Celecoxib Toxicity Is Cell Cycle Phase Specific

Jonathan M. Bock, Sarita G. Menon, Lori L. Sinclair, Nichole S. Bedford, Prabhat C. Goswami, Frederick E. Domann, and Douglas K. Trask

Abstract

Celecoxib inhibits proliferation and induces apoptosis in human tumors, but the molecular mechanisms for these processes are poorly understood. In this study, we evaluated the ability of celecoxib to induce toxicity in head and neck squamous cell carcinomas (HNSCC) and explored the relationships between celecoxib-induced cell cycle inhibition and toxicity in HNSCC. Celecoxib inhibited the proliferation of UM-SCC-1 and UM-SCC-17B cells both in vitro and in vivo, accompanied by G1 phase cell cycle arrest and apoptosis. Celecoxib induced p21waf1/cip1 at the transcriptional level independent of wild-type p53 function, leading to decreased expression of cyclin D1 and hypophosphorylation of RB, with subsequent marked downstream decreases in nuclear E2F-1 protein expression and E2F transactivating activity by luciferase reporter assay. Cell cycle phase–specific cytometric sorting showed that celecoxib induced clonogenic toxicity in vivo in UM-SCC-1 and UM-SCC-17B cells both in vitro and in vivo cell cycle growth, G1 cell cycle phase accumulation, and apoptosis. Levels of p21waf1/cip1 and cyclin D1 protein were reduced in the S phase compared with the G1 and G2 phases, suggesting a possible protective role for p21waf1/cip1 expression in celecoxib toxicity. In conclusion, we show that celecoxib has marked antiproliferative activity against head and neck cancer cells through transcriptional induction of p21waf1/cip1 and G1 phase accumulation leading to S phase–specific clonogenic toxicity. We additionally show that a profound inhibition of the E2F transactivating activity and subsequent cell cycle inhibition of nuclear E2F function provides a possible mechanism for this S phase–specific toxicity. [Cancer Res 2007;67(8):3801–8]

Introduction

Over 27,000 new cases of squamous cell carcinoma of the head and neck (HNSCC) will be diagnosed in the United States this year, and surgery, chemotherapy, and radiation therapy remain the hallmarks of modern HNSCC treatment (1). Despite dramatic improvements in surgical technique, radiation delivery, and chemotherapy regimens, overall survival rates for this disease have remained nearly constant for the last 40 years. Any therapeutic modalities that would increase curative outcomes without additional toxicity would be of great benefit.

Cyclooxygenase-2 (COX-2) inhibition has emerged as a novel molecular target for the prevention and treatment of head and neck cancers with this goal in mind (2). COX-2 is the key inducible enzyme in the conversion of arachidonic acid into prostaglandins (3) and seems to be elevated in a number of human cancers (4–8), including HNSCC (9). Celecoxib, a selective COX-2 inhibitor, is currently Food and Drug Administration–approved for chemoprevention in familial adenomatous polyposis based on demonstrable inhibition of colon polyp formation, further solidifying the use of this agent in cancer therapy (10). Recent large-cohort clinical trials have shown the significant activity that celecoxib has in vivo against colorectal adenomas (11). These same studies have also shown a risk of cardiac toxicity with long-term administration of celecoxib at high doses, and authors of these studies suggest that the significant antineoplastic benefits of celecoxib need to be balanced against these toxicities in human cancer treatment protocols (12, 13). It seems likely that long-term use of nonsteroidal anti-inflammatory drugs (NSAID) for chemoprevention may not be possible due to these toxicity issues, but short-term combination therapies with standard agents may significantly augment standard cancer treatments. A clearer understanding of the mechanism of the toxicity of celecoxib will allow for improved clinical outcomes while limiting treatment morbidity.

NSAIDs have been shown to markedly alter cell cycle kinetics in a number of human tumors, and cell cycle alteration seems to be a major contributing factor in celecoxib-mediated cancer toxicity. Celecoxib usually inhibits cell cycle progression through the G1 phase, and this inhibition of proliferation seems to be independent of COX-2 enzyme inhibition in breast cancer models (14). Ovarian tumors are similarly blocked in G1 following treatment with NS-398 (a COX-2–specific inhibitor), and this effect also seems to be independent of COX-2 inhibition (15). This implies that the actual mechanism underlying the antiproliferative activity of NSAIDs may be independent of COX-2 inhibition. The cellular factors involved in this cell cycle inhibition and how these changes affect the subsequent toxicity of celecoxib and other NSAIDs are not clearly known. Celecoxib alters the levels of several important G1 cell cycle checkpoint proteins including p21waf1/cip1 (p21) and cyclin D1, and this may mediate the activity of this drug (14, 16). NSAIDs have additionally been shown to induce peroxisome proliferator–activated receptor (PPAR) transcription factor family members (17), and PPAR-γ antisense oligonucleotides have been shown to abrogate the antiproliferative effects of NSAID compounds. These data propose a possible mechanism for the antineoplastic activity of NSAIDs apart from COX-2 inhibition through cell cycle inhibition by a transcriptional activation system. Unfortunately, few studies have confirmed these mechanistic patterns in HNSCC, and the connections between these pathways in any cell system remain unclear at best.

In this paper, we show that celecoxib has strong antiproliferative and toxic activity against HNSCC cells, shown by decreased in vitro and in vivo cellular growth, G1 cell cycle phase accumulation, and apoptosis. We show that celecoxib induces expression of both PPAR-γ and p21 protein independent of wild-type p53 activity, and that the induction of p21 occurs at the transcriptional level. We further show that these G1 checkpoint protein alterations lead to a marked decrease in E2F activity and subsequent cell cycle...
phase-specific toxicity in the S phase, correlating with p21 expression changes across the cell cycle phases. To date, no one has shown cell cycle phase-specific toxicity of any NSAID agent, and this describes a connection between the cytostatic and toxic effects of celecoxib treatment. This cell cycle phase-specific toxicity also points toward a potential avenue for therapeutic synergism by combining celecoxib with treatment modalities that are active in the S and G2 phases, including radiation therapy and chemotherapy.

**Materials and Methods**

**Cells and reagents.** All drugs and biochemicals were supplied by Sigma-Aldrich Fine Chemical Co. (St. Louis, MO), unless otherwise specified. Celecoxib was purchased from LKT Laboratories (St. Paul, MN). Celecoxib was prepared as a 0.1 mol/L stock solution in DMSO. Tissue culture reagents were supplied by Invitrogen Corporation/Life Technologies Life Sciences (Carlsbad, CA). The UM-SCC-1 and UM-SCC-17B oral cancer cell lines were generously provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). All cell lines were maintained as monolayer cultures in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, and 1% non-essential amino acids.

**Proliferation assays.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were done according to standard published protocols (18). Cells were incubated with drug and harvested after 96 h of drug exposure, followed by exposure to the MTT reagent (500 µg/mL) and data analysis. Relative MTT absorbance was measured at 570 nm on a Syva Autotak ELA Autoreader (Bio-Tek Instruments, Inc., Winooski, VT). Growth curve assays were done according to standard parameters. For xenograft assays, 6- to 8-week-old athymic nu/nu female mice (National Cancer Institute, Bethesda, MD) received bilateral flank injections of 1 × 10^6 HN5 SCC cells. For growth analysis, mice were immediately started on dietary celecoxib Chow (1,500 ppm in irradiated NIH 31 Chow, Harlan Teklad, Madison, WI) or control Chow (NIH 31, Harlan Teklad). For gavage experiments, mice received daily oral gavage with a solution of 1,500 mg/kg/day celecoxib in 0.5% methylcellulose or control methylcellulose gavage for 14 days. Tumor volume was calculated using the following equation: tumor volume = π/6 × (large diameter) × (small diameter)^2. All animal experiments were done according to University of Iowa Animal Care Facility protocols.

**Flow cytometry assays.** For cell cycle phase-sorting experiments, cells were incubated with 5 µmol/L Hoechst 33342 (Calbiochem, San Diego, CA) for 1 h at 37°C and filtered through 70-µm mesh. Cells were sorted on a Becton Dickinson (Mountain View, CA) FACS Diva using 100 µW at 488 nm from a Coherent 90C-4 argon ion laser for generation of forward- and side-scatter signals and 100 mW of multi-line UV from a Coherent I90C-4 argonion laser (Coherent, Santa Clara, CA). Images were captured using an Olympus BX51 microscope (Olympus, Center Valley, PA) and analyzed using FlowJo software. For cell cycle phase analysis, cells were harvested after 96 h of celecoxib treatment, fixed in 70% ethanol, and stained with propidium iodide. Flow cytometry was done according to published protocols (19). DNA content was measured by propidium iodide (PI)-staining for sub-G1 DNA accumulation which was done using a FACs Canto (BD Biosciences, San Jose, CA) flow cytometer.

**Immunoblotting.** Protein lysates were generated in reducing lysis buffer and boiled for 7 min. Nuclear extracts were prepared according to standard parameters. Protein content was measured by Bradford assay. A total of 20 µg of protein was loaded on 12% agarose gels (except 7.5% for Rb gels) according to standard SDS-PAGE protocol. Proteins were transferred to a nitrocellulose membrane for 1 h according to standard protocol. Equal protein loading was confirmed via Ponceau S staining of nitrocellulose. Blots were probed for 1 h with primary antibody diluted in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T), followed by 3 × 10 min washes. Primary antibodies included COX-1 (1:200 dilution, Cayman Chemical Company, Ann Arbor, MI), COX-2 (1:250, Cayman Chemical Company), p21^Waf1/Cip1 (1:250, BD PharMingen), p53 (1:500, BD PharMingen), Rb (1:250, BD PharMingen), cyclin D1 (1:333, BD PharMingen), E2F-1 (1:250, Santa Cruz Biotechnology, Santa Cruz, CA), PPAR-γ (1:250, sc-7273, Santa Cruz Biotechnology) actin (1:2,000, Sigma) and α-tubulin (1:3,000, Oncogene Research Products, San Diego, CA). Blots were then probed with appropriate peroxidase-conjugated secondary antibody (1:5,000–10,000, Jackson Immunoresearch Laboratories, West Grove, PA) in 5% nonfat dry milk in TBS-T for 1 h at room temperature. Blots were washed 5 × 8 min in TBS-T and exposed to enhanced chemiluminescence reagents (Amersham Biosciences, Buckinghamshire, England) followed by radiographic exposure and development. Relative band intensities were determined using CCD camera-based densitometry of bands (Alpha Imager 2200, Alpha Innotech Corporation, San Leandro, CA). Band intensities were first normalized to α-tubulin in each sample, and results were calculated relative to untreated controls.

**Quantitative analysis of p21^Waf1/Cip1 mRNA levels by real-time quantitative reverse transcription-PCR.** p21 mRNA levels were analyzed using TaqMan Universal RT-PCR Master Mix (PE Applied Biosystems, Foster City, CA), and reactions were done and analyzed using an ABI Prism 7700 sequence detector equipped with a 96-well thermal cycle (PE Applied Biosystems). RNA was isolated by TRizol and chloroform extraction (Invitrogen Corporation). About 100 ng of RNA was loaded per PCR reaction. RNA was reverse transcribed (30 min at 48°C, denaturation at 95°C for 10 min), followed by 40 cycles of PCR (92°C for 30 s and 60°C for 30 s). Fluorogenic probes were labeled at 5’ and 3’ ends with a reporter [6-carboxy-fluorescein (FAM)] and a quencher dye [6-carboxy-tetramethylrhodamine (TAMRA)]. Primers for PCR amplification of p21 were 5’-CTGGAGACTCTTACGGTGC-GAA-3’ (forward), 5’-GGCGTTGGAGTGGTAGAATT-3’ (reverse), and 5’-FAM-ACGGCGGCAGACAGATGAAATCT-3’ (fluorescent reporter construct, p21 mRNA levels were then evaluated using real-time quantitative real-time reverse transcription-PCR (RT-PCR) techniques as described above.

**E2F promoter luciferase reporter assays.** Dr. Mark May (University of Michigan, Ann Arbor, MI) graciously provided the 3× E2F luciferase reporter construct. pRL-TK Renilla luciferase reporter plasmid was purchased from Promega (Madison, WI). UM-SCC-1 cells were cultured in 24-well plates and pretreated with a range of celecoxib doses for 6 h. A 4:1 ratio of E2F reporter plasmid to pRL-TK vector was transfected into UM-SCC-1 cells using Effectene transfection reagent (Qiagen, Chatsworth, CA) according to manufacturer's instructions. E2F promoter activity was assayed using the Dual Luciferase Reporter kit from Promega according to manufacturer's instructions. Luciferase reactions were done on a Luminoskan Ascent Luminometer (Thermo Electron Corporation, Helsinki, Finland). Luciferase activity was reported as a ratio of E2F activity relative to untreated control UM-SCC-1 cells in triplicate experiments.

**Data analysis.** All experiments were repeated at least in triplicate, and representative data from a single experiment are presented in this manuscript as means, SD, and SE. Statistical comparisons between experimental groups included the use of a two-tailed Student’s t test and ANOVA. Data points marked with an asterisk indicate P < 0.05.
Results

Celecoxib inhibits HNSCC viability. UM-SCC-1 and UM-SCC-17B cells were treated with a dose range of celecoxib (0–100 μmol/L) for 96 h and assayed for viability using a standard MTT assay (Fig. 1A). Celecoxib induced a strong dose-dependent decrease in MTT absorbance in UM-SCC-1 and UM-SCC-17B cells, with an IC50 of 39.9 ± 1.1 and 35.2 ± 2.3 μmol/L, respectively (averaged from triplicate experiments). We also evaluated the ability of celecoxib to inhibit HNSCC proliferation by standard growth curve assays (Fig. 1B). UM-SCC-1 cells were incubated with a range of doses of celecoxib, and samples were harvested 3, 5, and 7 days after drug addition. Celecoxib administration produced a dose-dependent inhibition of cellular proliferation across a wide dose range. These data show that celecoxib treatment produces strong in vitro inhibition of HNSCC viability and proliferation.

In vivo celecoxib antiproliferative activity was assayed using a mouse xenograft assay (Fig. 1C). Bilateral UM-SCC-1 xenograft tumors were grown on the flanks of athymic nu/nu mice, and treatment mice consumed celecoxib chow (1,500 ppm). Celecoxib administration began on the day of tumor injection, and all mice were allowed continuous access to either drug or control chow throughout the experimental period. Celecoxib strongly inhibited in vivo tumor xenograft growth, inducing nearly a 5-fold decrease in overall tumor size (82%). This decrease in tumor size was statistically significant after 14 days of treatment and remained throughout the duration of the experimental period.

Celecoxib induces apoptosis in HNSCC. The antiproliferative effect of celecoxib can occur through the induction of apoptosis or cell cycle arrest. Therefore, we assessed the ability of celecoxib to induce apoptosis in our HNSCC model by evaluating the levels of active caspase-3, a central and irreversible component of both the cytosolic and mitochondrial apoptosis cascades, following treatment with celecoxib. UM-SCC-1 cells were treated for 36 h with a dose range of celecoxib, fixed in paraformaldehyde, and incubated with a phycoerythrin (PE)-conjugated antibody against active caspase-3. Dose-dependent increases in active caspase-3 levels were seen between 100 and 200 μmol/L celecoxib (Fig. 2A). We also assayed for apoptosis by analyzing sub-G1 DNA formation. Cells were treated with a similar dose range of celecoxib, and dose-dependent accumulation of sub-G1 DNA was observed again between doses of 100 and 200 μmol/L celecoxib (Fig. 2B).

Celecoxib inhibits progression through the G1-S transition via induction of p21. The effects of celecoxib on cell cycle progression in HNSCC were determined using BrdUrd pulse-chase experiments, allowing for clear visualization of G1, S, and G2 populations as previously described (20). UM-SCC-1 cells were treated with 0 to 100 μmol/L celecoxib for 24 h before ethanol fixation and staining. Celecoxib treatment caused a dose-dependent inhibition of cell cycle progression through the G1-S transition, leading to >77% G1 phase accumulation after 24 h of treatment with 100 μmol/L celecoxib (Fig. 3A [histograms] and B [graphic data]). A concomitant decrease in both S phase and G2 phase distributions was noted as G1 populations increased.

Further studies were done to evaluate the specific cellular proteins involved in these observed G1 phase cell cycle rearrangements. Celecoxib administration induced COX-2 expression at high doses, but COX-1 levels did not change appreciably following celecoxib administration. This increase in COX-2 expression was not associated with increased prostaglandin E2 (PGE2) levels by
enzyme-linked PGE$_2$ immunoassay (data not shown). Celecoxib induced p21 in a dose-dependent fashion, increasing to more than 16-fold of baseline expression by 50 µmol/L (Fig. 3C). This increase in p21 protein expression levels was seen within 6 h of initiation of treatment (data not shown). UM-SCC-1 cells have previously been reported to have wild-type p53 activity (22). Wild-type p53 levels were expressed at very low levels and did not change appreciably following celecoxib treatment. Induction of p21 was accompanied by a decrease in cyclin D1 expression and a dose-dependent increase in the predominance of hypophosphorylated Rb, consistent with the previously observed G$_1$ phase accumulation (Fig. 3A). PPAR-γ may also be involved in the activity of NSAIDs (23, 24), and we therefore evaluated the expression of this transcription factor following celecoxib treatment. PPAR-γ protein levels increased in a dose-dependent fashion by more than 40-fold at the highest doses of celecoxib and were increased by even low doses of celecoxib (1–5 µmol/L). These data show that celecoxib strongly induces p21 and PPAR-γ without significant changes in p53 expression, leading to decreased cyclin D1 expression and a shift to the hypophosphorylated state of Rb.

We also evaluated the effects of celecoxib therapy on G$_1$ checkpoint protein expression in a head and neck tumor xenograft model (Fig. 3D). Four athymic nude mice were injected with bilateral xenograft tumors of UM-SCC-17B cells, and tumors were
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Celecoxib induces p21 expression by transcriptional activation. Celecoxib strongly induced p21 expression in our model system of HNSCC, and this seemed to be independent of wild-type p53 activity. To determine the mechanism of this increase in p21 protein levels, we evaluated the expression of p21 mRNA following celecoxib administration using quantitative real-time RT-PCR analysis. Subconfluent UM-SCC-1 cells were treated with 100 μmol/L celecoxib or 0.1% DMSO for 14 h. After drug exposure, actinomycin D was added to all plates to arrest transcription. RNA was extracted at 4, 8, and 12 h, and relative p21 mRNA levels were determined by real-time quantitative RT-PCR. Data are from a representative experiment repeated in triplicate. Bars, SD.

was nearly identical, indicating that the mechanism for the observed increase in p21 mRNA levels was due to transcriptional induction and not to decreased RNA degradation.

Celecoxib treatment induces profound inhibition of E2F activity. We showed above that p21 induction induced a predominance of the hypophosphorylated form of Rb following celecoxib treatment. In this state, E2F should be highly bound to Rb in the cytoplasm of the cell, thereby inactivating E2F activity. We therefore evaluated the transcriptional transactivating activity of the E2F promoter using a luciferase reporter assay (Fig. 5A). UM-SCC-1 cells were pretreated with celecoxib for 6 h before transfection of a synthetic 3× E2F-driven luciferase reporter plasmid and then incubated with the reporter construct overnight along with the drug. Celecoxib treatment produced a dose-dependent decrease in E2F transactivating activity that roughly paralleled the amount of hypophosphorylated Rb present on Western blots (Fig. 3C). At maximal dosing of 100 μmol/L celecoxib, E2F activity decreased by more than 50-fold compared with DMSO-treated controls.

We further evaluated the nuclear expression of E2F-1 following treatment with celecoxib. UM-SCC-1 and UM-SCC-17B cells were treated with 0.1% DMSO or 100 μmol/L celecoxib for 24 h, followed by nuclear protein extraction and Western blotting for E2F-1 (Fig. 5B, inset). Baseline levels of nuclear E2F-1 expression were similar for both cell lines. Celecoxib produced a significant decrease (4–5-fold) in the amount of E2F-1 protein in the nucleus in both cell lines. Thus, celecoxib treatment reduced overall levels of E2F-1 protein within the nucleus and produced a profound decrease in E2F transactivating activity.
Celecoxib specifically kills S phase cells, which correlates with phase-specific alterations in p21 induction. Celecoxib induced apoptosis at doses where we also observed strong inhibition of E2F function and cell cycle rearrangement. To further evaluate the effects of these observed cell cycle rearrangements on celecoxib-induced cytoxicity, we treated UM-SCC-1 cells with 0.1% DMSO and 50 or 100 μmol/L celecoxib for 24 h and then did vital cellular staining with 5 μmol/L Hoechst 33342, followed by cell cycle phase-specific cytometric sorting. This technique allowed for identification and collection of pure cell populations from the G₁, S, and G₂ phases (see Fig. 6D for representative sorting histograms). Cells from each phase were then plated at clonogenic density in the absence of drug and evaluated for growth potential by standard clonogenic survival assay techniques. Figure 6A shows representative clonogenic survival plates seeded at similar density and treated for 24 h with 100 μmol/L celecoxib before sorting and plating, whereas Fig. 6B shows overall experimental data presented in graphic form for celecoxib 50 and 100 μmol/L dosing compared with control plating efficiency. Treatment with 50 μmol/L celecoxib for 24 h before sorting did not cause significant changes in plating efficiency across all cell cycle phases, although a general trend was noted toward decreased plating efficiency in the S phase at this dose (Fig. 6B). In contrast, 24-h treatment with 100 μmol/L celecoxib produced significant changes in plating efficiency across all cell cycle phases, with the most profound toxicity in the S phase (Fig. 6A and B). G₁ phase clonogenic survival was 43.2 ± 12.3% of control survival efficiency, a statistically significant decrease. G₂ phase cells had even lower plating efficiency, with 24.1 ± 2.0% of control efficiency. In contrast, cells from the S phase had a plating efficiency of only 3.64 ± 2.6% of control, more than a 27-fold decrease in plating efficiency compared with control.

We further hypothesized that changes in p21 expression may affect this celecoxib-induced phase-specific toxicity due to effects of p21 expression on cell cycle transition out of the G₁ phase. Using standard SDS-PAGE techniques on protein extracts from pure sorted cell cycle populations, we observed faint expression of p21 in the G₁ and G₂ phase of control cells (0.1% DMSO treated, Fig. 6C). Strong induction of p21 was seen in the G₁ and G₂ phase for 24 h, followed by 30 min of incubation with Hoechst 33342. Cells were trypsinized and sorted by FACS DiVa for cell cycle distribution, allowing for identification and collection of pure cell populations from the G₁, S, and G₂ phases (see Fig. 6D for representative sorting histograms). Cells from each phase were then plated at clonogenic density in the absence of drug and evaluated for growth potential by standard clonogenic survival assay techniques. Figure 6B shows overall experimental data presented in graphic form for celecoxib 50 and 100 μmol/L dosing compared with control plating efficiency. Treatment with 100 μmol/L celecoxib for 24 h before sorting did not cause significant changes in plating efficiency across all cell cycle phases, although a general trend was noted toward decreased plating efficiency in the S phase at this dose (Fig. 6B). In contrast, 24-h treatment with 100 μmol/L celecoxib produced significant changes in plating efficiency across all cell cycle phases, with the most profound toxicity in the S phase (Fig. 6A and B). G₁ phase clonogenic survival was 43.2 ± 12.3% of control survival efficiency, a statistically significant decrease. G₂ phase cells had even lower plating efficiency, with 24.1 ± 2.0% of control efficiency. In contrast, cells from the S phase had a plating efficiency of only 3.64 ± 2.6% of control, more than a 27-fold decrease in plating efficiency compared with control.

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populations after 24 h of treatment with 50 and 100 μmol/L celecoxib, 6- and 8.5-fold, respectively, compared with controls. Expression of p21 in the S phase was barely detectable at both doses. Further blotting for cyclin D1 showed a general trend toward decreased cyclin D1 expression as doses of celecoxib decreased, especially within the S phase. These data show that p21 and cyclin D1 are relatively underexpressed in the S phase of celecoxib-treated cells and suggest that p21 and cyclin D1 expression may perhaps be protective against celecoxib-induced toxicity in the G1 and G2 cell cycle phases.

Discussion

We show for the first time in this study that celecoxib toxicity is targeted specifically to the S phase of the cell cycle. This is of considerable significance because celecoxib is currently being used in clinical trials with agents most active in the S and G2 phases, such as chemotherapeutic agents (docetaxel, ref. 26; cisplatin/5-fluorouracil, ref. 27) and radiation (28, 29). This demonstration of marked toxicity specifically limited to cells within the S phase following celecoxib administration provides a clear basis for additive or synergistic toxicity with agents that function specifically in the S and G2 phases. We also show that this specific toxicity is likely mediated at least in part through profound downstream inhibition of E2F-1 activity within the nucleus because celecoxib induces derangements of G1 checkpoint proteins and downstream sequestration of E2F by hypophosphorylated Rb. Without active nuclear E2F-1 function, crucial S phase activities such as DNA repair are impaired, leading to eventual cell death (30). Indeed, celecoxib has been previously shown to inhibit DNA repair enzymes such as Ku70 through the inhibition of nuclear factor κB, leading to radiosensitization (31). Of note, decreased expression of Ku70 is seen at doses of celecoxib where we routinely observe strong E2F inhibition, suggesting that this E2F inhibition may be involved in downstream inhibition of intracellular DNA repair activity. Further work is needed to clarify these connections between toxicity and E2F activity and how these relate to the observed G1 arrest following celecoxib treatment.

The mechanism for celecoxib-induced specific S phase toxicity may also be related to the relative inability of S phase cells to manage reactive oxygen stress. NSAID compounds are able to markedly increase the formation of intracellular reactive oxygen species (ROS; ref. 32). Celecoxib has previously been shown to increase the formation of ROS in osteosarcoma cells (33). G1 and G2 populations are able to perform silent repair of oxidative DNA lesions through the base excision repair pathway, whereas the S phase remains highly sensitive to perturbations of peroxide and other ROS (34). It is possible that the generation of ROS by celecoxib leads to pronounced toxicity to S phase cells through reactive oxygen toxicity and DNA damage, while leaving G1 and G2 phases relatively protected. Additional studies are needed to clarify the exact relationship between the E2F inhibition that we observe and S phase progression following celecoxib treatment and how this may specifically relate to DNA replication and repair.

We also show for the first time that levels of p21 and cyclin D1 change markedly across the cell cycle phases following treatment with celecoxib, correlating with observed levels of toxicity. In sorted cell cycle populations, p21 was strongly induced in the G1 phase and, to a lesser extent, the G2 phase, and this expression roughly correlates with clonogenic survival within these cell cycle phases. Cells that are overexpressing p21 will arrest in the G1 phase and are then inhibited from further cell cycle progression. We routinely observe significant G1 phase accumulation only at doses of celecoxib sufficient to induce p21 and inhibit E2F function, suggesting that p21 function is important in this G1 blockade. Our data show that it is transit through S phase that is most toxic to celecoxib-treated cells; therefore, p21 induction would offer a protective effect by inhibiting cells from progressing into S phase. One previous study has also shown a similar protective effect of p21 expression in NSAID toxicity. Jung et al. (35) showed that murine thymocytes deficient in p21 were more susceptible to apoptosis following sulindac administration, another NSAID compound. This protective component of p21 expression has also been observed in chemotherapeutic toxicity studies, where cells expressing p21 are inhibited from progressing through S phase and developing polyplody (36). Conversely, it is also likely that overexpression of p21 is at least partly responsible for the inhibition of E2F function that we observe, which may contribute to S phase toxicity (31). In this way, p21 induction may have a dual role in celecoxib toxicity, protecting cells from toxicity by inducing G1 arrest, but inhibiting S phase E2F activity and thereby causing toxicity to cells progressing through S phase.

In conclusion, we show that celecoxib treatment produces marked toxicity in HNSCC cells through transcriptional induction of p21 and E2F-1 inhibition. This toxicity is specifically targeted to S phase cells, leading to a direct mechanism for synergistic toxicity with many common anticancer agents such as chemotherapeutics and radiation. This provides a rationale for the continued use of celecoxib in clinical cancer trials and dictates a basis for developing further combination therapies that exploit this facet of celecoxib-induced toxicity.

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