Cyclooxygenase-2 Inhibition Inhibits c-Met Kinase Activity and Wnt Activity in Colon Cancer

Jurriaan B. Tuynman, Louis Vermeulen, Elles M. Boon, Kristel Kemper, Aeilko H. Zwinderman, Maikel P. Peppelenbosch, and Dirk J. Richel

Abstract
Activity of receptor tyrosine kinases (RTK) in colorectal cancer (CRC) is associated with enhanced tumor growth and a poorer prognosis. In addition, cyclooxygenase-2 (COX-2) expression contributes to tumor growth and invasion. COX-2 inhibitors exhibit important anticarcinogenic potential against CRC, but the molecular mechanism underlying this effect and the relation with RTK signaling remain the subject of intense research effort. Therefore, the rapid effects of COX-2 inhibition in CRC on the complement of all cellular kinases were investigated using a kinase substrate peptide array, Western blotting, transfection, small interfering RNA assays, and CRC cell lines. The resulting alterations in the kinase profile revealed that celecoxib, a selective COX-2 inhibitor, impairs phosphorylation of substrates for the RTKs c-Met and insulin-like growth factor receptor, resulting in decreased downstream signaling. The decrease in c-Met activation is accompanied with an increase in glycogen synthase kinase 3β kinase activity together with a rapid increase in phosphorylation of β-catenin. In agreement, a significant reduction of β-catenin-T-cell factor–dependent transcription is observed both with celecoxib and selective inhibition of c-Met phosphorylation by small molecules. Hence, corepression of c-Met–related and β-catenin–related oncogenic signal transduction seems a major effect of celecoxib in CRC, which provides a rationale to use c-Met inhibitors and celecoxib analogous to target c-Met and Wnt signaling in a therapeutic setting for patients with CRC.

Introduction
Colorectal cancer (CRC) remains a leading cause of cancer-related death worldwide despite recent advances in adjuvant chemotherapeutic regimens. In the last decade, extensive research has been made in the development of new therapeutic regimens for CRC. Targeted therapy against receptor tyrosine kinases (RTK), such as epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor, has been shown to improve survival in patients with CRC (1, 2). In addition, a large amount of data is available describing both clinically chemopreventive and preclinically anticancer activities of selective cyclooxygenase-2 (COX-2) inhibitors and nonsteroidal anti-inflammatory drugs (NSAIDs; refs. 3–5). However, precise molecular mechanisms still remain to be established.

The main event and the most characterized mechanism of carcinogenesis in CRC is the disruption of a functional adenopolyposis coli (APC) complex due to a mutation of the APC gene or the β-catenin gene, leading to inappropriate activation of the Wnt signaling pathway (6, 7). This results in nuclear accumulation of β-catenin, which associates with the T-cell factor (TCF)-4, leading to transcription of genes involved in dedifferentiation, cell proliferation, and survival, events involved in carcinogenesis. In addition, overexpression or activating mutations of RTKs, such as the EGFR, the insulin-like growth factor receptor (IGFR), and c-Met, a receptor for hepatocyte growth factor (HGF), contribute to the survival and progression of CRC (8–11). c-Met is frequently overexpressed in advanced stages of CRC and is correlated with a poor prognosis (12). c-Met activation by HGF or by amplification results in cell dissociation, migration, scattering, and invasion, important processes in cancer progression and metastasis (9–11, 13).

Evidence has emerged suggesting functional interaction between COX-2 enzymatic activity, RTK signaling, and Wnt activity (14–17). COX-2 activity enhances the cross-talk between the membrane tyrosine kinases c-Met and EGFR, resulting in nuclear accumulation of β-catenin; however, precise mechanisms remain unclear (18). Previous studies have shown that relative high concentrations of NSAIDs are able to down-regulate the Wnt signaling cascade in colon cancer cells (19–22). Although c-Met activation has been shown to enhance Wnt signaling in non–APC-mutated, non-CRC cells, neither the relationship between c-Met activation and its downstream signal transduction and Wnt signaling nor the effect of COX-2 inhibition on c-Met signaling in CRC has been clarified (23).

Because COX-2 has a crucial role in the carcinogenesis and progression of CRC, exploration of its mechanisms can lead to new therapeutic targets to improve survival of CRC. Celecoxib, a selective COX-2 inhibitor, has been shown to possess the highest anticarcinogenic capacity of NSAIDs and coxibs. Therefore, we have explored the effects of the selective COX-2 inhibitor celecoxib on signal transduction in CRC cells. To this end, a peptide array was used, which contains 1,176 different kinase substrate consensus sequences, generating a comprehensive overview of the total complement of cellular kinase activity and thus enabling analysis of celecoxib effects without a priori assumptions as to signaling pathways affected by the compound. The generated data have been extensively validated (24–28). Our results suggest that celecoxib represses c-Met–dependent signaling, resulting in down-regulation of oncogenic Wnt signaling in CRC. These findings both stress the importance of the tyrosine kinase receptor c-Met in carcinogenesis and provide a rationale to target c-Met in therapeutic modalities.
Materials and Methods

**Reagents.** Aspirin and NS398 were purchased from Sigma. Celecoxib (pure reagent) was kindly provided by Pfizer. Primary antibodies against the following targets were used: phosphospecific β-catenin (Ser33/37/Thr41), non-phospho-β-catenin, phospho-insulin receptor (INSR; Tyr1161), phospho-EGFR (Tyr1068), EGFR, phospho-c-Met (Ser1234/5), c-Met, phospho-Gab-1 (Tyr6270), Gab-1, phospho-Akt (Ser473), Akt, phospho-Lck (Tyr505), phospho-Src (Tyr416), phospho-glycogen synthase kinase 3β (GSK3β; Ser9), GSK3β, phospho-PTEN (Ser380), and PTEN (Cell Signaling Technology). Antibodies against c-Myc (9E10), anti-c-Met (c12), and β-actin (c19; Santa Cruz Biotechnology) were also used. LY294002 and small interfering RNA (siRNA) GSK3α/β were purchased from Cell Signaling Technology. A novel small molecule inhibiting c-Met phosphorylation (PHA665752) was kindly provided by Pfizer, Inc. Horseradish peroxidase (HRP)-conjugated secondary antibodies were all derived from DAKO.

**Cell culture.** Human colon cancer cell lines DLD1 (COX-2 negative) and HT29 (COX-2 positive; American Type Culture Collection) were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FCS (Integro), 5 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen Corp.) at 37°C in a 5% CO2 atmosphere. Cells were passaged 20 times maximally. The concentration range of c-Met phosphorylation in the experiment was chosen to resemble the in vivo situation (5–50 μmol/L).

**Cell viability in cell culture.** The cell viability was assessed by mitochondrial function, measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity. Briefly, cells were seeded in a 14-well plate and stimulated with increasing concentrations of celecoxib (5–50 μmol/L). After 48 h, 0.5 mg/mL MTT (Sigma-Aldrich) was added for 30 min at 37°C. Subsequently, the medium was aspirated and the cells were lysed in isopropanol/0.04 mol/L HCl.

**Kinome array analysis.** A kinase substrate peptide array consisting of 1,176 peptides with specific phosphorylation sites was used to comprehensively evaluate the effects of aspirin and celecoxib on the kinome, as have been previously described (24–26). After celecoxib treatment, the cells were washed in ice-cold PBS and harvested in lysis buffer (Cell Signaling Technology) supplemented with protease and phosphatase inhibitors (Roche). Protein concentrations were determined using Bradford analysis (Bio-Rad). To study kinase activity, 50 μL of cell lysate (5 mg/mL protein) were cleared on a 22-μm filter on ice, after which 10 μL of activation mix were added, containing 50% glycerol, 50 μmol/L ATP, 60 mmol/L MgCl2, 0.05% (v/v) Brij-35, 0.25 mg/mL bovine serum albumin, and 2,000 μCi/mL [γ-32P]ATP. The resulting samples were incubated on the PepChip arrays (Pepscan) containing 1,176 kinase substrate peptides in duplicate for 2 h in a humidified stove at 37°C. Subsequently, the arrays were washed in 2 mol/L NaCl, 1% Triton X-100, PBS, 0.1% Tween 20, and H2O, after which slides were dried and exposed to a phosphoimaging screen for 24 h. Experiments were performed in duplicate and repeated independently (n = 3). The robustness of this array technology was confirmed by repeating each experiment (each time with a different print run of the array) and by using different dilutions of the same lysate and different exposure times to ensure that spots were analyzed in the linear range of the enzymatic kinase reaction.

**Data acquisition and statistical analysis.** Imaging of the incorporated [γ-32P]ATP was performed using a phosphoimager (Fuji) at 50 μm/pixel. Data analysis was performed using Microsoft Excel and Statistical Package for the Social Sciences 11.5 (SPSS, Inc.). After image acquisition and quantification using ArrayVision software 6.0 (Molecular Dynamics), each spot was corrected for its individual background. To reduce variation induced by incubation and imaging conditions, the data of each array were corrected with a linear trimmed median normalization method for each array and each individual experiment. After excluding inappropriate duplicates for further analysis, the resulting data matrix consisted of the phosphorylation levels of 1,176 peptides induced by the HT29 lysate treated with celecoxib relative to the control. To identify biologically relevant activity changes, we calculated the differences in variance relative to the regression curve (SD > 1.96). Data were considered relevant for analysis when the signal to noise ratio in both conditions was >1.8 and a ≥1.7 fold change was observed in both conditions.

**Transfection and luciferase activity assays.** The HT29 and DLD1 colon carcinoma cells were transiently transfected with Renilla constructs and 5 μg of either TOPflash or FOPflash TCF reporter constructs (Upstate Biotechnology) using Lipofectamine and Plus reagent (Invitrogen). Five hours after transfection, complete culture medium was added and the cells were stimulated. Forty-eight hours after stimulation, cells were lysed in lysis buffer (Luciferase Assay System, Promega), and 20 μL of each lysate were

![Figure 1](https://example.com/figure1.png)
monitored for luciferase activity using luciferase assay substrate buffer (Promega). Light units were recorded using a luminometer. Transfection efficiency was determined by cotransfection with the Renilla luciferase reporter vector pRL-CMV (Promega). The resulting transfection efficiency was used to correct for differences in transfection in each culture well in each experiment. All experiments were performed in duplicate and repeated independently thrice.

Western blot analysis. Equal amounts of lysates of HT29 and DLD1 cells were loaded onto SDS-PAGE and subsequently transferred to a polyvinylidene difluoride membrane (Millipore Corp.). The membranes were blocked with TBS supplemented with 0.05% Tween 20 (wash buffer) and 1% vinylidene difluoride membrane (Millipore Corp.). The membranes were independently thrice.

Each sample was corrected for loading by comparison with the appropriate β-actin signal.

Small interfering RNA transfection. SignalSilence GSK3 siRNA kit (Cell Signaling Technology) was used to down-regulate GSK3α/β protein in CRC cells. In brief, HT29 cells were transfected with siRNA (final concentration of 100 nmol/L) using transfection reagent (Cell Signaling Technology). After 12 h, the cells were treated with celecoxib. After 2 days, cells were harvested and subjected to Western blot analysis. Control cells were transfected with nonspecific siRNA and cultured under the identical conditions.

Results

The effects of celecoxib on viability of CRC cell lines. Before embarking on peptide array analysis of celecoxib effects on cellular physiology, we decided to first characterize the effects of NSAID treatment on cell proliferation and survival in our model system using a MTT assay. Celecoxib potently reduced mitochondrial activity on 72 h of incubation, which was observed in both HT29 cells (COX-2 positive) and DLD1 cells (COX-2 negative), whereas the administration of aspirin or NS398, another selective COX-2 inhibitor, had little effect (Fig. 1A). This supports the well-described phenomenon that the anticancer activity of celecoxib is more potent than other NSAIDs and is COX-2 independent (3).

Interestingly, it was noted that the effects of celecoxib on cellular viability were dependent on the amount of FCS present in the medium. Higher amounts of FCS reduced the celecoxib-induced reduction in mitochondrial activity significantly (Fig. 1B). We concluded that our experimental setup was valid for investigating celecoxib-specific effects on CRC cell physiology.

Analysis of kinome alterations on aspirin and celecoxib administration in HT29 cells. Because almost all cellular biochemical pathways are under strict control of reversible phosphorylation of rate-limiting enzymes, we assumed that the effects of celecoxib would be reflected in altered activity of cellular kinases. Therefore, we have examined the effects of celecoxib on rapid signal transduction in cell lysates of HT29 cells after short-term treatment with 25 μmol/L celecoxib using a peptide array containing 1,176 different, spatially addressed oligopeptide kinase substrates in duplex as described (24–26). After in vitro phosphorylation of the peptide substrates by kinase activities in cell lysates and [γ-33P]ATP and substrate phosphorylation quantification, the Spearman’s correlation between independently performed experiments was always in excess of 0.85. Therefore, the data were considered as valid for analysis of the celecoxib-induced effects on CRC cell kinase activity (n = 3). Thirty minutes of celecoxib treatment resulted in significant changes in the cellular phosphorylation patterns of HT29 cells, reflecting the potent effects of celecoxib on CRC cellular signal transduction (Fig. 2). Table 1 provides the top 20 affected substrates. Strikingly, a celecoxib-dependent, significant decrease in phosphatidylinositol 3-kinase (PI3K)-associated signal transduction, including reduced phosphorylation of substrates for IGFR/c-Met, protein kinase Cζ (PKCζ), and Akt. In agreement, a concomitant increase in phosphorylation of a substrate for GSK3β was apparent after celecoxib treatment. Taken together, the peptide array analysis suggests that celecoxib acts on growth factor–related signal transduction by inhibition of IGFR-induced and c-Met–induced kinase activity.

Dephosphorylation of c-Met on the Y1234 autophosphorylation site is induced by celecoxib in HT29 CRC cells. The results of the peptide array indicate that celecoxib inhibits IGFR and c-Met autophosphorylation followed by diminished activation of kinases downstream in the PI3K signaling pathway (Table 1). To confirm
Table 1. Peptide substrates with significantly altered phosphorylation by lysates from CRC cells treated with selective COX-2 inhibitor for 30 min (Cont’d)

<table>
<thead>
<tr>
<th>Fold change</th>
<th>PEP_NR</th>
<th>Sequence</th>
<th>PH_SITE</th>
<th>Kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.77</td>
<td>961</td>
<td>PRKGSPKRG</td>
<td>S-23</td>
<td>Sperm-specific</td>
</tr>
<tr>
<td>3.20</td>
<td>184</td>
<td>LRRASLGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.70</td>
<td>69</td>
<td>GDSSYKNI</td>
<td>Y-697</td>
<td>c-Fms</td>
</tr>
<tr>
<td>5.64</td>
<td>891</td>
<td>EAVTSPRF</td>
<td>S-31</td>
<td></td>
</tr>
<tr>
<td>5.65</td>
<td>845</td>
<td>YTRFSLARQ</td>
<td>S-24</td>
<td>PKC</td>
</tr>
<tr>
<td>17.99</td>
<td>242</td>
<td>VTPRTPPPPS</td>
<td>T-97</td>
<td>ERT</td>
</tr>
</tbody>
</table>

NOTE: The fold induction of each substrate, their corresponding protein kinases, and chip peptide number are provided. Abbreviation: PKA, protein kinase A.

this observations, short-term (30 min) incubation experiments were performed in HT29 cells and phosphorylation levels were evaluated by Western blot analysis using phosphospecific antibodies (Fig. 3A). In agreement with the peptide array analysis, celecoxib completely eliminated c-Met Y1234/5 autophosphorylation within a 30-min time frame. As the peptide substrate used in the array does not distinguish well between the c-Met receptor and INSR, the autophosphorylation status of the INSR was investigated, providing functional validation. Only a minor decrease in INSR autophosphorylation was observed (possibly the result of cross-reactivity of the INSR antibody with pY1234/5 c-Met; data not shown). The EGFR phosphorylation was not affected. Important regulating kinases involved in other pathways, such as Src and Lck, were altered in activation, as shown by their phosphorylation status. Moreover, the activity of PTEN, the phosphatase regulating the PI3K signal transduction, was not changed (Fig. 3A) on celecoxib administration, indicating a direct and specific effect on c-Met and validating the results of the peptide array.

Kinetic analysis of celecoxib action on impaired c-Met Y1234/5 autophosphorylation showed that, already after 2 min after celecoxib treatment, a markedly reduced autophosphorylation is evident (Fig. 3B). In addition, a concentration-dependent decrease of phospho-c-Met by celecoxib was observed (Fig. 3C). This effect of celecoxib on c-Met autophosphorylation was corroborated by experiments in which we directly addressed the phosphorylation status of the adaptor protein Gab-1, which is activated on c-Met signal transduction, by Western blot analysis using phosphospecific antibodies (Fig. 3A). In agreement with the peptide array analysis, celecoxib completely eliminated c-Met Y1234/5 autophosphorylation within a 30-min time frame. As the peptide substrate used in the array does not distinguish well between the c-Met receptor and INSR, the autophosphorylation status of the INSR was investigated, providing functional validation. Only a minor decrease in INSR autophosphorylation was observed (possibly the result of cross-reactivity of the INSR antibody with pY1234/5 c-Met; data not shown). The EGFR phosphorylation was not affected. Important regulating kinases involved in other pathways, such as Src and Lck, were altered in activation, as shown by their phosphorylation status. Moreover, the activity of PTEN, the phosphatase regulating the PI3K signal transduction, was not changed (Fig. 3A) on celecoxib administration, indicating a direct and specific effect on c-Met and validating the results of the peptide array.

Kinetic analysis of celecoxib action on impaired c-Met Y1234/5 autophosphorylation showed that, already after 2 min after celecoxib treatment, a markedly reduced autophosphorylation is evident (Fig. 3B). In addition, a concentration-dependent decrease of phospho-c-Met by celecoxib was observed (Fig. 3C). This effect of celecoxib on c-Met autophosphorylation was corroborated by experiments in which we directly addressed the phosphorylation status of the adaptor protein Gab-1, which is activated on c-Met signal transduction, by Western blot analysis using phosphospecific antibodies (Fig. 3A). In agreement with the peptide array analysis, celecoxib completely eliminated c-Met Y1234/5 autophosphorylation within a 30-min time frame. As the peptide substrate used in the array does not distinguish well between the c-Met receptor and INSR, the autophosphorylation status of the INSR was investigated, providing functional validation. Only a minor decrease in INSR autophosphorylation was observed (possibly the result of cross-reactivity of the INSR antibody with pY1234/5 c-Met; data not shown). The EGFR phosphorylation was not affected. Important regulating kinases involved in other pathways, such as Src and Lck, were altered in activation, as shown by their phosphorylation status. Moreover, the activity of PTEN, the phosphatase regulating the PI3K signal transduction, was not changed (Fig. 3A) on celecoxib administration, indicating a direct and specific effect on c-Met and validating the results of the peptide array.
Together, these results show that the findings of the peptide array correspond with those of conventional techniques. Selective COX-2 inhibition by celecoxib efficiently down-regulates c-Met receptor activity, resulting in both decreased activity of the extracellular signal-regulated kinase (ERK) kinases and PI3K pathway, the latter evident from decreased Akt and GSK3β phosphorylation. These results also strongly suggest that the celecoxib-induced inhibition of c-Met–dependent signaling decreases the level of Wnt activity in colon cancer cells because the levels of phospho-β-catenin are increased on celecoxib administration.

Wnt signaling is decreased by celecoxib. The results described above suggest that c-Met activation by phosphorylation is a relevant target for celecoxib with respect to CRC cell cytotoxicity but leave the mechanism by which diminished c-Met activation may have an anticancer effect unexplored. As it is well established that activity of the Wnt pathway is critical factor for CRC cell survival and proliferation, the effects of celecoxib on the activity of this pathway were determined. The major defect in the regulation of the Wnt pathway in APC-mutant cells is the decrease in levels of phospho-β-catenin, causing diminished breakdown of β-catenin by the proteosome and subsequent increased nuclear translocation of the protein. Interestingly, celecoxib-induced c-Met dephosphorylation was followed by a substantial increase of the levels of phospho-β-catenin (Fig. 3C). Because phosphorylation of β-catenin enables breakdown of the protein and thus to a decrease of TCF-β-catenin–dependent transcription, the effect of celecoxib on the transcription activity of TCF-β-catenin was explored. Therefore, DLD1 and HT29 cells were transfected with a specific TCF reporter (TOP) construct and corrected results for cell death using a constitutive Renilla firefly construct as well as by using a construct containing a scrambled TCF-responsive motif (FOP). Following celecoxib treatment for 36 h, β-catenin/TCF–activated TOPflash activity was abrogated in both DLD1 and HT29 cells (Fig. 4A). The inhibition of TCF-mediated transcription was observed after treatment of relative low concentrations (12.5 μmol/L) of celecoxib by HT29 cells, whereas 25 μmol/L was needed in DLD1 cells.

This indicates a specific effect of celecoxib on the TCF-mediated transcription in both HT29 and DLD1 cell lines, which coincides with the increase in β-catenin phosphorylation. In agreement, a dose-dependent, celecoxib-induced down-regulation of the established Wnt targets, Myc and cyclin D1, was observed on protein levels (Fig. 4B). Thus, the inhibition of c-Met signaling by

---

**Figure 3.** Western blot analysis confirms kinomic analysis and identifies Met and downstream Akt, GSK3β as a target for COX-2 inhibition. A, Western blot analysis with phosphospecific antibodies of HT29 cells incubated with celecoxib or control for 30 min confirms the results of the peptide array. B, kinetic analysis of celecoxib action on impaired Met Tyr1234/5 phosphorylation showed that, already after 2 min after celecoxib treatment, a markedly reduced phosphorylation is evident. C, the decrease of phospho-Met by celecoxib is concentration dependent. D, GSK3β mediates the celecoxib-induced phosphorylation of β-catenin (Ser37-39). HT29 cells were transfected with siRNA against GSK3β and subsequently treated with celecoxib for 30 min. It is shown that celecoxib treatment did not result in increased β-catenin phosphorylation in cells transfected with GSK3β siRNA (left three lanes) compared with cells transfected with scrambled siRNA as a control (right three lanes). When transfected with siRNA, GSK3β is still present but, after 36 h, in less quantity. Celecoxib did not induce β-catenin phosphorylation in combination with GSK3β siRNA, whereas the cells transfected with scrambled siRNA show phosphorylation of β-catenin on celecoxib treatment.
celecoxib coincides with reduced activity of Wnt\(/-\)-catenin pathway.

**Celecoxib induces decreased activity of GSK3\(\beta\) resulting in increased phospho-\(\beta\)-catenin.** The observed increase in the phosphorylation of \(\beta\)-catenin together with decreased phosphorylation of both Akt and one of its downstream targets, GSK3\(\beta\), suggest that the GSK3\(\beta\) enzyme has remaining functional activity to the phosphorylation of \(\beta\)-catenin in cell lines with an APC mutation. To investigate whether GSK3\(\beta\) is essential for the observed increase in phosphorylation of \(\beta\)-catenin on COX-2 inhibition, despite the presence of the APC mutation, HT29 cells were transfected with siRNA against GSK3\(\beta\) and subsequently treated with celecoxib for 30 min. In Fig. 3D, it is shown that celecoxib treatment did not result in increased \(\beta\)-catenin phosphorylation in cells transfected with GSK3\(\beta\) siRNA compared with cells transfected with scrambled siRNA. These results show that GSK3\(\beta\) is functionally active and necessary to induce phosphorylation of \(\beta\)-catenin in these cells. In addition, treatment with LiCl, an inhibitor of GSK3\(\beta\), did result in similar decreased \(\beta\)-catenin phosphorylation (data not shown). As shown in Fig. 3D, celecoxib did not induce \(\beta\)-catenin phosphorylation in combination with GSK3\(\beta\) siRNA. This confirms that GSK3\(\beta\) mediates the phosphorylation of \(\beta\)-catenin and suggests that the effects of celecoxib on \(\beta\)-catenin/TCP-dependent transcription are mediated upstream of GSK3\(\beta\).

**c-Met activation enhances Wnt signaling in colon cancer cell lines.** To determine whether HGF was able to stimulate TCF transcription, by stimulating c-Met, HT29 cells were transfected with a TCF reporter vector, TOPflash, which contains a combination of TCF-binding elements. Exposure to HGF increased the transcriptional activity of TOPflash \(\sim 10\)-fold (Fig. 5). As a positive control, cotransfection with a LEF-1 expression vector increased the activity of TOPflash \(\sim 40\)-fold. In contrast, the reporter construct containing mutated TCF sites, FOPflash, had very low activity in HT29 cells; treatment with HGF did not significantly increase luciferase activity of this construct. To investigate the effect of endogenous HGF on TCF activity, we treated HT29 cells with PHA665752, a selective c-Met kinase inhibitor. Previous studies have shown that the PHA665752 is

![Figure 4](image-url)

**Figure 4.** Wnt activity is decreased in DLD1 and HT29 cells on celecoxib administration. A, TCF reporter activity following treatment of HT29 and DLD1 cells. Treatment with increasing concentrations of celecoxib for 36 h. Cells were lysed and TOPflash and FOPflash activities were recorded in a luminometer. Both figures display the relative luciferase activity of cells transfected with TOPflash or FOPflash. B, after 48 h, a dose-dependent celecoxib-induced down-regulation of the established Wnt targets, Myc and Met, was observed in protein levels as determined by Western blot analysis. Actin levels are shown as a loading control.

![Figure 5](image-url)

**Figure 5.** Met activation stimulates Wnt activity. HT29 and DLD1 cells were treated with PHA665752, a selective Met kinase inhibitor, to investigate the effect of endogenous HGF and subsequent Met activation on TCF/\(\beta\)-catenin activity as displayed by the TOP/FOP ratio. All transfections were corrected for Renilla transfection efficiency. The addition of Met inhibition in the presence of HGF completely abrogated the effect of HGF on TCF transcriptional activity.
specific for c-Met, not blocking other kinases. It is shown that the addition of PHA665752 in the presence of HGF completely abrogated the effect of HGF on TCF transcriptional activity, thereby confirming that the HGF-induced effect is specific and acts by activation of c-Met.

Discussion

Although the antagonistic effect of COX-2 inhibitors on the mortality from CRC is undisputed, the nature of the underlying molecular mechanism remains unestablished. In the present study, we have aimed to clarify this issue by investigating the effects of celecoxib, a COX-2 inhibitor with both preventive and therapeutic properties, on phosphoregulated signal transduction in CRC cells without a priori assumptions as to the kinase pathways affected by the treatment. To this end, we used a relatively novel peptide array approach investigating multiple kinase activities in parallel in combination with conventional techniques to validate the findings and to translate kinase activity to mechanisms in CRC. The results obtained suggested that celecoxib targets c-Met autophosphorylation, resulting in impaired downstream activity of the Gab/PI3K pathway.

The identification of c-Met autophosphorylation and subsequent signaling through the PI3K pathway as a target for celecoxib was unexpected but makes sense as viewed from the body of currently available literature. The expression of c-Met is an important prognostic factor for CRC progression and metastasis (12, 31, 32). Similar results are found for other adenomatous cancer types. Furthermore, PI3K activity is a well-known phenomenon in CRC and literature is available showing that celecoxib treatment reduces activity of this pathway (33). The results presented in the current study suggest that activity of this pathway is partly driven by the basal c-Met activity. We have previously shown that c-Met expression can be regulated by the Wnt pathway, whereas also data have been presented that activation of c-Met results in enhanced activity of the Wnt pathway (12, 23, 32). Thus, a positive feedback between activation of the pathway on one hand and activity of c-Met signaling on the other hand seems to exist. This notion is reinforced by our observation that celecoxib treatment resulted in a rapid reduction of c-Met phosphorylation and subsequent downstream signaling through both ERK and Akt and GSK3β. This decrease is followed by a diminished transcriptional activity of the Wnt pathway as assessed by the TOP/FOP assay and expression levels of established Wnt targets as well as by the stimulation of β-catenin phosphorylation in c-Met-treated cells. Although we have shown that presence of GSK3β is essential for the c-Met-induced phosphorylation of β-catenin, these findings are in disparity with the presence of APC mutations in these cells, which are generally assumed to abolish the capacity of GSK3β to interact and thus phosphorylate β-catenin (34). However, recently, Li et al. (35) showed that GSK3β is still capable of regulating phosphorylation of β-catenin despite the presence of an APC mutation in CRC cells, supporting our findings. In mammary epithelial cells with a functional APC complex, HGF-dependent inhibition of GSK3β activity results in diminished phosphorylation of β-catenin and increased nuclear accumulation of the protein (36). In our study, we observed an increased GSK3β activity following celecoxib treatment in the peptide array experiments. These results were confirmed by Western blot analysis showing that celecoxib diminishes phosphorylation of GSK3β, an event associated with increased GSK3β activity (37). Thus, it is possible that also in CRC cells harboring APC mutation increased GSK3β activity mediates the increase of β-catenin phosphorylation and subsequent decreased transcriptional activity of the Wnt pathway. Hence, a possible interpretation of the present study is that inhibition of c-Met autophosphorylation followed by reduced c-Met activation contributes to celecoxib-dependent growth reduction and cytotoxicity in CRC cells.

To translate the results shown in vitro, we have chosen celecoxib concentration that can be achieved in vivo as shown in serum from patients (38). Furthermore, we have previously shown in a clinical phase 2 trial in patients with esophageal adenocarcinoma that both COX-2 and Met expression in cancer tissue, both highly expressed in esophageal adenocarcinoma, were down-regulated after 4 weeks of celecoxib treatment (17). A phase 2 trial in which patients with CRC have been treated with celecoxib neoadjuvantly is currently under investigation to show molecular effects in tumor tissue in vivo.

The mechanism by which celecoxib influences c-Met autophosphorylation remains unclear as the effect of celecoxib is overcome by high concentrations of HGF. Diminished prostaglandin E2 (PGE2) production by inhibition of the COX-2 activity may be implicated. Indeed, PGE2 enhances activation of c-Met, possibly via activation of prostanoid receptors, in turn cross-linking growth factor receptors and subsequent downstream signal transduction (18, 36). The observation that HGF is capable of inducing Wnt signaling, which can be reversed on c-Met inhibition, provides evidence that c-Met inhibition is essential for the effects described in this study regardless of PGE2.

The present study shows that corepression or c-Met and β-catenin oncogenic signal transduction is a major effector of c-Met-induced effects in CRC cells. Despite the negative reports showing an increased cardiovascular risk of COX-2 inhibitors, which indeed negates the use of COX-2 inhibitors for CRC prevention, celecoxib administration as (neo-) adjuvant therapy or in combination with other drugs for advanced disease could be of therapeutic benefit for patients with CRC. The importance of c-Met as a target for selective therapy in patients with CRC will need further clinical investigation.

Acknowledgments

Received 9/5/2007; revised 11/28/2007; accepted 12/19/2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank J. Joore (Pepscan Systems, Lelystad, the Netherlands) for the technical support.

References


Cyclooxygenase-2 Inhibition Inhibits c-Met Kinase Activity and Wnt Activity in Colon Cancer


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/68/4/1213

Cited articles
This article cites 38 articles, 20 of which you can access for free at:
http://cancerres.aacrjournals.org/content/68/4/1213.full#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/68/4/1213.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.