Molecular mechanisms in colon cancer
Hardwick, J.C.H.

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Chapter IV

Aspirin
and
Indomethacin
act directly
on the
Wnt/β-catenin
pathway

James Hardwick
Gijs van den Brink
Sander Diks
Marije van Santen
Leon Tertoolen
Sander van Deventer
Maikel Peppelenbosch

Submitted
Abstract

Background and aims: NSAIDs show chemopreventative efficacy in colon cancer but the mechanism behind this remains unclear. Elucidating this mechanism is seen as vital to the development of new chemopreventative agents. We studied the effects of Aspirin and Indomethacin on the oncogenic Wnt/β-catenin pathway, investigating their effects on different elements within this pathway.

Methods: We studied the effects of Aspirin and Indomethacin on β-catenin and glucose synthetase kinase 3 beta (GSK3β) by immunoblotting using phospho-specific antibodies, and assessments of ubiquitination and cytoplasmic levels of β-catenin. We assessed Wnt/β-catenin pathway activity using the TOP-Flash reporter assay and cellular localization of β-catenin using GFP-tagged β-catenin and time-lapse fluorescent imaging.

Results: We show that Aspirin has direct effects on the phosphorylation of β-catenin but not GSK3β. This in turn leads to enhanced ubiquitination and reduced cytoplasmic levels of β-catenin. We confirm inhibition of the Wnt/β-catenin pathway by reporter assay and that this effect is dependent on the proteasome.

Conclusions: These results support the theory that NSAIDs act on colon cancer through direct effects on the Wnt/β-catenin pathway, acting at or above the level of β-catenin to promote its phosphorylation and breakdown in a GSK3β independent fashion.
NSAIDs act directly on the Wnt/β-catenin pathway

Introduction

Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce death rates due to colon cancer and to reduce the size of colonic adenomatous polyps. These findings suggest that chemoprevention of colon cancer may be a possible way of reducing the impact of this increasingly common and frequently fatal disease. There remain, however, serious problems with the widespread use of NSAIDs in those at risk for the development of colon cancer. The incidence of side effects such as gastrointestinal haemorrhage and renal damage preclude their use in this setting. The development of drugs with similar efficacy in colon cancer with acceptable side effects is in process. Understanding the mechanism of action of NSAIDs in colon cancer is an essential step in this process.

The original and still the leading theory is that NSAIDs act through their ability to block cyclooxygenase (COX) enzymes. However, related compounds with no COX-inhibitory activity retain their antitumour activity and cancer cells lacking COX-2 enzyme are still sensitive to these compounds. Therefore COX-independent effects of NSAIDs in colon cancer have been postulated (reviewed in) such as their ability to block peroxisome proliferation activator delta, inhibition or activation of nuclear factor kappa beta and effects on the TGF-beta family member, macrophage inhibitory cytokine. A more direct explanation of the effects of NSAIDs in colon cancer would be that they target elements within the most essential oncogenic pathway in colon cancer, the Wnt/β-catenin pathway.

An activating mutation of the Wnt/β-catenin pathway is the first step in almost all colorectal cancers. Here the essential feature is an increase in the levels of free cytoplasmic β-catenin (reviewed in). In most cases Wnt/β-catenin pathway activation is brought about by a mutation in the adenomatous polyposis coli (APC) gene (reviewed in). This leads to a truncated APC protein that cannot perform its function as part of the β-catenin destruction complex. This leads to reduced β-catenin degradation and abnormally high levels of cytoplasmic β-catenin. This results in increased β-catenin translocation to the nucleus where it binds with members of the TCF/LEF family of transcription factors, and activates the transcription of essential genes such as Cyclin D and Myc. In many of the remaining cases, mutations in β-catenin itself at sites of Glycogen Synthetase Kinase-3β (GSK-3β) phosphorylation, leads to its cytoplasmic accumulation and Wnt/β-catenin pathway activation.
We set out to further investigate the effects of NSAIDs on the Wnt/\(\beta\)-catenin pathway in colon cancer cells. A reduction in the constitutive activity of this pathway in colon cancer, for example by reducing levels of signaling \(\beta\)-catenin, reverses this earliest and most essential oncogenic signal and has been shown to be an effective anti-tumour strategy \textit{in vitro} and \textit{in vivo}.\textsuperscript{161,162}

Evidence has been building that there may be direct effects of NSAIDs on this pathway.\textsuperscript{163,164,165} This would concur with the epidemiological data that suggest NSAIDs act at a very early stage in colon cancer and must be given at this earliest point in the disease process to be effective.\textsuperscript{166} Here we provide evidence that NSAIDs inhibit the Wnt/\(\beta\)-catenin pathway by stimulating the phosphorylation and breakdown of \(\beta\)-catenin.

\textbf{Materials and methods}

Anti-phospho \(\beta\)-catenin (Thr41, Ser45) and anti-phospho GSK-3\(\beta\) (Ser9, Ser21) were purchased from Cell Signaling (Beverley, MA). Anti \(\beta\)-catenin antibodies were from Upstate (Lake Placid, NY) and Ubiquitin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). ALLN was from Sigma (St. Louis, MO). TOPFlash and FOPFlash were gifts from Dr. Hans Clevers (Utrecht University, The Netherlands). GF-109203X was purchased from Calbiochem (La Jolla, CA) and MG-132 was from Sigma. Aspirin and Indomethacin were from Sigma. Stock 1M Aspirin and 400mM Indomethacin solutions were prepared in DMSO and stored at -20°C.

\textbf{Cell culture}

SW 480 and DLD-1 colon cancer cell lines were obtained from the ATCC, and cultured in Dulbeccos Modified Eagles Medium (DMEM) (Gibco, Paisley, Scotland) with 4.5g/L Glucose and l-Glutamine. This was supplemented with penicillin (50U/ml) and Streptomycin (50\(\mu\)g/ml) and, where serum was used, with 10% foetal calf Serum (Gibco). Cells were grown in monolayers in a humidified atmosphere containing 5% CO\(_2\). We chose these cell lines because SW480 and DLD-1 cells have mutations in both alleles of the \(APC\) gene\textsuperscript{167} and neither express COX-2 protein.\textsuperscript{168,163}
Immunoblotting

Cells at from 60 to 80% confluence from 6 well plates (Nalge Nunc, Denmark) were washed in ice cold PBS and scraped into 150μl of 2x sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 2% β-mercapto ethanol; 20% glycerol, 1mg bromphenol blue). Protein concentration was measured using the RC DC protein assay kit (Biorad, CA) according to the manufacturers instructions. Samples were loaded onto SDS-PAGE and blotted onto PVDF membrane (Millipore). Equal loading was confirmed by Coomassie brilliant blue staining. The blots were blocked with 5% low fat milk powder in TBST (Tris Buffered Saline with 1% Triton) for one hour at room temperature and washed 3x 10 mins in TBST before overnight incubation at 4°C with primary antibody in primary antibody buffer (TBST with 5% Bovine Serum Albumin for phospho-specific antibodies and 5% milk for all other antibodies). Blots were then washed 3x 10 mins in TBST and incubated for 1 hour at room temperature in 1/2000 Hosome Radish Peroxidase (HRP) conjugated secondary antibody in block buffer. After a final 3x 10 minute wash in wash buffer, blots were incubated for 5 minutes in Lumilitre plus (Boehringer-Mannheim, Mannheim, Germany) and then chemiluminescence detected using a Lumi-Imager (Boehringer-Mannheim). Blots were stripped as necessary with strip buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercapto ethanol).

Immunoprecipitation

Cells monolayers were washed in ice cold PBS and then scraped into ice-cold lysis buffer (150mM NaCl, 0.5% Triton, 5mM EDTA, 0.1% SDS, 0.5% Sodiumdeoxycholate, 1mM Pefabloc (Boehringer-Mannheim)) and incubated at 4°C for 15 minutes. Cell lysates were then centrifuged for 15 minutes at 15000g and the supernatant was incubated overnight at 4°C with anti beta catenin antibodies (1:500). This was followed by incubation for one hour with protein-A sepharose beads. After centrifugation for 15 minutes at 15000g at 4°C the beads were washed 5 times in ice cold PBS before the addition of an equal volume of 2x sample buffer. Samples were then further prepared as for immunoblotting.
Cytoplasmic extraction

These were performed essentially as described previously. Briefly, treated cells from 6 well plates were washed with ice cold PBS and scraped into 200μl ice-cold hypotonic lysis buffer (10mM Tris pH 7.4, 0.2 mM MgCl2 and 1 mM Pefabloc). The lysate was passed ten times through a 27G gauge needle and then centrifuged for one hour at 100,000 g. The supernatant, designated S100, was removed and added to an equal volume of sample buffer and then further prepared as for immunoblotting. The pellet, P100, was diluted in sample buffer to the same dilution as the S100 fraction for immunoblot analysis.

TOP-Flash assay

SW480 cells were seeded in 24-well plates at $1 \times 10^5$ cells/well. The following day cells were transiently transfected with 0.4 μg of the TCF reporter, pTOPFLASH (optimal motif), or pFOPFLASH (‘Far from OPTimal’ motif), and 0.008 μg of pCMV-Renilla luciferase (Promega) per well, using LipofectAMINE-Plus reagent according to the manufacturers instructions (Life Technologies, Inc.) for 5 h. Cells were then incubated for 48 hours in DMEM with 10% serum and no antibiotics containing various concentrations of NSAIDs. Samples were further prepared using the dual luciferase reporter kit (Roche) according to the manufacturers instructions and luciferase levels measured using a dual injector luminometer.

Real-time imaging of GFP-tagged β-catenin

SW480 cells were seeded out overnight onto glass coverslips. The following day cells were transiently transfected with GFP-tagged β-catenin using LipofectAMINE-Plus reagent according to the manufacturers instructions and then grown overnight in DMEM with 10% serum. The following day single GFP-expressing cells were centred under a fluorescent microscope in a chamber containing medium at 37°C. The medium was changed for medium with 10mM Aspirin 37°C after one minute, and the cells were followed for a further 3000 seconds with one image taken every 60 seconds.
Results

Aspirin inhibits β-catenin/TCF transactivation.

The Wnt/β-catenin pathway acts to control the transcription of genes through the binding of a complex of β-catenin and TCF/LEF to a specific promoter. The activity of this final step in the Wnt/β-catenin pathway can be measured using a luciferase reporter construct. SW480 cells were transiently transfected with the pTOPFLASH or pFOPFLASH (containing a ‘far from optimal’ TCF binding site) construct together with a pCMV-Renilla luciferase construct to act as an internal control for transfection efficiency and potential toxicity of treatments. Figure 1a shows that Aspirin down-regulates the constitutively active β-catenin-TCF signaling found in SW480 cells in a dose-dependent manner. The specificity of the Aspirin effect on TCF reporters was confirmed by the fact that pFOPFLASH was not influenced by Aspirin. Similar results were obtained in a second colon cancer cell line, DLD-1, and using a second NSAID, Indomethacin (Figure 1b).

![Figure 1a](image)

**Figure 1a** TCF reporter assay in SW480 cells treated with various concentrations of Aspirin. Cells were transiently co-transfected with either pTOPFLASH or pFOPFLASH luciferase reporter constructs as well as pCMV-Renilla luciferase construct to serve as an internal control for transfection efficiency and potential toxicity of treatments. Cells were then treated with Aspirin for 48 hours, lysed and analysed using the dual luciferase assay system (Roche). Each treatment was performed in triplicate and experiments were repeated at least three times. Results are presented as light units normalised to the internal control.
Aspirin leads to increased Serine/Threonine phosphorylation of β-catenin by a GSK3β independent mechanism.

Having shown that Wnt/β-catenin pathway activity is downregulated by Aspirin, we looked further at which elements within this pathway are affected. A central element within this pathway is β-catenin. It is the excess of cytoplasmic β-catenin that leads to the over activity of the Wnt/β-catenin pathway seen in colon cancer. Control of the size of this pool is regulated by the Serine/Threonine phosphorylation of β-catenin. This phosphorylation then targets β-catenin for ubiquitination and degradation by the 26S proteasome. In most cells this phosphorylation is brought about by GSK3β. However GSK3β can only phosphorylate β-catenin in the context of a complex with full-length APC protein. In most cases of colon cancer and in SW480 cells a mutant, truncated APC protein is formed. GSK3β is unable to complex with this mutant APC and is thus unable to phosphorylate β-catenin. We assessed the effect of Aspirin on β-catenin (Thr41, Ser45) phosphorylation and as seen in figure 2 show a dose and time related increase in β-catenin phosphorylation with Aspirin.
Figure 2 Immunoblot for SW480 cells treated with Aspirin for various times and at various concentrations using antibodies raised against (A and D) phospho β-catenin (Thr41, Ser45), (B) phospho GSK-3β (Ser9, Ser21), and (C) β-catenin. A, B and C were performed on the same blot which was stripped between successive antibodies. Cells at 60-80% confluence were treated with (blot A) DMSO (0.5%), various concentrations of Aspirin and the phosphatase inhibitor Calyculin A (50nM) as a positive control (+) for the phospho β-catenin antibody, all for one hour. In D, cells at 60-80% confluence were treated with Aspirin 5mM for various times shown in minutes. The positive control (+) was Calyculin A (50nM) for 60 minutes.

The activity of GSK3β is controlled by its phosphorylation. We therefore assessed the activity of this kinase using phosphospecific antibodies in SW480 cells treated with Aspirin. No alteration in the phosphorylation levels of GSK3β was observed with Aspirin (figure 2, blot C), suggesting that the increase in phospho-β-catenin observed is GSK3β independent. These experiments were repeated with similar results in the DLD-1 colon cancer cell line suggesting the effect is not cell type specific (not shown).

Aspirin leads to a reduction in cytoplasmic β-catenin levels.

Phosphorylation of β-catenin classically leads to its ubiquitination and breakdown. We initially tried to confirm this by blotting for total β-catenin in whole cell lysates of SW480 cells treated with Aspirin. As shown in figure 2, total β-catenin levels remained unchanged while there was a convincing increase in β-catenin phosphorylation seen on the same blots.

β-catenin exists in two main cellular pools, a structural pool important for the cytoskeleton and a free cytoplasmic pool. It is only this latter pool that plays a part in the
Wnt/β-catenin pathway. We therefore analysed the effect of Aspirin on the cytoplasmic pool of β-catenin (S100 cellular fraction), according to the method of Orford et al.,\textsuperscript{169} and as shown in figure 3, the cytoplasmic pool decreases in a dose dependent manner upon the addition of Aspirin. This effect is also seen with Indomethacin and in the DLD-1 cell line (not shown).

![Figure 3](image1)

**Figure 3** Immunoblot analysis of cytoplasmic extracts (S100) and the non-cytoplasmic fraction (P100) of SW480 cells treated with Aspirin using anti β-catenin or anti β-actin antibodies. The graph shows the relative amounts of β-catenin corrected for β-actin loading controls. The cytoplasmic pool of β-catenin is only one quarter of the size of the non-cytoplasmic pool demonstrating why β-catenin analysis in whole cell lysates may not adequately reflect changes in the cytoplasmic pool.

To further confirm that this effect is independent of GSK3β, we preincubated cells with the GSK3β inhibitor LiCl and then treated them with Aspirin at various concentrations and observed, in figure 4, the same reduction in the cytoplasmic pool of β-catenin.

![Figure 4](image2)

**Figure 4** Immunoblot of cytoplasmic extracts from SW480 cells treated with Aspirin or Aspirin and Lithium Chloride using anti-β-catenin antibodies. Cells at 80% confluence were treated for 90 minutes with various concentrations of Aspirin or Aspirin and Lithium Chloride (10mM) as indicated. Cytoplasmic extracts were made as described and equal amounts of total protein were immunoblotted for β-catenin.
NSAIDs act directly on the Wnt/β-catenin pathway

Aspirin leads to an increase in ubiquitinated β-catenin.

β-catenin is marked for degradation by the 26S proteasome by the covalent addition of ubiquitin. This task is performed by the SCF ubiquitin ligase complex, which contains a component, β-TrCP, that binds specifically to phosphorylated β-catenin. To confirm that the reductions in cytoplasmic β-catenin levels are indeed brought about by this mechanism we assessed the effect of Aspirin treatment on the appearance of higher molecular weight ubiquitinated β-catenin protein. We immunoprecipitated β-catenin from SW480 cells and then probed the blotted immunoprecipitate with anti-ubiquitin antibodies according to the method of Kitagawa et al.¹⁷¹ Figure 5 shows the appearance of an ubiquitin immunoreactive ladder extending upwards from the 97 Kd height of non-ubiquitinated β-catenin, induced by the addition of Aspirin.

![Figure 5](image)

**Figure 5** Cell lysates of SW480 cells with or without Aspirin treatment were immunoprecipitated with anti-β-catenin antibodies and then immunoblotted using anti-ubiquitin antibodies. The blot was then stripped and reprobed using anti-β-catenin antibodies to show that equal amounts of protein had been immunoprecipitated.

The proteasome inhibitor MG132 blocks Aspirin mediated inhibition of β-catenin/TCF transactivation.

If Aspirin reduces β-catenin levels by promoting its proteasomal degradation, disruption of proteasome activity would be expected to abolish the Aspirin effect. In figure 6 we show that addition of the proteasome inhibitor MG132 activates β-catenin/TCF transactivation above the high constitutive levels already found in colon cancer cells, and abolishes the effect seen with the addition of Aspirin.
Several authors have demonstrated the importance of Protein Kinase C in controlling cytoplasmic β-catenin stability. We investigated whether the effect we see with Aspirin is mediated via PKC by assessing the effect of a bisindoylmaleimide PKC inhibitor on the TOPFLASH assay. PKC inhibition fails to block the effect of Aspirin on the TOP-FLASH reporter assay (figure 7) suggesting that the Aspirin effect on the Wnt/β-catenin pathway is PKC independent.
NSAIDs act directly on the Wnt/β-catenin pathway

**Aspirin leads to a visible reduction in cytoplasmic GFP-tagged β-catenin.**

To further examine the effect of NSAIDs on β-catenin we used GFP-tagged β-catenin and visualized the effect of Aspirin in real time using a fluorescent microscope and time-lapse imaging in a single living colon cancer cell. Addition of Aspirin led to a 40% reduction in cytoplasmic β-catenin levels as seen in figure 8. This can be seen visually in figure 9. No change was seen in control experiments with the addition of vehicle over the same time period.

![Figure 8](image1)

**Figure 8** Analysis of the mean fluorescence of the cytoplasm and nucleus of a single SW480 cell transiently transfected with GFP-tagged β-catenin followed over time after treatment with Aspirin (10mM) at time=0. Cells were grown to 80% confluence on glass coverslips and then transiently transfected with GFP-tagged β-catenin. The following day single GFP-expressing cells were centred under a fluorescent microscope and a series of images taken automatically using time-lapse fluorescent imaging. The mean fluorescence of a selected area within the nucleus or cytoplasm was analysed using image analysis software for the series of images.

![A and B](image2)

**Figure 9** Fluorescent imaging of a single SW480 cell transiently transfected with GFP-tagged β-catenin. A shows the cell at time=0 and B the same cell at time=3000 seconds after treatment with Aspirin (10mM). Cells were transiently transfected with GFP-tagged β-catenin and further cultured overnight. The following day single GFP-expressing cells were centred under a fluorescent microscope and a series of images taken automatically using time-lapse fluorescent imaging.
Investigation into the mechanism of action of NSAIDs in colon cancer has gradually become a field in its own right. Understanding of the mechanisms involved is seen as the key to developing new more effective preventative treatments with fewer side effects. Most of the investigation has focused on COX enzymes 1 and 2 with the accumulation of genetic and pharmacologically derived data to support the importance of COX-2 in the progression of colon cancer. However, it has recently become clear that NSAIDs have a wide range of targets other than COX and this coupled with evidence mitigating against an essential role for COX-2, has led several investigators to propose COX-independent mechanisms for the action of NSAIDs in colon cancer.

We set out to investigate whether NSAIDs have direct effects on the Wnt/β-catenin pathway and elements within it. Constitutive activation of this pathway is found in almost all colon cancers. Mutations leading to truncation of the APC protein or in β-catenin itself lead to disruption of normal β-catenin breakdown, increased free cytoplasmic β-catenin, which translocates to the nucleus to bind with TCF and switch on Wnt/β-catenin target gene expression. The level of free cytoplasmic β-catenin is controlled largely by its ubiquitination and breakdown by the proteasome. This is in itself controlled by the phosphorylation of β-catenin at one or more conserved serine and threonine residues in the amino-terminal region of β-catenin. The phosphorylation of β-catenin occurs in a complex formed principally by APC, GSK-3β, axin and β-catenin. Only in the context of this complex can GSK3β phosphorylate β-catenin. The kinase activity of GSK3β is itself regulated by phosphorylation. Phosphorylation at Ser 9 or Ser21 leads to inactivation of GSK3β and therefore reduced phosphorylation and breakdown of β-catenin.

We first used a TCF reporter assay to assess the effects of NSAIDs on the activity of the pathway as a whole. We narrowed down the possible site of action of NSAIDs within this pathway by showing that a proteasome inhibitor inhibits the action of NSAIDs on the reporter assay. Since cytoplasmic β-catenin levels are controlled by the proteasome this suggests that NSAIDs act at or above the level of β-catenin.

We then used immunoblotting using phospho-specific antibodies to detect changes in the level of phosphorylated β-catenin and GSK3β. We found a time and concentration dependant increase in β-catenin phosphorylation. Importantly, this effect was seen at micromolar concentrations of Aspirin. Proponents of the COX-2 theory have voiced doubts
over the relevance of COX-independent actions of NSAIDs which require high doses (millimolar) that they feel are not achievable in vivo. However, human subjects given a short analgesic dose (600mg qid) attain millimolar (0.05-1.13mM) concentrations of Salicylate and up to 10mM Aspirin has been used by these investigators in cell culture.

In the intact Wnt/β-catenin pathway β-catenin phosphorylation is brought about by GSK-3β, a kinase inactivated by phosphorylation. We looked for changes in the phosphorylation status of GSK-3β at Serine 9 and 21, but it remained unchanged suggesting that β-catenin phosphorylation in this system is GSK-3β independent. This is not surprising in colon cancer cell lines with mutant APC, as used in this study, as GSK-3β requires the presence of wild-type APC and Axin in order to phosphorylate β-catenin. Thus phosphorylation of β-catenin seen in APC mutant cells is likely to be GSK-3β independent.

We then assessed levels of total β-catenin to confirm that the observed phosphorylation leads to β-catenin breakdown. We initially performed this in whole cell lysates where we saw no change in total β-catenin levels. β-catenin exists in two pools within the cell; one close to the cell membrane where it serves a structural function linking elements of the cytoskeleton to the cell membrane, and another free in the cytoplasm where it serves as a signal transduction element in the Wnt/β-catenin pathway. Analysis of total β-catenin will combine all β-catenin from both locations and thus major changes in intracytoplasmic levels of β-catenin may be masked. We therefore assessed the effect of NSAIDs on the cytoplasmic fraction of β-catenin and found a time and concentration dependent reduction. We confirmed that the effects that we see are independent of GSK-3β by the addition of Lithium Chloride, an inhibitor of GSK-3β, which failed to block the Aspirin-mediated reduction in cytoplasmic β-catenin levels.

Finally we confirmed the appearance of poly-ubiquitinated β-catenin in cells treated with NSAIDs. Cytoplasmic β-catenin levels are regulated by its breakdown by the 26s proteasome. This is preceded by polyubiquitination by the SCF (Skp1/cullin/F-box) protein complex.

The exact mechanism of NSAID-induced β-catenin phosphorylation remains to be elucidated. In cell lines containing wild type APC, protein kinase C has been shown to be essential for the regulation of cytoplasmic β-catenin levels. However, blocking PKC with the Bisindoylmaleimide inhibitor GF-109203X failed to block NSAID mediated reduction of TCF reporter activity, or reductions in cytoplasmic β-catenin. This may be
because the action of PKC is on APC-dependant elements such as GSK-3β, elements that play no role in Wnt/β-catenin pathway activity in APC mutant cells. Another possibility is that NSAIDs promote β-catenin phosphorylation through the inhibition of protein phosphatases such as PPA2. There is evidence that these play a critical role in β-catenin phosphorylation and breakdown.

In summary, we provide new evidence that NSAIDs directly inhibit the Wnt/β-catenin pathway by promoting the breakdown of cytoplasmic β-catenin through increasing β-catenin phosphorylation. This may provide an explanation for the action of NSAIDs in colon cancer.