Evaluation of Cell Death Caused by Triterpene Glycosides and Phenolic Substances from *Cimicifuga racemosa* Extract in Human MCF-7 Breast Cancer Cells

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We previously reported that the antiproliferative effect of an isopropanolic-aqueous extract of black cohosh (iCR) on MCF-7 estrogen-responsive breast cancer cell line was due to the induction of apoptosis. Here we address the question to what extent apoptosis induction can be ascribed to one of the two major fractions of iCR, the triterpene glycosides (TTG) or the cinnamic acid esters (CAE). Furthermore, as black cohosh is routinely administered orally, we studied whether its pharmacological effects would withstand simulated liver metabolism. The antiproliferative activity of TTG and CAE as well as of rat liver microsomal S9 fraction-pretreated iCR on MCF-7 cells were investigated by WST-1 assay. The features of cell death induced were tested for apoptosis by flow cytometry (light scatter characteristics, Annexin V binding). Irrespective of S9-pretreatment, 72 h iCR treatment induced a dose-dependent down regulation of cell proliferation with the same IC_{50} of 55.3 μg/ml dry residue which corresponds to 19.3 μg/ml TTG and 2.7 μg/ml CAE. The degree of apoptotic MCF-7 cells was also comparable. Both, isolated TTG and CAE fractions inhibited cell growth, the IC_{50} being 59.3 μg/ml and 26.1 μg/ml, respectively. Interestingly, whereas IC_{50} and apoptosis induction correspond well for the whole extract, TTG and CAE fractions induced apoptosis at concentrations (25 and 5 μg/ml) well below those required for significant growth inhibition. Observation of this study firstly showed that the cell death induced by iCR withstood a metabolic activation system. In addition, TTG and CAE compounds significantly contributed to its apoptotic effect, CAE being the more potent inhibitor of proliferation and apoptosis inducer.

Key words apoptosis; triterpene glycoside; cinnamic acid ester; breast cancer cell; black cohosh

Extracts of the rhizomes of black cohosh (*Actaea* syn. *Cimicifuga racemosa* L. NUTT), have since long been used as a popular herbal medicine to alleviate menopausal symptoms.[1—3] Apart from the use of *Cimicifuga racemosa* extracts (CR) for treating menopausal symptoms mainly in the European countries[4] and United States,[1,5] CR have long been used for the treatment of rheumatism, and as antipyretic and analgesic agents in Japan and China.[6] An isopropanolic extract of black cohosh (iCR) has been shown repeatedly to be devoid of proliferative activity on ER positive breast cancer cells,[7,8] even iCR inhibited the growth of both estrogen-dependent MCF-7 and estrogen-independent MDA-MB231 human breast cancer cells.[9,10] We recently demonstrated that apoptosis, the essential regulatory mechanism associated with many autoimmune disorders, malignant tumours and viral infections,[11] is one underlying mechanism responsible for the observed inhibition of the proliferation of ER^+ and ER^- breast cancer cells by iCR.[9] As herbal extracts must be considered multi-component drugs, any influence on the balance between cell growth and programmed cell death should ideally be attributable to a single ingredient or at least an enriched fraction of closely related compounds. Two main classes of compounds have been isolated from the rhizomes of CR, triterpene glycosides (TTG, *e.g.* actein, 26-deoxyactein, cimigenol, cimiracemoside)[12,13] and aromatic acids (*e.g.* caffeic, ferulic, isoferulic, fukiic, piscidic) and their derivates (cinnamic acid esters-cimicifugic acid A-H)[14,15] which differ in their biological activity. According to recently published reports, these include cytotoxic,[16] antitumor,[17] antiviral,[18] antiproliferative[10,16] effects of some triterpenene glycosides and the inhibitory effect of neutrophil elastase[19] and collagenolytic activity by cinnamic acid derivates.[20]

We therefore chose these two fractions, the fairly black cohosh-characteristic TTG[12] and, due to their similarity to synthetic estrogenic compounds, the cinnamic acid esters (CAE).[21] Furthermore, as the commercially available iCR preparations are exclusively for oral ingestion, we also tested whether the observed inhibition of proliferation and apoptosis induction would withstand simulated liver metabolism. Therefore we incorporated an incubation step in that we added rat liver S9 mix containing P450 enzymes to the cytotoxicity and apoptosis assays of iCR.[22—24] The aim of our study in ER^+ MCF-7 breast cancer cells was therefore twofold. Firstly, we compared the proliferation inhibiting and apoptosis inducing potential of iCR before and after S9-incubation. Then we tested two major fractions of iCR, namely TTG and CAE, for their share in the overall effect on programmed cell death as observed with unmodified iCR. We could verify that the cell growth inhibition exerted by iCR on ER^+ breast cancer cells was due to apoptosis induction, that these effects withstood a metabolic activation system with S9 fraction and that a significant part of the extract's activity was due to both TTG and CAE fractions.

MATERIALS AND METHODS

Reagents An isopropanolic-aqueous extract of *Rhizoma Actaeae* (=*Cimicifugae*) *racemosae* (iCR, B.Nr. 231050) was provided by Schaper & Brümmer GmbH & Co.KG. Salzgitter, Germany. The concentration of the extract was

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77.4 mg/ml in relation to the dry residue. The concentration of the total triterpene glycosides in iCR, calculated as standard triterpene glycoside 26-deoxyactein ($C_{37}H_{56}O_{10}$; MW: 660.8), amounted to 27 mg/ml. Concentration of CAE was measured according to the calibration of isoferulic acid. In addition to the free acids caffeic, ferulic, and isoferulic acids, fukiic acid esters as well as piscidic acid esters were easily detectable. Of these, fukinolic acid showed the highest concentration (max. 0.3%) in iCR. Cimicifugic acids A (max. 0.15%), B (max. 0.25%), E, and F were also frequently found in varying amounts. Based on the above method and composition, the total concentration of CAE was 3.7 mg/ml. The alcohol concentration was 40% (v/v). The characteristic pattern of iCR-compound classes, mainly TTG and their degradation products, was determined by TLC before and after incubation with S9 (Fig. 1). TTG and CAE fractions were obtained from the iCR-batch (B.Nr. 231050) by freeze-drying. The lyophilisate was then separated in methanol on a Sephadex LH20 column (3×42 cm) thus yielding the two fractions. The compound groups CAE and TTG were resuspended in ethyl acohol 60% (v/v).

Tamoxifen citrate (TAM) and cyclophosphamide (CPA) from (Sigma, Buchs, Switzerland) were used as controls in proliferation assays. The final concentration of ethanol and isopropanol in culture medium during treatment did not exceed 0.5% (v/v). This concentration did not affect the apoptosis or proliferation of the investigated cells. Cell proliferation reagent WST-1 was purchased from Roche Diagnostica (Rotkreuz, Switzerland). Aroclor 1254 induced S9 rat liver homogenate with 36.2 mg/ml protein concentration was from ICN Biomedicals (Eschwege, Germany).

Cell Lines and Culture Conditions ER^+ breast-adenocarcinoma cell line MCF-7, from ATCC (Rockville, MD), was cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% FBS and 2 mM L-glutamine, 1% penicillin/streptomycine (PenStrep) under a fully humidified atmosphere, 5% CO_2 at 37 °C. The cytotoxicity and apoptosis assays were performed in DMEM medium without phenol red, containing, 5% charcoal stripped FCS (CCS, Sigma) and 0.2% PenStrep (test medium). In all experiments untreated and vehicle treated cells were included as controls. The studies were carried out using cells from passages 3—7, and preincubated 24 h in test medium to remove exogenous estrogens. Cell viability, as determined by trypan blue exclusion, exceeded 91%. For proliferation and apoptosis assays, seeded cells (3000 and 20000/well) were exposed for 72 h to different concentrations of iCR, TTG or CAE. The influence of metabolic activation on the cytotoxicity of iCR on MCF-7 cells was studied in simultaneous experiments performed with S9-pretreated iCR. For this purpose, iCR and S9 homogenate (30%) were mixed 1 : 1, preincubated for 1 h at 37 °C and inactivated subsequently for 5 min at 80 °C. CPA (50 μg/ml) a pro-drug with slow metabolism and low cytotoxicity in MCF-7 cells was used as positive control.[25]

Cell Growth Assays Crystal Violet (CV) Staining: CV staining is a colorimetric determination of cell numbers. Cells at a density of 3000/well, that had been treated for 72 h, were centrifuged and supernatant was removed. Cells were stained with 0.5% CV (in 20% methanol) for 10 min at room temperature. After extensive washing with tap water, plates were blotted dry. Cell-bound dye was dissolved in citrate-buffered ethanol (0.05 M in 50% ethanol) and scanned at 540 nm in an ELISA reader.

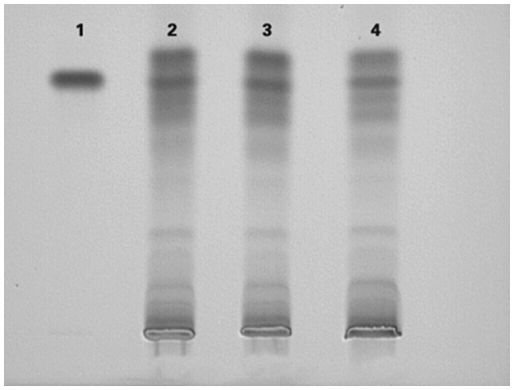

Fig. 1. Thinlayer Chromatography Investigations of iCR

TLC chromatograms of iCR (231050) before (lane 2) and after S9-mix pretreatment (lane 4) are presented. BSA concentration in control sample (lane 3) was normalized to the protein content in S9-mix preparation. As reference standard for CR extract triterpene glycoside 26-deoxyactein (lane 1) was used.

WST-1 Assay: In order to determine the effect of individual compounds on cell growth, treated cells at a density of 3000 cells/well were incubated in microtiter plates for 68 h followed by 4 h with a tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate). The cleavage of WST-1 to formazan by metabolically active cells was quantified by scanning the plates at 450 nm and 650 nm reference wavelenght in a microtiter plate reader.[26] Test medium was used as background control. Three independent set of experiments performed in triplicate were evaluated. Controls with the solvents ethanol and isopropanol (0.5% v/v) were tested in parallel. The growth inhibition rate was calculated as percentage of parallel untreated controls. TAM (50 μM) was used as positive control. The IC_{50} values were evaluated from the dose–response curves of each experiment using Microsoft-Excel computer software. The direct reductive potential of the test substances was tested concomitantly in a cell free system.

Apoptotic Death Assay In addition to the very sensitive Annexin V adherence assay,[27] cell size and granularity scatter characteristics were also investigated by flow cytometric (FCM) analysis. Apoptosis was detected in cells (2×10^4) after 72 h treatment by staining with Annexin V-FITC (Roche Diagnostics) as previously described.[28] Plasma membrane permeability was estimated by propidium iodide (PI). All experiments were performed using FACScalibur flow cytometer (BD Biosciences, San Jose, CA, U.S.A.). 5000 events per sample were collected and analyzed with the CellQuest program. Histograms of treated cells were compared to untreated controls. Vehicle treated cells (0.5% v/v ethanol, isopropanol) were always included and were not found to affect apoptosis.

Statistical Analysis All treatment experiments were repeated at least three times to generate statistically relevant data. Student's two-tailed test was employed to assess the statistical significance of differences between the respective means. IC_{50} values were obtained by linear or exponential regression analysis of data using the Microsoft Excel software. Probability values equal to or less than 0.05 were considered significant. The comparison of histograms was evaluated sta-

tistically using the Kolmogorov–Smirnov (K–S) two samples test for overlaid histograms.

RESULTS

Influence of a S9 Rat Hepatic Microsomal Fraction on Cytotoxicity Exerted by iCR Because of a reported interference of natural substances with another tetrazolium salt MTT,[29] we first measured the direct reductive potential of all substances tested in a cell-free system. None of them showed interaction with WST-1 reagent in the concentration range used and differed from the background control significantly. The $A_{(450—650)}$ nm values of substances were between 0.106—0.118 compared to the 0.107 value of the test medium. Further, S9 hepatic microsomal fraction was used to discern the mediating influence of metabolic bioactivation on iCR cytotoxicity. Bioactivation is an important aspect when evaluating cytotoxicity, as some test agents are biotransformed by hepatic enzymes to intermediates exerting toxicities different from the parent compounds. As most cell lines eventually lose their P450 enzymatic activity when maintained in culture, we used a common and accepted protocol[22] *i.e.* by adding a post mitochondrial S9 fraction from the liver of rats treated with Aroclor 1254, an inducer of mixed-function oxidase activity to the test system. The toxicity and apoptosis-inducing activity was neither potentiated nor reduced in the presence of the S9 fraction after 72 h continuous exposure of MCF-7 cells to iCR at concentrations between 1.55 and 154.8 μg/ml (Table 1). The concentration required to inhibit cell growth of MCF-7 to 50% obtained by regression analysis of dose-response curves was the same for iCR and S9 mix-pretreated iCR namely 55.3 μg/ml, which corresponded to 19.3 μg/ml and 2.7 μg/ml CAE. The cytotoxicity of CPA (50 μg/ml) as positive control in the presence of S9 mix was elevated 3.2-fold compared to its effect without S9. The percentage of apoptotic cells expressed as annexin V positive cells quantified by flow cytometry was very similar (about 50%) for iCR (77.4 μg/ml) with or without S9 pretreatment and about 37% of cells took up PI (Fig. 3). As shown in Fig. 1 no relevant changes in the pattern of TTG/CAE and their degradation products were found in TLC of both extracts. Bovine serum albumin (BSA) concentration in control sample was normalized to the protein content in S9-mix preparation.

Growth Inhibitory Effect of TTG and CAE Fractions on MCF-7 Cells Cell proliferation was measured indirectly by the cleavage of WST-1 to formazan by cellular enzymes of metabolically active cells after 72 h exposure of MCF-7 cells to TTG (10—100 μg/ml) and CAE (5—50 μg/ml). During this exposure period the viability of cells was also inspected with microscope. As positive control TAM (50 μM) inhibited the growth of MCF-7 cells up to 79%. The inhibition of cell proliferation by TTG fraction was strongly dose dependent and correlated well with microscopical assessment of cell viability. The IC_{50} concentration of TTG was 59.3±2.1 μg/ml. In comparison to typical adherent untreated MCF-7 cells, treatment with CAE in concentrations above 5 μg/ml led to shrinkage and detachement of most of the cells from the bottom of the wells. In contrast, no cytotoxicity of CAE could be determined using WST-1 in spite of lack of interference between CAE and WST-1 agent in the cell-free system. Therefore, we used CV staining as a second assay for assessment of cell number. CAE dose-dependently reduced the cell number and its IC_{50} value was calculated to be 26.1±1.3 μg/ml. To compare this value with

Table 1. Cytotoxicity to MCF-7 Cells of a 72 h Exposure to iCR as Mediated by an S9 Hepatic Microsomal Fraction

Test agent		Survival, % of control growth	
		Without S9	With S9
CPA (μg/ml)			
50		81.6±2.5	40.7+7.4[a]
iCR (231050)			
(μg/ml)	Dilution		
1.5	1/10000	101.4±2.1	99.8±3.4
15.5	1/5000	95.3±8.2	91.2±12.4
31.0	1/2500	75.9±10.3	78.9±5.7
77.4	1/1000	4.3±1.2	2.9±1.8
154.8	1/500	0.4±0.6	0.0±0.8

a) $p<0.01$; no significant differences in toxicity were estimated between iCR with and without S9-pretreatment, as determined with WST-1 assay. Means±S.D. from three independent experiments are shown. Survival of vehicle (0.5% isopropanol) treated cells was 101.2±0.9%.

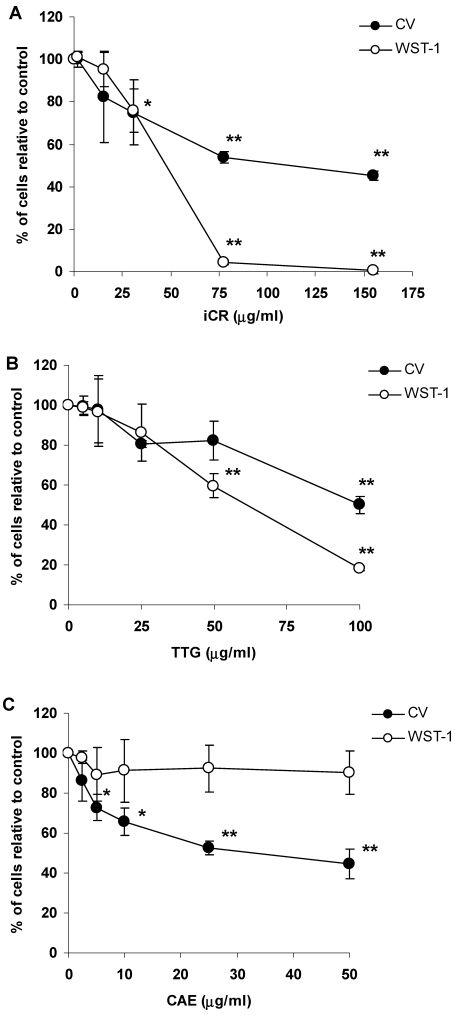

Fig. 2. Decrease of MCF-7 Human Breast Cancer Cells Growth by iCR, TTG and CAE

Cells were treated for 72 h with different concentrations of iCR (A), TTG (B) and CAE (C) determined by WST-1 reduction and CV stain. Measurements were done in triplicate in three different cultures. Means±S.D. are shown (* $p<0.05$; ** $p<0.01$).

the IC_{50} values determined with WST-1 assay, we also investigated the viability of MCF-7 cells exposed to iCR and TTG by CV stain. As shown in Fig. 2, the WST-1 assay was a more sensitive method than CV staining by iCR and TTG.

Flow Cytometric Analysis of the Type of Cell Death Induced by TTG and CAE In a previous study we showed that iCR induced apoptosis[9] in MCF-7 cells. To characterize the cell death induced by the iCR fractions TTG and CAE in MCF-7 cells, we used annexin V adherence to phosphatidylserine (PS) exposed on the outer leaflet of the cell membrane and PI uptake. Simultaneously, analysis of cell morphology based on light scattering characteristics, cell size according to forward (FSC) and granularity to side scatter (SSC) were investigated. We exposed cells to TTG at concentrations below and close to its IC_{50} (25, 50 μg/ml) and CAE fraction at three (5, 10, 25 μg/ml) concentrations. The two-parameter histograms in the left column of Fig. 3 show scattering properties of treated MCF-7 cells typical for apoptosis, namely the reduction of cell size and no change or enhancement on SSC characteristics in comparison to untreated cells. Morphological changes were confirmed with adherence of FITC-conjugated annexin V to PS expressing cells. The percentage of early apoptotic cells, as shown by annexin V binding, was significantly increased after treatment with either 25 μg/ml TTG or 5 μg/ml CAE. Differences amounted to 3.2% *vs.* 68.5% for TTG-treated and 3.2% *vs.* 74.1% for CAE-treated cells (Fig. 3). Compared to untreated controls (25.4%), plasma membrane permeability of MCF-7 cells was not significantly increased after 72 h exposure to TTG in concentrations of 25 (30.7%) and 50 (33.4%) μg/ml. With increasing concentrations of CAE, the number of cells expressing PS did not increase. However, there was an increase in

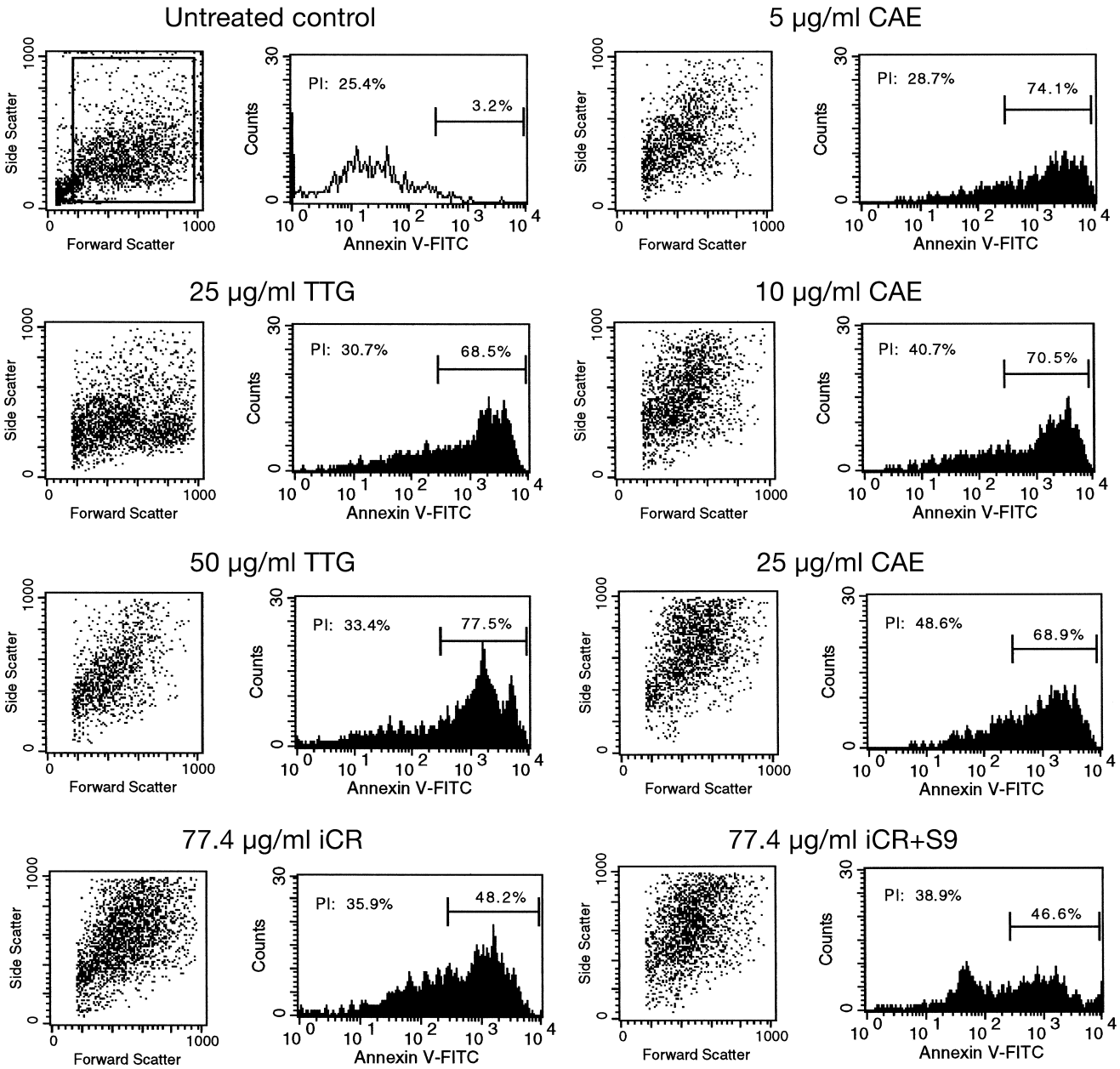

Fig. 3. Flow Cytometric Analysis of Morphological Changes Accompanied with Increased PS Exposure

MCF-7 cells were exposed (72 h) to indicated concentrations of TTG, CAE and iCR without and with S9-pretreatment. Biparametric histograms in the left column represent the scattering properties of the cells, cell size (measured as forward scatter) *vs.* cell granularity (measured as side scatter). PS externalization was assessed by staining with annexin V-FITC and is indicated in each histogram. The corresponding percentage of permeable cells (PI) is expressed. The experiment was repeated twice with similar results.

the percentage of cells taking up PI namely 28.7, 40.7 and 48.6% at concentrations 5, 10 and 25 μg/ml, respectively. The number of permeable cells compared to control (48.6% *vs.* 25.4%) almost doubled at a concentration of 25 μg/ml CAE. These changes, as evidenced by PI uptake, are indicative of late-stage apoptosis or necrosis.

DISCUSSION

Numerous reports have described the modulating action of phytochemicals on various enzyme systems including those associated with P450. Phytochemicals may affect the metabolism of substrates of P450[30,31] including the activation/detoxification on their substrates. There are, however, very few data regarding the effect of metabolism on CR. Although the formation of quinoid metabolites *in vitro* was demonstrated, these were not detected in urine samples from women consuming a black cohosh extract.[32] In that respect, it was important to evaluate the cytotoxicity of iCR based biomaterials after metabolism. We tested iCR in a test system, which has been employed as the xenobiotic activating system in *in vitro* cytotoxicity assays[22] with Aroclor 1254 induced rat liver microsomes containing P450 enzymes. As shown in the Table 1 we observed no differences in inhibition of proliferation or induction of apoptosis (Fig. 3) either before or after incubation of iCR with rat S9 liver homogenate. These results were in accordance with the TLC fingerprints investigations of both extracts as shown in Fig. 1.

Discovery of active compounds from natural products with cytocidal (*e.g.* apoptosis-inducing) rather than cytotoxic ability is of great interest for cancer treatment. Of the two major classes of substances of iCR, the TTG and CAE have been in the focus of research due to structural similarities with the natural estrogens.[33] We first investigated the ability of both iCR fractions to inhibit MCF-7 cancer cell proliferation using the WST-1 assay. Despite the fact that CAE did not interfere with WST-1 agents in a cell free system, there were discrepancies between morphological changes observed by microscopy and the viability results from the WST-1 assay. The latter gives an indication of the integrity of both mitochondrial and extramitochondrial NADH- and NADPH-dependent redox enzyme systems. Of course, that some compounds of CAE, *e.g.* caffeic acid, ferulic acids posses a free radical scavenging effect[34,35] which could explain our WST-1 results by CAE. In comparison, CV stain which is based on the cell number, was less sensitive than the WST-1 assay which measures the dehydrogenase activity of viable cells. There was about 2-fold variation between the IC_{50} (WST-1 *vs.* CV) of iCR (55.3 *vs.* 92.7 μg/ml) and TTG (55.9 *vs.* 100 μg/ml) (Fig. 2). Based on comparative IC_{50} values for 72 h exposure to iCR, TTG and CEA (taking into consideration the differences between WST-1 and CV assay), the MCF-7 cells were about 4 times more sensitive to CAE (13.0 μg/ml) than to TTG (55.9 μg/ml). Inhibition of ER^+ MCF-7 cell proliferation by TTG showed a linear dose-effect relationship probably utilizing a mechanism which does not require ER. However, by continuous 3-d exposure of MCF-7 cells to CAE fraction an exponential relationship was established (Fig. 2). This finding corresponds with studies of others[10] as well as our previous results,[9] where growth inhibition[9,10] and apoptosis induction[9] was demonstrated in breast cancer cell lines by iCR. Recently, several studies reported on antitumor[17] and antiproliferative activity[16] of TTG *e.g.* actein in different cell lines, however only a few studies were conducted to the mechanisms of action[10] and mode of cell death. To further substantiate the cell growth inhibitory effect of TTG and CAE, the apoptotic cells were stained by Annexin V adherence and plasma membrane permeability was estimated with PI. Both fractions of iCR, TTG (25 μg/ml) and CAE (5 μg/ml) elevated the number of apoptotic cells significantly up to about 70%, compared to 25% in untreated cells. However, the effect of CAE was superior to that of TTG regarding the dose and stage of apoptosis as reflected by PI permeated cells. Flow cytometric analysis of morphological changes reflected in the cell size (FSC) and granularity (SSC) confirmed apoptotic morphology (Fig. 3). Increasing data suggest that cell cycle control, particularly at G1/S and G2/M transitions, is fundamentally important for the cell to ensure accurate cell division. Research with breast cancer MCF-7 cells has shown, that triterpene glycosides actein, 26-deoxyactein and cimiracemoside A induced cell arrest by suppressing progression through the G1 phase of the cell cycle and that there is induction of differential changes in the cell cycle regulatory proteins cyclin D1, cdk4 and $p21^{cip1}$.[10] In this study we show that CAE as well as TTG alone can induce apoptosis, suggesting an additional explanation for their inhibitory properties on cell proliferation.

Apoptosis, the early stages of which can be detected with the very sensitive Annexin V binding method,[27] preceedes inhibition of proliferation as measured by WST-1 or dye exclusion assays. Therefore our results showing apoptosis induction by TTG and CAE fractions at concentrations well below those required to detect significant inhibition of proliferation is not surprising. Different effect levels obtained with isolated classes of compounds or whole iCR either when comparing different substances or comparing proliferation to apoptosis are most probably a consequence of matrix effects, *i.e.* synergistic or inhibitory effects between ingredients.

Interestingly, the minimally invasive MCF-7 cells, that express wild-type p53 and are caspase-3 deficient,[36] are highly responsive to treatment with iCR fractions. In our previous study we showed that iCR-induced apoptosis is associated with the cleavage of cytokeratin 18 and is comparable with staurosporine a potent non-selective protein kinase inhibitor.[9] In addition, iCR was as effective as the estrogen antagonist tamoxifen and even superior to isoflavonic genistein,[9] a phytoestrogenic compound of soy and red clover extracts. The caspase-3 deficiency is responsible for the relative insensitivity of MCF-7 cancer cells to many chemotherapeutic agents. Many of the present chemopreventive and chemotherapeutics against breast cancer can have undesirable side effects. Therefore, there has been interest in the use of natural alternative substances to avoid these problems.

Our results show for the first time that the apoptosis-inducing properties of iCR can be attributed to its TTG and CAE fractions and are not restricted to *in vitro* cell culture environments, but may also persist after metabolic pharmacokinetic pathways. However, such *in vitro* studies provide basic information concerning the nature and/or cellular response to the test agent, additional research using *in vitro* and *in vivo* experiments is necessary to elucidate the role that mutual interactions between defined extract fractions contribute to its

apoptosis-inducing mechanism.

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