Induction of Apoptosis and Inhibition of Proliferation in Human Tumor Cells Treated with Extracts of *Uncaria Tomentosa*

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Abstract. *Growth inhibitory activities of novel water extracts of Uncaria tomentosa (C-Med-100^{TM}) were examined in vitro using two human leukemic cell lines (K562 and HL60) and one human EBV-transformed B lymphoma cell line (Raji). The proliferative capacities of HL60 and Raji cells were strongly suppressed in the presence of the C-Med-100^{TM} while K562 was more resistant to the inhibition. Furthermore, the antiproliferative effect was confirmed using the clonogenic assay, which showed a very close correlation between C-Med-100^{TM} concentration and the surviving fraction. The suppressive effect of Uncaria tomentosa extracts on tumor cell growth appears to be mediated through induction of apoptosis which was demonstrated by characteristic morphological changes, internucleosomal DNA fragmentation after agarose gel electrophoresis and DNA fragmentation quantification. C-Med-100^{TM} induced a delayed type of apoptosis becoming most dose-dependently prominent after 48 hours of exposure. Both DNA single and double strand breaks were increased 24 hours after C-Med-100^{TM} treatment, which suggested a well-established linkage between the DNA damage and apoptosis. The induction of DNA strand breaks coupled to apoptosis may explain the growth inhibition of the tumor cells by Uncaria tomentosa extracts. These results provide the first direct evidence for the antitumor properties of Uncaria tomentosa extracts to be via a mechanism of selective induction of apoptosis.*

Uncaria tomentosa (Willd.) DC (also known as "Uña de gato" or "Cat's claw") has been used in South American traditional medicine. The native Indians particularly of the Amazon region use teas made of the bark or roots for the treatment of a variety of health disorders including cancer, arthritis and infectious diseases. Organic solvent extracts of this plant were shown to have cytostatic, contraceptive, and anti-inflammatory activity (1). Components isolated from these types of extracts have been shown to enhance phagocytosis (2), antiviral, anti-inflammatory and anti-mutagenic activities (3-6). Although a few cases of cancer treatment have been reported from internet sources, so far there has been little scientific data regarding the antitumor effect of Uncaria tomentosa.

Plant constituents have been important sources for anticancer drug development. Taxol, camptothecin, vinblastine and vincristine represent some of the most important drugs currently utilized for the treatment of human cancers. These lead compounds have been used as models for the development of novel chemotherapeutic drugs (7, 8). New discoveries of anticancer components from plant sources have been reported recently including betulinic acid as a selective inhibitor of human melanoma via induction of apoptosis (9), and combretastatin A-4 as an agent that displays potent and selective toxicity toward tumor vasculature (10).

It has become increasingly evident that apoptosis (programmed cell death) is an important mode of action for many cancer treatments including ionizing radiation (11), alkylating agents such as cisplatin and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (12), topoisomerase inhibitor etoposide (13), cytokine tumor necrosis factor (14), taxol (15), and N-substituted benzamides such as metoclopramide and 3-chloroprocainamide (16). Apoptotic induction has been a new target for innovative mechanism-based drug discovery (17, 18). In this study, the antitumor effect from a unique low molecular weight water extract of *Uncaria tomentosa* was investigated using *in vitro* cell models. Moreover the mechanism was related to recent cell and molecular biological development of cancer research.

Materials and Methods

Materials. A C-MED-100™ concentrate was prepared from C-Med-100™ water extracts supplied commercially by CampaMed, Inc.,

Arlington, Vermont. CampaMed's extracts were dialyzed against distilled water and the dialyzed portion concentrated to a stock solution of 8 mg/ml for all the *in vitro* cell assays. RPMI 1640 medium and fetal calf serum (FCS) were obtained from Gibco BRL (Gaithersburg, MD, USA). Proteinase K and RNAse were purchased from Sigma, Inc. The 100 base-pair DNA ladder marker is available from Pharmacia BioTech and polycarbonate filters were from Millipore. [6-^{3}H]thymidine (2 Ci/mmol) was supplied by Amersham Life Science.

Cell culture. Cells from the human leukemic cell lines HL60 and K562 as well as the lymphoma cell line Raji were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (R10) in a 5% CO_2, 80% humidity and 37°C incubator. The cells used in all the experiments were first subcultured for 2 days at an initial density of 2×10^5 prior to use in the *in vitro* assays. This resulted in an exponential growth stage and the cell viability was > 95% by trypan blue exclusion.

Cell proliferation by MTT assay. The anti-proliferative capacity of C-Med-100™ was determined by a colorimetric MTT assay as already described (19). Briefly, 10 μl of serial duplicate dilutions of C-Med-100™ were added to 190 μl of HL60 cells (0.05×10^6 cells/ml) in 96-well, flat-bottomed plates (Corning, NY) to give a final concentration range of C-Med-100™ from 1.6 μg/ml to 400 μg/ml. Plates were incubated for 72 hours at 37°C and then pulsed with 20 μl MTT (5 mg/ml, Sigma) and incubated for an additional 3 hours at 37°C. Reduced MTT was measured spectrophotometrically with an automated plate reader at 540 nm after lysis of cells with 150 μl of dimethylsulfoxide (DMSO) and 25 μl 0.1 M glycine buffer (pH 10.5).

DNA synthesis determined by [^{3}H]-thymidine incorporation. Exponentially growing cells suspended in fresh R10 at a density of 0.05×10^6/ml were plated in 96-well, flat-bottomed plates together with equal volume but different concentrations of C-Med-100™ (final concentration range 0-200 μg/ml) and cultured at 37°C and 5% CO_2 for 3 days. The cells were then incubated with 1 μCi/well of [^{3}H]thymidine for 1 hour. Labeled nuclear material was collected on glass fiber filters in a microtiter plate cell harvester, dried and counted in scintillation fluid (Ready Safe™, Beckman, USA). Each dose was done in quadruplicate, and the data are presented as mean ± SD.

Morphological measurement of apoptosis. HL60 cells were routinely cultured at a density of 0.5×10^6/ml up to 3 days in R10 in a 5% CO_2 atmosphere at 37°C before they were harvested by centrifugation and resuspended in fresh medium at a concentration of 0.5×10^6 cells/ml in 15 ml sterile SARSTEDT test tubes. Next the cells were exposed to a C-MED-100™ preparation at a concentration of 0.397 mg/ml in final concentration for 3, 6, 24, 48 and 72 hours at 37°C. Hydrogen peroxide (50 μM) and γ-radiation (5 Gy) were used as positive controls. The cytotoxicity was evaluated as percentages of apoptotic and necrotic cells, where apoptotic cells were counted by morphological criteria (as having chromatin condensation, shrinkage, membrane blebbing, fragmentation and appearance of "apoptotic bodies") using phase contrast microscopy (400 x magnification), and necrotic cells by trypan blue staining.

DNA fragmentation assayed by agarose gel electrophoresis. After incubation with C-Med-100™ for 3 days at different concentrations, 2×10^6 Cells were pelleted and suspended in 0.25 ml TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and 0.25 ml lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). The sample was then vortexed and incubated for 30 minutes at 4°C and spun at 13,000 g for 15 minutes. The DNA of low molecular weight in the supernatant was transferred to a new tube where 1 ml cold ethanol and 25 ml 5M NaCl were added and stored in the freezer (-20°C) over night. The DNA sample was then centrifuged at 13,000 g for 15 minutes, vacuum-dried and resuspended in 30 μl TE-buffer and enzyme digested consecutively by addition of 1 μl RNAse A (50 mg/ml) and 1 μl proteinase K (25 mg/ml) in a waterbath at 37°C for 1 hour. After the addition of 5 μl sample buffer (0.5% SDS,

0.25% bromphenolblue and 40% sucrose in TE-buffer), the DNA sample was run in a 1.8% agarose gel for 3 hours and visualized under uv light. The image of DNA ladder was photographed and analyzed by the software Gel-Pro™ (Media Cybernetics).

Quantitative DNA fragmentation assay. DNA fragmentation was quantified as described by Matzinger (20) and modified by Rokhlin (21). Briefly, 2×10^5 cells were incubated over night in T-25 flasks with 5 ml of R10 in the presence of 10 μCi of [^{3}H]-thymidine, then washed and resuspended in fresh R10 at a density of 0.05×10^6 cells/ml. 190 μl of the cell suspension was added to each well of the 96-well flat-bottomed plates in the presence of 10 μl C-Med-100™ in different concentrations (final concentrations: 0 - 100 μg/ml). The cells were cultured for 0, 6, 24 and 48 hours and the DNA of the cells were harvested on a glass fiber filter. Fragmented DNA was washed through the filter, while the intact DNA from living cells was retained there. The incorporated radioactivity of the DNA retained on filter was measured by liquid scintillation counting and the results were calculated as % of control at the same time point.

Cell survival by clonogenic assay. The assay used is based on a semisolid microtiter culture system with 0.6% (wt/vol) methyl cellulose and 10% fetal calf serum in culture medium (19). HL60 cells were seeded at a density of 800,1600 and 3200 cells per culture and cultured for 7 days in a 96-well flat-bottomed microtiter plate. After 7 days of incubation under standard culture conditions, colonies (> 40 cells) were counted by an inverted light microscope. Clonogenic survival data was processed and analyzed according to Gupta *et al* (22). The surviving fraction (SF) is calculated as the plating efficiency (PE) of treated cells divided by the PE of untreated control cells, and while the PE is calculated as the number of colonies per well divided by the number of cells originally seeded.

DNA single and double strand breaks. Exponentially growing HL60 cells at a density of 1×10^6 were incubated together with C-Med-100™ for 24 hours at 37°C. After the treatment, about 1×10^6 cells were layered directly onto 25 mm diameter and 2 μm pore size polycarbonate filters (Millipore). DNA single strand (SSB) and double strand breaks (DSB) were estimated by alkaline and neutral filter elution as described by Kohn *et al* (23) with modifications to measure the unlabeled DNA by microfluorometry (24).

Results

Effects on cell proliferation. Figure 1 displays the dose dependent inhibition of cell proliferation in two human leukemic cell lines (K562 and HL60) and one lymphoma cell line (Raji) by C-Med-100™. Using the [^{3}H]-thymidine DNA synthesis technique, C-Med-100™ was a more potent proliferation inhibitor in HL60 and Raji cells than K562 cells, with IC$_{50}$ values of 71, 84 and 200 μg/ml, respectively. The MTT proliferation assay showed a similar distinguishable difference of growth inhibition between HL60 (IC$_{50}$ = 100 μg/ml) and K562 (IC$_{50}$ = 267 μg/ml) although the DNA synthesis assay seems to be more sensitive.

Clonogenic Assay. The antitumor effect of C-Med-100™ was also demonstrated by clonogenic assay. As seen in Figure 2, there is a very close dose-response relationship between C-Med-100™ concentration and the colony survival fraction (R^2 = 0.9924, p < 0.0001 by exponential curve fitting). The IC$_{50}$ is about 83 μg/ml for this assay in HL60 cells.
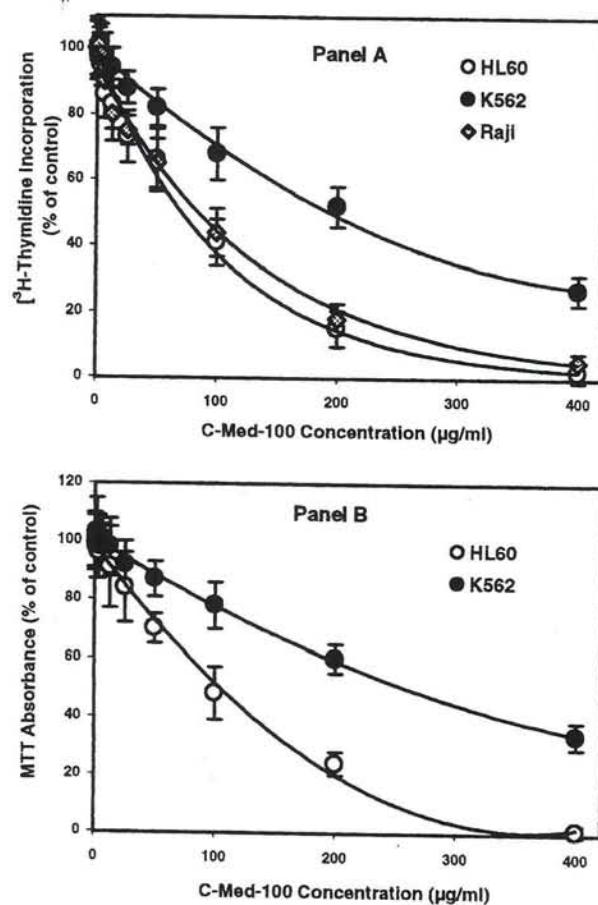
Figure 1. *Cell proliferation inhibition by C-Med-100™ in HL60, K562 and Raji Cells. Cell growth were assessed by both [³H]-thymidine incorporation and MTT assay. Results are shown as mean ± SD (n ≥ 4) and fitted by exponential or polynomial curves.*

$$y = 100e^{-0.0084x}$$
$$R^2 = 0.9924$$
$$IC_{50} = 83 \ \mu g/ml$$

Figure 2. *Clonogenic survival of HL60 cells treated with C-Med-100™. The cells were cultured in a 96-well flat-bottomed microtiter plate for 7 days and the colonies (> 40 cells) were counted by an inverted light microscope. Data points are averages from 4 samples and error bars represent the upper and lower limits of the 95% confidence interval for the point estimated with Poisson probability. Trend line was fitted by exponential curve with the IC_{50} = 83 μg/ml.*

Induction of apoptosis. Previous work in our laboratory and others have shown that HL60 is a good *in vitro* model for induction of apoptosis (16). Using this model we found that C-Med-100™ could induce a delayed type of apoptosis. The data in Figure 3 compares the dose response and time course of the induction of apoptosis by C-Med-100™ to two well-known apoptosis inducers, hydrogen peroxide and γ-radiation. Although C-Med-100™ induced apoptosis as early as after 6 hours at higher doses (200 and 400 μg/ml), it induced massive apoptosis only after about 24-36 hours incubation whereas classic apoptotic inducing agents such as hydrogen peroxide and radiation had completed their cycle of induction of apoptosis before this time interval. There was also a dose response of C-Med-100™ from 50 to 400 μg/ml, and the dose dependent induction of apoptosis remained up to 72 hours. Unlike hydrogen peroxide that induced necrosis as well as apoptosis with increasing time of incubation, C-Med-100™ did not significantly increase necrosis but massively induced apoptosis. These data show that C-Med-
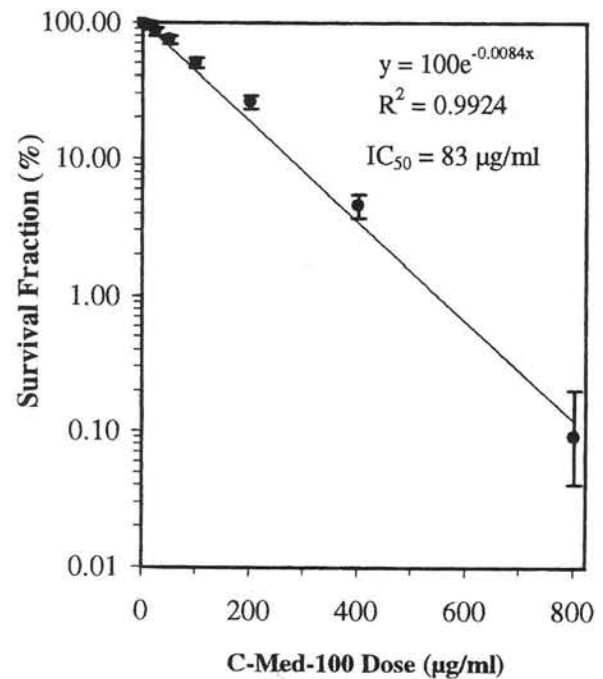
100™ inhibits the tumor cell growth mainly by induction of apoptosis.

The apoptotic morphological change after C-Med-100™ treatment was further confirmed by the agarose gel electrophoresis of DNA fragmentation from HL60 treated with C-Med-100™ for 72 hours (Figure 4). Here, with an increase of C-Med-100™ dose, the DNA fragmentations (180 bp, 380 bp or 600 bp) typical of apoptosis could be visualized at a dose of 80 μg/ml and became more evident at the higher dose of 200 μg/ml (Figure 4).

Apoptosis was also quantified using the DNA fragmentation assay in relation to C-Med-100™ dose in both HL60 and K562 (Figure 5). As expected, the apoptosis resistant cell line K562 showed only minor changes in apoptotically mediated DNA fragmentation over time while the apoptosis sensitive cell line HL60 revealed dose dependent induction of DNA fragmentation over time (Figure 5).

DNA strand breaks. Apoptotically mediated DNA damage by C-Med-100™ was further confirmed by measuring single strand breaks with alkaline elution and double strand breaks with neutral elution 24 hours after the drug treatment. It is clear from Figure 6 that C-Med-100™ induced DNA damage
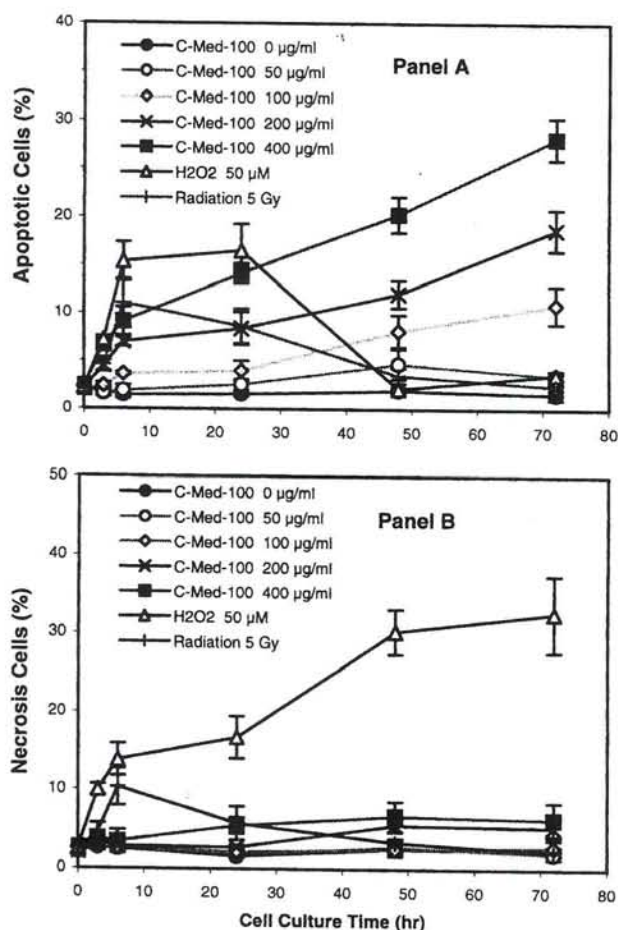
Figure 3. *Dose response and time course of induction of apoptosis and necrosis in HL60 cells by different doses of C-Med-100™ (50, 100, 200 and .400 µg/ml) and controls (negative control and positive controls as radiation and hydrogen peroxide). Apoptosis was assessed by light microscopic examination of morphological changes and counting at least 200 cells per sample. Necrosis was defined as trypan blue stained cell. The results are shown as mean ± SD (n ≥ 3).*

Figure 4. *Induction of DNA fragmentation by different doses of C-Med 100 in HL60 cells. The cells were cultured in the presence of different concentrations of C-Med-100™ for 3 days and DNA extracted and analysed in a 1.8% agarose gel electrophoresis. Lane A, 100 base-pair DNA ladder marker (Pharmacia Biotech); lane B-F, C-Med-100™ at a dose of 0, 8, 40, 80 and 200 µg/ml.*

in the human leukemic HL60 cell line in a dose-dependent pattern. At the dose of 200 µg/ml, C-Med-100™ induced about 50% single strand breaks and 25% double strand breaks.

Discussion

The C-Med-100™ water extracts clearly demonstrated a dose-dependent inhibition of cell proliferation in two human leukemic cell lines (K562 and HL60) and one lymphoma cell line (Raji) using two proliferation assays (Figure 1). The cell growth inhibition caused by C-Med-100™ was also confirmed by the clonogenic assay, the gold standard for determining cytotoxicity induced by physical (e.g. radiation) and chemical agents (25). These results are in agreement with a previous report (26) on the inhibition of the proliferation by oxindole
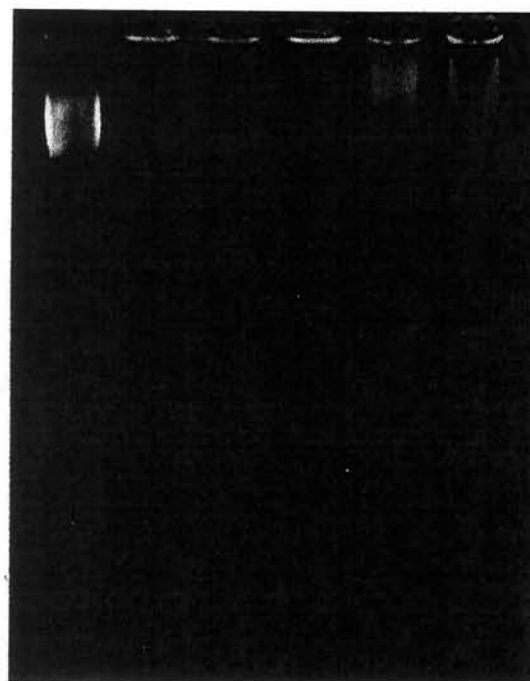
alkaloids obtained from *Uncaria tomentosa* (Willd.) DC whereby the mechanism of action was by induction of differentiation towards macrophages in U-937 cells (lymphoma cell line) and towards granulocytes in HL60 cells. However, C-Med-100™ is a water extracts and oxindole alkaloids are not particularly soluble in water.

The significant difference in IC_{50} values between HL60 and K562 has prompted us to further investigate the mechanism of growth inhibition by C-Med-100™ preparations. It is well known that HL60 cells are sensitive to different apoptotic stimuli whereas K562 cells are not (13, 27, 28). HL60, an acute myeloblastic leukaemic cell line, although lacking p53 (29), could still cleave poly(ADP-ribose) polymerase (PARP) at the onset of apoptosis, while such cleavage was not detectable in K562, a chronic myelogenous leukemic derived cell line (30). The resistance of K562 to cell proliferation inhibition by C-Med-100™ in the present study correlated well with previous findings that this cell line is particularly resistant to apoptosis induced by cytotoxic agents (31). Indeed, we could demonstrate apoptosis induction by C-Med-100™ in a dose dependent pattern in HL60 cells by morphological changes, DNA fragmentation by gel electrophoresis and DNA fragmentation quantification by [^{3}H]-thymidine releasing, while K562 was much more resistant to apoptosis induction by C-Med-100™ (Figure 5). These data suggest that C-Med-100™ inhibits cell
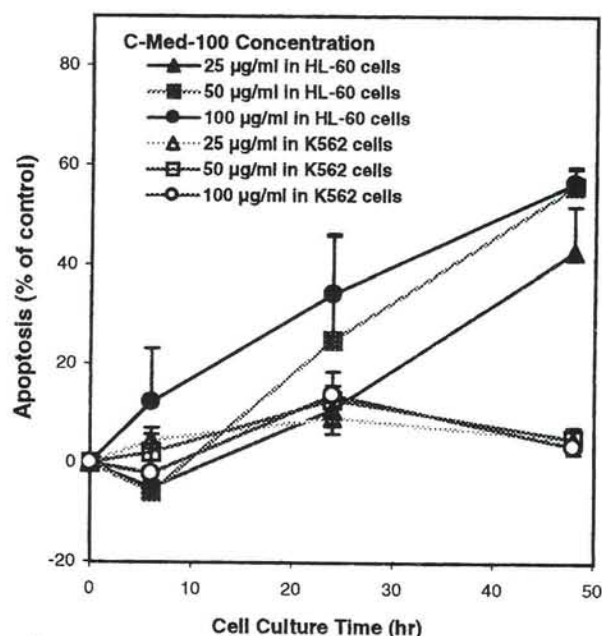
Figure 5. *Induction of apoptosis by C-Med-100™ treatment assessed by the quantitative DNA fragmentation assay in HL60 and K562 cells. The cells were first labeled with [³H]-thymidine overnight, washed and suspended in fresh medium and seeded in 96-well flat-bottomed microtiter plate at a density of 50,000 cells/ml and cultured for the indicated time periods in the presence of different concentrations of C-Med-100™. The unfragmented DNA of intact cells was retained on glassfibre filters while the fragmented DNA was washed away. The radioactivity was measured by liquid scintillation counting. All samples were measured in quadruplicate and the results are expressed as means ±SD.*
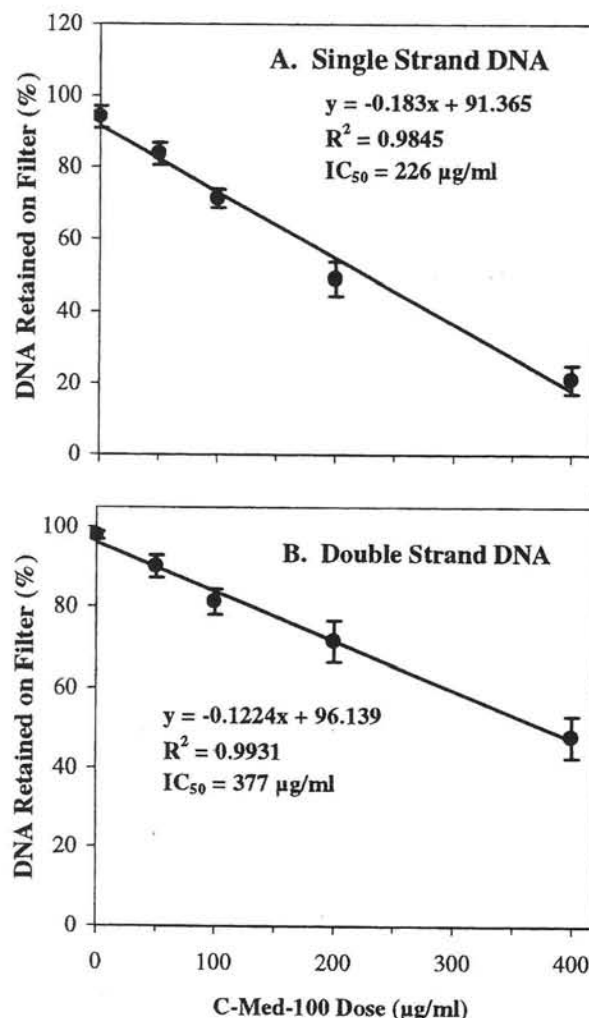

Figure 6. *DNA single strand breaks (SSB) and double strand breaks (DSB) in HL60 cells treated with C-Med-100™ extracts. SSB and DSB were estimated by alkaline and neutral filter elution 24 hours after the drug treatment. Results are expressed as means ± SD (n ≥ 3).*

proliferation mainly by the induction of apoptosis instead of by induction of necrosis as has been observed with the treatment of radiation or hydrogen peroxide (Figure 3).

Another important characteristic of apoptosis induction by C-Med-100™ is its delayed onset. Unlike the positive control agents like radiation (5 Gy) and hydrogen peroxide (50 μM) which induced peak apoptosis within 24 hours, C-Med-100™ induced maximum apoptosis much later (48 or 72 hours, Figure 3 and 5). The time interval between commitment to cell death and the appearance of the first cellular characteristics of apoptosis varies according to cell type and lethal stimulus. However, once the first structural changes occur it only takes a few hours to proceed the last stage of the programmed cell death and final resorption by phagocytosis (the execution phase) (32, 33). For most chemotherapeutic drugs, typical apoptosis usually can be detected within 3-24 hours. There has been at least one report where induction of apoptosis occurred mainly at a later stage (peaked at 52-72 hours) and that was induced by betulinic acid, another natural product from beech bark (9).

DNA single strand breaks and double strand breaks were both closely related to C-Med-100™ concentration (Figure 6) in the present study. Although C-Med-100™ induced SSB at

a much higher frequent rate than DSB as shown by the IC_{50} values (SSB = 226 μg/ml; DSB = 377 μg/ml), DSB are the most common lesions induced by radiation or chemotherapy that lead to tumor cell death (34). The fact that C-Med-100™ can induce a higher proportion of SSB to DSB seems to favor an apoptotic cytotoxicity instead of necrotic death, although the relationship between DNA strand breaks and apoptosis is complicated. The DNA fragments in the present study were observed to be multimers of approximately 180 base pairs. These fragments are consistent with internucleosomal cleavage of chromatin by an endonuclease, which generates a ladder of small fragments of double-stranded DNA, one of the hallmarks of apoptosis in agarose gel electrophoresis. However, it has also been suggested that DNA fragmentation induced during apoptosis is not due to a double-strand cutting

enzyme as previously postulated, but rather is the result of single-strand breaks (35). Although the formation of DNA DSB appears to enhance both apoptotic and necrotic cell death (34), they mainly induced apoptosis in the case of C-Med-100™ treatment (Figure 3).

The fact that DNA damage could be detected before the appearance of apoptosis induced by C-Med-100™ treatment and that the IC_{50} of strand breaks is much higher than those obtained from cell proliferation assays suggests that the growth inhibition cannot be completely explained by the direct DNA damage on the cells. It is quite possible that the apoptosis signal pathway was triggered by DNA damage, including the lethal DSB that was beyond the cell repair capacity.

Acknowledgements

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