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Antimicrobial Treatment with the Fixed-Dose Antibiotic Combination RHB-104 for *Mycobacterium avium* subspecies *paratuberculosis* in Crohn's Disease: Pharmacological and Clinical Implications

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Abstract

Introduction: Crohn's disease (CD) is an inflammatory bowel disease of unknown etiology. However, increasing evidence suggests *Mycobacterium avium* subspecies *paratuberculosis* (MAP) as a putative causative agent: 1) MAP is the etiological agent of Johne's disease, a granulomatous enteritis affecting ruminants, which shares clinical and pathological features with CD; 2) MAP has been detected in tissues and blood samples from CD patients; 3) case reports have documented a favorable therapeutic response to anti-MAP antibiotics.

Area covered: This review provides an appraisal of current information on MAP characteristics, diagnostic methodologies and emerging drug treatments. The authors focus on RHB-104, a novel oral formulation containing a fixed-dose combination of clarithromycin, clofazimine and rifabutin, endowed with synergistic inhibitory activity on MAP strains isolated from CD patients.

Expert opinion: Based on encouraging in vitro data, RHB-104 has entered recently the clinical phase of its development, and is being investigated in a randomized, placebo-controlled phase III trial aimed at evaluating its efficacy and safety in CD. Provided that the overall clinical development will support the suitability of RHB-104 for inducing disease remission in CD patients with documented MAP infection, this novel antibiotic combination will likely take a relevant position in the therapeutic armamentarium for CD management.

1. Introduction

Crohn's disease (CD) is a chronic transmural inflammation that may involve any part of the alimentary tract, from mouth to anus, and can be associated with several extra-intestinal co-morbidities. The disease onset usually falls within the second and fourth decade of life, with a smaller second peak described from 50 to 60 years.[1] There is no gender-specific distribution in adult CD. Its incidence and prevalence are higher in economically developed than emerging countries, as well as in urban rather than rural areas.[2] Asia, where some countries are undergoing a rapid urbanization, is witnessing an increase in the annual incidence of CD.[3]

CD is a chronic relapsing inflammatory disease, which presents frequently with abdominal pain, diarrhea, fever, and is often complicated by clinical signs of bowel obstruction, intestinal fistulization or both.[4] The most frequent sites affected by the disease include the ileum, colon and perianal region, with a large prominence for the terminal ileum. The distribution of inflammatory activity throughout the intestine is characteristically segmental, and has a trend towards a life-long recurrence, even after resective surgery. The etiology of CD is unknown and several factors, both genetic and environmental in nature, have been implicated in its pathogenesis. As CD occurs frequently after infectious gastroenteritis,[5] and it is characterized by a peculiar enteric microbial flora in terms of dysbiosis[6] and an increased number of intramucosal bacteria[7], the efforts for identifying specific causative infectious agents have been endless. In this context, it is of interest that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has been identified as the etiological agent of Johne's disease, a severe granulomatous chronic enteritis affecting ruminants, which shares a number of clinical and pathological features with CD, including intermittent diarrhea, body weight loss, primary disease site in the ileocecal area, mucosal ulcerations and intramural granulomas.

In addition to pathological and clinical analogies, MAP elicits also a similar pattern of immune response to CD[8] and has been detected in both tissues and blood samples of adult[9] and pediatric CD patients,[10] thus raising problems of differential diagnosis in endemic areas.[11] On the other hand, several case reports have documented a clearly favorable therapeutic response of CD patients to anti-MAP antibiotics[12] and, given the close similarities of CD with Johne's disease, MAP has been hypothesized as a potential etiological agent of CD. However, even though a significant clinical association between MAP and CD has been established, the actual causative role of this agent in CD remains undetermined.[13,14]

Acknowledging the argumentation that a specific infectious agent, such as MAP, could act as the sole or major cause of CD would have dramatic implications for the prevention and therapeutic management of this challenging disease. At present, the ECCO guidelines[15] do not support the use of systemically acting antibiotics (such as cyprofloxacine and metronidazole), due to the high incidence of adverse effects and the lack of clear evidence in support of their effectiveness. Likewise, these guidelines do not support antimycobacterial treatments, since a recent meta-analysis[16] demonstrated that the only two trials that affected positively the disease activity included the administration of steroids to induce remission. Thus current international guidelines, based on a low level of evidence (*conditional recommendation*), do not recommend the use of anti-mycobacterial drugs as a first line therapy in CD[15,17]. Indeed, two previous meta-analyses reported that anti-mycobacterial therapy alone was not effective for the induction or maintenance of remission[18,19].

In the present article, we have reviewed current information on MAP characteristics and the appropriate diagnostic methodologies for its detection in humans. The available data on emerging antibiotic formulations and novel anti-MAP treatment regimens in CD patients with MAP infection have been discussed as well.

2. Overview of the market

Current therapeutic approaches, based on treatments with biotechnological drugs, have remarkably increased our expectations of favorable outcomes in the management of patients with severe forms of CD.[20,21] However, up to 30-40% of naïve CD patients display a primary refractoriness to both traditional and biologic therapies.[22] To overcome this issue, it has been suggested that patients with primary resistance should be investigated for MAP and, in case of confirmation, be treated with antimicrobial drugs targeted against mycobacteria. Currently, there is only one medicinal product directed against MAP under clinical development, that is RHB-104 (RedHill Biopharma).

3. Introduction to the product

3.1 MAP: microbiological features, animal and human isolation and potential transmission routes

MAP is a bacterium belonging to the family of *Mycobacteriaceae* and is included in the *Mycobacterium avium* complex (MAC), together with *M. avium* and *M. intracellulare*. MAP is a Gram-positive obligate intracellular pathogen, acid-fast and dependent on mycobactin for its replication (a lipo-soluble compound that allows iron metabolism).[23] MAP cannot replicate outside of host cells and has a particular tropism for the colonization of intestinal tissues. Following oral ingestion, it undergoes internalization into the small bowel wall, and is carried to mesenteric or regional lymph nodes by dendritic cells or macrophages.[24] At the subclinical stage of infection, the bovine immune response begins with the activation of cell-mediated Th1 pathway, while, at later stages, the immune response switches from Th1 to an antibody-mediated humoral Th2 pathway, which is not able to eliminate intracellular pathogens.[25]

MAP was first isolated and described as the etiological agent of a chronic inflammatory bowel disease in cattle by Heinrich Albert Johne, a German veterinarian, in 1895.[24] The infection is transmitted primarily by fecal-oral route and is followed by a long latency period.

Indeed, the majority of animals become infected within their first month of life, but the onset of clinical symptoms occurs after 3-5 years of age. The Johne's disease is known to develop and progress over four consecutive stages: silent, subclinical, clinical, and advanced. The infected animals do not display symptoms during the subclinical stage, while the infection becomes evident in the clinical stage, with diarrhea and weight loss. The advanced disease stage is associated with decreased milk production and presence of granulomatous formations both in the regional lymph nodes and small bowel tissues, followed by wasting and death.[24,25]

Humans can be exposed to MAP through a variety of routes, including food ingestion and environment. The environmental spread is related to shedding of MAP into the feces of infected ruminants, especially cows, and is the most likely route of transmission in Western countries, due to the ingestion of contaminated water: MAP DNA was indeed detected in over 80% of domestic water samples in Ohio (USA).[26] MAP can also contaminate human alimonies, mainly meat and dairy products[27]. Pasteurization can reduce significantly, but not abolish this risk, as shown also in a study by Wynne et al.[28], where MAP could be cultured from 1.8% of samples of pasteurized milk from MAP-positive cows in UK. Indeed, the thick lipid bacterial cell wall of MAP allows it to survive pasteurization, and live MAP has been detected in retail milk and cheese products.[17]

The serious concern for MAP transmission from cattle to humans is that MAP-infected cows remain asymptomatic in the subclinical stage. Then, infected cows do not undergo early identification and removal, and may continue to be harvested for milk and meat. As a consequence, MAP can spread unnoticed through fecal matters to the rest of herd. In addition, once released into the environment, MAP can survive for 12 weeks, and up to one year in soil or water.[29]

MAP has been suspected to be involved in many autoimmune disorders in humans:[30,31] CD susceptibility has been related to genes CARD15, SLC11A1,[31] but there is a lack of data regarding the risk of developing CD in case of MAP infection.

3.2 Diagnostic assessment of MAP infection

Standard methods for detection of MAP infection in cattle rely on fecal culture or ELISA assays for anti-MAP antibodies. The sensitivity of both methods is low in subclinically infected animals, making difficult the eradication by culling.[29] ELISA testing for antibodies has been often negative when used only once; therefore, this technique has been suggested as a tool for longitudinal follow-up.[32] MAP suppresses actively the IFN-γ- dependent immune response, and therefore the IFN-γ release assay has been proposed as one of the most promising diagnostic tests for the early diagnosis of MAP infection. However, it has been observed that this assay can document only a condition of exposure to MAP, and that, when used in farm cattle, it is not able to discriminate infected from exposed animals.[33]

In the setting of CD patients, MAP takes a cell-wall deficient spheroplast-like form, which complicates the requirements for culturing and does not allow the use of the gold standard Ziehl-Nilsen mycobacterial staining test.[34] Moreover, in human tissues MAP is difficult or often impossible to identify microscopically. Its presence can be identified by culturing, and indeed histopathological findings and tissue cultures can display high degrees of concordance (up to 80%). However, the in vitro growth of MAP is very slow, and therefore from three to several months are required for colonies to appear. As suitable alternatives, molecular techniques, such as polymerase chain reaction (PCR) or in situ hybridization, can be employed also.[35]

3.3 Therapeutic management of MAP infection

Clarithromycin (CLA), clofazimine (CLO), and rifabutin (RIF) have been used individually or in combination with other drugs to treat diseases associated with mycobacterial infections, including CD.[13,36]

CLA is one of the most prominent member of macrolides, a class of antibiotics widely employed for treatment of upper and lower respiratory tract infections.[37] Macrolides share the same core molecular structure consisting of a macrocyclic lactone ring with various amino sugar side groups. Macrolides approved for clinical use are endowed with 14-, 15- or 16-membered lactone ring. These antibiotics can be sub-classified into natural compounds (e.g., erythromycin) and semi-synthetic derivatives (e.g. CLA or roxithromycin).[38] Macrolides exert bacteriostatic effects when employed at clinically relevant concentrations, and act by inhibition of protein biosynthesis through binding to the large 50S ribosomal subunit.[39] The condition of resistance to macrolides is very often associated with a mutation in the 23S rRNA gene leading to a base change at either position 2058 or 2059 (*E. coli* numbering); these are critical rRNA residues involved in the binding of macrolides to bacterial ribosomes, including mycobacteria.[40] Although 23S rRNA gene mutations are believed to account for most of the acquired macrolide resistance, there is also evidence that other, yet-uncharacterized, ribosome mutations may explain some acquired macrolide resistances in *M. avium*.[41] Generally, all wild-type strains (antibiotic naïve) of *M. avium* are susceptible to macrolides.[42] When tested in vitro against *M. avium*,[43] CLA was 8- to 32-fold more active than erythromycin, probably because the former can penetrate the lipid coat of mycobacteria more readily than erythromycin.[44] In vitro, minimum inhibitory concentrations (MICs) ranging from 0.25 to 0.5 µg/ml have been estimated for CLA against MAP.[45,46] Resistance of MAP to macrolides has been defined as CLA MICs of 64 µg/ml at pH 6.8 and 32 µg/ml at pH 7.3-7.4.[47] There is some evidence that CLA can induce clinical remission in CD patients, as compared with placebo in a randomized trial.[48] However, in another randomized controlled

study, CLA conferred some benefit to CD patients only in the first month of therapy, while it appeared to be ineffective for the following two months.[49]

A recent review[37] evaluated the possible role of macrolides in the management of multi-drug resistant *M. tuberculosis*, and concluded that CLA may serve as a complementary drug, increasing mycobacterial permeability to other antimicrobial agents and exerting its own immune-modulating effects. On these bases, macrolides should never be employed as the sole therapeutic agent for treatment of mycobacterial diseases.[50]

CLO is a R-imino-phenazine antibiotic, originally developed for treatment of tuberculosis.[51] The key structural feature of R-imino-phenazines is the phenazine nucleus, bearing an alkylimino (R-imino) group at the C-2 position and phenyl substituents at the C-3 and N-10 positions of the phenazine nucleus.[52] The basic nitrogen atom of the isopropylimino group at position C-2 of clofazimine contributes to its cationic amphiphilic properties.[53]

CLO has demonstrated impressive activity against various mycobacterial species.[54,55] In addition, CLO can act synergistically with other antimicrobial agents, such as CLA, against several mycobacterial species, including *M. avium*.[56] CLO exerts mainly bacteriostatic effects, while bactericidal actions against actively replicating bacilli are regarded as minor pharmacodynamic properties, with MIC values ranging from 0.06 to 2 μg/ml for *M. tuberculosis*.[52,57] With regard for *M. avium*, a CLO MIC of 0.12 μg/ml has been estimated in vitro.[58] The actual anti-mycobacterial activity of CLO remains unclear. For sure, its high lipophilicity enables an efficient transmembrane penetration and accumulation in fatty tissues, with a subsequent uptake by human mononuclear phagocytes, which are the same cells infected by mycobacteria.[52] Moreover, CLO displays a redox potential of 20.18 V at pH 7.25, thus favoring intracellular redox cycling.[59,60] Above all, CLO has a peculiar property that is highly relevant to the management of mycobacterial diseases; indeed, despite its long half-life

and extensive accumulation in tissues, it is associated with a poor risk of microbial resistance.[61] that might depend on the availability of multiple targets.[52,62]

Despite its impressive antimicrobial activity against in vitro isolates of *M. tuberculosis*, CLO monotherapy was found to be unsuccessful in early studies on higher primates and humans. The combination of this inefficacy with serious adverse events, such as skin discoloration and psychiatric disturbances, including depression, was also a deterrent to clinical application.[61] However, in 1981 CLO was recommended by the WHO for its inclusion as a component of multidrug treatment regimens for leprosy due to its useful combination of antimicrobial and anti-inflammatory properties.[63] Moreover, CLO is being used as a replacement for rifampicin, with good outcomes in up to 67% of patients with lung disease caused by the *M. avium* complex.[64] In a randomized, placebo-controlled trial, CLO was found to be ineffective in inducing CD remission.[65] Interestingly, in a retrospective study, Van Ingen et al.[66] observed that CLO concentrations were enhanced upon concomitant administration of a macrolide (azithromycin), and these findings have been taken as a basis to suggest the suitability of a combination of CLO with CLA for treatment of MAP.

RIF, previously designated as ansamycin or LM 427, is a spiropiperidyl-rifamycin that shares several properties of the rifamycin family members, such as rifampicin and rifapentine.[67] Rifampicin is commonly used for treatment of non-tuberculous mycobacterial infections.[50] Like other rifamycins, it binds prokaryotic DNA-dependent RNA polymerases causing their inhibition.[68] In *Mycobacterium spp*, RNA polymerase comprises five subunits, and the binding site of rifamycins lies within the β-subunit, which is the catalytic core of this enzyme. As a consequence, acquired resistance to rifamycin – that has been well documented in *M. tubercolosis* –[69] is conferred primarily by mutations in the *rpoB* gene, which encodes the β-subunit of RNA polymerase,[70] even though a significant proportion of resistant *M. avium* strains may lack a missense mutation in this gene.[71] Current evidence suggests that a similar

clustering of resistance-associated mutations occurs also in other mycobacteria.[48,72] In an interesting Dutch study, which evaluated the effects of various antimicrobial agents on non-tubercular mycobacteria, CLA and RIF were the most active, with 87% and 83% of all isolates, respectively, that were found to be susceptible.[73] In this setting, RIF displayed considerably lower MIC values than CLA for *M. avium* (MIC ratio 5–10), *M. tuberculosis* (MIC ratio 2–4) and *M. leprae* (MIC ratio 10).[73-75] A recent study estimated a MIC ≤ 0.25-16 μg/ml of RIF for *M. avium* in clinical isolates.[76] Same results are observed for MAP in an interesting Italian study.[77] Synergistic effects against *M. avium*, leading to an increase in the permeability of the outer cell envelope, have been observed with combinations of RIF and ethambutol.[78] In addition, studies on patients with HIV and *M. avium* infection have reported that RIF could protect against the development of CLA resistance.[79,80] For this reason, in lung diseases due to *M. avium*, RIF is always associated with CLA.[50,81]

Of interest, in 2009 Krishanan et al.[82] evaluated the susceptibility of MAP strains to different classes of drugs using MGIT (mycobacterial growth indicator tube) 960 system and the results were compared with those of radiometric (BACTEC) and agar dilution methods. The authors found that the MIC values determined by MGIT displayed a 80-100% agreement (+1 log2 dilution) and a 80% agreement (+1 log2 dilution) with those estimated by BACTEC and agar dilution methods for CLO and RIF, respectively. For CLA, the MGIT method had greater agreement with the agar dilution method (70% at the same dilution) than the BACTEC method (60% at +1 log2 dilution); the agreement rate increased to 100% at +2 log2 dilutions in all cases. Based on these findings, the authors concluded that the MGIT system can be used for rapid and reliable drug susceptibility testing of MAP. In another study, the same authors employed the MGIT method to evaluate the synergistic effects of 6-mercaptopurine against MAP when combined with other conventional antibacterial agents, including CLO, CLA and

RIF. The results showed that 6-mercaptopurine might be synergistic with macrolides, and that the inhibitory activity of CLA against MAP seems to be enhanced by rifampicin.[83]

3.4 Combination therapy: clinical studies

The putative efficacy of antibiotic treatment regimens, based on combined administration of CLA, CLO and RIF, has been investigated in previous clinical experiences (Table 1). In 2007, Selby et al.[13] reported the results of their phase III, parallel-group, placebo-controlled, double-blind trial, designed to assess the efficacy and safety of a combined therapy with CLA, CLO and RIF in patients with active CD. In this study, 213 patients under treatment with prednisolone (40 mg/day with a dose tapering in 16 weeks) were randomized to antibiotic therapy (CLA, CLO, RIF) or placebo for 2 years. CLA was started at the dose of 250 mg daily for the first week, followed by 250 mg twice daily for the second and third week, and then 750 mg/day from week 4; the RIF dosage was escalated at the same time points using daily doses of 150, 300 and 450 mg; CLO was given at the dose of 50 mg daily. The results showed a benefit of the antibiotic therapy over the first 16 weeks in combination with prednisolone; indeed, 66% of patients receiving antibiotic therapy were in remission at week 16, while in the placebo group the remission rate was 50% (P=0.02). From week 16 to 52, at least 1 relapse was observed in 39% of patients in the antibiotic arm and 56% of the placebo group (P= 0.054). The differences among the two study groups decreased at week 104 (26% and 43%, respectively. P=0.14) and at 1-year follow-up after treatment (59% and 50%, respectively, P= 0.54). The study treatments were well tolerated, with discontinuations for adverse events only in 16 patients (8 in each arm). However, several adverse events were significantly more frequent in the antibiotic group, as compared with placebo: abnormal liver function (2.3% vs 0.3%), vaginal candidiasis (4.0% vs 0.8%), abdominal distension (3.4% vs 0.8%), myalgia (2.3% vs 0.3%), and urine discoloration (2.8% vs 0.3%,). Moreover, from

week 17 to 52, arthralgia (3.5% vs 1.2%) and tooth discoloration (2.3% vs 0.2%) were the only adverse events significantly more common in the antibiotic than in the placebo group. The overall conclusion of this study was that the antibiotic treatment displayed a significant benefit in terms of remission induction after 16 weeks, even though in combination with a steroid, but there was no evidence for a prolonged benefit.

It is worthy to mention that, subsequently, Behr et al.[84] performed a re-analysis of benefits in the early and late time points of the above trial. In particular, they performed an intention-to-treat analysis based on all patients recruited into the two study groups, and obtained a significant difference in favor of the antibiotic therapy not only at week 16, but also at weeks 52 and 104 (P=0.003 and P=0.005, respectively). Some limitations of the trial carried out by Selby et al. must be acknowledged. First, no attempt was made to culture or perform polymerase chain reaction for MAP, and therefore no definite conclusions could be drawn about the effect of the antibiotic combination on the putative MAP infection. In particular, no assessment was allowed to record for the putative development of MAP resistance to the combined antibiotic treatment. Second, some concerns might pertain to the dosing and delivery of test drugs that might have been not adequate to obtain therapeutic efficacy (in particular, CLO was administered as a double encapsulated form, potentially hampering its appropriate delivery to the target sites). Third, the trial by Selby et al. was not designed to exclude the possibility of MAP reinfection during the course of therapy or in the follow-up period. Lastly, the study may have lacked sufficient statistical power to demonstrate a more substantial advantage for the subset of CD patients harboring the MAP infection, who were expected to benefit more likely from the combined antibiotic therapy.[85]

4. Emerging therapeutic approaches: RHB-104, a multi-drug oral formulation

RHB-104 (RedHill Biopharma) is a novel formulation that combines in a single pill the three active antibiotics tested in previous studies against mycobacteria. As mentioned above, their combination was used to avoid the development of antibiotic resistance (see Drug summary box). RHB-104 contains a fixed combination of 95 mg CLA, 10 mg CLO and 45 mg RIF. At variance with the regimen employed by Selby et al., this formulation allows taking the three antibiotics in a single pill and at lower antibiotic dosages.

5. Clinical Efficacy

5.1 Pre-clinical studies: in vitro efficacy of RHB-104

Alcedo et al. examined the in vitro activity of RHB-104 against mycobacterial strains isolated from CD patients.[46] In particular, these authors estimated the MIC of CLA, CLO and RIF individually, in two-drug combinations, and as present in the RHB-104 formulation, and they observed that the three-drug combination displayed the lowest effective concentration (0.25–10 μg/mL) as a result of the synergistic anti-bacterial activity exerted by the three antibiotics. The MIC for CLA-CLO-RIF in the RHB-104 formulation against 13 of 16 isolated MAP strains was 0.25 μg/mL (0.158 μg/mL CLA, 0.017 μg/mL CLO, and 0.075 μg/mL RIF). When tested individually, at the above concentrations, CLA, CLO and RIF were not able to inhibit the MAP growth. The inhibition for the CLA–CLO and CLA–RIF regimens was 70% and 15%, respectively. A complete inhibition of MAP growth for CLA–CLO and CLA–RIF regimens could be achieved only at higher levels than their concentrations in 0.5 μg/mL RHB-104. The CLO–RIF pair inhibited MAP growth by 90% when tested at their individual concentrations in 1.0 μg/mL RHB-104. Of note, the MICs of RHB-104 against non-MAP strains were higher, with the MIC against *M. avium* strains being 4.0 μg/mL. Like for MAP strains, at these concentrations the individual drugs were unable to inhibit bacterial growth. Overall, current data support the view that combined low doses of

CLA, CLO and RIF can exert a synergistic in vitro anti-MAP activity. The suitability of lower concentrations of a triple synergistic antibiotic combination could then allow the clinical use of more tolerable dose regimens, and this would translate into an improvement of the risk-to-benefit ratio of treatments for CD, along with a decreased risk of the onset of bacterial resistance, particularly in the long-term setting.

In the study by Alcedo et al.,[46] the contents of RHB-104 capsule formulation could not be dissolved in a single compatible solvent. Therefore, each of the three antibiotics was dissolved in individual solvents, and the respective proportions of the active ingredients contained in the RHB-104 formulation were then recreated in vitro by applying appropriate concentrations of each antibiotic to bacterial cultures. Subsequently, the same authors succeeded in dissolving the contents of RHB-104 capsule formulation in a single solvent, and therefore they performed a further in vitro study where the RHB-104 formulation dissolved in a single solvent was compared with the combination of appropriate concentrations of CLA, CLO and RIF dissolved in individual solvents. The results showed that the MIC values estimated for the RHB-104 formulation against several MAP clinical strains were fairly similar to those yielded by the combined application of the three antibiotics dissolved in individual solvents (MIC $\leq$ 0.2 μg/ml), thus confirming the contention that the multi-drug RHB-104 formulation can exert a potent synergistic anti-microbial activity.[86]

5.2 Clinical efficacy of RHB-104: phase III study

Following the encouraging in vitro results obtained with RHB-104,[86] this fixed-dose antibiotic combination is being tested in a multicenter, randomized, double-blind, placebo-controlled, parallel group phase III trial (clinicaltrials.gov NCT01951326) to evaluate its efficacy and safety in about 330 patients with moderately to severely active CD. Patients are randomized to receive five RHB-104 capsules administered orally BID, containing 95 mg

CLA, 45 mg RIF, and 10 mg CLO for 26 weeks. The primary outcome of the trial is clinical remission at week 26, but there are various secondary outcomes, among which the reduction of Crohn's Disease Activity Index (CDAI) score by a minimum of 100 points and the remission rate from week 26 through week 52 are the most important ones. Of note, in this study various background treatments are allowed: mesalazine and related compounds; corticosteroids; azathioprine, 6-mercaptopurine or methotrexate; infliximab or adalimumab. This could result in a selection bias. However, according to the study design, all the concomitant medications must be at stable doses prior patient randomization, and all the included patients must have a CDAI score from 220 to 450, meaning that they must display a clinically active disease despite the intake of concomitant drugs. Among the various outcomes, changes in MAP blood status will be assessed by PCR, even though MAP positivity will not represent a main criterion for patient inclusion in the study. This might be regarded as a relevant limitation, but, on the other hand, an evaluation of MAP concentration changes – only in patients with ongoing infection – should provide evidence that RHB-104 is effective against MAP in vivo as well. Of note, a long-term population pharmacokinetic study is being implemented also as a part of the Phase III MAP US trial (clinicaltrials.gov NCT01951326). This trial has been estimated to be completed on April 2019.

As compared to the Selby study,[13] the main strength of the ongoing trial on RHB-104 is the low dosage of the three antibiotics; indeed, due to their in vitro synergistic effect, the efficacy of RHB-1004 could be good and the adverse effects would probably be less, in terms of frequency and/or severity. Another important point is the detection of MAP: if this were an inclusion criterion, the data obtained with the ongoing trial would likely allow to understand whether the effect of RHB-104 depends actually on MAP suppression or result from other mechanisms. In this respect, if a good proportion of the included patients will carry a

concomitant MAP infection, some information on the role of MAP in CD are expected to be obtained, as compared to the Selby study, where MAP was not detected at all.

An open label study, designed to assess the efficacy and safety of RHB-104 in about 100 patients with active CD (CDAI≥150), after 26 weeks of participation in the MAP US study, is also ongoing, with an estimated completion on October 2019 (clinicaltrials.gov NCT03009396). In this trial, patients are treated with RHB-104 for 52 weeks with an open-label regimen. This study is evaluating also the proportion of patients with MAP positive blood PCR at baseline and changes in MAP blood PCR associated with RHB-104 treatment. Like above, background treatments with anti-inflammatory/immune-modulating drugs are allowed. However, after 8 weeks of open-label therapy with RHB-104, these medications could be discontinued by the investigator, as clinically indicated. In addition, concomitant corticosteroids must undergo tapering after 4 weeks of treatment with RHB-104. In this open-label trial, the primary outcome is the remission after 16 weeks of treatment with RHB-104, whereas the time to remission, the duration of remission, the duration of response and the time of response are secondary outcomes. Preliminary results have been released and the drug showed a good efficacy: 42% of patients achieved disease remission at week 16,[87] but extended data have not been published yet.

6. Safety

Both the ongoing phase III and open label studies have been designed to evaluate also the safety of RHB-104 in patients with moderate-to-severe active CD. Data are not yet available. However, no major concerns about safety are expected from these trials, mostly because the dosage of the three antibiotics is lower as compared to the Selby study.

7. Conclusions

The etiological agent accounting for the onset of CD remains largely unknown. However, there is some evidence to suggest that infectious microorganisms might act as stimuli for triggering and maintaining the condition of excessive immune activation that underlies the pathophysiology of CD. In this context, much attention is being paid to MAP, basically for three main reasons: 1) this mycobacterium has been identified as responsible for the etiology of Johne's disease, a severe chronic enteritis of ruminants that shares with CD several clinical, pathological and immune activation features;[24] 2) MAP has been repeatedly detected in blood and gut tissues from CD patients;[9,34] 3) a number of case reports have documented a favorable impact of anti-MAP antibiotics on the clinical course of patients with CD.[36,48] Based on this knowledge, it has been conceived that treatments with antibiotics targeting MAP might lend significant benefits in the therapeutic management of patients with CD. In vitro tests have identified CLA, CLO and RIF as antimicrobial agents endowed with good inhibitory activity on the growth of various mycobacterial species, including MAP.[46] These observations encouraged the implementation of clinical trials to evaluate the efficacy of treatment regimens, based on combinations of CLA, CLO and RIF, in inducing the remission of active CD, but the results of initial studies have not been fairly promising.[13] More recently, a renowned interest is revolving around the possible combined clinical use of CLA, CLO and RIF for treatment of CD, since in vitro tests on MAP strains isolated from CD patients showed that RHB-104, a novel oral ~~10-mg~~ formulation, containing 95 mg CLA, 10 mg CLO and 45 mg RIF, was significantly more effective than each individual antibiotic and displayed a remarkable synergistic activity in suppressing mycobacterial growth.[83] These interesting findings have prompted the design of novel clinical studies deputed to investigate the efficacy and safety of RHB-104 in patients with active CD. As a result, the first controlled clinical trial in this setting is currently ongoing, and it is expected to be concluded by middle 2019. In conclusion, current knowledge, suggesting a possible involvement of MAP in the

etiopathogenesis of CD, taken together with the strong synergistic anti-MAP activity displayed in vitro by the fixed antibiotic combination contained in RHB-104, provides a solid rationale supporting the clinical program aimed at testing the efficacy of RHB-104 in CD patients.

8. Expert opinion

Owing to the lack of evidence on the etiological agents underlying CD, all current options available for pharmacological interventions in CD patients, including traditional drugs and biologics, are devoted to counteract the inflammatory activity and downregulate the abnormal immune hyper-activation, through a variety of mechanisms, in an attempt of achieving clinical remission and, whenever possible, bowel tissue healing.[17] Of note, as anticipated in the above sections, current guidelines do not recommend the use of systemically acting antibiotics in the management of CD[15]. However, if one acknowledges the theory that a specific infectious agent, such as MAP, could be the primary cause of CD, this assumption might have a dramatic impact on the way physicians manage the disease, in terms of diagnostic procedures and, above all, therapeutic approaches. Indeed, the pharmacological suppression of the etiological agent would be expected to result in a definitive biological healing of the disease.

To recognize a specific etiological role for MAP in CD would translate into two main obvious consequences: a) importance of MAP detection in CD patients, as a part of the routine diagnostic work-up, commonly implemented for this disease in clinical practice; b) the need for identifying antibiotic-based treatment regimens, suitable to allow MAP eradication, with a consequent resolution of both clinical and pathological signs of disease activity. With regard to the latter point, the novel oral formulation RHB-104, containing a fixed combination of CLA, CLO and RIF, appears to be quite promising, due to the evidence

from in vitro studies showing a synergistic inhibitory activity of this combination on MAP strains isolated from CD patients.[46,86] Although the results of initial clinical studies on the possible remission of active CD by combined administration of CLA, CLO and RIF were not fairly encouraging,[13] novel clinical trials to test the efficacy and safety of RHB-104 in CD patients are currently ongoing and, in case of positive outcomes, their results would likely have a significant impact on the therapeutic strategies for CD. Indeed, the demonstration of efficacy for RHB-104 might dramatically change the therapeutic and clinical research scenarios in CD, since MAP eradicating regimens by antibiotics might become either first-line therapeutic approaches or complementary interventions to be employed in combination with current anti-inflammatory/immune-modulating drug therapies. Likewise, should the reliability of RHB-104 for treatment of CD be demonstrated, it is likely that physicians would trust and prescribe such a new therapeutic option, perhaps giving initial priority to those patients with clear demonstration of MAP infection or primary resistance to current standard pharmacological treatments.

As discussed above, at present the clinical development program of RHB-104 is being focused on a phase III trial aimed at evaluating its ability of inducing clinical remission in patients with active CD, as compared to placebo. However, the experimental design of this trial does not take into account some relevant arguments that deserve careful consideration. In particular, given the uncertainties on the causative role of MAP in CD and considering that the abnormal immune activation in CD seems to depend also on individual genetic determinants, it cannot be ruled out that in some patient subgroups CD might be triggered either by non-infectious causative factors or by infectious agents other than MAP. On this basis, a trial design where RHB-104 is intended to be compared with placebo irrespectively of the MAP status of CD patients does not appear satisfactory. We propose instead that, prior to enrolment, CD patients should be investigated for the presence of MAP, and RHB-104 should

then be tested comparatively in a double-blind way in CD patients with and without MAP infection. Indeed, a favorable outcome with RHB-104 would be expected mainly in the presence of MAP infection and, clearly, the achievement of favorable results in patients without MAP infection would lead to hypothesize the involvement of non-MAP infectious agents or alternative mechanisms of actions for RHB-104 unrelated to antimicrobial activities. An appropriate interpretation of clinical data generated by future trials could take also advantage by the isolation of MAP strains from CD patients at the time of diagnosis with the purpose of evaluating their in vitro susceptibility to RHB-104. In our opinion, this information could actually prompt physicians to include this antibiotic combination in their therapeutic armamentarium in CD patients, particularly in cases of primary resistance to standard regimens. In this respect, provided that a stringent program of clinical development is implemented over the near future, in order to link causally the efficacy of RHB-104 to the diagnostic evidence of MAP infection, it can be envisaged that in the next 5 years this novel antibiotic combination might likely be at the end of its registration path.

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Declaration of interest

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Drug Summary Box

Drug name	RHB-104 (fixed-dose combination of clarithromycin, clofazimine and rifabutin)
Phase	III
Indication	Crohn's disease
Pharmacology description/mechanism of action	Well documented in vitro synergistic inibitory activity on clinical isolates of *Mycobacterium avium* subspecies*, paratubercolosis*
Route of administration	Oral
Pivotal trial	MAP US Study: multicenter phase III trial to evaluate the efficacy and safety of RHB-104 in patients with moderate-severe active Crohn's disease

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Table 1. Clinical studies on antimicrobial treatment for MAP

Study	Study Design	Drugs used	Study Population	Results
Selby 2007 Behr 2008 (reanalysis)	Phase III, parallel-group, placebo-controlled, double-blind trial	CLA was started at the dose of 250 mg daily for the first week, followed by 250 mg twice daily for the second and third week, and then 750 mg/day from week 4; RIF dosage was escalated at the same time points using daily doses of 150, 300 and 450 mg; CLO was given at the dose of 50 mg daily.	213 patients under treatment with prednisolone	66% of patients receiving antibiotic therapy were in remission at week 16 vs 50% in the placebo group. A significant difference in favour of the antibiotic group was also observed at weeks 52 and 104
clinicaltrials.gov NCT01951326	Multicenter, randomized, double-blind, placebo-controlled, parallel group phase III trial	Five RHB-104 capsules administered orally BID (95 mg clarithromycin, 45 mg rifabutin, and 10 mg clofazimine)	330 patients with moderately to severely active CD	Expected on April 2019
clinicaltrials.gov NCT03009396	Open label study, phase III	RHB-104; a fixed-dose combination of 95 mg clarithromycin, 45 mg rifabutin, and 10 mg clofazimine	100 patients with active CD	Expected on October 2019