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TOPIC HIGHLIGHT

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Novel methylxanthine derivative-mediated anti-inflammatory effects in inflammatory bowel disease

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Abstract

Family 18 chitinases have a binding capacity with chitin, a polymer of N-acetylglucosamine. Recent studies strongly suggested that chitinase 3-like 1 (CHI3L1, also known as YKL-40) and acidic mammalian chitinase, the two major members of family 18 chitinases, play a pivotal role in the pathogenesis of inflammatory bowel disease (IBD), bronchial asthma and several other inflammatory disorders. Based on the data from high-throughput screening, it has been found that three methylxanthine derivatives, caffeine, theophylline, and pentoxifylline, have competitive inhibitory effects against a fungal family 18 chitinase by specifically interacting with conserved tryptophans in the active site of this protein. Methylxanthine derivatives are also known as adenosine receptor antagonists, phosphodiesterase inhibitors and histone deacetylase inducers. Anti-inflammatory effects of methylxanthine derivatives have been well-documented in the literature. For example, a beneficial link between coffee or caffeine consumption and type 2 diabetes as well as liver cirrhosis has been reported. Furthermore, theophylline has a long history of being used as a bronchodilator in asthma therapy, and pentoxifylline has an immuno-modulating effect for peripheral vascular disease. However, it is still largely unknown whether these methylxanthine derivative-mediated anti-inflammatory effects are associated with the inhibition of CHI3L1-induced cytoplasmic signaling cascades in epithelial cells. In this review article we will examine the above possibility and summarize the biological significance of methylxanthine derivatives in intestinal epithelial cells. We hope that this study will provide a rationale for the development of methylxanthine derivatives, in particular caffeine, -based anti-inflammatory therapeutics in the field of IBD and IBD-associated carcinogenesis.

Key words: Adherent-invasive *Escherichia coli*; Chitinase 3-like 1; Chitinase inhibitors; Intestinal epithelial cells; Host-microbial interactions; Inflammatory bowel disease

Core tip: The involvement of family 18 chitinases in the pathogenesis of inflammatory bowel disease has been increasing characterized. The discovery of methylxanthine derivatives as an effective inhibitor of family 18 chitinases provides a good tool to control the pathogenic effects of these proteins. This review discusses the underlying inhibitory mechanisms of the different methylxanthine derivatives and how these compounds have been shown to be effective in the amelioration of animal colitis models. As such, this mode of application can be extended to target other family 18 chitinases associated disorders such as asthma.

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INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a group of intestinal inflammatory disorders that affect millions people worldwide. IBD is associated with increased risk of colorectal cancer 8-10 years after initial diagnosis[1]. The chronic colitis in IBD is asssociated with inappropriate activation of the immune system by abnormal interactions between host and enteric luminal microbes. Our group have previously identified an unexpected role for chitinase 3-like 1 (CHI3L1) in enhancing bacterial adhesion and invasion on/into intestinal epithelial cells (IECs) and have demonstrated that CHI3L1 specifically activates protein kinase B (AKT) phosphorylation in IECs[2,3]. Given these roles, the ability of a host to produce CHI3L1 and other enzymatic active mammalian chitinases [*e.g.*, chitinase-1 and acidic mammalian chitinase (AMCase)] could be a critical factor in regulating the innate immune responses against microorganisms that exist in normal intestinal flora[4]. However, exaggerated production of these chitinases could cause highly pathogenic effects in mucosal tissues, directly initiating and perpetuating chronic inflammation[2,5-7]. CHI3L1 also has been identified as a potential autoantigen driving T cell-mediated immune responses in rheumatoid arthritis, suggesting that mammalian chitinases are highly associated with chronic inflammation[8,9].

As shown by Rao *et al*[10], methylxanthine derivatives, including caffeine, theophylline and pentoxifylline, are competitive inhibitors against a family 18 chitinase expressed by a fungal pathogen. Crystallographic analysis of chitinase and methylxantine derivative complexes revealed specific interactions with the active site of the chitinase protein, mimicking the binding of allosamidin, a well-known pan-chitinase inhibitor isolated from *Streptomyces* species[10]. Currently, most known family 18 chitinase inhibitors are natural products, including pseudotrisaccharide allosamidin[11]. However, this inhibitor is unsuitable as a therapeutic lead because of its high cost and high molecular weight. In contrast, methylxanthine derivatives are inexpensive and have much lower molecular weight as compared to allosamidin. In particular, caffeine is found in a wide variety of foods and beverages (*e.g.*, coffee, tea, cola, chocolates) and dietary supplements/ingredients (including botanicals such as guarana, yerba mate, and green tea extract)[11]. At physiological concentration, caffeine shows only minor adverse effects on the cardio-respiratory system and other health outcomes[12-14]. Therefore, caffeine is thought to be the most reasonable, least expensive, and safest compound among known chitinase inhibitors. In fact, our group recently demonstrated the beneficial effects of a medium dose of caffeine (2.5 mmol/L; equivalent to the concentration of caffeine in 2-3 cups of coffee) in the development of acute dextran sulfate sodium (DSS)-induced colitis by down-regulating the expression of CHI3L1 in the colon[15]. Although anti-inflammatory effects of caffeine is considered to be mediated, at least partially, *via* chitinase inhibition, it is still largely unknown whether the other methylxanthine derivatives, such as theophylline and pentoxifylline, also exert their anti-inflammatory activities by downregulating CHI3L1 expression. In this review article, we will discuss the important biological functions of caffeine, theophylline and pentoxifylline laying a special emphasis on the CHI3L1-mediated AKT/β-catenin signaling activation in IECs.

CHI3L1, BACTERIAL INFECTION AND IBD

It has been postulated that dysregulated host-microbial interactions play a central role in the development of intestinal inflammation[16-18]. In humans, the ileocecal region and colon are colonized by a group of anaerobic bacteria, many of which cannot be cultured using standard microbial techniques[19]. Altered epithelial barrier functions, mucosal immune responses and microbial defense are major factors of host susceptibility against these commensal bacteria[19]. Therefore, abnormal adhesion and invasion of commensal bacteria on/into IECs may be highly involved in the pathogenesis of IBD in patients with the mutations in IBD-susceptibility genes[20,21]. The development of excess bacterial adhesion and/or perpetuation of intestinal inflammation seems to be closely associated with the induction of several molecules on IECs[22,23].

Previous studies have addressed the possibility that chronic bacterial infections are involved in the pathogenesis of IBD[24-26]. An involvement of *Escherichia coli* (*E. coli*) in the pathogenesis of CD has been suggested by the detection of *E. coli* antigens and DNA in granulomatous and peri-ulcerative lesions in CD[27]. In addition, circulating antibodies against the porin protein C of *E. coli* outer membrane have been detected in CD patients with severe inflammation[28]. In fact, the terminal ileum of CD patients is sometimes heavily colonized by a special type of *E. coli* strain, adherent-invasive *E. coli* (AIEC), which is able to survive extensively within IECs and macrophages without inducing apoptosis[29-32]. Interestingly, AIEC can be detected only in 6% of ilea in healthy individuals, but is present in 36% of the newly formed terminal ilea (with early and acute inflammation) of post-surgical patients[31]. It has been demonstrated by Carvalho *et al*[33] that abnormal expression of specific host receptor, carcinoembryonic antigen-related cell adhesion molecule 6, is one of the inducible molecules enhancing the interaction between host cells and AIEC[32,33].

Utilizing DNA microarray analysis, our group also identified that CHI3L1 is specifically up-regulated on IECs under intestinal inflammatory conditions. Although

Table 1 Methylxanthines compared with common chitinase inhibitors in IC_{50}

Compound name	Chemical formula	IC_{50}	Ref.
Caffeine	$C_8H_{10}N_4O_2$	469 ± 23 μmol/L against *Aspergillus fumigatus* (*A. fumigatus*) chitinase	[10]
Pentoxifylline	$C_{13}H_{18}N_4O_3$	126 ± 7 μmol/L against *A. fumigatus* chitinase	[10]
Theophylline	$C_7H_8N_4O_2$	1500 ± 90 μmol/L against *A. fumigatus* chitinase	[10]
Allosamidin	$C_{25}H_{42}N_4O_{14}$	10 μmol/L against *Candida albicans* chitinase	[42]
Argifin	$C_{29}H_{41}N_9O_{10}$	3.7 μmol/L against *Lucilia cuprina* (*L. cuprina*) chitinase	[78]
Argadin	$C_{29}H_{42}N_{10}O_9$	3.4 nmol/L at 20 ℃ against *L. cuprina* chitinase	[79]

IC_{50}: The half maximal inhibitory concentration.

CHI3L1 entirely lacks glycohydrolase enzymatic activity, it has a functional chitin-binding motif acting as chilectin[34,35]. Chitin is an *N*-acetylglucosamine polymer and is the second most abundant polysaccharides in nature next to cellulose. In spite of lacking of chitin and chitin synthase, mammals can constitutively or inducibly produce several chitinases, including CHI3L1, which show a high degree of sequential homology to the bacterial and plant chitinases[36]. The expression of CHI3L1 is highly up-regulated in IECs and macrophages with inflammation and specifically enhances potentially pathogenic, but not non-pathogenic, bacterial adhesion and invasion on/into IECs[2,37]. Our recent studies further revealed that a specific adhesion between CHI3L1 and 5 distinct amino acids in the AIEC Chitinase A (ChiA) protein, which includes chitin-binding domains (CBDs), play critical roles in the initial host-microbial interaction[7]. Furthermore, *N*-glycosylation of a single amino acid residue (68th Asparagine) in the mouse CHI3L1 protein is crucial for the adhesion of potentially pathogenic *E. coli* on IECs[7]. Interestingly, similar to CHI3L1, bacterial CBDs have been found to bind directly to chitin[38,39]. Therefore, the specific interaction between glycosylated CHI3L1 and *E. coli* ChiA seems to be enhancing the bacterial adhesion and invasion on/into IECs under inflammatory conditions. These excess and abnormal host-microbial interactions *via* the above two chitinases may further perpetuate chronic intestinal inflammation as well as colitis-associated carcinogenic change of IECs, presumably by interacting with toll-like receptor-4 signaling[40,41].

METHYLXANTHINE DERIVATIVES AS PAN-CHITINASE INHIBITORS

Methylxanthines are a group of alkaloid chemicals which are derived from the purine base xanthine. Xanthine is a result of purine degradation from either guanine by guanine deaminase or hypoxanthine by xanthine oxidoreductase. Methylxanthines are methylated derivatives and include the compounds caffeine, aminophylline, 3-isobutyl-1-methylxanthine, paraxanthine, pentoxifylline, theobromine (found in chocolate), theophylline. Traditionally, they are used as stimulants, to increase athletic performance, and as brochiodilators, most notably in the case of asthma.

Through the use of drug screening tools, it was demonstrated that several methylxanthine derivatives, namely caffeine, theophylline, and pentoxifylline were potential chitinase inhibitors[10]. Subsequent analysis confirmed the inhibitory effects of all 3 methylxanthines, with pentoxifylline having the highest K_i of 37 μmol/L. In terms of another parameter IC_{50} (half maximal inhibitory concentration), pentoxifylline was almost 4 fold lower, and thus 4 times more effective, than caffeine (126 μmol/L *vs* 469 μmol/L, respectively), and was almost 12 times more powerful than theophylline (1500 μmol/L)[10]. In contrast, allosamidin is reported of having an IC_{50} of 10 μmol/L towards *Candida albicans*-derived chitinase[42]. Therefore, methylxanthines including pentoxifylline, caffeine and theophylline do show a significantly lower affinity against fungal chitinase as compared to allosamidin. The inhibitory strength of methylxanthines compared with common chitinase inhibitors is summarized in Table 1.

Interestingly, the chemical structures involved in binding between methylxanthines and the family 18 chitinases were found to be very similar for all 3 compounds (caffeine, pentoxifylline and theophylline) and mimicked chitinase binding to allosamidin. X-ray diffraction analysis revealed a common position for the methylxanthine substructure. The additional inhibition by pentoxifylline is suspected to be due to increased interactions including hydrogen bonding, and extensive π-π stacking with the active site[10].

Methylxanthines have the potential to be very useful as chitinase inhibitors and disease treatments as summarized in Table 2. They are typically very safe in low doses and represent a class of favorable drugs due to low cost, low molecular weight, and easy availability. As demonstrated, they exhibit significant biological activity against mammalian chitinases which have been implicated in several inflammatory disorders and cancers. Therefore, their use as immune-modulators will surely provide new therapeutic approaches.

ANTI-INFLAMMATORY EFFECTS OF CAFFEINE, THEOPHYLLINE, AND PENTOXIFYLLINE

The major anti-inflammatory effects of caffeine, pentoxifylline, and theophylline result from 2 main mechanisms; the non-selective inhibition of phosphodiesterases (PDEs) and as a non-selective adenosine receptor antagonist. Through the inhibition of PDEs, a rise in intracellular cyclic adenosine mono-phosphate, activation of protein kinase A, inhibition of tumor necrosis factor alpha (TNFα) and leukotriene synthesis, and reductions in in-

Table 2 Major biological effects of methylxanthine derivatives

Compound	Molecular weight (g/mol)	Biological effects	Side effects
Caffeine	194.19	Increases alertness, slightly increases metabolic rate[80], increases blood pressure, is a diuretic, improves sports performance[81]	Caffeine dependency, restlessness, insomnia, and anxiety at high levels (250-500 mg daily[82]) In extreme amounts (> 600 mg daily chronically): Gastrointestinal (GI) disturbance[1], irregular/rapid heartbeat, mania, depression, and psychosis[83]
Pentoxifylline	278.31	Improves blood circulation through peripheral blood vessels, prevents nausea/ altitude sickness, improves red blood cell deformability (*i.e.*, sickle cell anemia), reduces blood viscosity, reduces formations of platelet aggregation/thrombus[84]	Irregular heartbeat, chest pain, dizziness, edema in extremities Acute toxicity in rats determined at 1772 mg/kg[85]
Theophylline	180.164	Relaxes bronchial smooth muscle, increases heart contractility, rate, and efficiency, increases blood pressure, increases renal circulation, stimulates respiratory center of CNS, treatment for COPD, asthma, infant apnea[86,87]	Interactions with many drug (cimetidine and phenytoin), and causes nausea, arrhythmias, insomnia, irritability, dizziness, seizures and tachyarrhythmias at toxic concentrations (> 20 mg/mL)[86]

[1]GI disturbance is marked by nausea, vomiting, abdominal pain, diarrhea, bowel incontinence, and anorexia. COPD: Chronic obstructive pulmonary disease; CNS: Central nervous system.

flammation and innate immunity are observed[43-46]. Deree *et al*[43] reported that pentoxifylline successfully reduced TNFα production after human mononuclear cells were stimulated with lipopolysaccharide. A similar result was found in peripheral blood monocytes and alveolar macrophages from sarcoidosis patients, in which pentoxifylline also inhibited the spontaneous TNFα production associated with this disease[44]. Therefore these compounds may be useful in reducing LPS-induced inflammation and as a treatment for sarcoidosis. Methylxanthine derivatives demonstrate non-selective inhibition of all PDEs by competitive inhibition and therefore they likely bind to the active site of PDEs, however their exact molecular mechanism of inhibition is still uncertain. Caffeine and theophylline were shown to inhibit several PDE isozymes to a similar extent, and the two compounds showed an almost equal affinity for each of the PDE isozymes[47].

Through the inhibition of leukotrienes, which are known as pro-inflammatory mediators involved in asthma and bronchoconstriction and which play pivotal roles in innate immunity, asthma symptoms are relieved and inflammation is reduced[46]. Leukotrienes enhance inflammation by increasing leukocyte infiltration, phagocyte microbial ingestion, and generation of pro-inflammatory cytokines including IL-5, TNFα, and macrophage inflammatory protein-1β[48]. It was proven that theophylline effectively reduced leukotrience synthesis and reduced chemotaxis of complement 5a-and platelet-activating factor-stimulated human eosinophils obtained from normal and atopic donors[49].

As a non-selective receptor antagonist for adenosine, methylxanthine derivatives, most notably, caffeine, are well known as wakefulness aids, as adenosine is a known inducer of sleep. Caffeine, theophylline, and pentoxifylline non-selectively affect several adenosine receptors, including A_1, A_{2A}, A_{2B}, and A_3. A_1 receptor is found ubiquitiously throughout the body and studies demonstrated inhibition of this receptor with the novel compound *L*-97-1 [3-[2-(4-aminophenyl)-ethyl]-8-benzyl-7-{2-ethyl-(2-hydroxy-ethyl)-amino]-ethyl}-1-propyl-3,7-dihydro-purine-2,6-dione] reduce histamine and/or adenosine-induced hyperresponsiveness and early and late allergic responses in a rabbit model of house dust mite-induced allergic reactions[50]. The A_{2A} receptor is similar to the A_1 receptor in that it is found throughout the body. Mice deficient in A_{2A} receptor had significantly higher levels of the pro-inflammatory cytokines TNFα, IL-12 p40, and IL-6 in an LPS-induced model of septic shock as compared to wild-type mice. Therefore, it is suggested that this receptor plays a pivotal role in controlling excess inflammation/tissue damage[51]. In a mouse model of allergic asthma induced by AMP or 5-*N*-ethylcarboxamidoadenosine, antagonizing the A_{2B} receptor with the compound CVT-6883 resulted in decreased cellular infiltration in bronchoalveolar lavage fluid including eosinophils and lymphocytes and reduced bronchoconstriction. Interestingly, a similar but slightly blunted response was also seen in theophylline treatment at 36 mg/mL aerosolization for 5 min[52]. Therefore, this receptor is a target for asthma patients and CVT-6883 is currently undergoing clinical trials.

A novel area of investigation of methylxanthine derivatives is the anti-inflammatory effects through the inhibition of mammalian chitinases, including CHI3L1. As previously discussed, methylxanthine derivatives are effective pan-chitinase inhibitors[10]. CHI3L1 has been shown to play a role in many inflammatory disorders including rheumatoid arthritis, asthma, hepatitis, and IBD[2,6]. CHI3L1 increases inflammation in human bronchial epithelium by inducing IL-8 and activating the MAPK and nuclear factor-κB pathways, which are involved in cell survival[53]. IL-8 inhibition was hypothesized to be an effective treatment for asthma-related inflammation/remodeling. In a model of DSS-induced colitis, caffeine treatment at 2.5 mmol/L was shown to decrease TNFα, INFγ, IL-4 in mesenteric lymph nodes, and IL-17F in mesenteric lymph nodes and colon and increased the anti-inflammatory IL-10 production in spleen, mes-

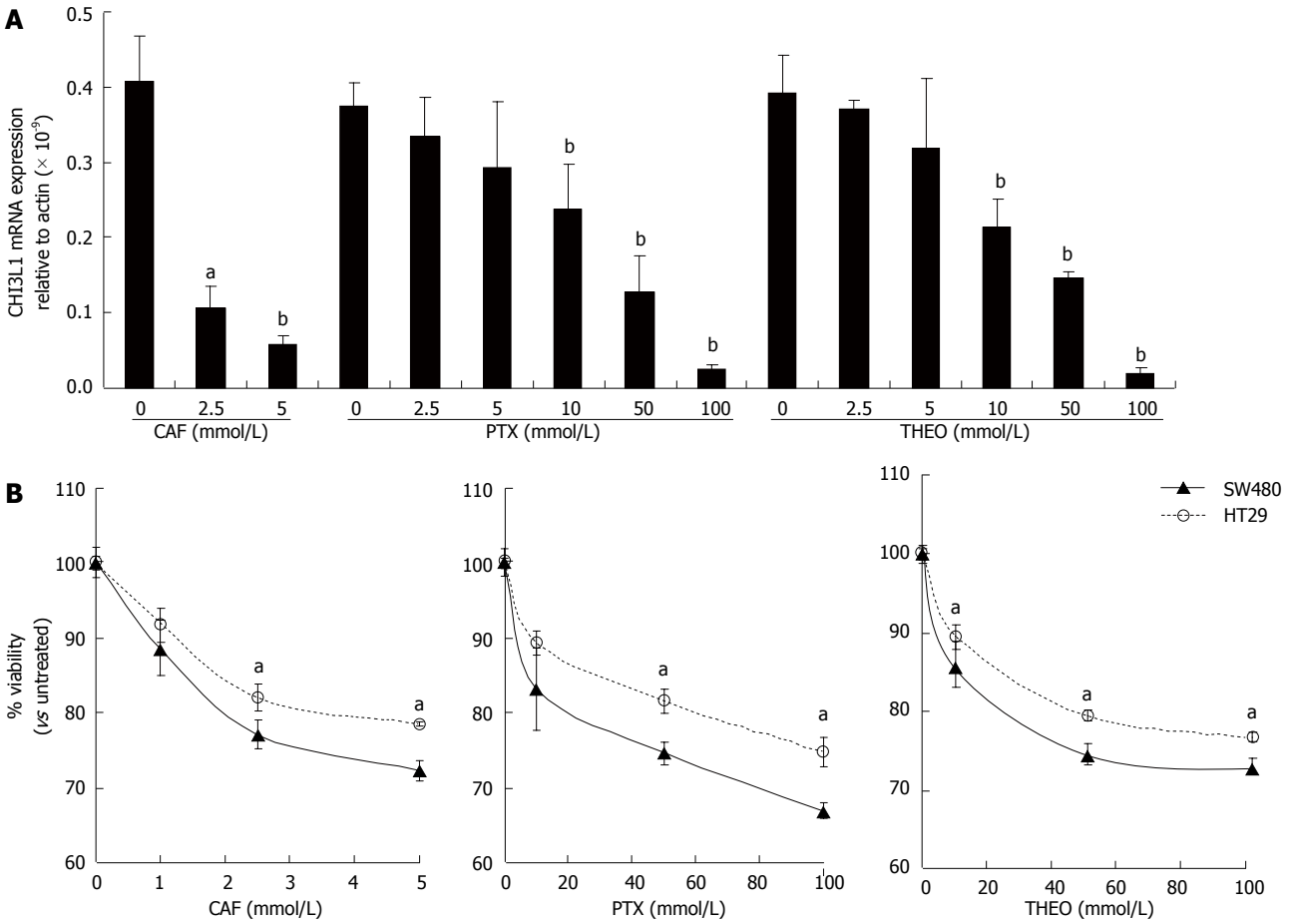

Figure 1 Caffeine, pentoxifylline and theophylline down-regulate chitinase 3-like 1 mRNA expression and reduce cell viability in human colonic epithelial cells. A: SW480 cells were stimulated with caffeine (CAF) at 0, 2.5 or 5 mmol/L or pentoxifylline (PTX) or theophylline (THEO) at 0, 2.5, 5, 10, 50 or 100 mmol/L for 48 h and detected for the chitinase 3-like 1 (CHI3L1) mRNA expression by quantitative-polymerase chain reaction. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control; B: SW480 and HT29 cells were treated with either CAF (0, 1, 2.5 or 5 mmol/L), PTX or THEO (0, 10, 50 or 100 mmol/L) for 48 h and cell viability were determined using trypan blue exclusion test. CAF, PTX and THEO were purchased from Sigma-Aldrich (St Louis, MO, United States). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

enteric lymph nodes, and colon[7,15].

Taken together, methylxanthine derivatives have demonstrated efficacy against the inflammatory disorders, and were shown to reduce inflammation in mice treated with DSS. Therefore, the efficacy of methylxanthine derivatives as potential anti-inflammatory and anti-cancer agents should be further elucidated in other inflammatory conditions and inflammation-associated cancers.

EFFECTS OF METHYLXANTHINE DERIVATIVES ON IBD MOLECULAR PATHWAYS ASSOCIATED WITH CHI3L1

The pathological involvement of CHI3L1 in many diseases, including autoimmune diseases (*e.g.*, IBD, asthma and RA), as well as many forms of solid tumors (*e.g.*, colorectal cancer) are becoming increasingly apparent at this time. The most direct evidence is a significant amount of CHI3L1 induction during the disease state (*e.g.*, IBD and IBD-associated cancer) which activates several important cellular pathways, including AKT and the β-catenin signaling pathway, thus playing crucial roles in disease pathogenesis[2,3,6,54]. Characterization of these CHI3L1-mediated pathological pathways can facilitate a better understanding on the molecular mechanisms behind how methylxanthine derivatives can ameliorate diseases through the inhibition of CHI3L1.

In addition to direct protein inhibition of the family 18 chitinases, as determined by X-ray crystallography, *in vitro* methylxanthine treatment in SW480 colonic epithelial cells (CECs), a human colon cancer cell line, directly results in a down-regulation of CHI3L1 mRNA levels (Figure 1A)[15]. The effective dose of caffeine that is optimal for achieving such down-regulation ranges from 2.5 to 5 mmol/L (Figure 1A). Nevertheless, it was previously shown that 1.0 mmol/L caffeine treatment is sufficient to cause a down-regulation of CHI3L1 in SW480 CECs[15]. Caffeine treatment also results in the down-regulation of other mammalian chitinases including AMCase, but not chitinase 1[15]. The effective dose of pentoxifylline and theophylline to down-regulate CHI3L1 in SW480 cells ranges from 10 to 100 mmol/L, whereby any concentration below that did not show any effects on CHI3L1

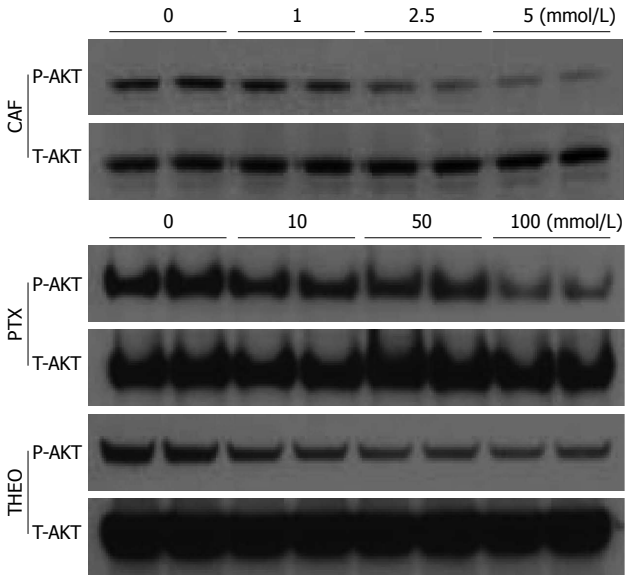

Figure 2 Caffeine, pentoxifylline and theophylline suppress protein kinase B signaling pathway activation in mouse colonic epithelial cells. CMT93 mouse colonic epithelial cells were stimulated with caffeine (CAF) (0, 1, 2.5 or 5 mmol/L), pentoxifylline (PTX) or theophylline (THEO) (0, 10, 50 or 100 mmol/L) for 48 h. Twenty five micro grams of total protein were resolved using SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using anti-phospho/total protein kinase B (AKT) Abs purchased from cell signaling technology (Danvers, MA, United States) .

mRNA expression (Figure 1A). Since rabbit anti-CHI3L1 antibody administration to mice has been shown to have an ameliorating effect in acute DSS-induced colitis development, the direct down-regulation of CHI3L1 using methylxanthine derivatives also achieves a similar therapeutic effect in IBD *in vivo*[2,15].

Furthermore, methylxanthine derivative treatment also reduces colon cancer cell viability in a CHI3L1 expression dependent manner. *In vitro* treatment with caffeine (1-5 mmol/L), pentoxifylline (10-100 mmol/L) or theophylline (10-100 mmol/L) in SW480 cells that express high endogenous CHI3L1 greatly reduces the viability of cells (Figure 1B). However, methylxanthine derivative treatment in HT29 CECs, a human colon cancer cell line that does not express endogenous CHI3L1, has minimal effect on cell viability, indicating a direct involvement of cell survival that is mediated by CHI3L1 expression, at least in part. This has important implications in carcinogenesis since many solid tumors, including colorectal cancer and breast cancer, exhibit exaggerated expression of CHI3L1[6]. Mechanistically, CHI3L1 directly contributes to tumorigenesis by exerting excessive cell proliferation and angiogenesis[54,55]. Thus, methylxanthine derivative treatment provides a proof-of-concept in controlling carcinogenic changes and progression by regulating cell viability *via* targeting ectopic CHI3L1 expression and function.

Several studies have demonstrated that AKT signaling is up-regulated in the IEC crypts of chronic UC and CD patients, as well as in a murine DSS-induced colitis model[56,57]. In contrast, colitis patients that had undergone 5-aminosalicylic acid (5-ASA) treatment showed reduced AKT-phosphorylation in inflamed tissues, suggesting a direct relationship between AKT signal activation and disease severity[58]. A progressive increase in the densities of phosphorylated AKT in tumor-associated macrophages was observed in normal, colitic and dysplastic to cancer patient specimens[59]. This expression pattern is in parallel to that of colonic CHI3L1 levels, which showed almost undetectable expression in normal colon, but is induced during colitis that further up-regulates during colitis-associated cancer development[2,54]. CHI3L1 can directly activate colonic AKT signaling, specifically *via* the 325th-339th amino acid residues within the chitin-binding motif[3]. This enhanced up-regulation of CHI3L1 during colitis-associated cancer development may provide a plausible explanation for the exaggerated enhancement of AKT phosphorylation. In the context of tumorigenesis, activation of this AKT signaling in the colon induces proliferative signals in IECs that is critical for G1 cell cycle progression[60]. Thus such constitutive activation of AKT, at least in part mediated by CHI3L1, might result in the uncontrolled cell proliferation. With this in mind, reducing AKT activation by targeting CHI3L1 using methylxanthines seemed to be a possible therapeutic strategy for inflammatory disorders. The combinatory effect of CHI3L1 protein inhibition, as well as direct down-regulation of CHI3L1 mRNA expression by caffeine, pentoxifylline and theophylline, was shown to significantly reduce AKT phosphorylation (Figures 1 and 2 and data not shown)[15]. The minimum dose of caffeine to achieve a reduction in AKT activation appears to be 2.5 mmol/L, whereas the effective dose of pentoxifylline and theophylline ranges from 10-100 mmol/L.

Another important signaling pathway in the colon that can be activated by CHI3L1 is the β-catenin pathway. Stimulation of SW480 CECs using low dose of CHI3L1 results in an apparent β-catenin nuclear translocation[6]. In contrast to SW480 cells stimulated with CHI3L1 (80 ng/mL) that predominantly showed a nuclear localization of β-catenin, cells that were stimulated with CHI3L1 and concurrently treated with caffeine (5 mmol/L), but less significant with pentoxifylline (100 mmol/L) or theophylline (100 mmol/L), showed cytoplasmic β-catenin localization (Figure 3). Canonical activation of β-catenin requires the binding of the Wingless (Wnt) ligand onto the Frizzled receptor that subsequently stabilizes cytoplasmic β-catenin by destroying a protein complex (AXIN, GSK3β and APC) which usually cause the proteolysis of β-catenin under steady-state. This then facilitates the free β-catenin to migrate into the nucleus and subsequent activates transcription of target genes including *c-Myc* and *cyclin D1* (Figure 4). In a cohort study, high activation of β-catenin was found in 100%, 55% and 50% in IBD with colitis-associated cancer, IBD with dysplastic and IBD with remote dysplasia patients, respectively[61]. Recently, Lee *et al*[62] identified phosphatidylinositide 3-kinase (PI3K)/AKT signaling as the crucial factor mediating β-catenin during mucosal

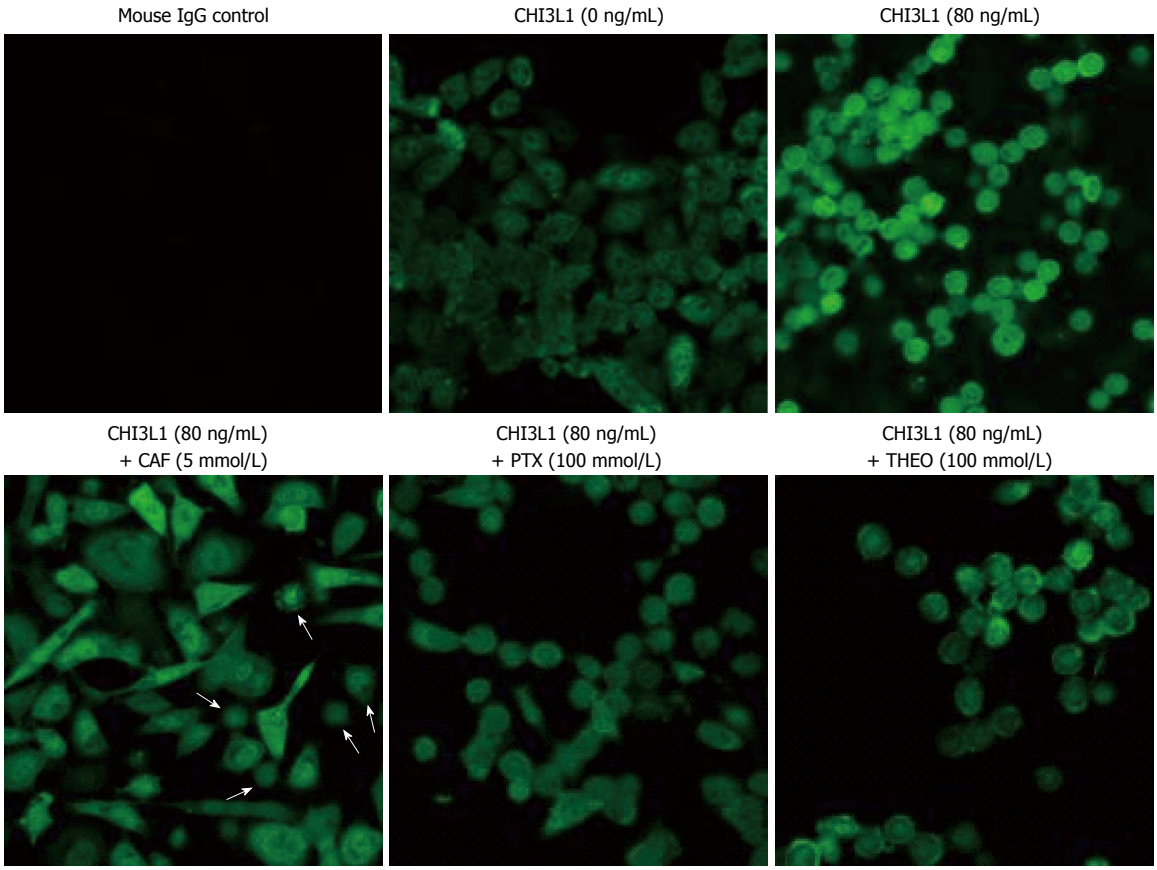

Figure 3 Caffeine, pentoxifylline and theophylline inhibit β-catenin nuclear translocation with different degrees. SW480 colonic epithelial cells were cultured on lab-tec chamber slide. After reached to 90% confluency, the cells were stimulated with or without purified human chitinase 3-like 1 (CHI3L1) (80 ng/mL) in combination with caffeine (CAF) (5 mmol/L), pentoxifylline (PTX) (100 mmol/L) or theophylline (THEO) (100 mmol/L) for 24 h. Human CHI3L1 protein was purchased from Quidel (San Diego, CA). β-catenin was then detected using mouse anti-human β-catenin monoclonal primary Ab (BD Biosciences, CA) and FITC-horse anti-mouse Immunoglobulin G (Vector Labs, Burlingame, CA) and analyzed by confocal microscope (magnification, objective 40 ×). White arrows show the limited numbers of completely nuclear translocated β-catenin positive cells after caffeine treatment.

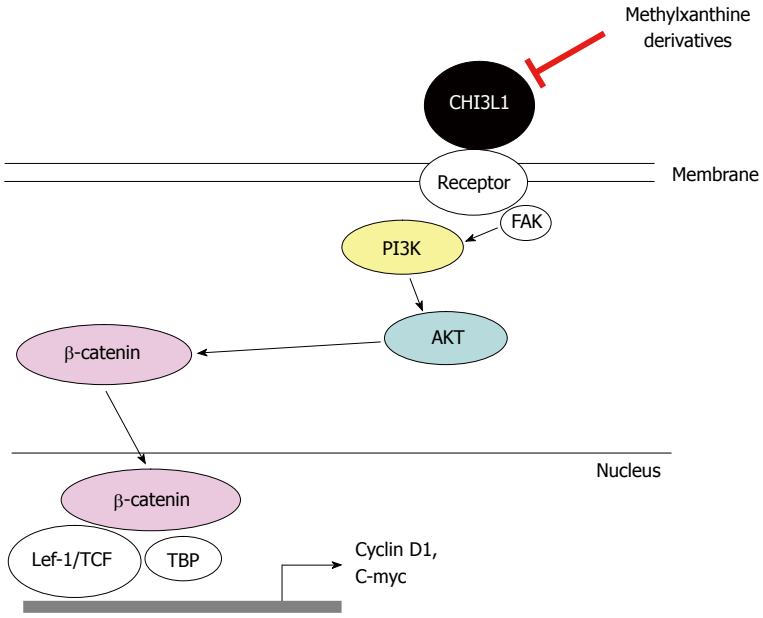

Figure 4 Schematic representation of chitinase 3-like 1-associated β-catenin activation signaling pathway, which is inhibited by methylxanthine derivatives. Binding of extracellular chitinase 3-like 1 (CHI3L1) to a putative receptor on plasma membrane activates the intracellular phosphatidylinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, which leads to β-catenin activation by translocating this protein from cytoplasm into nucleus. Methylxanthine derivatives, including caffeine, pentoxifylline and theophylline, directly down-regulate CHI3L1 mRNA expression and inhibit CHI3L1 protein functions, leading to reduced CHI3L1-associated AKT activation and prevent down-stream β-catenin nuclear translocation with different degrees of efficacy.

inflammation. They reported that IEC-specific PI3K conditional knockout mice showed reduced AKT and β-catenin signaling in the intestinal stem and progenitor cells and limits the extent of crypt epithelial proliferation. Inhibiting PI3K in IL-10 knockout mice, which develop spontaneous colitis, also impairs colitis-induced

epithelial AKT and β-catenin activation. Furthermore, a report by Fukumoto *et al*[63] also supports that viewpoint that AKT increases β-catenin activity by interfering with the AXIN/GSK3β complex. Chronic UC patients that have undergone 5-ASA treatment also show reduced AKT-mediated β-catenin phosphorylation in the middle and upper crypts in colon. This observation was recapitulated in 5-ASA treated IL-10 knockout colitic mice[64]. However, whether CHI3L1 mediated β-catenin activation is exerted directly through the Wnt or AKT pathway, or both but at different temporal time points or cell specific manner, remains to be investigated.

Currently, only a few receptors are known to bind to CHI3L1. Recently, He *et al*[65] identified that CHI3L1 binds to the interleukin-13 receptor α2 (IL-13Rα2) and activates both AKT and β-catenin signaling in the IL-13Rα2 dependent pathway. Therefore, exploring the use of methylxanthine derivatives for inhibiting CHI3L1 may block any downstream effects pertaining to AKT and/or β-catenin signaling and will provide direct mechanistic insights (Figure 4).

POTENTIALLY THERAPEUTIC/PROPHYLACTIC EFFECTS OF METHYLXANTHINE-DERIVATIVES IN IBD

IN VIVO

Recently, our group performed in depth analysis of the role of caffeine treatment in a DSS-induced colitis model in mice[15]. Our *in vivo* analysis involved prophylactic-, simultaneous-, and post-treatment of mice with caffeine at 2.5 mmol/L in this animal model of intestinal epithelial damage. After initial caffeine treatment for 7 d, we challenged the mice with DSS in the drinking water for 5 d, and then returned to normal drinking water for 7 d before sacrificing. Mice which received the caffeine treatment protocol showed significantly improved symptoms as demonstrated by less percentage bodyweight loss and improved clinical scores. Colons of the mice were isolated, and it was shown that CHI3L1 and AMCase expressions were both significantly decreased after caffeine treatment. In contrast, chitinase 1 expression remained stable after the treatment. Colonic sections were also analyzed for histological changes. Mice in the caffeine-treated group demonstrated improved histological scores, with markedly decreased accumulation of immune cells, including $F4/80^+$, $CD4^+$, or $CD11b^+$ cells. Interestingly, bacterial colony forming units from homogenized mouse spleens, mesenteric lymph nodes, liver, cecum and colon were all significantly reduced after 2.5 mmol/L caffeine treatment. In addition, as we described in the previous section, the levels of several pro-inflammatory cytokines were significantly decreased in spleen, mesenteric lymph nodes, and colon, with an increase in the anti-inflammatory cytokine IL-10 in tissues. A major factor in IBD development is host-microbial interactions including adhesion/invasion of bacteria into the CECs and lamina propria. Caffeine treatment at both 2.5 and 5 mmol/L effectively prevented AIEC from invading into SW480 CECs, as well as in mouse-derived peritoneal macrophages. This result provides a possible explanation on therapeutic potential of caffeine in IBD through the prevention of CHI3L1-mediated bacterial adhesion/invasion.

In vivo testing using pentoxifylline to study the effects on IBD was also reported. Peterson *et al*[66] demonstrated that intra-rectal administration of pentoxifylline or 1-(5-hydroxyhexyl)-3,7-dimethylxanthine (so called metabolite-1 or M-1) in a murine 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis model, which showed an attenuation of colonic inflammation and intestinal fibrosis. M-1 is a chiral molecule derived from pentoxifylline by the reduction of a single ketone group to a corresponding hydroxy group. They reported that 64 mg/kg of pentoxifylline or M-1 is an ideal therapeutic dose in mice, whereas mice treated with 32 mg/kg showed varied effects in disease-associated phenotype. Another study also showed similar amelioration by pentoxifylline in TNBS-colitis in rats[67]. Interestingly, Murthy *et al*[68] showed that combining pentoxifylline with anti-TNFα antibody in DSS-induced colitic mice can reduce the side effects that is associated with anti-TNFα antibody treatment alone. *Ex vivo* studies also showed that peripheral mononuclear cells, which are obtained from the inflamed mucosa of CD and UC patients, reduce TNFα secretion by 50% in the presence of pentoxifylline (up to 100 μg/mL) for 24 h[69].

FUTURE OPTIMIZATION OF METHYLXANTHINE DERAVATIVES FOR IMPROVED SPECIFICITY AND EFFICACY INHIBITION OF FAMILY 18 CHITINASES

The early discovery of the allosamidin-derived from *Streptomyces* as a chitinase inhibitor has opened up opportunities to test the inhibitory effect on controlling chitinase-associated diseases[70]. For instance, as demonstrated in a study on AMCase-associated asthmatic Th2 inflammation mouse model, allosamidin, or anti-AMCase antibody, both independently can reduce bronchoalveolar lavage inflammation[71]. However, the concern over using allosamidin is its broad range of activity against all family 18 chitinases and less than ideal chemical properties (*e.g.*, high molecular weight and poor ligand efficiency)[72]. In addition, allosamidin has a stronger inhibitory effect on chitinase 1 than AMCase and therefore, since chitinase 1 is highly regarded as a molecule involved in host-defence system against a chitin-containing pathogen rather than a driver molecule involved in allergic inflammation, there is a need to identify or develop other chitinase inhibitor with higher specificity[73-75]. The discovery of the methylxanthine derivative inhibitory effects on family 18 chitinases appears to represent a promising alternative for its more suitable chemical properties and advantages as

described above sections. Yet, being a pan-chitinase inhibitor, it still faces a similar challenge in target specificity. Therefore, the next step is to optimize both specificity and efficacy of these methylxanthine derivatives.

In order to improve the inhibitory properties of the methylxanthine derivatives, Schüttelkopf *et al*[76] has developed a virtual algorithm method to create better family 18 chitinases. The algorithm, named as LIGTOR, basically fixed the methylxanthine substructure while performing torsional evaluation of the substitution based on previous published chitinase-pentoxifylline complex. Upon identification of the most desirable chemical features using this algorithm, the group then subsequently developed a low micromolar chitinase inhibitor that is composed of a two linked caffeine moieties that binds in the active site of the target extensively in a manner that was not previously reported. This di-caffeine compound, subsequently named as bisdionin B, showed the desired drug-like structure, as demonstrated by X-ray crystal structure analysis, and provides a general scaffold for future development/optimization of the family 18 chitinase inhibitors.

Another major concern in drug design is target specificity. As a pan-chitinase inhibitor, one of the major drawbacks of methylxanthine derivatives is the discrimination between the different chitinases (*e.g.*, CHI3L1, AMCase and chitinase 1). To address this issue, Sutherland *et al*[77], utilized the LIGTOR algorithm derived di-caffeine scaffold and modified the caffeine linker length and subsequently analysed it against the AMCase crystal structure. They then developed a derivative of the di-caffeine scaffold, termed as bisdionin F, that showed a high selectivity for human AMCase up to 20-fold over chitinase 1. The exact orientation/coordinates were confirmed by crystal structure of the human AMCase-bisdionin F complex. The group further validated the efficacy of bisdionin F in a murine model of allergic inflammation. All these suggest that further improvements can be made to develop a molecule with improved inhibitory efficacy and higher specificity against the targeted molecule of the chitinase 18 family.

CONCLUSION

CHI3L1 is an important inducible molecule on IECs and acively participates in the pathogenesis of chronic inflammation and inflammation-associated malignant transformation of epithelial cells. Methylxanthine derivatives, including caffeine, theophylline and pentoxifylline, can potentially suppress inflammation *via* CHI3L1 inhibition. The result in this study may provide the conceptual framework for a new class of therapeutic agents, which will effectively prevent chronic inflammatory diseases with minimal side effects.

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