Apoptosis and Cytolysis Induced by Giganteosides and Hederacolchisides in HL-60 Cells

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Abstract. *The viability, cytolysis and apoptosis-mediated cellular death induced by giganteosides D and E (Gig-D and Gig-E) and hederacolchisides A and A1 (Hcol-A and Hcol-A1) were analysed in HL-60 cells. Materials and Methods: The end-point metabolic (WST1) and lactate dehydrogenase (LDH) assays were used. Cell cycle analysis and apoptosis were measured by flow cytometry, DNA laddering and caspase-3 analyses. Results: the HL-60 cell line was more sensitive to Hcol-A1 and Gig-D (IC_{50} 3-5 μM) than to Gig-E and Hcol-A (IC_{50} 8-13 μM; WST1 assay). This was related to LDH release. The induction of apoptosis could be detected without caspase-3 activation after 24 h of treatment. DNA fragmentation could be detected only with Gig-D. With Hcol-A1 and Gig-D, an accumulation of cells in the S-phase and an increase of cells in sub-G1 peak were observed. By the annexinV-fluorescein isothiocyanate (FITC)/7-amino-actinomycin D (AAD) assay, the majority of cells were in late apoptosis with Gig-D, and in necrosis with Hcol-A1. Conclusion: Hcol-A1 is more cytotoxic than Gig-D, followed by Gig-E and finally Hcol-A. This is related to a membrane permeabilization effect, leading to cytolysis.*

In this work, saponins extracted from two plants: *Cephalaria gigantea L.* (Dipsacaceae) and *Hedera colchica* K. Koch (Araliaceae) were studied. *Hedera colchica* K. Koch, an ivy species endemic in Georgia, has been traditionally used as a bronchospasmolytic, secretolytic and an anti-inflammatory remedy (1). Species from *Cephalaria* have been used as folk medicines for their anti-infectant, hypothermic, alleviative and relaxant activities (2). They contain a great variety of compounds with pharmaceutical activity such as alkaloids, iridoids, flavonoids and triterpenoid saponins (3). Several bidesmosidic or monodesmosidic triterpene saponins have been isolated from this plant (1-4). The biological activity of giganteosides D and E (Gig-D and Gig-E) and hederacolchisides A and A1 (Hcol-A and Hcol-A1), have shown interesting antileishmanial, antifungal and antiprotozoal activities modulated by the nature of the aglycone, as well as by the number, the sequence and the type of sugar residue (5-7).

Saponins are not yet used in human chemotherapy, even though some of them have been demonstrated to have anti-tumor activities *in vivo* (8-11) and antiproliferative activity against neoplastic cells *in vitro* (12-14). The apoptotic activity of triterpene saponins in leukemia HL-60 cells has been demonstrated (15-17). Some saponins are able to induce apoptosis and necrosis (15). Interesting antiproliferative effects on human carcinoma cell lines have been demonstrated with Hcol-A1 (IC_{50} values from 4.5 to 12 μM) and early membrane damaging effects were the main cause of toxicity (18). Gig-D and Gig-E have also shown promising antiproliferative effects on human MEL-5 and leukemia HL-60 cells with IC_{50} values in the range 3.15-7.5 μM (19).

In this work, the cytotoxic properties of Gig-D and Gig-E were compared to those of Hcol-A and Hcol-A1 using leukemia HL-60 cells. Cell growth/viability was determined by the end-point metabolic WST1 assay and membrane permeabilization was detected by the lactate dehydrogenase (LDH) release assay. Apoptosis-mediated cellular death induced by the saponins was also evaluated.

Materials and Methods

Drugs. Isolation from the root of *Cephalaria gigantea* or from the leaves of *Hedera colchica* K. Koch and purification of the active compounds were carried out as described in detail previously (1, 7, 20).

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Sterile stock solutions were made in DMSO (100 μg/100 μl). Freshly prepared solutions were then diluted in culture media at various concentrations (1 to 26 μM) and used immediately. In non-treated cells, the same final concentration of DMSO (0.5%) was added to the culture medium. Camptothecin (Sigma-Aldrich, Bornem, Belgium; 1 mM stock solution) was used as positive control for apoptosis detection.

Cell line. Human leukemia HL-60 cells (ATCC CCL 240) were cultured in T25 culture flasks containing 8 ml of RPMI-1640 supplemented with 10% heat inactivated (1 h at 57˚C) fetal bovine serum (FBS), 1% L-glutamine, 1% non-essential amino-acids, 1% sodium pyruvate (stock solutions from BioWhittaker Europe, Vervier, Belgium).

Cellular viability (WST1 test). The HL-60 cells ($50x10^3$) were seeded in 200 μl of medium containing 1 to 26 μM saponins in 96 well-plates and incubated for up to 72 h. The WST1 colorimetric assay (Boerhinger, Mannheim, Germany) was applied to the cell suspensions as described previously (19). Corresponding controls with analogous concentrations of DMSO were carried out in parallel.

Cell lysis (LDH test: cytotoxicity detection kit; LDH, Boehringer Mannheim, Germany). The HL-60 cells ($50x10^3$) were treated with 1-25 μM saponin in RPMI with 1% FBS (serum contains natural LDH activity). After a 2 h incubation at 37˚C, the plates were centrifuged at 1000 rpm (Jouan; CR 1000, St. Herblain, France) for 10 min. Fifty μl aliquots of the supernatants were collected and the instructions for the commercial assay were applied as described previously (21). Controls of background level (Bc = LDH activity of the medium without cell), of low level (natural release by cell = Lc), of high level (maximum release by disrupting cells with Triton X-100 = Hc) and of the substance (in the medium without cell = Sc) were realized. The percentage of LDH release in relation to the control was calculated as: (treated mean-Lc/Hc-Lc) x100.

Optical microscopy. Living cells were observed under a Nikon inverted phase contrast microscope. For apoptotic body detection, the HL-60 cells were centrifuged with cytospin 2 (Shandon, Cheshire, UK) at 100 g for 3 min, fixed in methanol and stained with Giemsa solution.

Sub-G1 peak detection and cell cycle analysis. HL-60 cells ($1x10^6$) in exponential growth were treated with the drugs (1 to 26 μM in 1 ml medium) for 24 h. Sample preparations were carried out as described previously (21). The samples were analysed on a FACSscan flow cytometer, using Cell Quest software, and then treated with Fit mode on WinMDI 2.8 version (Becton Dickinson BD Biosciences, San Jose, USA). A total of $10x10^3$ cells were counted. The experiment was repeated twice.

Detection of phosphatidylserine residues by Annexin V-FITC/7-AAD test. This test was performed using the Annexin V-FITC/ 7-AAD kit (Beckman Coulter, Fullerton, CA, USA). One ml of cell suspension containing $1x10^6$ HL-60 cells was treated with the drugs (1 to 26 μM) for 24 h. The cells were prepared as described previously (22). The sample preparations were analysed using a flow cytometer as described above.

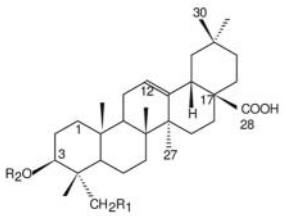
DNA laddering analysis. HL-60 cells ($1x10^6$) in exponential growth were treated with the drugs (1 to 26 μM in 1 ml medium) for 24 h. The cells were then collected into tubes, centrifuged at 170g for 5 min. The cells were washed with phosphate-buffered saline (PBS) and centrifuged at 170g for 5 min. The cells were lysed with an aqueous solution of NaCl (100 mM), TRIS-HCl (10 mM, pH 8), EDTA (25 mM, pH 8), SDS (0.5%) and proteinase K (0.2 mg/ml) at 50˚C for 3 h. One ml of lysate was extracted once with 1 ml of phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v) and twice with 1 ml of chloroform/isoamyl alcohol (24:1, v:v). The DNA was precipitated overnight at 4˚C by adding ammonium acetate to 2.5 M and 2 volumes of absolute ethanol. The DNA was isolated by centrifugation at 12,000 g for 30 min at room temperature. The pellet was washed with ethanol (70%). The dried pellet was dissolved in 100 μl of TE (10 mM TRIS-HCl [pH 8], 5 mM EDTA [pH 8]). Electrophoresis was performed on 15 μl of the DNA solution for 4 h in TBE buffer (50 mM TRIS-HCl [pH 8], 50 mM boric acid, 1 mM EDTA [pH 8]). The photographic film of the electrophoresis gel was overexposed to assure visualization of the ladder.

Caspase-3-like protease activity. HL-60 cells ($1x10^6$) in exponential growth were treated with the drugs (1 to 26 μM in 1 ml medium) for 24 h. The cells ($2x10^6$) were homogenized in 1 ml of lysis buffer (HEPES [50 mM, pH 7.4], CHAPS [0.1% w:v], DTT [1 mM], EDTA [0.1 mM], TRITON-X 100 [0.1%, w:v] and aprotinin [10 U/ml]) using a potter homogenizer. The homogenates were kept on ice for 15 min and then centrifuged at 10,000 g for 10 min at 4˚C. The supernatants were maintained at −70˚C until analysis. Before analysis, the supernatants were diluted 10 times in lysis buffer and 10 μl of this solution was combined with 10 μl of Ac-DEVD-pNA (2 mM) as substrate for caspase-3 activity. The volumes were brought to 100 μl with assay buffer (HEPES [50 mM, pH 7.4], NaCl [100 mM], CHAPS [0.1%, w:v], DTT [10 mM], EDTA [1 mM] and glycerol [10%, v:v]). The specificity of the assay was tested by addition of 10 μl of Ac-DEVD-CHO (1 μM) to the homogenate. Absorbance was monitored at room temperature for 60 min at 405 nm with a microplate reader. The results were expressed as nmol/min and g protein. Acetyl-Asp-Glu-Val-Asp-pNitroaniline (Ac-DEVD-pNa), was obtained from Alexis (Zandhoven, Belgium) and Ac-DEVD-aldehyde (Ac-DEVD-CHO) from Biomol (Exeter, UK).

Statistical analysis. Eight wells were used for each concentration, and at least two experiments were performed for each cellular assay (WST1, LDH). The statistical analysis was performed by means of the paired Student's *t*-test (*p*-values lower than 0.05 were considered as significant). The results of the cellular assays were expressed as percentages (controls taken as 100%). The determination of concentrations inducing 50% viability (IC_{50}) or 50% LDH (C_{50}) release was achieved by a regression analysis of the results at different concentrations of each drug.

Results

Cellular viability. The structure of the saponins is shown in Figure 1. Cell growth and viability were reduced in a dose-dependent manner. The concentrations of the saponins inducing 50% cell survival are given in Table I.



R_1	R_2	Name	Mw
OH	Rha^1 - $^2Rha^{1-2}Xyl^1$-^2Ara	Giganteoside E	992
H	Rha^1 -^2Xyl	Giganteoside D	734
H	Rha^1 -^{2}[Glc^1 -^{4}]Ara	hederacolchiside A1	896
OH	Rha^1 -^{2}[Glc^1 -^{4}]Ara	hederacolchiside A	912

Figure 1. *Structure of monodesmosides with rhamnose residues. R_1 = H: oleanolic acid; R_1 = OH: hederagenin; Glc = glucose; Xyl = xylose; Rha = rhamnose; Ara = arabinose.*

Table I. *Concentrations inducing 50% cellular viability (IC_{50} μM from WST1 assay) after 72 h treatment in HL-60 in RPMI 10% FBS and in the presence of monodesmosides.*

	Concentration μM
Gig-E	7.8
Gig-D	3.3
Hcol-A1	3.3
Hcol-A	11.5
Camptothecin	0.05

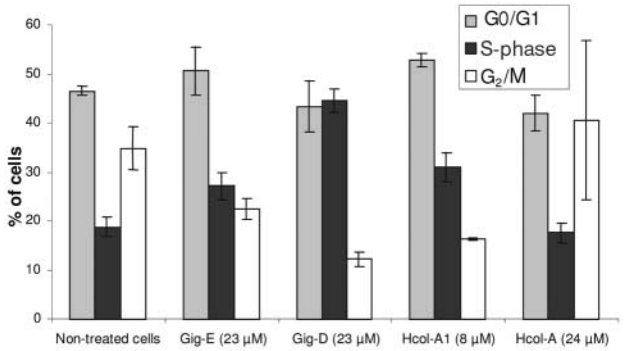


Figure 2. *Cell cycle analysis of HL-60 cells treated for 24 h with monodesmosides. 10,000 cells were counted. Means ± standard errors (n=3).*

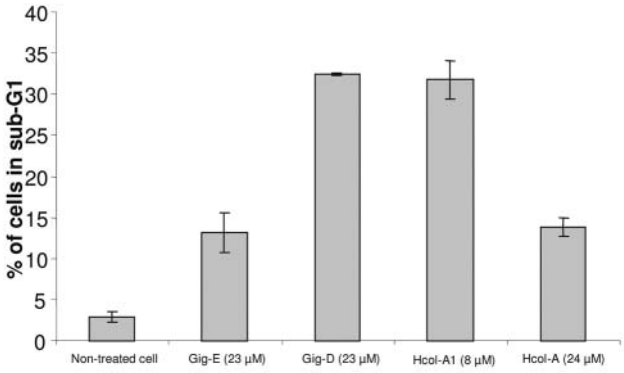


Figure 3. *Percentages of cells in sub-G1 peak in HL-60 cells non-treated or treated for 24 h with monodesmosides. 10,000 cells were counted. Means ± standard errors (n=3).*

The saponins with an oleanolic type pentacyclic aglycone were 2 to 3 times more toxic than the saponins with a hederagenin type pentacyclic aglycone. No great differences were observed between 24 h, 48 h or 72 h treatment indicating that the cytostatic effects of these compounds occurred within the first 24 h of treatment. Camptothecin was used as reference compound for inducing apoptosis and was 100x more cytotoxic than the tested saponins.

Apoptosis-mediated cell death and cell cycle analysis. Twenty-four h treatment with the test compounds was necessary to observe the formation of apoptotic bodies in the Giemsa stained cytospin HL-60 preparations. In contrast, 1 μM camptothecin induced many apoptotic bodies, which were already formed after 4 h treatment. Greater disturbances in the HL-60 cells cell cycle were observed after 24 h treatment with Gig- D and Hcol-A1 than with Gig-E and Hcol-A. An accumulation of the cells in the S-phase of the cell cycle was observed in the population of live cells (Figure 2). A sub-G1 peak appeared at concentrations 21-23 μM Gig-D or Gig-E and 6-9 μM Hcol-A1 or 21-24 μM Hcol-A (Figure 3). All cells were destroyed at higher concentrations.

At these concentrations, a high proportion of cells in the population treated with Hcol-A was identified as undergoing apoptosis (Annexin V-FITC positive, 7-AAD negative). Many cells in the population had already died with Hcol-A1 (Annexin V-FITC negative, 7-AAD positive) or were in late apoptosis with Gig-D (Annexin V-FITC positive, 7-AAD positive; Figure 4). The sub-G1 peak thus corresponded to late apoptotic cells for Gig-D and to necrosis for Hcol-A1. The degradation of nuclear DNA into nucleosomal units was observed only with Gig-D (Figure 5). No caspase-3 activity could be detected with the four saponins.

LDH release. Membrane permeabilization detected by analyzing LDH release into the culture medium was found to be dose-dependent. Concentrations inducing 50% (C_{50}) release of LDH into the medium from the HL-60 cells were in the same range as the IC_{50} concentrations (Table II).

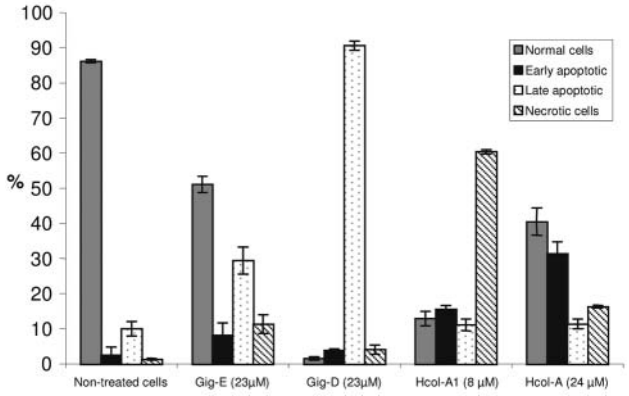


Figure 4. *Determination of necrotic vs. apoptotic cells by Annexin-V and 7-AAD assay in 10,000 non-treated or monodesmoside treated HL-60 cells after 24 h. Normal cells are living cells. Means ± standard errors (n=3).*

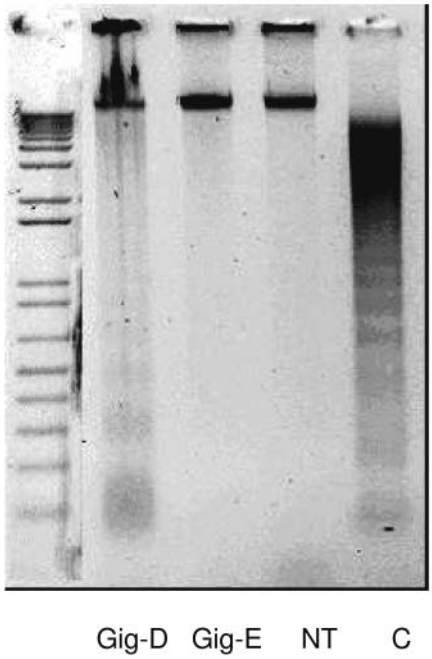


Figure 5. *DNA laddering of HL-60 cells after 24 h treatment with 21 μM of Gig-D, 23 μM Gig-E or 1 μM of camptothecin (C). The first column is a standard. NT: non- treated cells.*

Discussion

Inhibition of cell viability was higher for the monodesmosides of oleanolic acid than those of hederagenin. Gig-E has one hederagenin type pentacyclic aglycone analogous to Hcol-A and α-hederin (7) and possesses two rhamnose residues, and one branched xylose. Gig-D has one oleanolic type pentacyclic aglycone analogous to Hcol-A1. The active saponins also had interesting antiproliferative effects at low concentrations on the HL-60 cells (IC_{50} value <12 μM). In normal cells, Hcol-A1 was not toxic to THP1 human monocytes at 0.05-0.1 μM while at higher concentrations, it induced cell death by altering the cellular membrane, with an IC_{50} of 0.45 μM (6). Inhibition of human umbilical vein endothelial cells (HUVEC) tubulogenesis has been observed at μM concentrations (23).

HL-60 cells are known to be highly sensitive to any apoptotic stimuli. Camptothecin is a well-known potent apoptosis inducer in HL-60 cells, as also shown here by morphological analysis, DNA laddering assay or caspase-3 activity. After 24 h treatment, phosphatidylserine residues were exposed at the cell surface and a sub-G1 peak was detected with the four saponins. These effects were time- and dose-dependent, but in a narrow range of concentrations. DNA fragmentation could only be clearly detected with Gig-D, and here also in a narrow range of high concentrations. This could be related to the higher proportion of cells in late apoptosis. The four saponins thus seemed to be able to induce cell death by apoptosis, but longer times of treatment and higher concentrations than for camptothecin were required. However, as with betulinic acid, they did not induce caspase-3 activation. This is analogous to the saikosaponins from *Bupleurum falcatum* (15). The induction of apoptosis has recently been reported for other saponins from *Gleditsia sinensis* (16) or triterpene saponins from *Albizia adianthifolia* (17), from *Polygonatum zonlanscianense* in HL-60 cells (24) and for avicins from *Acacia victoriae* which induce apoptosis by mitochondrial perturbations (25). This confirms the results of Debiton *et al.* on melanoma cells treated with hederacolchisides (26). Delayed cytolysis was proposed to explain the sub-G1 peak in melanoma cells. This is confirmed here in the HL-60 cells by the annexin V-FITC/7-AAD assay with Hcol-A1.

Table II. *Concentrations inducing 50% LDH release into RPMI 1% FBS (C_{50} μM) after 2 h treatment of HL-60 cells in the presence of monodesmosides.*

	Concentration μM
Gig-E	7.9
Gig-D	4.5
Hcol-A1	3.7
Hcol-A	12.6

The cytotoxic activity of *Gleditsia* saponins in the HL-60 cells was in agreement with the sub-G1 population detected after 24 h (27). G_2/M cell cycle arrest has also been observed (16). The observed accumulation of the cells in the S-phase with Gig-D and Hcol-A1 in the HL-60 cells in the present study suggested that this phase had been elongated

and that this was related to a cytostatic effect.

The specific target of the saponins is primarily the plasma membrane. Cytosolic LDH release into the medium has been considered a sign of plasma membrane alteration leading to cellular lysis (28) and related to necrosis. The test must be carried out with a low level of serum in the medium. The release of lactate dehydrogenase into the culture medium was dose-dependent. Gig-D and Hcol-A1 were equally cytotoxic, they had the same C_{50} on the HL-60 cells in our LDH test, but they induced cell death by apoptosis at different concentrations. The result suggests that the interaction with the plasma membrane is more efficient and leads to cytolysis with Hcol-A1. Comparison of Tables I and II indicates that cell death may be due primarily to membrane permeabilization rather than due to an active process such as apoptosis. Both the sugar and the structure of the genin may explain the differences in cytotoxicity (29). Membrane injury of melanoma cells has been observed in human melanoma cells treated with Hcol-A1 (24), similar to that observed previously in B16 cells treated with α-hederin (14). Rapid cell membrane impairment through a saponin/cholesterol interaction that forms micelles could result in long transmembrane pores (24). For avenacin A-1, it is suggested that the sugar chain interacts and causes the aggregation of the sterol-avenacin A-1 complex. This may lead to rearrangement of the lipid bilayer and to the subsequent formation of pores (30).

In conclusion, Gig-E, Gig-D, Hcol-A and Hcol-A1 induced membrane permeabilization in the HL-60 leukemia cells leading to cytolysis. Apoptosis was also suggested for all the saponins, but at high concentrations and without caspase-3 activity. The mechanisms are not fully understood. Further work on the detergent-like properties and pore formation is in progress. The induction pathways of apoptosis for these monodesmosides in normal cells and other cancer cell lines could be compared to investigate potential anticancer properties.

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