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REVIEW

The human gut sterolbiome: bile acid-microbiome endocrine aspects and therapeutics

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Abstract The human body is now viewed as a complex ecosystem that on a cellular and gene level is mainly prokaryotic. The mammalian liver synthesizes and secretes hydrophilic primary bile acids, some of which enter the colon during the enterohepatic circulation, and are converted into numerous hydrophobic metabolites which are capable of entering the portal circulation, returned to the liver, and in humans, accumulating in the biliary pool. Bile acids are hormones that regulate their own synthesis, transport, in addition to glucose and lipid homeostasis, and energy balance. The gut microbial community through their capacity to produce bile acid metabolites distinct from the liver can be thought of as an "endocrine organ" with potential to alter host physiology, perhaps to their own favor. We propose the term "sterolbiome" to describe the genetic potential of the gut microbiome to produce endocrine molecules from endogenous and exogenous steroids in the mammalian gut. The affinity of secondary bile acid metabolites to host nuclear receptors is described, the potential of secondary bile acids to promote tumors, and the potential of bile acids to serve as therapeutic agents are discussed.

Abbreviations: APC, adenomatous polyposis coli; BA, bile acids; BSH, bile salt hydrolases; CA, cholic acid; CDCA, chenodeoxycholic acid; COX-2, cyclooxygenase-2; CRC, colorectal cancer; CYP27A1, sterol-27-hydroxylase; CYP7A1, cholesterol 7α-hydroxylase; CYP8B1, sterol 12α-hydroxylase; DCA, deoxycholic acid; EGFR, epidermal growth factor receptor; FAP, familial adenomatous polyposis; FGF15/19, fibroblast growth factor 15/19; FXR, farnesoid X receptor; GABA, γ-aminobutyric acid; GPCR, G-protein coupled receptors; HMP, Human Microbiome Project; HSDH, hydroxysteroid dehydrogenase; LCA, lithocholic acid; LOX, lipooxygenase; MetaHIT, Metagenomics of the Human Intestinal Tract; NSAIDs, non-steroidal anti-inflammatory drugs; PKC, protein kinase C; PSC, primary sclerosing cholangitis; PXR, pregnane X receptor; UDCA, ursodeoxycholic acid; VDR, vitamin D receptor

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1. Introduction

The "omics" revolution and the systems biology approach are fundamentally reshaping thought about the human body. Microbiome specialists now regard the epithelial surfaces of body (skin, oral and gastrointestinal, respiratory, and reproductive tract) as an interconnected network of ecosystem harboring all three domains of life, the eukarya, the prokarya, and the archaea. The gut microbiome is now regarded as a virtual organ[1]. It is unique among organs as it is composed of hundreds of species and thousands of strains of prokaryotes and their viruses[2]. The Human Genome Project is only part of the metagenome that comprises the human ecosystem. Indeed, work on the "second human genome"[3] has been undertaken by the Human Microbiome Project (HMP) in the United States, and by Metagenomics of the Human Intestinal Tract (MetaHIT) in Europe[4,5]. A major goal of much of this research is to understand the structure and function of the gut microbiome, and how diet, antibiotics, and pharmaceuticals perturb it. These studies also aim to uncover fundamental host-microbe interactions.

Research over the past several decades highlights the expanding role of bile acids (BAs) as hormones regulating lipid, glucose, lipoprotein, energy metabolism in addition to inflammatory responses[6,7]. BAs are also known to fundamentally shape the gut microbiome and vice versa. We will argue here that our gut microbiome should now be thought of as an "endocrine organ"[8,9] and we will focus on BAs, although this concept applies to many other classes of molecules including catecholamines[9], short-chain fatty acids[9], amino acids (GABA, γ-aminobutyric acid)[10] and steroid hormones[11].

2. Prokaryote-eukaryote influences on BA pool

Host primary BAs are synthesized in the liver from cholesterol and modified by prokaryotes in the gut[12] (Fig. 1). In humans, there are two separate pathways forming two primary BAs. The neutral pathway is thought to be the major pathway of BA synthesis under healthy conditions in humans. The liver is the only organ capable of producing the 14 enzymes which facilitate *de novo* synthesis of the dihydroxy BA chenodeoxycholic acid (CDCA; 3α, 7α), and the trihydroxy BA cholic acid (CA; 3α, 7α, 12α)[13]. The rate-limiting step of BA synthesis from cholesterol is initiated by cholesterol 7α-hydroxylase (CYP7A1). The synthesis of CA and the ratio of CA/CDCA are regulated by sterol 12α-hydroxylase (CYP8B1)[6]. Both CYP7A1 and CYP8B1 are tightly regulated by BAs through feedback repression mediated by farnesoid X receptor (FXR)-dependent induction of fibroblast growth factor 15/19 (FGF15/19) in the intestines[14]. FGF15/19 binds to FGF receptor 4/β-Klotho complex in hepatocytes which in turn activates the JNK1/2 and ERK1/2 signaling cascades, down-regulating *CYP7A1* mRNA expression in the liver[15–17].

The acidic pathway is initiated by mitochrondrial sterol-27-hydroxylase (CYP27A1) in the inner mitochondrial membrane[13]. CYP27A1 is expressed extra-hepatically in numerous tissues. The acidic pathway is thought to be a minor pathway for BA synthesis under the normal physiological state, but appears to predominate in patients with cholestatic liver disease as CYP7A1 is down-regulated by the inflammation produced as a consequence of small bowel overgrowth in these patients[18].

The BA pool of rodents, in addition to CA, converts a significant quantity of CDCA to muricholic acids by 6β-hydroxylation. Rodents, unlike humans, are capable of making 7α-hydroxylating secondary BAs return to the liver during enterohepatic circulation, thus maintaining a highly hydrophilic biliary pool. By contrast, secondary BAs can predominate in some humans approaching 60% of the biliary pool[12].

As bile salts enter the terminal ileum and the proximal colon, they are rapidly deconjugated by prokaryotic enzymes known as bile salt hydrolases (BSH)[12,19]. BSH have different affinities for

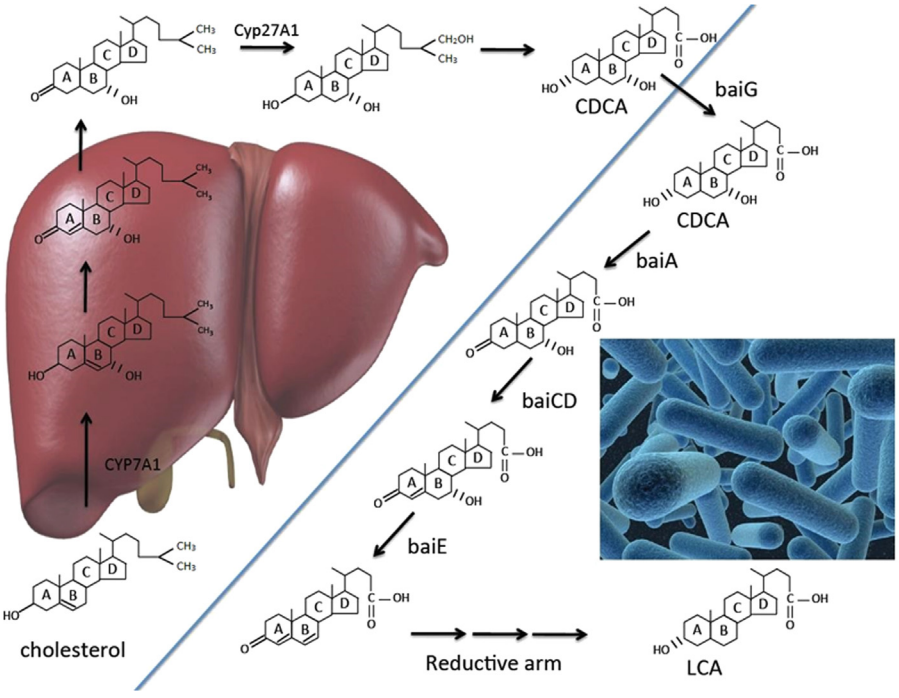

Figure 1 Pathway from cholesterol to lithocholic acid in the human ecosystem. CDCA is synthesized in the liver *via* the neutral pathway through a series of oxidative steps. LCA is produced by members of the gut microbiome through a multi-step biochemical pathway, the first half of which is oxidative followed by a net 2 electron reduction.

taurine or glycine conjugates[20]. BSH are widespread amongst prokaryote taxa. Functional metagenomic screening demonstrated that BSH located in the three major bacterial divisions Firmicutes (30%), Bacteroidetes (14.4%) and Actinobacteria (8.9%)[20]. *Methanobrevibacter smithii* and *Methanosphaera stadmanae*, the only two archaeal species known to inhabit the human gut possess BSH[20]. Bile salts, particularly glycine conjugates, have anti-microbial properties. BSH functions largely as a detoxification mechanism to reduce the levels of bile salts in the colonic environment[19].

The 3α, 7α, and 12α-hydroxy groups can be oxidized and epimerized by microbial hydroxysteroid dehydrogenase (HSDH) enzymes[12]. It is thus possible to generate 27 different oxo- and hydroxyl-metabolites of CA. Bokkenheuser and Winter[11] reflect on the difficulty in understanding the benefit a bacterium would incur through many steroid biotransformations, which they note do not appear to enhance growth *in vitro*: "These observations make it hard to understand why a bacterial species in the first place should have taken the trouble to develop these highly unusual and specialized enzymes." In the same review article, they note the peculiarity of producing oxo-derivatives through HSDH enzymes: "What benefit obligate anaerobes derive from this oxidation reaction is hard to understand". It is difficult to understand because in the anaerobic environment where gut bacteria inhabit, reactions tend to be reductive in nature for energetic considerations. The concept of gut microbiome as endocrine "organ" may make sense of these observations. One hypothesis is that HSDH enzymes may have evolved to generate signaling molecules that affect other microbes (microbe-microbe interactions) or alter host physiology in a way that benefits the organism which possess the sterolbiome gene(s) (interkingdom signaling).

Indeed, conversion of CDCA to 7-oxo-LCA by microbial 7α-HSDH is reduced in the liver by human 11β-HSDH-1, an enzyme whose function is primarily to convert cortisone to the active glucocorticoid, cortisol[21]. Microbial derived 7-oxo-LCA acts as a competitive inhibitor of 11β-HSDH-1, and thus may influence the ratio of cortisone/cortisol. Perhaps alteration of the cortisol/cortisone ratio induces physiological effects on the host that translates into a more favorable environment to the bacterium possessing 7α-HSDH. Determining the physiological significance of oxo- and primary BA epimers on both the host and other members of the microbiome may be an important, and as yet largely untapped area of research.

The secondary BAs, deoxycholic acid (DCA; 3α, 12α) and lithocholic acid (LCA; 3α) are produced solely by a few species of anaerobic gut bacteria in the genus *Clostridium* (Fig. 1). Removal of the 7α-hydroxy group requires multiple steps including ligation to CoA (*baiB* gene), oxidation of the 3α-hydroxy (*baiA* gene), oxidation of the C_4–C_5 (*baiCD* gene), 7α-dehydration (*baiE* gene), followed by sequential NAD(P)H-dependent reductions at C_6–C_7, C_4–C_5, 3-oxo, followed by export of the secondary BAs[12]. The genes encoding enzymes in the reductive arm of this pathway have yet to be identified. In addition, the BA 7α-dehydroxylation pathway can generate secondary "allo" BAs[22]. Secondary allo-BAs (5α-) are structural epimers of secondary BAs DCA and LCA. The A/B rings of allo-bile acids are planar, similar to steroid hormones, and more hydrophobic. Few studies have looked at the physiological effects of secondary allo-bile acids. However, there are a few reports of significant increases of fecal secondary allo-BAs in colon cancer patients[23].

Colonic BA composition has recently been shown to regulate germination of *Clostridium difficile*, a microbe responsible for billions of dollars in health costs yearly[24]. Primary BAs induce germination, while secondary BAs, whose formation is often inhibited by antibiotics, suppress *C. difficile* germination[24]. Indeed, recent work has shown that *Clostridium scindens,* presumably through the production of the secondary BA DCA also inhibits the growth of *C. difficile*[25]. Thus, under certain circumstances, such as antibiotic-associated diarrhea, secondary BA formation may be protective to infection by *C. difficile*.

The gut microbiome is thus an organ capable of producing a cocktail of BA hormones that affect the composition and function of the gut microbiome, and interact with cells of the gastro-intestinal tract during the enterohepatic circulation, in addition to distant targets including heart, kidney and even adipose tissue[26] (Fig. 2). Further elucidating the gut microbial genes involved in BA and other steroid hormones introduced in the gut by both endogenous and exogenous (diet and pharmaceuticals) is an important research direction simply because by this capacity, the gut microbiome is an endocrine organ. We propose the term "sterolbiome" to describe the repertoire of human gut microbiome genes involved in the uptake and metabolism of host, pharmaceutical and diet derived steroids.

3. Sterolbiome genes and host nuclear receptors

BAs are activators of several mammalian nuclear receptors. The sterolbiome interacts with these receptors by producing secondary BAs with altered affinities to these receptors. BA affinities for FXRα are as follows: CDCA > LCA = DCA > CA[6]. FXRα plays important roles in regulation of BA synthesis, regulation of the enterohepatic circulation of BAs, in addition to regulation of glucose, lipoprotein, lipid metabolism, inflammation, and tumor suppression[27]. One of the most dramatic recent observations of the effect of FXR activation by secondary BAs was the demonstration in mice that the gut sterolbiome could control the size of the BA pool by removing the potent FXR antagonist tauro-β-muricholic acid[28]. FXR activation is also an indirect regulator of the structure of the microbiome through the BA induced FXR-dependent expression of anti-microbial peptides[29]. Thus the types of BAs present, through their activation of FXR, have the potential to modulate the BA pool size, the microbiome composition, and host glucose and lipid metabolism. It may be speculated that members of the microbiome gain a selective advantage by altering cellular signaling *via* production of endocrine molecules in the form of secondary BAs.

BA affinities for pregnane X receptor (PXR) LCA > DCA > CA and vitamin D receptor (VDR) 3-oxo-LCA > LCA > DCA > CA appear important in detoxification of toxic LCA[30]. LCA has been shown to induce double-strand breaks in DNA[31]. The mammalian host responds by metabolizing LCA, mainly through sulfation, allowing for more efficient excretion and reduced hydrophobicity. Chimpanzees fed CDCA which is therapeutic in humans die as a result of the inability to detoxify LCA. These observations underscore the co-evolution among sterolbiome, activation of host nuclear receptors PXR, and downstream detoxification mechanisms in *Homo sapien* to cope with the toxicity of secondary BAs.

BAs also activate G-protein coupled receptors (GPCR) such as TGR5, sphingosine-1-phosphate receptor 2, and muscarinic receptor M2[6]. TGR5 expression is widespread in human tissues including gallbladder, spleen, intestinal neuroendocrine cells, macrophages, cholangiocytes, and brown adipose tissue[32]. Indeed,

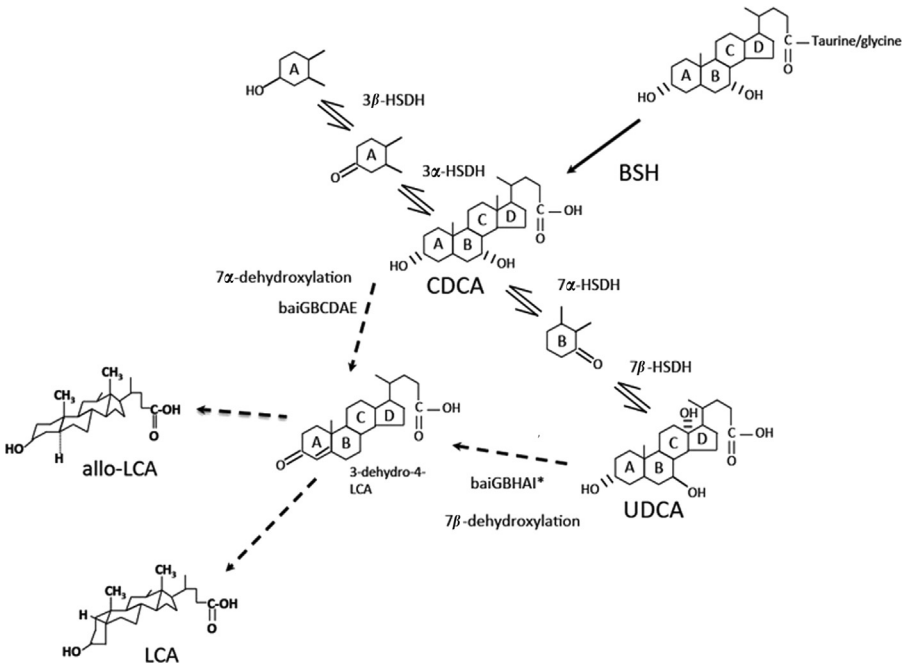

Figure 2 Metabolism of CDCA by the gut microbiome. As bile salts enter the gut and are rapidly deconjugated by BSH enzymes expressed by a diverse group of gut bacteria and archaea. Hydroxy groups at C3 (A ring) and C7 (B ring) can be epimerized through the concerted action of both α- and β-hydroxysteroid dehydrogenase enzymes. Epimerization at the C7 position results in the 7β-hydroxy bile acid UDCA, a therapeutic molecule used in treatment of GI disorders. Both CDCA and UDCA result in formation of a common 3-dehydro-4-LCA structure during the oxidative arm of the bile acid 7α-dehydroxylating and 7β-dehydroxylating pathways, each of which overlaps in many enzymatic steps. This 3-dehydro-4-LCA structure can be reduced at the C4 position yielding either LCA (5β-hydrogen) or allo-LCA (5α-hydrogen).

BAs are suggested to play an important role in host energy metabolism in brown adipose tissue through the activation of TGR5, resulting in activating type-2 iodothyroxine deiodinase and the stimulation of energy metabolism through increased levels of thyroid hormone[33]. This suggests TGR5 plays an important role in host glucose homeostasis, and may be a target for development of therapies for type 2 diabetes and other metabolic diseases. The order of activation of TGR5 by BAs LCA > DCA > CDCA > CA indicates yet another BA receptor with higher affinity for BA metabolites of the human gut sterolbiome.

4. Deoxycholic acid, a microbial co-carcinogen

In the United States, cancers of the colon and rectum were the second leading cause of cancer death in 2010[34]. In 2013, new cases of colorectal cancer in the US were estimated to be 143,820[35]. Patients classified as high-risk for colon cancer including those with familial adenomatous polyposis (FAP), hereditary nonpolyposis or inflammatory bowel disease comprise only 5% – 15% of all colorectal cancer (CRC) incidence[36]. The majority of CRC incidence is nonhereditary and thus sporadic, suggesting the importance of environmental influences. Epidemiologically, CRC incidence is significantly increased in nations consuming a "Western" diet high in protein and fat[37]. Western diets result in increased levels of secondary BA in both the colon and biliary pool[38]. In this regard, individuals with high levels of secondary BA in feces are shown in epidemiological studies to be at significant risk for CRC[39]. Colon cancer patients and patients with adenomas polyps have higher levels of DCA in blood and bile as compared to control patients. DCA levels in sera of these patients were significantly higher in men with colorectal adenomas (1.70 ± 0.59 *vs.* 1.16 ± 0.39 μmol/L, $P < 0.0005$) and in a combined analysis of both men and women compared to age and sex-matched controls (1.47 ± 0.78 *vs.* 1.08 ± 0.39 μmol/L, $P < 0.0025$)[40]. DCA is a logical candidate for promoting colon carcinogenesis for the following reasons: (1) DCA is found in high levels (> 100 μmol/L) in fecal water; (2) it can cross biological membranes *via* passive diffusion; (3) it activates mammalian cell signaling pathways that are known to be involved in promoting colon carcinogenesis[41].

Mechanisms by which DCA and LCA promote colon carcinogenesis have been largely elucidated. Mutations at the adenomatous polyposis coli (*APC*) locus are a common and early somatic event in polyp formation and colon cancer[42]. *APC* regulates β-catenin levels in the cell, failure of which results in transcription of proto-oncogenes and loss of cell-cell adhesion[43,44]. One particular downstream target of β-catenin is cyclooxygenase-2 (COX-2). A large body of evidence suggests that non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX isozymes, significantly reduce polyp formation and colon cancer risk, as well as other cancers of the GI tract[45]. $Apc^{\Delta 716(+/-)}$ mice, a model of human FAP, develop polyposis and CRC, however $Apc^{\Delta 716(+/-)}$, $Ptg2^{(+/-)}$ mice, which contain only a single *COX-2* gene developed significantly less frequent and smaller polyps[46].

DCA induces expression of COX-2 through transactivation of the epidermal growth factor receptor (EGFR)[47]. Qiao et al.[48] first reported that DCA can activate EGFR in primary hepatocytes. This observation has been confirmed by several laboratories in other intestinal epithelial cell types[49,50]. DCA has also been shown

to induce the β-catenin signaling pathway resulting in colon cancer cell proliferation and invasiveness[51]. β-catenin is also known to stabilize *COX-2* mRNA resulting in positive feedback[52].

The detergent properties of DCA cause membrane perturbations resulting in activation of protein kinase C (PKC) isoforms[53] as well as release of arachidonic acid which is the substrate for both COX-2 and lipooxygenase (LOX), resulting in pro-inflammatory and pro-angiogenic prostaglandins, and reactive oxygen species which damage DNA and inhibit DNA repair enzymes[54]. In this regard, DCA has been shown to activate proteosomal degradation of the tumor suppressor p53[55] selecting for cells resistant to apoptosis in spite of DNA damage. Taken together, DCA at physiological concentrations activates signaling pathways that lead to selective resistance to apoptosis, angiogenesis (PGE2 through VEGF), proliferation and oxidative stress[41,54].

Recent evidence also suggested an important role of DCA in the development of liver cancer[56]. While not sufficient, DCA was necessary to cause tumors in a mouse model of obesity-associated hepatocellular carcinoma by inducing the senescence-associated secretory phenotype in hepatic stellate cells, which induced expression of pro-inflammatory and tumor-promoting factors in the liver[56].

5. Metabolism of ursodeoxycholic acid (UDCA) by human intestinal bacteria

Close to 1000 metric tons of UDCA is produced globally each year for pharmaceutical use or in some countries as an over-the-counter supplement[57]. UDCA, the 7β-hydroxy isomer of CDCA, is used to treat a subset of cholesterol gallstone patients as well as a number of other diseases of the liver and GI tract including: primary biliary cirrhosis, primary sclerosing cholangitis (PSC), a prophylaxis for colonic adenomas recurrence, and as a protective agent in recurrent pancreatitis. It is generally recognized that UDCA has potential in preventing the progression of adenoma to adenocarcinoma; however, the results have varied[58,59]. A major mechanism by which UDCA acts therapeutically is by increasing the hydrophilicity of the biliary pool, and by diluting the concentration of toxic secondary BAs DCA and LCA in the biliary pool. Reduced secondary BA concentration modulates the membrane and inflammatory effects of secondary BAs on the colonic mucosa by the colonic BA milieu[58]. In part, UDCA acts similarly NSAIDs by blocking COX-2, which is activated by DCA and induces inflammation[60]. Problematic in these efforts is the microbial conversion of UDCA to LCA.

In humans, UDCA can be introduced exogenously *via* therapies (Ursodiol), or endogenously produced by epimerization of the 7α-hydroxy of CDCA by 7α-HSDH and 7β-HSDH found in single species (*Clostridium absonum*) or two separate species expressing one or the other HSDH[61]. In rodents, UDCA is synthesized in the liver, and forms a small part of the biliary pool.

We hypothesize that improvement of UDCA therapy, particularly when used in treatment of adenomatous polyps, will require prevention of LCA formation from UDCA by gut bacteria (Fig. 3). Gut bacteria capable of converting UDCA to LCA are found in the genus *Clostridium* and also have BA 7α-dehydroxylating activity against CA and CDCA[12]. It may be hypothesized that 7β-dehydroxylation proceeds through epimerization of UDCA to CDCA followed by 7α-dehydroxylation in *C. scindens* and related organisms. *C. scindens* encodes a constitutively expressed 7α-HSDH[62]; however, 7β-HSDH activity has not been observed in

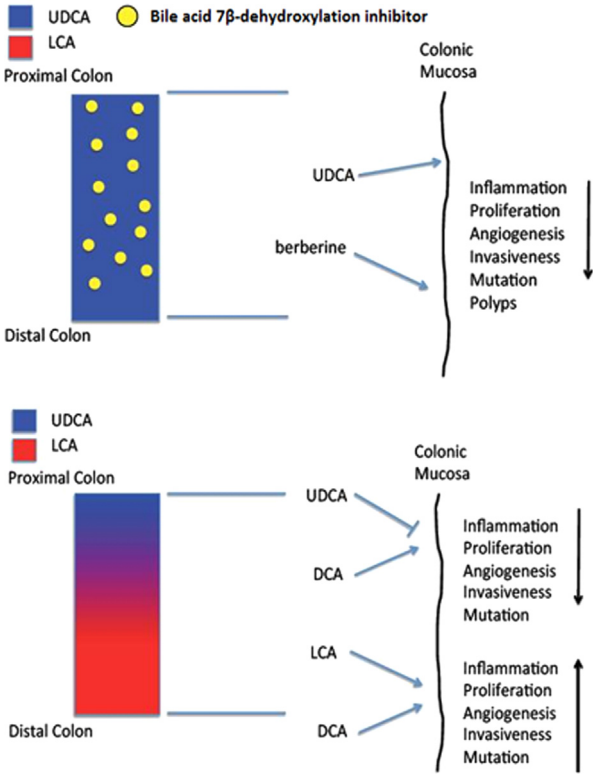

Figure 3 Targeted inhibition of bile acid 7β-dehydroxylating pathway hypothesized to improve therapeutic potential of UDCA in prevention of colon cancer development.

7α-dehydroxylating strains. The BA 7α-dehydratase encoded by the *baiE* gene has no activity against 7β-hydroxy BA substrates, and indeed 7α-dehydratase and 7β-dehydratase activities separate during gel filtration chromatography[63]. Interestingly, UDCA is a poor inducer of BA 7β-dehydroxylating genes, but 7α-hydroxy BAs are good inducers of this activity[64]. Thus a far more likely scenario is the presence of a separate BA inducible 7β-dehydratase that is co-expressed with the BA 7α-dehydroxylating pathway.

Previously, we provided evidence suggesting two homologous NAD^+-dependent flavin oxidoreductases introducing C_4–C_5 double bonds in 7α-hydroxy and 7β-hydroxy BAs (*baiCD* and *baiH*, respectively) are coexpressed on a CA-inducible *baiBCDAEFGHI* operon[65]. The *baiH* thus represents the first BA 7β-dehydroxylating gene identified. Directly downstream of the *baiH* gene is the yet uncharacterized *baiI* gene. The deduced amino acid sequence of the *baiI* gene is similar in size to the *baiE* gene and both are members of the SnoaL_4 superfamily of proteins (amino acid similarity 40%, 20% identity). The BA 7α-dehydroxylation and 7β-dehydroxylation pathways seem to share common core enzymes (BaiG, BaiB, BaiA, BaiF, reductive arm) but are differentiated at the 3-dehydro-4-oxidoreducatase (BaiCD *vs.* BaiH) and 7-dehydration step (BaiE *vs.* BaiI). Thus, the evolution of the 7β-dehydroxylation pathway appears to be a function of two gene-duplication events, duplication of the *baiCD* and *baiE*, and subsequent divergence of gene sequence and function resulting in formation of *baiH* and *baiI*. This adaptation would open up a new niche, as 7β-BAs, once formed would persist as BA 7α-dehydroxylating bacteria would be unable to remove the 7β-hydroxy group, and equilibrium constants favor formation of UDCA by bacteria possessing 7β-HSDH enzymes or combinations of

7α-HSDH and 7β-HSDH, likely owing to the reduced toxicity of UDCA[66]. We speculate that targeting the BaiI pharmacologically during UDCA treatment will lead to improved outcome in adenomatous polyp therapy, and other GI disorders such as PSC.

6. Conclusions

The BA composition observed in mammals is the result of equilibria between enzymes encoded in the mammalian genome, and the gut sterolbiome. BAs are now viewed as hormones with both hepatic and extra-hepatic effects ranging from inflammation to glucose and lipid homeostasis. Given that microbial secondary BAs activate the nuclear receptors that recognize host primary BAs, the gut sterolbiome represents an important class of enzyme encoding genes that give gut microbiome the ability to act as an endocrine organ with far-reaching effects in the host. Perturbing effects of the sterolbiome, such as diet, antibiotics, probiotics, and so forth, have the potential to affect many physiological effects that result from the composition of the BA pool. A major current goal of BA-sterolbiome research is identifying members of the gut microbiome that encode sterolbiome genes, and characterizing microbial BA metabolizing gene products and the metabolites that induce their expression. Future research will seek to build models to predict the pattern of BAs when certain sterolbiome members and metabolites are present in the gut, and the physiological effects on the host. A significant potential exists with the current therapeutic BA UDCA used for treating several disorders of the liver and GI tract. Our hypothesis, consistent with the current literature and opinions in the field is that by targeting the microbiota capable of producing DCA, and inhibiting the microbial conversion of UDCA to LCA, UDCA will be shown to be a more effective therapy. Specific inhibitors against the BA 7α-dehydroxylating pathway, which eliminate secondary BA productions, would provide further advances in understanding how a shift in BA pool composition impacts both host physiology, as well as microbiome structure-function.

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