Benzylamide Sulindac Analogue Induce Changes in Cell Shape, Loss of Microtubules and G2-M Arrest in a Chronic Lymphocytic Leukemia (CLL) Cell Line and Apoptosis in Primary CLL Cells

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ABSTRACT

Given our interest in cyclic nucleotide phosphodiesterase inhibitors in chronic lymphocytic leukemia (CLL), we studied the effects of sulindac sulfone (exisulind), a non-cyclooxygenase-inhibitory end metabolite of the NSAID sulindac that has been reported to inhibit cGMP phosphodiesterases. We focused on a novel benzyamide analogue of sulindac sulfone, CP461, which is in clinical trials as a chemotherapeutic agent. As previously reported for colon carcinoma cell lines, we found that CP461 induced a rise in cGMP levels and blocked cell proliferation in the CLL cell line WSU-CLL. Surprisingly, however, cell cycle analysis revealed that CP461 caused G2-M arrest with an EC50 of 1.1 μM. G2-M arrest was associated with phosphorylation of Bcl2 (but not BAD, Bax, or Bcl-XL): both of these end points were abrogated by treatment with a calcium chelator. Although CP461 induces p53 up-regulation, G2-M arrest and Bcl2 phosphorylation were independent of p53. Because microtubule-active drugs such as vincristine also induced G2-M arrest and Bcl2 phosphorylation in WSU-CLL, whereas the genotoxic drugs etoposide and doxorubicin did not, we examined the effect of CP461 on microtubules by indirect immunofluorescence microscopy. CP461 eliminated microtubules rapidly, with reduction detected within 30 min of drug treatment. CP461 also induced marked changes in cell shape. Neither sulindac sulfide (a cyclooxygenase inhibitor) nor sulindac sulfone induced G2-M arrest, Bcl2 phosphorylation, microtubule disassembly, or cell shape changes. Treatment with 30 μM CP461 induced greater than 50% apoptosis in 10 of 10 primary CLL leukemic cell samples, whereas the same drug concentration had only marginal effects (14% apoptosis) on whole mononuclear cells. Our work demonstrates that addition of a benzyamide moiety to sulindac compounds results in markedly altered pharmacological properties that may be of use in the therapy of lymphoid malignancies.

INTRODUCTION

NSAIDs1 are a family of compounds the anti-inflammatory activity of which parallel their ability to inhibit the COX enzymes, COX1 and COX2 (1). NSAIDs are also cancer chemopreventive agents, but the identification of the therapeutic target responsible for this pharmacological property remains more controversial. The NSAID sulindac was first reported almost 20 years ago to cause regression of adenomatous colon polyps in four patients with FAP (2). Trials with drugs of which parallel their ability to inhibit the COX enzymes, COX1 and COX2, have suggested that sulindac sulfone and a series of sulindac analogues induce apoptosis as a result of their ability to inhibit cGMP PDEs. In a study of SW480 colon tumor cells, they found that sulindac sulfone and three analogues, CP78 (a trimethoxy analogue) and CP461 and CP248 (benzyamide analogues), maintain rank order of potency for induction of apoptosis (EC50 of 557, 167, 15, and 0.6 μM, respectively) and inhibition of SW480 cGMP PDE activity (IC50 of 128, 24, 3.6, and 0.3 μM, respectively; Ref. 10). In a related study, CP248 and CP461 were reported to induce JNK activation in colon cancer cell lines, and cells stably transfected with dominant negative JNK were relatively resistant to CP248-induced apoptosis (11).

Given our interest in the therapeutic use of cyclic nucleotide PDE inhibitors in CLL, we report here on the effects of sulindac metabolites and their analogues on primary CLL cells and a CLL cell line (12, 13). Our studies demonstrate that in a CLL cell line, the benzyamide analogues of sulindac sulfone, CP248 and CP461, induce cell shape changes, G2-M arrest, Bcl2 phosphorylation, and microtubule depolymerization, whereas the parental compound does not. CP461 also potently induces apoptosis in primary CLL cells.

MATERIALS AND METHODS

Reagents. Sulindac sulfide, sulindac sulfone, CP248, and CP461 were provided by Cell Pathways (Horsham, PA). Drugs were dissolved and diluted in DMSO so as to allow direct addition to cell cultures at a 1:500 ratio (final DMSO was 0.2% v/v). Unless otherwise indicated, all of the other chemicals were purchased from Sigma (Sigma Chemical Company, St. Louis, MO).

Cell Lines and Leukemic Cell Samples. Cells were cultured in RPMI 1640 (Cellgro) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Inc.). WSU-CLL cells were generously provided by R. M. Mohammad (Wayne State University, Detroit, MI; Ref. 14). TK6 was obtained from Dr. W. Thilly (Massachusetts Institute of Technology, Cambridge, MA). WI-L2-NS...
was from the American Tissue Cell Collection (ATCC CRL 8155). Leukemic cells were obtained from the peripheral blood of patients previously diagnosed as having B-cell CLL (CD5+/H11001/C19+/H11001) by immunophenotyping using an Institutional Review Board-approved protocol. Mononuclear cells were obtained by density gradient centrifugation over Histopaque 1077, resulting in cell preparations that were ≥90% leukemic cells. The modified Rai stage and treatment history of the 10 patients shown in Fig. 7 were as follows: patient 1, high risk, chlorambucil/prednisone, fludarabine; patient 2, intermediate risk, no treatment; patient 3, high risk, IVIG; patient 4, high risk, chlorambucil, fludarabine, and rituximab; patient 5, low risk, no therapy; patient 6, high risk, chlorambucil and fludarabine; patient 7, high risk, chlorambucil, fludarabine, and rituximab; patient 8, high risk, chlorambucil and fludarabine; patient 9, high risk, chlorambucil and fludarabine; patient 10, high risk, chlorambucil. Samples were obtained at least 2 weeks after any cytotoxic therapy (15).

**MTT Assay.** Cell density was quantified using a colorimetric assay previously described for measuring intracellular succinate dehydrogenase content with MTT (16). WSU-CLL cells (2 × 10^5 in 200 µl) were cultured in the presence of each drug for 48 h in triplicate, then incubated with MTT (50 µg/ml) for 4 h. Plates were centrifuged and formazan dissolved in 100 µl of DMSO. Absorbance (A570 nm) was measured by an ELISA reader (Bio-Rad) and was in the linear range for experimental samples.

**cGMP Assay.** cGMP was measured using a RIA kit from NEN (Boston, MA). Fifteen million WSU-CLL cells were cultured in 1.5 ml of medium with or without drug treatment for the indicated period of time. Cells were collected by microcentrifugation at 1,200 × g, resuspended in 100 µl of PBS, followed by the addition of 400 µl of ethanol with vortexing. After 5 min on ice, cellular debris was removed by centrifugation at 16,000 × g. The supernatant was stored at −80°C. The supernatants were dried in a Speedivac, then resuspended in 120 µl of sodium acetate buffer as supplied in the RIA kit. The RIA was carried out according to the manufacturer’s instructions using 100 µl of sample for each assay. Samples were acetylated to increase the sensitivity of the assay, as described by the manufacturer. The SE of triplicate samples was calculated.

**Propidium Iodide Cell Cycle and Apoptosis Analysis (WSU-CLL, TK6 and WI-L2-NS).** One million cells were used for apoptosis and cell cycle analyses. Cells were fixed in 40% ethanol on ice for 30 min and then incubated with propidium iodide (50 µg/ml) and RNase (25 µg/ml) at 37°C for 30 min. FACS analysis was performed with a FacScan (Becton Dickinson, San Jose, CA). Apoptosis was measured in medium containing reduced FCS (2.5%). Cell cycle analysis was performed in medium with 10% FCS.

**Western Analysis.** Monoclonal anti-Bcl2 antibody (6C8) was from Pharmingen; antitubulin was from Sigma; polyclonal anti-cyclin B1, cyclin D1, p21 WAF1/CIP1, p53 (C19) and HRP-conjugated goat antimouse, rabbit antigoat, and donkey antigoat antibodies were from Santa Cruz; and HRP-conjugated goat antihamster IgG was from ICN Pharmaceuticals. WSU-CLL cells were lysed in ice-cold lysis buffer containing 0.5%...
NP40 (v/v) in 20 mM Tris-HCl (pH 8.3), 150 mM NaCl, protease inhibitors (2 
μg/ml aprotinin, pepstatin, and chymostatin, 1 μg/ml leupeptin and pepstatin, 
and 1 mM phenylmethylsulfonyl fluoride), and 1 mM Na4VO3. Lysates were 
incubated for 30 min on ice before centrifugation at 14,000 rpm for 5 min at 
4°C. Proteins in the supernatant were denatured by boiling for 5 min in SDS 
sample buffer, separated by 10–12% SDS-PAGE, and transferred to nitrocel-
 lulose membranes for immunoblotting. The membranes were blocked with 5% 
skim milk in Tris-buffered saline-Tween 20 [10 mM Tris-HCl (pH 7.6), 150 
mM NaCl, 0.5% Tween] and incubated with the indicated antibodies. Bound 
antibodies were revealed with HRP-conjugated secondary antibodies using 
ECL (Pierce).

Staining of Microtubules, F-Actin, and Nuclei. Microtubules were de-
tected by the method of Anand and Chou (17). Briefly, WSU-CLL or primary 
CLL cells were pelleted onto glass slides in a Cytospin 2 (Shandon) centrifuge, 
then fixed with 3.7% formaldehyde in a microstabilizing buffer [0.1 M PIPES, 
1 mM MgSO4, 2 mM glycerol, 2 mM EGTA (pH 6.9)]. After quenching in 0.1 M 
glycine buffer, cells were gently extracted with 0.5% NP40 in PBS. Microtu-
bules were indirectly detected by incubation with a primary mouse antitubulin 
 antibody (Sigma) and subsequent staining with FITC-conjugated goat anti-
ti-mouse IgG antibody (Santa Cruz). For staining of F-actin and nuclei, WSU-
CLL or primary CLL cells were fixed with 2% paraformaldehyde in PBS for 
30 min and permeabilized with 0.1% Triton X-100. Cells were then stained 
with Alexa 594 phalloidin (Molecular Probes) followed by DAPI (Sigma). 
Cells were mounted and visualized using a Nikon Eclipse 300 microscope.

Hoechst 33342 Apoptosis Assay (Primary CLL Cells). One million cells/
well were incubated in triplicate in 48-well tissue culture plates (Costar, 
Cambridge, MA) with or without drug treatment for 48 h in 1 ml of culture 
medium. Cells were transferred to 12 × 75 mm polystyrene Falcon 2054 
FACS tubes (Becton Dickinson Labware, Lincoln Park, NJ) and incubated for 
ten min at 37°C with Hoechst 33342 (Molecular Probes, Eugene, OR) at a final 
concentration of 0.25 μg/ml. Cells were stored on ice until analysis on a FACS 
Vantage flow cytometer (Becton Dickinson, San Jose, CA). Hoechst 33342 
dye fluorescence was excited with a UV laser and detected using a 450 
bandpass filter. Data were analyzed using FlowJo software.

RESULTS

Benzyamide Derivatives of Sulindac, but not Parental Sulindac 
Derivatives, Induce G2-M Arrest in a CLL Cell Line. We initially 
examined the effects of sulindac metabolites and their analogues 
(structures shown in Fig. 1A) on lymphoid cells by treating a CLL cell 
line, WSU-CLL, with such drugs and assessing growth and survival 
by MTT tetrazolium assay (16). As shown in Fig. 1B, the sulindac 
metabolites, sulindac sulfone and sulindac sulfide, inhibited the 
metabolic reduction of MTT with an EC50 of 519 and 59.8 μM, respec-
tively. In contrast, the sulindac sulfone benzyamide analogues, 
CP461 and CP248, were approximately 50- and 5000-fold more

Fig. 2. Sulindac analogues induce both G2-M arrest and apoptosis in WSU-CLL cells. The G2-M arrest is reversed by cotreatment with the divalent cation chelator EDTA. WSU-CLL 
cells were ethanol fixed, stained with propidium iodide, and analyzed for cell cycle by FACS. A, WSU-CLL cells were treated for 18 h with the indicated concentration of CP461. 
B, WSU-CLL cells were treated for the indicated time period with 10 μM CP461. C, WSU cells were treated with medium (Control), 5 mM EDTA, 10 μM CP461, or both agents 
for 18 h.
potent, respectively, with an EC₅₀ of 1.1 and 0.01 μM, respectively. As previously reported in human colon carcinoma cell lines, treatment with 10 μM CP461 for 24 h significantly augmented WSU-CLL cell cGMP levels (P < 0.05; Fig. 1C).

To assess the mechanism by which sulindac analogues inhibit WSU-CLL MTT reduction, we performed propidium iodide cell cycle analysis. Unexpectedly, we found that CP461 induced G₂-M arrest of WSU-CLL cells with a dose-response curve that resembled that observed for the MTT assay. The induction of G₂-M arrest was sharply dose dependent, because a concentration of 0.313 μM CP461 had no effect on cell cycle, whereas a concentration of 1.25 μM CP461 resulted in almost complete arrest (Fig. 2A). In contrast, the parental sulindac compounds, sulindac sulfone and sulindac sulfide, did not induce G₂-M arrest at any dose tested (data not shown). In a time-course experiment performed on a culture of initially unsynchronized WSU-CLL cells, a small (17%) increment in the percentage of cells with 4N DNA content was first detectable 6 h after addition of 10 μM CP461 (Fig. 2B). By 18 h, the majority of cells (88%) were arrested in the G₂-M phase (Fig. 2B). Lowering extracellular calcium below 10⁻³ m has been reported to block lymphoid cell cycle progression (18). Treatment of WSU-CLL cells with the divalent cation chelator EDTA (2 mM) markedly reduced CP461-mediated G₂-M arrest (Fig. 2C). Similar results were obtained with the calcium-specific chelator EGTA (data not shown).

**CP461 Up-Regulates p53 and p21 WAF1/CIP1.** Consistent with our observation that CP461 augmented the number of WSU-CLL cells with 4N DNA by propidium iodide FACS analysis, we observed CP461-induced alterations in the expression or posttranslational modification of several cell cycle-related proteins. Whereas the early G₁ cyclin, cyclin D1, was not clearly altered by treatment, concentrations of ≥2.5 μM CP461 augmented levels of the mitotic cyclin, cyclin B1 (Fig. 3; Ref. 19). Unsynchronized WSU-CLL cells contained three species of p107 Rb of varying mobility, consistent with known phosphorylation and reduced mobility of Rb in G₁ (20). After treatment with CP461, a marked diminution of the most slowly migrating Rb species was observed that roughly correlated with the observed reduction in the percentage of cells in G₀-G₁ by propidium iodide FACS analysis.

In the same Western blot dose-titration experiments, treatment of WSU-CLL cells with lower CP461 concentrations (0.625 μM) raised levels of both p53 and p21 WAF1/CIP1, a cyclin-dependent kinase inhibitor that can be transcriptionally up-regulated by p53 (Fig. 3; 21). p53 has been reported to play a regulatory role during the G₂-M transition, particularly after DNA damage (22). To address the role of such p53 up-regulation in CP461-induced G₂-M arrest, we used two isogenic lymphoblastoid cell lines that differ in p53 status. TK6 is a p53 wild-type B cell line whereas WI-L2-NS is p53 mutant as a result of a point mutation at codon 237 (23). Treatment of both cell lines with 10 μM CP461 induced G₂-M arrest, demonstrating that despite the ability of CP461 to up-regulate p53, CP461-induced G₂-M arrest is p53 independent (Fig. 3). These results also confirmed that the CP461-induced G₂-M arrest was not specific to the WSU-CLL cell line.

**CP461 Induces Cell Shape Changes and Apoptosis.** Examination of WSU-CLL cells after CP248 or CP461 treatment by phase contrast microscopy revealed that these drugs markedly altered cell shape. Within 2 h of treatment with either drug, a subpopulation of WSU-CLL cells developed elongated angular forms as well as cellular protrusions (data not shown). This effect was more pronounced at 18 h (Fig. 4, lower left panels). Of note, WSU-CLL shape changes were also seen after treatment with vincristine (Fig. 4, upper left panel), vinblastine, and paclitaxel, but not with doxorubicin or etoposide (data not shown). In contrast, neither sulindac sulfone nor sulindac sulfide induced cell shape changes (Fig. 4, center left panels).

CP461 also induced a modest degree of apoptosis in WSU-CLL cells when they were cultured in a lower FCS concentration (2.5%) as detected by a rise in the percentage of hypodiploid cells from 2% in control cells to a maximum of 26.1% at 2.5 μM (Fig. 2A and not shown). Consistent with this finding, DAPI staining of WSU-CLL cells demonstrated that, 18 h after treatment with 10 μM CP461, the nuclei of a fraction of cells became pyknotic in a manner characteristic of apoptosis (Fig. 4, middle right panels). Staining of WSU-CLL cells for F-actin with Alexa-phalloidin demonstrated that, although control cells or CP461-treated cells with cytoplasmic protrusions but without pyknotic nuclei contained cytoplasmic F-actin, cells with pyknotic nuclei lost all such staining (Fig. 4, far right panels).

**CP461-induced Bcl2 Phosphorylation Correlates with G₂-M Arrest.** To assess the potential mechanism by which apoptosis occurs, we performed immunoblot analysis of CP461-treated WSU-CLL cells to determine whether there were alterations in known lymphoid Bcl2 family members. Whereas Bcl-XL, Bad, and Bax were easily detectable, there were neither appreciable changes in total levels of these proteins nor obvious posttranslational modifications in CP461-treated cells (data not shown). In contrast, 30–50% of immunoreac-
tive Bcl2 migrated with retarded mobility characteristic of phospho-
hydration after treatment with concentrations of CP461 of \( \geq 2.5 \) \( \mu M \) (Fig. 5A). Bcl2 phosphorylation was detectable at concentrations as low as 0.625 \( \mu M \). A time course of Bcl2 phosphorylation showed that this posttranslational modification was detectable within 2 h but only became substantial at between 6 and 18 h (Fig. 5B). CP461-induced Bcl2 phosphorylation and cell cycle arrest were also observed in the human Burkitt’s lymphoma cell line, Ramos (data not shown).

At least two broad classes of chemotherapeutic agents, genotoxic drugs and microtubule active drugs, have been reported to induce G2-M arrest in cells. In WSU-CLL cells, microtubule-active drugs (vincristine, vinblastine, and paclitaxel) but not genotoxic compounds (doxorubicin, etoposide) induced Bcl2 phosphorylation (Fig. 5, C and D). As we had previously determined for G2-M arrest, the Bcl2 phosphorylation observed after treatment with the sulindac benzylamide derivatives CP248 and CP461 was not observed for the parental compounds, sulindac sulfide and sulindac sulfone (Fig. 5D). Consistent with prior reports that Bcl2 normally undergoes phosphorylation at the G2-M phase of the cell cycle, we found that blocking CP461-induced WSU-CLL G2-M arrest with a calcium chelator (1 or 2 mm EGTA) also blocked CP461-induced Bcl2 phosphorylation (Fig. 5E; Ref. 24).

**Benzylamide Sulindac Derivatives Induce Loss of Microtubules.** Drugs that either disrupt or stabilize microtubules, such as the Vinca alkaloids or the taxanes, have previously been reported to induce G2-M arrest and Bcl2 phosphorylation in a manner similar to what we had observed for the benzylamide sulindac derivatives CP461 and CP248 (25, 26, 27). For this reason, we next examined the effect of these drugs on WSU-CLL cell microtubules by indirect immunofluorescence microscopy. Treatment with 10 \( \mu M \) CP461 for 5 h resulted in loss of detectable microtubule staining (Fig. 6, bottom left panel). A time course demonstrated that loss of microtubules was fairly rapid, with the first clearly detectable decline observed within 30 min of CP461 treatment (Fig. 6, middle left panel). Similar effects were observed with CP248 treatment but not with sulindac sulfone (Fig. 6, upper right panel, and data not shown). Loss of microtubule staining was also seen with vincristine treatment (Fig. 6, bottom right panel). The changes observed after treatment with CP461 and CP248 contrasted markedly with the numerous abnormally thick microtubules detected after treatment with the microtubule-stabilizing drug paclitaxel (Fig. 6, middle right column).

**CP461 Effects on Primary Leukemic Cells.** Unlike leukemic cell lines, most primary leukemic cells do not enter the cell cycle when cultured in vitro. Consistent with this, treatment of leukemic cells
WMCs (10% at 10 of nine CLL patients tested (Fig. 7). Ten B augmentation of apoptosis in freshly isolated leukemic cells from six freshly isolated WMCs, a predominantly T-cell population (14% at 30 these malignant cells, because CP461 had only marginal effects on apoptosis in eight of eight patients. These effects were specific to/H9262 or 2/H9262 only at relatively high concentrations (26 and 25% at 100 and 800 sulindac sulfone induced a significant increase in WMC apoptosis 7/H9251 in vitro on April 13, 2017. © 2002 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from primary leukemic cells, as expected, given that CP461-induced Bcl2 phosphorylation in WSU-CLL cells was cell-cycle dependent (Fig. 7D). Thus, apoptosis induced in primary leukemic cells by CP461 is independent of G2-M arrest and Bcl2 phosphorylation but is associated with loss of microtubules.

**DISCUSSION**

This study demonstrates that the sulindac benzylamide analogues CP248 and CP461 have acquired novel pharmacological properties relative to sulindac sulfone and sulindac sulfide. These properties include: (a) induction of G2-M arrest and Bcl2 phosphorylation in a proliferating CLL cell line; (b) destabilization of microtubules and alterations in cell shape; and (c) a marked increase in potency in inducing apoptosis in primary CLL cells. Although the mechanism by which these benzylamide analogues achieve these effects remains unknown, several of the phenomena observed after CP461 and CP248 treatment (p53 induction, G2-M arrest, Bcl2 phosphorylation, loss of microtubules, change in cell shape) are shared by drugs that act by altering microtubule polymerization. Although primary CLL cells are not in cell cycle in tissue culture and, therefore, do not undergo a "mitotic death," such primary cells are known to be markedly more sensitive than normal lymphocytes to the induction of apoptosis by agents that cause microtubule dissociation, such as *Vinca* alkaloids (28).

Paclitaxel, a drug that binds to and stabilizes microtubules, induces Bcl2 phosphorylation in a 60-amino-acid loop region between the α1 from patients with CLL with 10 μM CP461 for 48 h did not augment the percentage of cells in G2-M, although such treatment did increase the percentage of hypodiploid cells, a characteristic of apoptosis (Fig. 7A). Using a Hoechst 33342 FACS assay to quantitate apoptosis, treatment for 48 h with 3 μM CP461 induced a small but significant augmentation of apoptosis in freshly isolated leukemic cells from six of nine CLL patients tested (Fig. 7B). Ten μM CP461 induced >40% apoptosis in 8 of 10 such samples. At 30 μM, CP461 induced >50% apoptosis in eight of eight patients. These effects were specific to these malignant cells, because CP461 had only marginal effects on freshly isolated WMCs, a predominantly T-cell population (14% at 30 μM). Similarly, CP248 also did not significantly induce apoptosis in WMCs (10% at 10 μM). The parental compounds sulindac sulfide and sulindac sulfone induced a significant increase in WMC apoptosis only at relatively high concentrations (26 and 25% at 100 and 800 μM, respectively).

As with WSU-CLL cells, treatment of primary CLL cells with CP461 induced the rapid loss of microtubule staining (Fig. 7C). Although DAPI staining of primary leukemic cells after CP461 treatment demonstrated nuclear pyknosis, staining of the same cells for F-actin with Alexa-phalloloid demonstrated that leukemic cells with pyknotic nuclei had lost all detectable F-actin structures (data not shown). Unlike WSU-CLL cells, however, CP461 treatment did not cause dramatic cell shape changes in primary leukemic cells (Fig. 7C). Assessment of cGMP levels in leukemic cells from three CLL patients after treatment *in vitro* with 10 μM CP461 for 0, 1, 6, or 24 h showed no CP461-induced alterations in two cell samples and a modest 2-fold augmentation in cGMP levels in a third at 6 h only (from 16 to 32 fmol cGMP/million cells). Immunoblotting of CLL whole-cell lysates revealed high basal levels of Bcl2, as previously reported by others. Treatment with CP461 failed to induce Bcl2 phosphorylation in
and α2 helices (29). Similarly, drugs that cause net tubulin depolymerization, such as vincristine and vinblastine, also induce Bcl2 phosphorylation. The kinase responsible for phosphorylation of Bcl2 has been a subject of some controversy with reports supporting roles for CDC2 (27), protein kinase A (25), Raf (26), and JNK (24). The functional importance of such Bcl2 phosphorylation in drug-induced apoptosis also remains controversial. Several groups have reported that transfection of Bcl2 mutants lacking the loop region protects cell lines from paclitaxel-induced apoptosis, which suggests that paclitaxel-induced phosphorylation and inactivation of Bcl2 is relevant to drug-induced apoptosis (29, 30). However, a double thymidine block induces M phase arrest and Bcl2 phosphorylation in HeLa cervical carcinoma cells, whereas such treatment does not induce apoptosis (31). Similarly, G2-M phase Jurkat cells that are isolated by elutriation have an activated Ask1/JNK pathway that results in physiological Bcl2 phosphorylation and inactivation (24). Thus, the functional importance of Bcl2 phosphorylation induced by novel compounds (such as CP461 and CP248) that induce G2-M arrest needs to be evaluated carefully before attributing a role to such phosphorylation in apoptotic processes. In keeping with these studies, we find that although CP461 and CP248 induce apoptosis both in a CLL cell line and in primary leukemic cells, Bcl2 phosphorylation is observed only in the proliferating cell line in which it is associated with G2-M arrest.

p53 activation plays an important role in some forms of G2-M
arrest, particularly those that are mediated by genotoxic compounds (22, 32). Both CP461 and CP248 induce p53 up-regulation in WSU-CLL cells, raising the question as to whether the G2-M arrest mediated by these compounds was p53 dependent. Using a pair of isogenic lymphoblastoid cell lines that differ in p53 status, TK6 (p53 wild-type) and WI-L2-NS (p53 mutant), we found that CP461 induced G2-M arrest in both cell types (23). The microtubule active drugs Taxol, vinblastine and nocodazole increase levels of transcriptionally active, phosphorylated p53 (33, 34). Unlike agents that cause DNA damage, however, microtubule active drugs cause G2-M arrest in p33-defective cells, presumably because they are required for spindle formation. Thus, the CP461-mediated p53 up-regulation and CP461-induced G2-M arrest in p53 mutant cells that we observe are similar to results obtained previously by other groups with microtubule-active drugs. p53 is responsible for a “spindle checkpoint” that prevents DNA reereplication in the presence of drugs that interfere with spindle assembly, such as Colcemid or nocodazole (35, 36). We did not see evidence of octaploid cells in our experiments, which may be a function of either the limited time in which the cells were cultured in CP461 or the fact that this phenomenon appears to be somewhat cell-type specific (35).

Consistent with prior studies demonstrating that CP461 and CP248 potently inhibit cGMP PDE activity in epithelial cell lines, we find that CP461 augments cGMP levels in the WSU-CLL cell line (10). Lymphoid cells express a variety of PDEs known to catabolize cGMP, including PDE1, PDE2, and PDE9, although PDE2 has not been detected in human lymphoid cells, and only PDE9 transcript has thus far been demonstrated in human lymphocytes (37–41). Whether the morphological and cytoskeletal changes observed in this report are the result of cGMP-mediated signaling will require further study. Of note, Fischer et al. recently demonstrated that freshly isolated peripheral blood T cells undergo arrest of cellular proliferation after treatment with membrane-permeant cGMP analogues as a result of activation of type 1 cGMP-dependent kinase (PKG), whereas T-cell lines are devoid of detectable type 1 (or type 2) PKG and maintain normal proliferation in the presence of high concentrations of cGMP analogues (42). Thus, future studies of the potential impact of the cGMP PDE inhibition of CP461 and CP248 will have to ensure that the model systems studied contain an array of PKG-effector enzymes comparable with those present in freshly isolated leukemic cells.

The molecular mechanism by which the benzylamide sulindac derivatives induce cell shape changes and disassembly of microtubules remains to be established. Although several of the alterations observed after treatment of WSU-CLL cells with CP461 resemble those induced by Vinca alkaloids such as vincristine or vinblastine, which induce disassembly of microtubules by binding to free tubulin and inhibiting their polymerization, it is possible that CP461-induced microtubule disassembly is a secondary phenomenon. Alterations in cell morphology are usually associated with changes in actin filament organization. Of note, treatment of cells with the microtubule-dissociating agent nocodazole has been reported to result in Raf1 activation by Pak1, a kinase downstream of the small GTPases Rac/Cdc42 (43). Activated Rac and Cdc42 induce cell shape changes by formation of lamellipodia and filopodia, respectively. It will be of interest to determine whether CP461 and CP248 activate a Rac/Cdc42/Pak1/Raf1 signaling pathway, as well as to determine directly whether these drugs alter tubulin polymerization.

Despite growing knowledge as to the genetic abnormalities underling CLL as well as a better appreciation in molecular and immunological terms of the clinical heterogeneity of this disease, CLL remains an illness that most typically progresses despite a variety of therapeutic measures. Because CP461 has been well tolerated in Phase 1 human studies, its antiproliferative and apoptotic activities in vitro will make it an interesting compound to study in the treatment of CLL and other lymphoid malignancies.

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