Regular Article

Rifamycin SV exhibits strong anti-inflammatory in vitro activity through pregnane X receptor stimulation and NFκB inhibition

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1. Introduction

Rifamycin SV (rifamycin), is a member of the ansamycin family of antimicrobial compounds which kills bacteria commonly associated with infectious diarrhea and other enteric infections. Rifamycin has been found to be effective in experimental animal models of gut inflammation and its efficacy in these settings has been attributed partially to immunomodulatory non-bacterial activities. This study aimed to further evaluate the anti-inflammatory activities of rifamycin by analyzing its effect on two key regulators of inflammation: PXR and NFκB. Rifamycin stimulated PXR transcriptional activity in two PXR reporter cell lines and induced expression of two genes known to be regulated by PXR and are directly involved in cellular detoxification: CYP3A4 and PgP. Moreover, CYP3A4 metabolic activity was induced by rifamycin in HepG2 cells. Rifamycin also antagonized TNFα and LPS-induced NFκB activities and inhibited IL1β-induced synthesis of inflammatory chemokine, IL8. Although reciprocal regulation of PXR and NFκB by rifamycin was not directly addressed, the data suggest that in the absence of PXR, inhibition of NFκB by rifamycin is not dependent on PXR stimulation. Thus, rifamycin exhibits potent anti-inflammatory activities, characterized by in vitro PXR activation and concomitant CYP3A4 and PgP induction, in parallel with potent NFκB inhibition and concomitant IL8 inhibition. © 2019 The Japanese Society for the Study of Xenobiotics. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

A B S T R A C T

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1. Introduction

Rifamycin SV (rifamycin) is a poorly absorbed antibiotic and exhibits low systemic availability following oral administration. In vitro, rifamycin kills bacteria commonly associated with infectious diarrhea and with other enteric infections, including Escherichia coli, Yersinia enterocolitica, Enterococcus faecalis, Staphylococcus aureus, and Clostridium difficile [1]. In vivo, rifamycin has been found to be effective in experimental animal models of gut inflammation. The efficacy of rifamycin in these settings has been attributed partially to immunomodulatory non-bacterial activities [2].

Inflammation and immune dysregulation contribute to the pathophysiology of several gastrointestinal (GI) diseases, such as inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), and diverticular disease. Alterations in the reciprocal crosstalk between two major regulators of inflammation, PXR and NFκB, may play a role in the dysregulation of the inflammatory mechanisms in IBD [3–5]. Activated PXR reduces the activity of NFκB [6] while activated NFκB reduces the expression and activity of PXR [3,7]. The reciprocal repression between PXR and NFκB signaling has also been suggested from studies using the antibiotic and gut specific PXR agonist, rifaximin [8]. Rifaximin antagonized TNFα-induced inflammation in experimental colitis models by activating PXR and concomitantly inhibited NFκB-driven cytokine production [4,9–11]. However, there are reports of endotoxin-induced NFκB that is independent of PXR activation in the context of an acute liver inflammation [12]. Thus, there are instances where PXR and NFκB are closely interconnected, while in others, each act independently of the other.

PXR plays a significant role in the pathogenesis of IBD [13]. PXR is a nuclear receptor and transcription factor with an established role in regulating drug clearance in the liver and intestine via induction of genes involved in drug and xenobiotic metabolism. Stimulation of PXR transcriptional activity induces expression of metabolic enzymes like CYP3A4, which oxidizes xenobiotics, such
as bacterial metabolic products, environmental toxins or drugs, for clearance from the body. Notably, CYP3A4 activity is significantly reduced in patients with Crohn's Disease (CD) [14,15]. Stimulation of PXR also induces expression of xenobiotic transporters like P-glycoprotein P (Pgp) in the intestine and liver [16]. Pgp mediates efflux of compounds from the mucosa to the gut lumen, thereby mediating protective mechanisms against endobiotics, xenobiotics and toxins. Changes in Pgp function and/or expression contribute to the pathogenesis of inflammatory disorders of the GI tract since IBD susceptibility loci have been found in Pgp gene loci. Pgp deficiency promotes intestinal inflammation [17] and Pgp expression is decreased in inflamed intestinal epithelia of CD, ulcerative colitis, collagenous colitis, and diverticulitis patients [18]. Thus, through the induction of CYP3A4 and Pgp, PXR plays a key role in detoxification mechanisms that help maintain intestinal barrier function [19–21].

Constant exposure to xenobiotics is not only capable of producing intestinal inflammation and injury [3,22] but can also impair immune function [23]. In addition to its role in detoxification mechanisms, PXR has been shown to indirectly inhibit inflammation through repression of NFκB activity. NFκB is the primary transcription factor that regulates gene expression of pro-inflammatory cytokines and chemokines. Patients with IBD have significantly higher expression of NFκB compared to non-IBD patients [24] and abnormal activation of NFκB leads to excessive production of pro-inflammatory cytokines that cause chronic inflammation in the bowel.

The aim of the present study is to evaluate the effect of rifamycin on PXR and two of its target genes, CYP3A4 and Pgp, and additionally its capability to influence NFκB signaling, thereby defining the in vitro anti-inflammatory characteristics of rifamycin. These characteristics are compared to rifaximin.

2. Materials and methods

2.1. Materials

Both PXR and NFκB reporter cell line kits were purchased from Indigo Biosciences (State College, PA, USA) and were used per manufacturer’s protocol. TNFα and PMA used in the NFκB reporter assay, as well as IL1β for Caco2 stimulation, were from R&D systems (Minneapolis, MN, USA), while LPS was from Sigma Aldrich (St. Louis, MO, USA). Costar 96 well assay plates and white opaque plates used to measure luminescence were from Corning (Tewksbury, MA, USA). The BMG Labtech ClarioStar plate reader (Ortenberg, Germany) was used to quantify luminescence in the reporter cell line assays. The CYP3A4 P450-Glo assay used to measure PXR enzymatic activation and CellTiter-Glo reagent used to measure cell viability, were both from Promega (Madison, WI, USA). The CYP3A4 assay was performed using HepG2 liver cells (wt and overexpressing PXR). HepG2 (wt), Caco2 and HT-29 colonic epithelial cells were all from ATCC (Manassas, VA, USA). Assays involving HepG2 overexpressing PXR were performed by Puracyp, Inc. (Carlsbad, CA) [25,26]. Fetal bovine serum (FBS) was from Omega Scientific (Tarzana, CA, USA) and Atlas Biologicals (Fort Collins, CO, USA). Cell culture medium and DMEM were from Sigma-Aldrich. Rifamycin was acquired from Cosmo S.p.A. (Lainate, Milan, Italy), rifaximin was from Tocris (Minneapolis, MN, USA), and rifampicin was from Indigo Biosciences (State College, PA, USA).

2.2. Cell culture

HepG2, Caco2, and HT-29 cell lines were cultured in T75 cm² flasks and kept healthy at approximately 70% confluence in complete media (Dulbecco’s Modified Eagle Medium/Nutrient mixture F12 (DMEM/F12k) with 10% FBS). Experiments with these cell lines were performed in treatment media containing DMEM/F12k with 2% charcoal stripped FBS. Caco-2 cells were cultured in DMEM/F12k with 20% FBS. All cell lines were kept in a 37 °C, 5% CO₂, and adherent cells were passaged using trypsin-EDTA to detach cells from flasks. Reporter cell lines were thawed using warm cell recovery medium upon use.

2.3. Test compound preparation

Test compounds (rifamycin, rifaximin, and rifampicin) were dissolved at a high-concentration (30 mM) in DMSO and stored under low humidity conditions at 4 °C. Concentrated DMSO stocks were stored at 4 °C for less than a month. Immediately prior to an assay, master stocks were serially diluted in media to achieve desired assay concentrations.

2.4. PXR transcriptional reporter assay

The PXR reporter cells are mammalian cells expressing a hybrid form of human PXR in which the DNA binding domain (DBD) has been substituted with yeast GAL4-DBD. As per manufacturer recommendation, cells were seeded onto 96 well collagen flat bottom plates, placed into a 37 °C, 5% CO₂ incubator, and rested for 4 h. Cell media was aspirated, replaced with titrated test compounds and treated for 24 h at 37 °C. Luminescence, which is proportional to PXR ligand activity, was quantified using a BMG Labtech ClarioStar luminometer (Ortenberg, Germany).

PXR activation assays were performed by Puracyp, Inc., utilizing their proprietary HepG2 cells stably overexpressing full-length human PXR. These cells harbor the human PXR gene and a luciferase reporter gene linked to two promoters identified in the human CYP3A4 gene. Cells were treated with antibiotics and incubated for 48 h in a 37 °C, 5% CO₂ atmosphere. Luminescence proportional to PXR activity was quantified using Promega OneGlo reagent and read in a Synergy 2 plate reader by BioTek (Winooski, VT).

2.5. PXR and CYP3A4 qRT-PCR

HepG2 cells were seeded in 6-well plates at a density of 5 × 10³ cells/mL in a total of 3 mL complete media. After 24 h, cells were treated in triplicate with 0.1% DMSO or antibiotic (2 × 10⁻³ M) in treatment media for 96 h. Cells were harvested by trypsinization, washed with complete media, centrifuged, then supernatant discarded to obtain a dry cell pellet that was stored at −80 °C. RNA was extracted from cell pellets using Promega SV Total RNA Isolation System (Madison, WI) in combination with Qiagen QIAshredder (Germantown, MD) and cDNA synthesized using Quantabio qScript XLT 1-Step RT-qPCR ToughMix (Beverly, MA). Each RNA sample was plated in duplicate and assayed for genomic cDNA specific for each primer set using Power SYBR Green detection system (ABI 7900HT).

Relative mRNA levels were calculated using the comparative ΔΔCt method, where each gene of interest was normalized to the endogenous housekeeping gene beta 2 microglobulin (B2M). Probe and primer sets were from ThermoFisher (Carlsbad, CA) PXR assay id# Hs01114267_m1, NM_001015786.1, at nucleotide 2994 spanning exons 8/9, 103 bp amplicon; CYP3A4 assay id#Hs00430021_m1, NM_001015786.1, at nucleotide 252 spanning exons 3/4, 92 bp amplicon; B2M assay id#Hs99999907_m1, NM_004048.2, at nucleotide 409 spanning exons 2/3, 75 bp amplicon).
2.6. CYP3A4 protein metabolic activity assay

HepG2 liver cells were seeded at 60,000 cells/well in complete media in a 96 well flat bottom plate and incubated for 4 h at 37 °C. Media was aspirated, replaced with antibiotics in treatment media or 0.1% DMSO. Cells were incubated for 48–72 h at 37 °C. CYP3A4 pro-luciferin substrate was added as per manufacturer instructions and luminescence proportional to CYP3A4 enzymatic activity was quantified. The Promega system described above was also used by Puracyp in their assays to detect CYP3A4 activity in HepG2 cells overexpressing PXR [26]. The same experiment wells from the PXR assays described in section 2.3 were simultaneously tested for CYP3A4 activity. Cells were treated with antibiotics and incubated for 48 h in a 37 °C, 5% CO2 incubator before analysis of CYP3A4 protein metabolic activity.

2.7. PgP protein expression

HepG2 and Caco2 cells were seeded in 96-well plates at a density of 18,000 cells/well. After 24 h, cells were treated in triplicate with 0.1% DMSO or antibiotic. After 96 h, cells were trypsinized, washed with complete media, then incubated for 30 min at 4 °C with an antibody to PgP that is fluorescently conjugated with phycoerythrin (Becton Dickinson, 10 μg/mL final concentration). Cells were washed and resuspended with FACs buffer (PBS containing 0.1% BSA) then analyzed through a flow cytometer (Miltenyi MACsQuant Analyzer, Bergisch Gladbach, Germany).

2.8. Cell viability assay

Cell Titer-Glo™ (CTG) luminescent cell viability assay reagent (Promega) was used to measure cell proliferation and determine cytotoxicity of antibiotics. Caco2 were seeded in 96-well plates at a density of 60,000 cells/well. After 24 h, cells were treated in triplicate with antibiotic, 0.1% DMSO (negative control) or 10% DMSO (positive control). After 96 h treatment, media was removed, and the remaining cells were lysed in CTG reagent for 10 min at room temperature and luminescence quantified. Promega CellTiterFluor was used by Puracyp to determine cell viability of HepG2 cells overexpressing PXR. The PXR and CYP3A4 activity assays simultaneously performed on these cells were normalized to the number of viable cells remaining after the 48 h incubation period with the tested antibiotics.

2.9. NFkB transcriptional reporter assay

The NFkB reporter cell line are HEK293t (human embryonic kidney epithelial) cells that express NFkB and contain the luciferase reporter gene functionally linked to upstream NFkB genetic response elements. As per manufacturer’s protocol, cells were plated in 96 well flat bottom plate and rested for 4 h at 37 °C. Media was aspirated then replaced with agonist and antibiotic, or 0.1% DMSO. Cells were incubated for 24 h at 37 °C, then assayed as per manufacturer protocol to quantify luminescence.

2.10. Cytokine expression

Caco2 cells were activated with 5 ng/mL IL1β for 48 h in the presence of rifamycin, rifaximin or DMSO. Secreted cytokines were quantified from the supernatants utilizing a bead-based ELISA to measure IL5 levels (Becton Dickinson cytokine bead array, La Jolla, CA). Anti-cytokine–associated fluorescence was quantified using Miltenyi MACsQuant Analyzer and data analyzed using FlowJo software (Ashland, OR). Cytokine levels were normalized to the number of viable cells remaining after the 48 h treatment period.

2.11. Data analysis

In vitro data were fitted using log agonist or antagonist versus normalized response, variable slope, 4 parameter curve settings. Data were plotted in GraphPad Prism software (version 7.03, San Diego, CA). EC50 and IC50 values were obtained from fitted curves. Background values were subtracted from all readings. Each independent experiment was repeated three times and performed in either duplicates or triplicates. Reproducibility between experiments was calculated by p value determination using paired 2-tailed t-test or ANOVA with Bonferroni multiple comparisons test using Prism software.

3. Results

3.1. Stimulation of PXR transcriptional activity and mRNA expression

To evaluate rifamycin effect on PXR transcriptional activity, rifamycin, rifaximin and the control antibiotic rifampicin were tested in a HEK293t reporter cell line expressing a hybrid PXR protein. Based on calculated EC50 values, rifamycin (EC50 = 6.08 × 10⁻⁹ M) is 300 times more potent than rifaximin (EC50 = 1.80 × 10⁻⁶ M), and 180 times more potent than rifampicin (EC50 = 1.10 × 10⁻⁶ M) in stimulating PXR transcription (Fig. 1A). In addition, the extent of PXR activity is 3-fold higher with rifamycin than with rifaximin at the respective highest tested concentrations of these antibiotics (Eₘₐₓ at 1800 vs. 600 relative luciferase units, respectively), while rifamycin and rifampicin achieved comparable Eₘₐₓ values (1800 for both).

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**Fig. 1.** Stimulation of PXR transcriptional activity in reporter cell line and HepG2. (A) PXR reporter cell line. Plot showing a dose titration of antibiotics in a mammalian PXR reporter cell line. Rifamycin was titrated from 3 × 10⁻¹² to 3 × 10⁻⁷ M while rifaximin and rifampicin were titrated from 3 × 10⁻¹¹ to 3 × 10⁻⁸ M, in half log increments. Data points are MEAN ± SEM, relative luciferase units, experiment was repeated 3 times in triplicate wells (*p < 0.05 dose response between respective antibiotics as indicated). (B) HepG2 reporter cells overexpressing human PXR. Rifamycin and rifaximin were titrated from 1.2 × 10⁻⁷ to 3 × 10⁻⁶ M while rifampicin was titrated from 1 × 10⁻⁷ to 2 × 10⁻⁶ M. Data points are MEAN ± SEM, relative luciferase units, experiment was repeated 3 times in duplicate wells (*p < 0.05 dose response between respective antibiotics as indicated).
PXR activity was also measured in HepG2 cells overexpressing PXR (Fig. 1B). While there were differences in absolute EC50 values when compared to the hybrid PXR reporter cell line assay reported in Fig. 1A, the finding that rifamycin has higher potency than rifaximin in activating PXR is confirmed in this reporter cell line expressing full length PXR (EC50 9.29 × 10^−6 M for rifamycin vs. 2.79 × 10^−5 M for rifaximin). In addition, the extent of PXR activity is 1.5-fold higher with rifamycin than with rifaximin at the respective highest tested concentrations of these antibiotics (E_{max} at 6700 vs. 4400 relative luciferase units, respectively). In contrast to the reporter cell line, rifampicin has the highest potency in these cells with E_{max} 2-fold higher than rifamycin.

To clarify the mechanism of the increased PXR activation, PXR mRNA expression was quantified in HepG2 cells treated with 0.1% DMSO, 20 μM rifamycin, rifaximin, or rifampicin (Fig. 2). The data indicate that rifamycin and rifaximin induced PXR mRNA expression 2.5-fold relative to the DMSO control while rifampicin did so by 1.5-fold. More importantly, mRNA expression of one of PXR’s target genes, CYP3A4, was quantified. Both antibiotics increased CYP3A4 mRNA expression (2.2-fold with rifamycin vs. 2.4-fold with rifaximin, relative to the DMSO control, Fig. 2). Despite the marginal induction of PXR mRNA by rifampicin, this control antibiotic induced CYP3A4 mRNA 4-fold relative to DMSO, suggesting that potent stimulation of PXR transcriptional activity in these cells is due largely to post-translational modification of PXR rather than positive feedback regulation via induction of PXR mRNA.

### 3.2. Stimulation of CYP3A4 protein metabolic activity in wild type HepG2 cells versus HepG2 cells overexpressing PXR

Since an increase in mRNA expression does not necessarily lead to increased function of a protein while an increase in metabolic activity of an enzyme is an excellent indication of induced biologic function, CYP3A4 protein metabolic activity in HepG2 cells in the presence of rifamycin was also quantified. In these cells, rifaximin induced CYP3A4 protein activity slightly better than rifamycin (E_{max} 1.3 vs. 1.4 relative luciferase units, Fig. 3A). These results are consistent with the equivalent induction of PXR mRNA expression in these HepG2 cells by both antibiotics (Fig. 2). In contrast to the modest stimulation of CYP3A4 activity in HepG2 cells, rifamycin induced high levels of CYP3A4 protein metabolic activity in the HepG2 cells overexpressing PXR (Fig. 3B). Rifamycin was a more potent agonist than rifaximin (EC50 6.58 × 10^−6 M vs. 1.40 × 10^−5 M and E_{max} 39 vs. 26 relative luciferase units), while rifampicin was the most potent antibiotic (EC50 1.67 × 10^−6 M) but with comparable E_{max} as rifamycin. The data regarding CYP3A4 activity in response to antibiotics are consistent with their respective potency as PXR agonists in this cell line (shown in Fig. 1B). CYP3A4 activity was also measured in Caco2 cells but were undetectable (data not shown).

### 3.3. Stimulation of PgP protein expression in HepG2 and Caco2 cells

To analyze induction of another PXR target gene besides CYP3A4, PgP protein expression was also analyzed in HepG2 and Caco2 cells. PgP protein expression was quantified in whole cells using an antibody to an extracellular epitope of PgP then stained cells were analyzed by flow cytometry (FACS). The FACS plots in Fig. 4 show representative histograms. Rifamycin at 20 μM induced PgP expression 2.4-fold higher than the negative control DMSO, while rifaximin at 20 μM induced PgP expression by 1.2-fold and rifampicin by 1.6-fold higher than control (Fig. 4A). Rifamycin’s induction of PgP protein at the highest concentration is significantly better than rifampicin’s, in contrast to their respective induction of CYP3A4 mRNA (Fig. 2). PgP expression in Caco2 cells was induced by an average of 1.8, 1.4, and 1.4 -fold with 20 μM rifamycin, rifaximin, and rifampicin, respectively (Fig. 4B). These values are not significantly different from each other, indicating equivalent stimulation of PgP protein expression by all three antibiotics in these cells.

### 3.4. Effect on viability of HepG2 cells overexpressing PXR and Caco2 cells

To evaluate cytotoxicity of the antibiotics on the HepG2 cells overexpressing PXR, cells that were used for the PXR activation and
Fig. 4. Stimulation of PgP protein expression in HepG2 and Caco2 cells. (A) HepG2 and (B) Caco2 cells were stimulated with 1–20 μM antibiotic for 96 h. FACs histograms are representative of 3 experiments each in triplicate. Data points are MEAN ± SEM, relative to DMSO of the mean fluorescence intensity associated with anti-PgP fluorescence, proportional to the PgP protein expression (\( p < 0.05 \), \( **p < 0.0005 \) vs. DMSO and \( *p < 0.05 \), \( **p < 0.005 \) between respective antibiotics as indicated).
CYP3A4 protein metabolic assays were analyzed to detect constitutive protease activity within live cells as a biomarker of cell viability. All antibiotics, at the highest concentration of 30 μM, were only cytotoxic to 10% of the cells after 48 h incubation (Fig. 5A).

All three antibiotics induced Caco2 intestinal cell proliferation after a 96 h treatment with all 4 tested concentrations and were significantly different from the negative control vehicle, 0.1% DMSO. The differences at the highest concentration (20 μM) were not significant between each antibiotic but were at the lowest concentration (1 μM), where the average fold-differences relative to 0.1% DMSO were 1.5, 1.8, and 2.4 for rifamycin, rifaximin, and rifampicin, respectively. (Fig. 5B). No cells survived the positive control, 10% DMSO.

3.5. Inhibition of NFκB transcriptional activity in a reporter cell line

In many published cases, activation of PXR leads to inhibition of NFκB transcriptional activity. However, in an acute liver inflammation model, it was shown that NFκB inhibition is not linked to PXR activation [12]. To establish if PXR activation by rifamycin is required for NFκB activation, a reporter cell line which does not express PXR but expresses full length NFκB and an NFκB-dependent luciferase reporter gene, was stimulated with TNFα or LPS. Antibiotics were dose-titrated onto these stimulated cells. After 24 h incubation, luminescence correlating to NFκB activity was quantified. Rifamycin antagonized TNFα-induced NFκB activity with IC50 value of 1.48 × 10^{-5} M (Fig. 6A) and LPS-induced NFκB activity with IC50 value of 3.49 × 10^{-6} M (Fig. 6B). In stark contrast, rifaximin did not inhibit TNFα- nor LPS-induced NFκB activity up to the highest concentration tested. Rifampicin inhibited TNFα and LPS-induced NFκB activity with IC50 value > 3 × 10^{-5} M. These results establish that in the absence of PXR expression, rifamycin and rifampicin are still capable of inhibiting NFκB. These results however do not preclude the possibility that PXR and NFκB activities are reciprocally regulated in cells that do express PXR, or in chronic disease states perpetuated by LPS or inflammatory cytokines such as TNFα.

3.6. Inhibition of cytokine synthesis in Caco2 cells

Our laboratory previously reported that rifamycin strongly inhibits synthesis of inflammatory chemokine, IL8, from colonic HT-29 cells stimulated with IL1[2]. In that publication, IFNγ, IL2, IL6, and IL17A were significantly inhibited from activated CD4 T cells, RANTES from monocytes, and TNFα from CD4 T cells, macrophages, and monocytes. To evaluate if rifamycin inhibits cytokine production in the colonic cell line that showed induction of PXR target genes, we tested for this inhibitory activity in Caco2 cells. Caco2 were activated with 5 ng/mL IL1β for 48 h in the presence of 3–20 μM rifamycin, rifaximin or 0.1% DMSO. Rifamycin inhibited IL8 by a maximum of 61% at 20 μM, while rifaximin inhibited by 45% at 10 μM (Fig. 7). The lack of inhibition with 20 μM rifaximin is a conundrum but was confirmed in several experiments, highlighting the complexity of the signaling mechanisms that these antibiotics regulate. Rifampicin was not tested in this assay.

3.7. Summary of rifamycin characterization compared to rifaximin

All results related to characterization of rifamycin’s anti-inflammatory activities are summarized in Table 1. PXR
transcriptional activation and induction of PXR and CYP3A4 mRNA, induction of CYP3A4 metabolic activity, induction of Pgp protein synthesis, inhibition of NFκB transcriptional activity, and inhibition of IL8 protein synthesis were compared to rifaximin activities. In 11 out of 14 parameters, rifamycin showed higher potency than rifaximin, and in the remaining 3 parameters, rifamycin and rifaximin showed comparable activities. The rifampicin data which were useful as positive control in most of the assays, are not included in this table because of its potential systemic toxicity as a drug to treat gastrointestinal or hepatic diseases.

4. Discussion

In the present study, the in vitro anti-inflammatory activity of rifamycin was characterized by PXR transcriptional activation and NFκB transcriptional inhibition. Rifamycin induced a strong PXR activation in two PXR reporter cell lines. The difference in efficacy was cell line dependent, where activity was higher in the kidney cell line expressing a hybrid PXR than in the liver cell line expressing full length PXR. Nonetheless, in both cell lines, rifamycin was more potent than rifaximin in activating PXR. To understand the mechanism of the increased PXR activation, PXR mRNA expression was quantified in HepG2 cells. Stimulation of PXR mRNA expression by rifamycin and rifaximin was not as dramatically different in HepG2 cells. This is contradictory to the much higher stimulation of PXR activity by rifamycin in the two reporter cell lines. This may due to differences in the extent of post translational modifications and their contribution to the overall stimulation of PXR activity. Indeed, transcription factors are often already expressed at high protein levels but in an inactive/stand-by mode that can quickly become activated in response to external stimuli [27]. Thus, activation via post translational modification rather than induction at the mRNA level is the more common paradigm of transcription factor activation, including PXR [28]. This is the case for rifampicin in HepG2 cells which was the most potent inducer of PXR activity but did not induce PXR mRNA.

However, consistent with the level of PXR activity, all 3 antibiotics induced PXR target genes: CYP3A4 mRNA and Pgp protein from HepG2 cells. More studies are needed to explore why the extent of stimulation of these target genes varied between the different antibiotics and did not directly correlate with the rank order of potency as PXR agonists. One possible explanation is differential regulation of the PXR dimer or heterodimer composition [16].

PXR protein is abundantly expressed in the liver and in tissues along the GI tract [29]. In the reporter cells overexpressing PXR, rifamycin was clearly better than rifaximin in stimulating PXR activity and inducing CYP3A4 protein metabolic activity. Presumably, these would apply to the homologous PXR signaling network in colonic epithelial cells and translate clinically to enhanced detoxification and maintenance of gut barrier function.

Furthermore, since PXR can indirectly regulate inflammation through inhibition of NFκB, the data show that rifamycin induces an alternate or parallel pathway to normalize gut immune function via antagonism of NFκB activity. Rifamycin exhibited strong antagonism of TNFα and LPS-induced NFκB activity. When the TNFα receptor was specifically activated to stimulate NFκB, rifamycin potently antagonized TNFα-induced signaling while rifaximin was ineffective. More importantly, there was robust inhibition by rifamycin of LPS-induced NFκB, while rifaximin was ineffective. In both cases, rifampicin did inhibit NFκB but to lower extents than rifamycin. Although reciprocal regulation of PXR and NFκB by rifamycin was not directly addressed here, these data suggest that in the absence of PXR, inhibition of NFκB by rifamycin is not dependent on PXR stimulation. On the other hand, the lack of inhibition by rifaximin in these cells that lack PXR, is consistent with reports that rifaximin inhibition of TNFα and endotoxin-induced NFκB require stimulation of PXR [30,31].

### Table 1

**Summary of rifamycin characteristics compared to rifaximin.** The 14 parameters analyzed based on all experimental assays are listed. Potency was calculated as a ratio of the value for rifamycin vs. rifaximin where the numerator/denominator of the ratio is rifaximin/rifamycin for EC50 or IC50 while for all other values, the potency was the ratio rifamycin/rifaximin.

<table>
<thead>
<tr>
<th>Experimental Assay</th>
<th>Cell Line</th>
<th>Rifamycin SV</th>
<th>Rifaximin</th>
<th>Potency Rifamycin over Rifaximin</th>
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<td>N.A. (Rifamycin much more effective)</td>
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<td>Inhibition LPS-induced NFκB transcriptional activity (IC50)</td>
<td>HEK293t reporter</td>
<td>3.5 μM</td>
<td>&gt;30 μM</td>
<td>N.A. (Rifamycin much more effective)</td>
</tr>
<tr>
<td>Inhibition of IL1β-induced IL8 protein synthesis</td>
<td>Caco2</td>
<td>61%</td>
<td>45%</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Beyond the well-recognized antimicrobial activity of rifamycin, these results support a possible additional immunomodulatory effect on key factors involved in the inflammatory cascade. We previously demonstrated that rifamycin [2] inhibits cytokine and chemokine synthesis from HT-29 colonic epithelial cells. The current results reported here extend this anti-inflammatory pathway analysis to upstream transcription factors (PXR and NFκB) that regulate synthesis of these cytokines/chemokines. Most importantly relevant in IBS or IBD, NFkB biologic function was confirmed in colonic epithelial cells where PXR target genes were induced by rifamycin. Rifamycin significantly inhibited IL-1β-induced synthesis in colon cell lines of an NFκB-regulated chemokine, IL8. Coupled with our previous report in other cell types, these cell-dependent and stimulus-dependent requirements for inducing inflammation imply that by acting on various target cells, rifamycin's anti-inflammatory potential is broad because dysregulated immune and epithelial cell interactions contribute to the pathogenesis of several GI disorders [32].

An additional result of this study is that rifamycin is not cytotoxic to liver and colonic cells. This property is suggestive of a great safety profile for the compound which is important for long-term treatment in chronic GI diseases. Indeed, rifamycin induced proliferation of colonic epithelial cells, as has been shown also for rifampicin [33], and reciprocal crosstalk between PXR and NFκB was demonstrated recently in various experimental GI disease models (reviewed in Ref. [34]).

In summary, rifamycin exhibits strong in vitro PXR activation with concomitant CYP3A4 and PgP induction, as well as potent NFκB inhibition with concomitant inhibition of IL8 synthesis. For most of these activities, rifamycin is superior to rifaximin. Therefore, coupled with its potential short term but immediate clinical benefit of reducing inflammation, and with rifamycin's longer term clinical benefit of bacterial infection eradication, these findings support rifamycin as a useful non-systemic drug candidate for IBS-D treatment and other GI diseases.

Conflicts of interest
All 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.

Author contributions
CR and MG authored the paper, designed and analyzed the study; FJA and NR performed the experiments; LM, AM and CH contributed to critical revision of the paper. All authors approved the submission of the manuscript.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.dmpk.2019.01.002.

References