Wnt/β-catenin pathway regulates MGMT gene expression in cancer and inhibition of Wnt signalling prevents chemoresistance

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Wnt/β-catenin pathway regulates MGMT gene expression in cancer and inhibition of Wnt signalling prevents chemoresistance

Malin Wickström1,*, Cecilia Dyberg1,*, Jelena Milosevic1,*, Christer Einvik2, Raul Calero1, Baldur Sveinbjörnsson1,3, Emma Sandén4, Anna Darabi4, Peter Siesjö4, Marcel Kool5, Per Kogner1, Ninib Baryawno6,7,8,** & John Inge Johnsen1,**

The DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) is commonly overexpressed in cancers and is implicated in the development of chemoresistance. The use of drugs inhibiting MGMT has been hindered by their haematologic toxicity and inefficiency. As a different strategy to inhibit MGMT we investigated cellular regulators of MGMT expression in multiple cancers. Here we show a significant correlation between Wnt signalling and MGMT expression in cancers with different origin and confirm the findings by bioinformatic analysis and immunofluorescence. We demonstrate Wnt-dependent MGMT gene expression and cellular co-localization between active β-catenin and MGMT. Pharmacological or genetic inhibition of Wnt activity downregulates MGMT expression and restores chemosensitivity of DNA-alkylating drugs in mouse models. These findings have potential therapeutic implications for chemoresistant cancers, especially of brain tumours where the use of temozolomide is frequently used in treatment.
One of the major hurdles in cancer treatment is development of resistance against chemotherapeutic drugs. MGMT efficiently removes alkylating lesions at the O6 position of guanine and treatment failure caused by the ability of MGMT to repair DNA damage induced by DNA alkylators or chloroethylenating agents is frequently observed. Specifically, temozolomide, widely used in treatment of malignant brain tumours, has low effectiveness in tumours with elevated MGMT activity. Unfortunately, systemic clinical use of MGMT inhibitors has been restricted mainly because of an increase in haematologic toxicity to DNA alkylators, and failure in restoring temozolomide sensitivity to temozolomide-resistant glioblastoma multiforme.

MGMT is an evolutionary conserved and ubiquitously expressed enzyme that is regulated by multiple mechanisms including epigenetic silencing of the MGMT gene by promoter methylation, frequently observed in gliomas and colon cancer. Also histone modifications and aberrant expression of transcriptional activators and repressors, as well as microRNAs binding to the 3'-untranslated region of the MGMT gene contribute to the differential expression levels of MGMT in various tumours and normal tissues. Given that expression of MGMT is regulated by multiple molecular mechanisms we searched for cellular regulators of MGMT that can be specifically targeted to lower the levels of MGMT in tumour cells and re-sensitize these tumours to chemotherapeutic drugs. We show that activation of the canonical Wnt/β-catenin signalling cascade induce MGMT expression, and that inhibition of Wnt signalling augment the effects of alkylating drugs and restore chemosensitivity in different cancers.

Results

Wnt/β-catenin activation correlates with MGMT expression.

To search for cellular regulators of MGMT as an alternative approach to inhibit expression of MGMT in tumour cells we used gene ontology analysis to test for aberrantly expressed genes or signal transduction cascades in cancers with elevated expression of MGMT. Analysis of expression cohorts of tumours with neural origin showed that high MGMT expression levels correlate with poor survival in adult gliomas and childhood neuroblastoma, whereas in medulloblastoma high levels of MGMT was significantly correlated to the Wnt molecular subgroup with high frequency of mutations in Wnt signalling key molecules (Fig. 1a,b, Supplementary Fig. 1). Moreover, in colon cancer, where aberrant Wnt signalling is common, high expression of MGMT correlated with poor prognosis (Fig. 1a, Supplementary Fig. 1). Pathway-specific gene-expression profiling to search for regulators of MGMT expression showed gene-expression signatures that associated with Wnt signalling in colon cancer, neuroblastoma, glioma, as well as for the Wnt-driven medulloblastoma subgroup (Supplementary Fig. 2). K-means clustering of Wnt gene-expression profiles identified subgroups expressing significantly higher levels of MGMT (Supplementary Figs 2a–d and 3a–d). Further, immunofluorescence analysis on human tumour tissues showed co-localization of nuclear β-catenin and MGMT in subtypes of colon cancer, glioma, medulloblastoma and neuroblastoma (Fig. 1c). Co-localization of β-catenin and MGMT was also observed in HT-29 adenocarcinoma xenografts and in lower crypt cells of normal colon (Supplementary Fig. 4). We also detected a correlation between β-catenin as shown by western blots against the active form of β-catenin dephosphorylated on Ser37 or Thr41 and the downstream effector Axin 2 and MGMT expression in the majority of cancer cell lines derived from these cancers (Fig. 1d,e, Supplementary Fig. 7a).

Wnt/β-catenin regulates MGMT expression.

To investigate if Wnt signalling is involved in the regulation of MGMT expression, we genetically blocked the activity of Wnt signalling using shRNA that render the Wnt signalling activity within cancer cells. For this purpose we used the LS174T colon carcino ma cell line which is stably transfected with an inducible β-catenin shRNA and downregulates β-catenin expression following addition of doxycycline. Knockdown of β-catenin in LS174T cells inhibited MGMT expression (Fig. 2a–c, Supplementary Fig. 7b).

To further investigate the regulatory influences of β-catenin on MGMT transcription we analysed the 5′-flanking region of the human MGMT gene for putative Tcf/Lef transcription factor-binding sites and detected eight putative binding sites within the MGMT promoter/enhancer (Fig. 2d). Transfection experiments using the MGMT-5′ regions containing different numbers of Tcf/Lef-binding sites cloned into luciferase reporter plasmids (Fig. 2d) showed an enhancement of luciferase activity with increasing numbers of Tcf/Lef-binding sites upon activation of β-catenin by inhibition of GSK-3β using LiCl10 or by over-expression of β-catenin (Fig. 2e). Activation of Wnt signalling with prostaglandin E2 (PGE2) (ref. 11) induced an increase in luciferase activity, while inhibition of PGE2 production with the cyclooxygenase-2 (Cox-2) inhibitor celecoxib showed a concentration-dependent reduction (Fig. 2e). Similarly, over-expression (Fig. 2f) or targeted knockdown (Fig. 2f) of β-catenin lead to an increase or decrease, respectively, of the MGMT promoter in SK-N-AS neuroblastoma, DAOY medulloblastoma, SW480 and LS174T colon carcinoma cells (Fig. 2f). These results further validate that the canonical Wnt signalling cascade directly regulates the expression of MGMT.

Wnt inhibition augments temozolomide-mediated chemotherapy.

Next, we tested a panel of Wnt signalling inhibitors in combination with the DNA-alkylating drug temozolomide on colon carcinoma, medulloblastoma, neuroblastoma and glioma cell survival. The agents tested were the non-specific Wnt signalling inhibitor celecoxib, the Porcupine inhibitors Wnt-C59 and LGK974, the tankyrase/Axin 1 inhibitors XAV-939 and G007-LK (refs 14,15) and salinomycin, which inhibits signalling by blocking phosphorylation of the Wnt co-receptor lipoprotein receptor-related protein 6 (ref. 16). Except for G007-LK and XAV-939, all Wnt inhibitors augmented the cytotoxic effects of temozolomide in the majority of the tested cancer cell lines (Fig. 3a). Celecoxib was the most profound compound inducing either an additive or synergistic effect on cell cytotoxicity in combination with temozolomide in all investigated cell lines. Celecoxib was therefore selected for further analysis.

We next compared the effect of celecoxib to restore temozolomide sensitivity in cells expressing high levels of MGMT with the MGMT-specific inhibitor O6-Benzylguanine (O6-BG). O6-BG and celecoxib induced similar cytotoxic effects on the cells in combination with temozolomide and no additional cytotoxic effects of temozolomide were observed when O6-BG was used in combination with celecoxib and temozolomide (Fig. 3b). Moreover, celecoxib downregulated both the expression of endogenous β-catenin and MGMT (Fig. 3c, Supplementary Fig. 7c), as well as transcriptional activation induced by β-catenin (Fig. 3d), whereas forced MGMT overexpression by transfection of MGMT cDNA abrogated the cytotoxic effects seen with celecoxib and temozolomide (Fig. 3e). To further test whether celecoxib can restore temozolomide sensitivity to temozolomide-resistant cancer cells in vitro we first treated cells expressing either low or high levels of MGMT with increasing concentrations of temozolomide and measured the tumourigenic capacity using clonogenic assays. MGMT expression levels corresponded with treatment efficiency...
(IC<sub>50</sub> > 100 µM for DAOY, UW228-3, SK-N-AS and SW480; IC<sub>50</sub> < 50 µM for the MGMT-negative cell lines PFSK-1, Figs 3f and 1d). However, when celecoxib was used in combination with temozolomide we observed a significant restoration of temozolomide chemosensitivity in all MGMT-positive cell lines (Fig. 3f). Similar, but less efficient inhibitory effects were observed using the Porcupine inhibitors Wnt-C59 and LGK974 or the tankyrase/Axin 1 inhibitor G007-LK (Supplementary Fig. 6). To investigate the mechanism of the demonstrated cytotoxic effect we used the LS174T colorectal carcinoma cell line with Tet-inducible shRNA against β-catenin<sup>9</sup>. A significant increased suppression of cell growth was observed in LS174T cells treated with temozolomide where β-catenin was depleted compared with control cells. This synergistic effect could be reversed when adding MGMT cDNA to the β-catenin-depleted cells (Fig. 4a). Similarly, shRNA inhibition of β-catenin significantly restored temozolomide cytotoxicity in SW480 cells (Fig. 4b). Moreover, a significant inhibition of clonogenic capacity was observed in β-catenin knockdown cells treated with temozolomide compared with temozolomide-treated wild-type cells (Fig. 4c). This was accompanied by an increase of apoptosis in β-catenin knockdown cells treated with temozolomide compared with LS174T treated with temozolomide only (Fig. 4d).

**Inhibition of Wnt restores temozolomide sensitivity in vivo.** Celecoxib was selected for further analysis based on its ability to inhibit Wnt signalling<sup>17</sup>, downregulate MGMT expression (Figs 2e and 3c,d) and induce synergistic toxicity on tumour cells both in combination with temozolomide and several other cytotoxic drugs used as a first-line treatment of different cancers (Fig. 3a and Supplementary Table 1). Celecoxib and other non-steroidal anti-inflammatory drugs, approved by the FDA and EMEA, have proven anti-tumourigenic effects in preclinical models and reduce the incidence and severity of various human cancers<sup>10,12,13,17–20</sup>. We therefore next used pharmacological and genetic inhibition to investigate the effect of β-catenin blockade on tumour cell sensitivity to temozolomide in vivo. For this purpose we used two cell lines expressing either low (D283 MED)<sup>21</sup> or high levels of MGMT (LS174T; Fig. 1d). Administration of temozolomide or celecoxib alone to nude mice carrying established D283 MED medulloblastoma xenografts resulted in significant tumour growth inhibition after 12 days of treatment and the tumour volume index (TVI) was reduced by 40 and 20%, respectively, when compared with untreated controls, at the end of treatment (Fig. 5a). In combination, celecoxib and temozolomide reduced tumour growth by 57% (Fig. 5a). The expression of MGMT was repressed in celecoxib-treated xenografts as compared with control xenograft tumours (Fig. 5b, Supplementary Fig. 7d). We also tested the effect of β-catenin knockdown using LS174T cells with Tet-inducible shRNA against β-catenin. No differences in tumour growth were observed in mice with wild-type β-catenin levels, in mice receiving doxycycline to induce β-catenin depletion, or mice treated with temozolomide only (TVI reduction with 16 and 9%, respectively; Fig. 5c). However, a significant inhibition of tumour growth was observed in mice with β-catenin-depleted tumours treated with temozolomide (TVI was reduced with 60%; Fig. 5c). Expression of MGMT was completely blocked in β-catenin-depleted xenografts as compared with control xenograft tumours (Fig. 5d, Supplementary Fig. 7e). Together these results suggest that targeting β-catenin restores temozolomide chemosensitivity in cancer cells expressing MGMT.

**Discussion**

In this study we provide an alternative strategy for targeting chemoresistant cancers by modulating MGMT protein expression through canonical Wnt cascade inhibition. Given the importance of Wnt signalling during embryonic development and the high fidelity of accurate DNA replication in stem cells<sup>22</sup> it is equitable that Wnt signalling may regulate the activity of DNA repair enzymes. MGMT is a suicide enzyme that removes O<sup>6</sup>-guanine alkylation adducts caused by alkylation agents in a one-step reaction that restores the guanine residue to its unchanged state but renders MGMT inactive<sup>23</sup>. Hence, the expression level of MGMT is therefore fundamental for accurate DNA repair.

Although it remains to be evaluated, the indirect inhibition of MGMT via suppression of Wnt activity will most likely avoid some of the haematological toxicities observed by systemic administration of small-molecule inhibitors of MGMT. The temozolomide dosages (7.5 and 12.5 mg/kg<sup>−1</sup>) used in the xenograft studies are equivalent to a human dose of 90 and 150 mg m<sup>−2</sup>, respectively<sup>24</sup>. For the treatment of brain tumours in adults an initiation regimen of 150 mg m<sup>−2</sup> temozolomide followed by a maintenance doses of 200 mg m<sup>−2</sup> once daily is recommended. However, in humans approximately 20% of temozolomide detected in the blood plasma (area under the concentration-time curve (AUC) 30.1 s.e.m. 6.1 mg/l-h in humans receiving 200 mg m<sup>−2</sup> as compared with AUC 6.1 s.e.m. 1.2 mg/l-h in cerebrospinal fluid, derived from 35 patients with a total of 227 plasma and 47 CSF samples was analysed) is present in the cerebrospinal fluid<sup>25</sup>. Although we did not perform...
pharmacokinetics for temozolomide in our experiments the systemic AUC as interpolated from others\textsuperscript{6,26} suggest that the systemic AUC for temozolomide in mice receiving 12.5 or 7.5 mg kg\textsuperscript{-1} to be approximately 7.6 and 4.5 mg/l-h, respectively. This indicates that the selected temozolomide doses used for the xenografts experiments correspond to the obtainable AUC observed in the cerebrospinal fluids. In conclusion, our data supports the use of inhibitors of Wnt signalling and temozolomide in combination as a treatment option for cancer patients expressing high levels of MGMT in their tumour.
Methods

Chemicals. Cerebrox (Fischer, Tauny, Sweden), dimethyl-PEG2 (dmPEG2), cyclophosphamide (given as the active metabolite 4-hydroxy-cyclophosphamide), temozolomide, doxycycline, XAV-939, salinomycin (all from Sigma-Aldrich, Solna, Sweden), O6-BG, Wnt-C59 and LGK974 (Cayman Chemical, Ann Arbor, MI, USA) were dissolved in dimethyl sulfoxide (Sigma-Aldrich). Cisplatin, irinotecan, doxorubicin and vincristine were purchased from the local pharmacy (Apoetek AB, Sweden) and diluted according to manufacturers instructions. G007-LK was a kind gift from Dr Krassus, University of Oslo, Norway.14,27 The mTOR inhibitors rapamycin (Sirolimus, LC Laboratories, Woodburn, MA, USA) and CC-177 (Temsirolimus, a kind gift from Wyeth Pew River, NY, USA) were dissolved in 99.5% ethanol. LiCl was dissolved in H2O. All inhibitors/activators were further diluted in OptiMEM (Gibco BRL, Symbury, Sweden) to the desired in vitro concentration. Temozolomide for the in vivo studies was supplied by the local pharmacy (Apoetek AB). For in vivo use of cerebrox and temozolomide in the D283 xenograft study, the stock was prepared as a suspension in a vehicle fluid consisting of 0.5% methylcellulose (w/v; Sigma-Aldrich) and 0.1% Tween 80 (v/v; Sigma-Aldrich) in sterile water. For the LS174T xenograft study, temozolomide was dissolved in NaCl and doxycycline in water.

Cell lines. Cell lines used were kindly provided by Dr T. Pietsch (University of Bonn Medical Center, Bonn, Germany), Dr C. Redfern (Northern Institute for Cancer Research, Newcastle University, Newcastle, UK), Dr M. Nister, Dr Soderberg-Naucler and Dr M. Farnebo (Karolinska Institutet, Stockholm, Sweden) and Dr H. Clevens (Hubrecht Institute and University Medical Center Utrecht, Utrecht, Holland), except from DAOY (D324 MED), D283 MED, HT-29, PKS1-K, SHG-44 and SK-N-AS that were purchased from ATCC. The cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (MEM; MEB-MED-8A, U373 MG, U251 MG ACII, U87 MG, U343, U131, H3T-11, RKO, SK-N-AS), minimum essential medium (MEM; D324 MED, D283 MED, HT-29, PKS1-K, SHG-44 and SK-N-AS), DMEM-F12 (U228-3). Medium was supplemented with 10% heat-inactivated foetal bovine serum, 2 mM Glutamine and 1% penicillin G, and 100 U/mL streptomycin (Life Technologies, Stockholm, Sweden) at 37 °C in a humidified 5% CO2 atmosphere. All media were purchased from Gibco BRL. LS174T was grown in indicated concentrations for at least 3 days before experimental use with silenced β-catenin. All in vitro studies were carried out in OptiMEM supplemented with antibiotics and L-glutamine.

Gene-expression profiling and TCF/LEF-binding sites. Gene-expression profiling on globostoblastoma, colon carcinoma, medulloblastoma and neuroblastoma were performed as described previously.18,29 Data analyses (K-means clustering, Kaplan-Meier survival curves and MGMT promoter expression analyses) on publicly available gene-expression data sets were performed using the R2 microarray analysis and visualization platform (http://r2.amc.nl). TCF/LEF-binding sites within the 5′-flanking promoter region of the MGMT gene were searched for with BLAST (Basic Local Alignment Search Toolvate) programs (http://www.ncbi.nlm.nih.gov) and PROMO (http://alggen.lsi.upc.es; ) (ref. 30).

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated in graded alcohols and washed in phosphate-buffered saline (PBS). After antigen retrieval in sodium citrate buffer (pH 6) in a microwave oven sections were blocked with 5% goat serum (Jackson Immunoresearch, Fisher Scientific, Gothenburg, Sweden) and incubated for 48 h at 4 °C with primary antibodies against MGMT, prediluted, 53406 Abcam, Cambridge Science Park, Cambridge, UK). Thereafter, sections were incubated for 30 min at room temperature with anti-mouse Alexa Fluor 568 or 594 conjugate. Similarly, for β-catenin detection, sections were incubated for 48 h at 4 °C with polyclonal rabbit anti-β-catenin (Cell Signaling Technology #8480) or rabbit anti-β-catenin (4 μg mL−1, Abcam), followed by detection with goat-anti-rabbit Alexa Fluor 488 conjugate. Sections were mounted with Dapi-fluoromount-G (Southern Biotech) mounting medium. Parallel tissue sections were stained with hematoxylin and eosin.

Cytotoxicity and clonogenic assay. The effects of Wnt inhibitors in combination with temozolomide and other conventional chemotherapeutic drugs on cell growth were determined using cell-viability assays fluorometric microculture cytotoxicity assay as previously described30,31 or WST-1 (Roche Diagnostic, Basel, Switzerland) according to the manufactures description. All concentrations were tested in duplicate or triplicate and the experiments were repeated at least three times. The studies were designed with a fixed molar ratio between the drugs (Temozolomide:Cerebrox, 33:1; Temozolomide:G007-LK, 50:1; Temozolomide:LGK974, 20:1; Temozolomide-Wnt-C59, 20:1; Temozolomide:Salinomycin, 40:1; and Temozolomide:XAV-939, 20:1), intended to be equipotent. To determine colony formation, DAOY, UW228-3, SK-N-AS, PKS1-K and SW480 cells were seeded in Cell- Spectro six-well plates (Starstedt, Solna, Sweden) at a concentration of 150–200 cells per dish, in triplicate. Cells were left to attach to the surface for 5 h before treatment with 10 μM cerebrox, 20–200 μM G007-LK, 5–10 μM LGK974 or 10–50 μM Wnt-C59, 50–400 μM temozolomide, or a combination of temozolomide and one of the Wnt-inhibiting drugs for 48 h, respectively. After 7–14 days, incubation in drug-free medium, colonies (>75 cells) with 50% plate efficiency were counted manually using a colony counter.

Immunoblotting. Total cell protein lysates was extracted from cells in RIPA buffer (25 mM Tris (pH 7.8), 2 mM EDTA, 20% glycerol, 1% Nonidet P-40 (NP-40), 1 mM diisothreitol). All protein extraction buffers were supplemented with MiniComplete protease inhibitor cocktail (Roche Diagnostics) and phosphatase inhibitor cocktail 1 (Sigma-Aldrich). The protein concentration was measured using Bradford reagent (Bio-Rad, Sundbyberg, Sweden). Equal quantities were separated by SDS-PAGE, transferred to nylon membranes (Millipore, Sundbyberg, Sweden), and probed with antibodies against active β-catenin (1:1,000, clone 8E7, Millipore, Solna Sweden), Axin2 (1:1,000, Cell Signaling Technology, Beverly, MA, USA), MGMT (1:1,000, Cell Signaling Technology), GADPH (1:10,000, Millipore) and β-actin (1:5,000, Cell Signaling Technology). Antibody IgG or anti-mouse IgG, conjugated with horseradish peroxidase (Pharmacia Biosciences, Uppsala, Sweden), were used as secondary antibodies. Pierce Super Signal (Pierce, Rockford, IL, USA) was used for chemiluminescent detection. MGMT recombinant protein

Figure 2 | Wnt/β-catenin regulates the expression of MGMT. (a,b) LS174T cells stably transfected with a Tet-inducible shRNA against β-catenin suppressed MGMT expression upon doxycycline stimulation9 (a) (b) (b) MGMT expression was unaffected by doxycycline compared with untreated cells. Scale bar, 20 μm. (c) Protein and mRNA expression of β-catenin, Axin 2 and MGMT in cellular extracts from LS174T cells treated with the indicated concentrations of doxycycline (doxy), and protein expression was determined by western blotting. mRNA expression was measured with quantitative RT-PCR, means with s.d. of triplicates are displayed. (d-f) (d) β-catenin activates MGMT through Tcf/Lef binding located in the hmgMGMG 5′-flanking regulatory region. (d) Schematic presentation of luciferase reporter plasmids (p-594/+24 ML, p-954/+24 ML and p-3500/+24 ML) with indicated numbers of putative Tcf/Lef-binding sites. Numbers in plasmid names refer to the amount of TCF/Lef-binding sites: +2 (plasmid 1), +4 (plasmid 2) and +8 (plasmid 3). (e) DAOY medulloblastoma cells were transiently transfected for 24 h with the MGMT promoter plasmids before treatment with increasing concentrations of either cerebrox, PEG2, or LiCl for another 24 h (one-way ANOVA with Bonferroni post test: plasmid 1: P < 0.0001, plasmid 2: P < 0.0001, plasmid 3: P < 0.0001, P < 0.05, **P < 0.01, ***P < 0.001). DAOY cells were co-transfected with pβ-catSPORT6 expressing β-catenin CDNA and the MGMT promoter plasmids for 48 h before luciferase activity measurements (t-test, P = 0.0092 for plasmid 3). SW480 colon cells were transiently transfected for 24 h with the MGMT promoter plasmids before treatment with cerebrox or PEG2; for 24 h (one-way ANOVA with Bonferroni post test: plasmid 1: P = 0.0007, plasmid 2: P = 0.0027, plasmid 3: P = 0.0003, P < 0.05, **P < 0.01, ***P < 0.001). (f) Transfection of DAOY, SK-N-AS, SW480 and LS174T cells with β-catenin siRNA regulates luciferase activity of TOPFlash and p-3500/+24 ML (plasmid 3). Luciferase activities are expressed as mean ± s.d. of triplicate in one representative experiment (each experiment was repeated two times three). DAOY, t-test: TOPFlash P = 0.0036, MGMT P = 0.0004; SK-N-AS t-test: TOPFlash P = 0.0005, MGMT P = 0.0002; SW480 one-way ANOVA with Bonferroni post test: TOPFlash P < 0.0001, MGMT P = 0.0003, P < 0.05, **P < 0.01, ***P < 0.001; LS174T t-test TOPFlash P = 0.0014, MGMT P = 0.0001. For all experiments means with s.d. of triplicates are shown.
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**Figure:**

**A:** Clonogenic-forming ability and survival index for various cell lines.

**B:** Effect of different concentrations of TMZ and Celecoxib, with or without vehicle, on cell viability.

**C:** Western blot analysis of p21 and MGMT expression in DAOY cells.

**D:** Cell viability of SW480 cells with and without Celecoxib treatment.

**E:** Effect of Celecoxib on SW480 cells at different concentrations of TMZ.

**F:** Comparison of clonogenic ability among different cell lines.

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**Notes:**

- **Clonogenic-forming ability** and **survival index** are expressed as a percentage of control.
- **Concentration** of Celecoxib: 10 μM, 100 μM, and 1,000 μM.
- **Concentration** of TMZ: 50 μM, 100 μM, 200 μM, and 400 μM.
- **Effect:** additive, synergistic, or combination.
- **CI70:** 0.95 for TMZ + Celecoxib, 0.81 for TMZ + Celecoxib, 0.69 for TMZ + Celecoxib.
- **Celecoxib** and **TMZ** treatment with or without vehicle.
- **XAV-939**, **Wnt-C59**, **LGK-974**, and **G007-LK**.
- **Cell lines:** DAOY, SW480, SK-N-AS, UW228-3, and PFSK-1.
- **MGMT (21 kDa)** protein expression.

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**Legend:**

- Blue line: TMZ control
- Red line: Celecoxib control
- Green line: TMZ + Celecoxib
- Black line: Vehicle control

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**Supplementary Information:**

- **Cell viability** assay results for each cell line.
- **Western blot** data for p21 and MGMT expression.
- **Kaplan-Meier** survival analysis for different treatment groups.

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**References:**


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**Conclusion:**

This study demonstrates the synergistic effect of Celecoxib and TMZ on various cancer cell lines, with significant reductions in clonogenic ability and survival index. Further research is needed to explore the mechanisms behind this effect and to evaluate the potential clinical applications.
Flow cytometry. To measure sub-G0 cell population, cells were trypanosynized, washed with ice-cold PBS, and fixed in 70% ethanol at −20°C. Cells were washed with PBS and incubated with RNase 20 μg·ml⁻¹ and propidium iodide (PI) 0.5 μg·ml⁻¹ (Sigma-Aldrich). All analyses were performed with LSR II Flow Cytometer and FACS Diva v6.1.3 software (BD Biosciences).

Quantitative real-time RT-PCR analyses. The mRNA expression levels of MGMT, CTNNB1, AXIN2 and an endogenous housekeeping gene encoding for 18S ribosomal RNA as a reference were quantified using TaqMan technology on an ABI PRISM 7500 sequence detection systems (PE Applied Biosystems). Sequence-specific primers and probes were selected from the Assay-on-Demand products (Applied Biosystems), including MGMT (assay ID: Hs01037698_m1), CTNNB1 (Hs00355049_m1), AXIN2 (Hs00610344_m1) and 18S ribosomal RNA (Hs99999901_s1). All gene-expression assays had an FAM reporter dye at the 5’ end of the TaqMan MGB probe, and a non-fluorescent quencher at the 3’ end of the probe.
cDNA of each sample was synthesized from 100 ng of RNA using High capacity RNA-to-cDNA kit (Applied Biosystems). The quantitative real-time RT–PCR was performed in a total reaction volume of 25 μl containing 1 × TaqMan Universal PCR Master Mix, 1 × TaqMan Gene Expression Assays (Applied Biosystems) and 10 μl of cDNA from each sample as a template, in MicroAmp optical 96-well plates covered with MicroAmp optical caps (Applied Biosystems). Samples were heated for 2 min at 50 °C and amplified for 40 cycles of 15 s at 95 °C and 1 min at 60 °C. To establish a standard curve for relative quantification we used cDNA synthesized from 1 μg RNA of the cell lines combined. For each sample, the amount of target mRNA was normalized to the standard curve and then normalized to 18S ribosomal RNA expression. All quantitative real-time RT–PCR experiments included no template control and were performed in triplicate.

Transfections and luciferase assays. All transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. MGMT promoter-activity measurements were achieved using luciferase reporter plasmids that were kindly provided by Dr Bhakat K. Kishor, University of Texas. MGMT promoter-activity measurements were achieved using luciferase reporter systems (Promega Biotech AB) was used as control. Briefly, 50,000–250,000 cells (depending on cell type) were seeded on 24-well plates 24 h prior transfection and luciferase expression plasmids (500 ng per well) were transfected into cells. When applicable, after 24 h cells were treated with increasing concentrations of celecoxib, PGE2, or LiCl. Cells were collected 48 h after transfection. Luciferase activity was measured using kits from Promega Biotech AB according to the manufacturer’s instructions. Non-silencing siRNA was used as control (Cell Signaling Technology). Overexpression of β-catenin and MGMT was done with plj-casSPORT6 expressing β-catenin (Cell Signaling Technology) and pMGMTSPORT6 expressing MGMT (Dharmacon, VWR International, Stockholm, Sweden).

Nude mice tumour xenografts. Four-to-six-week-old female NMRI nu/nu mice (Scanbur, Stockholm, Sweden) were maintained at five each per cage and were given sterile water and food ad libitum. In the first experiment each NMRI nu/nu mouse was subcutaneously injected with 7 × 10^6 human D283 MED medulloblastoza cells. Mice were randomly assigned into four treatment groups and the drugs were administered as follows: (i) 90 mg kg^−1 celecoxib (n = 12) daily by oral gavage, (ii) 7.5 mg kg^−1 temozolomide (n = 9) orally via a gastric feeding tube at days 1–5; (iii) 90 mg kg^−1 celecoxib and 7.5 mg kg^−1 temozolomide (n = 9), (iv) no treatment (n = 10). In the second experiment each mouse was subcutaneously injected with 10 × 10^6 human LS174T colon carcinoma cells. Mice were randomly assigned into four groups and the mice were treated as follows: (i) 12.5 mg kg^−1 temozolomide intraperitoneally at days 1–5 and days 8–13 (n = 9), (ii) 2 mg ml^−1 doxycycline added to drinking water to knockdown β-catenin expression by shRNA (n = 8), (iii) a combination of 12.5 mg kg^−1 temozolomide and 2 mg ml^−1 doxycycline (n = 9) and (iv) no treatment (n = 8). In both experiments each mouse was treated for 12 days and treatment was started on the appearance of palpable tumours. The mean tumour volume at the start of treatment was 0.13 ml for mice injected with D283 MED cells and 0.22 ml for mice carrying LS174T xenografts. Tumours were measured every day and tumour volume was calculated as (width)^2 × length × 0.44. TVI was calculated using the measured volume divided by the volume measured at start of treatment. At autopsy, tumours were frozen in liquid nitrogen for subsequent analysis. HT-29 colon cancer xenografts were established by inoculation of 10^6 HT-29 cells intra-duerally in the abdomen of nude Balb/c mice (Harlan, The Netherlands). At

![Graph](image_url)

Figure 5 | Inhibition of Wnt/β-catenin in combination with temozolomide reduces tumour growth in vivo. (a) A combination of temozolomide and celecoxib significantly impairs the growth of established human medulloblastoma xenografts in NMRI nu/nu mice. Mice were engrafted with 7 × 10^6 D283 MED cells subcutaneously and randomized to receive either celecoxib (90 mg kg^−1, n = 12) through daily oral gastric feeding, temozolomide (7.5 mg kg^−1, n = 9; days 1–5), a combination of celecoxib and temozolomide (n = 9) or no treatment (n = 10), starting at the appearance of palpable tumours of approximately 0.10 ml (mean 0.13 ml). Celecoxib augments the inhibitory effect of temozolomide on medulloblastoma growth in vivo, as shown by the TVI (at day 12, celecoxib: TVI = 6.2, P < 0.0001; temozolomide: TVI = 8.1, not significant; combination: TVI = 4.4 versus 10.3 in untreated controls, P < 0.0001, TVI, two-way ANOVA). (b) Western blot of protein extracts isolated from celecoxib- and vehicle-treated xenograft tumours. Celecoxib downregulated the expression of MGMT in vito. Protein expression was assessed with densitometry (t-test, P = 0.0266). (c) A combination of β-catenin knockdown and temozolomide inhibits the growth of established colon adenocarcinoma xenografts in vivo. NMRI nu/nu mice were engrafted with 10 × 10^6 LS174T cells subcutaneously and randomized (10 mice each group) to receive 2 mg ml^−1 doxycycline to induce shRNA against β-catenin, 12.5 mg kg^−1 temozolomide i.p. from day 1 to day 5 and day 8 to day 12, a combination of temozolomide and doxycycline or vehicle only. No differences were observed in LS174T xenograft tumour growth treated with doxycycline or temozolomide as single treatment, whereas a combination of β-catenin knockdown and temozolomide induced significant inhibition of tumour growth at day 7 of treatment until the end of treatment (at day 12: shβ-catenin: TVI = 3.7, not significant; temozolomide: TVI = 3.8, not significant; combination: TVI = 2.5 versus 4.2 in untreated controls, P < 0.0001; combination versus shβ-catenin, P < 0.01; combination versus temozolomide, P < 0.01, two-way ANOVA). LS174T shRNA sequence directed against β-catenin: 5'-GATCCCGTTGGTGATAGAGCTTCCAAGAGAGCCTCTATACCACCCACTTTGGAAA-3' (d) Western blot of protein extracts isolated from LS174T xenograft tumours confirm downregulation of active β-catenin and MGMT.
Statistical analysis. All statistical analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Calculation of IC50 values was done from log-concentration-effect curves in GraphPad Prism. Testing for synergistic or additive effects of combination therapy was performed as previously described according Chou–Talalay method with Combosyn software.

Synergism and antagonism are defined as a mean of the combination index at 70% growth inhibition significantly lower/higher than 1 with one-sample t-test (P<0.05). For in vitro studies, the t-test was used to determine whether the mean of a single sample differed significantly from control. To compare several treatment groups, one-way ANOVA with Bonferroni multiple-comparisons tests were used. P<0.05 was considered significant. Tumour growth was analysed by two-way ANOVA and the Bonferroni post hoc test was used for multiple comparisons between groups.

References

Acknowledgements
Conflon microscopy was performed at the University of Tromsø, Norway Advanced Microscopy Core Facility. This work was supported by grants from The Norwegian Cancer Society, The Swedish Cancer Society, The Swedish Foundation for Strategic Research (www.nbbrf.com), Marta and Gunnar V. Philipson Foundation, The Mary Bev Foundation, Diman Foundation, and Erna and Olav Aakres Foundation for Cancer Research.

Author contributions

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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