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ORIGINAL ARTICLE

Selective matrix metalloproteinase inhibition increases breaking strength and reduces anastomotic leakage in experimentally obstructed colon

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Abstract
Purpose Colonic obstruction causes loss of collagen and impairment of anastomotic integrity by matrix metalloproteinases (MMPs). Unexpectedly, pharmacological MMP inhibition increased anastomotic leakage (AL) in obstructed colon possibly due to the non-selective nature of these compounds and the experimental model applied. We therefore studied the effects of selective MMP inhibition on the healing of anastomoses in colon obstructed by a novel laparoscopic technique.
Methods Left colon was obstructed in 38 male Sprague-Dawley rats (226–284 g). After 12 h, stenoses were resected and end-to-end anastomoses constructed. Baseline breaking strength was determined in 6 animals on day 0. The remaining 32 rats were randomized to daily treatment with the selective MMP-8, MMP-9, and MMP-12 inhibitor AZD3342 (n = 16) or vehicle (n = 16). On day 3, anastomoses were evaluated for AL and breaking strength. Isolated anastomotic wound tissue was analyzed on total collagen and pepsin-insoluble and pepsin-soluble collagen by hydroxyproline. The soluble collagens were further differentiated into native, measured by Sircol, and fragmented forms.
Results Baseline breaking strength was maintained with AZD3342 but decreased by 25% (P = 0.023) in the vehicle group. The anastomotic breaking strength of AZD3342-treated rats was 44% higher (P = 0.008) than the vehicle-treated rats. Furthermore, the AL rate was reduced (P = 0.037) with AZD3342 compared with vehicle treatment. AZD3342 treatment influenced neither the total or insoluble collagen concentrations nor the degree of fragmentation of the soluble collagen triple helices.
Conclusion Selective MMP inhibition increased anastomotic breaking strength and reduced AL after resection of colonic obstruction.

Keywords Anastomosis · Anastomotic leak · Breaking strength · Colonic obstruction · Collagen

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Background

Anastomotic leakage (AL) is a devastating complication after colorectal surgery. The risk of AL increases in the presence of colonic obstruction to about 15% of the patients operated for colorectal cancer [1–3].

Obstruction changes the morphology of the colonic wall rapidly after onset with pronounced edema and infiltration of the mucosa and submucosa first by neutrophils then by macrophages, lymphocytes, and plasma cells [4]. Submucosal collagen is also markedly reduced leading to a destabilized anastomosis [4–6]. These cellular and biochemical changes of the obstructed bowel wall may be responsible for the increased AL rate.

The degradation of collagen has been attributed to the action of matrix metalloproteinases (MMPs) [6, 7]. The MMP family comprises more than 20 different enzymes with both physiological and pathophysiological functions [8]. Treatment

with non-selective MMP inhibitors improves the biomechanical strength of elective anastomoses [9–13]. The mechanisms for the beneficial effects are poorly understood. Paradoxically, the total collagen concentration of the anastomoses seems unaffected by the treatment with these drugs [9, 11]. This observation indicates that MMP inhibitors possibly influence the quality rather than the quantity of collagen during anastomotic healing. One non-selective MMP inhibitor increased the amount of soluble but not insoluble collagen in colonic anastomoses [10]. However, both native and fragmented collagens were included in their analyses [10]. In another study, the non-selective MMP inhibitor BB-94 protected the recently deposited collagen triple helices from MMP-mediated damage [14].

We recently investigated the effect of the non-selective MMP inhibitor GM6001 on anastomotic healing in a rat model complicated by colonic obstruction. Contrary to our expectations, GM6001 treatment *increased* AL [15]. Because epithelial coverage of wounds depends on the activity of specific MMPs [16], inhibition of these obligatory MMPs may have impaired epithelialization. In addition, the use of non-selective MMP inhibitors is associated with musculoskeletal adverse effects, bone destruction, and Dupuytren-like disease [17–19].

Another explanation for the unexpected results with GM6001 is perhaps the animal model. In this model, the breaking strength of anastomoses in obstructed colon *increases* compared with anastomoses in non-obstructed colon [20]. This is remarkable considering that the complications were, as anticipated, more common in anastomoses made in the obstructed colon than in the non-obstructed colon. This contradictory finding can be explained by the priming effect of the inflammatory reaction elicited by the laparotomy. Blood levels of the pro-inflammatory cytokine TNF-α were also higher in rats undergoing laparotomy compared with rats subjected to laparoscopy [21]. Another important finding is that pneumoperitoneum itself does not seem to influence the strength of colonic anastomoses [22].

Our primary objective was to investigate the effect of selective MMP inhibition on anastomotic repair in the obstructed colon. To circumvent the disadvantage with the previous experimental models [15, 20], we developed a novel laparoscopic method to induce obstruction. For the intervention studies, we chose the selective MMP-8, MMP-9, and MMP-12 inhibitor AZD3342 with proven efficacy in the repair of anastomoses in the normal, non-obstructed colon [23]. To study the effect of the AZD3342 on collagen metabolism specifically, total as well as fractionated collagens present in the anastomoses were analyzed.

Methods

The study adhered to the ARRIVE guidelines for reporting animal research [24].

Animals Thirty-eight inbred male Sprague–Dawley albino rats (Taconic, Ry, Denmark), weighing 226–284 g, were acclimatized for at least 7 days prior to surgery and kept in type III cages at room temperature with a 12-h light cycle. The rats were transferred to individual cages after the initial surgical procedure. The animals had free access to tap water and a highly digestible diet (TransWean; Special Diets Service, Essex, UK). The experiments were approved by the Animal Ethics Committee of the Danish Ministry of Justice (2010/561-1775).

Study design Acute colonic obstruction was induced by a novel laparoscopic technique. After 12 h of obstruction on day 0, the stenotic segment was resected and a primary anastomosis constructed. In 6 of the 38 rats, the anastomotic breaking strength was measured immediately after construction of the anastomosis day 0. The remaining 32 rats were assigned to daily treatment with AZD3342 (n = 16), or with vehicle (n = 16) by randomization. Three days after anastomotic surgery (day 3), the anastomoses were evaluated for AL, breaking strength, and total and fractionated collagens.

Selective MMP inhibitor AZD3342 (AstraZeneca Research and Development, Mölndal, Sweden) is a 403 D, synthetic non-hydroxamate MMP-8, MMP-9, and MMP-12 inhibitor [23]. The half-maximal inhibitory concentration (IC_{50}) for AZD3342 with respect to MMP-8 was determined to 16 nM, for MMP-9 10 nM and for MMP-12 6 nM. Accordingly, AZD3342 presents a greater than three orders of magnitude selectivity for MMP-8, MMP-9, and MMP-12 over MMP-1 and tumor necrosis factor-α (TNF-α) converting enzyme (TACE) [23].

AZD3342 at 50 mg/kg in vehicle (10% 2-hydroxypropyl-β-cyclodextrin; Sigma-Aldrich, St. Louis, MO) or vehicle alone were injected subcutaneously (s.c.) daily for three consecutive days starting day 0 directly after construction of anastomoses. All individuals handling the rats were blinded to the group allocation.

Anesthesia and analgesics Anesthesia was introduced with a mixture of isoflurane (Baxter, Deerfield, IL) 3.5%/O_2 (1 l/min) for 2 min and maintained with isoflurane (2%)/O_2. Bupivacaine (Marcain®; AstraZeneca, London, UK) 2 mg/kg was injected s.c. at the incisional site for local analgesia. Preoperative analgesia was provided by s.c. injections of 5 mg/kg carprofen (Rimadyl®; Pfizer Animal Health, New York, NY) and 0.03 mg/kg buprenorphine (Temgesic®; Schering-Plough, Brussels, Belgium). After completion of the anastomoses, rats were given 5 ml saline s.c. and 5 ml saline intraperitoneally. Postoperative analgesia in the treated rats was provided by buprenorphine (0.4 mg/kg) per os in a

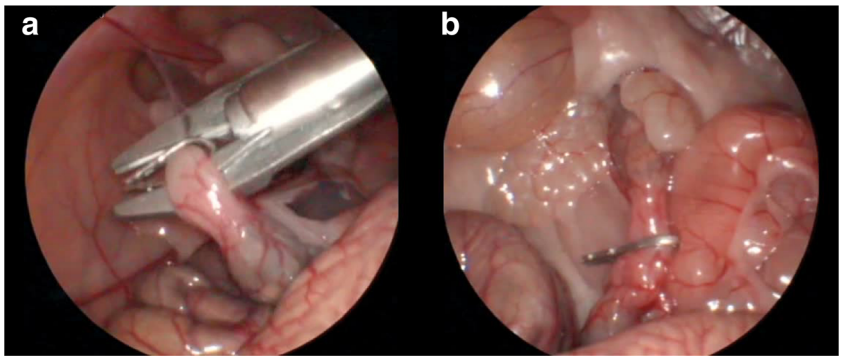

Fig. 1 Laparoscopic induction of experimental colonic obstruction in rats. **a** Application of a titanium clip on the left colon 30 mm from the peritoneal reflection using a 5-mm clip applier. **b** The titanium clip in situ on the left colon just after its application

hazelnut butter mixture at 8-h intervals and with daily s.c. injections of carprofen (5 mg/kg).

Induction of colonic obstruction and construction and evaluation of the anastomoses A 2.7-mm, 30° TrueView II arthroscope (A70963A; Olympus Danmark, Ballerup, Denmark) with a working distance of 70 mm was used to induce acute colonic obstruction. The scope was connected to a VISERA OTV-S7 N-H camera head, OTV-S7 video processor, and a CLV-S40 light-source (Olympus Danmark). A 2-mm skin incision was made in the epigastrium, and a suture was placed tangentially through the abdominal wall. Pneumoperitoneum was established with a 21-gauge needle as the holding suture was used to retract the abdominal wall. The abdominal cavity was then inflated with CO_2 to a maximal pressure of 4 mmHg using the UHI-2 insufflation unit (Olympus Danmark). Hereafter, a sheet and trocar were introduced through a 1-mm incision in the linea alba. The videoscope was inserted through the sheet. In the right lower quadrant, a 3-mm skin incision was made and the muscle fibers were split with a straight hemostat. The hemostat was forced through a 1-mm incision in the peritoneum where, after the laparoscopic, instruments were inserted directly into the abdominal cavity between the branches of the hemostat. The incision was tightened with a purse string suture (Ethicon, Somerville, NJ). A titanium clip was applied around the colon 30 mm from the peritoneal reflection, between two marginal veins using a 5-mm EndoClip (Autosuture; Covidien, Dublin, Ireland) shown in Fig. 1. Feces in the distal colon were cleared. The abdominal wall at the lateral incision was closed with two single Ethilon® 3/0 sutures (Ethicon). The skin incision was closed with titanium clips (Appose ULC 35 W; Covidien).

After 12 h of colonic obstruction (day 0), the peritoneal cavity was exposed through a 30-mm midline incision. A 15-mm colonic segment with the obstructive clip in the middle was resected. The colon was opened at the anti-mesenteric border and the inner circumference of the colon measured 10 mm proximal and distal to the stenosis. An end-to-end single-layer anastomosis was constructed with nine interrupted Ethilon® 6/0 polyamide sutures (Ethicon). The abdominal muscles and the transverse fascia were closed with a continuous Ethilon® 3/0 suture (Ethicon) and the skin incision with titanium clips (Appose ULC 35 W).

The return of bowel function was taken as the time to first defecation determined through daily inspection of the cages.

On day 3, a re-laparotomy was commenced, and the anastomoses were evaluated macroscopically and meticulously. AL was defined as a visible defect in the anastomotic suture line or an abscess in conjunction with the suture line [25]. The anastomoses were then freed of adhesions, excised with a 20-mm margin on each side of the suture line, and placed in saline. The colonic segment was fastened with clamps positioned 10 mm apart in a material testing machine equipped with a 10 N XLC load cell (LF Plus; Lloyd Instruments, Bognor Regis, UK). The specimen was pulled apart within 10 min after excision and vertically at 10 mm/min and the breaking strength in Newton (N) determined from the load-deformation curve [23]. The healing zone of the anastomoses was dissected from macroscopically uninjured colon. Two excisional biopsies of the anastomotic line were snap-frozen on dry ice and stored at −80 °C until analyzed for total collagen concentration and collagen fractions.

Collagen assessments Total anastomotic collagen was determined by hydroxyproline. One of the two anastomotic wound biopsies was lyophilized (11.4 ± 4.6 mg dry tissue), hydrolyzed in 6 N hydrochloric acid at 110 °C for 18 h, and assayed for hydroxyproline colorimetrically [26]. Hydroxyproline was converted into collagen by multiplying with 7.46 [27].

To further characterize the collagen present in the anastomotic wounds, collagens were isolated into pepsin-insoluble

Table 1 Colonic circumference (mm) after 12 h of obstruction

	Controls	Vehicle	AZD3342	*P*
Proximal	18.3 ± 2.4	18.6 ± 2.0	19.2 ± 2.0	.597[a]
Distal	10.3 ± 2.1	10.4 ± 1.3	10.8 ± 1.2	.603[a]
	P < .001[b]	*P* < .001[b]	*P* < .001[b]	

Mean ± SD

[a] One-way ANOVA

[b] Paired *t* test

Table 2 Study flow (number of rats)

	Vehicle	AZD3342	*P*
Starting population	16	16	
Withdrawn from study[a]	0	1	.999
Intention-to-treat population	16	15	
Mortality	3	4	.685
Per-protocol population	13	11	

[a] Trocar hernia day 0. Fisher's exact test

and pepsin-soluble collagen. Technically, the other anastomotic wound biopsy (46.7 ± 17.3 mg wet tissue) was finely dispersed for 10 s at 30,000 r.p.m. using a T10 Ultra-Turrax® instrument (IKA-Werke, Staufen, Germany) equipped with a 5-mm dispersing tool (S10 N-5G) in 5 ml of 0.5 N acetic acid with 1 mg pepsin (P7012; Sigma-Aldrich) per 10 mg wet tissue [28]. A pre-study showed that the amount of solubilized collagen determined by the Sircol collagen assay (Biocolor, Carrickfergus, Northern Ireland, UK) doubled in homogenized colonic tissue compared without prior homogenization. The homogenate was incubated for 24 h at 4 °C with continuous stirring at 100 r.p.m., then centrifuged at 16,000×*g* for 10 min and supernatant aspirated. One aliquot (0.5 ml) of the supernatant representing the total soluble collagen fraction was mixed with 0.5 ml 12 N hydrochloric acid, hydrolyzed at 110 °C for 18 h, and assayed for hydroxyproline [6]. Another aliquot (1.0 ml) representing intact soluble collagen was analyzed by Sircol per the manufacturer's collagen isolation and concentration protocol and with the rat type I collagen standard. The addition of AZD3342 (100 nM) [23] during the 24-h incubation period did not influence the soluble collagen levels by Sircol. A third aliquot (0.5 ml) was lyophilized, reconstituted in 0.05 ml NuPAGE® LDS sample buffer (Thermo Fisher Scientific, Carlsbad, CA) without or with 50 mM dithiothreitol (70 °C for 10 min), and electrophoresed on NuPAGE® 4–12% Bis–Tris gels (Thermo Fisher Scientific). The gels were stained with Colloidal Blue (Thermo Fisher Scientific). The pellet, representing insoluble collagen, was analyzed by hydroxyproline after hydrolysis in 6 N hydrochloric acid at 110 °C for 18 h.

Table 3 Body weight (g) after obstruction but before anastomotic surgery day 0 and 3 days after anastomotic surgery (per-protocol)

	Controls	Vehicle	AZD3342	*P*
Day 0	269 ± 12	262 ± 15	264 ± 10	.958[a]
Day 3		244 ± 19	252 ± 16	.240[b]
		$P < .001$[c]	$P < .001$[c]	

Mean ± SD

[a] One-way ANOVA

[b] Unpaired *t* test

[c] Paired *t* test

Sample size calculation and statistical analyses The sample size was based on the results of a previous study on colonic obstruction in rats, where each group comprised 12 animals [15]. Because of the unknown effects of the laparoscopic technique, we decided to include 16 animals in the vehicle group and 16 animals in the AZD3343 group.

Colonic circumference and complication rates were analyzed according to the intention-to-treat principle using one-way analysis of variance (ANOVA) or Fisher's exact tests. Body weight, anastomotic breaking strength, and collagen levels were evaluated per-protocol with the unpaired or paired *t* test or ANOVA. Collagen levels were log-transformed before performing the statistical analyses. All analyses were two-sided and carried out using IBM® SPSS® Statistics Version 20 (IBM Corporation, Armonk, NY). Data are presented as mean ± standard deviation (SD) unless stated otherwise. The level of statistical significance chosen was $P < 0.05$.

Results

Effects of acute colonic obstruction After 12 h of colonic obstruction, the circumference of the colon proximal to the obstruction increased 1.8-fold in the three groups (Table 1). The body weight after 12 h of obstruction increased from 263 ± 10 to 272 ± 12 g ($P < 0.001$) in the control group, from 250 ± 10 to 261 ± 15 g ($P < 0.001$) in the vehicle group, and from 250 ± 17 to 261 ± 13 g ($P < 0.001$) in the AZD3342 group.

Mortality and bowel function following anastomotic surgery and treatments One rat in the AZD3342 group was excluded because of a postoperative trocar hernia. Three of the 16 vehicle-treated rats and 4 of the 15 AZD3342-treated rats died prematurely (Table 2). One vehicle-treated animal died on postoperative day 3 with a completely dehisced anastomosis, which disqualified this animal from breaking strength determination. One animal in the AZD3342 group died in conjunction with anesthesia on day 0. The remaining 5 rats, 2 from the vehicle group and 3 from the AZD3342 group, died between days 1 and 2. Autopsy of these animals did not reveal any obvious causes.

The bowel function had returned in 8 of the 13 vehicle-treated animals compared with 8 of 11 animals treated with AZD3342 ($P = 0.562$) that survived the 3-day postoperative period.

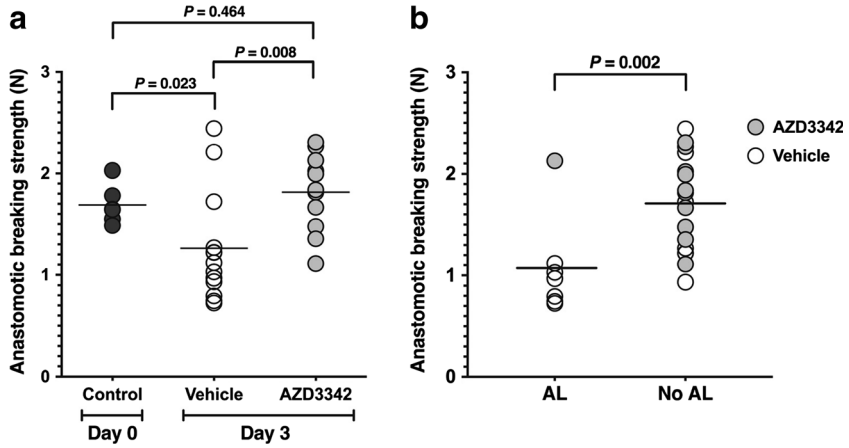

Fig. 2 Anastomotic breaking strength directly after construction of the anastomosis in obstructed colon day 0, and 3 days later in vehicle and AZD3342-treated rats (**a**). **b** Anastomotic breaking strength in rats with (n = 7) or without (n = 17) anastomotic leakage (AL). *Horizontal bars* indicate group mean values

Effect of AZD3342 on body weight, anastomotic breaking strength, AL, and anastomotic collagen

Body weight After anastomotic surgery, the body weight decreased in both groups (Table 3).

Breaking strength Anastomotic breaking strength was analyzed by the per-protocol principle because of the animal deaths before day 3 (Fig. 2a). Breaking strength day 3 was lower (P = 0.023) in the vehicle-treated rats (1.26 ± 0.54 N) but not (P = 0.464) in the AZD3342-treated rats (1.82 ± 0.38 N) compared with initial breaking strength day 0 (1.69 ± 0.19 N). AZD3342 treatment increased (P = 0.008) anastomotic breaking strength by 44% compared with vehicle treatment day 3.

Anastomotic leak To account for all animals included in this high-risk model, the intention-to-treat principle was applied for the statistical assessment of complications. One animal of the 15 animals in the AZD3342 group developed AL compared with 7 of 16 vehicle-treated (P = 0.037) animals (Fig. 3). The anastomotic breaking strength was lower in rats showing AL (P = 0.002) compared with non-AL rats (Fig. 2b).

Analyses of different collagens The total collagen concentration in the anastomotic wounds decreased from day 0 to day 3 with no statistical difference between the vehicle and AZD3342 groups day 3 (Fig. 4). After fractionation of the anastomotic wound tissue with pepsin treatment, the resulting insoluble collagen fraction of the anastomoses did not differ significantly between the vehicle and the AZD3342-treated animals either. Furthermore, the total concentration of soluble collagens and the proportion of intact collagen to total collagen in the soluble fraction was not significantly different between the vehicle and AZD3342 groups either (Table 4). The soluble collagen fractions were also analyzed after

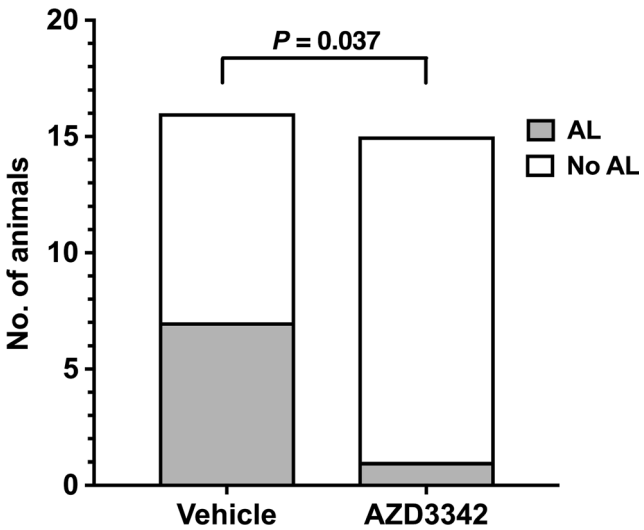

Fig. 3 Effect of the selective MMP inhibitor AZD3342 on the occurrence of anastomotic leakage (AL) on day 3

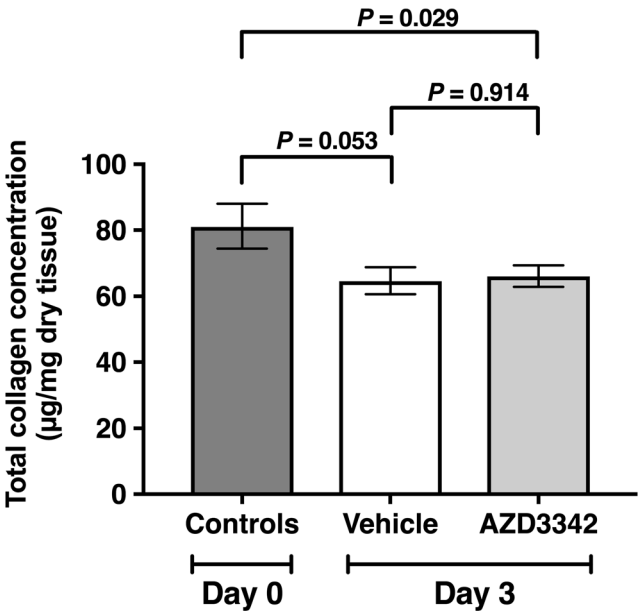

Fig. 4 Total collagen (converted from hydroxyproline) concentration in the anastomosis. Geometric mean ± backtransformed standard error

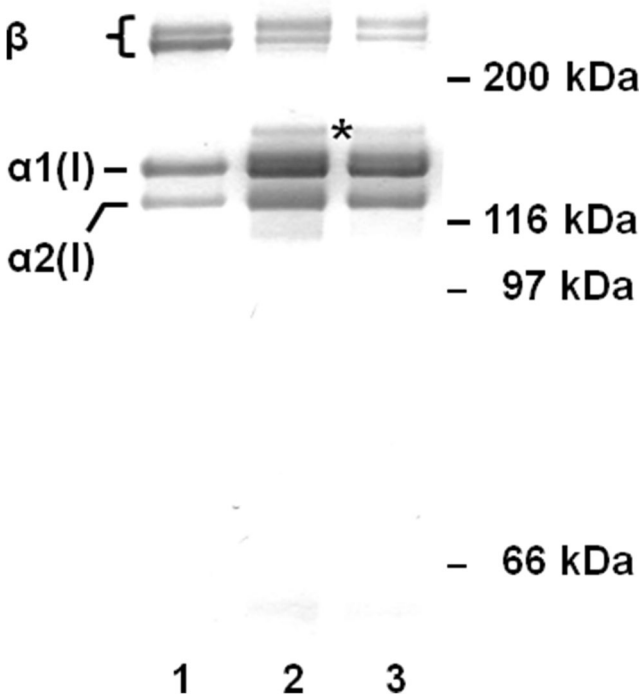

Fig. 5 Analysis of the pepsin-soluble collagen fraction of anastomotic wound tissue by electrophoresis (non-reduced). Lane *1*, rat type I collagen standard (1 μg, Biocolor); *2*, pepsin extract from the vehicle group; *3*, pepsin extract from the AZD3342 group. The corresponding determinations of collagen by the Sircol assay were 1.1 μg (lane 2) and 0.7 μg (lane 3). *Asterisk* (*) indicates unidentified band

electrophoretic separation. Sircol and electrophoretic collagen determinations showed good concordance. The pepsin extracts contained primarily monomers of type I collagen. The band above α1 chains indicated with asterisk (*) in Fig. 5 was also present when gel was run at reduced conditions indicating that it was not the α1 chain of type III collagen but possibly the α1 chain of type I procollagen. No apparent differences were found between anastomotic pepsin extracts of vehicle and AZD3342-treated animals.

Discussion

AL is a serious complication following emergency surgery for colonic obstruction. There are no available therapeutic agents to prevent AL [13, 29]. Although overexpression of certain MMPs is detrimental to anastomotic wound healing indiscriminate MMP inhibition *increases* AL in experimental models of colonic obstruction [15]. In the present study, we have demonstrated that by using a selective MMP inhibitor, AL is *reduced* perhaps by increasing the anastomotic biomechanical strength following experimental colonic obstruction.

In established experimental models of acute colonic obstruction, typically in animals undergoing laparotomy, suture materials or silicone rings are placed around the colon for periods of 24 h or longer [4, 7, 20, 29]. One drawback with these procedures is that the laparotomy per se increases MMP levels in the colon [7]. To correct for this model artifact, we developed a novel laparoscopic model of acute colonic obstruction. To avoid complications from prolonged obstruction by the titanium clip, the duration of obstruction was reduced to 12 h. This approach produced almost a 2-fold increase in the colonic circumference, corresponding to the circumference of using a silicone ring for 24 h [4]. Anastomoses constructed in the 12-h obstructed colon showed the anticipated high AL rate (44%). These observations suggest that by reducing the systemic response to trauma, the expected complications were manifested clinically using our laparoscopic technique.

The increased breaking strength with AZD3342 treatment was not accompanied by increased total collagen concentration of the anastomoses. Moreover, AZD3342 treatment increased neither the ratio of insoluble collagen to total soluble collagen nor the intact forms of soluble collagen. These findings suggest that AZD3342 did not alter the cross-linked, existent collagen of the anastomoses [30] or protected the susceptible soluble collagens from MMP-mediated fragmentation. This disagrees with the effects of non-selective MMP inhibition [14] indicating that other MMPs than MMP-8, MMP-9, and MMP-12 are responsible for collagen remodeling in the anastomoses. Taken together, these findings indicate that AZD3342 improved anastomotic healing by mechanisms other than by influencing collagen metabolism. It should be emphasized though, that AZD3342 possibly impacted other parameters important for tissue strength such as the type I collagen to type III collagen ratio, and/or collagen fiber diameter or orientation [31].

We speculated that the unfavorable effects of GM6001 [15] were due not only to inhibition of epithelialization-requiring MMPs [16] but also to reduced levels of TNF-α via inhibition of TACE. AZD3342 is a poor inhibitor of TACE and primarily inhibits inflammation-associated MMPs directly but spares the MMPs required for epithelialization [16].

The optimal MMP selectivity for AL prevention is unknown. The concept of selective MMP inhibition to improve complicated anastomotic wound healing was recently applied to an ischemic anastomotic rat model [32]. Shogan et al. observed a substantial reduction in AL from 50% in non-treated animals to almost zero in animals treated with an MMP-9 inhibitor [32]. Our results with AZD3342 suggest that by targeting up-regulated MMP-8, MMP-9, and MMP-12, the anastomotic wound healing complicated by an acute obstruction is significantly improved. We cannot exclude that inhibition of additional MMPs would produce a superior effect compared with AZD3342. For example, MMP-13 mRNA levels are highly up-regulated in day-3 colonic anastomoses [23]. From an overall clinical benefit-risk perspective, an additional advantage with selective MMP inhibitors is the expected reduced occurrence of adverse effects as compared with non-selective MMP inhibitors [17–19].

Table 4 Collagen forms in the day-3 anastomoses (per-protocol)

	Vehicle	AZD3342	*P*
Insoluble collagen (μg/mg wet tissue)	5.1 ± 1.5	3.9 ± 1.4	.071
Total soluble collagen (μg/mg wet tissue)	11.0 ± 0.5	10.7 ± 0.7	.320
Intact soluble collagen/total soluble collagen	0.12 ± 0.11	0.12 ± 0.10	.878
Insoluble collagen/total soluble collagen	0.46 ± 0.14	0.37 ± 0.14	.131

Geometric mean ± back-transformed standard error. Unpaired *t*-test

At least four different compounds have previously been investigated in experimental models of obstructed colon [29]. Iloprost, a synthetic prostacycline analog, was the most promising compound [29, 33]. Iloprost treatment decreased MMP-13 protein levels, more than doubled anastomotic bursting pressure but did not reduce the AL rates of 4-day-old anastomoses in obstructed colon [33].

In conclusion, selective MMP inhibition increased anastomotic breaking strength and decreased the AL rate in the acutely obstructed colon. The mechanism for the beneficial effects appears unrelated to the quantity or MMP susceptibility of existent or nascent collagen molecules and thus requires further elucidation. Nevertheless, it seems worthwhile exploring this class of therapeutics further for AL prevention after acute colonic obstruction.

Compliance with ethical standards The experiments were approved by the Animal Ethics Committee of the Danish Ministry of Justice (2010/561-1775).

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