Gong et al., 2016; Stakenborg et al., 2019). Only one study used pre-operative administration (Stakenborg et al., 2019); prucalopride was then able to significantly reduce inflammatory parameters in patients upon surgery. This anti-inflammatory effect has been reported earlier for mosapride in a study in rats, in which inflammatory mediator expression and leukocyte infiltration were measured at 24 h after IM (Tsuchida et al., 2011) and was

ascribed to increased acetylcholine release from nerve endings in contact with activated monocytes/macrophages, resulting in activation of α7 nicotinic ACh receptors (nAChRs) on these immune cells, subsequently leading to inhibition of their inflammatory reactions in the muscle layer. Data supporting this anti-inflammatory effect measured at 24 h post-surgery and the nAChR-pathway were also found in mice (Stakenborg et al., 2019).

I.2.3.2.5. Laxatives

Theoretically, laxatives could benefit the recovery of POI due to their direct or indirect stimulatory action on the GI motility. Prospective clinical trials using laxatives such as bisacodyl and magnesium oxide already demonstrated beneficial effects with respect to gastrointestinal recovery after abdominal surgery (Fanning & Yu-Brekke, 1999; Wiriyakosol et al., 2007; Zingg et al., 2008). However, more clinical studies with larger patient groups are needed to further substantiate these findings. Magnesium oxide was also used as part of a multimodal postoperative treatment regimen with encouraging results, indicating that postoperative laxatives can be added to the multimodal approach for POI (van Bree et al., 2011).

I.2.4. New targets for management of POI

Now that it is known that the inflammatory phase of POI is clinically most relevant, treatment of POI should aim to prevent or reduce the inflammatory cascade in response to gastrointestinal manipulation. One should interfere early in the inflammatory cascade when inflammation is not yet established and only a limited number of inflammatory mediators are released. Still, one should take in account that interference with the immune response may influence wound healing and even may increase the risk of anastomotic leakage and bacterial infection; therefore minimal handling of the small intestine and minimal invasive or laparoscopic procedures remain key in preventing POI (Boeckxstaens & de Jonge, 2009). In order to find new pharmacological treatment options that could possibly prevent or reduce the gastrointestinal inflammation seen in POI, multiple strategies are currently under investigation.

I.2.4.1. Mast cell stabilization

As activation of mast cells might play a role in triggering the inflammatory cascade in POI, mast cell stabilizers such as ketotifen could be useful to treat POI. A dose-finding pilot study in 60 patients undergoing major abdominal surgery, demonstrated that pre-treatment with ketotifen reduced gastric retention 1 h after liquid intake and improved abdominal cramps (The et al., 2009). However, the exact underlying mechanism remains unclear as ketotifen exerts both mast cell stabilizing and H1 histamine receptor blocking effects. Furthermore, prolonged postoperative treatment with ketotifen may even have an inhibitory effect on enteric smooth muscle contraction (Reisinger et al., 2013).

I.2.4.2. Activation of the cholinergic anti-inflammatory pathway

As mentioned above, pharmacological stimulation of cholinergic neurons with 5-HT_4 agonists exerts an anti-inflammatory effect via activation of α7 nAChRs located on monocytes and macrophages (Tsuchida et al., 2011; Stakenborg et al., 2019). The same effect can be obtained via electrical stimulation of the vagus nerve, leading to vagal activation of myenteric cholinergic neurons. In a mouse model of POI, it was shown that both cervical and abdominal vagus nerve stimulation improved gastrointestinal transit and dampened intestinal inflammation (Stakenborg et al., 2017). Moreover, abdominal vagus nerve stimulation can be safely performed in humans. Besides electrical stimulation, stimulation of the vagus nerve via enteral feeding of lipid-rich nutrition (Lubbers et al., 2009) or via intracerebroventricular administration of the MAPK p38 inhibitor semapimod (The et al., 2011) also reduced the influx of immune cells into the muscularis, dampened cytokine production, and improved transit in rodents. Finally, direct activation of nAChRs in a vagal-independent manner via the specific α7 nAChR agonist AR-R17779, prevented POI in mice as well (The et al., 2007).

I.2.4.3. Inhibition of intracellular signaling pathways

Given the fact that IM activates macrophages with concomitant activation of MAPKs p38, ERK1/2 and JNK, interference with these pathways may be an interesting strategy to prevent POI. Indeed, peripheral administration of the p38 inhibitor semapimod dampened muscular IL-

6, ICAM-1 and MCP-1 mRNA levels, and prevented suppression of smooth muscle function and gastrointestinal motility upon IM in mice. Furthermore, its beneficial effect was strengthened by a strong suppression of NO production in the muscularis externa (Wehner et al., 2009). Also inhibition of protein tyrosine kinases (PTKs), which are needed for the phosphorylation of cytosolic transcription factors involved in the activation of the pro-inflammatory NF-κB signaling pathway, may provide protection against POI. A single bolus injection of the PTK inhibitor tyrphostin AG 126 before surgery was already shown to attenuate the surgically induced impairment of colonic contractility in a murine model of POI; this effect was associated with reduction of the transcription of genes encoding IL-1β, MCP-1, iNOS and COX-2, and inhibition of the activation of the transcription factor NF-kB (Moore et al., 2004). However, caution is needed as interference with the activation of transcription factors and signaling pathways might compromise the immune response in general, leading to serious side effects.

I.2.4.4. Mimicking the heme oxygenase/carbon monoxide pathway

Heme oxygenase (HO) is the rate-limiting enzyme in the degradation of heme into ferrous iron (Fe^{2+}), biliverdin and carbon monoxide (CO). HO-1 contributes to the protection of tissues against oxidative stress and these cytoprotective effects have to a great extent be ascribed to the anti-apoptotic, anti-inflammatory and antioxidant properties of CO. Consequently, administration of CO via inhalation or peritoneal lavage was shown to be effective in reducing intestinal inflammation and preventing impaired transit in rodent and porcine models of POI (Moore et al., 2003; Moore et al., 2005; Nakao et al., 2006a). In our laboratory, similar results were obtained by De Backer et al. (2009) in the murine POI model via intraperitoneal (i.p) injection of the water-soluble CO-releasing molecule (CORM)-3. Furthermore, it was shown that the protective effects of CORM-3 were, at least in part, mediated through induction of HO-1, in a p38-dependent manner. A more detailed explanation on the HO-1/CO pathway and its protective effects on inflammation is given in the next section.

I.3. The heme oxygenase/carbon monoxide system

I.3.1. The heme oxygenase enzymes

HO catalyzes the rate-limiting step in the oxidative catabolism of heme (Fe-protoporphyrin-IX) as it cleaves heme at the α-methene bridge carbon atom which is liberated as CO, with generation of biliverdin and Fe^{2+}. This reaction occurs in association with cytochrome P450 reductase and in the presence of dihydronicotinamide-adenine dinucleotide phosphate (NADPH) and three molecules of molecular oxygen (O_2) per heme molecule. Biliverdin can then be further converted into bilirubin by the cytosolic enzyme biliverdin reductase and Fe^{2+} can be bound by the iron storage protein ferritin (Figure I.8). HO activity occurs in many organs, but its highest activity is reported in testes, spleen and brain. In mammals two major isoforms of HO have been identified to date: HO-1 and HO-2. Although HO-1 and HO-2 share 40% homology and catalyze the same reaction, they substantially differ with respect to regulation and expression pattern. HO-1 is highly inducible under conditions of stress such as oxidative stress and inflammation, allowing it to play a protective role in modulating tissue responses to injury in several diseases including sepsis, organ transplant rejection and others. Under basal conditions it is undetectable in most tissues; only in the spleen HO-1 is the predominant form in normal, unstressed conditions, likely due to the constant exposure to hemoglobin heme. In the gut, HO-1 is only expressed at very low levels unless induced by disease, injury and/or inflammation. HO-2 on the other hand is constitutively active and is expressed highly in many tissues including testes, spleen, liver, kidney, cardiovascular and nervous systems, where it regulates normal physiological functions. For example, in the testes HO-2 is involved in the modulation of the ejaculatory activity by monitoring the reflex activity of the bulbospongiosus muscle (Burnett et al., 1998). Although HO-2 activity does not respond to environmental stress, it was shown to respond to adrenal glucocorticoids in the brain (Maines, 1997; Maines et al., 1996). Within the gastrointestinal tract, HO-2 can be found in neuronal cell bodies and fibres within the myenteric plexuses, but also in non-neuronal cell types in the mucosal epithelium, in the smooth muscle and endothelium of blood vessels and in the interstitial cells of Cajal. Its

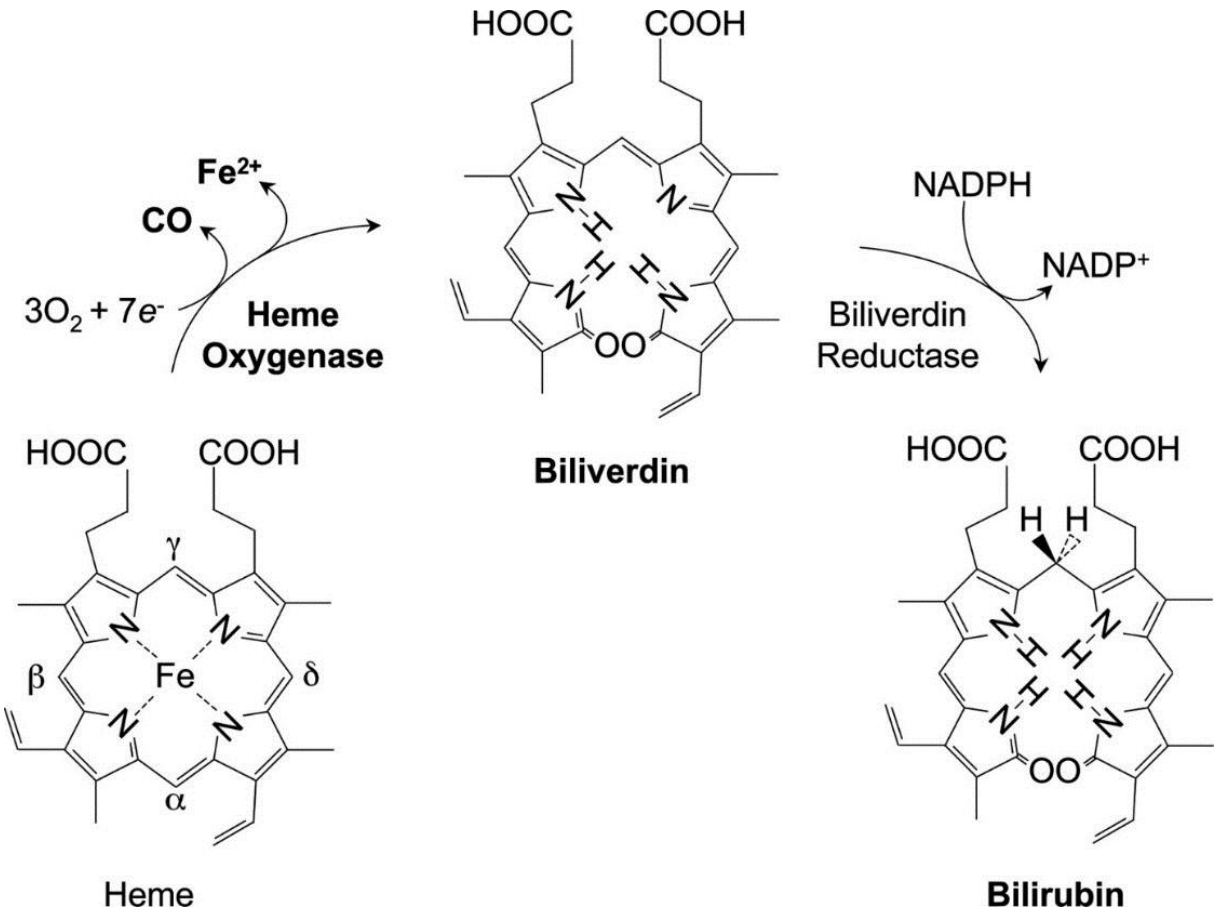


Figure I.8 Schematic representation of the heme oxygenase enzymatic activity (Stocker & Perrella, 2006).

proposed role within the gut is the constitutive generation of CO and bilirubin as signaling molecules. CO would then serve as an activator of soluble guanylate cyclase (sGC), leading to hyperpolarization and subsequent relaxation of smooth muscle cells; whereas bilirubin might potentiate inhibitory neurotransmission via preventing the oxidation of NO (Gibbons & Farrugia, 2004). The importance of HO-2 derived CO as hyperpolarizing factor was illustrated by Xue et al. (2000), as a loss of inhibitory neurotransmission upon electrical stimulation was observed in the gastric fundus in HO-2 knock-out mouse. The existence of a third HO isoform, called HO-3, has been reported in rat tissue, although it may be a pseudogene derived from HO-2 transcripts; its physiological role remains unclear (Hayashi et al., 2004).

I.3.2. Tissue protective effects of HO-1 induction

HO-1 is induced in response to a variety of stimuli such as hypoxia, oxidative stress and cytokines, in order to counteract stress conditions and prevent tissue injury. Its protective effects, which are modulated via anti-inflammatory, anti-apoptotic, anti-oxidant and anti-

proliferative actions, have been demonstrated in many disease models including colitis (Wang et al., 2001), atherosclerosis (Ishikawa et al., 2001), lung fibrosis (Zhou et al., 2004) and cardiac (Csonka et al., 1999), hepatic (Devey et al., 2009) and renal (Voelckel et al., 2000) ischemia/reperfusion (I/R) injury. The mechanism underlying such protection involves multiple factors, as HO-1 not only removes toxic oxidant moieties, but also protects the cells by providing anti-oxidant and anti-inflammatory molecules (Maines, 1997):

I.3.2.1. Degradation of free heme

Although heme (iron protoporphyrin IX), as the prosthetic group of various hemoproteins, is important for oxygen and mitochondrial electron transport, heme in its "free" non-protein bound form can be toxic. Free heme exerts pro-oxidant effects due to its catalytic active iron atom and can subsequently lead to generation of reactive oxygen species (ROS), membrane lipid peroxidation and damage to DNA and protein (Kumar & Bandyopadhyay, 2005). Free heme has also been shown to exhibit potent pro-inflammatory properties as it is recognized by TLR4, triggering the production of pro-inflammatory cytokines by innate immune cells such as macrophages (Figueiredo et al., 2007); as an example, Wagener et al. have demonstrated that a single i.v. injection of heme in mice caused experimental inflammation with a major influx of leukocytes in multiple organs including the intestines (Wagener et al., 2001). HO-1 induction will result in breakdown of these toxic free heme molecules and prevent the accumulation of heme in biological membranes.

I.3.2.1.1. Production of CO

Although CO is commonly known as a toxic air pollutant, it exerts important biological functions such as anti-inflammatory effects (Otterbein et al., 2000), protection against cell death (Brouard et al., 2000) and oxidative injury (Otterbein et al., 1999), and inhibition of cell proliferation (Duckles et al., 2015) and platelet aggregation (Brüne & Ullrich, 1987). Therefore, many protective effects of HO-1 induction have been attributed to this enzymatic byproduct. (see *I.3.3. Role of carbon monoxide in the protective effects of HO-1 induction*)

I.3.2.1.2. Production of biliverdin/bilirubin

Biliverdin and bilirubin are reducing agents with antioxidant properties and have the ability to efficiently scavenge peroxyl radicals and inhibit lipid peroxidation (Stocker et al., 1987). They have also been shown to exert important anti-inflammatory effects; for example, treatment with biliverdin in a rat model of small intestinal transplants decreased the mRNA expression of iNOS, COX-2, and the inflammatory cytokines IL-6 and IL-1β, as well as decreased neutrophil infiltration into the jejunal muscularis (Nakao et al., 2004). Although biliverdin has a short half-life and is rapidly converted to bilirubin, bilirubin as a potent antioxidant oxidizes itself back to biliverdin, thereby creating a recycling process (Sedlak & Snyder, 2004).

I.3.2.1.3. Co-induction of ferritin

The release of Fe^{2+} during HO-1 induction can amplify oxidative stress through ROS generation; however, Fe^{2+} is safely sequestered by ferritin that is co-induced with HO-1 (Maines, 1997). This protection has been demonstrated in various cell culture models, in which the synthesis of HO-1 and ferritin was correspondingly upregulated and prevented iron-mediated cell toxicity (Balla et al., 1992; Vile et al., 1994).

I.3.2.2. Endogenous induction of HO-1 in stress situations

HO-1 is endogenously induced by a wide range of diverse stimuli such as hypoxia, ischemia, heat shock, endotoxins, hydrogen peroxide, cytokines, UV light, heavy metals, and many more; which have in common only the ability to cause oxidative stress (Maines, 1997). The mechanisms by which HO-1 is induced, are primarily regulated at the transcriptional level (Figure I.9). Studies of the mouse HO-1 gene promoter have revealed two major regulatory enhancer regions which are located at −4 kb and −10 kb upstream of the transcriptional start site, termed SX2 and AB1. These enhancer elements are essential for the transcriptional regulation of the HO-1 gene in response to stress stimuli and contain tandem repeats of stress-responsive elements (StRE) such as the antioxidant response element (ARE) (Alam et al., 1994; Alam et al., 1995).

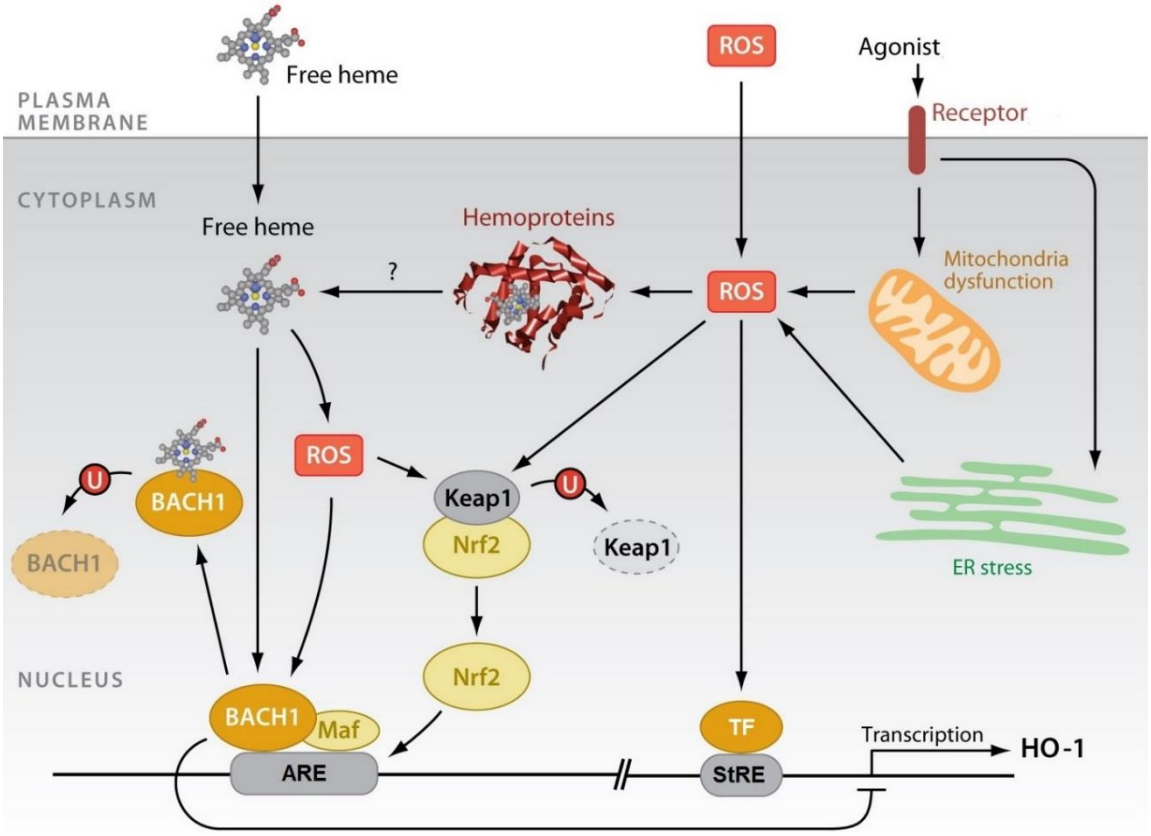


Figure I.9 Schematic representation of the transcriptional regulation of HO-1. HO-1 transcription can be induced by free heme, reactive oxygen species (ROS) and a variety of other agonists recognized by specific receptors. The activated receptors trigger signal transduction pathways that are associated with the production of ROS by the mitochondria and/or the endoplasmic reticulum (ER). ROS can elicit heme release from hemoproteins, leading to oxidative stress. The HO-1 promoter contains multiple stress-responsive elements (StRE) recognized by specific transcription factors (TF) activated in response to oxidative stress. Under basal conditions, Bach1/small Maf dimers bind constitutively to the antioxidant response element (ARE) in the HO-1 promoter and inhibit HO-1 transcription. In response to oxidative stress, Bach1 is exported from the nucleus, ubiquitinated and degraded. Oxidative stress also induces Keap1 ubiquitination-degradation, allowing the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) to translocate into the nucleus. Nrf2/small Maf protein heterodimers bind to ARE and promote HO-1 transcription (Adapted from Gozzelino et al., 2010).

The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) recognizes and binds to these ARE motifs and represents the major transcriptional regulator of the *HO-1* gene in response to oxidative stress (Alam et al., 1999). Nrf2 is anchored in the cytoplasm by the Kelch-like ECH-associated protein (Keap1), which functions as a sensor for oxidative stress. Under basal conditions, Keap1 forms a complex with Nrf2 and prevents its nuclear translocation, thereby inhibiting its transcriptional activity. It also facilitates the targeted ubiquitination and subsequent proteolytic degradation of Nrf2 as it acts as a substrate-recognition subunit for a cullin3-based ubiquitin E3 ligase. In the presence of oxidative stress, cysteine residues of Keap1 become modified which results in the dissociation of Nrf2 from Keap1, preventing the ubiquitination and

degradation of Nrf2. This allows Nrf2 to translocate to the nucleus where it forms a heterodimer with one of the small Maf proteins (MafF, MafG, MafK) and binds to the ARE motif to initiate the transcription of *HO-1* (Schmoll et al., 2017). The heme binding protein BTB and CNC homologue 1 (Bach1) has been identified as a negative transcriptional regulator of *HO-1*. Bach1 forms a complex with small Maf proteins and competes against Nrf2 for binding at the ARE motifs. Free heme and ROS can interact with Bach1 and inhibit its binding to ARE, promoting nuclear export and degradation of Bach1. Release of Bach1 from the ARE motifs in the *HO-1* promoter allows Nrf2 to access these StRE (Zhang et al., 2018).

Various other transcription factors have been identified to bind to different StRE of the *HO-1* gene, including NF-κB, activator protein-1, and heat-shock factor. Each transcription factor regulates different aspects of the cellular stress response and thus is activated under differing sets of circumstances. The heat-shock factor for instance is activated under stress conditions characterized by intracellular accumulation of non-native proteins, whereas NF-κB is an important regulator of inflammatory responses that provide protection against bacterial and viral infections. However, a single stimulus can simultaneously cause multiple types of "molecular" stress, resulting in a functional overlap between these different transcription factor pathways (Alam & Cook, 2007).

I.3.2.3. Induction of HO-1 by administering exogenous compounds

Since HO-1 is induced as a protective mechanism in response to oxidative stress and inflammation, targeted pharmacological induction of HO-1 may be considered as an important therapeutic strategy for protection against inflammatory processes and oxidative tissue damage. It is known for a long time that several compounds, including heme and heavy metals such as zinc, cadmium and cobalt, have the ability to induce HO-1 in different cell types and tissues. However, it was curcumin, a component of curry found in the South Asian root *Curcuma longa*, that was discovered as the first prototype of a small exogenous and natural HO-inducing molecule as it enhanced HO-1 expression in endothelial cells and renal proximal tubule cells. These findings stimulated a search for novel molecules with similar properties and

many new compounds have been reported to similarly affect HO-1 expression since then. However, not all compounds that exert HO-1 inducing properties are applicable for clinical interventions. For example, metalloporphyrins such as cobalt protoporphyrin IX (CoPP), which are prototypical inducers of HO-1 and are commonly used in experimental cell culture and animal models, lack cell-specificity and are severely toxic (Paine et al., 2010; Motterlini & Foresti, 2014). In 2013, Foresti et al. (2013) performed a screening of compounds reported in the literature as Nrf2 activators/HO-1 inducers; they selected 56 compounds and analyzed them for HO-1 induction and cytotoxicity in BV2 microglial cells in vitro, using hemin as internal positive. They found that, besides curcumin and carnosol, also dimethyl fumarate (DMF) was a very potent HO-1 activator exhibiting a good HO-1 expression/low toxicity profile. In the interest of the current thesis, only hemin and DMF will be described in detail:

I.3.2.3.1. Hemin

Chemically, hemin differs from heme chiefly in that the iron ion in heme is Fe^{2+}, whereas in hemin it is ferric (Fe^{3+}) stabilized by a chloride ion (Figure I.10). Hemin acts as a substrate for HO-1, leading to HO-1 activation and induction. It has been used in a variety of animal models, such as renal (Rossi et al., 2018) and cardiac (Lakkisto et al., 2009) I/R injury, septic shock (Yu & Yao, 2008), ventilator-induced lung injury (An et al., 2011) and cadmium induced-testicular injury (Fouad et al., 2009) to study the potential protective effects of HO-1 induction. Its benefecial effect has also been shown in gastrointestinal inflammation (see *I.3.2.3 Influence of HO-1 induction on gastrointestinal inflammation*). In the clinical setting; it is available under the form of hematin (Panhematin®), which differs from hemin in that the coordinating ion is a hydroxide ion instead of chloride ion, and heme arginate (Normosang®), in which heme is stabilized as a complex with arginine. These drugs are approved respectively by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for treatment of acute attacks of porphyria; however, their mechanism of action for this indication is inhibition of δ-aminolevulinic acid synthase and not induction of HO-1 (Bickers, 1981; Mustajoki & Nordmann, 1993; Siegert & Holt, 2008). More recently, it was shown in phase II clinical trials that both

Normosang® and Panhematin® are able to safely induce HO-1 in patients (Bharucha et al., 2016; Thomas et al., 2016).

I.3.2.3.2. Dimethyl fumarate

Dimethyl fumarate (DMF; Figure I.10) is a lipophilic ester of fumaric acid, a component of the intracellular citric acid cycle. The anti-inflammatory and immunosuppressive effects of fumaric esters have been studied since 1959 and a mixture of fumaric esters (Fumaderm®), also containing DMF, has been used in the treatment of psoriaris, an autoimmune skin disease. In 2013, an oral formulation of DMF (BG-12, Tecfidera®) has been approved for treatment of multiple sclerosis (MS) upon the demonstration of the efficacy and safety for management of relapsing forms of MS in phase III trials (Kees, 2013; Xu et al., 2015). When taken orally, DMF is quickly hydrolyzed into monomethyl fumarate (MMF) by esterases in the small intestine. However, it is suggested that DMF is not completely hydrolyzed but absorbed from the small

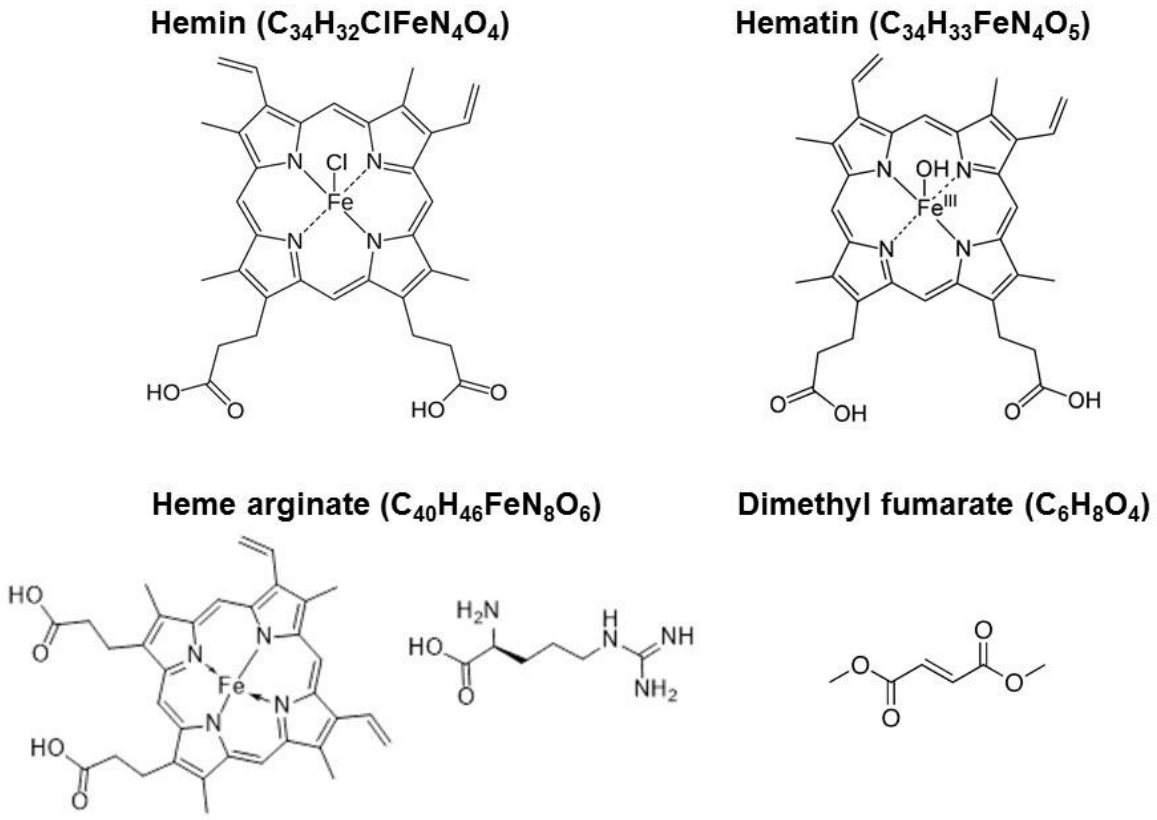


Figure I.10 The chemical structure and formula of hemin, hematin (Panhematin®), heme arginate (Normosang®) and dimethyl fumarate.

intestine into the presystemic circulation, where it reacts with intracellular molecules containing thiols or cysteine residues such as glutathione (GSH). It is therefore probable that absorbed DMF rapidly translocates into blood cells, including immune cells, due to its lipophilic character and then immediately reacts with GSH or other molecules (Schmidt et al., 2007; Rostami-Yazdi et al., 2010). Subsequently, both DMF and MMF can covalently bind to cysteine residues of Keap1 protein, which is the inhibitor of Nrf2, resulting in Nrf2 activation and increased transcription of anti-oxidant target genes, including HO-1 (see Figure I.9); this effect of fumaric esters was shown both *in vitro* and *in vivo* in multiple animal models (Linker et al., 2011; Kobayashi et al., 2015; Kunze et al., 2015; Zhao et al., 2015). Furthermore, DMF can cause depletion of circulating GSH levels by binding to its free cysteines and forming GSH conjugates. This GSH depletion can contribute to the immunosuppressive and anti-inflammatory properties of DMF, as it leads to induction of HO-1 and reduces pro-inflammatory cytokine secretion in human peripheral blood cells (Lehmann et al., 2007). Although activation of Nrf2 is the main reported pharmacological target of DMF, it cannot explain its immune modulating effects reported in Nrf2 knockout mice (Schulze-Topphoff et al., 2016). It is suggested that DMF is able to inhibit the pro-inflammatory transcription factor NF-kB independently of Nrf2. In an inactivated state, NF-kB dimers (e.g. p65/p50) are located in the cytosol complexed with the inhibitory protein IκB (Inhibitor of κB). Through the intermediacy of integral membrane receptors such as TLR and cytokine receptors, extracellular signals can activate the enzyme IκB kinase (IKK). IKK, in turn, phosphorylates the IκB protein, which results in ubiquitination, dissociation of IκB from NF-kB, and eventual degradation of IκB by the proteasome. This will allow the NF-kB dimer to translocate to the nucleus where it can interact with DNA-binding sites of specific genes, resulting in the transcription of pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 (Figure I.11) (Tornatore et al., 2012). Gillard et al. (2015) have shown that DMF, but not MMF, inhibited NF-kB-driven cytokine production and nuclear translocation of p65 and p52 in splenocytes of $Nrf2^{-/-}$ mice. Recent in vitro evidence in breast cancer cells suggests that DMF prevents the NF-kB p65 nuclear

translocation and attenuates its DNA binding activity via covalent modification of NF-kB p65, with cysteine 38 being essential for the activity of DMF (Kastrati et al., 2016).

Another possible therapeutic target of DMF involves the hydroxycarboxylic acid receptor 2 (HCAR2), which is a G protein-coupled receptor that is expressed by immune cells such as macrophages and dendritic cells. While HCAR2 is commonly known for its anti-lipolytic properties, activation by endogenous and exogenous ligands has also been associated with anti-inflammatory effects in numerous disease states (Graff et al., 2016). In such manner, Chen et al. (2014) observed that HCAR2 deficiency prevented the beneficial effects of DMF treatment in acute experimental autoimmune encephalomyelitis in mice, suggesting that also HCAR2 can contribute to the therapeutic effects of DMF.

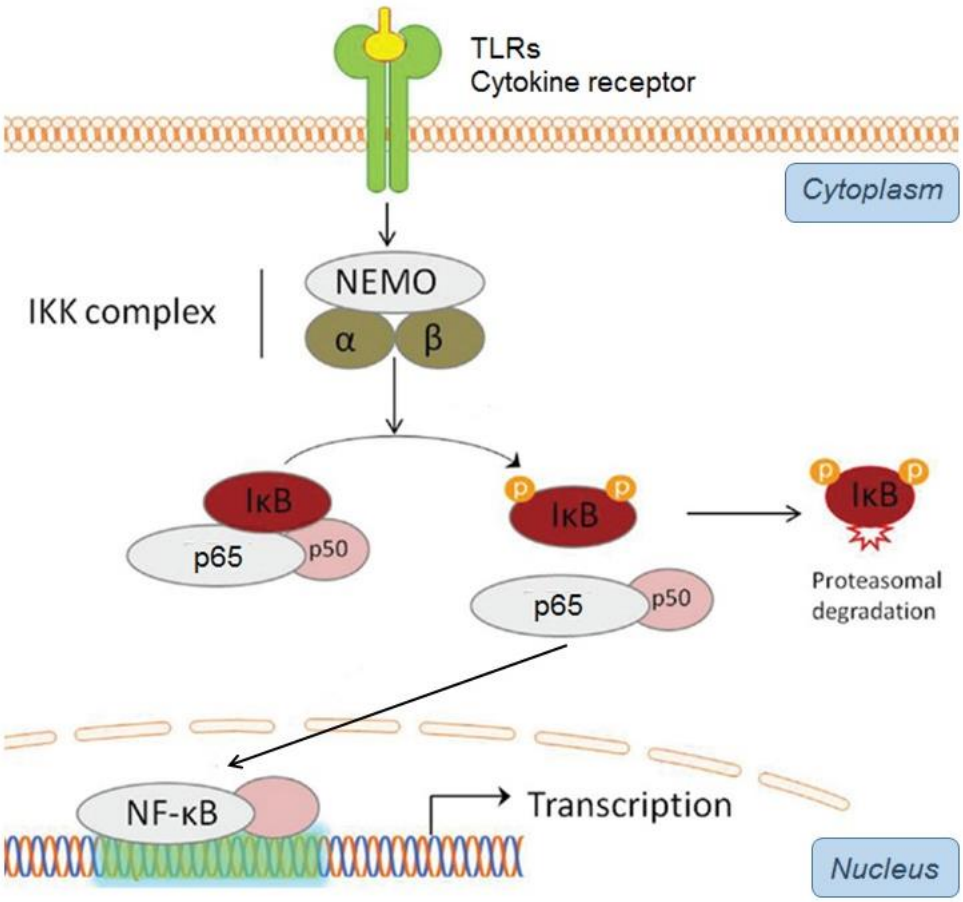


Figure I.11 Schematic representation of the activation of NF-kB. In unstimulated cells, the NF-kB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκBs (Inhibitor of κB). Ligation of Toll-like-receptor (TLR)/Cytokine receptors will activate the IKK complex, containing NF-kB essential modulator (NEMO) and IKKα/IKKβ, leading to phosphorylation of IκB and subsequently proteasomal degradation of IκB. This will allow the NF-kB dimers to translocate towards the nucleus where they can interact with DNA-bindings sites of specific genes (Adapted from Godwin et al., 2013).

I.3.2.4. Influence of HO-1 induction on gastrointestinal inflammation

A beneficial effect of HO-1 induction has been shown for many gastrointestinal inflammatory conditions, both acute and chronic; for review see Chang et al. (2015).

I.3.2.4.1. Intestinal ischemia/reperfusion injury

Intestinal I/R injury is considered to be a major and frequent problem in many clinical conditions, including intestinal mechanical obstruction, liver and intestinal transplantation, mesenteric artery occlusion, shock and severe trauma. It is a complex, multifactorial pathophysiological process characterized by an increase in ROS production, leading to a subsequent initial inflammatory cascade which is amplified by the recruitment of circulating neutrophils. It has been shown that induction of HO-1 has anti-inflammatory and cytoprotective effects in I/R mediated intestinal injuries. For example, upregulation of HO-1 by CoPP administration 24 h before induction of intestinal I/R in rats resulted in a significant reduction of intestinal tissue injury, IL-6 mRNA level and myeloperoxidase (MPO) activity (Wasserberg et al., 2007). Similarly, hemin treatment 2 h before superior mesenteric artery occlusion in rats was associated with increased HO-1 protein expression, lessened mucosal injury, decreased MPO activity and improved intestinal transit following gut I/R (Attuwaybi et al., 2004). Furthermore, it was also shown that HO-1 is mechanistically involved in the beneficial effect of intestinal ischemic preconditioning on systemic inflammation in shock models; this might prove to be an effective strategy to prevent intestinal I/R injury and its inflammatory response (Tamion et al., 2002; Tamion et al., 2007).

As I/R injury has been associated with early graft dysfunction after intestinal transplantation, the approach of HO-1 induction might have a dramatic impact in reducing the occurrence of primary non-function of the transplanted organ. Previous studies have shown that treatment of the graft recipients with CO-inhalation at a low concentration (250 ppm) protects against I/R injury of intestinal grafts. CO was able to significantly reduce intestinal inflammation and to improve the microvascular blood flow within the intestinal wall and the gastrointestinal transit (Nakao et al., 2003a; Nakao et al., 2003b; Nakao et al., 2003c). Also treatment with biliverdin

can significantly decrease inflammation in the jejunal muscularis in rat syngeneic small intestinal transplants (Nakao et al., 2004). Although the effect of treatment with HO-1 inducers has not yet been investigated for I/R injury of intestinal grafts, this evidence suggests that it might be beneficial as the different products of HO-1 activity exert protective effects that are additive or synergistic. Moreover, overexpression of HO-1 obtained via administration of CoPP was already shown to exert potent protective effects in both rat hepatic (Kato et al., 2001) and cardiac (Katori et al., 2002) cold I/R models resulting in increased transplant survival.

I.3.2.4.2. Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is among the most common diseases in neonates and is characterized by severe necrosis of the small intestine, resulting in symptoms such as blood in the stool, diarrhea, poor tolerance of feeding and weight loss. Although its pathophysiology is not completely understood, epidemiologic observations indicate that genetic factors and several immature characteristics of the fetal intestine (such as altered microbiota, inadequate barrier function and an excessive inflammatory response) contribute to the severe necrosis of the small intestine. The excessive inflammatory process initiated in the highly immunoreactive intestine extends the effects of the disease systemically, affecting distant organs such as the brain and heart, possibly resulting in an increased risk for neurodevelopmental delay, multiorgan failure and even death (Neu & Walker, 2011). HO-1 activity has been linked to the development of NEC as mice pups heterozygous for the HO-1 gene ($HO\text{-}1^{Het}$) appeared predisposed to injury, with higher histological damage scores and a significant reduction in muscularis externa thickness upon induction of NEC-like intestinal injury. Due to the fact that $HO\text{-}1^{Het}$ mice have deficient upregulation of HO-1, they have a compromised immune function and an exaggerated inflammatory response under stress conditions, observed by increased intestinal expression of IL-1β and P-selectin and resulting in higher incidence and onset of NEC. Moreover, treatment of wild-type pups with heme at 24 and 3 h before the initiation of NEC induction, reduced intestinal damage scores and NEC incidence (Schulz et al., 2013; Schulz et al., 2015).

I.3.2.4.3. Intestine as triggering organ in sepsis

Sepsis is characterized by a systemic inflammatory reaction in response to intensive and massive infection that failed to be locally contained by the host. The most widely used experimental model for sepsis is cecal ligation and puncture (CLP)-induced sepsis, which consists of the perforation of the cecum allowing the release of fecal material into the peritoneal cavity to generate an immune response induced by polymicrobial infection (Toscano et al., 2011). When challenged with CLP, markers of liver (asparate aminotransferase), kidney (blood urea nitrogen) and muscle (creatinine phosphokinase) dysfunction were significantly increased in HO-1-deficient mice resulting in a higher mortality rate compared to wild-type mice. The HO-1-deficient mice also displayed increased levels of free circulating heme, which is cytotoxic to red blood cells and causes hemolysis, and reduced levels of the heme binding protein hemopexin. As administration of heme at 2, 12 and 24 h after CLP to wild-type mice also promoted tissue damage and severe sepsis, the increase in free heme was associated with increased susceptibility to sepsis-induced mortality (Larsen et al., 2010). In contrast, hemin has been used as a therapeutic conditioning agent, as it can provide protection by inducing HO-1 expression in tissues prior to injury. Pre-treatment with hemin at 24 h before CLP was shown to partly protect mice from sepsis-induced skeletal muscle wasting, as HO-1 induced by hemin reduced CLP-mediated skeletal muscle atrophy by reducing the expression of pro-inflammatory cytokines and ROS activation (Yu et al., 2018). Similarly, Fei et al. (2012) have shown that pre-administration of hemin to septic mice increased the expression and activation of HO-1, leading to reduced plasma levels of IL-6 and TNF-α, and to inhibited thrombosis in liver, kidney and lung tissues; all of these beneficial effects were abolished by co-administration of the HO-1 inhibitor zinc protoporphyrin IX. Furthermore, hemin also reduced mitochondrial fission, promoted mitochondrial quality control, and stimulated mitochondrial autophagy and biogenesis in the murine CLP model (Park et al., 2018).

I.3.2.4.4. NSAID-induced gastric and intestinal injury

NSAIDs, such as indomethacin, can cause severe gastrointestinal damage and lead to ulceration and bleeding. Treatment of mice with hemin 6 h before indomethacin administration significantly reduced the increase in MPO activity and mRNA expression of TNF-α and MCP-1 in the intestinal mucosa, as well as the development of intestinal lesions, as seen in non-treated animals. These beneficial effects of hemin were accompanied by a significant increase in HO-1 protein expression in the intestinal mucosa; and were reversed by co-administration of tin protoporphyrin, an HO-1 inhibitor (Yoriki et al., 2013). Similar results were obtained by Uc et al. (2012), as pre-treatment with the HO-1 inducer cobalt protoporphyrin was able to reduce gastric inflammation, number of stomach ulcers, tissue neutrophil activation, and proinflammatory cytokine expression caused by indomethacin administration.

Despite the clear evidence pointing towards a protective effect of HO-1 induction in the above described gastrointestinal conditions characterized by acute inflammation, the potential anti-inflammatory effect of HO-1 inducers has not yet been studied in animal models of POI.

I.3.2.4.5. Inflammatory bowel disease

Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel disease (IBD), are multifactorial immune disorders characterized by chronic relapsing inflammation of the intestine causing symptoms such as diarrhea, hematochezia and abdominal pain (Kim & Cheon, 2017). Dextran sulfate sodium (DSS)- and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis are among the most commonly used experimental models that have yielded major insights into the pathogenesis of IBD (Kiesler et al., 2015). HO-1 mRNA and protein expression was shown to be increased in inflamed colonic tissue in TNBS-induced colitis in rats and the colonic damage was potentiated, along with a decrease in HO-1 activity and increase in ROS, after administration of the HO-inhibitor tin mesoporphyrin. Mechanistically, the protective effect of HO-1 could be the result of radical scavenging and inhibition of NO production and iNOS expression since HO-1 induction by hemin lowered iNOS mRNA and protein expression in the affected colon (Wang et al., 2001). Similarly, in a DDS-induced colitis

model in mice, HO-1 mRNA is markedly induced in inflamed colonic tissue and co-administration with the HO-inhibitor zinc protoporphyrin enhanced intestinal inflammation and increased the disease activity index (Naito et al., 2004). Administration of the HO-1 inducer CoPP before the onset of intestinal inflammation in DSS-induced colitis, significantly reduced the histological degree of intestinal inflammation and colonic MPO activity compared to control mice. However, when CoPP was administered after the onset of colonic inflammation, the authors did not observe these beneficial effects on the extent of colonic inflammation, indicating that HO-1 counteracts early events in the initiation of inflammation and requires a preventive approach (Paul et al., 2005). Similar results were obtained with the HO-inducer hemin in both TNBS- (Mateus et al., 2018) and DSS-induced murine colitis (Zhang et al., 2014), as pretreatment with hemin increased the colonic expression of HO-1 and reduced the histological damage. Furthermore, mice deficient of Nrf2, have low HO-1 expression and are more susceptible at developing colitis in the DSS model (Khor et al., 2006); whereas mice deficient of the Bach1 transcription factor have higher expression of HO-1 mRNA and protein in colonic mucosa and have significantly attenuated colonic inflammation after induction of TNBS colitis (Harusato et al., 2013). In a clinical setting, an increase in HO-1 mRNA and protein expression was shown in inflamed colonic mucosa of IBD patients, comparable to that of patients suffering from intestinal ischemia (Paul et al., 2005; Takagi et al., 2008).

I.3.2.4.6. Diabetic gastroparesis

Diabetic gastroparesis is a gastric complication of diabetes mellitus defined as a delay in gastric emptying without any mechanical obstruction and is more prevalent in patients with type 1 diabetes than in those with type 2 diabetes. Although still not fully elucidated, the suggested pathophysiology of diabetic gastroparesis mainly involves the loss of interstitial cells of Cajal, being the electrical pacemakers involved in stimulating motor function and neurotransmission in the stomach (Young et al., 2020). Animal studies have increasingly highlighted a role of oxidative stress and inflammation in the development of gastroparesis. For example, it was shown in nonobese diabetic mice that development of delayed gastric

emptying was associated with high levels of markers of oxidative stress, failure to maintain up-regulation of gastric HO-1 expression as observed in diabetic mice with normal gastric emptying, and loss of expression of Kit, a marker for interstitial cells of Cajal. Furthermore, the development of delayed gastric emptying was reversed by induction of HO-1 in all diabetic mice receiving hemin, whereas inhibition of HO-1 activity by chromium mesoporphyrin (CrMP) caused increased oxidative stress, loss of Kit and development of delayed gastric emptying. These data strongly suggest that HO-1 is essential to protect interstitial cells of Cajal against oxidative injury (Choi et al., 2008). The same authors have demonstrated in nonobese diabetic mice that the proportion of anti-inflammatory $CD206^+$ M2 macrophages expressing HO-1 increased with the development of diabetes and markedly decreased with the onset of delayed gastric emptying; the latter being accompanied by an increased expression of pro-inflammatory M1 macrophages. Induction of HO-1 by hemin in diabetic mice that had developed delayed gastric emptying reversed the decrease of $CD206^+$ M2 macrophages, whereas inhibition of HO activity by chromium mesoporphyrin decreased the number of $CD206^+$ M2 macrophages (Choi et al., 2010). Summarized, HO-1 may play a dual role in diabetic mice, acting both as a signal to initiate phenotypic switching from M1 macrophages to M2 and to protect against oxidative stress. Although it has to be noted that there might be a sequential relation between these mechanisms proposed by Choi et al. (2010) as reduction of oxidative stress by HO-1 upregulation may promote an M2 macrophage phenotype and thus blunt M1 macrophage phenotype; similar to the dampened M1 and increased M2 phenotype seen in mice deficient in NADPH oxidase-derived superoxide anion radicals (O_2^-) leading to protection against type 1 diabetes (Padgett et al., 2015).

The beneficial effects of hemin on diabetic gastroparesis could not yet be demonstrated in a clinical setting. In a placebo-controlled, randomized 8-week clinical trial with a total of 20 patients, treatment with i.v. Panhematin® on day 1, 3 and 7, followed by weekly infusions for 7 weeks, did not significantly improve gastric emptying or symptoms of diabetic gastroparesis. Although there was an increase in HO-1 plasma levels and activity on day 2, Panhematin®

failed to sustain increased HO-1 levels beyond a week. These findings suggest that perhaps more frequent Panhematin® infusions or other inducers are needed to obtain a more sustained increase in HO-1 levels, resulting in a beneficial effect on gastric emptying. Another possible explanation might be that other biochemical mechanisms cause irreversible enteric neuromuscular and/or autonomic nervous dysfunctions in patients (Bharucha et al., 2016). Further studies are necessary to elucidate these possibilities.

I.3.3. Role of carbon monoxide in the protective effects of HO-1 induction

I.3.3.1. Mechanisms of the anti-oxidative/anti-inflammatory effects of carbon monoxide

As mentioned earlier, the products of the HO pathway exert unique biological functions and their contribution to the protective effects of the HO system have attracted great interest. Despite its reputation as the “silent killer”, especially CO has gained a lot of interest in the past few decades as a possible therapeutic agent for conditions with inflammation and oxidative stress. As CO binds to hemoglobin forming CO-hemoglobin, exposure to high concentrations of exogenous CO will compromise the oxygen transport in the body. The basal blood CO-hemoglobin (COHb) level in humans is 1%; in smokers it can increase till 18%. From 10% on, toxicity symptoms can occur and high levels can result in oxygen shortage, seizures and coma with fatal consequence (Von Burg, 1999).

However, CO is produced endogenously via the breakdown of heme by HO at a rate of about 16 µmol/h per human body, without causing any harmful effects (Heinemann et al., 2014). This involves both a detoxification process, as free heme derived from death red blood cells is cytotoxic due to the reactivity of iron, and functional production of CO with physiological or pathophysiological purposes. Since the 1990s, multiple research groups have shown that low concentrations of CO exert biological functions as diverse as neurotransmission and protection against apoptosis, inflammation and oxidative stress (Maines, 1993; Otterbein et al., 1999; Brouard et al., 2000; Otterbein et al., 2000). CO is able to exert such effects through different cellular targets; many of those targets are heme-containing proteins as CO binds preferentially

and almost exclusively to transition metals (Foresti & Motterlini, 2010). Mechanisms implied in the physiological function of CO involve the activation of sGC and/or NO synthase, leading to the production of respectively cGMP and NO and subsequently to smooth muscle relaxation, vasoactive effects and effects on neurotransmission (Utz & Ullrich, 1991; Suematsu et al., 1994; Thorup et al., 1999). sGC-independent mechanisms of vasoactive effects have also been suggested; CO may directly activate calcium-dependent K^+ channels, resulting in dilation of blood vessels (Wang et al., 1997). Other mechanisms, listed below, come in to play in response to oxidative stress and inflammation and exert a protective role; it has to be noted that these are mainly based on data obtained in cell lines and non-gastrointestinal tissue (Otterbein et al., 2000; Morse et al., 2013; Babu et al., 2015):

I.3.3.1.1. Activation of sGC/p38 MAPK and downstream target molecules

Activation of sGC was also proposed as an anti-inflammatory mechanism of CO. The inhibitory effects of CO on leukocyte adhesion in mesenteric venules and on neutrophil migration into the peritoneal cavity induced by carrageenan administration in mice was indeed reduced by an sGC-inhibitor (Freitas et al., 2006). CO also plays an important role as a signaling molecule in modulating MAPK, especially p38. CO has been shown to regulate the downregulation of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 together with augmentation of the anti-inflammatory cytokine IL-10 via the p38 and JNK pathways (Otterbein et al., 2000; Morse et al., 2003). However, CO is unlikely to activate p38 MAPK directly as p38 lacks a transition metal centre in the protein structure that would function as a binding site for the gaseous molecule. Thus, the p38 MAPK activation by CO might be the result of another upstream target. Downstream target molecules of CO-dependent p38 activation have also been identified. In vitro and in vivo data demonstrate that activation of the p38 signaling pathway by CO leads to increased expression of heat shock protein 70 (Kim et al., 2005b) or caveolin-1 (Kim et al., 2005a), resulting in respectively cytoprotective and anti-proliferative effects. Furthermore, Zhang et al. (2005) showed that the anti-apoptotic effects of CO involve both

phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and p38 MAPK signaling pathways in endothelial cells in a model of anoxia-reoxygenation injury.

I.3.3.1.2. Interaction with iNOS

CO is able to regulate NO synthesis via its interaction with iNOS. It was shown in cultured IL-1β-stimulated hepatocytes that exposure to CO (250 ppm) resulted in a significant decrease in iNOS protein, cytosolic iNOS activity and nitrite production through a process involving MAPK p38 activation. Similar results on NO synthesis were obtained via HO-1 induction with an adenoviral vector carrying HO-1 (Kim et al., 2008). Also in LPS-stimulated macrophages a reduction of iNOS expression was observed after HO-1 induction or exposure to the CO-releasing molecule (see *I.3.3.2 Strategies to deliver CO – I.3.3.2.3 CO-releasing molecules*) CORM-2 (Tsoyi et al., 2009); however in the same model, CORM-2 or CORM-3 decreased nitrite levels without changing iNOS protein expression (Sawle et al., 2005), suggesting that CO might only inhibit iNOS activity.

I.3.3.1.3. Anti-/pro-oxidant effect

Several studies suggest that CO exerts an anti-oxidant effect, probably mediated by binding to the heme-containing unit in NADPH oxidases, an enzyme family solely producing O_2^- (Nakahira et al., 2006). In such manner CO derived from CORM-2 inhibits NADPH oxidase activity and suppresses O_2^- overproduction and accumulation of ROS in LPS-stimulated macrophages (Srisook et al., 2006). Similarly, exposure of human airway smooth muscle cells to CORM-2 leads to inhibition of cytochrome B558 of NADPH oxidase, resulting in a reduced production of O_2^- and possibly explaining the anti-proliferative effect of CORM-2 on human airway smooth muscle cells (Taillé et al., 2005). In contrast, low concentrations of CO may partially inhibit the mitochondrial electron transport chain and lead to an accumulation of electrons, facilitating O_2^- generation. The mild oxidative stress on mitochondria, so-called preconditioning, ultimately enhances the energy production in mitochondria by accelerating the oxidative phosphorylation and improving the mitochondrial respiration. By these

preconditioning effects on mitochondrial biogenesis, CO could have a positive role in cellular survival (Oliveira et al., 2016).

I.3.3.1.4. Induction of HO-1

Also induction of HO-1 might contribute to the beneficial effects of CO, providing a positive feedback loop and allowing the ROS scavenging properties of biliverdin/bilirubin to come into play. In human hepatocytes, exposure to CO resulted in HO-1 induction via nuclear translocation and accumulation of Nrf2. This has been shown to be associated with upstream activation of MAPKs (p38, ERK and JNK) as they regulate the ARE promoter activity (Lee et al., 2006). Also in human umbilical vein endothelial cells, exogenous CO activated Nrf2 and subsequently induced HO-1, which correlated with protection against endoplasmic reticulum stress-induced endothelial cell apoptosis (Kim et al., 2007).

Although the exact underlying signaling mechanisms and precise molecular targets of CO are thus only partially elucidated, it is clear that CO exerts cytoprotective effects and is able to modulate inflammatory responses. Therefore, administration of controlled amounts of CO might be of use to counteract conditions characterized by inflammation such as POI. However, the therapeutic use of CO is impeded by safety challenges as a result of high COHb formation following non-targeted, systemic administration. This prompted scientists to develop new strategies to deliver CO for therapeutic purposes in a controlled and safe manner.

I.3.3.2. Strategies to deliver carbon monoxide

I.3.3.2.1. CO gas

CO administered as gas was firstly used in vivo to demonstrate that it protects against hyperoxic lung injury in rats; Otterbein et al. (1999) exposed the animals to doses in the order of 50 – 500 ppm (particles per million) which do not compromise the oxygen carrying capacity of hemoglobin. Since then, inhalation of CO gas at doses of 250 ppm for a short period of time has shown efficacy in many animal models of disease. Furthermore, the Covox delivery system for administering CO gas by inhalation in humans was developed. This device was used for a

Phase I trial in healthy volunteers showing that at a dosing of 3.0 mg/kg/hour, COHb levels in the blood were elevated to 12% in a predictable manner with no documented severe adverse events in comparison to placebo and no need for supplementary oxygen (Motterlini & Otterbein, 2010). Still, a phase II clinical trial to study the safety and tolerability of inhaled CO, administered via Covox, in kidney transplant patients, was withdrawn early without results being reported (NCT00531856; Goebel & Wollborn, 2020). The degree of tissue distribution should be considered; it was indeed demonstrated that the distribution of CO after 1 h CO inhalation in rats (at a dose of 250 ppm) is tissue specific and results in increased CO levels ranging from 32-fold in spleen to 2-fold in intestine (Vanova et al., 2014). The lack of tissue specificity and the undesired effect of CO gas inhalation on the oxygen-carrying capacity of hemoglobin are crucial aspects that need to be addressed before CO gas can be approved as a therapeutic agent.

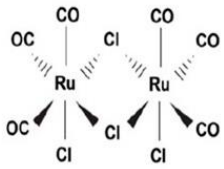
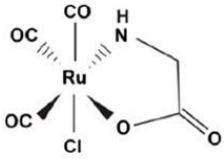
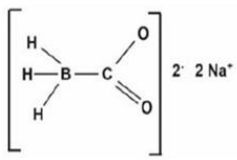
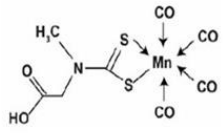
I.3.3.2.2. CO-saturated solutions

Organ transplantation procedure obligates cold preservation and induces certain degrees of I/R injury in all grafts. Therefore CO has been proposed to be used ex vivo as an adjuvant to preservation solution in which organs are normally stored prior to grafting. Indeed, cold storage of rat intestinal grafts in University of Wisconsin solution bubbled with 5% CO for 5 min before transplantation in recipient animals has been reported to markedly reduce the up-regulation of inflammatory mediators and improve graft blood flow and mucosal barrier function (Nakao et al., 2006b). Comparable data have been obtained for rat kidney and lung grafts (Kohmoto et al., 2008; Nakao et al., 2008) and for renal transplantation in pigs (Yoshida et al., 2010). However, it has to be mentioned that in contact with air, the CO-bubbled storage solution quickly loses CO by release into air; therefore the CO-bubbled solution must be kept in a tightly sealed container without an air layer to keep the CO concentration constant (Kohmoto et al., 2008).

I.3.3.2.3. CO-releasing molecules

CORMs were developed to deliver CO in a more practical, controllable and accurate way to the target site in comparison to CO gas inhalation. The structure of the most frequently investigated CORMs is shown in Table I.1. While the original transition-metal carbonyl complexes (CORM-1 and CORM-2) had to be dissolved in organic solvents like dimethyl sulfoxide (DMSO) (Motterlini et al., 2002), water-soluble CORMs such as CORM-A1 and CORM-3 have been subsequently developed with different CO release kinetics (Clark et al., 2003; Motterlini et al., 2005). In recent decades CORM development has expanded introducing a wide range of compounds with modifiable release stoichiometry and kinetics which can be classified by the manner CO release is triggered, including solvent-triggered CORMs, photo-CORMs, enzyme-triggered CORMs and pH-triggered CORMs (Schatzschneider, 2015). In many animal models of inflammation, I/R injury and oxidative stress, parenteral administration (i.p., i.v.) of the CORMs shown in Table I.1, was demonstrated to have beneficial effects similar to CO gas (Motterlini, 2007). One major shortcoming of CORMs is that after the CO release process, their CO-absent analogues are left in situ, and the heavy metal core backbone often

Table I.1 Formula, structure and properties of the most frequently investigated Carbon Monoxide Releasing Molecules (CORMs; adapted from Ryter, 2020)

CORM	Chemical Formula	Properties	Structure
CORM2	Tricarbonyl dichloro Ruthenium (II) dimer. $[Ru(CO_3)Cl_2]_2$	Fast CO release kinetics. Activatable by ligand substitution. Lipid/ DMSO soluble.	
CORM3	Tricarbonylchloro (glycinato) ruthenium (II). $C_5H_4ClNO_5Ru$	Fast CO release kinetics. Activatable by ligand substitution. Water soluble.	
CORM-A1	Sodium boranocarbonate. $CH3BNa_2O_2$	Slow CO release kinetics. Activatable by pH changes. Water soluble.	
CORM-401	Tetracarbonyl[N-(dithiocarboxy-?S,?S′)-N-methylglycine] manganate. $(Mn(CO)_4(S2CNMe(CH_2CO_2H)))$	Releases 3 mol CO per molecule. Activatable by oxidants, Water / DMSO soluble.	

harbors cofactors that may generate uncontrolled reactions with adjacent cells, thereby leading to severe cell damage. For example, it was demonstrated in primary rat cardiomyocytes and two cell lines (HeK 293 and MDCK) that both CORM-2 and its CO-depleted metabolite (i-CORM-2) induced significant cellular toxicity in the form of reduced cell viability, abnormal cell cytology, increased apoptosis and necrosis, cell cycle arrest, and suppressed mitochondrial enzyme activity (Winburn et al., 2012). Interestingly, it is also believed that the transition metal in CORMs appears to be influential in transferring efficiently CO into the cells and limiting the potential toxic effects of free CO gas (Foresti & Motterlini, 2010).

I.3.3.2.4. Oral Carbon Monoxide Release System

In 2014 Steiger et al. (2014) developed the oral CO release system (OCORS), providing precise, controlled, tunable and targeted CO delivery for the treatment of gastrointestinal diseases. OCORS is an oral tablet based on sulfite induced CO release from CORM-2. Mechanistically, OCORS links CO generation and release rates to controlled water/medium influx across the tablet shell (see Figure I.12), thereby locally generating high concentrations

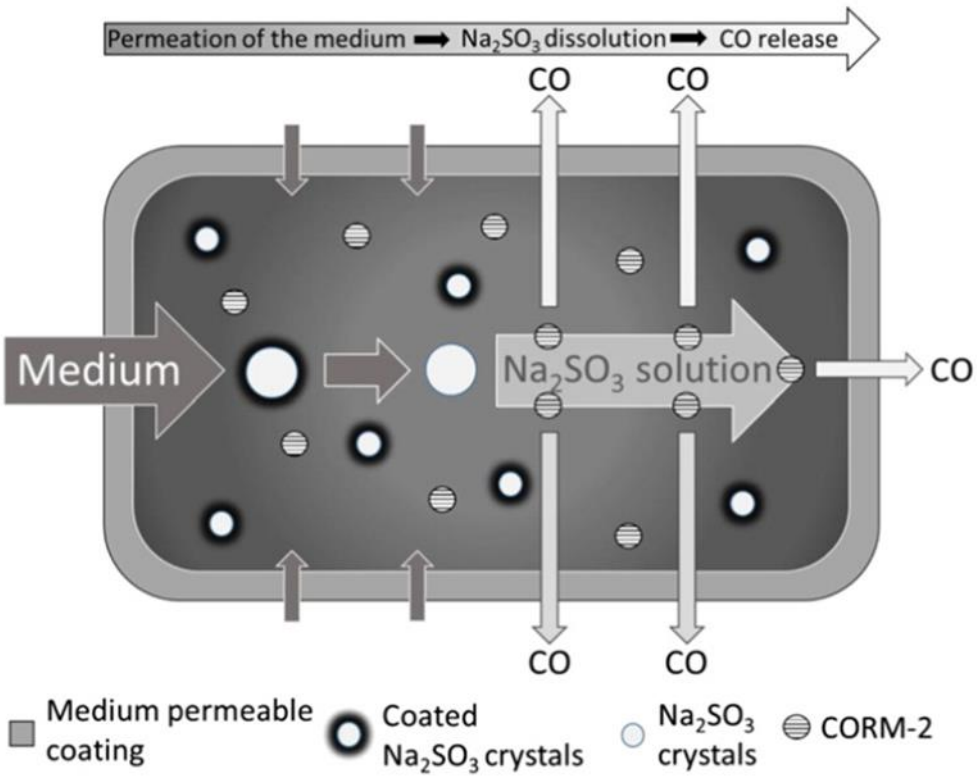


Figure I.12 Schematic representation of the oral carbon monoxide release system (OCORS). Medium permeates the semi-permeable cellulose acetate shell, dissolving the swelling coating layer around the sodium sulfite crystals which readily dissolve. The Na_2SO_3 subsequently interacts with CORM-2, thereby triggering CO release (Steiger et al., 2014).

within the gastrointestinal tract while overall patient exposure is marginal. Adaptation of the porosity and number of coating layers of the semipermeable tablet shell allows for tailored CO release profiles and thus CO delivery to different parts of the gastrointestinal tract (stomach, small or large intestine). Furthermore, the tablet core is blended with citrate buffer making its functionality independent of environmental fluids such as gastric fluids, thereby providing a reliable, easy to use pharmaceutical platform. Recently, it was demonstrated that repetitive intragastric (i.g.) administration of micro-scale OCORS tablets with a 24 h release profile reduced histologic damage and inflammation in a murine model of colitis induced by intrarectal administration of TNBS (Steiger et al., 2016). Although these first results are promising, additional animal studies involving other gastrointestinal disease models are needed to explore the therapeutic effectiveness of OCORS.

I.3.3.3. Influence of carbon monoxide on gastrointestinal inflammation

Experimental administration of CO is beneficial in as good as all conditions with gastrointestinal inflammation, where induction of HO-1 was shown to be efficient (see *I.3.2.4 Influence of HO-1 induction on gastrointestinal inflammation*). This further underlines the important contribution of HO-1-derived CO to the beneficial effects of HO-1 induction. Relevant examples are given in Table I.2.

Table I.2 Relevant examples of studies showing a protective effect of CO in experimental models characterized by intestinal inflammation

Experimental model	Application of CO	Animal	Reference
Intestinal I/R injury	CORM-2	mouse	(Katada et al., 2010)
Intestinal I/R injury	CO inhalation (250 ppm)	rat	(Nakao et al., 2003b)
Necrotizing enterocolitis	CO inhalation (250 ppm)	rat	(Zuckerbraun et al., 2005)
CLP-induced sepsis	CORM-2	mouse	(Liu, Wang, Xu, Qin & Sun, 2019)
CLP-induced sepsis	CO inhalation (250 ppm)	mouse	(Lee et al., 2014)
DSS-induced colitis	CO inhalation (250 ppm)	mouse	(Joe et al., 2014)
DSS-induced colitis	CORM-3	mouse	(Fukuda et al., 2014)
TNBS-induced colitis	CO inhalation (250 ppm)	mouse	(Takagi et al., 2010)
Diabetic gastroparesis	CO inhalation (100 ppm)	mouse	(Joe et al., 2014; Kashyap et al., 2010)

As the pathophysiology of POI is characterized by inflammation of the gastrointestinal tissue, many of the earlier mentioned strategies to deliver CO were already studied for potential therapeutic effect in preclinical POI models (Babu et al., 2015)

I.3.3.3.1. CO gas

Inhaled CO (250 ppm for 1 h before and for 24 h after surgery) in mice improved the IM-induced suppression in spontaneous and betanechol-induced circular smooth muscle contractility and significantly attenuated the impairment in intestinal transit compared to non-treated animals. Although CO is known to relax gastrointestinal smooth muscle in vitro, CO inhalation in control non-operated animals did not significantly influence transit. Surgery increased the mRNA expression of pro-inflammatory IL-1β, IL-6 and COX-2, and of anti-inflammatory IL-10 and HO-1; CO reduced IL-1β expression and further enhanced IL-10 and HO-1. This supports that the beneficial effects of CO are at least partially due to increased HO-1 expression. Interestingly, the increase in muscular leukocyte infiltration by surgery was not suppressed by CO. Furthermore, surgery-induced increases in both iNOS gene expression and NO production were reduced by approximately 75% in CO-treated animals, suggesting that CO could act at the level of gene transcription to modulate iNOS expression (Moore et al., 2003). The same group showed in rats that a shorter exposure time and lower concentrations of CO were equally protective, as inhaling 250 ppm CO for only 3 h before surgery was sufficient to fully reverse surgery-induced retardation in transit, with even 75 ppm being sufficient. This certainly means a more feasible schedule for testing in humans than prolonged inhalation. They also showed that 250 ppm for 3 h before surgery is effective in pigs that received repetitive opioid analgesia after surgery, to mimic the human condition where the gastrointestinal inhibitory effects of opioid analgesics also contribute to ileus. Blood COHb levels reached 6% just before surgery and progressively declined thereafter. Although CO inhalation suppressed the increase in total blood white cell count at 4 h after surgery, it did not suppress muscular leukocyte infiltration at 24 h, again suggesting that CO by inhalation is not able to inhibit additional leukocyte recruitment during the pathogenesis of POI (Moore et al., 2005).

I.3.3.3.2. CO-saturated solutions

Following the report that intestinal graft performance is enhanced by preserving them in organ preservation solutions saturated with CO, Nakao et al. (2006a) found that a single i.p. dose of

CO-saturated Ringer's lactate solution just before closing the abdomen ameliorated POI in mice as it improved gut motility after surgery. In these experiments, 100% CO gas was bubbled into Ringer's lactate solution in a 15-mL plastic tube for 5 min and the peritoneal cavity was injected with 1.5 mL of this solution after induction of ileus. This led to COHb levels of almost 8% at 5 min after administration of CO which decreased below 4% within 30 min. In contrast to CO via inhalation (Moore et al., 2003; Moore et al., 2005), CO by i.p. injection suppressed muscular leukocyte infiltration. The effects of treatment with CO-saturated solution on the surgery-induced mRNA expression of pro- and anti-inflammatory parameters were as reported with inhaled CO, with the exception that CO did not further enhance HO-1 induction. Mechanistically, the study showed that surgery induced activation of the MAPK p38, ERK and JNK, and of the transcription factor NF-κB; all of these effects, except for p38, were suppressed by CO. Furthermore, the involvement of activation of sGC by CO was suggested as the majority of the beneficial effects of CO were diminished by pretreatment with the sGC inhibitor ODQ (Nakao et al., 2006a).

I.3.3.3.3. CORMs

An i.p. injection of the “fast” CO-releaser CORM-3 (40 mg/kg at 3 and 1 h before surgery) and an equimolar dose of the “slow” CO releaser CORM-A1 (15 mg/kg at 4.5 and 1.5 h before surgery) both provided partial protection against retarded transit by surgery in mice; while the inactive compounds i-CORM-3 and i-CORM-A1 were not effective (De Backer et al., 2009). Due to their different kinetics, CORM-3 induced maximal blood COHb levels of 2.3% at 10 min after injection, whereas CORM-A1 reached a maximum COHb level of 8.4% at 20 min after injection. Similar to the data obtained with CO saturated solutions described by Nakao et al. (2006a), i.p. administration of CORM-3 resulted in a significant reduction of leukocyte infiltration and ICAM-1 expression in the muscularis externa at 6 and 24 h after surgery, as well as a suppression of ERK activation. MAPK p38 activation on the other hand was increased upon CORM-3 administration and was shown to play a role in the progressive increase in protein expression of HO-1 and total HO activity at 1 and 6 h after surgery, as simultaneous

administration of a p38 MAPK inhibitor prevented this CORM-3 induced effect. Finally, the study showed that CORM-3 suppresses the progressive increase in muscular oxidative stress levels induced by surgery, illustrating that also anti-oxidative effects of CO might contribute to its beneficial effect in POI. De Backer et al. (2009) also demonstrated that molecular changes in the intestinal mucosa play an important role in the pathogenesis of POI. Surgery did not induce pronounced expression of pro- or anti-inflammatory cytokines in the mucosa, but an early and transient increase of oxidative stress was measured in the mucosa at 1 h after surgery. CORM-3 was able to significantly reduce this early mucosal oxidative burst in a fully HO-1 dependent manner, while the anti-inflammatory/anti-oxidative effects of CORM-3 in the muscularis were only partially HO-1 dependent.

Thus, CO administered via inhalation or directly into the abdominal cavity, and i.p. administered CORMs all reduce POI. OCORs is an orally applicable formulation developed particularly to treat gastrointestinal disease and was shown to reduce TNBS-induced colitis (Steiger et al., 2016). The effect of OCORS on POI has not yet been investigated.

I.4. Hydrogen sulfide

I.4.1. Hydrogen sulfide producing enzymes

The pathways for endogenous production of hydrogen sulfide (H_2S) are outlined in Figure I.13. Endogenous production of H_2S is a result of direct enzymatic desulfhydration of cysteine, catalyzed by cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS), and indirect desulfhydration catalyzed by 3-mercapto-sulfurtransferase (3-MST) in the presence of reductants. H_2S can also accumulate in the lumen of the gastrointestinal tract via production from bacteria of the microbiome, including members of the group of Enteroccocci and Enterobacteria, which degrade the sulfur-containing amino acids cysteine and methionine (via conversion to homocysteine) through the expression of desulfhydrases. Other bacteria, such as δ-Proteobacteria genera, utilize sulfate as a terminal electron acceptor in the production of ATP to produce H_2S. In addition, there is a small contribution of assimilatory sulfite-reducing

bacteria to the production of luminal H_2S via the expression of the iron flavoprotein sulfite reductase (Linden, 2013).

I.4.1.1. CSE

CSE catalyzes the pyridoxyl 5'-phosphate (PLP) dependent reaction of cysteine and homocysteine to pyruvate and α-ketoglutarate, resulting in the release of ammonia (NH_3) and H_2S. It is located solely in the cytoplasm of cells and is mostly present in the cardiovascular system, making it a possible drug target to treat cardiovascular diseases. In such manner, cardiac-specific overexpressing of CSE significantly limits the extent of myocardial I/R injury in transgenic mice (Elrod et al., 2007). Besides its activity in the cardiovascular system, CSE was also shown to contribute to cellular signaling and homeostasis in other organs. For example, Bos et al. (2013) showed that CSE knockout mice had markedly reduced renal production of H_2S, which was associated with increased damage and mortality after renal I/R injury.

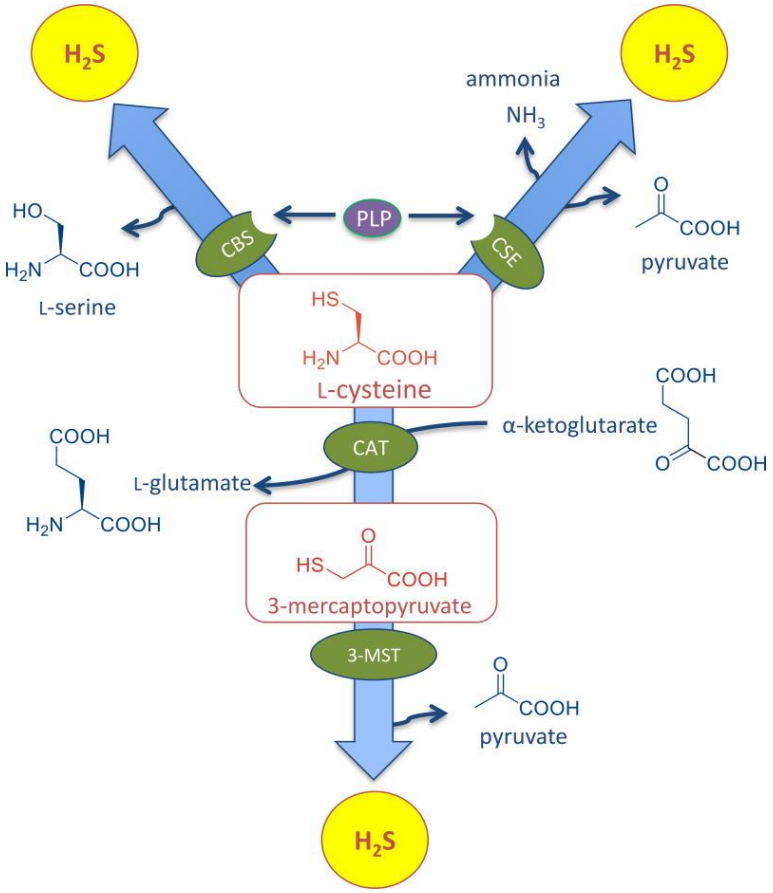


Figure I.13 Schematic overview of endogenous H_2S production in mammalian cells (Powell, Dillon & Matson, 2018). CBS: cystathionine-β-synthase, PLP: pyridoxyl 5'-phosphate, CSE: cystathionine-γ-lyase, CAT: cytoplasmic aspartate aminotransferase, 3-MST: 3-mercapto-sulfurtransferase.

I.4.1.2. CBS

The normal function of CBS is to catalyze the condensation of serine with homocysteine in a PLP dependent manner, resulting in the formation of cystathionine and water; a key reaction in the transsulfuration. In the presence of L-cysteine, CBS is able to perform four alternate reactions (including β-replacement, β-elimination and α,β-elimination) which are all capable of producing H_2S (Chen et al., 2004). CBS is located in the cytosol and highly expressed in the brain, where H_2S has very complex roles acting as a neuromodulator (e.g. enhancing NMDA-mediated responses and facilitating long-term potentiation) (Abe & Kimura, 1996). The physiological importance of CBS is illustrated by CBS-deficient patients who suffer from various pathologies including mental retardation, thrombosis and osteoporosis (Kruger, 2017).

I.4.1.3. 3-MST

PLP-independent 3-MST is the least studied out of the three H_2S-generating enzymes. It is predominantly located in mitochondria and it catalyzes the conversion of cysteine to pyruvate with the assistance of cytoplasmic aspartate aminotransferase (CAT) in a two-step reaction (Shibuya et al., 2009). In addition to producing H_2S, 3-MST catalyzes the formation of various sulfur oxides in perthiol redox cycles. In rats 3-MST is distributed in all tissues, but it is mostly found in the kidney, followed by the liver and heart. Furthermore, mutations in 3-MST causing reduced levels of H_2S and sulfur oxides in the brain correlate with behavioral abnormalities and increased anxiety (Nagahara et al., 2013; Nagahara, 2018).

I.4.2. Tissue protective effects of H_2S

H_2S, primarily known as a toxic environmental gas, belongs just like CO and NO to the family of labile mediators called gasotransmitters. Gasotransmitters are small gas molecules which typically have high lipid solubility allowing penetration through cell membranes without requiring a specific transporter or receptor. Gasotransmitters are generated endogenously by specific enzymes and can generate various functions at physiologically relevant concentrations by targeting specific cellular and molecular targets (Yang et al., 2016). It is present in various tissues, including the cardiovascular, nervous and digestive system, and its concentration in

blood ranges from 30 to 300 µmol/L (Olson, 2009). H_2S plays important roles in both physiological and pathophysiological conditions. It functions as a neuromodulator in the central nervous system, but also serves as a neuroprotectant against oxidative stress (Kimura et al., 2006). In the cardiovascular system, H_2S relaxes vascular smooth muscle by activation of ATP-dependent K^+-channels (K_{ATP} channels), lowering blood pressure (Yang et al., 2008; Sun et al., 2015) and inhibits smooth muscle cell proliferation via MAPK (Du et al., 2004). In the digestive system, it is suggested that high levels of endogenous H_2S have a reducing effect on insulin secretion via activation of K_{ATP} channels in pancreatic β-cells (Yang et al., 2005) and that it inhibits pacemaker activity in interstitial cells of Cajal (Parajuli et al., 2010). In the interest of the current thesis, we will focus on the possible anti-inflammatory, anti-oxidant and gastroprotective effects of H_2S.

I.4.2.1. Anti-inflammatory effect

There has been much controversy whether H_2S is an anti- or in contrast a pro-inflammatory agent. A lot of this controversy can be ascribed to studies with the H_2S-donor sodium hydrosulfide (NaHS). For example, the study of Zang et al. (2006), who used a mouse model of CLP-induced sepsis, implicates H_2S as a pro-inflammatory mediator. Mice were treated i.p. with either propargylglycine (PAG; 50 mg/kg), a CSE inhibitor, or NaHS (10 mg/kg). Upon induction of sepsis, mRNA production of CSE in the liver was significantly upregulated. Inflammation was significantly downregulated after treatment with PAG, as evidenced by reduced MPO activity and histological changes in lung and liver. Injection of NaHS significantly aggravated sepsis-associated systemic inflammation. Similar pro-inflammatory effects of H_2S were observed by Li et al. (2005) in endotoxin-induced inflammation in mice. However, the majority of studies now point to an anti-inflammatory effect of H_2S; for example the more recent study of Ahmad et al. (2016) which demonstrates the anti-inflammatory effect of H_2S in CLP-induced sepsis in rats. Following NaHS administration (3 mg/kg i.p.), MPO levels in heart and lung and plasma levels of IL-1β, IL-6 and TNF-α were reduced. Similarly, administration of NaHS caused a significant reduction in gastric mucosal lesions and of pro-inflammatory

cytokines in a rat model of gastric I/R injury (Mard et al., 2012). This difference in pro- and anti-inflammatory effects of exogenous H_2S is most notably dose-dependent as low doses of NaHS are more likely to mimic the endogenous H_2S level produced by the controlled enzymatic H_2S synthesis. Moreover, sulfide salts, like NaHS, dissolved in aqueous solutions, will release large amounts of H_2S within seconds which may result in toxic effects (Li et al., 2008; Rose et al., 2015).

The ability of H_2S to reduce inflammation has been further demonstrated in a variety of animal models including kaolin/carrageenan-induced monoarthritis in rats (Andruski et al., 2008), tobacco-smoke induced lung inflammation in mice (Han et al., 2011) and synovitis in rats (Ekundi-Valentim et al., 2010). Mechanistically, H_2S was shown to inhibit leukocyte adherence to the vascular endothelium and reduce migration of leukocytes into the subendothelial space, resulting in reduced edema formation in a rat model of carrageenan-induced hind paw edema (see Figure I.14). This effect is mediated via activation of K_{ATP} channels on both leukocytes and endothelial cells, resulting in downregulation of CD11/CD18 integrins on leukocytes and P-selectin and ICAM-1 on endothelial cells (Zanardo et al., 2006).

Another major anti-inflammatory effect of H_2S involves its ability to suppress the activation of NF-kB, resulting in a reduced production of proinflammatory cytokines such as IL-6, IL-1β and TNF-α and chemokines such as MCP-1 (Gao et al., 2012; Chen & Liu, 2016; Wu et al., 2017). Also the MAPK intracellular signaling pathway, which plays a crucial role in inflammatory reactions and regulation of inflammatory and cell adhesion factors, is affected as *in vitro* studies in microglia (Hu et al., 2007) and cardiac cells (Xu et al., 2013; Wu et al., 2015) have shown that exogenous H_2S results in inhibition of p38, EKR1/2 and/or JNK. Promotion of tissue repair by H_2S is mediated via its vasodilatory actions, facilitated by its inhibiting effect on phosphodiesterases resulting in elevated tissue levels of cGMP (Bucci et al., 2010), and enhancement of angiogenesis (Katsouda et al., 2016).

I.4.2.2. Anti-oxidant effect

H_2S has anti-oxidant properties via direct scavenging of ROS (see Figure I.14). *In vitro* experiments in myocytes revealed that NaHS exerts antagonizing effects on the increased level of malondialdehyde induced by oxygen-free radicals and is able to directly scavenge O_2^- and hydrogen peroxide (H_2O_2), explaining the reducing effect of NaHS on lipid peroxidation observed in a rat model of myocardial I/R injury (Geng et al., 2004). Similarly, tyrosine nitration and cell toxicity induced by peroxynitrite ($ONOO^-$) can be significantly inhibited by NaHS pretreatment in human neuroblastoma cell line SH-SY5Y (Whiteman et al., 2004). H_2S can also exert anti-oxidant effects indirectly via upregulation of the antioxidant defense system. Research using a mouse model of myocardial I/R injury, has indicated that i.v. administration of sodium sulfide (Na_2S) is able to increase HO-1 and thioredoxin-1 production through a Nrf2-dependent signaling pathway (Calvert et al., 2009).The underlying mechanism for the latter effect may be the sulfhydration of the Keap1 protein which tonically suppresses the Nrf2 activity (Yang et al., 2012). Furthermore, mitochondria can also utilize H_2S as an electron donor in

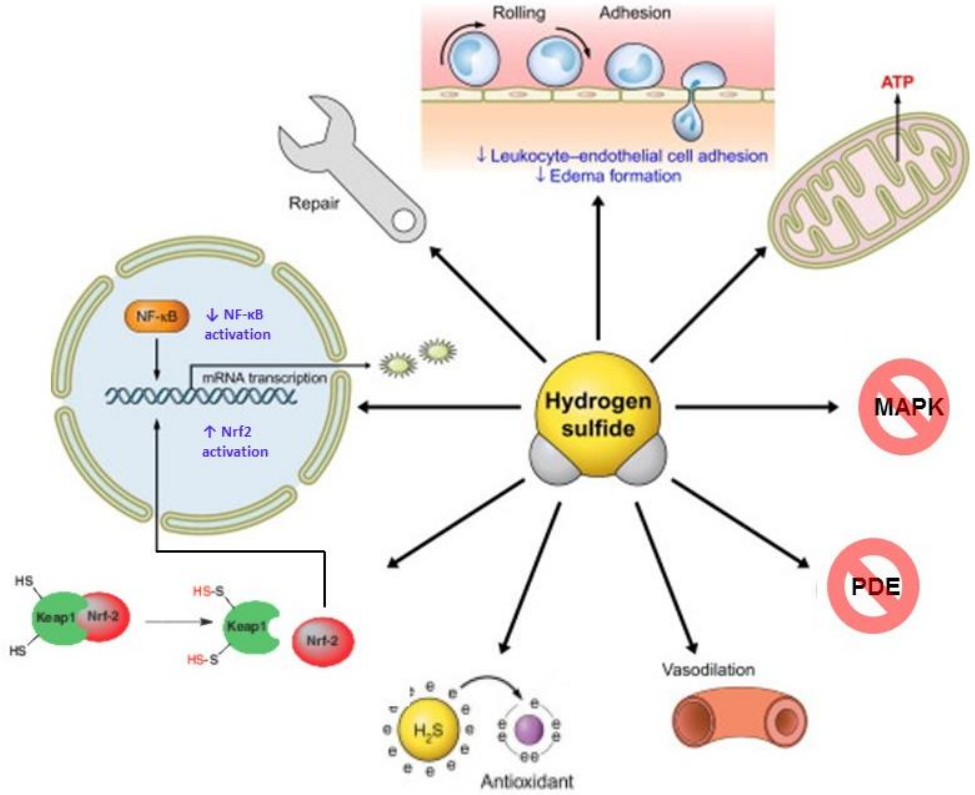


Figure I.14 The anti-inflammatory and anti-oxidant effects of H_2S (Adapted from Chan & Wallace, 2013).

ATP production, particularly during anoxia/hypoxia, and in doing so reduce generation of tissue-damaging oxygen-derived free radicals (Fu et al., 2012; Chan & Wallace, 2013) .

I.4.2.3. Gastroprotective effect

It is well known that stress, alcohol and NSAIDs can cause mucosal damage leading to gastric and duodenal ulceration. Evidence suggests that H_2S can protect the gastric mucosa, most likely through mechanisms that involve anti-oxidant and anti-inflammatory actions and preservation of the gastric microcirculation (Shen et al., 2019). For example, it was shown that exogenous H_2S exerts protective effects against cold restraint stress-induced gastric mucosal injury in rats as pre-treatment with NaHS significantly attenuated ulcer index, decreased ROS production and reduced the serum level of TNF-α (Aboubakr et al., 2013). Similarly, Fiorucci et al. (2005) demonstrated in a rodent model of gastropathy that pre-treatment with NaHS reduced gastric mucosal injury and reduced the mRNA upregulation of TNF-α and ICAM-1 following administration of NSAID or acetyl salicylic acid. Moreover, NaHS administration increased gastric mucosal blood flow by ± 20%; this effect was reversed by glibenclamide suggesting that K_{ATP} channels are the targets of this effect. An important finding of this study is the demonstration that exposure of gastric mucosa to NSAIDs also decreases the endogenous H_2S production by modulating CSE expression, which contributes to the gastric injury caused by NSAIDs. Furthermore, H_2S stimulates the secretion of bicarbonate in the stomach (Takeuchi et al., 2015) and duodenum (Takeuchi et al., 2012) and inhibits gastric secretion (Mard et al., 2014), thereby reducing the potential damaging effects of gastric acid.

I.4.3. Strategies to deliver H_2S

The anti-oxidant and anti-inflammatory effects of H_2S, make it a potential agent for therapeutic applications in for example ischemic diseases. Many different H_2S donor drugs have been created, aiming to mimic H_2S production under physiologic or diseased conditions. At present it is difficult to define a universal best donor for all pathological applications as many donors face problems such as an uncontrolled pattern of release and unknown by-product profiles. An ideal H_2S donor for clinical application will have the ability to be easily measured, to work locally

without systemic effects, to show controlled and sustained release, and to have a minimal toxic side effect profile (Jensen et al., 2017). In the next section we will describe some of the most common available H_2S donors.

I.4.3.1. H_2S-donors

I.4.3.1.1. Sulfide salts

The inorganic salts, NaHS and Na_2S, are classic H_2S donors and were the first donors to be studied in the cardiac system (Elrod et al., 2007). They have the advantage that they do not produce any by-products. However, the major problem is that release is very rapid, leading to an uncontrollable boost in H_2S concentration and a lack of sustained effect. Both inorganic substances release H_2S within seconds and completely as soon as they are dissolved in aqueous buffers. Since endogenous production of H_2S through CSE and CBS is relatively slow and sustained, it is highly unlikely that cells or tissues are ever exposed to such high H_2S concentrations caused by the rapid H_2S release from NaHS and Na_2S (Whiteman et al., 2010). Furthermore, because H_2S is a gaseous molecule, H_2S concentration can diminish quickly in an open stock solution due to volatilization (DeLeon et al., 2012). These properties make sulfide salts far from perfect H_2S donors and may be a partial explanation for the emergence of divergent results, even when the same function, e.g. their effect on inflammation, is investigated (as discussed in section *I.4.2.1 Anti-inflammatory effect*).

I.4.3.1.2. Precursors for endogenous H_2S synthesis

N-acetylcysteine and L-cysteine are precursors for endogenous H_2S synthesis. As cysteine availability is the rate limiting step in endogenous H_2S synthesis, administration of these precursors might be expected to increase endogenous H_2S production via CSE and CBS (DiNicolantonio et al., 2017; Ezeriņa et al., 2018). In such manner, Zanardo et al. (2006) showed that i.p. administration of N-acetylcysteine dose dependently reduced the number of leukocytes infiltrating into the air pouch in response to carrageenan in rats. Pre-treatment with the CSE inhibitor β-cyanoalanine completely reversed this effect, indicating that the protective effects of N-acetylcysteine are mediated by H_2S. Besides their ability to increase H_2S levels,

N-acetylcysteine and L-cysteine are also important precursors for the production of GSH, which is known for its anti-oxidant properties. Therefore, many studies ascribe the protective effects of N-acetylcysteine and L-cysteine to their ability to increase the levels of GSH. For example, administration of N-acetylcysteine was shown to restore the depletion of GSH tissue levels observed in experimental models of radiotherapy-induced small intestine injury (Mercantepe et al., 2019) and renal I/R injury (Nitescu et al., 2006), resulting in reduction of oxidative stress. Similarly, Giustarini et al. (2018) have shown that N-acetylcysteine treated rats were protected against paracetamol overdose via restoration of GSH tissue levels. However, no increase in hepatic, renal or cardiac tissue levels of protein thiols was observed after treatment with N-acetylcysteine; also administration of N-acetylcysteine to healthy animals did not result in an increase in H_2S plasma levels. These results may question the effectivity of precursors like N-acetylcysteine as H_2S-donors. Moreover, convincing evidence of the benefit of N-acetylcysteine is lacking as mainly small clinical trials with variable doses have yielded inconsistent results in a wide variety of diseases, including chronic obstructive pulmonary disease and ischemic liver injury (Aitio, 2006).

I.4.3.1.3. Synthetic H_2S donors

Currently there are many organic, synthetic substances under investigation for their H_2S releasing properties. For example, Lawesson's reagent [2,4-Bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane-2,4-disulfide], which is a widely used sulfurization reagent in organic synthesis, releases H_2S in an uncontrollable manner upon hydrolysis. In addition, the poor solubility of Lawesson's reagent in aqueous solutions limits its applications. To overcome these shortcomings, more recent compounds have been designed with the specific intent to deliver H_2S gas to tissues in more controlled manner, aiming at a means to manipulate endogenous H_2S levels over a longer time course and within the naturally achieved concentration range. Of particular interest are molecules that release H_2S in aqueous environment. GYY4137 [morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate], is a water soluble derivative of Lawesson's reagent, and was the first organic small molecule, to be reported and

characterized as a slow releasing H_2S donor (Zhao et al. 2014; Powell et al., 2018). The in vitro H_2S release from GYY4137 was confirmed by spectrophotometric and amperometry assays, showing a sustained release of H_2S peaking at 6 to 10 minutes, whereas NaHS showed a peak signal within 5 to 8 seconds. This difference in release kinetics between GYY4137 and the classic H_2S donor NaHS was further illustrated by Lee et al. (2011), as they investigated the H_2S release in the medium of cultured MCF-7 cells after adding NaHS or GYY4137. NaHS rapidly caused a peak after administration whereas GYY4137 caused a lower, but more sustained increase in H_2S concentration, better mimicking the time course of naturally produced H_2S. These data were confirmed in an in vivo study as i.v. or i.p. administration of GYY4137 to anesthetized rats led to increase in plasma H_2S concentration at 30 min which remained elevated over a 180-min time course; NaHS on the other hand did not elevate H_2S plasma levels at these time points (Li et al., 2008).

I.4.3.2. H_2S-releasing non-steroidal anti-inflammatory drugs

NSAIDs, such as aspirin and diclofenac, are among the most commonly used anti-inflammatory drugs, but have significant side effects including gastrointestinal ulceration and bleeding. The key mechanism underlying the ability of NSAIDs to cause ulceration is inhibition of the COX enzymes and their production of gastroprotective prostaglandins, causing damage to the gastrointestinal mucosa via several mechanisms including reduced bicarbonate and mucus secretion and increased neutrophil-endothelium adherence (Wallace, 2000; Wallace, 2008). Therefore, H_2S-NSAID hybrids (see Figure I.15) have been developed expecting that the suppression of leukocyte adherence and inflammation by the released H2S might counteract the NSAID-induced ulceration. For example, an equimolar dose of ATB-337 (an H_2S-releasing derivative of diclofenac) did not induce the formation of hemorrhagic erosions in the stomach, did not stimulate leukocyte adherence and did not elevate gastric mRNA-expression of TNF-α or ICAM-1 in rats, as seen with diclofenac; however, ATB-337 suppressed COX-1 and COX-2 activity to a similar extent as diclofenac in a rat air pouch model illustrating the maintained inhibitory effect on COX enzymes (Wallace et al., 2007). Moreover, released

H_2S will contribute to the overall anti-inflammatory effect; ATB-337 was shown to exert better anti-inflammatory effects over diclofenac in LPS-induced endotoxic shock in rats as the increase in plasma levels of IL-1β and TNF-α was significantly lower in animals treated with ATB-337 compared to those treated with diclofenac (Li et al., 2007). Similar gastroprotective and anti-inflammatory effects were obtained with other H_2S-releasing derivatives such as ATB-429 (mesalamine derivative) (Fiorucci et al., 2007), ATB-352 (ketoprofen derivative) (Gugliandolo et al., 2018; Costa et al., 2020) and ATB-346 (naproxen derivative) (Wallace et al. 2010; Dief et al., 2015; Magierowski et al., 2017).

The safety of ATB-346 was already shown in a phase 1 study in healthy subjects and a phase 2 clinical trial demonstrated pain relief with ATB-346 in patients with osteoarthritis (Wallace et al., 2018). A recent phase 2 clinical study also investigated the gastrointestinal safety of a 14-day dosing regimen of ATB-346 in comparison to naproxen. Patients treated with a daily single dose of 250 mg ATB-346 showed a dramatic reduction in ulcer incidence compared to patients treated with sodium naproxen twice a day (550 mg/dose), while the two drugs produced comparable suppression of COX activity. In addition, subjects treated with ATB-346 exhibited a significantly lower incidence of several gastrointestinal symptoms associated with the use of

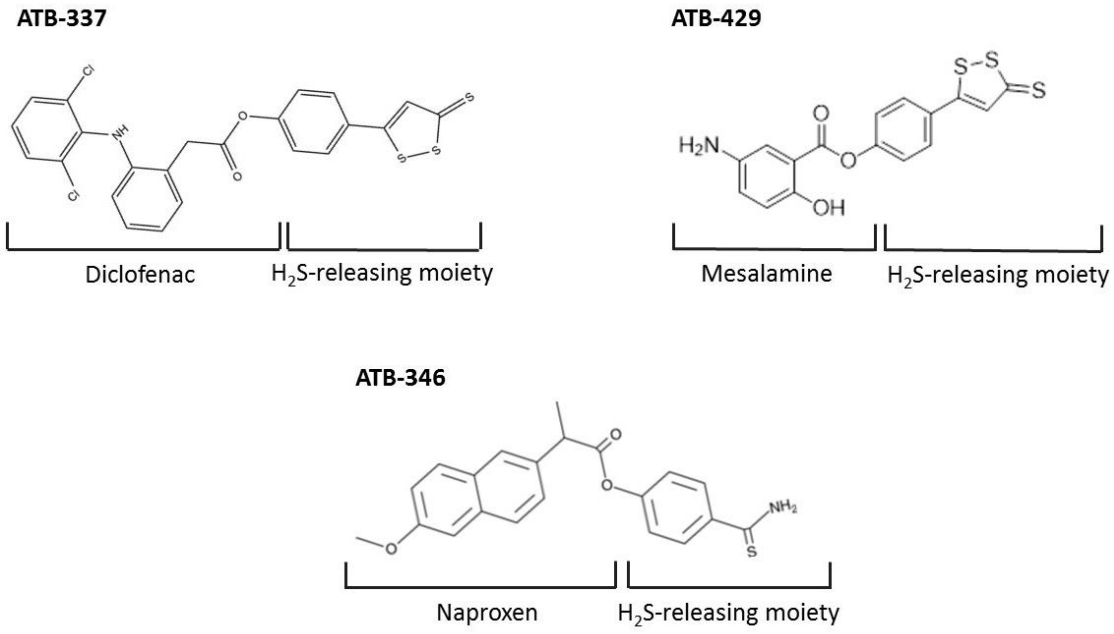


Figure I.15 Examples of H_2S-releasing NSAIDs: ATB-337, ATB-429 and ATB-346.

conventional NSAIDs such as abdominal pain, gastro-oesophageal reflux and nausea, all symptoms often leading to patient non-compliance. Furthermore, a significant increase in plasma H_2S levels of ~ 50% was measured in the patient group treated with ATB-346, confirming that the drug produced a biologically relevant elevation of this mediator in humans, which could contribute to both its analgesic and cytoprotective effects (Wallace et al., 2020). These results suggest that H_2S-releasing NSAIDs have considerable promise to replace convential NSAIDs in the future.

I.4.4. Influence of H_2S-releasing strategies on gastrointestinal inflammation

As mentioned earlier, evidence demonstrates that H_2S exerts anti-inflammatory effects at physiological concentrations. Administration of exogenous H_2S has been proposed as a therapeutic means for many gastrointestinal inflammatory conditions and has been shown to be effective in disease models with acute gastrointestinal inflammation:

I.4.4.1. Intestinal ischemia/reperfusion injury

Studies have shown that H_2S has an anti-ischemic activity in intestinal I/R injury (Kong et al., 1998; Wu et al., 2015). In a rat model of intestinal I/R injury, NaHS significantly reduced the severity of histological damage and the levels of malondialdehyde activity in serum and intestine, which correlated with increased activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase (Liu et al., 2009). Other evidence suggests that vasodilatation via endothelial NO plays a crucial role, as certain beneficial effects of NaHS, such as improved mesenteric perfusion and reduced histological injury, were abolished in endothelial NOS (eNOS) KO mice (Jensen et al., 2017). Similar results were obtained using i.p. administration of GYY4137, including the involvement of superoxide dismutase and eNOS dependent pathways as mediators for its beneficial effects (Drucker et al., 2019; Cui et al., 2020). In addition, a study in mice revealed that the i.p. application of GYY4137 was superior to i.v. administration of GYY4137 and inhalation of H_2S gas, resulting in a better restoration of mesenteric perfusion, better preservation of the histological structure of the ileum and a significant reduction in IL-6 tissue levels. This is most likely explained by the compromised

blood flow to the intestines as they are ischemic or recovering from acute ischemia; although it is also likely that there is a therapeutic benefit of direct application of the drug to the bowel wall with i.p. application. Pre-ischemic i.p. application of GYY4137 was found to be slightly more beneficial than post-ischemic application (Jensen et al., 2018).

I.4.4.2. Necrotizing enterocolitis

Drucker et al. (2018) have shown that i.p. injections of GYY4137, Na_2S or AP39 (the latter being a mitochondria-targeted H_2S donor), protect against development of experimental NEC in mouse pups. Pups treated with these H_2S donors experienced only marginal weight gain, but improved intestinal perfusion and reduced histological injury. Only GYY4137 was able to significantly reduce the increase in IL-6 and IL-1β levels in liver and lung caused by the systemic inflammation following NEC. These results were confirmed in a more recent study by the same authors in which they also investigated the involvement of eNOS in the protective effects of GYY4137 in NEC. Similar to the I/R injury model, the improved intestinal perfusion and reduced histological injury was abolished in eNOS KO mice, indicating eNOS as a major mediator of the protective effects of H_2S in NEC through vasodilation by NO (Drucker et al., 2019).

I.4.4.3. Intestine as triggering organ in sepsis

Initial studies using NaHS (10 mg/kg ~ 178 µmol/kg i.p.) as H_2S donor illustrated a pro-inflammatory effect of H_2S in CLP-induced sepsis, with increased cytokine and chemokine tissue levels in lung and liver via upregulation of NF-κB (Zhang et al., 2006; Ang et al., 2011). However, more recent studies using lower doses of NaHS (in the range of 10 – 100 µmol/kg i.p.), show the opposite with reduced systemic inflammation, reduced myocardial, intestinal, lung and renal injury and improved survival rate (Ahmad et al., 2016; Li et al., 2017; Zhang et al., 2017; Liu et al., 2019). For example, following NaHS administration in a dose of 3 mg/kg (~ 53 µmol/kg i.p.), Ahmad et al. (2016) observed reduced MPO levels in heart and lung and reduced plasma levels of IL-1β, IL-6 and TNF-α; blood flow was increased in heart, liver and kidney. The underlying mechanism of action involves NF-κB as both Li et al. (2017) and Liu et

al. (2019) have shown a reduction in the expression of NF-κB p65 in the myocardial tissue of septic rats following treatment with NaHS. Liu et al. (2019) also suggest that H_2S attenuates sepsis via a PI3K/Akt-dependent mechanism as simultaneous administration of the PI3K inhibitor LY294002 almost completely abolished the anti-inflammatory effects, as well as the activation of Akt seen in rats treated with NaHS.

Despite this growing evidence for the beneficial effects of H_2S in gastrointestinal conditions characterized by acute inflammation, the potential anti-inflammatory effect of H_2S donors has not yet been studied in animal models of POI.

I.4.4.4. Chronic gastrointestinal conditions

Treatment with H_2S donors was also proposed for chronic gastrointestinal inflammatory conditions such as IBD. For example, i.p. injection of NaHS (14 μmol/kg) twice a day ameliorates the negative effects, such as diarrhea and rectal bleeding, of DSS exposure in mice. This was accompanied by a significant alleviation of the DSS-induced increase in colonic tissue levels of IL-6, IL-1β, IL-17 and TNA-α, mediated via inhibition of the NF-κB signaling pathway (Chen & Liu, 2016). Similar results on DSS-induced inflammation and injury in mice were obtained with i.v. injection of GYY4137 by Zhao et al. (2016), who also illustrated an improvement in intestinal barrier integrity with GYY4137 correlating with a less pronounced increase in LPS serum level induced by DSS exposure. In TNBS-induced colitis in rats, per os administration of Lawesson's reagent twice a day significantly decreased the extent of colonic lesions and inflammation as assessed via the degree of intestinal MPO activity and TNF-α level. Mechanistically, HO-1 was believed to be involved as HO-1 expression was upregulated upon treatment with Lawesson's reagent and co-administration of the HO-1 inhibitor tin protoporphyrin IX resulted in reduction of its protective activity on colitis (Kupai et al., 2018). Of particular interest is a study of Fiorucci et al. (2007) in which they demonstrated in murine TNBS-induced colitis that the H_2S-releasing NSAID ATB-429 was considerably more effective than the parent drug mesalamine, which is currently used in the clinic to treat mild-to-moderate IBD. In addition to reducing mucosal injury and disease activity (body weight loss, fecal blood,

diarrhea), ATB-429 markedly reduced the colonic MPO activity and expression of mRNA for several important pro-inflammatory cytokines. Importantly, these effects of ATB-429 were obtained with a dose that, on a molar basis, was only half the dose of mesalamine. These observations clearly show a contributing protective effect of H_2S derived from ATB-429.

Finally, there has also been suggested that H_2S might play some role in diabetic gastroparesis, Mard et al. (2016) showed that the increase in mRNA and protein expression of CSE in response to gastric distention is significantly less pronounced in diabetic rats compared to normal rats. I.p. injection of NaHS or L-cysteine accelerated the gastric emptying rate in diabetic rats up to 80% of the rate in normal control animals, suggesting potential for treatment of diabetic gastroparesis.

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CHAPTER II

AIMS

CHAPTER II

Aims

POI refers to the transient impairment of gastrointestinal motility commonly seen after abdominal surgery. When prolonged, POI leads to symptoms as abdominal distension, nausea and vomiting and the inability to tolerate an oral diet; this is a frequent reason for prolonged hospital stay and has an important socio-economic impact. The pathophysiology of POI is characterized by an acute neurogenic phase with activation of inhibitory motor pathways during surgical handling of the intestines, followed by a prolonged inflammatory phase. Activated resident macrophages in the muscular layer of the intestine release inflammatory cytokines such as IL-6 and chemokines, resulting in increased expression of adhesion molecules and recruitment of circulatory leukocytes. These leukocytes produce prostaglandins and NO, inhibiting the neuromuscular contractile apparatus (Boeckxstaens & de Jonge, 2009). Laparoscopic procedures, enhanced recovery pathways, and pharmacological treatment options such as prokinetics and NSAIDs are advocated to prevent prolonged POI but no full protection is obtained (Bragg et al., 2015). Therefore, multiple strategies for treatment of POI are currently under investigation, focusing on reduction of the inflammatory cascade; most of the studies are performed in mice as the experimental model of POI is well established within this species.

HO is the rate-limiting enzyme in the degradation of heme into Fe^{2+}, CO and biliverdin, which is subsequently converted into bilirubin. Two major isoforms have been identified of which HO-1 is upregulated by a wide variety of stimuli including heme but also non-heme stimuli such as ROS and pro-inflammatory cytokines to contribute to anti-oxidant and anti-inflammatory protection; HO-2 is constitutively active and regulates normal physiological functions (Maines, 1997). The protective effects of HO-1 induction have been mainly attributed to the beneficial effects of CO, raising the interest in CO as a potential therapeutic agent. In such manner, CO was shown to exert a protective anti-inflammatory effect in experimental models of POI upon

inhalation of CO (Moore et al., 2005) and upon i.p. administration of CO-satured solutions (Nakao et al., 2006) or CORMs (De Backer et al., 2009). A matter of concern with general administration of CO remains the formation of COHb in the systemic circulation. Steiger et al. (2014) therefore developed an oral tablet containing CORM-2, named OCORS, which allows a tailored CO release rate via different coatings covering key elements of OCORS; this facilitates the release of CO to specific parts of the gastrointestinal tract. OCORs is primarily developed for treatment of gastrointestinal inflammatory conditions. The first aim was therefore to evaluate the possible effect of OCORS in the murine model of POI. These results are described in **Chapter III**.

The data obtained in chapter 3 showed no beneficial effect of OCORS in our experimental model of POI. Besides administration of CO, pharmacologic induction of HO-1 may be an effective strategy to modulate inflammation as the other byproducts of heme degradation, being biliverdin/bilirubin, also exert anti-oxidant effects (Jansen & Daiber, 2012). Moreover, it was previously shown in our laboratory that administration of CO via i.p. injection of CORM-3 in mice leads to induction of HO-1 (De Backer et al., 2009), illustrating that CO itself provides a positive feedback loop allowing the ROS scavenging properties of biliverdin/bilirubin to come into play. A large number of compounds have been demonstrated to induce HO-1 (Foresti et al., 2013); hemin is the most commonly used in experimental studies and was already shown to exert anti-inflammatory effects via HO-1 induction in various gastrointestinal disease models such as I/R injury (Attuwaybi et al., 2004). In vitro, DMF has been found to be a very effective HO-1 inducer. In 2013, DMF has been approved for treatment of MS upon completion of phase III clinical trials implying it is safe to use in humans. Experimental and human data suggest that an important mechanism of action of DMF is the stabilization of Nrf2, leading to transcription of antioxidant genes such as HO-1 (Bomprezzi, 2015). Our second aim was therefore to investigate the protective effect of hemin and DMF in POI, with particular attention for the role of induction of HO-1 (**Chapter IV**).

The use of NSAIDs is already advocated as part of multimodal postoperative analgesic strategy to reduce opioid consumption upon POI. However, NSAIDs are a major cause of gastric and duodenal ulceration due to the inhibition of the COX enzymes, with concomitant reduction in gastroprotective prostaglandins (Wallace, 2000). Therefore, the use of H_2S–releasing NSAIDs such as ATB-346, a naproxen derivative, is of interest as the simultaneous release of H_2S will counteract the NSAID-induced ulceration and will contribute to the overall anti-inflammatory effect. Moreover, the safety and pain relieving effect of ATB-346 was already shown in phase 1 and 2 clinical trials (Wallace et al., 2018). H_2S is produced endogenously via direct and indirect enzymatic desulfhydration of cysteine by CSE, CBS and 3-MST. H_2S acts as an important gasotransmitter in various tissues, including the cardiovascular, nervous and digestive system. Exogenous H_2S was shown to exert both anti- and pro-inflammatory effects depending on the dose; low doses that are more likely to mimic the physiological H_2S concentrations, result in an anti-inflammatory effect. Multiple synthetic substances such as GYY4137 have therefore been designed to release H_2S in a more controlled and sustained manner compared to the classic H_2S donors like sulfide salts (Rose et al., 2015; Powell et al., 2018). Our third aim was therefore to investigate the protective effect of the H_2S donor GYY4137 and the H_2S-releasing naproxen derivative ATB-346 in POI (**Chapter V**).

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CHAPTER III

INVESTIGATION OF ORALLY DELIVERED CARBON MONOXIDE FOR POSTOPERATIVE ILEUS

Jonas Van Dingenen[a], Christoph Steiger[b,c], Markus Zehe[b], Lorenz Meinel[b], Romain A. Lefebvre[a]

[a] Heymans Institute of Pharmacology, Ghent University, Corneel Heymanslaan 10 - Blok B, B-9000 Gent, Belgium

[b] Institute for Pharmacy and Food Chemistry, University of Wuerzburg, Am Hubland, DE-97074 Wuerzburg, Germany

[c] Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, US-02140 Cambridge MA, USA

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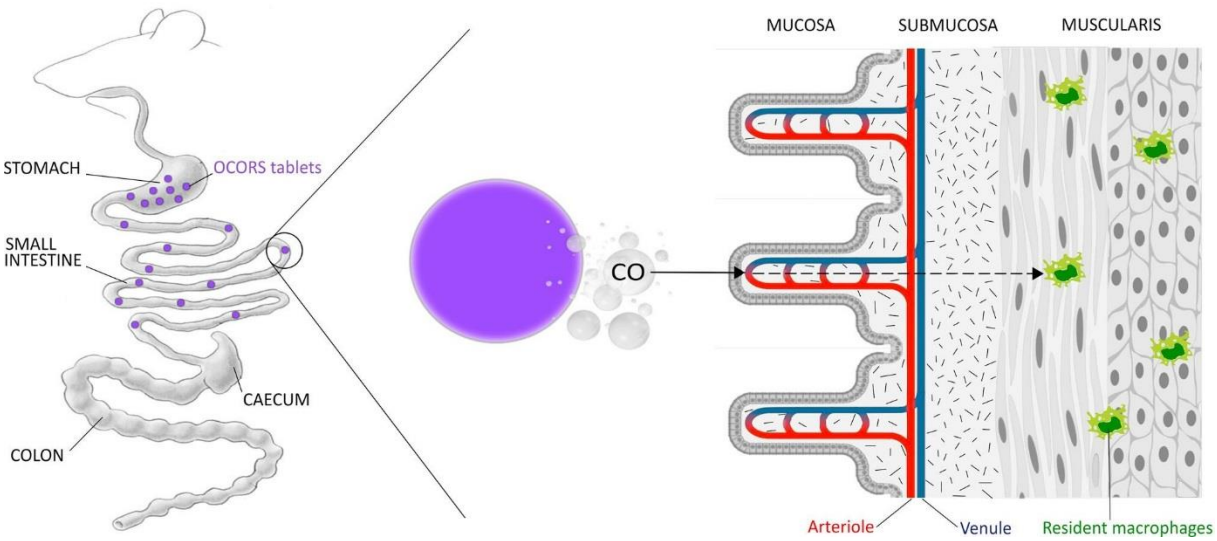
CHAPTER III

Investigation of orally delivered carbon monoxide for postoperative ileus

III.1. Abstract

Endogenously produced carbon monoxide (CO) has antioxidant and anti-inflammatory effects which is why CO has been investigated as a possible therapeutic agent for inflammatory disorders in different body systems, including the gastrointestinal (GI) tract. In an effort to develop an easy to use platform for CO delivery to the GI tract, we recently introduced the Oral CO Release System (OCORS) and demonstrated its preventive effect for experimental colitis in a rodent model. Building off on a comprehensive preclinical dataset on efficacy of inhaled and intraperitoneal CO in reducing postoperative ileus (POI), which is being defined as GI transit retardation after abdominal surgery, we evaluated an adapted OCORS platform to ameliorate POI by local CO delivery to the murine small intestine. To match design characteristics of OCORS with the murine physiology we developed a miniaturized version of the OCORS and tailored its release pattern to release CO for 2 h following first order kinetics. Upon intragastric gavage of 20 tablets, 55 % of the tablets reached the murine small intestine after 1 h while triggering a blood carboxyhemoglobin rise to 5.2 %. Although this is in line with previous systemic CO dosing protocols, GI muscular inflammation and transit retardation by small intestinal manipulation, performed at 1 h after gavage of 20 tablets, was not prevented while the positive control - intravenous nitrite - prevented POI. The results show that local CO treatment of POI is insufficient - suggesting a strong systemic component for effective therapy - thereby providing critical insight into effective design of CO drug delivery in POI.

III.2. Graphical abstract



III.3. Introduction

Postoperative ileus (POI) refers to impaired gastrointestinal motility occurring after abdominal surgery. When prolonged, POI leads to symptoms as nausea and vomiting, abdominal distension, absence of flatus and inability to tolerate an oral diet [1]; this is a frequent reason for prolonged hospital stay. Different definitions are used to indicate prolonged POI versus “normal” but a recent meta-analysis reported an overall incidence of 10 % of prolonged POI after colorectal surgery with great variability according to definition, type of surgery, type of access (open versus laparoscopic) and duration of the operation [2]. Prolonged POI has an important socioeconomical impact, with estimations for the annual cost ranging from 0.75 to 1.5 billion US$ in the United States [3,4]. The pathogenesis of POI is multifactorial with an iatrogenic component due to perioperative medicines in particular opioid analgesics. The mechanisms induced by the intra-abdominal manipulation per se include an acute neurogenic phase with activation of inhibitory neuronal pathways and a more important prolonged inflammatory phase [5]. Activation of resident macrophages in the muscularis plays a critical role. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), chemokines such as monocyte chemoattractant protein-1 (MCP-1) and adhesion molecules

as intercellular adhesion molecule-1 (ICAM-1) are released, the latter recruiting circulatory leukocytes. Nitric oxide (NO) and prostaglandins released by the inflammatory cells inhibit gastrointestinal motility [6,7].

Multimodal perioperative fast-track protocols (enhanced recovery after surgery; ERAS) with optimal perioperative fluid management and analgesia as well as early mobilization and feeding are now advocated. In patients undergoing colonic surgery, the shortest total postoperative hospital stay was obtained with fast-track management combined with laparoscopy [8]; this combination was also shown to lead to quicker recovery of colonic transit [9]. However adherence to ERAS protocols does not at all fully prevent the occurrence of prolonged POI [10]. As for drugs, prokinetics are most frequently used but their effectiveness has been questioned [11]. As it is accepted as the major mechanism of POI, intestinal inflammation should be primarily targeted when considering new strategies [12]. Heme oxygenase 1 (HO1) is an adaptive gene expressed in response to oxidative stress and inflammation, providing antioxidant, cytoprotective and anti-inflammatory effects [13,14]; its protective role was also established in the gastrointestinal tract [15,16]. Heme oxygenase metabolizes heme to ferrous iron, biliverdin and carbon monoxide (CO) and CO seems to contribute most significantly to the beneficial effects of the HO1 pathway [17]. CO protects the gastrointestinal tract against noxious injury and is explored as therapy for several gastrointestinal disorders including diabetic gastroparesis, POI and inflammatory bowel disease [18,19]. Inhalation of CO was shown to reduce surgery-induced retardation of gastrointestinal transit in models of POI in mice and rats [20,21]. In the mouse model with manipulation of the small intestine to induce POI, intraperitoneal injection of the CO releasing molecule CORM-3 also protected against retarded transit correlating with a reduction in inflammatory parameters [22]. Application of CO by inhalation lacks targetability as the delivery of CO to the diseased tissue depends on diffusion of CO from the blood into the tissue [23]. In the blood, CO binds to hemoglobin forming carboxyhemoglobin (COHb), on the one hand excluding CO from diffusing into the tissue and on the other hand reducing oxygen transport

capacity of hemoglobin. In humans, toxic symptoms occur from COHb levels of 10 % [24] and higher levels than 10 % should thus be avoided. Also systemic administration of CORMs will increase blood COHb; additionally most CORMs are metal based and cellular damage by the CO-depleted molecule has been shown in *in-vitro* cell assays [25].

The gastrointestinal tract is particular in that it can be targeted directly with CORMs via the oral route to treat gastrointestinal disease from the luminal side. For this purpose, Steiger et al. [26] developed an oral tablet with CORM-2 (oral carbon monoxide release system; OCORS). The CO release rate of OCORS can be tailored as a function of different coatings covering key elements of OCORS (*vide infra*). With CO release being adjustable from minutes to days, OCORS allows CO delivery to different parts of the gastrointestinal tract (stomach, small or large intestine). Repetitive intragastric administration of micro scale OCORS tablets with a 24 h release profile reduced histologic damage in a murine model of colitis induced by intrarectal administration of 2,4,6-trinitrobenzesulfonic acid (TNBS;[27]). The aim of the present study was to evaluate whether OCORS tablets can be developed to prevent POI in the murine model with manipulation of the small intestine. As the inflammation of the gastrointestinal muscle layer starts immediately upon manipulation of the bowel [28], the OCORS tablets should have reached the small intestine at that moment, fully releasing CO. The gastric emptying and passage through the gastrointestinal tract upon intragastric application of the developed tablets was therefore studied, together with their *in-vitro* CO release profile after recuperation of the tablet rests as well as the blood COHb levels induced; finally the effect of OCORS tablets with 0.5 mm diameter versus POI was tested.

III.4. Materials and methods

III.4.1. Materials

Carbon monoxide releasing molecule 2 (CORM-2; tricarbonyldichlororuthenium-(II)dimer; $[Ru(CO)_3Cl_2]_2$), dibutyl phthalate, sodium hydrogen phosphate dibasic dodecahydrate, sodium citrate tribasic dihydrate, sodium dodecyl sulfate, ruthenium (III) chloride hydrate, and fumed

silica (Aerosil®) were purchased from Sigma-Aldrich Chemie (Schnelldorf, Germany). Sodium sulfite was purchased from Gruessing (Filsum, Germany). Citric acid monohydrrate was from Jäkle Chemie (Nuernberg, Germany). Talcum was from Caelo (Hilden, Germany). Tabletose 80 and premixed tableting powders ("Tablettiermischung, technisch") consisting of lactose, cellulose, aluminum oxide and magnesium stearate were from Meggle (Wasserburg am Inn, Germany). Sam specracol erythrosine lk was from Sensient (Geesthacht, Germany). Eudragit E PO was a generous gift from Evonik Industries (Essen, Germany). CORM-A1 (sodium boranocarbonate; $Na_2[H_3BCO_2]$; gift of Roberto Motterlini, University Paris Est, France) was studied in comparison with OCORS to determine blood COHb levels induced. Sodium nitrite (Sigma-Aldrich, Diegem, Belgium) was used in comparison with OCORS tablets in the POI model. Isoflurane (IsoFlo, Abbott Laboratories Ltd, Maidenhead, Berkshire, UK) was used to anesthetize the mice when inducing POI. Fluorescein-labelled dextran (70 kDa; Invitrogen, Merelbeke, Belgium) was used to measure postoperative gastrointestinal transit.

III.4.2. Development of Oral Carbon Monoxide Release System (OCORs)

Two OCORS formulations with a diameter of 1.0- and 0.5 mm (indicated as OCORS-1mm and OCORS-0.5mm, respectively) were developed as previously described, with modifications ([26]; for detailed manufacture protocol for OCORS-1mm see Formulation 2 (F2) in [27]). In short, 1 mm- and 0.5 mm tablet cores were compressed and coated, respectively. The OCORS-1mm tableting powder blend was prepared by mixing 128 mg pulverized trisodium citrate, 64 mg pulverized citric acid monohydrate, 75 mg coated sodium sulfite (100 – 125 µm, *vide infra*), 75 mg CORM-2, 5 mg magnesium stearate, 2.5 mg Aerosil® and 143 mg tableting mixture in a Turbula T2Fmixer (WAB AG, Muttenz, Switzerland) for 30 minutes. The OCORS-0.5mm powder blend contained overall identical constituents but was adapted with respect to particle size and compressibility. Therefore, only the < 50 µm fraction of citric acid, trisodium citrate, and tableting mixture was used after sieving with an AS 200 analytical sieve tower from Retsch (Haan, Germany). In addition, 30 mg 50 – 80 µm coated sodium sulfite crystals were used (instead of 75 mg 100 – 125 µm, *vide supra*), accordingly. Sieved sodium sulfite crystals

were coated using a Mini-Coater from Glatt with coating solution A and B for OCORS-1mm and OCORS-0.5mm, respectively (Binzen, Germany – see coating protocol and solution below).

The OCORS-1mm blend was compressed in an FE136SRC eccentric tableting machine from Korsch (Berlin, Germany) equipped with a custom-made 1 mm tablet punch (Franz-Oberthuer School for mechanical engineering, Wuerzburg, Germany). The OCORS-0.5mm blend was compressed using a custom-made tableting machine prepared by the engineering toolshop of the department for pharmaceutics and biopharmaceutics (Fig. III.1).

OCORS-0.5mm and OCORS-1mm cores were subsequently fluid bed coated with the coating solution B using the Mini-Coater. In all settings the coater was operated in top-spray configuration at 21 °C, a fluid bed pressure of 0.6 bar, and an atomizing pressure of 0.76 bar. Coating solution A) contained 8.6 g Eudragit E PO, 0.9 g sodium dodecyl sulfate, 1.3 g magnesium stearate, 4.3 g talcum, 50 ml distilled water, and 50 ml absolute ethanol. Coating solution B) contained 2.32 g dibutyl phthalate, 4 g talcum, and 11.6 g cellulose acetate butyrate

Figure III.1 Custom-made tableting machine used to compress the OCORS-0.5mm blend.

dissolved in 460 ml acetone. OCORS-1mm contained 98 ± 1 µg of CORM-2 while the OCORS-0.5mm tablet contained 47 ± 5 µg of CORM-2. OCORS-0.5mm is shown in Fig. III.2.

III.4.3. Amperometric CO detection system

The CO release pattern of the OCORS systems *in-vitro* and *in-vitro* after retrieval from the murine gastrointestinal tract (*vide infra*) was assessed as described before [27]. In brief, CO release was analysed in a reaction chamber consisting of 2 sealed DN 40 flanges. Therefore, CO release was initiated by injecting 15 ml water into the reaction chamber. CO headspace gas concentration was monitored for 3 h thereafter using an X-am 5000 CO sensor from Draeger (Luebeck, Germany). OCORS tablets retrieved from the murine gastrointestinal tract, were placed on murine gastrointestinal tract within the reaction chamber to simulate physiological conditions (preliminary tests indicated that presence of fluid/tissue is mandatory to maintain CO release from OCORS; not shown).

III.4.4. In vivo studies in mice

III.4.4.1. Animals

Seven weeks old male C57BL/6J mice were purchased from Janvier, Le Genest St-Isle, France and were used between 9 and 12 weeks of age (25-28 g). Mice were housed in an animal care facility with a 12 h light/dark cycle and had free access to water and commercially available chow. Animal care and experimental procedures were approved by the Ethical

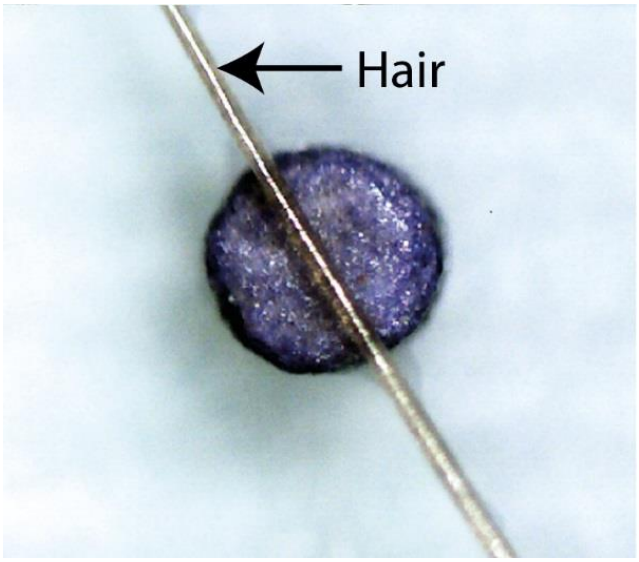


Figure III.2 OCORS-0.5mm in comparison to a human hair.

Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

III.4.4.2. Evaluation of gastrointestinal distribution and CO release

In a preliminary test, 5 mice were fasted for 1 h and then received either 1 OCORS-1mm tablet intragastrically (n=3) or 15 mg/kg of CORM-A1 intraperitoneally (n=2). Mice receiving a tablet were sacrificed at 1- or 2 h after its administration, while mice receiving CORM-A1 were sacrificed 20 min after the injection. Blood was obtained by cardiac puncture in a heparin coated syringe (RAPIDLyte, Siemens Healthcare Diagnostics Inc, Tarrytown, NY, USA) and the blood COHb level was determined using the RAPIDPoint 500 Blood Gas System (Siemens Healthcare Diagnostics Inc).

Subsequently, 8 mice were fasted for 1 h, then received 1 OCORS-1mm tablet intragastrically and were sacrificed either 1- or 2 h after administration. The gastrointestinal tract was opened to determine the position of the tablet and to analyse the remaining *in-vitro* CO release with the amperometric CO detector.

As 4 OCORS-0.5mm tablets released a similar amount of CO as 1 OCORS-1mm tablet *in-vitro* (Fig. III.3A and Fig. III.4A), the gastric emptying of 4 tablets was tested. Four mice were fasted for 1- and 6 h, respectively, and then received the 4 tablets intragastrically. Mice were sacrificed 1- or 2 h after administration, blood was obtained by cardiac puncture for COHb analysis, and the gastrointestinal tract was opened to evaluate the distribution of the tablets and to analyse their remaining *in-vitro* release.

Finally, a similar protocol was performed in 12 mice, receiving 20 OCORS-0.5mm tablets after 6 h of fasting.

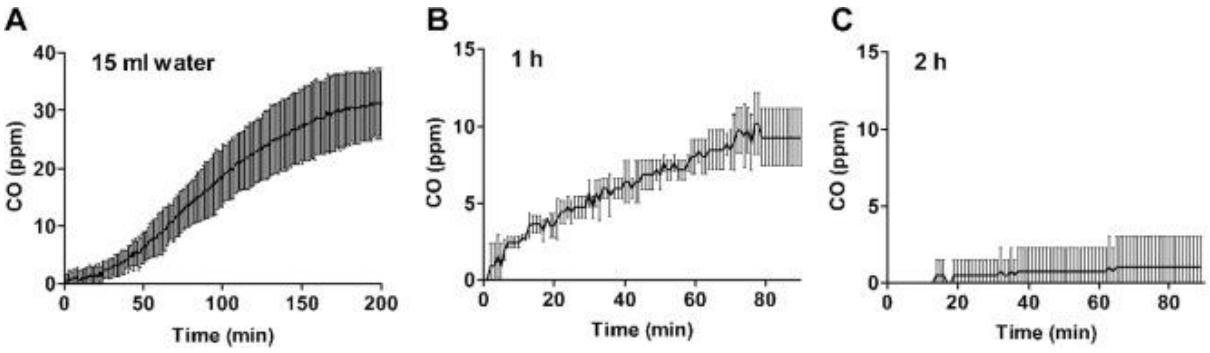


Figure III.3 In-vitro CO release from OCORS-1mm tablets. The CO release pattern of non-administered OCORS-1mm (A, n=6, release initiated with 15 ml water) and OCORS-1 mm retrieved from the murine gastrointestinal tract at 1 h (B, n=3) or 2 h (C, n=4) after intragastric administration; mean ± s. d. Release was measured during 200 min or 90 min (retrieved tablets). For the tablets retrieved in mouse No. 4 sacrificed at 1 h after gavaging, in-vitro release could not be determined in 1 tablet due to technical reasons.

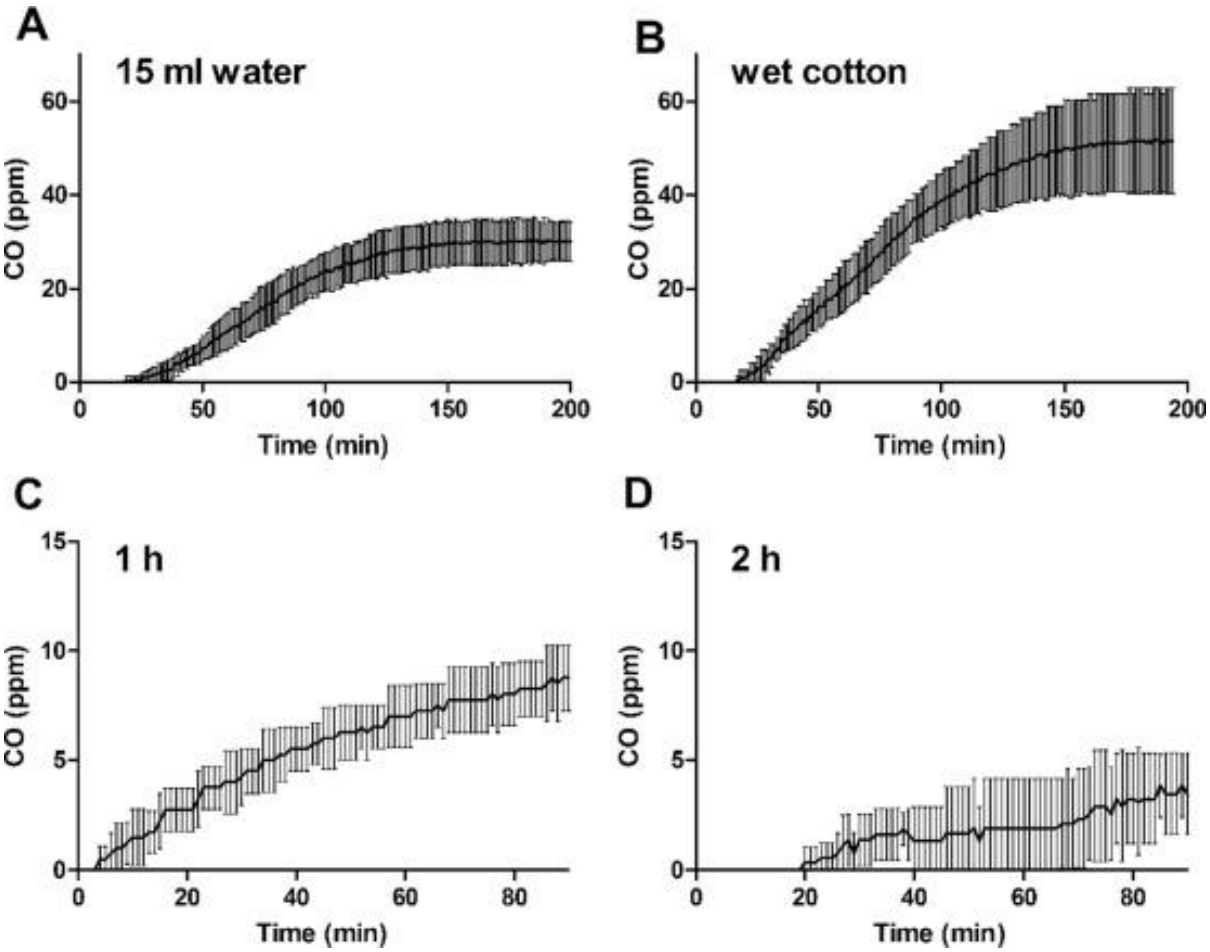


Figure III.4 In-vitro CO release from sets of 4 OCORS-0.5mm tablets. Release was measured from sets of non-administered tablets placed in the amperometer in 15 ml water (A, n=6 sets) or on a wet piece of cotton (C, n=3 sets) or of sets retrieved from the murine gastrointestinal tract at 1 h (C, n=4 sets) or 2 h (D, n=4 sets) after intragastric administration; mean ± s.d. Release was measured during 200 min or 90 min (retrieved tablets).

The application of the OCORS tablets was done by use of a custom-made gavage needle (outer diameter 1.651 mm, inner diameter 1.194 mm, for OCORS-1mm; outer diameter 1.067 mm, inner diameter 0.686 mm, for OCORS-0.5mm. The tablet(s) was (were) placed at the end of the needle, that was connected to a 1 ml syringe. After intragastric positioning of the end of the needle, the tablet(s) was (were) expelled via an intraluminal wire pushed forward exactly till the end of the needle.

III.4.4.3. Study of 20 OCORS-0.5mm tablets in POI

The effect of 20 gavaged OCORS-0.5mm tablets was evaluated in comparison with 48 nmol nitrite injected intravenously, as the latter was previously shown to be protective in POI [29]. The study was performed in 28 mice: 7 were non-operated controls, 7 were abdominally operated but not treated, 7 received 20 OCORS-0.5mm tablets by gavage 1 h before the operation; 7 were given 48 nmol nitrite intravenously into the vena cava inferior, immediately after laparotomy and just before starting small intestinal manipulation. All animals were fasted from 7 h before the operation, meaning that the OCORS tablets were administered after 6 h of fasting. POI was induced by surgical manipulation of the small intestine (intestinal manipulation, IM). Mice were anaesthetised with inhaled isoflurane (induction, 5%; maintenance, 2%) and the abdomen was opened by midline laparotomy. The small intestine was exteriorized and then compressed for 5 min along its entire length by using sterile moist cotton swabs. The bowel was repositioned in the abdominal cavity and the incision was closed by 2 layers of continuous sutures. The total duration of the procedure was approximately 20-25 min. After the operation, the mice had again access to food until 21 h after IM. Mice were sacrificed 24 h after induction of POI. The gastrointestinal tract from stomach till colon was then isolated. After measuring intestinal transit, the gastrointestinal tract was flushed with aerated (5 % CO_2 in O_2) ice-cold Krebs solution containing 1 mM phenylmethylsulfonyl fluoride and the small intestine was divided in 6 segments of equal length. After removal of the mucosa using a glass slide, the muscular layer was stored at -80°C till further analysis of myeloperoxidase activity as a marker of leukocyte infiltration or of IL-6 protein level.

III.4.4.4. Measurement of intestinal transit

Intestinal transit was measured by evaluating the distribution of non-absorbable fluorescein-labeled dextran (70kDa, FD70) along the GI tract [30]. Twenty-one h after IM, the mice were deprived of food and 1.5 h hereafter a liquid fluorescein-labelled dextran meal (200 µl of a 25 mg/ml solution) was gavaged. One and a half h later, i.e. 24 h after surgery, the animals were sacrificed. After excision of the gastrointestinal tract, 2 full-field images (one in normal illumination mode and another in fluorescent mode) were taken with a CCD camera and subsequently matched to determine the fluorescence intensity along the GI tract. The fluorescent intensity was analysed and calculated for 14 consecutive segments: stom, stomach; sb, small bowel segments 1–10; caec, caecum; col, colon segments 1–2. For each segment, fluorescence intensity was expressed as percentage of totally recovered fluorescence. The geometric center (GC) of the fluorescence distribution was calculated by the formula: ∑(% FD70 per segment*segment number)/100).

III.4.4.5. Biochemical analyses

Analysis of myeloperoxidase (MPO): In mucosa-free segment 1 of the small intestine, myeloperoxidase activity was measured as an index of leukocyte infiltration by optical densitometry using 3,3',5,5'- tetramethylbenzidine (TMB). Frozen tissue samples were homogenized with a Mikro-Dismembrator and dissolved in 10 volumes of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 % hexadecyl-trimethylammonium bromide (HETAB). The homogenate was sonicated on ice (15 pulses of 0.7 s at full power) and subsequently subjected to freeze/thaw. The suspension was centrifuged (14000 *g*, 20 min, 4 °C) and 10 µl of the supernatant was added to 200 µl of assay mixture, containing ready-to-use TMB substrate, 0.5% HETAB, and 10 mM EDTA (on ice). The optical density was immediately read at 620 nm (Biotrak II). The reaction was then allowed to proceed for 3 min at 37 °C. The reaction was stopped by placing the 96-well plate on ice, and the optical density was measured again. One unit of MPO activity was defined as the amount of enzyme that produces a change

in optical density of 1.0 per minute at 37 °C. Results were normalized to total protein content (Pierce BCA Protein Assay Kit) and expressed as U/mg protein.

Analysis of IL-6 protein level: Protein expression levels of IL-6 in mucosa-free segment 2 of the small intestine were determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol (Invitrogen, Merelbeke, Belgium). Briefly, frozen tissue samples were homogenized with a Mikro-Dismembrator, dissolved in 10 volumes of 20 mM PBS buffer (pH 7.4) containing protease inhibitors (Complete Mini Protease Inhibitor EDTA-free tablets, Roche, Basel, Switzerland), and centrifuged at 10000 *g* for 15 min at 4°C. The supernatant (100 µl) was added to the appropriate microtiter wells, after which the plate was covered and incubated at room temperature. After 2 h, the solution was thoroughly aspirated from the wells and wells were washed with Wash Buffer. 100 µl of IL-6 Biotin Conjugate Solution was added to each well, after which the plate was covered and incubated at room temperature. After 30 min, the solution was aspirated from the wells and wells were washed with Wash Buffer. Streptavidin-HRP Working Solution (100 µl) was then added to each well, after which the plate was covered and incubated at room temperature. After 30 min, the solution was thoroughly aspirated from the wells and wells were washed with Wash Buffer. Stabilized Chromogen (100 µl) was then added to each well and incubated at room temperature in the dark. After 30 min, Stop Solution (100 µl) was added to each well and the absorbance was measured at 450 nm. Results were normalized to total protein content (Pierce BCA Protein Assay Kit) and expressed as pg/mg protein.

III.4.4.6. Statistical analysis

Data are given as mean ± s.d. or mean ± s.e.m. as indicated. The results in the 4 groups in the POI study were compared by one-way ANOVA followed by Bonferroni's multiple comparison t-test. Results were considered different for a P-value less than 0.05. In the group treated with nitrite, the results in 1 animal were aberrant. Therefore, we used the Grubbs' test to determine significant outliers and excluded data with a Z-value greater than the critical Z-value of 2.02 (in a group with n = 7) for further statistical analysis [31].

III.5. Results and Discussion

III.5.1. Development and in-vitro characteristics of OCORS-1mm and OCORS-0.5mm

The OCORS platform contains CORM-2, as well as sodium sulfite serving as CO release triggering molecule. Following oral application, gastric fluid permeates through the cellulose acetate shell surrounding OCORS, in a second step, dissolves sodium sulfite and ultimately triggers CO release form CORM-2. Coating procedures modifying the permeability of i) the cellulose acetate shell surrounding the system, and ii) the coating surrounding sodium sulfite hence allow to precisely tailor the permeation of GI fluid and subsequently the release profile of OCORS [27]. We hence tailored the release profile of both OCORS formulations as such that it approximates the murine small intestinal residence time of particles with comparable size (0.5 - 1 mm; [32]) to ensure local CO delivery to manipulated tissue. *In-vitro* CO release followed first order kinetics for 172 ± 33 minutes (OCORS-1mm) and 121 ± 25 minutes (OCORS-0.5mm), respectively (Fig. III.3A and Fig III.4A). The release pattern plateaued thereafter at 31 ± 5 ppm and 29 ± 4 ppm for 1 x OCORS-1mm and 4 x OCORS-0.5mm, respectively. Technical constraints limit the CORM-2 loading capacity to 98 ± 1 µg and 47 ± 5 µg for OCORS-1mm and OCORS-0.5, respectively. The higher quantity of CO release per CORM-2 in OCORS-1mm as compared to OCORS-0.5mm suggests further design space for system modifications. Noteworthy, independence of CO release kinetics from environmental factors such as pH or ionic strength was confirmed by monitoring the CO release pattern following exposure to different simulated gastrointestinal fluids in two frontrunner studies (7 mm [26] and 1 mm [27] OCORS).

III.5.2. Gastrointestinal distribution and CO release of OCORS

In the preliminary test, 3 mice received 1 OCORS-1mm tablet intragastrically after 1 h of fasting. The blood COHb level was 0.3 % in the mouse studied at 1 h after gavage and 0.1 and 0.2 % in the 2 mice where blood was sampled at 2 h after gavage; these values are similar to basal values in untreated control C57BL/6J mice [22,33]. In contrast, the blood COHb level

was 7.4 and 6.5 % in 2 mice at 20 min after intraperitoneal administration of 15 mg/kg CORM-A1, confirming previously reported values and illustrating the in vivo release of CO from CORM-A1 [22].

In the next step, 1 OCORS-1mm tablet was gavaged in 8 mice fasted for 1 h. Both at 1 h (n=4) and 2 h (n=4) after administration, the tablet was systematically found in the stomach. When *in-vitro* release was studied from the retrieved tablets, the release was decreased in comparison to that from non-used tablets placed in water; the release was smallest for tablets that had stayed in the gastrointestinal tract for 2 h (Fig. III.3B and C) suggesting that the OCORS-1mm tablets were effectively releasing CO in the gastrointestinal medium. But the CO released did either not reach the circulation or the amount reaching the circulation was too small to increase blood COHb levels.

As the aim was to deliver CO to the small intestine at the site of intestinal manipulation, the size of the OCORS tablets was downsized to 0.5 mm. As the amount released from 4 OCORS-0.5mm tablets equaled that of 1 OCORS-1mm tablet (see description in III.5.1.), the gastrointestinal distribution of 4 OCORS-0.5mm tablets was tested in mice. Although the influence of food on gastric emptying is not easy to predict, experiments in dogs showed that the presence of food can delay gastric emptying of tablets [34]; gastric emptying of 4 OCORS-0.5mm tablets was therefore performed in mice fasting for 1 h or for 6 h. When administering the tablets after 1 h of fasting, they were mainly found in the stomach even at 2 h after administration. However, in mice fasted for 6 h before gavaging the tablets, they all had left the stomach already at 1 h after administration; at 2 h after administration some or all had reached the caecum (Table III.1). This suggests that also in mice, food in the stomach can delay gastric emptying of tablets. Blood COHb levels measured at 1- or 2 h after administration were again similar to levels observed in control C57BL/6J mice (Table III.1).

Table III.1 Gastrointestinal location (number of tablets) and blood COHb levels after administration of 4 OCORS-0.5mm tablets intragastrically in 8 mice fasted for 1 h of for 6 h.

Mouse nr.	Time of retrieval	Gastrointestinal location			COHb (%)
		Stomach	**Small intestine**	**Caecum**	
1h fasting					
1	1h	3	1	0	ND
2	1h	4	0	0	ND
3	2h	2	2	0	0.3
4	2h	4	0	0	ND
6h fasting					
5	1h	0	4	0	0.1
6	1h	0	4	0	0.1
7	2h	0	2	2	0.1
8	2h	0	0	4	0.1

ND: Not determined as the minimum volume of blood needed for analysis (0.2 ml) was not obtained.

Analysis of the *in-vitro* CO release from the retrieved tablets illustrated that the OCORS-0.5mm tablets had also released CO while in the gastrointestinal tract; the longer the tablets had been in the gastrointestinal tract, the smaller the remaining release was in comparison to tablets placed in 15 ml of water (Fig. III.4C and D). Non-administered tablets, placed in the CO detection system without water but in contact with a piece of wet cotton (Fig. III.4B), released a similar amount of CO as those in combination with 15 ml of water (Fig. III.4A) excluding that the absence of water explained the low CO release from retrieved tablets.

The study with 4 OCORS-0.5mm tablets made clear that this size of tablets was already emptied from the stomach at 1 h after gavage, when mice were fasting for 6 h, and that the tablets released CO during their stay in the gastrointestinal tract. Still, the complete absence of any change in blood COHb levels, raised the question whether a sufficient amount of CO

was released to possibly counteract the acute muscular inflammation starting after intestinal manipulation and inducing POI. The number of OCORS-0.5mm tablets was therefore increased to 20 in an attempt to increase the total amount of CO released. Evaluation of the gastrointestinal distribution of 20 gavaged OCORS-0.5mm tablets in mice fasted for 6 h showed that at 1 h after gavage, mean gastric emptying attained 55 % of the tablets (Table III.2). This was not increased at 2 h after gavage; still at 2 h, 60 % of the tablets had already reached the caecum in 1 mouse. The incomplete gastric emptying of 20 OCORS-0.5mm tablets contrasts with the 100 % gastric emptying of 4 OCORS-0.5mm tablets in the same conditions. With 20 tablets in the stomach, some competition might occur at the level of the pylorus to leave the stomach. De Filippis et al. [35] reported 75 % of gastric emptying at 2 h after gavaging 25 glass beads (0.4-0.5 mm in diameter) in fasted male Swiss mice of higher body weight (30-40 g) than the C57BL/6J mice used in the present study (25-28 g). At 1 h after gavaging 20 OCORS-0.5mm tablets, blood COHb level was increased to 5.2 % illustrating that a larger total amount of CO is released than from 4 tablets, now reaching the general circulation. The blood COHb level already declined to 1.9 % at 2 h after gavage. As the study showed that on average, 55 % of the administered tablets had reached the small intestine at 1 h after administration in mice fasted for 6 h and the tablets are maximally releasing CO at this time point, the influence of 20 gavaged OCORS-0.5mm tablets was tested in the murine POI model, operating the animals at 1 h after administration; the small intestine can then be expected to be fully exposed to CO at the moment of surgical manipulation, inducing the inflammatory reaction leading to POI.

Table III.2 Gastrointestinal location (number of tablets) and blood COHb levels after administration of 20 OCORS-0.5mm tablets intragastrically in 12 mice fasted for 6 h.

Mouse nr.	Time of retrieval	Gastrointestinal location			COHb (%)
		Stomach	**Small intestine**	**Caecum**	
1	1h	0	19	0^{NF}	ND
2	1h	0	20	0	5.5
3	1h	17	2	0^{NF}	6.2
4	1h	15	5	0	4.0
5	1h	14	6	0	5.3
6	1h	4	16	0	5.1
Mean ± s.e.m.		8 ± 3	11 ± 3	0 ± 0	5.2 ± 0.4
7	2h	20	0	0	0.8
8	2h	11	9	0	2.3
9	2h	7	13	0	3.3
10	2h	1	19	0	2.9
11	2h	0	8	12	0.4
12	2h	15	5	0	1.4
Mean ± s.e.m.		9 ± 3	9 ± 3	2 ± 2	1.9 ± 0.5

ND: Not determined as the minimum volume of blood needed for analysis (0.2 ml) was not obtained.

^{NF}: In mice nr 1 and 3, 1 of the 20 tablets was not found throughout the gastrointestinal tract or in the faeces.

III.5.3. Influence of 20 OCORS-0.5mm tablets in murine POI.

At 1.5 h after intragastric administration, the major part of fluorescein-labelled dextran had moved to the distal region of the small bowel in non-operated control animals (Fig. III.5). At 24 h after small bowel manipulation, the major part of fluorescein-labelled dextran was present in the upper 3 segments of the small bowel, inducing a significant reduction of the geometric centre of transit to 3.4 ± 0.3 (n=7) from 8.2 ± 0.3 in control animals (n=7, $P < 0.001$). As previously reported [29], intravenous injection of 48 nmol of nitrite just before small bowel manipulation prevented the delay in transit. In the 7 mice gavaged with 20 OCORS-0.5mm tablets at 1 h before surgery, an attempt was done to count the number of tablets that had already left the stomach upon operation. Some tablets were indeed clearly visible inside the small intestine due to their purple color. This is a rough estimation as some tablets may not have been observable in the small intestine because they were covered with chyme or had already passed into the caecum. This estimation of tablets visible through the small bowel was < 5 tablets in 1 mouse, 5-15 tablets in 5 mice and > 15 tablets in 1 mouse. However, the delay in gastrointestinal transit by surgery was not influenced by administration of the OCORS-0.5mm tablets (Fig. III.5).

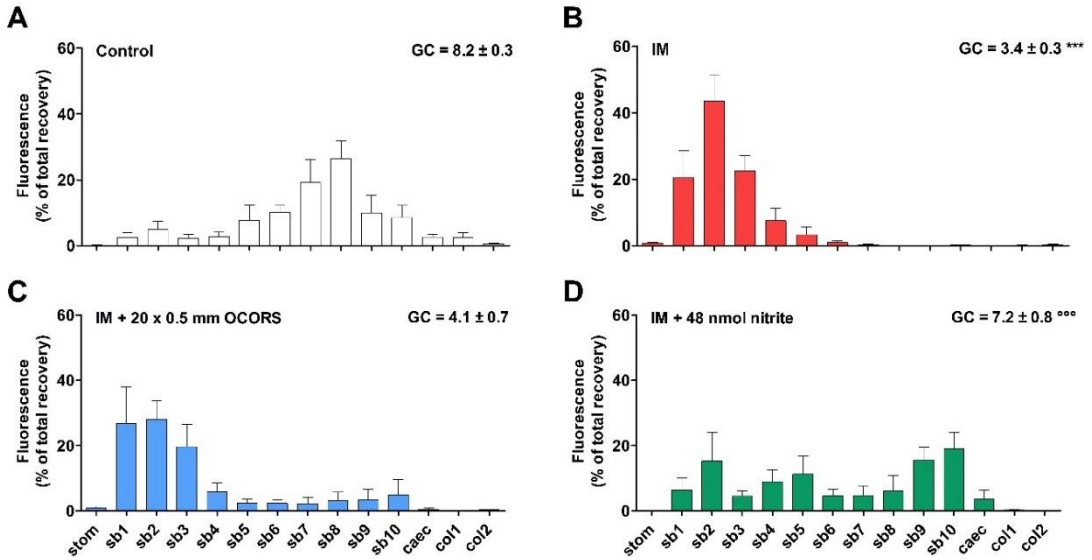


Figure III.5 Transit histograms and derived values for the geometric centre (GC) for the distribution of fluorescein-labelled dextran along the gastrointestinal tract (stom, stomach; sb, small bowel segments 1-10; caec, caecum; col, colonic segments 1-2) measured 24 h after intestinal manipulation in non-treated mice (IM; panel B) and in mice gavaged with 20 OCORS-0.5mm tablets at 1 h before surgery (panel C) or treated intravenously with 48 nmol nitrite just before surgery (panel D). Controls (panel A) were not operated. Mean ± s.e.m. of n = 7. *** P<0.001 for intestinal manipulated mice versus control mice; °°° P<0.001 for nitrite-treated mice versus non-treated intestinal manipulated mice (one-way ANOVA followed by A Bonferroni multiple comparison test).

Surgical manipulation of the small intestine induced a significant increase in MPO activity activity and in the protein level of the inflammatory cytokine IL-6. While pretreatment with nitrite significantly reduced these markers of surgery-induced inflammation, they were not influenced by gavaging the OCORS-tablets (Fig. III.6).

We hypothesize that three potential mechanisms are contributing to lack of efficacy of CO delivery from the luminal side. These include i) sub-therapeutic CO levels in the muscularis due to sink conditions in the more luminal and highly blood perfused parts of the intestine, ii) necessity of sustained, or iii) necessity of high systemic CO levels in order to trigger therapeutic effects in POI models. Numerous effective CO-delivery approaches for POI have been introduced before, most of which translate, as assessed from blood COHb levels, either into high systemic CO peak levels ≥ 8 % COHb or into medium systemic CO levels (5 % COHb) for a prolonged perioperative period of time (≥ 3 h). It should be taken in account that blood COHb levels do not necessarily provide exact information on CO delivery to organs and tissues [36]. Indeed, with the ruthenium based CORM-3, COHb levels < 3 % were sufficient to reduce POI (Fig. III.7). Ruthenium based CORMs release CO intracellularly, in response to sulfite

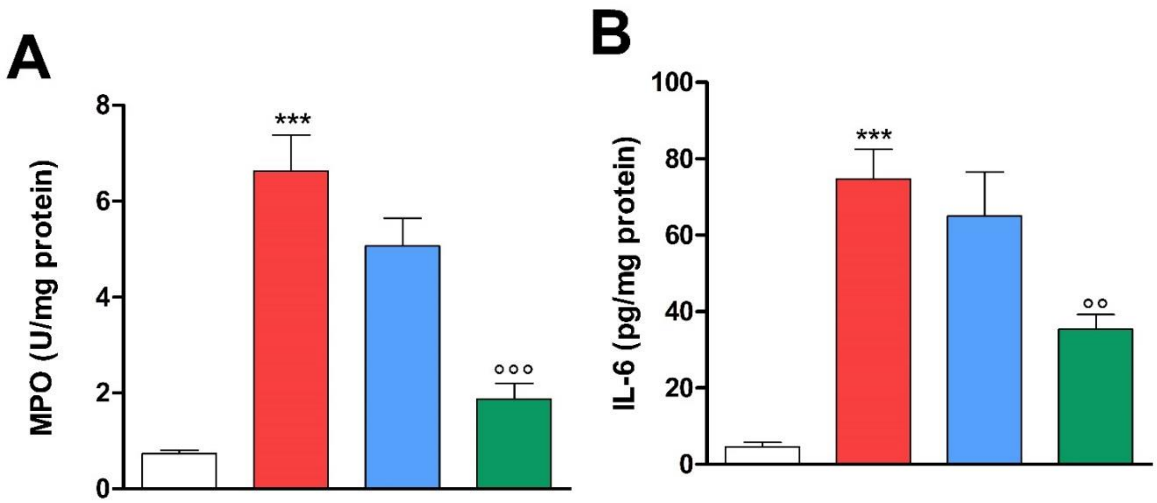


Figure III.6 MPO activity (panel A) and protein level of IL-6 (panel B) in small intestine measured 24 h after intestinal manipulation in non-treated mice ( ) and in mice gavaged with 20 OCORS-0.5mm tablets at 1 h before surgery ( ) or treated intravenously with 48 nmol nitrite just before surgey ( ). Controls ( ) were not operated. Mean ± s.e.m. of n = 7 except for MPO and IL-6 in the nitrite-treated group where n=6. *** P<0.001 for intestinal manipulated mice versus control mice; °° P<0.01, °°° P<0.001 for nitrite-treated mice versus non-treated intestinal manipulated mice (one-way ANOVA followed by A Bonferroni multiple comparison test).

species [37] and over prolonged period – thereby decreasing COHb while maintaining intracellular CO levels [38]. Importantly, most approaches used intraperitoneal administration including injection of CO-enriched saline at the end of surgery just before closure of the abdomen or injection of CORM-A1 at 90 min before starting surgery. With intraperitoneal administration, CO can enter from the peritoneal cavity into the whole gastrointestinal tract via the serosal side then also quickly reaching the muscular layer, which is the site of inflammation in POI [6,7]. With intragastric administration of OCORS-tablets, CO has to enter the gastrointestinal tract from the mucosal side. An important part of CO entering the mucosa may hence be absorbed in the mucosal capillary network, explaining the clear increase in blood

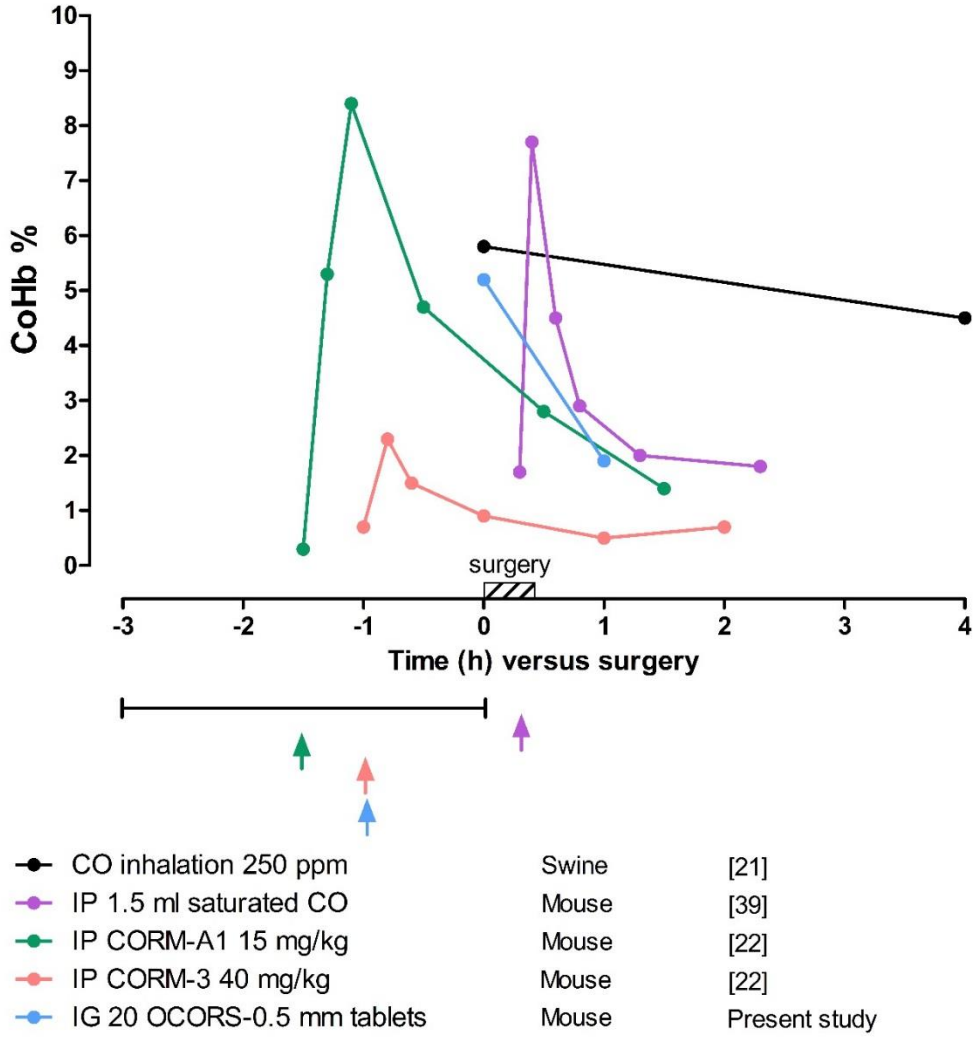


Figure III.7 Systemic CO exposure as assessed via blood COHb levels, reported in experimental studies with different strategies of CO delivery to prevent POI. The COHb levels and the period or moment of administration of each CO delivering strategy are shown versus the period of surgery, arbitrarily taken as 20 min. (See above-mentioned references for further information).

COHb levels. Although the absorption of CO into mucosal capillaries will increase the amount of CO that might reach the muscular layer via the systemic circulation, mucosal capillaries at the same time form then a sink for CO preventing direct exposure of the muscular layer to high concentrations of CO. Additionally, the exposure of the gastrointestinal tract to CO from the intraluminal tablets may also be less homogeneous than with the intraperitoneal delivery strategies, as CO release will be very local when a tablet is stuck at a given site for a longer period. Although this should be balanced by the number of tablets and by the propulsing activity of the gastrointestinal tract, it cannot be excluded that some sites of the small intestine receive clearly less CO than others so that inflammation will be less counteracted at these sites. The acute muscular inflammation induced by abdominal surgery might be too severe to be counteracted by the less pronounced and more dispersed CO. Indeed, the degree and primary location of the inflammation in POI plays a role as repetitive administration of OCORs tablets with a slow CO release profile was able to reduce the more protracted inflammation starting from the mucosal side in murine colitis, induced by intraluminal exposure to TNBS [27]. Noteworthy in this frontrunner study we matched in-vivo with in-vitro data by pharmacokinetic modeling: In three different OCORS formulations with overall release patterns spanning from minutes to almost one day CO release kinetics matched absorption kinetics suggesting that CO (following release) immediately reaches the mucosa and is readily and completely absorbed thereafter.

III.6. Conclusion

OCORS oral tablets of 0.5 mm diameter, releasing their CO content in 150 min were developed and studied to prevent murine POI. Although reaching the small intestine at the moment of surgical manipulation, and releasing an amount of CO leading to systemic CO exposure comparable to intraperitoneal delivery strategies, the tablets were unable to reduce surgery-induced muscular inflammation and delay in gastrointestinal transit. Although gastrointestinal diseases are very suitable for treatment with oral OCORS tablets, the study illustrates

limitations of the concept. Hence, effectiveness should be tested for each type of gastrointestinal disease where CO was already reported to be effective when administered by inhalation or intraperitoneal injection; further optimization will be required for particular conditions.

III.7. Declarations of interest

Lorenz Meinel and Christoph Steiger filed a provisional patent application for the OCORS technology (PCT/EP2015/001187).

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CHAPTER IV

HEMIN REDUCES POSTOPERATIVE ILEUS IN A HEME OXYGENASE 1-DEPENDENT MANNER WHILE DIMETHYL FUMARATE DOES WITHOUT HEME OXYGENASE 1-INDUCTION

Jonas Van Dingenen[1], Leen Pieters[2], Elien Van Nuffel[3,4], Romain A. Lefebvre[1]

[1] Department of Basic and Applied Medical Sciences, Faculty of Medicine & Health Sciences, Ghent University, Ghent, Belgium

[2] Department of Human Structure and Repair, Faculty of Medicine & Health Sciences, Ghent University, Ghent, Belgium

[3] Unit of Molecular Signal Transduction in Inflammation, Center for Inflammation Research, VIB, Ghent, Belgium

[4] Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

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CHAPTER IV

Hemin reduces postoperative ileus in heme oxygenase 1-dependent manner while dimethyle fumarate does without heme oxygenase-1 induction

IV.1. Abstract and keywords

Background: Postoperative ileus (POI), the impairment of gastrointestinal motility after abdominal surgery, is mainly due to intestinal muscular inflammation. Carbon monoxide (CO)-releasing compounds were shown to exert an anti-inflammatory effect in murine POI partially through induction of heme oxygenase-1 (HO-1). The influence of hemin and dimethyl fumarate (DMF), currently used for multiple sclerosis (MS), were therefore tested in murine POI.

Methods: C57BL/6J mice were anesthetized and after laparotomy, POI was induced via intestinal manipulation (IM). Animals were treated with either 30 mg kg^{-1} hemin intraperitoneally (i.p.), 30 mg kg^{-1} DMF i.p. or 100 mg kg^{-1} intragastrically (i.g.) 24 h before IM. Intestinal transit was assessed 24 h postoperatively and mucosa-free muscularis or whole segments of the small intestine were stored for later analysis. Intestinal HO-1 protein expression was studied at 6, 12 and 24 h after administration of hemin or DMF in non-manipulated mice.

Key Results: Pretreatment with hemin and DMF, both i.g. and i.p., prevented the delayed transit seen after IM. Concomitantly, both hemin and DMF significantly reduced the increased interleukin-6 levels and the elevated leukocyte infiltration in the muscularis. Hemin but not DMF caused a significant increase in intestinal HO-1 protein expression and co-administration of the HO-1 inhibitor chromium mesoporphyrin abolished the protective effects of hemin on POI; DMF reduced the IM-induced activation of NF-κB and ERK 1/2.

Conclusions & Inferences: Both hemin and DMF improve the delayed transit and inflammation seen in murine POI, but only hemin does so in a HO-1-dependent manner.

Keywords: Dimethyl fumarate; Heme oxygenase-1; Hemin; Mouse; Postoperative ileus

IV.2. Key points

- Induction of heme oxygenase (HO)-1 can prevent intestinal inflammation and tissue damage; this treatment strategy was now investigated in a murine model of postoperative ileus (POI) with hemin and dimethyl fumarate (DMF) as HO-1 inducers.
- Both hemin and DMF prevent the delayed transit and inflammation caused by POI, but only the protective effect of hemin is HO-1 dependent; DMF reduced the activation of NF-κB and ERK 1/2 during ileus.
- The anti-inflammatory effects of hemin and DMF may be useful for the management of POI in the clinical setting.

IV.3. Introduction

Heme oxygenase (HO) is the rate-limiting enzyme in the degradation of heme into Fe^{2+}, carbon monoxide (CO) and biliverdin, which is subsequently converted into bilirubin. The HO-1 isoform is upregulated by stimuli that cause oxidative stress and inflammation such as heavy metals[1,2], ischemia[3,4] and reactive oxygen species[5,6] to contribute to antioxidant and anti-inflammatory protection. Pre-clinical data indicate that CO mediates many of the biological actions of HO-1.[7,8] Exogenous administration of CO was investigated to protect the gastrointestinal tract upon diabetic gastroparesis, inflammatory bowel disease and organ transplantation.[9] Also bilirubin and biliverdin demonstrated a cytoprotective effect in experimental models of ischemia/reperfusion (I/R) injury[10,11], sepsis[12] and colitis.[13]

POI is a transient impairment of gastrointestinal motility, commonly seen after abdominal surgery. Normally it resolves within 3 days, but when prolonged, POI leads to increased morbidity and length of hospital stay.[14] The pathophysiology of POI is characterized by an acute neurogenic phase with activation of inhibitory motor pathways followed by a prolonged inflammatory phase. Activated resident macrophages in the muscular layer release inflammatory cytokines such as interleukin-6 (IL-6) and chemokines, resulting in increased expression of adhesion molecules and recruitment of circulatory leukocytes. These leukocytes produce prostaglandins and nitric oxide, inhibiting the neuromuscular contractile apparatus.[15,16] Laparoscopic procedures, enhanced recovery pathways and pharmacological treatment options such as prokinetics are advocated to prevent prolonged POI but no full protection is obtained.[17] Intestinal inflammation is now considered to be the primary target for novel treatment options.[18] Inhalation of CO was shown to suppress POI in mice and rats.[19,20] Also the CO releasing molecule CORM-3 improved the delay in transit and inflammation in murine POI, partially via intestinal upregulation of HO-1.[21]

The protective effects of CO and bilirubin/biliverdin suggest that pharmacologic induction of the HO-1 system may be the most general effective strategy to modulate inflammation. The

effectiveness of HO-1 induction via administration of hemin was already shown in gut I/R injury[22], colitis[23,24] and indomethacin-induced small intestinal injury[25] but was not yet explored for POI.

Dimethyl fumarate (DMF) has been approved for treatment of MS upon the demonstration of the efficacy and safety for management of relapsing forms of MS in phase III trials.[26] Experimental and human data suggest that DMF stabilizes nuclear (erythroid-derived 2) related factor (Nrf2), leading to transcription of antioxidant genes such as HO-1.[27,28] Other evidence suggests that DMF, independently of Nrf2, inhibits nuclear factor-κ B (NF-κB) and therefore reduces the production of proinflammatory cytokines.[29-31] Furthermore, in vitro studies in macrophages have shown that DMF is able to modulate the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase 1 and 2 (ERK 1/2), c-Jun N-terminal kinase (JNK) and p38.[32] In an in vitro study in BV2 microglial cells DMF showed one of the best HO-1 inducing/cytotoxicity profiles amongst the 56 compounds tested.[33] In murine colitis, oral administration of DMF resulted in inhibition of NF-κB translocation and reduction of inflammatory parameters.[34] The aim of the actual study was to investigate the influence of DMF and hemin on murine POI, and the involvement of HO-1; preliminary accounts of the results have been presented.[35,36]

IV.4. Materials and methods

IV.4.1. Materials

DMF (Sigma-Aldrich, Diegem, Belgium) was diluted in 0.08% (m/v) methocel (methylcellulose, Sigma-Aldrich)/H_2O when given intragastrically (i.g.) via gavage or dissolved in DMSO (26.8 mg mL^{-1}) followed by dilution in PBS when given intraperitoneally (i.p.). Hemin (Sigma-Aldrich) was solubilized in NaOH (0.1M) and diluted in PBS to 2.5 mg mL^{-1} after lowering the pH to 7.4 using HCl (0.1M). Cr(III) Mesoporphyrin IX chloride (CrMP; Frontier Scientific, Logan, UT, USA) was dissolved in 0.25% ammonium hydroxide and diluted in saline to 0.625 mg mL^{-1}. Isoflurane (IsoFlo®, Abbott Laboratories Ltd, Maidenhead, Berkshire, UK) was used to

anaesthetize the mice when inducing POI. Fluorescein-labelled dextran (70 kDa; Invitrogen, Merelbeke, Belgium) was used to measure postoperative gastrointestinal transit.

IV.4.2. Animals

Seven weeks old male C57BL/6J mice were purchased from Janvier, Le Genest St-Isle, France and were used between 8 to 12 weeks of age (22-25 g). Mice were housed in an animal care facility with a 12 h light/dark cycle and had free access to water and commercially available chow. Animal care and experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

IV.4.3. Surgical procedure

POI was induced by surgical manipulation of the small intestine (intestinal manipulation, IM). Mice were anaesthetized with inhaled isoflurane (induction, 5%; maintenance, 2%) and the abdomen was opened by midline laparotomy. The small intestine was exteriorized and then compressed for 5 min along its entire length by using sterile moist cotton swabs. The bowel was repositioned in the abdominal cavity and the incision was closed by two layers of continuous sutures. The total duration of the procedure was approximately 20-25 min. After the operation, mice were sacrificed at 1, 3, 6 or 24 h after induction of POI. The gastrointestinal tract from stomach until colon was then isolated. After measuring intestinal transit (see IV.4.5), the gastrointestinal tract was flushed with aerated (5 % CO_2 in O_2) ice-cold Krebs solution containing 1 mM phenylmethylsulfonyl fluoride. The small intestine was divided in 6 segments of equal length. After removal of the mucosa using a glass slide, the muscular layer was stored at -80°C until further analysis of neutrophil infiltration, IL-6 protein level or HO-1 protein level. Whole tissue sections of segment 3 of animals sacrificed 3 h after IM were fixed in formalin for 24 – 48 h before they were imbedded in paraffin in order to perform immunohistochemical staining of NF-κB p65; whole tissue sections of segment 3 of animals sacrificed 1 and 6 h after IM were stored at -80°C for later analysis of p-NF-kB p65 via Western blot.

IV.4.4. Experimental protocol

Mice were randomly assigned to four experimental groups. Group I served as control (non-treated, non-operated). Group II underwent surgical manipulation of the small intestine (IM). Group III and IV received DMF, respectively, i.p. (30 mg kg^{-1}) or i.g. (100 mg kg^{-1}) at 24 h before the surgical procedure. The dose/schedule selection was based on literature[34,37]. Mice were killed at 3, 6 or 24 h after surgery.

In a second set of experiments, seven groups were studied in parallel. Groups I-III were the same as in the first set. Group IV consisted of mice treated with the HO-1 inducer hemin (30 mg kg^{-1} i.p.) at 24 h before IM. Group V consisted of manipulated mice pre-treated with the HO-1 inhibitor CrMP (2.5 mg kg^{-1} i.p.) alone at 3 h before IM; group VI and VII consisted of manipulated mice pre-treated respectively with DMF or hemin i.p at 24 h before IM, and with CrMP at 3 h before IM. Mice were killed at 24 h after surgery. The dose/schedule selection for hemin[38] and CrMP[39] was based on literature.

In a third set of experiments, groups I-III of the first set were again studied to investigate MAPK and NF-kB activation. Mice were killed at 1 or 6 h after surgery.

The possible time-dependent effect of DMF and hemin on intestinal HO-1 expression was also studied. Mice were treated with DMF (i.p. or i.g.) or hemin (i.p.) and sacrificed at 6, 12 or 24 h after administration without undergoing IM.

IV.4.5. Measurement of intestinal transit

Intestinal transit was measured by evaluating the distribution of non-absorbable fluorescein-labeled dextran (70kDa; FD70) along the gastrointestinal tract.[40] 21 h after IM, the mice were deprived of food and 1.5 h hereafter a liquid fluorescein-labelled dextran meal (200 µl of a 25 mg mL^{-1} solution) was gavaged. 1.5 h later, i.e. 24 h after surgery, the animals were sacrificed. After excision of the gastrointestinal tract, two full-field images (one in normal illumination mode and another in fluorescent mode) were taken with a CCD camera and subsequently matched for calculation of fluorescence distribution along the gastrointestinal tract. The fluorescent

intensity throughout the entire gastrointestinal tract was analyzed and calculated. Data were expressed as the percentage of fluorescence intensity per segment (stom, stomach; sb, small bowel segments 1–10; caec, caecum; col, colon segments 1–2); the geometric center (GC) of the fluorescence distribution was calculated by the formula: ∑(% FD70 per segment*segment number)/100).

IV.4.6. Biochemical analyses

IV.4.6.1. MPO activity

Myeloperoxidase (MPO) activity in mucosa-free segments of the small intestine was measured as an index of neutrophil infiltration by optical densitometry using 3,3',5,5'-tetramethylbenzidine (TMB). This method was based on a previously described protocol.[41]

Frozen tissue samples were homogenized with a Mikro-Dismembrator and dissolved in 10 volumes of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 % hexadecyl-trimethylammonium bromide (HETAB). The homogenate was sonicated on ice (15 pulses of 0.7 s at full power) and subsequently subjected to freeze/thaw. The suspension was centrifuged (14000 g, 20 min, 4 °C) and 10 µL of the supernatant was added to 200 µL of assay mixture, containing ready-to-use TMB substrate, 0.5% HETAB, and 10 mM EDTA (on ice). The optical density was immediately read at 620 nm (Biotrak II). The reaction was then allowed to proceed for 3 min at 37 °C. The reaction was stopped by placing the 96-well plate on ice, and the optical density was measured again. One unit of MPO activity was defined as the amount of enzyme that produces a change in optical density of 1.0 per minute at 37 °C. Results were normalized to total protein content (Pierce BCA Protein Assay Kit) and expressed as U mg^{-1} protein.

IV.4.6.2. IL-6 protein level

Protein expression levels of IL-6 were determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol (Invitrogen, Merelbeke, Belgium).

Frozen tissue samples were homogenized with a Mikro-Dismembrator, dissolved in 10 volumes of 20 mM PBS buffer (pH 7.4) containing protease inhibitors (Complete Mini Protease Inhibitor EDTA-free tablets, Roche, Basel, Switzerland), and centrifuged at 10000 *g* for 15 min at 4°C. The supernatant (100 µL) was added to the appropriate microtiter wells, after which the plate was covered and incubated at room temperature. After 2 h, the solution was thoroughly aspirated from the wells and wells were washed with Wash Buffer. 100 µL of IL-6 Biotin Conjugate Solution was added to each well, after which the plate was covered and incubated at room temperature. After 30 min, the solution was aspirated from the wells and wells were washed with Wash Buffer. Streptavidin-HRP Working Solution (100 µL) was then added to each well, after which the plate was covered and incubated at room temperature. After 30 min, the solution was thoroughly aspirated from the wells and wells were washed with Wash Buffer. Stabilized Chromogen (100 µL) was then added to each well and incubated at room temperature in the dark. After 30 min, Stop Solution (100 µL) was added to each well and the absorbance was measured at 450 nm. Results were normalized to total protein content (Pierce BCA Protein Assay Kit) and expressed as pg mg^{-1} protein.

IV.4.6.3. HO-1 protein level

Protein expression levels of HO-1 were determined by ELISA, according to the manufacturer's protocols (Takara Bio, Shiga, Japan).

Frozen tissue samples were homogenized with a Mikro-Dismembrator, dissolved in 10 volumes of 1% NP40 in PBS containing protease inhibitors (Complete Mini Protease Inhibitor EDTA-free tablets, Roche, Basel, Switzerland), and centrifuged at 10000 *g* for 15 min at 4°C. The supernatant (100 µL), after dilution (1/10), was added to the appropriate microtiter wells, after which the plate was covered and incubated at room temperature. After 1 h, the solution was thoroughly aspirated from the wells and wells were washed with Wash Buffer. 100 µL of Avidin-POD Conjugate Solution was added to each well, after which the plate was covered and incubated at room temperature. After 1 h, the solution was aspirated from the wells and wells were washed with Wash Buffer. Substrate Solution (100 µL) was then added to each

well, after which the plate was covered and incubated at room temperature. After 15 min, the solution was thoroughly aspirated from the wells and wells were washed with Wash Buffer. Stop Solution (1N H_2SO_4, 100 µL) was added to each well and the absorbance was measured at 450 nm. Results were normalized to total protein content (Pierce BCA Protein Assay Kit) and expressed as pg mg^{-1} protein.

IV.4.6.4. ERK 1/2, p38 and JNK protein levels

Protein expression levels of ERK 1/2 (pT202/Y204 + total), p38 (pT180/Y182 + total) and JNK (pT183/Y185 + total) were determined by ELISA, according to the manufacturer's protocols (Abcam, Cambridge, UK).

Frozen tissue samples were homogenized with a Mikro-Dismembrator, dissolved in 5 volumes of chilled 1X Cell Extraction Buffer PTR containing protease inhibitors (Complete Mini Protease Inhibitor EDTA-free tablets, Roche, Basel, Switzerland), and incubated on ice for 20 min. Samples were centrifuged at 18000 *g* for 20 min at 4°C and the supernatant (50 µL), after dilution (1/50), was added to the appropriate microtiter wells. 50 µL of the Antibody Cocktail, containing both capture and detector antibodies, was added to each well after which the plate was covered and incubated at room temperature on a plate shaker set to 250 rpm. After 1 h, the solution was aspirated from the wells and wells were washed with Wash Buffer. TMB substrate (100 µL) was then added to each well, after which the plate was covered and incubated at room temperature on a plate shaker set to 250 rpm protected from light. After 15 min, Stop Solution (100 µL) was added to each well and the absorbance was measured at 450 nm. Results were expressed as the percentage of phosphorylated protein to total protein of ERK1/2, p38 or JNK.

IV.4.6.5. p-NF-κB p65 protein level

Protein expression levels of p-NF-κB p65 in whole tissue sections were determined by Western blot.

50 µg of protein from whole tissue homogenates were separated by 10% SDS-PAGE and proteins were then transferred to nitrocellulose membrane with 0.45 µM pores (Protran, Perkin Elmer, Zaventem, Belgium). The membranes were blocked in TBS/0.2% Tween 20 (TBST) with 5% bovine serum albumin (BSA) for 1 h at room temperature and probed with specific primary antibodies. The following antibodies were used: anti-p-NF-κB p65 (Ser536, 1/1000 in TBST with 5% BSA, #3033, Cell Signaling Technology, Beverly, MA, USA) and anti-β-actin (1/5000 in TBST with 5% milk powder, sc-47778, Santa Cruz biotechnologies, Santa Cruz, CA, USA). Secondary HRP-conjugated anti-rabbit IgG antibody was purchased from Thermo Fisher Scientific (1/5000 in TBST, 31464; Waltham, MA, USA). Proteins were detected using the Western Lightning ECL detection system (Perkin Elmer) according to the manufacturer's instructions. ImageJ (version 1.50i, Bethesda, MD, USA) was used to quantify each protein band and data were expressed as the ratio of p-NF-κB p65/β-actin.

IV.4.7. Immunohistochemical staining for NF- κB p65

Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded 5 µm tissue sections of the small intestine. The sections were deparaffinized and antigen retrieval was performed with citrate buffer (1M, pH 6.0) in a microwave oven for 2 x 5 min. The sections were incubated in 3% hydrogen peroxide for 10 min to quench endogenous tissue peroxidase and treated with blocking serum, containing 5% NSS, 1% BSA and 0.2% Tween 20, for 30 min. The tissue sections were then incubated with rabbit polyclonal antibody against NF-κB p65 (Abcam; ab16502), at 1:800 dilution overnight at 4°C. Sections were then covered with biotinylated swine anti-rabbit secondary antibody and incubated at room temperature for 30 min followed by incubation with streptavidin peroxidase solution for 30 min at room temperature. Immunohistochemical reactions were developed for 10 min with diaminobenzidine as the chromogenic peroxidase substrate, and slides were counterstained with hematoxylin.

Translocation of NF-κB p65 to the nuclei was analyzed by determining the ratio of the area of NF-κB p65 stained nuclei vs the total area of nuclei present within the selected region of the

muscular layer of the small intestine, using the Color Deconvolution ImageJ (version 1.50i, Bethesda, MD, USA) plugin; for each sample four images were analyzed and data were expressed as the percentage of NF-κB p65 staining of the total nuclear area.

IV.4.8. Statistical analysis

Data are given as mean ± s.e.m. as indicated; n refers to tissues obtained from different animals. The results were compared by one-way analysis of variance (ANOVA) or two-way ANOVA with time and treatment as the factors, followed by Bonferroni's multiple comparison t-test. Results were considered different from a P-value less than 0.05 on (Graphpad version 5.03). The Grubbs' test was used to determine significant outliers ($P<0.05$), that were excluded for further statistical analysis[42].

IV.5. Results

IV.5.1. Effect of DMF on manipulation-induced intestinal dysmotility

In non-manipulated control mice, fluorescein-labelled dextran had mainly moved to the middle part of the small bowel and to further segments. In mice that underwent intestinal manipulation, fluorescein-labelled dextran was mostly present in the more proximal parts of the small bowel, pointing out a delay in transit (Fig. IV.1A and B). This delay was confirmed by a significant reduction in GC (Fig. IV.1E). Pre-treatment with both 30 mg kg^{-1} DMF i.p. and 100 mg kg^{-1} DMF i.g. reduced the delay in transit caused by IM, as indicated by the shift of fluorescein-labelled dextran to the distal part of the small bowel (Fig. IV.1C and D) and the significant increase in GC (Fig. IV.1E).

IV.5.2. Effect of DMF on manipulation-induced inflammation

Leukocyte infiltration, measured as MPO activity, into the muscularis progressively increased from 3 to 24 h after IM. This IM-induced accumulation of leukocytes in the muscular layer was

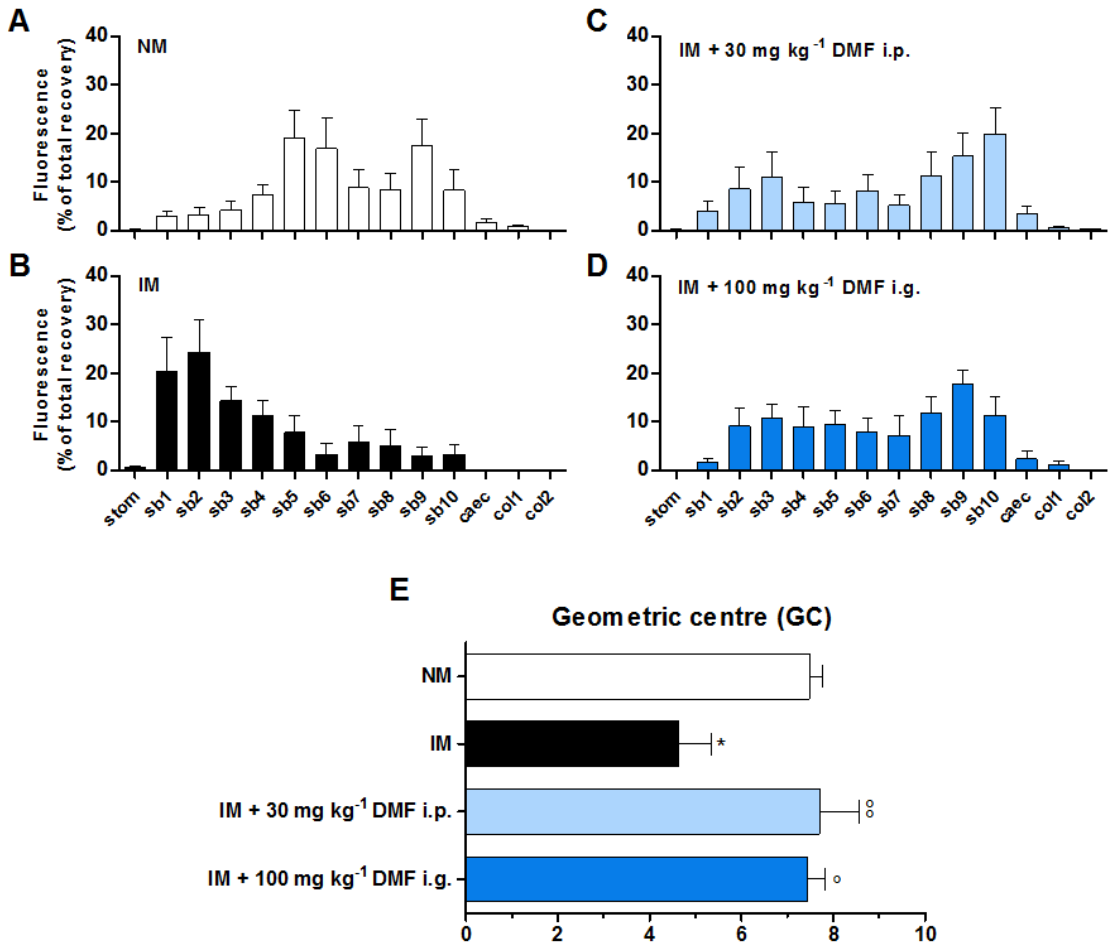

Figure IV.1 Influence of DMF on the delay in gastrointestinal transit caused by postoperative ileus. Transit histograms (A-D) and geometric centre (E) for the distribution of fluorescein-labelled dextran (70 kDa) along the gastrointestinal tract (stom, stomach; sb, small bowel segments; caec, caecum; col, colon segments), measured 24 h after intestinal manipulation (IM). Data represent the means ± S.E.M. of n = 6. *P<0.05 for comparison with non-manipulated (NM) control mice; °P<0.05, °°P<0.01 for comparison with non-treated IM mice: one-way ANOVA followed by a Bonferroni multiple comparison test.

markedly reduced when mice were pre-treated with DMF. 30 mg kg^{-1} DMF i.p. significantly reduced the increased MPO activity at 6 and 24 h after IM, whereas the reduction by 100 mg kg^{-1} DMF i.g. reached significance at 6 h after IM (Fig. IV.2A).

IL-6 protein level in the muscular layer was also significantly increased at 3, 6 and 24 h after IM, peaking at 3 h and then declining. Pre-treatment with both 30 mg kg^{-1} DMF i.p. and 100 mg kg^{-1} DMF i.g. resulted in a significant reduction of these elevated IL-6 levels at all three time points (Fig. IV.2B).

IV.5.3. Effect of DMF and hemin on HO-1 protein expression

Intestinal HO-1 protein expression in the muscularis was significantly upregulated at 24 h after IM when compared to control mice shown. Administration of DMF, both i.p. and i.g., had no significant effect on this elevated HO-1 protein level (Fig. IV.3A). To further explore whether DMF might increase the intestinal HO-1 protein level within the time interval between administration and the moment of surgery, we studied the effect of 30 mg kg^{-1} DMF i.p. and 100 mg kg^{-1} DMF i.g. on HO-1 protein expression in non-manipulated animals at 6 (only investigated for 30 mg kg^{-1} DMF i.p.), 12 and 24 h after administration. The influence of 30 mg

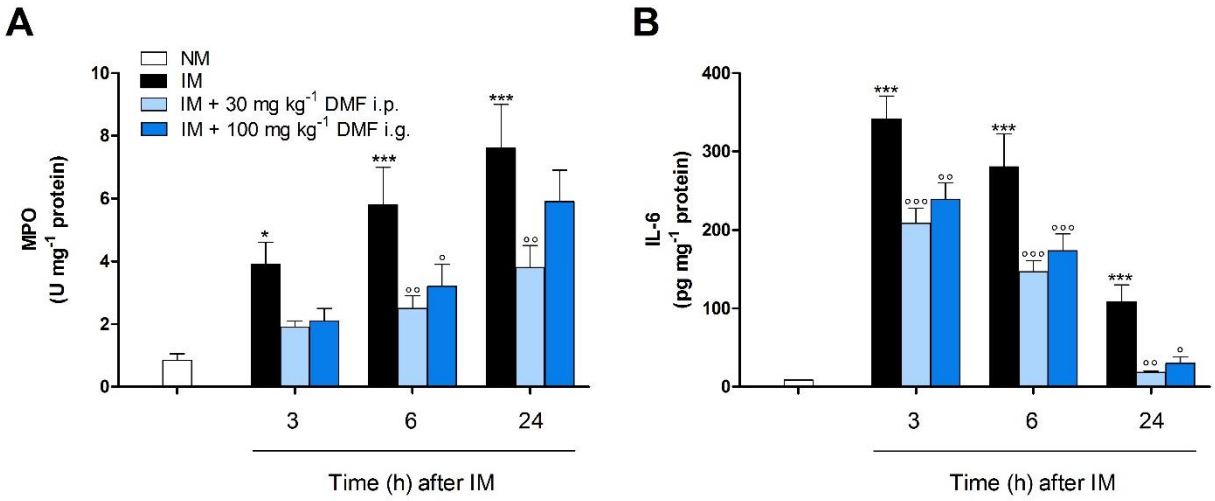

Figure IV.2 Influence of DMF on intestinal manipulation induced inflammation. Leukocyte infiltration (A; myeloperoxidase activity, MPO) and IL-6 protein level (B) in the muscular layer, measured 3, 6 and 24 h after intestinal manipulation (IM). Data represent the means ± S.E.M. of n = 6. *P<0.05, ***P<0.001 for comparison with non-manipulated (NM) control mice; °P<0.05, °°P<0.01, °°°P<0.001 for comparison with non-treated IM mice: two-way ANOVA followed by a Bonferroni multiple comparison test.

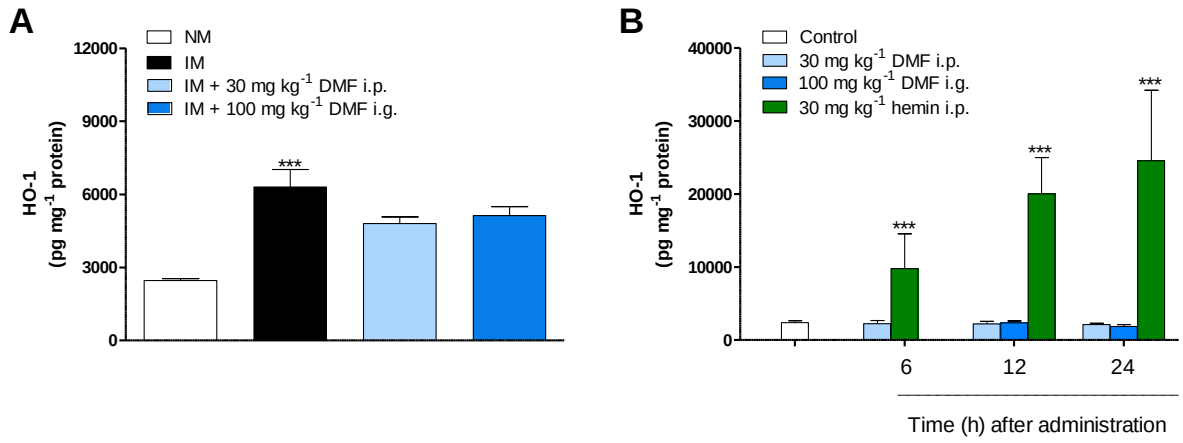

Figure IV.3 Influence of DMF and hemin on intestinal HO-1 expression. Effect of DMF on intestinal manipulation (IM) induced changes in HO-1 expression in the muscular layer, measured 24 h after IM (A). HO-1 protein expression in the intestinal muscularis at 6, 12 and 24 h after administration of DMF or hemin to non-manipulated animals (B). Data represent the means ± S.E.M. of n = 4-6. ***P<0.001 for comparison with non-manipulated (NM) control mice: one-way (A) or two-way (B) ANOVA followed by a Bonferroni multiple comparison test.

kg^{-1} hemin i.p. was studied in comparison. DMF did not increase HO-1 expression at 6, 12 and 24 h after administration whereas hemin resulted in a significant increase of HO-1 protein level at 6 h after administration, further increasing at 12 and 24 h (Fig. IV.3B).

IV.5.4. Investigation of the possible protective effect of hemin on murine POI and the role of HO-1

Similar to DMF, administration of 30 mg kg^{-1} hemin i.p. significantly improved the delayed transit and reduced the increased MPO activity and IL-6 protein level in the muscularis, observed in non-treated IM mice. The HO-1 inhibitor CrMP did not have an influence per se on transit and inflammation in operated mice but it completely inhibited the hemin-induced protection of gastrointestinal motility and inflammation in manipulated mice. In contrast to the results with hemin, co-administration of CrMP did not influence the protective effects of 30 mg kg^{-1} DMF i.p. in POI (Fig. IV.4A-C).

IV.5.5. Investigation of the possible role of MAPK in the protective effect of DMF

IM resulted in activation of the three subtypes of MAPK in the muscularis at 1 h after surgery, whereas at 6 h after IM, only ERK 1/2 activation was still significantly increased. Pre-treatment with 30 mg kg^{-1} DMF i.p. significantly reduced IM-induced ERK 1/2 activation both at 1 and 6 h after IM; it did not significantly influence p38 and JNK activation (Fig. IV.5A-C).

IV.5.6. Investigation of the possible role of NF-κB p65 in the protective effect of DMF

Immunohistochemical staining for NF-κB p65 showed that surgical manipulation of the intestine led to translocation of NF-κB p65 to the nuclei in the muscular layer at 3 h after IM. Administration of 30 mg kg^{-1} DMF i.p. significantly reduced this translocation of NF-κB p65 (Fig. IV.6A-D). Western blot analysis of p-NF-κB p65 in whole tissue segments confirmed the activation of NF-κB at 1 and 6 h after IM; 30 mg kg^{-1} DMF i.p. significantly reduced the IM-induced phosphorylation of NF-κB p65 both at 1 and 6 h after IM (Fig. IV.6E-F).

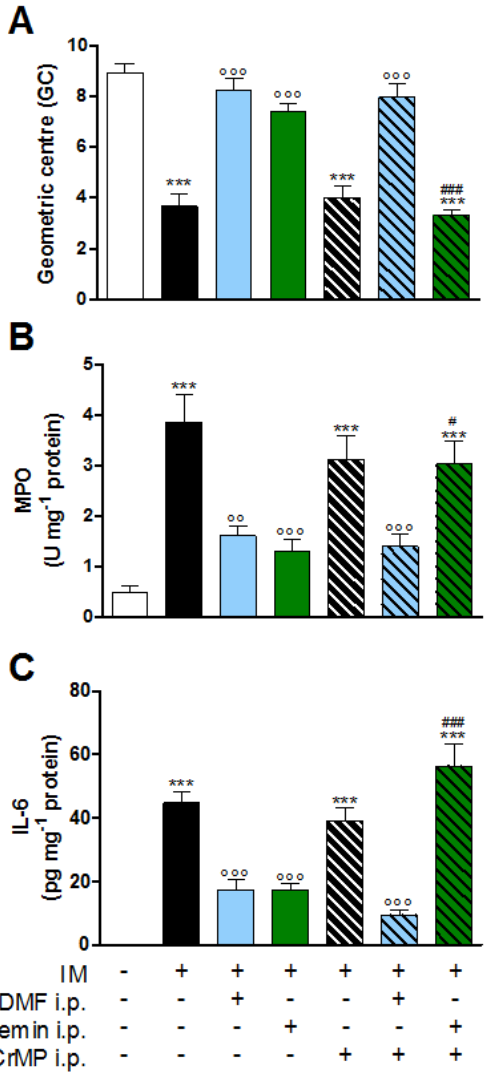

Figure IV.4 Influence of the HO-1 inhibitor CrMP on the effect of DMF and hemin versus manipulation-induced changes in gastrointestinal transit and inflammation. Geometric centre (A) for the distribution of fluorescein-labelled dextran (70 kDa) along the gastrointestinal tract, leukocyte infiltration (B, myeloperoxidase activity, MPO) and IL-6 protein level (C), measured 24 h after intestinal manipulation (IM). Data represent the means ± S.E.M. of n = 5-6. ***P<0.001 for comparison with non-manipulated (NM) control mice; °°P<0.01, °°°P<0.001 for comparison with non-treated IM mice; #P<0.05, ###P<0.001 for comparison with hemin-treated IM mice: one-way ANOVA followed by a Bonferroni multiple comparison test.

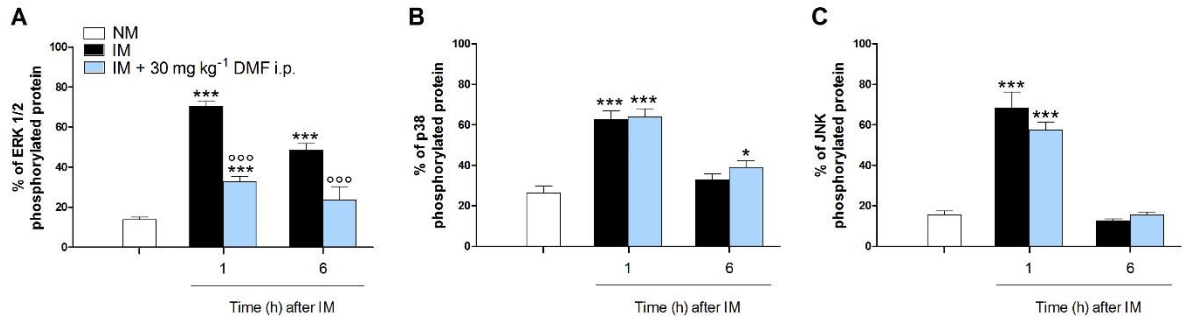

Figure IV.5 Influence of DMF on intestinal manipulation induced activation of MAPK. Percentage of phosphorylated protein of ERK 1/2 (A), p38 (B) and JNK (C) in the muscular layer, measured 1 and 6 h after intestinal manipulation (IM). Data represent the means ± S.E.M. of n = 6. *P<0.05, ***P<0.001 for comparison with non-manipulated (NM) control mice; °°°P<0.001 for comparison with non-treated IM mice: two-way ANOVA followed by a Bonferroni multiple comparison test.

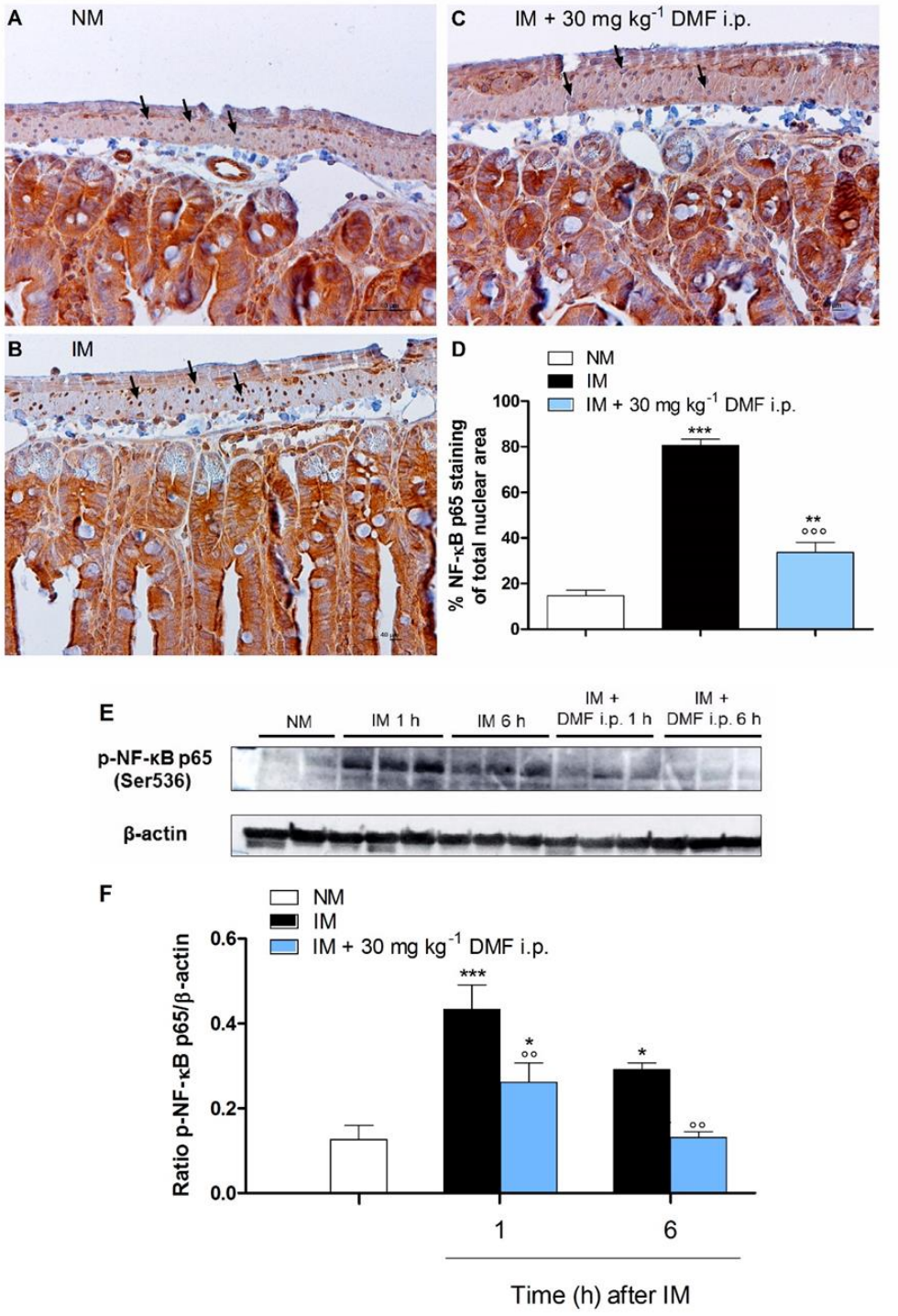

Figure IV.6 Influence of DMF on intestinal NF-κB p65 activation. Effect of DMF on intestinal manipulation (IM) induced changes in NF-κB p65 translocation to the nuclei in the intestinal muscular layer (D) and in phosphorylation of NF-κB p65 in whole tissue sections (F). Immunohistochemical staining of intestine sections of non-manipulated (NM) control mice (A), IM mice (B) and DMF-treated IM mice (C) measured at 3 h after IM. Black arrows indicate non-stained nuclei in panel A and C and positively stained nuclei in panel B. Representative Western blots for p-NF-κB p65 (Ser536) and β-actin in whole intestinal tissue of NM control mice, IM mice and DMF-treated IM mice measured at 1 and 6 h after IM (E). Data represent the means ± S.E.M. of n = 4-6; one sample of the non-treated IM group was excluded from the immunohistochemistry data as only the counterstaining with hematoxylin was visible and it did not show any staining for NF-κB p65, indicating a technical error. *P<0.05, **P<0.01, ***P<0.001 for comparison with NM control mice; °°P<0.01, °°°P<0.001 for comparison with non-treated IM mice: one- or two-way ANOVA followed by a Bonferroni multiple comparison test.

IV.6. Discussion

POI, a transient impairment of gastrointestinal motility, is a common complication seen after abdominal surgery in which intestinal inflammation plays a crucial role. Previous studies have shown that, upon surgical manipulation of the small intestine, activation of the dense network of resident macrophages in the muscularis externa leads to the production and release of inflammatory cytokines, chemokines and adhesion molecules.[15,16] This local molecular inflammatory response is followed by a cellular inflammatory phase with the recruitment of circulatory leukocytes into the muscularis and subsequent release of more pro-inflammatory mediators.[43] As reported previously for the murine model of POI[19,21,44], the inflammatory response when manipulating the murine small intestine was also confirmed in the present study. When measured at 3, 6 and 24 h after IM, the influx of neutrophils progressively increased with the highest value at 24 h while the intestinal level of the inflammatory cytokine IL-6 peaked already at 3 h after manipulation and then decreased, still being significantly enhanced at 24 h. The same time-dependent induction pattern of IL-6 protein/mRNA expression and MPO activity after IM is observed in other studies.[19,41,44]

Because of the importance of the inflammatory cascade in the pathogenesis of POI, it has become the primary target in the development of novel treatment options for POI. Induction of HO-1, the enzyme producing CO and biliverdin/bilirubin, with hemin has been shown to reduce inflammation in different body systems in a variety of animal models.[45-47] This also included gut inflammatory models such as I/R injury, indomethacin induced injury of the small intestine and colitis.[22,24,25] Hemin might also be efficient in postoperative ileus as inhalation of CO or administration of the CO releasing molecule CORM-3 improved the delay in transit and reduced intestinal inflammation upon IM in mice[19,21]; and biliverdin/bilirubin are effective in intestinal inflammatory models.[13,48]

In the present study, hemin was used in a dose of 30 mg kg^{-1} hemin i.p. as this dose was shown to counteract inflammation in different animal models.[22,25,38,45] As the time interval of

administration of hemin before the inflammatory stimulus varied in these studies, we first investigated the HO-1 protein expression in intestinal muscular tissue at 6, 12 and 24 h after administration of 30 mg kg^{-1} hemin i.p. in non-manipulated mice. This resulted in a time-dependent increase in HO-1 protein levels with the highest induction of HO-1 at 24 h after administration of hemin. We therefore administered 30 mg kg^{-1} hemin i.p. 24 h before intestinal manipulation; this reduced the development of POI in mice, as evidenced by the improvement in transit and the marked reduction of IL-6 protein level and leukocyte infiltration. To determine whether induction of HO-1, and, hence, the endogenous production of CO and bilirubin/biliverdin, is essential for hemin-mediated protection in POI, we investigated the influence of the HO inhibitor CrMP in our study. The dose of CrMP was selected according to the study of Liu et al. in which co-administration of 2.5 mg kg^{-1} CrMP completely abolished the protective effects of tropisetron, which correlated with HO-1 induction, on the increased hepatic inflammatory parameters seen 24 h after induction of hemorrhagic shock in rats.[39] Co-administration of CrMP completely abolished the previously shown protective effects of hemin on the delay in transit and increase in IL-6 and leukocyte infiltration caused by POI. These findings are in line with the study of Yoriki et al. in which co-administration of the HO inhibitor tin protoporphyrin abolished the beneficial effect of hemin on the ulceration seen in indomethacin-induced small intestinal injury in mice.[25] Similarly, co-administration of tin mesoporphyrin prevented the anti-inflammatory effect of hemin in retroperitoneal adipose tissue in obese rats with type 2 diabetes.[45] These results illustrate that HO-1 induction is indeed effective in preventing POI.

Hemin itself has not yet been tested in a clinical setting; only hematin, which differs from hemin in that the coordinating ion is a hydroxide ion instead of chloride ion, and heme arginate, in which heme is stabilized as a complex with arginine, are available for clinical use in the form of respectively Panhematin® and Normosang®. These drugs are approved respectively by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for treatment of acute attacks of porphyria, but their mechanism of action for this indication is inhibition of δ-

aminolevulinic acid synthase and not induction of HO-1.[49-51] Therefore, we also included DMF in our study as it has been approved for the treatment of MS by the FDA and EMA in 2013 and preclinical studies suggest that its immunosuppressive and neuroprotective effects are related to induction of HO-1.[27,52] Moreover, DMF was shown to exert one of the best HO-1 inducing/low toxicity profiles among 56 compounds in an in vitro study in BV2 microglia cells, in which hemin was used as an internal positive control.[33] DMF has been primarily tested in animal models of chronic diseases such as MS, requiring a daily administration of DMF for several weeks[53,54]. For prevention/treatment of POI, an acute dosing scheme would be optimal. Hence, we opted for a single administration of 30 mg kg^{-1} DMF i.p. at 24 h before IM, based on the study of Kunze et al. in which they tested the effect of DMF on focal cerebral ischemia in mice and illustrated that the greatest induction in HO-1 gene expression occurs after one day of treatment with 15 mg kg^{-1} DMF i.p. twice a day.[37] We also included 100 mg kg^{-1} DMF i.g. because of its proven beneficial effect in an experimental model of colitis, which is, similar to POI, also characterized by inflammation of the gastrointestinal tract.[34] In our study we have shown that pre-treatment with both 30 mg kg^{-1} DMF i.p. or 100 mg kg^{-1} DMF i.g. had a protective effect on POI in mice, equaling that of hemin. That the effect of the i.g. dose of DMF is only similar to that of the 3-fold lower i.p. dose might be related to the fact that DMF, when given i.g., is more exposed to esterases and enteric enzymes synthesized by epithelial cells of the small intestine, resulting in a higher degradation rate of DMF compared to DMF given i.p.[55] Although the metabolite monomethyl fumarate is also able to reduce the inflammatory response in murine splenocytes after stimulation with lipopolysaccharides, it is less potent and, unlike DMF, not able to inhibit NF-κB activity.[29]

In contrast to hemin, DMF did not increase HO-1 protein expression in the intestinal muscular layer of non-manipulated mice at 6, 12 or 24 h after administration; it also did not further increase the elevated HO-1 protein levels seen in manipulated mice 24 h after IM as previously has been shown with administration of 40 mg kg^{-1} CORM-3 i.p.[21] Additionally, as tested for i.p. DMF, the HO-1 inhibitor CrMP did not influence the preventive effect on POI. The lack of HO-1

induction by DMF is in contrast with many in vitro and in vivo studies showing a HO-1 inducing effect of DMF. As mentioned above, most of these in vivo studies were performed in chronic animal models with progressive chronic inflammation such as MS. In these studies animals were treated with DMF for up to 36 days after induction of MS, resulting in increased expression of HO-1 and beneficial anti-inflammatory effects.[53,56-58] POI on the other hand is characterized by an acute inflammatory cascade, similar to for example I/R injury. Oral administration of 25 mg kg^{-1} DMF twice a day for 2 days before induction of liver I/R injury in rats resulted in a significant reduction of MPO activity and NF-κB protein expression in liver tissue of DMF treated animals compared to non-treated animals. However, administration of DMF for only 2 days did not result in a significant increase in HO-1 protein levels.[59] Similar beneficial effects of DMF were observed in a rat model of myocardial I/R injury after administration of 10 mg kg^{-1} DMF intravenously 90 minutes, and immediately before induction of myocardial ischemia. The authors did however not measure and/or report any effect of DMF on HO-1 induction, but assigned the protective effects of DMF to its potential to inhibit NF-κB activity.[30] Furthermore, in a murine model of experimental colitis, it was shown that administration of both 30 and 100 mg kg^{-1} of DMF by oral gavage for 4 days after induction of colitis caused a substantial reduction in the degree of colon injury and MPO activity. But only the dosage of 100 mg kg^{-1} of DMF resulted in a significant increase in colonic nuclear Nrf2 protein levels, which regulate the expression of HO-1; whereas both doses were able to significantly reduce colonic NF-κB p65 activity. Taken together, these data might indicate that in vivo, induction of HO-1 by DMF requires a higher dose and/or longer treatment duration; whereas inhibition of NF-κB might already occur at a lower dose and/or shorter treatment duration.[34] The NF-κB inhibiting effect of DMF was also reported by Gillard et al.; in their study DMF inhibited nuclear translocation of NF-κB p65 in U-2 OS cells and inhibited NF-κB-driven cytokine production in murine splenocytes, and doing so in an Nrf2-independent manner.[29] Correspondingly, DMF is equally effective in an experimental model of autoimmune encephalomyelitis in both wild-type and Nrf2-deficient mice.[31] Although little is known about the exact mechanism of action by which DMF inhibits NF-κB p65, recent in vitro evidence in

breast cancer cells suggests that DMF prevents NF-κB p65 nuclear translocation and attenuates its DNA binding activity via covalent modification of NF-κB p65 and has no effect on upstream proteins in the NF-κB pathway.[60] Immunohistochemical staining for NF-κB p65 in whole tissue intestinal sections in the present study also suggests that 30 mg kg^{-1} DMF i.p. has an inhibiting effect on the increase in nuclear NF-κB p65 translocation in the intestinal muscularis at 3 h after IM. The increase in nuclear NF-κB p65 translocation by IM seen with immunohistochemistry corresponds with studies where activation of NF-κB p65 during POI was measured at 1.5 and 6 h after IM via electrophoretic mobility shift assay.[61,62] This result could not be confirmed by analysis of NF-κB p65 in the nuclear fraction of muscular intestinal tissue via ELISA or Western blot in preliminary experiments (results not shown). However, evaluation of the phosphorylation of NF-κB p65 in whole tissue segments showed that 30 mg kg^{-1} DMF i.p was able to reduce the IM-induced increase in phosphorylation of NF-κB p65 at 1 and 6 h after IM, confirming that DMF inhibits IM-induced NF-κB p65 activation.

To further explore the molecular mechanisms underlying the beneficial effects of DMF in POI, we examined the possible involvement of ERK 1/2, p38 and JNK MAPK signaling pathways. These MAPK are activated by a wide range of extracellular stimuli and have been shown to be key regulators of pro-inflammatory mediators, such as cytokines and chemokines.[63] In the present study administration of 30 mg kg^{-1} DMF i.p. led to a significant reduction of the IM-induced activation of ERK 1/2 at 1 h and 6 h after IM, but showed no effect on the IM-induced activation of p38 and JNK at 1 h after IM. These findings for ERK 1/2 are in line with previous in vitro studies showing an inhibiting effect of DMF on ERK 1/2 activation in microglial cells[64] and neutrophils[65] stimulated with LPS. Inhibition of both NF-κB and ERK 1/2 activation by DMF as observed in the present study was reported for LPS-stimulated bone marrow-derived and RAW264.7 macrophages.[32] In summary, we demonstrated that both DMF and hemin prevent POI in mice by reduction of IM-induced intestinal muscle inflammation. The protective effects of hemin are mediated through induction of HO-1, supporting the proposed strategy of HO-1 induction to counteract the inflammation caused by IM. The beneficial effects of DMF on the

other hand seem to occur independently of HO-1, but are related to inhibition of NF-κB and ERK 1/2 activation. Taken together, these data indicate that hemin and DMF may be useful compounds for the management of POI in the clinical setting.

IV.7. Acknowledgments, Conflict of interest and Author contribution

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No competing interests declared

RL designed the study. JVD performed the experiments and data analysis. Immunohistochemical staining was done by LP and Western blotting by EVN. RL and JVD interpreted the findings and prepared and completed the manuscript.

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CHAPTER V

THE H_2S-RELEASING NAPROXEN DERIVATIVE ATB-346 AND THE SLOW RELEASE H_2S DONOR GYY4137 REDUCE INTESTINAL INFLAMMATION AND RESTORE TRANSIT IN POSTOPERATIVE ILEUS

Jonas Van Dingenen[1], Leen Pieters[2], Anne Vral[2], Romain A. Lefebvre[1]

[1] Department of Basic and Applied Medical Sciences, Heymans Institute of Pharmacology, Faculty of Medicine & Health Sciences, Ghent University, Gent, Belgium

[2] Department of Human Structure and Repair, Faculty of Medicine & Health Sciences, Ghent University, Gent, Belgium

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CHAPTER V

The H_2S releasing naproxen derivative ATB-346 and the slow release H_2S donor GYY4137 reduce intestinal inflammation and restore transit in postoperative ileus

V.1. Abstract and keywords

Objective: Intestinal inflammation triggers postoperative ileus (POI), commonly seen after abdominal surgery and characterized by impaired gastrointestinal transit; when prolonged, this leads to increased morbidity. Hydrogen sulfide (H_2S) is recognized as an important mediator of many (patho)physiological processes, including inflammation, and is now investigated for anti-inflammatory application. Therefore the aim of this study was to investigate the effect of the H_2S-releasing naproxen derivative ATB-346, developed to reduce gastrointestinal injury by naproxen, and the slow-release H_2S donor GYY4137 on intestinal inflammation and delayed gastrointestinal transit in murine POI.

Methods: C57Bl6J mice were fasted for 6 h, anesthetized and after laparotomy, POI was induced by compressing the small intestine with two cotton applicators for 5 min (intestinal manipulation; IM). GYY4137 (50 mg/kg, intraperitoneally), ATB-346 (16 mg/kg, intragastrically) or naproxen (10 mg/kg, intragastrically) were administered 1 h before IM. At 24 h postoperatively, gastrointestinal transit was assessed via fluorescent imaging, and mucosa-free muscularis segments were prepared for later analysis. Inflammatory parameters and activity of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase (COX)-2 were measured. Histological examination of whole tissue sections was done on hematoxylin-eosin stained slides.

Results: Pre-treatment with GYY4137 (geometric center; GC: 7.6 ± 0.5) and ATB-346 (GC: 8.4 ± 0.3) prevented the delayed transit induced by IM (GC: 3.6 ± 0.5 versus 9.0 ± 0.4 in non-operated controls) while naproxen only partially did (GC: 5.9 ± 0.5; n = 8 for all groups).

GYY4137 and ATB-346 significantly reduced the IM-induced increase in muscular myeloperoxidase (MPO) activity and protein levels of interleukin (IL)-6, IL-1β and monocyte chemotactic protein 1; the reduction by naproxen was less pronounced and only reached significance for MPO activity and IL-6 levels. All treatments significantly reduced the increase in COX-2 activity caused by IM, whereas only GYY4137 significantly reduced the increase in iNOS activity. Naproxen treatment caused significant histological damage of intestinal villi.

Conclusion: The study shows that naproxen partially prevents POI, probably through its inhibitory effect on COX-2 activity. Both ATB-346 and GYY4137 were more effective, the result with GYY4137 showing that H_2S per se can prevent POI.

Key words: ATB-346, GYY4137, hydrogen sulfide, naproxen, postoperative ileus

V.2. Introduction

Postoperative ileus (POI) refers to the transient impairment of gastrointestinal motility, which commonly occurs after abdominal surgery. It presents clinically as abdominal distension, inability to tolerate an oral diet, absence of bowel sounds and lack of flatus and defecation. Normally it resolves within three days, but when prolonged, nausea and vomiting will further contribute to increased morbidity, length of hospital stay and increased healthcare costs (Boeckxstaens and de Jonge, 2009). In the United States alone, the annual socio-economic impact of prolonged POI is estimated up to 1.46 billion US$ (Goldstein et al., 2007). POI upon intra-abdominal manipulation is induced by an acute neurogenic reaction with activation of inhibitory neural pathways and more importantly by a following prolonged inflammatory reaction triggered in the muscularis externa. Activated resident macrophages in the muscular layer play a critical role as they release inflammatory cytokines such as interleukin (IL)-6 and chemokines such as monocyte chemoattractant protein-1 (MCP-1), resulting in an increased endothelial expression of adhesion molecules such as intercellular adhesion molecule-1 and recruitment of circulatory leukocytes. Nitric oxide and prostaglandins released from these inflammatory cells will directly impair the smooth muscle contractility causing the delay in gastrointestinal transit (Boeckxstaens and de Jonge, 2009; Wehner et al., 2012). Laparoscopic procedures, enhanced recovery pathways and pharmacological treatment options such as prokinetics and non-steroidal anti-inflammatory drugs (NSAIDs) are advised to prevent prolonged POI but no full protection is obtained (Person and Wexner, 2006; Augestad, 2010; Bragg et al., 2015). Therefore there is need for new treatment options, especially those targeting the intestinal inflammation (van Bree et al., 2012).

Hydrogen sulfide (H_2S), primarily known as a toxic environmental gas, is endogenously produced in mammalian tissue from cysteine, largely via three enzymes: cystathionine γ-lyase, cystathionine β-synthetase and 3-mercaptosulfurtransferase (Rose et al., 2017). H_2S biosynthesis has been identified in a variety of mammalian tissues including lung, liver, heart

and intestine and it has become clear that H_2S, next to other endogenous gases like nitric oxide and carbon monoxide, plays an important role in both physiological and pathophysiological processes (Chen et al., 2007; Olas, 2015). Its role in inflammation is possibly one of the more controversial areas of the H_2S biology as there are a lot of conflicting data concerning the pro- and/or anti-inflammatory properties of exogenous H_2S. For example, administration of sodium hydrosulfide (NaHS), an H_2S donor, was shown to inhibit aspirin-induced leukocyte adherence in mesenteric venules and reduced the leukocyte infiltration in an air pouch model in rats (Zanardo et al., 2006). In contrast, pre-treatment of mice with NaHS was shown to significantly enhance the lipopolysaccharide (LPS)-induced leukocyte adhesion, neutrophil migration and expression of adhesion molecules like P-selectin and intercellular adhesion molecule-1 in venular endothelium (Dal-Secco et al., 2008). One reason for the lack of clarity is the reliance on NaHS as an H_2S donor in many studies; sulfide salts like NaHS, dissolved in aqueous solutions, will release large amounts of H_2S within seconds (Li et al., 2008). Although the precise kinetic profile of endogenous H_2S release within individual tissues has yet to be evaluated, it is likely that the controlled enzymatic H_2S synthesis occurs at a much slower rate and in lesser amounts. Therefore, NaHS may not mimic the biological effects of endogenously produced H_2S and, depending on the used dosage, may even exert toxic effects (Rose et al., 2015). In contrast to sulfide salts, GYY4137 [morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate] releases H_2S slowly both *in vitro* and *in vivo* for several hours, better mimicking the time course of naturally produced H_2S, making this H_2S donor more suitable to investigate the effect of exogenous H_2S (Li et al., 2008; Lee et al., 2011). The anti-inflammatory effects of GYY4137 have already been shown in a variety of animal models including myocardial (Meng et al., 2015; Karwi et al., 2016; Qiu et al., 2018) and intestinal (Jensen et al., 2018) ischemia/reperfusion injury, LPS-induced endotoxemia (Li et al., 2009; Chen et al., 2016), atherosclerosis (Liu et al., 2013; Xie et al., 2016) and cisplatin-induced nephrotoxicity (Cao et al., 2018) whereby GYY4137 was able to reduce myeloperoxidase (MPO) activity and the level of pro-inflammatory cytokines such as IL-1β, IL-6, tumor necrosis factor-α and interferon (IFN)γ.

As mentioned earlier, NSAIDs are already used to treat pain and inflammation during prolonged POI allowing to spare opioids (Person and Wexner, 2006). However, NSAIDs, which suppress synthesis of prostaglandins by inhibiting cyclooxygenase (COX), are a major cause of gastric and duodenal ulceration and have been shown to also injure more distal parts of the small intestine, where the damage is more difficult to detect and treat (Wallace et al., 2011; Takeuchi and Satoh, 2015). It has been shown that H_2S-releasing NSAIDs like ATB-346 [2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester], being the H_2S-releasing derivative of naproxen, reduce the NSAID stimulated gastric leukocyte adhesion and protect the mucosa from ulceration (Wallace, 2007; Ekundi-Valentim et al., 2013). Moreover, released H_2S will contribute to the overall anti-inflammatory effect of ATB-346; multiple studies have demonstrated a superior anti-inflammatory effect of ATB-346 when compared to naproxen as ATB-346 was able to reduce several inflammatory parameters like leukocyte infiltration, COX-2 activity and expression of IL-1β and tumor necrosis factor-α more effectively (Wallace et al., 2010; Campolo et al., 2013; Dief et al., 2015; Magierowski et al., 2017).

In the present study, we investigate the effect of a single dose of GYY4137 and ATB-346 on the delay in transit and inflammation caused by POI in mice and compare their effect with that of naproxen.

V.3. Materials and methods

V.3.1. Materials

GYY4137 (Tocris Bioscience, Bristol, UK) was dissolved in saline (6.25 mg/mL) and ATB-346 (2 mg/mL; Axon Medchem, Groningen, Netherlands) and naproxen (1.25 mg/mL; Sigma-Aldrich, Diegem, Belgium) were suspended in 0.5% (m/v) methocel (methylcellulose, Sigma-Aldrich)/H_2O. Isoflurane (IsoFlo®, Abbott Laboratories Ltd, Maidenhead, Berkshire, UK) was used to anaesthetize the mice when inducing POI. Fluorescein-labelled dextran (70 kDa; Invitrogen, Merelbeke, Belgium) was used to measure postoperative gastrointestinal transit.

V.3.2. Animals

Seven weeks old male C57BL/6J mice were purchased from Janvier, Le Genest St-Isle, France and were used between 8 to 12 weeks of age (22-25 g). Mice were housed in an animal care facility with a 12 h light/dark cycle and had free access to water and commercially available chow. Animal care and experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University (ECD 16-48).

V.3.3. Surgical procedure

POI was induced by surgical manipulation of the small intestine (intestinal manipulation, IM) as previously applied by several groups (Kalff et al., 2000; De Jonge et al., 2003; Cosyns et al., 2015). Mice were anaesthetized with inhaled isoflurane (induction, 5%; maintenance, 2%) and the abdomen was opened by midline laparotomy. The small intestine was exteriorized and then compressed for 5 min along its entire length by using sterile moist cotton swabs. The bowel was repositioned in the abdominal cavity and the incision was closed by two layers of continuous sutures. The total duration of the procedure was approximately 20-25 min. After the operation, mice were sacrificed at 24 h after induction of POI. The gastrointestinal tract from stomach until colon was then isolated. After measuring intestinal transit (see Section V.3.5 “Measurement of Intestinal Transit”), the gastrointestinal tract was flushed with aerated (5 % CO_2 in O_2) ice-cold Krebs solution containing 1 mM phenylmethylsulfonyl fluoride. The small intestine was divided in 6 segments of equal length. Whole tissue sections of segment 3 were fixed in formalin for further histological examination. The mucosa was removed in the other segments using a glass slide and the muscular layer was stored at -80°C until further analysis.

V.3.4. Experimental protocol

Mice were randomly assigned to five experimental groups (n = 8 per group). Group I served as control (non-treated, non-operated). Group II underwent surgical manipulation of the small intestine (IM). Group III to V received respectively GYY4137 intraperitoneally (i.p., 50 mg/kg), naproxen intragastrically (i.g., 10 mg/kg) and ATB-346 i.g. (16 mg/kg) at 1 h before the surgical

procedure. All animals were deprived of food from 6 h before IM but had again access to food after IM. At 21 h after surgery, mice were again deprived of food to allow measurement of intestinal transit (see section V.3.5 “Measurement of Intestinal Transit”). Mice were sacrificed at 24 h after surgery. Figure V.1 shows a schematic overview of the protocol.

V.3.5. Measurement of intestinal transit

Intestinal transit was measured by evaluating the distribution of non-absorbable fluorescein-labeled dextran (70kDa; FD70) along the gastrointestinal tract as previously described (De Backer et al., 2008). 21 h after IM, the mice were deprived of food and 1.5 h hereafter a liquid fluorescein-labelled dextran meal (200 µl of a 25 mg/mL solution) was gavaged. 1.5 h later, i.e. 24 h after surgery, the animals were sacrificed. After excision of the gastrointestinal tract, two full-field images (one in normal illumination mode and another in fluorescent mode) were taken with a CCD camera and subsequently matched for calculation of fluorescence distribution along the gastrointestinal tract. The fluorescent intensity throughout the entire gastrointestinal tract was analyzed and calculated. Data were expressed as the percentage of fluorescence intensity per segment (stom, stomach; sb, small bowel segments 1–10; caec, caecum; col, colon segments 1–2). The geometric center (GC) of the fluorescence distribution, which can be described as the center of gravity for the distribution of fluorescein, was calculated by the formula: ∑(% FD70 per segment*segment number)/100). It can range from

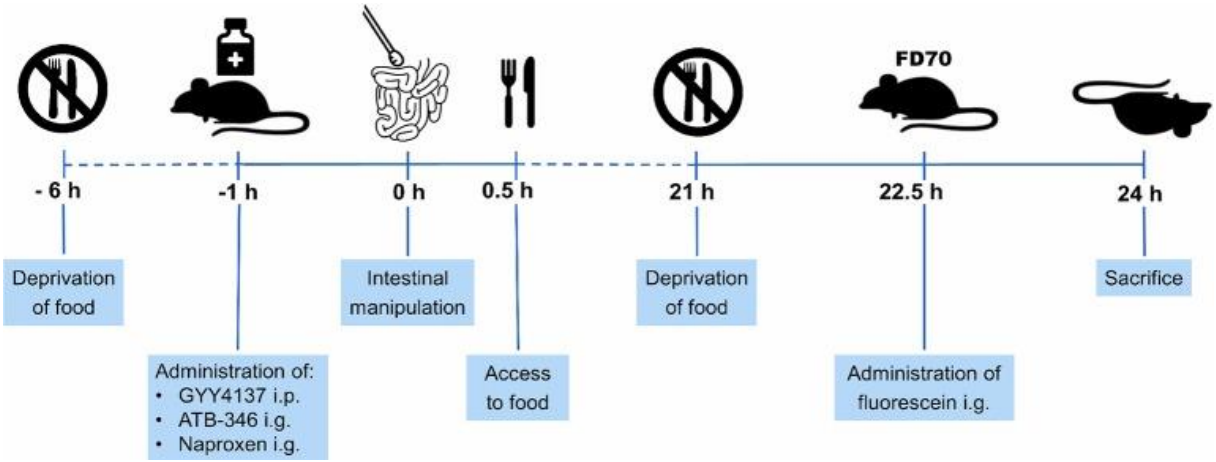

Figure V.1 Schematic overview of experimental protocol. Mice were deprived of food from 6 h before intestinal manipulation (IM) and given GYY4137, ATB-346 or naproxen 1 h before IM. When mice recovered from surgery, they had again access to food. Mice were deprived of food at 21 h after IM and received 200 µl of fluorescein-labeled dextran (FD70) at 22.5 h after IM. 1.5 h later, mice were sacrificed followed by measurement of the gastrointestinal transit and collection of tissue samples of the small intestine.

1, if all fluorescein were found in the stomach, to 14, if all fluorescein were found in the second segment of the colon.

V.3.6. Biochemical analyses

V.3.6.1. MPO activity

MPO activity in mucosa-free segments of the small intestine was measured as an index of neutrophil infiltration, based on a previously described protocol (De Jonge et al., 2003).

Frozen tissue samples were homogenized with a Mikro-Dismembrator and dissolved in 10 volumes of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 % hexadecyl-trimethylammonium bromide (HETAB). The homogenate was sonicated on ice (15 pulses of 0.7 s at full power) and subsequently subjected to freeze/thaw. The suspension was centrifuged (14000 *g*, 20 min, 4 °C) and 10 µL of the supernatant was added to 200 µL of assay mixture, containing ready-to-use 3,3',5,5'- tetramethylbenzidine substrate, 0.5% HETAB, and 10 mM EDTA (on ice). The optical density was immediately read at 620 nm (Biotrak II). The reaction was then allowed to proceed for 3 min at 37 °C. The reaction was stopped by placing the 96-well plate on ice, and the optical density was measured again. One unit of MPO activity was defined as the amount of enzyme that produces a change in optical density of 1.0 per minute at 37 °C. Results were normalized to total protein content (Pierce BCA Protein Assay Kit) and expressed as U/mg protein.

V.3.6.2. IL-1β, IL-6, IL-10, IFNγ and MCP-1 protein levels

Protein expression levels of IL-1β, IL-6, IL-10, IFNγ and MCP-1 in mucosa-free segments of the small intestine were assessed by a Luminex magnetic bead assay according to the manufacturer's guidelines (Bio-Rad, Temse, Belgium); the samples were processed using the BioPlex Pro Reagent Kit (Bio-Rad).

Frozen tissue samples were homogenized in PBS, containing protease and phosphatase inhibitors. The homogenate was sonicated on ice (5 pulses of 0.7 s) and after centrifugation (15000 rpm, 15 min, 4 °C), 50 µL of the supernatant was added to the corresponding well of a

96-well plate loaded with coupled beats for IL-1β, IL-6, IL-10, IFNγ and MCP-1. After placing the plate for 30 min on a shaking platform at 850 rpm protected from light, wells were washed and 25 µL of the mixture of detection antibodies for IL-1β, IL-6, IL-10, IFNγ and MCP-1 was added to each well. After placing the plate for another 30 min on the shaking platform at 850 rpm protected from light, wells were washed and 50 µL of Streptavidin Phycoerythrin solution was added to each well. After placing the plate for 10 min on the shaking platform at 850 rpm protected from light, wells were washed and the beads in each well were re-suspended in 125 µL assay buffer. After a last incubation step of 30 sec, the wells were analyzed for IL-1β, IL-6, IL-10, IFNγ and MCP-1 protein concentration using Bio-Plex Manager Software (Bio-Rad). Data from tissue samples were normalized to the total protein content (Pierce BCA Protein Assay Kit).

V.3.6.3. COX-2 activity

COX-2 enzyme activity was measured in mucosa-free segments by COX activity assay (Cayman Chemical) using arachidonic acid as a substrate and 10-acetyl-3,7-dihydroxy-phenoxazine (ADHP) as a fluorometric co-substrate. The reaction between COX-derived prostaglandin G2 and ADHP produces the highly fluorescent compound resorufin, which was measured.

Frozen tissue samples were homogenized with a Mikro-Dismembrator, dissolved in 5 volumes of 100 mM Tris-HCl buffer (pH 7.5), containing protease inhibitors, and centrifuged at 10000 *g* for 15 min at 4 °C. 10 µL of the supernatant was transferred to the appropriate well containing 140 µL of 1X Assay Buffer (100 mM Tris-HCl, pH 8.0), 10 µL of Heme in DMSO and 10 µL of the COX-1 inhibitor SC-560 (66µM). After an incubation period of 5 min, 10 µL of ADHP Assay Reagent was added to each well. Administration of 10 µL of a 2 mM arachidonic acid solution to each well initiated the reaction; 1 min later the plate was measured using a spectrofluorometer (excitation wavelength: 530-540 nm, emission wavelength: 585-595 nm; Victor Wallac, PerkinElmer Life and Analytical Sciences). Background values were measured by not adding 10 µL of the arachidonic acid solution to the assigned wells and were deducted

from the corresponding sample values. Results were normalized to total protein content (Pierce BCA Protein Assay Kit) and COX-2 activity was expressed as % of mean fluorescence in tissue from non-manipulated control mice.

V.3.6.4. iNOS activity

Inducible NO synthase (iNOS) enzyme activity in intestinal muscularis samples was assayed by measuring the conversion of [^{3}H]-arginine to [^{3}H]-citrulline by use of a NOS activity assay (Cayman Chemical), according to the manufacturer's protocol. The assay was conducted in calcium-free conditions to measure only iNOS.

Frozen tissue samples were homogenized with a Mikro-Dismembrator, dissolved in five volumes of 1X Homogenization Buffer and centrifuged at 10000 *g* for 15 min at 4 °C. 10 µl of the supernatant was added to 40 µl reaction mix (22.72 µL of Reaction Buffer [50 mM Tris-HCl (pH 7.4), 6 µM tetrahydrobiopterin, 2 µM flavin adenine dinucleotide, 2 µM flavin adenine mononucleotide], 4.55 µL of 10 mM NADPH (prepared in 10 mM Tris-HCl), 0.90 µL of [^{3}H]-arginine (1 µCl/µL), 4.55 µL 8 mM Mg acetate, 3.64 µL calmoduline and 3.64 µL ddH_2O). The reaction samples were incubated for 1 h at room temperature, and the reaction was stopped by adding 400 µL of Stop Buffer to the reaction sample. 100 µL of the equilibrated resin was added into each reaction sample and the reaction samples were then transferred in the provided spin cups, which were centrifuged for 30 s in a microcentrifuge at full speed. The eluate was then transferred to scintillation vials, and, after adding 2 mL scintillation solution (Ultima Gold, Canberra Packard, USA) to each vial, the radioactivity was quantified in a liquid scintillation counter (Packard Tri-Carb 2100 TR, Canberra Packard, USA). Results were normalized to total protein content (Pierce BCA Protein Assay Kit) and iNOS activity was expressed as % of [^{3}H]-citrulline in tissue from non-manipulated control mice.

V.3.7. Intestinal histological evaluation

Intestinal samples were fixed in formalin, dehydrated in a graded ethanol series and embedded in paraffin. Tissue sections of 5 µm were cut with a microtome and then stained with

hematoxylin-eosin. Jonas Van Dingenen and Leen Pieters independently scored the slides to evaluate intestinal mucosal damage using the Chiu score (Chiu et al., 1970): grade 0, normal mucosa villi; grade 1, development of sub-epithelial Gruenhagen's space at the apex of the villi; grade 2, extension of the sub-epithelial space with moderate epithelial lifting; grade 3, massive epithelial lifting, possibly with a few denuded villi; grade 4, denuded villi with lamina propria and exposed capillaries; and grade 5, disintegration of the lamina propria, ulceration and hemorrhage. For each sample, 100 villi were scored by both evaluators and the mean score per sample was calculated. Good correlation between the scores of the two evaluators is illustrated by a Pearson's r-value of 0.8324.

V.3.8. Statistical analysis

Data are given as mean ± s.e.m. as indicated; n refers to tissues obtained from different animals. The results were compared by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison t-test for all manipulated groups versus the non-operated control group and for the treated manipulated groups versus the non-treated manipulated group. Results were considered different from a P-value less than 0.05 on (Graphpad version 5.03, San Diego, CA, USA). The Grubbs' test was used to determine significant outliers ($P<0.05$), that were excluded for further statistical analysis (Grubbs, 1969). The Pearson correlation test was used to investigate the correlation between the scores of the two evaluators performing the intestinal histological evaluation.

V.4. Results

V.4.1. Effect of GYY4137, naproxen and ATB-346 on manipulation-induced intestinal dysmotility

In non-manipulated control mice, fluorescein-labelled dextran had mainly moved to the distal part of the small bowel (Fig. V.2A). In mice that underwent intestinal manipulation, fluorescein-labelled dextran was mostly present in the proximal part of the small bowel, pointing out a delay in transit (Fig. V.2B), which was confirmed by a significant reduction in GC (Fig. V.2F). Pre-treatment with 50 mg/kg GYY4137 i.p. or 16 mg/kg ATB-346 i.g. reduced the delay in

transit caused by IM, as indicated by the shift of fluorescein-labelled dextran to the distal part of the small bowel (Fig. V.2C-E); the GC was significantly increased versus that in untreated manipulated animals and no longer significantly different from that in non-manipulated control mice (Fig. V.2F). Pre-treatment with naproxen did not completely prevent the delay in transit caused by IM as the GC-value was still significantly different from that in non-manipulated control mice (Fig. V.2F).

V.4.2. Effect of GYY4137, naproxen and ATB-346 on manipulation-induced inflammation

The leukocyte infiltration into the muscularis, measured as MPO activity, was increased 24 h after IM. This IM-induced accumulation of leukocytes in the muscular layer was significantly reduced when mice were pre-treated with 50 mg/kg GYY4137 i.p., 10 mg/kg naproxen i.g. or

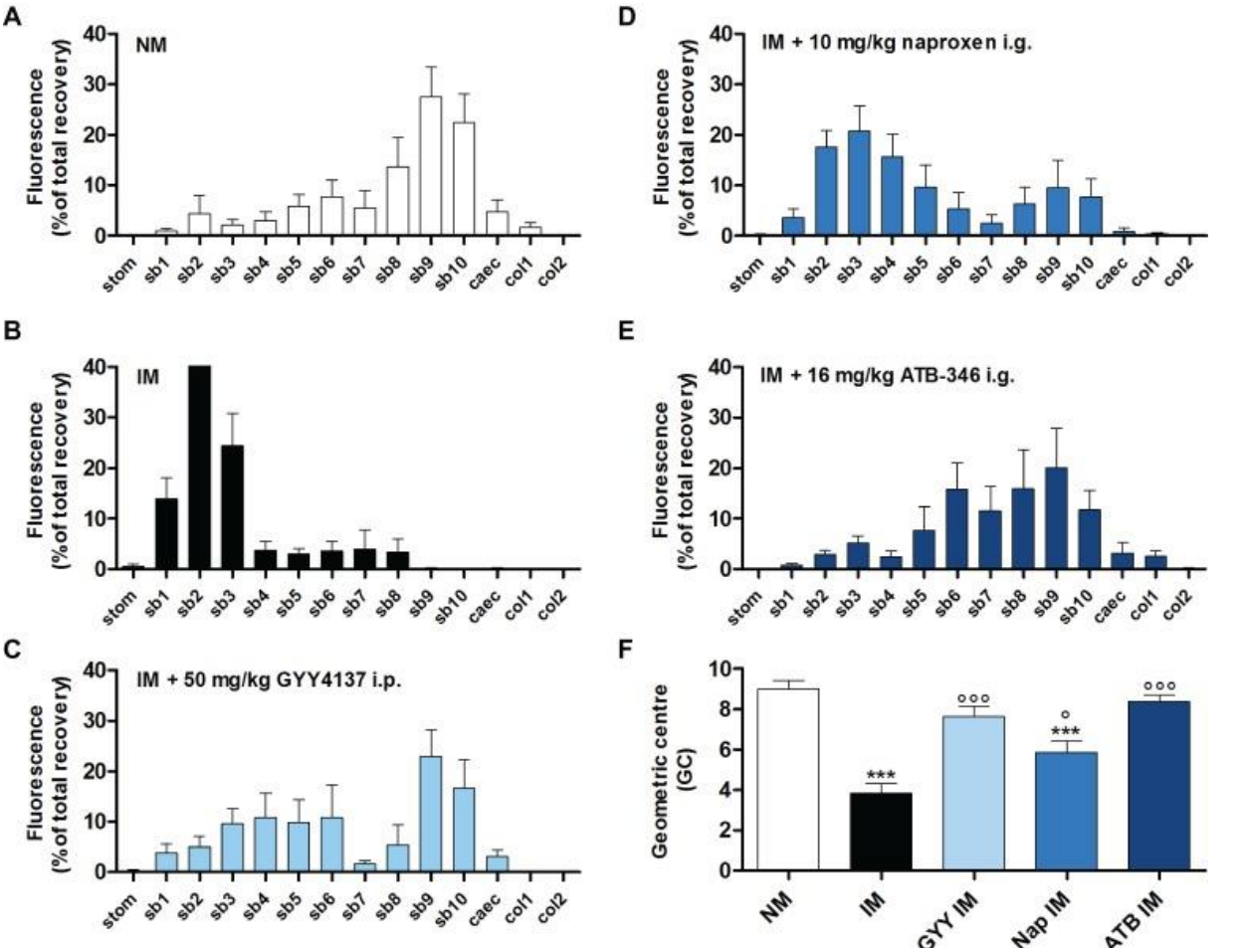

Figure V.2 Influence of GYY4137, naproxen and ATB-346 on the delay in gastrointestinal transit caused by intestinal manipulation. Transit histograms (A–E) and geometric center (F) for the distribution of fluorescein-labeled dextran (70 kDa) along the gastrointestinal tract (stom, stomach; sb, small bowel segments; caec, caecum; col, colon segments), measured 24 h after intestinal manipulation (IM). Data represent the means ± S.E.M. of n = 8. ***p < 0.001 for comparison with non-manipulated (NM) control mice; °p < 0.05, °°°p < 0.001 for comparison with non-treated IM mice: one-way ANOVA followed by a Bonferroni multiple comparison test.

16 mg/kg ATB-346 i.g. (Fig. V.3A). However, the MPO activity in the naproxen treated group was still significantly increased compared to the non-manipulated control group.

As shown in Figure V.3B-D, surgical manipulation of the small intestine markedly increased IL-1β, MCP-1 and IL-6 protein expression in the intestinal muscular layer at 24 h after IM. Pre-treatment with all three compounds resulted in a significant reduction of the elevated IL-6 levels but the level of IL-6 in the group treated with naproxen remained significantly different from that in non-manipulated control animals (Fig. V.3D). Only GYY4137 and ATB-346 were able to significantly reduce the increase in IL-1β and MCP-1 levels caused by IM (Fig. V.3B and C). No significant increase in protein expression of IL-10 and IFNγ was observed in the muscularis of manipulated mice with the Luminex magnetic bead assay (Fig. V.3E and F).

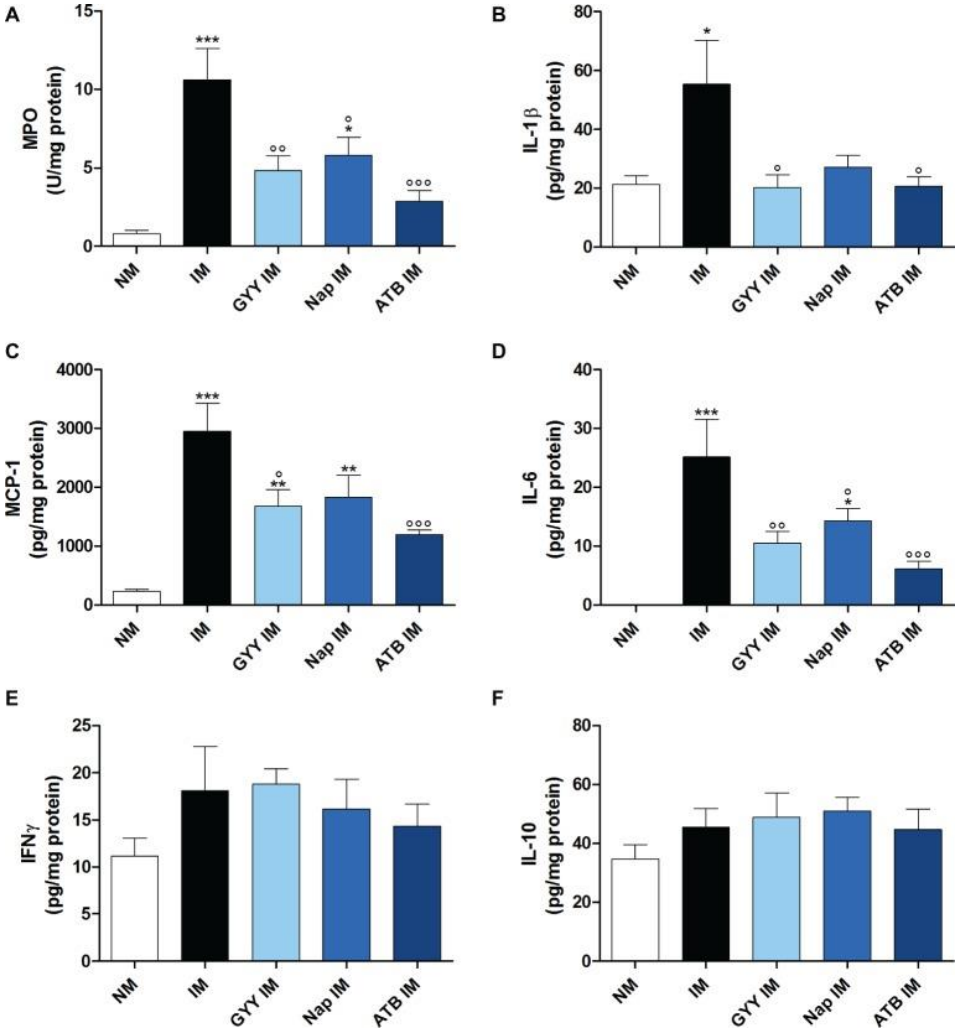

Figure V.3 Influence of GYY4137, naproxen and ATB-346 on intestinal manipulation induced inflammation. Leukocyte infiltration ((A); myeloperoxidase activity, MPO) and IL-1β (B), MCP-1 (C), IL-6 (D), IFNγ (E) and IL-10 (F) protein levels in the muscular layer, measured 24 h after intestinal manipulation (IM). Data represent the means ± S.E.M. of n = 7–8. *p < 0.05, **p < 0.01, ***p < 0.001 for comparison with non-manipulated (NM) control mice; °p < 0.05, °°p < 0.01, °°°p < 0.001 for comparison with non-treated IM mice: one-way ANOVA followed by a Bonferroni multiple comparison test.

V.4.3. Effect of GYY4137, naproxen and ATB-346 on intestinal iNOS and COX-2 activity

Surgical manipulation of the small intestine caused a significant increase in iNOS enzyme activity in the muscularis. Only GYY4137 significantly reduced this increase in iNOS activity. Treatment with ATB-346 also tended to reduce the elevated iNOS activity, but this effect was not significant (Fig. V.4A). Surgery also caused a pronounced increase in COX-2 activity. All three compounds were able to significantly reduce the increased COX-2 activity caused by IM (Fig. V.4B).

V.4.4. Effect of GYY4137, naproxen and ATB-346 on morphology of intestinal villi

No injury was seen in the non-manipulated control group, which demonstrated normal mucosal villi (Fig. V.5A). However, in the non-treated (Fig. V.5B) and GYY4137 treated (Fig. V.5C) manipulated mice, development of sub-epithelial Gruenhagen's space in the tip of the villi was observed although the increase in Chiu score did not reach significance. The intestinal structure was damaged most severely in the naproxen treated IM group (Fig. V.5D), regularly showing moderate to massive epithelial lifting down the sides of the villi leading to a significant increase in Chiu score. Compared to the naproxen treated IM group, villi were less damaged when animals were treated with ATB-346 (Fig. V.5E), showing Gruenhagen's space at the apex of the villi and moderate epithelial lifting.

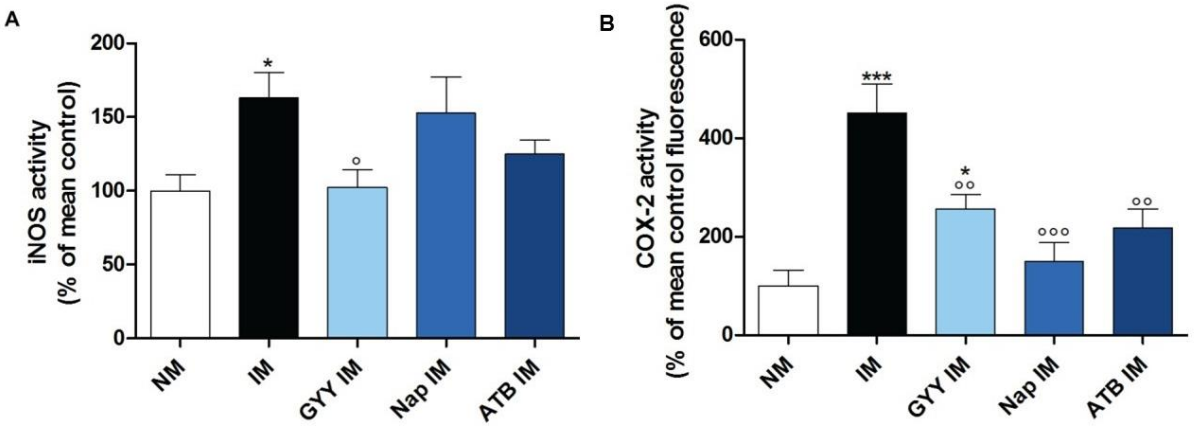

Figure V.4 Influence of GYY4137, naproxen and ATB-346 on intestinal iNOS and COX-2 activity. iNOS (A) and COX-2 (B) activity in the muscular layer, measured 24 h after intestinal manipulation (IM). Data represent the means ± S.E.M. of n = 8. *p < 0.05, ***p < 0.001 for comparison with non-manipulated (NM) control mice; °p < 0.05, °°p < 0.01, °°°p < 0.001 for comparison with non-treated IM mice: one-way ANOVA followed by a Bonferroni multiple comparison test.

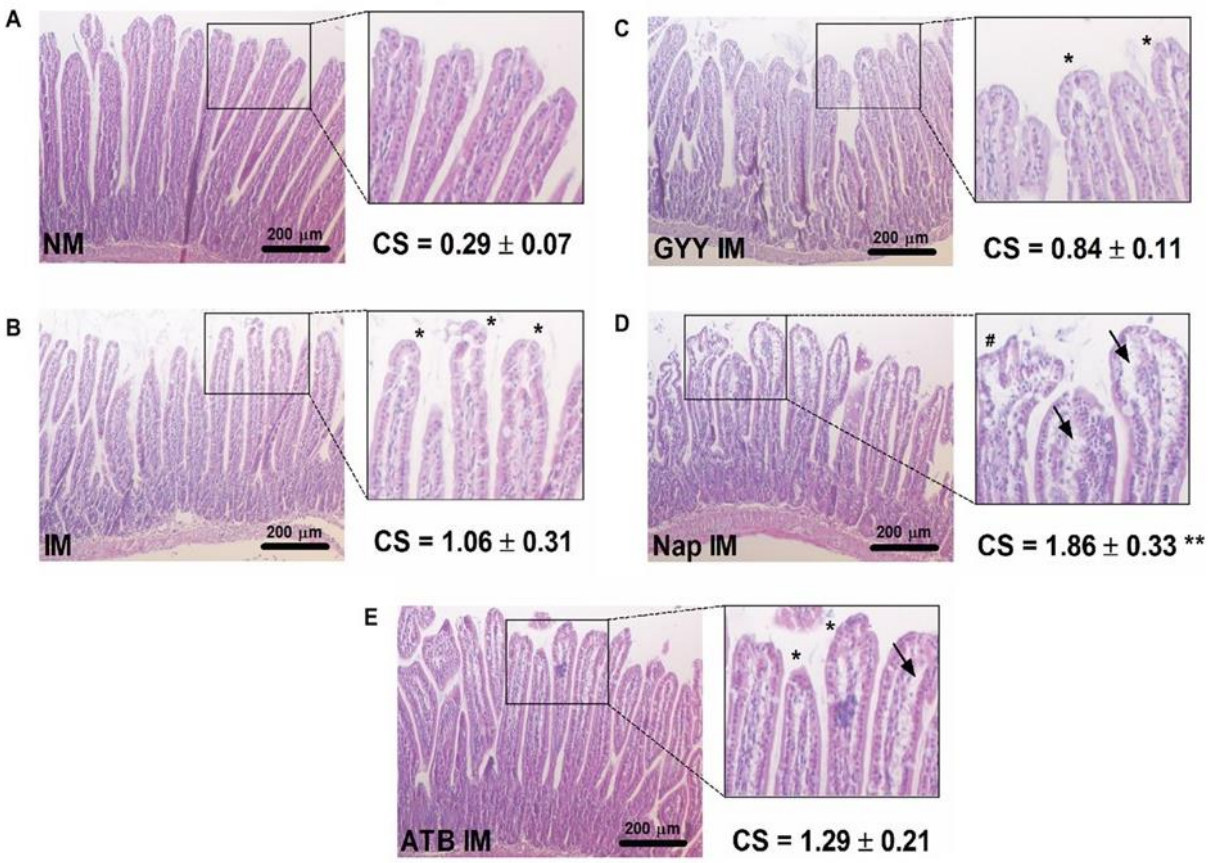

Figure V.5 Influence of GYY4137, naproxen and ATB-346 on morphology of intestinal villi. Histological analysis and Chiu score (CS;A–E) of small intestinal mucosa following hematoxylin-eosin staining, analyzed 24 h after intestinal manipulation (IM). Asterisks (*) mark the development of sub-epithelial Gruenhagen's space at villus apex; arrows (→) indicate further epithelial lifting down the sides of the villi and the number signs (#) mark massive epithelial lifting. Data represent the means ± S.E.M. of n = 4. **p < 0.01 for comparison with non-manipulated (NM) control mice: one-way ANOVA followed by a Bonferroni multiple comparison test.

V.5. Discussion

Hydrogen sulfide therapy has attracted significant attention as a potent biological mediator. Although its benefits have been appreciated in different gastrointestinal disease models like colitis (Fiorucci et al., 2007; Chen and Liu, 2016) and intestinal ischemia/reperfusion injury (Henderson et al., 2010; Jensen et al., 2018), its potential effect on POI has not yet been investigated. POI is a serious complication seen after abdominal surgery, leading to impairment of the gastrointestinal transit that traditionally was thought to result from neuronal dysfunction. Presently, it is clear that the immune response in the intestinal muscular layer upon abdominal surgery, characterized by the activation of resident macrophages and upregulation of inflammatory cytokines, adhesion molecules and chemokines, is the main

mechanism of POI (Boeckxstaens and de Jonge, 2009; van Bree et al., 2012; Bragg et al., 2015). Many of the data leading to the pathophysiological explanation of POI were obtained in the murine model of POI with manipulation of the small intestine. Throughout the years, this model yielded consistent results for transit and inflammation in our and other laboratories (Moore et al., 2003; Nakao et al., 2006; De Backer et al., 2009; Engel et al., 2010; Snoek et al., 2012; Cosyns et al., 2015). Also in the present study an inflammatory response is observed after manipulation of the murine small intestine, as shown by the increased leukocyte infiltration and elevated protein levels of the pro-inflammatory cytokines IL-6 and IL-1β and the chemokine MCP-1 in the intestinal muscularis externa at 24 h after induction of POI. Although previous reports (De Backer et al., 2009; Engel et al., 2010) show elevated IL-10 protein and IFNγ mRNA levels at 24 h after IM, we could not measure a significant increase in protein expression for these cytokines with the Luminex magnetic bead assay used in this study.

GYY4137 is an H_2S-releasing molecule that was shown to have a pronounced anti-inflammatory effect in rodent models of myocardial (Qiu et al., 2018) and intestinal (Jensen et al., 2018) ischemia/reperfusion injury, endotoxemia (Chen et al., 2016) and atherosclerosis (Liu et al., 2013). The usual dose applied is 50 mg/kg by i.p. route.(Liu et al., 2013; Chen et al., 2016; Jensen et al., 2018; Qiu et al., 2018) I.g. administration of GYY4137 has to be avoided since the release of H_2S from GYY4137 will be enhanced under acidic conditions (Li et al., 2008), possibly leading to toxic H_2S levels as seen with fast-release H_2S donors such as NaHS. H_2S-releasing naproxen ATB-346 showed beneficial anti-inflammatory effects in rodent models of colorectal cancer (Elsheikh et al., 2014), melanoma (De Cicco et al., 2016), zymosan-induced arthritis (Dief et al., 2015), spinal cord injury (Campolo et al., 2013) and carrageenan-induced synovitis (Ekundi-Valentim et al., 2013); the usual dose is 16 mg/kg by i.g. route (Ekundi-Valentim et al., 2013; Elsheikh et al., 2014; Dief et al., 2015). The studied route of administration for ATB-346 in rodents is indeed the i.g. one, as the compound is developed to improve the efficacy and decrease the side effects of the NSAID naproxen, used perorally in clinical practice. As neither GYY4137 nor ATB-346 have been tested before for

POI, this was done in the actual study. I.g. naproxen was studied in comparison with ATB-346; the dose used (10 mg/kg) is the molar equivalent of the amount of naproxen contained within 16 mg/kg ATB-346 and has already been shown to exert anti-inflammatory effects in a rat model of carrageenan-induced synovitis (Ekundi-Valentim et al., 2013).

In the present study we show that both GYY4137 and ATB-346 were able to completely reverse the delay in gastrointestinal transit seen after IM, whereas naproxen was only able to partially restore the delayed transit. The findings for the NSAID naproxen are similar to previous work, in which the authors show the beneficial effects of pre-treatment with the non-selective COX inhibitors indomethacin and ketorolac and the selective COX-2 inhibitor 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl) phenyl-2(5H)-furan on the impaired transit in a rat model of POI (De Winter et al., 1998; Schwarz et al., 2001). Treatment with GYY4137 and ATB-346 was also found to reduce the IM-induced inflammatory response, as evidenced by a marked reduction of the inflammatory mediators IL-1β, IL-6 and MCP-1 and leukocyte infiltration; whereas the reduction caused by naproxen only reached significance for IL-6 protein levels and leukocyte infiltration. These data are consistent with previous evidence showing that the H_2S-releasing moiety of ATB-346 adds complimentary anti-inflammatory effects to the COX inhibiting effect of naproxen (Wallace et al., 2010; Campolo et al., 2013; Dief et al., 2015; Magierowski et al., 2017). The functional importance of the kinetically active substances NO and prostaglandins, that directly inhibit smooth muscle contractility, in the pathogenesis of POI has been demonstrated in earlier studies (Kalff et al., 2000; Schwarz et al., 2001). Therefore, we further investigated whether GYY4137, naproxen or ATB-346 could alter iNOS and COX-2 enzyme activity levels. Only GYY4137 was able to significantly reduce both the increased iNOS and COX-2 activity. Similarly, in an *in vitro* study in human synoviocytes and articular chondrocytes GYY4137 was able to dose-dependently reduce the LPS-induced increase in both iNOS and COX-2 expression and activity (Li et al., 2013). ATB-346 and naproxen did only cause a significant reduction in COX-2 activity, although ATB-346 tended to reduce iNOS activity. These findings are in line with the study of Campolo et al. in

which ATB-346, but not naproxen, was able to attenuate iNOS expression in a murine model of spinal cord injury; it has to be noted that naproxen did also not affect COX-2 expression in this model (Campolo et al., 2013). However, the same research group also reported that in a model of traumatic brain injury in mice, administration of both ATB-346 and naproxen led to a significant reduction of COX-2 and iNOS expression (Campolo et al., 2014). The absence of any effect with naproxen on iNOS activity in the actual study might contribute to the less pronounced effect of naproxen on retarded transit.

As mentioned earlier, NSAIDs are a major cause of gastric and duodenal ulceration and have been shown to also injure more distal parts of the small intestine. The protective effect of H_2S-releasing NSAIDs like ATB-346 on gastric and intestinal lesions has been well described in the literature and a double-blind, controlled clinical study to compare the gastrointestinal safety of ATB-346 versus naproxen in healthy subjects is ongoing (ClinicalTrials.gov[1]; Wallace, 2007; Wallace et al., 2010; Magierowski et al., 2017). In this study intestinal manipulation per se tended to induce moderate mucosal damage although not reaching significance; a similar Chiu score was seen in manipulated mice pretreated with GYY4137. In manipulated mice, pretreated with naproxen, a significant increase in Chiu score was seen, confirming the well-known intestinal mucosal injury by NSAIDs. However in manipulated mice pretreated with ATB-346, the Chiu score was similar to that in non-treated manipulated mice showing that the H_2S-release from this molecule was able to counteract mucosal injury of the naproxen moiety.

Follow-up studies can be performed to unravel the underlying mechanisms by which these H_2S-releasing compounds exert their beneficial effects in the murine model of POI. The most frequently described mechanisms by which exogenous H_2S exerts its anti-inflammatory effect are inhibition of the pro-inflammatory nuclear transcription factor-κB (Oh et al., 2006; Li et al., 2013; Chen and Liu, 2016), activation of the anti-oxidant nuclear factor erythroid 2–related

[1] Clinicaltrials.gov. *To Compare the Gastrointestinal Safety of a 14-Day Oral Dosing Regimen of ATB-346 to Sodium Naproxen in Healthy Subjects* (2018). Retrieved October 10, 2018, from https://ClinicalTrials.gov/show/NCT03291418

factor 2 (Shimada et al., 2015; Xie et al., 2016; Qiu et al., 2018) and inhibition of the mitogen-activated protein kinase signaling pathways like p38 and extracellular signal-regulated kinase 1/2 (Hu et al., 2007; Xu et al., 2013). Furthermore, it can be investigated whether the endogenous H_2S pathway is upregulated during POI as a protective mechanism, whereby exogenous H_2S donors then strengthen this reaction; enhanced H_2S synthesis with increased expression of H_2S synthetizing enzymes at sites of mucosal ulceration was indeed demonstrated in rat colitis (Wallace et al., 2009; Flannigan et al., 2013).

This study demonstrates that pre-treatment with GYY4137, ATB-346 or naproxen reduces the impaired transit and suppresses the IM-induced inflammatory response in the muscularis seen during POI in mice, with naproxen being the least effective compound. Furthermore, the intestinal mucosal damage with naproxen alone was prevented by administering it as a H_2S-releasing compound. The data suggest that delivery of H_2S may represent a new pharmacological approach to prevent POI or to improve the effect of NSAIDs in POI. NSAIDs are indeed already studied for postoperative ileus with some improvement of time to gut recovery (Milne et al., 2018). The current data suggest that ATB-346 is preferable over naproxen per se for application in POI. The step to a clinical trial with ATB-346 to prevent POI in patients undergoing colorectal surgery seems easily feasible as safety of ATB-346 was already shown in a phase 1 study in healthy subjects and a phase 2 clinical trial demonstrated pain relief of ATB-346 in patients with osteoarthritis; in both studies whole blood COX activity was profoundly suppressed (Wallace et al., 2018).

V.6. Ethics Statement

This study was carried out in accordance with the recommendations of the most recent national legislation (Belgian Royal Decree, 29/05/2013) and European Directive (Directive 2010/63/EU). The protocol was approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University (ECD 16-48).

V.7. Author Contributions

RL designed the study. JD performed the experiments and data analysis. Intestinal histological evaluation was done by LP and JD under supervision of AV. RL and JD interpreted the findings and prepared and completed the manuscript. All authors approved the final version of the manuscript.

V.8. Acknowledgments

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Preliminary accounts of the study were presented at the Belgian Week of Gastroenterology, February 22th 2018, Antwerp, Belgium (Van Dingenen and Lefebvre, 2018a) and at the Meeting of the Federation of Neurogastroenterology and Motility, August 31st 2018, Amsterdam, The Netherlands (Van Dingenen and Lefebvre, 2018b).

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V.10 Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER VI

GENERAL DISCUSSION

CHAPTER VI

General discussion

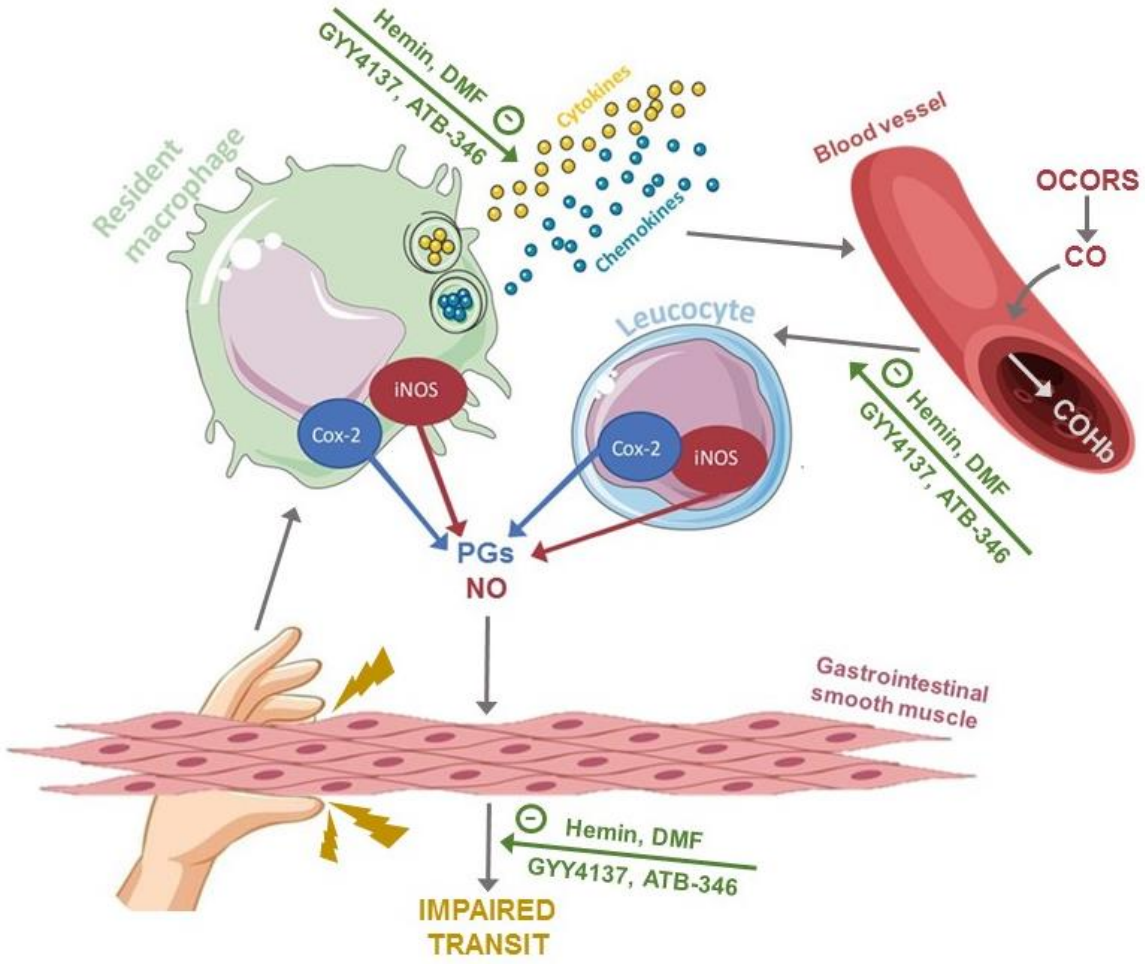


Figure VI.1 Overarching graphical abstract (adapted from Buscail & Deraison, 2022).

POI refers to the transient impairment of gastrointestinal motility, which commonly occurs after abdominal surgery and normally resolves within three days. However, when prolonged, symptoms like nausea and vomiting will contribute to increased morbidity and length of hospital stay. The pathophysiology of POI is characterized by an acute neurogenic phase followed by a prolonged, and clinically more relevant, inflammatory phase triggered in the muscularis externa. Activated macrophages play a crucial role as they will release inflammatory cytokines (e.g. IL-6) and chemokines (e.g. MCP-1), stimulating the expression of adhesion molecules

and the recruitment of circulatory leukocytes. These inflammatory cells will release NO and prostaglandins which impair the neuromuscular contractile apparatus and cause the delay in gastrointestinal transit (Boeckxstaens & de Jonge, 2009). Laparoscopic procedures, enhanced recovery pathways and pharmacological treatment options such as prokinetics and NSAID's are advocated to prevent prolonged POI but no full protection is obtained (Augestad, 2010). Due to the importance of the prolonged inflammatory phase, intestinal inflammation is now considered to be the primary target for novel treatment options (van Bree et al., 2012).

CO and H_2S are traditionally seen as harmful gases due to their toxic effects in humans and animals at high concentrations. This perception has changed because of the demonstrated endogenous production and signaling roles of these molecules, playing an important role in both physiological and pathophysiological processes in multiple organs including the gastrointestinal tract. Both CO and H_2S were shown to exert anti-inflammatory and anti-oxidant effects, leading to the investigation of their potential therapeutic applications (Farrugia & Szurszewski, 2014). As for exogenous administration of CO, inhalation of CO gas has been successfully used in a variety of disease models in animals, including POI, but it is less feasible for human use as it lacks the possibility to target specific tissues and affects the oxygen-carrying capacity of hemoglobin. CORMs were developed to deliver CO in a more practical and controllable manner, but contain heavy metal cores which may induce significant cellular toxicity. To specifically treat gastrointestinal diseases, Steiger et al. (2014) developed an oral tablet named OCORS which contains CORM-2 and allows tunable and targeted CO delivery within the gastrointestinal tract. Alternatively, the endogenous release of CO can be enhanced via induction of HO-1, leading to the co-production of biliverdin and subsequently bilirubin which have pronounced antioxidant capacity and also exert protective effects (Chang et al., 2015). Experimentally, the classic HO-1 inducer hemin was shown to be protective in several gastrointestinal inflammatory diseases; DMF, approved for use in patients with MS, was in vitro shown to be a potent HO-1 inducer. H_2S can be administered via classic H_2S donors such as sulfide salts, but synthetic H_2S donors such as GYY4137 are preferred as their release kinetics

better mimic the time course of naturally produced H_2S (Li et al., 2008). Another group of H_2S donors consists of H_2S-releasing NSAID's such as ATB-346, which is the H_2S-releasing derivative of naproxen. The H_2S released by these NSAID-hybrids will contribute to the overall anti-inflammatory effect of the NSAID and simultaneously counteract the NSAID-induced gastrointestinal ulceration (Wallace et al., 2010).

Using the murine model of POI, the aims of our 3 consecutive studies were therefore to investigate the possible protective effect of

1. OCORS.
2. Hemin and DMF, with particular attention for the role of induction of HO-1.
3. The H_2S donor GYY4137 and the H_2S-releasing naproxen derivative ATB-346.

VI.1. Orally delivered CO did not prevent POI

From a theoretical point of view, pre-operative administration of OCORS seems ideal to prevent POI as it allows tunable release of CO within the gastrointestinal tract. Previous studies have shown that CO, administered either via inhalation of CO gas (Moore et al., 2003; Moore et al., 2005) or i.p. injection of CORM-3 (De Backer et al., 2009), is able to successfully counteract the gastrointestinal inflammation during POI. As the local inflammation of the gastrointestinal muscle layer starts almost immediately upon manipulation of the bowel, OCORS-derived CO present within the lumen at the time of manipulation could diffuse towards the muscle layer to exert its protective effects without the need for absorption and subsequent increase in blood COHb levels.

After preliminary tests, OCORs tablets with a diameter of 0.5 mm were developed for further investigation. In mice fasted for 6 h before administration of 20 OCORS-0.5mm tablets, 55% of tablets had left the stomach and the blood COHb level was increased to 5.2% at 1 h after gavage. This value of blood COHb is quite similar to that seen 20 min after i.p. injection of 15 mg/kg CORM-A1. As demonstrated previously by De Backer et al. (2009), i.p. injection of 15

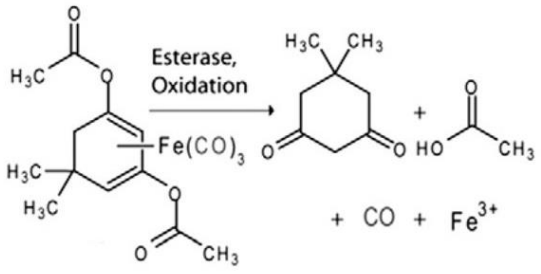
mg/kg CORM-A1 at 4.5 h and 1.5 h before surgery is able to partially prevent the intestinal dysmotility induced during POI. However, the inflammatory response and the delay in gastrointestinal transit induced by IM was not influenced by administration of the 20 OCORS-0.5mm tablets at 1 h before surgery. The lack of efficacy to counteract POI in our study may be caused by non-sustained and/or too low systemic COHb levels which are not able to trigger therapeutic effects in our POI model. Two h after administration of the OCORS tablets, the blood COHb level was indeed already declined to 1.9%; whereas most other studies showing a beneficial effect of CO-delivery in POI reached high systemic COHb peak levels (≥ 8%) or medium systemic COHb levels (≥ 5%) for a prolonged perioperative period of time (≥ 3 h) (Moore et al., 2005; Nakao et al., 2006; De Backer et al., 2009). Still, it needs to be taken into account that blood COHb levels do not directly reflect the amount of CO that it is delivered towards the organs and tissues. Although the OCORS-derived CO is released locally within the small intestine, CO diffusing into the gastrointestinal wall, might be absorbed quickly by the mucosal capillary network explaining the observed increase in blood COHb level but at the same time preventing pronounced direct diffusion into the outer muscularis, which is the site of inflammation in POI. Whereas with i.p. injection, CO can enter from the peritoneal cavity into the gastrointestinal tract via the serosal side then also quickly reaching the muscle layer. So for OCORS to be effective, the primary location of the inflammation in the gastrointestinal wall may play a role. The release kinetics of OCORS tablets match the absorption kinetics suggesting that CO immediately reaches the mucosa; repetitive administration of OCORS tablets with a slow CO release profile was indeed able to reduce the more protracted inflammation starting from the mucosal side in murine colitis, induced by intraluminal exposure to TNBS (Steiger et al., 2016). This further indicates that, if locally released CO is able to reach the site of inflammation easily, the OCORS delivery system might be effective.

Although we cannot exclude that the current OCORS-0.5mm formulation might be effective in other gastrointestinal disease models, and other OCORS formulations with a more sustained and/or higher CO release profile might be able to protect against POI, there are still limitations.

Despite the fact that the visual integrity of the OCORS tablets remains unchanged, approximately 90% of the cytotoxic ruthenium present within the CO-donating CORM-2 molecules in the OCORS tablets, escapes from the tablets after submerging them into water for 13 h (Steiger et al., 2014). This indicates that upon in vivo administration of OCORS tablets ruthenium will be released in the gastrointestinal tract, limiting the clinical application of the CORM-2 based OCORS. A modified OCORS was developed in which the sulfite/CORM-2 system was replaced by an esterase/*rac*-1 system to avoid ruthenium toxicity, *rac*-1 being an iron based CORM (Figure VI.2). These esterase-triggered OCORS (E-OCORS) still require future in vivo studies to assess the effectiveness and possible toxicity, but seem promising as the concept of *rac*-1 has been expanded allowing introduction of various linkers cleavable by enzymes that have been associated with inflammation. Future modifications of E-OCORS might therefore adopt this approach and aim for an E-OCORS that selectively releases CO in response to inflammation in the gastrointestinal tract (Steiger et al., 2016).

VI.2. Hemin and dimethyl fumarate prevent POI

Pharmacologic induction of HO-1 might offer an effective strategy to counteract POI as the by-products of heme degradation, being CO and biliverdin/bilirubin, exert anti-inflammatory and/or anti-oxidant effects. Hemin, which is chemically very similar to heme, acts as a substrate for HO-1 leading to HO-1 activation and induction. It has already been widely used in experimental studies: HO-1 induction via hemin was shown to protect against gut I/R injury (Attuwaybi et al., 2004), colitis (Berberat et al., 2005; Zhong et al., 2010) and indomethacin-induced small



***Rac*-1**

Figure VI.2 Scheme of the esterase triggered release of CO from *rac*-1. .

intestinal injury (Yoriki et al., 2013). In the clinical setting, the hemin-derivatives hematin (Panhematin®) and heme arginate (Normosang®) are approved by respectively the FDA and EMA for treatment of acute attacks of porphyria, but their mechanism of action for this indication is inhibition of δ-aminolevulinic acid synthase and not induction of HO-1 (Bickers, 1981; Mustajoki & Nordmann, 1993; Siegert & Holt, 2008). More recently, it was shown in phase II clinical trials that both Normosang® and Panhematin® are able to safely induce HO-1 in patients (Bharucha et al., 2016; Thomas et al., 2016). DMF has been approved by the FDA and EMA for use in MS and experimental studies suggested that its immunosuppressive and neuroprotective effects in the treatment of MS might be related to induction of HO-1 (Lin et al., 2011; Bomprezzi, 2015). Moreover, DMF was shown to exert one of the best HO-1 inducing/low toxicity profiles among 56 compounds in an in vitro study in BV2 microglia cells (Foresti et al., 2013). Therefore, we studied the potential beneficial effects of both hemin and DMF in our murine model of POI.

VI.2.1. Hemin prevents POI via HO-1 induction

Hemin was tested in a dose of 30 mg/kg i.p. based upon the reported effectiveness of this dose in different experimental disease models (Attuwaybi et al., 2004; Ndisang & Jadhav, 2013; Yoriki et al., 2013; Chi et al., 2015). A preliminary study, in which we investigated the HO-1 protein expression in intestinal muscular tissue at 6, 12 and 24 h after i.p. administration of 30 mg/kg hemin in non-manipulated mice, showed a time-dependent increase in HO-1 protein levels with the highest induction at 24 h. We therefore opted for a time interval of 24 h between administration of 30 mg/kg hemin i.p. and IM; this reduced the development of POI in mice, as evidenced by the significant improvement in transit and the significant reduction in IL-6 protein level and leukocyte infiltration in muscular tissue. The importance of HO-1 induction was demonstrated by the abolishment of the benefical effect of hemin on POI upon co-administration of the HO-1 inhibitor CrMP. These findings clearly illustrate that HO-1 induction is effective in preventing POI. Correspondingly, Bortscher et al. (2012) showed that pretreatment of rats with 5 mg/kg hemin i.p. 24 h before induction of LPS-induced septic ileus

improved the delay in transit and the intestinal muscular inflammation induced by LPS. Moreover, HO-1 mRNA levels in muscular tissue was increased upon hemin administration and co-administration of the HO-1 inhibitor zinc protoporphyrin 2 h before LPS completely abolished the protective effects of hemin. Interestingly, the dose of hemin used by Bortscher et al. was six times lower than our dose (5 mg/kg vs 30 mg/kg), suggesting that a lower dose might be sufficient to counteract POI.

Although hemin itself is not approved for clinical use, the transition of HO-1 induction for the treatment of POI towards patient use is feasible via the use of Panhematin® or Normosang®. Thomas et al. (2016) already demonstrated in patients that Normosang® is able to upregulate HO-1 mRNA and protein levels in peripheral blood mononuclear cells and to increase HO-1 protein levels in renal tissue of transplant recipients. However, it needs mentioning that the observed transient HO-1 induction did not translate into the structural or functional cytoprotection of the renal grafts. Similarly, Panhematin® was able to increase the HO-1 protein concentration in venous plasma and the HO-1 activity in venous leukocytes of patients suffering from diabetic gastroparesis; but the increase in HO-1 protein concentration and activity was not maintained in the course of the treatment and gastric emptying, autonomic functions, and symptoms did not differ significantly in the Panhematin® group relative to the placebo group (Bharucha et al., 2016). Although both studies were not able to confirm a protective effect of HO-1 induction in a clinical setting, they do deliver a first insight in HO-1 induction upon hemin treatment in humans. Future studies with higher sample sizes, different dosing schemes and different pathophysiological conditions are needed to further explore its full potential.

VI.2.2. DMF prevents POI via HO-1-unrelated mechanisms.

In our study we have shown that pre-treatment with both 30 mg/kg DMF i.p. or 100 mg/kg DMF i.g., 2 dose schedules previously shown to be effective in other disease models, had a protective effect on POI in mice. In contrast to hemin, DMF did not increase HO-1 protein expression in the intestinal muscularis of non-manipulated mice within 24 h after administration

and the HO-1 inhibitor CrMP did not influence its preventive effect on POI. The lack of HO-1 induction by DMF might be explained by the fact that we opted for a single dose of DMF as an acute dosing scheme would be more feasible in the clinical setting of POI. In many studies DMF was administered daily for 7 days or more to investigate its effect on chronic animal models with progressive chronic inflammation such as MS, resulting in increased expression of HO-1 and beneficial anti-inflammatory effects (Linker et al., 2011; Kobayashi et al., 2015; Han et al., 2016; Kasarełło et al., 2017). Recently, Gendy et al. (2021) also reported HO-1 inducing and protective effects of DMF in a murine model of intestinal I/R injury, which similar to POI is characterized by an acute inflammatory phase, after administering DMF daily for 14 days. In animal studies of myocardial (Meili-Butz et al., 2008) and liver (Takasu et al., 2017) I/R injury, in which rats were treated with DMF for only one or two days, DMF was reported to exert protective anti-inflammatory effects despite the fact that no upregulation of HO-1 was observed or reported; both studies assigned the protective effects of DMF to its potential to inhibit NF-κB activity. Furthermore, in a murine model of experimental colitis, it was shown that administration of both 30 and 100 mg/kg of DMF by oral gavage reduced colonic injury; both doses were able to significantly reduce colonic NF-κB p65 activity, whereas only the dosage of 100 mg/kg of DMF resulted in upregulation of nuclear Nrf2 protein levels, which regulate the expression of HO-1 (Casili et al., 2016). These data might suggest that in vivo induction of HO-1 by DMF requires a higher dose and/or longer treatment duration, whereas inhibition of NF-κB might already occur at a lower dose and/or shorter treatment duration.

We therefore further explored the role of NF-κB in the protective effects of DMF. Evaluation of the phosphorylation of NF-κB p65 in whole tissue segments showed that 30 mg/kg DMF i.p was able to reduce the IM-induced increase in phosphorylation of NF-κB p65 at 1 and 6 h after IM, confirming that DMF inhibits IM-induced NF-κB p65 activation. Although the exact mechanism of action by which DMF inhibits NF-κB p65 is incompletely characterized, in vitro (Kastrati et al., 2016) and in vivo (Takeda et al., 2020) evidence suggests that DMF prevents the nuclear translocation of NF-κB p65 and attenuates its DNA binding activity via covalent

modification of NF-κB p65, but does not affect upstream proteins in the NF-κB pathway. In our study, immunochemistry showed that DMF indeed reduced the nuclear translocation of NF-κB p65. Besides NF-κB p65, also ERK 1/2, p38 and JNK MAPK signaling pathways have been shown to be key regulators of pro-inflammatory cytokines. In our study, administration of 30 mg/kg DMF i.p was able to significantly reduce the IM-induced activation of ERK 1/2 at 1 h and 6 h after IM while not influencing the activation of the 2 other MAPKs. The inhibition of both NF-κB and ERK 1/2 signaling pathways by DMF was also observed in LPS-stimulated bone marrow-derived and RAW264.7 macrophages (McGuire et al., 2016).

DMF was already shown to be safe in patients and is also in Belgium available for the treatment of MS under the name of Tecfidera®, making the step towards exploring its potential to counteract POI in humans more feasible. However, it needs mentioning that little is known about the exact immunologic and neuroprotective effects of DMF in humans. A phase IV clinical trial to investigate the antioxidant and immunologic changes within the central nervous system and blood of MS patients in relation to DMF therapy was set up in 2016, but was withdrawn within the same year (ClinicalTrials.gov Identifier: NCT02675413).

VI.3. H_2S-releasing naproxen and slow-release H_2S donor GYY4137 prevent POI

Delivery of exogenous H_2S has shown beneficial effects in different gastrointestinal inflammatory disease models like colitis (Chen & Liu, 2016; Fiorucci et al., 2007) and intestinal I/R injury (Henderson et al., 2010; Jensen et al., 2018). The preventive effect of exogenous H_2S on POI was proven in our study by use of the slow-releasing H_2S donor GYY4137 in a classic experimental dose schedule (50 mg/kg i.p.). GYY4137 completely reversed the delay in gastrointestinal transit seen after IM and significantly reduced the IM-induced increase of the inflammatory mediators IL-1β, IL-6 and MCP-1 and of leukocyte infiltration in the intestinal muscularis. GYY4137 also significantly reduced the IM-induced increase of iNOS and COX-2 enzyme activity in the intestinal muscularis.

NSAIDs are already used in POI and H_2S-releasing NSAIDs are developed to enhance the anti-inflammatory effect on the one hand and to counteract the gastrointestinal side-effects on the other hand via the released H_2S. We therefore also tested the naproxen-hybrid ATB-346 in a common experimental dose schedule (16 mg/kg i.g.) and compared this to the equimolecular amount of naproxen (10 mg/kg i.g.) as present in the hybrid. Although naproxen significantly reduced IM-induced COX-2 activity (but not iNOS activity) in the intestinal muscularis, it only partially restored the delayed transit by IM and the reduction of inflammatory parameters in the muscularis only reached significance for IL-6 and leukocyte infiltration. Furthermore, it induced significant histological mucosal damage in the intestine. ATB-346 however had the same effects as GYY4137 except that it only tended to reduce iNOS activity. Additionally, intestinal mucosal damage was not significantly increased in the ATB-346-treated group and similar to that in non-treated operated control animals. The results illustrate a moderate effect of the NSAID naproxen in the murine POI model and clearly show that the H_2S-releasing moiety of ATB-346 1) adds complimentary anti-inflammatory effects to the COX inhibiting effect of naproxen, as seen in previous studies (Wallace et al., 2010; Campolo et al., 2013; Dief et al., 2015; Magierowski et al., 2017) and 2) counteracts the mucosal injury by the naproxen moiety (Wallace et al., 2010; Magierowski et al., 2017).

Follow-up studies are needed to unravel the exact underlying mechanisms by which these H_2S-releasing compounds exert their beneficial effects in the murine model of POI. The most frequently proposed mechanisms for the anti-inflammatory effects of exogenous H_2S in the current literature are inhibition of NF-κB (Chen & Liu, 2016; Wu et al., 2017; Sousa et al., 2021), activation of the anti-oxidant Nrf2 system (Xie et al., 2016; Cui et al., 2021; Zhao et al., 2021) and inhibition of the MAPK signaling pathways like p38 and ERK 1/2 (Hu et al., 2007; Xu et al., 2013; Wu et al., 2015; Wu et al., 2017). Furthermore, it might also be of interest to investigate whether endogenous H_2S generation is upregulated during POI as a protective mechanism, which is then strengthened by administration of exogenous H_2S donors.

Our data suggest that delivering H_2S might be tested per se to prevent POI, or added to an NSAID to improve the effect of the latter. GYY4137 has not been tested yet in a clinical setting but other H_2S-donors have. The H_2S prodrug SG-1002, which is >90 % α-sulfur with the remainder being oxidized sulfur species, was evaluated as well tolerated and safe in an investigation aimed at increasing circulating H_2S levels after heart failure (Polhemus et al., 2015). The step to a clinical study in patients undergoing abdominal surgery might be best feasible with ATB-346. The safety of the compound was already demonstrated in healthy volunteers as well as in patients with osteoarthritis (Wallace et al., 2018). Recently, the marked reduction of gastrointestinal toxicity of ATB-346 as compared to naproxen was also evidenced in a phase II clinical trial in healthy volunteers. After two weeks of treatment with either ATB-346 or naproxen, a very pronounced reduction in ulcer incidence (94%) was observed in subjects treated with ATB-346 versus those treated with naproxen. It needs mentioning that the used dose of ATB-346 (250 mg/day) contains only one sixth the amount of naproxen that was administered daily (550 mg twice/day) to the subjects treated with naproxen, as this might contribute to the difference in ulcer incidence. Still, the dose of ATB-346 produced a profound suppression of COX activity (measured as whole blood thromboxane synthesis), comparable to that by naproxen (Wallace et al., 2020).

Finally it can be mentioned that adding a H_2S-releasing moiety to improve the effect is also investigated for trimebutine, a noncompetitive spasmolytic agent with moderate affinity to μ- and κ-opioid receptors which is used for relief of symptoms associated with irritable bowel syndrome and in postoperative paralytic ileus. Trimebutine 3-thiocarbamoylbenzenesulfonate (GIC-1001), an orally administered trimebutine maleate salt developed to enhance the analgesic effects of trimebutine with the release of H_2S, was found to be well tolerated and safe in a phase I clinical trial (Paquette et al., 2014).

VI.4. Future perspectives

In our study, OCORS showed no beneficial effects in POI. It would therefore be of interest to compare the CO tissue levels in the muscle layer after oral administration of OCORS versus i.p. injection of CORM-A1 or CORM-3, which were both able to improve the delay in gastrointestinal transit upon induction of POI (De Backer et al., 2009). The potential difference in CO tissue levels upon administration might explain the discrepancy in effectivity between i.p and p.o. administration of CO. This could be obtained via a new method, designed by Mao et al. (2021) to quantify CO tissue levels with a simple colorimetric assay. The outcome will help to decide whether it is useful to pursuit investigation of OCORS for treatment of POI using other formulations with longer/more CO release.

Furthermore, OCORS might be effective in the treatment/prevention of other gastrointestinal diseases, as shown previously in a TNBS-induced model of colitis (Steiger et al., 2016). Gastrointestinal I/R injury might be a better candidate as inflammation within the mucosa and dysfunction of the mucosal barrier function play a more prominent role in the pathophysiology of intestinal I/R injury compared to POI; OCORS-derived CO can easily enter the mucosa via the luminal side to protect it against I/R injury. Recently, it was shown that intraluminal administration of a CORM-3 containing solution in intestinal grafts during cold preservation reduced the gastrointestinal I/R injury upon transplantation (Obara et al., 2021) further underlining that gastrointestinal disease can be treated with luminally delivered CO.

Despite the negative result with OCORS, other oral delivery forms of CO might be of interest for further investigation in POI. Oral administration allows CO release locally within the gastrointestinal tract directly targeting the site of inflammation and thus limiting the need for a higher dose and the subsequent systemic exposure to CO. Moreover, it is a preferable and convenient route for drug administration due to ease of administration and patient compliance (Yang et al., 2021). To avoid metal-related issues with compounds such as CORM-2, which is also embedded in OCORS tablets, organic CO prodrugs with tunable CO release have been

designed. For example, oral administration of the CO prodrug BW-CO-111 was shown to exert significant gastroprotective and anti-inflammatory activity against aspirin and 75% ethanol-induced mucosal injuries in rats, while the byproduct CP-111 did not show cytotoxic effects (Bakalarz et al., 2021). A new type of sweetener-based CO prodrugs, such as the saccharine-based BW-CO-306 and the acesulfame-based BW-CO-307, might be of particular interest as the FDA has already approved these sweeteners as food additives, largely mitigating the safety concerns of byproducts. Oral administration of both BW-CO-306 and BW-CO-307 resulted in a significant increase in COHb blood levels in mice and BW-CO-306 was shown to exert protective effects in a mouse rhabdomyolysis-induced acute kidney injury (De La Cruz et al., 2021).

In this thesis, we have shown that hemin, DMF, ATB-346 and GYY4137 exert anti-inflammatory effects and were able to completely or partially reverse the delay in transit in a murine model of POI. Although a single dose of DMF did not result in upregulation of HO-1 in our study, it was still able to partially counteract the POI-induced inflammation via inhibition of NF-κB and ERK 1/2. Possibly administration of DMF 100 mg/kg p.o. for four days could result in upregulation of the Nrf2/HO-1 signaling pathway, as seen in the study of Casili et al. (2016), and strengthen the already observed effects of DMF in our POI model.

As our laboratory already demonstrated the efficacy of both DMF (see *Chapter IV*) and CORMs (De Backer et al., 2009) in murine POI, the dual activity hybrid CO-releasing molecule (HYCO)-3 combining CORM-401 and DMF (Figure VI.3) might be beneficial in the murine POI model. In mice challenged with LPS Motterlini et al. (2019) already found that orally administered HYCO-3 reduced levels of the pro-inflammatory cytokines TNF, IL-1, and IL-6 in the brain, liver, heart and lungs. Regarding Nrf2, they showed that HYCO-3 could induce the expression of Nrf2-dependent genes, including the gene for HO-1, in several organs. Preferentially, proof of concept that peroral administration of CO is able to counteract POI should be obtained before proceeding with the investigation of HYCO-3 in POI.

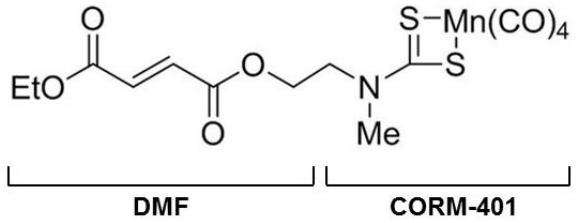


Figure VI.3 Chemical structure of HYCO-3.

As the crucial role of inflammation in the pathophysiology of POI has been well established over the past decades, research interest has broadened to the barrier function of the gut, gastrointestinal permeability and the translocation of bacterial material to the inflammation. Snoek et al. (2012) demonstrated in a murine model of POI that IM leads to epithelial barrier dysfunction of the small intestine, resulting in increased bacterial translocation to the draining mesenteric lymph nodes measured at 24 h after surgery. Furthermore, they found that decontamination of the gut using a 6-day regiment of antibiotic treatment reduced the number of infiltrated leukocytes in the muscularis externa of the small intestine and improved postoperative transit after IM. Similarly, Stein et al. (2018) also observed bacterial translocation into mesenteric lymph nodes and the liver at 24 h after induction of POI in mice . Recent evidence suggests that HO-1/CO and H_2S are both able to protect the gastrointestinal barrier function. Niu et al. (2022) demonstrated that CORM-2 preserves intestinal mucosal barrier function by reducing the epithelial tight-junction damage in rats undergoing cardiopulmonary resuscitation. This effect was accompanied with a downregulation of pro-inflammatory markers such as TNF-α and NF-κB p65 in the ileum. Similarly, CORM-2 suppresses the sepsis-induced intestinal epithelial permeability changes and reduces the mortality rate of rats undergoing CLP (Zhang et al., 2015). HO-1 is also of importance in protecting the gut barrier function as illustrated by in vitro and in vivo data in a study of Zhang et al. (2021). After induction of gut dysfunction by injecting carbon tetrachloride in mice, administration of the HO-1 inducer CoPP reduced the increased pathology score of colon sections in non-treated mice, whereas the score was exaggerated in intestinal HO-1-deficient mice or mice treated with the HO-1 inhibitor zinc protoporphyrin. In Caco-2 cells, HO-1 overexpression was shown to protect against epithelial barrier loss after TNF-α stimulation. Also H_2S is believed to protect the

gastrointestinal barrier function; NaHS was shown to exert a protective effect on TNF-α and IFN-γ-induced injury of intestinal epithelial barrier function in Caco-2 monolayers by blocking the NF-κB p65 signaling pathway (Chen et al., 2015). In mice with LPS-induced colitis, administration of GYY4137 was shown to attenuate the injury of colon epithelium whereas a more remarkable increase was observed in non-treated $CBS^{-/+}$ mice with reduced capacity to produce H_2S (Guo et al., 2022). Furthermore, Motta et al. (2018) showed that oral administration of ATB-429, the H_2S-releasing derivative of mesalamine, reduces the severity of colitis and inhibits the translocation of commensal bacteria to the liver in mice with DNBS colitis, compared with mice given vehicle. These data suggest that hemin, GYY4137 and ATB-346 might also exert a protective effect on the epithelial barrier dysfunction during POI. However, it needs mentioning that we did not observe a significant reduction in intestinal mucosal damage in mice treated with either GYY4137 or ATB-346 compared to non-treated operated mice (see V.4.4. Effect of GYY4137, naproxen and ATB-346 on morphology of intestinal villi). More in depth research with focus on bacterial translocation and the expression of tight junction proteins, such as occluding and claudin, is needed to further elucidate the potential effect of hemin, GYY4137 and ATB-346 on the intestinal barrier function in the murine POI model.

Interaction between the CO and H_2S pathways has been described. Studies show that under physiological conditions CO is able to inactivate CBS by combining with Fe^{2+}-CBS, leading to a decrease in H_2S generation by CBS (Puranik et al., 2006; Vicente et al., 2014). Also CSE is believed to be downregulated by CO, as inhibition of HO-1 in vascular smooth muscle cells results in upregulation of the CSE protein level and H_2S concentration. Similarly, H_2S inhibits the HO/CO pathway under physiological conditions. Treatment of vascular smooth muscle cells with an inhibitor of CSE was shown to enhance the COHb concentration and HO-1 protein level whilst treatment with NaHS inhibited them (Jin et al., 2006). However, under pathophysiological conditions, H_2S and CO seem to enhance each other pathways. Qingyou et al. (2004) have demonstrated that H_2S increased the plasmatic CO concentration and the

HO-1 expression in hypoxic rat pulmonary artery, while the inhibition of H_2S production downregulated the HO/CO pathway. Similar results were obtained in a rat model of recurrent febrile seizures in which upregulation of both H_2S and CO alone could reduce hippocampal damage. Administration of NaHS augmented the plasma CO content as well as the mRNA and protein expression of HO-1 in hippocampal neurons, whereas administration of hemin to promote CO generation increased the plasmatic H_2S content as well as mRNA and protein expression of CBS in hippocampal neurons (Han et al., 2006; Huang et al., 2021). In a rat model of gastric ulcers, the gastroprotective effect of NaHS was shown to be abolished by administration of the HO-1 inhibitor zinc protoporphyrin suggesting a crucial role for the HO/CO pathway. However, although an upregulation of Nrf2 was observed, no increase in HO-1 mRNA expression was measured at the margin of the gastric ulcers. Furthermore, CO-induced acceleration of ulcer healing via administration of CORM-2 seems to be H_2S-independent in this model (Magierowski et al., 2018). Although little is known about the exact mechanisms, these data indicate that there is cross-talk between the CO and H_2S pathways. It would therefore be interesting to see whether H_2S respectively CO contribute to the protective effect of hemin and GYY4137/ATB-346 in the murine model of POI.

At this moment, the department of surgery of Ghent University hospital supervises a multicenter clinical trial to investigate the possible beneficial effect of exogenous nitrite in patients undergoing colonic surgery (ClinicalTrials.gov Identifier: NCT03772444). This is based on a previous study in our laboratory, showing positive effects of exogenous nitrite in the murine POI model as used in this thesis (Cosyns et al., 2015). On the basis of the results described in this thesis, the step to a clinical trial in patients undergoing abdominal surgery might be taken for DMF and for ATB-346. Indeed DMF is already on the market under the name of Tecfidera® for the treatment of MS. The recommended dose of 240 mg twice/day (Saida et al., 2019) (corresponding to a daily dose of 8 mg/kg for a 60 kg person) is almost similar to the applied dose of 100 mg/kg p.o. in mice as this corresponds to a human dose of 8.13 mg/kg taking into account the human equivalent dose calculation factor of 12.3 for mice

(Nair & Jacob, 2016). ATB-346 on the other hand is not yet approved for clinical use, but the first clinical trials are promising as the ulcer incidence in healthy volunteers is significantly lower compared to naproxen whilst being equally effective in the suppression of COX activity. Moreover, the already effective dose used in our experimental murine model (16 mg/kg or a human equivalent dose of 1.30 mg/kg) is ± 3 times lower than the dose used in the first clinical trials (250 mg/day or 4.17 mg/kg for a 60 kg person) (Wallace et al., 2020).

VI.5. References

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CHAPTER VII

SUMMARY

CHAPTER VII

Summary

In the gastrointestinal tract, four main physiological processes, secretion, digestion, absorption and motility, lead to the extraction of necessary nutrients and water from the swallowed food and to the elimination of remaining waste. The coordinated activity of the muscular layer in the gastrointestinal wall results in the movement of the luminal content from mouth to anus and is regulated by the chemical input from nerves, hormones and paracrine signals. Postoperative ileus (POI) is defined as transient impairment of this coordinated bowel motility after abdominal surgical intervention, which prevents effective transit of intestinal content. POI normally resolves within three days but when prolonged, symptoms like nausea and vomiting, abdominal distension and inability to tolerate an oral diet contribute to the increased morbidity and prolonged hospital stay. Pharmacological factors contribute to the pathophysiology of POI, but the process is mainly driven by two endogenous processes: acute neurogenic inhibitory reflexes, followed by a more important sustained inflammatory response. This inflammatory cascade is triggered by activation of resident macrophages within the muscular layer, resulting in the release of inflammatory cytokines and chemokines which stimulate the expression of adhesion molecules and the recruitment of circulatory leukocytes. These cells upregulate inducible nitric oxide (NO) synthase and cyclo-oxygenase 2, and the released NO and prostaglandins impair the neuromuscular contractile apparatus and cause the delay in gastrointestinal transit. Despite the availability of laparoscopic procedures, enhanced recovery pathways and pharmacological treatment options, no full protection for POI is obtained explaining the continuous search for new treatment possibilities.

Carbon monoxide (CO) and hydrogen sulfide (H_2S), which are commonly known for their toxic effects in humans and animals at high concentrations, are endogenously produced and play an important role as signaling molecules in both physiological and pathophysiological

processes of multiple organs, including the gastrointestinal tract. The evidence that both CO and H_2S exert anti-oxidant and anti-inflammatory effects led to the investigation of their potential therapeutic application for inflammatory diseases in different organ systems. The aim of this thesis was to investigate the possible protective effect of different compounds, developed to administer/provide CO or H_2S, in a murine model of POI. In this model the small intestine is manipulated after laparotomy leading to intestinal muscular inflammation and delayed transit; transit is measured at 24 h after surgery.

Exogenous CO, administered via inhalation or via intraperitoneal (i.p.) injection of CO-releasing molecules (CORMs), has been successfully used in a variety of disease models, including POI. However, these administration routes are less feasible for human use as they lack the possibility to target specific tissues and are potentially toxic. To specifically treat gastrointestinal diseases, an oral tablet named “oral carbon monoxide release system (OCORS)” was developed. The coating layers around the CORM-2 core of the tablet can be adapted to change the release rate of CO from OCORS, in order to deliver CO preferentially to a particular part of the gastrointestinal tract. We aimed to evaluate whether OCORS is able to counteract POI in the murine model **(Chapter III)**. OCORS tablets with a diameter of 0.5 mm, that in vitro showed CO release following first order kinetics for 2 h after which CO release plateaued, were developed. Gastric emptying studies showed that upon intragastric (i.g.) gavage of 20 tablets, more than 50% reached the small intestine after 1 h; administration was therefore done 1 h before surgery. Despite having reached the small intestine at the moment of intestinal manipulation (IM) and leading to systemic CO exposure comparable to i.p. delivery strategies, the tablets were unable to reduce surgery-induced muscular inflammation and delay in gastrointestinal transit. Possibly, CO released in the small intestine diffuses into the gastrointestinal wall, but is quickly absorbed by the mucosal capillary network explaining systemic CO exposure but preventing pronounced diffusion of CO into the muscular layer where the inflammation triggering POI resides. The effectiveness of OCORS tablets with a 24 h release profile was indeed already demonstrated in an experimental model of colitis, where

the inflammation starts from the mucosa. Our study illustrates the limitations of the concept and the optimization needs for particular gastrointestinal conditions.

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the degradation of heme into ferrous iron, CO and biliverdin, the latter being subsequently converted to bilirubin. It is upregulated in conditions of oxidative stress and inflammation, providing anti-oxidative and anti-inflammatory protection mainly by the produced CO, but with a contribution of biliverdin/bilirubin. HO-1 induction by exogenous compounds was shown to be protective in inflammatory conditions in different body systems including the gastrointestinal tract but it was not yet investigated for POI. The effect of administration of the HO-1 inducers hemin and dimethyl fumarate (DMF) 24 h before surgery was therefore investigated in the murine model of POI **(Chapter IV)**. I.p. pretreatment with hemin, shown to be effective in many experimental studies, significantly reduced the increase in the intestinal inflammatory parameters and improved the delayed transit seen after IM. The protective effect of hemin was HO-1 dependent as co-administration of the HO-1 inhibitor chromium mesoporphyrin abolished the beneficial effects of hemin. DMF is used for the treatment of multiple sclerosis and was in vitro shown to have an optimal HO-1 induction/cytotoxicity profile. As well i.p. as i.g. administration of a single dose of DMF prevented the delayed transit and significantly reduced the intestinal muscular inflammation induced by IM but this effect was not influenced by the HO-1 inhibitor. While hemin was able to induce an increase in intestinal HO-1 expression in non-manipulated mice, DMF did not. Further investigation showed that the protective effects of DMF on POI are the result of a significant reduction of the IM-induced activation of the pro-inflammatory nuclear factor kB (NF-kB) and extracellular signal–regulated kinases (ERK) 1/2. Our data indicate that both induction of HO-1 and DMF could be further investigated as a therapeutic possibility for the management of POI.

Although initial studies, investigating the potential effects of H_2S, used classic sulfide salts as H_2S donors, synthetic donors as GYY4137 are now preferred as their release kinetics better mimic the time course of endogenously produced H_2S. Non-steroidal anti-inflammatory drugs

(NSAIDs) are already used in POI but cause gastrointestinal mucosal injury. H_2S-releasing NSAIDs are now under development, aiming at reducing the gastrointestinal mucosal damage and enhancing the anti-inflammatory effect by released H_2S. The naproxen derivative ATB-346 was already shown to be safe in healthy volunteers and patients, and to reduce pain in patients with osteo-arthritis. Treatment with both GYY4137 (i.p.) and ATB-346 (i.g.) at 1 h before surgery had a beneficial effect on POI in the murine model (**Chapter V**) as they prevented the delay in transit and suppressed the muscular inflammatory response induced by IM. The protective effects of naproxen on both transit and inflammatory parameters were less pronounced. All three treatments were able to reduce the increase in cyclo-oxygenase 2 activity, whereas only GYY4137 significantly reduced the increase in inducible NO synthase activity. Naproxen caused histological damage of the intestinal villi but this was not seen with ATB-346. Delivery of H_2S can thus be considered to prevent POI or to improve the benefit/side effect balance of NSAIDs in POI.

Conclusions

Despite an increase in systemic CO levels, the release of CO in the gastrointestinal tract obtained via i.g. administration of particular OCORS tablets was not able to exert a protective effect in a murine POI model. Indirect upregulation of endogenous CO levels via pretreatment with the HO-1 inducer hemin resulted in HO-1 dependent protection against POI, whilst the beneficial effects of DMF were HO-1 independent and relied on inhibition of NF- kB and ERK 1/2. Exogenous H_2S per se can reduce POI as demonstrated via administration of the H_2S-donor GYY4137. The same degree of protection was obtained with the H_2S-releasing naproxen derivative ATB-346, while naproxen had a less pronounced beneficial effect on POI and caused intestinal mucosal damage, demonstrating the synergistic/protective effect of the simultaneous released H_2S with ATB-346.

CHAPTER VIII

SAMENVATTING

CHAPTER VIII

Samenvatting

In de gastro-intestinale tractus zijn er vier voorname fysiologische processen, zijnde secretie, digestie, absorptie en motiliteit, die leiden tot de extractie van de nodige nutriënten en water uit het ingenomen voedsel en tot de eliminatie van de resterende afvalstoffen. De gecoördineerde activiteit van de spierlaag van de darmwand resulteert in de beweging van de inhoud van de mond naar de anus en wordt gereguleerd door de chemische input van zenuwen, hormonen en paracriene signalen. Postoperatieve ileus (POI) wordt gedefinieerd als een voorbijgaande storing van deze gecoördineerde darmmotiliteit na een abdominale operatie, waarbij de darmtransit wordt geremd. POI duurt normaal maar een drietal dagen, maar wanneer verlengd kunnen symptomen zoals misselijkheid, braken, abdominale distensie en intolerantie voor oraal voedsel bijdragen tot een verhoogde morbiditeit en een verlengde ziekenhuisopname. Farmacologische factoren dragen bij tot de pathofysiologie van POI, maar de conditie is vooral te wijten aan twee endogene processen: acute neurogene inhiberende reflexen, gevolgd door de meer belangrijke en aanhoudende inflammatoire respons. Deze inflammatoire cascade wordt veroorzaakt door activatie van macrofagen, die resideren in de spierlaag. Dit resulteert in de vrijstelling van inflammatoire cytokines en chemokines die op hun beurt de expressie van adhesiemoleculen stimuleren en leukocyten uit de bloedsomloop aantrekken. Deze cellen zorgen voor opregulatie van stikstofmonoxide (NO) synthase en cyclo-oxygenase 2. De vrijgestelde NO en prostaglandines remmen het neuromusculair mechanisme dat zorgt voor de gecoördineerde spiercontracties van de darm en leiden bijgevolg tot een vertraagde darmtransit. Ondanks de beschikbaarheid van laparoscopische operaties, specifieke herstelprogramma's en farmacologische behandelingsopties, kan er op heden geen volledige bescherming tegen POI bekomen worden, wat de continue zoektocht naar nieuwe behandelingsstrategieën verklaart.

Koolstofmonoxide (CO) en waterstofsulfide (H_2S), welke algemeen gekend zijn voor hun toxische effecten in hoge concentraties bij mens en dier, worden endogeen geproduceerd en spelen een belangrijke rol als signaalmolecules bij zowel fysiologische als pathofysiologische processen in meerdere organen, waaronder het gastro-intestinaal stelsel. De ontdekking dat zowel CO als H_2S anti-oxidatieve en anti-inflammatoire effecten kunnen uitoefenen leidde tot onderzoek van hun potentieel therapeutisch nut bij inflammatoire ziekten in verschillende orgaansystemen. Het doel van deze thesis was het bestuderen van het potentieel beschermend effect van verschillende chemische verbindingen, die ontwikkeld werden om CO of H_2S toe te dienen of vrij te stellen, in een muismodel van POI. In dit model wordt de dunne darm na laparotomie gemanipuleerd wat leidt tot inflammatie van het musculair weefsel van de darm en een vertraagde darmtransit; hierbij wordt de darmtransit gemeten op 24 u na de operatie.

Exogeen CO, toegediend via inhalatie of via een intraperitoneale (i.p.) injectie van CO-vrijstellende moleculen (CORMs), werd reeds met succes gebruikt in verschillende ziektemodellen, waaronder POI. Echter zijn deze toedieningsmethoden minder geschikt voor gebruik bij patiënten, aangezien het niet mogelijk is om via deze wegen specifieke weefsels te gaan behandelen en ze bovendien potentieel toxisch zijn. Om specifiek gastro-intestinale aandoeningen te behandelen, werd een tablet ontwikkeld genaamd “oraal koolstofmonoxide afgifte systeem (OCORS)”. De samenstelling van de deklaag rondom de kern met CORM-2 van deze tablet kan worden gewijzigd, om de snelheid waarmee CO wordt vrijgesteld te kunnen aanpassen en bijgevolg CO af te geven ter hoogte van een specifiek deel van de gastro-intestinale tractus. We evalueerden of OCORS in staat is om POI tegen te gaan in het muismodel van POI **(Hoofdstuk III)**. OCORS tabletten met een diameter van 0,5 mm werden ontwikkeld die in vitro een CO afgifte vertoonden, die eerste orde kinetiek volgde gedurende 2 u waarna de vrijstelling van CO stagneerde. Maagledigingstudies toonden aan dat na intragastrische (i.g.) toediening van 20 tabletten, meer dan 50% van de tabletten de dunne darm had bereikt na 1 u; om deze reden werden de tabletten 1 u voor de operatie toegediend.

Ondanks het feit dat de OCORS tabletten de dunne darm bereikt hadden op het moment van intestinale manipulatie (IM) en er ook een systemische blootstelling aan CO werd waargenomen gelijkaardig aan die van i.p. toedieningsstrategieën, was het gebruikte type van OCORS niet in staat om de musculaire inflammatie en de vertraging van de darmtransit tegen te gaan. Dit kan mogelijk verklaard worden door het feit dat bij vrijstelling van CO in de dunne darm er diffusie optreedt in de darmwand waarbij het snel wordt geabsorbeerd door het capillair netwerk van de mucosa; dit resulteert in een systemische blootstelling aan CO, maar voorkomt dat er voldoende CO doordringt tot in de spierlaag waar de inflammatie ontstaat die POI veroorzaakt. Deze verklaring wordt ondersteund door een eerder uitgevoerde studie waarbij OCORS tabletten met een 24 u vrijstellingsprofiel wel effectief waren in een diermodel van colitis, waarbij de inflammatie start vanuit de mucosa. Onze studie illustreert de beperkingen van het OCORS-concept en de noodzaak voor optimalisatie voor specifieke gastro-intestinale aandoeningen.

Heem oxygenase-1 (HO-1) is het snelheidsbepalende enzym in de degradatie van heem tot ijzer(II)-ion, CO en biliverdine, waarna biliverdine verder wordt omgezet tot bilirubine. Het wordt opgereguleerd in omstandigheden van oxidatieve stress en inflammatie, waarbij het via voornamelijk CO, en in mindere mate biliverdine/bilirubine, anti-oxidatieve en anti-inflammatoire effecten uitoefent. Er werd reeds aangetoond dat inductie van HO-1 via exogene producten een beschermend effect heeft bij inflammatoire aandoeningen in verschillende organen, waaronder het gastro-intestinale stelsel, maar dit werd nog niet onderzocht voor POI. Om deze reden werd het effect van inductie van HO-1 op het muismodel van POI onderzocht door middel van toediening van hemine en dimethylfumaraat (DMF) 24 u voor operatie; beide stoffen zijn gekend voor hun HO-1 inducerende eigenschappen **(Hoofdstuk IV)**. I.p. toediening van hemine, waarvan de effectiviteit reeds werd aangetoond in meerdere experimentele studies, leidde tot een significante daling van de toename in intestinale inflammatoire parameters en verbeterde de vertraagde darmtransit die werd waargenomen na IM. Dit effect was afhankelijk van HO-1 aangezien een gelijktijdige toediening van de HO-1 remmer chroom

mesoporfyrine de voordelige effecten van hemine teniet deed. DMF wordt reeds gebruikt voor de behandeling van multiple sclerose en vertoonde in vitro een optimaal HO-1 inductie/cytotoxiciteit profiel. Zowel i.p. als i.g. toediening van een enkele dosis van DMF verhinderde de ontwikkeling van vertraagde transit en zorgde voor een significantie reductie van de intestinale inflammatie in de spierlaag, geïnduceerd door IM, maar dit effect werd niet beïnvloed door de HO-1 remmer. Bovendien kon enkel hemine, en niet DMF, de intestinale HO-1 expressie doen stijgen bij niet-gemanipuleerde muizen. Verder onderzoek toonde aan dat de protectieve effecten van DMF bij POI het gevolg zijn van een significante reductie van de IM-geïnduceerde activatie van de pro-inflammatoire nucleaire factor kB (NF-kB) en de extracellulaire signaal-gereguleerde kinases (ERK) 1/2. Onze data tonen aan dat zowel de inductie van HO-1 als DMF verder onderzocht kan worden als een therapeutische mogelijkheid voor het voorkomen van POI.

Hoewel initiële studies, die het potentieel effect van H_2S onderzochten, klassieke sulfidezouten als H_2S donoren hanteerden, wordt tegenwoordig de voorkeur gegeven aan synthetische donoren zoals GYY4137 aangezien de vrijgavekinetiek van deze donoren beter het tijdsverloop van endogeen geproduceerd H_2S nabootst. Niet-steroïdale anti-inflammatoire geneesmiddelen (NSAIDs) worden reeds gebruikt bij POI, maar veroorzaken schade aan de gastro-intestinale mucosa. NSAIDs, die H_2S vrijstellen, zijn momenteel in ontwikkeling met het doel om via de afgifte van H_2S de schade aan de mucosa te reduceren alsook het anti-inflammatoir effect te verstrekken. Voor het naproxen derivaat ATB-346 werd reeds aangetoond dat het veilig is in gebruik bij gezonde vrijwilligers en patiënten, en dat het een pijnstillende werking heeft bij patiënten met artrose. Behandeling met zowel GYY4137 (i.p.) als ATB-346 (i.g.) 1 u voor operatie had een positief effect op POI in het muismodel **(Hoofdstuk V)** aangezien beiden de IM-geïnduceerde verstoring van de darmtransit en inflammatoire respons in de spierlaag konden verhinderen. De protectieve effecten van naproxen op zowel de darmtransit als de inflammatoire parameters waren minder uitgesproken. Alle drie de behandelingen waren in staat om de stijging in cyclo-oxygenase 2

activiteit te reduceren, terwijl enkel GYY1437 kon zorgen voor een significante reductie van de activiteit van NO synthase. Bovendien leidde toediening van naproxen tot histologische schade aan de intestinale villi, wat niet werd waargenomen met ATB-346. Behandeling met H_2S kan dus beschouwd worden als een strategie om POI te voorkomen, of om het evenwicht tussen de voordelen en bijwerkingen van NSAIDs bij POI te verbeteren.

Conclusies

Ondanks een toename in de systemische blootstelling aan CO, was de afgifte van CO in de gastro-intestinale tractus na i.g. toediening van het gebruikte type OCORS tabletten niet in staat om een protectief effect uit te oefenen op een muismodel van POI. Indirecte opregulatie van de endogene CO spiegels via voorbehandeling met de HO-1 inductor hemine resulteerde in een HO-1 afhankelijke protectie tegen POI, terwijl de voordelige effecten van DMF onafhankelijk waren van HO-1 en steunden op de inhibitie van NF- kB en ERK 1/2. Exogeen H_2S per se kan POI reduceren zoals aangetoond via administratie van de H_2S donor GYY4137. Dezelfde graad van protectie werd bekomen met het naproxen derivaat ATB-346 dat H_2S vrijstelt, terwijl naproxen een minder uitgesproken voordelig effect had op POI en leidde tot schade aan de intestinale mucosa; dit toont het synergetisch/protectief effect van het door ATB-346 gelijktijdig vrijgestelde H_2S aan.

CURRICULUM VITAE

Curriculum Vitae Jonas Van Dingenen

Particulars

Name	Jonas Van Dingenen
Date of birth	December 4th, 1990
Place of birth	Antwerp, Belgium
Nationality	Belgian
Marital status	living together

Professional career

2021 – now QA consultant at Farma Consulting & Partners, Diest, Belgium

Projects:

- Quality Release Responsible COVID-19 Vaccine at The Janssen Pharmaceutical Companies of Johnson & Johnson
- Quality Release Responsible Eye Care at The Janssen Pharmaceutical Companies of Johnson & Johnson

2019 – 2021 Quality Specialist Operations at Pfizer, Puurs, Belgium

Education

2014 – 2022 *PhD* in Health Sciences

Department of Basic and Applied Medical Sciences, Heymans Institute of Pharmacology, Faculty of Medicine & Health Sciences,

Ghent University, Gent, Belgium

Dissertation: Application of the heme oxygenase-1/carbon monoxide pathway and the hydrogen sulfide pathway as new approach to prevent postoperative ileus

Promotors: Prof. Dr. Lindsey Devisscher & Prof. Dr. Romain Lefebvre

2011 – 2014 Master of Science in Drug Development – *cum laude*

University of Antwerp, Antwerp, Belgium

Thesis: Effect of neuregulin-1 on the collagen synthesis in cardiac fibroblasts (Laboratory of Physiology, University of Antwerp, Antwerp, Belgium)

Promotor: Prof. Dr. Gilles De Keulenaer

2008 – 2011 Bachelor in Pharmaceutical Sciences – *cum laude*

University of Antwerp, Antwerp, Belgium

Additional certificates

- Introductory Statistics (2018, Ghent University, Ghent, Belgium)
- Federation of European Laboratory Animal Science Associations (FELASA) C attest (2015, Ghent University, Ghent, Belgium)

List of publications

Journal articles:

1. Van Dingenen, J., Steiger, C., Zehe, M., Meinel, L., & Lefebvre, R.A. (2018). Investigation of orally delivered carbon monoxide for postoperative ileus. EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS, 130, 306–313. https://doi.org/10.1016/j.ejpb.2018.07.009
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1. Van Dingenen, J., Steiger, C., Zehe, M., Meinel, L., & Lefebvre, R. (2017). Investigation to prevent postoperative ileus via peroral CO. In NEUROGASTROENTEROLOGY AND MOTILITY (Vol. 29, pp. 77–77)

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5. Van Dingenen, J., & Lefebvre, R. (2018). Influence of the slow- release H2S donor GYY4137 and the H2S- releasing naproxen derivative ATB-346 on postoperative ileus. In NEUROGASTROENTEROLOGY AND MOTILITY (Vol. 30, pp. 23–23).
6. Van Dingenen, J., & Lefebvre, R. (2018). Dimethyl fumarate vs hemin for the induction of heme oxygenase-1 as treatment strategy for murine postoperative ileus. In NEUROGASTROENTEROLOGY AND MOTILITY (Vol. 30, pp. 176–176).

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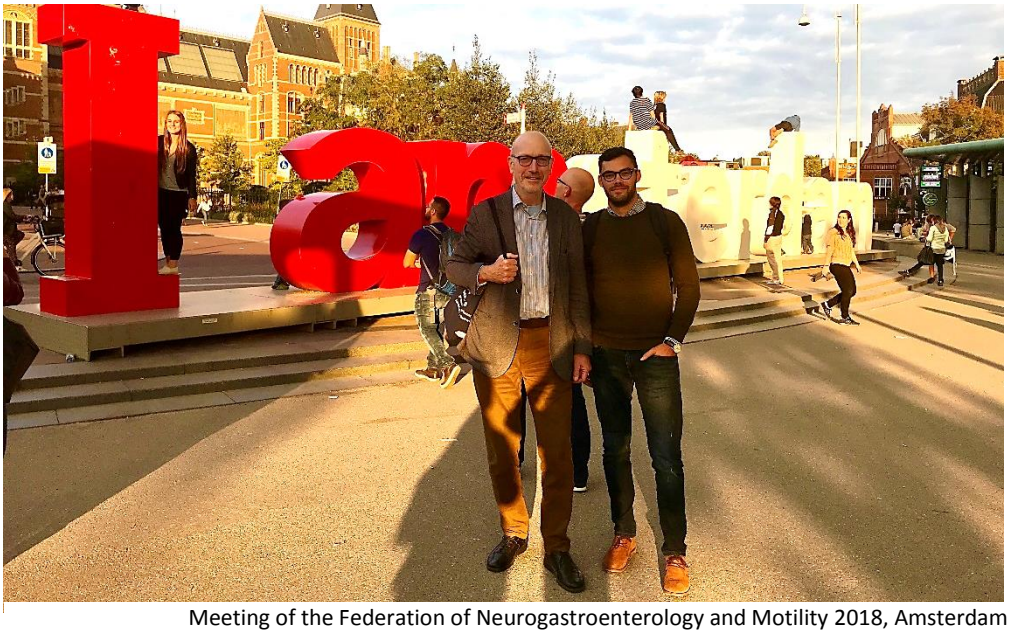

Meeting of the Federation of Neurogastroenterology and Motility 2018, Amsterdam

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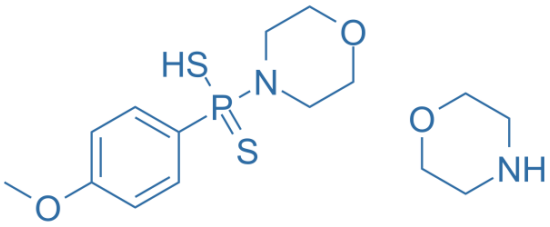
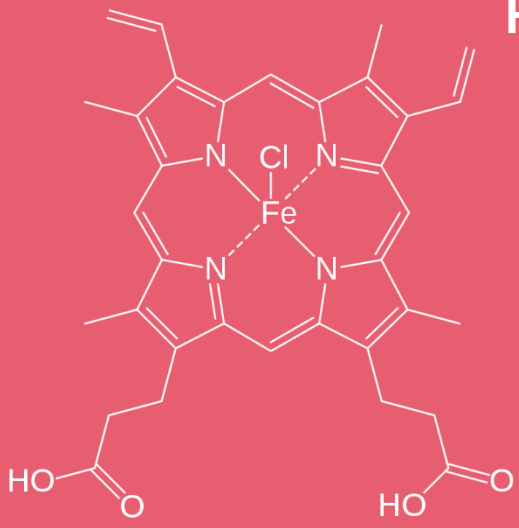
- Jonas out -

Adapted from B. Obama, 2016

GYY4137



HEMIN



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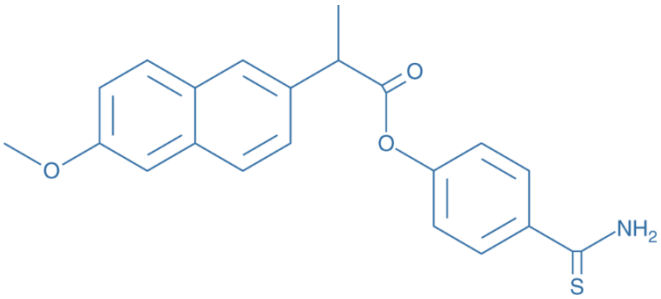

DIMETHYL FUMARATE