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The curcuminoid CLEFMA selectively induces cell death in H441 lung adenocarcinoma cells via oxidative stress

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Summary

CLEFMA or 4-[3,5-bis(2-chlorobenzylidene-4-oxo-piperidine-1-yl)-4-oxo-2-butenic acid] is a curcuminoid being developed as an anticancer drug. We recently reported that it potently inhibits proliferation of various cancer cells. In this project, we investigated the effect of CLEFMA on gene expression profile in H441 lung adenocarcinoma cells, and studied its mechanism of action. In microarray data, we observed a deregulation of genes involved in redox and glutamate metabolism. Based on the affected ontologies, we hypothesized that antiproliferative activity of CLEFMA could be a result of the induction of reactive oxygen species (ROS). We tested this hypothesis by determining the level of glutathione (GSH) and ROS in H441 cells treated with CLEFMA. We observed a rapid depletion of intracellular GSH/GSSG ratio. Using a cell-permeable fluorogenic substrate, we found that CLEFMA significantly induced ROS in a time- and dose-dependent manner ($p < 0.05$). Flow-cytometry with a mitochondria-selective fluorescent reporter of ROS indicated that the CLEFMA-induced ROS was of mitochondrial origin. In contrast to the cancer cells, the normal lung fibroblasts (CCL-151) did not show any increase in ROS and were resistant to CLEFMA-induced cell death. Furthermore, the addition of antioxidants, such as catalase, superoxide dismutase and N-acetylcysteine, rescued cancer cells from CLEFMA-induced cell death. Gene expression pathway analysis suggested that a transcription factor regulator Nrf2 is a pivotal molecule in the CLEFMA-induced deregulation of redox pathways. The immunoblotting of Nrf2 showed that CLEFMA treatment resulted in phosphorylation and nuclear translocation of Nrf2 in a time-dependent fashion. Based on these results, we conclude that induction of ROS is critical for the antiproliferative activity of CLEFMA and the Nrf2-mediated oxidative stress response fails to salvage H441 cells.

Keywords

CLEFMA; Curcumin; Cancer; Reactive oxygen species; Oxidative stress

Introduction

Chemotherapeutic drugs are the mainstay in the management of cancer patients; however, the emergent chemoresistance, morbid toxicities and overall inefficacy of current drug portfolios in many cancers necessitate the development of new drugs with novel mechanisms of action and therapeutic selectivity in cancer cells. Our laboratory performed a structure-activity relationship on several synthetic diphenyldihaloketone analogs [1,2]. Because of the structural similarity with curcumin, these compounds are also known as curcuminoids. As a chemical class, such compounds belong to chalcones, in which two aromatic rings flank a three-carbon enone fragment on either side. The lead chalcone derivative, 3,5-Bis(2-fluorobenzylidene)-4-piperidone (also known as EF24), was first reported by Adams, et al. [3] and possesses potent antiproliferative activity against colon [2], breast [4] and ovarian cancer cell lines [5]. The exact mechanism of action of EF24 is unclear, but it appears to suppress cancer cell proliferation and angiogenesis by downregulating various cancer-promoting genes, such as COX-2, IL-8 and VEGF [2].

In our previous work, we reported the synthesis of 4-[3,5-bis(2-chlorobenzylidene)-4-oxo-piperidine-1-yl]-4-oxo-2-butenic acid or CLEFMA as a novel diphenyldihaloketone analog (Fig. 1). CLEFMA potently inhibited the proliferation of H441 lung adenocarcinoma cells by inducing autophagic cell death [6]. Lung cancers are typified by the downregulation of the apoptotic pathway resulting in an inherent chemoresistance. Specifically, protooncogenic mutations in the tumor suppressor p53 are found in ~50% of non-small cell lung carcinomas [7], and K-Ras is mutated in approximately 30% of lung adenocarcinomas [8]. Both the PTEN-PI3K-AKT-mTOR and the Ras-RAF-MEK-ERK pathways bear mutations conferring antiapoptotic and survival advantages in lung cancer cells [9,10]. Other molecular prognostic markers, such as p53, bcl-2, p21WAF1 and their associated pathways, are also defective in lung cancer [11–13]. The altered expression of these apoptosis regulators renders many apoptosis-inducing drugs ineffective in lung cancer. Therefore, there is merit in designing drugs that induce the alternate modes of cell death, such as macroautophagy.

Cancer cells have a unique ability of maintaining reactive oxygen species (ROS) at levels conducive to growth and proliferation [14,15]. However, a further increase in ROS can promote cell death secondary to the widespread oxidative damage of macromolecules [14,16]. In this work, we employed a combination of gene expression profile, pathway analysis and biochemical assays to associate CLEFMA-induced antiproliferative response with phenotypic markers of oxidative stress.

Materials and methods

Cell culture

The human lung adenocarcinoma cell line NCI-H441 (ATCC Number: HTB-174) and normal lung fibroblasts CCL-151 were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained at 37°C with 5% CO₂ in RPMI 1640 medium (Invitrogen, Carlsbad, California) supplemented with 10% heat-inactivated fetal bovine serum and gentamicin at 50 µg/ml.

Cell proliferation assay

CLEFMA was synthesized and analyzed for purity by the methods detailed elsewhere [6]. Doxorubicin (DOX, GBiosciences, Maryland Heights, MO), Paclitaxel (PAX, EMD Chemicals, Gibbstown, NJ), Curcumin (CUR, Sigma, St. Louis, MO) and Gemcitabine

(GMCB, Acros Organics, Morris Plains, NJ) were obtained from commercial sources. For cell proliferation assays, the cells were seeded in 96-well, flat-bottom tissue culture plates at a density of 5,000 cells per well. The cells were allowed to adhere and grow overnight, followed by treatment with 0–20 μ M CLEFMA as sterile solutions in cell culture grade dimethylsulfoxide (DMSO); control wells received identical amounts of DMSO without CLEFMA. The inhibition of cell proliferation was determined after 24–48 h by measuring the decrease in hexosaminidase activity as described by Landegren [17]. To assess the effect of antioxidants, the cells were co-treated with catalase (CAT, 1000 U), superoxide dismutase (SOD, 500 U) and N-acetylcysteine (NAC, 1 mM). A non-toxic concentration of CAT (Worthington, NJ), SOD (Sigma, St. Louis, MO) and NAC (Enzo Life Sciences, Plymouth Meeting, PA) was established in H441 cells prior to these experiments.

RNA Extraction and microarray sample processing

H441 cells were seeded in 6-well tissue culture plates at a density of 1×10^6 cells per well. The cells were allowed to attach and grow overnight and then treated with 1 μ M CLEFMA ($n=5$) in culture medium supplemented with 5% FBS. The DMSO concentration was maintained at 0.1% per well. Control wells ($n=5$) received equivalent volumes of DMSO without any CLEFMA. The cells were kept in the treatment medium for 12 h, after which the cells were washed twice with ice-cold Dulbecco's phosphate buffered saline (PBS). The total RNA was extracted using the RNeasy kit (Qiagen, CA) as per the manufacturer's instructions. The quality of RNA ($OD_{260/280} \geq 1.8$) was ascertained by Nanodrop 2000 spectrophotometry (NanoDrop products, Wilmington, DE).

We compared the expression of approximately 48,000 genes using Illumina Human WG-6 v3 arrays. Hybridization to Illumina microarrays was performed at the Oklahoma Medical Research Foundation (Oklahoma City, USA). RNA (250 ng) was processed using the Illumina Total Prep RNA Amplification Kit according to the manufacturer's directions (Ambion, Austin, TX). Briefly, cDNA was reverse transcribed from RNA after priming with T7-oligo-dT, and cRNA was synthesized from the T7 promoter while incorporating biotinylated UTP. The cRNA was then hybridized overnight to Illumina BeadChips, and the microarray chips were washed with high stringency and labeled with streptavidin-Cy3 (Amersham Biosciences; Piscataway, NJ) prior to scanning on an Illumina BeadArray Reader.

Microarray data analysis

The Illumina WG-6_v3 array data were normalized as described previously [18,19] using the variability of low-expressing genes as a reference point. In order to find genes expressed above the level of technical noise, a frequency histogram of raw expression values was examined for each array. The histogram yielded a right-skewed unimodal distribution curve with a mode of approximately 75. A normal distribution curve representing the variability of the data around zero was then fitted to the mode, mirroring the Gaussian profile of the left part of the histogram. The data were normalized to the standard deviation of the noise after subtraction of the mean. The arrays were $\log_{10}$ -transformed and adjusted by robust linear regression under the assumption that the expression of most genes does not change. The data were then filtered to remove genes with an expression value less than 3.0. This is equivalent to setting a threshold at 3 SD above the noise level. The genes that expressed below the noise level under all experimental conditions (approximately 1,600) were excluded from consideration, as their expression could not be reliably assessed. Full microarray data were deposited in the Gene Expression Omnibus (GEO, GSE23420) and are accessible on the GEO web site (<http://www.ncbi.nlm.nih.gov/geo/>).

To identify differentially expressed genes between the two experimental groups, we used the associative analysis as described elsewhere [19]. Briefly, a reference group of genes expressed above background with low variability of expression in pooled microarray datasets was identified by an F-test. An associative *t*-test was applied to investigate if a given gene belongs to a certain group. To identify an evaluable number of differentially expressed genes, a stringent criterion was applied: only the genes that were expressed below the noise level in one group (control or treated) and greater than 5 SD above the noise level in another group (treated or control) were selected for further analysis. The genes that were expressed above the noise level in both groups and the ones that expressed greater than 10 SD above the noise level in at least one group were selected. The genes that showed greater than a 1.5-fold difference in their expression level were also selected. These genes were considered to be the “beacons” that pointed to the affected pathways or gene networks.

To classify each group of genes by their ontological properties, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) [20]. A medium stringency was used to yield a comprehensive set of ontological groups and to categorize the genes with similar functions. Increasing or decreasing the stringency resulted in the identification of fewer or more groups of genes with similar functions but did not provide any additional information.

The lists of genes from individual clusters were submitted to Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, Redwood City, CA). IPA performs statistical computing to identify the most significant ontologies, canonical pathways, and networks over-represented in a given gene list. By default, $p < 0.05$ was used in all calculations. Gene lists from each group were analyzed for over-represented general functions, canonical pathways, and the networks that could be assembled from them.

Reactive oxygen species (ROS) assay

The CLEFMA-induced generation of ROS was measured using the OxiSelect assay kit (Cell Biolabs, Inc., San Diego, CA). Briefly, a cell-permeable fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was added to the cells cultured in 96-well plates and the plate was incubated for 30–60 min. The wells were washed twice with PBS and treated with CLEFMA for 24 and 48 h. After the incubation period, the medium was removed and the cells were gently washed 2–3 times with PBS. The fluorescence was measured at 480 nm/530 nm (excitation/emission wavelengths) using the Cytofluor 2300 (Millipore, Billerica, MA).

MitoSOX Red mitochondrial superoxide imaging

The cells were grown to 70% confluency in 24-well tissue culture plates and treated with CLEFMA (0–20 μ M) for 12–48 h; treatment with hydrogen peroxide (H_2O_2 , 10 μ M) served as a positive control. At the end of the incubation, both the non-adherent and adherent cells were incubated with 5 μ M MitoSOX Red solution (Invitrogen, Carlsbad, CA) for 30 min at 37°C. For flow cytometric analysis, the MitoSOX-loaded cells were trypsinized and collected in Hank's balanced salt solution (with Ca/Mg) containing 1% bovine serum albumin and suspended at a density of $1-2 \times 10^7$ cells/ml. Approximately 5–10 million cells were aliquoted into a binding buffer and run through an automated dual-laser-excited FACS Calibur (Flow and Imaging Core Facility, OUHSC, Oklahoma City) measuring at 488 nm excitation wavelength. The data were collected at the FSC, FL2 (585/42 nm) and FL3 (670LP) channels for at least 10,000 events, and the histogram and dot-plot charts were analyzed using Summit V4.3 software (Dako Colorado Inc, Carpinteria, CA). For fluorescence microscopy (Nikon TE2000-E inverted epi-fluorescent microscope), the

MitoSOX-loaded cells were counterstained with 1 µg/ml Hoechst 33342 dye (Molecular Probes, Carlsbad, CA).

Glutathione-to-glutathione disulfide (GSH/GSSG) assay

We used an assay kit from Oxford Biomedical Research (Oxford, MI) and followed a modification of the method reported elsewhere (NCL Method GTA-3, Nanotechnology Characterization Lab, Frederick, MD). H441 cells were seeded in a 12-well plate (1 million cells/well) and treated with CLEFMA for 12 and 24 h. The cell layer was washed twice with ice-cold PBS, collected in 100 µl of ice-cold 5% m-phosphoric acid, and centrifuged at 1000×g at 4°C for 10 min. For GSSG determination, approximately 30 µl supernatant was mixed with 20 µl thiol scavenger and 130 µl ice-cold assay buffer, whereas for GSH assay, approximately 5 µl of the supernatant was diluted in 180 µl with ice-cold assay buffer. The diluted GSSG and GSH samples (50 µl) were allowed to react with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) solution in the presence of glutathione reductase and NADPH. The plate was read by microplate reader at 405–414 nm at 0 and 10 min after DTNB addition. The GSH and GSSG concentrations were determined following a linear regression analysis and the values were normalized to total protein. The ratio (GSH–2GSSG)/GSSG was calculated and expressed as the percent of untreated cells.

Nrf2 phosphorylation

Nuclear extract was prepared using kit from Active Motif (Carlsbad, CA) according to the manufacturer's instructions. To immunoblot nuclear factor-erythroid 2-related factor 2 (Nrf2), the nuclear protein (10 µg) was separated on a Novex 4–20% SDS-PAGE gel (Invitrogen, Carlsbad, CA), transferred onto nitrocellulose membrane and probed with 1:2,000 rabbit anti-phospho-Nrf2 antibody (Epitomics, Burlingame, CA) and 1:2,000 HRP-conjugated goat anti-rabbit IgG anti-body (Sigma, St. Louis, MO). The chemiluminescence signal was developed by Pierce SuperSignal West Femto reagent (Thermo Scientific, Rockford, IL). Equal protein loading was confirmed by stripping the blot and reprobing it with 1:1,000 anti-actin antibody (Sigma, St. Louis, MO).

Data analysis

The results are expressed as the mean ± standard error of the mean. They are either representative or the average of at least three independent experiments. The statistical analysis of data was performed using Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). The differences were accepted as significant for $p < 0.05$.

Results

The antiproliferative action of CLEFMA is comparable to that of other anticancer drugs

The antiproliferative capability of CLEFMA was compared to anticancer drugs, such as paclitaxel (PAX), gemcitabine (GMCB), and doxorubicin (DOX); CLEFMA was also compared to curcumin (CUR), the natural congener of CLEFMA. As shown in Fig. 2, CLEFMA exhibited antiproliferative activity comparable to that shown by these standard drugs.

CLEFMA upregulates genes related to cellular redox status

We identified 95 genes that were upregulated (>1.5-fold) and 59 genes that were downregulated in cells treated with CLEFMA. The main ontology over-represented in 95 upregulated genes was of oxidation/reduction, containing 23 genes (Table 1). These genes belonged to more specific ontologies, namely, aldo-keto reductase, NADP or NADPH binding, and glutathione metabolism. We observed that nearly complete sets of Phase I and

II enzymes were upregulated by CLEFMA. The Phase I enzymes included several members of the aldo-keto reductase family: AKR1B1, AKR1B10, and AKR1C2-4. Phase II enzymes included glutathione peroxidase (GPX2), glutathione reductase (GSR), glutamate cysteine ligase (GCLC, GCLM), glutathione S-transferase (GSTA4), NAD(P)H:quinine oxidoreductase 1 (NQO1), and UDP glucuronyl transferase (UGDH). Other known antioxidants were also upregulated either to a lesser extent or not stably enough to pass the strict criteria of associative analysis. These enzymes included superoxide dismutase (SOD1, 1.3-fold), heme oxygenase 1 (HMOX1, 5.3-fold), and sulfotransferases (SULTA1, 3.8-fold and SULTA2, 8.5-fold). We also observed an increase in glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1.3-fold), the redox-sensitive activity of which indirectly leads to increased NADPH production [21].

ROS production is specifically induced in cancer cells

We hypothesized that the upregulation of the redox pathway was due to the enhanced production of ROS by H441 cells in response to CLEFMA. As shown in Fig. 3, CLEFMA treatment generated ROS in a dose- and time-dependent fashion. Importantly, CLEFMA did not increase ROS production in normal lung fibroblasts (Fig. 3). We further tested whether CLEFMA-induced ROS was generated in mitochondria. Both flow cytometric data as well as inverted fluorescent micrographs of MitoSOX-loaded cells confirmed the mitochondrial origin of CLEFMA-generated ROS in H441 cells (Fig. 4).

We further hypothesized that supplementation with ROS scavengers would counteract antiproliferative effect of CLEFMA. The addition of catalase (CAT), superoxide dismutase (SOD) and N-acetylcysteine (NAC) was able to partially rescue CLEFMA-induced cell death (Fig. 5a). We also observed a simultaneous reduction in CLEFMA-induced ROS generation in the presence of these free radical scavengers (Fig. 5b).

CLEFMA depletes glutathione in H441 cells

Next we submitted the microarray data to the Ingenuity Pathway Analysis (IPA) of up- and downregulated genes. Two main functional annotations over-represented by up- and downregulated genes (Table 2), namely, glutathione (GSH) metabolism and cell death were identified. In GSH metabolic pathway, CLEFMA induced upregulation of glutathione peroxidase 2 (GPX2) and glutathione reductase (GSR). We also found that CLEFMA treatment resulted in a depletion of intracellular GSH levels in H441 cells (Fig. 6). These results suggest that by depleting GSH, CLEFMA induces a feedback upregulation of enzymes responsible for GSH homeostasis.

CLEFMA activates Nrf2

The canonical pathway analysis in H441 cells treated with CLEFMA identified an Nrf2-mediated oxidative stress response. Other pathways included glutathione metabolism, pyruvate and glycerolipid metabolism, and xenobiotic metabolism (Table 3). Network analysis also identified Nrf2, also called NF2E2L, as a central player regulating the majority of oxidative stress-related genes (Fig. 7). We found that Nrf2 is highly expressed in our system. Although its expression did not significantly change at the mRNA level, CLEFMA treatment post-transcriptionally activated Nrf2. The phosphorylated form of Nrf2 translocated into the nuclear fraction (Fig. 8).

Discussion

We identified the induction of oxidative stress as a major mechanism of cell death induced by CLEFMA in H441 lung cancer cells. The activation of Nrf2-mediated protective redox mechanisms appeared to be insufficient to combat the oxidative damage, resulting in cell

death. It is known that moderate oxidative stress can trigger apoptosis [22,23]. The observation that CLEFMA failed to cause cell death and did not result in ROS generation in normal lung fibroblasts suggests that the selectivity of CLEFMA in cancer cells might be ROS-mediated. Several other ROS-generating agents have been reported to preferentially kill cancer cells while sparing the normal cells from significant toxicity [24,25]. For instance, overproduction of ROS is implicated in the anticancer activity of vinblastine, cisplatin, mitomycin C, doxorubicin, camptothecin, inostamycin and neocarzinostatin [26].

Oxidative stress causes a wide spectrum of cellular responses, mainly geared towards protecting the cell from damage. Even apoptosis induction at high oxidant exposures appears to protect surrounding cells and tissues [27]. The balance between oxidative stress and cell-mounted antioxidant response determines the extent of cellular damage caused by a pro-oxidant stimulus [27,28]. The early response to oxidative stress in mammalian cells is regulated by Nrf2 [29]. Inactive Nrf2 is retained in the cytoplasm by association with Kelch-like ECH-associated protein 1; however, under oxidative stress, Nrf2 is phosphorylated and translocates to the nucleus. Once in the nucleus, Nrf2 heterodimerizes with a small musculoaponeurotic fibrosarcoma (MAF) protein [31,32]. Activated Nrf2 binds to the ARE within the promoter region of these enzymes and activates their transcription [30]. Our immunoblot results show that CLEFMA treatment induces Nrf2 phosphorylation and translocation into the nucleus (Fig. 8). It is noteworthy that the CLEFMA-induced genes GST, NQO1/NQO2 [32,33], GCLC and GCLM [34], members of AKR family [35,36], and SRNX1 [37] share ARE sequences in their promoter region.

Through microarray analysis, we identified the activation of both Phase I and Phase II metabolic enzymes by CLEFMA treatment. The Phase I and Phase II enzymes are responsible for metabolism of xenobiotics by catalyzing functionalization and conjugation reactions, respectively. Seldom, the Phase I reactions may promote the conversion of a relatively non-toxic molecule into a toxic species (toxification). In CLEFMA-treated H441 cells, two aldo-reductases (AKR1B1 and AKR1B10) and three hydro-steroid dehydrogenases (AKR1C2-4) were upregulated. AKRs convert carbonyl groups to primary or secondary alcohols. It is likely that these enzymes introduce reactive groups in CLEFMA, a process responsible for ROS producing intermediates. The Phase II conjugation reactions generally involve reactions between the Phase I metabolites and endogenous conjugating molecules, such as GSH. GSH is an important cellular antioxidant, and severe oxidative stress depletes its intracellular level. The GSH conjugation reactions are catalyzed mostly by glutathione-S-transferase (GST). GST is synthesized from cysteine by glutamate cysteine ligase (GCL). Our microarray data indicate that CLEFMA treatment of H441 cells results in induction of multiple mechanisms geared towards maintenance of intracellular GSH. Both catalytic as well as modifier subunits of the GCL heterodimer (GCLC and GCLM, respectively) [38,39] were highly induced upon CLEFMA treatment. Similarly, GSR, which reduces GSSG back to GSH at the expense of NADPH, is upregulated. As shown in Table 3, three major pathways for replenishing NADPH-pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD) and glyceraldehyde 3-phosphate dehydrogenase (GADPH) are activated by CLEFMA treatment [21]. These observations suggest that the CLEFMA-induced oxidative stress results in a rapid activation of cellular mechanisms responsible for GSH synthesis. Several members of the AKR family represent an alternative GSH-independent route for the reductive elimination of CLEFMA, as in the case of 4-hydroxynonenal [40].

It is noteworthy that the two members of the NAD(P)H dehydrogenase (quinone) family, NQO1 (NAD(P)H dehydrogenase, quinone 1) and NQO2, were also highly induced in our system. NQO1 mediates the two electron transfer for the cellular defense against various damaging quinones [41]; however, some nontoxic quinones are reduced to free radical-

generating and toxic semiquinones via one electron transfer by NQO1. For instance, NQO1 induces the formation of semiquinone form of β -lapachone, depleting the cells of NAD(P)H in the process and resulting in the generation of DNA-damaging hydroxyl radicals [42]. It has also been shown that NQO1 stabilizes p53, especially under oxidative stress [43]. Other Phase II xenobiotic-metabolizing enzymes that were upregulated in our system are mainly involved in the network of interconnected metabolic reactions that eliminate reactive species at the sites of origin. Such cellular antioxidants included GPX2, peroxiredoxin (PRDX1), and GSTA4. We also observed the upregulation of thioredoxin reductase 1 (TXNRD1). Several other important Phase II enzymes that did not pass rigorous statistical criteria include SOD1, HMOX1, epoxide hydrolase (EPHX1), and sulfiredoxin 1 (SRNX1). HO-1 mRNA and protein expression are commonly upregulated following oxidative stress and cellular injury [44]. However, expression of biliverdin reductase B (BLVRB) and peroxiredoxin (PRDX1) was altered. HMOX1 catalyzes the rate-limiting step in the catabolism of the pro-oxidant heme to biliverdin, whereas SRNX1 catalyzes the reduction of peroxiredoxin, converting it to an active state.

We recapitulate the observed interplay among altered enzymes and detoxification systems in Fig. 9. The majority of the Phase I and II genes identified in our system were found to be regulated by the Nrf2 transcription regulator. Besides Nrf2-mediated transcriptional activation, the levels of certain antioxidant enzymes may be regulated post-transcriptionally in a redox-sensitive manner. For example, the stability of both the GCLC and GCLM mRNAs is redox-regulated, and their half-lives increase under oxidative stress [45,46]. In case this first line of defense is insufficient to prevent oxidative damage to the DNA, several other signaling processes are initiated [47]. Dividing cells are particularly susceptible to oxidative damage, because their DNA is uncoiled and naked as it undergoes fast replication [28]. In order to allow more time for damage repair, cell proliferation could be blocked at several phases of the cell cycle, such as G1-S, S and G2-M [28,48]. A prolonged arrest or irreparable damage results in cell death associated with apoptosis, macroautophagy, necrosis or senescence. This study provides preliminary evidences that the actions of CLEFMA in H441 cells follow this mechanistic pathway.

Acknowledgments

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Fig. 1.
The molecular structure of CLEFMA

Fig. 2.

The antiproliferative action of CLEFMA in H441 cells (5,000 cells/well) is comparable to that exhibited by other anticancer drugs. The extent of cell proliferation after 48 h of treatment was assayed by measuring hexosaminidase activity. PAX = Paclitaxel, GMCB = Gemcitabine, DOX = Doxorubicin, and CUR = Curcumin

Fig. 3. Treatment with CLEFMA induces the generation of reactive oxygen species (ROS) in H441 cells but not in CCL-151 normal lung fibroblasts. ROS was estimated based on the fluorescence intensity of the cell-permeable, fluorogenic 2',7'-dichlorodihydro-fluorescein diacetate

Fig. 4. CLEFMA-induced ROS generation is mitochondrial in origin. A mitochondria-specific indicator of ROS (MitoSOX) was loaded into H441 cells for (a) flow cytometry and (b) inverted microscopy. In both assays, there was a time- and dose-dependent increase in red fluorescence in CLEFMA-treated H441 cells

Fig. 5.

ROS scavengers rescue H441 cells from CLEFMA-induced cell death. **a** The simultaneous treatment with CLEFMA and various ROS scavengers rescues H441 cells from cell death. Cells were treated with CLEFMA (10 and 20 μM) in the presence of catalase (CAT, 1000 Units), superoxide dismutase (SOD, 200 Units), and N-acetylcysteine (NAC, 1 mM). Cell proliferation was measured by hexoaminidase assay. **b** The ROS scavengers also inhibit ROS generation in H441 cells. Asterisk (*) denotes $p < 0.001$ compared to control (100%)

Fig. 6. CLEFMA reduces the GSH/GSSG ratio in H441 cells. The GSH and GSSG levels were assayed as described in Materials and Methods

Fig. 7.
CLEFMA alters the expression of genes involved in redox pathways. Red and green highlight up- and downregulated genes, respectively. The complete microarray data have been deposited in the Gene Expression Omnibus (GEO, GSE23420) for access on the GEO web site

Fig. 8. CLEFMA induces nuclear translocation of phosphorylated Nrf2 in H441 cells. A representative immunoblot shown from two sets of experiments at multiple time points

Fig. 9.

The failure of protective cellular redox mechanisms (in shaded block) appears to be responsible for CLEFMA-induced cell death in H441 cells. G6PD—glucose-6-phosphate dehydrogenase; GCL—glutamate cysteine ligase; GCLC—GCL catalytic subunit; GCLM—GCL modifier subunit; GPX2—glutathione peroxidase 2; GSH—glutathione; GSR—glutathione reductase; GSSG—disulfide-oxidized form of glutathione; GSTA4—glutathione S-transferase; NQO1/2—NAD(P)H dehydrogenase, quinone 1/2; PRDX1—peroxiredoxin; SOD1—superoxide dismutase; TXNRD1—thioredoxin reductase

Table 1

The ontologies affected in H441 cells treated with CLEFMA

Functional annotation	Genes in ontology	<i>p</i> -value *
Oxidation reduction	AKR1B1, AKR1C2, AKR1C3, AKR1C4, ALDH3A2, BLVRB, FTHL3, FTL, G6PD, GCLM, GPX2, GSR, HTATIP2, LOC441282, ME1, NOS3, NQO1, NQO2, PRDX1, SEPX1, TP53I3, TXNRD1, UGDH	3.50E-12
Aldo/keto reductase	AKR1B1, AKR1C2, AKR1C3, AKR1C4, GCLM, LOC441282 (AKR1B10)	1.30E-06
NADP or NADPH binding	G6PD, GSR, ME1, NOS3, TP53I3, TXNRD1	4.50E-05
Glutathione metabolism	G6PD, GCLC, GCLM, GPX2, GSR, GSTA4	1.30E-03

* Benjamini-Hochberg corrected

Table 2

The ontologies over-represented by up- and downregulated genes affected in H441 cells treated with CLEFMA. Upregulated genes are in **bold** font

Functional annotation	Genes in ontology	<i>p</i> -value
Metabolism of glutathione	G6PD, GCLC, GCLM, GSR	2.99E-05
Cell death	ADM, EPHX1, FTH1, G6PD, GCLC, GCLM, GPC1, GPX2, GSR, HTATIP2, IGFBP3, IKBKG, NCF2, NOS3, NQO1, NQO2, PLEKHF1, PRDX1, RAP1GAP, RASD1, SLC7A11, TXNRD1, ALDH1A3, APP, CDC42EP3, DUSP10, DUSP6, EGR1, EIF2AK3, FOS, IGF1R, IL1R1, LMNB1, PEG10, PLAT, RNF19A, SNX33, TAF9B, TIA1, TNFRSF6B	9.36E-05

Table 3

The canonical pathways affected by CLEFMA. The upregulated genes are in **bold** font

Ingenuity canonical pathways	Molecules	p-value
Nrf2-mediated oxidative stress response	EPHX1, FTH1, FTL, GCLC, GCLM, GPX2, GSR, GSTA4, NQO1, NQO2, PRDX1, SQSTM1, TXNRD1, FOS, EIF2AK3	1.00E-11
Glutathione metabolism	G6PD, GCLC, GCLM, GPX2, GSR, GSTA4	9.33E-06
Pyruvate metabolism	AKR1B1, AKR1B10, AKR1B15, ALDH3A2, ME1, ALDH1A3	3.16E-05
Metabolism of xenobiotics by cytochrome P450	AKR1C2, AKR1C3, AKR1C4, EPHX1, GSTA4, ALDH1A3	1.41E-04
Glycerolipid metabolism	AKR1B1, AKR1B10, AKR1B15, ALDH3A2, GLA, ALDH1A3	1.48E-04
Xenobiotic metabolism Signaling	ABCC3, ALDH3A2, FTL, GCLC, GSTA4, NQO1, NQO2, ALDH1A3, EIF2AK3	2.63E-04
Pentose and glucuronate interconversions	AKR1B1, AKR1B10, AKR1B15, UGDH	4.37E-04