A Nucleotide Analog Prevents Colitis-Associated Cancer via Beta-Catenin Independently of Inflammation and Autophagy

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SUMMARY
Colitis increases the risk of bowel cancer. We show in mouse models that a thioguanine nucleotide can inhibit Wnt/b-catenin signaling via Rac1 in colonic epithelial cells to prevent carcinogenesis. The novel mechanism is independent of inflammation and autophagy.

BACKGROUND & AIMS: Chronic bowel inflammation increases the risk of colon cancer; colitis-associated cancer (CAC). Thiopurine treatments are associated with a reduction in dysplasia and CAC in inflammatory bowel disease (IBD). Abnormal Wnt/β-catenin signalling is characteristic of >90% of colorectal cancers. Immunosuppression by thiopurines is via Rac1 GTPase, which also affects Wnt/β-catenin signalling. Autophagy is implicated in colonic tumors, and topical delivery of the thiopurine thioguanine (TG) is known to alleviate colitis and augment autophagy. This study investigated the effects of TG in a murine model of CAC and potential mechanisms.

METHODS: Colonic dysplasia was induced by exposure to azoxymethane (AOM) and dextran sodium sulfate (DSS) in wild-type (WT) mice and mice harboring intestinal epithelial cell-specific deletion of autophagy related 7 gene (Atg7<sup>-ΔIEC</sup>). TG or vehicle was administered intrarectally, and the effect on tumor burden and β-catenin activity was assessed. The mechanisms of action of TG were investigated in vitro and in vivo.

RESULTS: TG ameliorated DSS colitis in wild-type but not Atg7<sup>-ΔIEC</sup>IEC mice, demonstrating that anti-inflammatory effects of locally delivered TG are autophagy-dependent. However, TG inhibited CAC in both wild-type and Atg7<sup>-ΔIEC</sup> mice. This was associated with decreased β-catenin activation/nuclear translocation demonstrating that TG’s inhibition of tumorigenesis occurred independently of anti-inflammatory and pro-autophagic actions. These results were confirmed in cell lines, and the dependency on Rac1 GTPase was demonstrated by siRNA knockdown and overexpression of constitutively active Rac1.

CONCLUSIONS: Our findings provide evidence for a new mechanism that could be exploited to improve CAC chemoprophylactic approaches. (Cell Mol Gastroenterol Hepatol 2021;11:33–53; https://doi.org/10.1016/j.jcmgh.2020.05.012)

Keywords: Colon Cancer; β-Catenin; Thioguanine; Autophagy.
Inflammatory bowel disease (IBD) is becoming increasingly prevalent in the developed and developing world. The subgroup of IBD patients with extensive colitis has an increased risk of developing colitis-associated colorectal cancer (CAC). Although the pathogenesis of CAC in the setting of IBD is being more strongly linked to chronic inflammation and is difficult to detect endoscopically because of arising from flat dysplastic lesions, it has many features in common with the pathogenesis of sporadic and hereditary colon cancers, which are the second leading cause of cancer death globally.

Hyperactivated Wnt/β-catenin signalling is considered a hallmark of colorectal tumorigenesis and has been recently implicated in immune evasion, invasion, and metastasis. With colorectal cancer (CRC), 90% of all tumors have a mutation(s) in the Wnt/β-catenin pathway. However, the role of Wnt/β-catenin signalling in CAC is less well-understood. Initial studies indicated that the Wnt/β-catenin pathways were activated late during pathogenesis. However, more recent reports have suggested early Wnt/β-catenin signalling activation similar to that observed in other CRCs. Nuclear localization of β-catenin in response to Wnt signalling, which is controlled by a large number of binding partners that include Rac1, is essential for its canonical signalling and similarly for the redistribution of β-catenin and T-cell factor/lymphoid enhancer factor (TCF/LEF) transcriptional activation in colon cancer cells.

Epidemiologic studies have shown a reduced incidence of dysplasia and CAC in those IBD patients treated with thiopurines, either azathioprine or mercaptopurine (MP). These are pro-drug purine base analogues, whose action is thought to relate to their conversion to active thioguanine nucleotides (TGN) in circulating activated T lymphocytes, many of which home to the gut. Thioguanine nucleotide triphosphate (TGTP) is the predominant intracellular species of TGN, which binds to the small guanosine triphosphatase (GTPase) Rac1, leading to inhibition of downstream signalling (Figure 1A). The cancer chemopreventive action of thiopurines has been thought to relate nonspecifically to their overall anti-inflammatory actions. Although MP and azathioprine (which is non-enzymatically converted to MP) are associated with reduced CAC, there are no epidemiologic data with respect to the less commonly prescribed thiopurine, thioguanine (TG).

Autophagy is an evolutionarily conserved homeostatic system that eliminates targeted cytoplasmic contents through the formation of a characteristic double-membrane autophagosome, followed by lysosomal fusion and cargo degradation. In addition to its homeostatic function, autophagy has roles in maintaining cell survival during stress and is critical in inflammation and cancer among other cellular processes. Polyomorphisms in autophagy genes have been associated with IBD through genome-wide association studies. In the context of cancer, autophagy has both antitumorigenic activity in early stages of oncogenesis where activation of autophagy can inhibit colitis and CAC initiation and protumorigenic activity in larger tumors and metastatic disease under nutrient limiting conditions, hypoxia, and in the face of accumulated damage to cellular organelles where autophagy is required. ATG7 is an evolutionarily conserved core autophagy protein that is essential for mammalian autophagy. Disruption of ATG7 in the intestinal epithelial cells in the murine APC<sup>min</sup> model of sporadic CRC leads to decreased tumor burden in a microbiome-dependent manner due to enhanced immunosurveillance.

In this article, we show that TG, but not MP, acts directly on intestinal epithelial cell lines in vitro to inhibit β-catenin activity in a TGN-dependent manner. In a mouse model of CAC, daily intrarectal TG prevented colitis in wild-type (WT) mice but not in mice with deficient autophagy in intestinal epithelial cells. Administration of TG reduced CAC tumor development in both WT and autophagy-deficient mice. The largely autophagy-independent action of TG to prevent CAC involves inhibition of β-catenin signalling and its downstream nuclear translocation, which at physiological concentrations was dependent on Rac1.

Results

Thioguanine, but not Mercaptopurine, Inhibits β-Catenin Transcriptional Activity in Colonic Cell Lines

To determine whether thiopurine pro-drugs can affect β-catenin signalling, the effects of the commonly used thiopurine MP, as well as TG, were studied by using 2 cell lines, HCT116 and Caco2, harboring a β-catenin transcriptional reporter. Both cell lines, which are derived from colon cancer cells, bear an activating mutation in β-catenin. The reporter cell line cultures were exposed to TG or MP in the presence of 1 μmol/L CHIR-99021 (CHIR) for 16 hours. CHIR stimulates the Wnt signalling pathway by inhibiting glycogen synthase kinase 3. TG, but not MP, inhibited β-catenin transcriptional activity in both cell lines in a dose-dependent manner (Figure 1B). The inhibitory concentration of 50% (IC50) of TG inhibition of β-catenin transcriptional activity in these cultures was ~2 μmol/L.

Immunofluorescence staining of β-catenin corroborated TG-mediated inhibition of β-catenin activity in cells with or without CHIR. The staining indicated that TG treatment markedly reduced cellular β-catenin (Figure 1C).
with the observation that TG inhibited Wnt signalling. TG treatment also inhibited cell proliferation in the presence and absence of CHIR stimulation in these cultures (Figure 1D). Neither MP nor TG treatment for ≤24 hours in cultures of HCT116 or Caco2 was associated with cytotoxicity (data not shown). We subsequently had the opportunity to examine the effect of TG on epithelial spheroids grown from a colon cancer resected from a patient with pancolitis. TG inhibited both total β-catenin and activated β-catenin (Figure 1E).

TG was converted to intracellular TGN by the HCT116 and Caco2 colon cells cultures (Figure 1F). TGN were not detected in any MP cultures at similar concentrations (data not shown), which was expected because conversion of MP to TGN is rate-limited by inosine-5’-monophosphate dehydrogenase (IMPDH). These results are consistent with a requirement for TGN for the observed effect and may explain the lack of effect observed with MP treatment, because MP’s, but not TG’s, conversion to TGN is rate-limited by IMPDH15 (Figure 1A).
**Intrarectal Thioguanine Inhibited the Initiation of Colitis-Associated Tumorigenesis**

Having established that TG inhibits β-catenin signalling in colonic cells in vitro at an IC50 of 2 μmol/L (0.33 mg/kg), we next ascertained the effect of TG on colon tumorigenesis in vivo by using an established mouse model of CAC induced by intraperitoneal azoxymethane (AOM) administration, followed by cycles of dextran sodium sulfate (DSS) exposure to induce colonic inflammation (Figure 2A). To specifically examine the effect of TG on CAC oncogenesis we use localized delivery via intrarectal gavage. A large daily dose of TG 1.5 mg/kg was selected for the in vivo mouse experiments because the contact time of intrarectal drug with colonic mucosa in the mice was short because of rapid expulsion of enema fluid by the ambient mice. Consistent with our previous findings with TG, mice treated with intrarectal TG exhibited fewer clinical signs of colitis during and immediately after the DSS cycles than control vehicle treated mice, as evidenced by less diarrhea, absence of rectal bleeding, and lower disease activity index scores (DAI) during periods both on and off DSS treatment (Figure 2B).

Vehicle-treated mice had a mean 14 (standard error, 2.0) colonic tumors (Figure 2C). Tumors were associated with an increase in proliferating bromodeoxyuridine (BrDU) + cells (Figure 2D). Areas of high-grade dysplasia and high-grade dysplasia with tumor invasion into submucosa were twice as frequent as areas with low-grade dysplasia. Remarkably, intrarectal TG treatment completely prevented colonic tumorigenesis (Figure 2C and F). TG-treated mice had fewer proliferating (BrDU+) cells in comparable regions of the colon than vehicle-treated mice (Figure 2D). There were no tumors and no dysplasia in the WT mice treated with TG (Figure 2E and F).

**Oncoprotection by Intrarectal Thioguanine Was Mainly Independent of Thioguanine’s Effect on Autophagy**

We had previously reported that TG induced a rapid augmentation of autophagy by intestinal epithelial cells in vitro, which could contribute to how TG effects a rapid amelioration of colitis. Under normal physiological conditions Wnt/β-catenin signalling has been implicated in suppression of autophagy, and β-catenin is itself subject to proteasome-independent degradation by autophagy. Intestinal epithelial autophagy also impacts CRC growth once a tumor is initiated in some murine models of colon cancer. Therefore, we investigated the role of TG in the absence of intestinal epithelial autophagy in the mouse CAC model by using conditional Atg7Cre/null;ColnCre mice with Atg7 selectively absent in intestinal epithelial cells (Atg7fl/fl;ColnCre) (Figure 3A).

Intrarectal TG did not improve the colitis associated with exposure to each cycle of DSS in this model, as indicated by similar DAI scores in TG-treated and vehicle-treated mice (Figure 3B), which is consistent with the anti-inflammatory effect of intrarectal TG being autophagy dependent. Rectal bleeding was significantly increased in the vehicle-treated Atg7fl/fl;ColnCre mice from day 70, which was consistent with tumor formation; there was a mean of 8 tumors per colon in these vehicle-treated Atg7fl/fl;ColnCre mice. On the other hand, TG treatment was associated with a significant reduction of tumor load (<4 tumors/colon) in the Atg7fl/fl;ColnCre mice (Figure 3C; P = .006) despite the ongoing inflammatory burden with the TG treatment. TG-treated tumors in Atg7fl/fl;ColnCre mice had less proliferative (BrDU+) cells than control-treated Atg7fl/fl;ColnCre tumors (Figure 3D). Conventional H&E histopathologic assessment of intrarectal vehicle-treated Atg7fl/fl;ColnCre mice revealed less high-grade dysplasia and less high-grade dysplasia with invasion into submucosa, compared with vehicle-treated WT mice (Figures 2E and 3E). TG-treated Atg7fl/fl;ColnCre mice had low-grade dysplasia but no areas of high-grade dysplasia or invasion (Figure 3E). Whereas tumors in vehicle-treated WT mice tended to be ≥5 mm, those in the vehicle-treated Atg7fl/fl;ColnCre mice were mainly ≤5 mm. The tumors in the TG-treated Atg7fl/fl;ColnCre mice were all ≤2 mm (Figures 2F and 3C). Figure 3F illustrates tumor load data from the 2 experiments (Figures 2C and 3C); there was a trend for fewer tumors in vehicle-treated Atg7fl/fl;ColnCre mice compared with vehicle-treated WT mice (P = .054), and although TG treatment was effective in the Atg7fl/fl;ColnCre mice (P = .003), it was less effective in these mice compared with WT where TG treatment was associated with a complete absence of tumors.

Rectal delivery of TG was associated with scant systemic toxicity in these models; there was no evidence of hepatic sinusoidal obstructive syndrome in any of the mice treated with daily intrarectal TG. Figure 4A shows a representative histology image from vehicle-treated and TG-treated animals. Intrarectal TG during the 85-day treatment duration in the WT mice was not associated with peripheral blood immunosuppression (Figure 4B), but mild leukopenia was demonstrated in the Atg7fl/fl;ColnCre mice at day 85 (P < .05). Intrarectal TG treatment did not appear to alter total mesenteric lymph node leukocyte numbers. However,

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**Figure 2.** (See previous page). TG inhibited colitis and colitis-associated colorectal tumor development and progression in mice. (A) Scheme showing induction procedure for AOM/DSS model of CAC. C57BL/6J WT were treated with DSS 1.5% in drinking water for 5 days, 3 cycles, ± daily intrarectal TG 1.5 mg/kg for 85 days (n = 5 each group). (B) Daily assessments in TG or vehicle-treated mice (n ≥5) for diarrhea, body weight, rectal bleeding, and DAI score. (C) At death day 85: number of colon tumors (left panel); representative macroscopic images of colon mucosa (right panel). (D) Average number of BrdU-staining cells per field of view in tumors and comparable areas of TG-treated mice, and representative immunohistochemical staining for BrdU in tumors (right panel). (E) Severity of dysplasia vs TG treatment in tumors 85 days after injection of AOM with representative H&E–stained sections of colonic tissue with tumors (scale bars are shown, right panel). (F) Tumor diameter and frequency in vehicle-treated mice versus TG-treated mice. Statistics B–D: F: mean ± standard error of the mean, Mann-Whitney U test. *TG vs vehicle, ****P < .0001 (B), **P < .01 (F) are P values shown (C and D).
intrarectal TG was associated with a small but significant change in the CD4:CD8 ratio in mesenteric lymph nodes at day 85 (Figure 4C; P < .05).

In summary, although the size of the dose and the duration of TG administration for 85 days were significantly greater than previously reported46,48 there was negligible systemic toxicity, reinforcing previous observations showing a lack of toxicity with rectally administered TG.46–48 The anti-inflammatory effects of rectally delivered TG in colitis is dependent on intestinal epithelial cell autophagy, a mechanism that is in addition to recognized immunomodulatory actions on adaptive T-lymphocyte–mediated immune responses and innate immunity from systemically absorbed TG.48 Furthermore, inhibition of tumor formation by daily intrarectal TG is largely independent of intestinal epithelial cell autophagy, even in the absence of its anti-inflammatory effects. The lower tumor load in vehicle-treated Atg7IEC mice than in vehicle-treated WT mice is consistent with the reported role of intestinal epithelial cell autophagy in the development of CRC.32

**Thioguanine Treatment Was Associated With Decreased Beta-Catenin Nuclear Accumulation and Oncogenic Transcriptional Activity in Colitis-Associated Cancer Tumors**

Because TG treatment of epithelial cells reduced β-catenin activity in vitro, we investigated the effects of TG administration on β-catenin in the CAC model. Beta-catenin in tumors was assessed by immunohistochemistry in WT mice; greater cytoplasmic staining intensity and particularly strong nuclear β-catenin localization were observed in vehicle-treated tumors compared with comparable regions in the colons of TG-treated mice in which β-catenin was typically restricted to discrete areas of the cytoplasm/cellular junctions (Figure 5A). Quantitative analysis confirmed significantly higher β-catenin staining as indicated by both staining area and intensity in vehicle-treated tumors (Figure 5A). Western blot analysis of tumor tissue from the distal colon using an antibody specific for active nonphosphorylated β-catenin confirmed higher levels in vehicle-treated tumors compared with distal colon from TG-treated mice in a similar location (Figure 5B). These data corroborate that intrarectal TG treatment inhibited activation and nuclear translocation of β-catenin in the AOM/DSS-treated mice.

To gain insight into the consequences of TG-inhibited β-catenin activity in the tumors, we assessed the relationship between β-catenin expression and expression of oncogenic target genes. Higher expression levels of cyclin D1 and C-Jun (drivers of cell cycle G1/S-phase transition and involvement in DNA damage repair), matrix metalloproteinase-7 (believed to have an oncogenic function in colonic tumorigenesis49), CD44 (a marker for cancer stem cells50), and survivin (cell survival marker51,52) were generally observed within the vehicle-treated tumors (Figure 5B) in comparison with TG-treated animals. Taken together with the BrdU measurements, these results suggest that TG inhibits tumor cell proliferation and invasion by actions on β-catenin transcriptional activity and downstream target gene expression.

To determine whether TG also affected β-catenin activation in the setting of autophagy disruption, we further examined β-catenin levels in Atg7iec tumors by immunohistochemistry. As with WT tumors, we found much higher cytoplasmic and particularly strong nuclear β-catenin localization in vehicle-treated tumors compared with TG-treated tumors (Figure 6A). In addition, there appeared to be more cytoplasmic β-catenin staining in TG-treated Atg7iec tumors than in TG-treated WT tumors (Figure 6A), which is consistent with reports that intestinal epithelial cell autophagy normally downregulates cytosol β-catenin level.53 Tumors in Atg7iec mice treated with TG had markedly reduced expression of oncogenic target genes (Figure 6B). When these data are considered along with the similar measurements in WT tumors, they indicate that TG’s inhibition of β-catenin oncogenic transcriptional activity is independent of autophagy.

Extending the relevance of these mechanistic findings to human cancer, we examined the effect of ATG7 silencing on β-catenin transcriptional activity in response to TG treatment in the human colon cancer derived HCT116 and Caco2 cell lines using the luciferase reporter assay. Two independent sets of ATG7 small interfering RNAs (siRNAs) were used to control for potential off-target effects. ATG7 siRNA reduced ATG7 protein expression by ~85% in Caco2 and 95% in HCT116 cell lines (Figure 7A). Similar to our in vivo finding, we noted higher β-catenin transcriptional activity in ATG7 knockdown cells than in control cells under basal conditions (Figure 7B), which is consistent with the previously reported autophagy-mediated degradation of β-catenin43; however, CHIR treatment still resulted in additional activation. Treatment with TG in conjunction with CHIR activation resulted in significantly reduced β-catenin activity in a dose-dependent manner (Figure 7C). This occurred despite knockdown of ATG7 and was consistent with the in vivo findings of TG’s oncoprotective effect being largely independent of autophagy (Figure 5).

**Thioguanine Suppresses β-Catenin Activity via Conversion to Thioguanine Nucleotides**

The cellular metabolism of thiopurines results in the production of many metabolites. To confirm that the action of TG in the cell lines involved TGN (Figure 1),
we examined the effect of hypoxanthine phosphoribosyltransferase gene (HPRT) silencing on β-catenin transcriptional activity in response to TG treatment in HCT116 and Caco2 cells using the luciferase reporter assay. Disruption of HPRT results in an inability for cells to metabolize TG to TGN. Two independent sets of HPRT siRNAs were used to control for potential off-target effects. Each set of HPRT siRNA reduced HPRT protein expression by \(\approx 85\%\) in these cell lines (Figure 8A). Inhibition of β-catenin transcription activity

**Figure 5.** TG inhibited β-catenin activity in CAC tumors in vivo. Measurements in TG or vehicle-treated tumors at day 85 (N ≥ 5 each group). (A) Immunohistochemical (IHC) analysis of β-catenin in WT mice and in Atg7\(^{ΔIEC}\) conditional knockout mice. Left panels: representative IHC staining for β-catenin. Right panels: β-catenin protein levels assessed using IHC and Visiopharm software (where higher intensity has a lower value) and expressed as percentage of β-catenin positive cells within the tumors (upper) and tumor β-catenin intensity (bottom) from each mouse (5 mice per group). (B) Immunoblot analysis of non-phosphorylated β-catenin and its downstream tumor promoting gene products, cyclin D1, C-Jun, CD44, MMP7, survivin in WT mice and (C) in Atg7\(^{ΔIEC}\) mice. Each lane represents distal colon or pooled tumors from distal colon in an individual mouse. Densitometry of proteins corrected for β-actin in bottom right panel. Statistics: box plots show median, quartiles, and range; Mann-Whitney U test. *TG vs vehicle, **P < .01. Data representative of 3 independent experiments.

**Figure 4.** (See previous page). Long-term daily intrarectal TG treatment was not associated with clinically significant systemic toxicity. (A) Sinusoidal obstructive syndrome was not present at day 85. Representative H&E liver histology for vehicle and TG-treated animals. (B) Hematologic parameters (white blood cell counts, hemoglobin, and platelets) in peripheral blood at day 85 from WT and Atg7\(^{ΔIEC}\) mice. (C) Fluorescence-activated cell sorter plots showing cell gating strategy and subsets of CD4\(^+\)CD3e\(^+\) (T lymphocyte) and CD4\(^+\), CD8\(^+\), CD8\(^+\)populations. There was a small but statistically significant increase in CD8: CD4 mesenteric node lymphocytes with TG treatment in both WT and Atg7\(^{ΔIEC}\) mice.
was seen in both TG-treated cells treated with control siRNA, but there was no inhibition of β-catenin with knockdown of HPRT, thus indicating that the effect was mediated by TGN (Figure 8B). To corroborate these findings we examined the effect of TG on fibroblasts harvested from Hprt−/− mice. Fibroblast cell cultures derived from both WT and Hprt−/− mice were incubated for 16 hours with vehicle control or 10 μmol/L TG ± CHIR. As expected, cellular and nuclear β-catenin was significantly reduced in TG-treated WT-derived fibroblasts, but there was no effect in the absence of Hprt (Figure 8C). Immunoblots confirmed decreased

Figure 6. TG inhibited β-catenin activity in Atg7−/IEC conditional knockout mice. Measurements in TG or vehicle-treated tumors at day 85 (N ≥ 5 each group). (A) Immunohistochemical (IHC) analysis of β-catenin in WT mice and in Atg7−/IEC conditional knockout mice. Left panels: representative IHC staining for β-catenin. Right panels: β-catenin protein levels assessed using IHC and Visiopharm software (where higher intensity has a lower value) and expressed as percentage of β-catenin positive cells within the tumors (upper) and tumor β-catenin intensity (bottom) from each mouse (5 mice per group). (B) Immunoblot analysis of non-phosphorylated β-catenin and its downstream tumor promoting gene products, cyclin D1, C-Jun, CD44, MMP7, survivin in WT mice and (C) in Atg7−/IEC mice. Each lane represents distal colon or pooled tumors from distal colon in an individual mouse. Densitometry of proteins corrected for β-actin in bottom right panel. Statistics: box plots show median, quartiles, and range; Mann-Whitney U test. *TG vs vehicle, **P < .01. Data representative of 3 independent experiments.
cytoplasmic and nuclear fractions of β-catenin on TG treatment in WT fibroblasts; the decrease was more marked, not unexpectedly, in the nuclear fractions from the CHIR-stimulated WT fibroblasts. As expected, TG was not associated with decreased cytoplasmic and nuclear fractions of β-catenin from Hprt-/− fibroblasts (Figure 8D), thus confirming that TG’s inhibition of β-catenin activity is mediated by TGN, most likely involving TGTP (Figure 1A).

**Figure 7.** TG inhibits β-catenin activity in vitro independently of autophagy. (A) Knockdown of ATG7 in Caco2 and HCT116 cells 48 hours after transfection with ATG7 (#3) or ATG7 (#4) or control (-) siRNA was assessed by Western blotting. (B) Basal β-catenin–driven transcriptional activity in Caco2 and HCT116 cells transfected with ATG7 or control siRNA for 48 hours was increased with inhibition of the autophagy gene. (N = 4). (C) Treatment of the same cells in presence of 1 μmol/L CHIR-99021 and indicated dose of TG for 16 hours inhibited β-catenin–driven transcriptional activity. Data are representative of 4 independent experiments. RLU, relative light units.

**Thioguanine Suppression of β-Catenin Activity Is Dependent on Rac1 at Lower Concentrations**

TGN interact with Rac1, a Rho family GTPase that binds to guanosine triphosphate (GTP) in its active form, to inhibit proliferation of activated blood lymphocytes.24,25 To determine whether TGN also inhibit β-catenin activity in colonic epithelial cells via Rac1, we examined the effect of Rac1 silencing on β-catenin transcriptional activity in HCT116 and Caco2 cells using the luciferase reporter assay.
in Rac1Q61L active mutant transfected cells. However, higher transcriptional activity was inhibited by TG in an expected intrinsic GTPase activity and thus unresponsive to TG.

Western blotting of cytosol and nuclear compartments of WT and catenin, we studied CHIR only in WT cells.

activity in Rac1-Q61L active mutant transfected cells (Figure 9A). The siRNA silencing of Rac1 induced a decrease in β-catenin transcriptional activity in these cells. At the IC50 dose of TG (2 μmol/L), there was no additional inhibition of β-catenin transcriptional activity in the presence of Rac1 silencing, indicating that at this dose, TG-mediated inhibition is Rac1 dependent. However, a higher concentration of TG (20 μmol/L) did also inhibit β-catenin transcriptional activity (Figure 9B), indicating an additional Rac1 independent effect of TG on β-catenin activity at a supraphysiological higher concentration.

To better define the role of Rac1 mediated activation of β-catenin, we studied β-catenin activity in HCT116 and Caco-2 cells expressing mutant Rac1Q61L, in which Rac1 exists constitutively in the GTP-bound (activated) form. Normally, hydrolysis of bound GTP by Rac1 GTPase to Rac1-guanosine diphosphate (GDP) results in its inactivation (Figure 9C.1). When TGTP binds Rac1 and is hydrolyzed to TGDP, it cannot be exchanged for GTP or TGTP by guanosine exchange factor (Figure 9C.2), leading to a decreased pool of Rac1 that can be activated to Rac1-GTP. In cells expressing mutant Rac1Q61L, we hypothesized that β-catenin activity would be constitutively up-regulated (Figure 9C.3) because of defective intrinsic GTPase activity and thus unresponsive to TG.

In WT cells transfected with vector only, β-catenin transcriptional activity was inhibited by TG in an expected dose-response fashion, but 1–4 μmol/L TG was ineffective in Rac1Q61L active mutant transfected cells. However, higher dose TG (>4 μmol/L) inhibited β-catenin transcriptional activity in Rac1-Q61L active mutant transfected cells (Figure 9D). Taken together with the siRNA results, these data indicate that suppression of β-catenin transcriptional activity via inhibition of activated Rac1 occurs at concentrations of TG that correspond to the IC50, but that higher concentrations of TG inhibit β-catenin transcriptional activity via Rac1-independent mechanisms.44 One possibility is that TGTP may directly inhibit β-catenin. To determine whether there is a potential binding site, molecular docking experiments were performed by using the crystal structure of the armadillo repeat region of human β-catenin.45 In these modeling experiments, TGTP docked with β-catenin protein with a binding free energy ΔG value of -47.11 kcal/mol (Figure 9E). The docking of TGTP ligand in the conserved Rac1 nucleotide binding site is shown in Figure 9E with corresponding ΔG value of -58.53 kcal/mol.

Discussion

In this study, we provide evidence to indicate that TG but not MP acts directly on intestinal epithelial cells in vitro to inhibit β-catenin activity. This effect of TG was also confirmed in ex vivo epithelial cells from N = 1 colon cancer, which was resected from a patient with ulcerative colitis (Figure 1D). The action on β-catenin activity is in addition to TG’s known actions on immune cells. The inability of MP to inhibit β-catenin activity is likely related to its rate-limited conversion to TGN as well as other MP metabolites. We further demonstrate that daily intrarectal TG inhibits colonic tumorigenesis in vivo in a murine CAC model, that the mechanism of action to inhibit CAC is dependent on TG’s conversion to TGN, that the mechanism is largely independent of TG’s action on intestinal inflammation, which requires autophagy induction in intestinal epithelial cells, and that the antitumorigenic activity is via suppression of Rac1-mediated β-catenin nuclear translocation, which results in both reduced proliferation of colonic epithelial cells and transcription of oncogenic genes.

Our finding that TG but not MP effects a rapid inhibition of β-catenin activity advances current concepts about the mechanism of action of TG in the treatment of IBD and prevention of CAC. Unlike azathioprine and MP, the mechanism of rectal TG in abrogating colitis and preventing CAC involves a direct effect on epithelial cells. Conversion to TGN from MP and azathioprine46 for their therapeutic effects is rate-limited by IMPDH and occurs mainly in liver and circulating leukocytes.34 While systemic conversion of thiopurine pro-drugs to TGTP inhibits proliferation and promotes apoptosis of activated immune cells via Rac1 to reduce overall inflammation, our data suggest that the effect of locally administered TG on colonic epithelium relies on rapid local conversion to TGN in the epithelium.

We found that the anti-inflammatory properties of topical TG were dependent on intact autophagy machinery, which is consistent with a TG-mediated up-regulation of autophagy leading to decreased colitis. This is significant because the common ATG16L1T300A polymorphism is associated with decreased autophagy and a higher risk of developing IBD.47 Because there was a >50% reduction of tumor burden in animals with disrupted autophagy machinery in the epithelial cell compartment (Figure 3), the largely autophagy-independent mechanism of action to prevent CAC is entirely consistent with (i) the demonstrated mechanism of action via inhibition of β-catenin, which occurs despite a role for autophagy in the development of CRC48 (ii) reports that disruption of Atg7 in a model of sporadic CRC (APCmin) decreases tumor initiation,32 and (iii) evidence that TG’s anti-inflammatory effect in epithelial cells involves autophagy.36 As in previous studies of tissue-specific ATG7 disruption in intestinal epithelial cells,32 our

Figure 8. (See previous page). TG inhibits β-catenin activity via TGN. (A) Knockdown of HPRT in Caco2 and HCT116 cells 48 hours after transfection with HPRT (#1) or HPRT (#2) or control (-) siRNA, assessed by Western blotting. (B) TG in presence of 1 μmol/L CHIR-99021versus β-catenin-driven transcriptional activity in cells silenced with HPRT siRNA. (C) WT and Hprt-/- derived primary murine fibroblasts treated with 5 μmol/L CHIR ± 10 μmol/L TG or vehicle for 16 hours. Immunofluorescence staining for β-catenin (green) and nuclei (DAPI, blue). Data are representative of 4 independent experiments. (D) Western blotting of cytosol and nuclear compartments of WT and Hprt-/- derived primary murine fibroblasts treated with 5 μmol/L CHIR ± 10 μmol/L TG or vehicle for 16 hours showing TG inhibiting β-catenin activity and nuclear translocation with CHIR only in WT cells.
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Caco2

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**B**

Caco2

![Graph showing RLU vs CHIR+TG µM](image)

HCT116

![Graph showing RLU vs CHIR+TG µM](image)

**C**

1) Normal GTP

GEF

RAC1-GDP → RAC1-GTP

GAP

RAC1-TGDP → RAC1-TGTP

Active Rac1+ CHIR

β-catenin

Transcription of Wnt target gene

2) TGTP

GEF

RAC1-TGDP → RAC1-TGTP

GAP

Accumulation of inactivated Rac1

3) *Rac1(Q61L)

Constitutively activated Rac1

Active Rac1+ CHIR

β-catenin

Transcription of Wnt target gene

**D**

Caco2

![Western blot showing Flag and β-Actin](image)

HCT116

![Western blot showing Flag and β-Actin](image)

**E**

![Molecular structure of β-catenin and Rac1](image)
data (Figure 3) demonstrate a lower rate of tumor initiation and growth in the absence of autophagy.

In addition to the mechanism of prevention of colon tumorigenesis by TG elucidated in the present study, the magnitude of the effect was strikingly large, compared with the magnitude of the cancer chemopreventive effect of azathioprine and MP reported in large epidemiologic studies.\textsuperscript{23,25,48,49} The current paradigm is that their oncoprotective property relates to their anti-inflammatory effect, although not all studies confirm the effect.\textsuperscript{25} Similarly, it has been proposed that targeting of the interleukin 23 pathway should prevent CAC in IBD by treating inflammation.\textsuperscript{28} Also consistent with this paradigm, there are large epidemiologic studies that report a cancer chemoprophylaxis effect with mesalamine in ulcerative colitis,\textsuperscript{51} although this is also contested.\textsuperscript{52}

An alternative possibility for the reported cancer-chemoprophylactic benefit of azathioprine and MP is that their beneficial action is mediated by a similar mechanism of action as the present study demonstrates for intrarectal TG, ie, by RAC1-mediated inhibition of $\beta$-catenin activity in epithelial cells. However, although azathioprine and MP are converted in vivo via IMPDH and HPRT to TG in circulating blood cells, it is difficult to envisage how TG might exchange into intestinal epithelial cells from circulating red and white blood cells because the TGN are intracellular, and TGN do not exist extracellularly.

Constitutive nuclear $\beta$-catenin expression and over-activated Wnt/$\beta$-catenin signalling have been reported in several cancers, including CRC, where they promote growth, invasion, and metastasis in cancer.\textsuperscript{53,54} Although systemic conversion of thiopurine pro-drugs to TGTP inhibits proliferation and promotes apoptosis of activated immune cells via Rac1 to so reduce overall inflammation, we show that the effect of locally administered TG on colonic epithelium requires local conversion to TGN to inhibit Wnt/$\beta$-catenin signalling. Rac1, which binds to TGTP, mediated the action of low-dose TG on $\beta$-catenin activity in colon cancer cell lines, but there were other actions with higher doses of TG in the colon cancer cell lines such as might occur with binding of TGTP to $\beta$-catenin (Figure 9E) or other mechanisms. In summary, the novel findings regarding the potency and pharmacologic mechanism of action of TG in epithelial cells add to our understanding of the biology of colon cancer prevention in the setting of chronic colonic inflammation and suggest that local administration of TG may independently reduce the risk of CAC through a novel mechanism involving RAC1 GTPase and canonical $\beta$-catenin signalling.

**Materials and Methods**

**Cell Culture, Transfection, and Infection**

Human HCT116 and Caco2 intestinal epithelial cells were obtained from the American Type Culture Collection (Rockville, MD) and propagated in Dulbecco modified Eagle medium (DMEM) (Life technologies, Carlsbad, CA) with 10% heat inactivated fetal calf serum, 100 U mL\textsuperscript{-1} penicillin, 100 $\mu$g mL\textsuperscript{-1} streptomycin, and 2 mmol/L L-glutamine. All these cell lines were authenticated by short tandem repeat profiling, were mycoplasma-free, and underwent 5 or fewer passages.

**Cignal Lenti T-Cell Factor/Lymphoid Enhancer Factor Reporter Luciferase Assay**

Cignal Lenti transcriptional response element reporter lentivirus particles, which express a luciferase gene driven by multiple TCF/LEF (AGATCAAGGCGGTGA) repeats and Cignal Lenti Renilla control lentivirus particles, were purchased from QIAGEN (Hilden, Germany) (#CLS-01BL). HCT116 and Caco2 cells were transduced with TCF/LEF promoter along with Renilla luciferase (selected with puromycin). Stable HCT116 and Caco2 pooled clones over-expressing reporter construct were treated with 1 $\mu$mol/L CHIR 99021(Selleckchem, Houston, TX) for 16 hours. Luciferase activity was measured on TiterTek Berthold luminometer by using the Dual Luciferase reporter assay (Promega, Madison, WI) according to the manufacturer’s protocol.

**Immunofluorescence**

For immunofluorescence, cells were seeded on coverslips in 24-well plates and fixed with 4% paraformaldehyde for 20 minutes at ambient temperature. After 3 phosphate-buffered saline with 0.1% Tween-20 (PBS-T) washes, cells were blocked with 5% bovine serum albumin (BSA) in PBS-T washes, cells were blocked with 5% BSA in PBS-T for 20 minutes at ambient temperature. After 3 washes (10 minutes each), the slides were incubated with Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA) 30 minutes at 37°C and then blocked with the Image-It FX Signal Enhancer Ready Probes Reagent (Life Technologies, Carlsbad, CA) 30 minutes at 37°C. Cells were incubated with the anti-$\beta$-catenin (8480, clone D10A8; Cell Signaling Technology, Danvers, MA) in PBS-T with 1% BSA overnight at 4°C. After 3 washes (10 minutes each), the slides were incubated with Alexa Fluor 594 goat anti-rabbit (Molecular Probes, Eugene, OR) prepared in PBS-T with 2% BSA. The slides were counterstained with DAPI (Molecular Probes) and imaged by using the confocal laser-scanning microscope Olympus FV1200 (Tokyo, Japan) microscopy.

Figure 9. (See previous page). TG at low concentrations suppressed $\beta$-catenin activation via inhibition of Rac1. (A) Knockdown of Rac1 in Caco2 and HCT116 cells 48 hours after transfection with Rac1 siRNA #6 or Rac1 siRNA #7 versus control (+) siRNA, assessed by Western blotting. (B) [TG] versus $\beta$-catenin–driven transcriptional activity in control cells versus Rac1-siRNA silenced cells. (C) Cartoon showing role of Rac1 in $\beta$-catenin–driven transcription of Wnt target gene: C1, normal physiology with GTP; C2, TG with WT Rac1; C3, TG with genetically inactivated GTPase Q61L Rac1. (D) Left panel: Western blotting for Flag in HCT116 and Caco2 cells confirms overexpression of Rac1 WT and Rac1 mutant in these cells. Right panels: [TG] versus $\beta$-catenin–driven transcriptional activity in WT and Q61L (ie, constitutively active Rac1). Caco2 and HCT116 cells. Data are representative of 3 independent experiments. (E) Modelling experiments showing TGTP docked with $\beta$-catenin protein crystal structure (left panel) and docked in the conserved Rac1 nucleotide binding site (right panel). RLU, relative light units.
Spheroids

Ulcerative colitis–associated CRC specimen used in this study was collected with written informed consent from one patient undergoing surgical resection under the approval of the Mater Research Ethics Committee. Tumor tissue freshly resected from the patient was dissociated into single cells and grown as a suspension culture in a serum-free medium that consisted of Advanced DMEM/F12 medium (Thermo Scientific, Mulgrave, Australia), 0.4% BSA (Sigma, Castle Hill, Australia), 5 mmol/L HEPES (Thermo Scientific), 1× GlutaMax (Thermo Scientific), 4 μg/mL heparin (Sigma), 0.3% D-glucose (Sigma), 1% B27 supplement without vitamin A (Thermo Scientific), 1% N2 supplement (Thermo Scientific), 10 μg/mL insulin (Sigma), 1 mmol/L N-acetyl-L-cysteine (Sigma), 10 mmol/L nicotinamide (Sigma), 20 ng/mL EGF (Thermo Scientific), and 20 ng/mL FGF (R&D Systems, Minneapolis, MN) for 14 days in low-adsorption plates (Corning, Tewksbury, MA) at a density of 1 × 10^6 cells per mL. These conditions select for cells with the capacity to grow as spheroids that display stemness properties including the ability to reinstate tumor growth in vivo. The established spheroids were treated with 10 μmol/L TG or vehicle control for 4 days, and then spheroids were harvested by centrifugation at 30g for 4 minutes, washed twice with PBS, and fixed in 4% paraformaldehyde in PBS for 20 minutes. Fixed spheroids were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes, washed 3 times with PBS, and then residual aldehyde quenched by addition of ammonium chloride (50 mmol/L) for 10 minutes. Nonspecific binding sites on PBS washed spheres were blocked with 0.2% albumin in PBS for 30 minutes. Spheres were incubated overnight in blocking buffer containing rabbit anti-active β-catenin antibody D13A1 (1:100; Cell Signaling #8814) and mouse anti-β-catenin (1:100; BD #610154; San Jose, CA) antibodies. After 3 washes with PBS, spheres were incubated for 30 minutes in blocking buffer containing 46-diamidino-2-phenylindole (DAPI) and fluorescently tagged secondary antibodies. Fluorescent signals from spheres were acquired by using an Olympus FV1200 microscope confocal microscope.

Immunoblot Analysis

Protein concentrations were measured by using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) with BSA as a standard. Aliquots of the lysate containing 30 μg total protein were mixed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis Laemmli buffer (0.05 mol/L Tris-HCl, pH 6.8, 0.1% 2-mercaptoethanol, 1.0% sodium dodecyl sulfate, 5% glycerol, and 0.15% bromophenol blue), boiled for 5 minutes, and resolved on 4–12% acrylamide gels. Resolved proteins were transferred to polyvinylidene difluoride membranes for Western blotting, probed with appropriate antibodies at the manufacturer recommended dilutions, and detected by dual-label infrared analysis, imaged on Odyssey XL scanning system (LI-COR BIOSCIENCES, Lincoln, NE), and analysis on Image Studio software version 5.2 (LI-COR BIOSCIENCES).

Atg7-floxed C57Bl/6 Mice

Atg7 is an autophagy gene whose expression is essential for the biogenesis of autophagosomes; homozygous loss of Atg7 is lethal at birth. Therefore, for these experiments we used conditional targeting of the Atg7 locus (Atg7flox/flox) bred with villin-Cre-expressing mice to enable analysis of epithelial cell specific functions of Atg7flox/flox (Atg7flox/flox x villin-Cre) mice and its littermates, henceforth referred to as Atg7flox/flox and Atg7flox/+ mice, respectively.

Tumor Induction

Colon tumors were induced by AOM and DSS model. Briefly, 4- to 5-week-old WT or Atg7flox/flox C57Bl/6 mice were injected intraperitoneally with 10 mg AOM (A5486; Sigma-Aldrich, St Louis, MO) per kg body weight. Seven days later, 1.5% DSS (molecular mass 36–40 kDa) was given in the drinking water for 5 days, followed by regular drinking water for 2 weeks, and the DSS cycle was repeated twice. TG-treated and vehicle-treated mice were housed separately. Symptoms of colitis were assessed by the DAI. The DAI is a score based on sum of subscores for diarrhea, rectal bleeding, and loss of body weight. Mice were killed on day 85, and the person dissecting the colons was blinded to the treatment. Colon tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. The number of proliferating cells in the intestinal epithelium was detected by BrdU as described below and previously.

β-Catenin Immunohistochemistry Staining and Histology Image Analysis

Sections from paraffin-embedded tissues or tumors were deparaffinized, rehydrated, and antigen-retrieved by heating for 20 minutes in 10 mmol/L citric acid (pH 6), cooled to room temperature, and then treated with 3% hydrogen peroxide in PBS for 10 minutes. Sections were then stained with the anti-β-catenin antibody (#8480, clone D10A8; Cell Signaling Technology) at 1:200 dilution and detected with anti-rabbit antibody-horseradish peroxidase (BD Pharmingen) and DAB. Histology sections stained with β-catenin were analyzed by using Visiopharm software version 2017.2 (Hoersholm, Denmark). The software allowed for quantification of specific tissue areas and measurement of percentage of positive cells and intensity (0–255) of the staining in these areas.

Growth Curves Assay

Cells (7500 cells/well) were plated in a 96-well plate and visualized by using a real-time cell imaging VL21 system (Phase Focus Limited, Sheffield, UK), which uses a method known as ptychography in image formation. Growth curves were built from confluence measurements acquired during round-the-clock kinetic imaging.

Proliferation Assays

To assess proliferation in vivo, mice were given 100 μg/g body weight of BrdU intraperitoneally 1.5 hours before euthanasia for tissue or tumor sampling. Paraffin sections
were processed, and antigen was retrieved as detailed above. Sections were then stained with the biotinylated anti-BrdU antibody (#ab6326, clone ICR1; Abcam, Cambridge, UK) at 1/100 dilution and detected with streptavidin-horseradish peroxidase (BD Pharmingen) and DAB.

Reagents and Antibodies

All reagents and chemicals were purchased from Sigma-Aldrich unless otherwise stated. AOM (A5486; Sigma-Aldrich, DSS; molecular mass 40–50 kDa) was purchased from Affymetrix (Santa Clara, CA). Complete protease inhibitor cocktail and phosphate stop were purchased from Roche (Basel, Switzerland). CHIR 990211(CHIR) was purchased from Selleckchem (Houston, TX).

The antibodies used in this study were anti-β-catenin (#8480, clone D10A8; Cell Signaling Technology), anti-Non-Phospho-β-catenin (#19807, clone D208Y; Cell Signaling Technology), anti-Cyclin D1 (#2978, clone 92G2; Cell Signaling Technology), anti-C-Jun (#9165, clone 60A8; Cell Signaling Technology), anti-CD44 (#3570; Cell Signaling Technology), anti-MMP7 (#3801, clone D4H5; Cell Signaling Technology), anti-Survivin (#2808, clone 71G4B7; Cell Signaling Technology), anti-ATG7 (#MAB6608, clone 683906; R&D Systems), anti-β-actin (#MAB1501; Novus Biologicals, Littleton, CO), anti-HPRT (#ab16048; Abcam), anti-Rac1 (#05-389, clone 23A8; Millipore, Burlington, MA), and anti-lamin B (#ab16048; Abcam).

Disease Activity Index Scores

The DAI scores are defined as the sum of subscores for diarrhea, rectal bleeding, and loss of body weight. Diarrhea scores ranged from 0 to 3, with 0 being none and 3 being frank diarrhea; rectal bleeding ranged from 0 to 3, with 0 being no bleeding and 3 being frank rectal bleeding. Body weight percent loss subscores were 0 = <5% loss; 1 = 5%–15% loss; 2 = 15%–20% loss; and 3 >20% loss.

Small Interfering RNAs

Small interfering RNAs targeting ATG7 (FlexiTube GeneSolution GS10533 for ATG7, catalog #1027416), HPRT (FlexiTube GeneSolution GS3251 for HPRT1, catalog #1027416), and RAC1 (FlexiTube GeneSolution GS5879 for RAC1, catalog #1027416), and non-targeted control siRNA (Negative Control siRNA negative (non-silencing) control siRNA, catalog #SI03650325) were purchased from QIAGEN. Oligonucleotide transfection was performed with Lipofectamine 2000 Reagent (Life Technologies) at a final concentration of 50 nmol/L, and cells were harvested for the assays 48 hours after transfection.

Generation of Stable Rac1 Wild-type and Mutant Rac1Q61L Cell Lines

WT or activated mutant Rac1Q61L flag-tagged constructs in pcDNA3.1 were from GenScript (Piscataway, NJ). DNA fragments encoding RAC1 WT or activated mutant Rac1Q61L flag-tagged were then cloned into a Lentiviral construct pLenti CMV Hygro DEST vector (Addgene, Cambridge, MA) using Gateway LR recombination cloning technology (Life Technologies) (Figure 9D). For stable cell lines, 5 × 10^6 amphototropic (HEK293) packaging cells were transfected with 10 μg DNA of the appropriate construct by Lipofectamine 2000 Reagent according to the manufacturer’s protocol. Culture supernatants were collected 36–48 hours after transfection and filtered through 0.45 μm filter. HCT116 and Caco2 cells were infected with the filtered viral supernatants in the presence of 4 μg/mL polybrene (Sigma-Aldrich) for 12 hours, after which the medium was changed. Fresh viral suspensions were added after a 24-hour interval for an additional 12 hours. Infected cells were selected for 5 days in 200 μg mL^-1 hygromycin.

Measurement of Thioguanine Nucleotides

HCT116 or Caco2 colon cells (25,000 cells/well) were cultured for 16 hours in 96-well plates with TG or MP or vehicle control substrate. TGN were measured in the washed colon cells by the Queensland Pathology Service’s laboratory using an automated dervieux and Boulieu method with liquid chromatography–mass spectrometry detection. The limit of quantification for measuring TGN in our cultures without an additional concentration step was 0.5 μmol/L.

Instrumentation and reagents.

An Acquity UPLC system with a binary solvent manager, sample manager, QDa single quadrupole mass detector, and Empower 3 Chromatography data manager were used. An Atlantis T3 C18 column (3.0 μm particles; 2.1 x 100 mm) was used to optimize the chromatographic conditions. The equipment and reagents mentioned above were sourced from Waters Corporation (Milford, MA). Formic acid (Sigma-Aldrich) and acetonitrile (Thermo Fisher) were MS-grade.

Sample preparation.

We mixed 200 μL sample, 100 μL Internal Standards (6TG-13C2 15N), 100 μL dithiothreitol, and 100 μL 35% perchloric acid in 1-mL-deep 96-well plate on Biosoan Laboratories (Warren, MI) 96-well heater/mixer at 100°C for 60 minutes. The 96-well plate was centrifuged and placed into the sample manager.

Optimized chromatography and mass spectrometry conditions.

The column temperature was 45°C, flow rate of 0.4 mL/min, with the initial conditions of 99% mobile phase A (0.1% formic acid in water) and 1% mobile phase B (0.1% formic acid in acetonitrile). After 1 μL sample is injected, a linear gradient (curve 6) is applied to 97% A at 1 minute, followed by 80% A at 2 minutes, and 1% A at 2.1 minutes before returning to initial conditions at 2.50 minutes. The MS detector was programmed to collect single ion reactions of selected atomic masses in positive ionization mode during the expected time interval (6TG, 167.93 Da and 6TG IS, 170.92 Da). The probe temperature was 600°C, the capillary voltage was 0.8 kV, cone voltage was 5V, and the sampling rate was 5 points/second.

Cell Viability WST-1 Colorimetric Assay

WST-1 (4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate; cat. no. 11644807001; Roche Applied Science) colorimetric assay was carried out...
to determine the effects of 16 hours of TG treatment on cell viability. The stable tetrazolium salt WST-1 is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bioreduction is largely dependent on the glycolytic production of reduced nicotinamide adenine dinucleotide phosphate in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. The assay was performed in 96-well plates, with seeding of ~25,000 cells in each well. After 16 hours of TG treatment, 20 µL of the WST-1 solution was added to the culture medium and incubated for 2.5 hours at 37 °C. Absorbance was subsequently determined by using a microplate enzyme-linked immunosorbent assay reader (VERSAmax; Molecular Devices, Sunnyvale, CA) and analyzed with SoftMax Pro 5 Software (Molecular Devices) by applying the wavelengths 450 nm for measurements and 650 nm as reference. All experiments were conducted in 4 wells for each condition and replicated at least 3 times.

**Molecular Docking**

Crystal structures of β-catenin (1G3J.pdb) and Rac1 (1MIH.pdb) were retrieved from the RSCB Protein Data Bank and prepared for modelling by using the Protein Preparation Wizard in Schrödinger Release 2019-2 software (New York, NY). The crystal structures were assigned bond orders, hydrogens were added, and protonation states of ionizable groups were assigned at pH = 7.4 ± 1.0 with Epik. The OPLS3e force field was used to minimize protein hydrogen atoms with restraints on heavy atoms. In preparation for docking experiments all solvent molecules were removed from the binding sites. Ligand structures were retrieved from the PubChem database and prepared for docking by using LigPrep. LigPrep assigned bond orders, stereochemistry, added hydrogens, and set protonation states of ionizable groups at pH = 7.4 ± 1.0 using Epik. Final ligand structures were minimized with the OPLS3e force field ready for docking.

Docking experiments were performed by using the Schrödinger Suite Glide module. A Glide docking grid of β-catenin was prepared by using default parameters and centered on Arg469. Similarly, a Glide docking grid was prepared for Rac1 by using default parameters centered on the co-crystallized ligand guanosine-5’-(beta gamma-imino) triphosphate (GMPPNP). The docking grid for Rac1 also included interaction constraints to the Mg²⁺ ion and a core atom constraint applied to the 6-membered ring of the purine group. Glide standard precision docking with default parameters was used to dock TMGP into the binding site of β-catenin with the top 10 docked conformers kept. Similarly, Glide standard precision was used to dock ligands TMGP and GMPPNP into the binding site of Rac1. Docked conformations were constrained with a core constraint to the 6-membered ring of the purine group, and the ligand had to interact with the Mg²⁺ ion. The top 3 docked poses of TGTP in β-catenin were submitted to a Prime MMGBSA calculation to determine a calculated ΔG of binding. Prime MMGBSA calculations included the docked ligand and sidechain atoms of residues within 6 Å of the ligand. Prime used the OPLS3e force field with the updated solvent model VSGB2.141 to perform all optimizations and calculations. Similarly, crystal pose of GMPPNP and the top Glide docked poses of TGTP and GMPPNP were submitted to a Prime-MMGBSA calculation to determine their calculated Prime-MMGBSA ΔG of binding.

**Ethics**

All experiments were approved by the University of Queensland Animal Ethics Committee.

All authors had access to the study data and reviewed and approved the final manuscript.

**Statistical Analysis**

All statistical analyses were performed by using Prism v5 (Graphpad Software, San Diego, CA). Sample sizes for experiments were determined by power analyses based on the variation shown in previous experiments and predicted effect sizes considered to be of biological relevance. No data were excluded from any analyses. The normal distribution of data was assessed by probability plots, and where a normal distribution could not be established, nonparametric testing was used. The statistical test used and the sample sizes for individual analyses are provided within the figure legends.

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Received March 25, 2020. Accepted May 27, 2020.

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Acknowledgments
The authors thank Prof Andrew Clouston (Envoi Pathology & Berghofer Queensland Institute of Medical Research) for reporting on liver sections, Prof Allison Pettit for providing the IHC reagents, and Dr Kuan Yan Wong, Dr Ran Wang, and Ms Rhea Menon for help with experiments. The authors also recognize the technical assistance of the TRI core facilities for histology, flow cytometry, and microscopy.

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Rabina Giri (Investigation: Equal; Methodology: Supporting; Visualization: Supporting)
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Michael McGuckin (Funding acquisition: Supporting; Supervision: Equal; Validation: Supporting)
Timothy H. J. Florin (Conceptualization: Equal; Project administration: Equal; Writing – original draft: Equal; Writing – review & editing: Lead)
Jakob Begun (Conceptualization: Lead; Funding acquisition: Lead; Project administration: Equal; Writing – original draft: Supporting; Writing – review & editing: Equal)

Conflicts of interest
The authors disclose no conflicts.

Funding
The Translational Research Institute (TRI) is supported by a grant from the Australian Government. Mater Research Institute is supported by Mater Foundation. JB has a UQ Reginald Ferguson Fellowship. AJL is supported by an ARC grant and DPF by the NHMRC.
Minerva Access is the Institutional Repository of The University of Melbourne

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Title:
A Nucleotide Analog Prevents Colitis-Associated Cancer via Beta-Catenin Independently of Inflammation and Autophagy

Date:
2021

Citation:

Persistent Link:
http://hdl.handle.net/11343/251487

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