Celecoxib Prevents Neuroblastoma Tumor Development and Potentiates the Effect of Chemotherapeutic Drugs In vitro and In vivo

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Abstract

Purpose: Neuroblastoma is the most common and deadly solid tumor of childhood. Cyclooxygenase-2 is expressed in clinical neuroblastoma tumors and cell lines and inhibitors of this enzyme induce apoptosis in human neuroblastoma cells in vitro and in neuroblastoma xenografts in vivo. We hypothesized that the cyclooxygenase-2-specific inhibitor celecoxib could enhance the cytotoxic effect of chemotherapeutic drugs currently used in neuroblastoma treatment. Furthermore, we investigated if prophylactic treatment with celecoxib could prevent neuroblastoma tumor development in vivo.

Experimental Design: Neuroblastoma cell cytotoxicity of chemotherapeutic drugs in combination with celecoxib was examined. In vivo, athymic rats carrying established SH-SY5Y xenografts were treated with celecoxib in combination with irinotecan, doxorubicin or etoposide, or with either drug alone. For prevention studies, rats received celecoxib in the diet, 250 to 2,500 ppm, from the time of tumor cell injection.

Results: Celecoxib induced a synergistic or an additive cytotoxic effect in combination with doxorubicin, etoposide, irinotecan or vincristine in vitro. In vivo, treatment with celecoxib in combination with irinotecan or doxorubicin induced a significant growth inhibition of established neuroblastoma tumors. Rats receiving celecoxib in the diet showed a distinct dose-dependent delay in tumor development compared with untreated rats. Plasma levels of celecoxib were comparable with levels obtainable in humans.

Conclusions: Celecoxib potentiates the antitumor effect of chemotherapeutic drugs currently used in neuroblastoma treatment, which argues for clinical trials combining these drugs. Celecoxib could also be a potential drug for treatment of minimal residual disease.

Neuroblastoma, the commonest solid tumor diagnosed in infancy, accounts for 15% of deaths from all childhood cancers. The prognosis is both age and stage dependent, with metastases frequently occurring in older children. For these high-risk neuroblastoma patients, the prospect of long-term survival is poor despite intensive multimodal therapy (1). As survival rates for stage IV disease are still <50%, it is important to develop more effective modes of therapy. Cytotoxic agents currently used in the treatment of neuroblastoma mediate cell death by activating key elements of the apoptotic signaling pathways. The use of new drug combinations that have synergistic effects, particularly if cell specificity in response to the drug combination facilitates better targeting to tumor cells, may therefore lead to important developments in the treatment of neuroblastoma.

Cyclooxygenase-2 (COX-2) and the constitutively expressed equivalent COX-1 are key enzymes responsible for the generation of prostaglandins from arachidonic acid. Whereas COX-1 is expressed constitutively in most tissues and is responsible for the production of prostaglandins controlling normal physiologic functions, COX-2 is induced by mitogenic and inflammatory stimuli. This results in enhanced synthesis of prostaglandins in neoplastic and inflamed tissues (2–5). COX-2 is expressed in several adult epithelial tumors and is linked to tumor-promoting effects, including tumor growth and metastasis, by stimulating invasiveness and angiogenesis, inhibiting apoptosis and immune surveillance, and enhancing drug resistance (3–6). Epidemiologic studies, clinical observations and experimental investigations in transgenic rodents suggest that cyclooxygenases are important in tumor development.
(7–11) and that nonsteroidal anti-inflammatory drugs, which mediate their effects by inhibiting COX-1 and COX-2, may be important drugs for cancer therapy (3–5, 11). Several nonsteroidal anti-inflammatory drugs are now under study for therapeutic effects in adult cancers both in preclinical studies and in clinical phase 2 and phase 3 trials (6, 9, 11–16). Recently, it was shown that neuroblastoma tumors and cell lines express high levels of COX-2 and that nonsteroidal anti-inflammatory drugs have significant effect on neuroblastoma growth in vitro and in vivo (17). These results suggest that COX-2 may be an important target for the therapy of neuroblastoma and possibly other pediatric malignancies.

Angiogenesis, the formation of new blood vessels from an already established microvasculature, is a key contributor to the pathogenesis of several diseases including cancer. There are several studies reporting that COX-2 inhibition is a potent mechanism to reduce angiogenesis, and that celecoxib effectively can decrease tumor angiogenesis and reduce tumor growth of a variety of experimental primary tumors (18–25). Recently, a study showed that treatment of pediatric patients with recurrent or progressive cancer with an oral antiangiogenic chemotherapy regimen (a combination of celecoxib and chemotherapeutic drugs) resulted in a prolonged or persistent disease-free status (26).

The aim of the present study was to investigate the therapeutic potential of celecoxib in combination with chemotherapeutic drugs on neuroblastoma cells in vitro and using an experimental xenograft model of neuroblastoma in vivo. Furthermore, the possibility of using celecoxib as a treatment for minimal residual disease of neuroblastoma was examined.

**Materials and Methods**

**Chemicals.** High-performance liquid chromatography grade methanol, analytic grade ammonium acetate, n-hexane, and diethyl ether were supplied by Merck (Darmstadt, Germany). Sodium dihydrogen phosphate dihydrate was from Fluka (Sigma-Aldrich, St. Louis, MO). Celecoxib and valdecoxib were supplied by Pfizer (La Jolla, CA). A stock solution for liquid chromatography was prepared by dissolving celecoxib in methanol to obtain a concentration of 1,630 μmol/L. Plasma standard samples were prepared by dilution of the stock solution with drug-free plasma at the following concentrations: 50, 100, 200, 400, 800, 1,600, 3,200, and 6,400 nmol/mL. Quality control plasma samples at 300 and 3,000 nmol/L were prepared in the same manner. The samples and standard solutions were stored at −70°C before use.

For in vitro combination experiments, celecoxib was dissolved in DMSO (Sigma-Aldrich, Stockholm, Sweden) and further diluted in PBS (Life Technologies, Inc., Sandy, UT; final DMSO concentration, 0.06-1%). Doxorubicin, etoposide, irinotecan, vincristine, and cisplatin were supplied by the local pharmacy (Apoteket AB, Solna, Sweden) and were further diluted in PBS to the appropriate concentrations. For in vivo oral administration via a gastric feeding tube, celecoxib was dissolved in DMSO to 100 mg/mL and further diluted 10 times in fetal bovine serum (Life Technologies) immediately before administration. For dietary administration, celecoxib was incorporated at 250, 500, 1,500, and 2,500 ppm in the diet equivalent to 35, 70, 210, and 350 mg/kg/d orally, respectively. Irinotecan (20 mg/mL) was diluted in NaCl to a final concentration of 2 mg/mL.

**Blood collection and pharmacokinetic study.** Blood was collected by cardiac exsanguinations in 5-mL EDTA tubes (Apoteket) and kept on ice until centrifugation. Plasma samples were stored at −80°C until analysis. For the pharmacokinetic study, a single dose of 10-mg celecoxib was given to rats by oral gavage and blood samples were collected after 5, 15, 30, 45 min, 1, 1.5, 2, 4, 6, 8, 12, and 24 h, with two rats for each time point. Pharmacokinetic analysis was done by pharmacokinetic modeling. Initial estimates were obtained with the JANA stripping program (27). The final estimates of the pharmacokinetic variables were obtained with the MIMODEL program (version 3.30, Biosoft, Cambridge, United Kingdom).

**Extraction chromatography and liquid chromatography mass spectrometry.** Samples were prepared by adding 25 μL of 0.1 mol/L phosphate buffer solution (pH 2.4) and 50 μL of internal standard (valdecoxib, 4 μmol/L) to 50 μL of plasma in a 4-mL polypropylene tube (Sarstedt, Darmstadt, Germany). To the same tube, 1 mL of n-hexane/diethyl ether (1:1, v/v) was added as the extractant. The tubes were capped, mixed by vortexing for 1 min, and centrifuged at 3,000 × g for 150 s. The supernatant (800 μL) was transferred to a second set of clean tubes and evaporated to dryness under a stream of nitrogen at 40°C. The residue was then reconstituted in 100-μL mobile phase. The chromatographic system consisted of a Waters Acuity UPLC system with an autosampler and a binary solvent delivery system (Waters, Milford, MA). Liquid chromatography was done on a 50 × 2.1-mm Waters Acuity BEH C18 1.7-μm column. Mass spectrometry was done using a Micromass Quattro Premier (Waters, Manchester, United Kingdom) tandem mass spectrometer. The system was controlled by MassLynx version 4.0. The liquid chromatography-tandem mass spectrometry method showed good reproducibility and linearity, with a coefficient of variation of <6% and a correlation coefficient (r) of >0.99. The isotropic mobile phase consisted of 80% methanol in 2 mol/L aqueous ammonium acetate with a flow rate of 0.2 mL/min. Ionization was achieved using electrospray in both positive and negative ionization modes. The injection volume was 2 μL, and the injection interval was 1.2 min. Liquid chromatography-tandem mass spectrometry was done with an autosampler temperature of 8°C, a desolvation gas temperature of 280°C, a source temperature of 120°C, and with a desolvation gas flow of 800 L/h, a cone gas flow of 40 L/h, a collision gas pressure of 3 × 10−3 mbar (argon), and ion energies of 0.9 V for both quadrupoles. Quantitative analysis was done in the multiple reaction monitoring mode with the following transitions: m/z 380→316, 380→296, and 382→362 for celecoxib, and m/z 313→210 and 313→118 for valdecoxib. The dwell time was set to 80 ms for each transition.

**Cell lines and culture conditions.** Neuroblastoma cell lines were grown in Eagle’s MEM (SH-SYSY) or RPMI 1640 [SK-N-BE(2)] supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 μmol/L penicillin G, and 100 μmol/mL streptomyacin (Life Technologies) at 37°C in a humidified atmosphere of 5% CO2. When incubated with drugs, cells were grown in Optimem supplemented with 2 mmol/L L-glutamine, 100 μmol/L penicillin G, and 100 μmol/L streptomyacin (Life Technologies).

**Animals and xenografting.** Male athymic nude rats (HsdHan:Rnunu, Harlan, Horst, the Netherlands), 4 to 6 weeks old, were used for all in vivo xenograft experiments. Rats were housed and maintained in laminar flow cabinets under specific pathogen-free conditions and given sterile water and food ad libitum. For the pharmacokinetic study, male Sprague-Dawley rats (B&K Universal, Sollentuna, Sweden) with an average weight of 299 g (range, 267-318 g) were used. All animal studies done were approved by the regional ethics committee for animal research in accordance with the Animal Protection Law (SFS 1988:541). Doxorubicin (2 mg/mL) was diluted in NaCl to 0.2 mg/mL, and etoposide (20 mg/mL) was diluted in NaCl to a final concentration of 2 mg/mL.

Celecoxib Potentiates Cytotoxic Drugs in Neuroblastoma

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Cytotoxicity assay and analysis of apoptosis by fluorescence-activated cell sorting. The effects of celecoxib in combination with cisplatin, doxorubicin, etoposide, irinotecan, or vincristine were investigated in SH-SY5Y and SK-N-Be(2) neuroblastoma cells. For this purpose, the previously described fluorometric microculture cytotoxicity assay was used (29, 30). Briefly, 384-well microtiter plates (Nunclon surface, NUNC Brand Products, Roskilde, Denmark) were prepared with drug solutions in duplicate at 10 times the desired final drug concentration. The plates were stored at −70°C until use and protected from light during all experimental steps. Cell suspensions were seeded into the drug-prepared microtiter plates at a cell density of 5,000 cells per well and incubated for 72 h at 37°C in a humidified 5% CO2 atmosphere. After incubation, the fluorometric microculture cytotoxicity assay was done using the automated Optimized Robot for Chemical Analysis (Orca, Beckman Coulter, Fullerton, CA) programmed through the software SAMI (Beckman Coulter). The plates were washed; fluorescein diacetate (Sigma-Aldrich) was added; and after 50 min of incubation, fluorescence generated was measured at 485/520 nm in a fluorometer in Fluoroscan II (Labsystems Oy, Vantaa, Finland). The fluorescence is proportional to the number of intact cells in the well. A successful assay required a ratio of >5 between the signal in the control wells and the blank wells and a coefficient of variation of <30% in the control wells. All concentrations were tested in duplicate and the experiments were repeated five to six times.

To investigate whether the combination of celecoxib and chemotherapeutic drugs induced apoptosis in neuroblastoma cells, SH-SY5Y and SK-N-Be(2) cells were seeded in six-well plates and treated with sublethal doses of celecoxib in combination with doxorubicin, etoposide, irinotecan, cisplatin, and vincristine, or each drug alone. Apoptosis was evaluated by flow cytometry of propidium iodide–stained cells recovered from the wells by trypsinization and pooled with apoptotic bodies and nonadherent cells recovered from the culture medium as previously described (31). In short, fluorescence resulting from excitation at 488 nm with a 15-mW argon laser was monitored at 585 ± 21 nm using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). Events were triggered using a double-discriminator parameter to exclude aggregates and a threshold of forward and side scatter was used to exclude debris.

Treatment with celecoxib in combination with chemotherapeutic drugs in vivo. Two independent therapeutic experiments were carried out. In the first experiment, nude rats with established neuroblastoma xenografts (n = 30) were randomized into six groups (five rats in each treatment group) and the drugs were given as follows: (a) celecoxib 10 mg orally daily via a gastric feeding tube; (b) irinotecan 2 mg/kg i.p. every second day; (c) doxorubicin 1 mg/kg i.p. every third day; (d) both celecoxib 10 mg orally daily and irinotecan 2 mg/kg i.p. every second day; (e) both celecoxib 10 mg orally daily and doxorubicin 1 mg/kg i.p. every third day; or (f) no treatment. In the second experiment, nude rats with established tumors (n = 30) were randomly assigned into six groups (five rats in each treatment group) and the drugs were given as follows: (a) celecoxib 10 mg orally daily via a gastric feeding tube; (b) irinotecan 0.75 mg/kg i.p. every second day; (c) etoposide 10 mg/kg i.p. for 5 days; (d) both celecoxib 10 mg orally daily and irinotecan 0.75 mg/kg i.p. every second day; (e) both celecoxib 10 mg orally daily and etoposide 10 mg/kg i.p. for 5 days; or (f) no treatment. Each rat was treated for 12 days, starting when a tumor in the rat had reached a volume of 0.2 to 0.3 mL (mean, 0.26 mL). Tumor dimensions were measured every second day and tumor volume was calculated as previously described (28). Tumor weight was recorded at autopsy and all animals were monitored for signs of toxicity including weight loss and diarrhea during treatment. Blood samples from animals treated with celecoxib were collected at sacrifice.

Effects of dietary celecoxib on the growth of established neuroblastoma tumors. Two independent experiments were done. In the first experiment, nude rats with established neuroblastoma tumors (n = 20) were randomly assigned to receive 500 ppm (n = 5) or 1,500 ppm (n = 5) of celecoxib in the diet, or no treatment (n = 5), for 12 days. For comparison, five animals were randomized to receive 10 mg celecoxib orally daily for 12 days. In the second experiment, nude rats (n = 15) were randomized to receive 12 days of treatment with 250 ppm (n = 5) or 2,500 ppm (n = 5) of celecoxib in the diet, or no treatment (n = 5). Treatment of each rat started when a tumor had reached a volume of 0.2 to 0.3 mL (mean, 0.28 mL). Tumor volume was measured every second day. Tumor weight was recorded at autopsy and all animals were monitored for signs of toxicity including weight loss and diarrhea during treatment. Blood samples were collected at sacrifice.

Effects of dietary celecoxib on neuroblastoma tumor development. Two independent experiments were carried out. In the first experiment, nude rats were randomly assigned to receive 500 ppm (n = 5) or 1,500 ppm (n = 5) of celecoxib in the diet, or no treatment (n = 10). In the second experiment, nude rats were randomized to receive 250 ppm (n = 6) or 2,500 ppm (n = 6) of celecoxib in the diet, or no treatment (n = 19). Treatment was started 2 to 7 days before tumor cell injection and was continued until sacrifice or, in case of no tumor development, until 40 days after tumor cell injection. Tumor development was monitored daily to 60 days after tumor cell injection; a tumor was considered to be established once it had reached a volume of 0.3 mL. Blood samples were collected and tumor weight was recorded at autopsy.

Immunohistochemistry. Proliferation and angiogenesis were evaluated in 5-μm paraffinized tumor sections from treated and untreated rats by staining for Ki-67 and BS-1. The specific Ki-67 (SP6) antibody (NeoMarkers, Fremont, CA) was incubated overnight at 4°C, before a SuperPicture polymer detection kit with appropriate secondary antibodies was used together with diaminobenzidine tetrahydrochloride substrate chromogen system to visualize immunopositivity (Zymed, Invitrogen, Stockholm, Sweden). Proliferation was assessed by counting the number of Ki-67 positively stained nuclei and the total number of cancer cells in four representative regions in three tumors at ×400 magnification. The results are expressed as the proportion of positively stained cells. Biotinylated Bandeiraea Simplicifolia-1 (BS-1, L3759, Sigma-Aldrich) lectin was used for highlighting endothelial cells. BS-1 was diluted 1:50 and incubated overnight at 4°C. Detection was done with ABCComplex conjugated to horseradish peroxidase (K355, Dako A/S, Glostrup, Denmark) diluted 1:100 and incubated for 30 min at room temperature. Sections were developed using diaminobenzidine tetrahydrochloride (SK-4100, Vector Laboratories, Inc., Burlingame, CA), counterstained with Harris’ hematoxylin, and mounted with Kaiser’s glycerol gelatin (Merck). Cell pellets of bovine endothelial cells were used as a positive control and omission of BS-1 was used as a negative control. Three tumor slides per treatment group and four fields per slide were quantified for vessel density at ×200 magnification. Fields were randomly chosen by blindly advancing the goniometer stage and with the inclusion criteria that it had to consist of viable tissue. The results are expressed as average number of microvessels per field.

Statistical analysis. To test for synergistic effects of combination therapy in vitro, data were analyzed using the median-effect method of Chou and Talalay (32) using the software CalcuSyn (version 2; Biosoft). Each dose-response curve (individual agents as well as combinations) was fitted to a linear model using the median effect equation, allowing calculation of a median effect value D (corresponding to IC50) and slope. Goodness of fit was assessed using the linear correlation coefficient and r > 0.85 was set as the criterion for a successful analysis. The extent of interaction between the drugs was expressed using the combination index (CI) for mutually exclusive drugs: CI = (D1 / D1*) + (D2 / D2*), where D1* and D2* represent the concentration of drug 1 and 2 alone required to produce a certain effect, and D1 and D2 are the concentration of drugs 1 and 2 in combination required to produce the same effect. A CI close to 1 indicates an additive effect; CI significantly lower than 1 was defined as synergy; and CI significantly higher than 1 as antagonism. Mutual exclusivity was assumed. One-sample t tests were used to determine if
Celecoxib Potentiates Cytotoxic Drugs in Neuroblastoma

Results

Celecoxib significantly augmented the cytotoxic effect of chemotherapeutic drugs in vitro. To study potential synergistic or additive effects of celecoxib in combination with chemotherapeutic drugs, we used the SH-SY5Y and SK-N-BE(2) neuroblastoma cell lines. Unlike SH-SY5Y cells, the SK-N-BE(2) cell line carries an amplified MYCN, a p53 mutation, and is relatively drug resistant. Both cell lines were treated with increasing concentrations of celecoxib and chemotherapeutic drugs. Single-drug activities in SH-SY5Y and SK-N-BE(2) cells were determined in initial experiments (data not shown). Fixed concentration ratios of the drugs were used with 2-fold serial dilutions in five steps for combination and single-drug treatments. As summarized in Table 1, showing the CI at IC70, celecoxib induced a synergistic or an additive cytotoxic effect in SH-SY5Y and SK-N-BE(2) neuroblastoma cells when used in combination with doxorubicin (IC70; 0.45-0.59; 0.62-0.90), etoposide (IC70; 0.98-1.44; 0.97-1.57), irinotecan (IC70; 0.82-1.36; 0.94-1.37), or vincristine (IC70; 0.45-0.85; 0.74-1.25), respectively. In contrast, celecoxib in combination with cisplatin had an antagonistic effect in both cell lines at the doses used (IC70 2.07-3.63; 1.42-2.68).

To investigate further the cytotoxic effect of treating neuroblastoma cells with the combination of celecoxib and chemotherapeutic drugs, cells were stained with propidium iodide and analyzed for the induction of apoptosis by flow cytometry. An accumulation of cells in sub-G1 phase of the cell cycle was detected in SH-SY5Y and SK-N-BE(2) cells treated with celecoxib in combination with cytotoxic drugs, as exemplified in Fig. 1.

Treatment with celecoxib in combination with chemotherapeutic drugs significantly inhibited growth of established neuroblastoma tumors. After having shown that celecoxib was able to potentiate the effect of chemotherapeutic drugs in vitro, we examined the in vivo activity of these drug combinations. Irinotecan, regardless of dose, inhibited the growth of established neuroblastoma xenografts in terms of tumor volume when used as a single agent (Fig. 2A). The most pronounced growth inhibitory effect was achieved by coadministration of celecoxib and irinotecan (Fig. 2A). Celecoxib, irinotecan, and the combination of both drugs showed a 43%, 86%, and 93% decrease, respectively, in mean tumor volume at day 12, compared with tumors from untreated controls (Fig. 2A). Treatment with doxorubicin alone induced a 42% reduction in tumor growth (Fig. 2B). However, doxorubicin in combination with celecoxib reduced tumor growth by 65% compared with controls (Fig. 2B). These data were analyzed by two-way ANOVA with drug treatment (no drug, irinotecan, and doxorubicin) and in the presence or absence of celecoxib as fixed effects. Results showed that celecoxib had a significant effect of reducing tumor volume regardless of treatment (control, irinotecan, and doxorubicin: ANOVA, effect of celecoxib F1,28 = 8.04, P = 0.008; treatment × celecoxib interaction F2,28 = 0.25, P = 0.78, not significant). However, because of sample size and variability between animals, only the individual comparison of irinotecan versus irinotecan and celecoxib reached statistical significance (F1,28 = 4.84, P = 0.036). Celecoxib did not potentiate the tumor-inhibiting effect of etoposide at the current dose (data not shown). All rats, regardless of treatment, gained in weight during the experiments and showed no signs of toxicity.

Celecoxib inhibited neuroblastoma growth in vivo at plasma concentrations obtainable in humans. To investigate if celecoxib could inhibit neuroblastoma growth in vivo at plasma concentrations obtainable in humans, two therapeutic experiments were done. In the first experiment, nude rats with established neuroblastoma tumors received either 10 mg celecoxib through daily oral gavage or 500 or 1,500 ppm of celecoxib supplemented in their diet corresponding to a daily oral intake of 10 to 15 or 30 to 45 mg of celecoxib, respectively. Blood samples from time of sacrifice showed that celecoxib plasma levels were highest in the animals receiving 1,500 ppm of celecoxib in their diet (Fig. 3A). The area under the curve during 24 h was 78 μmol/L for animals receiving 500 ppm and 145 μmol/L for animals receiving 1,500 ppm in their diet. Rats receiving 10 mg celecoxib daily via a gastric feeding tube as a bolus dose showed low plasma concentrations, as expected, because samples were assessed 24 h after the last oral gavage (Fig. 3A). This low level, measured after 24 h, was confirmed in

<table>
<thead>
<tr>
<th>Combination</th>
<th>SH-SY5Y</th>
<th>SK-N-BE(2)</th>
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<tbody>
<tr>
<td>Celecoxib + doxorubicin</td>
<td>0.52 (0.45-0.59)</td>
<td>0.76 (0.62-0.90)</td>
</tr>
<tr>
<td>Celecoxib + etoposide</td>
<td>1.21 (0.98-1.44)</td>
<td>1.27 (0.97-1.57)</td>
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<tr>
<td>Celecoxib + irinotecan</td>
<td>1.09 (0.82-1.36)</td>
<td>1.15 (0.94-1.37)</td>
</tr>
<tr>
<td>Celecoxib + cisplatin</td>
<td>2.85 (2.07-3.63)</td>
<td>2.05 (1.42-2.68)</td>
</tr>
<tr>
<td>Celecoxib + vincristine</td>
<td>0.65 (0.45-0.85)</td>
<td>1.00 (0.74-1.25)</td>
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*Synergistic and antagonistic effects are defined as a CI mean statistically significantly lower or higher than 1, respectively (one-sample t test, P < 0.05).
a pharmacokinetic study (Fig. 3B) in rats given one single oral dose of 10 mg celecoxib. The half-life of celecoxib was 6.67 h, with a $C_{\text{max}}$ of 11.5 $\mu$mol/L at ~3.2 h (dose dependent) and an area under the curve during 24 h of 116 $\mu$mol/L (Fig. 3B). At sacrifice, mean tumor volumes from celecoxib-treated animals were reduced by 30% to 43% compared with controls (Fig. 3C). In the second experiment, nude rats with established neuroblastoma tumors received 250 or 2,500 ppm of celecoxib supplemented in their diet corresponding to a daily oral intake of 5 to 7.5 or 50 to 75 mg of celecoxib, respectively. At sacrifice, tumor volumes were reduced by 33% or 65%, respectively, compared with controls (Fig. 3D). To assess the efficacy of celecoxib delivered by oral gavage compared with different dietary concentrations, data from the two experiments were combined by expressing tumor growth (expressed as tumor volume) as a percentage of the mean control in the experiment. A significant reduction in tumor volume in response to celecoxib delivered by oral gavage was found when compared with control animals (ANOVA, $F_{5,43} = 2.53$, $P = 0.043$; Dunnett’s test, $P = 0.025$). This reduction was not significantly different from dietary celecoxib at all concentrations (Dunnett’s

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**Fig. 1.** Induction of apoptosis in SH-SY5Y (A–D) and SK-N-Brain (E–H) neuroblastoma cells by celecoxib in combination with vincristine as determined by flow cytometry of propidium iodide–stained cells. Flow cytometry profiles of untreated control cells (A and E), cells treated with 7.5 $\mu$mol/L celecoxib (B and F), cells treated with 6.8 $\mu$mol/L (C) or 890 $\mu$mol/L (G) of vincristine, and cells treated with the combination of celecoxib and vincristine (D and H). Each bar represents the percentage of apoptotic cells. SH-SY5Y cells were exposed to drugs for 48 h, whereas SK-N-Brain cells were exposed for 24 h.

**Fig. 2.** Treatment with celecoxib in combination with cytotoxic drugs induces a significant neuroblastoma tumor growth inhibition in vivo. A and B, neuroblastoma SH-SY5Y xenograft tumor volumes in nude rats. Mean tumor volumes (error bars are not plotted for simplicity but increased in proportion to tumor volume) at tumor take (day 0) and 2 to 12 d from start of treatment. A, ○, untreated control rats (SE, 11-39%); ■, rats treated with celecoxib (SE, 19-71%); ▲, rats treated with irinotecan (SE, 9-46%); ▲, rats treated with the combination of the two drugs (SE, 6-53%). B, ○, untreated control rats (SE, 11-39%); ■, rats treated with celecoxib (SE, 19-71%); ○, rats treated with doxorubicin (SE, 14-58%); ▲, rats treated with the combination of the two drugs (SE, 8-46%). Celecoxib had a significant tumor-reducing effect when used in combination with any of the treatments ($P = 0.008$). The combination treatment with celecoxib and irinotecan was more effective in inhibiting tumor growth than irinotecan alone ($P = 0.036$).
Blood samples at the end of the experiment showed that the concentration of celecoxib in plasma was higher in the animals receiving 2,500 ppm compared with rats receiving 250 ppm (Fig. 3A), with an area under the curve test, $P > 0.25$). Blood samples at the end of the experiment showed that the concentration of celecoxib in plasma was higher in the animals receiving 2,500 ppm compared with rats receiving 250 ppm (Fig. 3A), with an area under the curve test, $P > 0.25$).

Fig. 3. Celecoxib inhibits neuroblastoma tumor growth in vivo at plasma levels obtainable in humans. A, columns, median plasma levels of celecoxib, at sacrifice, for five different treatment groups; bars, SE. B, plasma concentration-time curve of celecoxib after one oral dose of 10 mg celecoxib. Solid line, concentration-time data estimated by pharmacokinetic modeling. C and D, neuroblastoma SH-SY5Y xenograft mean tumor volumes (error bars are not plotted for simplicity but increased in proportion to mean tumor volume) in nude rats at tumor take (day 0) and 2 to 12 d from start of treatment. C, ○, untreated control rats (SE, 11-39%); ■, rats treated with 10 mg of celecoxib once daily by oral gavage (SE, 19-71%); ▲, rats receiving 500 ppm celecoxib in their diet (SE, 9-91%); ●, rats receiving 1,500 ppm of celecoxib in their diet (SE, 17-73%). D, ○, untreated rats (SE, 5-56%); ◀, rats receiving 250 ppm celecoxib in their diet (SE, 22-48%); ▲, rats receiving 2,500 ppm of celecoxib in their diet (SE, 17-51%). Tumors in rats receiving celecoxib through oral gavage grew significantly slower compared with tumors in control animals ($P = 0.025$). There was no significant difference in tumor growth inhibition when celecoxib given as a daily bolus dose was compared with celecoxib provided in the diet at any concentration ($P > 0.25$).

Fig. 4. Celecoxib prevents neuroblastoma tumor development in vivo. A and B, Kaplan-Meier curves showing the percentage of tumor free rats from the day of tumor cell injection. A, untreated rats (solid line), rats receiving 500 ppm (dashed line), and rats receiving 1,500 ppm (dotted line) of celecoxib in their diet from 2 d before tumor cell injection and throughout the experiment. Rats treated with 500 ppm of celecoxib showed a significant delay in tumor development compared with controls ($P = 0.03$); however, treatment with 1,500 ppm was not significantly different from controls ($P = 0.068$). B, untreated rats (solid line), rats receiving 250 ppm (dashed line), and rats receiving 2,500 ppm (dotted line) of celecoxib in their diet from 7 d before tumor cell injection until sacrifice or until 40 d after tumor cell injection. Rats treated with 2,500 ppm of celecoxib showed a significant delay in tumor development compared with controls ($P < 0.001$); however, treatment with 250 ppm was not significantly different from controls ($P = 0.06$). There was a significant dose-dependent delay in tumor development in rats treated with up to 2,500 ppm of celecoxib ($P < 0.001$).
during 24 h of 118 versus 76 μmol/L, respectively. All oral celecoxib treatments were nontoxic and induced tumor growth inhibition at plasma levels that can be readily obtained in humans (33) and at a lower concentration needed to suppress neuroblastoma cell growth in vitro (17, 34).

Prophylactic treatment with celecoxib prevented neuroblastoma development. To investigate whether celecoxib could prevent neuroblastoma tumor development, two different experiments were done wherein rats received celecoxib in their diet from 2 to 7 days before tumor cell injection. The median time to tumor take, defined as the number of days for a tumor in an animal to reach a volume of 0.3 mL, was delayed in all treatment groups compared with tumors in the control group (Fig. 4A and B) and was statistically significant for rats treated with 500 and 2,500 ppm of celecoxib (log-rank tests, $P = 0.03$ and $P < 0.001$, respectively). Dose-dependent effects of celecoxib were investigated by combining the two experiments to test for linearity. There was a significant dose-dependent delay in tumor development in rats treated with up to 2,500 ppm of celecoxib ($P < 0.001$). Fifty percent of rats receiving the highest dose of celecoxib, 2,500 ppm in their diet, had still not developed tumors at the end of the experiment, 60 days after tumor cell injection, although they had been without treatment since day 40 after tumor cell injection (Fig. 4B).

Treatment with celecoxib reduced cell proliferation and inhibited angiogenesis in vivo. Because inhibition of angiogenesis can contribute to a reduced cell proliferation, tumors from animals treated with celecoxib, supplemented in the diet or given once daily by oral gavage, were stained with the proliferation marker Ki-67 and the endothelial cell marker BS-1 and analyzed by two-way ANOVA with respect to drug and celecoxib effects. Treatment with celecoxib resulted in a significant decrease in cell proliferation ($P < 0.001$). Furthermore, celecoxib significantly potentiated the antiproliferative effect of doxorubicin ($P < 0.006$) but not that of irinotecan ($P = 0.65$). 

Fig. 5. Celecoxib reduces cell proliferation and inhibits angiogenesis in vivo. A, percentage of Ki-67 positively stained cells and immunohistochemistry of Ki-67 expression (×400 magnification for all samples) in tumors from untreated control animals and from rats treated with celecoxib (10 mg, once daily by oral gavage), irinotecan (2 mg/kg i.p. every second day), doxorubicin (1 mg/kg i.p. every third day), and a combination of celecoxib/irinotecan or a combination of celecoxib/doxorubicin. Treatment with celecoxib resulted in a significant decrease in cell proliferation ($P < 0.001$). Furthermore, celecoxib significantly potentiated the antiproliferative effect of doxorubicin ($P < 0.006$) but not that of irinotecan ($P = 0.65$). B, microvessel density in tumors from untreated controls and rats treated with celecoxib (10 mg, once daily by oral gavage), irinotecan (2 mg/kg i.p. every second day), doxorubicin (1 mg/kg i.p. every third day), and a combination of celecoxib/irinotecan or a combination of celecoxib/doxorubicin. Immunohistochemistry of BS-1 staining in tumor sections from an untreated control rat and a rat treated with celecoxib (10 mg, once daily by oral gavage), irinotecan (2 mg/kg i.p. every second day), doxorubicin (1 mg/kg i.p. every third day), and a combination of celecoxib/irinotecan or a combination of celecoxib/doxorubicin. Treatment with celecoxib significantly reduced the microvessel density compared with tumors from untreated rats ($P = 0.01$).
compared with control ($F_{1,12} = 21.75, P < 0.001$). The interaction term was significant ($F_{2,12} = 4.22, P = 0.04$), indicating a difference in effect of celecoxib between drug treatments: celecoxib had a significant effect in reducing proliferation of tumors in control or doxorubicin-treated animals (hypothesis tests, $F_{1,12} > 11, P < 0.006$) but not in irinotecan-treated animals ($F_{1,12} = 0.2, P = 0.65$; Fig. 5A). There was also a significant effect of celecoxib in reducing microvessel density (two-way ANOVA, effect of celecoxib, $F_{1,9} = 6.09, P = 0.031$; treatment $\times$ celecoxib interaction nonsignificant, $P > 0.2$). There was a 55% decrease in microvessel density in tumors from animals receiving celecoxib compared with tumors from animals receiving no treatment (hypothesis test $F_{1,9} = 10.41, P = 0.01$; Fig. 5B). For the other drug combinations, the decrease in microvessel density was not significant compared with celecoxib as a single-drug treatment with the present sample size. There was no significant difference in microvessel density between tumors from rats receiving celecoxib by gastric feeding and those from animals receiving celecoxib in the diet (ANOVAs, $F_{1,12} < 2.2, P > 0.16$; data not shown).

**Discussion**

COX-2 is expressed in human malignant tumors (3–6, 35), and we have shown that human neuroblastoma tumors and cell lines express COX-2 and that treatment with COX-inhibitors inhibits neuroblastoma growth both in vitro and in vivo (17). In the present study, we evaluated the potential role of the specific COX-2 inhibitor celecoxib as an adjuvant therapy in human neuroblastoma by asking whether celecoxib could enhance the cytotoxic effect of chemotherapeutic drugs currently used in neuroblastoma treatment (1). Furthermore, we evaluated if prophylactic treatment with celecoxib could prevent neuroblastoma tumor development.

Celecoxib significantly potentiated the cytotoxic effect, by inducing either synergistic or additive cytotoxicity in neuroblastoma cell lines, when used in combination with doxorubicin, etoposide, irinotecan, or vincristine. Interestingly, celecoxib induced an antagonistic effect in combination with cisplatin in neuroblastoma cell lines at the doses used. The accumulation of cells in the sub-G$_1$ phase of the cell cycle in cells treated with celecoxib in combination with chemotherapeutic drugs was an indication that these drug combinations induced apoptosis in neuroblastoma cells in vitro. This is consistent with previous studies showing that chemotherapeutic drugs and celecoxib used as single agents induce apoptosis in neuroblastoma cells in vitro (31, 34). Furthermore, celecoxib has been shown to augment chemotherapeutic drug–induced apoptosis in prostate cancer cells (36).

The potentiating effect of celecoxib was further confirmed in SH-SY5Y xenografts in vivo, wherein celecoxib had a significant effect of reducing tumor growth regardless of treatment combination. Similar results have been shown in human colon cancer xenografts in which celecoxib enhanced the antitumor effect of irinotecan (CPT-11) and reduced the severity of late diarrhea in a dose-dependent manner (37). Furthermore, preliminary results from studies in humans indicate that treatment with COX-2 inhibitors together with conventional chemotherapy may be beneficial for adult cancer patients (38–40). However, recent concerns about cardiovascular risks being associated with selective COX-2 inhibitors have caused some clinical studies to shut down (41–43).

To evaluate different drug administration regimens in vivo, we asked if there was any difference in giving celecoxib as a daily bolus dose (10 mg) compared with a continuous supply in the diet in terms of tumor growth inhibition of established tumors, achieved plasma concentrations, and estimated drug exposure. Average plasma concentrations of celecoxib were highest in rats given dietary celecoxib (1,500 ppm) but this was significantly lower than the peak plasma concentration after the bolus dose. All rats gained in weight during treatment, indicating that neither a high plasma concentration nor a high steady state plasma level of celecoxib was toxic. The pharmacokinetic study showed that maximal plasma concentrations were achieved ~3 h after the oral dose. Interestingly, the area under the curve obtained with 10 mg celecoxib delivered by oral gavage was comparable to that resulting from higher amounts of celecoxib, 5 to 75 mg, continuously provided in the diet. Although celecoxib delivered by oral gavage induced a significant tumor growth inhibition similar to dietary celecoxib at all concentrations, from these results we cannot conclude that a high peak plasma concentration per se is important for the observed antitumor effects in vivo. Clearly, continuously providing celecoxib in the diet, resulting in a steady-state plasma concentration, was also an effective treatment regimen. By supplementing the diet with 250 to 2,500 ppm of celecoxib, we obtained plasma concentrations up to 6,373 nmol/L (2,447 μg/L), which is within the same range as in children receiving 250 mg/m$^2$ celecoxib orally twice daily (33). The plasma levels of celecoxib were not significantly higher in animals receiving 2,500 ppm compared with animals receiving 1,500 ppm of celecoxib in their diet, which may be explained by a large variability in plasma concentrations between different animals within the same treatment group.

The antiangiogenic properties of celecoxib are well established (44–47), and we obtained a significant decrease in microvessel density in tumors from rats treated with 10 mg daily by oral gavage or receiving celecoxib in their diet. Furthermore, the bolus dose was as effective in inhibiting angiogenesis because there was no significant difference in microvessel density between the different treatment regimens. We also detected a significant decrease in tumor cell proliferation in xenografts isolated from rats treated with celecoxib as compared with tumors from untreated rats, and celecoxib significantly increased the antiproliferative effects of chemotherapeutic drugs. These findings are consistent with prior evidence that selective COX-2 inhibitors can suppress the growth of experimental tumors by inhibiting angiogenesis and cell proliferation (44–46, 48–50), and provide further evidence that celecoxib added in the diet or as a bolus dose is equally effective.

COX-2 inhibitors have been implied to have tumor-preventive effects in several human cancers, and evidence suggests that celecoxib reduces the occurrence of polyps in the colon and rectum (43). One way of using celecoxib in the treatment of neuroblastoma may be to target minimal residual disease. To test this hypothesis, we measured the time to tumor development in celecoxib-treated animals compared with untreated controls. The significant, dose-dependent delay in tumor development in animals receiving celecoxib suggests that this drug or other COX-2 inhibitors could have an
chemotherapeutic drugs currently used in neuroblastoma treatment both in vitro and in vivo. Furthermore, we have shown that celecoxib can prevent neuroblastoma tumor development and inhibit the growth of already established neuroblastoma tumors at plasma levels obtainable in humans. These results show that celecoxib is a potential drug for neuroblastoma treatment, and that further clinical trials should define the role of celecoxib as a single drug or in combination with established chemotherapeutic drugs for the prevention and treatment of neuroblastoma.

References


Celecoxib Prevents Neuroblastoma Tumor Development and Potentiates the Effect of Chemotherapeutic Drugs  

"In vitro" and "In vivo" 

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