Chapter 4

Farnesoid X Receptor activation aggravates methotrexate-induced gastrointestinal mucositis

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ABSTRACT

Introduction: Gastrointestinal mucositis is a severe side effect of chemotherapy. Bile salts, present in the intestinal lumen in high (millimolar) concentrations to promote fat absorption, can be cytotoxic depending on their chemical structure and act as signaling molecules via Farnesoid X Receptor (FXR, NR1H4). Alterations in the amount and composition of intestinal bile salts may contribute to diarrhea, inflammation, or bacterial overgrowth and thus to the etiology of mucositis. Therefore we aimed to determine the effect of a potent FXR-agonist, known to modulate bile salt metabolism, on the severity and recovery of methotrexate (MTX)-induced mucositis in rats.

Methods: Male Wistar rats (n=44) received 45 mg/kg, 60 mg/kg MTX or saline (control) as single intravenous injection. A potent FXR-agonist (PX20606) was daily administered by gavage starting seven days before MTX injection until termination at days 6 or 12 after MTX. Bodyweight, food intake, presence of diarrhea and plasma citrulline levels were determined continuously. At day 6 or day 12, bile cannulation was performed to assess biliary parameters. In addition, caecal microbiota, villus length and MTX plasma levels were determined.

Results: FXR activation aggravated the severity of mucositis, associated with a steeper decrease in bodyweight and food intake, more diarrhea and a lower survival rate, despite the fact that FXR activation markedly increased the hydrophilicity and thereby reduced the cytotoxic potential of the bile salt pool. However, FXR activation also led to elevated plasma citrulline levels and increased villus length in control rats as well as in MTX-treated rats during their recovery phase, indicating trophic actions of FXR in the intestine. The diversity of the microbiome was reduced by MTX, whereas the FXR-agonist, surprisingly, did not significantly affect microbiome composition in control or MTX treated rats.

Conclusions: Our data indicate that FXR activation worsens MTX-induced mucositis in rats; a more hydrophilic bile salt pool does not provide protection. Since FXR activation promotes intestinal growth, future studies should determine whether FXR activation might be useful in clinical situations that benefit from stimulation of intestinal repair.
INTRODUCTION

Gastrointestinal mucositis, further referred to as mucositis, is a severe side effect of both chemotherapy and radiotherapy and is associated with high morbidity [1,2]. The proposed 5-phase pathophysiological model of mucositis development includes an increase in transcription factors including NF-κb, release of pro-inflammatory cytokines, apoptosis, and ulcerations which are potential portal entries for several microorganisms [1,3]. However, the exact mechanisms underlying the onset and propagation of mucositis have not been elucidated [4-9]. Bile salts have so far not been implicated as causative factors in the etiology of mucositis. Bile salts are synthesized in the liver from cholesterol [10]. Upon their release in the intestinal lumen, they play an important role in the solubilization, digestion and absorption of dietary lipids and lipid-soluble vitamins [10,11]. Primary bile salts, de novo synthesized in the liver, can be converted by commensal bacteria into secondary bile salts [12,13]. Primary and secondary bile salts can be reabsorbed in the ileum, leading to a circulating bile salt pool comprising of different bile salt species of which the physicochemical characteristics (i.e., detergent activity) are determined by number and location of hydroxyl groups on the steroid moiety and conjugation of either taurine or glycine to the side chain [14,15]. Also the amount of bile salts entering the intestine is important: high bile salt concentration can be toxic for the intestinal wall and cause diarrhea and inflammation [16]. On the other hand, a decreased supply of bile salts can lead to bacterial overgrowth, translocation across mucosal barrier and, consequently, to systemic infections [10,16]. Bile salts regulate their own synthesis, to a large extent, via activation of the Farnesoid X Receptor (FXR, NR1H4). FXR is a member of the nuclear receptor superfamily and highly expressed in liver and small intestine [11,16-19]. Upon activation by bile salts, FXR binds RXR (Retinoid X receptor, NR2B1), to modulate the transcription of its target genes in enterocytes and in liver cells [11,16]. Activation of FXR by endogenous ligands (i.e., bile salts) or synthetic non-steroidal FXR agonists in liver and small intestine, in the latter case through stimulation of FGF 15/19 production, suppresses hepatic bile salt synthesis and modulates bile salt pool composition [20-26]. Moreover, in animal models of inflammatory bowel disease and total parenteral nutrition-related mucosal atrophy, FXR activation has been shown to inhibit inflammatory cytokine production, affect the microbiota, improve the villus and crypt lengths, stimulate intestinal growth and reverse mucosal atrophy [25,27-29]. Therefore, targeting FXR might interfere at different levels in the 5-phase pathophysiological model of mucositis and may be of potential use in the prevention and treatment of mucositis. In this study we aimed to determine the effects of the potent FXR agonist Px20606 on the severity and recovery of Methotrexate (MTX)-induced mucositis in rats.
MATERIAL AND METHODS

Animals and housing
Male Wistar outbred rats (4 weeks old, 84-101 gram) were obtained from Charles River (Sulzfeld, Germany) and allowed to acclimatize for one week. They were individually housed at a humidified temperature of 21 °C and kept on a 12 hours day-night cycle (7:00 AM-7:00 PM). Water and AIN93G diet where available ad libitum. The study protocol was approved by the Ethics committee for Animal Experiments, University of Groningen, The Netherlands.

Materials
Methotrexate was obtained from Pharmachemie Holding B.V. (Haarlem, the Netherlands). AIN93G diet (composition macronutrients: fat 17%, protein 19%, carbohydrates 64%) was obtained from Research Diet Services (Wijk bij Duurstede, the Netherlands). Px20606, a FXR agonist [22,23], was kindly provided by Dr C. Kremoser (Phenex Pharmaceuticals, AG, Heidelberg, Germany).

Experimental methods
Short-term experiment
Male Wistar rats (n=28) were randomized by weight into four groups to receive 5 mg/kg Px20606 or vehicle by gavage in combination with 60 mg/kg MTX or its solvent saline by intravenous injection, leading to four groups, i.e., Control, Px, MTX60 and MTX60+Px(n=6-8). Px20606 was dissolved in 0.5% Polyvinylpyrrolidon (PVP k3 0), 0.1%Tween 80 and 1.5 % benzyl alcohol in Phosphate Buffered Saline (PBS)) as described [20,22].
From day -7 until day 5 (12 days) the rats received daily 5 mg/kg Px20606 or vehicle by gavage. At day 0, the rats were iv injected with either MTX 60 mg/kg or NaCl 0.9% in the dorsal penile vein under isoflurane anesthesia. Daily measurements of food intake, bodyweight, illness (bad fur, red nose, decreased activity) and diarrhea were documented. Plasma citrulline was measured as marker for the severity of mucositis in blood samples obtained via the tail tip at day -7, day 0, day 4 and day 6 [30-33]. At day 1, an additional blood sample was collected to measure MTX plasma levels. At day 6, the rats were anesthetized using isoflurane and bile was collected for 10 minutes. Thereafter, the rats were euthanized by bleeding from the vena cava inferior followed by cervical dislocation. Blood was centrifuged for 10 minutes at 2,000 g and stored at -20 °C until further analysis. The small intestines were excised, flushed with PBS and smaller parts of the small intestine were fixed in formalin, dehydrated and embedded in paraffin according to standard procedures for histology. In order to investigate the microbiota of the animals, caecal contents were collected and stored at -20 °C until further analysis.
Long-term experiment
Male Wistar rats were randomized by weight into two groups, i.e., the MTX45 group (n=8) was intravenously injected with 45 mg/kg MTX during vehicle treatment by gavage while group MTX45+Px (n=8) received 45 mg/kg MTX iv during Px20606 treatment by gavage. The dose of MTX was lowered to induce a less severe mucositis and the recovery was followed during a longer period of time, according to a previous study [34]. Experimental procedures were identical to those described for the short-term experiment.

Analytical methods
Plasma citrulline concentrations
Citrulline concentrations were measured in 30 µl plasma using automated ion exchange column chromatography, as described before [35-37].

Histology
Hematoxylin and eosin (H&E) staining was performed on 3 µm thick sections of formalin-fixed duodenal, jejunal and ileal segments, as done previously [37]. The H&E slides were scanned using Aperio scanscope (Aperio Technologies, Vista, CA, USA). Villus length and crypt length were blindly measured manually in well-orientated sections from digitized images. The digitized images were analyzed at 10 x magnification, with 10 measurements per rat using Aperio Imagescope software (Aperio Technologies).

Composition and concentration of bile salts in bile and in plasma
Levels of individual bile salts were measured in bile and plasma samples using ultra high performance liquid chromatography, as described previously [38].

DNA extraction, MiSeq preparation and analysis of sequences.
DNA was extracted from 0.20-0.25 gram caecal content precisely as described before by de Goffau et al. [39]. Next, a polymerase chain reaction (PCR) was performed to amplify the V3-V4 region of the 16S rRNA gene using the 341F and 806R primers with a 6 nucleotide barcode as described earlier [40]. Here, also a description of the PCR, cleanup of the PCR product and preparation of the MiSeq library were described. To analyze the data received from Illumina paired-end sequencing different software programs were used including PANDAseq [41], QIIME and ARB [42]. QIIME was used to identify bacteria to family and genus level, whereas ARB identified the bacteria to species level.
**Methotrexate level**

In 50-60µl plasma and bile the MTX concentration was measured using the enzyme-multiplied immuno assay (EMIT) on an automated drug analyzer (Abbott Architect C8000), as described previously [43].

**Statistical analysis**

Statistical analysis was done using SPSS version 23.0 for Windows (SPSS Inc., Chicago, IL, USA). Values are expressed as medians and ranges in text, or interquartile ranges in figures. Non-parametrical data analysis between more than two groups was assessed by using the Kruskal Wallis test with post hoc Mann-Whitney U-test with Bonferoni correction. Data analysis with non-parametrical data between two groups was performed by using a Mann-Whitney U-test. Survival analysis was assessed by using Cox Regression analysis. A p-value of <0.05 was considered to be statistical significant.

**RESULTS**

**FXR activation worsens the clinical parameters in MTX-induced mucositis**

MTX (60mg/kg) induced mucositis in all rats in the short-term experiment, as shown in Figure 1. Bodyweight was significantly decreased in both MTX60 groups as compared to the respective control groups, without difference between the MTX60 and MTX60-Px groups. Food intake was significantly decreased after induction of mucositis in both MTX-treated groups compared to the control groups, again without differences between the MTX and MTX-Px groups. Diarrhea was present during at least two days in 60% of the rats in both the MTX and MTX-Px groups. The survival in the MTX-Px group (62.5%) tended to be lower (p=0.108) than the survival in the MTX group (87.5%) and both control groups (100%).

Supplemental Figure 1 shows the MTX plasma levels measured at day 1, 24 hours after MTX60 injection and the MTX levels in bile collected at day 6. The MTX plasma levels were significantly higher in the MTX60-Px group compared to the MTX60 group (105 µg/l, 59-228 vs 65.5, 40-154 vs, respectively, p<0.05). The MTX levels in bile collected at day 6 were similar in the MTX60-Px group compared to the MTX60 group (39.5 µg/l, 16-113 vs 110, 35-156, respectively, p=0.2). To evaluate the effects of Px treatment in more subtle MTX-induced mucositis as well as its potential influence on recovery, a lower dose of MTX (45 mg/kg) was injected and rats were
followed for 2 weeks after MTX. The lower dose of MTX also induced mucositis in all rats, more severely in the MTX45-Px group than in the MTX45 group, as shown in Figure 2. Bodyweight was significantly lower in the MTX45-Px group at day three, four and five after injection and normalization of bodyweight was delayed in these animals. Food intake was significantly lower at day two in the MTX45-Px group compared to the MTX45 group. Moreover, significantly more rats in the MTX45-Px group (i.e., 90%) developed diarrhea as compared to only 30% in MTX45 group (p<0.05). Importantly, the survival of 62.5% in the MTX45-Px group was significantly lower compared to 100% survival in the MTX45 group (p<0.05).
Plasma citrulline levels are increased upon FXR activation

Plasma citrulline levels, as a measure of enterocyte mass, was significantly increased after 7 days of treatment with Px20606, as measured just prior to MTX60 injection (Figure 3). At day 4 and 6 plasma citrulline levels were decreased significantly in the MTX60-treated groups compared to the respective control groups, while the levels in the Px group remained elevated when compared to untreated controls throughout the experiment. In the long-term experiment plasma citrulline level was measured before the start of Px, immediately before MTX45 injection at day 0 and subsequently every 2 days after MTX injection, as shown in Supplemental Figure 2a. Seven days of Px treatment again resulted in significantly higher plasma citrulline levels prior to MTX injection at day 0. Induction of mucositis led to a similar reduction in citrulline levels in both groups while during the recovery phase these levels were again significantly higher in the MTX45-Px group compared to the MTX45 group.

Figure 2. Clinical parameters long-term experiment. A. Relative bodyweight was compared to bodyweight at day 0, which was set at 100%. B. Food intake in gram per day. C. Diarrhea incidence, in amount of days with diarrhea. D. Survival curve analyzed with the cox regression analysis (n=9). Data represent medians and interquartile ranges. *p<0.05, **p<0.01
FXR activation improves small intestinal morphology in MTX-induced mucositis

Morphology of three parts of the small intestine was evaluated at the end of both experiments, i.e., at days 6 and 12, respectively as shown for the short-term experiment in Figure 4. Px significantly increased the villus length in jejunum and ileum in the control group. The villus length in duodenum was significantly decreased in the MTX60 group compared to the Control group whereas the crypt lengths in jejunum and ileum were significantly increased in MTX60 group compared to the Control group. The jejunal crypt length was significantly increased in MTX60-Px group compared to Px. However, there were no significant differences in villus and crypt lengths between MTX60 and MTX60-Px groups.

Results of histological examination in the long-term experiment are summarized in Supplemental Figure 2b,c. The villus length was significantly increased in the MTX45-Px group in comparison with the MTX45 group in the duodenum, jejunum and ileum. The crypt length was not significantly different between both groups.
Modulation of bile salt hydrophobicity by FXR activation does not ameliorate MTX-induced mucositis

Px treatment was associated with a reduction of plasma bile salt levels at day 6. Plasma bile salt levels showed a large variability in the MTX60 group and tended to be reduced in the MTX60+Px group (Supplemental Figure 3). The contribution of individual bile salt species (Supplemental Figure 4a) shifted to a preponderance of more hydrophilic species. Bile was collected to gain insight in the composition of the bile salt pool entering the intestine. Bile flow was significantly increased in the Px group compared to the Control group, as shown in Figure 5. As expected, Px decreased the biliary bile salt concentration (data not shown) but, due to the increased bile flow,

Figure 4. Histology short-term experiment. A. Villus length measured at day 6 in duodenum, jejunum and ileum (n=5-7). B. Crypt length measured at day 6 in duodenum, jejunum, ileum (n=5-7). Data represent medians and interquartile ranges. * p<0.01. C. Control jejunum day 6. D. Px jejunum day 6. E. MTX60 jejunum day 6. F. MTX60-Px jejunum day 6. Bars represent 300 µm
the biliary bile salt excretion was not significantly altered. Analysis of the bile salt profile revealed an increase in more hydrophilic species as a consequence of Px treatment (Supplemental Figure 4b), as evident from a significantly more negative Heuman’s index [44] in the Px group compared to the Control group. MTX itself had only slight effects on bile salt composition: bile salt pool composition was similar in MTX60-Px group compared to the MTX60 group (Supplemental Figure 4b). Yet, the MTX60 group had more glycine conjugated bile salts (+32.9%) and more secondary bile salts (+9.9%) compared to the Control group.

MTX modulates caecal microbiota composition, no additional effects of FXR activation

Figure 6 shows the composition of microbiota in caecum at day 6 in the short-term experiment. The proteobacteria, specifically the gammaproteobacteria, were proportionally increased in both the MTX60 group and the MTX60-Px group compared to the respective control groups. The diversity, represented by the Shannon index, was significantly lower in the MTX60 group compared to the Control group as well as in the MTX60-Px group compared to the Px group. Thus, Px treatment by itself did not modulate diversity of the microbiome despite markedly changed bile salt pool composition. No pattern in the variation of microbiota between the Px group compared to control group or between MTX60 and MTX60-Px group was evident, as shown by Principal Component Analysis (Supplemental Figure 5).
DISCUSSION

In this study, we aimed to determine the effect of a potent FXR agonist, i.e., Px20606, on the severity of and recovery from MTX-induced mucositis in rats. The results indicate that, in contrast to our expectation, continuous FXR activation markedly aggravated the severity of clinical parameters of mucositis in our established rat model. Thus, the increase in hydrophilicity of the circulating bile salt pool upon chronic FXR activation did not ameliorate MTX-induced mucositis in the rat. In addition, FXR activation did not modulate MTX-associated changes in microbiome composition: altered microbiome composition may contribute to the development or propagation of MTX-induced mucositis. Furthermore, our results also show that the FXR agonist significantly increased the plasma citrulline level and villus length in healthy rats, indicative for increased enterocyte mass. Also during the recovery phase of mucositis, FXR activation was associated with higher citrulline levels and an increased villus length. This suggests that the FXR-agonist acts as an intestinal growth factor that increases cellular proliferation.

Currently, the pathophysiology of mucositis has not been completely elucidated. Bile salts have so far not been implicated as causative factors in the etiology of mucositis. Alterations in the
amount and composition of bile salts may result in toxic effects leading to diarrhea and inflammation, or in bacterial overgrowth [10,16]. FXR agonists have been shown to decrease bile salt synthesis and cytokines secretion as well as to improve the intestinal barrier function and alter the microbiota in the intestine in other intestinal animal models [10-12,17,25,27,28,45]. Therefore, we hypothesized that a potent FXR agonist would target more than one phase of the 5-phase mucositis model in a beneficial way [27-29]. However, in contrast to our expectations, FXR activation significantly increased the severity of mucositis. There is a number of potential explanations that will be discussed in the following paragraphs.

First, our results show higher MTX plasma levels in Px-treated rats at day one after MTX injection, suggesting altered MTX pharmacokinetics due to FXR activation, via modulation of ABCC transporters which function as cellular exporters [46-49]. This would result in longer exposure of enterocytes to circulating MTX and hence more damage. However, MTX plasma levels did not correlate with any parameter, which is comparable to the clinical practice in which MTX plasma levels do not correlate with toxicity in pediatric cancer patients [50]. Therefore, it can not be concluded from the current results that this interaction between FXR and MTX actually influences the severity of mucositis.

Secondly, MTX treatment changed the bile salt composition towards more glycine conjugated and more hydrophobic species. This altered bile salt pool is potentially more cytotoxic to the intestine [14,15]. As expected, FXR activation markedly increased the hydrophilicity of the bile salt pool. In contrast to our expectations, this did not lead to a reduction in the severity of mucositis [16,51], although it may have ameliorated other, unrelated aggravating consequences of Px treatment.

Thirdly, the composition of the caecal microbiome was altered and the diversity was decreased due to MTX, as shown before [52]. FXR activation did not modulate MTX-associated changes in microbiome composition. This is unexpected, since bile salts have been shown to interact with microbiota in other studies [11,13,53-57], and bile salt composition was markedly altered upon FXR activation (see above). However, the interaction between microbiota and bile salts is complex and still incompletely understood. Hence, it is possible that subtle changes in bacterial species or metabolites produced by the microbiome were actually induced by FXR activation and contribute to aggravation of MTX-induced mucositis. Further studies on this issue are warranted.

Finally, our study shows for the first time that a potent FXR agonist functions as an intestinal growth factor. This was shown by increased villus lengths and elevated plasma citrulline levels upon FXR activation both in healthy and mucositis rats, the latter particularly during their recovery phase. This intestinal trophic action possibly led to an increased intestinal proliferation rate at the moment of MTX administration, which might have made the intestine more prone to MTX and resulted in an aggravated severity of mucositis. This would be comparable to the effect of Palifermin, a keratinocyte growth factor, that has been shown to act as an epithelial growth
factor. It was advised not to administer Palifermin within 24 hours before or after MTX injection, because it may lead to increased severity and/or prolonged duration of mucositis [58]. Since we did not expect these results, we have activated FXR continuously prior to and after MTX injection. Based on this knowledge, alternative dosing regimens may be worthwhile to evaluate. A limitation of this study is that we did not determine the effect of FXR activation on the inflammatory cytokine production. Yet, proinflammatory cytokines were reduced by FXR activation in previous experiments [18,27,28], which would affect mucositis in a beneficial way. A second limitation is that the rat model is not directly translatable to the human situation, since humans have a different, more hydrophobic bile salt composition and rats have no gall bladder and hence an enterohepatic circulation with different dynamics. Furthermore, MTX is mainly excreted via the bile in rats in contrast to humans in whom it is primarily excreted via the urine [47,59].

In summary this is the first study to document the influence of a potent FXR agonist on the severity of and recovery from mucositis induced by MTX in rats. Continuous activation of FXR markedly aggravated the severity of mucositis and a more hydrophilic bile salt pool did not provide protection. Furthermore, the FXR agonist significantly increased the plasma citrulline level and villus length in healthy rats as well as in mucositis rats during their recovery phase. Therefore, we suggest that the FXR agonist functions as an intestinal growth factor and should not be administered directly prior, during, or directly after MTX. Since FXR activation promotes intestinal growth, future studies should determine whether FXR activation might be useful in clinical situations that benefit from stimulation of intestinal repair.
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Supplemental Figure 1. Methotrexate level short-term experiment A. MTX plasma level in ug/l, measured at day 1, 24 hours after MTX injection (n=8). B. MTX level in bile in ug/l, measured at day 6 after MTX injection (n=4). Tukey boxplot, Data represent median and interquartile ranges. *p<0.01. N.D.=not detectable.

Supplemental Figure 2. Plasma citrulline and histology long-term experiment
A. Plasma citrulline level in µmol/l at day -7 before Px, at day 0 before MTX injection (N=8). B. Villus length in µm perin duodenum, jejunum, ileum at day 12 (MTX45: n=8, MTX45+Px: n=3-5). C. Crypt length in µm in duodenum, jejunum, ileum at day 12 (MTX45: n=8, MTX45+Px: n=3-5). Data represent medians and interquartile ranges. *p<0.05, ** p<0.01.
Supplemental Figure 3. Plasma bile salt levels short-term experiment. Plasma bile salt levels measured at day 6 after MTX (n=6-8). Tukey boxplot, data represent medians and interquartile ranges. *p<0.01

Supplemental Figure 4. Composition of bile salts short-term experiment. A. Composition of bile salts in plasma (n=6-8). Ursodeoxycholic salt (UDCA); Glycoursodeoxycholic salt (GUDCA); Tauroursodeoxycholic salt (TUDCA); Cholic salt (CA); Glycocholic salt (GCA); Taurocholic salt (TCA); Chenodeoxycholic salt (CDCA); Glycochenodeoxycholic salt (GCDCA); Taurochenodeoxycholic salt (TCDCA); Deoxycholic salt (DCA); Glycodeoxycholic salt (GDCA); Taurodeoxycholic salt (TDCA); Lithocholic salt (LCA); Taurolithocholic salt (TLCA); Alpha-muricholic salt (α-MCA); Beta-muricholic salt (β-MCA); Omega-muricholic salt (ω-MCA); Hyodeoxycholic salt (HDCA); Glycohyodeoxycholic salt (GHDCA); Taura-alpha-muricholic salt (Tα-MCA); Tauro-beta-muricholic salt (Tβ-MCA).

B. Composition of bile salts in bile (n=6-8).
Supplemental Figure 5. Microbiota caecum short-term experiment. A. Principal Component 1 and Principal Component 2 explaining the variation. B. Principal Component 2 and Principal Component 3 explaining the variation.
Clinical care

Risk, diagnosis and feeding strategy