6-(methylsulfinyl)hexyl isothiocyanate (6-MITC) from Wasabia japonica alleviates inflammatory bowel disease (IBD) by potential inhibition of glycogen synthase kinase 3 beta (GSK-3β)

Lohning, Anna; Kidachi, Yumi; Kamiie, Katsuyoshi; Sasaki, Kazuo; Ryoyama, Kazuo; Yamaguchi, Hideaki

Published in:
European Journal of Medicinal Chemistry

DOI:
10.1016/j.ejmech.2021.113250

Licence:
CC BY-NC-ND

Link to output in Bond University research repository.

Recommended citation (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

For more information, or if you believe that this document breaches copyright, please contact the Bond University research repository coordinator.
6-(methylsulfinyl)hexyl isothiocyanate (6-MITC) from *Wasabia japonica* alleviates inflammatory bowel disease (IBD) by potential inhibition of glycogen synthase kinase 3 beta (GSK-3β).

Anna Lohning¹, Yumi Kidachi², Katsuyoshi Kamiie², Kazuo Sasaki³, Kazuo Ryoyama², Hideaki Yamaguchi⁴

¹ Faculty of Health Sciences & Medicine, Bond University, Gold Coast, Australia
² Department of Pharmacy, Aomori University; 2-3-1 Kobata, Aomori 030-0943, Japan
³ Department of Food and Life Sciences, Toyo University; 1-1-1 Izumino, Itakura, Gunma 374-0193, Japan
⁴ Department of Applied Biological Chemistry, Meijo University; 1-501 Shiogamaguchi, Tempaku, Nagoya 468-8502, Japan

*Corresponding author: (First three authors contributed equally to this work) Dr. Anna Lohning; Tel: +61-7-5595 4779. E-mail: alohning@bond.edu.au

Abstract

Inflammatory bowel disease (IBD) describes a set of disorders involving alterations to gastrointestinal physiology and mucosal immunity. Unravelling its complex pathophysiology is important since many IBD patients are refractory to or suffer adverse side effects from current treatments. Isothiocyanates (ITCs), such as 6-(methylsulfinyl)hexyl ITC (6-MITC) in *Wasabia japonica*, have potential anti-inflammatory activity. We aimed to elucidate the pathways through which 6-MITC alleviates inflammation by examining its role in the nuclear factor-kappa B (NF-κB) pathway through inhibition of glycogen synthase kinase 3 beta (GSK-3β) using a chemically induced murine model of IBD, cell-based and *in silico* techniques. The effects of 6-MITC and two NF-κB inhibitors, sulfasalazine (SS), pyrroldine dithiolcarbamate
(PDTC) were investigated on a dextran sulfate sodium (DSS)-induced murine mouse model of acute and chronic colitis using macroscopic measurements and pro-inflammatory markers. The effect of 6-MITC on NF-κB induction was assessed using a murine macrophage cell line. Complexes of GSK-3β-6-MITC and GSK-3β-ATP were generated in silico to elucidate the mechanism of 6-MITC direct inhibition of GSK-3β. Changes in pro-inflammatory markers, inducible nitric oxide synthase (iNOS) (increased) and interleukin-6 (IL-6) (decreased) demonstrated that iNOS regulation occurred at the translational level. Intraperitoneal (ip) injection of 6-MITC to the colitis-induced mice ameliorated weight loss whereas oral administration had negligible effect. Fecal blood and colon weight/length ratio parameters improved on treatment with 6-MITC and the other NF-κB inhibitors. Levels of NF-κB decreased upon addition of 6-MITC in vitro while structural studies showed 6-MITC acts competitively to inhibit GSK-3β at the ATP binding site.

In this study we demonstrated that 6-MITC inhibits NF-κB signaling via GSK-3β inhibition ameliorating fecal blood, colonic alterations and DSS-induced weight loss indirectly indicating reduced intestinal stress. Taken together these results suggest a role for 6-MITC in the treatment of IBD acting to alleviate inflammation through the GSK-3β/NF-κB pathway. Furthermore, the GSK-3β-6-MITC model can be utilized as a basis for development of novel therapeutics targeting GSK-3β for use in other disorders including cancer.

Keywords: Inflammatory bowel disease (IBD); Ulcerative colitis (UC); Crohn’s disease (CD); 6-MITC; GSK-3β inhibitor; NF-κB regulation

Abbreviations: AMP-PNP, adenylyl imidodiphosphate; ASE-Dock, alpha sphere and excluded volume-based ligand-protein docking; CD, Crohn’s disease; DSS, dextran sodium sulphate;
GSK-3β, glycogen synthase kinase 3 beta; IBD, inflammatory bowel disease; IFN-γ, interferon gamma; IκB, inhibitor kappa B; IKK, inhibitory kappa B kinase; IL, interleukin; iNOS, inducible nitric oxide synthase; ITC, isothiocyanate; LBS, ligand binding site; LPS, lipopolysaccharide; MOE, Molecular Operating Environment; NF-κB, nuclear factor-kappa B; NO, nitric oxide; PDTC, pyrrolidine dithiocarbamate; SS, sulfasalazine; UC, ulcerative colitis; 6-MITC, 6-(methylsulfinyl)hexyl isothiocyanate.

1. Introduction

Inflammatory bowel disease (IBD) is a global disease of the 21st century with evolving epidemiology in which the incidence is rising in newly industrialized countries while stabilizing in western countries [1]. IBD has been described as a complex disease underpinning a heterogeneity of clinical manifestations exemplified by prototypical conditions such as ulcerative colitis (UC) and Crohn’s disease (CD) [2]. Such complexity in pathogenic mechanisms has hindered the elucidation of its etiology and subsequent treatment. These chronic and recurrent conditions, however, are unified by the presence of inflammation of the gastrointestinal tract. It is generally accepted that IBD is associated with an uncontrolled mucosal immune response to intestinal microflora infiltration of the epithelial lining in genetically susceptible hosts [3-6]. In a recent review of the pathophysiology of IBD (therein described as Irritable Bowel Syndrome or IBS), potential factors that determine the manifestation of IBD symptoms are described [7]. These often-overlapping implicating factors include gut-brain axis disorders, genetic factors, infections and disturbances in the intestinal microbiota, low-grade mucosal inflammation, immune activation, altered intestinal permeability, diet and environment. The term, IBD interactome, was recently used in a review of the etiopathogenesis of IBD suggesting such pathophysiologic complexity requires a
systems biology approach to fully elucidating the network of dynamic mechanisms underpinning such complex diseases [2].

The immune response in IBD patients results in inflamed intestinal tissue infiltrated by large numbers of immune cells which secrete pro-inflammatory mediators, such as cytokines and nitric oxide (NO). The persistent recruitment of large numbers of leukocytes from the blood to the intestinal mucosa is largely mediated by adhesion molecules - integrins and their ligands [8]. The processes that derail the immune response in IBD are among the mechanisms being widely investigated to improve our understanding of IBD which underpin its physiopathology and, importantly, to identify new treatment therapies or cures.

A useful tool for identifying novel therapeutics has been the development of experimental colitis models which have enabled preclinical evaluation of the efficacy of new therapeutics prior to clinical trial [9]. A number of chemically-induced models have been employed to investigate the pathogenesis of IBD in humans including 2,4,6-trinitro-benzene sulfonic acid, oxazolone and dextran sodium sulphate (DSS) [10]. Collective data from these models have been essential in understanding the underlying mechanisms of IBD pathogenesis [11]. The DSS model is the most widely used murine model for colitis and, unlike the immunogenic agents, causes damage to epithelial cells when added to drinking water [12, 13]. The advantages of DSS over other chemical colitogens include its reproducibility, controllability, and ease of use. The DSS model can be used to study acute and chronic colitis by varying the duration of treatment.

Classic therapies for IBD centre around treating symptoms and include aminosalicylates and corticosteroids. The recent introduction of immunosuppressive (eg. azathioprine) and biologic agents (eg. anti-tumour necrosis factor alpha (TNF-α) antibodies) has markedly reduced the need for corticosteroids [14]. For those patients refractory to broad spectrum...
immunotherapies which can increase the risk of infection and cancer other strategies currently in the pipeline bring hope for symptom alleviation. Agents targeting adhesins involved in leukocyte trafficking (vedolizumab) [8] and egression from lymph nodes (etrolizumab) [15] are among an array of future therapeutic strategies to treat IBD including plant-derived agents.

Isothiocyanates (ITCs) are found in a variety of cruciferous vegetables, such as Brassica and Raphanus species and are derived from glucosinolates. Unlike other members of the Brassicaceae family, *Wasabia japonica* (Japanese horseradish) has particularly high levels of ITCs [16], such as 6-(methylsulfinyl)hexyl ITC (6-MITC) and, although well known as a condiment, the ITCs contained therein have received attention due to their pharmacological effects on the proteins and factors involved inflammation, cell growth and apoptosis [17, 18]. 6-MITC has shown anti-inflammatory and anti-oxidant activities [19-23] as well as inhibiting growth and metastasis in various cancer cells [24-26]. We have been studying 6-MITC as a multi-functional agent in the prevention, treatment and management of cancer and some of our findings include a strong suppression of NO production [27] and interaction with inducible NO synthase (iNOS), p-glycoprotein and multi-drug resistance-associated protein-1 [28-30].

Glycogen synthase kinase 3 beta (GSK-3β) inhibitors have become promising targets for anti-inflammatory research [31]. Active GSK-3 is an essential positive regulator of a wide range of pro-inflammatory cytokines such as TNF-α, interleukin 1 beta (IL-1β), IL-6 as well as pro-inflammatory mediators such as NO or prostaglandin E2. An inhibitory effect of 6-MITC on GSK-3β activity was reported in relation to new treatments for type 2 diabetes [32]. GSK-3s are ubiquitous, multi-tasking serine/threonine kinases, existing in two isoforms (GSK-3α and GSK-3β) that, despite their name, are involved in a wide variety of cellular processes beyond inhibiting glycogen synthase [33]. GSK-3 has been implicated in a diverse variety of diseases, such as cancer [34], cardiovascular diseases [35], neurodegenerative diseases [36],
and psychiatric diseases [37]. A number of signaling pathways, but especially PI3K/Akt, converge on GSK-3 to facilitate phosphorylation at particular serine residues (Ser21 for GSK-3α and Ser9 for GSK-3β) although other regulatory mechanisms are at play including cellular localization and protein-protein interactions [38]. The GSK-3β isoform regulates proliferation, apoptosis, inflammatory responses, migration, microtubules, and a variety of other cellular processes [39] and has been shown to be essential for either promoting [40] or repressing nuclear factor-kappa B (NF-κB) [41]. NF-κB signaling plays a pivotal role in the induction of inflammatory factors in macrophages in IBD [42].

The molecular events regulated by GSK-3 which mediate pro- or anti-inflammatory effects have been reviewed in detail [31] (Supplemental Fig. 1). Briefly, inflammatory stimuli induce phosphorylation of the inhibitor kappa B (IκB) by the inhibitory kappa B kinase (IKK) complex. This allows translocation of NF-κB heterodimer (p50/p65) to the nucleus to activate proinflammatory gene expression. GSK-3β plays a regulatory role in phosphorylation of p65 amongst other targets [43]. NF-κB is itself a central mediator of inflammation therefore a regulatory role for GSK-3β in inflammation, acting upstream of NF-κB, is clear [44]. Studies have shown that NF-κB is highly expressed in intestinal mucosal epithelium, crypt epithelial cells and lamina propria monocytes of patients with UC and that its expression is much higher in the nucleus compared to the cytoplasm [45] indicating its activation in UC. Understanding the complex modulatory role of GSK-3β on NF-κB-induced gene expression will aid therapeutic anti-inflammatory use of GSK-3 inhibitors since this apparent dichotomy suggests selective inhibition of NF-κB action (Fig 1).
Fig. 1. Molecular events regulated by GSK-3β leading to NF-κB activation which mediate pro-inflammatory effects and site of action by GSK-3β inhibitors such as 6-MITC.

Following the report that 6-MITC exhibited an inhibitory effect on GSK-3β enzyme activity [32] we hypothesized that 6-MITC could be utilized as a GSK-3β inhibitor in the treatment of a variety of health conditions however mechanistic details remain unclarified. The binding of 6-MITC to GSK-3β has never been reported and therefore required elucidation.

In this study we exploited an array of in vivo, in vitro and in silico techniques to investigate a potential role for 6-MITC as a GSK-3β inhibitor for the alleviation of IBD.

2. Materials & Methods

2.1. Animal model

Pathogen-free BALB/c mice, 5-10 weeks old, were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were kept in a conventional animal facility with 40-60% humidity, 23-25°C temperature and 12-hour light/dark cycles illumination at Faculty of Pharmacy, Aomori University (Aomori, Japan). Mice had free access to a standard pellet (CLEA Japan,
Inc.) diet and tap water and were acclimatized for 1 week or more before entering the study. The study was conducted in accordance with Notification Concerning Animal Experimentation Conducted by Universities etc. (Ministry of Education, Japan) and Guidelines for Animal Experimentation (Faculty of Pharmacy, Aomori University, Japan).

2.2. Induction of colitis and histology of colon

Tap water with 3% DSS (36-50 kDa; MP Bio, Tokyo, Japan) was prepared for female BALB/c mice for 5 days to induce colitis, followed by tap water without DSS for 7 days. Control mice had tap water without DSS.

Mice were sacrificed after the 12-day treatment and the colon removed. The colon was thoroughly rinsed before representative samples were fixed in 4% formalin (Merck KGaA, Darmstadt, Germany) for overnight and embedded in paraffin. Paraffin sections (10 μm) were cut and de-waxed for hematoxylin/eosin (Merck KGaA) staining. The stained tissue (the regions 20 mm from the rectum) was analyzed with a microscope (Olympus BX2, Tokyo, Japan).

2.3. Initiation of colitis and analysis of gene and protein expression in colon

Tap water with DSS at 3% was prepared for female BALB/c mice for 7 days. After this 7-day treatment the mice were sacrificed prior to their colon being removed. Total RNA of the distal colon was extracted from the regions 10 mm from the rectum using ISOGEN (NIPPON GENE, Tokyo, Japan). cDNA was synthesized from 1 μg total RNA using the TaKaRa RNA PCR kit (Takara Bio, Shiga, Japan) and subjected to PCR using Takara Taq™ (Takara Bio). The protocol for PCR was as follows:

Oligonucleotide primer sequences were as follows: β-actin F: 5’-GTG GGC CGC TCT
AGG CAC CAA-3’ R: 5’-CTC TTT GAT GTC ACG CAC GAT TTC-3’ (540 bp); iNOS F: 5’-
TGC AAG CTG ATG GTC AAG AT-3’ R: 5’-ATT CTG CAT GTG CTT CAT GA-3’ (422 bp);
IL-1β F: 5’-GCC CAT CCT CTG TGA CTC AT-3’ R: 5’-AGG CCA CAG GTA TTT TGT CG-
3’ (230 bp); IL-6 F: 5’-CCG GAG AGG AGA CTT CAC AG-3’ R: 5’-TTC TGC AAG TGC
ATC ATC GT-3’ (166 bp)

PCR protocol (TaKaRa PCR Thermal Cycler MP, Takara Bio) included the following
procedure: 95˚C 10 min, 1 cycle; 95˚C 45 sec.; 60˚C 45 sec.; 72˚C 45 sec.; 30 cycles (for IL-
6: 35 cycles); 72˚C 1 min, 1 cycle; 4˚C hold

PCR products were subjected to agarose gel electrophoresis and dyed with ethidium
bromide. The size of the PCR products was calculated using a size marker (100 bp DNA
Ladder) (Takara Bio) and Luminescent Image Analyzer LAS-3000 (FUJIFILM, Tokyo, Japan).

Note: 3% DSS was enough to induce iNOS gene.

Proteins of the distal colon were extracted from the regions 5 mm from the rectum using
CelLytic™ MT Cell Lysis Reagent (Merck KGaA). For western blotting analysis, aliquots of
proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and probed with a
primary antibody followed by a secondary antibody. The primary antibodies used were mouse
monoclonal anti-β-actin (Merck KGaA), rabbit polyclonal anti-iNOS (Santa Cruz
Biotechnology, Santa Cruz, CA, USA), goat polyclonal anti-IL-1β (Merck KGaA) and rabbit
polyclonal anti-IL-6 (Thermo Fisher Scientific, Waltham, MA, USA). The secondary
antibodies used were HRP-conjugated anti-mouse, anti-rabbit and anti-goat IgG (Santa Cruz
Biotechnology). Visualization of the antigen-antibody complexes was performed with ECL
plus Western Blotting Detection Reagents (GE Healthcare Japan, Tokyo, Japan). The size of
the positive bands was calculated using Perfect Protein™ Markers 15-150 kDa (Merck KGaA)
and Luminescent Image Analyzer LAS-3000 (FUJIFILM).
2.4. Induction of colitis and assessment of body weight, fecal matter and colon

Tap water with DSS at 3% for male and 5% for female BALB/c mice was prepared for 7 days to induce colitis (an acute phase), followed by the tap water with DSS at 1% for 7 days to maintain the colitic condition (a chronic phase). During the 2nd week the mice were treated with NF-κB inhibitors with either per os (po) (once a day for 7 days of the second week) or ip (twice: day 1 and 3 of the second week). Preparation of NF-κB inhibitors was as follows: for po, sulfasalazine (SS) (Merck KGaA) or 6-MITC (Shiratori Pharmaceuticals, Chiba, Japan) were dissolved in 1% carboxymethylcellulose sodium salt (Merck KGaA) and po-administered at 100 mg/kg or 10 mg/kg/day, respectively; for ip, pyrrolidine dithiocarbamate (PDTC) (Merck KGaA) or 6-MITC were dissolved in physiological saline and ip-injected at 100 mg/kg or 10 mg/kg/day, respectively.

Body weight (% increase) was measured at the beginning and end of the 2nd week of the treatment (the chronic phase). At the end of the 2nd week of the treatment the fecal blood score was characterized on a scale of 0-4 (0: no blood; 1: very slightly bloody; 2: slightly bloody; 3: bloody; 4: extremely bloody (blood in whole colon)). The state of the fecal matter was characterized in accordance with the Bristol stool chart. After the mice were sacrificed and the colon was removed and rinsed, the length and weight of the colon was measured.

2.5. Protein expression induced by interferon gamma (IFN-γ) and lipopolysaccharide (LPS) in macrophages

The macrophage-like murine cell line, J774.1, was obtained from Riken Cell Bank (Tsukuba, Japan). The cells were cultured in the nutrient medium RPMI-1640 (Merck KGaA) supplemented with fetal bovine serum (5%; JRK Biosciences, Lenaxa, KS, USA) and gentamicin sulfate (10 μg/ml; Wako Pure Chemical Industries Ltd., Osaka, Japan) in a
humidified atmosphere containing 20% O$_2$ and 5% CO$_2$ at 37°C. Then culture medium was replaced with fresh medium with IFN-γ (100 U/ml; Chemicon International Inc., Temecula, CA, USA) and LPS (0.5 μg/ml; Difco Laboratories, Detroit, MI, USA) and then incubated with or without 6-MITC (10 μM) at 37°C for 15, 30, 60 and 120 min. Proteins were extracted with CellLytic™ M Cell Lysis Reagent (Merck KGaA). For western blotting analysis, aliquots of proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and probed with a primary antibody followed by a secondary antibody. The primary antibodies used were mouse monoclonal anti-β-actin (Merck KGaA), rabbit polyclonal anti-IκB-α (Santa Cruz Biotechnology), rabbit polyclonal anti-IκB-β (Merck KGaA) and rabbit polyclonal anti-NF-κB p65 (Cell Signaling Technology, Danvers, MA, USA). The secondary antibodies used were HRP-conjugated anti-mouse and anti-rabbit IgG (Santa Cruz Biotechnology and Cell Signaling Technology, respectively). Visualization of the antigen-antibody complexes was performed with ECL plus Western Blotting Detection Reagents (GE Healthcare Japan). The size of the positive bands was calculated using Perfect Protein™ Markers 15-150 kDa (Merck KGaA) and Luminescent Image Analyzer LAS-3000 (FUJIFILM).

2.6. Modeling and 3D construction of GSK-3β

Modeling and 3D construction of GSK-3β were performed as previously reported [28]. In brief, the crystal structure coordinates for human GSK-3β (PDB codes: 1PYX and 1I09) [46, 47] along with their amino acid sequences were loaded into the Molecular Operating Environment (MOE) 2017.11 software program (Chemical Computing Group Inc., Montreal, Canada).

After alignment of the primary structure of GSK-3β, each amino acid was carefully checked to avoid deletions or insertions in conserved regions and necessary correction was executed. Full energy minimization and further inspection were applied to the model with the best
2.7. Structural justification of the modeled GSK-3\(\beta\)

The structural models of GSK-3\(\beta\) were assessed and justified as previously reported [28]. In brief, Ramachandran plots were utilized for the overall geometric and stereochemical qualities of the final modeled structure of GSK-3\(\beta\) in MOE. The effective atomic contact energies were calculated with the MOE for evaluation of the qualities of the protein folds of the modeled GSK-3\(\beta\). Quality of the protein folds was evaluated by calculating the effective atomic contact energies, comprising the desolvation free energies required to transfer atoms from water to the interior of the protein. Briefly, the calculation was performed for obtaining the contact desolvation energies for 18 different atom types of the 20 common amino acids that were resolved based on the clustering pattern of their properties. By explicitly accounting for neighbouring interactions, the contact potentials for each atom type were estimated within a 6 Å contact range.

2.8. Binding site selection and exploration for the modeled GSK-3\(\beta\)

The binding site selection and exploration for GSK-3\(\beta\) were executed as previously reported [28]. In brief, to identify possible substrate or inhibitor binding pockets within the newly generated 3D structures of GSK-3\(\beta\), the Site Finder module of the MOE was utilized. To denote zones of tight atom packing, hydrophobic or hydrophilic alpha spheres served as probes. Applying these alpha spheres, we were able to define potential ligand binding sites (LBSs) and centroids for the creation of dummy ligand atoms. Fitting of the dummy atoms was implemented to the corresponding alpha spheres during the characterization of the LBSs in GSK-3\(\beta\) and the bound conformations that approach crystallographic resolutions were generated.
2.9. Alpha sphere and excluded volume-based ligand-protein docking (ASE-Dock) of the modeled GSK-3β

Subsequently, the docking simulation and structural analysis of the ligand-receptor interaction between 6-MITC and GSK-3β were also implemented with ASE-Dock [48] in the MOE. In brief, ligand atoms have alpha spheres within 1 Å in the ASE-Dock module, and based on this property, concave models are created. By evaluating and scoring maximum overlap with alpha spheres and minimum overlap with the receptor atoms, ligand atoms from many conformations generated by superimposition with these points could be fitted. Ligand-receptor interaction energies were the basis for the scoring function in ASE-Dock, and the score was expressed as a $U_{\text{total}}$ value. For the energy minimization of the ligand conformations, MMFF94S force field was used. As a result, 500,000 poses were created and the 200 poses with the lowest $U_{\text{total}}$ values were selected for further optimization with the MMFF94S force field. The ligand was free to move within the binding pocket during the refinement step. $U_{\text{dock}}$ values were also adopted to determine the most reasonably docked GSK-3β-6-MITC complex.

3. Results

Acute colitis was induced in BALB/c mice (female) by administering a 5-day treatment with 3% DSS followed by one week tap water. Abnormal enlargement of the intestinal mucosal cells (thickening of mucosa) was observed with detachment of mucosa from the muscularis. DSS-induced histopathological changes (Figs. 2B and D) were evident in comparison to healthy control intestinal mucosal cells (Figs. 2A and C).
Fig. 2. Histological changes in intestinal tract of female BALB/c mice following treatment with 3% DSS for 5 days. A and C (healthy controls, n=3, C=enlarged view). B and D (treated group, n=3, D=enlarged view). Similar results were produced using 3% and 5% DSS for male BALB/c and C57BL/6 mice respectively (data not shown).

Colon tissue from female BALB/c mice was analyzed for the presence of pro-inflammatory-marker related genes including iNOS, IL-1β and IL-6 using gel electrophoresis. iNOS expression in the colon tissues of the mice treated with 3% DSS was markedly increased compared to control (Fig. 3) whereas no significant changes in levels of IL-1β and IL-6 were observed.
Fig. 3. Gel electrophoresis of PCR products showing detection of pro-inflammatory marker genes (iNOS, IL-1β and IL-6) in colon tissue from female BALB/c mice. Control (n=3), treatment group (n=3). β-actin used as a housekeeping gene.

Western blot analysis (Fig. 4) showed increased levels of iNOS correlating with the marked increase in iNOS expression observed in Fig. 3. Levels of IL-6 decreased compared to control. The observed increase in IL-1β in one lane was most likely an anomaly since this result was not able to be subsequently reproduced in any other subsequent experiment.

Fig. 4. Western blot analysis showing detection of pro-inflammatory markers in colon tissue
from female BALB/c mice. Control (n=3), treatment group (n=3). β-actin used as a loading control.

DSS treatment was extended for a further 7 days at 1% DSS to establish a chronic state of colitis. NF-κB inhibitors were administered during the chronic phase of the experiment either via a po (SS and 6-MITC) or ip route (PDTC and 6-MITC).

**Fig. 5.** Percent change in body weight in DSS-induced BALB/c mice in model (n=18, 9 male, 9 female) or test groups (n=8-10, 4-5 male, 4-5 female per group) following either po or ip administration of NF-κB inhibitors or 6-MITC during chronic phase.

The effect on body weight was determined following administration of NF-κB inhibitors and 6-MITC during the chronic phase of the study (Fig. 5). The results were expressed as percentage change in body weight. Administration of 6-MITC via the ip route to the DSS-induced mice increased their body weight in 14 days whereas no increase was observed with
the po route of administration for 6-MITC. Ip injection of a NF-κB inhibitor PDTC increased their body weight but the po administration of the other NF-κB inhibitor, SS, did not increase their body weight.

In addition to inflammatory markers, disease progression was assessed by monitoring changes in the degree of fecal blood (Fig. 6A) and fecal stool consistency measured according the Bristol stool chart (Fig. 6B).

Both 6-MITC and the NF-κB inhibitors ameliorated the fecal blood caused by DSS (Fig. 6A). Fecal state was measured using the Bristol stool chart in which a score of 1 to 2 indicate constipation, 3 to 4 are normal stools and 5 to 7 are associated with diarrhea and urgency. Our results showed slight softening of the feces by both 6-MITC and the NF-κB inhibitors
Fig. 7. A Colon length for DSS-induced BALB/c mice. Model (n=18, 9 male, 9 female) against test groups (n=9-10, 4-5 male, 4-5 female per group). B Colon weight/length ratio for DSS-induced BALB/c mice. Model (n=16, 8 male, 8 female) against test groups (n=9-10, 4-5 male, 4-5 female per group). NF-κB inhibitors or 6-MITC was po or ip administered during chronic phase.

No apparent change to colon length was observed between model and test groups in the DSS-induced mice (Fig. 7A). However, po administration of the NF-κB inhibitor SS appeared to decrease colon wet weight/length as did 6-MITC when administered orally (Fig. 7B).

The direct effect of 6-MITC on NF-κB expression as well as the inhibitory κB proteins (IκB-α and IκB-β) was investigated using a murine macrophage cell line. Cells were cultured to confluency prior to NF-κB induction by IFN-γ and LPS and treatment with or without 6-MITC. Measurements were taken over a 2-hour period. The induction of NF-κB was confirmed by western blot analysis showing a significant increase in NF-κB confirming activation of the inflammatory process. Cells treated with 6-MITC produced significantly less NF-κB within
Fig. 8. Western blot analysis (3 replicate experiments) of NF-κB induction by IFN-γ and LPS in J774.1 macrophage cells in the absence (left) and presence (right) of 6-MITC showing changes in levels of NF-κB and the inhibitory IκB isoforms, α and β. Increased NF-κB (red circle) decreased with 6-MITC treatment (blue circle).

A structural analysis was conducted to examine the potential of 6-MITC as a direct GSK-3β inhibitor. In this study we took advantage of two available high-resolution crystal structure coordinates for GSK-3β in the apo form (PDB code: 1I09; 2.7Å) and with bound competitive inhibitor, adenylyl imidodiphosphate (AMP-PNP, PDB code: 1PYX; 2.4Å). Currently there are no 3D crystal structures available depicting the conformations of the native ligand, ATP within the substrate binding pocket. A detailed analysis of the binding interactions between inhibitor, AMP-PNP, and protein were provided by the authors [47].

The primary sequence for GSK-3β is shown in Fig. 9. The N-terminal domain (25-138) of
GSK-3β consists predominantly of β-sheets connected to an α-helix rich C-terminal domain (139-384). The N-terminal domain includes a seven-stranded β-sheet structure forming a closed orthogonal barrel (Fig. 10A) and resembles the N-termini of tyrosine kinases [49-51].

Fig. 9. An excerpt from the primary structure of GSK-3β (PDB code: 1I09) showing the N-terminal β-sheet domain (25-138) connected to an α-helix-rich C-terminal domain (139-384). β-sheets depicted by yellow arrows; α-helices in red cylinders.

The C-terminal domain is connected to the end of the 7th strand of the N-terminal domain where an α-helix (139-148) extends to the rest of the GSK-3β structure. The C-terminal domain shows some similarity in its structure to the equivalent domain in mitogen activated protein kinases [52, 53]. Inhibitors have been reported to bind with similar topology to the ATP binding site in the GSK-3β active site [47] such as the ATP-mimetic and competitive inhibitor, AMP-PNP, (Fig. 10B). The 3D structure of 6-MITC is also presented in Fig. 10C.
Structural analysis of the GSK-3β-AMP-PNP complex (PDB code: 1PYX) showed that Lys85 formed 3 hydrogen bonds with the non-bridging oxygen atoms of the α and β phosphoryl groups of the inhibitor and the bridging oxygen between the α and β phosphoryl groups (Fig. 11A). The presence of a hydrogen bond was found between Tyr134 and the N1 of adenine. Val135 also formed a hydrogen bond with the N1 of adenine. The catalytic amino acid residue Asp200 was located in the proximity of AMP-PNP [54]. The Ligand Interactions module of MOE was used to create ligand-receptor interaction plots for the GSK-3β-AMP-PNP complex. These plots clearly display the key intermolecular interactions facilitating accurate interpretation of the 3D juxtaposition of AMP-PNP within the ATP binding site in GSK-3β (Fig. 11B). Among the 21 interacting residues Gly63, Asn64, Gly65, Lys83 and Gln185 also
formed hydrogen bonds with the oxygen of ribose, the C5’ of ribose, the non-bridging oxygen atoms of the β and γ phosphoryl groups, the non-bridging oxygen of the γ phosphoryl group and the OH3’ of ribose, respectively.

**Fig. 11.** A The 3D structure of the GSK-3β-AMP-PNP complex at the ATP binding site. Lys85, Tyr134 and Val135 formed hydrogen bonds with AMP-PNP. The catalytic amino acid residue Asp200 was in the proximity of AMP-PNP. B The ligand-receptor interaction plots for the GSK-3β-AMP-PNP complex created by the Ligand Interactions module of the MOE. Twenty-one residues were identified as the preferred interaction residues. In addition to Lys85, Tyr134 and Val135, Gly63, Asn64, Gly65, Lys183 and Gln185 also formed hydrogen bonds with AMP-PNP.
Molecular docking was undertaken to elucidate mechanistic details of the interaction between 6-MITC and the GSK-3β model. The results are depicted in Fig. 12A. The 4 interacting amino acid residues identified in the GSK-3β-AMP-PNP complex (Fig. 11A) were also found in the proximity of 6-MITC (Fig. 12B). The Ligand Interactions module of MOE identified 11 residues in total as the preferred interaction residues for the GSK-3β-6-MITC complex (Fig. 12C). A hydrogen bond was observed between Lys85 and the oxygen of the sulfinyl group in 6-MITC. The sulphur of isothiocyanate in 6-MITC was identified as the element that formed the hydrogen bonds with Tyr134 and Val135. Asp200 was located on the proximity contour line.
Fig. 12. A Docking simulation of 6-MITC to the GSK-3β model. 6-MITC binds to the ATP binding site in the GSK-3β model. The hydrogen-bond (purple), hydrophobic (green) and hydrophilic (blue) regions are shown on the molecular surfaces of the ATP binding site (4.5 Å from 6-MITC) in the GSK-3β model. B Enlarged Fig. of the ATP binding site in the GSK-3β-6-MITC complex. Lys85, Tyr134, Val135 and Asp200 are found in the proximity of 6-MITC as in the GSK-3β-AMP-PNP complex (Fig. 11A). C The ligand-receptor interaction plots for the GSK-3β-6-MITC complex. Ten residues out of the 11 interacting residues in the GSK-3β-6-MITC complex are also found in the GSK-3β-AMP-PNP complex (Fig. 11B). Of the 11 interacting residues in the GSK-3β-6-MITC complex (Fig. 12C), 10 residues (Gly65, Val70, Ala83, Lys85, Asp133, Tyr134, Val135, Leu188, Cys199 and Asp200) but not Phe67, were also found in the GSK-3β-AMP-PNP complex (Fig. 11B).

The GSK-3β-AMP-PNP complex (Fig. 11) (PDB code: 1PYX) [47] was the most relevant structure available in the PDB to surmise the binding mode of the native ligand ATP. Upon removal of the AMP-PNP ligand from the model, ATP was subsequently docked into the ATP binding pocket of GSK-3β to generate the GSK-3β-ATP complex.

The present analysis identified 15 interacting residues including Lys85 and Thr138, and the ATP binding site within the newly generated 3D structure of the GSK-3β-ATP complex was further analyzed. The catalytically important, Asp200 formed a hydrogen bond with the hydroxyl of the α phosphoryl group (Fig. 13A) later confirmed by the ligand-receptor interaction plots (Fig. 13B). The presence of a hydrogen bond was found between Val135 and the amino group of adenine. Pro136 also formed a hydrogen bond with the NH2 of adenine (Fig. 13B). Among the 15 interacting residues Asn186 formed hydrogen bonds with the OH2’ of ribose (Fig. 13B).
Fig. 13. A The 3D structure of the GSK-3β-ATP complex at the ATP binding site. Val135 and the catalytic amino acid residue Asp200 form hydrogen bonds with ATP. B The ligand-receptor interaction plots for the GSK-3β-ATP complex created by the Ligand Interactions module of
the MOE. Fifteen residues are identified as the preferred interaction residues. In addition to Val135 and Asp200, Pro136 and Asn186 also form hydrogen bonds with ATP.

4. Discussion

In this study we assessed the anti-inflammatory activity of 6-MITC for the alleviation of IBD using a DSS-induced murine model of colitis and provide evidence for its mechanism of action via NF-κB inhibition. It is well documented that GSK-3β controls the activity of NF-κB [55]. Our results demonstrate NF-κB-mediated signal transduction following induction of colitis in the murine model and suggest that 6-MITC acts as a competitive GSK-3β inhibitor, operating upstream of NF-κB, with therapeutic potential for IBD.

Administration with 3% DSS (acute phase) followed by lower and more prolonged dose (chronic phase) of DSS was performed to mimic the more relevant chronic inflammation observed in human IBD [56]. Chronic colitis has been reported to be induced in mice either by continuous treatment of low concentrations or by cyclical administration of DSS [57]. The mechanism of DSS toxicity occurs through association with medium-chain fatty acids present in the colonic lumen. Particles thus formed penetrate colonocyte membranes thereby diminishing the integrity of the barrier [58, 59]. Importantly, the immune response to DSS is mediated through effector cytokines rather than by the adaptive immune system [60].

DSS-induced acute colitis exhibits several morphological and pathophysiological features resembling human UC, including superficial ulceration, mucosal damage, production of cytokines and other inflammatory mediators as well as leukocyte infiltration [61]. Our results demonstrate the macroscopic changes associated with chronic colitis including shortening of the colon [62].
Development of colitis was demonstrated histologically by abnormally enlarged intestinal mucosal cells and detachment of the mucosa from the muscularis (Fig. 2). Associated inflammation was evidenced by the observed increase in gene expression of the pro-inflammatory marker iNOS as well as its associated gene product (Figs. 3 and 4, respectively). The results shown in Fig. 4 suggest a differential pattern of regulation for iNOS and IL-6 with iNOS regulation occurring at the transcriptional level following DSS treatment whereas IL-6 appears to be regulated at the translational level with repression of expression. Apart from being an important component of the host defense system, iNOS-mediated NO production may occasionally become part of a dysregulated immune response, resulting in chronic inflammatory disorders [63]. Our results support those cited in other studies in which altered iNOS, as well as other adhesion determinants, were altered in IBD [64]. The increased vascular permeability, due to NO release, likely exacerbates the deterioration of the intestinal barrier.

iNOS is known to be increased by NF-κβ, a central mediator of inflammation [65-67]. Therefore, to elucidate the mechanism of activation of inflammation in the DSS-induced model, anti-inflammatory mediators, including NF-κβ inhibitors, SS and PDTC, as well as 6-MITC were administered during the chronic phase of the experiment either orally or by ip injection.

Direct ip administration of NF-κβ inhibitor PDTC and 6-MITC (chronic phase) resulted in an increased body weight over the 14 days compared to models which suggests that NF-κβ inhibition contributed to the weight gain when administered directly without first entering the gastrointestinal system. Weight loss is a characteristic marker of DSS-induced colitis therefore these results support a role for 6-MITC inhibition of NF-κβ signaling. 6-MITC has been reported elsewhere to inhibit NF-κβ thereby releasing its control on the PI3K/AKT pathway [68].

The severity of colitis can be scored macroscopically by colonic bleeding, fecal blood,
shortening of the colon and loosening of stool consistency [69]. 6-MITC and NF-κβ inhibitors alleviated fecal blood compared to DSS-induced mice models. A slight softening of the stool was evident following administration of 6-MITC and NF-κβ inhibitors (Figs. 6A and B, respectively). Small changes in stool form may not be significant. Loose stools may arise from a range of other factors including changes in gut microbiota [70] to emotional stress [71].

No apparent changes in colon length were observed in DSS-induced mice between model and test groups. While these results differ from other studies where treatment groups showed similar colon lengths to untreated models in 14 days [72, 73] we note that in such cases treatment was administered from Day 1 whereas our treatment was administered only at Day 7 and using a higher dose throughout which may explain why a similar effect was not observed in our study. However, when considering the more commonly used indicator, colon wet weight to length ratio, we observed that oral administration of the NF-κβ inhibitor SS and 6-MITC decreased colon wet weight to length ratio (Fig. 7B) which suggests NF-κβ inhibition through the gastrointestinal system can alleviate adverse intestinal effects induced by DSS. These results are in agreement with those cited in the aforementioned studies.

Increased expression of NF-κβ in macrophage cells leads to increased levels of proinflammatory cytokines TNF-α, IL-1 and IL-6 resulting in damaged epithelia [74]. Increased levels of these cytokines in colonic epithelia cells lead to increased expression of intercellular adhesion molecule-1 which contributes to the recruitment of neutrophil granulocytes to the site of inflammation. Cytokine activity leads to stimulation, activation and differentiation of lamina propria immune cells further aggravating mucosal inflammation. The observed decreased production of IL-6 following 6-MITC administration supports the assertion that 6-MITC acts by inhibition of NF-κβ.

Addition of 6-MITC subsequent to induction of inflammation by LPS in the macrophage
cell line caused a significant decrease in NF-κβ levels as well as increased levels of the IκB proteins, including isoforms IκB-α and IκB-β. In an inactive state, the IκB proteins sequester the NF-κβ isoforms in the cytosol. Upon inflammatory stimulus, phosphorylation of the IκB proteins by IKK leads to their proteasomal degradation and nuclear localization of NF-κβ and subsequent altered gene expression according to the canonical pathway of NF-κβ activation [42]. The increased levels of IκB isoforms observed in Fig. 8 upon addition of 6-MITC implies less proteasomal degradation allowing NF-κβ to remain sequestered in the cytosol and that 6-MITC acts upstream of this regulatory step.

Structural analysis of the high-resolution structures of human GSK-3β showed that the GSK-3β structure conforms to that observed for the “activation-segment” protein kinases consisting of a β-sheet-rich amino-terminal domain coupled to a carboxy-terminal α-helical domain. The C-terminal domain shows some similarity in its structure to the equivalent domain in mitogen activated protein kinases [52, 53]. Structures of the apoenzyme (PDB code: 1I09) and that bound by the inhibitor, AMP-PNP (PDB code: 1PYX) within the ATP-binding site, were selected for this analysis due to their high resolution and structure quality. Inhibitors have been reported to bind in a similar topology to that of ATP in the ATP binding site in the GSK-3β [47]. We docked 6-MITC into the binding site and compared the interactions made to those of the AMP-PNP competitive inhibitor. As no crystal structure currently exists with the native ligand ATP bound, we generated a model of the GSK-3β-ATP complex and further compared the interactions to determine the mode of inhibition of 6-MITC.

Previous reports have discussed the importance of a few amino acid residues which interacted with inhibitors, such as Thr138 [47] and Lys85, Thr138 and Arg141 [75]. 21 interacting residues were identified in this study including Lys85 and Thr138 but not Arg141 using Ligand Interactions Module of MOE which provides a more distinct arrangement of the
important intermolecular interactions that assists in the interpretation of the 3D juxtaposition of AMP-PNP and the ATP binding site in GSK-3β (Fig. 11B). The charged amino sidechain group of Lys85 formed multiple hydrogen bonds with both the bridging and non-bridging oxygens of the phosphoryl groups of the inhibitor. The catalytically important, Asp200, was also found close to AMP-PNP. This analysis identified a further five residues, Gly63, Asn64, Gly65, Lys183 and Gln185, forming hydrogen bonds with the polar part of the ligand.

The generated model of the GSK-3β-6-MITC complex (Fig. 12) showed 6-MITC to adopt an extended conformation within protein binding site despite the 6 carbon alkyl spacer of 6-MITC conferring a high degree of rotational freedom [76]. This conformation facilitated additional stabilization due to hydrogen bonding at each end of the ligand and the nonpolar allyl groups owing to the hydrophobic surroundings at the ATP binding site. The same 4 interacting amino acid residues (Lys85, Tyr134, Val135 and Asp200) in the GSK-3β-AMP-PNP and GSK-3β-ATP complexes (Fig. 11A and Fig. 13A) were found in the proximity of 6-MITC (Fig. 12B) along with a further 11 preferred interaction residues (Fig. 12C). Lys85 was important for providing stabilization through hydrogen bonding to the sulfinyl oxygen of 6-MITC. The isothiocyanate sulphur in 6-MITC was stabilized by two additional hydrogen bonds from Tyr134 and Val135 which could contribute to its binding affinity. Importantly, the catalytically important residue, Asp200 was found within the proximity contour area indicating close contiguity with ligand atoms with potential for interaction. 10 out of the 11 interacting residues in the GSK-3β-6-MITC complex are also found in the GSK-3β-AMP-PNP complex (Fig. 11B) which further suggests that 6-MITC may act as a competitive ATP inhibitor of GSK-3β.

In the absence of available 3D coordinates we generated the GSK-3β-ATP complex and compared its binding interactions to that previously discussed with the two inhibitors. Among
the 15 interacting residues, Lys85 and Thr138 have been previously shown to be crucial for stabilizing AMP-PNP and 6-MITC in the ATP-ligand binding site. As expected, Asp200 was within interacting distance for catalysis stabilized by hydrogen bonding (Fig. 13A), further supported by the ligand interaction plots (Fig. 13B). Hydrogen bond stabilization was further provided by Val135 and Pro136 similar to that observed in the GSK-3β-6-MITC complex.

Results from our structural analysis provide considerable insight into the binding mechanism of 6-MITC at the ligand binding site of GSK-3β. Analyses of the structural properties of the GSK-3β-6-MITC complex and the docking simulation suggest that 6-MITC competitively inhibits GSK-3β interfering with ATP binding and the key amino acids for this inhibition are Lys85, Tyr134, Val135 and Asp200. To the best of our knowledge, this is the first report of the in silico GSK-3β model with 6-MITC as a potential GSK-3β inhibitor. Analyses of the GSK-3β-6-MITC complex can be of great significance in designing in silico GSK-3β-inhibitor models for successful development of various drugs with medicinal benefits.

5. Conclusion

Novel approaches to new therapeutics for IBD are in demand. The main objectives in the present study were to determine the protective effects of 6-MITC on a murine colitis model, elucidate details of the signaling pathway involved and finally to investigate the mode of GSK-3β inhibition. We employed a murine model of colitis to explore the mechanistic aspects of inhibition of 6-MITC from wasabi as a potential therapeutic for IBD. 6-MITC improved fecal blood scores and DSS-induced weight loss indicating reduced intestinal stress. This was reinforced by the observed reduction in colon weight/length through oral administration of 6-MITC. We found that 6-MITC inhibits NF-κβ signaling by competitive inhibition of GSK-3β. A structural analysis provided insight into the competitive nature of inhibition of 6-MITC at the ATP binding site of GSK-3β. Taken together our results demonstrate a role for 6-MITC as
a potential anti-inflammatory agent for the alleviation of IBD.

6. Acknowledgements

This study was partly supported by a Grant-in-Aid from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

7. References


