Cancer Therapy: Preclinical

COX-2-Independent Effects of Celecoxib Sensitize Lymphoma B Cells to TRAIL-Mediated Apoptosis

Anne-Sophie Gallouet1,2,3,5, Marion Travert6, Laurence Bresson-Bepoldin7, Fabien Guilloton1,2,3,5, Céline Pangault1,2,3,4, Sylvie Cautel-Maugendre6,1, Thierry Lamy1,2,3,4, Karin Tarte1,2,3,4, and Thierry Guillaudeux1,2,3,5

Abstract

**Purpose:** Despite therapeutic advances, non-Hodgkin lymphomas (NHL) remain incurable. They form a group of neoplasms strongly dependent on their inflammatory microenvironment, which plays an important supportive role in tumor B-cell survival and in the resistance to antitumor immune response. New therapies must consider both tumor cells and their surrounding microenvironment.

**Experimental Design:** Stromal cells, derived from bone marrow or lymph nodes, and B cells from follicular lymphoma patients were cocultured or cultured alone with celecoxib treatment, a nonsteroidal anti-inflammatory drug, and/or TRAIL, a promising cytotoxic molecule for cancer therapy.

**Results:** In this study, we show that follicular lymphoma stromal cells produce large amounts of PGE2. This production is abrogated after celecoxib treatment, targeting the COX-2 isoenzyme involved in PGE2 synthesis. Furthermore, we demonstrate that celecoxib increases apoptosis in NHL B-cell lines and in primary follicular lymphoma B cells cocultured with stromal cells, but independently of the PGE2/COX-2 axis. Finally, celecoxib increases the apoptotic activity of TRAIL. We provide evidence that celecoxib affects proliferation and sensitizes NHL B-cell lines to apoptosis through COX-2–independent effects by slowing down the cell cycle and decreasing the expression of survival proteins, such as Mcl-1.

**Conclusions:** These data suggest new potent strategies for NHL therapy combining drugs targeting both tumor B cells and survival signals provided by the tumor microenvironment. Clin Cancer Res; 20(10); 2663–73. ©2014 AACR.

Introduction

Chronic inflammation represents a major pathologic step for tumor development in agreement with its implication in multistage carcinogenesis. Inflammatory signaling triggers the secretion by immune and stromal cells of cytokines, chemokines, and a cocktail of other soluble factors like prostaglandins, which can contribute to tumor development and drug resistance. One of them, prostaglandin E2 (PGE2), is synthesized through the catalytic activity of two COX isoenzymes COX-1 and COX-2. Unlike COX-1, which is constitutively expressed and involved in tissue homeostasis, COX-2 expression is induced by several agents, including inflammatory cytokines, tumor promoters, growth factors, and viral transformation. PGE2 is a prominent bioactive mediator involved in key physiologic functions, including induction of IL-6 and haptoglobin, both of which are important regulators of angiogenesis; but it is also implicated in several pathologic conditions like fever, inflammation, pain, and cancer (1). Moreover, PGE2 can modulate tumor microenvironment to support angiogenesis and induce an immunosuppressive environment. In addition, COX-2 overexpression has been associated with resistance to apoptosis in several cancer cell types (1). The hypothesis that COX-2 is involved in cancer growth is further supported by animal studies showing that tumorigenesis is inhibited in COX-2 knockout mice (2, 3).

Nonsteroidal anti-inflammatory drugs (NSAID) are commonly used in anti-inflammatory therapies as well as to treat fever and pain and are now also used for their potential antitumor properties (4). One major molecular mechanism of NSAIDs is related to the inhibition of COX-1 and COX-2 (5). Thus, COX inhibitors, including celecoxib, have shown significant promise as anticancer agents. There is indeed strong evidence from epidemiologic and animal studies that long-term use of NSAIDs can prevent cancer development. This is particularly striking in the case of colorectal cancer, for which the incidence is reduced by 40% to 50% (4).
Non–Hodgkin B-cell lymphomas (NHL) arising from the malignant transformation of germinal center (GC)-derived B cells include follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL), which are by far the two most frequent lymphomas and represent the sixth most common cause of cancer-related deaths in the occidental countries (6). Follicular lymphoma is an indolent NHL highly dependent on a specific supporting microenvironment. Most researches on lymphomas have focused on oncogenic events occurring in B cells, but tumor microenvironment recently appeared as playing a crucial role in lymphomagenesis with dynamic bidirectional interactions between cancer cells and their microenvironment (7, 8). CD4+ T Follicular helper cells, monocytes/macrophages, and stromal cells contribute to tumor progression via cell–cell interactions and soluble factor secretion providing a permissive environment for lymphoma B cells to proliferate in lymph nodes. In another niche of follicular lymphoma, the bone marrow, where tumor extension is observed in more than 70% of the cases, the emergence of an ectopic lymphoid supportive microenvironment was also described (7–11). Inflammatory factors and cytokines secreted by tumor stromal cells contribute to lymphoma B cells survival and drug resistance.

Standard therapies combined cyclophosphamide–adriamycin–vincristine–prednisone (CHOP) regimen with the anti-CD20 antibody rituximab. Nonetheless, despite these therapeutic advances, NHL-B-cell lymphomas remain incurable and are characterized by multiple relapses and resistances. New therapies must now consider both tumor cells and their surrounding microenvironment. The use of alternative new molecules, like death receptor ligands belonging to the TNF superfamily, as therapeutic agents is of potential importance (12, 13). TRAIL, a member of the TNF family, induces apoptosis in a variety of transformed cells following ligation to TRAIL-Receptor-1/DR4 and -2/DR5 (14). TRAIL can induce apoptosis in transformed cells of diverse origin with little or no effects on normal cells (15) and exhibits antitumor activity in several xenograft studies without systemic toxicity (16, 17). On the basis of these findings, clinical trials are currently in progress to assess the potential efficacy of TRAIL itself, or agonistic monoclonal anti-DR4 and anti-DR5 antibodies, in treating human cancers and among them hematologic cancers (18). However, some tumor cells exhibit resistance to TRAIL-mediated apoptosis (19–22) either due to intrinsic modifications of cancer cells or due to mediators present in the tumor microenvironment (18). We have notably shown, in our previous work on FL-B cells, that T-cell–dependent CD40 signaling partially counteracts TRAIL efficacy (23). Consequently, strategies that circumvent resistance to TRAIL-mediated apoptosis may further enhance its activity in human lymphoma, in particular, new drugs targeting tumor microenvironment itself or blocking the crosstalk between tumor microenvironment and lymphoma B cells. High levels of PGE2 have been found in the plasma of patients with lymphoma (24) and recent studies have revealed that patients with DLBCL also exhibit high COX-2 expression (25).

We thus studied the impact of a COX-2 inhibitor in B-cell lymphomas and particularly the role played by celecoxib in modulating TRAIL-induced apoptosis in follicular lymphoma, considering the stromal cell compartment as an important player. Here, we have determined that the combination of these two drugs leads to a significant increase in the induction of lymphoma B-cell apoptosis.

**Materials and Methods**

**Patient samples and cell lines**

For primary cells, patient recruitment followed written informed consent process according to the Declaration of Helsinki. Lymph nodes were collected from follicular lymphoma patients and patients with reactive nonmalignant disease, considered as healthy donors. Biopsies were performed at the University Hospital of Rennes. Cell suspensions were obtained as previously described (23). For coculture experiments with stromal cells, B cells are purified by negative selection (B cell isolation Kit; Miltenyi Biotec). Stromal cells were obtained from tonsils from children undergoing routine tonsillotomy (HD-RESTO) or from follicular lymphoma lymph nodes (FL-RESTO) as previously described (7). Mesenchymal stromal cells (MSC) were generated from bone marrow aspirates obtained from either follicular lymphoma patients or healthy donors undergoing cardiac surgery as previously described (26). Cell lines used in this study are Burkitt lymphomas cell lines (BL2, RAMOS, Daudi, and Raji) and follicular lymphoma-transformed cell lines (SU5D4, DOHH2, and RL). All these cell lines were tested every 6 months with HLA typing. The BL2 cell line was provided by J. Wied (IGR, Villejuif, France). RAMOS, Daudi, Raji, and SU5D4 cell lines were a generous gift from C. Bastard (Centre Becquerel, Rouen, France). DOHH2 and RL cell lines were obtained from the DSMZ.
Chemicals and antibodies

KillerTRAIL was purchased from Alexis Biochemicals. Celecoxib was obtained from LKT Laboratories. FITC-CD19 and FITC-CD20 antibodies were purchased from Beckman Coulter, Alexa-Fluor 647-CD105 antibody was from Biolegend, 7-AAD, FITC-BrdU flow kit, and anti-active caspase-3 apoptosis kit were from Becton Dickinson. The McI1, Bax, and active-Bax antibodies were obtained from BD Biosciences and survivin antibody from R&D Systems. CHOP antibody was from Cell Signaling Technology and β-actin antibody was from Sigma-Aldrich. The peroxidase-conjugated-goat anti-mouse or anti-rabbit antibodies were purchased from Bio-Rad. Rituximab (Roche) was a gift from T. Lamy (CHU, Rennes, France).

Apoptosis assay

B-cell lines or primary B-cells were cultured alone or with 30 μmol/L celecoxib, with or without rituximab (1 μg/ml). After 1 hour, cells were cotreated or not with killerTRAIL during 24 hours. B-cell apoptosis was evaluated with a PE-conjugated anti-active caspase-3 apoptosis kit (BD Biosciences). Apoptotic B cells were analyzed by flow cytometry on selectively gated CD19<sup>pos</sup>CD20<sup>pos</sup>CD105<sup>neg</sup> active caspase-3<sup>pos</sup> cells. Stainings were analyzed using a Gallios cytometer and Kaluza (Beckman Coulter).

B-cell proliferation

After treatment with 30 μmol/L celecoxib or dimethyl sulfoxide (DMSO) for 24, 48, 72, and 96 hours, absolute number of TOPRO-3<sup>neg</sup>CD19<sup>pos</sup>CD20<sup>pos</sup> viable B cells was harvested for each condition, fixed, and permeabilized (FITC-BrdU Flow Kit, BD Pharmingen). After 7-AAD staining, cell-cycle phases were analyzed on BrdUrd<sup>pos</sup> cells using ModFit software (Verity Software House).

PGE<sub>2</sub> quantification

PGE<sub>2</sub> were quantified using the Prostaglandin Screening EIA Kit (Cayman chemical) in the supernatants of stromal cells.

Quantitative real-time PCR

RNA was extracted using RNeasy Mini Kit (Qiagen). cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using the TaqMan Universal PCR Master Mix and Assays on Demand from Applied Biosystems. Gene expression levels were quantified using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control.

Western blot analysis

After treatment, cells were lysed in radioimmunoprecipitation assay buffer and 30 μg proteins were separated by 4%–12% gradient SDS-PAGE and transferred to a PVDF membrane (Millipore).

Protein array

Modification of protein expression was evaluated with protein array kits delivered by R&D systems. For the human phospho-MAPK array kit, SUDHL4 cells were stimulated or not with 30 μmol/L celecoxib for 20 minutes. For the human apoptosis array kit, 30 μmol/L celecoxib was added for 6 hours.

ROS detection

To detect ROS production after 30 μmol/L celecoxib treatment for 24 hours, cells were incubated with CM-H<sub>2</sub>-DCFDA probes (Invitrogen) for 45 minutes. Analysis of reactive oxygen species (ROS) production was performed by flow cytometry on viable cells (DAPI<sup>neg</sup>). Results are expressed as the ratio of mean fluorescence intensity (RMFI) obtained from celecoxib-treated cells and control cells.

Statistical analysis

Statistical analyses were performed with the Wilcoxon test for matched pairs or Mann–Whitney U tests, using Prism software (GraphPad Software Inc.). The significance is shown as follows: *; P ≤ 0.05; **; P ≤ 0.01; and ***; P ≤ 0.001.

Results

Stromal cells secrete high levels of PGE<sub>2</sub>

NHLs are characterized by close interactions between tumor B cells and their stromal microenvironment. These cells interact and influence each other via cell–cell interactions and soluble factors. PGE<sub>2</sub> was previously documented as being secreted in B-cell lymphomas where it could contribute to tumorigenesis (27). Therefore, we have first evaluated whether stromal cells obtained from healthy donors and follicular lymphoma patients produced PGE<sub>2</sub>. We studied PGE<sub>2</sub> production by stromal cells derived either from lymph nodes (RESTO) or from bone marrow (MSC). We generated HD-RESTO, FL-RESTO, HD-MSC, and FL-MSC from different donors. After 72 hours of culture, supernatants were collected and PGE<sub>2</sub> concentration was confirmed the COX inhibitory activity of this molecule on stromal cells (Fig. 1B). The concentration of celecoxib used, 30 μmol/L, was based on several published <i>in vitro</i> dose–response experiments (28, 29) and was nontoxic neither for normal B cells (Fig. 3D) nor for stromal cells (data not shown). Moreover, this concentration is in the range of what obtained in vivo in the peripheral blood of patients with familial adenomatous polyposis that are...
treated with 800 mg celecoxib and reach a 10 μmol/L plasmatic concentration (30, 31). Both COX-1 and COX-2 are involved in the production of PGE2 and are expressed in FL-RESTO and FL-MSC (Supplementary Fig. S1). Therefore, our results showed that celecoxib can inhibit PGE2 secretion produced after COX-1 or COX-2 expression.

The COX-2 inhibitor celecoxib potentiates TRAIL-induced apoptosis in lymphoma B cells

To mimic the germinal center microenvironment and to evaluate new drug efficacy on lymphoma B cells, we cocultured B-cell lines or primary B cells with lymphoid stromal cells (RESTO), previously described in our lab as being able to support malignant B-cell growth (7) and that secrete PGE2 (Fig. 1). Combination therapies with drugs targeting intracellular molecular cancer switches or prosurvival signals delivered by the tumor microenvironment are of interest in the development of new NHL treatments. Then, a combination of the proapoptotic molecule TRAIL and the COX-2 inhibitor celecoxib was used in this study. We have shown in a previous work that TRAIL, while it does not exhibit any toxicity on normal resting or activated B cells, could induce apoptosis in NHL B-cell lines and primary lymphoma B cells (23). We first evaluated TRAIL efficiency and showed that it was significantly reduced in the presence of stromal cells compared with lymphoma B-cells cultured alone (Fig. 2, where dotted lines represent apoptosis levels obtained in culture alone). Similar results were observed with bone marrow MSCs (Supplementary Fig. S2). These results indicate that factors produced by these supportive stromal cells interfered with TRAIL-mediated apoptosis. Using Transwell experiments and supernatants collected from HD-RESTO cells, we demonstrated that this effect was partly due to soluble factors (Supplementary Fig. S3A and S3B). In coculture, celecoxib treatment enhanced B-cell apoptosis. Moreover, when TRAIL and celecoxib were combined, their proapoptotic effects were significantly increased on malignant B cells. This was strongly demonstrated on lymphoma B-cell lines where more than 60% of tumor B cells were dead after 24 hours of treatment with a low concentration of TRAIL (50 ng/mL; Fig. 2A and B). This combination was also effective on purified primary follicular lymphoma B-cells, even if they are intrinsically more resistant to proapoptotic drugs than their lymphoma B-cell lines counterpart (ref. 23; Fig. 2C and D). We checked in parallel that TRAIL, celecoxib, and combinatory treatment did not exhibit any toxicity on the stromal compartment (data not shown).

Celecoxib acts on B cells through COX-2–independent effects and sensitizes lymphoma B cells to TRAIL-mediated apoptosis

Among the soluble factors produce by stromal cells that could contribute to lymphoma B-cell survival, PGE2 targeted by celecoxib could be a good candidate. Moreover, the receptors for PGE2 (EP1–EP4) were detected in lymphoma B cells (Supplementary Fig. S4A) and can therefore provide PGE2-mediated survival signals to B cells. We then directly evaluated the potential of PGE2 produced by stromal cells to modulate B-cell proliferation and survival. We observed that PGE2 did not affect proliferation or apoptosis in BL2 and SUDHL4 cell lines (Supplementary Fig. S4B and S4C). Moreover, we detected no expression of COX-2 in all B-cell lines tested. Similar results were obtained in normal lymph node B cells and primary follicular lymphoma B cells (Fig. 3A). These data were confirmed by quantitative PCR analysis (Fig. 3B). We have directly treated seven germinal center–derived lymphoma B-cell lines with 30 μmol/L celecoxib (Fig. 3C), a dose devoid of deleterious activity on normal B cells (Fig. 3D). B-cell death was weakly but significantly induced after celecoxib treatment with DOHH2 being as one of the most sensitive cell line (30%) and RL...
Figure 2. Celecoxib potentiates TRAIL-induced apoptosis even in the presence of lymph node stromal cells. A, NHL-B cell lines (10⁵ cells) were previously treated with 30 μmol/L celecoxib for 1 hour and cotreated or not with 50 ng/mL of TRAIL for 24 hours. Apoptosis was evaluated with an anti-active caspase-3 staining by flow cytometry. Mean ± SD, n = 7. B, TRAIL sensitive NHL-B cell lines (10⁵ cells) were cocultured on HD-RESTO, seeded previously during 3 days at 0.5 × 10⁵ cells/cm². Coculture was treated with 30 μmol/L celecoxib for 1 hour and cotreated with or without 50 ng/mL of TRAIL for 24 hours. Apoptotic B cells (active caspase-3pos) were analyzed by flow cytometry. Apoptosis level of B-cell lines without stromal cells (A) was reported with dotted lines. Mean ± SD, n = 4. C and D, eight samples of FL-B cells derived from patient biopsies obtained from 8 different patients and identified as individual symbols. B cells were cultured alone (C) or seeded on HD-RESTO cells (D). FL-B cells were treated or not with 30 μmol/L celecoxib and with or without 1 μg/mL TRAIL for 24 hours. Apoptotic B cells were analyzed on the criteria of CD19pos CD20pos CD105neg active caspase-3pos. Mean ± SD, *P ≤ 0.05; **P ≤ 0.01; and ***P ≤ 0.001. Statistical tests were performed between B cells alone and B cells cocultured with HD-RESTO.
the most resistant one (10%; Fig. 3C). Altogether, these data underlined that the direct action of celecoxib on lymphoma B cells was independent of the COX-2/PGE2 axis.

Then, we evaluated whether the combination of TRAIL and celecoxib on isolated follicular lymphoma B cells also sensitized them to apoptosis. In this context, we first confirmed that TRAIL was devoid of proapoptotic activity towards normal B cells. In addition, TRAIL treatment combined with celecoxib did not promote normal B-cell death (Fig. 3D). We then demonstrated that celecoxib potentiated TRAIL-induced apoptosis in lymphoma B cells, including TRAIL-resistant B-cell lines like RAJI and RL, which are much more resistant to cell death (ref. 32; Fig. 2A). More importantly, celecoxib also induced efficient killing of purified follicular lymphoma B cells and, when combined to TRAIL, celecoxib potentiates its proapoptotic effect (21% ± 13% for the control, which is a normal condition after 24 hours of culture; 47% ± 15% for TRAIL + celecoxib; 34% ± 10% for celecoxib alone; and 34% ± 14% for TRAIL alone; Fig. 2C). We complete our study introducing rituximab, the commonly used molecule in follicular lymphoma treatment. When rituximab was combined with celecoxib and TRAIL, we demonstrate that primary FL-B cells were efficiently killed by this new combination of antineoplastic molecules (Fig. 3E). Altogether, these results demonstrate that COX-2–independent effects of celecoxib sensitize lymphoma B cells to rituximab and TRAIL-mediated apoptosis.

Molecular targets of celecoxib in lymphoma B cells

To evaluate in more detail the COX-2–independent mechanisms of action of celecoxib on lymphoma B cells,
we treated lymphoma B-cell lines with 30 μmol/L celecoxib. We showed that B-cell growth was significantly reduced after treatment with DOHH2 being as one of the most sensitive cell line (79% ± 2% of growth inhibition) and RL the less sensitive one (39% ± 5% of growth inhibition; Fig. 4A and B). We then studied the effects of celecoxib on malignant B-cell proliferation in two representative lymphoma B-cell lines, BL2 and SUDHL4. These cell lines showed a reduced cell-cycle progression, as demonstrated by a reduction of BrdUrd dilution after 24-hour treatment with celecoxib. These results were more obvious at 48 hours (Fig. 4C). Indeed we could observe a complete dilution of BrdUrd signal in control condition at 48 hours, whereas in treated cells a second peak was still present, revealing a deceleration of cell proliferation. Furthermore, dilution of BrdUrd signal in control condition at 48 hours, at 48 hours (Fig. 4C). Indeed we could observe a complete dilution of BrdUrd signal in control condition at 48 hours, revealing a deceleration of cell proliferation. Furthermore, 7-AAD labeling on BrdUrd-positive cells indicated that the progression of the cell cycle was slowed down with an accumulation of lymphoma B cells in the G2–M phase (for example, 71% of treated BL2 cells are in G2–M vs. 56% for control), explaining the reduction of cell growth for these cell lines. We thus showed that celecoxib induced an inhibition of cell growth, due to an induction of apoptosis and a slowing down of the cell cycle.

To provide better understanding on celecoxib effects on apoptosis and cell cycle, we have investigated few molecular processes that could be affected in lymphoma B cells. As BL2 and SUDHL4 were affected in their cell-cycle progression by celecoxib, we evaluated by Western blot analysis cell-cycle proteins like Cyclin B1, Cyclin D1, p21, p16, and p27. We did not observe significant differences between treated and untreated cells (data not shown). It could be explained by the coexistence of cells at different stages in their cell cycle. However, using protein microarrays we could identify a slight increase of p21 and p27, two well-known inhibitors of the cell cycle (Fig. 5A). As apoptosis was induced in all cell lines, we have screened Bcl-2 family proteins. While Bcl-2 and Bcl-xl expression did not change (data not shown), we observed a decrease of Mcl-1 expression, which is known to be an important protein for B-cell survival in germinal center (33) (Fig. 5B). Moreover, for several cell lines, we also observed a reduction of Survivin and an increase of the active form of the proapoptotic protein Bax. We also showed that celecoxib induced a reticular stress with a strong induction of the DNA damage-inducible gene 153 (CHOP/GADD153) protein, implicated in the ER stress, and simultaneously the production of ROS (Fig. 5C and D) contributing to apoptosis.

Altogether, these data show that celecoxib promotes lymphoma B-cell death via modulation of key proteins of apoptosis and induction of a cellular stress.

Discussion
In this study, we show that celecoxib reduces B-cell growth through COX-2-independent mechanisms, by modulating cellular stress, apoptosis, and cell cycle in tumor B cells. Simultaneously, celecoxib suppresses, through a COX-1/COX-2-dependent mechanism, the production of PGE2 by the stromal microenvironment that could otherwise contribute to tumor development.

Overexpression of COX-2 is a hallmark of numerous tumor malignancies. This specific enzyme is known to increase tumorigenic potential by promoting cell proliferation and resistance to apoptosis. In germinal center-derived B-cell lymphomas, we have shown that COX-2 or COX-1 are overexpressed in the stromal microenvironment surrounding the tumor as well as in MSC stromal cells derived from the bone marrow where follicular lymphoma invasion frequently occurs.

The production of PGE2 by stromal cells is exacerbated in tumor (FL-MSC and FL-RESTO producing up to 10-fold more PGE2 than their healthy counterparts). Moreover, PGE2 delivered by stromal cells was previously described as a prosurvival signal for B-cell in germinal center (34). PGE2 not only support survival and local inflammation but it can also control the activation of CTL-. Th1-, and NK cell-mediated type 1 (cytotoxic) immunity, and favor local enhancement of regulatory T cells and myeloid-derived suppressor cell-mediated suppressive events (35, 36). The consequence is the establishment of an immunosuppression that will favor tumor progression. These data reflect the ability of COX-2 inhibitors to enhance the immune and therapeutic activity of cancer treatment.

We have recently demonstrated that follicular lymphoma-associated stromal cells, locally induced during tumor development, display an inflammatory gene signature (26). This has also been highlighted in fibroblasts obtained from solid tumors in mice and humans, where they promote angiogenesis and inflammatory cell recruitment (37). It is worth noting that these follicular lymphoma-associated stromal cells supported more efficiently the growth of malignant B cells than their normal counterpart. These data underline the complexity of follicular lymphoma tumors where stromal cells directly promote tumor growth through the production of soluble molecules like PGE2 that will cooperate with other survival signals supplied by the immune environment, notably IL15 and CD40L, previously characterized as survival signals in B-lymphoma pathogenesis (9, 23). These survival signals give a beneficial advantage for lymphoma B-cell survival and development, but they also counteract the efficacy of drugs targeting apoptosis like TRAIL.

In this study, we have shown that most effects of celecoxib treatment in B cells, with or without the presence of stromal cells, are nevertheless independent of its ability to inhibit COX-2.

We showed that these COX-2-independent effects play a role in sensitizing tumor lymphoma B cells to TRAIL cytotoxicity (Figs. 2 and 4 and Supplementary Data S2). The molecular mechanisms involved in COX-2-independent effects mediated by celecoxib have been investigated. We have shown that celecoxib promotes B-cell death through the downregulation of antiapoptotic proteins, like Mcl-1 and survivin, and the activation of Bax. These proteins are notably involved in TRAIL resistance. Their modulation by celecoxib gives more impact in TRAIL-induced apoptosis.
Figure 4. Celecoxib affects NHL-B cell line growth. A, NHL-B cell lines (10^5 cells/mL) were treated with 30 μmol/L of celecoxib or DMSO (control) for 24, 48, 72, and 96 hours. Absolute number of viable cells (TOPRO-3^®^CD19^®^CD20^®^) was determined by flow cytometry with FlowCount beads. Mean ± SD, n = 3. B, percentage of cell growth inhibition was deduced with NHL-B cells treated with celecoxib versus control at 96 hours. Mean ± SD; *, P ≤ 0.05 and **, P ≤ 0.01. C, BL2 or SUDHL4 were pulsed with 10 μmol/L of BrdUrd during 20 minutes. One million cells were then harvested at different time points (0, 6, 24, and 48 hours). BrdUrd-labeled cells were followed through the cell cycle by 7-AAD staining. Results are representative from one of three independent experiments.
Moreover, celecoxib acts on cell-cycle progression probably by modulating key proteins like p21, p38α, and GSK3β kinases whose expressions were slightly modulated on protein microarrays after treatment (Fig. 5). As a consequence, blockage in the G2–M mitotic phase is taking place (Fig. 4). Celecoxib treatment of lymphoma B cells is also associated with cellular stress. Stress-related transcription factors have acquired a pivotal role in the sensitization of highly resistant cancer cells to TRAIL-mediated cell death like in pancreatic and glioblastoma cancer cells (38). CHOP transcription factor, a critical initiator of ER stress-induced apoptosis, was found to be upregulated after celecoxib treatment (Fig. 5C). It is known that CHOP regulates the expression of two proapoptotic proteins, PUMA and BIM, in response to ER stress (39). Moreover, downregulation of Mcl-1 associated with CHOP-induced apoptosis represent additional opportunities distinct from Bim induction for synergy in cell death pathway. Mcl-1 phosphorylation by GSK3 (glycogen synthase kinase-3), a central regulator of a stress-activated network, is necessary for its further degradation by the proteasome (40). Therefore, the slight GSK-3 upregulation observed by protein microarrays after

Figure 5. Celecoxib enhances apoptotic pathway and ER-stress. A, expression of apoptotic, cell cycle (Bax, Bcl-xL, pro-caspase-3, caspase-3 cleaved, p21 and p27), and signaling (p38α and GSK3β) proteins from SUDHL4 was analyzed after celecoxib treatment (30 μmol/L). Pixel density was determined with ImageJ software. Variation of protein level expression was normalized with positive control on the array. Representative of two experiments. B and C, NHL-B cell lines were treated with 30 μmol/L celecoxib (CX) or control DMSO (ø) for 24 hours. Analyses of proapoptotic proteins (Bax), antia apoptotic proteins (Mcl1, Survivin) and ER stress–related protein (CHOP) were performed by Western blot analysis. β–Actin was used as loading control. D, ROS production was evaluated with CM-H2-DCFDA probe by flow cytometry. RMFI was calculated after ratio between MFI of 30 μmol/L celecoxib and control cells (n = 5); P < 0.05; **, P < 0.01; and ***, P < 0.001.
treatment with celecoxib could contribute to Mcl-1 degrada-
tion (Fig. 5A). The role of Sax in CHOP-induced apo-
poptosis was suggested from studies using macrophages (41, 42) where Sax level increased with ER stress in a CHOP-
dependent manner (43). Prolonged ER stress can hyperox-
idize the ER lumen and directly induce cytoxic ROS in the
cytoplasm. We highlighted a strong production of ROS on
lymphoma B-cell lines after treatment with celecoxib (Fig.
5D). Oxidation of the ER lumen is induced by the CHOP-
transcriptional target ER oxidoase-1α (ERO1α; ref. 44).
ERO1 promotes a hyperoxidizing environment that leads
to cell death. Interestingly, ROS is also part of a positive
feedback cycle that activates PKR and amplifies CHOP
expression (45). Moreover, it was previously described
that CHOP could upregulate the TRAIL death receptor DR5.
However, we could not observe any modification in protein
expression levels of DR4 and DR5 in BL2 and SUDHL4
(data not shown). This treatment seems to contribute to
cell death sensitization via ER stress. Moreover, recent
studies have shown that ER stress is already increased in
lymphoma B cells compared with normal B cells, due to
numerous factors, including oncogene activation and an
inflammatory microenvironment (46, 47). Then, celecoxib
treatment can exacerbate this cellular stress explaining why
normal primary B cells were not affected by celecoxib (Fig.
3D) when lymphoma B cells died after celecoxib treat-
ment (Fig. 2). Modulation of numerous apoptotic proteins after
treatment by celecoxib can slightly induce B-lymphoma cell
death. Then, celecoxib can play the role of a sensitizer to cell
death and acts synergistically with different chemothera-
pic drugs (48, 49) or the new therapeutic proapoptotic
molecule TRAIL in the induction of tumor cell cytotoxicity.
It could be interesting to further evaluate whether the ability
do cycliclooxygenase (COX) and PGE2. COX inhibition can
be administered over a long period of time. However, celecoxib, which can be administered over a long period of
times, targets lymphoma B cells through its COX-2–
deleted effects, sensitizing tumor B cells to cell death
and on the other hand it inhibits PGE2 production by
stromal cells, abrogating the contribution of the tumor
microenvironment to chronic inflammation and immuno-
modulatory status.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.-S. Gallouet, M. Travert, L. Bresson-Bepoldin, T. Guillaudeux
Development of methodology: A.-S. Gallouet, T. Guillaudeux
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.-S. Gallouet, L. Bresson-Bepoldin, F. Guilloton, S. Cautel-Maugendre, T. Lamy, T. Guillaudeux
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.-S. Gallouet, M. Travert, L. Bresson-Bepoldin, K. Tarte, T. Guillaudeux
Writing, review, and/or revision of the manuscript: A.-S. Gallouet, M. Travert, L. Bresson-Bepoldin, S. Cautel-Maugendre, T. Lamy, K. Tarte, T. Guillaudeux
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.-S. Gallouet, F. Guilloton, C. Panguel, S. Cautel-Maugendre, T. Lamy, T. Guillaudeux
Study supervision: T. Guillaudeux

Acknowledgments
The authors thank the Centre de Ressources Biologiques (CRB)-Sanlé de Rennes hospital, Pr. Thierry Fest and Patrick Tas for providing nonmalignant and follicular lymph nodes, Christophe Ruaux for providing tonsil samples, and the BREHAT network for follicular lymphoma bone marrow samples. The authors also thank Severine Moullé and J-Philippe Stéphane for technical assistance.

Grant Support
This work was supported by research funding from the Association pour le Développement de l’Hématologie-OncoLogie (ADHO) and the Ligue contre le cancer. A.-S. Gallouet was supported by a PhD studentship from INSERM, Région Bretagne and Université de Rennes 1.

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.

Received August 20, 2013; revised February 3, 2014; accepted February 27, 2014; published OnlineFirst March 17, 2014.

References
6. Armitage JO, Weisenburger DD. New approach to classifying non-
Hodgkin’s lymphomas: clinical features of the major histologic sub-


COX-2–Independent Effects of Celecoxib Sensitize Lymphoma B Cells to TRAIL-Mediated Apoptosis

Anne-Sophie Gallouet, Marion Travert, Laurence Bresson-Bepoldin, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-2305

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2014/03/17/1078-0432.CCR-13-2305.DC1

Cited articles
This article cites 50 articles, 21 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/20/10/2663.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/20/10/2663.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.