Celastrol and an EGCG pro-drug exhibit potent chemosensitizing activity in human leukemia cells

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Abstract. Chemotherapy remains the staple of treatment for many types of leukemia. Despite the positive impact on extending overall survival in patients with hematological malignancies, new treatment strategies are needed to reduce the nonspecific toxicity and improve the efficacy of treatment. Celastrol, derived from the 'Thunder God Vine' and Pro-EGCG, a pre-drug version of green tea polyphenol EGCG have shown potent biological activity *in vitro* and *in vivo*. Whether these natural products augment the efficacy of conventional chemotherapy in the treatment of leukemia cells has yet to be demonstrated. Here we demonstrate that these natural products could sensitize the effect of chemotherapy in both K-562 and Jurkat T human leukemia cells. Accordingly, this potent biological activity was associated with increased levels of leukemia cell killing, caspase 3 activation, and poly(ADP-ribose) polymerase cleavage. Furthermore, the higher levels of apoptotic indices were associated with decreased levels of Bcr-Abl oncoprotein in K-562 cells. Taken together, our findings present a compelling rationale for the development of combination strategies using natural products in the treatment of hematological malignancies.

Introduction

Chemotherapy and radiation remain at the forefront of treatment options for many patients afflicted with hematological malignancies. Daunorubicin, derived from the anthracycline family is a commonly used cytotoxic agent in the treatment of various cancers including leukemia (1). It has been widely reported that Daunorubicin exerts its activity by preventing DNA replication and inhibiting transcription (2). Cytarabine, otherwise known as Ara-C, is a widely employed chemotherapeutic agent for the treatment of various types of leukemia (3). One mode of action of Ara-C is due to its rapid conversion to triphosphate derivative (Ara-CTP), which incorporates into DNA resulting in DNA chain termination and S phase arrest. Other modes of action consist of inhibition of DNA and RNA polymerases required for DNA synthesis (4). Despite the positive clinical impact of Ara-C, the collateral damage to normal cells and acquisition of drug resistance are limiting factors that impede a favorable clinical outcome (5). Therefore, new treatment strategies are warranted that can reduce non-specific toxicity, overcome drug resistance and improve the overall efficacy of treatment.

Epidemiological studies have indicated that green tea consumption is associated with cancer-preventative effects (6,7). Among the polyphenols of green tea, (-)-EGCG is the most abundant and biologically active constituent with respect to anticancer activity in several types of human cancer (8,9). Various mechanisms underlying its anticancer effects have been described, including proteasome inhibition (10). However, (-)-EGCG is relatively unstable under neutral or alkaline conditions. In an attempt to enhance the stability of (-)-EGCG, peracetate-protecting groups were introduced to the reactive hydroxyls to form Pro-EGCG (11).

Celastrol is a natural compound extracted from *Tripterygium wilfordii Hook F* and has been used for medicinal purposes in China for hundreds of years (12). Due to its anti-inflammatory effects, Celastrol has been effectively used in the treatment of various diseases (13,14). Within physiological concentrations, Celastrol inhibits cancer cell proliferation and induces cell death in a broad range of cancer lines including, leukemia cells (15,16). Additionally our laboratory has reported that proteasome inhibition induced by Celastrol is one mechanism responsible for its potent biological activity (17).

In this study we report that Celastrol and Pro-EGCG can sensitize two different leukemia cell lines to conventional chemotherapy. K-562 and Jurkat T leukemia cells showed increase cell killing activity when incubated with both natural compound (Celastrol or Pro-EGCG) and chemotherapy compared to natural compound or chemotherapy alone.

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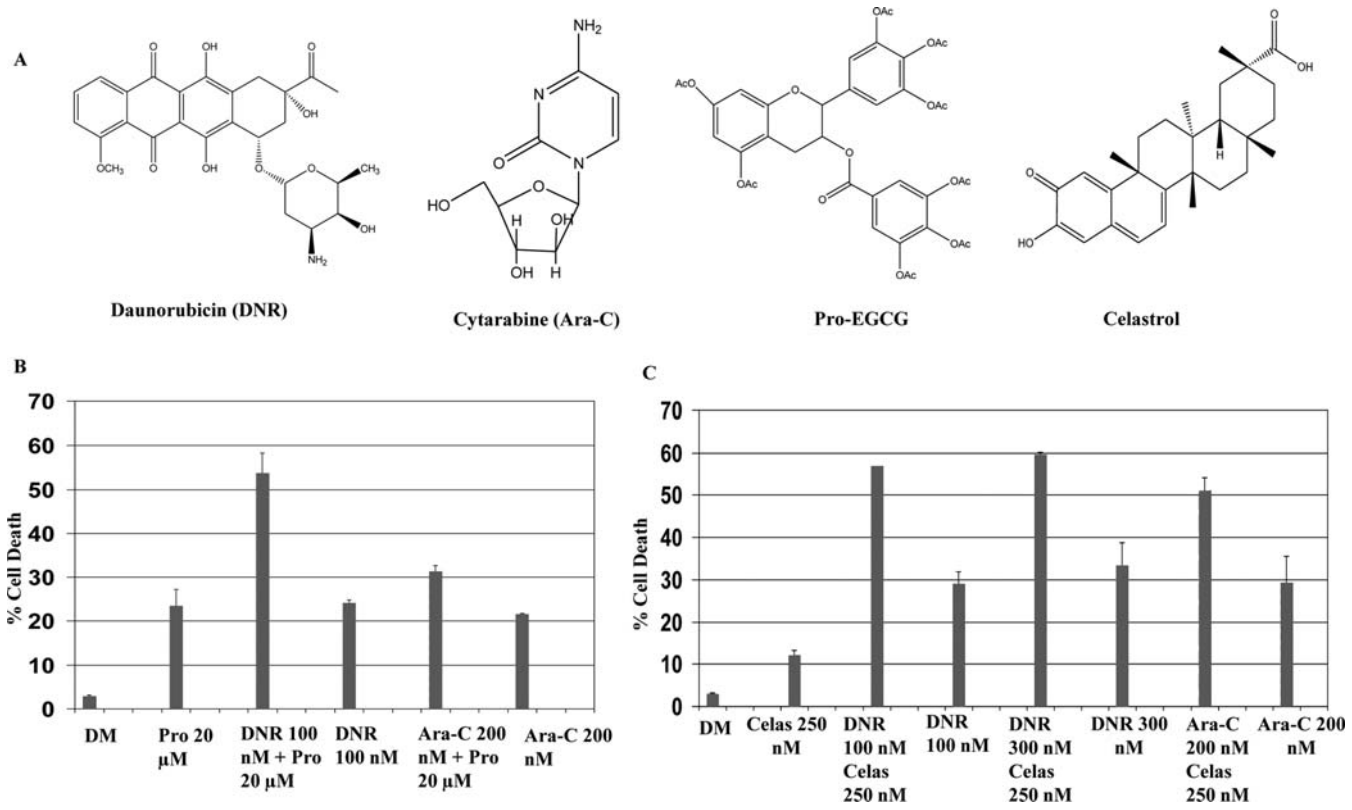

Figure 1. Cytotoxicity profile of natural products as chemosensitizing agents in K-562 cells. (A) Chemical structures of chemotherapeutic agents Daunorubicin (DNR) and cytarabine (Ara-C), and natural products Pro-EGCG (Pro) and Celastrol (Celas). (B) K-562 cells were treated with either 20 μM Pro-EGCG (Pro), 100 nM DNR, 200 nM Ara-C alone, or in combination for 72 h, followed by trypan blue exclusion assay. (C) K-562 cells were treated with 250 nM Celastrol (Celas), 100 nM DNR, 200 nM Ara-C alone, or in combination for 72 h, followed by trypan blue exclusion assay. The numbers given are percentages of nonviable cells to total cells.

Consistent with increased cell killing, higher levels of caspase 3 activity and PARP cleavage and decreased levels of Bcr-abl protein (in K-562 cells) were observed when treated in combination. Our results show that natural compounds have the ability to sensitize human leukemia cells to chemotherapy and provide evidence for the rational design of new combination treatments with less toxic agents, such as natural products in the treatment of hematological malignancies.

Materials and methods

Materials. Human Jurkat (T-cell leukemia) and K-562 (CML at blast crisis) cells were purchased from ATCC (Manassas, VA). Purified Celastrol (>98%) was purchased from Calbiochem, Inc., (San Diego, CA). Synthesis of Pro-EGCG from (-)-EGCG was done as previously described (11,18). Daunorubicin, Cytarabine (Ara-C), Dimethylsulfoxide (DMSO) and trypan blue dye were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Aleken Biologicals (Texarkana, AK). Mouse monoclonal antibody against PARP was from Biomol International (Plymouth Meeting, PA). Mouse monoclonal antibody to Abl, and rabbit polyclonal to Bcr were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse and anti-goat secondary antibodies were from BioRad (Hercules, CA). HyGLO chemiluminescent reagent was purchased from Denville Scientific (Metuchen, NJ).

Cell cultures and whole cell extract preparation. Jurkat T and K-562 cells were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum and penicillin/streptomycin, and maintained in a humidified incubator at 37˚C and 5% CO_2. A whole-cell extract was prepared as previously described (19).

Trypan blue assay. The trypan blue dye exclusion assay was performed by mixing 100 μl of cell suspension with 50 μl trypan blue dye before injecting into a hemocytometer and counting. The number of cells that absorbed the dye and those that excluded the dye were counted, from which the percentage of nonviable cell number to total cell number was calculated.

Caspase 3 activity assay. Leukemia cells were treated with each drug or combination at indicated concentrations for 72 h, harvested and lysed. The substrate Ac-DEVD-AMC (40 μmol/l) was then incubated with the prepared cell lysates for 24 h, followed by measurement of caspase 3 activity as previously described (20).

Western blot analysis. Human leukemia cells were treated, harvested, and lysed. Cell lysates (40-50 μg) were separated

by SDS-PAGE and transferred to a nitrocellulose membrane, followed by visualization using the HyGLO reagent (Denville Scientific; Metuchen, NJ).

Results

Celastrol and Pro-EGCG sensitize K-562 human leukemic cells to chemotherapy. Celastrol and Pro-EGCG (Fig. 1A) have been shown to possess potent antitumor activity *in vitro* and *in vivo*, including growth suppression and apoptosis induction (17,21). Whether these non-toxic agents sensitize leukemia cells to chemotherapy has yet to be reported. These natural compounds were first tested for their ability to enhance the activity of chemotherapeutic agents, Ara-C and Daunorubicin (Fig. 1A) toward human leukemia cells by measuring cell death induction in a trypan blue assay. The procedure was undertaken by treating K-562 cells with natural compounds, clinically relevant doses of Ara-C or Daunorubicin, or combination of natural compound and chemotherapy for 72 h, followed by measurement of non-viable cells. When 20 μM Pro-EGCG was combined with 100 nM Daunorubicin, ~53% cell death was achieved (Fig. 1B). This observation is significant since only 22 and 23% cell death was achieved when incubated with Pro-EGCG or Danuorubicin alone under the same experimental conditions (Fig. 1B). Additionally, when 20 μM Pro-EGCG was combined with 200 nM Ara-C, cell death induction was increased to ~31% compared to 22 and 21% when either of these agents was used alone (Fig. 1B). It is notable to point that when the unprotected parent compound (-)-EGCG was used no significant cell death was observed either alone or in combination with chemotherapy (data not shown), which was probably due to its low bioavailability.

To test whether the natural compound Celastrol could sensitize K-562 cells to chemotherapy, a similar approach was taken by measuring cell death in a trypan blue exclusion assay. Interestingly, we found when 250 nM Celastrol was combined with 100 nM Daunorubicin, ~ 57% cell death was achieved (Fig. 1C). This result was significant, since cells treated with either Celastrol or Daunorubicin alone was only able to impart ~12 and 28% cell death, respectively (Fig. 1C). Additionally when increasing the concentration of Daunorubicin to 300 nM and using the same dose of Celastrol, cell death was increased to ~60% in combination, while Daunorubicin treatment alone induced ~32% cell death (Fig. 1C). When investigating the sensitizing effect of 250 nM Celastrol with 200 nM Ara-C, ~51% cell death was achieved compared to 29% cell death when Ara-C was treated alone (Fig. 1C). These results clearly show that natural compounds Celastrol and Pro-EGCG are able to sensitize leukemic cells to chemotherapy resulting in increase cytotoxicity. However, whether these interesting results illustrate an additive or synergistic effect is rather speculative at this point and premature.

Chemosensitization by natural compounds is associated with apoptosis induction and decreased Bcr-Abl expression in K-562 human leukemic cells. We next set out to investigate whether apoptosis is responsible for chemosensitization by these natural compounds by measuring caspase 3 activity, apoptosis-specific PARP cleavage and Bcr-Abl protein expression. Caspase 3 is a critical determinant in the initiation of apoptosis and is associated with the cleavage of many critical

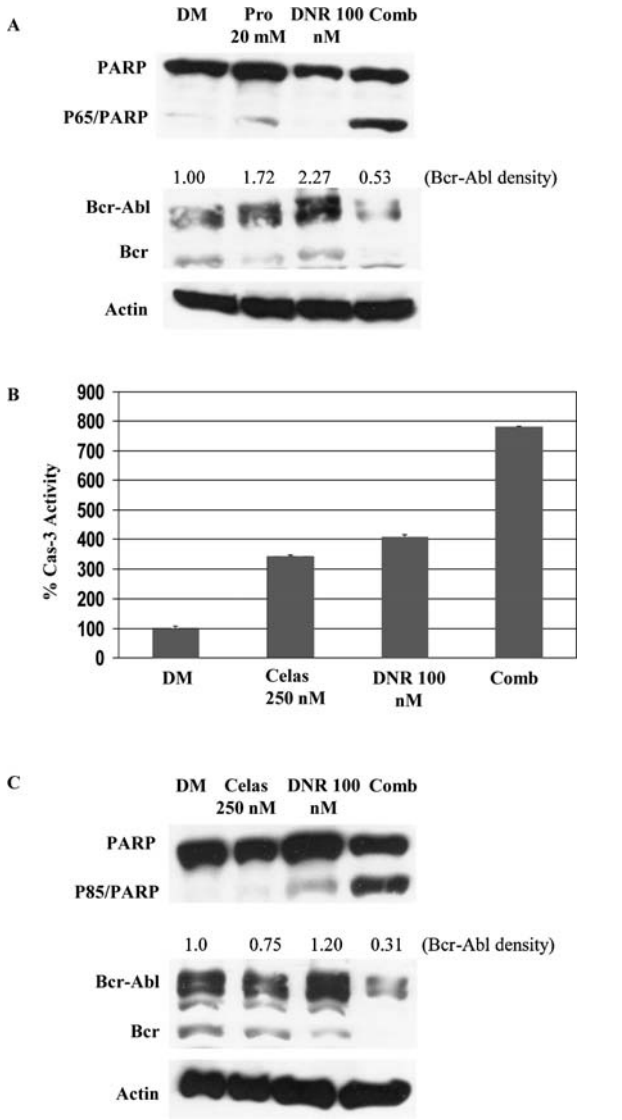

Figure 2. Induction of K-562 cell apoptosis by combination treatment of natural compounds and chemotherapy. (A) K-562 cells were treated with either DMSO control, Pro-EGCG (Pro) at 20 μM, Daunorubicin (DNR) at 100 nM, or combination for 72 h, followed by measurement of PARP cleavage and expression of Bcr-Abl protein levels. (B-C) K-562 cells were treated with either DMSO control, Celastrol (Celas) at 250 nM, Daunorubicin (DNR) at 100 nM or in combination, followed by measurement of caspase 3 activity (B) and PARP cleavage and expression levels of Bcr-Abl protein in Western blot analysis (C). Actin was used as a loading control.

cellular substrates, including poly(ADP-ribose) polymerase or PARP (22). K-562 cells were first treated with either 20 μM Pro-EGCG, 100 nM Daunorubicin, or combination of the two for 72 h, and their cell lysates were measured for caspase 3 activity and PARP cleavage. Our results show when both agents were used in combination, caspase 3 activity increased more significantly compared to each treatment alone and DMSO solvent control (data not shown). Consistent with higher levels of caspase 3 activity in co-treated cells, significantly higher levels of PARP cleavage was observed, compared to either agent treated alone (Fig. 2A). In another experiment, combining 250 nM celastrol with 100 nM Daunorubicin elicited 7.8-fold increase in caspase 3 activity, compared to only 3.4- or 4.0-fold increase for either treatment alone (Fig. 2B). This notable increase in caspase 3 activity

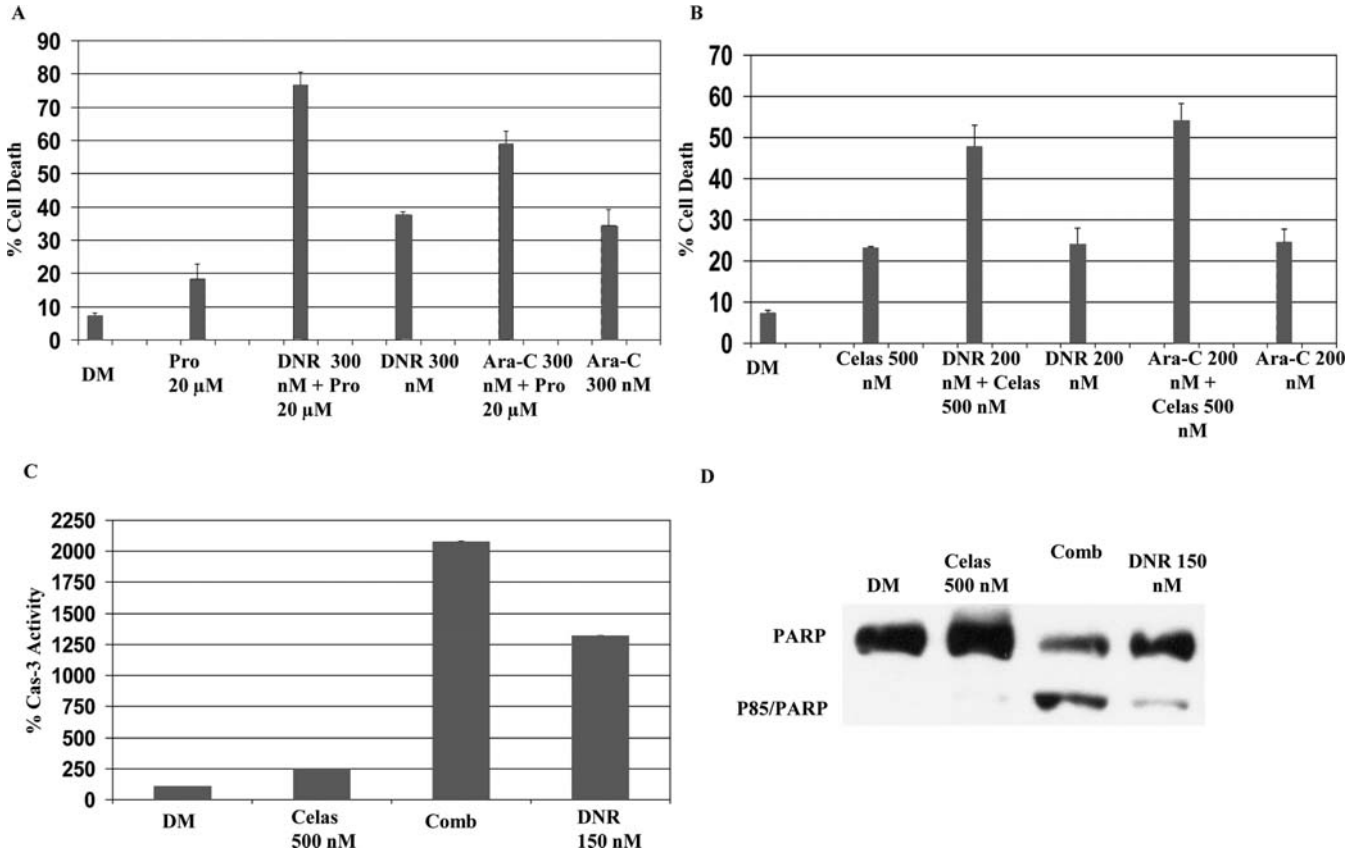

Figure 3. Combination treatment of natural compounds and chemotherapy was associated with higher cell killing and indices of apoptosis in Jurkat T cells. (A) Jurkat T cells were treated with either DMSO control, Pro-EGCG (Pro) at 20 μM, DNR at 300 nM or Ara-C at 300 nM, or combination for 72 h, followed by trypan blue exclusion assay. (B) Jurkat T cells were treated with Celastrol (Celas) at 500 nM, DNR at 200 nM, Ara-C at 200 nM or in combination for 72 h, followed by trypan blue assay. The numbers given are percentages of nonviable cells to total cells. (C-D) Jurkat T cells were treated with either Celastrol (Celas) at 500 nM, Daunorubicin at 150 nM, or in combination for 72 h, followed by measurement of caspase 3 activity (C) and PARP cleavage by Western blot analysis (D). Actin was used as loading control.

from the combination treatment was accompanied by significant levels of PARP cleavage (Fig. 2C). We next tested whether the higher apoptosis indices found in combination treatment is associated with decreased levels of Bcr-Abl protein in CML-derived K-562 cells. This molecular abnormality functions as a constitutively active receptor tyrosine kinase and represents the underlying pathologic event in the formation of CML (23). Interestingly, when K-562 cells were co-treated with Pro-EGCG and Daunorubicin, we found ~50% decrease in Bcr-Abl protein levels (Fig. 2A). Additionally, when cells were treated with combination of Celastol and Daunorubicin, we found ~70% decrease in Bcr-abl protein levels (Fig. 2C). These results show that chemosensitization of K-562 cells by natural compounds is associated with decreased levels of Bcr-Abl protein.

Chemosensitization of human Jurkat T leukemic cells by natural compounds is associated with cell killing and apoptosis induction. After we showed the ability of natural compounds to sensitize CML-derived K-562 cells to chemotherapy, we next investigated whether this effect is consistent against human Jurkat T cells. We first treated Jurkat T cells with 20 μM Pro-EGCG, 300 nM Daunorubicin or Ara-C, or in combination for 72 h, followed by measurement of nonviable cells. Our results show that treatment with Pro-EGCG alone only induced cell killing by ~18%, but when combined with Daunorubicin, cytotoxicity reached ~76% (Fig. 3A). In comparison, treatment with Daunorubicin alone only reached ~38% cell death (Fig. 3A). Additionally, when Jurkat cells were treated in combination with Pro-EGCG and Ara-c, cell death reached ~59% compared to only ~33% cell death when treated with Ara-C alone (Fig. 3A).

We next tested the chemosensitizing effect of Celastrol toward chemotherapy in Jurkat T cells by measuring the number of nonviable cells. Cells were treated with Celastrol at 500 nM with or without Daunorubicin or Ara-C at 200 nM for 72 h, followed by the measurement of nonviable cells. Our results show that when Celastrol or Daunorubicin were used as single agents, cell killing only reached ~23 and 24%, respectively (Fig. 3B). However, in combination treatment, their ability to induce cell death reached ~47% (Fig. 3B). Similarly, when Celastrol was used in combination with Ara-C, cell death was significantly increased to ~53% (Fig 3B). In comparison, when Celastrol and Ara-C were used singularly, cytotoxicity only reached ~23 and 24%, respectively (Fig. 3B).

To determine whether this enhanced cell killing by combination treatment is associated with apoptosis, caspase 3 activity and apoptosis-specific PARP cleavage was measured. Jurkat cells were treated with either 500 nM Celastrol, 150 nM Daunorubicin, or combination followed by the measurement

of caspase 3 activity and PARP cleavage. Our results show that treatment with Celastrol and Daunorubicin alone induced ~2.5- and 13-fold caspase 3 activity, respectively, compared to DMSO control (Fig. 3C). However, when both agents were treated in combination, caspase activity increased by ~21-fold (Fig. 3C). In addition to the significantly higher levels of caspase 3 levels, apoptosis-specific PARP cleavage was much more apparent when both agents were treated in combination (Fig. 3D). As demonstrated with K-562 cells, Pro-EGCG and Celastrol have significant ability to sensitize Jurkat T cells to conventional chemotherapy, as illustrated by their potent cell killing activity leading to higher levels of caspase 3 activities and PARP cleavage.

Discussion

The use of chemotherapy remains one of the important approaches among cancer therapies. Unfortunately, in many malignancies, acquisition of drug resistance and toxicity during chemotherapy constitutes a major hurdle and challenge in cancer therapy (24). Therefore, combination strategies that include natural compounds that sensitize tumor cells to chemotherapy may represent a more promising approach to cancer therapy. Emerging evidence has shown that some natural compounds, including Pro-EGCG and Celastrol have potent growth inhibitory and apoptosis-inducing effects on cultured human cancer cells and xenografts (17,21). Therefore, these non-toxic agents derived from natural resources could be useful in combination with conventional chemotherapeutic agents for the treatment of human malignancies, including leukemia. In fact, increasing evidence from pre-clinical *in vitro* and *in vivo* studies have shown some success in support of conventional chemotherapeutic agents in combination with natural products (25,26).

Our laboratory has previously shown that both Pro-EGCG and Celastrol exhibit potent growth suppression and apoptosis inducing effects, and that the proteasome is a critical target underlying their biological effects (17,21). However, whether these natural compounds could sensitize the effect of conventional chemotherapy in human leukemia cells has yet to be demonstrated. In this study, we investigated the chemosensitivity of Pro-EGCG and Celastrol toward two human leukemia cell lines, namely CML-derived K-562 cells and Jurkat T-cell leukemia cells. Interestingly, we found that both Celastrol and Pro-EGCG were able to sensitize both leukemia cell lines to the effects of chemotherapy, resulting in a significant increase in cell killing (Figs. 1-3). Our data also show that both natural compounds are much more effective in sensitizing leukemia cells to Daunorubicin compared to Ara-C (Fig. 1B,C; Fig. 2A). It is important to point out that whether this chemosensitizing effect is the result of an additive or synergistic effect is rather speculative and further investigation is needed in order to establish a firm conclusion.

An important aspect of the current study was to determine whether chemosensitization by natural products is associated with apoptosis induction and decreased levels of Bcr-Abl in leukemia cells. Bcr-abl is a constitutively active kinase that represents a critical molecular determinant in the development of CML (23). Our results show that associated with increased cell killing, both Pro-EGCG and Celastrol could sensitize K-562 cells to Daunorubin as shown by higher levels of caspase 3 activity and PARP cleavage (Fig. 2B,C). Additionally, associated with the higher apoptotic indices in combination treatment were decreased levels of Bcr-Abl protein (Fig. 2A,C). This represents a significant achievement since many CML patients become refractory to TKI therapy and eventually relapse upon cessation of treatment (27). Furthermore, higher levels of PARP cleavage were apparent in Jurkat T cells when Celastrol is used in combination with daunorubicin, but at higher concentrations (Fig. 3D).

While the precise mechanism responsible for chemosensitization by natural compounds is not clearly understood, our current results and previous studies validate the need for new combination strategies that incorporate natural compounds as chemosensitizing agents in the treatment of hematological malignancies.

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