Nrf2 responses and the therapeutic selectivity of electrophilic compounds in chronic lymphocytic leukemia

Raymond P. Wu[a], Tomoko Hayashi[a], Howard B. Cottam[a], Guangyi Jin[a], Shiyin Yao[a], Christina C. N. Wu[a], Michael D. Rosenbach[a], Maripat Corr[b], Richard B. Schwab[a], and Dennis A. Carson[a,1]

[a]Moores Cancer Center, University of California San Diego, La Jolla, CA 92093; and [b]University of California San Diego School of Medicine, La Jolla, CA 92093

Contributed by Dennis A. Carson, March 10, 2010 (sent for review January 29, 2010)

Recent studies show that redox-active small molecules are selectively cytotoxic to chronic lymphocytic leukemia (CLL). Although elevated levels of reactive oxygen species in CLL cells have been implicated, the molecular mechanism underlying this selectivity is unclear. In other cell types, the nuclear factor erythroid 2–related factor 2 (Nrf2) signaling pathway regulates the oxidative stress response. We found elevated Nrf2 signaling in untreated CLL cells compared with normal lymphocytes. Therefore, we tested 27 known electrophilic and antioxidant compounds with drug-like properties and determined their CLL-selective cytotoxicity and effect on Nrf2 signaling. The selected compounds were from five distinct structural classes; α-β unsaturated carbonyls, isothiocyanates, sulfhydryl reactive metals, flavones, and polyphenols. Our results show that compounds containing α-β unsaturated carbonyls, sulfhydryl reactive metals, and isothiocyanates are strong activators of Nrf2 in a reporter assay system and in primary human CLL based on increased expression of the Nrf2 target heme oxygenase–1. α-β Unsaturated carbonyl–containing compounds were selectively cytotoxic to CLL, and loss of the α-β unsaturation abrogated Nrf2 activity and CLL toxicity. The α-β unsaturated carbonyl containing compounds ethacrynic acid and parthenolide activated Nrf2 in normal peripheral blood mononuclear cells, but had a less potent effect in CLL cells. Furthermore, ethacrynic acid bound directly to the Nrf2-negative regulator Kelch-like ECH-associated protein 1 (Keap1) in CLL cells. These experiments document the presence of Nrf2 signaling in human CLL and suggest that altered Nrf2 responses may contribute to the observed selective cytotoxicity of electrophilic compounds in this disease.

antioxidant response | electrophilic stress response activators | natural product | dietary component

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the Western world. Despite advances in our understanding of the biology of CLL (1), its current management includes monitoring without treatment of asymptomatic patients until disease progresses by clinical criteria (2). Improving the care of indolent CLL will require the development of compounds with minimal or no toxicity. Recent studies with low-toxicity compounds including 2-methoxyestradiol (3) and phenethyl isothiocyanate (PEITC) (4) implicate elevated oxidative stress as a biochemical basis for their selective toxicity with CLL cells.

Compared with normal lymphocytes, CLL cells have increased levels of reactive oxygen species (ROS) and, by definition, are under constant oxidative stress (5). This observation is not specific for CLL, and cancer cells often possess an imbalanced redox state as a result of elevated metabolism and expression of oncogenic pathways (6, 7). This redox imbalance could have significant effects on the function of cellular proteins and signaling pathways implicated in cancers (8). High levels of ROS normally kill cells by damaging structural components such as DNA, lipids, and mitochondria (9, 10). However, low-level oxidative stress has been reported to promote cell growth and survival by modulating signal transduction pathways, potentially explaining the common finding of elevated ROS in cancer (6). Oxidative modification of the sulfur containing amino acids of cellular proteins is known to effect transcriptional regulation of key signaling pathways including NF-κB, p53, and nuclear factor erythroid 2–related factor 2 (Nrf2) (8, 11).

The main signaling pathway involved in the oxidative stress response is the Keap1-Nrf2-ARE pathway (12). Nrf2 signaling is the major cellular defense to relieve oxidative and electrophilic stress (13, 14). Nrf2 is a transcription factor that induces antioxidant responsive genes such as heme oxygenase–1 (HO-1), catalase, glutathione dismutase, and superoxide dismutase (SOD1). In addition, Nrf2 also regulates phase II drug metabolism genes such as NAD(P)H dehydrogenase, quinone 1 (NQO1). In the absence of oxidative stress, Nrf2 protein is continually degraded in the cytoplasm by an E3 ubiquitin ligase complex containing the regulatory protein Kelch-like ECH-associated protein 1 (Keap1) (15). Keap1 contains multiple cysteine residues that, when modified by oxidation or electrophiles, accelerate its dissociation from Nrf2 (16). This change allows Nrf2 protein to accumulate in the cytoplasm and translocate into the nucleus where it binds to the antioxidant response element (ARE) present in target gene promoters and enhancers. The Nrf2 pathway may be the most sensitive pathway for the presence of thiol-modifying molecules such as ROS or electrophilic small molecules as a result of the presence of multiple, highly reactive, functionally important cysteine residues in Keap1 (17). The role of Nrf2 activation in protecting cells from environmental insults including electrophilic compounds has already been well characterized (18).

Small molecules that activate Nrf2 signaling are under active study for their potential to become cancer preventive or therapeutic agents. To date, 10 chemically distinct classes of compounds have been found to induce Nrf2 target genes, including toxins, e.g., heavy metals (12). However, several classes such as Michael acceptors, isothiocyanates, and trivalent arsenicals are being studied or have been shown to be cancer preventive or therapeutic agents. Many of these compounds, including PEITC (4), parthenolide (19), and arsenic trioxide (20), were shown to selectively kill CLL cells compared with normal peripheral blood mononuclear cells (PBMCs). However, the role of the Nrf2 pathway in this therapeutic selectivity has not previously been evaluated to our knowledge.

In this article, we demonstrate Nrf2 signaling in primary human CLL compared with normal PBMCs at baseline and in response to various Nrf2-activating compounds. Most of the tested compounds were originally isolated from natural sources or previously

Author contributions: R.P.W. and D.A.C. designed research; R.P.W., T.H., S.Y., C.C.W., and M.D.R. performed research; G.J. contributed new reagents/analytic tools; R.P.W., T.H., H.B.C., M.C., R.S., and D.A.C. analyzed data; and R.P.W., T.H., H.B.C., R.S., and D.A.C. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. E-mail address: dacarson@ucsd.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/1002890107/DCSupplemental.

found to possess drug-like properties (Table S1). Many of these compounds have previously been shown to have either anti-inflammatory or anticancer activity (21). As aberrant oxidative stress has been observed in CLL (4), we compared steady-state levels of Nrf2 mRNAs and proteins in PBMC from patients with CLL versus those from healthy donors. We then measured Nrf2 activation and CLL-selective cytotoxicity of electrophilic and antioxidant small molecules from five structural classes. Based on our previous work showing that the α-β unsaturated carbonyl group of ethacrynic acid (EA) is necessary for CLL cytotoxicity (22), we evaluated the requirement of α-β unsaturation of this group for Nrf2 activation. We also compared Nrf2 induction between normal and CLL PBMCs to evaluate the relationship between Nrf2 induction and CLL-specific cytotoxicity.

Results

Nrf2 Signaling Is Up-Regulated in CLL. Previous studies show that PBMCs from CLL have higher levels of ROS than their normal counterparts (3, 4). One study found that CLL cells also express higher levels of SOD1 compared with normal PBMCs (5). As Nrf2 is the main regulator of responsive antioxidant genes such as SOD1, we compared the Nrf2 expression level between PBMCs isolated from patients with CLL versus those from healthy donors. The quantitative PCR (qPCR) analysis showed that Nrf2 mRNA levels were significantly higher in CLL samples ($P < 0.05$ by one-way ANOVA) compared with the normal counterparts (Fig. 1*A*). The data displayed in Fig. 1*A* are from mRNA isolated from frozen and freshly isolated PBMCs. We initially compared six frozen normal PBMC samples and six frozen CLL PBMC samples. Repeat analysis using freshly isolated normal and CLL PBMCs confirmed that Nrf2 activation was not a freezing artifact. Immunoblot analysis also revealed that the protein levels of Nrf2 and its downstream target HO-1 were highly elevated in CLL compared with normal samples (Fig. 1*B*). Moreover, the negative regulator of Nrf2 protein, Keap1, was also highly expressed in CLL PBMCs compared with normal PBMCs, showing that Nrf2 mediates Keap1 expression to auto-regulate its activity. These data suggest that, although Nrf2 activity is highly up-regulated in CLL PBMCs, the compensatory induction of Keap1 by Nrf2 remains intact.

As PBMCs from patients with CLL are highly enriched in B cells, we also compared the Nrf2 expression between freshly isolated normal B cells and PBMCs. Normal B cells were isolated using a negative selection technique from buffy coat layers of healthy donors, yielding at least 85% CD19-positive cells. After isolation, the cells were assayed for Nrf2 expression by qPCR. The results show that Nrf2 is also elevated in normal B cells compared with normal PBMCs by qPCR ($P < 0.05$ by one-way ANOVA) and immunoblot analysis (Fig. 1). These results suggest that the increased Nrf2 signaling in PBMCs from patients with CLL may be a general finding in B cells.

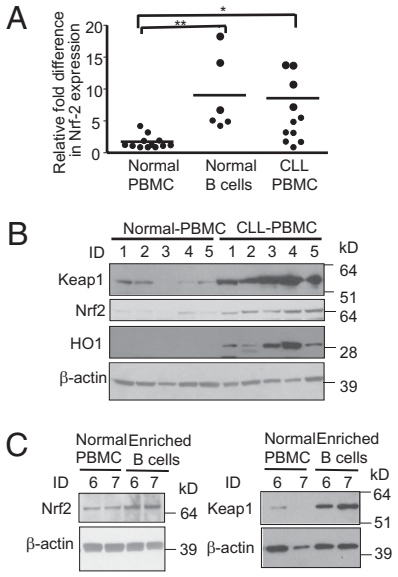

Fig. 1. (*A*) qPCR analysis of Nrf2 expression levels in normal PBMCs ($n = 12$), normal B cells ($n = 6$), and CLL PBMCs ($n = 12$). Normal B cells were enriched from buffy coats of healthy donors, as described in *Materials and Methods*. Nrf2 expression was measured by qPCR assay. The qPCR results were analyzed by the $2^{\Delta\Delta CT}$ method. $^{*}P < 0.05$ and $^{**}P < 0.01$ by one-way ANOVA with Dunnett post hoc testing. (*B*) Immunoblot analysis of Nrf2 and HO-1 proteins in normal PBMCs and CLL PBMCs. Frozen normal ($n = 5$) and CLL PBMCs ($n = 5$) were lysed and immunoblotted with anti-Nrf2 and HO-1–specific antibodies. β-Actin antibody was used as an internal control. (*C*) Immunoblot analyses of Nrf2 and Keap1 in normal PBMCs and normal B cells. Normal B cells were isolated and the cells were lysed and Western blot analysis was performed with indicated antibodies.

Electrophilic and Antioxidant Compounds Activate Nrf2 Signaling in a Cell-Based Assay. Electrophilic small molecules, such as 2-methoxyestradiol (3), PEITC (4), parthenolide (19), and arsenic trioxide (20) selectively kill CLL cells. Hypothesized mechanisms for their selective cytotoxicity include increasing ROS levels or other alterations of redox state. Modification of free thiols on cellular proteins is known to lead to redox environment–related signaling in cells (11). Therefore, we tested a panel of small molecules with oxidative or electrophilic properties capable of modifying free thiol groups for activation of Nrf2, the master regulator of the oxidative stress response (13, 14). These compounds were from five structural classes: α-β unsaturated carbonyls, isothiocyanates, sulfhydryl reactive metals, flavones, and polyphenols. The polyphenols were included because they have been shown to activate the Nrf2 pathway in some systems, presumably as a result of oxidation to form quinones or other electrophilic products (23). Initially, Nrf2 activation by these compounds was assessed in a cell-based reporter assay (GeneBlazer; Invitrogen). The assay uses a FRET β-lactamase reporter system under the control of the ARE, the DNA sequence specifically bound by activated Nrf2 (24). The reporter element has been stably integrated into the HepG2 cell line, a human carcinoma cell line of liver origin. The results show that, of 27 compounds tested, 18 compounds induced ARE with a response ratio of 2 or more at concentrations less than 12 μM (Table 1). The response ratio is the ratio of ARE-dependent β-lactamase enzymatic activity compared with baseline fluorescent activity. α-β Unsaturated carbonyls, isothiocyanates, and sulfhydryl reactive metals were the most active groups of compounds in this assay. All six tested α-β unsaturated carbonyl containing compounds activated Nrf2. Three of four sulfhydryl reactive metals, all of which contained arsenic, potently activated Nrf2. Both of the tested isothiocyanates, including PEITC, activated Nrf2. Finally, two of five flavones and five of eight polyphenols activated Nrf2 signaling. In summary, we used a cell-based assay to measure which of, and with what potency, a group of electrophilic and antioxidant thiol-modifying compounds activated Nrf2.

Selective Toxicity in CLL. The cytotoxicity of the same panel of electrophilic and antioxidant compounds was assessed in primary human PBMCs from normal donors and patients with CLL. Freshly isolated normal and CLL PBMCs were exposed to compounds for 48 h and cell viabilities were measured by a tetrazolium (MTT) uptake assay. As fresh CLL cells from a small number of subjects were available for each experiment, we first tested compounds in samples from three subjects. Nontoxic compounds were excluded in subsequent testing with samples from additional subjects. The LD_{50} values were calculated and were compared using Mann-Whitney *U* tests. The results show that five α-β unsaturated

Table 1. Nrf2 activity and cytotoxicity of electrophilic and antioxidant compounds

Compound class/name	Nrf2 activity C_{RR2}* ± SEM (N†), μM	LD_{50}‡ ± SEM (N), μM		Selectivity index§
	ARE-bla HepG2	CLL-PBMC	Normal PBMC	
α−β Unsaturated carbonyl				
Parthenolide	1.46 ± 0.09	0.9 ± 0.07 (8)	3.1 ± 0.8 (6)¶	3.6
Hypoestoxide	0.93 ± 0.06	19 ± 1.9 (10)	25 ± 3 (6)	1.3
EA	1.97 ± 0.25	7.3 ± 1 (12)	20 ± 2 (6)¶	2.7
Curcumin	5.16 ± 1.53	16 ± 1.5 (10)	29 ± 5 (6)¶	1.8
15-OH-PG-J2	0.36 ± 0.076	7.8 ± 0.7 (10)	22 ± 4 (6)¶	2.9
Dimethyl fumarate	5.02 ± 0.47	28 ± 2 (11)	31 ± 2 (6)‖	1.1
α−β Saturated carbonyl				
Hypoestoxide reduced	31.62 ± 5.52	>50 (3)	>50 (3)	1
EA-reduced	NA	>50 (3)	>50 (3)	1
Linomide	NA	>50 (3)	>50 (3)	1
Capsaicin	34.76 ± 9.35	>50 (3)	>50 (3)	1
Isothiocyanates				
PEITC	11.81 ± 0.22	8 ± 5 (7)	15 ± 2 (6)‖	1.8
Sulforaphane	0.43 ± 0.06	10 ± 3 (9)	19 ± 5 (6)‖	2.0
Sulfhydryl reactive metals				
PAO	0.023 ± 0.002	0.02 ± 0.01 (5)	0.04 ± 0.01 (6)	1.7
FlAsh-EDT_2	0.034 ± 0.01	0.8 ± 0.2 (5)	5 ± 2 (6)¶	6.9
ReAsh-EDT_2	0.021 ± 0.01	0.7 ± 0.1 (5)	5 ± 1 (6)¶	7.6
Mersalyl acid	>50	>50 (3)	>50 (3)	1
Flavones				
Tangeretin	39.4 ± 13	16 ± 2 (5)	>50 (6)	>3.0
Apigenin	11.0 ± 0.2	14 ± 2 (8)	40 ± 5 (6)¶	2.9
Luteolin	26 ± 15	20 ± 7 (5)	29 ± 6 (6)	1.4
Quercetin	>50	>50 (3)	>50 (3)	1
b-naphthoflavone	0.08 ± 0.03	>50 (3)	>50 (3)	1
Polyphenols				
TBHQ	0.95 ± 0.06	6 ± 3 (8)	65 ± 47 (6)	11
Gossypol	>50	8 ± 2 (10)	8 ± 3 (6)	1.0
Resveratrol	8.2 ± 0.2	49 ± 2 (5)	40 ± 7 (6)	1.4
Piceatannol	10.6 ± 0.5	>50 (3)	>50 (3)	1
Hydroquinone	0.43 ± 0.05	>50 (3)	>50 (3)	1
Sudan-1	0.12 ± 0.02	>50 (3)	>50 (3)	1

NA, not applicable.

*C_{RR2}, Concentration at response ratio of 2. Response ratio is defined as FRET activity of Nrf-2 GeneBlazer assay of inducers divided by FRET activity at baseline. The value of C_{RR2} is inversely correlated with the Nrf2 activity of a given compound.

†N is 3 replicates for all compounds except for FlAsh-EDT_2 and ReAsh-EDT_2, n is 2.

‡LD_{50}, lethal concentration that induces 50% toxicity of PBMC. The cytotoxicity was determined by MTT assay.

§Selectivity Index was calculated by LD_{50} value of normal PBMC divided by LD_{50} value of CLL-PBMC.

¶$P < 0.01$ and ‖$P < 0.05$ vs. CLL-PBMC [nonparametric one-tailed Student *t* test (Mann-Whitney test) in Prism (Graphpad)].

carbonyl containing compounds and both isothiocyanates were more toxic to CLL PBMCs than to normal PBMCs ($P < 0.05$; Table 1). Phenylarsine oxide (PAO) was cytotoxic at nanomolar concentration to CLL, but its selectivity versus normal PBMCs was not statistically significant (Table 1). Two other arsenic-containing compounds, FlAsh-EDT_2 and ReAsh-EDT_2, were selectively cytotoxic to CLL cells ($P < 0.01$). Two flavones, tangeretin and apigenin, were selectively toxic to the CLL, but with low potency (LD_{50} values of 16 μM and 14 μM, respectively).

Comparison of the cytotoxicity and Nrf2 activation data reveals that compounds that are selectively cytotoxic to CLL almost always activate Nrf2. However, not all Nrf2 activators in the cell-based assay were cytotoxic to CLL. Specifically, resveratrol, piceatannol, and β-naphthoflavone were not toxic to CLL even though they activate ARE in the cell-based assay. The divergent results may reflect differences in the oxidation of the phenolic compounds by lymphocytes and liver cells.

The α-β Unsaturated Carbonyl Functional Group Is Essential for Nrf2 Activation and CLL Toxicity. Previously, we reported that reduction of the α-β unsaturated carbonyl of EA abrogated this compound's toxicity in CLL (22). In this report, we have shown that a series of α-β unsaturated carbonyl containing compounds, including EA, activate Nrf2 in a HepG2 cell-based assay. Therefore, we tested this functional group's role in both Nrf2 activation and CLL cytotoxicity. Both reduced EA and reduced hypoestoxide lacking only α-β unsaturation (Table S1) were tested. These compounds were not toxic to CLL (Fig. 2*A*) and did not activate Nrf2 (Fig. 2*B*). In addition, compounds tested that contain a carbonyl with saturated α-β carbons, specifically capsaicin and linomide (Table S1), also were not toxic to CLL and did not activate Nrf2 (Table 1). These results show that carbonyl compounds with reduced α-β carbons can neither activate Nrf2 nor elicit toxic effect in CLL cells. Together, the results support a general activity of compounds containing α-β unsaturated carbonyl groups in both Nrf2 activation and CLL cytotoxicity.

Induction of HO-1 in Human Immune Cells. To further characterize Nrf2 induction in immune cells, HO-1 protein induction in normal PBMCs was assessed (Fig. 3). Nrf2 is the primary transcription factor responsible for HO-1 induction (25). We selected compounds and concentrations based on our results from the Nrf2

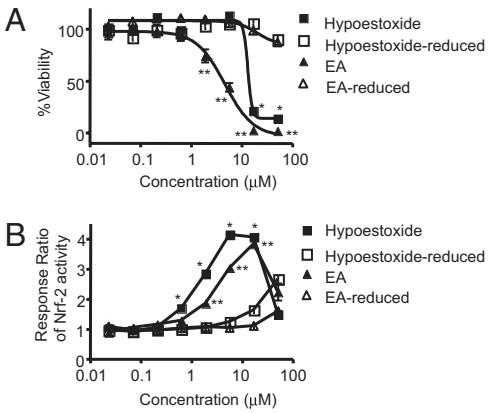

Fig. 2. Necessity of the α-β unsaturated carbonyl group for ARE activity and CLL toxicity. (*A*) Cytotoxicity of EA and hypoestoxide and their reduced analogues on CLL PBMCs. CLL PBMCs were incubated with compounds for 48 h and viability was tested with MTT assay. Data shown are representative of the three independent experiments using PBMCs from three patients with CLL. Asterisks denote $P < 0.001$ vs. corresponding reduced drugs by two-way ANOVA with Bonferroni post hoc testing. (*B*) Nrf2 activity measured by ARE assay. Induction of Nrf2 activity was measured by β-lactamase reporter assay (GeneBlazer) in ARE-bla HepG2 cells. The cells were treated with compounds for 16 h and the assay was performed according to the manufacturer's protocol. The response ratios were plotted against various concentrations of the drugs. Asterisks denote $P < 0.001$ vs. corresponding reduced drugs by two-way ANOVA with Bonferroni post hoc testing.

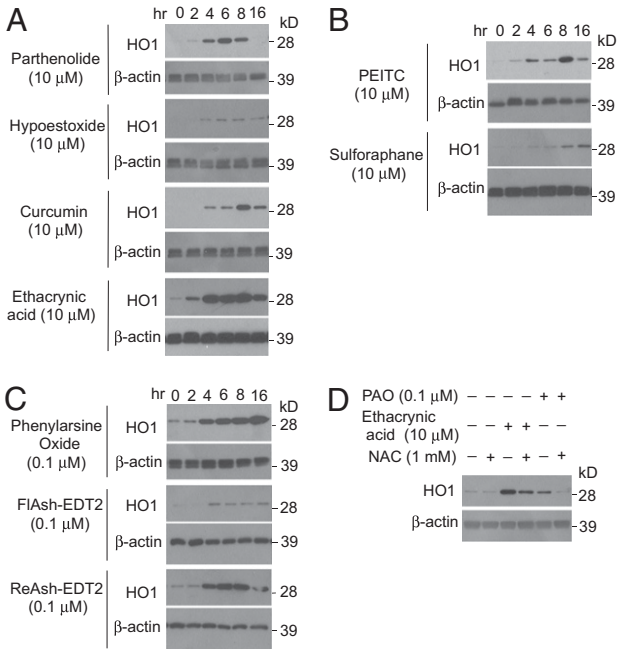

Fig. 3. HO-1 protein induction in PBMCs is a response to oxidative stress. (*A–C*) Normal PBMCs were incubated with selected compounds: α-β unsaturated carbonyl (*A*), isothiocyanates (*B*), and sulfhydryl reactive metals (*C*) at indicated concentrations. The incubation was terminated at different time points. (*D*) Normal PBMCs were treated with 10 μM of EA and 0.1 μM of PAO together with 1 mM NAC for 6 h. The cells were lysed at the end of the treatments. The lysates were separated on a SDS/PAGE gel and immunoblot analysis was performed with anti–HO-1 antibody. Anti–β-actin antibody was used for internal control.

cell-based reporter assay. Except for the very potent sulfhydryl reactive metals, we tested compounds at 10 μM, an effective concentration for most active compounds in the Nrf2 reporter assay. Similarly, we chose 0.1 μM for PAO, FlAsh-EDT_2, and ReAsh-EDT_2 as an empirically found active concentration for this group of compounds in the cell-based assay. PBMCs from a healthy donor were treated with these agents and HO-1 protein levels were measured at various time points by immunoblotting. The results show that most of these compounds are active in normal PBMCs. We found HO-1 induction with α-β unsaturated carbonyl–containing compounds, isothiocyanates, and sulfhydryl reactive metals in PBMCs. However, the magnitude of HO-1 induction did not correlate with Nrf2 activation potency in the reporter assay. For example, although hypoestoxide was more potent in the Nrf2 reporter assay, it induced HO-1 protein in PBMCs with less magnitude than the other α-β unsaturated carbonyl–containing compounds. Conversely, EA induced HO-1 protein expression in PBMCs with the greatest magnitude even though it had only moderate potency in the Nrf2 reporter assay. The complex relationship between the reporter assay and an Nrf2 target protein expression confirms the need to assay this signaling pathway using the primary cells that are the proposed target of drug treatment.

Induction of HO-1 in PBMC Is Reduced with N-Acetylcysteine Cotreatment. The induction of HO-1 in PBMCs observed with various electrophilic and antioxidant compounds may be attributable to changes in ROS production and the redox environment, and/or to direct thiol modification of Keap1 and other proteins (26). To better characterize HO-1 induction, we treated normal PBMCs with high concentrations of N-acetylcysteine (NAC) along with EA and PAO (Fig. 3*D*). The NAC cotreatment partially inhibited EA-induced HO-1 protein expression, but fully inhibited the lower-magnitude PAO-induced HO-1 expression. Presumably, reaction of PAO with a large excess of NAC results in irreversible formation of the thiol-PAO product, leaving no PAO available for interaction with the Keap1 thiols. In contrast, Michael addition of NAC to EA results in formation of a reversible adduct that may allow for a limited amount of free EA to be available for interaction with Keap1. This could explain the observation that HO-1 induction is abrogated by cotreatment of cells with PAO and NAC, but only partially abrogated by EA/NAC cotreatment.

Selective Induction of HO-1 in Normal PBMCs. Nrf2 signaling is the normal cellular response to oxidative damage and induces protective antioxidant genes and phase II metabolizing enzymes (12). Nrf2 signaling can protect cells from oxidant-induced cell death, whereas disruption of Nrf2 increases sensitivity to acetaminophen-induced hepatotoxicity (27). Because previous studies show that compounds that induce oxidative stress are selectively cytotoxic to CLL cells, we compared Nrf2 induction in CLL cells and normal PBMCs. Using the strongest inducers of HO-1 in PBMC (parthenolide, EA, curcumin, and phenylarsine oxide), we compared their ability to induce HO-1 protein expression in fresh PBMCs from subjects with CLL and normal donors. The results confirmed that the basal level of Nrf2 activity, as measured by HO-1 protein expression, is lower in normal PBMCs compared with CLL PBMCs (Fig. 4). Parthenolide induced HO-1 protein in normal PBMCs in a dose-dependent manner (Fig. 4*A*). In CLL PBMCs, HO-1 protein induction by parthenolide was dramatically reduced. Similarly, we observed greater induction of HO-1 expression in normal PBMCs treated with EA compared with CLL PBMCs (Fig. 4*B*). However, treatment with curcumin and PAO induced HO-1 expression in normal PBMCs only at the highest dose and had minimal effect on CLL PBMCs (Fig. 4 *C* and *D*). Overall, the data are consistent with the general observation that normal PBMCs have a more robust Nrf2 response to electrophilic agents, presumably as a result of their lower levels of Keap1.

EA Binds to Keap1. Based on our observation of incomplete inhibition of EA-induced Nrf2 signaling with NAC cotreatment

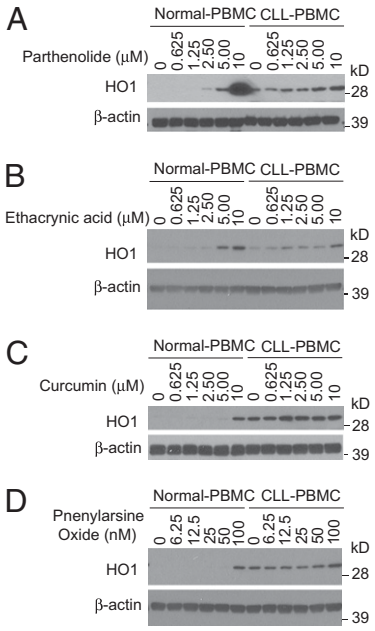

Fig. 4. HO-1 protein induction in normal and CLL PBMCs. Dose response of HO-1 induction by parthenolide (*A*), EA (*B*), curcumin (*C*), and PAO (*D*) in CLL and normal PBMCs. Freshly isolated cells were treated with 10, 5, 2.5, 1.25, and 0.625 μM of parthenolide, EA, and curcumin and 100, 50, 25, 12.5, and 6.25 nM of PAO. DMSO was used as the solvent control. The cells were treated for 4 h with parthenolide, EA, and PAO, and for 8 h with curcumin. The lysates were separated on an SDS/PAGE gel and Western blot analysis was performed with indicated antibodies. Anti–β-actin antibody was used as an internal control.

and the previously reported chemistry of α-β unsaturated carbonyl groups (17, 28), we hypothesized that compounds containing α-β unsaturated carbonyl groups inactivate Keap1 through a Michael addition reaction. Keap1 is rich in cysteine residues, which are susceptible to thiol modification by electrophiles. To test this prediction, we tested for EA binding of Keap1 with a pull down assay. Following treatment with biotinylated EA, CLL PBMCs were lysed and mixed with streptavidin beads. The pull-down complex was separated on a SDS/PAGE gel and immunoblotted for the presence of Keap1. The results show that Keap1 protein was pulled down by biotin-EA in a dose-dependent manner (Fig. 5), indicating that EA directly interacts with Keap1 to activate the Nrf2 pathway in CLL cells.

Discussion

Redox-active small molecules have been proposed as potential treatments for CLL based on their selective in vitro cytotoxicity toward the malignant B lymphocytes compared with normal PBMCs. The molecular mechanism underlying this selectivity is unclear. In this work, we focused on Nrf2 signaling as a possible mechanism because the Keap1-Nrf2-ARE pathway is known to control the oxidative stress response. Although CLL cells were known to have high intracellular ROS levels, to our knowledge this is the first report of Nrf2 signaling in primary human CLL. The Nrf2 pathway is intact in CLL cells and constitutively up-regulated in response to these high ROS levels. Additionally, the negative regulator of Nrf2, Keap1, was also elevated, indicating that this is the chronic state of CLL cells. Notably, normal B cells were also found to have up-regulated Nrf2 signaling, potentially indicating that this is a preserved phenotype from the normal progenitor cell as opposed to a malignant dysregulation. Regardless, based on the success of current therapies that target mature B cells, this finding does not alter the clinical potential of this approach.

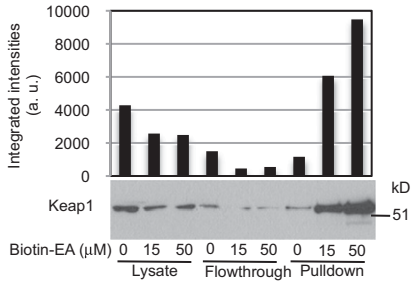

Fig. 5. Binding of EA to Keap1. CLL PBMCs were treated with biotinylated EA for 4 h. The cells were lysed and equal concentration of total proteins was incubated with streptavidin beads. The lysate, flow-through, and pull-down were collected and separated on a SDS/PAGE gel and immunoblot analysis was performed with Keap1 antibody. The band intensities were quantitated with ImageJ software (NIH).

The small molecules examined in this work were selected for study based on their electrophilic nature, as in the case of the α-β unsaturated carbonyl–containing compounds, or based on their potential chemical or enzymatic conversion to electrophiles, as in the case of the flavonoids and phenolic compounds. The mechanism of Nrf2 activation by these different classes of compounds may differ but ultimately result in the disruption of the Keap1–Nrf2 complex, most likely through interaction with the thiols present on Keap1. This interaction involves thiol modification either by direct alkylation by the electrophilic compound or by oxidation to the disulfide form. Excluding other unlikely mechanisms such as phosphorylation of Nrf2, compounds in this study that were capable of activating Nrf2 were those that can modify the Keap1 thiols or be converted to compounds capable of such modifications. Some phenols are good activators of Nrf2 because they can undergo a two-step process in which they are first oxidized under physiological conditions (biochemically or enzymatically) to a 1,2- or 1,4-quinoid structure that is then capable of Keap1 thiol modification (23). A good example of this in the present study is tert-butylhydroquinone (TBHQ). This potent diphenolic activator must first be oxidized to the corresponding quinone before it can effect covalent thiol modification and subsequent Nrf2 induction. Other polyphenols and flavones follow the same pattern (29). Good electron donors become good electrophilic compounds and the relative potency of activation by different classes of inducers has been shown to be correlated with their ability to release an electron (23). In fact, the pro-oxidant activity of the inducers after donating electrons may be of greater importance in Nrf2 induction than any direct antioxidant effect derived from simple free radical scavenging properties. Indeed, up-regulation of hepatic Nrf2 is more important for detoxification and elimination of electrophiles than ROS (30).

Given Nrf2 pathway up-regulation in CLL, we tested these electrophilic and antioxidant drug-like compounds for both Nrf2 activation and CLL selective cytotoxicity. Compounds containing α-β unsaturated carbonyl groups robustly activated Nrf2 signaling in a reporter system and normal PBMCs whereas they had a less potent effect in CLL cells. Additionally, they were found to selectively kill CLL cells. Based on known chemistry and our prior work, we tested the requirement for the α-β unsaturation by using both reduced versions of active compounds and alternative carbonyl compounds with saturated α-β carbons and confirmed this requirement. To further characterize this activity, cotreatment with high concentrations of NAC was studied. Although this treatment completely abrogated the activity of a much more potent compound, PAO, it only partially inhibited EA activation of Nrf2 signaling. Possibly, the difference is caused by the formation of irreversible thiol-PAO products in contrast to reversible EA-NAC adducts that may allow for a limited amount of

free EA to be available for interaction with Keap1. A direct interaction between EA and Keap1 was hypothesized, in part, based on this finding, and subsequently confirmed by pull-down assay.

Collectively, our results demonstrate that electrophilic compounds with CLL-selective cytotoxicity can interact with redox-sensitive proteins in primary CLL cells, resulting in induction of Nrf2 signaling. Therefore, future efforts to develop electrophilic and antioxidant compounds as treatments for CLL should examine known, and seek to identify additional, redox-sensitive pathways to optimize the compounds to be used and to identify predictive and responsive biomarkers.

Materials and Methods

PBMC Isolation and B Cell Enrichment. Blood was collected from consenting subjects with CLL at the Moores Cancer Center at the University of California San Diego with the approval of the University of California San Diego Institutional Review Board. Normal PBMCs and B cells were isolated from anonymous samples from the San Diego Blood Bank. PBMCs were isolated by Ficoll-density gradient centrifugation and resuspended in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. B cells were enriched using RosetteSep Human B cell enrichment antibody mixtures according to manufacturer's instructions (Stemcell Technologies).

qPCR. After treatment, total RNA was isolated with PureLink RNA mini kit (Invitrogen). The mRNA was converted to cDNA with SuperScript II RNaseH-Reverse Transcriptase first strand synthesis kit (Invitrogen). The resulting cDNA was amplified by Taqman Universal Master Mix (Applied Biosystems) for GAPDH, Nrf-2, and HO-1 (see *SI Materials and Methods* for details). The qPCR results were analyzed by the $2^{\Delta\Delta CT}$ method (31).

Cell-Based Assays to Measure ARE Activities and Cytotoxicity. The GeneBlazer assay for ARE activity was measured after 16-h compound treatments in ARE-bla HepG2 cells according to the manufacturer's instructions (Invitrogen). For cytotoxicity, the MTT assay was performed after 48-h compound treatments as previously described (32). (See *SI Materials and Methods* for details.)

Immunoblotting. Cells were lysed on ice with Phosphosafe Extraction buffer (EMD Biosciences) containing protease inhibitors for 10 min. The lysate was denatured by boiling in NuPAGE sample buffer (Invitrogen) supplemented with 1 mM DTT. After separating the proteins in SDS/PAGE and transferring the proteins onto PVDF membranes, immunoblot analysis was performed with specific antibodies (*SI Materials and Methods*) and developed by ECL (Pierce Biotechnology).

Streptavidin Pull-Down Assay. Biotin was conjugated to EA using EZ-Link Biotin-LC-Hydrazide (Thermo Scientific) and isolated by HPLC. The cells were treated with biotinylated EA at 15 and 50 μM for 4 h. At the end of the treatments, the cells were lysed in Phosphosafe Extraction Buffer and EA-bound proteins were pulled down by Streptavidin beads (Thermo Scientific). The beads were boiled in 4× NuPAGE Sample Buffer (Invitrogen) supplemented with 1 mM DTT (see *SI Materials and Methods* for details).

Statistics. All statistical analyses were performed with Prism software (Graphpad).

ACKNOWLEDGMENTS. The authors thank Charlene Gutierrez, Laura Rassenti, and Thomas J. Kipps for providing CLL samples; and Stephen Adams and Roger Tsien for providing FlAsh-EDT_2 and ReAsh-EDT_2. We also appreciate Nancy Noon for help with preparing this manuscript. This study was supported by the Leukemia and Lymphoma Society and National Institutes of Health Grants CA113318 and AR007608.

1. Rassenti LZ, et al. (2008) Relative value of ZAP-70, CD38, and immunoglobulin mutation status in predicting aggressive disease in chronic lymphocytic leukemia. *Blood* 112:1923–1930.
2. Hallek M, et al. (2008) Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood* 111: 5446–5456.
3. Zhou Y, Hileman EO, Plunkett W, Keating MJ, Huang P (2003) Free radical stress in chronic lymphocytic leukemia cells and its role in cellular sensitivity to ROS-generating anticancer agents. *Blood* 101:4098–4104.
4. Trachootham D, et al. (2008) Effective elimination of fludarabine-resistant CLL cells by PEITC through a redox-mediated mechanism. *Blood* 112:1912–1922.
5. Hileman EO, Liu J, Albitar M, Keating MJ, Huang P (2004) Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. *Cancer Chemother Pharmacol* 53:209–219.
6. Valko M, et al. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39:44–84.
7. Trachootham D, et al. (2006) Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. *Cancer Cell* 10:241–252.
8. Trachootham D, Alexandre J, Huang P (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discov* 8:579–591.
9. Chandra J, Samali A, Orrenius S (2000) Triggering and modulation of apoptosis by oxidative stress. *Free Radic Biol Med* 29:323–333.
10. Halliwell B (2007) Oxidative stress and cancer: have we moved forward? *Biochem J* 401:1–11.
11. Trachootham D, Lu W, Ogasawara MA, Nilsa RD, Huang P (2008) Redox regulation of cell survival. *Antioxid Redox Signal* 10:1343–1374.
12. Kensler TW, Wakabayashi N, Biswal S (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* 47:89–116.
13. Nguyen T, Nioi P, Pickett CB (2009) The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem* 284:13291–13295.
14. Itoh K, et al. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 236:313–322.
15. Kobayashi A, et al. (2006) Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Mol Cell Biol* 26:221–229.
16. Zhang DD, Hannink M (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol Cell Biol* 23:8137–8151.
17. Dinkova-Kostova AT, et al. (2002) Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci USA* 99:11908–11913.
18. Wakabayashi N, et al. (2004) Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc Natl Acad Sci USA* 101:2040–2045.
19. Steele AJ, et al. (2006) The sesquiterpene lactone parthenolide induces selective apoptosis of B-chronic lymphocytic leukemia cells in vitro. *Leukemia* 20:1073–1079.
20. Merkel O, et al. (2008) Arsenic trioxide induces apoptosis preferentially in B-CLL cells of patients with unfavourable prognostic factors including del17p13. *J Mol Med* 86: 541–552.
21. Surh YJ (2003) Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 3:768–780.
22. Jin G, et al. (2009) Amide derivatives of ethacrynic acid: synthesis and evaluation as antagonists of Wnt/beta-catenin signaling and CLL cell survival. *Bioorg Med Chem Lett* 19:606–609.
23. Bensasson RV, Zoete V, Dinkova-Kostova AT, Talalay P (2008) Two-step mechanism of induction of the gene expression of a prototypic cancer-protective enzyme by diphenols. *Chem Res Toxicol* 21:805–812.
24. Zlokarnik G, et al. (1998) Quantitation of transcription and clonal selection of single living cells with beta-lactamase as reporter. *Science* 279:84–88.
25. Alam J, et al. (1999) Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J Biol Chem* 274:26071–26078.
26. Motohashi H, Yamamoto M (2004) Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol Med* 10:549–557.
27. Chan K, Han XD, Kan YW (2001) An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proc Natl Acad Sci USA* 98:4611–4616.
28. Dinkova-Kostova AT, Massiah MA, Bozak RE, Hicks RJ, Talalay P (2001) Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proc Natl Acad Sci USA* 98: 3404–3409.
29. Lee-Hilz YY, et al. (2006) Pro-oxidant activity of flavonoids induces EpRE-mediated gene expression. *Chem Res Toxicol* 19:1499–1505.
30. Reisman SA, Yeager RL, Yamamoto M, Klaassen CD (2009) Increased Nrf2 activation in livers from Keap1-knockdown mice increases expression of cytoprotective genes that detoxify electrophiles more than those that detoxify reactive oxygen species. *Toxicol Sci* 108:35–47.
31. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.
32. LU D, et al. (2009) Ethacrynic acid exhibits selective toxicity to chronic lymphocytic leukemia cells by inhibition of the Wnt/b-catenin pathway. *PLoS One* 4:e8294.