UNIVERSITE LILLE NORD DE FRANCE

FACULTE DES SCIENCES PHARMACEUTIQUES ET BIOLOGIQUES

ECOLE DOCTORALE BIOLOGIE-SANTE

THESE DE DOCTORAT

Spécialité « Pharmacie en Sciences physico-chimiques et Ingénierie appliquée à la santé »

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UN NOUVEL AGENT ANTIBACTERIEN: CARACTERISATION BACTERIOLOGIQUE *IN VITRO* ET PERFORMANCE DES FORMULATIONS A LIBERATION PROLONGEE *IN VITRO/IN VIVO*

A NEW ANTIBACTERIAL AGENT: *IN VITRO* BACTERIOLOGICAL CHARACTERIZATION AND *IN VITRO/IN VIVO* PERFORMANCE OF SUSTAINED RELEASE FORMULATIONS

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Soutenue le 08 septembre 2015

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Acknowledgments

I would like to acknowledge Mrs. **Christine ROQUES-CESCHIN**, Professor at the University Paul Sabatier in Toulouse, and Mr. **Abdul BASIT**, Professor at the University College London, for accepting to be reporters of my thesis work, for evaluating mi work, as well as for being present as jury members in my dissertation. Please accept the assurances of my highest consideration and respect.

I am very thankful to Mr. **Raphael DUVAL**, Professor at the University of Lorraine in Nancy, for accepting to participate as jury member in my dissertation and for evaluating mi work. Pease accept the assurances of my highest consideration and respect.

Je remercie Madame **Christel NEUT**, maître de conférences à l'Université Lille 2, de m'avoir accueillie au sein du laboratoire de Bactériologie clinique pendant ces quatre dernières années. Je tiens à vous exprimer toute ma gratitude pour l'hospitalité que vous m'avez témoignée lors de mon arrivée en France, pour votre encadrement académique inestimable et pour avoir partagé avec moi votre vaste connaissance de la bactériologie. Vos conseils et critiques envers mon travail, ainsi que votre temps et votre patience seront toujours très appréciés. Soyez assurée de mon profond respect et de ma très sincère gratitude.

Également, Monsieur **Luc DUBREUIL**, Professeur à l'Université Lille 2, je vous suis très reconnaissante de m'avoir reçue dans votre laboratoire et de m'avoir octroyé cette opportunité de financer mes études de doctorat en France. Merci de votre confiance et de vos conseils avisés. Merci notamment de votre grande disponibilité, malgré votre agenda chargé. Je vous exprime ma profonde gratitude et mon très grand respect.

J'aimerais aussi remercier de façon très spéciale Monsieur **Juergen SIEPMANN**, Professeur à l'Université Lille 2, de m'avoir acceptée au sein de son laboratoire. Merci de tout le savoir que vous m'avez transmis et de votre patience. Merci avant tout, d'avoir toujours été disponible pour moi avec tant de gentillesse, et finalement, de votre encouragement et vos conseils. Veuillez trouver ici, l'expression de mon profond respect et ma sincère gratitude.

Je tiens à remercier Monsieur **Nicolas TESSE**, Président de Neteos Groupe SAS, de m'avoir donné l'opportunité de travailler sur un de ses brevets ainsi que d'avoir participé au financement de mes études de doctorat. Merci de votre confiance et de vos conseils. Je voudrais remercier **ADRINORD**, pour le financement de ce travail de thèse.

L'équipe INSERM U995

Je veux aussi remercier Monsieur **Pierre DESREUMAUX**, Directeur de l'unité INSERM U995, de m'avoir procuré l'opportunité de travailler dans son équipe.

Je remercie Monsieur **Laurent DUBUQUOY** de votre aide au cours des manips chez la souris, de vos conseils et vos remarques dans mes manuscrits et de votre disponibilité. Merci à **Madjid DJOUINA** de m'avoir appris des techniques en biologie moléculaire et histologie, ainsi que l'expérimentation animale (malgré les nombreuses fois que les souris m'ont mordue). Merci de ta patience et de ton immense disponibilité à chaque fois que j'avais des questions.

L'équipe de Bactériologie : merci à **Séverine MAHIEUX** de m'avoir transmis tes connaissances en bactériologie, pour ta grande disponibilité et la confiance que tu m'as accordée dès ma première journée au labo. Merci à **Carole LEBRUN, Oumaira RAHMOUNI, Fabiola GARCIA, Charlotte MAILLET** de votre aide au quotidien et de la bonne ambiance au sein du labo. Un grand merci à **Isabelle HOUCKE** de m'avoir transmis tes connaissances en bactériologie, de m'avoir aidée avec les difficultés de la langue dans mes présentations autant orales qu'écrites tout au début de mon séjour en France, mais surtout de ton écoute et ton encouragement constant dans les moments les plus difficiles.

L'équipe INSERM U1008

Je remercie Madame **Florence SIEPMANN** de m'avoir encadrée dans le domaine de la galénique, de vos conseils et d'avoir toujours gardé le sourire, la patience et l'optimisme au cours de mes manips avec les pellets et les comprimés. Merci de votre écoute et soutien au-delà de la thèse.

Merci à Monsieur **Jean-Francois WILLART,** Directeur de recherches CNRS à l'Université Lille 1, d'avoir partagé avec moi vos vastes connaissances reliées à la DSC, de votre patience à m'expliquer ces nouveaux domaines et de votre disponibilité.

Merci à **Susanne MUSCHERT, Mounira HAMOUDI, Youness KARROUT**, de vos conseils, votre aide et votre soutien au laboratoire.

Merci à **Hugues FLORIN** et **Muriel DEUDON** de votre aide technique et de nos discussions toujours intéressantes aux pauses café.

Merci aux filles de l'équipe U1008 : **Hanane GASMI, Maria GEHRKE, Julie FAHIER, Oriane CANTIN, Esther MARTINEZ**, pour ces 3 années pleines d'anecdotes, pour votre aide au quotidien et surtout pour la bonne humeur au sein du labo. Je suis contente d'avoir fait partie d'une équipe si dynamique et internationale.

Deux ex-doctorantes que j'apprécie beaucoup **: DO MINH Phuong** et **HOANG Huong**. Merci de votre écoute, de vos conseils dans tous ces moments où nous n'avions pas la moindre idée de comment résoudre un problème au laboratoire. J'admire votre travail ardu et votre humilité.

Merci à **Berengère DEKYNDT** pour ton amitié, ton écoute et tes encouragements. Un merci spécial à **Carine VELGHE**, pour m'avoir intégrée à l'équipe lors de mon arrivée chez vous, pour ton amitié inconditionnelle dans les moments difficiles, y compris les maladies et les déménagements. Enfin merci à vous 2 pour avoir partagé avec moi les longues et inoubliables journées avec le coater, les pellets collés, le triage des pellets, le nettoyage des machines à l'annexe et le cher hplc.

Mes dernières remerciements en français et anglais, et non des moindres, vont à Anna et Chivy de votre aide avec les corrections en anglais-français. Thank you so much, I really appreciate it!

Mis seres queridos, gracias a:

Mi hermano **Oscar**, por tu cariño y estar siempre disponible cuando necesito ayuda. Por estar a mi lado en este dia importante.

Maximilian, por tu amor y apoyo incondicional. Por esas carcajadas que podían desencadenar una crisis de asma.

Mi mamá **Hilda**, por tu amor, la valentía a prueba de todo con la que me criaste y tu desprendimiento absoluto por darme una vida mejor. Por las innumerables noches durmiendo en el sofá atenta a lo que yo pueda necesitar.

Mi padre **Oscar**, por tu amor y sacrificio. Tu eres mi motivación para seguir adelante, pues te debo todo lo que tengo. Por esa Visa expirada, yo te dedico este trabajo con todo mi cariño.

Finalmente, dedico un pensamiento especial a los momentos más tristes y los más difíciles que viví durante estos cuatro años. A mis pastillas. Porque los recuerdo hoy como experiencias que me ayudaron a crecer como persona, mucho más allá de los fríos conocimientos científicos y los vacíos grados profesionales.

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Abbreviations

5-ASA	5-aminosalicylic acid
AIEC	Adherent-Invasive *Escherichia coli*
APC	Antigen presenting cells
ATG	Autophagy-related gene
ATP	Adenosine triphosphate
BH	Brain-heart
BLA	β-lactam antibiotics
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CAP	Cellulose acetate phthalate
CARD	Caspase recruitment domain
CD	Crohn's disease
cDNA	complementary deoxyribonucleic acid
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CODES	Colon-specific drug delivery system
CTDC	Colon-targeted delivery capsule
DAI	Disease activity index
DCs	Dendritic cells
DLG	Drosophilia disc larg
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulfate
EC	Ethylcellulose
ECDC	European Center for Disease prevention and Control
EEN	Exclusive enteral nutrition
EN	Enteral nutrition
EOs	Essential oils
ESBL	Extended-spectrum β-lactamases
FDA	Food and Drug Administration
FIC	Fractional inhibitory concentration

FMT	Fecal microbiota transplantation
FOS	Fructo-oligosaccharide
GBF	Germinated barley foodstuff
GI	Gastrointestinal
HIMEC	Human intestinal microvascular endothelial cells
HLA	Human leukocyte antigen
HP-β-CD	Hydroxypropyl- β-cyclodextrin
HPC	Hydroxypropyl cellulose
HPMC	Hydroxypropyl methylcellulose
HPMCAS	Hydroxypropyl methylcellulose acetate succinate
HTS	High-throughput screening
IBD	Inflammatory bowel diseases
IBS	Intestinal bowel syndrome
IECs	Intestinal epithelial cells
IFNγ	Interferon gamma
IL	Interleukin
IRGM	Immunity-related guanosine triphosphatase family M
JAK	Janus kinase
L-HPC	Low-substituted Hydroxypropyl cellulose
LP	Lamina propria
MAGUK	Membrane-associated guanylate kinase
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MCC	Microcrystalline cellulose
MDP	Muramyl dipeptide
MDR	Multiple-drug resistant
mDSC	modulated Differential Scanning Calorimetry
MH	Mueller-Hinton
MIC	Minimal inhibitory concentration
MMPS	Matrix metalloproteinases
MMX	Multi-matrix system
MRSA	Methicillin-resistant *Staphylococcus aureus*

NCF	Neutrophil cytosolic factor
NFκB	Nuclear factor kappa B
NOD	Nucleotide-binding oligomerization domain
NPs	Natural products
OCTN	Organic cation-carnitine transporter
OM	Outer membrane
PAE	Post-antibiotic effect
PBP	Penicillin-binding protein
PCDC	Pressure-controlled colon delivery capsules
PEN	Partial enteral nutrition
PLGA	Poly(lactic-co-glycolic acid)
PPARγ	Peroxisome proliferator-activated receptor gamma
PSM	Plant secondary metabolite
qPCR	quantitative Polymerase Chain Reaction
QS	Quorum sensing
R&D	Research and development
RNA	Ribonucleic acid
RXR	Retinoid X receptor
SCFA	Shorty-chain fatty acids
SD	Standard deviation
SLC	Soluble carrier
SLS	Sodium lauryl sulfate
subMIC	sub-Minimal inhibitory concentration
TEM	Transmission electron microscopy
TES	Time-controlled explosion system
Tg	Glass transition temperature
TGFB	Transforming growing factor B
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonic acid
TNFα	Tumor necrosis factor alfa
UC	Ulcerative colitis

UFA	Unsaturated fatty acids
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization

Research context

Bacterial antibiotic resistance constitutes a worldwide threat to public health. The propagation of multiple-drug resistant bacteria, especially Gram-negative bacteria, risks causing hospital and community infections for which there are no effective antibiotics. The human and economic costs of the antibiotic resistance burden are enormous. In Europe at least 25 000 patients die per year because of resistant infections which are not treatable with available antibiotics, and the estimated costs rise to more than 1.5 billion EUR annually.

These facts emphasize the urgent need of new antibiotics able to target resistant bacteria. However, the antibiotic research and development pipeline from pharmaceutical companies is in decline. Several factors have led to the absence of antibiotic discovery, between them, the lack of return on investments, the scientific challenges to obtain approval and the restrictions on antibiotic use because of the risk of developing resistance. New models encouraging the investment on antibiotic research with intelligent measures to avoid their misuse have to be established.

In the search for new antibiotics, nature represents a vast reservoir of bioactive compounds that merit to be exploited. Plant secondary metabolites are provided of an inherent antimicrobial activity as a result of long evolutionary process aiming at the protection and survival of plants in their environment. Essential oils (EOs) for example, are complex mixtures of compounds where all, major and minor components, seem to cooperate in the antibacterial activity. Indeed, synergistic interactions between EO components might thus occur, potentiating their antibacterial action. Furthermore, as EO affects multiple bacterial targets, the risk of resistance development is lower than single-targeted antibiotics. With the purpose of antibiotic discovery based on natural products, a pharmaceutical company has developed CIN-102, a synthetic and synergistic blend that resembles the composition of cinnamon EOs. The fact that CIN-102 is a defined blend with known concentrations for each active component, avoids the problem of unknown concentration and composition variability typically found in botanic drugs. Preliminary studies showed that CIN-102 is an interesting new antibacterial candidate with potential to treat infections caused by resistant bacteria. Among all the possible therapeutic applications for this new agent, we were interested in targeting Inflammatory Bowel Diseases (IBD).

IBD comprises Crohn's disease and ulcerative colitis, which are chronic diseases whose etiology has not yet been completely understood. Nevertheless, there is abundant evidence about the role of enteric bacteria in the origin and the course of these diseases. Even though the main treatment is basically focused on an immunomodulatory approach, the use of drugs capable of modulating the intestinal microbiota has an increasing interest. Among them, antibiotics could be useful to reduce increased bacterial loads commonly found in the ileum and colon of IBD patients; as well as to target specific bacteria with potential pathogenic

activity like enterobacteria. This way, regulation of intestinal microbiota disorders might decrease the aggressive immune response to bacterial antigens, alleviating the outcomes of intestinal inflammation.

Drugs that intend to have a local action in the lower part of the gastrointestinal tract, like in the case of IBD, present the challenge of an appropriate design of the pharmaceutical dosage form. Given that an oral dosage form has to pass through the stomach and the small intestine before arriving to the colon, release or absorption of the drug in the upper part of the gastrointestinal tract might occur. This could limit the amount of drug available at the site of action, requiring administration of high drug concentrations to ensure a therapeutic effect, increasing the risk of adverse effects. In this context, colonic targeted drug delivery technologies permit oral forms to resist degradation and release of the drug in the stomach and small intestine, while enhancing drug delivery at the colon overcoming the aforementioned shortcomings. For this, colonic targeted systems should be able to sense their arrival into the colon in order to trigger drug release. Specific physiological characteristics found in the colon, in comparison to those of the upper gastrointestinal tract can thus be used in the fabrication of colonic oral dosage forms.

Objectives

The present work has 3 main objectives:

- To characterize the *in vitro* bacteriological activity of CIN-102.
    - A long evaluation of CIN-102 activity to confirm its spectrum of activity using a wide panel of clinical bacterial isolates.
    - Determination of the logarithmic reduction time of CIN-102 against a panel of bacterial isolates.
    - Determine if CIN-102 possess a post-antibiotic effect and if sub-inhibitory concentrations have an effect on bacterial growth.
    - A study of the interactions between CIN-102 and currently commercialized antibiotics against a wide panel of clinical bacterial isolates, searching for future therapeutic applications in combined therapies.

- To fabricate colon targeted multi-particulate formulations of CIN-102 aiming a future therapeutic application against IBD.
    - Fabrication and optimization of sustained release reservoir formulations of CIN-102: *in vitro* evaluation of drug release from coated pellets.
    - Fabrication and optimization of sustained release matrix formulation of CIN-102: *in vitro* evaluation of drug release from mini-tablets.

- *In vivo* evaluation of the efficacy of multi-particulate sustained release formulations of CIN-102, as well as CIN-102 antibacterial activity using a model of colitis.

- To analyze the *in vitro* antibacterial activity of peroxisome proliferator-activated receptor gamma (PPARγ) agonists and antibiotics commonly used in the treatment of IBD.
  - Determination of the antibacterial activity of 5-aminosalicylic acid and GED-0507-34-Levo against anaerobic clinical isolates.
  - Determination of the antibacterial activity of narrow and broad spectrum antibiotics against anaerobic clinical isolates.

Presentation of the work

The present work is composed of four chapters: the first one is an introduction describing the problem of antibiotic resistance, the actual role of pharmaceutical companies in antibiotic discovery, the potential of natural products as new antibacterial agents, the relevance of bacteria in the etiology and treatment of IBD; and the different approaches to deliver drugs into the colon. The second, third and fourth chapters correspond to the research work done to achieve the 3 aforementioned objectives, respectively. Each of these three chapters presents a brief introduction about the subject, the materials and methods used, the results obtained and discussions; and a concise conclusion. Finally a general conclusion will regroup the goals achieved in this work, and the perspectives will throw new light on the continuation of this research project.

CHAPTER I

INTRODUCTION

1. The threat of resistant bacteria

Antibiotic resistance has emerged as one of the greatest **global health challenges** to be addressed in the 21st century. In its first technical report, the European Center for Disease prevention and Control (ECDC) rated resistant bacterial infections as **one of the most important health threats** in Europe because of the increase in infections due multidrug-resistant bacteria in Europe[1]. The risk of widespread antibiotic resistance threatens to mitigate the positive changes made in modernizing healthcare systems; therefore, new approaches as well as new and effective antibacterial drugs are essential. Presently, at least 25 000 patients in Europe die per year because their bacterial infections are not treatable with available antibiotics at the estimated cost of more than 1.5 billion EUR annually[1,2].

1.1. Principles of antimicrobial resistance

Bacteria can be intrinsically resistant to certain antibiotics but can also acquire resistance via mutations in chromosomal genes and by horizontal gene transfer (HGT)[3,4].

- The **intrinsic resistance** is given by the ability to resist the action of an antibiotic as a result of inherent structural or functional characteristics determined by naturally occurring genes in bacteria's chromosome[3,4].
- The **acquired resistance** involves mutations in genes targeted by the antibiotic and the transfer of resistance determinants borne on mobile genetic material[3].

  Three mechanisms of bacteria gene transfer have been identified[5]:

  - **Transformation**, which involves the uptake and incorporation of naked DNA. It depends on bacterial competence which is variable between species;
  - **Conjugation**, a cell contact-dependent DNA transfer mechanism found to occur in most bacterial genera. The vast majority of reports of HGT in the environment concern conjugation; and
  - **Transduction,** whereby host DNA is encapsulated into a bacteriophage that acts as the vector for its injection into a recipient cell.

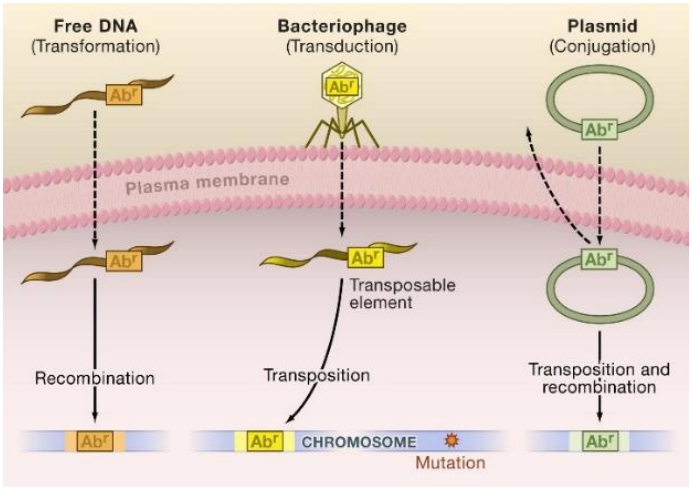

***Fig. 1**: **Acquisition of antibiotic resistance** (Abr)[3]*

HGT shows that drug resistance is mobile. Indeed, genes for resistance traits can be transferred among bacteria of different taxonomic and ecological groups by means of mobile genetic elements (bacteriophage, plasmids, transposons, integrons or naked DNA)[6]. Resistant plasmids are not only maintained stable, but also usually transferred between bacterial cells at a very high efficiency[7]. Plasmids may carry resistance to several antibiotics, selection by one antibiotic may result in selection for resistance to other antibiotics carried by the same plasmid. Transposons and integrons have evolved as a mean for microorganisms to change more rapidly than possible by only mutations. They are frequently carried on plasmids, but can also have a chromosomal location[8]. Bacteriophages are very common in the environment and are relatively stable, being protected by the protein coat. Phages are also more compact and thus more diffusible than naked DNA[5].

Bacterial resistance can be mediated by several **mechanisms**, which fall into the following main groups[4,8]:

- Those that minimize the intracellular concentrations of the antibiotic as a result of poor penetration (such as lack of porins) into the bacteria or antibiotic efflux;
- Those that modify or inactivate the antibiotic target by genetic mutation or post-translational modification of the target;
- Those that introduce a new resistant target, overproduction of target or other by-pass mechanisms; and
- Those that inactivate the antibiotic by hydrolysis or modification.

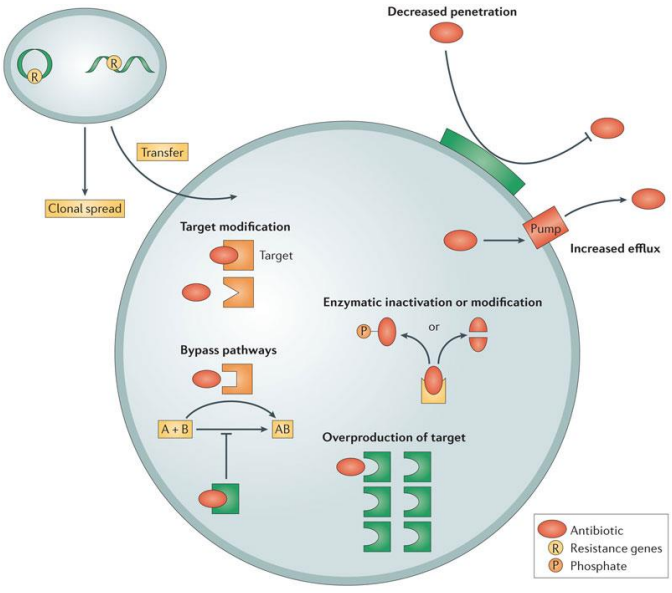

***Fig. 2*: Mechanisms of antibiotic resistance**[9]

In addition, the same kind of drug resistance mechanism can be specified by many different genes, and more than one type of mechanism may provide resistance to the same antibiotic[6].

Antibiotics target important bacterial functions, so it is not surprising that resistant mutants usually suffer a decrease in biological fitness. The acquisition of resistance genes by HGT also carries a **fitness cost**. The decreased fitness caused by resistance may be rapidly and efficiently counterbalanced by compensatory mutations without a loss of resistance. Compensatory evolution can stabilize resistant bacterial populations in the absence of antibiotics by making them as fit as susceptible clones[10]. And this explains why many types of resistance may never disappear in bacterial populations[5]. Thus, reversibility in clinical settings is expected to be slow or non-existent, compensatory evolution and cost-free resistances can reduce the cost and thereby reduce the driving force for reversibility[10].

However, antibiotics do more than select for resistance. In fact, subpopulations of bacteria can survive lethal doses of antibiotics becoming resistant by a transient and non-hereditary mechanism, called **persistence**[11]. This persister population is genetically identical with the susceptible cells. The presence of persisters is now thought to be an example of the strategy whereby bacteria naturally generate mixtures of phenotypically different populations, so that one of them can be advantageous to a changing environmental demand[7]. Antibiotic pressure also selects for bacteria with an elevated mutation rate, this **hypermutable** clones can indirectly select for increased probability of resistance to non-related antibiotics[11].

1.2. Sources of resistant bacteria

The antibiotic **resistome** is the collection of all genes that directly contribute to antibiotic resistance, both in the environment and in the clinic[12]. The source of resistance genes is composed of producing organisms (antibiotic-producing microbes, which must defend themselves from the antibiotics produced) and microorganisms in the environment, especially those found in soil (which have been exposed to various antibiotics throughout their evolutionary history). Soil bacteria typically undergo higher rates of gene transfer[7].

Even though the number and diversity of resistance genes in clinical pathogens are relatively small compared to their diversity in the environment, numerous studies have confirmed the fact that antibiotic resistance genes have an environmental reservoir[12]. Changes in this **environmental resistome** might be relevant for the emergence of previously unknown resistance determinants in bacterial pathogens[13].

The predominant role of human activities in the generation of environmental reservoir of antibiotic resistance cannot be disputed. Since the 1930s, ever-increasing amounts of antibiotics have been manufactured, used, released into the environment, and widely disseminated. This way, anthropogenic changes provide constant selection and maintenance pressure for populations of resistant strains in all environments[14]. This might enrich the population of resistant bacteria and facilitate the transfer of resistance genes to human pathogens[13]. It should be noted that therapeutic use in humans accounts for

less than half of all applications of antibiotics produced commercially. Alternative uses of antimicrobial agents are: growth, promotion and prophylactic use in animals; therapeutic and prophylactic use in aquaculture and in household pets; pest control and cloning for plants and agriculture; use as biocides in toiletries and in hand care; household cleaning products; culture sterility, cloning, and selection in research and industry[14]. Large amounts of antibiotics used for human therapy and for **anthropogenic activities** including agriculture, veterinary medicine, animal husbandry, aquaculture, waste disposal and other nonhuman applications create major environmental reserves of resistance[7,14]. Another important issue is the potential effect of contamination by antibiotics themselves[13]. Taking into consideration the large-scale disposal of **toxic wastes** and residues of manufacturing processes, the amounts of noxious xenobiotics released into the biosphere are inestimable. The fact that many of the chemicals disposed are recalcitrant to biodegradation makes the issue worse[14]. Additionally, the increase in human population and the widespread lack of efficient wastewater treatment bring with them a risk of transfer of antibiotic resistance[13].Antibiotics and bacteria resistant to them in waste water, surface water, ground water, sediments and soils are being reported with increasing frequency and are potentially important contributors to the environmental selection of antibiotic-resistance organism[7,15].

Besides these sources, the human gut, containing a densely populated microbial system, offers ample opportunities for HGT of resistance genes. Antibiotic resistance genes harboured by the gut microbiota constitutes the **gut resistome**[16]. Furthermore, **animal** contributions to the resistance problem in human infections are small but not insignificant; they have a major role if enteric organisms are involved[6]. All these factors resulted in the selection of pathogenic bacteria resistant to multiple drugs[7,14].

1.3. Multiple-drug resistant (MDR) bacteria

Bacteria resistant to multiple families of antibiotics constitute a worrisome threat to public health. In this regard, *Chang et al*[17]explained the **mechanisms** that have led to an excess of MDR. Unexpectedly high rates of origin and high rates of spread of MDR strains or determinants, or both, cause this excess. Certainly, a major complicating factor is the possibility of HGT, which can disseminate resistance to multiple antibiotics in a single step.

Which is the origin of MDR strains?[7,17]

- Single biochemical mechanisms conferring resistance to multiple drugs;
- Genetic linkage: MDR strains may arise because determinants of resistance to multiple drug classes are genetically linked;
- Highly mutable or recombinogenic bacterial lineages; and
- Multidrug therapy with accelerated treatment failure in resistant infections.

Once MDR have emerged in one or more hosts, their proliferation depends on their ability to survive and to be transmitted to other hosts. **How can MDR strains proliferate?**[5,17]

- Associated linkage selection: multi-resistance proliferation does not need to be consequence of direct selection but may result from selection of other genes that are inherited along with it.
- Bystander selection: when a drug is used to treat infection with a particular species, other species carried by the same host might be affected by the treatment.
- Positive epistasis between drug resistance determinants: if the fitness cost of MDR in the absence of antimicrobial use is smaller than the total cost of each resistant determinant on its own, MDR strains may outcompete strains with a limited number of resistance elements and spread more quickly.
- Niche differentiation: aggregation of multiple drug selection pressures within specific populations.
- Importation of MDR strains and geographic source-sink dynamics: MDR strains in source populations may be introduced into other sink populations, where they continue to spread. In clinical practice, widespread use of antimicrobials in the intensive care units and for immunocompromised patients has resulted in the section of MDR organisms.

1.4. There is no ESKAPE?

As reported by Rice[18] and further described by Boucher *et al*.[19], a group of bacteria has emerged that escape the lethal action of antibiotics: vancomycin-resistant ***E****nterococcus faecium*, methicillin-resistant ***S****taphylococcus aureus* (MRSA), extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* and ***K****lebsiella pneumoniae*, carbapenemase-hydrolyzing β-lactamases *K. pneumoniae*, MDR ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa* and ***E****nterobacter* species **(ESKAPE)**. These bacteria are a threat to immunocompromised patients and are primarily, although no longer exclusively, associated with the nosocomial environment[18].

Even though infections caused by resistant Gram-positive bacteria like MRSA and vancomycin-resistant enterococci (VRE) have been partially controlled, it is worth mentioning that rates of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) capable of causing hospital outbreaks and developing multiple resistance, are increasing. Furthermore, glycopeptide-resistant enterococci are of concern because of their ability to transfer resistance to other species. The rare appearance of vancomycin-resistance in MRSA is worrying, but the transfer of resistance to vancomycin to virulent and highly transmissible strains of CA-MRSA would be calamitous[20]. And also the fact that animals are a reservoir of MRSA clonal complex 398, able to colonize and infect humans could undermine existing MRSA control programs[21,22].

Now, the increased burden of **multidrug-resistant Gram-negative bacteria** is a real phenomenon[23]. These bacteria remain an important threat as few antimicrobial agents targeting Gram-negative are reliably active and very little is expected to be available in the near future[24]. The increased dissemination of Gram-negative resistance outside hospitals is attributed to several factors: mobile genes that can readily spread through bacterial populations, human travel and migration[20,25], contact with animals and food products[26,27]. Of particular interest is that much of this dissemination is undetected, with resistant clones **carried in the normal human flora** and only becoming evident when they are the source of endogenous infections[25].

Asymptomatic fecal carriage of **β-lactamase and ESBL-producing enterobacteria** in healthy people has been reported[28,29,30,31]. These facts show that the community is a reservoir of ESBL-producing enterobacteria, which has dramatically increased worldwide, and that might constitute a sustaining source for nosocomial infections and compromise the efficacy of hospital infection-control measures[29]. The growing prevalence of ESBL producers is sufficient to drive a greater reliance on carbapenems. Consequently, there is selection pressure for **carbapenem resistance in *Enterobacteriaceae***, and its emergence is a global public health concern since there are few antibiotics in reserve beyond carbapenems[25,32]. Indeed, it is alarming to confirm the spread of New Delhi metallo-B-lactamase (NDM)-producing enterobacteria in community infections[25,33]. The findings of *Arpin et al.*[34] require special attention since **resistance to colistin**, one of the last-resort drugs against carbapenemase-producing enterobacteria, was found in a strain of NDM- *K. pneumoniae* in a community patient. Other cases of resistance to colistin have also been reported in the community[35]. The possibility that polymyxin resistant, community-acquired strains exist increases the dilemma of treating patients with infections caused by such organisms[35]. These findings only emphasize the **crisis of multi-resistant bacteria** not only in hospitals, but also in the community, and highlight the urgent need for new antimicrobials able deal with these microorganisms.

1.5. Costs of Bacterial Resistance

Antimicrobial resistance results in increased morbidity, mortality, length of hospitalization, evolution of new pathogens, frequency of surgical interventions required to control infection, isolation procedures resulting in **escalating healthcare costs**[36,37,38]**.**

Patients with infections due to antimicrobial resistant organisms have higher costs than do patients with infections due to antimicrobial susceptible organisms[36,38]. Even though hospital studies have shown an association in the order of a 1.3 to 2-fold increase in mortality, morbidity, and cost of patients for resistant versus susceptible infections, its perspective offers only a limited view of the health care impact

of resistance. Indeed, significant portions of clinical care are now provided in rehabilitation facilities in nursing homes, and at home[36].

Furthermore, increasing antimicrobial resistance and MDR have resulted in bigger difficulties in their treatment. Resistance leads to **inappropriate empirical therapy**, delay in starting effective treatment, and the use of less effective, more toxic and more expensive drugs[36,37]. In fact, the factor with the greatest effect on attributable mortality is the efficacy of initial empirical antibiotic therapy[38].

Considering the **patient perspective**, infections caused by resistant bacteria lead to loss of work and family time associated with increased hospitalization time and subsequent recovery, and even an emotional impact[36,37].

1.6. Pharmaceutical industry and antibiotic discovery

Infectious diseases are the second leading cause of death worldwide[39]. In 2004, a report from the World Health Organization (WHO) on "priority medicines for Europe and the World" identified infections caused by resistant bacteria as the number one therapeutic area requiring priority medicines based on the potential public health impact[39]. Therefore, the main rationale for the development of novel antibacterial agents is the emergence and dissemination of resistant strains[40].

1.6.1. The need of new antimicrobials

Since their discovery, antibiotics have served society by saving tens of millions of lives[41]. The use of antibiotics has brought huge changes to the world that we live in, especially our expectations of survival of children into adulthood, the numbers of people that are able to be productive in the workforce, and ability to live to an old age[42]. These drugs are the cornerstone of infectious disease therapy and are the hidden backbone of advanced medical care[43]. Despite all of this, antibiotics are not perceived as essential to health or to the practice of medicine[42] and there is a substantial **gap in the discovery of new antibacterials**, which is responsible for the current lack of newly approved systemic antibacterial agents[43].This might carry terrible consequences in a world where bacteria have developed resistance to all current types of antibiotics.

The number of new antibacterials that make it through the complete development process and ultimately receive the Food and Drug Administration (FDA) approval has precipitously decreased over the past 25 years. Recent reports about the decrease in discovery research efforts in large pharmaceutical companies and the decrease in antibacterial clinical trials, further highlight the diminishing industry focus on antibacterial drug research and development[19].

To better illustrate this, *Freire-Moran et al.*[44] published an interesting review compiling available information about antibacterial agents in clinical development, evaluating their novelty and potential use against MDR. In this study, **66 new active agents** were found, of which 15 could be

systemically administered. Of these agents, 12 were active against Gram-positive, and only **4** had documented activity **against Gram-negative resistant bacteria**. Furthermore, only **2** acted on **new or possibly new targets** and crucially, **none** acted via **new mechanisms of action**. These results clearly show the current paucity of development of active antibacterial agents, especially against the multi-resistant Gram-negative bacteria that are of particular concern.

This reflects the difficulties encountered in identifying new bacterial targets and the possibility that the majority of targets amenable to antibacterial activity have already been identified[44]. Indeed, examination of the current status of potential novel antibacterial drugs indicates that there are only a few compounds in development by the large pharmaceutical companies, with the majority of candidates coming from the smaller biotechnology pharmaceutical companies. The observed trend is a combination of the acquisition of niche products that have not been developed by larger pharmaceutical companies, the exploitation of scientific discoveries not successfully applied to drug discovery by larger pharmaceutical companies and an incremental improvement in an existing class of agents[45].

Novel intravenous and oral drugs to treat both hospitalized and community-based patients are needed, as opposed to “me too” drugs that provide minimal improvement over existing therapies. Priority should be given to antimicrobials with the potential to treat serious infections that are resistant to current antibacterial agents. There is an urgent, **immediate need for new agents** with activity against pan-resistant organisms[19]. A wide range of measures is needed to ensure both: that currently available antibiotics remain effective as long as possible and to promote research in novel drugs and alternatives to traditional antibiotics to combat MDR.

1.6.2. Why have the pharmaceutical companies abandoned antibiotic R&D?

There are several reasons why development of new antimicrobial drugs has stalled in the world. Among them is the difficulty to develop antibiotics targeting pathogens endowed with antimicrobial resistance[2]. Additionally, antibiotic discovery and development has evolved into an expensive, time consuming, cumbersome and bureaucratic process involving multiple interest groups such as pharmaceutical manufacturers, governmental regulatory authorities, patent officers, academic and clinical researchers and trial lawyers[40].

First, a vast underestimation of the implications of antibiotic resistance has in part, contributed to this situation. Since antibiotics were discovered, they have been used excessively and with little attention to the inevitable consequence of resistance, without full appreciation that they are a limited resource that can be renewed only with great difficulty and expense[41].

Another reason is that it is **scientifically challenging** to discover new antibiotics that are active against resistant bacteria of current clinical concern[43]. Unlike most drugs, an antibacterial usually has to be active against many targets (i.e. bacteria), have activity for many infections, with low toxic effects on humans and be effective at different body sites. This is exacerbated with the difficulty of obtaining approval for use when candidate antibacterial wants to be translated into drugs that can be administered to patients. In addition, the FDA has stated that novel antibacterials should demonstrate frank **superiority for approval**. These challenges on the part of regulators is self-defeating in an age of antibacterial drug resistance[40]. To show non-inferiority to the comparator drug, large numbers of patients are needed in clinical trials[42].

However, the main hurdle is **diminished economic incentives**[43], the perceived **lack of return on investments** by pharmaceutical companies with several factors contributing to this problem. Among them, the shift of investment towards the target of chronic diseases, a more cost-effective area than antimicrobials used for short course therapies in most cases[40,42]. Pharmaceutical companies need to recognize that many expensive medicines in their portfolio and in development might be useless if patients succumb to fatal infections. Therefore, their return on investment for products to treat cancer or chronic diseases depends, in part, on effective treatment of infections[42]. Furthermore, the **expense of clinical trials** have increased dramatically, especially for antibacterials. These trials become very expensive as larger numbers of patients are needed to show non-inferiority to the comparator drug[40,42]. Therefore, to achieve reasonable levels of income, it is necessary to demand prices that appear to be excessive[40]. Besides that, the availability of **generic forms** is a clear market pressure likely to result in lower revenues[40].

Moreover, use of antibiotics is associated with **fear of selecting resistance**, calling for **restrictions on their use**. These factors have led to reduced use and hence low return on investment[42]. It is undeniable that the level of antibiotic use is directly related to the emergence and dissemination of resistant strains, but the goal of preventing resistance cannot be elevated above the primary responsibility of treating infected patients[40].

These factors have coalesced to make development of new antimicrobials a largely unattractive prospect for drug companies[2].

1.6.3. What can be done to encourage antibiotic R&D?

Several approaches to overcome these barriers to antibiotic development have been proposed. All stakeholders have a part in this general industry term, including those that discover and develop drugs, those that approve drugs for licensing, prescribe drugs for infections, the patients, and the administrator and/or payers of the cost expenditures for antimicrobial therapy[45].

Most certainly, the regulatory issues relating to the licensing of new antimicrobials are extremely important[42]. Lack of **clear regulatory guidance** is a major disincentive to anti-infective drug development, therefore clarity about both trial design and overall program requirements for the development of new antimicrobial agents is needed[19]. Allowing for smaller sized clinical trials coupled with faster and easier regulatory approvals[40].

Wise et al.[46] stated that changes in the economic field related to antibiotic development are most likely to yield results. Antimicrobial development must allow pharmaceutical companies realistic returns on their investment. This is crucial if the society is to obtain new agents. There should be a rebalancing of risks and, more importantly, benefits. The **price of antibiotics** should be related to their value. Antibiotics are substantially cheaper than many other life-saving drugs such as cancer drugs, which sometimes only extend life for weeks or months, whereas antibiotics can extend life for years[42]. *Wise et al.*[46] suggested that antimicrobials should be at a higher premium.

In the same way, better reimbursement rates are necessary as well as to support pharmaceutical companies' intellectual property. **Extending market exclusivity** could be an incentive through **prolonged patents**[40]. Furthermore, research and development **tax credits** to developers of new infectious diseases products need to be fostered[19]. To make developing antibacterial a viable option for companies, the creation of new incentives for antibiotic discovery and the elimination of disincentives are needed[19].

Thus, efforts to stimulate new antibiotic development must be paired with programs and policies to limit inappropriate use of antibiotics[41]. A shared understanding of antibiotics as precious drugs but with a diminishing activity due to resistance, as well as the creation of favorable environments for discovery of new agents must be fostered. **New economic models** capable of reconciling these goals are imperative[43].

All the initiatives come to the same conclusion: antibiotic resistance and a need for new antibiotics is a global problem and need multistakeholder and multifaceted action[42]. The solutions call for changes in public/social policy and a culture of **cooperation between stakeholders**: one that recognizes that there must be a balance between public health/clinical needs and the commercial realities of drug discovery and development[40].

2. Natural products (NPs) as source of new antibiotics

There is a renewed interest in NPs as a source of chemical diversity and lead generation in drug discovery. The availability of new analytical chemistry and molecular biology methods and the gradual transition away from

the mono-substance therapy towards multi-targeted therapy have resumed research in NPs[47]. The Plant Kingdom for example, represents an enormous reservoir of the most structurally diverse compounds, offering rich mines of biologically valuable molecules. In this respect, **plant secondary metabolites (PSMs)** are proven to be active against a vast number of microbial species, making these molecules an interesting starting point in drug development[48].

2.1. Why NPs as new antibacterials?

There are several reasons why NPs are of main interest in the discovery of drugs as new antibacterials:

- The abundant scaffold diversity of NPs is coupled with a "**purposeful design**". Most microbes and plants make by-products with a purpose, usually to afford advantages for survival in environments threatening their growth. It is reasoned that these defense systems produced would have an inherent antimicrobial activity, giving an evolutionary advantage to the producer organism. In the search for novel antibiotics, it would be difficult to imagine a more specific source of naturally occurring antimicrobials than nature itself[49].
- NPs structures have a high **chemical diversity**, **biochemical specificity** and other molecular properties that make them favorable as lead structures for drug discovery. This serves to differentiate them from libraries of synthetic and combinatorial compounds[50].
- **Suitability** of NPs for modulating chemical reactions and protein interactions: NPs can be viewed as a population of privileged structures selected by evolutionary pressures to interact with a wide variety of proteins and other biological targets for specific purposes[50].
- NPs identified as antibacterial leads typically possess **bacterial permeability**, i.e. access to the target. Hence, avoiding the need for engineering in bacterial membrane and cell wall permeability, a situation often encountered with synthetic leads[49].

Therefore, it seems that the main task in NPs research is to identify and isolate appropriate PSMs from a rich pool provided by the Plant Kingdom. The fact that one of the reasons plants biosynthesize these compounds is the defense against microorganisms justifies the efforts aimed towards finding PSMs with antimicrobial activity[48].

2.2. Which are the shortcomings of NPs?

Despite the above-mentioned advantages, NPs have some limitations that could explain the fact that these mixtures are currently not commercialized as antibacterials:

- NPs library and high-throughput screening (HTS): The heterogeneity of NPs library samples adds two additional levels of **complexity to the screening process**[50]:
    - Once a response for the sample is detected by HTS, one or more rounds of chemical purification and biological assays might be necessary for identifying and isolating the active components in the mixture.

    - The complexity of crude or semi-pure NPs libraries challenges the robustness of HTS technology.
- **Usefulness of *in vitro* data**: Active concentrations in *in vitro* conditions frequently cannot be reached *in vivo* and so *in vitro* results cannot correlate with *in vivo* activities of the tested compounds[48].
- **Therapeutic window**: most of the time therapeutic windows of active PSMs are not specified as they are for other drugs, leaving a risk of appearance of side effects. Toxicity studies, for example towards host cells, have to be carried out. Side effects of NP drugs with synthetic drugs in co-medication must also been studied[48].
- **Variable composition of NP drugs**: relative concentrations of individual components in a sample are not know precisely, and are highly susceptible to external geographical, climatic and ecological factors that influence plant metabolism[48,50].

In such way, biological activity is just one of the necessary prerequisites for NP substances to be applied as pharmaceuticals. Despite of the shortcomings, PSMs are still considered a good starting point in the search for/design/development of new antimicrobial agents[48]. Biological activities of NPs aiming a therapeutic application must also be verified in controlled clinical trials, before they can be submitted as conventional drugs[47].

2.3. Intelligent mixtures

Plant species often respond to stress by increasing the biosynthesis of different classes of molecules, rather than just an individual PSM[48]. In light of the cost-benefit aspect, it is beneficial for a plant to produce mixtures of small molecules made of simple and ubiquitous building blocks that easily diffuse to reach a maximal number of potential targets. **Networks of synergic molecules** could greatly enhance the chance for fitness, closer to a trial and error-type of selection[51].

Mixtures of bioactive compounds are widely claimed to be superior over monosubstances, and a synergistic therapeutic effect might be mainly responsible for this[48]. Thus, a PSM does not need to completely inhibit a target but partially inhibit different targets within a network. Other authors state that multi-target effects predominate over synergistic mechanisms[51]. Of importance is the fact that production of diverse compounds is an evolutionary advantage developed by plants to overcome emerging resistance. It is much harder for microorganisms to shield themselves simultaneously against all these different PSMs. In this way, it is highly likely that the mixture of these compounds is in fact responsible of the onset of their activity[48].

Therefore, in the search for new potent antimicrobials, research should move towards the investigation of combination of substances to achieve efficacy[48]. However, we need to scientifically address the therapeutic potential of NPs. The **reengineering** of botanical drugs would make the area of medicinal plant research more reproducible, and may even lead to the *de novo* engineering of more intelligent mixtures than the ones provided by plants. By doing so, our understanding of pharmacological efficacy would be based on measurable parameters, and also avoid the problem of variable concentrations[51].

Probably the best way of corroborating the pharmacological efficacy of NP drugs would be to **reengineer the mixtures**. By taking apart and reassembling all bioactive constituents, one would be able to find out which ones contribute to the final pharmacological effect either directly (interacting with particular targets) or indirectly (modulating solubility bioavailability)[51].

Mechanisms of synergy in NPs: Based on studies, the following mechanisms can be observed[47]:

- **Synergistic multi-target effects**: The single constituents of a natural blend affect not only one single, but also several targets; cooperating therefore in an agonistic, synergistic way.
- **Pharmacokinetic or physicochemical effects**: Certain components that do not possess specific pharmacological effects themselves may increase the solubility and/or the resorption rate of other constituents; enhancing thereby its bioavailability and resulting in higher effectiveness of the mixture rather than an isolated constituent.
- **Interactions with resistance mechanisms of bacteria:** some PSMs are able to partly or completely suppress bacterial resistance mechanisms working synergistically with antibiotics.
- **The elimination or neutralization of adverse effects by compounds in the mixture**: even if this is not a real synergistic effect, it generates better effectiveness in therapy. It occurs when a constituent of the mixture neutralizes or destroys another possessing toxicity.

2.4. Essential oils (EOs) as antibacterial agents

EOs are PSMs composed of complex mixtures of several components at quite different concentrations. They are characterized by 2 or 3 major components at fairly high concentrations compared to the others. Generally, these major components determine the biological properties of the EOs. The EOs components include 2 groups of distinct biological origin: terpenes and terpenoids; and phenylpropane derivatives[52].

EOs can be present in all plant organs, but are generally stored by the plant in secretory cells, cavities, canals, glandular trichomes or epidermic cells[53]. They do not appear to participate directly in plant growth and development. Instead, EOs play an important role in the **protection of plants** against microbial infection[48].

EOs possess **antibacterial properties** and have been screened worldwide as potential sources of **novel antimicrobial compounds** as alternatives to treat infectious diseases and promote food preservation, and for the fight against resistant bacteria[54].

Mechanisms of action of EOs: The activity of an EO can affect both the external envelope of the cell and the cytoplasm. The antimicrobial activity of EOs, similar to all natural extracts, is dependent on their chemical composition and on the concentration of the components. Generally, the antimicrobial activity is not attributable to an unique mechanism, but is instead a **cascade of reactions** involving the **entire bacterial cell**[53].

- **Targeting the membranes:**

  The **lipophilicity** typical of EOs is responsible for their interaction with bacterial membranes[55]. The hydrophobicity of EOs enable them to partition with lipids of bacterial cell membrane, disturbing the phospholipid bilayers, leading to an **increased permeability**[53,54,56,57].

  This might cause degradation of the cell wall, damage of the cytoplasmic membrane, damage of membrane proteins involved in active transport, inactivation of enzymatic mechanisms like reduction of the intracellular adenosine triphosphate (ATP) pool via decreased ATP synthesis, **leakage of cell contents**, reduction of the proton motive force and membrane potential, disruption of the electron transport system and coagulation of cell contents[53,55,58].

  Furthermore, some EOs can cause a change on the fatty acids profile of the bacterial cell membrane. This is induced by an increase in the percentage of unsaturated fatty acids (UFAs) responsible for the fluidity of the membrane, causing membrane structural alterations[59]. Indeed, Kwon et *al.*[60] showed that EO components caused **elongation and filamentation** in *B. cereus* because septa formation was incomplete. Other effects on bacterial cell morphology, like swelling of bacterial surfaces, have also been reported[61].

- **Other targets**
  - Components of EOs can also act on **cell proteins** embedded in the cytoplasmic membrane. ATPases are located in the cytoplasmic membrane and bordered by lipid molecules. Hence two mechanisms have been suggested whereby EOs components could act: lipophilic hydrocarbons could accumulate in the lipid bilayer distorting the lipid-protein interaction; or a direct interaction of the lipophilic compounds with hydrophobic parts of the protein[55,62]. Components of EOs can directly **bind to proteins** affecting cell division[63] and to **enzymes**[64]. EOs may also affect the enzymes that are involved in fatty acid synthesis[65].
  - EOs can also inhibit bacterial **toxin production**[66].

- Intracellular processes such as **DNA/RNA synthesis** can also be affected[67] as well as **protein expression**[68].
- **Effect on ATP**: EOs disrupt the cell membrane alter the intracellular and external ATP balance such that ATP is lost through the disturbed membrane[69,70]. Other events may contribute to the intracellular ATP decrease like disrupted balance of K^+ and H^+ by EOs[71]and decrease ATP synthesis as mentioned before[53].
- **Effects on the metabolome**: *Picone et al.*[72] found that glucose tends to accumulate when microbial cells are treated with EOs components and that cells are unable to metabolize the glucose, leading to a loss of viability. An important change in the production of bacterial metabolites has also been shown[73].
- **Communication** between bacterial cells involves the production and detection of diffusible signal molecules and it is known as **Quorum Sensing (QS)**. The discovery that many pathogenic bacteria employ QS to regulate their virulence makes this system interesting as target for antimicrobial therapy[74]. EOs components can affect QS in bacteria[75,76].

Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are **several targets in the cell**. Not all of the mechanisms are separate targets; some are affected as a consequence of another mechanism being targeted. Hence, studies have concluded that whole EOs have a greater antibacterial activity than the major components alone, suggesting that the minor components are critical to the activity and many have a **synergistic effect** or potentiating influence[58].

Furthermore, EOs in combination with other antimicrobial can improve antimicrobial effectiveness. EOs could have important implications for the development and implementation of therapeutic antimicrobial strategies[54].

Moreover, the presence of multiple compounds and a possible multiplicity of action in EOs is favorable since drugs that interact with multiple targets are highly desirable because they have a **low likelyhood** for or can **delay development of bacterial resistance**[77,78]. It is likely more difficult for bacteria to develop resistance to the multi-component EOs than to common antibiotics that are often composed of only a single molecular entity[54].

2.5. EOs vs. MDR bacteria

In the fight against MDR strains, multidrug therapy has become of paramount importance[48] and EOs are good sources for combination therapy[79]. In this context, EOs can overcome several mechanisms of bacterial resistance:

- **Receptor or active site modification**: Nicolson *et al.*[80], have shown that the phenolic diterpene totarol potentiated the activity of methicillin against MRSA by significantly reducing the expression of penicillin-binding protein 2a (PBP 2a). This protein, encoded by MecA has reduced affinity for β-lactam antibiotics (BLA).
- **Enzymatic degradation and modification of the drug**: ESBL are enzymes that confer resistance to BLA like third generation cephalosporins. Cinnamaldehyde and eugenol hydrogen bonded with catalytic and other crucial amino acid residues of ESBL enzymes of pathogenic bacteria[81], which may restore BLA activity.
- **Decreased outer membrane (OM) permeability:** The OM of Gram-negative bacteria functions as a permeability barrier for many agents. EOs like thymol and carvacrol are membrane permeabilizers[57] that could enhance penetration of antibiotics.
- **Active efflux**: Efflux pumps are one of the defense mechanisms employed by bacteria to reduce the accumulation of antibiotics inside the cell. EOs were able to block efflux pumps in Gram-negative bacteria[82].

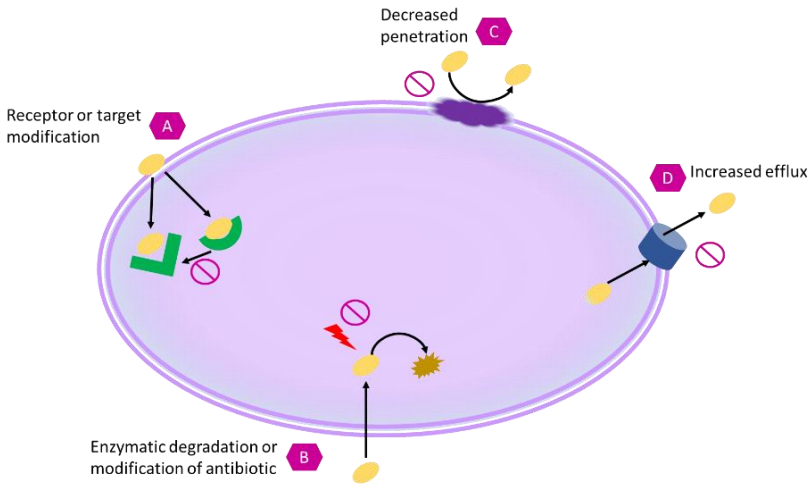

Fig. 3: EOs as modifiers of multidrug resistance mechanisms[79]

3. Inflammatory Bowel Diseases (IBD) and its link to bacteria

Ulcerative colitis (UC) and **Crohn's disease (CD)** are collectively called the IBDs because of such similarities as a **chronic remitting and relapsing course**, their inflammatory nature, and their **unknown causes**[83]. CD manifests itself as a chronic granulomatous inflammation of the gastrointestinal (GI) tract able of affecting its entire length with the presence of "skip" lesions. It preferentially affects the terminal ileum. UC, on the contrary, presents as a continuous inflammatory lesion affecting the rectum and colon, lacking granulomatous characteristics[84].

3.1. Etiology of IBD

The most widely held hypothesis on the pathogenesis of IBD is that they result from an overly of aggressive **immune response** to a subset of **commensal enteric bacteria** in **genetically susceptible** hosts, and **environmental factors** precipitate the onset or reactivation of disease[85].

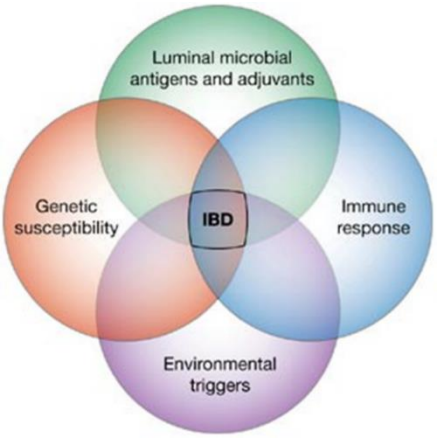

Fig. 4: Multi-factorial etiology of IBD[85]

3.1.1. Genetic factors

More than 100 genes have now been involved in conferring genetic susceptibility to CD. The most important ones are described here:

3.1.1.1. NOD2/CARD15 (Nucleotide-binding oligomerization domain / Caspase recruitment domain)

NOD2/CARD15 is an intracellular receptor responsible for the direct recognition of bacterial peptidoglycan through the binding of muramyl dipeptide (MDP). It is expressed in various cells including monocytes, dendritic cells (DCs), Paneth cells and intestinal epithelial cells (IECs)[86,87]. This was the first gene to be associated with CD. There are three mutations found within the region of NOD2/CARD15 that encodes a leucine-rich repeat, which is responsible for bacterial recognition through binding of MDP present in almost all bacteria. The binding of MDP by dimerized NOD2/CARD15 activates nuclear factor kappa B (NFκB), eliciting pro-inflammatory reactions[86].

Mutation in this region may explain the findings of higher NFkB tissue levels in IBD patients[88]. Mutant NOD2/CARD15 affects the clearance of invasive bacteria that is dependent on NFκB activation[89]. CD patients with NOD2/CARD15 mutations present a decrease in α-defensin production, as this gene is expressed in Paneth cells, the source of secreted antimicrobial peptides[90].

3.1.1.2. ATG16L1 and IRGM (Autophagy-related 16-like 1 and Immunity-related GTPase family M)

Recent genome-wide association studies identified polymorphisms in autophagy genes: ATG16L1 and IRGM as important risk factors for the onset of CD. These genes are critically involved in the innate immune response to invading pathogens. Dysfunction of these molecules results in the increased survival of intracellular bacteria, defective antigen presentation and pro-inflammatory cytokine secretion. Furthermore, levels of these two proteins are significantly decreased in CD patients[91,92].

3.1.1.3. SLC22A4/OCTN1 and SLC22A5/OCTN2 (Soluble carrier family / Organic cation-carnitine transporter)

One of the most important findings in the genetics of IBD is the identification of OCTN1/SLC122A4 and OCTN2/SLC22A5 genes coding for integral membrane proteins. The function of these proteins is multispecific in bidirectional transmembrane transport of carnitine and organic cations[93].

Two functional variants of these transporters have been associated with CD in association with NOD2 mutations[94]. Mutations in SLC22A4 and SLC22A5 affect the transcription and function of these transporters. These variants are most actively expressed in the intestinal epithelium, macrophages and T cells, causing decreased carnitine transport[95].

Carnitine is an essential mediator of fatty acid oxidation. Inhibition of fatty acid oxidation can evoke clinical and pathologic signs of colitis[96] which may explain why impairments in carnitine transport may confer an increased risk for IBD.

3.1.1.4. DLG5 (Drosophilia discs large homolog)

DLG5 is a member of the membrane-associated guanylate kinase (MAGUK) gene family that encodes a scaffolding protein involved in the maintenance of epithelial integrity and regulation of cell growth[97]. The expression of DLG5 variants associated with CD causes increased permeability and disease[93]. A mutation in DLG5 is associated with NOD2 mutation in CD patients[85].

3.1.1.5. HLA (Human leukocyte antigen)

These genes are candidates for a role in the pathogenesis of IBD because their products play a central role in the immune response[93]. Several studies have addressed the possible association between certain HLA polymorphisms and the risk for IBD. The association of UC with HLA genes is stronger than CD[98].

3.1.1.6. MDR-1 (multidrug resistance gene)

This gene encodes a membrane transporter glycoprotein that governs efflux of drugs and possibly xenobiotic compounds from cells. Several documented human polymorphisms having effects on intestinal absorption and drug pharmacokinetics and MDR1 variants have been associated with UC and CD[99].

3.1.2. Immune factors

3.1.2.1. Innate immunity

There is an increase in the number of macrophages and DCs with an activated phenotype in the lamina propria (LP) of both forms of IBD. As well, the production of proinflammatory cytokines, chemokines, adhesion molecules and co-stimulatory molecules is enhanced[85].

Toll-like receptors (TLRs) play a major role both in detecting microbes and in initiating innate immune responses. A disturbance in its function predisposes to infections with Gram-negative pathogens, possibly influencing the advancement of IBD. In IBD, mucosal DCs are activated, express increased levels of TLR2, TLR4 and produce more cytokines[100]. Also alterations in the expression of TLR3 and TLR4 by IECs have been described in IBD, suggesting that abnormal bacterial sensing contributes to disease pathogenesis[101]. Another TLR that binds to and recognizes bacterial DNA, TLR9, may also play an important role in the pathogenesis of IBD. A synergy between TLR9 and NOD2 is lost in the CD patients carrying NOD2 mutation, indicating impairment in the innate immunity[102].

Furthermore, there is a decreased production of α-defensins by ileal paneth cells of CD patients carrying NOD mutations, perhaps leading to an impaired resistance against enteric microorganisms[103]. These facts show a generalized major defects of innate immune responses mediated via pattern recognition receptors in IBD[104].

3.1.2.2. Adaptive immunity

The production of antibodies in the systemic as well as mucosal compartments is drastically increased as a consequence of chronic gut inflammation[105]. Studies suggested that IgG antibodies against a structural protein of colonocytes were selectively produced in UC[106]. However, the focus of immune investigation in IBD shifted to T helper (T_H) cell subsets and their soluble mediators.

In contrast to innate immune responses, which are similarly activated in all forms of IBD, T-cell profiles are disparate in CD and UC.

- **CD:** Traditional T_H1 responses are mediated by interferon gamma (IFN-γ), the production of which is stimulated by interleukin (IL)-12[85]. On the other hand, IL-17

mediates T_H17 responses. The production of this cytokine is stimulated by the production of IL-6, transforming growth factor beta (TGFB) and IL-23[107]. Bacterial colonization stimulates IL-23 expression[108]. The levels of both IL-23[109] and IL-17 are increased in CD tissues[110].

- **UC:** This disease was considered to have a T_H2 profile, but concentrations of IL-4 and IL-5, which are normally elevated in T_H2 responses, have been variable in UC tissues[111].

3.1.2.3. Non-immune cells

It was shown that **IECs** expressed high levels of the class II antigens HLA-DR in actively inflamed mucosa of UC and CD patients[112]. IECs from IBD mucosa fail to induce suppressor T-cells, and instead activate $CD4^+$ T-cells, and thus potentially amplify intestinal inflammation[113].

Fibroblasts are also involved in gut injury because they represent a major source of matrix metalloproteinases (MMPs), a family of proteolytic enzymes directly responsible for tissue destruction during inflammation[114]. Activation of fibroblasts induces upregulation of cell adhesion molecules and production of chemokines, inducing migration of T-cells through local microvascular cells[115].

Human intestinal **microvascular endothelial cells** (HIMEC) from CD and UC mucosa exhibit higher cytokine-mediated leukocyte binding capacity[116], secondary to their chronic exposure to the inflammatory milieu of the IBD mucosa[117].

Platelets trigger an inflammatory response in the microvasculature of IBD patients[118] thus linking this cell type to process of IBD pathogenesis.

3.1.3. Environmental factors

A variety of environmental factors are considered risk factors for IBD, including smoking, diet, drugs, geography, social status, stress and appendectomy[119]. Although the epidemiological evidence linking environmental factors to IBD is fairly solid, it is widely believed that no environmental factor alone can directly cause CD or UC, and an intrinsic disease predisposition must also be present[104]. The mechanisms by which these factors initiate the onset of disease or reactive quiescent IBD are not well understood. From a broad perspective, these triggering factors alter mucosal barrier integrity, immune responses, or the luminal microenvironment, each of which have an impact on susceptibility to inflammation[85].

Because **dietary antigens** are, next to bacterial antigens, the most common type of luminal antigen, it is logical to surmise that diet might play a role in the expression of IBD[120]. Several studies showed the association between sugar intake and CD[121] as well as monounsaturated and polyunsaturated fats in both CD and UC[122,123]. Dietary additives such as **aluminum and iron** have a well-described adjuvant activity and stimulate bacterial virulence[124]. **Smoking** is perhaps the most thoroughly documented environmental contributor to IBD, but its opposite effect on CD and UC is difficult to understand. In contrast to UC, several studies have implicated cigarette smoking as a risk factor for CD[125]. **Appendectomy** might reduce the risk of UC but increase the risk of CD[120,126]. Some studies have suggested a dose response, with higher risks for IBD seen among longtime users of **oral contraceptives** or among users of high estrogen preparations[127]. It has been proposed that the expression of IBD may be influenced by events in **early childhood**, such as mode of feeding, domestic hygiene, or perinatal infections[120]. A history of frequent childhood infections or exposure to antibiotics has also been proposed as a risk factor for IBD[128].

3.1.4. Bacterial factors

The intestinal microbiota play a crucial role in IBD, it participates in the initiation, maintenance, and determination of the phenotype of IBD[129]. The complex microbiota of the distal ileum and colon provide a source of adjuvants or antigens triggering immunological responses causing chronic intestinal injury[85,130]:

- **As adjuvants** (lipopolysaccharides, peptidoglycan, flagellin) they activate innate immune responses including DCs and other antigen presenting cells (APCs)[129].
- **As antigens** they stimulate the expansion of T cells, which are stimulated by APCs selectively recognized by their receptor[129].

3.1.4.1. Evidence of the microbial influences in chronic mediated intestinal inflammation

An important role for microbial agents in the pathogenesis of IBD has been reported both, in IBD patients and in animal studies. This evidence is summarized in Table 1.[131]:

Table 1: Evidence of microbial influences in intestinal inflammation[131]

Clinical Evidence	Experimental Evidence
• IBDs are localized in intestinal segments with the highest bacterial concentrations. • Abnormal microbial composition in IBD • Altered metabolic activity of microbiota in IBD • Increased mucosal-associated bacteria in IBD patients • Serologic and T-cell responses to enteric microbial antigens in IBD patients • Enhanced *E. coli* virulence in CD • Enhanced mucosal invasion and translocation in IBD • Polymorphisms of genes that encode bacterial receptors and processing in CD • Fecal stream diversion prevents and treats CD, inflammation recurs upon restoration fecal flow • Antibiotics treat CD • Probiotics prevent relapse of UC	• Luminal commensal bacteria are required for chronic inflammation in most rodent models • Systemic immunoregulatory defects can have a colonic phenotype • Bacterial antigens stimulate pathogenic T-cell responses • Increased mucosal association and translocation of bacteria • Broad spectrum antibiotics, probiotics, and prebiotics prevent and treat disease • Defective bacterial signaling in epithelial cells induce chronic, immune-mediated intestinal inflammation • Defective immunoregulation, mucosal barrier function, or bacterial killing induce chronic, immune-mediated intestinal inflammation or potentiate acute mucosa injury

3.1.4.2. Role of microbes in the pathogenesis of IBD

Four broad mechanisms explain the complex relationship between microbiota and IBD[129]:

- **Dysbiosis of conventional microbiota**

  Increased bacterial concentrations in the ileum and colon of IBD patients revealed a direct relationship between bacterial numbers and the severity of the disease[132].

  - **Changes in composition:** There is an altered composition of the mucosally associated and fecal microbiota in IBD patients. Most studies have shown a decreased microbial diversity in active IBD, increased numbers of *Enterobacteriaceae*, including *E. coli*, and decreased *Firmicutes*, including *Clostridium* species[132,133,134].
  - **Metabolic consequences:** Altered composition of luminal microbiota can lead to important metabolic changes in the intestinal environment. Enteric bacteria metabolize dietary constituents to produce both beneficial and detrimental products. For example, reduced levels of *Clostridium*, responsible for the production of shorty-chain fatty acids (SCFA), determined reduced levels of

butyrate in IBD patients. These SCFA are important source of energy of colonic epithelial cells[135,136].

- **Induction of intestinal inflammation by pathogens and functionally altered commensal bacteria**
  - *Mycobacterium avium* subspecies *paratuberculosis* **(MAP)**: CD has been associated with Johne's disease in cattle caused by MAP, an spontaneous granulomatous enterocolitis[137]. Proposed mechanisms of transmission include infected milk, meat and water[131]. MAP is an intracellular pathogen that evades killing within phagolysosomes[138] and can infect hosts with defective innate immune killing, such as NOD2 polymorphisms[139]. Indeed, Feller *et al*. [140] showed the specific association between MAP and CD in a systematic analysis.
  - Functional changes in commensal bacteria: High prevalence of adherent/invasive *E. coli* **(AIEC)** in genetically susceptible CD patients support the hypothesis that this disease can result from AIEC abnormally colonizing of the gastrointestinal tract and parasitizing IECs and macrophages[141]. Enterotoxins from colonic commensal bacteria like *Clostridium difficile*[142] and *Bacteroides fragilis* can induce intestinal inflammation[143]. Furthermore, *C. difficile* infection is increased in IBD patients[144].

- **Host genetic defects in containing commensal bacteria**

  As already mentioned, host disturbances that affect innate immunity to microbial agents as NOD2 mutations and TLR altered expression, can contribute to dysbiosis in IBD patients. Furthermore, mutations in autophagy results in increased intracellular bacterial survival.

  Another susceptibility factor for CD are mutations on the neutrophil cytosolic factor (NCF) 4, a gene that encodes an oxidase that is responsible for the generation of reactive oxygen species in response to bacterial infections, resulting in altered killing of phagocytosed bacteria[145].

  These defects result in altered antigen presentation, reduced luminal and intracellular bacterial killing, leading to increased bacterial persistence in gut mucosa and dysbiosis[129].

- **Defective bacterial host immunoregulation**
  - **Mucosal barrier defects:** IBD is characterized by enhanced mucosal permeability. Defects in mucosal barrier exclusion causes permeation of pro-inflammatory

molecules, such as bacteria, toxins, antigens, from the luminal environment and enhancing inflammatory responses[146].

- **Defective microbial killing:** CD patients present defective microbial killing[147], as well as defective antimicrobial peptide production[148] resulting in abnormalities in killing bacteria. Moreover, NOD2 polymorphisms are associated with decreased defensing production by Paneth cells[90] and defective clearance of intracellular pathogens by colonic epithelial cells[149].
- **Defective suppressor cell activation:** IBD patients present serologic responses to a wide variety of microbial antigens[150,151] and fail to develop oral tolerance[152]. Molecular mimicry between microbial and host antigens has been reported, determining autoimmune responses by cross-reactive immunity[153].

All these mechanisms have a common theme: all increase the exposure of bacterial antigens to effector T cells and innate immune cells resident in the intestinal mucosa and/or alter the host immune response to commensal bacteria[129].

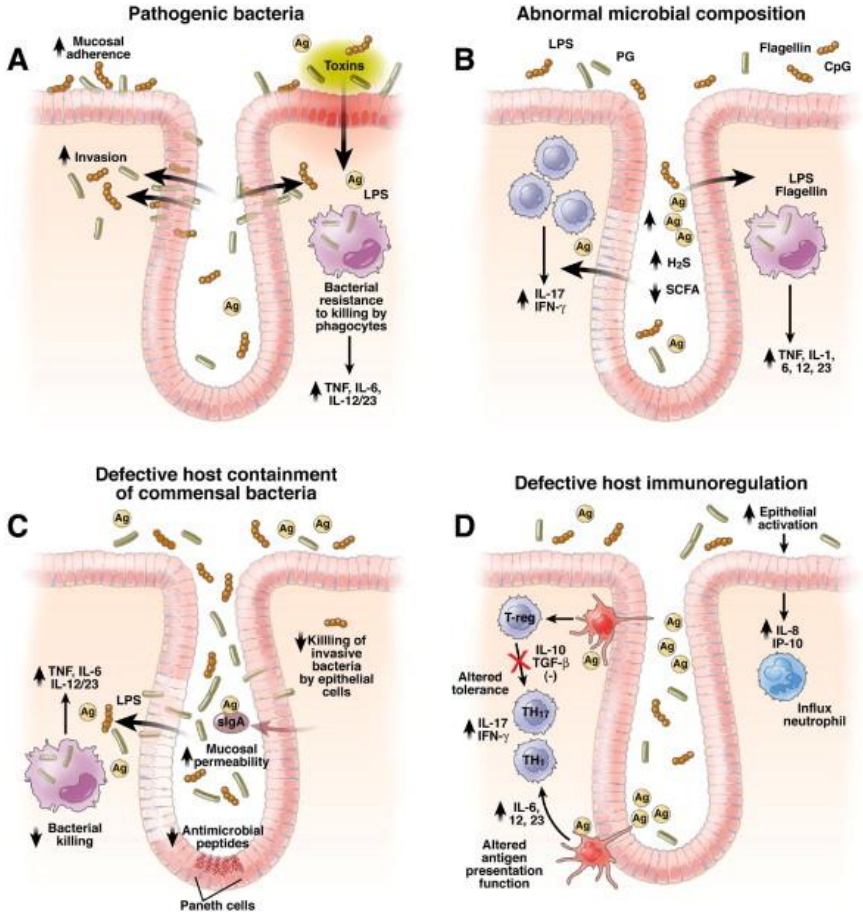

Fig. 5: Role of microbes in IBD pathogenesis[131]

Ag: antigen; LPS: lipopolysaccharide; PG: peptidoglycan; CpG: oligodeoxynucleotides; SCFA: short-chain fatty acid; sIgA: immunoglobulin A; IP-10: Inducible protein 10

3.2. Treatment of IBD

As curing is never obtained, the main goals of therapy in patients with IBD are **to induce a clinical remission** and then **maintain that remission** over time. Despite scientific advances, treatment for most patients with IBD continues to rely upon a **stepwise therapeutic approach**: using less potent, but safer medications for milder disease and moving to progressively more potent, but potentially toxic, medications as the diseases worsens. In this "step-up" approach, treatment for more patients begins with 5-aminosalicylates and antibiotics, next moves to corticosteroids and immunomodulators and finally progresses to anti-tumor necrosis factor therapy and surgical intervention[154].

3.2.1. Anti-inflammatory and immunomodulatory drugs

3.2.1.1. 5-Aminosalicylates (5-ASA)

5-ASA remains a mainstay of treating active UC, it is effective for maintenance[155] and induction therapy in UC[156]. Even though existing data show little benefit for 5-ASA, sulfasalazine presented a modest efficacy for the treatment of active CD[157] and controlled-release of mesalamine was effective as single agent in the treatment of active CD of ileum and colon[158].

3.2.1.2. Corticosteroids

Corticosteroids are effective in CD and UC for inducing remission[159]. Even if adverse effects for corticosteroids are reported and they are associated with presence of infections, they have remained a mainstay of therapy in both CD and UC[160]. In this regard, in a phase III trial, budesonide in a controlled release formulation induced remission in patients with active UC minimizing systemic side effects[161].

3.2.1.3. Thiopurines

Thiopurine analogs azathioprine and 6-mercaptopurine, may prevent relapse in quiescent UC and CD. These agents may therefore be useful steroid-sparing agents and as an adjunct to biological therapies[162]. The use of thiopurines further increased with the advent of antibody tumor necrosis factor therapy, as they have been shown to enhance the efficacy of this biological therapy either through complementary immunomodulatory mechanisms or by reducing the likelihood of forming neutralizing antibodies to these agents[160].

3.2.1.4. Methotrexate

Like thiopurines, this agent had been used in other immune disease before it was routinely used or studied in IBD. In a group of patients with chronically active CD, methotrexate was effective inducing remission and reducing requirements for prednisone[163]. In patients with CD

who enter remission after treatment with methotrexate, a low dose of this agent maintains remission[164].

3.2.1.5. Calcineurin inhibitors

Cyclosporine therapy was rapidly effective for patients with severe corticosteroid-resistant UC[165]. Moreover, cyclosporine has a beneficial therapeutic effect in patients with active chronic CD and resistance to or intolerance of corticosteroids[166]. However, cyclosporine is associated with adverse effects, and is mostly used as a bridge to thiopurine therapy considering the renal toxicity with long-term use. In this respect, infliximab has become a favored option over calcineurin inhibitors in severely active UC[160].

3.2.1.6. Anti-tumor necrosis factor alpha (anti-TNFα)

The pro-inflammatory cytokine TNF plays a key role in chronic intestinal inflammation found in IBD. Accordingly, the most efficient biological agents developed so far in IBD aimed at neutralizing TNF[167]. Anti-TNF agents have been a major advance in the management of acutely ill or corticosteroid-dependent individuals with CD or UC[160].

Infliximab is a chimeric anti-TNFα monoclonal antibody that binds to TNFα with high affinity, thereby neutralizing its biological activity. Patients with CD who respond to an initial dose of infliximab are more likely to be in remission, discontinue corticosteroids, and maintain their response for a longer period of time[168]. As well, infliximab was effective in moderate to severe active UC patients in achieving clinical remission and corticosteroid-sparing effects[169]. However, infliximab is immunogenic, and intermittent administration results in the development of human antichimeric antibodies, that lead to infusion reactions, loss of efficacy, and delayed hypersensitivity reactions[170].

Adalimumab is a recombinant human monoclonal antibody that binds with high affinity and specificity to human TNFα and is frequently less immunogenic than chimeric monoclonal antibodies. In CD patients, adalimumab was well tolerated and induced remission[171], maintenance and steroid sparing effects[172]. This agent is also effective in UC, inducing remission with a safety profile[173].

One of the major problems with anti-TNF therapy is the need for alternate therapy among initial responders, secondary to loss of response or intolerance. Individuals who lose response to one anti-TNFα agent may respond to a second, although at a lower response rate. Although anti-TNFα agents are quite effective and reasonably safe, they are not effective in up to one-third of individuals receiving this therapy, and they are quite expensive[160].

3.2.1.7. Anti-adhesion molecules

TNF antagonists, although efficacious, predispose patients to serious infection[174], showing the need of safer therapies. The α4β7 integrin is a cell-surface glycoprotein expressed on circulating T and B lymphocytes that interacts with a mucosal cell adhesion molecule on intestinal vasculature. **Vedolizumab**, a humanized monoclonal antibody that specifically recognizes the α4β7 heterodimer, is proposed as a therapy that selectively blocks gut lymphocyte trafficking. Vedolizumab is effective as both induction and maintenance therapies for patients with moderately to severely active UC[175]. As well, patients with moderately to severely active CD who were treated with vedolizumab were more likely to induce and maintained remission[176].

3.2.1.8. Agents in the pipeline

- **Anti-adhesion molecules**

  Etrolizumab is a humanized monoclonal antibody that selectively binds the β7 subunit of the heterodimeric integrins α4β7 and αEβ7. It led to remission in patients with moderately to severely active UC who had no responded to conventional therapy[177]. Phase III trials of this agent are ongoing[167].

- **Blockade of cytokines and signaling pathways**

  - **IL-12/IL-23:** IL-12 and IL-23 are inflammatory cytokines implicated in CD. **Ustekinumab** is a human monoclonal antibody against both cytokines. This agent was effective in maintenance therapy and increasing rates of remission in CD patients[178]. Interestingly, patients with moderate to severe CD that were resistant to TNF antagonists had an increased rate of response to induction with ustekinumab and remission in maintenance therapy[179]. This molecule, approved for the treatment of psoriasis by the FDA, is in a phase II trial[167].
  - **IL-6:** IL-6 is a pleiotropic cytokine with central roles in immune regulation and inflammation. IL-6 and its receptor are increased in both serum and intestinal tissue of CD patients[180]. Humanized anti-IL-6 receptor monoclonal antibody was well tolerated and normalized the acute-phase responses in active CD[181]. A phase II clinical trial is ongoing[167].
  - **IL-13:** IL-13 has been implicated as a key driver of UC. **Tralokinumab** is an IL-13 neutralizing antibody. Tralokinumab did not significantly improve clinical response in UC patients but induced higher remission rates than placebo. This

suggests a possible therapeutic effect of tralokinumab that requires further study[182].

- **JAK inhibitors:** The involvement of Janus kinase (JAK) 1 and JAK3 in the transduction processes of the receptors of several cytokines, has made JAK inhibition a potential therapeutic target in IBD. **Tofacitinib** inhibits JAK family members by blocking inflammation, interfering with several cytokine receptors[160]. Patients with moderately to severely UC treated with tofacitinib were more likely to have clinical response and remission[183]. A phase III trial is ongoing[167].

3.2.2. Microbial modulatory drugs

As mentioned before, there is evidence that components of the intestinal microbiota contribute to the pathogenesis of IBD. These facts provide the rationale for selective therapeutic manipulation of the enteric bacterial population.

3.2.2.1. Antibiotics

Antibiotics as primary or adjuvant treatment of IBD have been largely studied. There is a therapeutic rationale for using antibiotics active against enteric bacteria, and the mechanisms of action proposed are[184]:

- Decrease **luminal** and **adherent mucosal bacterial** concentrations
- Selectively eliminate **detrimental luminal bacterial** subsets (alter the ratio of beneficial to aggressive commensal bacteria)
- Decrease **tissue invasion**, treat micro-abscesses and secondary bacterial proliferation adjacent to mucosal ulcers and fistulas
- Decrease bacterial **translocation** and systemic dissemination of viable bacteria

Many antibiotics and their combinations have been evaluated in IBD. Conclusions about their effectiveness against CD and UC are difficult to state as current data are conflicting. In this regard, Khan *et al.*[185] conducted the first comprehensive systematic meta-analysis looking at all antibacterial therapies in IBD. Only well-defined controlled assays were considered, demonstrating that antibiotics are effective for:

- **Active CD**, especially rifamycin derivatives either alone or in combination with other antibiotics, inducing remission in active CD.
- **Perianal CD fistula**, reducing fistula drainage, using either ciprofloxacin or metronidazole.
- **Quiescent CD**, preventing relapse especially for combinations including antimycobacterials

- **Active UC**, different single or combination of antibiotics, inducing remission.

However, given the variety of antibiotics tested in the trials, it is not possible to recommend a specific antibiotic therapy for IBD. The only exception to this is the use of either ciprofloxacin or metronidazole for treating CD perianal fistulas[185].

Post-operatively, metronidazole[186] and ornidazole[187] were effective preventing CD recurrence, showing the efficacy of nitroimidazoles after surgical resection. Furthermore, concomitant use of azathioprine and metronidazole resulted in lower post-surgical recurrences and favorable outcomes. Thus, this combined treatment seems to be recommendable for operated CD patients with enhanced risk for recurrence[188].

Therefore, it is clear that altering gut microbial flora may have a role in modulating IBD activity. Further studies are needed to determine if antibiotics are having an effect acting on one bacterial species or changing the composition of gut microbiome in general. For that, analysis of **changes in intestinal microbiota** should be included in trials of antibiotic therapy to explore what effects these interventions are having on gut flora[162].

As suggested by *Pineton de Chambrun et al.*[189]*,* **antibiotic therapy in IBD** needs to be **personalized**. Patients need to be stratified according to pathological type, bacteriological and genetic markers. At the same time, antibiotics need to be selected based on what effect on microbiota is expected (reduction of total loads or specific pathogens), as well as evaluated for their use alone or in combination therapies (with immunomodulators). Additionally, well-established endpoints as markers of the therapy efficacy are needed. Thus, selecting the adequate antibiotic for the right type of patient may lead to better results.

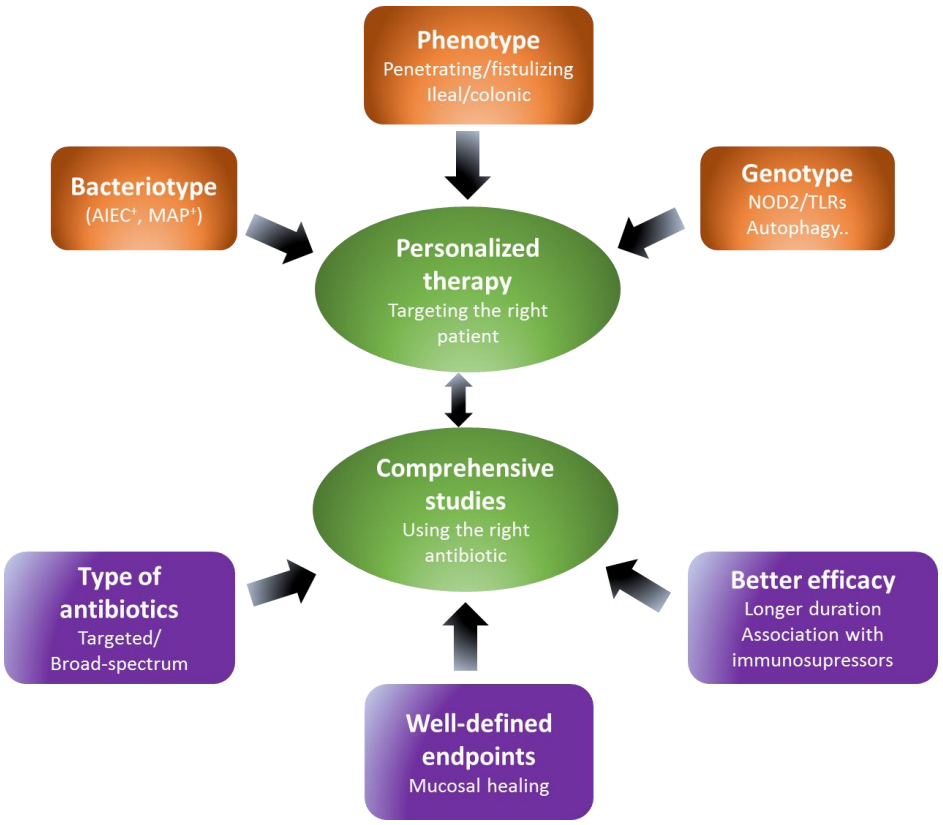

Fig. 6: New strategies for the use of antibiotics in IBD[189]

3.2.2.2. Probiotics

Probiotics are **viable microorganisms** with beneficial physiologic or therapeutic activities, originally derived from cultured foods, especially milk products[184]. Various species and bacterial strains that have been used in IBD clinical trials, are believed to have a potential beneficial role. The most evaluated probiotics are *E. coli Nissle*, *VSL #3* (mixture of 4 strains of *Lactobacillus*, 3 strains of *Bifidobacterium* and one strain of *Streptococcus salivarius thermophilus*), BIO-three (mixture of *S. faecalis, Clostridum butyricum, Bacillus mesentericus*), a mixture of *L. rhammosus* and *L. reuteri*, strains of *Bifidobacterium brevis, Bifidobacterium bifidum*, *L. acidophilus* and *Saccharomyces boulardii*[190].

Probiotic supplementation can **reestablish intestinal bacterial homeostasis** and downregulate gut inflammation characteristic of IBD patients, thus modulating the inflammatory/anti-inflammatory balance. Indeed, administration of probiotics can normalize altered intestinal microbiota in IBD patients, and **increase protective species by reducing the pathogen load**, positively affecting intestinal permeability, balancing local immune response, producing beneficial substances and disintegrating pathogenic antigens in the intestinal lumen[191].

In **UC** patients probiotics showed promising results: In patients not responding to conventional therapy, VSL #3 resulted in induction of remission with no adverse events[192]. Administration

of BIO-three improved the clinical symptoms and endoscopic findings[193]. *Lactobacillus* GG was effective and safe for maintaining remission and was more effective than standard treatment with mesalazine in prolonging the relapse-free time[194]. *E. coli Nissle* was as effective as 5-ASA in preventing relapse[195] and in maintenance therapy[195,196].

On the other hand, trials with the use of probiotics in **CD** are less concordant than in UC. There is insufficient evidence to make conclusions about the efficacy of probiotics for induction of remission in CD[197]. Results of trials are mixed: randomized controlled trials showed that *Lactobacillus johnsonii* did not prevent endoscopic recurrence in operated patients[198,199]. *Lactobacillus GG* neither prevented endoscopic recurrence at one year nor reduced the severity of recurrent lesions in operated patients[200]. On the contrary, *E. coli Nissle* reduced the risk of relapse and minimized the need of glucocorticoids[201]. *Guandalini*[202] reported benefit of *Lactobacillus GG* in pediatric patients, and *Saccharomyces boulardii* may represent a useful tool in maintenance therapy[203].

However, when the intestinal epithelial layer is inflamed like in IBD, it is important for probiotics to be rationally tested, to avoid causing harm. In fact, probiotics should only be contemplated if the integrity of the gastrointestinal tract is not severely compromised[160]. More recently, genetically engineered bacteria that secrete immunosuppressive substances such as IL-10 have been studied[204].

3.2.2.3. Prebiotics

Prebiotics are dietary supplementations, usually **non-digestible selectively fermented glucides**, which are **energy substrates** for protective intestinal organisms. They can stimulate for example *Lactobacillus* and *bifidobacterium* growth, thus inducing increasing bacterial fermentation resulting in SCFA production, in particular butyrate. Consequently, prebiotics promote **inhibition of harmful species** by decreasing the luminal pH, improve epithelial barrier function, reduce epithelial adhesion and produce bactericidal molecules[190]. The net result of prebiotic administration is functionally equivalent to administering probiotic bacteria[184].

Fructo-oligosaccharide **(FOS)** therapy was studied against **CD**. FOS treatment was well tolerated, led to a significant improvement in disease activity and increased fecal bifidobacteria concentration. The percentage of DCs producing IL-10, an anti-inflammatory cytokine was increased[205]. However, a randomized placebo-controlled trial showed no clinical benefit of FOS in inducing remission, despite impacting on DC function. This suggests that further studies of the effects of FOS perhaps in maintaining disease remission or in preventing the onset, are needed[206].

Germinated barley foodstuff **(GBF)**, which is derived from malt, consists mainly of dietary fiber and glutamine rich protein. GBF is a prebiotic foodstuff that effectively increases luminal butyrate production by stimulating the growth of protective bacteria[207]. Studies of GBF administration in **UC** patients have been carried out. After 4 weeks of GBF treatment, no side effects were observed and there was a decrease in clinical activity and increased fecal concentrations of *Bificobacterium* and *Eubacterium limosum*[208]. GBF was also effective in long-term therapy[209]. This suggests that GBF may have the potency to reduce clinical activity of UC, supporting its administration as a new adjunct therapy[208].

3.2.2.4. Enteral nutrition

Enteral nutritional (EN) therapy using **liquid formulas** (elemental, semi-elemental or polymeric) is often used in the management of **pediatric CD**. It can be administered in two ways: an exclusive enteral nutrition **(EEN)** as the sole dietary source, being used as primary medical therapy to induce remission; and as partial enteral nutrition **(PEN)** given in addition to a normal diet, with the primary goal to improve nutritional status or to maintain remission[210]. Clinical guidelines recommend EEN if a patient declines drug therapy or as an adjunctive therapy to support nutrition, rather than as a primary therapy[211].

EN has been shown to exert both a **change in gut microflora** and an anti-inflammatory effect. Hypotheses about the mechanism of action of EN include: elimination of dietary antigen uptake, correction of intestinal permeability, diminution of intestinal synthesis of inflammatory mediators via reduction in dietary fat and provision of important micronutrients to the diseased intestine[210,212].

EEN is well-established as a first line therapy **instead of corticosteroids** to treat active **CD in children**[211]. It has been shown that there is no difference in efficacy between EN and corticosteroid therapy in children. The absence of steroids side effects could make EN a better choice for first-line therapy in children[213]. Children treated with EEN alone had remission and modification of the fecal microflora. However, variations in bacterial species required time to achieve stability[214]. PEN can instead, be useful in the maintenance of remission and prevention of relapse, delaying the requirement for further therapy[210].

There is also evidence to support a possible role of EEN for adult CD patients, with newly diagnosed disease and ileal involvement[211]. *O'Morain*[215] demonstrated the benefit of EN on the induction of remission in adult CD patients, as a safer therapy than corticoids.

3.2.2.5. Fecal microbiota transplantation (FMT)

FMT is the **transfer of gut microbiota from a healthy donor**, via infusion of a liquid stool suspension, to restore the gut microbiota of a diseased individual. The objective of FMT is to introduce a complete, **stable community of gut microorganisms**, which are aimed at repairing or replacing the disrupted native microbiota of a patient[216,217]. FMT has recently become prominent as a treatment for refractory and recurrent *C. difficile* infection. Whilst the available evidence is limited, FMT could be an effective and safe treatment for IBD, at least when standard treatments have failed. However, controlled trials are still required to confirm its efficacy[217].

In a large pilot study, FMT was effective in patients with **refractory CD.** These results demonstrated that FMT through patient's mid-gut might be a safe, feasible, and efficient rescue therapy for refractory CD[218]. Concerning **UC**, a controlled trial showed that FMT through enema administration induced remission in active UC[219]. Nonetheless, long-term follow-up is still lacking.

Temporal fecal microbiota analysis represent an effective option for monitoring colonization efficacy and assessing FMT therapy success. In this respect microbiota of UC patients after FMT was analyzed. Results showed that healthy bacterial communities such as *Bacteroidetes, Firmicutes*, and *Verrucomicrobia* were decreased and enterobacteria were abundant. In some cases, there was a major shift in patient microbiota towards the donor microbiota that was transient, in other, this was maintained several weeks. These findings suggested that the **colonization of donor microbiota is a gradual process** of sequential establishment of individual organisms, and that UC patients in remission via conventional medical therapy or those with mild disease might be more likely candidates for future FMT trials[220].

Finally, there is no currently cure for IBDs, these diseases can persist sometimes even after surgery. Thus IBD patients will typically need treatment throughout their lives. However, understanding the causes of these pathologies will help to find a cure.

4. Colonic-targeted delivery of drugs

There is an increasing interest in developing oral dosage forms that effect primary drug release to the colon. Colonic-targeted drug delivery is of crucial importance for drugs that are unstable or unabsorbed in the upper GI tract, drugs that require a delay in absorption and those required for the **treatment of colonic pathologies**[221]. Therefore, in the **treatment of IBD**, oral forms providing a colonic drug delivery are of relevance.

Furthermore, for local colonic pathologies like IBD, a colon targeted approach not only **increases the bioavailability of the drug at the site of action**, but also reduces the amount of drug needed to be administered resulting in **decreased side effects**[221,222]**.**

For this purpose, oral dosage forms aiming a colonic drug delivery have to overcome many physiological barriers. **Absorption or degradation** of the drug in the **upper part of the GI tract** is one of the major obstacles that has to be circumvented for successful colonic drug delivery. For this, oral forms that can respond to the physiological changes in the colon to trigger the release of the drug in this site of action are needed[221,223]. There are several approaches to target drugs into the colon:

4.1. pH-dependent

pH dependent colonic targeted oral formulations should able to withstand the lower pH values of the stomach and of the proximal part of the small intestine, and to **disintegrate at neutral or slightly alkaline pH** in order to deliver the drug at the terminal ileum and colon[224,225].

The most commonly used pH-dependent polymers for coating of oral dosage forms are **methacrylic acid copolymers**, commonly known as **Eudragit®**. In this respect, Eudragit® L100-55, L100 and S100 which dissolve at pH 5.5, 6.0 and 7.0 respectively have been widely used[226,227]. Commercialized mesalazine tablets coated with Eudragit® L-100 available as Claversal, Salofalk, Mesasal and Rowasa. As well, Eudragit® S-100 mesalazine tablets are marketed as Asacol. These tablets release the drug at the terminal ileum and beyond for topical inflammatory action in the colon[225].

However, GI fluids might pass through the polymeric coating while the dosage form transits the small intestine, leading to premature drug release in the upper parts of the GI tract. Furthermore, failure of the coating to dissolve may also occur when the pH of the colon drops below normal in patients with IBD[225].

Coating formulations consisting of a **combination of pH-dependent polymers** have been used to overcome the issue of high GI pH variability among individuals[226]. Eudragit® L and Eudragit® S have frequently been mixed with each other in differing weight ratios in an attempt to fine-tune the overall dissolution performance of the coat[227]. There are also formulations based on the **combination of pH-dependent and pH-independent polymers**. Eudragit® RS and RL are copolymers of acrylic and methacrylic acid esters, which contain a low level of quaternary ammonium groups. Both copolymers are insoluble in water but they hydrate in GI fluids independent of pH[225]. The use of these insoluble materials combined with pH-dependent polymers, would result in a further delayed and slower release of the drug, acting as a pH-controlled pore formers[227].

Although polymethacrylates have most widely been employed as pH-dependent coating agents for colon delivery, the use of other polymers, such as hydroxypropyl methylcellulose acetate succinate **(HPMCAS)**, cellulose acetate phthalate **(CAP)**[228] and **shellac**[229], a pharmaceutically used resin of animal origin, was also investigated. These polymers possess a pH threshold of around 6.7; 5.8 and 7.3, respectively.

Determinants of luminal pH in the colon include mucosal bicarbonate and lactate production, bacterial fermentation of carbohydrates, mucosal absorption of SCFA, and possibly intestinal transit. Alterations in these factors as a result of mucosal disease and changes in diet, likely explain abnormal pH measurement in IBD[230]. Therefore, pH-sensitive polymers are not completely suitable for colon targeted drug delivery systems due to **poor site specificity**[231,232]. These issues have prompted the development of other types of delivery systems[225].

Some examples of pH-dependent technologies are described below:

Multi-matrix system (MMX™): This technology permits a sustained and homogeneous release of 5-ASA throughout the entire colon. It combines an outer pH-dependent polymethacrylate coat comprising a blend of Eudragit® S and L and an inner tablet core containing 5-ASA dispersed within a network of hydrophilic and lipophilic matrices made of carmellose sodium, carnauba wax and stearic acid. Once the outer coat dissolves at pH ≥7, normally in the terminal ileum, the interaction of intestinal fluids with the hydrophilic matrix causes the tablet core to swell and form a viscous gel mass that slows the diffusion of 5-ASA into the colonic lumen. Small pieces of the gel mass gradually break away in the colon, releasing 5-ASA in proximity to the colonic mucosa. The lipophilic matrix further slows penetration of intestinal fluids into the tablet core, reducing the rate of dissolution and thereby prolonging therapeutic activity[233,234].

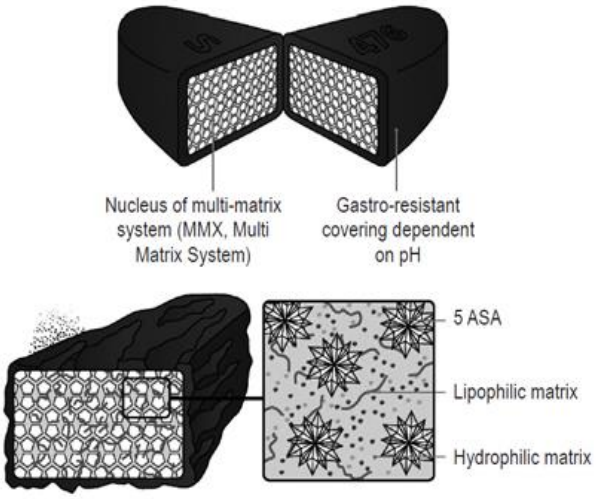

Fig. 7: Structure of the MMX ™ system[233]

Targit™: This technology is based on the application of pH-sensitive coating made of a mixture of Eudragit® S and L, onto injection-molded starch capsules. The blend of pH-sensitive polymers in Targit™ is specifically chosen to start dissolving at a relatively low pH to avoid the potential drawbacks associated with polymers that do not dissolve until a high pH is reached that is, incomplete or delayed disintegration. Due to the construction of Targit™, in which the walls of the starch capsule separate the enteric coating from the capsule contents, dissolution of the coating is essentially independent of the capsule fill. This avoids the need to reformulate the coating when changing from one drug compound or formulation to another[235].

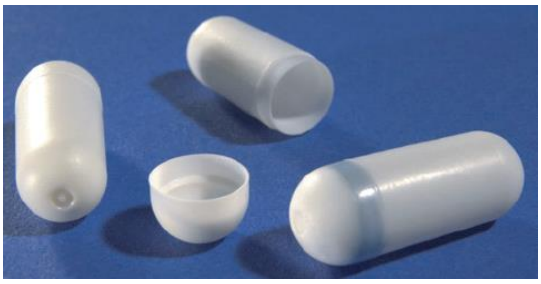

Fig. 8: Starch TARGIT™capsule component parts (lid and body) and assembled to form capsule [235]

Colopulse®: The tablet coating of the Colopulse® technology consists of an Eudragit® S film with dispersed croscarmellose, in a non-percolating mode, as a super-disintegrant [236,237].Release from the coated ColoPulse® system is triggered by the variation in the GI pH in the terminal ileum and occurs at pH >7. It differs from other pH responsive systems because of the non-percolating incorporation of a disintegrant in the coating, yielding a highly reliable and pulsatile release pattern in the targeted region[238]. The mechanisms are explained in Figure 9.

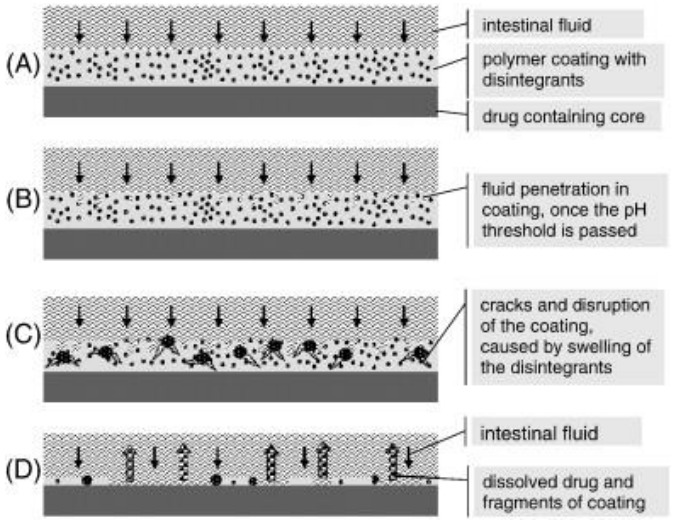

Fig. 9: Schematic representation of the mode of action of non-percolating incorporation of disintegrants in a pH-responsive polymer coating [237]

4.2. Time-dependent

In these systems the site of drug release is decided by the transit time of the formulation in the GI tract. On an average, an orally administered dosage form takes about 3h to travel through the length of the small intestine to the beginning of the colon. Compared to gastric emptying rate, the small intestinal transit time is relatively consistent. In principle, time-controlled systems rely on this **consistent small intestinal transit time**[225].

For this purpose, different combinations of hydrophilic and hydrophobic polymers have been used as coating materials on solid formulations[239]. The majority of coated systems intended for time-based colonic release consist in enteric dosage forms for **pulsatile delivery**, wherein erodible, rupturable, permeable or semipermeable functional layers are responsible for imparting the appropriate delay phase prior to the drug liberation[240]. **Swellable systems** for example, use hydrophilic polymers that swell when coming in contact with water and release the drug based on GIT time transit. Lag time can be adjusted by altering the thickness of the coating polymer. Combinations of water insoluble polymers like ethylcellulose **(EC)** and hydrophilic polymers like hydroxypropyl methylcellulose **(HPMC)**, hydroxypropyl cellulose **(HPC)**, have been used to produce time-dependent systems[239].

Generally time-controlled formulations for colonic delivery include a pH-dependent component because the transit of a formulation in the GI tract is **largely influenced by the gastric emptying time**. **Enteric coating** is also used for preventing the rapid swelling and disintegration of the other components in the upper GI tract[225].

Ideally, these formulations are designed such that the site of delivery is not affected by the individual differences in the gastric emptying time, pH of the stomach and small intestine or presence of anaerobic bacteria in the colon[225].

Due to the inter-subject variation in GI transit times, the onset of initial drug release could sometimes occur in the small intestine, or formulations could pass the ascending colon intact[223]. Additionally, the performance of a time-dependent formulation can be affected significantly by the pathophysiological conditions associated with the GI tract. Accelerated transit through different regions of the colon has been observed in the patients with irritable bowel syndrome, diarrhea, and UC. Therefore, these systems **might not be ideal** to deliver drugs colon-specifically for the treatment of colon-related diseases[223].

Some examples of time-dependent systems are:

Chronotopic™ system: The system consists in a drug-loaded core coated with a swellable hydrophilic polymer, which is responsible for a lag phase preceding the onset of release. When in contact with the aqueous fluids, the hydrophilic polymer undergoes a glassy-rubbery transition and, in the rubbery state, it becomes more permeable,

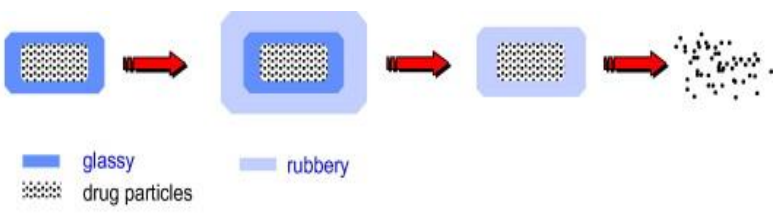

Fig. 10: Schematic mechanism of action of Chronotopic™ system [242]

dissolves and/or erodes. An outer gastro-resistant film is expected to remain the formulation intact as long as it is located in the stomach, overcoming the variability in gastric emptying time. The pH change occurring with the passage of the unit from the gastric to the duodenal environment determines the dissolution of the enteric coating, thus allowing the interaction between hydrophilic polymer and biological fluids to start. This interaction gives rise to the lag phase[241,242]. The delayed release behavior is mainly controlled by the viscosity grade and amount of the swellable polymer applied[227].

Time-Controlled Explosion System (TES): The TES could be in the form of a bead or granule, which has four-layered spherical structure: a core, the drug, the swelling agent and a water insoluble polymer membrane. TES is characterized by a rapid drug release with a precisely programmed lag time. This is given by the expansion of the swelling agent by water penetration through the outer membrane, destruction of the membrane by stress due to swelling force and subsequent rapid drug release. Low-substituted-HPC (L-HPC) and EC were used as the swelling agent and for the outer insoluble membrane, respectively. The lag time can be programmed by changing the outer membrane thickness[243].

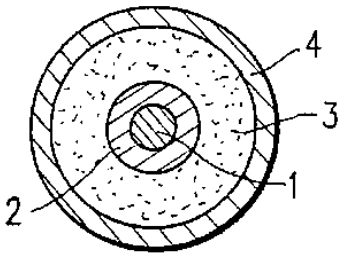

Fig. 11: Structure of TES
1: sucrose core; 2: drug; 3: swelling agent; 4: polymer membrane [*from: US 4871549 A patent*]

Time-controlled capsule: This capsule is composed of four parts: drug container, swellable substance, capsule body and cap. At the bottom of the body, micropores are made. After oral administration, GI fluid permeates through the micropores and causes swelling of excipients as L-HPC. This causes an inner pressure, which pushes the drug container. When the cap made of water-insoluble EC cannot resist the swelling pressure, it disintegrates and the drug in the container is released from the capsule[244]. The disintegration lag-time depends on the balance between the tolerability and thickness of the water-insoluble membrane and the amount of swellable excipient[245].

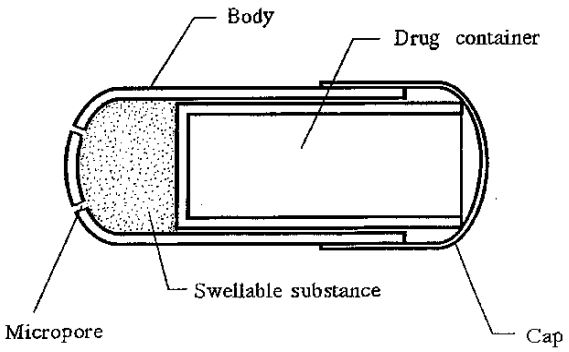

Fig. 12: Structure of Time-controlled capsule [*from US 5637319 patent*]

Time clock® system: This system is made of a coated solid dosage form, either tablet or capsule. The coating consists of a hydrophobic-surfactant layer, applied as aqueous dispersion, to which a hydrosoluble polymer is added to improve adhesion to the core. The coating is made of carnauba wax, beeswax, polyoxyethylene sorbitan monooleate and water. The dispersion, dried on the core during coating, retains the capacity to rehydrate and redisperse in an aqueous environment in a time proportional to the thickness of the film. Following redispersion, the core is available for dissolution[246]. To overcome the problem of inopportune drug release in the small bowel, an enteric coating can be applied to the formulation preventing dispersion of the hydrophobic layer in the stomach[247,248].

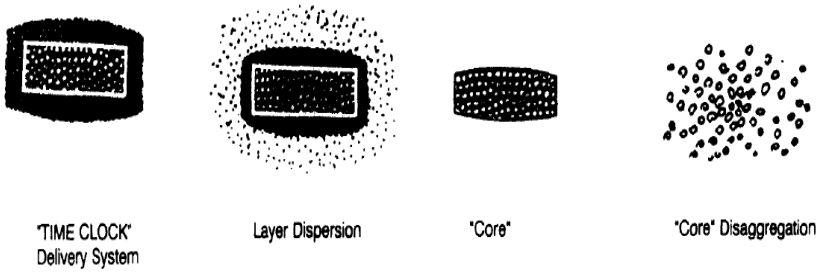

Fig. 13: Schematic mechanism of action of the Time clock® system [246]

Pulsincap™ system: The device is composed of a water insoluble body capsule enclosing the drug reservoir. The half capsule body is sealed at the open end with a swellable hydrogel plug that is covered by a water soluble cap. The whole unit is coated with an enteric polymer to avoid problems of variable gastric emptying. When the capsule comes in contact with fluids, the plug swells, and after a lag time, the plug pushes itself outside the capsule and rapidly releases the drug. The length of the plug and its point of insertion into the capsule control the lag time[249,250].

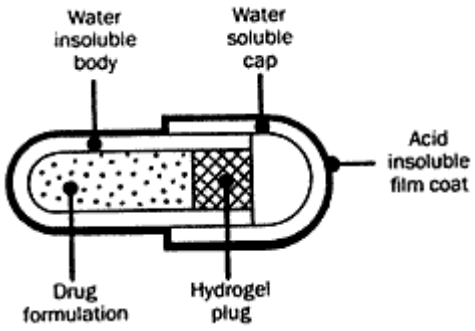

Fig. 14: Structure of Pulsincap system
(modified from [221])

Colon-targeted delivery capsule (CTDC): The system combines time and pH approaches to a hard gelatin capsule. It contains an organic acid together with an active ingredient in a capsule coated with a three-layered film consisting of an acid-soluble polymer, a water soluble polymer, and an enteric polymer. The

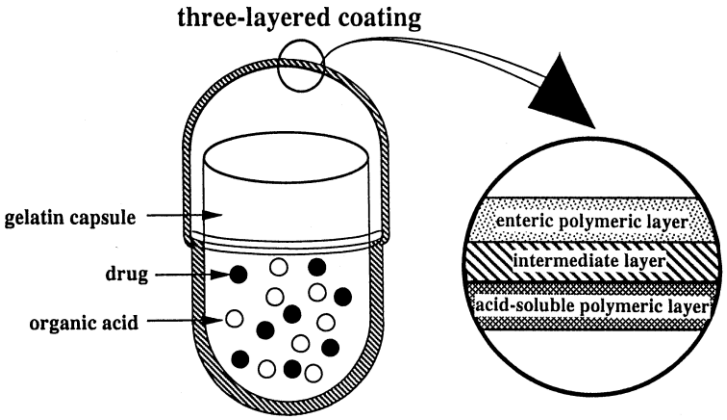

Fig. 15: Structure of CTDC[251]

intermediate layer prevents the direct contact of the cationic and anionic polymeric layer. After ingestion of the capsule, drug release is prevented in the stomach due to enteric polymeric layer. After gastric emptying, the outer layer and the intermediate layer quickly dissolve, but the inner polymeric layer still remains and prevents the drug release in the intestine. However, when the micro-environmental pH inside the capsule gradually decreases according to the dissolution of organic acid, and when the inner polymeric layer is finally dissolved by the acid fluid, the drug content is quickly released. The onset of the drug release, therefore, can be controlled by the thickness of the inner polymeric layer[251].

4.3. Microbial enzyme-dependent

Colon microbiota is increasingly recognized as a **preferable triggering componen**t in the design of colon-specific drug delivery systems since there is an abrupt increase of the bacterial population and corresponding enzyme activities in the colon, which is independent of the GI transit time. The colon has a population of **10^{11} – 10^{12} colony-forming units (CFU) mL^{-1}** with *Bacteroides, Bifidobacterium, Eubacterium* and *Lactobacillus* greatly outnumbering other species, converting the large intestine as the site of the most abundant microflora in the GI tract[252,253,254].

Hydrolytical and redox reactions are the predominant metabolic conversions triggered by the intestinal microflora. The main hydrolytic enzymes produced by the intestinal bacteria are β-glucuronidase, β-xylosidase, α-L-arabinosidase and β-galactosidase, whereas the reductive enzymes include nitroreductase, deaminase and urea dehydroxylase[253].

4.3.1. Prodrugs

These are therapeutic agents that have to undergo biotransformation before exerting a pharmacological action. Once in the colon, enzymes produced by colonic bacteria act on the prodrugs to release the active moiety[224]:

- **Azo-bond conjugates**: Through cleavage of the azo group by bacterial azoreductases. For example: sulfasalazine where sulfapyridine is linked to a salicylate radical by an azo bond; olsalazine that is a dimer of 5-ASA linked via an azo bond.
- **Glycoside conjugates**: Steroid glycosides, for example, are hydrophilic and thus poorly absorbed from the small intestine. One it reaches the colon it can be cleaved by bacterial glycosidases, releasing the free drug to be absorbed by the colonic mucosa.
- **Glucuronide conjugates**: Here the deglucuronidation process results in the release of active drug and enables its absorption. This is used for narcotic antagonists and steroids.
- **Cyclodextrin conjugates**: Cyclodextrins are cyclic oligosaccharides barely capable of being hydrolyzed and only slightly absorbed in the upper GI tract. However, they are fermented by

colonic microflora into small saccharides. The formation of drug conjugates with cyclodextrins might thus be used in colon-targeting delivery.

- **Dextran conjugates**: Ester prodrugs of this polysaccharide with naproxen and corticosteroids provided a colonic drug release.
- **Polymeric prodrugs**: Here, drug is linked directly to a high molecular weight polymeric backbone. This linkage is susceptible to enzymatic attack in the large intestine. The large size of the prodrug hinders absorption from the upper GIT. Poly-asa, in which sulfapyridine is linked to an inert polymer backbone to which 5-ASA is azo linked. The mechanism of colonic 5-ASA delivery is essentially that of sulfasalazine: reduction of azo bonds.

The prodrug approach is not very versatile as its formation depends upon the functional groups available on the drug moiety for **chemical linkage**. Furthermore, prodrugs are **new chemical entities** and need evaluation before being used as drug carriers. Toxicological studies need to be performed before their use as drug delivery systems[255]. Thus, dextrans and cyclodextrins remain the polysaccharides of choice as drug carriers for the formation of conjugates/prodrugs because of a safer toxicological profile[255].

4.3.2. Azo-polymeric coatings

Various azo polymers have also been evaluated as coating materials over drug cores. They are similarly susceptible to cleavage by the azoreductase in the colon[255]. Peptide drugs were coated with polymers cross-linked with azoaromatic groups to form a film degradable by colonic microflora[256]. Azopolymer-coated pellets containing budesonide were effective for colonic delivery in a rat model of colitis[257].

However, it has been shown the azoreductase activity in CD patients is decreased[258]. Furthermore, microbial degradation of azo crossed link polymers is generally slow, resulting in incomplete and irregular dug absorption. A limitation of azopolymer based formulation is the release of harmful substances on long-term use, generating a **toxicity concern**[239,259].

These limitations can be overcome by the use of natural polymer materials with glycosidic linkage[239]. Considering this, the use of naturally occurring polysaccharides has been investigated for colonic delivery of drugs.

4.3.3. Polysaccharide delivery systems

The primary **sources of carbon and energy** of the vast colonic microflora are **polysaccharides** present in dietary residues that have been left **indigested in the small intestine**[260]. Therefore,

colonic degradable polysaccharides have been employed in colon-targeted systems, generally as film or compression coatings or within matrix systems.

In **film coating** naturally occurring polysaccharides of plant, algal, animal or microbial origin, such as: pectin[261], inulin[262], chitosan[263], chondroitin sulphate[264], galactomannan[265], amylose[266], cyclodextrin[267] and starch derivatives[268,269], have been preferred as colon delivery aids primarily in view of their proven safety and biocompatibility characteristics. It should be noted that, because of the **water solubility** and **poor film-forming properties** of these polysaccharides, they might have to be mixed with other synthetic polymers in order to obtain a polymer film or to decrease the aqueous solubility[259]. That is the reason why coatings prepared from cellulosic (Aquacoat® ECD30, Surelease®), acrylic (Eudragit® NE30D and RS30D) or vinyl acetate (Kollicoat® SR30D) **insoluble polymer** aqueous dispersions are **incorporated** in appropriate amounts to polysaccharide solutions. This aims to prepare more suitable forms for targeting drugs to the colon. The identified release mechanism is drug diffusion through the coating, after leaching out or bacterial degradation of the polyssacharide, via the water-filled pores formed within the insoluble polymer network[227].

COLAL® technology involves a coating for drug pellets, tablets or capsules which is composed of EC and a 'glassy amylose', derived from starch. Human enzymes do not digest this amylose as the preparation moves through the upper GI tract, but is digested by bacterial enzymes found only in the colon. This technology was applied to the delivery of prednisolone: **COLAL-PRED®**. Clinical studies showed that prednisolone is consistently delivered in the ileocecal junction/ascending colon, regardless of the transit time and that treatment with COLAL-PRED® did not entail major systemic exposure to the corticosteroid[270]. COLAL-PRED® is in phase III clinical trials for treatment of mild to moderate UC[271].

Poor film-forming properties of natural substances can be overcome by applying the polysaccharides as **compression coatings**. Pectin[272] and guar gum[273,274] were for example, used as direct compression coatings for tablets aiming a colonic delivery.

Another alternative approach involves the compression of blends of the drug, a degradable polysaccharide and additives to form a monolithic or multiparticulate solid dosage form. The drug is thus embedded in the **matrix core of the degradable polymer.** For example bioerodible matrix systems of cross-linked chondroitin sulfate[275], guar gum[276], pectin[277], chitosan[278], starch derivatives[279] have been studied. However, matrices have the disadvantage that only a limited amount of drug can be incorporated[259].

Bacterially triggered colonic drug release seems to **avoid drawbacks of pH and time dependent approaches**. These systems have **better site specificity** and reliability in humans than pH-dependent formulations[280] and they are not influenced by the feeding state[281]. Moreover, a system than conciliates both, the pH and bacterial approach, provides also colon-specificity[282].

4.4. Newer technologies

4.4.1. Pressure-dependent

Intestinal pressure-controlled colon delivery capsules **(PCDCs)**, rely on the relatively strong peristaltic waves in the colon that lead to an **increased luminal pressure**. It consists of **capsular shaped suppositories** coated with a water-insoluble polymer like EC[283]. The PCDCs were originally prepared by coating the inner surface of gelatin capsules where the drug was introduced along with a suppository base[283,284]. In these systems, the drug is dissolved with a suppository base such as polyethylene glycol[285,284], propylene glycol[283] or an oily base[286]. Therefore, after oral administration, the system behaves like an **EC balloon** containing drug solution, since the suppository base liquefies at body temperature. In the upper GI tract, the EC balloon is not directly subjected to intestinal luminal pressures since sufficient fluid is present in the stomach and small intestine. However, reabsorption of water occurs in the colon and the viscosity of the luminal contents increases. As a result, intestinal pressures due to peristalsis directly affect the EC balloon. Since the EC balloon cannot tolerate these pressures, it disintegrates and releases the drug in the colon. The thickness of the EC membrane is the most important factor for the disintegration of the balloon in the colon[287]. Other EC coating methods like dipping[288] or direct suppository coating[285,287] have also been studied.

4.4.2. Osmotic-dependent

The **OROS®** system comprises 4 or 5 push-pull units within a hard gelatin capsule. Each push-pull unit is a bilayer tablet core containing the drug in the top layer and the osmotic push agent in the lower layer. The drug formulation contains the drug combined with osmotic and suspending agents. This bilayer tablet is coated with a semipermeable membrane that is drilled on the drug side to allow drug delivery through the orifice. The outside surface of the semipermeable membrane is then coated with

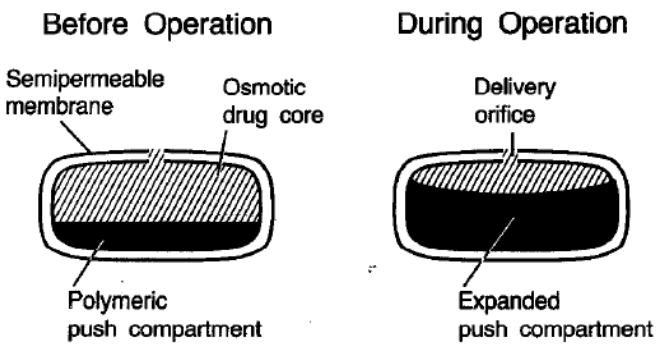

Fig. 16: Schematic mechanism of action of OROS® system[289]

Eudragit® S-100 to delay the drug release from the devise during the transit through the stomach. Upon arrival to the small intestine, when the pump is in operation, both the drug and osmotic layers **imbibe water** from the GI fluids across the membrane **by the process of osmosis** to formulate a suspension form in the drug layer. Simultaneously, by the pulling action of the water in the drug compartment, the drug suspension is pushed out of the drilled orifice by the dispersing action of the expanding osmotic, resulting in the release of the drug. The drug release kinetics is controlled by the rate of influx of water through the semipermeable membrane[289].

4.4.3. CODES™

This system consists of three components: a **core** containing **lactulose and the drug**, an **inner acid-soluble** material **layer**, and an **outer layer** of an **enterosoluble** material. The concept of CODES™ is shown in *Figure 17:* in the stomach, the drug is not released from CODES™ with the enteric coating layer. In the small intestine where the enteric coating layer dissolves, the drug is still not released from the tablet core because of its inner acid-soluble coating layer. However, GI fluids penetrate into the tablet core through the acid-soluble coating layer and then lactulose begins to dissolve inside the core during the small intestinal transit. When CODES™ arrives at the colon, **lactulose** leaches through the acid-soluble coating layer and is degraded by enterobacteria and **produces organic acids**. These organic acids should **dissolve the acid-soluble coating layer** to release the drug. The feature that distinguishes CODES™ from other strategies is the generation of organic acids by bacterial degradation of lactulose in the colon to dissolve the acid-soluble coating layer[290,291].

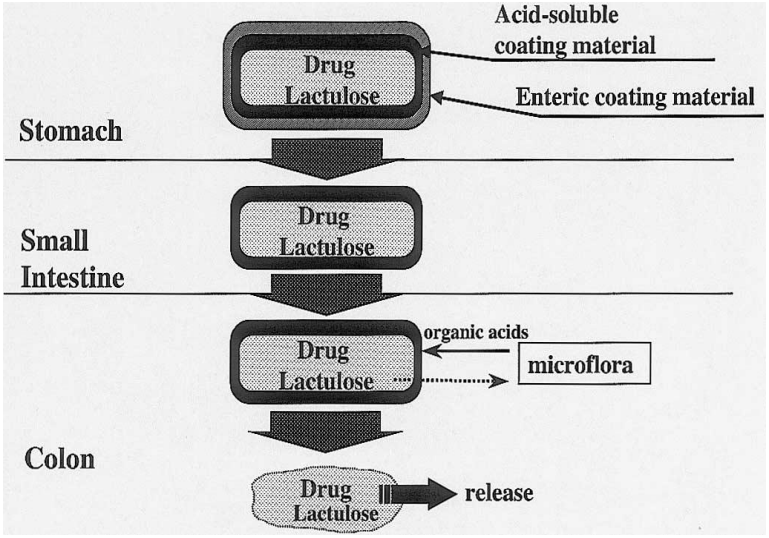

Fig. 17: Schematic mechanism of action of CODES™[290]

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CHAPTER II

ABOUT THE BACTERIOLOGICAL CHARACTERIZATION OF CIN-102

1. Introduction

Antimicrobial resistance is an increasingly global threat to public health and novel antimicrobials are urgently needed. In spite of this, in recent decades pharmaceutical companies have largely abandoned the discovery and development in this area. The perceived lack of return on investment is one of the main reasons for the abandon of this pipeline. However, antibiotics are essential for life because they are the cornerstone of infectious disease therapy and medical care rely on their effectiveness for successful clinical outcomes[1]. A group of bacteria (ESKAPE) has emerged that escape the lethal action of antibiotics: vancomycin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae*, carbapenemase-hydrolyzing β-lactamases producing *K. pneumoniae* (KPC), multi-drug resistant (MDR) *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species. These bacteria which are a threat to immunocompromised patients, are no longer exclusively associated with the nosocomial environment[2].

Even though infections caused by resistant Gram-positive bacteria like MRSA and vancomycin-resistant *Enterococcus spp.* (VRE) have been partially controlled, the actual increased burden of multidrug-resistant Gram-negative bacteria is a real phenomenon. These bacteria remain a considerable threat not only because of their faster dissemination compared to Gram-positive, but also because few antimicrobial agents are reliably active and very little is expected to be available in the near future[3]. Of particular interest is that much of this dissemination is undetected, with resistant clones carried in the normal human flora and only becoming evident when they are the source of endogenous infections[4]. Asymptomatic fecal carriage of ESBL-producing enterobacteria in healthy people[5,6], as well as the alarming spread of New Delhi metallo-β-lactamase (NDM)-producing enterobacteria[4] and colistin resistance in community infections[7] have been reported. These facts show that the community is a reservoir of multi-resistant bacteria that might constitute a sustaining source for nosocomial infections and compromise the efficacy of hospital infection-control measures[5]. All of these findings highlight the urgent need for new antimicrobials able to target these microorganisms.

Bush *et al.*[8], pointed out that in battle against antimicrobial resistant, the vast reservoir of bioactive compound available in nature merit to be exploited for novel antibiotic discovery Plant secondary metabolites (PSMs), like essential oils (EOs), do not appear to participate in plant growth and development; instead they possess inherent antimicrobial activity important for protection against microbial infections, permitting plants to compete for an ecological niche. EOs are complex mixtures characterized by 2 or 3 major components and composed of terpenes, terpenoids and phenylpropanoids[9]. EOs are of main interest since they possess antibacterial properties and have been screened as potential sources of novel antimicrobial compounds[10]. Furthermore, the presence of multiple compounds and a possible multiplicity of action in EOs is favorable since drugs that interact with multiple targets can delay development of bacterial resistance[11].

The pharmaceutical company Septeos (Paris, France) followed the aim of antibiotic discovery based on natural products. After an initial screening of the antibacterial activity of 33 EOs alone and in combination, cinnamon EOs were selected because of their broad-spectrum of action. However, the purpose was the fabrication of a reproducible synthetic blend that respects the concentrations of the compounds naturally found in cinnamon EOs. For that, chemical standards of the main components of these EOs were purchased and the known toxic compounds were excluded from the blend composition. This resulted in a reconstituted blend at well-defined concentrations of each compound to respect synergies observed in EOs combinations. Then, this blend and closely related ones were evaluated in order to confirm that all of the components were involved in the synergistic antibacterial effect. The one presenting the highest efficacy, called CIN-102 represents an interesting new antibacterial candidate with potential to treat infections caused by resistant bacteria (***See Annexe 1***).

In this work we confirm the broad-spectrum of activity of CIN-102 against a wide range of different subsets of bacteria, many of them multi-resistant. The logarithmic reduction time, the post-antibiotic effect and the impact of sub-inhibitory concentrations of CIN-102 on bacterial growth were also evaluated. Finally, an extensive study of the interactions between CIN-102 and several antibiotics is presented.

2. Materials and methods

2.1. Antibacterial agents and growth media

CIN-102 (Septeos, Paris, France) (Composition in Table 1); polyethylene sorbitan monooleate (Tween 80; Panreac, Barcelona, Spain); propylene glycol (Cooper, Melun Cedex, France); Ringer solution (Merck, Darmstadt, Germany); Rosenow broth (Bio-Rad, Marnes-la-Coquette, France); Mueller-Hinton agar; Columbia agar base (Oxoid; Hampshire, England); Brain-Heart broth; Brucella agar and Dey-Engley neutralizing broth (Becton, Dickinson and Company, Le Pont de Claix, France). Antibiotics were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Table 1: CIN-102 composition

Component	% (w/w)
Trans-cinnamaldehyde	86.7
Trans-2-methoxycinnamaldehyde	5.35
Cinnamyl acetate	2.5
Linalool	2.4
β-Caryophyllene	1.7
Cineol	1
Benzyl benzoate	0.35

2.2. Microorganisms, storage and growth conditions

Strains were isolated from human clinical samples and are listed in Table 2 and 3. Strains were stored at -20˚C in Brain-Heart (BH) and Rosenow broth for aerobic and anaerobic isolates, respectively.

Table 2: Aerobic bacterial strains

Bacterial strain	Wild or acquired resistance	N° of strains tested
	Enterobacteria	
Escherichia. coli	ATCC 25922	1
	Wild (8137, 8141, 8150, 8151, 8155, 8156)	6
	Penicillinase (8138, 8154)	2
	Penicillinase and nalidixic acid-resistant (8142)	1
	Penicillinase and fluoroquinolone-resistant (8157)	1
	ESBL (09003), NDM-1 (10269, 10385, 10386), KPC-2 (10269), CTX-M1 (11003), CTX-M3 (11004), CTX-M14 (11005), CTX-M15 (11006), TEM-12 (11007), SHV-2A (11008), SHV-12 (11009), OXA-30 (11010), CMY-2 (11011), OXA-48 (10012), VIM-1 (11013), VIM-2 (11014), VIM-19 (11015)	18
Enterobacter aerogenes	TEM-24 (09004)	1
Enterobacter cloacae	GES-5 (10274), KPC-2 (10281), NDM-1 (11053), NMC-A (11054), OXA-43 (11055)	5
	Wild (11050)	1
	Cephalosporinase (11051, 11052)	2
Serratia marcescens	KPC-2 (10267), SME-1 (10271), SME-2(10279)	3
	Wild (11056)	1
	Cephalosporinase high-level (11057, 11058)	2
Citrobacter freundii	KPC-2 (10268), TEM-3 (11028), CTX-M1 (11044), CTX-M15 (11045), NDM-1 (11046)	5
	Wild (11041)	1
	Cephalosporinase high-level (11016)	1
Klebsiella pneumoniae	VIM (10270), OXA-48 (10272), KPC-2 (10277), ACT-1 (11018), DHA-2 (11019), LAT-1 (11020), CTX-M2 (11021), CTX-M3 (11022), CTX-M14 (11023), CTX-M15 (11024), SHV-2A (11025), SHV (11026), SHV-12 (11027), TEM-3 (11028), TEM-2 (11029), KPC-3 (11030), NMD-1 (11031, 11032)	18
	Wild (11016)	1
	Penicillinase (11017)	1
Salmonella spp.	Wild (11033, 11034, 11035, 11036)	4
	CMY-2 (11037)	1
Providencia stuartii	Wild (11038, 11039)	2
	TEM-24 (11040)	1
Proteus mirabilis	Wild (11059, 11060)	2
	Penicillinase (11061)	1
	TEM-21 (11062), TEM-52 (11063), ACC-1 (11064)	3

Klebsiella oxytoca	Wild (11047)	1
	OXY (11048)	1
	CTX-M15 (11049)	1
Non-fermentative Gram-negative		
Pseudomonas aeruginosa	Fluoroquinolone-resistant (8127)	1
	Multi-resistant (8128, 8132)	2
	Cephalosporinase high-level (8129)	1
	Penicillinase (8130, 8136)	2
	Wild (8131, 8133, 8135)	3
	Fluoroquinolone- and aminoglycoside-resistant (8134)	1
	VIM-2 (09007), ESBL (09008), PER-1 (10276), GES-2 (10278), VIM-2 (10280)	5
	ATCC 27583	1
Acinetobacter baumanii	VEB-1 (09010), VIM-4 (10275)	2
	Multi-resistant (09011)	1
Burkholderia cepacia	Wild (10282)	1
Gram-positive		
Staphylococcus spp.	Methicillin-, kanamycin- and tobramycin-resistant (8143)	1
	Methicillin- and kanamycin-resistant (8146)	1
	Wild (8147, 8149, 8237, 8238)	4
	MRSA and fluoroquinolone-resistant (8148, 8240)	2
	MRSA (8239, 10168)	2
	Methicillin-, kanamycin-, tobramycin- and fluoroquinolone-resistant(8241)	1
Enterococcus spp.	Multi-resistant (09001, 09002)	2
	MLSB (8153)	1
	Aminoglycoside-resistant (8152)	1

ATCC: American Type Culture Collection
Other strains: Collection from the College of Pharmacy (University Lille Nord de France)

Table 3: Anaerobic bacterial strains

Bacterial strain	Wild or acquired resistance	N° of strains tested
	Gram-positive cocci	
Peptostreptococcus micros	Wild (11129, 11124)	2
Peptostreptococcus magnus	Clindamycin-resistant (11123)	1
	Wild (11135, 11131)	2
Anaerococcus prevotii	Wild (11104, 11125, 11313, TB47-B10)	4
Peptoniphilus asaccharolyticus	Wild (10315, 11315)	2
Peptostreptococcus anaerobius	Wild (11310)	1
Ruminococcus spp.	Wild (T46-B17, Y67-4)	2
	Gram-positive bacilli	
Clostridium difficile	ATCC 700057	1
	Wild (11178, 11179, 11180)	3
Clostridium ramosum	Wild (9361)	1
Clostridum perfringens	Wild (11115, 11116, Y66F6-4, 11117, 11126, ATCC 13124)	6
Clostridium scindens	Clindamycin- and penicillin G-resistant (11323)	1
	Wild (11318, 11321)	2
Clostridium innocuum	Clindamycin resistant (11327)	1
Bifidobacterium bifidum	Wild (Y56-7)	1
Clostridium clostridioforme	Wild (Y67-9)	1
Clostridium sporogenes	Clindamycin-resistant (11127)	1
	Gram-negative bacilli	
Fusobacterium nucleatum	Wild (11324, 8029, JD7)	3
Bacteroides fragilis	ATCC 25285	1
	Clindamycin-resistant (11105, 11108)	2
Bacteroides thetaiotamicron	ATCC 29742	1
	Clindamycin- and piperacilin + tazobactam-resistant (11134)	1
Fusobacterium necrophorum	Clindamycin-resistant (11320)	1
	Wild (8246)	1

Bacteroides distasonis	Clindamycin-resistant (11092)	1
	Wild (Y67-10, 11107)	2
Bacteroides vulgatus	Clindamycin-resistant (11118, 11326)	2
Bacteroides uniformis	Wild (11316, 9074)	2
Prevotella spp.	Wild (Y67-6)	1
	Gram-negative cocci	
Veillonella spp.	Wild (T18.8)	1

ATCC: American Type Culture Collection
Other strains: Collection from the College of Pharmacy (University Lille Nord de France)

2.3. Determination of the Minimal Inhibitory Concentration (MIC)

MICs of CIN-102 were determined following the reference agar dilution procedures of the Clinical and Laboratory Standards Institute (CLSI)[12,13]. The stock solution (1% v/v) of CIN-102, was prepared by solubilizing CIN-102 in a blend of Tween 80 : Propylene glycol 10% (38 : 62), then only propylene glycol 10% was used for the further dilutions. The absence of antibacterial activity of the surfactants at the concentrations used to perform the dilutions of CIN-102 was first verified. The inocula (2 to 3 µL) were delivered using a Steers replicator (Mast Systems, London, UK) giving a final 10^4 colony-forming units (CFU) per spot of inoculation onto Mueller-Hinton agar (MHA) plates and 10^5 CFU per spot onto Brucella blood agar plates for aerobes and anaerobes, respectively. The lowest concentration of CIN-102 that inhibits growth of bacteria is reported as the MIC

2.4. Time of logarithmic reduction

Inocula were prepared by making a suspension on Ringer solution of overnight sub-cultured bacteria in BH broth from 26 enterobacteria, 20 non-fermentative Gram-negative and 11 Gram-positive isolates with known MICs for CIN-102. Testing tubes containing 8mL of Ringer solution were inoculated with 1mL of the previous bacterial suspension (approximately 10^7 CFU mL^{-1}). Then, 1 mL of the dilution corresponding to either 1% or 4xMIC of CIN-102 was added. Next, aliquots were removed from the incubation mixture after 15, 60 min and 24h to assess the bacterial growth logarithmic reduction in time. For that, aliquots were ten-fold diluted in Dey-Engley neutralizing broth. Aliquots from the neutralized mixture were further diluted 1:10 and 1:100 in Ringer solution. Viable counts were performed by inoculation of these aliquots onto MHA plates subsequently incubated at 37°C for 24h.

2.5. Post-Antibiotic Effect (PAE)

The PAE describes the phenomenon of suppression of microbial growth that persists after antimicrobial exposure. The technique involves the measurement of microbial growth kinetics after rapid removal of the antimicrobial agent. The PAE of CIN-102 was determined by the viable plate count method described by Craig and Gudmundsson[14] for aerobes: *K. pneumoniae*-OXA 48 (enterobacteria), *B. cepacia* (non-fermentative, Gram-negative), multiresistant *E. faecium* (Gram-positive) and anaerobes: *C. perfringens*.

Testing tubes were prepared as previously but 1 mL of the dilution corresponding to either the MIC or 4xMIC of CIN-102 was added. They were incubated at 37°C for 2 hours and at the end of the exposure period cultures were diluted 1000-fold with Mueller-Hinton (MH) broth in order to remove CIN-102. Next, tubes were re-incubated at 37°C. Viable counts on MHA plates were determined before exposure to CIN-102, immediately after dilution (zero hour) and every 1 or 2h after dilution until turbidity developed and reached an intensity comparable at least to that of the control group (turbidity reached a 1 McFarland standard). Please note that for anaerobes all the culture media and Ringer solution used were previously reduced. As well, viable counts were performed in Columbia agar plates incubated under anaerobic conditions. The PAE was defined as PAE = T – C, where the T represents the time required for the viability count of CIN-102 exposed culture to increase by 1 $\log_{10}$ above the count obtained immediately after dilution and C represents the corresponding time for the growth control.

2.6. Bacterial growth at sub-inhibitory (subMIC) concentrations

The impact of subMICs of CIN-102 on bacterial growth was studied for aerobes: *E. coli* (enterobacteria), *P. aeruginosa* (non-fermentative Gram-negative,) *S. aureus* (Gram-positive) and anaerobes: *C. perfringens*. To evaluate bacterial growth at sub-inhibitory concentrations, we proceeded as previously, CIN-102 at either the CMI/2 or CMI/4 was added. Tubes were placed at 37°C and viable counts were determined before exposure to CIN-102 (zero time) and every hour after exposure on MHA plates. For anaerobes the methodology was modified as formerly mentioned.

2.7. Interaction with antibiotics

To perform the CIN-102 – antibiotic combinations assays, a checkerboard procedure was applied[15]. Antibiotic tested were: aminoglycosides (amikacin and gentamicin), polymyxin (colistin), lincosamide (clindamycin), macrolide (erythromycin), ß-lactam (imipenem), glycylcycline (tigecycline) and glycopeptide (vancomycin). Serial two-fold dilutions of the antibiotics and CIN-102 were used (final concentrations from 1- to 0.002-fold MIC for the antibiotic; and from 10 000 to 5 mg L^{-1} for CIN-102). All combinations of any antibiotic and CIN-102 concentrations were inoculated using a Steers replicator. After inoculation, plates were incubated for 24h at 37°C. For each combination, the fractional inhibitory concentration (FIC) index was calculated using the following formula: ΣFIC index = FIC A + FIC B = (MIC of A in combination / MIC of A alone) + (MIC of B in combination / MIC of B alone). ΣFIC index ≤ 0.5 indicates synergism; > 0.5 and ≤ 1 addition; >1 and ≤ 4 indifference and > 4 antagonism.

3. Results and discussion

3.1. MIC and spectrum of action of CIN-102

CIN-102 was tested against 123 aerobic bacterial strains many of them multi-resistant bacteria isolated from nosocomial infections. The lipophilic nature of CIN-102 demanded the adaptation and validation of a new solubilization procedure. All strains are inhibited by CIN-102 at 1250 mg L^{-1} (Table 4). For aerobic

bacteria MIC ranges between 15 and 600 mg/L (average MIC of 300 mg L^{-1}), *B. cepacia* is the most susceptible (75 mg L^{-1}) and *P. aeruginosa* has the highest MIC (1250 mg L^{-1}). Experimental animal models still under investigation (data not shown) allow us to assess that active concentrations are obtained in infected tissues Furthermore, 52 anaerobic bacterial strains were also tested (Table 5), they have lower CIN-102 MICs, within a range of 5 to 150 mg L^{-1}. *C. scindens* and *F. necrophorum* strains have the lowest MICs (5 to 37.5 mg L^{-1}) and *C. perfringens* strains the highest (75 to 150 mg L^{-1}). CIN-102 is confirmed as an antibacterial agent with a broad spectrum of action. A remarkable aspect is the relative stability of CIN-102 MICs within different isolates of the same species.

Table 4: MIC of CIN-102 against aerobic bacteria

Bacterial strain	N° of strains tested	MIC Range (mgL^{-1})
Enterobacteria		
Escherichia coli	29	300
Enterobacter aerogenes	1	300
Enterobacter cloacae	8	300
Serratia marcescens	6	300-600
Citrobacter freundii	7	300
Klebsiella pneumoniae	20	300-600
Salmonella spp.	5	150-300
Providencia stuartii	3	300
Proteus mirabilis	6	300
Klebsiella oxytoca	3	300
Non-fermentative Gram-negative		
Pseudomona aeruginosa	16	600-1250
Acinetobacter baumanii	3	150-300
Burkholderia cepacia	1	75
Gram-positive		
Staphylococcus aureus	11	300
Enterococcus spp.	4	300-600

Table 5: MIC of CIN-102 against anaerobic bacteria

Bacterial strain	N° of strains tested	MIC Range (mgL^{-1})
Gram-positive cocci		
Peptostreptococcus micros	2	37.5
Peptostreptococcus magnus	3	37.5 – 75
Anaerococcus prevotii	4	75
Peptoniphilus asaccharolyticus	2	75
Peptostreptococcus anaerobius	1	75
Ruminococcus sp	2	5 – 37.5
Gram-positive bacilli		
Clostridium difficile	4	75
Clostridium ramosum	1	75
Clostridum perfringens	6	75 – 150
Clostridium scindens	3	5 – 37.5

Clostridium innocuum	1	75
Bifidobacterium bifidum	1	150
Clostridium clostridioforme	1	37.5
Clostridium sporogenes	1	75
Gram-negative bacilli		
Fusobacterium nucleatum	3	20 – 37.5
Bacteroides fragilis	3	37.5
Bacteroides thetaiotamicron	2	37.5
Fusobacterium necrophorum	2	5 – 37.5
Bacteroides distasonis	3	37.5
Bacteroides vulgatus	2	20 – 37.5
Bacteroides uniformis	2	37.5 – 75
Prevotella spp.	1	37.5
Gram-negative cocci		
Veillonella spp.	1	75

The relative increased tolerance of *P. aeruginosa* towards CIN-102 correlated with the observations of Longbottom *et al*.[16], explaining the increased resistance of this bacteria towards essential oil compounds due to the barrier and energy functions of its outer membrane and a possible participation of efflux systems.

Cinnamaldehyde, a phenylpropanoid, is the major compound of CIN-102 and its antibacterial mechanism of action is not yet totally understood. Cinnamaldehyde does not seem to cause disruption of the bacterial membrane, indicating that it might interfere with intracellular targets[17,18]. However, it has also been observed that as cinnamaldehyde concentration increases, damage caused to membrane integrity and permeability might become more serious[19]. Domadia *et al*. [20] showed that cinnamaldehyde is able to bind FtsZ, the prokaryotic homolog of the eukaryotic tubulin, perturbing the assembly of the Z-ring, essential for bacterial division. Given the widespread conservation of FtsZ in the bacterial kingdom[21], it might be possible that the broad-spectrum activity of CIN-102 is due to the inhibition of cell division through binding of cinnamaldehyde to FtsZ. Furthermore, cinnamaldehyde inhibits quorum sensing (QS) processes[22] and alter gene expression in bacteria[23].

This evidence suggest that the antibacterial activity of cinnamaldehyde might not be attributed to only one mechanism. However, to elucidate the mechanism of action of CIN-102, the presence of the other minor compounds must also be taken into account. β-caryophyllene, cineole and linalool are terpenoids, and the accumulation of terpene hydrocarbons in lipid bilayers of the cytoplasmic membrane causes loss of membrane integrity, increased permeability and even proteins embedded in the membrane can be affected[24]. The presence of these compounds in the formulation of CIN-102 might favor the penetration of cinnamaldehyde into the bacterial cell to exert its activity, interact synergistically to perform a multi-targeted action against intra-cellular components and/or affect membrane permeability. Indeed,

targeting bacterial membranes is of especial interest in treating persistent infections involving slow-growing or non-growing bacteria[25]. Further studies are needed to elucidate the mechanism of action of CIN-102.

3.2. Logarithmic reduction time of CIN-102

The rapidity of the cidal effect of CIN-102 (time, in minutes, necessary to reduce bacterial counts by 1 log), at 1% and 4-fold the MIC of CIN-102 was investigated against 57 isolates. As shown in Figure 1, there is a dramatic and fast killing more important with Gram-negative bacilli.

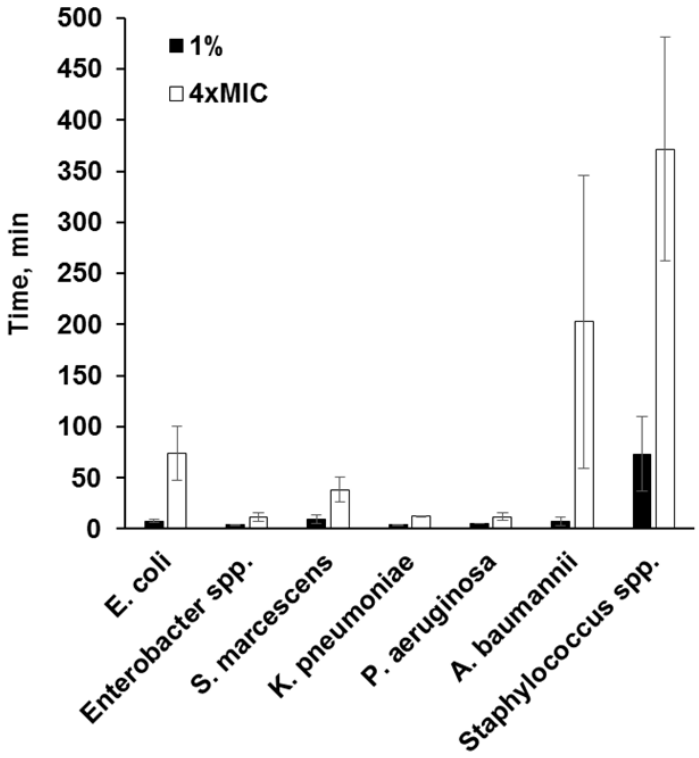

Figure 1: One log reduction time: The time to reduce bacterial counts by 1 log was tested at 1% and 4×MIC of CIN-102. Bacteria tested comprised: enterobacteria (E. coli, Enterobacter spp., S. marcescens and K. pneumoniae), non-fermentative Gram-negative (P. aeruginosa, A. baumannii) and Gram-positive (Staphylococcus spp) (mean values +/- SD are indicated).

The rapid log reduction time of CIN-102 might be related with disruption of cell wall or cell membranes caused by high concentrations of this agent. The advantages of bactericidal agents appear obvious, as rapid elimination of bacteria and decrease possibility of resistance development or recurrence of infection. Indeed, infectious of the central nervous system, endocarditis or infections in patients with cancer are some specific settings where the use of antibacterials with *in vitro* bactericidal activity is more important than a bacteriostatic activity[26]. However, some *in vitro* and experimental evidence suggest that rapid killing of bacteria may be undesirable when release of endotoxins could induce inflammatory responses[27,28].

3.3. PAE of CIN-102

The PAE of CIN-102 for *K. pneumoniae* (strain10272) is of 3.3h at the MIC and 4.0h at 4-fold the MIC (Fig. 2A). For *B. cepacia* (strain 10282), CIN-102 has a PAE of 2.17h at the MIC and, it is prolonged until 21.57h at 4-fold the MIC (Fig. 2B). *E. faecium* (strain 9002) has a PAE of 0.4h and 3.6h at the MIC and 4-fold the MIC of CIN-102, respectively (Fig. 2C). For *C. perfringens* (strain 11116) the PAE of CIN-102 is 1.7h at the MIC and 1.9h at 4-fold the MIC (Fig. 2D). The PAE for this strain does not seem to depend upon the concentration of CIN-102 used, as little variation between the PAE at the MIC and 4-fold the MIC is observed.

The PAE could represent the prolonged antibiotic persistence at the site of action, the time necessary to recover the normal metabolic activities of bacteria, or even to repair cell membrane damage that might occur[14]. Severe damage to the cellular wall as well as irreversible binding might explain the long PAE of an antibiotic[29]. Given the marked susceptibility of *B. cepacia* to CIN-102 reflected by its low MIC, it is possible that the damage produced by high CIN-102 concentrations caused more serious alterations than for the other bacterial strains and so the time required to recover a normal growth is longer.

The determination of the PAE is of importance for an optimal dosing frequency. If a drug produces a very short or no PAE, organism regrowth will begin soon after concentrations fall below the MIC and the goal of a dosing regimen might be to maximize the time of exposure. In contrast, for drugs producing PAE widely-spaced dosing intervals should be possible, since the PAE would prevent bacterial regrowth when levels fall below the MIC[14].

These results permit to state that CIN-102 present a PAE against different subsets of bacteria: Gram-negative and Gram-positive, aerobic and anaerobic, which can be of importance in the dosing of this agent.

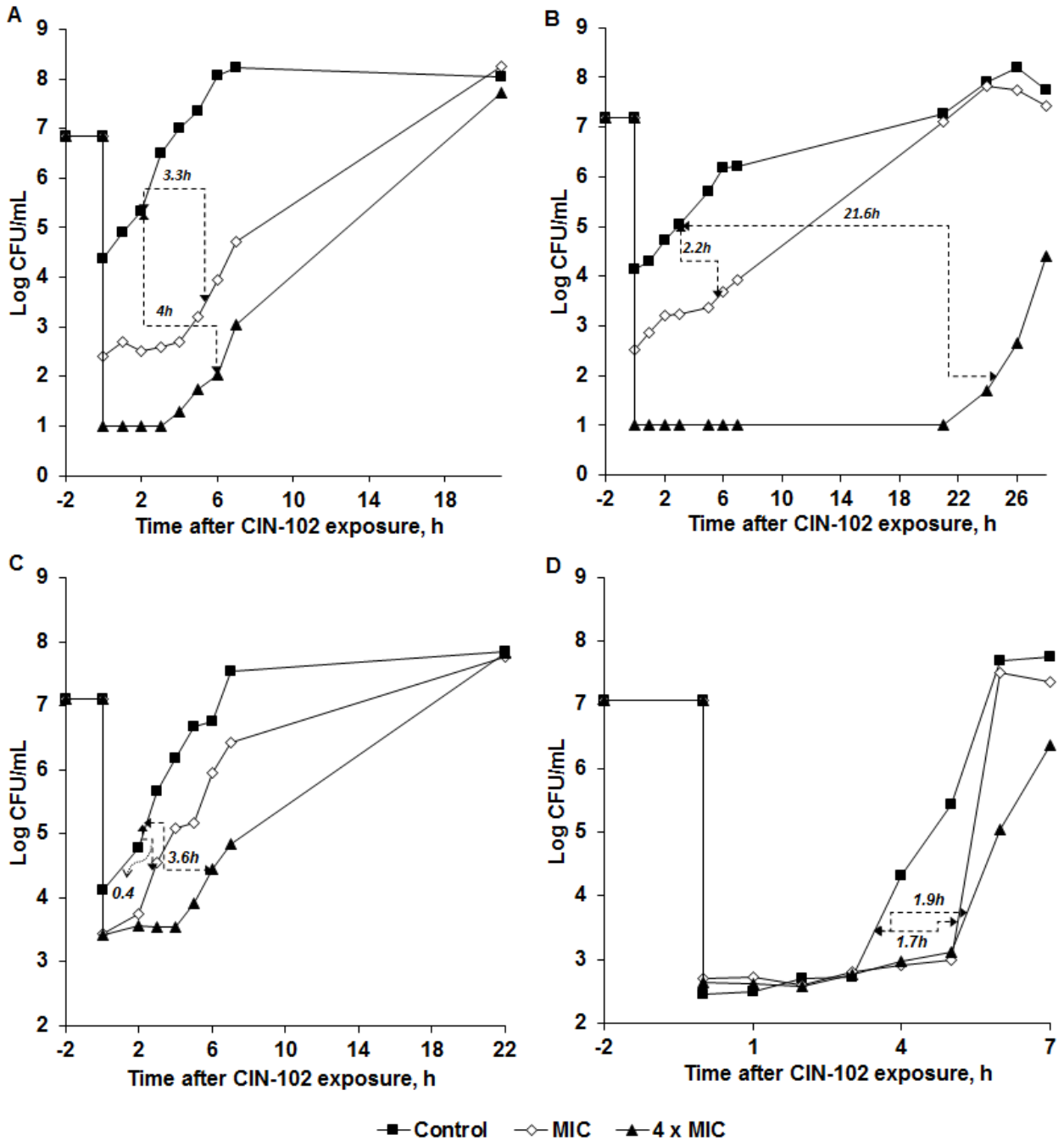

Figure 2: PAE of CIN-102: Growth curves of A) K. pneumoniae, B) B. cepacia, C) E. faecium and D) C. perfringens following a 2h exposure to CIN-102, at MIC and 4×MIC, which was then removed by dilution into fresh broth. Dashed lines denote the duration of the PAE for both concentrations.

3.4. Bacterial growth a CIN-102 subMICs

E. coli (strain 8154) (Fig. 3A) exposed to CIN-102 at MIC/2 presents a prolongation of the lag phase, but about 20h later the concentration of bacteria at the stationary phase reaches that of the control. At the MIC/4 the prolongation of the lag phase is shorter than at the MIC/2, the logarithmic growth is slower than the control, and also reaches a similar bacterial concentration at the stationary phase at about 18h. For *P. aeruginosa* (strain 09007) (Fig. 3B), it is observed that at the MIC/2 of CIN-102 the rate of the logarithmic growth is slower than in the control and there is also a decrease in the size of the population at the stationary phase. At the MIC/4, there is reduction in the concentration of bacteria at the stationary phase compared to control. *S. aureus* (strain 10168) (Fig. 3C), when exposed to CIN-102 at the MIC/2 and at the MIC/4 presents a decrease in the rate of logarithmic growth, however bacteria exposed at MIC/4 reach the same bacterial count of the control at the stationary phase. For *C. perfringens* (strain 11116), CIN-102 at the MIC and at the MIC/2 inhibits bacterial growth. At the MIC/4 a long lag phase of more than 20 hours is obtained (Fig. 3D).

It has been shown that subMICs of antibiotics cannot kill bacteria but can affect microorganisms in various ways like slower bacterial growth, induction of morphological changes, alterations of the cell surface structure, inhibition of enzyme and toxin production, reduction of bacterial adhesion and biofilm formation, sensitivity to stress conditions. These effects can consequently interfere with process of host-bacteria interaction like phagocytosis[30]. Cinnamaldehyde at subMICs causes bacterial cell elongation[23], interferes with the QS[22] and gene expression[23] altering virulent factors as biofilm formation, toxin production and/or susceptibility to stress. Therefore, in addition to the PAE, the success of discontinuous dosing regimens may be attributed to both the function of a normal host defense and to the effects of subMICs[30].

These results demonstrated that CIN-102 is able to slow down bacterial growth of Gram-positive, Gram-negative, aerobic and anaerobic bacteria even at sub-inhibitory concentrations (CMI/2 and CMI/4). It would be of interest to study if subMICs of CIN-102 can alter other characteristics of bacteria.

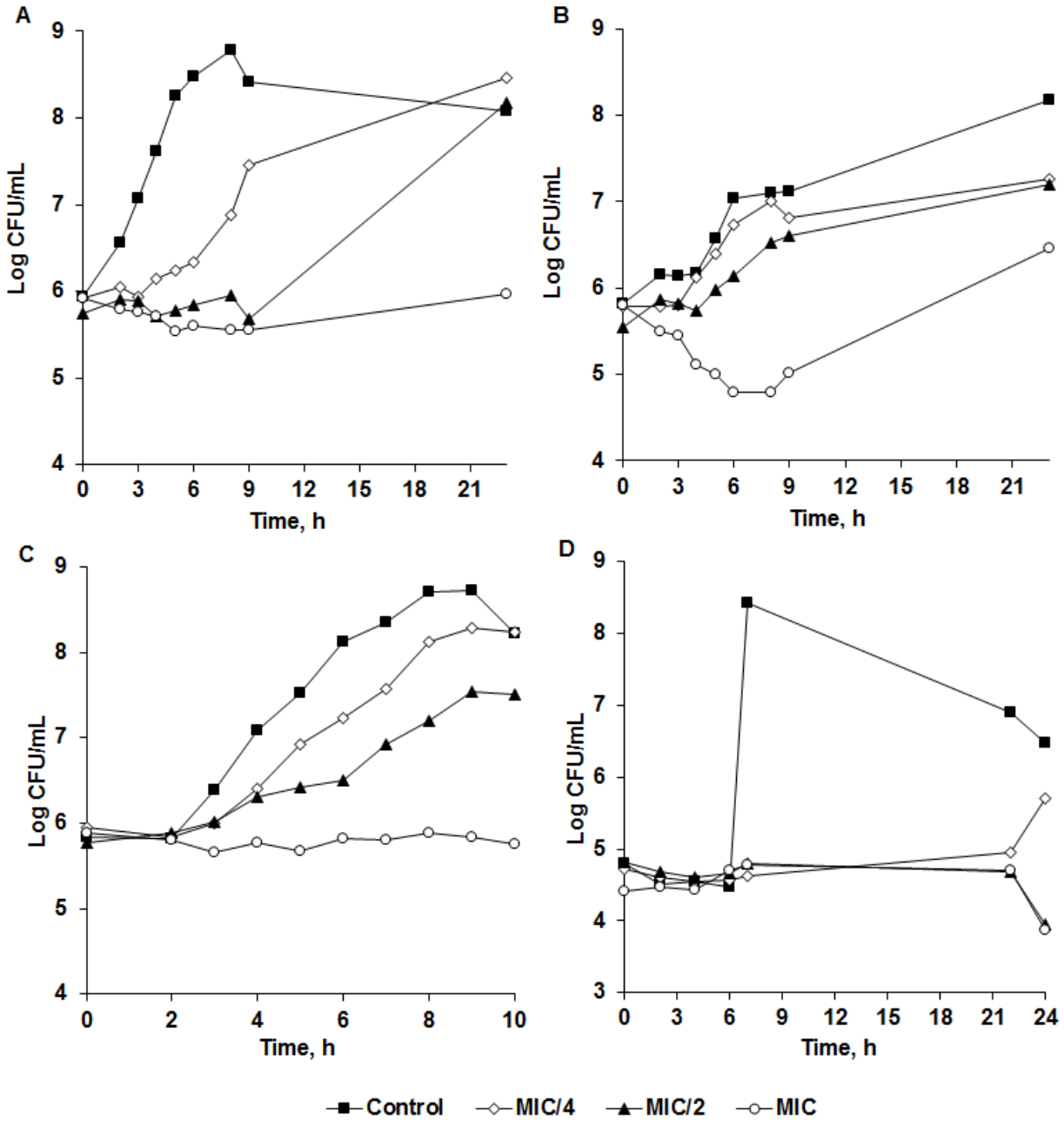

Figure 3: Effect of subMICs of CIN-102: Growth curves of A) E. coli, B) P. aeruginosa, C) S. aureus and D) C. perfringens exposed to CIN-102 at the MIC/2 and MIC/4. For reasons of comparison, CIN-102 at the MIC is also presented.

3.5. Interaction of CIN-102 with antibiotics

As clinicians would prefer to use a new antibacterial in combination with known antibiotics, rather than introducing a new wide spectrum monotherapy, the interaction of CIN-102 with commercialized antibiotics was studied. Among all the antibiotics tested in combination, no antagonism has been detected, and it was observed that colistin and aminoglycosides (gentamicin and amikacin) have often synergistic interactions with CIN-102 (Table 6).

Table 6: CIN-102 and antibiotic combinations (%)

	Amikacin N°strains=62				**Gentamicin** N°strains=57				**Tigecycline** N°strains=62				**Vancomycin** N°strains=62			
	S	**A**	**I**	**An**	**S**	**A**	**I**	**An**	**S**	**A**	**I**	**An**	**S**	**A**	**I**	**An**
Enterob	81	19	0	0	56	44	0	0	4	44	52	0	44	56	0	0
Non-ferm	45	40	10	0	47	47	7	0	20	20	60	0	55	45	0	0
Gpositive	93	7	0	0	87	13	0	0	7	87	7	0	13	73	13	0
Total	73	24	3	0	61	37	2	0	10	46	44	0	40	57	3	0
	Clindamycin N°strains=65				**Erythromycin** N°strains=62				**Imipenem** N°strains=61				**Colistin** N°strains=65			
	S	**A**	**I**	**An**	**S**	**A**	**I**	**An**	**S**	**A**	**I**	**An**	**S**	**A**	**I**	**An**
Enterob	73	20	7	0	22	78	0	0	38	42	19	0	93	7	0	0
Non-ferm	35	65	0	0	45	30	25	0	5	35	60	0	45	50	0	0
Gpositive	53	40	7	0	47	53	0	0	13	47	47	0	73	27	0	0
Total	57	38	5	0	36	56	8	0	21	41	38	0	74	25	1	0

S=synergy; A=addition; I=indifference; An=Antagonism; Enterob=Enterobacteria; Non-fermen=Non-fermentative Gram-negative bacteria and Gpositive=Gram-positive bacteria

Colistin possess a detergent-like mechanism, interfering with the structure and function of the outer and cytoplasmic membranes of bacteria. The insertion of colistin and its self-promoted uptake disrupts the outer membrane, release lipopolysaccharides and leads to leakage of intracellular components with subsequent death[31]. Possible mechanisms of synergism between CIN-102 and colistin might be a potentiation of the outer membrane disruption and cytoplasmic membrane permeabilization, leading to an important leakage of intracellular components. Another explanation could be the enhancement of the penetration CIN-102 components that would bind their intracellular targets, as a result of an increased membrane permeability by colistin. However, it has been shown that polymyxin-mediated cell death takes place apparently prior to cytoplasmic membrane depolarization. This suggests that there are multiple potential anionic targets, some of which are cytoplasmic[32]. Perhaps a possible synergism between the intracellular targets of CIN-102 and colistin exists besides outer membrane permeabilization.

Aminoglycosides cross the outer membrane by a self-promoted uptake and bind to the 30S ribosomal subunit leading to the production of misread proteins that cause membrane damage when incorporated into the membrane, which further stimulates the influx of the antibiotic. A rapid expanding cycle is triggered and the intracellular antibiotic eventually reaches a concentration that blocks all the initiating ribosomes preventing further protein synthesis[33]. The uptake of aminoglycosides could favor the penetration of CIN-102 components, which would be enhanced as the misreading proteins are further inserted into the membranes. In the same way, CIN-102 hydrophobic compounds could favor the penetration of aminoglycosides. Synergy might also occur from the different intracellular targets of aminoglycosides and CIN-102. Cinnamaldehyde can alter gene regulation reducing the expression of protein, RNA, 50S and 30S ribosomal protein synthesis[23], and this could potentiate the mechanism of aminoglycosides. In addition, it has been shown that gentamicin can interact with the outer membrane causing increased permeabilization[34]. This would further favor the penetration of CIN-102 components or lead to a more serious damage of the membranes when interacting with the terpenoid compounds of CIN-102. It has been proved that sub-lethal concentrations of amikacin interfere with cell division through an impairment in the formation of the Z ring[35]. This could explain the synergistic activity between amikacin and CIN-102, as cinnamaldehyde is able to bind FtsZ preventing the assembly of the Z ring[20].

Nevertheless, the most remarkable aspect for MDR strains, is that when antibiotics are combined with CIN-102, resistance is overpassed. To better illustrate these results, the CIN-102 – antibiotic interactions from 8 multi-resistant bacterial strains tested are represented in Table 7 and 8. First, the MIC of the antibiotics and CIN-102 alone are presented (Table 7). Then the MICs of a single combination CIN-102 – antibiotic and the respective Σ FIC index is shown (Table 8).

Table 7: MIC of antibiotics and CIN-102 for MDR strains

	MIC (mgL^{-1})								
	CIN-102	**Imipenem**	**Gentamicin**	**Vancomycin**	**Erythromycin**	**Clindamycin**	**Colistin**	**Amikacin**	**Tigecycline**
Escherichia coli	300	16*	64*	64	64	64	4*	64*	1
Klebsiella pneumoniae	600	16*	64*	64	64	64	16*	16	2
Serratia marcescens	600	64*	8*	64	64	64	32*	64	2
Enterobacter cloacae	300	16*	64*	64	64	64	8*	16	2
Pseudomonas aeruginosa	1250	16*	64*	64	64	64	8*	4	32
Acinetobacter baumannii	300	2	64*	64	4	64	8*	32*	4
Staphylococcus aureus	300	0.25	1	1	8*	32*	32	16	0.5
Enterococcus faecium	600	64*	64	64*	64*	64	32	64	1*

**:* Resistant Bacteria*,* according to: The European Committee on Antimicrobial Susceptibility Testing (EUCAST)[36].

Table 8: Interaction CIN-102 – Antibiotics for MDR strains

	MIC (mg/L) in combination (CIN-102/Antibiotic) Σ FIC index							
	Imipenem	**Gentamicin**	**Vancomycin**	**Erythromycin**	**Clindamycin**	**Colistin**	**Amikacin**	**Tigecycline**
Escherichia	37.5/4	5/16	300/0.03	150/4	150/0.03	20/1	37.5/0.5	5/0.5
coli	**0.38**	**0.27**	1.00	0.56	**0.50**	**0.32**	**0.13**	0.52
Klebsiella	150/4	150/1	300/0.03	300/0.03	150/0.03	37.5/2	37.5/0.06	300/0.03
pneumoniae	**0.50**	**0.27**	**0.50**	**0.50**	**0.25**	**0.19**	**0.07**	0.52
Serratia	150/16	150/1	150/0.06	300/4	150/0.25	75/4	75/0.06	300/0.03
marcescens	**0.50**	**0.38**	**0.25**	0.56	**0.25**	**0.25**	**0.13**	0.52
Enterobacter	37.5/4	5/0.25	300/0.03	150/16	150/0.03	75/1	5/0.25	300/0.03
cloacae	**0.38**	**0.02**	1.00	0.75	**0.50**	**0.38**	**0.03**	1.02
Pseudomonas	600/8	600/1	600/0.25	600/0.5	1250/0.03	150/4	600/0.06	5/16
aeruginosa	0.98	**0.50**	**0.48**	**0.49**	1.00	0.62	**0.5**	**0.50**
Acinetobacter	5/1	150/0.03	150/0.25	150/0.25	150/0.25	37.5/2	75/4	300/0.03
baumannii	0.52	**0.50**	**0.50**	0.56	**0.50**	**0.38**	**0.38**	1.01
Staphylococcus	5/0.125	5/0.25	150/0.06	75/0.03	75/8	75/1	5/4	5/0.25
aureus	0.52	**0.27**	0.56	**0.25**	**0.50**	**0.28**	**0.27**	0.52
Enterococcus	600/0.03	150/0.25	150/2	150/4	600/0.03	600/0.03	150/1	5/0.5
faecium	1.00	**0.25**	**0.28**	**0.31**	1.00	1.00	**0.27**	0.51

Synergy : ΣFIC index ≤0.5

As seen in Table 7, all these four enterobacteria (*E. coli, K. pneumoniae, S. marcescens* and *E. cloacae*) are resistant to imipenem, gentamicin and colistin, whose MICs decreases between 4- and 256- fold when combined with subMICs of CIN-102, resulting in synergistic interactions. For *E. coli*, also amikacin-resistant, a synergistic interaction with CIN-102 subMIC decreases the MIC of amikacin by a factor of 128. For non-fermentative Gram-negative bacteria: *P. aeruginosa* imipenem, gentamicin and colistin-resistant shows a decrease of 2-, 64- and 2-fold their MICs respectively in association with CIN-102 subMICs. And even though *A. baumannii* is resistant to colistin and amikacin, these antibiotics present synergistic interactions with subMICs of CIN-102, decreasing their MICs by a factor of 4 and 8 respectively. Furthermore, antibiotics like vancomycin, erythromycin and clindamycin with marginal activity against Gram-negative, exhibit an enhanced activity against these bacteria when associated with CIN-102, shown by a decrease between 4- and 2133-fold in their MICs. Multi-resistant Gram-positive bacteria (*S. aureus* and *E. faecium*) become susceptible when CIN-102 is combined with these antibiotics, reducing their MICs between 4- and 2133-fold. Additionally, against Gram-positive bacteria, antibiotics like gentamicin, colistin and amikacin decrease their MICs between 4- and 1066-fold when associated with subMICs of CIN-102.

PSMs are good sources for combination therapy with antibiotics, as there is evidence that they act as multidrug resistance modifiers, restoring the activity of antibiotics against resistant bacteria. These compounds can reduce the expression of modified targets, inhibit enzymes able to degrade the antibiotic, increase the permeability of the OM and inhibit efflux pumps facilitating the accumulation of antibiotic within bacterial cell[37]. Further studies are needed to understand how the components of CIN-102 can improve the effectiveness of antibiotics which might involve several targets. In this regard, Dhara *et al.*[38] have shown that cinnamaldehyde might be able to interact with ESBLs like TEM-2, SHV-2, CTXM-9 by cleaving this enzymes through hydrogen bonds with catalytic and other crucial amino acid residues in these proteins.

These interactions show that CIN-102 improves the effectiveness and restores the action of antibiotics. Through these combinations, the MICs of antibiotics can be reduced and then the potential side effects of antibiotics can be minimized or even the emergence of antibiotic resistance using monotherapy could be prevented.

4. Conclusion

CIN-102 is a broad-spectrum antibacterial agent of. All bacteria tested, including several multi-resistant clinical isolates, are susceptible to CIN-102. The MICs are higher as those found for antibiotics but animal studies carried out suggested that the active concentrations of CIN-102 are effective in infection models and seem to be compatible with a therapeutic use (data not shown). This activity is associated with a rapid 1 log-

reduction of the bacterial inoculum, an important PAE and the capacity of sub-inhibitory concentrations to slow down bacterial growth. The most important fact is that CIN-102 in combination, could allow the re-utilization of antibiotics that are ineffective against multi-resistant bacteria. Nowadays, aminoglycosides and colistin are sometimes the only antibiotics that possess activity against MDR bacteria. If these antibiotics would remain ineffective or if the resistance would become more important, CIN-102 alone or in combination could be an encouraging alternative. The promising *in vitro* activity has to be confirmed by further clinical studies.

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CHAPTER III

ABOUT CIN-102 SUSTAINED RELEASE FORMULATIONS

Submitted manuscript, revised version: *European Journal of Pharmaceutics and Biopharmaceutics*

1. Introduction

Research on Intestinal Bowel Diseases (IBD) suggests that these disorders occur as an inappropriate immune response in genetically susceptible individuals as a result of a complex interaction between environmental and microbial factors and the intestinal immune system. However, the exact causes and mechanisms of IBD are not yet completely understood[1]. Nevertheless, bacteria seem to play an important role in the pathogenesis of IBD, as patients suffering from these diseases show an increased number of bacteria in their intestinal mucus layer. Also, lesions are preferentially observed in segments with the highest concentrations of bacteria; and IBD patients show an enhanced systemic and mucosal immunological reactivity against bacterial antigens[2]. There is indeed major evidence that IBD is due to an abnormal immune response to the bacterial flora[3]. The perturbations in the homeostasis between the immune system and the commensal flora can lead to gastro intestinal tract inflammation. In this situation the commensal flora appears to act as a pathogenic community, starting a lifelong inflammation because the host responses are incapable of controlling the flora[4]. The increasing evidence that gastro intestinal bacteria play a role in the pathogenesis and the development of IBD and the persistence of inflammation, provides the rational for research on the use of antibacterial agents in the treatment of these diseases: as modulator of the gastro intestinal flora or as adjuvants in the treatment of gastro intestinal tract inflammation[2,5]. In this respect, antibiotics may act via different pathways: They may for instance inhibit bacteria linked to the pathological process of the disease, or they may lower the luminal and/or mucosal bacterial overgrowth. The modification of the intestinal flora may reduce the strength of certain symptoms, including pain and diarrhea. Research in this field suggests that antibiotics can indeed improve clinical outcomes in patients suffering from an acute relapse of IBD[6]. For example, significant beneficial clinical effects have been reported for ciprofloxacin[7] and rifaximin[8] (versus placebo) in the treatment of IBD patients.

However, antimicrobial resistance is a major concern for any antibiotic treatment. It is considered as an increasing threat to public health, with enormous economic and human costs, that requires a global action from the governments, academia and industry[9]. Despite the urgent need for new antimicrobials, there is a decline in the antibiotic research and in the industrial development pipeline, as evidenced by the decrease in discovery research and in antibacterial trials conducted by the pharmaceutical industry[10]. The perceived lack of return on investment in the field of antibacterial research has led industry to focus more on markets of chronic diseases, rather than acute infectious diseases. It seems that antibiotics are not perceived as being essential to health, however, they have saved millions of lives since these drugs were introduced in the early 1930s, permitting individuals to live for many years after infection[11].

As stated by Bush *et al.*[9], in the battle against antimicrobial resistance, natural products appear as a vast reservoir of bioactive compounds available in nature that merit to be exploited. Plant secondary metabolites, like essential oils, are provided of an inherent antimicrobial activity as defense mechanism to survive in a

competing environment. These antimicrobial defenses are the result of a long evolutionary process. Therefore, they should be reconsidered as a good starting point in the development of new antimicrobial agents[12]. Furthermore, it is known that antibiotics that act on more than one target are advantageous to avoid the development of bacterial resistances[13]. This is an inherent major advantage of many multi-component essential oils[14]. Although the antimicrobial activity of essential oils seems to be a property of the assembly of their components, it might be necessary to "reengineer" these botanical mixtures in order to render their production more reproducible[15]. Following this aim of antibiotic discovery based on natural products, a chemically well-defined, synergistic blend (called "CIN-102") has been developed by Septeos. The composition of CIN-102 resembles the composition of cinnamon essential oils (Table 1). Its major component is cinnamaldehyde (86.7 % w/w). CIN-102 has a broad-spectrum antibacterial activity and no resistance has been observed so far (unpublished data).

The hypothesis of this study was that the delivery of the broad-spectrum antibiotic CIN-102 to the lower gastrointestinal tract can ameliorate the state of colitis via a reduction in the total bacterial loads and/or the number of bacterial pathogens in the colon. Preliminary studies in trinitrobenzene sulfonic acid (TNBS) treated rats revealed that oral gavage of pure CIN-102 had only slight effects on bacterial flora (data not shown). Thus, it was concluded that adequate controlled oral drug delivery systems are required to assure minimal effective antibiotic concentrations at the site of action – the colon. The aim of these dosage forms is to minimize undesired premature drug release in the upper gastro intestinal tract and to control drug release in the colon[16,17,18,19,20]. Different types of multiparticulate dosage forms were prepared, offering major advantages compared to single unit dosage forms, such as a more homogeneous distribution within the contents of the gastro intestinal tract and a reduced risk of dose dumping[21]. Coated pellets as well as uncoated mini-tablets were studied. In the first case, an outer polymeric film coating (sensitive to colonic bacterial enzymes) was used to assure the onset of drug release at the site of action. In the case of mini-tablets, the incorporation of inulin (which is preferentially degraded by colonic bacterial enzymes[22] is intended to provide the site-specific delivery of CIN-102 along the gastro intestinal tract. A particular formulation challenge was the fact that CIN-102 is a volatile hydrophobic liquid.

The objective of this study was to prepare multiparticulate dosage forms for CIN-102, able to minimize undesired drug release in the upper gastro intestinal tract and to control the release of the novel broad spectrum antibacterial agent in the colon. The systems were tested *in vitro* as well as *in vivo* (in mice suffering from colitis). The idea was to reduce the animals' bacterial loads, especially in the intestinal mucosa in order to improve the state of intestinal inflammation.

2. Materials and methods

2.1. Materials

CIN-102, a well-defined blend of trans-cinnamaldehyde, trans-2-methoxycinnamaldehyde, cinnamyl acetate, linalool, β-caryophyllene, cineol and benzyl benzoate (see Table 1 for the exact composition) (Septeos, Paris, France); ethylcellulose powder (Ethocel Standard 10 FP Premium; Dow, Knoxville, TN; USA); microcrystalline cellulose (MCC, Avicel PH 101) and an aqueous ethylcellulose dispersion (Aquacoat ECD 30D, 30 % solids content) (FMC Biopolymer, Brussels, Belgium); an aqueous dispersion of poly(vinyl acetate) (27 %), containing also poly(vinyl pyrrolidone) (2.7 %) and sodium lauryl sulfate (0.3 %) [30 % solids content, Kollicoat SR 30D; BASF, Ludwigshafen, Germany]; triethyl citrate (TEC; Alfa Aesar, Karlsruhe, Germany); magnesium aluminometasilicate (Neusilin US2, neutral grade; Fuji Chemical Industry, Toyama, Japan); polyethylene sorbitan monooleate (Tween 80; Panreac, Barcelona, Spain); poly(vinyl alcohol) (Opadry; Colorcon, Dartford Kent, UK); lactose (Lactochem fine powder; Domo, Veghel, The Netherlands); inulin (Orafti HP; Beneo Orafti, Oreye, Belgium); peas starch (Peas starch; Roquette Freres, Lestrem, France); silicon dioxide (Syloid 244 FP; Grace Davison, Worms, Germany); glyceryl tristearate (Dynasan 118; Sasol, Witten, Germany); dextran sodium sulfate (DSS; TdB, Uppsala, Sweden); acetonitrile (VWR, Fontenay-sous-Bois, France); methanol (Fisher Scientific, Loughborough, UK); cysteinated Ringer's solution (Merck, Darmstadt, Germany).

Table 1: Composition of CIN-102

Component	% (w/w)
Trans-cinnamaldehyde	86.7
Trans-2-methoxycinnamaldehyde	5.35
Cinnamyl acetate	2.5
Linalool	2.4
β-Caryophyllene	1.7
Cineol	1
Benzyl benzoate	0.35

2.2. Preparation of coated pellets

2.2.1. Preparation of drug-laded pellet starter cores

Three types of CIN-102-loaded pellet cores were prepared by extrusion-spheronization. Table 2 shows their theoretical compositions. The respective excipients, CIN-102 and purified water (400, 600 and 800 mL for 1 kg MCC/lactose/Tween, MCC/lactose/Syloid, and MCC/Ethocel/Neusilin cores) were blended in a high speed granulator (Gral 10; Collette, Antwerp, Belgium). The mixtures were

passed through a cylinder extruder (SK M/R; 1 mm diameter holes, rotation speed = 96 rpm; Alexanderwerk, Remscheid, Germany). The extrudates were subsequently spheronized at 520 rpm for 30 s (Spheronizer Model 15; Calveva, Dorset, UK) and dried in a fluidized bed at 30°C for 30 min (Strea 1; Aeromatic, Bubendorf, Switzerland). Pellets in the size range of 710-1000 µm were obtained by sieving.

2.2.2. Pellet coating

Pellets were coated with peas starch: ethylcellulose or inulin: Kollicoat SR blends. Aquacoat ECD 30D was used as an aqueous ethylcellulose dispersion. Kollicoat SR 30D is an aqueous dispersion of poly(vinyl acetate) (27 %), containing also poly(vinyl pyrrolidone) (2.7 %) and sodium lauryl sulfate (0.3 %). Aquacoat ECD 30D and Kollicoat SR 30D were plasticized overnight with triethyl citrate (25 and 10 % w/w of the solid contents, respectively) under magnetic stirring. Peas starch and inulin were dissolved in purified water (5 and 7% w/w, respectively) and mixed with plasticized Aquacoat ECD 30 D or Kollicoat SR 30D dispersion at different ratios (w/w polymer mass, as indicated). The blends were stirred for 2 h prior to coating. Optionally, an Opadry sub-coating was applied. For this Opadry was dissolved in purified water (15% w/w).

CIN-102-loaded pellet cores were coated in a fluidized bed (Strea 1), equipped with a Wurster insert. The process parameters for polymer blends were as follows: inlet temperature = 38 °C, product temperature = 40 °C, spray rate = 1.5 g/min, atomization pressure = 1.2 bar, nozzle diameter = 1.2 mm. Pellets were coated until a weight gain of 15, 20, 25 or 30 % (w/w) was achieved (as indicated). Finally, the pellets were cured in an oven at 60 °C for 24 h. The process parameters for the Opadry sub-coating were as follows: inlet temperature = 30 °C, product temperature = 35°C, spray rate = 0.7 g/min, atomization pressure = 1.2 bar, nozzle diameter = 1.2 mm. In this case, the coating level was 7 % (w/w). At the end, the pellets were further fluidized in the coater without spraying any liquid during 15 min (for drying).

2.3. Preparation of mini-tablets

Mini-tablets were prepared by direct compression. Table 3 shows the theoretical compositions of the investigated systems (further compositions are described in the text). CIN-102 was adsorbed onto Neusilin to obtain a powder (upon manual mixing in a mortar). Ethocel, Dynasan 118 and/or inulin were added under continuous manual mixing. The obtained blends were compressed using a Frank Universalpruefmaschine 81816 (Karl Frank, Weinheim-Birkenau, Germany) into flat-faced cylinders (diameter = 2 mm, height = 2 mm). The compression force was 300 N and the compression time 10 s, unless otherwise stated.

2.4. *In vitro* drug release

To monitor the release of CIN-102, the release of its main component (cinnamaldehyde, 86.7 % w/w) was measured. In the case of coated pellets, 500, 80 and 85 mg samples (MCC/lactose/Tween, MCC/lactose/Syloid and MCC/Ethocel/Neusilin cores) were placed into 120 mL cylindrical flasks, filled with 100 mL release medium: 0.1 M HCl during the first 2 h, which was completely exchanged with phosphate buffer pH 7.4 for the following 6 h. The flasks were agitated in a horizontal shaker (80 rpm; GFL 3033, Gesellschaft fuer Labortechnik, Burgwedel, Germany) at 37°C. At pre-determinated time points, 3 mL samples were withdrawn, replaced with fresh medium, and analyzed UV-spectrophotometrically (λ = 290 nm in 0.1 M HCl and λ = 291 nm in phosphate buffer pH 7.4) (Shimadzu UV-1650, Champs sur Marne, France). In the case of mini-tablets, 1 mini tablet was placed in a 2.5 mL Eppendorf tube, filled with 2 mL 0.1 M HCl during the first 2 h, followed by phosphate buffer pH 7.4 for the subsequent 6 h. The tubes were horizontally shaken at 37°C (80 rpm, GFL 3033). At pre-determined time points, the release medium was completely replaced. The cinnamaldehyde content in the withdrawn samples was measured by UV-spectrophotometry, as described above. After the last measurement time point, the amount of cinnamaldehyde potentially remaining within the mini-tablet remnants was determined as follows: The residues were dissolved in 2 mL acetonitrile, and the cinnamaldehyde content in the liquid was determined by HPLC analysis (Varian ProStar 230, 410 autosampler, 325 UV-Vis detector, Galaxie software; Varian, Les Ulis, France). A reversed phase C18 column (Luna 5µm, 110 Å, 150mm x 4.6mm, Phenomenex, France) was used. The mobile phase consisted of an acetonitrile:methanol:water mixture (20:35:45, v/v/v). Thirty µL samples were injected, the flow rate was 1 mL/min, the detection wavelength 290 nm. Each experiment was conducted in triplicate and sink conditions were maintained throughout all experiments.

Table 2
Theoretical composition of the investigated pellet cores loaded with CIN-102 (%, w/w)

Compound \ Core type	MCC/lactose/Tween	MCC/lactose/Syloid	MCC/Ethocel/Neusilin
CIN-102	1	14.5	10
MCC	45	38.75	55
Lactose	45	38.75	-
Tween 80	9	-	-
Syloid 244 FP	-	8	-
Ethocel	-	-	25
Neusilin	-	-	10

Table 3
Theoretical composition of the investigated mini-tablets loaded with CIN-102 (%, w/w) (further compositions are described in the text)

Compound \ Mini-tablet type	Neusilin/Dynasan	Neusilin/Inulin/Dynasan				Neusilin/Ethocel/Inulin/Dynasan									
CIN-102	4	4				4					4				
Neusilin	1.33	1.33				1.33					1.33				
Ethocel						25					50				
Inulin		1	5	10	25	5	10	15	20	25	5	10	15	20	25
Dynasan	94.67	93.67	89.67	84.67	69.67	64.67	59.67	54.67	49.67	44.67	39.67	34.67	29.67	24.67	19.67

2.5. Equilibrium solubility measurements

The solubility at equilibrium of cinnamaldehyde was determined in agitated flasks in 0.1 M HCl and phosphate buffer pH 7.4 at 37 °C. An excess amount of CIN-102 was exposed to 100 mL bulk fluid under horizontal shaking (80 rpm; GFL 3033). Every day, samples were withdrawn, filtered and analyzed by UV for their cinnamaldehyde content (as described above) until equilibrium was reached. Each experiment was conducted in triplicate.

2.6. mDSC analysis

Modulated Differential Scanning Calorimetry (mDSC) experiments of polymeric film samples were performed with a DSC Q200 (TA Instruments, Guyancourt, France). Temperature and enthalpy readings were calibrated using pure indium at the same scan rates and with the same kind of pans as used for the measurements. Thin films were obtained by spraying the same formulations as used for the preparation of the coated pellets, onto Teflon plates and subsequent controlled drying at 60 °C for 24 h. Approximately 5 mg samples were heated in open pans (containers without lids) from −20 to 100 °C at 5 °C/min using a modulation amplitude of ±0.663 °C and a modulation period of 50 s. The samples were flushed with highly purified nitrogen gas. Two heating cycles were run, the reported data was obtained from the second cycle.

2.7. Mouse study

An *in vivo* study with seven-week-old, male mice (C57BL6; Janvier Laboratory, Le Genest St. Isle, France) was conducted in the animal facilities "Animalerie Haute Technologie" at the School of Medicine of the University of Lille (Lille, France), respecting all governmental guidelines (including n°2010/63/UE; Décret 2013-118; EC Directive 86/609/EEC) and ethical rules. The animals were housed with free access to commercial rodent food and water. *Fig. 1* shows the study design. At the beginning of the experiment (Day 1), the mice were divided into 3 groups (10 animals in each group): (i) One group received only standard chow (negative control). (ii) One group received standard chow mixed with CIN-102-loaded coated pellets (50 mg cinnamaldehyde/kg/day) [MCC/Ethocel/Neusilin cores; 7 % Opadry sub-coating; 30 % inulin:Kollicoat SR (1:4) outer coating]; (iii) One group received standard chow mixed with CIN-102-loaded mini-tablets (50 mg cinnamaldehyde/kg/day) [4 % CIN-102 (theoretical), 1.33 % Neusilin, 50 % Ethocel, 25 % inulin, 19.67 % Dynasan]. The disease was induced from Day 4 on by adding 2.5 % (w/v) dextran sodium sulfate to the drinking water. The course of the colitis was evaluated by monitoring the body weight, presence of blood in stools and occurrence of diarrhea. On Day 12 the animals were

sacrificed. Samples of the luminal content and of sections of the colon were used for bacteriological analysis and colonic tissue samples for immunological analysis.

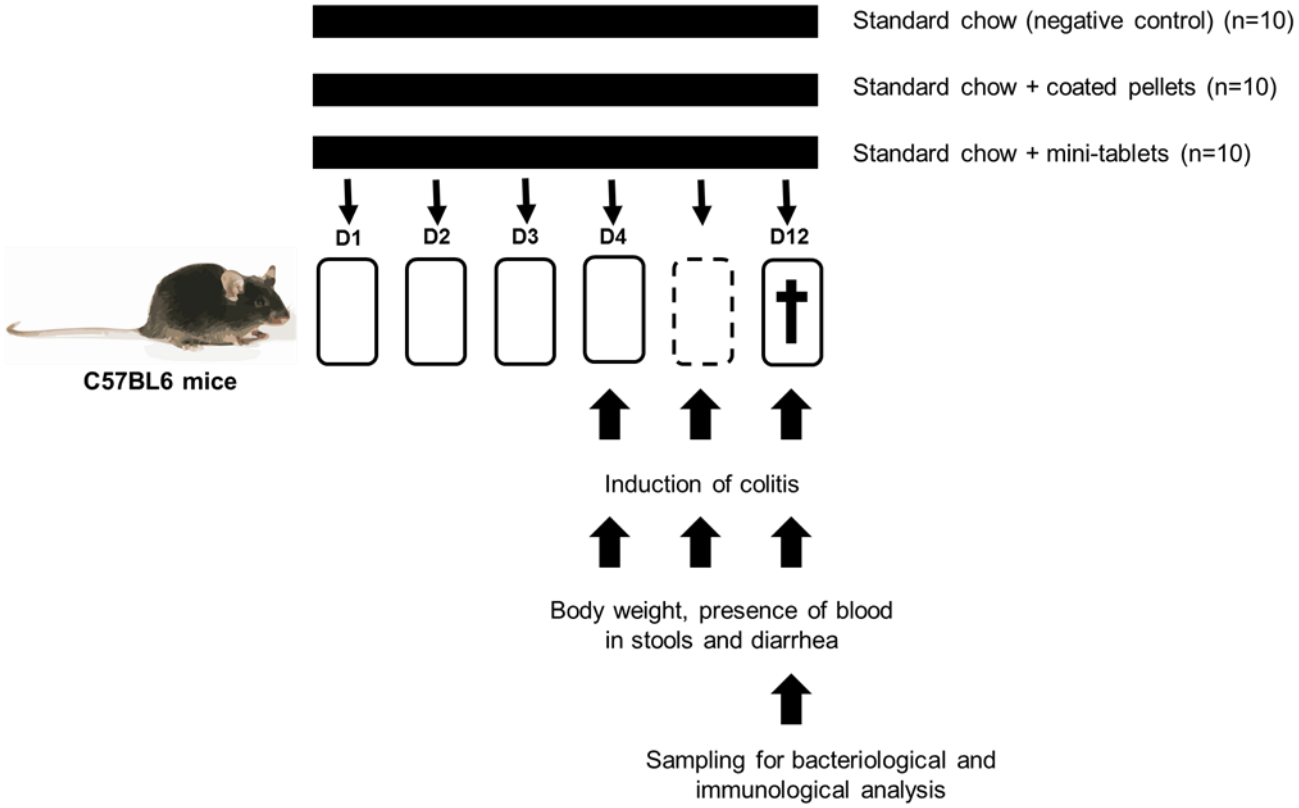

Fig. 1: ***In vivo mouse study design: Oral administration of coated pellets and mini-tablets began on Day 1. Colitis was induced by adding dextran sodium sulfate in drinking water from Day 4 on. The animals were sacrificed on Day 12. The course of colitis was evaluated by monitoring the body weight, presence of blood in stools and diarrhea, as well as by bacteriological and immunological analysis.***

The colonic tissue and luminal content samples were introduced into pre-weighed vials containing 1.5 mL cysteinated Ringer's solution. After manual physical disruption, 10-fold dilutions were made in the same diluent (until 10^{-4} for colonic section samples and 10^{-6} for luminal content samples, respectively). Each dilution was spread onto plates of non-selective blood agar: modified Columbia agar [Columbia blood agar base (Oxoid, Basingstoke, England), glucose (5 g L^{-1}), agar (5 g L^{-1}), cysteine hydrochloride (0.5 g L^{-1}) supplemented with defibrinated horse blood (5%)][23] and incubated at 37°C for 1 week under anaerobic conditions. McConkey (selective for enterobacteria) as well as Dcoccosel (selective for enterococci) plates (BioMerieux, Marcy l'Etoile, France) were incubated at 37 °C for 48 h under aerobic conditions. Man, Rogosa, Sharpe (selective for lactobacilli) plates were incubated at 37 °C for 48 h under CO2-enriched conditions. Total counts were performed, and different types of colonies were sub-cultured and identified based on established morphological and biochemical criteria. Quantitative results are expressed in "log colony forming

units per gram" (CFU g^{-1}). The threshold of detection was 2.5 x 103 CFU g^{-1} for colonic section samples and 4.8 x 102 CFU g^{-1} for luminal content samples, respectively.

Total RNA was extracted from colonic tissue samples using the NucleoSpin RNAII commercial kit (Macherey-Nagel, Hoerd, France), following the manufacturer's instructions. cDNA was prepared using the High Capacity cDNA Archive kit (Macherey-Nagel, Hoerd, France), and quantitative Polymerase Chain Reaction (qPCR) was performed using SyBrGreen (Applied Biosystems, Saint-Aubin, France). Expression levels of TNFα, IL-1β, IL-6, IL-10 were measured. Polr2A was used as a reference gene.

Two tail paired t-tests were used to determine if the mean of measurements from one condition is different from the mean of another condition, using 95% confidence intervals.

3. Results and discussion

In order to provide controlled release of CIN-102 in the lower part of the gastro intestinal tract, this volatile hydrophobic liquid blend was incorporated into: (i) pellet starter cores, which were subsequently coated with appropriate polymer blends, or (ii) mini-tablets. For reasons of simplicity, only the release of the main compound of the liquid mixture CIN-102 was monitored during the *in vitro* drug release studies: the release of cinnamaldehyde (the main compound with antibacterial activity, constituting 86.7 % w/w of the blend).

3.1. CIN-102-loaded coated pellets

Pellet starter cores were prepared by extrusion-spheronization and subsequently coated in a fluidized bed with blends of a water-insoluble polymer and a polymer, which is known to be preferentially degraded by bacterial enzymes in the colon. Since CIN-102 is volatile, 30-50 % of the liquid drug got lost during the manufacturing procedure (e.g., during pellet core drying in a fluidized bed for 30 min at 30°C, and pellet curing subsequent to coating in an oven at 60 °C for 24 h). Fig. 2A shows the resulting cinnamaldehyde release kinetics from pellets consisting of: (i) cores prepared with 1 % CIN-102, 45 % MCC, 45 % lactose and 9 % Tween 80, and a (ii) coating based on a peas starch:ethylcellulose (1:2) blend. The coating level was 15 or 20 % (as indicated). The release medium was initially 0.1 M HCl. After 2 h, it was completely exchanged against phosphate buffer pH 7.4. An ideal formulation should release no or only negligible amounts of cinnamaldehyde under these conditions, simulating the transit through the upper gastro intestinal tract. However, as it can be seen, the investigated formulations released more than 50 % of the drug within the first 2 h. The rest was rapidly released upon medium change. This was true for both coating levels and indicates that these pellets are not suitable for controlled CIN-102 delivery to the lower gastro intestinal

tract. The reasons for their failure probably include: limited drug affinity to the pellets' core, high affinity of the drug to the film coating, and high permeability of the drug through the film coating. In addition, the presence of the surfactant Tween 80 likely facilitates water penetration into the system, drug dissolution and drug release. The significant increase in the release rate of cinnamaldehyde upon medium change might be attributable to the fact that the aqueous ethylcellulose dispersion Aquacoat ECD was used for film coating, containing small amounts of sodium lauryl sulfate (SLS): This surfactant is protonated (and non-charged) at low pH, while un-protonated (and negatively charged) at pH 7.4. It has been reported in the literature that in the case of films prepared from aqueous polymer dispersions, the presence of SLS can be responsible for pH-depended drug release if the polymer particles are not completely fused together: At pH 7.4, the SLS (being negatively charged) more effectively facilitates the penetration of water into the system, water being mandatory for drug dissolution and subsequent drug release[24]. It has to be pointed out that for all the investigated formulations, sink conditions were provided throughout the experiments: The solubility of cinnamaldehyde in 0.1 M HCl and phosphate buffer pH 7.4 was determined to be equal to 1.1 ± 0.04 g L^{-1} and 1.4 ± 0.05 g L^{-1} at 37 °C, respectively. The higher drug solubility at neural pH can also partially explain the observed increase in the drug release rate upon medium change (Fig. 2A), but it cannot be the only reason.

In order to slow down cinnamaldehyde release, the surfactant Tween 80 was omitted, and parts of the MCC and lactose in the pellets' cores were replaced by Syloid (highly porous silicon dioxide particles with a high surface area and adsorption capacity) to increase the affinity of the drug to the core. At the same time, the theoretical CIN-102 content was increased to 14.5 %, in order to compensate drug loss during preparation. However, as it can be seen in Fig. 2B, these strategies did not work out: The release rates became even faster, irrespective of the coating level. This might at least partially be explained by the fact that after extrusion-spheronization the surface of the pellets' cores appeared to be oily, indicating that the drug was not effectively taken up the Syloid. Also, the increased drug loading might facilitate drug release[25].

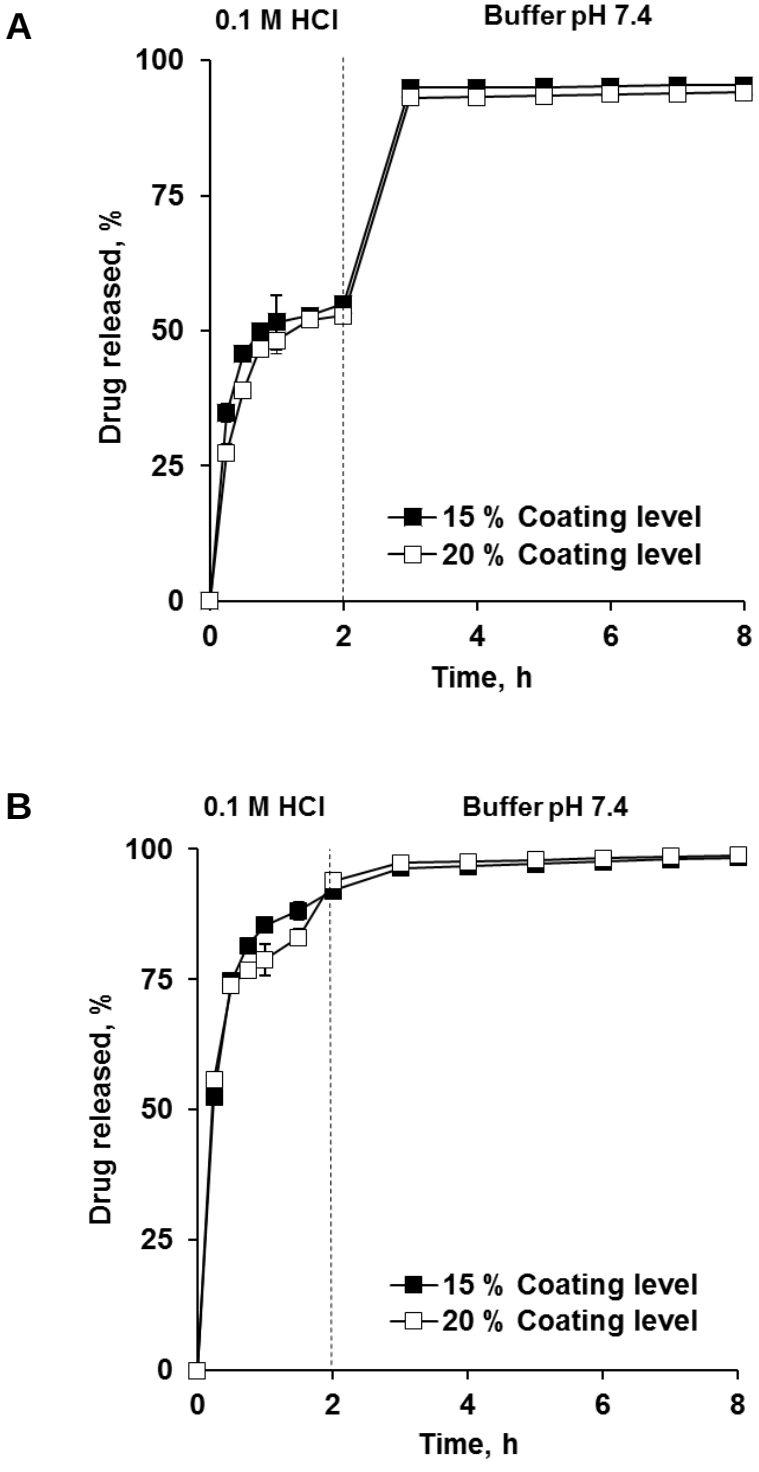

Fig. 2: Cinnamaldehyde release from CIN-102-loaded pellets, coated with peas starch:ethylcellulose (1:2) in 0.1 M HCl for 2 h, followed by phosphate buffer pH 7.4. The coating level was 15 or 20 % (as indicated), the pellet cores contained: A) MCC/lactose/Tween, or B) MCC/lactose/Syloid (exact compositions see Table 2) (mean values +/- SD are indicated).

To effectively slow down cinnamaldehyde release in 0.1 M HCl and phosphate buffer pH 7.4, the relative amount of ethylcellulose in the film coating was increased, the film coating thickness was increased, an Opadry-sub-coating was introduced, lactose omitted, Ethocel (ethylcellulose powder) introduced into the core, the drug and MCC loadings reduced, and Syloid replaced by Neusilin (magnesium aluminometasilicate, with a high adsorption capacity for oily compounds). The aim of the Opadry sub-coating [based on poly(vinyl alcohol)] was to reduce the risk of drug migration into the outer peas starch:ethylcellulose film coating. The introduction of ethylcellulose into the pellets' cores was intended to hinder water penetration into the system. Fig. 3 shows that these strategies were at least partially successful: Cinnamaldehyde release was sustained for 8 h at both coating levels. In addition, drug release was no more pH dependent (indicating improved film formation). But cinnamaldehyde release was still significant under these conditions.

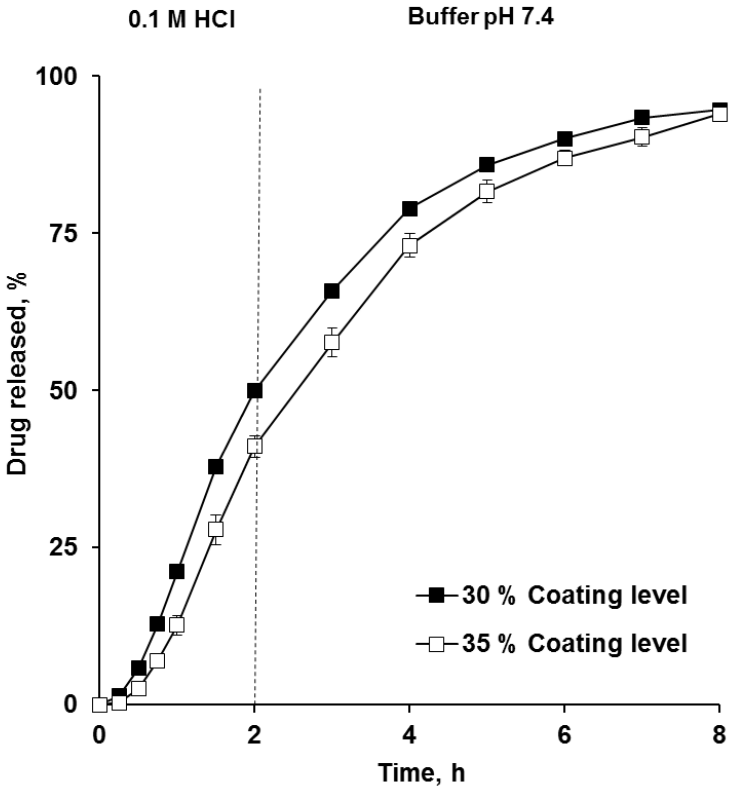

Fig. 3: Cinnamaldehyde release from CIN-102-loaded pellets, coated with peas starch:ethylcellulose (1:3) (and an Opadry sub-coating) in 0.1 M HCl for 2 h, followed by phosphate buffer pH 7.4. The coating level was 30 and 35 % (as indicated), the pellet cores contained MCC/Ethocel/Neusilin (exact composition see Table 2) (mean values +/- SD are indicated).

Thus, the coated pellets were further optimized: The controlled release film coating was replaced by a blend of inulin and Kollicoat SR (1:4). The pellet cores and the Opadry sub-coating remained unaltered. As it can be seen in Fig. 4, even at lower coating levels (20 and 30 %, respectively) drug release could be effectively further slowed down. This is probably due to a lower cinnamaldehyde affinity to these film coatings and/or a lower permeability of the latter for the drug. Fig. 5 shows the reversible signal of the mDSC scans of samples of thin film pieces of the same composition as the inulin:Kollicoat SR (1:4) outer film coatings. For reasons of comparison, inulin (pure substance) and Kollicoat SR films (plasticized or not) were also studied. As it can be seen, the plasticized inulin:Kollicoat SR films used for pellet coating showed a single glass transition temperature (Tg) at 33 °C. This corresponds to the Tg of the plasticized Kollicoat SR. Inulin did not show any thermal event in the investigated temperature range. This likely indicates that the polymeric pellet coating is in an amorphous state. Since water acts as an efficient plasticizer for many polymers[26], it can further be expected that the polymeric film coating is in the rubbery state during drug release.

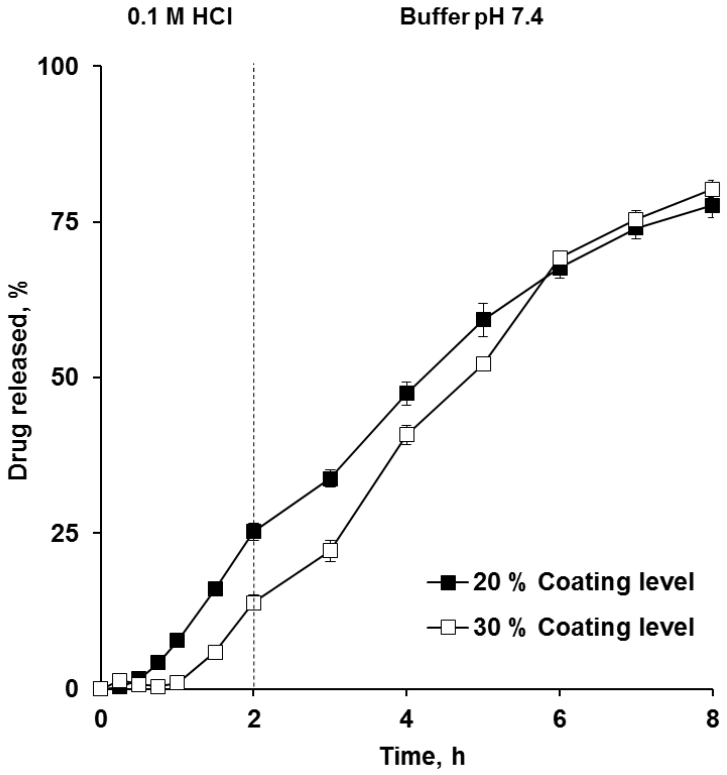

Fig. 4: Cinnamaldehyde release from CIN-102-loaded pellets, coated with inulin:Kollicoat SR (1:4) (and an Opadry sub-coating) in 0.1 M HCl for 2 h, followed by phosphate buffer pH 7.4. The coating level was 20 and 30 % (as indicated), the pellet cores contained MCC/Ethocel/Neusilin (exact composition see Table 2) (mean values +/- SD are indicated).

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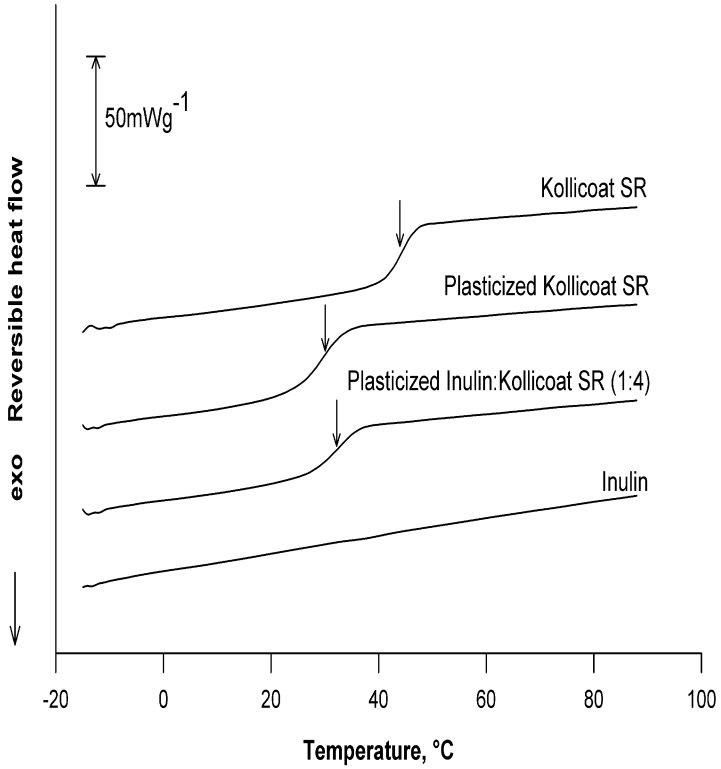


Fig. 5: mDSC Thermograms of film samples consisting of Kollicoat SR, plasticized Kollicoat SR, plasticized inulin:Kollicoat SR (1:4) or inulin (pure substance). The arrows mark glass transition temperatures

Based on these results, pellets containing MCC/Ethocel/Neusilin cores, a 7 % Opadry sub-coating and an inulin:Kollicoat SR (1:4) outer coating (30 %) were selected for *in vivo* studies.

3.2. CIN-102 mini-tablets

Mini-tablets were prepared by direct compression. To transform the liquid drug into a solid powder, CIN-102 was first adsorbed onto Neusilin (magnesium aluminometasilicate, with a high adsorption capacity for oily compounds). Then, Dynasan 118 (glyceryl tristearate) was added and flat-faced cylinders (diameter = 2 mm, height = 2 mm) prepared using a Frank Universalpruefmaschine 81816. The compression force was either 300 or 2000 N, the holding time either 10 or 120 s. Fig. 6 shows the resulting cinnamaldehyde release kinetics from these mini-tablets in 0.1 M HCl, followed by phosphate buffer pH 7.4. As it can be seen, drug release was slow in all cases and the impact of the investigated variations in the compression force and holding time were only minor. Based on these results, a compression at 300 N for 10 s was selected for all further experiments. Note that also in the case of mini-tablets, substantial amounts of CIN-102 (approximately 40 % in all cases) were lost during tablet preparation. This is due to the high volatility of the liquid blend. The analysis of the mini-tablet remnants at the end of the observation period revealed that the amounts of released

and non-released CIN-102 added up to 100 % of the practical tablet loading. This was true for all the investigated mini-tablets.

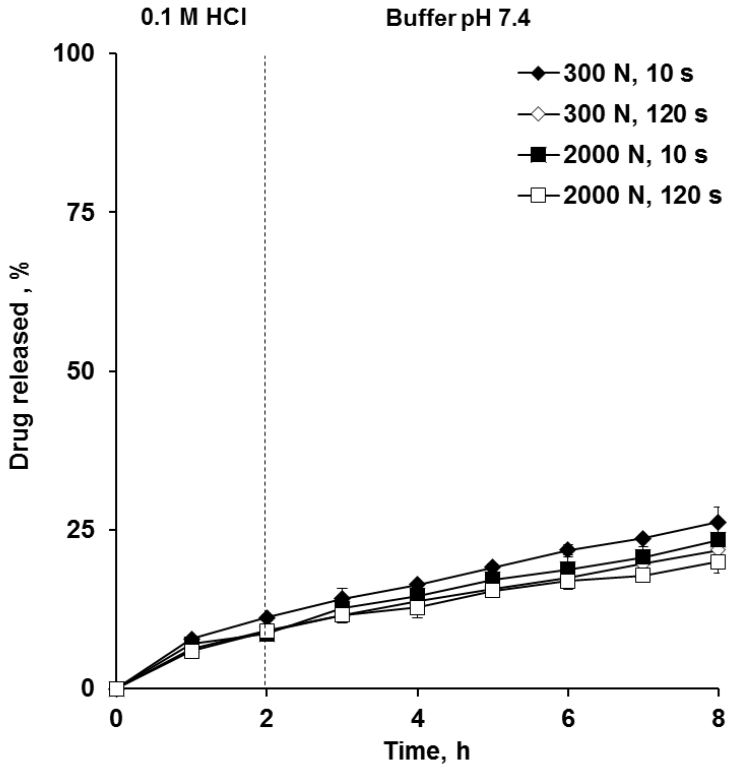


Fig. 6 Impact of the compression force and holding time (indicated in the diagram) on cinnamaldehyde release from CIN-102-loaded mini-tablets in 0.1 M HCl for 2 h, followed by phosphate buffer pH 7.4. The mini-tablets consisted of 4 % CIN-102 (theoretical), 1.33 % Neusilin and 94.67 % Dynasan (mean values +/- SD are indicated).

In order to provide an onset of drug release in the colon, different amounts of inulin were incorporated into the mini-tablets. As it can be seen in Fig. 7A, the introduction of up to 25 % inulin led to an undesired increase in the resulting drug release rate (due to the higher permeability of this hydrogel forming polymer compared to glyceryl tristearate). To reduce the cinnamaldehyde release rate in media simulating the upper gastro intestinal tract, Ethocel (ethylcellulose powder) was added to the mini-tablets. Figs. 7B and C show the observed drug release profiles from mini-tablets containing 25 or 50 % Ethocel and up-to 25 % inulin. Clearly, the release rate decreased upon Ethocel addition. Based on these results, mini-tablets consisting of 4 % CIN-102 (theoretical), 1.33 % Neusilin, 50 % Ethocel, 25 % inulin and 19.67 % Dynasan were considered as most promising formulation.

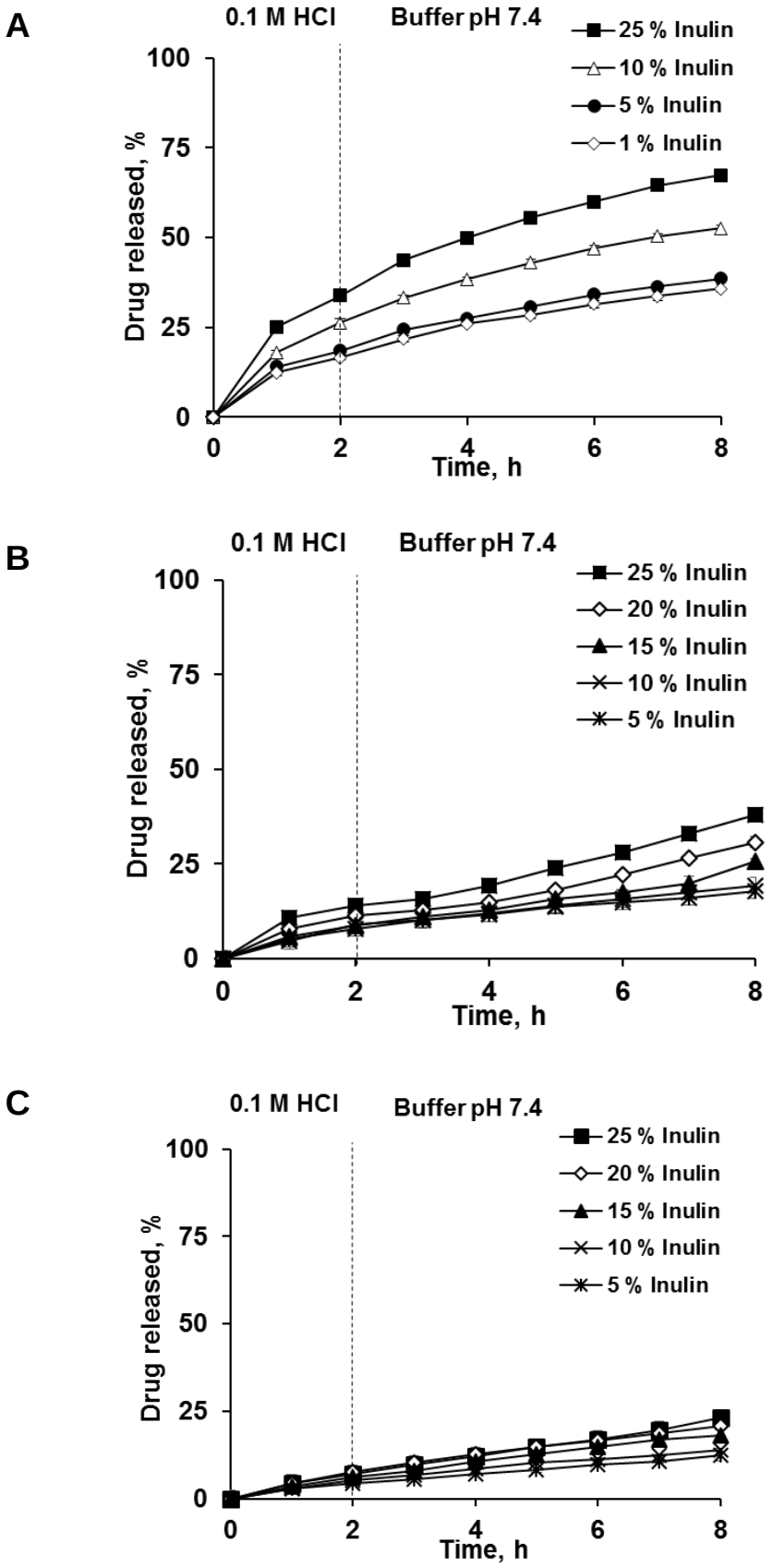


Fig. 7: Impact of the composition of CIN-102-loaded mini-tablets on cinnamaldehyde release in 0.1 M HCl for 2 h, followed by phosphate buffer pH 7.4. The mini-tablets contained 4 % CIN-102 (theoretical), 1.33 % Neusilin and: A) 1-25 % inulin and 70-94 % Dynasan; B) 25 % Ethocel, 5-25 % inulin and 65-45 % Dynasan; or C) 50 % Ethocel, 5-25 % inulin and 20-40 % Dynasan (as indicated) (mean values +/- SD are indicated; note that the SD are often too small to be visible).

In order to evaluate the impact of increasing initial drug contents on the resulting release kinetics, the CIN-102 content was increased up to 12 %. To be able to effectively adsorb the increasing amounts of liquid drug, the Neusilin content was increased accordingly. Fig. 8 shows the release of cinnamaldehyde from mini-tablets consisting of: (i) 4 % CIN-102 (theoretical), 1.33 % Neusilin, 50 % Ethocel, 25 % inulin and 19.67 % Dynasan, (ii) 8 % CIN-102 (theoretical), 2.67 % Neusilin, 50 % Ethocel, 25 % inulin and 14.33 % Dynasan, or (iii) 12 % CIN-102 (theoretical), 4 % Neusilin, 50 % Ethocel, 25 % inulin and 9 % Dynasan. As expected the release rate increased with increasing initial drug content, due to the reasons discussed above.

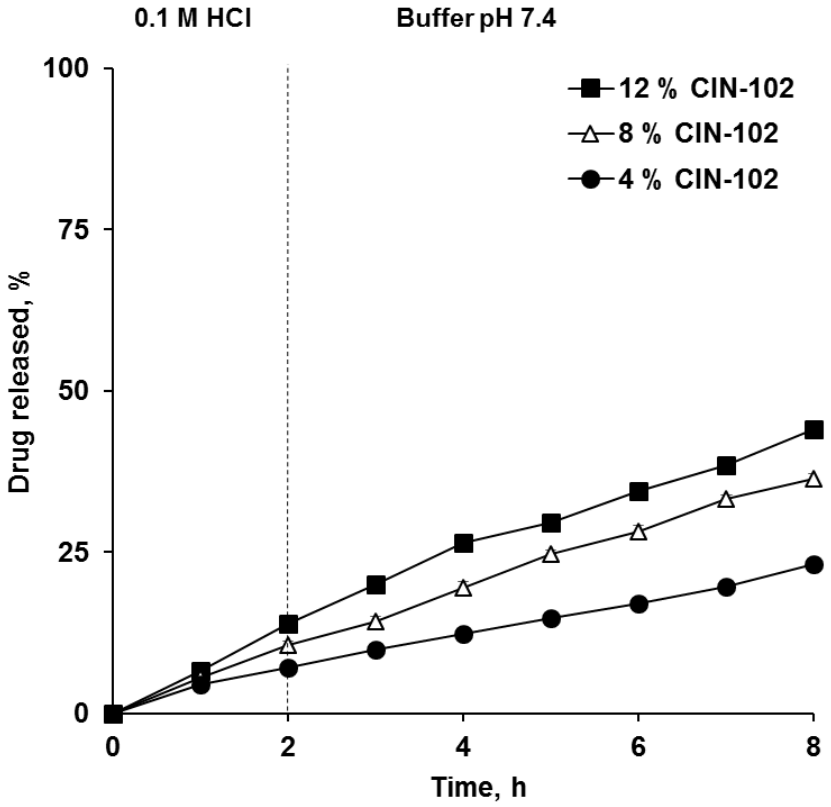


Fig. 8: Impact of the CIN-102 contents (indicated in the diagram) on cinnamaldehyde release from CIN-102-loaded mini-tablets in 0.1 M HCl for 2 h, followed by phosphate buffer pH 7.4. The mini-tablets consisted of 4-12 % CIN-102 (theoretical), 1.33-4 % Neusilin, 50 % Ethocel, 25 % inulin and 9-20 % Dynasan (as indicated) (mean values +/- SD are indicated; note that the SD are often too small to be visible).

Based on these results, mini-tablets consisting of 4 % CIN-102 (theoretical), 1.33 % Neusilin, 50 % Ethocel, 25 % inulin and 19.67 % Dynasan were selected for *in vivo* studies. No *in vitro* release studies were performed upon exposure to media simulating the conditions in the colon, since cinnamaldehyde rapidly degrades upon contact with fecal samples (the peak obtained by HPLC

analysis rapidly decreased and multiple new peaks appeared). This does not necessarily mean that CIN-102 has no chance to become active in a living colon: The survival time might be sufficient and/or the degradation products might be active. It was beyond the scope of this study to investigate this aspect in more detail. Instead, we focused on the *in vivo* efficiency of the most promising coated pellet and mini-tablet formulations.

3.3. *In vivo* evaluation

The objective of the CIN-102 treatment was two-fold: (i) to reduce total bacterial loads in the colon of the animals, and (ii) to reduce the pathogenic bacteria involved in colitis. Preliminary studies in TNBS treated rats revealed that oral gavage of the pure drug had only slight effects on bacterial flora (data not shown). In this study, colitis was induced by oral gavage of dextran sodium sulfate (DSS). The DSS treatment did not lead to a significant change in the bacterial counts of the total flora, which was true for the luminal and mucosal colonic bacteria (data not shown). However, the DSS-induced colitis was accompanied by a significant increase in the population of enterobacteria (luminal and mucosal) (data not shown). This is in good agreement with previous reports on colitis, where inflammation supported a high colonization by these bacteria on intestinal microbiota[27] and in IBD patients, the diversity of the microbiota was altered leading to an increase of the Gram-negative bacteria[28]. Also, an increase of enterobacteria in stool samples of patients suffering from IBD has been reported[29]. The critical role of this bacterial family in the pathogenesis of IBD might be explained based on the fact that lipopolysaccharides (present in enterobacteria) are specific significant contributors to Toll-like-receptors 4 (TLR4), and these receptors are abundantly expressed in intestinal epithelial cells in IBD[30], this interaction triggering important inflammatory cascades[31].

Fig. 9 shows the bacterial counts (luminal and mucosal) in mice, which were treated with the selected coated pellets and mini-tablets. For reasons of comparison, also mice not receiving CIN-102 were studied. Fig. 9A shows the bacterial counts of the total flora, Fig. 9B the bacterial counts of the enterobacteria. As it can be seen, the CIN-102 treatment (using coated pellets or mini-tablets) did not significantly alter the total flora counts (luminal or mucosal), but significantly decreased the enterobacteria counts: $P = 0.0003$ and 0.02 for luminal and mucosal counts (non-treated vs. coated pellets); $P = 0.0002$ and 0.02 for luminal and mucosal counts (non-treated vs. mini-tablets). This clearly demonstrates the *in vivo* efficacy of the novel CIN-102 formulations. It has to be pointed out that the decrease in the mucosal enterobacteria counts is particularly promising, since bacteria associated with the gastro intestinal tract wall are considered to be a more critical factor than luminal bacteria for the promotion of IBD pathogenesis, given their immediate proximity to affected tissues[32]. For example, increased enterobacterial levels were found in

mucosal tissue biopsies from Crohn disease patients, correlating with the severity of the inflammation[33]. Based on all these results, it can be expected that the reduction of enterobacteria by CIN-102 in stools (luminal bacteria) and more importantly, in the colonic mucosa, will have a positive impact on the IBD treatment. The explanation of the fact that the CIN-102 treatment did not significantly affect the total flora counts requires further research.

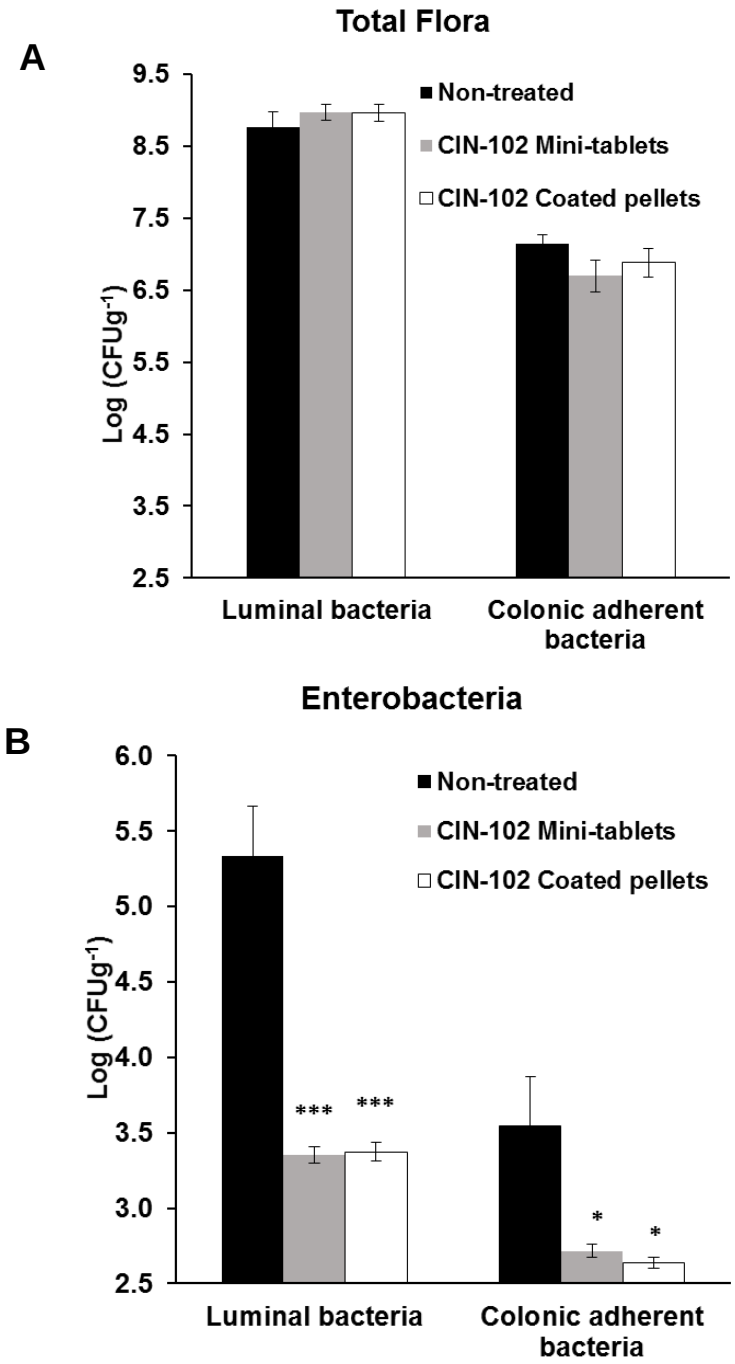
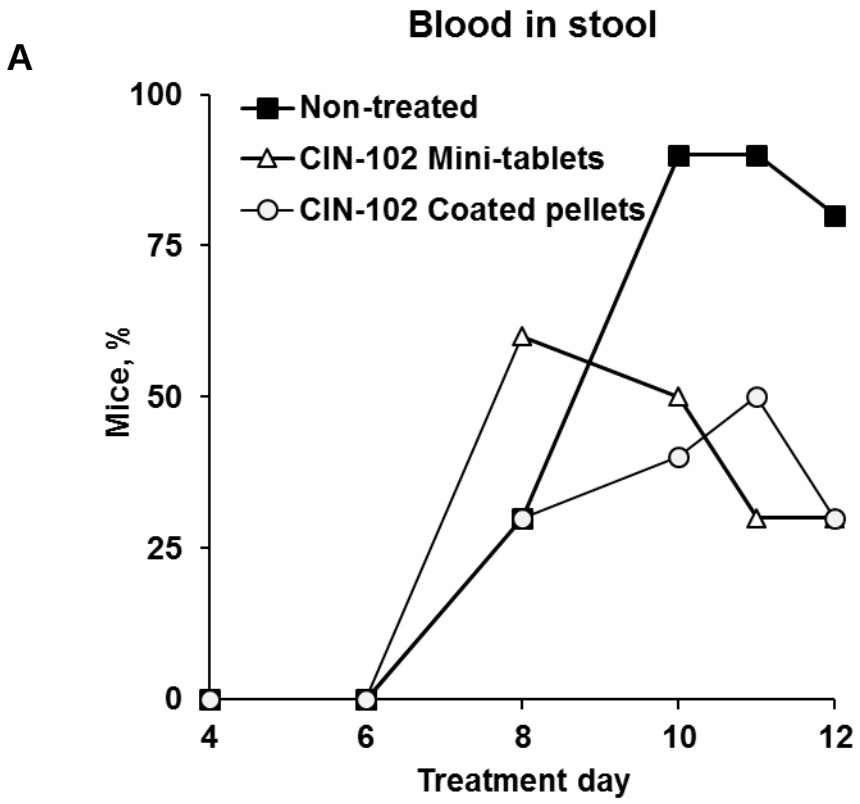


Fig. 9: Antibacterial activity of CIN-102-loaded coated pellets and mini-tablets (compositions see text) in a colitis mouse model, with respect to the: A) total flora, B) enterobacteria. For reasons of comparison, a control mouse group did not receive CIN-102 ("non-treated") (mean values +/- SD are indicated).

Importantly, the reduction in the enterobacteria counts upon treatment with CIN-102-loaded coated pellets and mini-tablets was accompanied by improved clinical scores of the DSS-colitis mice: Fig. 10 shows the percentages of animals with bloody stools and suffering from diarrhea as a function of time and treatment type. Very clearly, the presence of blood in stools and occurrence of diarrhea was much less frequent in mice receiving CIN-102-loaded pellets and mini-tablets compared to mice receiving standard chow only. However, there was no difference in the body weight loss between non-treated and CIN-102 treated mice (data not shown). It can be hypothesized that the observed amelioration of the course of the colitis is related to the above described reduction in the number of colonic enterobacteria. It has indeed been shown that broad-spectrum antibiotics or broad-spectrum antibiotic combinations are able to prevent, treat and/or reduce the severity of the inflammation in colitis animal models[34,35,36]. Also, in clinical practice antibiotic therapy has been reported to be promising for the treatment of acute Crohn's disease[37,38] and to be beneficial for the treatment of ulcerative colitis in combination with corticosteroids[39].



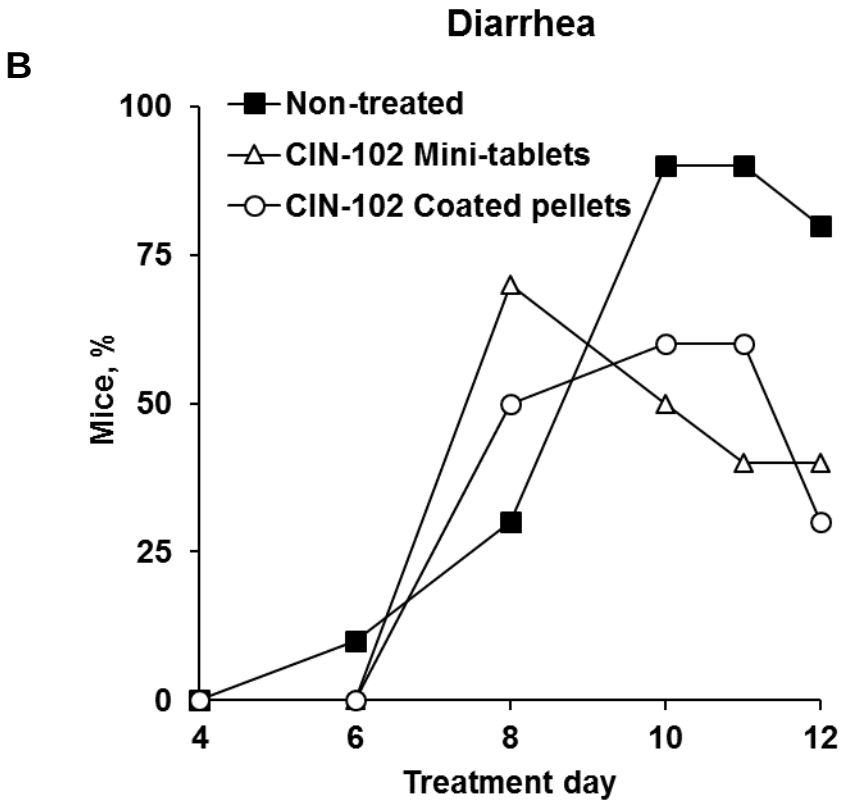


Fig. 10 Evolution of the course of colitis in the mouse model: A) Presence of blood in stools, and B) Occurrence of diarrhea. Ten mice were in each group, 100 % correspond to all 10 mice in the respective group. The mice received standard chow only ("non-treated"), standard chow with coated pellets, or standard chow with mini-tablets (as indicated).

To know whether the observed amelioration of the clinical course of colitis in the investigated mice was potentially related to immunomodulatory effects (as a consequence of the reduction in the levels of colonic enterobacteria), the colonic expression of several cytokines was measured. It has been shown that cinnamaldehyde is capable to decrease inflammatory mediators and to increase anti-inflammatory mediators[40]. As it can be seen in Fig. 11, the mRNA levels of the pro-inflammatory cytokines IL-1β and TNF-α in colonic tissue samples decreased upon CIN-102 treatment (in the case of coated pellets and mini-tablets). In contrast, the expression of IL-6, a cytokine involved in intestinal healing after inflammatory injury[41], increased upon CIN-102 treatment (in the case of coated pellets and mini-tablets). However, due to the high standard deviations the differences were not statistically significant. The levels of IL-10, an anti-inflammatory cytokine involved in intestinal homeostasis[42], remained about constant.

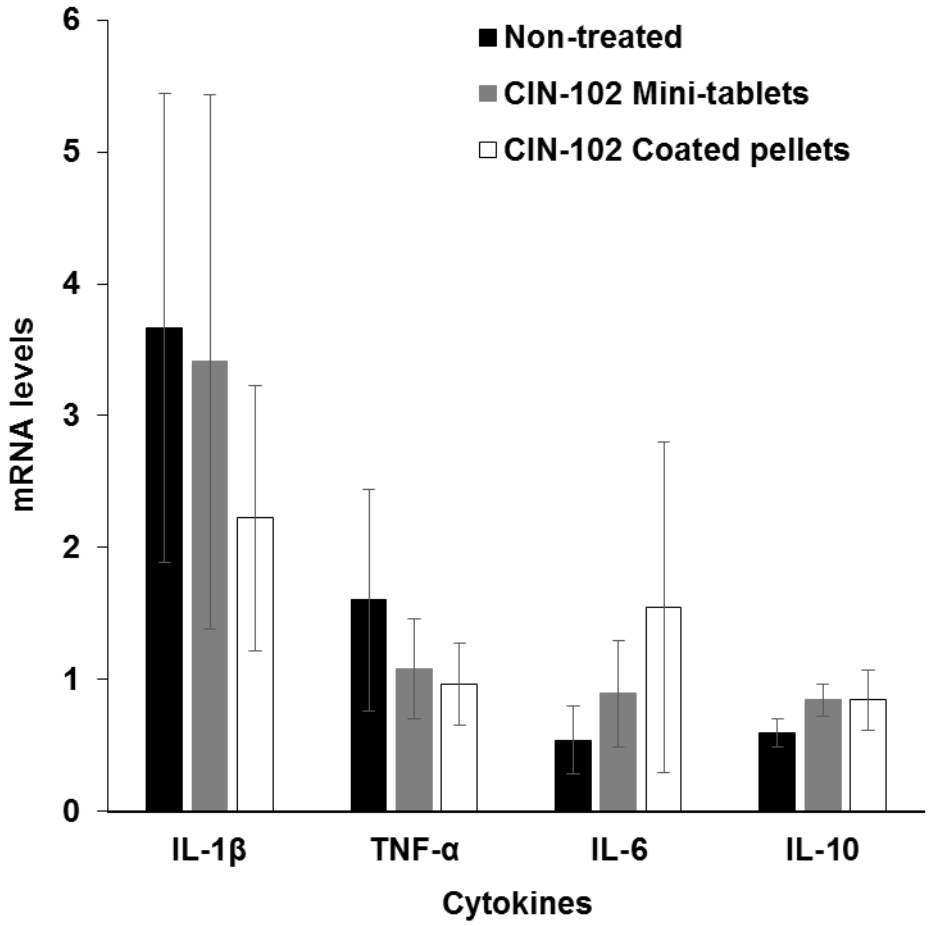


Fig. 11: Effects of CIN-102 treatment on the state of inflammation in the mouse colitis model: IL-1β, TNF-α, IL-6, and IL-10 mRNA expression levels in colonic tissue samples obtained on Treatment Day 12 (mean values +/- SD are indicated).

4. Conclusion

The proposed CIN-102 coated pellets and mini-tablets show a promising potential for the treatment of colitis. The controlled release of the new broad spectrum antibiotic was demonstrated to effectively reduce the luminal and mucosal enterobacteria in mice, accompanied by an amelioration of the clinical course of the intestinal inflammation (presence of blood in stools and occurrence of diarrhea). Further studies are needed to better understand the mechanisms of action of CIN-102 during colitis.

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CHAPTER IV

ABOUT THE ANTIBACTERIAL ACTIVITY OF PPARγ AGONISTS AND ANTIBIOTICS

1. Introduction

Intestinal Bowel Diseases (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), are chronic, relapsing, inflammatory pathologies that are immunologically mediated and with multi-factorial etiology. These idiopathic disorders appear to be caused by overly aggressive acquired immune responses to a subset of commensal enteric bacteria in genetically susceptible hosts, and that environmental factors precipitate their onset or reactivation. Indeed, increasing evidence suggest that the intestinal microbiota play a role in initiating, maintaining and determining the phenotype of these diseases[1].

The main goals of therapy in patients with IBD are to induce a clinical remission and then maintain that remission over time[2]. Mesalazine (5-aminosalicylic acid or 5-ASA) has a well-established role in the management of UC and a less clear role in the management of CD, and is likely to have multiple anti-inflammatory effects[3]. 5-ASA inhibit the production of prostaglandins and leukotrienes by interfering with cyclooxygenase and lipoxygenase pathways[4,5]. Mesalazine also inhibits tumor necrosis factor (TNF)-α effects on intestinal epithelial cell proliferation and activation of mitogen-activated protein (MAP) kinase and nuclear transcription factor κB (NF-kB)[6]. Furthermore, it has recently been demonstrated that the peroxisome proliferator-activated receptor-γ (PPARγ) is the key receptor that mediates the main effects of 5-ASA in the colon[7] .

The heterodimer of PPARγ with the retinoid X receptor (RXR) plays a crucial role in adipogenesis and insulin sensitization, has important immunomodulatory activities and affect cell proliferation/differentiation pathways. Adipose tissue, large intestine and hematopoietic cells express high levels of PPARγ[8]. PPARγ/RXR could inhibit two of the most important signaling pathways regulating expression of pro-inflammatory genes: NF-κB and stress kinase pathways[9].

This receptor seems to play a crucial role in the regulation of intestinal inflammation. In fact, PPARγ$^{+/-}$ mice have an exacerbation of intestinal inflammation[10,11] and PPARγ expression is reduced in colonic tissue of DSS-colitic mice[12] and in epithelial colonic cells from UC patients[13]. Furthermore, in murine models of colitis, PPARγ ligands like thiazolidinediones can inhibit the inflammatory response[10,11,14,15] and PPARγ gene therapy can restore or/and enhance endogenous anti-inflammatory processes[12]. In UC patients, PPARγ ligands as therapy were effective[16,17]. Furthermore, there is a close link between intestinal microbiota and regulation of PPARγ expression by colonic epithelial cells[18].

These facts have led to the development of 5-ASA analogs with stronger affinity for PPARγ and fewer side effects. In this context, a molecule belonging to the aminophenyl-methoxy-propionic acid family: GED-0507-34-Levo, has appeared as a new a new drug candidate with potent anti-inflammatory and

analgesic properties. GED-0507-34-Levo showed 100- to 150-fold higher PPARγ activation than 5-ASA *in vitro*; and 30-fold higher anti-inflammatory effect than 5-ASA in experimental models of colitis[19]. As well, no side-effects have been observed in phase I study in healthy subjects[20] and it is currently used in phase II clinical trials for IBD[21].

Given the fundamental role of intestinal bacteria in IBD and the increasing attention of PPARγ as a target against these diseases, a broad-spectrum evaluation of the inherent antibacterial activity of PPARγ ligands is presented in this work. These findings could give complementary information about the mechanism of action of PPARγ ligands used to combat intestinal inflammation. Moreover, the activity of several antibiotic families commonly used in IBD treatment were also submitted to a broad-spectrum evaluation for reasons of comparison.

2. Materials and methods

2.1. Antibacterial agents and growth media

Antibiotics, pioglitazone and 5-aminosalicylic acid were obtained from Sigma-Aldrich, France. GED-0507-34-Levo (INSERM U995, Lille, France); Columbia agar base and Defibrinated horse blood (Oxoid; Hampshire, England); Brucella agar base (Becton, Dickinson and Company; Le Pont de Claix, France).

2.2. Microorganisms, storage and growth conditions

Thirty two clinical isolates (see Table 1) and three standard strains: *Escherichia coli* ATCC 25922, *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotamicron* ATCC 29741 were considered in this study. They were stored at -20˚C in Rosenow broth. For the determination of the minimal inhibitory concentration all bacteria were thawed, subcultured in Wilkins West broth and then incubated under anaerobic conditions for 48 hours at 37°C. The day of the test, a suspension of approximately 10^6 CFU/mL was prepared in Brucella broth.

Table 1: Anaerobic strains

Bacterial strains	N° of strains
Escherichia coli	3
Klebsiella pneumoniae	1
Enterobacter cloacae	1
Proteus mirabilis	1
Bacteroides fragilis	2
Bacteroides thetaoitamicron	2
Bacteroides vultagus	2
Bacteroides distasonis	1
Bacteroides uniformis	1
Fusobacterium nucleatum	1
Fusobacterium necrophorum	1
Veillonella spp	1
Enterococcus faecalis	1
Staphylococcus aureus	2
Clostridium perfringens	2
Clostridium clostridioforme	1
Clostridium difficile	1
Clostridium innocuum	1
Clostridium ramosum	1
Ruminococcus spp	1
Finegoldia magna	1
Anaerococcus prevotii	1
Peptoniphilus asaccharolyticus	1
Bifidobacterium bifidum	1
Bifidobacerium spp	2
Propionibacterium acnes	2

2.3. Determination of the Minimal Inhibitory Concentration (MIC)

The MICs of all the 9 products tested (amoxicillin, amoxicillin + clavulanic acid, imipenem, ciprofloxacin, erythromycin, metronidazole, pioglitazone, 5-ASA, GED-0507-34-Levo) were determined by the agar dilution procedure described by the Clinical and Laboratory Standard Institute (CLSI)[22]. The tested concentrations of antibiotics, pioglitazone, 5-ASA and GED-0507-34-Levo were prepared by making serial two-fold dilution in water, ranging from 128 to 0.03 mg/L. Each concentration was added to molten Columbia blood agar, mixed, poured into a Petri dish, and allowed to solidify. The inocula from the Brucella broth bacterial suspension was delivered using a Steers replicator (Mast Systems, London, UK) onto the Columbia blood agar plates. Inoculated control plates (without the agents to be tested) were included at the beginning (pre-O_2: to check for aerobic contamination; and pre-Ana: anaerobic growth control) and at the end of the test (post-O_2

and post-Ana). Finally, plates were incubated under anaerobic conditions at 37°C for 48h, with the exception of control pre-O_2 and post-O_2 which were incubated under aerobic conditions at 37°C for 48h. The lowest concentration of each product tested that inhibits growth of bacteria was reported as the MIC of the agent.

3. Results

The antibacterial activity of 5-ASA and GED-0507-34-Levo was explored against a group of clinical anaerobic isolates regrouping different intestinal bacteria. For reasons of comparison, the activity of different subsets of antibiotics: β-lactam (amoxicillin), β-lactam + β-lactamase inhibitor (amoxicillin + clavulanic acid), carbapenem (imipenem), macrolide (erythromycin), fluoroquinolone (ciprofloxacin) and nitroimidazole (metronidazole); as well as the kwon PPARγ activator: pioglitazone, was also studied.

Concerning Gram-positives (Table 2), there is a general good activity of β-lactams, including imipenem. Only the *E. faecalis* isolate was resistant to these antibiotics. Erythromycin was not active *against E. faecalis, C. innocuum,* and *F. magna* (MIC>128 mg/L). Ciprofloxacin showed poor activity against all isolates from *Clostridium*, and also against *Ruminococcus, A. prevotii* and some *Bifidobacterium* isolates. For metronidazole, facultative anaerobes like *Enterococcus* and *Staphylococcus* were resistant; as well as the strict anaerobes: *Ruminococcus. Bifidobacterium* and *Propionibacterium*. For the PPARγ agonists 5-ASA and GED-0507-34-Levo there was an absence of antibacterial activity against all Gram positive anaerobes (MICs >128 g/mL). However, *C. clostridioforme* and *F. magna* were the only isolates susceptible to pioglitazone.

Table 2: MIC (mg/L) for Gram-positive strains

	Amoxicillin	Amoxicillin + clavulanic acid	Imipenem	Erythromycin	Ciprofloxacine	Metronidazole	5-ASA	GED-0507-34-Levo	Pioglitazone
Enterococcus faecalis	64*	32*	128*	>128	4	>128	>128	>128	>128
Staphylococcus aureus	0.5	0.125	0.03	2	0.5	>128	>128	>128	>128
Staphylococcus aureus	1	0.125	0.03	2	0.5	>128	>128	>128	>128
Clostridium perfringens	0.25	0.25	0.25	0.25	8	0.25	>128	>128	>128
Clostridium perfringens	0.125	0.25	0.125	0.25	4	0.125	>128	>128	>128
Clostridium clostridioforme	0.5	0.5	0.25	0.5	64	0.125	>128	>128	1
Clostridium difficile	0.5	0.5	4	1	8	0.25	>128	>128	>128
Clostridium innocuum	0.25	0.25	1	>128	4	0.5	>128	>128	>128
Clostridium ramosum	0.25	0.25	0.25	1	>128	0.5	>128	>128	>128
Ruminococcus sp	0.25	0.125	0.06	0.03	64	8*	>128	>128	>128
Finegoldia magna	0.25	0.125	0.06	>128	0.25	0.125	>128	>128	0.25
Anaerococcus prevotii	0.06	0.03	0.03	2	8	0.5	64	>128	>128
Peptoniphilus asaccharolyticus	0.25	0.25	0.03	4	2	0.5	>128	>128	>128
Bifidobacterium bifidum	0.03	0.03	0.03	0.03	8	4	>128	>128	>128
Bifidobacterium sp	0.03	0.03	0.03	0.03	8	>128*	>128	>128	>128
Bifidobacterium sp	0.03	0.03	0.03	0.03	0.5	8*	>128	>128	>128
Propionibacterium acnes	0.06	0.03	0.03	0.03	0.5	>128*	>128	>128	>128
Propionibacterium acnes	0.25	0.03	0.03	0.06	2	>128*	>128	>128	>128

*: Resistant Bacteria, according to: The European Committee on Antimicrobial Susceptibility Testing (EUCAST)[23]

Concerning the Gram-negative anaerobic-facultative enterobacteria (Tabe 3), *E. coli* ATCC and the *P. mirabilis* isolates were sensitive to β-lactams, and imipenem was only active against *E. coli* isolates. For ciprofloxacin, isolates of *E. coli, K. pneumoniae* and *E. cloacae* were resistant. As was expected, all of the facultative anaerobic enterobacteria were resistant to erythromycin and metronidazole. PPARγ agonists did not shown any antibacterial activity against these strains (MIC > 128 g/mL). Concerning the Gram-negative strict anaerobes (Table 3), most of the *Bacteroides*, as well as *F. nucleatum* and *Veillonella spp*. were resistant to amoxicillin, whereas only *B. thetaiotaomicron* and *B. distasonis* were resistant to amoxicillin + clavulanic acid. All of these clinical isolates were susceptible to imipenem and metronidazole, with the exception of one metronidazole-resistant strain of *Veillonella spp*. As expected,

erythromycin and ciprofloxacin did not show a good antibacterial activity against these isolates. All of these strains were not susceptible to 5-ASA nor GED-0507-34-Levo, however, pioglitazone was active against *Bacteroides* and *Fusobacterium*.

Table 3: MIC (mg/L) for Gram-negative strains

	Amoxicillin	Amoxicillin + clavulanic acid	Imipenem	Erythromycin	Ciprofloxaxin	Metronidazole	5-ASA	GED-0507-34-Levo	Pioglitazone
Escherichia coli	>128*	>128*	0.5	>128	>128*	>128	>128	>128	>128
Escherichia coli LF-82	>128*	>128*	1	>128	0.125	>128	>128	>128	>128
Escherichia coli ATCC	8	4	1	>128	0.06	>128	>128	>128	>128
Klebsiella pneumoniae	>128*	>128*	128*	>128	4*	>128	>128	>128	>128
Enterobacter cloacae	>128*	>128*	16*	>128	8*	>128	>128	>128	>128
Proteus mirabilis	2	1	16*	>128	0.06	>128	>128	>128	>128
Bacteroides fragilis ATCC	32*	0.125	0.06	8	4	0.25	>128	>128	>128
Bacteroides thetaiotaomicron ATCC	16*	0.5	0.125	8	16	1	>128	>128	1
Bacteroides fragilis	16*	0.25	0.06	>128*	8	0.25	>128	>128	32
Bacteroides thetaiotaomicron	>128*	16*	2	>128*	64	0.25	>128	>128	0.125
Bacteroides vulgatus	2	2	0.25	32*	8	0.5	>128	>128	16
Bacteroides vulgatus	4*	0.03	0.06	>128*	4	0.125	128	>128	0.03
Bacteroides distasonis	>128*	64*	0.5	>128*	>128	0.25	>128	>128	128
Bacteroides uniformis	64*	0.25	0.25	8	>128	0.5	>128	>128	2
Fusobacterium nucleatum	0.125	0.03	0.06	128*	16	0.25	>128	>128	1
Fusobacterium necrophorum	16*	0.03	0.06	32*	4	0.125	>128	>128	0.125
Veillonella sp	0.5	0.5	0.5	128*	2	>128	>128	>128	>128

*: Resistant Bacteria, according to: The European Committee on Antimicrobial Susceptibility Testing (EUCAST)[23]

4. Discussion

In recent years, PPARγ has become an interesting potential target in IBD, and there is an emerging interest by its role in intestinal homeostasis[18]. Given the importance of intestinal bacteria in the pathogenesis of IBD[1] and also in the expression of PPAR in colonic epithelial cells[13], an *in vitro* study of the inherent antibacterial activity of PPAR γ ligands is presented.

We have showed that 5-ASA did not possess antibacterial activity *in vitro* against a large range of anaerobic intestinal isolates. Nevertheless, *in vivo* studies have shown an antibacterial effect when 5-ASA was administered. In a murine model of colitis, 5-ASA decreased bacterial richness and diversity of luminal and mucosal intestinal bacteria[24]. In the same way, colonic mucosal bacteria of IBD patients[25] and fecal bacteria of Intestinal Bowel Syndrome patients (IBS)[26] were decreased when they were treated with 5-ASA.

However, the mechanism through which 5-ASA alter intestinal microbiota *in vivo* is still not clear. Our results correlate with the observations of Swidsinski *et al*.[25], for a lack of suppression of anaerobic or aerobic bacterial growth when 5-ASA was added to suspensions of fecal bacteria. It might be possible that the *in vivo* changes in the intestinal flora upon 5-ASA treatment are secondary to its effects on mucosal immune functions. Furthermore, it has been shown that 5-ASA *in vitro* altered bacterial gene expression involved in metabolism, invasiveness and antibiotic/stress resistance, without inhibiting bacterial growth. These changes could result in altered bacterial physiology, with a wider impact on the gastrointestinal bacterial community[27]. However, *in vitro* antimicrobial activity of 5-ASA against *Mycobacterium avium* subspecies *paratuberculosis*, an organism that may be an etiological factor for IBD, has been demonstrated[28].

For the novel PPARγ agonist GED-0507-34-Levo, this is the first study of its antibacterial properties *in vitro*. Pioglitazone, a kwon PPARγ agonist, was studied for reasons of comparison. However it has shown a beneficial role in a DSS model of colitis[11] and also a potential chemopreventive property in colon carcinogenesis[15,29]. In this work we have showed a narrow-spectrum antibacterial activity of pioglitazone mostly active against strict anaerobic Gram-negative strains like *Bacteroides* and *Fusobacterium*. Other studies demonstrated that *E. coli* was susceptible, while *S. pneumoniae* and *K. pneumoniae* were resistant to pioglitazone. However, its antibacterial activity was considered low compared to standard antibiotics, but when pioglitazone was used at sub-inhibitory concentrations it was able to enhance the antibacterial effect of some antibiotics[30].

We consider that a lack of inherent antibacterial activity for 5-ASA and GED-0507-34-Levo might avoid further disturbance of the intestinal microbiota during inflammation. In the case of pioglitazone, its narrow-spectrum of action might cause further imbalance in gut microbiota that could enhance intestinal inflammation. In contrast, it has been proved that antibiotics with a broad-spectrum of action were effective in animal models of colitis[31,32] and in CD patients[33]. In the context of IBD, broad-spectrum antibiotics are preferred as treatment adjuvants, since a reduction of the total bacterial loads might have a beneficial effect in intestinal inflammation. Surprisingly, besides of the lack of antibacterial activity of 5-ASA and GED-0507-34-Levo, none of the antibiotics used in this work had a broad-spectrum

of action against the anaerobic isolates tested. This fact could also denote the lack of antibacterials with a veritable broad-spectrum of activity.

5. Conclusion

These results show that the PPARγ agonists 5-ASA and GED-0507-34-Levo do not have inherent antibacterial activity against anaerobic clinical isolates *in vitro*. In the context of IBD, where an imbalance of the gut microbiota is involved in the pathogenesis, compounds with narrow-spectrum activity, like pioglitazone, could further enhance the dysbiosis. In these pathologies, broad-spectrum antibiotics would be preferred; however neither of the antibiotic families tested possess a veritable broad-spectrum of action.

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GENERAL CONCLUSION

In this work we present CIN-102 as a promising broad-spectrum antibacterial agent acting also against multi-resistant bacteria, this novel agent might have as well a therapeutic effect through modulation of the microbiota in case of intestinal inflammation.

Firstly, the broad-spectrum of action of CIN-102 has been confirmed. All the 175 bacterial clinical isolates tested, comprising aerobic and anaerobic bacteria, were susceptible to CIN-102. This new agent was active against multi-resistant bacteria of great interest in hospital and community-acquired infections as: NDM-1 producing *E. coli*, OXA-48 producing *K. pneumoniae*, MDR *A. baumannii* and *P. aeruginosa*, VRE and MRSA. CIN-102 also presented a fast logarithmic reduction time, especially against Gram-negative bacteria. Furthermore, the fact that CIN-102 exerted a PAE and slowed down bacterial growth at sub-MICs are of especial importance in future drug dosing. Importantly, it was demonstrated that CIN-102, when combined with other antibacterials: did not show antagonistic effects, resulted in synergistic interactions with all antibiotics tested (mostly with colistin and aminoglycosides) and was able to overcome resistance and restore antibiotic activity against resistant strains. These results suggest that CIN-102 has a promising broad-spectrum antibacterial activity and that further *in vivo* and clinical studies are needed to confirm these findings.

Second, sustained release CIN-102 dosage forms effectively delivered CIN-102 in the lower gastrointestinal tract *in vivo* in an experimental model of colitis. We have demonstrated that CIN-102 was released from coated pellets and mini-tablets at the appropriate site of action in order to exert is antibacterial activity. In this regard, CIN-102 was able to significantly reduce enterobacterial concentrations not only in luminal colonic contents (stools) but also in colonic tissue (adhered to mucosa). This targeted antibacterial effect seems to be linked to the improvement of the course of colitis in mice (reduction of bloody stools and diarrhea). These results suggest that CIN-102, when efficiently delivered into the colon, can modulate intestinal microflora which might ameliorate the state of inflammation. Further studies will better explain how does CIN-102 act in case of colitis.

In third place, the need of broad-spectrum antimicrobials in the treatment of IBD has been observed. PPARγ agonist with anti-inflammatory effects in IBD were not provided of inherent antibacterial activity *in vitro*. 5-ASA and GED-0507-34-Levo were not active against clinical isolates of common intestinal bacteria. Neither of the antibiotics tested showed a veritable broad-spectrum activity against the strains tested. This emphasizes the need of broad-spectrum antimicrobials able to modulate intestinal microbiota in case of IBD, recovering lost intestinal homeostasis.

Overall: given the lack of appropriate broad-spectrum antibiotics in IBD treatment, CIN-102, a novel broad-spectrum antibacterial agent, seems to positively modulate intestinal microflora aiming further therapeutic applications.

PERSPECTIVES

About CIN-102

We are searching to elucidate the mechanism of action of CIN-102. The lipophilic character of CIN-102 might indicate that bacterial membranes are one important target of action. In this regard, morphological changes of bacteria exposed to high CIN-102 concentrations were observed by transmission electron microscopy (TEM), in preliminary studies (Fig. 18). A leakage of intracellular material is clearly observed after bacterial exposure to CIN-102. Studies at lower CIN-102 concentrations (MIC, subMICs) will give further insights about the mechanism of action.

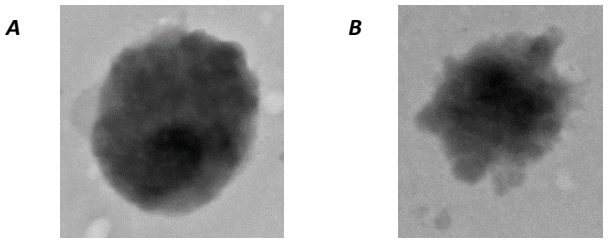

Fig. 18: TEM photos of S. aureus A) without exposure to CIN-102 B) after exposure to 1% of CIN-102

Another important point is the evaluation of the development of bacterial resistance to CIN-102. For this, the "serial passage" is a simple method that could be proposed[1,2]. Bacteria can be grown at different CIN-102 concentrations. After incubation, the culture below the MIC is used to prepare the inoculum for a new MIC determination. Again, after incubation, the culture below the MIC is used to prepare the inoculum for the next MIC determination. These "serial passages" can be repeated as many times as wanted. Variation of the values of MIC can be thus observed. Preliminary results for some strains showed little variability on CIN-102 MIC after several passages.

The study of subMICs of CIN-102 on virulence factors such as biofilm formation is also contemplated in a future approach. The crystal-violet biofilm formation assay could be proposed[3,4]. Bacteria are grown in polystyrene plates with or without CIN-102. Cells cultures are washed with water to remove not-adhered cells, biofilms are stained with crystal violet, extracted with ethanol and absorbance is measured at 570 nm.

About sustained release formulations

We are interested in the optimization of the sustained release formulations of CIN-102. A particular problem encountered during the fabrication of these formulations was the loss of CIN-102 due to volatilization. For this purpose, encapsulation of EOs can improve their stability through protection against volatilization, oxidation or heat damage; allowing the EOs to remain effective under environmental conditions for longer

time periods. Furthermore, micro- and nano-particles of encapsulated EOs can improve water solubility and enhance drug delivery through increased bioavailability. Finally, the release of encapsulated EOs can also be controlled. After this process, if a power is obtained by freeze-drying or directly by spray-drying, the sustained release formulation could be fabricated using the encapsulated CIN-102.

In this regard, microencapsulation with cyclodextrins is an attractive approach. β-cyclodextrins have already been used to efficiently encapsulate EOs[5] and even cinnamaldehyde[6,7]. Preliminary studies show that hydroxypropyl-β-cyclodextrin (HP-β-CD) can encapsulate cinnamaldehyde present in CIN-102 (Fig. 19). A solution of 0.05M of cinnamaldehyde was exposed to 0.5; 2.6; 5.3; and 25 mM of HP-β-CD. As seen in Fig. 19, the maximum absorption of cinnamaldehyde at 290 nm remains unchanged, but the absorbance is considerably decreased as increasing HP-β-CD concentrations, confirming the presence of an interaction between the cyclodextrin and cinnamaldehyde, possibly by the formation of an inclusion complex.

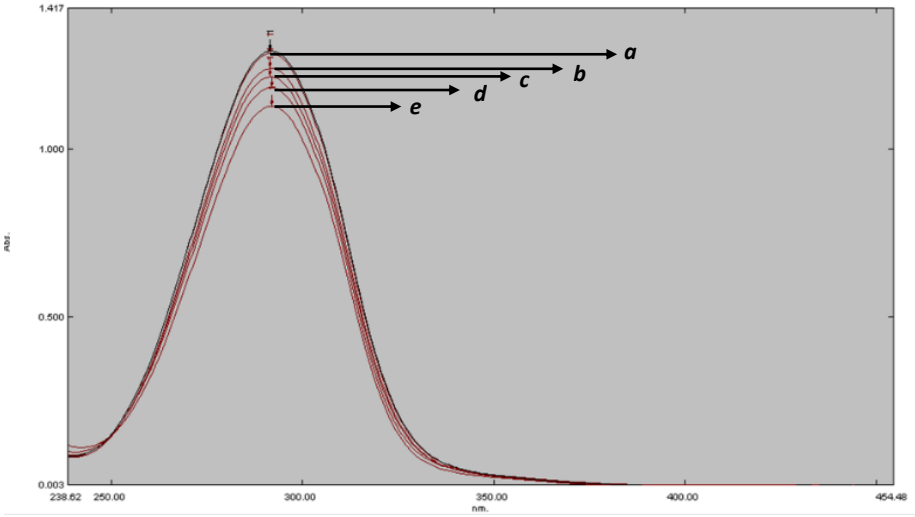

Fig. 19: Effect of cyclodextrin on the UV spectrum cinnamaldehyde 0.05 mM at (a) 0; (b) 0.5; (c) 2.6; (d) 5.2; (e) 26.4 mM of HP-β-CD

Nanoparticles for encapsulation of EOs is another methodology that could be applied. In this regard, biodegradable polymers like poly(lactic-co-glycolic acid) (PLGA)[8], alginate and cashew gum[9] or chitosan[10] have already been used to encapsulate EOs. Another option could be the fabrication of microparticles by spray drying using polysaccharides as wall materials. Lipophilic compounds have already been microencapsulated using arabic gum, maltodextrin[11] pectin and chitosan[12].

About potential treatment against IBD

In a first approach we are interested in studying the *in vivo* effect of CIN-102 in optimized protocols of acute models of colitis: use of placebo formulations, well-established criteria for colitis evaluation, different CIN-102 concentrations, use of CIN-102 as preventive or treatment agent.

In this regard, CIN-102 (50mg/Kg/day) sustained release formulations as well as placebos (formulations without CIN-102) are being tested in a DSS model of colitis. The Disease Activity Index (DAI) is used to assess the severity of colitis and is calculated as the sum of the diarrheal score and the bloody stool score. The colon weight/colon length ratio is used as a reliable indicator of the severity and extent of the inflammatory response in colitis. Preliminary results for CIN-102 coated pellets show significant reduction of DAI and colonic weight/length ratio compared to placebo coated pellets (Fig. 20). These studies will be completed by a bacteriological analysis of colonic flora, colonic cytokine expression and histological studies.

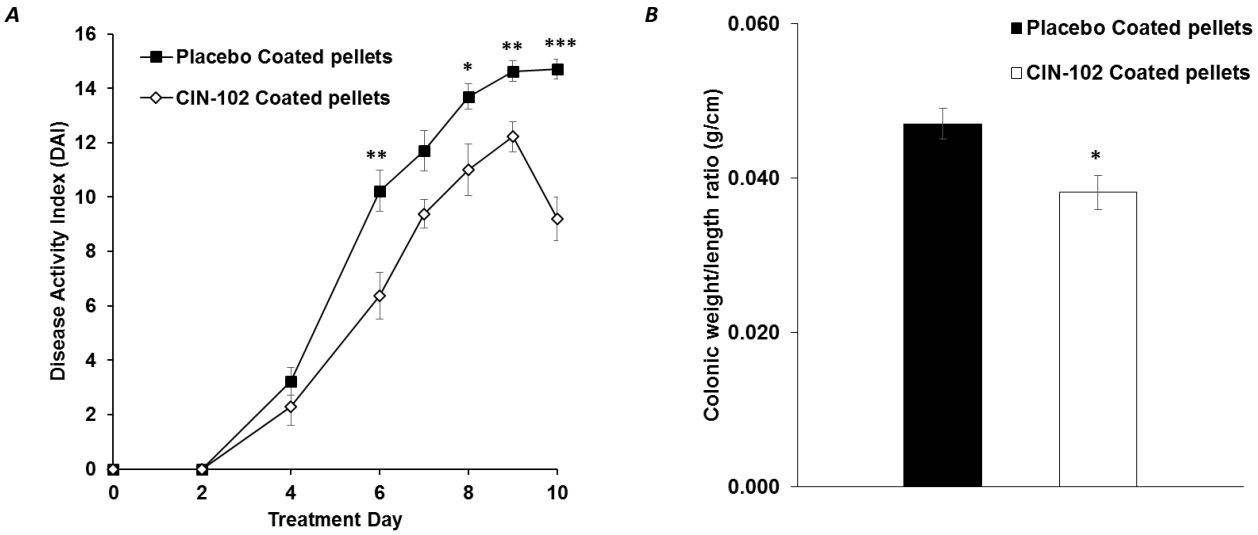

Fig. 20: CIN-102 and placebo treatment in DSS-induced colitis in C57BL6 mice: A) DAI and B) colonic weight/length ratio

A second approach will be the evaluation of a combined therapy of CIN-102 with immunomodulatory agents like 5-ASA, GED-0507-34-Levo and corticosteroids in experimental models of colitis

Future approaches will include evaluation of CIN-102 in chronic and infection induced-models of colitis. For example, HLA-27 transgenic rats spontaneously develop chronic inflammation mainly involving the colon, providing a powerful animal model for IBD. Furthermore, normal luminal bacteria play an essential role in initiating and perpetuating chronic colitis in HLA-B27 rats[13] Infection by *Citrobacter rodentium* is another robust model for studying IBD pathogenesis. *C. rodentium* is a murine mucosal pathogen, its increased growth in the colon results in a pronounced dysbiosis, causing colonic hyperplasia, inflammation and systematic dissemination of the pathogen[14,15].

About PPARγ agonists

In vivo studies of the impact of GED-0507-34-Levo on the intestinal flora of colitic mice are intended. This would explain if the anti-inflammatory activity of this molecule can indirectly impact the composition or concentrations of the intestinal microbiota during colitis.

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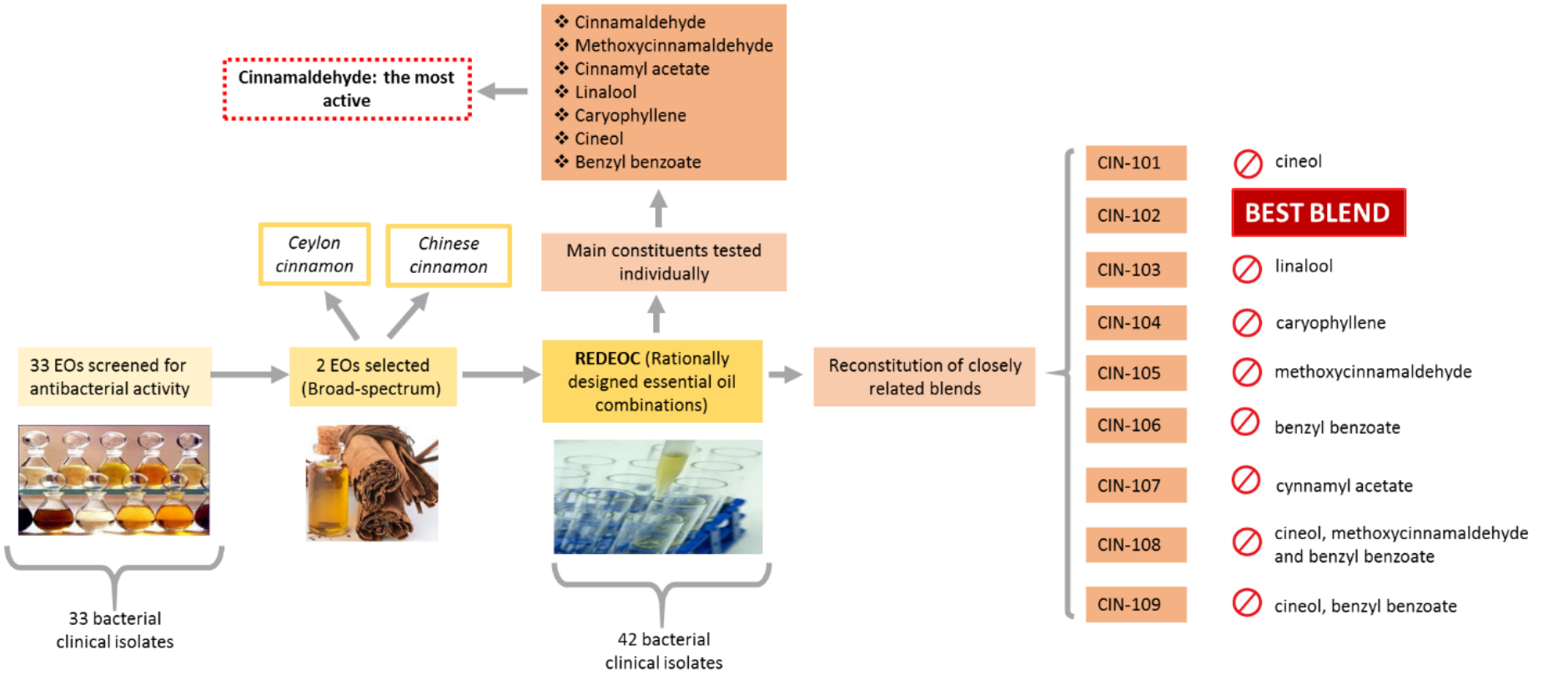
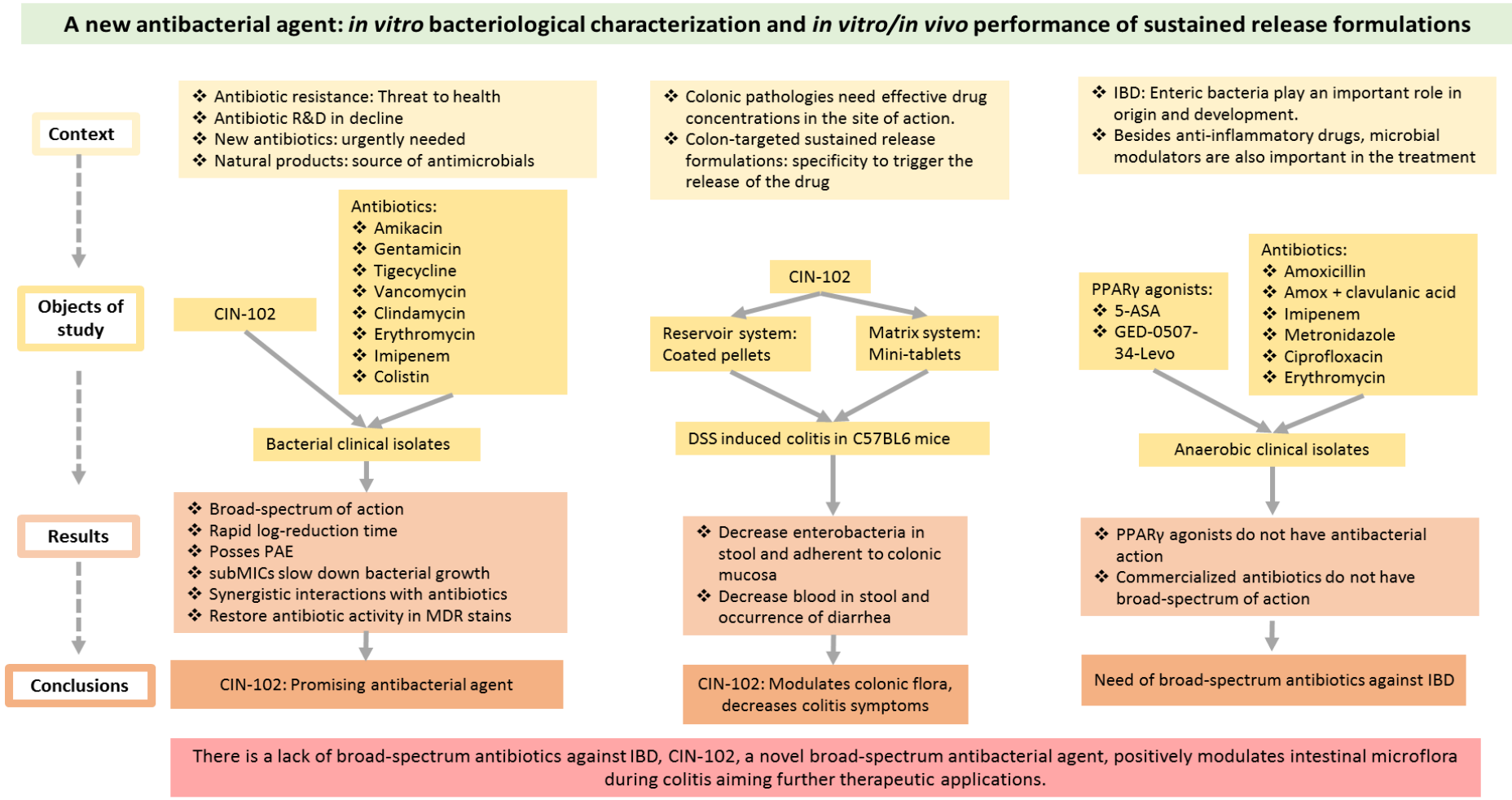
ANNEXES

Annexe 1: Fabrication of CIN-102



Annexe 2: Graphical abstract of the work



RÉSUMÉ EN FRANÇAIS

La résistance des bactéries aux antibiotiques constitue un problème majeur de santé. La dissémination des bactéries multi-résistantes, spécialement chez les bactéries à Gram négatif, risque de provoquer des infections hospitalières et communautaires pour lesquelles aucun antibiotique n'est effectif. Il a été estimé qu'en Europe, au moins 25 000 patients par an décèdent à cause d'infections provoquées par des bactéries résistantes aux antibiotiques. Les coûts liés à ces infections s'élèvent à plus de 1.5 billion d'euros chaque année.

Ces faits mettent en relief le besoin urgent de développer des nouveaux antibiotiques capables de cibler ces bactéries résistantes. Cependant, le domaine de la recherche et développement d'antibiotiques est peu entrepris par les compagnies pharmaceutiques. Plusieurs facteurs ont contribué à l'abandon de la découverte des antibiotiques, parmi eux, le manque de rendement sur les investissements, les défis scientifiques impliqués dans leur approbation et les appels à la restriction de l'usage d'antibiotiques pour éviter le développement de résistance. Il est donc nécessaire d'établir de nouveaux modèles encourageant l'investissement dans la recherche d'antibiotiques tout en proposant des mesures intelligentes afin d'éviter leur mauvaise utilisation.

Dans la recherche de nouveaux antibiotiques, un vaste réservoir de composants bioactifs qui mérite d'être exploité est retrouvé dans la nature. Les métabolites secondaires des plantes sont dotés d'une activité antimicrobienne inhérente résultant d'un long processus d'évolution visant la protection des plantes dans leur environnement. Les huiles essentielles sont des mélanges complexes dont les composants majoritaires ainsi que les mineurs, participent dans l'activité antibactérienne. En effet, il semble que des interactions synergiques entre les composants des huiles essentielles pourraient potentialiser l'effet antibactérien. Par ailleurs, les huiles essentielles, en agissant sur plusieurs cibles bactériennes, présentent un risque inférieur de développement de résistance par rapport aux antibiotiques qui agissent sur une seule cible. Dans la perspective du développement d'antibiotiques basé sur des produits d'origine naturelle, une compagnie pharmaceutique a développé CIN-102, un mélange synergique et synthétique à des concentrations définies pour chaque composant. Cela évite les inconvenants des concentrations inconnues et de

variabilités dans la composition, typiquement retrouvés dans les médicaments d'origine botanique. Des études préliminaires ont montré que CIN-102 est un agent antibactérien avec le potentiel de traiter les infections provoquées par des bactéries résistantes. Parmi toutes les potentielles applications thérapeutiques envisageables pour ce nouvel antibactérien, nous sommes intéressés à cibler des Maladies Inflammatoires Chroniques de l'Intestin (MICIs)

Les MICIs comprennent la maladie de Crohn (MC) et la recto-colite hémorragique (RCH), qui sont des pathologies dont l'étiologie n'est pas encore complètement connue. Néanmoins, il a été largement démontré que les bactéries entériques jouent un rôle important dans leur origine et développement. Bien que le traitement des MICIs soit essentiellement orienté vers une approche immuno-modulateur, l'utilisation d'agents capables de réguler le microbiote intestinal a un intérêt croissant. Parmi ces agents, les antibiotiques peuvent être utiles afin de réduire l'augmentation de la charge bactérienne généralement trouvée dans l'ileum et le colon des patients atteints de MICIS, aussi bien que de cibler des bactéries pathogènes spécifiques comme par exemple les entérobactéries. De cette façon, la réduction des déséquilibres au niveau de la flore intestinale, pourrait diminuer l'agressivité de la réponse immunitaire envers les antigènes bactériens et atténuer les signes cliniques d'inflammation intestinale.

Les principes actifs qui visent une action locale dans le bas tractus gastro-intestinale, comme dans le cas des MICIc, présentent le défi de la conception d'une formulation galénique appropriée. Étant donné qu'une forme galénique à administration orale doit parcourir l'estomac et l'intestin grêle avant d'arriver au colon, il pourrait se produire une libération ou absorption du principe actif dans le haut tractus gastro-intestinale. Ceci pourrait limiter la concentration de principe actif disponible au site d'action, exigeant donc l'administration de grandes concentrations de molécules actives pour assurer un effet thérapeutique, entrainant un risque d'apparition d'effets secondaires. Dans ce contexte, les systèmes à libération colique ciblée permettent que les formes galéniques à administration orale soient capables de contenir la libération et de résister à la dégradation des principes actifs dans le tractus gastro-intestinale, évitant les problèmes mentionnés ci-dessus. Pour cela, ces systèmes à libération colique doivent détecter leur arrivé dans le colon afin de déclencher la libération des molécules actives. Les caractéristiques physiologiques trouvées spécifiquement au niveau du colon, différentes de celles de l'estomac ou de l'intestin grêle, peuvent ainsi être utilisées dans la fabrication des formes orales à libération colique.

Les objectifs de ce travail de thèse sont les suivants :

- Caractériser l'activité antibactérienne *in vitro* du CIN-102
    - Une longue évaluation de l'activité antibactérienne du CIN-102 sur un large éventail d'isolats bactériens cliniques afin de confirmer son large spectre d'action.
    - Détermination du temps de réduction logarithmique du CIN-102 contre un éventail d'isolats bactériens.
    - Déterminer si le CIN-102 possède un effet post-antibiotique et si le CIN-102 exerce un effet sur la croissance bactérienne à des concentrations sub-inhibitrices.
    - Une étude des interactions entre le CIN-102 et des antibiotiques existant sur le marché contre un large éventail d'isolats bactériens cliniques, envisageant une future application thérapeutique dans des thérapies combinées.

- Fabriquer des formes galéniques multi-particulaires contenant le CIN-102 dans le but d'une future application thérapeutique contre les MICIs.
    - Fabrication et optimisation des systèmes réservoir du CIN-102 : évaluation *in vitro* de la libération du principe actif à partir des mini-granules pellicules.
    - Fabrication et optimisation des formes matricielles du CIN-102 : évaluation *in vitro* de la libération du principe actif à partir des mini-comprimés.
    - Evaluation *in vivo* de l'efficacité des formes galéniques multi-particulaires à libération prolongée du CIN-102 : étude de l'activité antibactérienne du CIN-102 dans un modèle expérimentale de colite.

- Analyser l'activité antibactérienne *in vitro* des agonistes du récepteur activé par les proliférateurs des peroxysomes gamma (PPARγ) et des antibiotiques communément utilisés dans le traitement des MICIs.
    - Détermination de l'activité antibactérienne de l'acide 5-aminosalicylique (5-ASA) et du GED-0507-34-Levo contre des isolats bactériens anaérobies.
    - Détermination de l'activité antibactérienne des antibiotiques à spectre étroit et large contre des isolats bactériens anaérobies.

Ce travail de thèse est composé de quatre chapitres : le premier est une introduction sur la problématique de la résistance aux antibiotiques, le rôle des compagnies pharmaceutiques dans la recherche et développement d'antibiotiques, le potentiel des produits d'origine naturelle comme source de nouveaux antibactériens, l'importances des bactéries entériques dans l'étiologie et le traitement des MICIs ; et les différentes approches utilisées pour cibler la libération des principes actifs dans le colon. Le deuxième, troisième et quatrième chapitre correspondent aux travaux de recherche effectués pour atteindre les objectifs décrits ci-dessus, respectivement. Chacun de ces trois derniers chapitres se compose d'une brève introduction sur le sujet à développer, les matériels et méthodes utilisés, les résultats obtenus ainsi que les discussions ; et une conclusion concise. Finalement une conclusion générale permettra de regrouper les buts atteints et les perspectives suggèreront de nouvelles idées pour la suite de ce projet de recherche.

Deuxième chapitre : **Sur la caractérisation de l'activité antibactérienne du CIN-102 *in vitro***

La problématique : La résistance aux antibiotiques est une menace de santé et de nouveaux antibactériens ont besoin d'être développés. Malgré ce besoin, la plupart des compagnies pharmaceutiques ont abandonné la recherche et développement d'antibiotiques.

Il existe dans l'actualité un groupe de bactéries résistantes qui pose des problèmes au niveau hospitalier mais aussi communautaire, parmi elles on retrouve : *Enterococcus faecium* résistant à la vancomycine, *Staphylococcus aureus* résistant à la méticilline*, Escherichia coli* et *Klebsiella pneumoniae* productrices de β-lactamases à spectre étendu, *Klebsiella pneumoniae* productrice de carbapénémase, ainsi qu'*Acinetobacter baumannii*, *Pseudomonas aeruginosa* et *Enterobacter spp.* multi-résistants. Néanmoins, un phénomène d'importance mondiale est la propagation des bactéries multi-résistantes à Gram négatif, pour lesquelles il n'y a pratiquement pas d'antibiotiques qui soient effectifs. En effet, plusieurs études ont montré que ces bactéries sont souvent retrouvées chez des individus sains, en faisant partie de la flore intestinale commensale.

Il a été suggéré que, dans la lutte contre la résistance aux antibiotiques, les composants bioactifs d'origine naturelle méritent d'être exploités dans le développement de nouveaux antibiotiques. Les huiles essentielles par exemple, sont des métabolites secondaires des plantes qui exercent une activité antibactérienne inhérente. De plus, grâce à la présence de

nombreux composants dans leur composition, le risque de développement de résistance contre ces mélanges est moins important

Dans ce contexte, une compagnie pharmaceutique française (Septeos), a élaboré un mélange synthétique qui ressemble à la composition des huiles essentielles de cannelle, mais à des concentrations définies pour chaque composant chimique. Ce mélange, appelé CIN-102, semble avoir une activité antibactérienne à large spectre et constitue donc un agent prometteur contre les bactéries résistantes. Les objectifs de ce travail étaient : caractériser l'activité antibactérienne *in vitro* du CIN-102 et étudier les interactions entre le CIN-102 et les différentes familles d'antibiotiques existantes sur le marché.

Matériels et méthodes : Un total de 175 isolats bactériens cliniques, comprenant des souches aérobies et anaérobies, a été étudié ; parmi eux, plusieurs bactéries multi-résistantes aux antibiotiques. La concentration minimale inhibitrice (CMI) a été déterminée en respectant les guides du *Clinical & Laboratory Standards Insitute (CLSI)*, cependant le CIN-102 a été solubilisé à l'aide des tensioactifs pour lesquels l'absence d'activité antibactérienne a été préalablement vérifiée. Le temps de réduction logarithmique du CIN-102 a été étudié à des concentrations supra-inhibitrices (4 fois la CMI et 1%). L'effet post-antibiotique a été déterminé par la méthode proposée par *Craig et Gudmundsson*, à la CMI et 4 fois la CMI du CIN-102. L'effet des concentrations sub-inhibitrices du CIN-102 sur la croissance bactérienne a été déterminé à la CMI/2 et à la CMI/4. Les interactions entre le CIN-102 et les antibiotiques ont été déterminées par la méthode du *checkerboard* et les résultats ont été interprétés à l'aide du *Fractional inhibitory concentration (FIC) index*.

Résultats et discussions : Toutes les souches testées sont inhibées par le CIN-102. Les bactéries anaérobies sont les plus sensibles au CIN-102, et *Pseudomonas aeruginosa* possède les CMIs les plus élevées. Le temps nécessaire pour réduire la concentration bactérienne d'un log est court pour le CIN-102, spécialement chez des bactéries à Gram négatif.

Ensuite, le CIN-102 présente un effet post-antibiotique et il ralentie la croissance bactérienne à des concentrations sub-inhibitrices sur plusieurs types de bactéries : aérobies, anaérobies, à Gram positif et à Gram négatif.

Finalement, quand le CIN-102 est utilisé en combinaison avec des autres antibiotiques : il y a une absence d'interactions antagonistes, il existe des interactions synergiques (notamment avec la colistine et les aminogycosides), les souches résistantes aux antibiotiques redeviennent sensibles.

Il semble que l'activité antibactérienne du CIN-102 est principalement due à la présence du cinnamaldehyde, composant majoritaire (86,7%). Il a été démontré que le cinnamaldehyde est capable de se lier à la protéine FtsZ, qui est un homologue de la tubuline chez les procaryotes. Le fait que cette protéine soit largement conservée chez les bactéries, pourrait expliquer le large spectre d'action du CIN-102. Néanmoins, il est important de prendre en considération la présence des composants minoritaires du CIN-102. Ces terpenoides sont capables de perméabiliser les membranes bactériennes, entrainant une fuite du contenu intracellulaire. Par conséquent, le mécanisme d'action du CIN-102 est un ensemble des différentes cibles bactériennes. Il est important de noter que, des études sur des modèles d'infection chez l'animal semblent indiquer que des concentrations actives du CIN-102 peuvent être atteintes *in vivo*.

De plus, la présence d'un effet post-antibiotique et la réduction de la croissance bactérienne à des concentrations sub-inhibitrices du CIN-102 sont importantes pour établir la future posologie du CIN-102 visant une application thérapeutique.

Enfin, la présence d'interactions synergiques avec des antibiotiques est d'importance capitale dans le cadre des thérapies combinées, ou des concentrations sub-inhibitrices du CIN-102 pourraient aider à rétablir l'activité des antibiotiques contre des bactéries multi-résistantes. Il est donc important de poursuivre des études *in vivo* et des essais cliniques afin de confirmer ces résultats encourageants *in vitro.*

Conclusion intermédiaire: CIN-102 est un antibactérien a large-spectre d'action et un agent prometteur dans la lutte contre des bactéries multi-résistantes.

Troisième chapitre* : Sur les formulations à libération prolongée du CIN-102, performance i*n vitro/in vivo

La problématique : Les MICIs sont des pathologies chroniques dont l'étiologie n'est pas encore complètement connue. Cependant, il a été largement démontré que les bactéries entériques

jouent un rôle primordial dans l'étiologie, le développement et le phénotype de ces maladies. Par exemple: les lésions sont localisées dans des endroits ou la charge bactérienne est la plus forte, il y a une composition et une activité métabolique anormales de la flore intestinale, il y a une augmentation de la concentration de bactéries adhérées a la muqueuse intestinale, il y a une réponse immunitaire exacerbée aux bactéries intestinales commensales, entre autres. Dans ce contexte, des antibiotiques peuvent agir soit en ciblant les bactéries pathogènes soit en diminuant les concentrations bactériennes au niveau de la lumière intestinale ou adhérentes à la muqueuse. La modulation de la microflore intestinale en cas d'inflammation pourrait donc améliorer les symptômes de la colite et réduire la réponse immunitaire aux antigènes bactériens. Il semble donc que l'utilisation d'agents antibactériens, seuls ou comme adjuvants des immunomodulateurs, pourrait être utile dans les traitements des MICIs.

Apres avoir confirmé l'activité antibactérienne à large spectre du CIN-102, nous nous sommes intéressés à l'utilisation de cet agent dans le cadre des MICIs. L'hypothèse de cette étude se base sur la délivrance du CIN-102 dans le colon, qui pourrait améliorer le cours de la colite à travers une diminution de la charge bactérienne totale du colon et/ou d'une activité ciblée sur des bactéries pathogènes. Des études préliminaires ont montré que des rats atteints d'une colite induite au trinitrobenzene sulfonate (TNBS) et traités avec le CIN-102 par gavage orale ont eu des effets mineurs sur la flore intestinale. Il a été conclu qu'une forme galénique appropriée pourrait être capable d'assurer la délivrance et donc l'activité antibactérienne du CIN-102 dans le site d'action souhaité : le colon.

Plusieurs approches sont décrites dans la littérature afin de cibler la libération des principes actifs au niveau du colon. Les principales comprennent des systèmes dépendants du pH, du temps de transit gastro-intestinal et de l'activité enzymatique des bactéries coliques. Etant donné que les patients atteints des MICIs ont souvent des variations du pH intestinal et des temps de transit altérés, l'approche enzymatique semble plus appropriée. En effet, la concentration bactérienne au niveau du colon est beaucoup plus grande (10^{11}- 10^{12} unités formant colonies (UFC)/g) par rapport à l'estomac (10^{2} UCF/g) ou l'intestin grêle (10^{3}-10^{4} UFC/g). Ces bactéries sont capables de dégrader des polysaccharides non-digestibles qui proviennent du haut tractus gastro-intestinal, et de les utiliser comme source d'énergie. Des systèmes à libération colique à base de ces polysaccharides d'origine naturelle ont été amplement étudiés.

Ce travail a comme objectif la fabrication des formulations à libération prolongée du CIN-102 (des mini-granules pelliculés et des mini-comprimés) en utilisant des polysaccharides dégradables par des bactéries coliques. Dans un deuxième temps, l'efficacité de ces systèmes a été étudiée dans un modèle de colite aigüe chez la souris, où l'activité antibactérienne du CIN-102 au niveau du colon a été analysée.

Matériels et méthodes : Mini-granules : les noyaux des granules contenant le CIN-102 ont été fabriqués par extrusion-spheronization. Différents excipients ont été utilisés : cellulose microcristalline, lactose, silice et/ou aluminosilicate de magnésium. Ensuite les mini-granules ont été pelliculés à l'aide des mélanges des polysaccharides (inuline ou amidon de pois) et des polymères insolubles dans l'eau (Aquacoat® ou Kollicoat®). Eventuellement, un enrobage intermédiaire à base d'un polymère hydrophile (Opadry®) a été incorporé. Finalement les mini-granules enrobés sont *curés* à 60° C pendant 24h.

Mini-comprimés : les mini-comprimés ont été fabriqués par compression directe. Des triglycérides solides, l'aluminosilicate de magnésium, l'inuline et l'ethylcellulose ont été utilisés comme excipients.

Tests de libération *in vitro* : la libération du cinnamaldehyde a partir des formulations à libération prolongée a été étudiée dans des milieux simulant le pH de l'estomac et de l'intestin grêle.

Etudes *in vivo* : les mini-granules enrobés et les mini-comprimés qui ont montré les meilleurs profiles de libération *in vitro* ont été soumis à des tests *in vivo*. Des souris de fond génétique B57CL6 ont été traitées au dextran sodium sulfate (DSS) afin d'induire une colite. Les formes galéniques ont été administrées par voie orale. Le cours de la colite a été évalué en observant la présence de sang dans les selles et l'occurrence de diarrhées. A la fin du traitement, les souris ont été sacrifiées et des échantillons de selles ainsi que des tissus coliques ont été récupérés pour suivre une analyse bactériologique. Une étude de l'expression des cytokines dans des tissus colique a été aussi effectuée.

Résultats et discussions : Mini-granules enrobés : les noyaux à base de cellulose microcristalline, lactose et/ou silice et enrobés avec un mélange d'amidon de pois et d'Aquacoat®, ont montré une libération prématurée du cinnamaldehyde *in vitro*. Pour cette raison, certains excipients ont été retirés de la formulation (lactose) et d'autres ont été incorporés (aluminosilicate de

magnésium) afin d'augmenter la rétention du CIN-102 dans les noyaux des mini-granules. Un enrobage intermédiaire a aussi été incorporé, et l'enrobage externe s'est composé d'un mélange d'inuline et de Kollicoat®. De cette façon, la libération prématurée du cinnamaldehyde dans des milieux mimant l'estomac a été significativement diminuée.

Mini-comprimés : la libération du cinnamaldehyde a été significativement réduite dans des milieux simulant l'estomac et l'intestin grêle. Cependant, l'incorporation de l'inuline déclenche une libération prématurée du principe actif. Afin de contourner ce problème, l'ethylcellulose a été additionnée dans la composition des mini-granules. Ainsi, la libération du cinnamaldehyde a été largement réduite dans les milieux simulant le haut tractus gastro-intestinal.

Etudes *in vivo* : Il a été montré que, les souris atteintes de colite et traitées avec les formulations à libération prolongée du CIN-102 présentaient une diminution de la présence de sang dans les selles ainsi qu'une diminution des diarrhées, par rapport aux souris non-traitées. D'ailleurs, les souris traitées avec les formulations à libération prolongée ont eu une diminution significative de la concentration d'entérobactéries dans les selles et dans la muqueuse colique, par rapport aux souris non-traitées. La diminution des entérobactéries, considérées comme des espèces pathogènes en cas de MICIs, semble donc être associée à l'amélioration du cours de la colite.

Conclusion intermédiaire: Les mini-granules enrobés et les mini-comprimés de CIN-102 ont un potentiel pour le traitement de la colite. La libération du CIN-102 au niveau du colon permet une réduction de la charge d'entérobactéries dans les selles et dans la muqueuse chez les souris atteintes de colite. Cela s'accompagne d'une amélioration clinique du développement de la colite. Des études supplémentaires sont nécessaires afin de mieux élucider l'action du CIN-102 en cas de colite.

Quatrième chapitre : Sur l'activité antibactérienne *in vitro* des agonistes des PPARγ et des antibiotiques utilisés contre les MICIs

La problématique : Les MICIs sont des pathologies chroniques dont l'étiologie est multifactorielle mais n'a pas encore été complétement élucidée. Ces maladies semblent être provoquées par une réponse immunitaire exacerbée aux bactéries commensales intestinales chez des individus génétiquement prédisposés. De plus, des facteurs environnementaux peuvent précipiter leur déclanchement ou leur réactivation.

Le but principal du traitement des MICIs est l'induction d'une rémission clinique ainsi que la maintenance de cette rémission au cours du temps. Le 5-ASA est très utilisé dans les thérapies des MICIs, notamment chez les patients atteints de RCH. Le 5-ASA inhibe la production de prostaglandines, de leucotriènes, et les effets du facteur de nécrose tumorale alpha, entre autres. Par ailleurs, il a été récemment démontré que le PPARγ est un récepteur clé qui détermine les principaux effets du 5-ASA dans le colon.

En effet, le PPARγ est capable d'inhiber deux des plus importantes voies de signalisation qui régulent l'expression des gènes pro-inflammatoires : les voies du facteur nucléaire-κB et des kinases activées par le stress. De cette façon, ce récepteur joue un rôle important dans la régulation de l'inflammation intestinale. Par exemple, l'expression de PPARγ est réduite dans les tissus coliques des souris atteintes de colite, ainsi que dans les cellules épithéliales du colon des patients atteints de RCH. De plus, dans des modèles expérimentaux de colite, des agonistes de PPARγ peuvent inhiber la réponse inflammatoire. Dans le contexte clinique, les agonistes PPARγ ont été effectifs en cas de RCH. D'ailleurs, il existe un lien étroit entre le microbiote intestinal et la régulation de l'expression du PPARγ par les cellules épithéliales du colon.

Ces faits ont amené au développement des analogues du 5-ASA mais en possédant une affinité plus forte pour le PPARγ et moins d'effets secondaires. Dans ce contexte, le GED-0507-Levo a été conçu en tant que nouveau candidat possédant des propriétés anti-inflammatoires et analgésiques très puissantes. Cette molécule est actuellement en essai clinique de phase 2 contre les MICIs.

Compte tenu de l'intérêt croissant pour le PPARγ comme cible d'action dans le traitement des MICIs, et son étroite relation avec la microflore intestinale, une étude de l'activité antibactérienne *in vitro* des agonistes du PPARγ est présentée dans ce travail. Ces résultats pourraient donner des informations supplémentaires sur le mécanisme d'action de ces agents contre l'inflammation intestinale. Egalement, plusieurs familles d'antibiotiques communément utilisées en cas de MICIs ont était soumises à une évaluation de leur activité antimicrobienne contre des isolats bactériens anaérobies.

Matériels et méthodes : Un total de 32 isolats bactériens cliniques comprenant des bactéries anaérobies à Gram positive et à Gram négatif a été considéré dans cette étude. Les agonistes du PPARγ utilisés étaient : le 5-ASA, le GED-0507-Levo et la pioglitazone. Les antibiotiques

testés : amoxicilline, amoxicilline + acide clavulanique, imipenème, érythromycine, ciprofloxacine et métronidazole. Pour la détermination des CMIs, les procédures décrites dans les guides du *CLSI* ont été suivies. La gélose columbia supplémentée avec du sang de cheval a été utilisée comme milieu pour la détermination des CMIs.

Résultats et discussions : Concernant les bactéries à Gram positif, les β-lactames y compris les carbapenemes ont été capables d'inhiber la plupart des souches testées. L'érythromycine, le ciprofloxacine et le métronidazole ont montré une activité moins efficace. Pour les agonistes du PPARγ : le 5-ASA et le GED-0507-34-Levo n'ont exercé aucune activité antibactérienne, cependant la pioglitazone a inhibé une souche de *C. clostridioforme* et de *F. magna*.

Concernant les entérobactéries anaérobies facultatives, aucun antibiotique n'a été capable d'inhiber toutes les entérobactéries. Les agonistes du PPARγ se sont montrés dépourvus d'activité antibactérienne contre ces bactéries.

Concernant les bactéries anaérobies strictes à Gram négatif, l'imipenème et le métronidazole ont été les antibiotiques les plus actifs. Le 5-ASA et GED-0507-34-Levo n'ont montré aucune activité contre ces bactéries, *Bacteroides* et *Fusobacterium* ont été inhibés par la pioglitazone.

Ces études montrent que le 5-ASA et GED-0507-Levo sont dépourvus d'activité antibactérienne *in vitro*. Malgré que d'autres études ont montré une activité antibactérienne *in vivo* du 5-ASA, il est possible que ceci soit dû à un effet indirect de la régulation de l'inflammation intestinale sur la microflore. Néanmoins, il a aussi été montré que le 5-ASA est capable d'altérer l'expression des gènes liés aux activités métaboliques et aux facteurs de virulences des bactéries. Ce constat pourrait aussi avoir un impact sur le microbiote intestinal en cas d'inflammation. Ceci est la première étude qui porte sur l'activité antibactérienne du GED-0507-Levo. L'analyse de la flore intestinale chez l'animal après administration de ces 2 agonistes du PPARγ pourrait expliquer si la réduction de la réponse inflammatoire a un effet indirect sur les bactéries entériques.

Conclusion intermédiaire : Ces résultats montrent que les agents anti-inflammatoires utilisés contre les MICIs (5-ASA et GED-0507-Levo) n'ont pas une activité antibactérienne inhérente *in vitro*. De plus, aucun des antibiotiques testés n'était capable d'inhiber toutes les souches

bactériennes étudiées. Cette observation témoigne d'un manque d'agents à large-spectre d'action, capables de réduire la charge bactérienne augmentée en cas de colite.

Conclusion générale

Dans ce travail nous présentons CIN-102 comme un agent antibactérien à large spectre d'action prometteur, aussi capable d'agir contre des bactéries multi-résistantes. Ce nouvel antibactérien pourrait également avoir un effet thérapeutique grâce à la modulation du microbiote intestinal en cas d'inflammation.

- Dans un premier temps, le large spectre d'action du CIN-102 a été confirmé. Les 175 isolats bactériens testés ont été inhibés par le CIN-102. Ce nouvel agent a ainsi inhibé des bactéries multi-résistantes suscitant un grand intérêt dans le cadre des infections hospitalières et communautaires. CIN-102 a exercé un temps rapide de réduction logarithmique, spécialement chez les bactéries à Gram négatif. De plus, CIN-102 a montré un effet post-antibiotique et a été capable de ralentir la croissance bactérienne a des concentrations sub-inhibitrices, ces deux derniers aspects sont d'importance capitale pour la posologie du médicament. Un aspect remarquable a été l'étude des interactions entre CIN-102 et antibiotiques, ces résultats ont montré que : il n'y a pas d'interaction antagonique, il y a des interactions synergiques avec tous les antibiotiques testés (surtout avec la colistine et les aminoglycosides), CIN-102 a rétabli l'activité des antibiotiques contre des souches résistantes. Ces résultats suggèrent que CIN-102 est un antibactérien à large spectre d'action prometteur mais des études *in vivo* et des essais cliniques permettront de confirmer son activité.

- Deuxièmement, des formes à libération prolongée du CIN-102 peuvent délivrer cet agent dans le tractus gastro-intestinal inférieur dans un modèle expérimental de colite. Nous avons démontré que le CIN-102 contenu dans des mini-granules enrobés et des mini-comprimés a été libéré dans le site d'action cible pour exercer son activité antibactérienne. Dans ce cadre, CIN-102 a réduit significativement la concentration d'entérobactéries présentes dans les contenus de la lumière colique (selles) ainsi que dans les tissus coliques (adhérentes à la muqueuse). Cet effet

antibactérien ciblé au colon, semble être lié à l'amélioration du développement de la colite chez la souris (réduction de la présence du sang dans les selles et des diarrhées). Ces résultats suggèrent que CIN-102, une fois libéré dans le colon, peut moduler la microflore intestinale, qui pourrait donc améliorer l'état de l'inflammation. Des études futures permettront de mieux comprendre le mécanisme d'action du CIN-102 en cas de colite.

- En troisième lieu, nous avons constaté le besoin d'antibiotiques à large-spectre d'actions dans le traitement des MICIs. Les agonistes des PPARγ utilisés en tant qu'anti-inflammatoires en cas des MICI sont dépourvus d'activité antibactérienne *in vitro*. 5-ASA et GED-0507-34-Levo n'ont pas été actifs contre des souches anaérobies testées. De la même façon, aucun des antibiotiques étudiés n'a montré une véritable action à large-spectre contre les souches anaérobies testées. Cette observation souligne le besoin d'antibiotiques à large spectre d'action capables de moduler la microflore colique afin de rétablir l'homéostasie intestinale en cas de MICIs

Perspectives

- ***Concernant le CIN-102***

  Nous sommes intéressés à l'élucidation du mécanisme d'action du CIN-102. Le caractère lipophile du CIN-102 semble indiquer que les membranes bactériennes constituent une cible d'action importante. A cet égard, des changements morphologiques chez des bactéries exposées au CIN-102 ont été observés par microscopie électronique en transmission. Les photos montrent une fuite du contenu intracellulaire après l'exposition des bactéries à une grande concentration de CIN-102. Des études à des concentrations inférieures permettront de mieux comprendre le mécanisme d'action.

  Ensuite, nous envisageons d'effectuer des études afin de détecter l'apparition de résistance bactérienne au CIN-102 et les effets des concentrations sub-inhibitrices sur des facteurs de virulence tels que la formation des biofilms.

- ***Concernant les formulations à libération prolongée :***

Un problème important retrouvé au cours de la fabrication des mini-granules pelliculés et des mini-comprimés du CIN-102 a été les pertes de cet agent à cause de sa volatilisation. Nous souhaitions, par conséquent, encapsuler le CIN-102 afin de renforcer sa stabilité au sein des formulations à libération prolongée. L'encapsulation des huiles essentielles, sous forme de micro- ou nanoparticules, permet non seulement d'améliorer leur stabilité mais aussi d'augmenter leur solubilité dans l'eau et donc leur biodisponibilité. Dans ce but, la micro-encapsulation des principes actifs à l'aide des cyclodextrines est une approche intéressante. Des études préliminaires montrent que le cinnamaldehyde, composant majoritaire du CIN-102, est capable d'interagir avec l'hydroxypropyl-β-cyclodextrines probablement à cause de la formation d'un complexe d'inclusion. Cela a été montré par spectroscopie UV, en observant une diminution de l'absorbance du cinnamaldehyde à 290 nm en présence des concentrations croissantes de cyclodextrine.

D'autres approches comprennent la fabrication des nanoparticules en utilisant des polymères biodégradables comme l'acide poly(lactique-co-glycolique) (PLGA) ou les microparticules composées des polysaccharides naturels fabriquées par *spray drying*.

- ***Concernant l'application thérapeutique contre les MICIs***

  Dans un premier temps, nous sommes intéressés à mieux analyser l'activité du CIN-102 dans des modèles expérimentaux de colite aigüe. Pour cela nous avons besoin des protocoles mieux structurés, par exemple : à travers l'utilisation des formulations placebo, des critères bien définis pour l'évaluation de la colite, l'effet à différentes concentrations du CIN-102. Dans ce cadre, des mini-granules placebo et contenant le CIN-102 ont été évaluées dans un modèle de colite aigüe chez la souris. Deux paramètres bien définis : l'indice d'activité de la maladie et le rapport poids/taille du colon ont permis d'évaluer les effets du CIN-102. Actuellement, ces résultats sont complétés par une analyse de la microflore colique, l'expression des cytokines et des études histologiques.

Dans un deuxième temps, nous souhaitions étudier l'activité du CIN-102 en combinaison avec des anti-inflammatoires dans des souris atteintes de colite. De la même façon, il est envisagé d'analyser dans le futur, l'effet de CIN-102 dans des modèles de colite chronique et infectieuse.

- ***Concernant les agonistes des PPARγ***

  L'étude *in vivo* de l'activité antibactérienne des agonistes des PPARγ confirmera si l'effet anti-inflammatoire de ces agents pourrait influencer indirectement la flore intestinale des souris.

PUBLICATIONS AND PRESENTATIONS

Original Research Articles

- M.S. Nieto-Bobadilla, F. Siepmann, M. Djouina, L. Dubuquoy, N. Tesse, J.F. Willart, L. Dubreuil, J. Siepmann, C. Neut. Controlled Delivery of a New Broad Spectrum Antibacterial Agent against Colitis: *In Vitro* and *In Vivo* Performance. Eur. J. Pharm. Biopharm. (*submitted-revised*).

- M.S. Nieto-Bobadilla, S. Mahieux, N. Tesse, C. Neut, L. Dubreuil. Bacteriological Characterization of a Novel Broad-Spectrum Antibacterial Agent of Natural Origin (*in preparation*).

Oral Presentations

- M.S. Nieto-Bobadilla, F. Siepmann, J. Siepmann, C. Neut. Towards Colon Targeting of a New Broad-Spectrum Antibacterial Agent, 7th Annual Pharmaceutical Solid State Research Cluster Meeting.
  Lille, France, 2013.

- M.S. Nieto-Bobadilla, F. Siepmann, M. Djouina, L. Dubuquoy, J. Siepmann, L. Dubreuil, C. Neut. Towards Colon Targeting of a New Broad-Spectrum Antibacterial Agent, 8th Annual Pharmaceutical Solid State Research Cluster Meeting.
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Poster Presentation

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