Published in final edited form as:

Mol Cancer Ther. 2010 November ; 9(11): 2869–2878. doi:10.1158/1535-7163.MCT-10-0517.

The synthetic caged *Garcinia* xanthone cluvenone induces cell stress and apoptosis and has immune modulatory activity

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Abstract

Several caged *Garcinia* xanthone natural products have potent bioactivity and a documented value in traditional eastern medicine. Previous synthesis and SAR studies of these natural products resulted in the identification of the pharmacophore represented by the structure of cluvenone. In the current study, we examined the anti-cancer activity of cluvenone and conducted gene expression profiling and pathway analyses. Cluvenone was found to induce apoptosis in T-cell acute lymphoblastic leukemia cells (EC₅₀ = 0.25 μ M) and had potent growth inhibitory activity against the NCI60 cell panel, including those that are multi-drug resistant, with a GI₅₀ range of 0.1 μ M - 2.7 μ M. Importantly, cluvenone was about 5-fold more potent against a primary B-cell acute lymphoblastic leukemia compared to peripheral blood mononuclear cells (PBMC) from normal donors suggesting that it has significant tumor selectivity. Comparison of cluvenone's growth inhibitory profile to those in the NCI database revealed that compounds with similar profile to cluvenone were mechanistically unlike known agents, but were associated with cell stress and survival signaling. Gene expression profiling studies determined that cluvenone induced activation of the MAPK and Nrf2 stress response pathways. *Furthermore, cluvenone was found to induce intracellular ROS formation.* Lastly, the modulation in expression of several genes associated with T-cell and NK cell activation and function by cluvenone suggests a role as an immune-modulator. The current work highlights the potential of cluvenone as a chemotherapeutic agent and provides support for further investigation of these intriguing molecules with regard to mechanism and targets.

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Conflict of Interest: None

Keywords

Cluvenone; apoptosis; cell stress

Introduction

According to the American Cancer Society, there were nearly 1.5 million men and women diagnosed with cancer in the US in 2009. It is projected that about 40% of these individuals will die of their disease. The current annual cancer incidence rate corresponds to one out of every two men, and one out of every three women being diagnosed with cancer within their lifetime. The main obstacle for successful treatment is resistance to current therapies. Not only are the current chemotherapeutic agents ineffective against many cancers, especially relapsed cancers, but there is significant toxicity associated with these therapies. Therefore, it is essential to intensively investigate novel agents that are not only effective against refractory cancers, but are also tumor selective and thus less toxic.

The tropical trees of the genus *Garcinia*, found in lowland rainforests of Southeast Asia, are widely known for their use in folk medicine (1,2). Phytochemically, these trees are recognized as a rich source of xanthone and xanthonoid natural products that hold high pharmaceutical potential. Gambogic acid (GA) is the main active component of gamboge, a dry resin secreted from *Garcinia hanburyi*. It has been well established that GA induces apoptosis in multiple tumor cell lines including T47D breast cancer cells and MGC-803 human gastric carcinoma cells at sub-micromolar to low micromolar concentrations (3-5). Furthermore, GA has demonstrated efficacy in several human tumor xenograft models and has entered clinical trials in China for the treatment of cancer (6-9).

Previously, we have reported a new strategy for the chemical synthesis of the *Garcinia* natural products (10,11). The synthesis is short, efficient, and stereo-selective, and allows access to a variety of these compounds and related analogs. This technology not only eliminates the drawbacks of natural supply and varying isomeric mixtures, typical of natural products, but also provides the opportunity to perform systematic biologic and pharmacologic studies resulting in the discovery of novel pharmacophores and the development of highly effective chemotherapeutic agents. More recently, we have evaluated the pharmacophoric motif of the caged *Garcinia* xanthenes and have identified the minimum bioactive motif of these compounds (12,13). Based on this information, we have generated a simple synthetic analog, cluvenone, which was found to induce apoptosis in multi-drug resistant promyelocytic leukemia cells (HL-60/ADR) at nanomolar concentrations, with EC₅₀ values equal to that found in the parental cells (HL-60) (13). It is believed that the unique structure of cluvenone and members of the *Garcinia* family of natural products represents a novel pharmacophore that accounts for the cytotoxicity of these compounds against multi-drug resistant cancer cells. In the current study, we describe the anti-cancer activity and tumor selectivity of cluvenone as well as the results of gene expression profiling and pathway analyses towards the identification of critical molecular determinants in the action of the caged *Garcinia* xanthenes.

Materials and Methods

Cell lines

T-cell acute lymphoblastic leukemia, CEM, and prostate cancer cells, PC3, cells were purchased from ATTC in 2008. These cell lines were authenticated by observation of morphology and by measuring sensitivity to known agent, gambogic acid, and then comparing IC₅₀ to that reported in the literature. This testing is performed routinely in our laboratory. The NCI60 cell lines screened by the NCI against cluvenone were not authenticated by the authors.

Apoptosis assay

CEM, cells were plated at 10,000 cells/well (96-well plate) in RPMI medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin/streptomycin (complete medium). Cells were then treated with increasing concentrations of cluvenone or with 0.1% DMSO, and incubated at 37° C for 7 h before apoptosis was measured using the Cell Death Detection ELISAPLUS kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. This method constitutes a photometric enzyme-immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated-DNA-fragments after induced cell death.

Determination of cytotoxicity of cluvenone in the NCI60 cell panel screen

Briefly, cell lines were treated with increasing concentrations of cluvenone for 48 h and total cell protein was then determined by Sulforhodamine B (SRB) staining. For additional details, please see <http://www.dtp.nci.nih.gov/branches/btb/ivclsp.html>. The NCI's COMPARE program was utilized to evaluate the correlation between the growth inhibitory profile (GI₅₀) of cluvenone and other compounds in the NCI chemical database. In addition, 3D Mind tools (<http://spheroid.ncifcrf.gov>) was used to determine where cluvenone mapped on a self organizing map (SOM).

Determination of primary acute lymphoblastic leukemia and peripheral blood mononuclear cell viability

Heparinized bone marrow or peripheral blood samples were obtained at diagnosis or relapse from T-cell acute lymphoblastic leukemia (ALL) patients enrolled in Pediatric Oncology Group Protocols #9000 and #9400 (ALL Biology Study). In addition, peripheral blood was obtained from normal donors. Mononuclear cells from bone marrow or peripheral blood were isolated by isopycnic sedimentation through Ficoll-Hypaque (specific gravity 1.077 g/ml; Pharmacia, Piscataway, NJ) at 400g for 30 min followed by two washes with RPMI 1640. The content of lymphoblasts in these patient samples, as determined by Wright stain, was ≥ 80%. Primary B-cell ALL and peripheral blood mononuclear cells (PBMC) obtained from normal donors were treated with increasing concentrations of cluvenone for 48 h and then viable cell numbers were determined by counting in a hemocytometer.

Agilent Whole Human Genome 4 × 44K arrays

Treatment of Cells with cluvenone and isolation of total RNA—CEM cells were treated in quadruplicate with 0.3 μM cluvenone, or with 0.1% DMSO (control) for 2 and 4.5 h. Total RNA was isolated from 5-10 × 10⁶ cells using the ArrayGrade™ Total RNA Isolation Kit (SuperArray Bioscience Corp., Frederick, MD) according to the manufacturer's recommendations. The integrity and concentration of the total RNA was assessed on a 2100 Bioanalyzer (Agilent Technologies), using the RNA 6000 Nano LabChip. The RNA integrity (RIN number) ranged from 9.5 to 10.0.

Microarray Analysis—RNA (500 ng/reaction) was amplified (1-round) and labeled with Cyanine 3 (Cy3, control) or Cyanine 5 (Cy5, treated) using the Quick-Amp Labeling Kit (Agilent) according to the manufacturer's recommendations. The labeled amplified RNA was hybridized at 65 °C for 17 h to Agilent Whole Human Genome 4 × 44K microarrays. The arrays were washed with Agilent wash buffers 1 and 2 and then placed in Agilent Stabilization and Drying Solution according to the manufacturers' directions. The arrays were scanned on a Perkin Elmer ProScanArray Express HT scanner. The scanned images were quantified using ImaGene 8.0.1 software from BioDiscovery (El Segundo, CA) and significantly differentially expressed genes were identified using GeneSifter from Geospiza (Seattle, WA). Probes that achieved a significance of P=0.005 or less in the ANOVA analysis with Benjamin-Hochberg

correction were subjected to pathway analysis. The genetic loci corresponding to each probe together with the fold-change at both the 2 h and the 4.5 h time points were analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems Inc., Redwood City, CA) and the Pathway-Express Tool, created by Sorin Draghici at Wayne State University (available at <http://vortex.cs.wayne.edu/ontoexpress>), to assess affected pathways.

Unlike other pathway analysis methods, The Pathway-Express Tool conducts an Impact analysis that includes the classical statistics, but also considers other crucial factors such as the magnitude of each gene's expression change, their type and position in the given pathways, and their interconnectedness, thus representing a systems biology approach (14). The Impact Factor is equivalent to the negative log of the global probability of having both a statistically significant number of differentially expressed genes, and a large perturbation in the given pathway. Thus, Impact Factor values indicate the likelihood that the listed pathway is truly affected in the treated case, and is not a spurious result.

Real-Time RT/PCR

cDNA was transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). All cDNA reaction mixtures included 50 ng of template RNA. The reverse transcriptase PCR reactions were performed on an Applied Biosystems GeneAmp PCR System 2700 using the temperature cycling conditions recommended in the iScript cDNA Synthesis Kit protocol. The temperature cycling conditions included 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. Real Time PCR reactions were performed on each cDNA sample to quantify selected genes. Primer sequences for HSPA1A (Forward: 5'-GCCGAGAAGGACGAGTTTGA-3', Reverse: 5'-TCCGCTGATGATGGGGTTAC-3') were determined using RTPrimerDB (www.rtprimerdb.org). Actin (Forward: 5'-CGTGGACATCCGCAAAGAC-3', Reverse: 5'-AGGGTGTAACGCAACTAAG-3') was used as a control for each cDNA sample. Primers were purchased from Integrative DNA Technologies (San Diego, CA). The primers were combined in equal volumes to create a primer mix with a final concentration of 5 μ M per primer. Each 25 μ l PCR reaction included 5 μ l water, 12.5 μ l of iQ Sybr Green Supermix (Bio-Rad, Hercules, CA), 5 μ l of 5 μ M primer mix and 2.5 μ l of cDNA sample. Real Time PCR reactions were performed on a MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The thermal cycler protocol consisted of an initial denaturation at 95°C for 30 sec, followed by 50 cycles of 95°C denaturation for 10 sec, annealing for 50 sec at gene-specific temperature, and by an extension at 60°C for 1 min. The final step of the protocol included a melt curve from 60°C to 100°C in 0.5°C interval at 10 sec per interval.

Measurement of intracellular ROS

The human prostate cancer cell line PC3 was plated in a 96-well plate at 5×10^4 cells/well in complete RPMI and incubated overnight. The cells were treated with up to 1 μ M cluvenone or with 0.1% DMSO (- control) for 1, 2, 5.5, 15 h. In addition, cells were treated with increasing concentrations of H₂O₂ as a control for ROS induction. To measure ROS, cells were loaded with 0.2 mM of DCFH-DA for 45 min at 37°C using the OxiSelect ROS Assay kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's recommendations. Fluorescence was measured on a Synergy Mx plate reader (BioTek Instruments Inc., Winooski, VT) with excitation/emission at 480/530 nm.

Measurement of cluvenone cytotoxicity in the presence of N-acetylcysteine

PC3 cells were plated in a 96-well plate at 1×10^4 cells/well in complete RPMI and incubated overnight. The following day, cells were treated for 48 h with 0.1% DMSO or 1 μ M cluvenone in the presence or absence of N-acetylcysteine at 0.1, 1, and 5 mM. Cells were pulsed for 6 h with ³H-thymidine and incorporation was measured in a scintillation counter (Beckman

Coulter Inc., Fullerton, CA) after cells were washed and deposited onto glass microfiber filters using a cell harvester M-24 (Brandel, Gaithersburg, MD).

Results

In a previous report (12), we screened a number of different caged *Garcinia* xanthones against solid and non-solid tumor cell lines including multidrug resistant HL-60 cells (HL-60/ADR) expressing P-glycoprotein (15). We found that cluvenone (Figure 1, inset) was the most potent of the synthetic analogs with equal or similar activity to the structurally more complex parent natural product, GA. More importantly, cluvenone, like GA, was equally toxic to HL-60/ADR as the parental HL-60 cell line. The mechanism of cytotoxicity of cluvenone was examined in leukemia cell lines using a cell death detection ELISA assay which measures histone associated DNA fragments. Cluvenone induced apoptosis in a dose-dependent manner in the T-cell acute lymphoblastic leukemia cell line, CEM, characterized by having mutated *p53* and deletion of *p15* and *p16*, genes frequently inactivated in multiple cancers (Figure 1). The EC₅₀ of cluvenone in CEM was 0.25 μ M after 7.5 h of treatment and characteristic morphology of apoptotic death, membrane blebbing, was visible as early as within 4 h of treatment (data not shown). Similar results were obtained using HL-60 and HL-60/ADR cells with an EC₅₀ of 0.25 and 0.32 μ M, respectively (13), indicating that the cytotoxicity of cluvenone is not affected by the expression of P-glycoprotein or by the absence of *p53* and cell cycle inhibitory genes.

Encouraged by these results, cluvenone was then screened against the NCI60 cell panel to extend the evaluation of its cytotoxicity and, more importantly, gain insight into its possible mode of action. The results of the NCI60 cell panel screen confirmed that cluvenone is cytotoxic to all cancer cell lines tested including those that are multi-drug resistant, with a mean GI₅₀ of 0.5 μ M (range 0.1 μ M - 2.7 μ M) (Figure 2). For example, even a robustly multi-drug resistant cell line such as NCI/ADR-RES (breast) as well as others such as HCT15 (colon) and U031 (renal) were sensitive to cluvenone with a GI₅₀ less than 0.5 μ M, with the exception of TK10 (renal) which had a GI₅₀ of 1.0 μ M. Additionally, tumor cells with very aggressive growth and metastatic potential such as NCIH460 (large cell lung cancer) and LOX (melanoma) were also sensitive to cluvenone with a GI₅₀ of 786 and 163 nM, respectively. Collectively, these results suggest that cluvenone and members of this class of *Garcinia* xanthones may overcome the drug-resistance typical of relapsed cancers.

Although cluvenone is cytotoxic to a broad range of tumor cells including multidrug-resistant cancer cells, it must demonstrate tumor selectivity in order to be an effective chemotherapeutic agent. To determine whether cluvenone has any selectivity for cancer cells over normal cells, we examined the effect of cluvenone in a primary human leukemia and PBMC obtained from a normal donor. Previously, we have found that cytotoxicity results from viability assays using primary cells from normal donors and patients correlates well with *in vivo* data, as opposed to data from similar assays using cell lines (15). For these experiments, primary cells were exposed to increasing concentrations of cluvenone for 48 h and then viable cell numbers were determined. The results shown in Figure 3 indicate that cluvenone is 4.7-fold more toxic to leukemia cells (IC₅₀=1.1 μ M) than PBMC from normal donors (IC₅₀=5.2 μ M). Interestingly, in the same experiment, GA was 3.9-fold more toxic to B-ALL cells than PBMC suggesting that it may have greater toxicity to normal cells compared to cluvenone (not shown). The observed differential cytotoxicity of cluvenone against leukemia cells versus PBMC from normal individuals is extremely encouraging as standard chemotherapeutic agents are generally similarly toxic to PBMC and tumor cells. Based on the observed tumor selectivity, it is anticipated that cluvenone will be well tolerated at the low doses which are therapeutically effective.

In order to gain insight into the mechanism of action of cluvenone, a COMPARE analysis was conducted using the results of the NCI60 cell panel screen. The results of this analysis determined that molecules with a similar cytotoxicity profile to cluvenone were of completely different chemical structures (Figure 4A), and were not similar to standard agents. Furthermore, using 3D Mind tools, the top 6 ranked molecules and GA were mapped to the Q region of a self organizing map (SOM), distal to agents with known mechanism of action (Figure 4B). The Q region of the SOM includes bioactive molecules with yet unknown mechanisms of action but which have recently been implicated to activate signaling pathways involved in cell stress and survival (16, 17). These findings highlight the structural uniqueness of cluvenone and members of the caged *Garcinia* xanthenes as well as demonstrate the potential of the current research for therapeutic application to cancers refractory to standard chemotherapy.

Intrigued by the results of the SOM suggesting a novel mechanism of action and to further investigate the mode of action of cluvenone, gene expression profiling experiments were conducted using Agilent Whole Genome $4 \times 44K$ arrays (*accession number GSE23536 in GEO database*). In these experiments, CEM cells were treated in quadruplicate with vehicle (DMSO) or with 0.3 μM cluvenone for 2 and 4.5 h. Analysis of the microarray data using GeneSifter software revealed that 191 or 250 genes were altered in expression at least 1.7-fold (adjusted p value < 0.005) by cluvenone treatment for 2 or 4.5 h, respectively. The fold change cutoff was selected based on a report of an independent laboratory demonstrating decreased expression of telomerase reverse transcriptase (TERT) in response to treatment with GA (18, 19). In agreement with this report, our data indicated that TERT expression was decreased 1.7-fold by cluvenone indicating that a 1.7-fold change in expression was a valid cutoff.

The list of significantly altered genes at both times (2 and 4.5 h) of cluvenone treatment were analyzed using Pathway-Express software to determine which cell signaling pathways would be affected. The results, summarized in Table 1, reveal that one of the most significantly affected pathways, at both time points of treatment, includes the MAPK cell stress signaling pathway as evidenced by the dramatic and progressive up-regulation of the heat shock gene, HSPA1A, in addition to up-regulation of HSPA8 and MAPK14, also known as p38. The dramatic up-regulation of HSPA1A mRNA by cluvenone treatment for 2 and 4.5 h, as determined by microarray analysis, was confirmed by real-time RT/PCR (data not shown). Analysis of the gene expression data using Ingenuity Pathway Analysis software confirmed oxidative cell stress signaling to be a predominant pathway activated in response to cluvenone. In particular, Nrf2-mediated oxidative stress signaling which is downstream of MAPK signaling, and a defense mechanism against oxidative stress (20), appeared to be activated ($P = 9.09 \times 10^{-4}$). *This result was based on changes in the expression (≥ 1.9 -fold) of the following stress associated genes; DNAJA1, DNAJA4, DNAJA6, DNAJB1, DNAJB2, FTL, FTH1, FKBP5.*

HSPA1A and other heat shock proteins, known to be associated with cell stress, are also involved in antigen processing and presentation (21), as well as in *apoptotic signaling in prostate cancer* (22), and possibly other cancer cells. Prostate cancer cells, especially at the advanced stages of prostate cancer, are constitutively under oxidative stress resulting in the induction of heat shock proteins which block apoptosis (23). In such cells, the additional oxidative stress imposed by cluvenone, would be expected to result in apoptosis. It is well established that when cell stress is too high for protective mechanisms to overcome damage, cells undergo apoptosis. The induction of MAPK14, Jun, and caspase 9 by cluvenone is consistent with the induction of apoptosis via the MAPK-dependent pathway (24). In summary, the induced expression of heat shock genes by cluvenone can affect antigen processing and presentation in antigen presenting cells, as well as apoptosis in cancer cells.

Treatment of CEM cells with cluvenone appears to significantly affect adherens junction signaling (Table 1A). Though this result is based on a small change (-1.7-fold) in the T-cell specific transcription factor (TCF7), this gene is central in adherens junction signaling involving the Wnt pathway. Furthermore, deregulated transcription of the full length TCF7 isoform appears to be involved in prostate and colon cancers (25-27). This may be due, in part, to the transcriptional activation of c-myc by complexes of B-catenin and members of the TCF family ultimately resulting in enhanced cell proliferation (28). Interestingly, cluvenone decreased the expression of both TCF7 and c-myc (greater than 2-fold) suggesting that it may have a role in suppressing signaling through the Wnt pathway associated with prostate and colon cancer (29).

Genes that are central to the regulation of the actin cytoskeleton, ITGAV and ITGB2, are significantly up-regulated by cluvenone at both time points (Table 1A and B) suggesting that cluvenone can affect activities associated with the actin cytoskeleton such as cell motility and adhesion. These activities are particularly important for T-cell function as is protein export (Table 1B), also found to be modulated by cluvenone. Further evidence suggesting regulation of T-cell function by cluvenone is the induction in the expression of CD69 (2.7-fold), an early leukocyte activation molecule expressed transiently in activated immune cells including T-cells (30). Other genes involved in immune function that were found to be up-regulated (about 2-fold) by cluvenone include IL11RA and IL2-inducible T-cell kinase, ITK.

Collectively, analyses of the gene expression array results indicate that cluvenone induces Nrf2-mediated oxidative stress and activation of the MAPK-dependent apoptotic pathway. Furthermore, cluvenone appears to have a role in modulating immune function and in regulating adherens junction signaling.

To determine whether cell stress induced by cluvenone is due to the formation of reactive oxygen species (ROS), the levels of ROS were measured in PC3 (prostate cancer) cells treated with cluvenone for various times up to 15 h using a fluorescence based assay. The results shown in Figure 5A, indicate that cluvenone induced ROS formation in a dose-dependent manner within 5.5 h. Although the level induced was only 30% above control at the highest concentration of cluvenone tested, this level was equivalent to that induced by 1 mM H₂O₂ and sufficient to induce apoptosis. As PC3 cells are under constitutive oxidative stress, the increase of ROS levels to 30% above control, appears to surpass the capability of protective mechanisms to prevent apoptosis. Induction of ROS by cluvenone was also time-dependent as ROS was not detectable at times tested earlier than 5.5 h (data not shown). To determine whether induction of ROS accounted for the cytotoxicity of cluvenone, PC3 cells were treated with cluvenone in the presence and absence of the antioxidant, N-acetylcysteine. Cell proliferation was then measured after 48 h in a ³H-thymidine incorporation assay. The results shown in Figure 5B demonstrated that N-acetylcysteine was able to reduce the cytotoxicity of cluvenone with complete inhibition of cluvenone cytotoxicity at 1 mM N-acetylcysteine. Collectively, these results suggest that the induction of ROS is responsible for the induction of cell stress and apoptosis by cluvenone in PC3 cells. How cluvenone induces ROS and whether additional mechanisms account for cluvenone's cytotoxicity in other cell types remains to be determined.

Discussion

The simple synthetic GA analog, cluvenone, has significant cytotoxicity at nanomolar concentrations against a broad range of tumor types including multi-drug resistant tumors with leukemia being the most sensitive tumor type, followed by colon, breast, prostate, and renal cancers. Since breast, prostate, and colon cancers are among the most common cancers in the U.S., cluvenone may have significant value as a chemotherapeutic agent. Our results indicate

that cluvenone is an effective inducer of apoptosis, a required attribute for an effective chemotherapeutic agent. Furthermore, the observed differential cytotoxicity between leukemia cells and PBMC from normal donors, suggesting that cluvenone has tumor selectivity, further adds to the potential value of cluvenone in the treatment of cancer.

The unique structure and activity profile of cluvenone is consistent with the results of the COMPARE analysis indicating that compounds most similar to cluvenone map to the Q region of a SOM, a region including molecules with unknown mechanisms of action, but associated with signaling involved in cell stress and survival. *Cellular stress generated by cluvenone appears to be due to the generation of ROS as determined for GA* (31). In a recent study, GA induced the formation of ROS in hepatoma cells resulting in loss of mitochondrial membrane potential, release of cytochrome c, and apoptosis. Furthermore, these activities were associated with phosphorylation of JNK and p38, key members of the MAPK stress signaling pathway. Interestingly, the anti-oxidant, N-acetylcysteine, only partly reversed the activation of JNK and p38, and the induction of apoptosis in GA-treated cells, suggesting that additional mechanisms account for the cytotoxicity of GA. Like many natural products, both GA and cluvenone likely have multiple targets and mechanisms of action. For instance, in addition to inducing ROS, GA has been shown to bind to and inhibit the anti-apoptotic activities of the Bcl-2 family of proteins (32). These activities would result in a two-pronged attack on tumor cells with the induction of cell stress and the release of an apoptotic block, not uncommon in tumor cells.

Most recently, it has been reported that the oxidative stress induced by GA results in DNA damage as detected by a comet assay and evidenced by the activation of p53/p21 through the ATR-Chk1 pathway (33). It is important to note, however, that activation of DNA damage response pathways can occur in the absence of DNA strand breaks and can occur in response to alteration in chromatin structure or, to depletion of nucleotide pools (34). Furthermore, it is important to appreciate that DNA damage measured in the comet assay is not necessarily a result of direct genotoxicity as mitochondrial or membrane damage can cause extensive DNA fragmentation via apoptosis or necrosis (35). Whether cluvenone induces DNA damage is yet to be determined. However, the results of the Agilent Whole Genome array analysis did not indicate increased expression, at either time point, of genes encoding DNA repair enzymes including but not limited to RAD17, RAD51, PARP1,2, POL B, POLG typically induced upon DNA damage (36). Furthermore, neither cluvenone nor GA map to a region to which known DNA damage inducing agents map on a SOM.

The mitochondria have recently emerged as an important target for cancer therapy due to the bio-energetic differences between normal and cancer cell mitochondria (37). In addition, the mitochondria are an invariant target common to all cells and in which mutations are very rare (37). The rare mutation event in mitochondria suggests a low probability for the development of resistance to agents targeting mitochondria. It is well established that many mitocans target components of the electron redox chain, leading to ROS production and activation of the intrinsic pathway of apoptosis in cancer cells. Examples of such drugs include the vitamin E analog, α -TOS, and N-(4-hydroxyphenyl) retinamide (4-HPR, fenretinide). The pro-oxidant mechanism of 4-HPR activity has not been clearly identified, although it is likely to act as an inhibitor of at least one of the complexes along the electron transport chain (38). We anticipate cluvenone to be a member of this class of mitocans since it appears to induce oxidative stress resulting in increased expression of caspase 9 (characteristic of intrinsic apoptosis pathway) and apoptosis. Furthermore, the slow induction of ROS by cluvenone, suggesting the presence of certain events prior to the formation of ROS, is consistent with the release of ROS as a consequence of disruption of the mitochondria. Future work is aimed at investigating this possibility.

In addition to the induction of oxidative cell stress, the results of our gene expression array data indicate the induction of several genes associated with T-cell activation and function, as well as NK cell function. These genes include those encoding adhesion molecules expressed in resting and activated T-cells (integrin alpha 5, integrin beta 2) as well as a differentiation marker (CD69), and ITK, a kinase involved in regulating NK cell mediated cytotoxicity (39). These results suggest that cluvenone may have significant immune-regulatory functions that may contribute to its anti-cancer activity. Previous reports on the effects of GA on immune function support our findings. For instance, in one study, GA was found to activate T lymphocytes to induce cancer cell apoptosis in H22 transplanted mice thereby inhibiting tumor growth and extending survival (40).

Clearly, cluvenone, like GA and other natural products, has multiple actions including induction of cellular stress and apoptosis, modulation of immune function, possibly resulting in T- and NK cell mediated anti-cancer responses. It is likely that additional mechanisms exist that contribute to the anti-cancer effects of cluvenone as several genes with unknown function are modulated by cluvenone. Hence, cluvenone appears to be a multi-target anti-cancer agent with apparent tumor selectivity and ability to circumvent known mechanisms of chemoresistance. These findings demonstrate the clinical potential of cluvenone as a chemotherapeutic agent and support further pre-clinical investigation. Importantly, however, the identification of the multiple targets of cluvenone will be critical to its development as a chemotherapeutic agent and may also lead to the discovery of even more effective anti-cancer agents.

Acknowledgments

We gratefully acknowledge The Developmental Therapeutics Program of the NCI for conducting screening of cluvenone against the NCI60 cell panel. In addition, we like to acknowledge Dr. James Zapf for his assistance in generating the SOM with positions of molecules having similar mechanism(s) of action to cluvenone.

This work was supported by an NIH grant (CA 133002) awarded to Emmanuel Theodorakis and, in part, by a fund from Academia Sinica (A. L. Yu). The gene expression profiling studies were conducted at the University of South Carolina DNA Microarray Facility, supported in part by NIH Grant Number P20 RR-016461 from the National Center for Research Resources.

References

1. Ollis WD, Redman BT, Sutherland IO, Jewers K. The constitution of bronianone. *J Chem Soc Chem Commun* 1969:879–880.
2. Kumar P, Baslas RK. Phytochemical and biological studies of the plants of the genus *Garcinia*. *Herba Hungarica* 1980;19:81–91.
3. Asano J, Chiba K, Tada M, Yoshii T. Cytotoxic xanthenes from *Garcinia hanburyi*. *Phytochemistry* 1996;41:815–820. [PubMed: 8835458]
4. Zhang HZ, Kasibhatla S, Wang Y, et al. Discovery, characterization and SAR of gambogic acid as a potent apoptosis inducer by a HTS assay. *Bioorg Med Chem* 2004;12:309–317. [PubMed: 14723951]
5. Zhao L, Guo QL, You QD, Wu ZQ, Gu HY. Gambogic acid induces apoptosis and regulates expressions of Bax and Bcl-2 protein in human gastric carcinoma MGC-803 cells. *Biol Pharm Bull* 2004;27:998–1003. [PubMed: 15256729]
6. Yang Y, Yang L, You QD, et al. Differential apoptotic induction of gambogic acid, a novel anticancer natural product, on hepatoma cells and normal hepatocytes. *Cancer Lett* 2007;256:259–66. [PubMed: 17693016]
7. Guo QL, You QD, Wu ZQ, Yuan ST, Zhao L. General gambogic acids inhibited growth of human hepatoma SMMC-7721 cells in vitro and in nude mice. *Acta Pharmacol Sin* 2004;25:769–774. [PubMed: 15169630]
8. Qiang L, Yang Y, You QD, et al. Inhibition of glioblastoma growth and angiogenesis by gambogic acid: An in vitro and in vivo study. *Biochem Pharmacol* 2008;75:1083–1092. [PubMed: 18070617]

9. Zhou ZT, Wang JW. Phase I human tolerability trial of gambogic acid. *Chinese J New Drugs* 2007;16:79–83.
10. Tisdale EJ, Vong BG, Li H, Kim SH, Chowdhury C, Theodorakis EA. Total Synthesis of Secolateriflorone. *Tetrahedron* 2003;59:6873–6887.
11. Tisdale EJ, Slobodov I, Theodorakis EA. Unified Synthesis of Caged Garcinia Natural Products Based on a Site-Selective Claisen/Diels-Alder/Claisen Rearrangement. *Proc Nat Acad Sci USA* 2004;101:12030–12035. [PubMed: 15210986]
12. Batova A, Lam T, Wascholowski V, Yu A, Giannis A, Theodorakis EA. Synthesis and evaluation of caged Garcinia xanthonoids. *Org Biomol Chem* 2007;5:494–500. [PubMed: 17252132]
13. Chantarasriwong O, Cho WC, Batova A, et al. Evaluation of the pharmacophoric motif of the caged Garcinia xanthonoids. *Org Biomol Chem* 2009;7:4886–94. [PubMed: 19907779]
14. Draghici S, Khatri P, Tarca AL, et al. A systems biology approach for pathway level analysis. *Genome Research* 2007;17:1537–1545. [PubMed: 17785539]
15. Batova A, Shao LE, Diccianni MB, et al. The histone deacetylase inhibitor AN-9 has selective toxicity to acute leukemia and drug-resistant primary leukemia and cancer cell lines. *Blood* 2002;100:3319–24. [PubMed: 12384433]
16. Rabow AA, Shoemaker RH, Sausville EA, Covell DG. Mining the National Cancer Institute's tumor-screening database: Identification of compounds with similar cellular activities. *J Med Chem* 2002;45:818–840. [PubMed: 11831894]
17. Covelle DG, Wallquist A, Huang R, Thanki N, Rabow AA, Lu XJ. Linking tumor cell cytotoxicity to mechanism of drug action: An integrated analysis of gene expression, Small-molecule screening and structural databases. *Proteins* 2005;59:403–433. [PubMed: 15778971]
18. Wu ZQ, Guo QL, You QD, Zhao L, Gu HY. Gambogic acid inhibits proliferation of human lung carcinoma SPC-A1 cells in vivo and in vitro and represses telomerase activity and telomerase reverse transcriptase mRNA expression in the cells. *Biol Pharm Bull* 2004;27:1769–1774. [PubMed: 15516720]
19. Yu J, Guo QL, You QD, et al. Repression of telomerase reverse transcriptase mRNA and hTERT promoter by gambogic acid in human gastric carcinoma cells. *Cancer Chemother Pharmacol* 2006;58:434–443. [PubMed: 16470410]
20. Owuor ED, Kong AN. Antioxidants and oxidants regulated signal transduction pathways. *Biochem Pharmacol* 2002;64:765–70. [PubMed: 12213568]
21. Javid B, MacAry PA, Lehner PJ. Structure and function: heat shock proteins and adaptive immunity. *J Immunol* 2007;179:2035–40. [PubMed: 17675458]
22. Byun J, Logothetis CJ, Gorlov IP. Housekeeping genes in prostate tumorigenesis. *Int J Cancer* 2009;125:2603–8. [PubMed: 19551858]
23. Kirshner JR, He S, Balasubramanyam V, Kepros J, Yang CY, Zhang M. Elesclomol induces cancer cell apoptosis through oxidative stress. *Mol Cancer Ther* 2008;7:2319–27. [PubMed: 18723479]
24. Sumbayev VV, Yasinska IM. Role of MAP kinase-dependent apoptotic pathway in innate immune responses and viral infection. *Scand J Immunol* 2006;63:391–400. [PubMed: 16764692]
25. Waterman M. Expression of lymphoid enhancer factor/T-cell factor proteins in colon cancer. *Curr Opin in Gaster* 2002;18:53–59.
26. Cronauer MV, Schulz WA, Ackermann R, Burchardt M. Effects of WNT/beta-catenin pathway activation on signaling through T-cell factor and androgen receptor in prostate cancer cell lines. *Int J Oncol* 2005;26:1033–40. [PubMed: 15753999]
27. Waterman M. Lymphoid enhancer factor/T cell factor expression in colorectal cancer. *Cancer and Metastasis Reviews* 2004;23:41–52. [PubMed: 15000148]
28. He TC, Sparks AB, Rago C, et al. Identification of c-myc as a target of the APC pathway. *Science* 1998;281:1509–1512. [PubMed: 9727977]
29. Chen S, Guttridge DC, You Z, et al. Wnt-1 Signaling Inhibits Apoptosis by Activating b-Catenin/T Cell Factor-mediated Transcription. *J Cell Biol* 2001;152:87–96. [PubMed: 11149923]
30. Sancho D, Gómez M, Sánchez-Madrid F. CD69 is an immunoregulatory molecule induced following activation. *Trends Immunol* 2005;26:136–40. [PubMed: 15745855]

31. Nie F, Zhang X, Qi Q, et al. Reactive oxygen species accumulation contributes to gambogic acid-induced apoptosis in human hepatoma SMMC-7721 cells. *Toxicology* 2009;260:60–7. [PubMed: 19464570]
32. Zhai D, Jin C, Shiao CW, Kitada S, Satterthwait AC, Reed JC. Gambogic acid is an antagonist of antiapoptotic Bcl-2 family proteins. *Mol Cancer Ther* 2008;7:1639–46. [PubMed: 18566235]
33. Rong JJ, Hu R, Song XM, et al. Gambogic acid triggers DNA damage signaling that induces p53/p21(Waf1/CIP1) activation through the ATR-Chk1 pathway. *Cancer Lett*. 2010 Apr 20; Epub ahead of print.
34. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 2003;421:499–506. [PubMed: 12556884]
35. Olive PL, Banáth JP. The comet assay: a method to measure DNA damage in individual cells. *Nature Protocols* 2006;1:23–29.
36. Lieberman HB. DNA Damage Repair and Response Proteins as Targets for Cancer Therapy. *Current Medicinal Chemistry* 2008;15:360–367. [PubMed: 18288990]
37. Ralph SJ, Rodríguez-Enríquez S, Neuzil J, Moreno-Sánchez R. Bioenergetic pathways in tumor mitochondria as targets for cancer therapy and the importance of the ROS-induced apoptotic trigger. *Mol Aspects Med* 2010;31:29–59. [PubMed: 20026172]
38. Ralph SJ, Neuzil J. Mitochondria as targets for cancer therapy. *Mol Nutr Food Res* 2009;53:9–28. [PubMed: 19123183]
39. Khurana D, Arneson LN, Schoon RA, Dick CJ, Leibson PJ. Differential regulation of human NK cell-mediated cytotoxicity by the tyrosine kinase Itk. *J Immunol* 2007;178:3575–82. [PubMed: 17339454]
40. Gu H, You Q, Liu W, et al. Gambogic acid induced tumor cell apoptosis by T lymphocyte activation in H22 transplanted mice. *Int Immunopharmacol* 2008;8:1493–502. [PubMed: 18573352]

Abbreviations

SOM	self organizing map
PBMC	peripheral blood mononuclear cells
ALL	acute lymphoblastic leukemia

Figure 1.

Structure of cluvenone (inset) and induction of apoptosis by cluvenone in CEM cells. CEM cells were plated into a 96-well plate at 10,000 cells well and were treated with 0.1% DMSO or Cluvenone at increasing concentrations. After incubation at 37° C for 7 h, apoptosis was measured using the Cell Death Detection ELISAPLUS kit. All conditions were conducted in triplicate. Error bars represent standard deviation.

Figure 2.

Cluvenone induces potent inhibition of proliferation in multiple solid and non-solid tumor cells. NCI60 cell lines were treated with increasing concentrations of cluvenone for 48 h and total cell protein was then determined by Sulforhodamine B (SRB) staining and GI₅₀ was determined.

Figure 3. Cluvenone has selective toxicity against leukemia cells compared PBMC form normal donors. Primary B-ALL cells and PBMC obtained from a normal donor were treated with increasing concentrations of cluvenone for 48 h and then viable cell numbers were determined by counting in a hemocytometer. All conditions were conducted in triplicate. Error bars represent standard deviation. ●, B-ALL; ▲, PBMC.

Figure 4.

GA and the top 6 ranked compounds in NCI data base with similar anti-proliferation activity profile to cluvenone map to the Q-region of a self organizing map (SOM). The NCI's COMPARE program was utilized to evaluate the correlation between the cytotoxicity profile (GI₅₀) of cluvenone and other compounds in the NCI chemical database. In addition, 3D Mind tools (<http://spheroid.ncicrf.gov>) was used to determine where the top ranked compounds mapped on a SOM. **A**, Structures of top 6 ranked compounds with NCI data base identifier number followed by numbers in parentheses corresponding to positions on the SOM. **B**, Self organizing map with the positions of top ranked compounds relative to those of known agents.

Figure 5.

Cluvenone induces cell stress via ROS formation in PC3 cells. **A**, Levels of intracellular ROS were measured by a fluorescence assay in cells treated with up to 1 μ M cluvenone or with 0.1% DMSO (- control) for 5.5 h. In addition, cells were treated with increasing concentrations of H₂O₂ as a control for ROS induction. Fluorescence was measured with excitation/emission at 480/530 nM. $\square$, cluvenone; $\blacksquare$, H₂O₂ **B**, Cytotoxicity of cluvenone (1 μ M) in PC3 cells was measured in the presence of increasing concentrations of N-acetylcysteine after a 48 h incubation in a ³H-thymidine incorporation assay.

Table 1

Cell signaling pathways modulated by cluvenone. Probes that achieved a significance of $P=0.005$ or less in the ANOVA analysis with Benjamin-Hochberg correction were subjected to pathway analysis using the Pathway-Express Tool created by Sorin Draghici at Wayne State University and available at <http://vortex.cs.wayne.edu/ontoexpress>. The genetic loci corresponding to each probe together with the fold-change at both the 2 h (**A**) and the 4.5 h (**B**) time point were analyzed to assess affected pathways.

A	Pathway	Impact Factor	P value	Genes (fold change)
	Adherens Junction	21.8	8.040×10^{-9}	TCF7(-1.7)
	MAPK	20.5	2.766×10^{-8}	HSPA1A(26.9), MAPK14(1.7)
	Antigen Processing and Presentation	9.6	6.968×10^{-4}	HSPA1A(26.9), HSP90AA1(2.6), HSP90AB1(2.2)
	Regulation of Actin Cytoskeleton	7.1	6.867×10^{-3}	ITGB2(1.8), ITGAV(2.4)
	Prostate Cancer	6.5	1.065×10^{-2}	TCF7(-1.7), HSP90AB1(2.2), HSP90AA1(2.6), CASP9(1.7)
	Pathways in Cancer	5.6	2.292×10^{-2}	MSH2(-1.7), CASP9(1.7), HSP90AB1(2.2), TCF7(-1.7), ITGAV(2.4), CEBPA(-1.8), HSP90AA1(2.6)

B	Pathway	Impact Factor	P value	Genes(fold change)
	MAPK	23.8	1.197×10^{-9}	HSPA1A(45.1), HSPA8(2.9), CASP3(-1.8)
	Antigen Processing and Presentation	11.5	1.214×10^{-4}	HSP90AB1(2.0), HSPA1A(45.1), HSP90AA1(3.4), HSPA8(2.9)
	Regulation of Actin Cytoskeleton	8.3	2.376×10^{-3}	ITGAV(2.8)
	Protein Export	6.0	1.716×10^{-2}	OXA1L(2.3), SRP9(1.8)