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Concurrent targeting of EP1/EP4 receptors and COX-2 induces synergistic apoptosis in KSHV and EBV associated non-Hodgkin lymphoma cell lines

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Abstract

The effective anti-tumorigenic potential of non-steroidal anti-inflammatory drugs (NSAIDs) and eicosonoid (EP; EP1–4) receptor antagonists prompted us to test their efficacy in Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) related lymphomas. Our study demonstrated that (1) EP1–4 receptor protein levels vary among the various non-Hodgkin's lymphoma (NHL) cell lines tested (BCBL-1:KSHV+/EBV–;BC-3: KSHV+/EBV–; Akata/EBV+: KSHV–/EBV+; and JSC-1 cells: KSHV+/EBV+ cells); (2) 5.0 μ M of EP1 antagonist (SC-51322) had a significant anti-proliferative effect on BCBL-1, BC-3, Akata/EBV+, and JSC-1 cells; (3) 50.0 μ M of EP2 antagonist (AH6809) was required to induce a significant anti-proliferative effect on BCBL-1, Akata/EBV+, and JSC-1 cells; (4) 5.0 μ M of EP4 antagonist (GW 627368X) had a significant anti-proliferative effect on BC-3, Akata/EBV+, and JSC-1 cells; (5) COX-2 selective inhibitor celecoxib (5.0 μ M) had significant anti-proliferative effects on BCBL-1, BC-3, Akata/EBV+, and JSC-1 cells; and (6) a combination of 1.0 μ M each of celecoxib, SC-51322 and GW 627368X could potentiate the pro-apoptotic properties of celecoxib or vice-versa. Overall, our studies identified the synergistic anti-proliferative effect of NSAIDs and EP receptor blockers on KSHV and EBV related B cell malignancies.

Keywords

cyclooxygenase-2; COX-2 inhibitors; celecoxib; PGE2; EP receptors; EP receptor antagonists; KSHV latency; KSHV lymphomas; EBV lymphomas; non-Hodgkin's lymphomas; AIDS linked lymphomas

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INTRODUCTION

Malignant lymphomas account for 3–4% of all human malignancies.^{1, 2} Several infectious agents are known to cause aggressive malignant lymphomas such as lymphotropic herpes viruses, KSHV and Epstein-Barr virus (EBV).² EBV and KSHV are the etiological agents underlying aggressive non-Hodgkin lymphomas (NHL) such as Burkitt's lymphoma (BL), and primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD), respectively.^{3–7} Interestingly, KSHV and EBV related lymphomas are often associated with HIV/AIDS or in the context of immune deficiency such as organ transplantation.^{3–7} The pathogenesis underlying KSHV and EBV related lymphomas is proposed to be the result of mutually dependent interactions between viral proteins, angiogenic factors, and proinflammatory mechanisms that under the conditions of immune suppression overcome host defenses against uncontrolled cell growth.^{4, 7} In recent years, several studies have suggested the role of a proinflammatory tumor microenvironment as a pivotal player in KSHV and EBV related oncogenesis.⁴ Consequently, drugs targeting viral infection and proinflammatory mechanisms are extensively studied to develop a disease specific treatment against KSHV and EBV associated lymphomas.^{4, 8–16}

Studies using non-steroidal anti-inflammatory drugs (NSAIDs) have demonstrated their anti-cancer potential due to the blockade of major angiogenic stress response protein cyclooxygenase-2 (COX-2).^{17–30} COX-2 and PGE2 are proposed to play a crucial role in the pathogenesis underlying several cancers.³¹ The tumorigenic properties of COX-2 are attributed to its metabolite prostaglandin E2 (PGE2) that exerts its effect through eicosonoid (EP) receptors belonging to the seven-transmembrane rhodopsin family of G protein coupled receptors (GPCRs) designated as EP1, EP2, EP3, and EP4.³² The signaling pathways downstream of the EP receptors consist of canonical G-protein coupled signal molecules such as Ca²⁺, cAMP, PI3K, cell survival pathways and transcription regulators (AKT, ERK, NFκB, and β-catenin).^{32–38} Several recent studies suggested that EP receptors could be linked to major pathways involved in cancer such as angiogenesis, apoptosis, cell proliferation, metastasis, and immune suppression.^{32–38} The link between EP receptors and tumorigenesis has also revealed the possibility of using highly specific EP receptor antagonists, such as SC-51322 (EP1 antagonist), AH6809 (EP2 antagonist), and AH23848 (EP4 antagonist), as anti-cancer drugs.^{32–38} Taken together, these studies have indicated that EP receptors are vital in COX-2/PGE2 mediated inflammation and oncogenesis.

Although the anti-cancer effects of COX-2 inhibitors in treating NHLs are well studied, the chemotherapeutic potential of EP receptor antagonists is still unexplored in any lymphoma. In addition, the potential of creating a synergistic chemotherapeutic effect on cancer cells by the simultaneous blockade of COX-2 and EP receptors is also unexamined. Interestingly, our results demonstrate the anti-proliferative effects of EP1, EP2, and EP4 antagonists and the COX-2 inhibitor celecoxib on KSHV+/EBV– (BCBL-1 and BC-3), KSHV–/EBV+ (Akata/EBV+), and KSHV+/EBV+ (JSC-1) cell lines. EP1 and EP4 receptor blockade and COX-2 inhibition by celecoxib also induced apoptosis in BCBL-1 and Akata/EBV+ cells but not KSHV–/EBV– cell lines BJAB and Akata/EBV–. Combining EP1 and EP4 antagonists with celecoxib at concentrations that were not anti-proliferative, however, drastically induced apoptosis in BCBL-1 and Akata/EBV+ cells.

METHODS

Cell cultures

Body cavity B cell lymphoma (BCBL-1; KSHV+/EBV-), Akata/EBV+ (KSHV-/EBV+), Akata/EBV- (KSHV-/EBV-), BC-3 (KSHV+/EBV-), JSC-1 (KSHV+/EBV+), and BJAB (KSHV-/EBV-) cells were cultured in RPMI 1640 (Gibco BRL, Grand Island, New York) medium with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, Utah), 2mM L-glutamine (Gibco BRL), and penicillin/streptomycin (Gibco BRL). Human microvascular dermal endothelial cells were cultured as described before.³⁹ All cell lines were tested for mycoplasma contamination prior to be included in the experiments using MycoAlert™ PLUS mycoplasma detection Kit (Lonza Walkersville, Maryland) as per the manufacturer's instructions. It is a selective biochemical test that exploits the activity of mycoplasma enzymes which are found in all six of the main mycoplasma cell culture contaminants and the vast majority of 180 mycoplasma species, but are not present in eukaryotic cells.

Reagents

Celecoxib was from Sigma, St. Louis, Mo. DAPI (4,6-diamidino-2-phenylindole) and rabbit-cleaved-caspase 3 antibody were from Invitrogen, Carlsbad, CA. GW 627368X (EP4 antagonist: 4-(4,9-diethoxy-1,3-dihydro-1-oxo-2H-benz[f]isoindol-2-yl)-N-(phenylsulfonyl benzenacetamide)), and AH6809 (EP2 antagonist: 6-isopropoxy-9-oxoxanthene-2-carboxylic acid) were purchased from Cayman Chemical, Ann Arbor, MI. SC-51322 (EP1 antagonist) was purchased from Enzo Life Sciences, Plymouth Meeting, PA.

Antibodies

Mouse monoclonal antibodies against human COX-2 and rabbit polyclonal antibodies against EP1, EP2, EP3, and EP4 receptors were purchased from Cayman Chemicals, Ann Arbor, MI. Mouse tubulin antibody was from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Rabbit-cleaved-caspase 3 antibody was from Invitrogen, Carlsbad, CA. Conjugates of anti mouse/rabbit-alkaline phosphatase and anti-mouse/rabbit-horseradish peroxidase were from Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD. Alexa-488/594-coupled anti-mouse antibody was from Molecular Probes, Eugene, OR.

Fluorescence activated cell sorting (FACS)

NHL cells after indicated treatments were permeabilized with permeabilization buffer (BD Biosciences, San Jose, CA), washed with permeabilization/wash buffer (BD Biosciences), blocked with 3% BSA (Sigma), and incubated with cleaved-caspase 3 antibody for 1h. In experiments with HMVEC-d cells, cells were serum starved for 24h, infected with KSHV (20 DNA copies per cell) for 2h or left uninfected (UN), washed with PBS, and supplemented with serum free DMEM throughout the experiment. At 24h, 48h, and 72h post-infection, cells were collected, permeabilized with permeabilization buffer, washed with permeabilization/wash buffer (BD Biosciences), blocked with 3% BSA, and incubated with EP1, EP2, EP3 or EP4 antibodies for 1h. The cells were washed again with permeabilization/wash buffer and stained with Alexa 488 secondary antibody and total protein levels were then measured with a FACSCalibur flow cytometer (Becton Dickinson,

Bedford, MA). 10,000 cells were analyzed by gating all samples by forward and side scatter to eliminate dead cells. The data was analyzed with CellQuest Pro software (Becton Dickinson) at the Rosalind Franklin University of Medicine and Science (RFUMS) flow cytometry core facility.

Western blotting

Total cell lysates prepared from respective cells were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The resolved proteins transferred to nitrocellulose membranes were immunoblotted with the indicated primary antibodies. α -tubulin was used as loading control. Species-specific horseradish peroxidase secondary antibodies were used for detection. Immunoreactive bands were developed by Pierce Super Signal West Pico or Femto reagents (Pierce, Rockford, IL). The bands were scanned and quantitated with ImageQuant software following standard protocols.³⁹

Real-time reverse transcription PCR (RT-PCR)

5×10^5 of BCBL-1, BC-3, Akata/EBV+, and BJAB cells were seeded with a combination of 1.0 μ M each of celecoxib, SC- 51322, and GW 627368X and were replenished with the drugs every 12h. RNA samples were collected at 8h and 24h to examine the gene expression levels of Bcl-2-associated death promoter (BAD), B-cell lymphoma-2/Bcl-associated $\times$ protein (BAX), Bcl-10, Bcl-2, Bcl-xL, BH3 interacting-domain death agonist (BID), cellular inhibitor of apoptosis-1/2 (cIAP-1 and cIAP-2), Fas-Associated protein with Death Domain or FAS antigen or TNFRSF6 (FADD), p53, p21, Survivin, tumor necrosis factor (TNF), and X-linked inhibitor of apoptosis protein (XIAP) transcripts were detected by real time RT-PCR as described before.³⁹ Normalization was done with respect to 18s. Primer sequences used for various genes were 18s (5'- AACCCGTTGAACCCATT-3' and 5'- CCATCCAATCGGTAGTAGCG-3'), BAD (5'- TGGATGACCTCGATGATGAAG-3' and 5'-GCACAGCAACGCAGATGCG-3'), BAX (5'- TCCCCCGAGAGGTCTTTT-3' and 5'- CGGCCCCAGTTGAAGTTG-3'), Bcl-10 (5'- CAGGCTGGTCTTGAAC-3' and 5'- GCTCGTGTCTGTAATCC-3'), Bcl-2 (5'- GAGGAGCTCTTCAGGGACGG-3' and 5'- GGTGCCGGTTCAGGTACTCA-3'), Bcl-xL (5'- TCCTTGTCTACGCTTTCCACG-3' and 5'-GGTCGCATTGTGGCCTTT- 3'), BID (5'- GTGAACCAGGAGTGAGTCGG-3' and 5'- GACATCACGGAGCAAGGAC-3'), cIAP-1 (5'- CCAGCCTGCCCTCAAACCCTCT-3' and 5'-GGGTCATCTCCGGGTTCCCAAC-3'), cIAP-2 (5'- GACAGGAGTTCATCCGTCAAGTTC-3' and 5'-CCTGGGCTGTCTGATGTGG-3'), FADD (5'-TGCAGAAGATGTAGATTGTGTGATGA-3' and 5'- GGGTCCGGGTGCAGTTTATT-3'), p53 (5'-TAACAGTTCCTGCATGGGCGGC-3' and 5'- AGGACAGGCACAAACACGCACC-3'), p21 (5'- GAGGCCGGGATGAGTTGGGAGGAG-3' and 5'- CAGCCGGCGTTTGGAGTGGTAGAA-3'), Survivin (5'- GCCCAGTGTCTTCTGCTT-3' and 5'-CCGGACGAATGCTTTTTATG-3'), TNF (5'- TGGCCCAGGCAGTCAGA-3' and 5'-GGTTTGCTACAACATGGGCTACA-3'), XIAP (5'- GAGAAGATGACTTTTAACAGTTTTGA-3' and 5'- TTTTTTGCTTGAAAGTAATGACTGTGT-3'). Statistics (t-test) were calculated with respect to 8h gene expression for each time point. Error bars represent mean $\pm$ SD (*) $p < 0.05$. Upregulation or downregulation in gene expression are indicated in Table1.

Measurement of PGE2

Secreted amounts of PGE2 were measured using a PGE2 ELISA kit as per the manufacturer's guidelines (R & D systems, Minneapolis, MN).

Immunofluorescence

Cells, after acetone fixation to glass slides, were washed with PBS and treated with the indicated primary antibodies, washed with PBS, followed by Alexa 488 secondary antibody treatment. After being washed with PBS, the cells were mounted with antifade reagent containing DAPI. The fluorescence-positive cells were observed under a fluorescence microscope equipped with a Nikon Metamorph digital imaging system.

Cell proliferation and viability assays

5×10^5 of respective cells were seeded with respective drugs at the indicated concentrations and the number of proliferating and viable cells as examined by measuring their metabolically active mitochondria (an index of cell proliferation) based on a colorimetric assay (ATCC, Manassas, VA) as per the manufacturer's instructions at the indicated time points. Fold cell proliferation and cell death was calculated with respect to 0h for each treatment. A student's t-test was employed ($p < 0.01$) for all data analysis.

Cytotoxicity assay

Doses and time of incubation with drugs were chosen based on preliminary cytotoxicity assays. Supernatants of Body cavity B cell lymphoma (BCBL-1; KSHV+/EBV-), Akata/EBV+ (KSHV-/EBV+), Akata/EBV- (KSHV-/EBV-), BC-3 (KSHV+/EBV-), JSC-1 (KSHV+/EBV+), and BJAB (KSHV-/EBV-) cells treated with various drugs (celecoxib, GW 627368X, AH6809, and SC-51322) were collected at different time points (1d, 2d, 3d, 4d, 5d) to assess cellular toxicity using a cytotoxic assay kit (Promega, Madison, WI) as described in our previous manuscripts.^{39, 40}

Real time PCR Array

1×10^6 BCBL-1 and Akata/EBV+ cells were seeded with a combination of 1.0 μ M each of celecoxib, GW 627368X, AH6809, and SC-51322 or left untreated and were replenished with the drugs every 12h for treated samples. RNA was collected at 8h and 24h post treatment, and was used to profile 84 key genes involved in lymphoma pathogenesis using RT² profiler lymphoma PCR array as per manufacturer's guidelines (SABiosciences, Valencia, CA).

RESULTS

EP1–4 receptor protein levels and PGE2 secretion vary between different NHL cell lines

Previous studies have reported the upregulation of COX-2 in NHL.¹⁷ Therefore, we examined the levels of EP1–4 receptors in KSHV-/EBV- (BJAB and Akata/EBV-), KSHV+/EBV- (BCBL-1 and BC-3), KSHV-/EBV+ (Akata/EBV+), and KSHV+/EBV+ (JSC-1) cell lines (Fig. 1a). Our results indicate that the protein levels of EP receptors varied between the cell types considerably and within the same cell type (Fig. 1a). In parallel

experiments, we also demonstrated the presence of PGE2 secretion in the supernatants of BJAB (56pg/ml), Akata/EBV⁻ (198pg/ml), BCBL-1 (274pg/ml), BC-3 (284pg/ml), Akata/EBV⁺ (313pg/ml), and JSC-1 (390pg/ml) cells (Fig. 1b). Immunofluorescence studies further confirmed the presence of EP1–4 receptors in all cell lines tested with a mainly cytoplasmic distribution (Fig. 1c).

KSHV infection upregulates EP receptors in primary HMVEC-d cells

Previous studies have clearly described the role of the COX-2/PGE2 pathway in the KSHV latency program.^{39–42} Therefore, we next examined the effect of *de novo* KSHV infection on EP1–4 receptor levels in primary HMVEC-d cells by measuring the mean fluorescent intensity (MFI) of each receptor, post infection by FACS. The MFI for EP1, EP2, and EP3 receptors per cell increased at 24h to 53.4, 112.8, and 413 and at 48h to 57.4, 135.2, and 419 from 45.2, 115.7, and 347, respectively (Fig. 1d). The MFI for EP4 receptor increased to 254.3 at 24h from 188.7 (untreated) and decreased to 131.3 and 99.3 at 48h and 72h p.i., respectively (Fig. 1d). At 72h p.i., the MFI for EP1, EP2, and EP3 receptors per cell decreased to 40.2, 96.3, and 263 compared to untreated cells, respectively (Fig. 1d). Overall, these results indicate that KSHV infection regulates EP1–4 receptor levels.

EP1, EP2, and EP4 antagonists downregulated KSHV⁺ and EBV⁺ cell proliferation in culture

Our earlier studies have strongly indicated the role of COX-2 and EP receptors on the KSHV latency program.^{39–41, 42, 43, 44} The anti-proliferative effects of EP receptor blockers have also been reported in other tumor model systems^{32–38} but never studied in KSHV related cancers. We first examined the effect of EP1 antagonist (SC-51322), EP2 antagonist (AH6809), and EP4 antagonist (GW 627368X) on human NHL cell lines BCBL-1 (KSHV⁺/EBV⁻), BC-3 (KSHV⁺/EBV⁻), Akata/EBV⁺ (KSHV⁻/EBV⁺), and JSC-1 (KSHV⁺/EBV⁺).

The EP1 antagonist (SC-51322) at 5.0μM induced significant proliferation arrest and cell death at day 5 post-treatment on BCBL-1 (Fig. 2a–b), BC-3 (Fig. 2c–d), and BJAB (Fig. 2i–j) cells. The drug at 5.0μM significantly downregulated cell proliferation and induced cell death at day 3 and sustained the effect on day 5 for Akata/EBV⁺ (Fig. 2e–f) and JSC-1 (Fig. 2g–h) cells. At 50.0μM concentration, SC-51322 induced proliferation arrest and cell death at day 2 for BCBL-1 (Fig. 2b), BC-3 (Fig. 2c–d), and JSC-1 cells (Fig. 2g–h), at day 1 for Akata/EBV⁺ (Fig. 2e–f) and BJAB (Fig. 2i–j) cells and was sustained until day 5. SC-51322 (0.5μM) induced significant cell death at day 5 in BCBL-1 (Fig. 2b) and BC-3 (Fig. 2d) cells, although we did not see a significant effect on cell proliferation.

The EP2 antagonist (AH6809) did not have any significant effect on the cell proliferation of BCBL-1, BC-3, Akata/EBV⁺, JSC-1, and BJAB cells at 0.5μM and 5.0μM concentrations (Fig. 3a, 3c, 3e, 3g, and 3i). However, AH6809 (0.5μM and 5.0μM) induced significant cell death at day 5 in BCBL-1 (Fig. 3b) and BC-3 cells (Fig. 3d). However, at 50.0μM, AH6809 induced significant proliferation arrest and cell death at day 3 for BCBL-1 (Fig. 3a–b), and JSC-1 cells (Fig. 3g–h), at day 2 for Akata/EBV⁺ cells (Fig. 3e–f) and was sustained until day 5 with no significant effect on BC-3 cells (Fig. 3c–d). 50.0μM AH6809 also induced

significant cell death at day 3 in BJAB cells and sustained it with no significant effect on cell proliferation (Fig. 3i–j).

EP4 antagonist (GW 627368X) at 5.0 μ M induced significant proliferation arrest and cell death at day 5 in BC-3 (Fig. 4–d), Akata/EBV+ (Fig. 4e–f), and JSC-1 cells (Fig. 4g–h) with no significant effect on BCBL-1 (Fig. 4a) and BJAB (Fig. 4i) cell proliferation. However, GW 627368X (0.5 μ M) induced significant cell death at day 5 in BCBL-1 cells (Fig. 3b). At 50.0 μ M, GW 627368X downregulated cell proliferation and induced cell death significantly at day 2 for BC-3 (Fig. 4c–d), Akata/EBV+ (Fig. 4e–f), and JSC-1 cells (Fig. 4g–h), at day 1 for BJAB cells (Fig. 4i–j), at day 3 for BCBL-1 cells (Fig. 4a–b) and sustained it till day 5. At 0.5 μ M concentration, GW 627368X had no significant effect on cell proliferation and cell death in any of the cell lines (Fig. 4a–j).

COX-2 inhibitor celecoxib downregulates the proliferation of KSHV+ and EBV+ cells in culture

Previous reports have demonstrated the anti-proliferative effects of celecoxib at concentrations greater than 10 μ M on NHL cells^{45, 46} and the role of COX-2 in the KSHV latency program.³⁹ Therefore, as a control for our cell proliferation studies we next examined whether we can replicate the effects of celecoxib on BCBL-1 (KSHV+/EBV), BC-3 (KSHV+/EBV), Akata/EBV+ (KSHV–/EBV+), JSC-1 (KSHV+/EBV+), and BJAB (KSHV–/EBV–). Celecoxib at 5.0 μ M concentration induced significant proliferation arrest and cell death in BCBL-1 (Fig. 5a–b), BC-3 (Fig. 5c–d), and JSC-1 cells (Fig. 5g–h) at 5 days post-treatment and at days 3 and 5 in Akata/EBV+ cells (Fig. 5e–f). There was no significant effect on any of the cell lines at 0.5 μ M (Fig. 5a–j). However, celecoxib (0.5 μ M and 5.0 μ M) induced significant cell death at day 5 in BCBL-1 cells (Fig. 5b). In contrast, at 50.0 μ M celecoxib induced sustained proliferation arrest and cell death in BCBL-1 cells (Fig. 5a–b) at day 2 and at day 1 in BC-3 (Fig. 5c–d), Akata/EBV+ (Fig. 5e–f), and JSC-1 (Fig. 5i–j) cells.

Celecoxib, SC-51322, and GW 627368X induces apoptosis in BCBL-1, BC-3, Akata/EBV+, and JSC-1 cells

We next examined whether the sustained anti-proliferative effects we observed with celecoxib, SC-51322, and GW 627368X were due to the induction of apoptosis by measuring the levels of cleaved-caspase 3. To investigate whether the anti-growth effects we observed were due to a specific anti-KSHV or anti-EBV effect or general cytotoxic effect, we selected 5.0 μ M, the lowest concentration of celecoxib, SC-51322, and GW 627368X that had a significant anti-proliferative effect and used KSHV/EBV– cell lines Akata/EBV– and BJAB cells as control cell lines. BCBL-1, Akata/EBV+, Akata/EBV–, and BJAB were seeded with 5.0 μ M celecoxib, SC-51322, and GW 627368X and supplemented with the drug every 12h for 5 days and the levels of cleaved-caspase 3 were compared between untreated and day 5 cells.

The COX-2 inhibitor celecoxib at 5.0 μ M, increased the percentage of cells positive for cleaved-caspase 3 at day 5 compared to day 1 in BCBL-1 (Fig. 6a) and Akata/EBV+ (Fig. 6b) cells, increased the most dramatically from 16.1% to 46.9% and 30.1% to 56.4%,

respectively with a modest effect on Akata/EBV– (Fig. 6c) and BJAB (Fig. 6d) cells from 13.6% to 27.0% and 47.5% to 51.7%, respectively. Similarly, 5.0 μ M of EP1 antagonist SC-51322 also induced apoptosis with an increase in the percent of cleaved-caspase 3 positive cells at day 5 compared to day 1 in BCBL-1 (Fig. 6a) and Akata/EBV+ (Fig. 6b) cells, from 21.0% to 50.5% and 20.3% to 39.7% with a modest effect on Akata/EBV– (Fig. 6c) and BJAB (Fig. 6d) cells from 49.5% to 67.3% and 37.9% to 44.4%, respectively. 5.0 μ M of EP4 antagonist GW 627368X had the most pro-apoptotic effect with an increase in the percent of cleaved-caspase 3 positive cells at day 5 compared to day 1 in BCBL-1 (Fig. 6a) and Akata/EBV+ (Fig. 6b) cells, from 22.9% to 80.3% and 17.8% to 43.8% with a modest effect on Akata/EBV– (Fig. 6c) and BJAB (Fig. 6d) cells from 31.5% to 61.2% and 29.5% to 38.0%, respectively.

Combinations of celecoxib, SC-51322, and GW 627368X potentiate the pro-apoptotic effects on BCBL-1 and Akata/EBV+ cells

PGE2 is the tumorigenic metabolite of COX-2 that exerts its effects through EP receptors.³² Therefore, we next examined whether treating BCBL-1 and Akata/EBV+ cells with a combination of 1.0 μ M each of SC-51322, GW 627368X, and celecoxib could induce additive pro-apoptotic effects. Although, we did not observe any additive effects with the combination of SC-51322 (5.0 μ M) and GW 627368X (5.0 μ M), compared to their individual pro-apoptotic effects, we observed an increase in the percent of cleaved-caspase 3 positive cells at day 5 compared to day 1 for BCBL-1 and Akata/EBV+ cells from 51.7% to 74.2% and 13.2% to 42.1%, respectively (Fig. 7a). We next investigated whether combining 1.0 μ M celecoxib with 1.0 μ M of SC-51322 and GW 627368X can potentiate the anti-proliferative effect of celecoxib or vice-versa. Although, 5.0 μ M of celecoxib, SC-51322 or GW 627368X was the least concentration required to induce proliferation arrest, the combination of celecoxib, SC-51322, and GW 627368X at 1.0 μ M increased the percent of cleaved-caspase 3 positive cells for BCBL-1 and Akata/EBV+ from 39.5% to 68.5% and 28.2% to 42.5%, respectively (Fig. 7b).

We next examined whether combinations of celecoxib, SC-51322, and GW 627368X at 1.0 μ M each affected the expression of various pro-apoptotic and anti-apoptotic genes. In Akata/EBV+ cells, concurrent targeting of COX-2 and EP1/EP4 antagonists at 8h and 24h post treatment significantly upregulated pro-apoptotic genes TNF, FADD, BID, BAD, and BAX and significantly downregulated anti-apoptotic genes cIAP-1, cIAP-2, Survivin, Bcl-2, Bcl-xL, Bcl-10, and XIAP with no significant change in p21 (8h) and XIAP (8h) (Table 1). At 8h and 24h post-treatment, concurrent targeting of COX-2 and EP1/EP4 antagonists significantly upregulated pro-apoptotic genes TNF, FADD, BID, BAD, BAX, p53, and significantly downregulated anti-apoptotic genes Bcl-10, Bcl-2, Bcl-xL, cIAP-1, cIAP-2, and Survivin in BCBL-1 cells with no significant change in p21 (8h) and p53 (8h) (Table 1). In BC-3 cells, significant upregulation of pro-apoptotic genes BAX, p53, and p21 and significant downregulation of anti-apoptotic genes cIAP-1 and cIAP-2 was seen at 8h (Table 1) post-treatment with celecoxib, SC-51322, and GW 627368X treatments. At 24h, celecoxib and EP1/4 receptor antagonist combination treatment in BC-3 cells significantly upregulated p53, p21 and downregulated cIAP-1, cIAP-2, Survivin, and Bcl-2 (Table 1). With the exception of the significant downregulation of general cell survival molecules

cIAP-1 and cIAP-2 at 8h and 24h and Survivin at 24h, concurrent targeting of COX-2 and EP1/EP4 antagonists had no significant effect on any of the apoptotic genes examined in BJAB cells (Table 1).

Concurrent targeting of COX-2 and EP1/EP4 receptors markedly alters the lymphoma gene expression profile of BCBL-1 and Akata/EBV+ cells

BCBL-1 and Akata/EBV+ cells are B cells transformed by latent infections of KSHV and EBV with overlapping gene expression profiles compared to other NHLs such as follicular lymphoma (FL) and diffused large B cell lymphoma (DLBCL).^{2–5, 7} Considering apoptosis pathway induction with concurrent inhibition of COX-2 and EP1/EP4 receptors (Table 1), we next examined the gene expression profile of key genes involved in lymphoma pathogenesis including lymphoma survival, immune and cell cycle regulation, cell adhesion, PI3K/Akt signaling, inflammation, gene fusion, differential methylation of promoters, and DNA repair, and also screened for key lymphoma therapeutic targets. BCBL-1 and Akata/EBV+ cells were seeded with a combination of 1.0 μ M each of celecoxib, SC-51322, and GW 627368X and fold regulation for untreated samples at 24h and treated samples at 8h and 24h was calculated with respect to untreated samples at 8h and are listed in supplement tables 1–3 (BCBL-1) and supplement tables 4–6 (Akata/EBV+) with respective p-values ($p < 0.05$ for significance). Clustergrams and dendrograms indicating co-regulated genes across groups (untreated samples at 8h and 24h and treated samples at 8h and 24h) for BCBL-1 and Akata/EBV+ cells are represented in supplemental figures 1–2. Drug treatments did not have a significant effect on any of the genes at 24h post treatment for BCBL-1 (Supplemental table 1–3) and Akata/EBV+ cells (Supplemental table 10–12).

Table 2 lists the respective genes significantly ($p < 0.05$) upregulated or downregulated at the indicated time points for BCBL-1 and Akata/EBV+ cells. Various genes were classified based on functionality using SA Biosciences pathway source references (Table 1). Genes significantly upregulated or downregulated upon concurrent inhibition of COX-2 and EP1/EP4 receptors in BCBL-1 or Akata/EBV+ cells are categorized based on fold regulation into three groups: a) 2–10 fold (shown in red), b) 10–100 fold (shown in green), and c) 100 fold (shown in blue) in Table 2.

We observed 10–100 fold induction of lymphoma therapeutic target genes (SYK, PTK2, SRC), DLBCL survival (PTK2), cell adhesion (SYK, SRC), cell cycle (MK167, MLH1) and T cell differentiation (SYK), PI3K/AKT signaling (PTK2, TP53, SYK), and tumor suppressor pathway (MLH1) genes in BCBL-1 cells at the time points indicated in Table 2.

10–100 fold induction was observed in the expression of lymphoma therapeutic target genes (MCL1, PARP1), DLBCL survival (ASB13, VGLL4, ITPKB), follicular lymphoma survival (TLR5, STAT4), inflammatory response (NFKB1, TLR5), immune regulation (ITPKB, PDLIM7), T cell differentiation (ITPKB, TP53), cell cycle (RASSF1), cell adhesion (NCAM1), differentially methylated promoters (ATM, FHIT, HIC1, RASSF1, RBP1, TIMP2, TIMP3, SLC19A1), PI3K/AKT signaling (TIMP2, TIMP3), and tumor suppressor pathway (ATM, FHIT, HIC1, MCL1, NCAM1, RASSF1, TIMP2, TIMP3, TP53) genes in BCBL-1 cells at the time points indicated in Table 2. We observed 2–10 fold induction in the expression of lymphoma therapeutic target genes (TGFB1, HSP90AA1),

follicular lymphoma survival (TLR5, TNFRSF1B), inflammatory response (TGFB1, TNF, TLR5, TNFRSF1B), immune regulation and T cell differentiation (TGFB1, PTPRC), cell cycle (TGFB1), cell adhesion (PTPRC, TNF), differentially methylated promoters (HRK), and PI3K/AKT signaling (HSP90AA1, TGFB1, TNFRSF1B) genes in BCBL-1 cells at the time points as indicated in Table 2. Celecoxib and EP1/EP4 receptor antagonist treatment downregulated genes of multiple pathways including lymphoma therapeutic targets (BTK, CD20, and CD40), follicular lymphoma survival (C1QA), inflammatory response (CD40, C1QA), cell adhesion (CD22), and PI3K/AKT signaling (BTK, CD40) at multiple folds at the time points indicated in Table 2.

Similar to BCBL-1 cells, we observed 10–100 fold induction of lymphoma therapeutic target genes (MCL1, MS4A1, mTOR, TNFSF13B, BIRC5), DLBCL survival (LMO2, LRMP, MYBL1, PAG1), follicular lymphoma survival (F8, FLT3LG, TNFSF13B), inflammatory response (CSF3, F8, IL6), immune regulation (IFNG, IL2, IL6, TNFSF13B), T cell differentiation (IL2), cell cycle (ATM, IFNG, MLH1, MK167, MYC, ID4, BIRC5), cell adhesion (CDH13, CDKN1C, CDKN2B, CDLN2C, F8, IL2), differentially methylated promoters (ATM, CDH13, CDKN1C, CDKN2B, CDKN2C, DAPK1, DLC1, GSTP1, HRK, ID4, LRP1B, MLH1, LRP1B), PI3K/AKT signaling (CSF3, IL2, IL6, mTOR, TNFSF13B), tumor suppressor pathway (ATM, CDH13, CDKN1C, CDKN2B, CDKN2C, DAPK1, DLC1, LRP1B, MLH1), gene fusion (MME), and DNA repair (DNMT) genes in Akata/EBV + cells at the time points as indicated in Table 2.

10–100 fold induction was observed in the expression of lymphoma therapeutic target genes (FCGR1A, HSP90AA1, MCL1, PTK2, PARP1, SYK), DLBCL survival (ITPKB, LMO2, PTK2), follicular lymphoma survival (FCGR1A, STAT4, FLT3LG), immune regulation (ITPKB, SYK), T cell differentiation (TP53, ITPKB, SYK), cell cycle (TP53, MLH1, MYC), cell adhesion (CDH1, SYK), differentially methylated promoters (CDH1, HIC, TIMP2, TIMP3), PI3K/AKT signaling (HSP90AA1, TP53, TIMP2, TIMP3, SYK), and tumor suppressor pathway (CDH1, HIC1, TIMP2, TIMP3) genes in Akata/EBV+ cells at the time points indicated in Table 2. We found 2–10 fold induction in the expression of lymphoma therapeutic target genes (CXCR4, SRC), DLBCL survival (VGLL4, LRMP), follicular lymphoma survival (TLR5), inflammatory response (CXCR4, TLR5, CSF3), T cell differentiation (SRC, TP53), cell cycle (RASSF1, TP53), differentially methylated promoters (RASSF1, SLC19A1), PI3K/AKT signaling (CXCR4, CSF3, TP53), and tumor suppressor pathway (RASSF1) genes in Akata/EBV+ cells at the time points indicated in Table 2. COXIB and EP1/EP4 receptor antagonist treatment downregulated genes of multiple pathways including, lymphoma therapeutic targets (BTK, CD22, CD40, CXCR4), follicular lymphoma survival (C1QA, CCR1), inflammatory response (C1QA, CADM1, CD2, CD40), T cell differentiation (Bcl-2), cell cycle (Bcl-2, CCND1), cell adhesion (Bcl-2, CADM1, CCR1, CD2, CD22), differentially methylated promoters (CADM1, CCND1, DAPK1), and PI3K/AKT signaling (Bcl-2, BTK2, CCND1, CCR1, CD2, CD40, CXCR4) in Akata/EBV+ cells at multiple folds at the time points indicated in Table 2.

DISCUSSION

KSHV and EBV associated lymphomas are characterized by fully transformed B cells exhibiting multiple aspects of oncogenesis such as downregulation of immune screening of cancerous cells, downregulation of pro-apoptotic mechanisms, and bypass of cell cycle check points, upregulation of metastatic and proliferative mechanisms and creation of a proinflammatory microenvironment.³⁻⁷ The proinflammatory tumor microenvironment is enriched with several inflammatory cytokines (ICs), growth factors (GFs), and angiogenic factors (AFs) that constantly nourish a condition of chronic reactive inflammatory hyperplasia that plays an important role in driving the pathogenesis of aggressive lymphomas.³⁻⁷ COX-2, its oncogenic metabolite PGE₂, and PGE₂ receptors, namely, EP receptors and associated signaling, forms one of most crucial pathways that regulate inflammation. Many viruses such as HTLV-1, HCV, and herpes viruses including CMV, EBV, and KSHV, have evolved mechanisms to regulate the COX-2/PGE₂/EP receptor pathway for creating a proinflammatory tumor microenvironment that provides them with a survival advantage.^{47, 48} Previous studies from our laboratory have demonstrated the role of EP receptors in the KSHV, latency program and the chemotherapeutic potential of NSAIDs in treating KSHV associated lymphomas.⁴¹ This prompted us to examine the therapeutic potential of EP receptor antagonists in treating KSHV and EBV related lymphomas and consequently examining whether combining EP receptor blockers with NSAIDs mutually potentiate their anti-proliferative effects.

Several cell lines such as BC-1, BC-3, BCBL-1, HBL-6 and JSC-1 have been established from primary effusion lymphoma tumors.^{4,7} BCBL-1 and BC-3 cells carry only the KSHV genome whereas multiple genome copies of KSHV and related EBV exist in BC-1, HBL-6 and JSC-1 cells.^{6,7} The Akata/EBV+ cell line is an EBV producing Burkitt's lymphoma cell line that harbors the EBV episome and a Burkitt's type chromosome translocation, t(8q-; 14q+).⁴⁹ However, the BJAB and Akata/EBV- cell lines possess the t(8q-; 14q+) translocation but not the EBV episome.⁵⁰ In our study, we chose KSHV+/EBV- (BCBL-1 and BC-3), KSHV-/EBV+ (Akata/EBV+), KSHV+/EBV+ (JSC-1), and KSHV-/EBV- (BJAB and Akata/EBV-) cell lines to cover the entire spectrum of KSHV and EBV associated lymphomas.

In the first part of our work, we screened for the specific EP receptor antagonist and the lowest concentration that had a specific effect on KSHV+/EBV- (BCBL-1 and BC-3), KSHV-/EBV+ (Akata/EBV+), and KSHV+/EBV+ (JSC-1) cell lines. EP receptor antagonists are reversible receptor blockers and therefore we supplemented the drug every 12h. The EC₅₀ of SC-51322 (EP1 antagonist), AH 6809 (EP2 antagonist), and GW 627368X (EP4 antagonist) are 7.75 μ M, 50 μ M, and 10 μ M, respectively.⁵¹⁻⁵³ We used an exponential range of 0.5 μ M, 5.0 μ M, and 50.0 μ M for all three EP receptor antagonists to delineate between a specific EP receptor mediated anti-proliferative effect and a non-EP receptor mediated anti-growth effect. The lowest concentration of SC-51322, and GW 627368X that had a significant sustained anti-proliferative effect on BCBL-1, BC-3, Akata/EBV+, and JSC-1 was 5.0 μ M. In comparison, 50.0 μ M AH6809 was required to induce a significant anti-proliferative effect. The anti-proliferative effects of SC-51322 and GW 627368X were initiated at earlier time points at 50.0 μ M compared to 5.0 μ M indicating the initiation of a

non-EP receptor mediated anti-proliferative effect in addition to EP receptor blockade. Furthermore, at higher concentrations, EP receptor antagonists are also shown to block thromboxane receptors as well, which might also have contributed to their anti-growth effect.

Our decision to use celecoxib as the COX-2 inhibitor was due to its specificity for blocking COX-2 with 30 fold biochemical selectivity for COX-2 compared to COX-1.^{54, 55} Celecoxib is also known to induce anti-proliferative effects through non-COX-2 blockade mediated anti-proliferative effects at concentrations greater than 10.0 μ M.⁵⁵ Therefore, our data strongly suggests that the anti-proliferative effects we observed with 5.0 μ M celecoxib were specifically due to an anti-COX-2 mediated effect and the dependency of BCBL-1 and Akata/EBV+ cells on COX-2 mediated cell survival mechanisms. This is further supported by our previous work where the COX-2 inhibitor nimesulide was selectively more pro-apoptotic to BCBL-1 compared to BJAB due to upregulation of the COX-2/PGE2 pathway in BCBL-1 cells.⁴⁰

In the second part of our study, we examined whether the anti-proliferation arrest we observed is due to the induction of apoptosis by measuring the levels of the apoptotic marker cleaved-caspase 3. We did not use AH6809 in our apoptotic studies because the drug did not induce significant proliferation arrest in BCBL-1, BC-3, and JSC-1 cells at earlier time points except for Akata/EBV+ cells. We chose BCBL-1 and Akata/EBV+ cells with BJAB and Akata/EBV- cells as control cell lines. SC-51322, GW 627368X, and celecoxib at 5.0 μ M were able to induce a modest level of apoptosis in BJAB cells compared to BCBL-1 and Akata/EBV+ cell lines. Our data on BCBL-1 cells is further supported by our earlier work that demonstrated the selective anti-proliferative effect of the COX-2 inhibitor nimesulide on BCBL-1 cells compared to BJAB due to the piracy of the COX-2/PGE2 receptor pathway for the maintenance of the KSHV latency program.⁴¹ Nimesulide downregulated the KSHV latency program resulting in the initiation of p53 mediated cell cycle check points and apoptosis in BCBL-1 cells.⁴⁰ Our earlier studies have also demonstrated the utilization of EP receptors by KSHV infection for maintenance of the latency program.⁴¹ Similarly, in Akata/EBV+ cells, EBV might be utilizing the COX-2/PGE2/EP receptor pathway for latency and survival related mechanisms.

The levels of PGE2 secreted by BCBL-1, BC-3, Akata/EBV+, JSC-1, and Akata/EBV cells were much higher than BJAB cells. Therefore, our data indicating the increase in susceptibility of Akata/EBV- cell lines compared to BJAB to the pro-apoptotic effects of SC-51322, GW 627368X, and celecoxib could be due to elevation of PGE2 secretion and therefore activation of EP receptors, although neither cell line harbors the EBV episome. Previous report⁴ have demonstrated the accumulation of multiple oncogenic mutations in BL cell lines in culture that once harbored the EBV episome, which might be responsible for the activation of the COX-2/PGE2/EP receptor pathway in Akata/EBV- cell lines.

Our earlier reports have illustrated the activation of COX-2 gene expression, and subsequent increased protein levels and PGE2 secretion in *de novo* infected primary endothelial cells.⁴² Further studies have strongly indicated the utilization of the COX-2/PGE2/EP receptor pathway by the KSHV latency program.³⁹⁻⁴² Therefore, our data demonstrating the

elevation of EP receptors by *de novo* KSHV infection in HMVEC-d cells suggests that primary KSHV infection induces EP receptors as well. However, at day 3 p.i., the levels of EP receptors were reduced to base line levels. Our earlier reports have also shown that the initial surge in COX-2 induction and PGE2 secretion is a process regulated by KSHV infection and a self-sustained positive feedback loop through EP receptors. Therefore, after establishment of the sustained activation of COX-2 and PGE2 secretion, the individual activation of EP receptors might be a process mediated by the ligand PGE2 rather than a receptor level driven process. Although, an identical process cannot be generalized in the NHL cell lines we used due to the variable levels of EP receptors, the susceptibility of the Akata/EBV⁻ cell line to the pro-apoptotic effects of SC-51322, GW 627368X, and celecoxib with PGE2 levels comparable to KSHV⁺ and EBV⁺ cell lines further supports our data. Similar work needs to be done with *de novo* EBV infection of primary endothelial cells.⁵⁶

One of the major issues associated with NSAIDs is their non-specific side effects such GI toxicity from the inhibition of COX-1.³² Although highly specific COX-2 inhibitors such as COXIBs were introduced to resolve this, they were associated with an increased risk of thrombotic and cardiovascular events due to the absence of aspirin-like platelet aggregation inhibiting properties.^{32, 57} Although the chemotherapeutic effects of celecoxib are reported in several studies, they were due to non-specific effects from blocking cell survival kinases such as PI3K/Akt at concentrations greater than their EC_{50s}.⁴⁶ Therefore, we proposed the simultaneous blockade of COX-2 and EP receptors at concentrations closer to their EC_{50s} to obtain the desired anti-COX-2/PGE2/EP receptor mediated chemotherapeutic effect without the non-specific anti-growth effect and associated side effects of NSAIDs. The anti-cancer potency of concurrent targeting of COX-2 and EP1/EP4 receptors is demonstrated by the induction of the apoptotic pathway and upregulation of the expression of several tumor suppressor genes in BCBL-1 and Akata/EBV⁺ cells such as ataxia telangiectasia mutated (ATM), fragile histidine triad gene (FHIT), hypermethylated in cancer 1 (HIC1), myeloid cell leukemia sequence 1 (MCL1), MutL homolog 1 (MLH1), ras association (RalGDS/AF-6) domain family member 1 (RASSF1), neural cell adhesion molecule 1 (NCAM1), TIMP2/3, tumor protein p53 (TP53), E-cadherin (CDH1), cadherin 13 (CDH13), death-associated protein kinase 1 (DAPK1), deleted in liver cancer 1 (DLC1), cyclin-dependent kinase inhibitor 1C (CDKN1C), cyclin-dependent kinase inhibitor 2B (CDKN2B), cyclin-dependent kinase inhibitor 2C (CDKN2C), and low density lipoprotein receptor-related protein 1B (LRP1B).^{58–71} Several genes upregulated in our PCR based gene array study have also been shown to be downregulated by either indicated viral infections such as chemokine (C-X-C motif) receptor 4 (CXCR4), LIM domain only 2 (LMO2), v-myc myelocytomatosis viral oncogene homolog (MYC), toll-like receptor 5 (TLR5), and inhibitor of DNA binding 4 (ID4) by EBV^{72–75} Genes otherwise downregulated by KSHV protein LANA-1 (TGFB1), and vIRF1 (ATM) were upregulated upon celecoxib, EP1 antagonist SC-51322, and EP4 antagonist GW 627368X treatment^{76, 77} We observed induction in the expression of LMP1 downregulated genes (TGFB1, CXCR4, LRMP, TP53, RASSF1, and TIMP3) and EBNA2 downregulated gene (MME) were induced upon combination of COX-2 inhibitor and EP/EP4 receptor antagonist treatment^{72, 73, 78–81} Similarly, expression of genes downregulated by EBV infection mediated methylation such

as MLH1, CDH1, DLC1, CDKN1C, glutathione S-transferase pi 1 (GSTP1), HIC1, and RASSF1 were also significantly upregulated in Akata/EBV+ cells treated with COX-2 inhibitor celecoxib, EP1 antagonist SC-51322, and EP4 antagonist GW 627368X.^{81–90} Expression of several genes induced by concurrent COX-2 and EP1/EP4 receptor inhibition has also been shown to be activated by either KSHV and EBV infections such as SRC, NFκB, mTOR, NFκB1, IL-2, IL-6, and BIRC5 (EBV) or by respective viral proteins such as SRC and SYK which are activated by KSHV proteins K1, K15, vGPCR and by EBV proteins LMP1 and LMP2A, and mTOR activation by EBV LMP1.^{90–103} The significance of such counter-intuitive results needs to be further delineated and it might very well be that the level of expression of these genes might be the deciding factor for survival versus cell death in BCBL-1 and Akata/EBV+ cells. The hijacking of the pro-survival effect of TNF via the TRAF pathway versus the pro-apoptotic effect by KSHV protein vFLIP is strongly suggestive of the existence of such mechanisms.¹⁰⁴ FHIT and CSF3 are genes proposed to be undetectable in PEL and BL.^{105–107} However, FHIT and CSF3 were significantly upregulated in BCBL-1 and Akata/EBV+ cells, respectively indicating the reversal of mechanisms that downregulates FHIT and CSF3 by the concurrent inhibition of COX-2 and EP1/EP4 receptors. Viral mimicry and hijacking of host proteins are common themes in KSHV and EBV mediated tumor pathogenesis such as the role of KSHV-Bcl-2 and EBV-BHRF1 in modulating Bcl-2 and MCL-1 mediated anti-apoptotic mechanisms and the role of EBV-LMP1 in regulating CD40 functions and inducing Btk mediated cell survival mechanisms.^{108–111} The downregulation of CD40, Btk, Bcl-2, Bcl-xL, Bcl-10 and MCL-1 along with the upregulation of BID, BAD, and Bax by concurrent COX-2 and EP1/EP4 receptor inhibition in BCBL-1 and Akata/EBV+ cells is strongly suggestive of the inhibition of the anti-apoptotic and pro-survival mechanisms initiated by viral proteins. In our studies with BCBL-1 and Akata/EBV+, TLR5 was significantly upregulated by concurrent COX-2 and EP1/EP4 receptor inhibition. KSHV genome copy number has been shown to be downregulated by TLR5 activation and EBV infection has been proposed to downregulate TLR5 signaling and therefore our data is strongly suggestive of the activation of host innate immune responses, which otherwise are subverted by viral infection.^{112,113} The upregulation of PARP1 in BCBL-1 cells and studies indicating the role of PARP1 in KSHV genome maintenance and replication plus blockade of the lytic cycle through poly-(ADP-ribosyl)-ation of LANA-1 and O-GlcNAcylation of KSHV-RTA raises the question of whether the lytic cycle is activated by COX-2 and EP receptor inhibition.^{114–116}

Overall, our gene array based data further augment our proposal that the anti-cancer effects of concurrent targeting of COX-2 and EP1/EP4 receptors is due to the simultaneous inhibition of viral and non-viral mediated tumorigenic mechanisms acting at multiple levels such as viral-host protein interactions, host and viral gene expression through regulation of epigenetic mechanisms such as methylation, host signaling, immune system activation, pro-inflammatory and cell survival processes. The novelty of our study lies in the exploration of PGE2 receptor antagonists and COX-2 specific inhibitor in the lowest concentration in lymphoma cells expressing COX-2 and PGE2 receptors. Though there are some reports indicating the role of combination therapy in colorectal carcinogenesis,^{19,20} liver cancer,¹¹⁷ skin cancer,¹¹⁸ esophageal cancer,¹¹⁹ breast cancer,¹²⁰ and non-small cell lung cancer (NSCLC)¹²¹ but has never been tested in any lymphoma. Our study has translational

significance as it explored the combination of safe and more effective EP receptor antagonists and COXIB which could hit multiple targets simultaneously. Our study tested this novel hypothesis and reported that combination of the COX-2 inhibitor celecoxib, EP1 antagonist SC-51322, and EP4 antagonist GW 627368X induced apoptosis at concentrations below the lowest concentration required to induce proliferation than when used alone. Studies to understand the relevance of COX-2 and EP receptors in viral latency and chemotherapeutic potential of these combination therapies in non-invasive and invasive PEL-SCID xenograft and KS xenograft tumors in murine models are currently ongoing in our lab. PEL and KS models will pave the way for identifying a unique arena of drug targets for treating KSHV linked B and endothelial cell malignancies. As the expectation of developing a "magic bullet" for curing virus linked neoplasia is low, designing better treatment modalities such as additive therapies might be effective and will provide promising targets at multiple levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

COX-2	cyclooxygenase-2
PGE2	prostaglandin E2
NSAIDs	non-steroidal anti-inflammatory drugs
EP	eicosonoid
GPCRs	G protein coupled receptors
PEL	primary effusion lymphoma

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SPECULATIONS

Our studies for the first time demonstrate the chemotherapeutic potential of EP1 and EP4 receptor antagonists and the synergistic pro-apoptotic effect of simultaneous blockade of COX-2 and EP1/EP4 receptors in treating KSHV+ and EBV+ NHL cell lines. Although our study was based on *in vitro* experiments using human cell lines, currently work is underway to test our hypothesis in animal models and to delineate the molecular and pathological mechanisms mediated by EP receptors in KSHV and EBV associated NHLs. Considering the role of inflammation in NHLs, we also predict that our model is applicable in developing a novel treatment modality in other NHLs as well.

Figure 1. EP receptors in non-Hodgkin lymphoma cell lines and *de novo* KSHV infected HMVEC-d cells

(a) Total lysates from 5×10^5 BJAB, Akata/EBV-, BCBL-1, BC-3, Akata/EBV+, and JSC-1 cells were immunoblotted for EP1, EP2, EP3, and EP4. Data represents three independent experiments. Tubulin was used as loading control. (b) In parallel experiments, supernatants from the indicated cells were collected to measure the amount of PGE2 secreted by each cell line. (c) The indicated cell lines were stained for EP1, EP2, EP3, and EP4 receptors by immunofluorescence. (d) Mean fluorescent intensity (MFI) of EP1, EP2, EP3, and EP4

receptors in HMVEC-d cells infected with KSHV measured by FACS. Indicated are the MFI for the respective receptor at each time point. The data is representative of three independent experiments.

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Figure 2. Effects of SC-51322 on NHL cell lines

5×10^5 BCBL-1 (a–b), BC-3 (c–d), Akata/EBV+ (e–f), JSC-1 (g–h), and BJAB (i–j) cells were seeded with the indicated concentrations of SC-51322 and were replenished with the drug every 12h for 5 days. The cells were not replenished with fresh media. Cell proliferation was measured by MTT assay at day 1 (1d), day 2 (2d), day 3 (3d), and day 5 (5d). The fold absorbance was calculated with respect to the untreated (UN) control before treatments. Statistics (t-test) were calculated with respect to untreated for each time point. Error bars represent mean $\pm$ SD.

Figure 3. Effects of AH6809 on NHL cell lines

5×10^5 BCBL-1 (a–b), BC-3 (c–d), Akata/EBV+ (e–f), JSC-1 (g–h), and BJAB (i–j) cells were seeded with the indicated concentrations of AH6809 and were replenished with the drug every 12h for 5 days. The cells were not replenished with fresh media. Cell proliferation was measured by MTT assay at day 1 (1d), day 2 (2d), day 3 (3d), and day 5 (5d). The fold absorbance was calculated with respect to the untreated (UN) control before treatments. Statistics (t-test) were calculated with respect to untreated for each time point. Error bars represent mean $\pm$ SD.

Figure 4. Effects of GW 627268X on NHL cell lines

5×10^5 BCBL-1 (a–b), BC-3 (c–d), Akata/EBV+ (e–f), JSC-1 (g–h), and BJAB (i–j) cells were seeded with the indicated concentrations of GW 627268X and were replenished with the drug every 12h for 5 days. The cells were not replenished with fresh media. Cell proliferation was measured by MTT assay at day 1 (1d), day 2 (2d), day 3 (3d), and day 5 (5d). The fold absorbance was calculated with respect to the untreated (UN) control before treatments. Statistics (t-test) were calculated with respect to untreated for each time point. Error bars represent mean $\pm$ SD.

Figure 5. Effects of celecoxib on NHL cell lines

5×10^5 BCBL-1 (a–b), BC-3 (c–d), Akata/EBV+ (e–f), JSC-1 (g–h), and BJAB (i–j) cells were seeded with the indicated concentrations of celecoxib and were replenished with the drug every 12h for 5 days. The cells were not replenished with fresh media. Cell proliferation was measured by MTT assay at day 1 (1d), day 2 (2d), day 3 (3d), and day 5 (5d). The fold absorbance was calculated with respect to the untreated (UN) control before treatments. Statistics (t-test) were calculated with respect to untreated for each time point. Error bars represent mean $\pm$ SD.

Figure 6. Effect of celecoxib, SC-51322, and GW 627368X on the apoptotic marker cleaved-caspase-3 in BCBL-1, Akata/EBV+, Akata/EBV-, and BJAB cells
 (a–b) 5×10^5 BCBL-1 (a), Akata/EBV+ (b), Akata/EBV- (c), and BJAB (d) cells were seeded with celecoxib (5 μM), SC-51322 (5 μM) or GW 627368X (5 μM) and were replenished with the drug every 12h for 5 days. Samples were collected at days 1 and day 5 to examine the levels of the apoptotic marker cleaved-caspase 3 by FACS. The data is representative of two independent experiments.

Figure 7. Effect of combinations of celecoxib, SC-51322, and GW 627368X on KSHV+/EBV- and KSHV-/EBV+ cells

(a–b) 5×10^5 BCBL-1 and Akata/EBV+ cells were seeded with a combination of 5.0 μ M each of SC-51322 and GW 627368X (a) or a combination of 1.0 μ M each of celecoxib, SC-51322, and GW 627368X (b) and were replenished with the drugs every 12h for 5 days. Samples were collected at days 1 and 5 to examine the levels of the apoptotic marker cleaved-caspase 3 by FACS. The data is representative of two independent experiments.

Table 1

Effect of concurrent targeting of COX-2 and EP1/EP4 receptors with 1.0μM each of celecoxib, SC-51322, and GW 627368X on various classes of NHL genes

	Akata/EBV+ (KSHV-/EBV+)		BCBL-1 (KSHV+/EBV-)		BC-3 (KSHV+/EBV-)		BJAB (KSHV-/EBV-)	
	8h	24h	8h	24h	8h	24h	8h	24h
Pro-apoptotic genes								
<i>TNF</i>	↑	↑	↑	↑	•	•	•	•
<i>FADD</i>	↑	↑	↑	↑	•	•	•	•
<i>BID</i>	↑	↑	↑	↑	•	•	•	•
<i>BAD</i>	↑	↑	↑	↑	•	•	•	•
<i>BAX</i>	↑	↑	↑	↑	↑	✖	✖	✖
<i>p53</i>	↑	↑	✖	↑	↑	↑	✖	✖
<i>p21</i>	✖	↑	✖	↑	↑	↑	✖	✖
Anti-apoptotic genes								
<i>CIAP-1</i>	↓	↓	↓	↓	↓	↓	↓	↓
<i>CIAP-2</i>	↓	↓	↓	↓	↓	↓	↓	↓
<i>Survivin</i>	↓	↓	↓	↓	✖	↓	✖	↓
<i>Bcl-2</i>	↓	↓	↓	↓	✖	↓	✖	✖
<i>Bcl-xL</i>	↓	↓	↓	↓	✖	✖	✖	✖
<i>Bcl-10</i>	↓	↓	↓	↓	•	•	•	•
<i>XIAP</i>	✖	↓	↓	↓	•	•	•	•

(↑) Increase (↓) Decrease (✖) No significant change (•) Undetermined

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Table 2

Effect of concurrent targeting of COX-2 and EPL/EP4 receptors with LQJMs each of celecoxib, SC-51322 and GW 6273681

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Gene grouping	BCBL-1 (KSHV+/EBV-)				Akata/EBV+ (KSHV+/EBV+)			
	Up-regulated		Down-regulated		Up-regulated		Down-regulated	
	8h	24h	8h	24h	8h	24h	8h	24h
Lymphoma therapeutic targets	SYK ⁺ , TGFB1 ⁺	MCL1, HSP90AA1, PARP1, PTK2, SRC, SYK ⁺ , TGFB1	BTX, CD22, CD40	BTX, CD22, CD40	CXCR4 ⁺ , FCGR1A, HSP90AA1, MCL1 ⁺ , MSAA1, MSAA1, mTOR, TNFSF13B	MCL1, mTOR ⁺ , PARP1, PTK2, SRC ⁺ , SYK ⁺ , BIRC5 ⁺	BTX	BTX, CD22, CD40 ⁺ , CXCR4
DLBCL survival	ASB13, VGLL4	ITPKB, PTK2, VGLL4			LMO2 ⁺ , LRMP ⁺ , MYBL1, VGLL4	ITPKB, LMO2, LRMP, MYBL1, PAG1, PTK2		
Follicular lymphoma survival	STAT4, TLR5	STAT4, TLR5, TNFRSF1B			F8, FCGR1A, FLT3LG, TLR5 ⁺	STAT4, FLT3LG	C1QA	CCR1, C1QA
Inflammatory response	NFKB1 ⁺ , TGFB1, TLR5	NFKB1, TGFB1, TLR5, TNF, TNFRSF1B	CD40	CD40, CD10A	CSF3, CXCR4, F8, IL6 ⁺ , TLR5	CSF3, IL6	C1QA	CCR1, CD40, CXCR4, C1QA
Immune regulation	SYK, TGFB1	ITPKB, POLM7, PTPRC, SYK, TGFB1	CD40, CD73A	CD40, CD73A, C1QA	IFNG, IL2 ⁺ , IL6, IL6, TNFSF13B	IFNG, IL2, IL6, ITPKB, SYK	C1QA	CAOM1, CD2, CD40, C1QA
T Cell differentiation	SYK, TGFB1	ITPKB, PTPRC, SYK, TGFB1, TP53 ⁺			IL2, TP53	IL2, ITPKB, SRC, SYK, TP53 ⁺		Bcl-2 ⁺
Cell cycle	ATM ⁺ , TGFB1	MLH1, MRE11, RASGE1 ⁺ , TGFB1, TP53			ATM ⁺ , IFNG, MLH1 ⁺ , MKI67, MYC ⁺ , ID4, TP53	IFNG, MLH1, MKI67, MYC, RASGE1 ⁺ , TP53, BIRC5		Bcl-2, CCND1
Cell adhesion	SYK	PTPRC, SYK, SRC, TNF, NCAM1	CD22	CD22	CDH1 ⁺ , CDH13, CDKN1C ⁺ , CDKN2B, CDKN2C, F8, IL2	IL2, SYK		Bcl-2, CAOM1, CCR1, CD2, CD22
Differentially methylated promoters	ATM, FHIT, HIC1	HIC1, HRK, RASGE1, RBP1, TP53, TP53			ATM, CDH1, CDH13, CDKN1C, CDKN2B, CDKN2C, DAPK1 ⁺ , DLC1 ⁺ , GSTP1 ⁺ , HIC1 ⁺ , HRK, ID4 ⁺ , LRP1B, MLH1	LRP1B, RASGE1, SLC19A1, TP53 ⁺		CAOM1, CCND1, DAPK1
PI3K/Akt signaling	SYK, TGFB1	PTK2, HSP90AA1, SYK, TGFB1, TP53, TP53	BTX, CD40	BTX, CD40	CSF3, CXCR4, HSP90AA1, IL2, IL6, mTOR, TP53, TNFSF13B	CSF3, IL2, mTOR, TP53, TP53	BTX	BCL2, BTX2, CCND1, CCR1, CD2, CD40, CXCR4
Tumor suppressor genes	ATM, FHIT, HIC1	HIC1, MCL1, MLH1, NCAM1, RASGE1, TP53, TP53			ATM, CDH1, CDH13, CDKN1C, CDKN2B, CDKN2C, DAPK1, DLC1, HIC1, LRP1B, MLH1, TP53	RASGE1, TP53, TP53		
Gene fusion					MME ⁺			
DNA repair								

(Red) 22-10 fold change, (Green) ≥10-100 fold change, (Blue) ≥100 fold change
(Underlined) Significant change in both BCBL-1 and Akata/EBV+ cells, (*) Up-regulated and (†) Down-regulated by the respective virus