Indole-3-Carbinol Synergizes with and Restores Fludarabine Sensitivity in Chronic Lymphocytic Leukemia Cells Irrespective of p53 Activity and Treatment Resistances

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Abstract

Purpose: Chronic lymphocytic leukemia (CLL) still is lacking a cure. Relapse and development of refractoriness to current treatments are common. New therapies are needed to improve patient prognosis and survival.

Experimental design: Indole-3-carbinol (I3C) is a natural product with antitumor properties already clinically tested. The effect of I3C, F-ara-A, and combinations of both drugs on CLL cells from patients representing different Rai stages, IGHV mutation status, cytogenetic alterations, p53 functionality, and treatment resistances was tested, as well as the toxicity of these treatments in mice.

Results: I3C induces cytotoxicity in CLL cells but not in normal lymphocytes. I3C strongly synergized with F-ara-A in all CLL cells tested, including those with p53 deficiency and/or F-ara-A resistance. The mechanism of cell death involved p53-dependent and -independent apoptosis. The combination of I3C + F-ara-A was equally effective in CLL cells irrespective of IGHV mutation stage and patient refractoriness. Moreover, CLL survival and treatment resistance induced by co-culturing CLL cells on stroma cells were overcome by the combinatory I3C + F-ara-A treatment. No toxicity was associated with the combined I3C + fludarabine treatment in mice.

Conclusions: I3C in combination with F-ara-A is highly cytotoxic in CLL cells from refractory patients and those with p53 deficiency. The striking dose reduction index for F-ara-A in combination with I3C would reduce fludarabine toxicity while having a similar or better anti-CLL effectiveness. Moreover, the low toxicity of I3C, already clinically tested, supports its use as adjuvant and combinatory therapy in CLL, particularly for patients with relapsed or refractory disease. Clin Cancer Res; 22(1); 134–45. ©2015 AACR.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia found in adults in Western countries. It is a heterogeneous disease characterized by a clonal expansion of mature CD5+ B lymphocytes that are resistant to apoptosis (1). The clinical course of CLL patients is highly variable. Two subgroups of CLL patients are defined attending to the IGHV mutation status, a group showing mutated IGHV that can survive an average of 15 years, and a second group with unmutated IGHV and worse prognosis. In addition to IGHV mutation status, CLL cells commonly show genomic aberrations such as deletions at 13q14, 11q22-q23, and 17p13 or trisomy 12 that affect the clinical outcome and constitute important prognostic markers (2, 3). Deletions and mutations in TP53 and/or in other genes controlling p53 functions also have an important impact on both the efficacy of the treatments and the outcome of the disease (4).

A variety of chemotherapy regimens are being used in CLL (5), but unfortunately patients will eventually become refractory to treatments and die. It is therefore a priority to develop new treatments and therapeutic approaches that improve patient prognosis and survival.

Indole-3-carbinol (I3C) (1H-indol-3-ylmethanol) is a glucobrassicine (or indole-3-glycosynolate) derivative that is found in edible plants of the Brassica genus, such as the broccoli, the Brussels sprouts, and the mustard greens (6, 7). Several studies have described I3C and its dimer 3,3′-indolylmethane (DIM), which is the major condensation product of I3C produced in the gastric acid milieu, as chemopreventive and antitumor agents. Indeed, these compounds have been shown to inhibit inflammation, cell proliferation, and tumor invasion (6, 7). Phase I and II clinical trials demonstrated that oral administration of these compounds is effective in promoting regression of precancerous cervix lesions (8), vulvar epidermal neoplasia (9), and recurrent respiratory papillomatosis (10), while lacking significant toxicity in humans (11).
I3C Synergizes with Fludarabine in Refractory CLL Cells

Translational Relevance

Chronic lymphocytic leukemia (CLL) is the most common elderly leukemia and still is lacking a cure. A variety of immunotherapy treatments are available, but relapse and development of refractoriness to treatment is common. In this report, we show that bioavailable concentrations of indole-3-carbinol (I3C) are cytotoxic to CLL cells but not to normal lymphocytes. I3C strongly synergized with F-ara-A in all CLL samples studied, irrespective of their \(I_{G}GH\) mutation status, \(p_{53}\) functionality, and patients’ refractoriness to multiagent chemoimmunotherapy. I3C even restored in vitro sensitivity to F-ara-A in fludarabine-resistant CLL samples. The low toxicity of I3C, already clinically tested in humans, was further confirmed in a combinatorial I3C/fludarabine treatment in mice, confirming the safety of this treatment. These results strongly support the use of I3C as a novel adjuvant therapy in CLL, or in combination with fludarabine and other chemotherapeutic drugs, particularly in patients with relapsed or refractory disease.

Previous results have shown the antitumor effect of I3C in acute myeloid leukemia cells (12) and EBV+ Burkitt lymphoma cell lines (13). These results prompted us to study whether I3C could also be active against CLL cells. In this report, we have shown that I3C shows a potent antitumor activity as single agent against CLL cells. More importantly, I3C strongly synergizes with F-ara-A in all types of CLL tested, including CLL cells with mutated \(p_{53}\) or from patients that have developed multiagent chemoimmunotherapy refractory disease, even those no longer responding to fludarabine, suggesting that I3C could restore sensitivity to fludarabine of refractory CLL patients.

Materials and Methods

Reagents

I3C, vincristine, chlorambucil, and F-ara-A, the active product of fludarabine, were from Sigma-Aldrich. Fludarabine phosphate was from Hospira. The caspase inhibitor Z-Val-Ala-DL-Asp-(OMe)-OMe (Z-VAD-fmk) was from Bachem. Protease inhibitors (Complete) and phosphatase inhibitors (PhosSTOP) were from Roche Diagnostics. Alexa Fluor 647-Phalloidin was from Life Technologies. Anti-mouse B220-FITC, Gr-1-PE, CD45-PerCP, and CD3-APC (HRP) mAbs were from BD Biosciences. Fludarabine phosphate (Fludara), vincristine, chlorambucil, and F-ara-A, the active product of fludarabine, were from Sigma-Aldrich. Fludarabine phosphate was from Hospira. The caspase inhibitor Z-Val-Ala-DL-Asp-(OMe)-OMe (Z-VAD-fmk) was from Bachem. Protease inhibitors (Complete) and phosphatase inhibitors (PhosSTOP) were from Roche Diagnostics. Alexa Fluor 647-Phalloidin was from Life Technologies. Anti-mouse B220-FITC, Gr-1-PE, CD45-PerCP, and CD3-APC (HRP) mAbs were from BD Biosciences.

Immunoblot analysis

PBMCs were lysed in incomplete Laemmli buffer (125 mmol/L Tris, pH 6.8, 4% SDS, and 20% glycerol) supplemented with a mixture of protease and phosphatase inhibitors. Lysates were diagnosed according to established morphologic and immunophenotypic criteria (17, 18). These cases were collected from Hospital General Universitario Gregorio Marañon and Hospital Universitario de La Princesa (Madrid, Spain). Experimental protocols were approved by the pertinent ethic committees following the guidelines of the Helsinki declaration.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from CLL patients and healthy donors by centrifugation in a density gradient with Lymphocyte Separation Medium (Lonza). For all experiments, PBMCs were cultured in complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mmol/L \(\cdot\)-glutamine, 100 U/mL penicillin, and 100 \(\mu_{g}\)/mL streptomycin) at 37°C in a humidified 5% CO\(_2\) atmosphere.

Bone marrow-derived human mesenchymal stem cells were immortalized by stably transfection with active human telomerase reverse transcriptase catalytic subunit (hMSC-TERT; ref. 19). hMSC-TERT cells were cultured in low-glucose DMEM (Sigma-Aldrich) supplemented with 10% inactivated FBS, 2 mmol/L \(\cdot\)-glutamine, 100 U/mL penicillin, and 100 \(\mu_{g}\)/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO\(_2\). Cells were grown until confluence and then detached by using the TrypLE Express reactive (Life Technologies). For co-culturing experiments, hMSC-TERT cells were seeded in 96-well plates until 80% confluence, then PBMCs isolated from CLL patients (1.5 × 10\(^6\) cells/mL) were cultured on the hMSC-TERT monolayer for 48 hours.

Viability assays

For viability assays, PBMCs were incubated in 96-well microtiter plates and cultured with different reagents as indicated. Viability was measured by using the CellTiter-Glo Kit (Promega Biotech Iberica). When indicated, 100 \(\mu_{g}/mL\) Z-VAD-fmk was added to the cell cultures 30 minutes before the addition of the drugs.

Apoptosis detection

Apoptosis was determined by Annexin V/propidium iodide (PI) staining (Biovision). Briefly, CLL cells (2–5 × 10\(^5\) cells) were harvested and incubated with FITC-labeled Annexin V and 1 pg/mL PI in binding buffer (2.5 mol/L CaCl\(_2\), 10 mmol/L Hepes, and 140 mmol/L NaCl). After 15 minutes in the dark, they were analyzed by flow cytometry. Cell viability was measured as the percentage of Annexin V and PI-double negative cells (AV\(^−/PI\)^−). For analysis, the percentage of viable cells was determined considering the control value as 100% viability.

For F-actin staining, PBMCs (10\(^7\)) were incubated with 50 \(\mu_{g}/mL\) \(\gamma\)-globulin for 10 minutes and then stained with anti-human CD3-FITC, CD5-PE, CD19-Cy7, and CD3-APC mAbs. After washing, cells were fixed for 20 minutes, washed, and permeabilized in the presence of Alexa Fluor 647 Phalloidin for 20 minutes. Cells were analyzed by flow cytometry. The percentage of T cells (CD3\(^+\)) and CLL B cells (CD19\(^+\)CD5\(^−\)) for each condition was calculated in Phalloidin-positive cells (viable cells).

Immunoblot analysis

PBMCs were lysed in incomplete Laemmli buffer (125 mmol/L Tris, pH 6.8, 4% SDS, and 20% glycerol) supplemented with a mixture of protease and phosphatase inhibitors. Lysates were...
sonicated and protein concentration was determined by the bicinchoninic acid method (Pierce). Protein samples (10–20 µg/condition) were supplemented with 2.5% 2-ME and 0.004% bromophenol blue, and subjected to SDS-PAGE analysis and immunoblotting, using the indicated primary antibodies and HRP-conjugated secondary antibodies. Proteins were detected by chemiluminescence and exposure on film. β-Actin expression was used as an internal loading control.

**FISH analyses**

FISH analyses were carried out following standard procedures on fixed cells to detect del. 13q14.3, del. 13q34, del. 17p13.1, del. 11q22.3, and trisomy 12p11.1-q11. The probe set consisted of a LSI Vysis CLL FISH Probe Kit (Abbott Molecular Inc.). The number of interphase nuclei analyzed was 200. Interphase FISH cutoff percentages were 7%.

**IGHV mutation status**

Total RNA was extracted from 5 to 10 × 10⁶ PBMCs using TRZOL reagent and the PureLink RNA mini Kit (Life Technologies). The obtained RNA was reverse transcribed into cDNA using 2 U Superscript II reverse transcriptase (Life Technologies), following the manufacturer’s instructions. Determination of IGHV mutational status was carried out as previously described (20). Nucleotide sequences were analyzed using Chromas 1.45 software (Technelysium) and compared with those available in the BLAST and IMGT databases. Sequences with more than 98% homology to the corresponding germ-line IGHV sequence were considered as unmutated (21, 22).

**In vivo toxicity assays**

The in vivo experimental procedures were approved by the pertinent ethic committees and carried out in accordance with the guidelines of the European Union directives and Spanish laws. Mice (3-month-old C57Bl/6 males) were housed in the animal facility of the Instituto de Investigaciones Biomedicas “Alberto Sols” under standard sterile conditions in air-filtered containers. Mice were administered vehicle alone (ethanol/corn oil, 1:9, n = 8) or vehicle containing 200 mg/Kg (600 mg/m²) of I3C (n = 8) by oral gavage. I3C or vehicle were daily administered for 5 consecutive days followed by 2 days of rest, repeating this schedule along the experiment. One week after the beginning of the I3C treatment, half of the mice treated with vehicle or I3C were administered with 35 mg/kg (105 mg/m²) of fludarabine by i.p. injection for 5 consecutive days, and this treatment was repeated 3 weeks later.

**Flow cytometry analyses**

Blood was collected from mice before and after the treatments. Whole blood was preincubated in the presence of 50 µg/mL γ-globulin for 10 minutes and then cells were stained with anti-mouse B220-ITC, Gr-1-PE, CD45-PerCP, and CD3-APC mAbs for 20 minutes at room temperature. Following, red cells were lysed with a lysis buffer (0.155 mol/L NH₄Cl, 10 mmol/L KHCO₃, and 0.1 mmol/L EDTA) for 15 minutes at room temperature. After washing twice with PBS, total white blood cell population (CD45⁺), neutrophils (GR-1⁺), B cells (B220⁺), and T cells (CD3⁺) were determined by FACS analysis. Quantification was achieved using Perfect Count Microspheres (Cyto gens).

**Analysis of transaminases and urea concentration**

Serum levels of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and urea were determined using Releff strips and the Releffron plus analyzer (Roche Diagnostics). To obtain the mouse serum, blood was collected from the mice and incubated in BD microtainer SST tubes (BD Diagnostics), followed by centrifugation. Serum was stored at -20°C until use.

**Data analysis**

SPSS v.19 software for Windows (SPSS) was used for statistical analysis. Statistical significance in cell viability assays was determined using the Student t test. Statistical significance between groups in toxicity assays was assessed by the Wilcoxon test. For synergy analyses, the combination index (CI) isobologram (23) was determined using the CalcuSyn software (Biosoft). CI values <1, = 1, and >1 indicate synergy, additivity, and antagonism, respectively.

**Results**

I3C downmodulates XIAP and cIAP1/2 and induces caspase 9–dependent apoptosis of CLL cells

To assess the effect of I3C on CLL cell viability, we used a cohort of CLL cells from 38 patients representing different Rai stages, IGHV mutation status, cytogenetic alterations, and responses to treatments (Table 1). Our results show that I3C potently reduced the viability of CLL cells with an LD₅₀ of approximately 37 µmol/L, whereas it was less cytotoxic in PBMCs from normal donors (LD₅₀ ~100 µmol/L; Supplementary Fig. S1). I3C treatment induced apoptosis in CLL cells, as demonstrated by the caspase-dependent binding of Annexin V on the surface of I3C-treated CLL cells (not shown). Moreover, as shown in Fig. 1 using cells from two representative patients with different in vivo sensitivity to F-ara-A, one of them sensitive (Fig. 1A, left) and the other one resistant (Fig. 1A, right), we observed that I3C efficiently induced activation of caspases 9 and 3 as well as PARP cleavage in both cases. Interestingly, I3C-induced caspase 9 cleavage and activation were not prevented by ZVAD-fmk and could still cleave caspase 3 to its p24 form even in the presence of the inhibitor. However, ZVAD-fmk prevented caspase 3 self-cleavage to its active form and consequently prevented PARP cleavage. These results, together with the lack of significant activation of caspase 8 by I3C (not shown), strongly suggest that I3C triggers caspase 9–mediated apoptosis of CLL cells. We also assessed whether increasing concentrations of I3C had an effect on the expression levels of key proteins regulating CLL tumorigenesis and survival. Our results showed that I3C consistently downregulated cellular inhibitor of apoptosis (cIAP)-1/2 and X-linked inhibitor of apoptosis (XIAP) expression, similar to what others and we have previously described in other leukemic cells (12, 13). Downmodulation of MCL1 expression was also observed in response to increasing I3C concentrations. In contrast, I3C caused no significant effect on the levels of expression of BCL2, BCLXL, BAX, and TGLC. Next, we wanted to determine whether I3Cinduced activation of p53 pathways. Interestingly, we observed that I3C (50–100 µmol/L) caused p53 accumulation, which did not correlate with p53 phosphorylation or upregulation of p53 transcription targets, including NOXA, PUMA, and CDKN1 (Fig. 1). In addition, I3C caused induction of γ-H2AX, but, since this induction was prevented by ZVAD-fmk, it was a
Table 1. Biologic characteristics and treatment information of the CLL patients under study

<table>
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<th>Gender</th>
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<th>IGHV status</th>
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<th>Cyto genetic alterations</th>
<th>Treatment</th>
<th>Treatment-free months after diagnosis</th>
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<td>M</td>
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<tr>
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<td>60</td>
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<td>1.FC 2.Ofat + FC 3.Alectem + DXM 4.Ofat</td>
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</table>

Gender: F, Female; M, Male; IGHV mutation status: M, Mutated; UM, Unmutated; del, deletion; ND, not detected. Treatments (by alphabetic order): Alemt, alemtuzumab; Benda, bendamustine; Chlor, chlorambucil; Cyclophosphamide + vincristine + prednisolone; DXM, dexamethasone; FC, Fludarabine + cyclophosphamide; FCR, Fludarabine + cyclophosphamide + rituximab; Flu, fludarabine; GEMOX, gemcitabine-oxaliplatin; No, untreated; Ofat, ofatumumab; R-Benda, rituximab + bendamustine; R-CHOP, rituximab + cyclophosphamide + hydroxydaunorubicin + Oncovin + prednisone; Rtx, rituximab; Rtx-sc, rituximab (subcutaneous).

consequence of caspase 3 activation (24) instead of ATM/ATR-mediated phosphorylation. A densitometry analysis of the effects of I3C on the expression of IAP-family proteins, p53 and -H2A.X, is shown in Fig. 1B. The effect of I3C on the viability of the CLL cells under study is shown in Fig. 1C.

I3C synergizes strongly with fludarabine in CLL cells irrespective of their IGHV mutation status, p53 functionality, and treatment resistances.

The striking effects of I3C in promoting CLL apoptosis prompted us to question whether I3C could have differential effects on different types of CLL cells and if it could cooperate with other chemotherapeutic drugs currently used for treating CLL patients. The effect of increasing concentrations of I3C, F-ara-A, and combinations of both drugs (keeping a constant 50:1 ratio) on CLL cell viability after 24-hour (Supplementary Fig. S2) and 48-hour treatment (Fig. 2), as well as the statistical analysis of these studies (Supplementary Table S1), is shown. CLL cells used in these experiments are described in Table 1. Thus, we studied the effect of these drugs on CLL cells from patients that were left untreated or received just one treatment (n = 28) compared with patients that received multiple treatment combinations (n = 10; Fig. 2A); patients having less than 70% of CLL cells with deleted TP53 (del17p13) or ATM (del11q22) (n = 29) versus patients with at least 70% of cells harboring deletions in any of these genes (n = 9; Fig. 2B); CLL cells showing in vitro sensitivity to F-Ara-A (n = 29) compared with CLL cells that were unaffected by F-Ara-A (n = 9; Fig. 2C); and finally CLL cells with mutated IGHV (n = 17) versus CLL cells with unmutated IGHV (n = 21; Fig. 2D). Our results confirmed that I3C as single treatment is effective in promoting apoptosis of all types of CLL, irrespective of p53 functionality and IGHV.
Figure 1. Analysis of the effect of I3C on the expression of different proteins implicated in caspase and p53 pathways or in apoptosis regulation. PBMCs isolated from CLL patients (1.5 × 10^6 cells/mL) were cultured in medium with vehicle control (0.02% ethanol/DMSO) or with increasing doses of I3C (10–100 μmol/L). When indicated, 100 μmol/L Z-VAD-fmk (ZVAD) was added to the culture. After 24 hours, cells were lysed in Laemmli buffer and sonicated. In all cases, 10 to 20 μg of proteins were subjected to 8% to 13% SDS-PAGE, followed by immunoblotting with antibodies specific for the proteins of interest. β-Actin expression was used as a loading control. Figure shows representative cases of CLL cells that are either sensitive to F-ara-A in vitro (left plots) or resistant to this drug (right plots). B. densitometry analysis of the effects of I3C on the expression of XIAP, cIAP1/2, p53, and pH2A.X Ser139 shown in A. Densitometry was performed on scanned images using Quantity One software (Bio-Rad Laboratories), and values were normalized for β-actin. We assign the 100% expression to the point with the larger expression for each protein. C. dose-dependent effects of I3C on the viability of F-ara-A-sensitive (left) and F-ara-A-resistant (right) CLL cells, measured at 24 and 48 hours by a luminescence assay.
I3C Synergizes with Fludarabine in Refractory CLL Cells

In all cases, LD50 for I3C ranged from 26 to 48 μmol/L at 48 hours. Even more importantly, I3C showed strong synergy with F-ara-A in inducing CLL cell death (Fig. 2, right plots and Supplementary Table S2). Statistical significance is observed after 48-hour treatment.

Figure 2.
I3C and F-ara-A show strong synergy against CLL cells irrespective of their treatment resistances, p53 functionality, in vitro sensitivity to F-ara-A, and IGHV mutation status. PBMCs isolated from CLL patients (1.5 × 10⁶ cells/mL) were cultured in medium in triplicates with vehicle alone (<0.02% ethanol) or with increasing doses of F-ara-A (0.1, 0.2, 0.5, and 1 μg/mL), I3C (5, 10, 25, and 50 μmol/L), or a combination of both compounds keeping a constant ratio of 1:50 (F-ara-A:I3C). After 48 hours, the percentage of viable cells was determined considering the control value as 100% viability. For analysis, samples were divided into two different groups based on different criteria, including (A) the in vivo treatment response (patients that received no treatment or treated once vs. patients treated twice or more), (B) the TP53/ATM deletion status [patients with <70% of CLL cells harboring either TP53 (del17p13) or ATM (del11q22) deletions vs. patients with ≥70% of CLL cells with TP53 or ATM deletions], (C) the in vitro F-ara-A sensitivity (<60% viable cells (sensitive) compared with ≥60% viable cells (resistant) in the presence of F-ara-A (1 μg/mL)), and (D) the IGHV mutation status [patients with ≥2% mutations in IGHV (mutated) vs. patients with <2% (unmutated)]. Left and middle plots show mean ± SEM. Plots at the right show the graphic representation of the CI for the fraction affected (Fa) for the corresponding groups, calculated with the CalcuSyn software. The results of this study at 24 hours are shown in Supplementary Fig. S2. Statistical significances are shown in Supplementary Table S1.
Figure 3.
Effect of I3C, F-ara-A, and the I3C/F-ara-A combination treatment on the p53 pathway. PBMCs isolated from CLL patients (1.5 × 10^6 cells/mL) were cultured in medium with either vehicle (< 0.02% ethanol/DMSO), 0.5 mg/mL F-ara-A, 25 mmol/L I3C, or the combination of both compounds. (Continued on the following page.)
treatment at dosages of I3C as low as 5 to 10 μmol/L. (Supplementary Table S1). Indeed, the average LD50 for I3C is 7.9 μmol/L if administered in combination with F-ara-A (Fig. 2), which are concentrations of I3C similar to and even smaller than those of I3C-condensation products detected in plasma and organs of human and rodents after oral administration of I3C or derivatives (25–27). Remarkably, I3C was able to synergize with F-ara-A in p53-deficient CLL cells (Fig. 2B) and even in CLL cells that showed in vitro resistance to F-ara-A (Fig. 2C). Thus, the combined treatment of I3C and F-ara-A needed to kill 90% of CLL cells resulted in an F-ara-A dose reduction index (DRI) of 29.8 for CLL cells harboring TP53/ATM deletions and of 85.8 for CLL cells showing in vitro resistance to F-ara-A (Supplementary Table S2). In addition, I3C could also synergize with vincristine and chlorambucil (Supplementary Table S2). Of note is that I3C cytotoxicity and its synergy with F-ara-A are equally active in the presence of human AB serum and FBS (Supplementary Fig. S3).

Because the p53 pathway has been shown to be involved in the antitumor effect of fludarabine (28, 29), we next studied whether this pathway was implicated in the synergistic antitumor effects of I3C and F-ara-A (Fig. 3). First, using cells from two untreated CLL patients, one showing in vitro resistance to F-ara-A (patient 16, panel A) and another one sensitive to this drug (patient 26, panel B), we observed that F-ara-A or I3C alone caused minimal p53 response, but the combined I3C and F-ara-A treatment strongly activated p53, causing p53 accumulation and phosphorylation as well as induction of PUMA and CDKN1, and potently induced cytotoxicity (panels A and B, bottom). However, as shown in plot C, when this study was performed using cells from a patient (n. 2) with deleted TP53 in more than 70% of PBMCs resulting in nonfunctional p53 accumulation, we found that the combined I3C/F-ara-A treatment could not activate the p53 response but still caused massive cell death (panel C, bottom). In addition, when CLL cells from 2 patients (patient 36, panel D and patient 1, panel E) with clinically refractory CLL were used, our results indicate that combined I3C/F-ara-A treatment could induce a partial p53 response, causing p53 accumulation and CDKN1 expression, and efficiently induced cell death of both CLL specimens. Of note is that CDKN1 accumulated in the presence of Z-VAD-fmk, confirming previous evidence (30) indicating that CDKN1 is a substrate for activated caspases.

Finally, we analyzed CLL cells from a clinically refractory patient (patient 38, panel F) in which the combined I3C/F-ara-A treatment failed to induce any p53 response. Indeed, I3C/F-ara-A did not induce p53 accumulation, p53 phosphorylation, or upregulation of CDKN1, NOXA, and PUMA (the latter only being observed in samples incubated with ZVAD-fmk in an overexposed film), whereas this treatment could still efficiently induce cell death in this CLL sample. Altogether, our results support that combined I3C and F-ara-A treatment induces cell death by p53-dependent and -independent mechanisms.

It is noteworthy that these experiments also showed that the combined I3C and F-ara-A treatment caused a more effective downmodulation of cIAP1/2 and XIAP compared with the single treatments of I3C or F-ara-A (Fig. 4D). Moreover, cIAP1/2 and XIAP downmodulation could not be prevented by Z-VAD-fmk, further supporting a possible role of these IAP family proteins in I3C-mediated toxicity. In contrast, MCL1 expression was not significantly affected by the combination treatment (Supplementary Fig. S4). We also analyzed the effect of I3C, F-ara-A, and the combined I3C/F-ara-A treatment on the mitochondria membrane potential and on reactive oxygen species (ROS) production. As shown in Supplementary Fig. S5A, both F-ara-A and I3C were able to induce mitochondria membrane depolarization in CLL cells that were sensitive to F-ara-A, although the effect on ΔΨm depolarization of the combined F-ara-A/I3C treatment was significantly higher. When F-ara-A-resistant CLL cells were used, only the combined F-ara-A/I3C treatment triggered ΔΨm depolarization. These results are consistent with our results showing that the combination treatment preferentially triggers caspase 9-dependent apoptosis (Supplementary Fig. S4). In contrast, neither of the treatments did induce ROS production (Supplementary Fig. S5B).

I3C in combination with fludarabine overcomes stroma-mediated drug resistance

Interactions between CLL cells and the tissue microenvironment promote leukemic cell survival, growth, and drug resistance, being critical for disease progression and development of treatment resistances (31, 32).

To determine whether I3C alone or in combination with F-ara-A was able to induce death of CLL cells in the presence of microenvironment protective signals, we cultured CLL cells over a monolayer of human bone marrow–derived mesenchymal stem (hMSC-TERT) cell line (19). As shown in Fig. 4A, culturing CLL cells in the presence of the stroma cells increased CLL survival. As expected, in the absence of the stroma cells, I3C and F-ara-A induced cell death of CLL cells as single treatment (Fig. 4B), while co-culturing of CLL cells over a monolayer of MSC-TERT cells conferred CLL cells resistance to both I3C and F-ara-A (Fig. 4C), thus further confirming the protective environment provided by the stroma cells. However, the combination I3C/F-ara-A treatment was still able to efficiently induce cell death of both stroma-free and stroma-protected CLL cells (Fig. 4B and C), keeping the synergy of both drugs in these conditions (Fig. 4D).

Lack of toxicity of I3C alone or in combination with fludarabine

Next, we wanted to determine whether the combination of I3C and F-ara-A has a broad toxicity or it was mostly restricted to tumor cells. First, using PBMCs from a representative CLL patient, we tested the effect of I3C, F-ara-A, and the combination I3C/F-ara-A treatment on CLL cells and T cells, by assessing the percentage of T (CD5+CD3+) and CLL (CD5−CD19+) cells that could be recovered after the treatments (Supplementary Fig. S6).
Indeed, we showed that the combination treatment had a striking cytotoxic effect on CLL cells while T cells were much less affected, as indicated by the accumulation of T cells. Incubation with ZVAD-fmk protected CLL cells from the cytotoxic effect of I3C + F-ara-A (Supplementary Fig. S6).

In addition, we tested the toxicity of the combinatory I3C + fludarabine treatment in mice. Thus, we treated mice with either I3C (oral gavage, 600 mg/m², a similar dose to that used in humans; ref. 11), fludarabine (i.p. bolus, 100 mg/m²), or the combined treatment (Fig. 5). The treatment with I3C had no effect on the weight of the mice, serum levels of liver enzymes and urea, and on white blood cell counts. Fludarabine caused instead an increase in GOT and urea and a significant reduction in granulocytes and B lymphocytes. However, combined I3C + fludarabine treatment did not worsen any of these parameters compared with the treatment with fludarabine alone, thus further emphasizing the limited toxicity of I3C.

Discussion

I3C is an orally bioavailable natural small chemical with potent antitumor activity in CLL cells

In this report, we have shown that I3C, a phytochemical found in broadly consumed cruciferous vegetables of the Brassica genus, such as the broccoli, the cauliflower, and the Brussels sprouts, shows potent antitumor activity against CLL cells. I3C is derived from glucosinolates produced by these plants, found at concentrations ranging from 0.4 to 4 mg/g (33). Although oral bioavailability of I3C is limited, I3C derives, in particular its condensation dimer DIM formed in the acidic gastric milieu, can reach μmol/L concentrations in plasma and organs similar to and even higher than those used in this study. In this regard, oral intake of 1 g of I3C allowed human plasma concentrations of I3C (in its dimer form, DIM) equivalent to 24 μmol/L × h (25). In mice administered with 250 mg/kg of I3C, this compound was found in plasma at concentrations of 28 μmol/L in plasma and 160 μmol/L in liver. It was also observed that I3C persisted longer in plasma and tissues converted into its dimer, DIM, reaching similar or even higher concentrations than I3C (26, 27). Furthermore, we have shown that oral administration of I3C and DIM to mice xenotransplanted with Burkitt lymphoma cells efficiently downmodulated cMYC expression, reduced tumor burden, and increased survival of the mice (13). It is worth noting that all these studies were based on the oral administration of a single I3C dose, thus opening the possibility that different regimes, venues of administration, or I3C-liposomal formulations might improve I3C systemic exposure. In this regard, it has been shown that I3C could be intravenously administered (34), and the improved clinical pharmacokinetics and tumor uptake of liposomal formulations of doxorubicin,
Figure 5.
Analysis of the in vivo toxicity of I3C, fludarabine, and the combined treatment. A, schematic representation of the drug administration schedules and weight of the mice during the treatment. C57bl/6 mice were administered vehicle alone (n = 8) or vehicle containing 200 mg/kg of I3C (n = 8) by oral gavage for 5 consecutive days followed by 2 days of rest, repeating this schedule during all experiment. One week after the beginning of the I3C treatment, half of the mice treated with vehicle (n = 4) or I3C (n = 4) were administered 35 mg/kg fludarabine by i.p. injection for 5 consecutive days, and this treatment was repeated 3 weeks later. Groups of mice were as follows: vehicle (n = 4), I3C (n = 4), vehicle + fludarabine (n = 4), and I3C + fludarabine (n = 4). B, the serum levels of GOT, GPT, and the concentration of urea were determined. Serum was obtained from the mice right before the treatment (pretreatment) and 12 days after the last fludarabine administration (posttreatment). Data represent mean ± SEM. Statistical significance was assessed by the Wilcoxon test. C, analysis of white blood cell populations before and after the treatments. Blood was collected from mice before and after the treatments as indicated in B. Total white blood cell population (CD45+), neutrophils (Gr-1+), B cells (B220+), and T cells (CD3+) were determined by FACS analysis. Quantification was achieved using Perfect Count Microspheres (Cytognos). Data represent mean ± SEM. The Wilcoxon test was used to assess statistical significances.
platiflex, and daunorubicin compared with that of the free drugs have been already demonstrated (35, 36).

Several studies have assessed the antitumor effects of I3C and derivatives in a variety of tumors, including breast, prostate, and colon cancer cell lines, and it has been proposed the use of these compounds as chemopreventive agents (reviewed in refs. 6, 7). Among the described activities of I3C and derivatives, there are reports describing an antagonistic effect on the estrogen and androgen receptors. On the other hand, there is evidence supporting an agonistic effect on the aryl hydrocarbon receptor. It has also been proposed that I3C could revert multiple drug resistance in K562 erythroleukemic cells by downregulating P-glycoprotein. Moreover, I3C and oligomers are potent AKT inhibitors and disrupt AKT-dependent survival pathways in breast and prostate cancer cell lines (reviewed in refs. 6, 7). In regard to hematopoietic malignancies, Takada and colleagues (12) have shown in multiple myeloma cells that I3C prevented TNF-mediated induction of NF-κB-regulated genes encoding proteins involved in the control of cell proliferation and apoptosis, including cyclin D1, IAP family, and BCL2 family proteins, among others. In addition, we have also observed potent antitumor effect of I3C and DIM in Epstein–Barr-positive Burkitt lymphoma cells, through a mechanism involving cMYC and IAP family downmodulation (13).

Interestingly, a role for IAP family in I3C cytotoxicity in CLL would be also supported by the fact that I3C downmodulates cIAP1/2 and XIAP in CLL cells. Furthermore, as shown in Supplementary Fig. S5, downmodulation of these IAP family proteins is stronger in the combinatory I3C + F-Ara-A treatments and could not be prevented by the caspase inhibitor Z-VAD-fmk, suggesting that IAP family downmodulation precedes and might have a role in caspase activation.

I3C strongly synergizes with F-Ara-A in all types of CLL cells and restored F-Ara-A sensitivity in fludarabine-resistant CLL cells

CLL that are resistant or become refractory to fludarabine or purine analogues constitute a major clinical problem because of poor patient survival and limited treatment options (reviewed in refs. 37, 38). There is a need of new treatment options that are effective on these patients and/or reduce or delay the development of refractoriness to these compounds.

Our results indicate that I3C was active against CLL cells as single treatment but, even more importantly, that it could synergize with other chemotherapeutic drugs currently used for treating CLL. Remarkably, I3C strongly synergized with F-Ara-A in tumor cells from all CLL variants, including those with different Rai stages, with mutated and unmutated IGHV, with p53 and ATM deletions and even in those from patients with relapsed or refractory disease. Indeed, in CLL samples that were resistant to F-Ara-A in vitro, I3C restored the sensitivity to F-Ara-A by reducing 85.8-fold the F-Ara-A concentration required to kill 90% of CLL cells. As expected, CLL cells with TP53 and ATM deletions (del17p13 and/or del11q22) were more resistant to F-Ara-A compared with CLL cells lacking 17p13 and/or 11q22 deletions. However, the combination treatment of I3C and F-Ara-A was effective inducing apoptosis in both groups. Indeed, I3C was able to reduce 29.8-fold the concentration of F-Ara-A needed to kill 90% of CLL cells with deficient p53 pathway.

The biochemical analysis of the p53 pathway showed that the combination treatment of I3C and F-Ara-A was more efficient than F-Ara-A alone triggering the p53 response in CLL cells with fully functional p53 pathway, including p53 phosphorylation and upregulation of PUMA and CDKN1. However, the combination treatment was equally effective inducing apoptosis of CLL cells with deficient p53 pathway and of CLL cells from refractory patients able to mount only a partial p53 response. Altogether, these results indicate that I3C and F-Ara-A work together to induce p53-dependent and -independent cell death.

Furthermore, we have shown that co-culturing CLL cells over a layer of stroma cells both increased CLL cell survival in vitro but also induced resistance of CLL cells to I3C and F-Ara-A as single treatments. However, even in these F-Ara-A–refractory CLL cells, the combination I3C/F-Ara-A treatment could efficiently induce CLL cell death keeping a strong synergy of both drugs. These results suggest that the I3C/fludarabine treatment would be effective in a nodular and tissue environment.

Finally, the assessment of toxicity of the combination I3C/fludarabine treatment in mice showed that I3C did not increase toxicity compared with the fludarabine treatment alone, thus further stressing the safety and adequacy of the combination I3C/fludarabine treatment.

From a clinical point of view, the striking DRI for F-Ara-A in combination with I3C would likely reduce the fludarabine dosages needed for treating patients while having a similar or better effectiveness. This reduction would likely minimize harmful secondary effects associated with fludarabine (39). Moreover, reducing the clinically effective dosage of fludarabine might also prevent or delay fludarabine-refractoriness development, thus improving overall survival of patients. On the other hand, the average DRI for I3C in combination with F-Ara-A is 5, and the LD₅₀ for I3C in the combination treatment is 7.9 μmol/L, which is a clinically relevant concentration (25).

In summary, the low toxicity of I3C and derivatives, already demonstrated in clinical trials, would support its clinical use as a novel anti-CLL chemotherapy either alone, as a neoadjuvant or adjuvant therapy, or in combination with other chemoimmuno-therapeutic agents, particularly with fludarabine, even in patients with relapsed or refractory CLL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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I3C Synergizes with Fludarabine in Refractory CLL Cells

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Indole-3-Carbinol Synergizes with and Restores Fludarabine Sensitivity in Chronic Lymphocytic Leukemia Cells Irrespective of p53 Activity and Treatment Resistances
