Targeting Cyclooxygenase-2 Reduces Overt Toxicity toward
Low-Dose Vinblastine and Extends Survival of
Juvenile Mice with Friend Disease

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ABSTRACT

Purpose: To test the efficacy of selective therapy
against cyclooxygenase-2 in combination with a low-dose
regimen of a cytotoxic agent in the treatment of juvenile
hematopoietic malignancies in the experimental model,
Friend disease.

Experimental Design: Juvenile erythroleukemic mice
(n = 8) received no treatment, celecoxib (1600 mg/kg/d),
v vinblastine (0.5 μg/g twice weekly), vehicle controls, or
clecoxib + vinblastine combination (n = 9) over a 6-month
period from time of tumor induction. Overt toxicity was
assessed daily and recorded weekly.

Results: Among randomly selected mice from celecoxib
treatment groups, plasma concentrations ranged from 2 to
6 μmol/L. As a single agent, celecoxib was not associated
with any apparent toxicity. Monotherapy with vinblastine,
however, caused early mortality marked by severe diarrhea,
lethargy, and weight loss. At the tested doses, neither
vinblastine nor celecoxib enhanced survival as monothera-
pies. Coadministration of these two drugs alleviated the overt
toxicity associated with vinblastine and resulted in a
significant increase in survival (P < 0.05). Survivors sampled
throughout the study showed a trend to decreased weight loss
and hematocrit levels among all groups, but significance was
evidenced earlier in the vinblastine monotherapy group
overall (P < 0.05). Despite similar degree of splenomegaly,
histologic analysis revealed preserved splenic mantle archi-
tecture from mice given combination therapy compared with
those sampled from mice on all other monotherapies,
exhibiting a more diffuse burden of blasts and destruction
of germinal centers.

Conclusion: We propose that addition of a selective
cyclooxygenase-2 inhibitor to a modified low-dose conven-
tional chemotherapeutic regimen protects juvenile mice with
Friend disease from succumbing to low-dose cytotoxicity, in
part, by neutralizing acute inflammatory responses.

INTRODUCTION

The need for improved clinical modalities in the treatment
of advanced forms of cancer becomes most apparent when one
considers the subsets of patients for whom the current standard
treatment offers little hope. Such is the case with patients
suffering from aggressive forms of acute myeloid leukemias
(AML), highly chemotherapy resistant tumors. A subtype of
AML, M6, first described by Di Guglielmo in 1917, has
experienced little advancement in effective treatments over the
years. The main difficulties associated with such hematologic
malignancies include a lack of correlation between morphologic
changes, cytogenetic abnormalities, and clinical outcome (1).
Despite some improved understanding of the disease, achieved
through retrospective studies of patients (2), even with newer
therapies, such as daunomycin and arabinoside-C combinations,
for a variety of AMLs including M6, complete remission of
patients (50-80%) still occurs in an age- and leukemic
karyotype–dependent manner (3, 4). The main reasons accounting
for failure of this therapy include side effects of
chemotherapy during induction phases, and drug resistance (5).

A murine model, Friend disease, whereby the stepwise
progression of a viral-induced erythroleukemia can be monitored
from an early polyclonal stage through to the proliferation of
tumorigenic clones leading to blast crisis and death of the
organism, is invaluable for testing new therapies in these
cancers. Pathophysiology of murine erythroleukemia was first
described by Charlotte Friend as a presentation of anemia,
thrombocytopenia, leukocytosis, increased liver and spleen
volume, and hematopoiesis (6). Subsequent studies over the
years have discerned the molecular mechanisms surrounding
the growth and survival advantages associated with Friend virus–
induced erythroleukemia cells (for review, see refs. 7, 8). The
pathology and aggressive nature of Friend disease are akin to the
human M6 variant.

The study presented herein is exploring a therapeutic
regimen of clinically available drugs. The anticancer activity of
cyclooxygenase (COX)-2-selective nonsteroidal anti-inflamma-
tory drugs (NSAID) has now been documented both in vitro and
in vivo (for review, see ref. 9). In particular, the selective COX-2
inhibitor celecoxib, which inhibits the conversion of arachidonic
acid to prostaglandin-H2, has shown promising results. Human
prostate LNCaP and PC-3 cancer cells exhibit sensitivity toward such COX-2 inhibitors through a proapoptotic mechanism mediated via inactivation of the antiapoptotic kinase (Akt; ref. 10). Similar effects are evident against the in vitro proliferation of colorectal cancer cells, in which the induction of apoptosis occurs by a currently undescribed mechanism, albeit independent of COX-2 inhibition (11). From an angiogenesis perspective, an interesting parallel has also been currently established whereby increased expression of vascular endothelial growth factor protein is associated with increased COX-2 activity in head and neck cancers (12). Recent findings from our laboratory (13) and others (14) have implicated a role for COX-2 in the proliferation and differentiation of murine and human leukemia cell lines through the use of COX-2-specific inhibitors; thus we embarked on evaluating the efficacy of COX-2 inhibitors as prevention or intervention therapies in hematologic malignancies.

Presented here is a study evaluating the efficacy of a COX-2-selective NSAID in conjunction with continuous, low-dose administration of the conventional cytotoxic agent, vinblastine, for the treatment of Friend disease, used here as a clinical surrogate of FAB M6 AML. Vinblastine was chosen based on the previous observations of Vacca et al. (15) and Klement et al. (16, 17), showing that low doses of vinblastine exhibited potent antiangiogenic properties in vitro and in vivo with sustained tumor regression and minimal toxicity. We show that as monotherapies neither celecoxib nor vinblastine provided survival benefits for juvenile erythroleukemic mice, whereas the combination of these two drugs provided significant results. Similar observations have recently been reported with the use of another variant of the COX-2-targeted drugs, rofecoxib, in the clinical management of metastatic melanoma (18). Only when combined with metronomic, low-dose treosulfan was stable disease achieved in these patients. Similar to their findings, the synergistic action of the agents used in our present study cannot be explained through presently known mechanisms of action for either drug. However, celecoxib may provide protection against the toxicity of low-dose, continuous antiangiogenic regimens with vinblastine, particularly evident in vulnerable, juvenile mice with aggressive disease, or it may further enhance lymphoid-activating capacity to effectively control the erythroleukemic blast population.

MATERIALS AND METHODS

Reagents and Pharmacologic Agents. Rodent chow containing 0.16% celecoxib [SC-58635; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzene-sulfonamide] was supplied by Searle Research and Development (St. Louis, MO). Antineoplastic vinblastine sulfate at 1 mg/mL was supplied by Searle Research and Development (St. Louis, MO) was added as the internal standard. All columns were preconditioned with 2 mL of water. Samples were kept at −70°C until analyzed. Plasma samples were thawed and analyzed for celecoxib by high-pressure liquid chromatography (HPLC) using a C18 SecurityGuard cartridge (Phenomenex, Torrence, CA). Celecoxib and SC-751 were detected using fluorescence detection (Shimadzu Corporation; Kyoto, Japan; excitation = 240 nm, emission = 380 nm). The mobile phase consisted of 1.0 N phosphoric acid and SC-751 (Pharmacia Corporation, Mississauga, Ontario, Canada) was added as the internal standard. Celecoxib and SC-751 were extracted using Bond Elute Certify 130-mg solid phase extraction columns (Varian Canada, Mississauga, Ontario, Canada). All columns were preconditioned with 2 × 1 mL of acetonitrile followed by 2 × 2 mL of water. Samples were eluted using 2 mL of 0.6% ammonium hydroxide in methanol. Extracts were evaporated to dryness using nitrogen and the residue was reconstituted in 250 μL of HPLC mobile phase. Separation was achieved by injecting 150 μL of sample onto a 150 × 4.6-mm Prodigy ODS3 column (5 μm) equipped with a C18 SecurityGuard cartridge (Phenomenex, Torrence, CA). Celecoxib and SC-751 were detected using fluorescence detection (Shimadzu Corporation; Kyoto, Japan; excitation = 240 nm, emission = 380 nm). The mobile phase consisted of acetonitrile and 0.01 mol/L sodium phosphate in a 1:1 ratio (v/v) and run at 1 mL/min. An untreated mouse was sampled whose plasma was analyzed in a similar manner as a negative control.
Body Weights, Hematocrit Measurements, and Blood Sampling. Body weight and overt toxicity of all animals were documented twice weekly, that is, at time of treatments. Unless otherwise indicated, mice were sacrificed at term as per institutional guidelines. Peripheral blood was collected on a weekly basis by tail clipping and smear preparations were prepared by wedge slide technique followed by standard Wright's staining. Whole blood (∼1 mL) was then collected by cardiac puncture with a 1-mL U-100 insulin syringe (Becton Dickinson) into a K$_2$-EDTA-coated, 4-mL Vacutainer (Becton Dickinson). Automated and manual peripheral differential cell counts were done to confirm presentation of erythroleukemic blasts. Hematocrits were measured weekly by clipping tails and collecting 50 to 60 μL of blood into heparinized capillary tubes, followed by centrifugation at 14,000 × g in an Ultra microhematocrit centrifuge.

Immunohistochemistry. Splenic size was assessed by weight and vernier caliper measurement before tissue processing. Splenic tissues of healthy and erythroleukemic, moribund mice (n ≥ 3) from the indicated treatment groups were sectioned (Leica CM3050) to a thickness of 7 μm and placed onto ProbeOn Plus microscope slides (FisherBiotech, Mississauga, Ontario, Canada) and fixed with cold acetone and 1.5% hydrogen peroxide/methanol. After blocking nonspecific sites with 10% rabbit serum, purified anti-mouse CD31 [platelet/endothelial cell adhesion molecule 1 (PECAM-1); PharmMingen, San Diego, CA] was incubated with a 1:400 dilution in antibody diluting buffer (Dako Diagnostic Canada, Mississauga, Ontario, Canada) overnight at 4°C. Anti-mouse IgG + IgM (H + L, Jackson ImmunoResearch Laboratories, Kirkland, Quebec, Canada) in a dilution of 1:200 served as a negative control. The following day, all specimens were washed with PBS and incubated with 1:200 dilutions of anti-rat IgG (Jackson ImmunoResearch Laboratories) for 30 minutes at room temperature. Parameters set out by streptavidin/biotin labeling via HistoStain SP kit (Zymed Laboratories, San Francisco, CA), using purified anti-mouse CD31 (PECAM-1; PharmMingen) were followed.

Survival and Data Statistical Analyses. Survival among all groups was computed and plotted according to the nonparametric Kaplan-Meier analysis. Comparison of monotherapy and combination therapy was made using the nonparametric Mann-Whitney $U$ test with two-sided $P$ values and significant differences reflecting a $P < 0.05$. Student’s $t$ test for one and two populations for body weights and hematocrit values were conducted within a given treatment group and between treatment groups, respectively ($P < 0.05$).

RESULTS

Coadministration of Celecoxib and Low-Dose Vinblastine Enhances Survival of Juvenile Mice with Friend Erythroleukemia

All mice infected with F-MuLV and subjected to the indicated treatments were sacrificed at term according to institutional guidelines. All deaths including those occurring unexpectedly throughout the experiment were documented. Assessment of peripheral smears, automated differentials, and degree of splenomegaly were our primary indicators of disease (data not shown), parameters that typically reflect murine erythroleukemia development. Nonparametric analysis using Kaplan-Meier probability of survival revealed a significant increase in survival among mice given celecoxib with low-dose vinblastine compared with mice receiving either monotherapy ($P < 0.05$; Fig. 1). A difference in survival of 42 days was observed in favor of mice treated with celecoxib + vinblastine over the next control group treated with celecoxib + vinblastine vehicle. Maximal death rates were observed at or below 85 days post infection for all treatment groups. This corresponds to the death rates typically observed for mice with Friend disease, as recorded by others (20). Significant toxicity due to vinblastine, both as a monotherapy and coadministration with celecoxib vehicle, was primarily evidenced through significant mortality within the first 5 days of treatment onset. With the exception of mice placed on celecoxib + vinblastine therapy, those obtaining vinblastine alone or in combination with celecoxib vehicle appeared dehydrated and lethargic and experienced persistent diarrhea. Necropsy revealed significant bowel destruction consistent with necrotizing enterocolitis, as well as moderate splenomegaly, consistent with viral induction of leukemia and tumor burden. Over a 14-week period, significant survival differences were evident between celecoxib/vinblastine-treated mice (77% survival) and remaining treatment groups (≤12% survival) and this trend was maintained for the duration of the experiment in favor of combination therapy.

Pharmacologic Analysis of Plasma Celecoxib Concentrations

To establish therapeutic levels of celecoxib in mice obtaining drug via chow, randomly selected samples were obtained and quantified by HPLC. Two samples were chosen from the celecoxib monotherapy group, four from the control group of celecoxib + vinblastine-vehicle, and two from the celecoxib + vinblastine group. The overall averaged concentration among all samples was 2198.46 ng/mL (5.77 μmol/L; Fig. 2).

Reduced Toxicity Is Associated with Coadministration of Celecoxib with Low-Dose Vinblastine in Juvenile Erythroleukemic Mice

Although celecoxib was given ad libitum, vinblastine was given according to body weight in a low-dose “metronomic” fashion adopted from previous studies (16, 17). Weights were measured and recorded twice weekly. Within the first 2 weeks of treatment a significant decrease in body weight was observed in young mice given vinblastine alone in comparison with healthy, nontreated mice (Fig. 3A, asterisk; $P < 0.05$). No such significance was evident at similar times in the remaining groups despite overall lower values. Coadministration of celecoxib led to moderately decreased weight loss as compared with control animals during this same period (Fig. 3A, double asterisk, $P > 0.05$). There were no significant differences between body weights between the monotherapy and combination therapy groups throughout this period ($P > 0.05$). Although there were no significant weight differences among survivors at the indicated sampling times, there was early mortality associated with the use of low-dose vinblastine alone (Fig. 1).

Hematocrits measured weekly were used as semiquantitative assessment of disease state in the study. We have
previously established a negative correlation between hematocrit values and spleen size. Hence, a decreasing hematocrit would be indicative of leukemic volume expansion. In this study, hematocrit values for nonleukemic mice averaged 50%, as depicted in Fig. 3B. Age-matched leukemic mice, however, presented with significant decreases in hematocrit values regardless of treatment regimen ($P < 0.05$). A marginal increase was observed at final sampling time for combination therapy group, although still significantly less than healthy controls. Among all treatments, hematocrits averaged 30%, in comparison with 48% from nonleukemic mice over the sampling period.

Conserved Splenic Architecture in Erythroleukemic Mice Given Celecoxib + Vinblastine Therapy

Splenomegaly is a hallmark presentation of murine erythroleukemia, resulting in splenic rupture and death in a large proportion of mice. When compared with healthy controls histologic inspection of spleens from vinblastine monotherapy revealed relatively homogeneous infiltration of blasts (Fig. 4A and B). Celecoxib-treated mice exhibited similar pathology, with dispersed, poorly defined germinal centers (Fig. 4C), but spleens from mice on combination therapy indicated considerable maintenance of healthy architecture, as depicted by the appearance of well-defined germinal centers with a prominent vasculature lining the mantle regions (Fig. 4D). Despite these observations in the combination therapy group, the extent of splenomegaly was similar across all groups (data not shown).

**DISCUSSION**

The difficulty in understanding the pivotal time points of natural leukemoid progression toward a malignant phenotype may be largely due to lack of suitable *in vivo* models. Most xenograft models exhibit inherent limitations due to the use of a clonal, *in vitro* selected human leukemia cell line in which many of the genetic changes are due to culturing conditions. In comparison, Friend disease is induced by viral infection of susceptible neonates and as such may represent a model more relevant to the clinical scenario. The defined genetic mutations in Friend disease have not been completely translated into its human equivalent, but its development, progression, and response to chemotherapy, as observed here, closely resemble its human counterpart. As such, the development of new

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Fig. 1 Kaplan-Meier survival analysis. At time of weaning mice infected with Friend virus were separated into the following six treatment groups [$n = 9$ for group receiving celecoxib (Clbrx.) + vinblastine (Vinblst.), $n = 8$ for remaining groups]: Clbrx. monotherapy; Vinblst. monotherapy; Clbrx. vehicle (Vhcl.) alone; Clbrx. + Vinblst. Vhcl. combination; Clbrx. Vhcl. + Vinblst. combination; and Clbrx. + Vinblst. combination at the indicated concentrations (for Clbrx.) or doses (for Vinblst.). Daily observations were made and moribund mice were sacrificed according to institutional guidelines. Significance between monotherapy and combination treatment groups was calculated using Mann-Whitney $U$ test with two-sided $P$ values and significant differences reflecting a $P < 0.05$.

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$^6$ D. Cervi, Y. Shaked, Y. Ben-David, unpublished material.
Assessment of body weights and hematocrit values as primary indicators of overt toxicity.

A. All surviving mice in each treatment group were weighed twice weekly, that is, at time of vinblastine administration. Biweekly averages are presented over a 14-week period.

B. In addition, hematocrits were measured biweekly over a 14-week sampling period and presented as percentages of whole blood collected. Both measurements in which error bars are lacking depict inadequate sample sizes (<3) to perform statistical analysis. Significance between monotherapy and combination treatments was determined by Student’s t test for one and two populations for body weights and hematocrit values, within and between treatment groups, respectively. *, P < 0.05, significance between groups tested at the indicated sampling times, as addressed in RESULTS; **, P > 0.05, insignificant differences from the common control.
therapies targeting alternative molecular pathways may overcome the current clinical difficulties with treating this disease, particularly overcoming toxicities associated with such therapies.

Although the concept of low-dose metronomic chemotherapy has become better understood among clinicians and scientists worldwide, the doses applicable for the treatment of leukemias are yet to be established. Given the recent works conducted on solid tumors with respect to effective concentrations against endothelial cells, but not tumor cells, we decided to adopt a dosing regimen of vinblastine previously shown to be effective in the sustained regression of solid tumors (15–17). We found that in young mice a low-dose regimen of vinblastine, if given as a single agent, resulted in significant toxicity. The early mortality in this group was not due to leukemic burden but rather to gastrointestinal and possibly other organ damage. Age-matched mice of the remaining treatment groups, particularly combination therapy, did not exhibit these side effects or significant weight loss when compared with healthy controls over the duration of the experiment. It is therefore quite likely that in young mice the use of antiangiogenic therapy may be associated with increased morbidity, or that in the early postnatal period the doses necessary to achieve antiangiogenic effects may be even lower than anticipated (21). We have observed that BALB/c neonates infected with F-MuLV experience a high rate of mortality at approximately 9 weeks post infection. Similarly, we have observed the greatest rate of mortality at 10 weeks among all treatment groups (median survival time of 68 days), with the exception of the combination-treated mice. Coadministration of celecoxib with low-dose vinblastine alleviated all such toxic side effects and resulted in a significant overall increase in survival. In subsequent studies we have observed that these toxicities are age dependent and do not occur if treatment commences once mice have reached a mean weight of $\geq 14$ g.7 In this study, however, treatments began at time of weaning (21 days) and resulted in a mean weight loss of 8 g (20% of total body weight) across all groups. Our study supports the advantages of coadministering a selective COX-2 inhibitor to a modified dosing protocol using a conventional chemotherapy, at least in juvenile mice with Friend disease, as it seems to increase tolerance toward potential vinblastine toxicity. Whether this may be the case with other conventional chemotherapeutics in the treatment of hematologic malignancies remains to be tested.

It has been shown that the median survival time after induction of chemotherapy for erythroleukemia patients is approximately 4 months (22). A past case report made reference to the monitoring of a patient with erythroleukemia

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7 D. Cervi, G. Klement, Y. Ben-David, unpublished material.
who was given indomethacin postchemotherapeutic intervention who eventually acquired stable disease (9). In our model of Friend disease we found that celecoxib monotherapy was relatively ineffective in extending survival, and only when coadministered with low-dose vinblastine was survival enhanced. It is possible that similar to our juvenile mice, the patient within the case report experienced the protective effects of COX-2 inhibition. Because these agents inhibit both cyclooxygenase isoforms, we cannot rule out the possibility that COX-1 may also be implicated, such that perhaps the use of combinations of nonspecific inhibitors with low-dose vinblastine could have further enhanced the survival profile observed.

In addition to the above, alternative targets for COX-2-specific NSAIDs have come into question recently (23), and we too have observed that the growth kinetics of murine erythroleukemic blasts overexpressing COX-2 is significantly inhibited when celecoxib is added to the growth medium (13). However, the inhibition of COX-2 at lower concentrations of drug did negatively affect the growth of cells in vitro; rather, the disruption of signaling pathways downstream of Epo and c-Kit receptors at higher concentrations of celecoxib was responsible for these inhibitory observations (13). In the past, pathologic assessment and mortality from erythroleukemia have been documented by way of correlating survival with age, sex, hepatomegaly, lymphadenopathy, infection or hemorrhaging, and splenomegaly (2). Interestingly, based on the results of a retrospective study of 134 patients, the spleen remains the most highly investigated organ in the pathology of erythroleukemia, and the degree of splenomegaly serves as a reliable prognostic factor in some, but not all, hematologic diseases. The spleen is a reflection of tumor burden in hematologic malignancies, but whether this is due primarily to its distensible nature or to a favorable microenvironment and extramedullary hematopoiesis remains elusive. In Friend disease, splenectomized mice did not develop leukemia despite viral infection (24), suggesting a principal role of splenic environment in this disease. Our staining for PECAM suggests a more preserved splenic architecture with combination treatment over that of either vinblastine or celecoxib alone. Similarly to the healthy controls, splenic mantle regions from mice given both celecoxib and low-dose vinblastine show a peripheral vasculature with a highly cellular core. This is in contrast to the homogeneous replacement of the splenic parenchyma by infiltrating blasts from mice given either vinblastine or celecoxib alone.

A potential explanation of the mechanism of action of celecoxib may be in the defined role of the E family of prostaglandins in the suppression of immunogenic responses in favor of T_{H2} (25–27). It has been observed that the phenotypically suppressed lymphocytes collected from Hodgkin’s patients become markedly activated in the presence of indomethacin (28). Although the presence of celecoxib alone in our murine model of erythroleukemia did not result in significant survival advantage, in combination with low-dose vinblastine it was seen to be most effective. Thus, celecoxib could be driving a T_{H2} cell–mediated immune response by decreasing prostaglandin E, thus increasing antigen presentation by the mutated erythroblast and leading to recognition and eradication of blasts by supra-activated T cells. Alternatively, there may exist a currently uncharacterized synergy between low-dose vinblastine and celecoxib, which controls the growth of malignant leukemic blasts.

In this study we have examined two very important issues related to the treatment of erythroleukemia. First, we have tested the suitability of using a viral-induced hematologic malignancy as a preclinical model of the human AML variant, M6. Second, we have tested the response of this model to a modified clinical regimen incorporating a selective COX-2-inhibiting NSAID with metronomic antiangiogenic dosing of a conventional chemotherapeutic drug. The premise of this work is based on the successes of several recent studies in various solid tumors, whereby the use of each regimen alone has revealed significant antitumor effects by both direct and indirect mechanisms. Coupled with an antiangiogenic dosing regimen of vinblastine we therefore have shown considerable efficacy in the use of such a combination therapy for the treatment of aggressive hematologic malignancies. The toxicity of this regimen in early postnatal period could be counteracted by combination therapy. Recent investigations in colorectal cancer models support celecoxib adjuvant therapy, as it has been shown to exhibit considerable antitumor effects in addition to decreasing the diarrhea side effect associated with topoisomerase inhibitors (26). Similarly, considerable benefits in using COX-2 inhibitors have been reported whereby the response of tumors to both radio- and chemoradiotherapy was enhanced, along with improving tumor growth inhibition (26). Most importantly, decreased damage of normal tissue was reported. Even though erythroleukemia represents only 1% to 3% of all leukemias, its aggressive nature and chemotherapy resistance makes for improved prognosis and alternative therapies a necessity (22, 29–31). As such, the antiangiogenic metronomic low-dose chemotherapy with its lowered toxic profile and improved efficacy provides a valid therapeutic alternative for these patients.

REFERENCES


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