Research Article

Cucurbitacin L 2-O-β-Glucoside Demonstrates Apoptogenesis in Colon Adenocarcinoma Cells (HT-29): Involvement of Reactive Oxygen and Nitrogen Species Regulation

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Emerging evidence suggests that reactive oxygen (ROS) and nitrogen (RNS) species can contribute to diverse signalling pathways of inflammatory and tumour cells. Cucurbitacins are a group of highly oxygenated triterpenes. Many plants used in folk medicine to treat cancer have been found to contain cucurbitacins displaying potentially important anti-inflammatory actions. The current study was designed to investigate the anti-ROS and -RNS effects of cucurbitacin L 2-O-β-glucoside (CLG) and the role of these signaling factors in the apoptogenic effects of CLG on human colon cancer cells (HT-29). This natural cucurbitacin was isolated purely from Citrullus lanatus var. citroides (Cucurbitaceae). The results revealed that CLG was cytotoxic to HT-29. CLG increased significantly ($P < 0.05$) RNA and protein levels of caspase-3 in HT-29 cells when verified using a colorimetric assay and real-time qPCR, respectively. The results showed that lipopolysaccharide/interferon-gamma (LPS/INF-γ) increased nitrous oxide (NO) production in RAW264.7 macrophages, whereas N(G)-nitro-L-argininemethyl ester (L-NAME) and CLG curtailed it. This compound did not reveal any cytotoxicity on RAW264.7 macrophages and human normal liver cells (WRL-68) when tested using the MTT assay. Findings of ferric reducing antioxidant power (FRAP) and oxygen radical absorption capacity (ORAC) assays demonstrate the antioxidant properties of CLG. The apoptogenic property of CLG on HT-29 cells is thus related to inhibition of reactive nitrogen and oxygen reactive species and the triggering of caspase-3-regulated apoptosis.

1. Introduction

The attractive association between chronic inflammation and cancer has been a fertile field for the growth of biomedical research. In particular, the development of colon cancer is a distinctive situation in which inflammatory conditions such as ulcerative colitis increase the risk of cancer by 20-fold [1, 2]. The presence of certain inflammation markers, such as the C-reactive protein circulating in the blood, is correlated with an increased risk of colon cancer [3]. In addition, over-expression of proinflammatory enzymes, such as inducible nitric oxide synthase and cyclooxygenase-2, has been
reported in human colon cancer and in an aoxymethane-induced colon cancer model in rats [4, 5]. More importantly, 
selective inhibitors of these inflammatory genes are effective 
in inhibiting experimental colon cancer of rodents [6, 7].

Epithelial cells express reactive nitrogen and oxygen 
radicals (free radicals) in response to inflammatory cytokines 
and the bacterial endotoxins. The initiation by NADPH 
(nicotinamide adenine dinucleotide phosphate) oxidase is 
always required for the production of free radicals. These 
free radicals were activated upon translocation of several 
sytosolic proteins to the membrane-bound complex carrying 
proteins. Moreover, the activation of NADPH oxidase 
can be caused by microbial products such as lipopolysac-
charide and lipoproteins, by IFN-γ (interferon-gamma), by 
IL-8 (interleukin-8), or by IgG binding to Fc-receptors. The 
primary product of the reaction catalyzed by the NADPH 
(3, (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), phosphate buffered saline (PBS), and Griess reagent were from Invitrogen (Carlsbad, USA). Fetal bovine serum (FBS), LPS from E. coli serotype 0111:B4, L-NAME, dimethylsulfoxide (DMSO), and 
sodium nitrite were obtained from Sigma (St. Louis, USA). IFNy was from BD Biosciences (New Jersey, USA). All other 
chemicals and reagents used were of HPLC grade.

2. Materials and Methods

2.1. Cell Lines and Reagents. All cell lines were obtained from American type culture collection (ATCC). Dulbecco’s Mod-
ified Eagle Medium (DMEM) both with and without phenol 
red, phosphate buffered saline and Hanks’ balanced salt 
solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-

tetrazoliumbromide (MTT), phosphate buffered saline (PBS), 
and Griess reagent were from Invitrogen (Carlsbad, USA). Fetal 
bovine serum (FBS), LPS from E. coli serotype 0111:B4, L-NAME, dimethylsulfoxide (DMSO), and 
sodium nitrite were obtained from Sigma (St. Louis, USA). IFNy 
was from BD Biosciences (New Jersey, USA). All other 
chemicals and reagents used were of HPLC grade.

2.2. Isolation of Cucurbitin L 2-O-β-Glucoside. Citrullus 
lanatus var. citroides was collected from AL-Musawwarat area, 
Northern Sudan, on February 2008. The voucher specimen 
was identified by Dr. Wail S. Abdalla, a senior botanist at 
the herbarium of Medicinal and Aromatic Plants Research 
Institute (MAPRI), Khartoum, Sudan, where the specimen 
was also deposited and coded with CL2-8. CLG was isolated 
from the dried fruit pulp according to the method described 
earlier [20]. The structure of this compound was established 
by spectroscopic methods and by comparison with the 
previous reported works [20–22]. Purity of the compound 
was found to be 98.5% using LC/MS.

2.3.3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bro-
mide Cell Viability Assay on Normal and Cancer Cells. 
All the cells were maintained in a 37°C incubator with 
5% CO2 saturation. Human hepatocellular carcinoma cells 
(HepG2), colon adenocarcinoma cells (HT-29), and normal 
hepatic cells (WRL-68) were maintained in Dulbecco’s 
modified Eagle’s medium (DMEM), whereas non–small-cell 

tumor cell lines (A549) and prostate adenocarcinoma cells 
(PC3) were maintained in RPMI-1640 medium. Both media 
were supplemented with 10% fetal bovine serum (FBS), 
100 units/mL penicillin, and 0.1 mg/mL streptomycin.

Different cell types from above were used to determine 
the cytotoxic effects of CLG and paclitaxel on cancer and 
normal cells using the MTT assay. For measurement of cell 
viability, cells were seeded at a density of 1 × 10^5 cells/mL 
in a 96-well plate and incubated for 24 h at 37°C and 5% 
CO2. Cells were treated and incubated for 24 h. After 24 h, 
MTT solution at 2 mg/mL was added for 4 h. Absorbance 
was measured at 570 nm. Results were expressed as a percentage 
of control giving percentage cell viability after 24 h exposure 
to test agents. The potency of cell growth inhibition for the 
tested compounds was expressed as an EC_{50} value, defined 
as the concentration that caused a 50% loss of cell growth.
Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

2.4. Chromatin Condensation Assay. For detection of apoptotic cells, apoptotic nuclear morphology was observed by staining with Hoechst 33342. Cells at a density 1 × 10^5 cells/mL were seeded on a 96-well culture plate. Cells were treated with IC_{50} of CLG and incubated for 24 and 48 h.

2.4.1. Colorimetric Assays of Caspase-3. The colorimetric protease assay of caspase-3 provides a simple and convenient method for quantifying the enzyme activity that recognizes the amino acid sequence, DEVD (A synthetic tetrapeptide, (Asp-Glu-Val-Asp), which is the upstream amino acid sequence of the caspase-3 (cleavage site), coupled with p-nitroanilide, which is released upon substrate cleavage. This assay was performed using the commercial kit ApoTarget (Code: KHZ0022; BioSource International, Inc., USA). 1 × 10^5 cells/mL were treated with IC_{50} of CLG and incubated for 24 and 48 h while untreated cells acted as control.

2.5. Real-Time Polymerase Chain Reaction. Total RNA was extracted using RNeasy Mini Kit following the manufacturer's instructions (Qiagen, Germantown, Maryland, USA). RNA concentrations were quantified using a spectrophotometer (Smart Spec, Bio-Rad). RNA quality and integrity were determined via the A260/A280 ratio and agarose gel electrophoresis, respectively. cDNA was synthesized with Revert Aid H Minus M-muLV reverse transcriptase (Biometra, Goettingen, Germany). Real-time RT-PCR was performed using an ABI 7700 Prism Sequence Detection System and TaqMan primer probes (Applied Biosystems, Foster City, CA). The total reaction volume was 20 μL containing 2 μL cDNA, 10 μL SYBR Premix ExTaq, 0.4 μL of each primer (10 μM; caspase-3 primer; sense, 5'-TIAATAAGGG-TACCCATGGAGAACACT-3'; antisense, 5'-TTAGTGATAAACATGATTCTTGGAG-3'), and 7.2 μL ultrapure water. Cycle parameters were as follows: activation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, and then annealing and extension at 60°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as an internal control for each sample. The primers for GAPDH were 5'-GGTTGCTCTCCTGTGACTCCAACA-3' (sense) and 5'-GCTTGGGTAGCCAAATTCTGGTG-3' (antisense). PCR products were detected using gel electrophoresis.

2.7. Effect of CLG on Nitric Oxide Production

2.7.1. Cell Culture and Stimulation. The murine monocytic macrophages cell line (RAW 264.7) was maintained in DMEM supplemented with 10% FBS, 4.5 g/L glucose, sodium pyruvate (1 mM), L-glutamine (2 mM), streptomycin (50 μg/mL), and penicillin (50 U/mL) at 37°C and 5% CO_{2}. Cells at confluency of 80–90% were centrifuged at 120 × g at 4°C for 10 min, and cell concentration was adjusted to (2 × 10^6) cells/mL, whereby the cell viability was always more than 90%, as determined by trypan blue exclusion. A total of 50 μL of cell suspension was seeded into a tissue culture grade 96-well plate (4 × 10^5 cells/well) and incubated for 2 h at 37°C, 5% CO_{2} for cell attachment. Then, the cells were stimulated by using 100 U/mL of IFN-γ and 5 μg/mL of LPS with or without the presence of CLG and L-NAME (250 μM) tested at the final volume of 100 μL/well. DMSO was used as vehicle, where the final concentration of DMSO was maintained at 0.1% of all cultures.

2.7.2. Griess Assay. To evaluate the inhibitory activity of CLG and L-NAME on nitric oxide (NO) production, culture media was assayed using Griess reaction [23]. Briefly, an equal volume of Griess reagent (1% sulphanilamide and 0.1% N-(1-naphthyl)-ethylene diaminedihydrochloride, dissolved in 2.5% H_{3}PO_{4}) was mixed with the culture supernatant, and color development was measured at 550 nm using a microplate reader (SpectraMax Plus, Molecular Devices Inc., Sunnyvale, CA, USA). The amount of nitrite in the culture supernatant was calculated from a standard curve (0–100 μM) of sodium nitrite freshly prepared in deionized water. Percentage of the NO inhibition was calculated by using nitrate level of IFN-γ/LPS-induced group as the control,

\[ \text{NO inhibitory (\%)} = \left( \frac{[\text{NO}_2^-]_{\text{sample}} - [\text{NO}_2^-]_{\text{control}}}{[\text{NO}_2^-]_{\text{control}}} \right) \times 100\%. \]  

2.7.3. Cell Viability of RAW 264.7 Macrophage. The cytotoxicity of CLG on cultured cells was determined by assaying the reduction of MTT reagents to formazan salts [24]. After removing the supernatant, the MTT reagents (5 mg/mL, dissolved in sterile PBS, pH 7.0) were added into each well. The cells remaining in the well were tested for cell viability 4 h, and the formazan salts formed were dissolved by adding 100 μL of 100% DMSO in each well. The absorbance was then measured at 570 nm using SpectraMax Plus microplate reader (Molecular Devices, USA). The percentage of cell viability was calculated by using the cell viability of IFN-γ/LPS-induced group as the control,

\[ \text{cell viability (\%)} = \left( \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \right) \times 100\%. \]
Table 1: Effect of CLG and paclitaxel on different cells type expressed as EC<sub>50</sub> values in MTT assay.

| Cell line | Tissue of human origin | Compound EC<sub>50</sub> ± SD (µg/mL) |
|-----------|------------------------|----------------------------------------|
| A549      | Non-small-cell lung cancer | >200                                   |
| PC-3      | Human prostate carcinoma | >200                                   |
| HepG2     | Hepatocellular carcinoma | >200                                   |
| HT-29     | Colon adenocarcinoma     | 79.76 ± 2.34                           |
| WRL-68    | Normal hepatic cells     | >200                                   |
|           |                        | 5.81 ± 1.03                            |
|           |                        | 0.08 ± 0.03                            |
|           |                        | 1.18 ± 0.24                            |
|           |                        | 0.06 ± 0.02                            |
|           |                        | 0.10 ± 0.05                            |

3. Results

3.1. Cytotoxicity of CLG. The panel of cell lines used in this study were human hepatocellular carcinoma cells (HepG2), prostate adenocarcinoma cells (PC3), colon adenocarcinoma cells (HT-29), non-small-cell lung cancer cells (A549), and normal hepatic cells (WRL-68). The cytotoxicity assay (MTT) performed in this study revealed that the CLG had demonstrated a dose-dependent effect on HT-29. The EC<sub>50</sub> value (the sample concentration reducing the absorbance of treated cells by 50% with respect to untreated cells) of CLG on the viability of HT-29 has been determined to be 79.76 ± 2.34 µg/mL (Table 1). All cells were observed to be resistant to CLG. Fortunately, CLG did not produce any toxic effects on normal hepatic cells until the concentration of 200 µg/mL. Paclitaxel showed high indiscriminate cytotoxicity to all cells used in this study.

3.2. Chromatin Condensation Assay. We then investigated apoptosis in cells that had been incubated with CLG using the DNA staining dyes Hoechst 33342. After a 24-hour incubation with CGL, the percentage of apoptotic cells was significantly increased compared with the control (medium alone) (P < 0.05). Following incubation of cells with CGL for 72 h, the apoptosis was also significantly increased (P < 0.05). The increased percentage of fluorescent intensity is the direct representation of chromatin condensation in the nucleus (Figures 1(a) and 1(b)). Figure 1(c) summarizes the results of CGL-dependent apoptosis, that is, the percentage of apoptosis above control levels.

3.3. Colorimetric Assay of Caspase-3. Since HT-29 has shown remarkable sensitivity to CLG, an in vitro colorimetric assay of caspase-3 was conducted to assess apoptosis between control and treated cells. As shown in Figure 2, CLG significantly (independent t-test, P < 0.05) stimulated caspase-3, the hallmark enzyme of apoptosis. The level of this enzyme is higher in treated HT-29 cells as compared to nontreated cells; this concludes that CLG induces cell death towards human colon cancer cells, HT-29.

3.4. Gene Expression. To examine whether the apoptotic effect of CLG is due to intervention with caspase-3-regulated apoptosis, mRNAs of HT-29 cells were studied using qRT-PCR. Caspase-3 mRNA level was significantly increased in cells exposed to CLG compared to levels in control cells (Figure 3).

3.5. Effect of CLG on Nitric Oxide from RAW 264.7 Macrophage. Stimulation with LPS and IFN-γ led to fortyfold
increase in nitrite concentrations in the cell supernatant with a concentration of 39.76 ± 1.2 μM as compared to the basal level of 1.0 ± 0.05 μM in untreated cells (Figure 4). NO release was effectively inhibited by CLG with an IC₅₀ of 26.5 ± 1.81 μM. Following 20 h of treatment and stimulation, CLG caused a 90% inhibition of NO at 75 μM, without affecting the cell viability (Figure 5). L-NAME, as a positive control anti-NO drug, caused an 84% inhibition of NO at 250 μM.

3.6. ORAC Antioxidant Activity Assay. To evaluate the antioxidant capacity of CLG, ORAC assay was used, and the potency of this natural compound was compared with the positive control, quercetin. The area under the curve (AUC) was calculated for the CLG, trolox, and quercetin. ORAC results are shown in Table 1. CLG displays considerable
antioxidant activity. Whereby, this compound at 20 μg/mL is equivalent to a concentration of 82.5 ± 0.53 μM of trolox. Quercetin at 5 μg/mL is equivalent to a concentration of 160.32 ± 2.75 μM of Trolox.

3.7. FRAP Assay. The total antioxidant activity was determined using FRAP assay. This assay measures the reduction of ferric iron (Fe³⁺) to ferrous iron (Fe²⁺) in the presence of antioxidants, which are reductants with half-reaction reduction potentials above Fe³⁺/Fe²⁺. On treatment, CLG exhibited a significant FRAP value, with a 241 ± 12.5 μmol/L, while the positive control used in this study exhibited a value of 350 ± 9.5 and 251 ± 5.7 for ascorbic acid and quercetin, respectively (Figure 6).

4. Discussion

The cucurbitacins are of great interest because of the wide range of biological activities they exhibit in plants and animals [20]. In the present study, we investigated for the first time the apoptogenic effects of cucurbitacin L 2-O-β-glucoside (CLG) on cancer cell (HT-29) and the involvement of reactive oxygen and nitrogen species regulation. Our results indicate that CLG is more cytoselective to colon cancer cells than the other tested cancer cells. The inhibitory effects of CLG on RNS and ROS are suggested to be the mechanism of apoptogenic effect of this natural compound. Based on our study, the anti-inflammatory and antioxidant properties of CLG may be regarded as a key attribute for its role against colon tumorigenesis.

Nitric oxide (NO) is a free radical gas with important immune, cardiovascular, and neurological second messenger functions that is implicated in sepsis, cancer, and inflammation. This molecule is synthesized from the amino acid L-arginine by a family of enzymes, the nitric oxide synthases (NOSs). NO in colonic mucosa is susceptible to manipulation by proinflammatory cytokines [27–30]. The obtained results suggest that the compound has dose-dependent anti-inflammatory activities related with their inhibition of NO production in macrophages without affecting the viability of these cells. Our results are in line with previous findings which showed that cucurbitacin compounds are able to inhibit the production of NO [27, 28].

Anticolorectal cancer agents have an inhibitory effect on nitrite production in colonic mucosa and could play an anti-inflammatory role in intestinal inflammation and malignancies [31]. NO has also been demonstrated to be involved in the inhibition of apoptosis in a number of cell types including leukocytes, hepatocytes, trophoblasts, and endothelial cells such as HT-29 [32]. Generally, the antiapoptotic effects of NO can be mediated through a number of mechanisms such as the nitrosylation and inactivation of many of the caspases including caspase 3. As shown in Figure 2, CLG significantly (independent t-test,
and antioxidant e in HT-29. Based on our studies, the anti-inflammatory regulation are very much correlated to cell death induced e cancer cells is selectively directed to HT-29. The suppressive colon tumorigenesis.

Selective apoptogenesis of potential anticancer drugs have been reported previously for various chemical entities with the aid of molecular modelling and structure activity relationship (SAR) studies [34–36]. Previous SAR study with the formation of nitrosamines may be important in the development of colorectal cancer in ulcerative colitis. High levels of nitrosamines have been demonstrated in rectal dialysates of patients with active inflammatory bowel disease [12]. Therefore, the obtained results warrant further research for the clinical application of CLG as chemopreventive agents for advanced ulcerative colitis patients.

Selective apoptogenesis of potential anticancer drugs have been reported previously for various chemical entities with the aid of molecular modelling and structure activity relationship (SAR) studies [34–36]. Previous SAR study with five cucurbitacin analogues led to a highly selective STAT3, a proliferation protein, and cucurbitacin Q; a highly selective inhibitor of JAK2 activation, cucurbitacin A; three dual inhibitors, cucurbitacin I, E, and B. From the chemical point of view, these findings indicate that addition of a single hydroxyl group to carbon 11 of the cucurbitacins results in loss of anti-STAT3 activity, whereas a simple conversion of a carbon 3 carbonyl to a hydroxyl leads to loss of anti-JAK2 activity [37].

In summary, the present study showed that the apoptogenic effects of cucurbitacin L 2-O-β-glucoside on the tested cancer cells is selectively directed to HT-29. The suppressive effects of CLG on reactive oxygen and nitrogen species regulation are very much correlated to cell death induced in HT-29. Based on our studies, the anti-inflammatory and antioxidant effects of CLG may be regarded as a key attribute for its role against chronic ulcerative colitis and colon tumorigenesis.

5. Conclusion

Cucurbitacin is one of the constituents of plants used in folk medicine. Till date, there was no study reporting the apoptogenic effects of CLG on human colon cancer cells (HT-29). The current study shows for the first time that the apoptogenic property of CLG on HT-29 cells is related to the inhibition of reactive nitrogen and oxygen species eventually leading to caspase-3-mediated apoptosis. At present, the pathways related to apoptosis are being studied in our lab.

Conflict of Interests

The authors declare no conflict of interests.

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