

Inhibitory Effect of an Isopropanolic Extract of Black Cohosh on the Invasiveness of MDA-MB 231 Human Breast Cancer Cells

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Abstract. *Background:* The isopropanolic extract of black cohosh (iCR)b has recently been reported to exert antiproliferative and apoptosis-inducing effects on estrogen receptor-positive MCF-7, as well as estrogen receptor-negative MDA-MB 231 human breast cancer cells. To broaden observations, the anti-invasive effects of iCR and its two major fractions triterpene glycosides (TTG) and cinnamic acid esters (CAE) were tested in highly invasive MDA-MB 231 cells. *Materials and Methods:* The effect of drugs upon the invasive potential of MDA-MB231 cells was studied in BD Biocoat Matrigel™ invasion chambers over a period of 24 h. *Results:* The suppression of invasion reached 51.8% at 77.4 µg/ml of iCR, an extract concentration where 89% of MDA-MB231 cells were viable. TTG and CAE reduced cell invasion by 34% and 25.5%, respectively, at a dose of 5 µg/ml. The motility of cells was only moderately reduced. *Conclusion:* In this study iCR was found to suppress tumor cell invasion without affecting cell viability. This result together with the antiproliferative and apoptosis-inducing effect of iCR suggest its use as a secure agent in postmenopausal hormone replacement therapy with additional chemopreventive activity.

Tumor cell invasion is a complex process which is characterized by alterations in cellular attachment, proteolytic and migratory activities. The invasive potential of malignant cells is linked to their capacity to degrade basement membrane and extracellular matrix to create a

path for migration (1). Any agent, which can inhibit the invasive process with low toxicity, even when chronically used, is therefore of great interest. An increasing number of phytopharmaceuticals have received major attention as promising chemopreventive agents (2-4).

Extracts of the rhizomes of black cohosh (*Actaea racemosa* L. syn. *Cimicifuga racemosa* L. Nutt.) have been recognized as a rational choice for the treatment and prevention of menopausal disorders (5, 6). Ethanolic and isopropanolic (iCR) extracts of black cohosh rhizomes inhibited the growth of both estrogen-dependent MCF-7 and estrogen-independent MDA-MB231 human breast cancer cells (7-9). Furthermore, the proliferation of hormone dependent LNCaP as well as hormone independent DU 145 and PC3 prostate cancer cells was down-regulated (10, 11). We recently demonstrated that apoptosis, the essential regulatory mechanism of programmed cell death, is one underlying mechanism responsible for the observed inhibition of the proliferation of breast and prostate cancer cells by iCR. Apoptosis induction by iCR involved cleavage of cytokeratin (CK) 18 and caspase activation (8, 10). However, in MCF-7 cells black cohosh extract also induced cell cycle arrest at G1 and G2/M which proposes as an additional pathway (9). Its antitumor activity was reported in *in vitro* and *in vivo* experiments. Black cohosh extract showed strong inhibition of TPA (12-O-tetradecanoylphorbol-13-acetate)-induced promotion of Epstein-Barr virus early antigen (EBV-EA) in Raji cells in *in vitro* assay (12) and *in vivo* inhibited tumor development following subcutaneous inoculation of LNCaP cells in immunodeficient nu/nu mice (13).

Herbal extracts like iCR typically contain a complex mixture of various constituents. From the rhizomes of black cohosh, two main classes of compounds have been isolated namely triterpene glycosides (TTG) and polyphenolic cinnamic acid esters (CAE) with different biological

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activities (14-18). We previously reported that TTG and CAE compounds contributed significantly to the apoptotic effect of iCR, CAE being the more potent inhibitor of proliferation an apoptosis inducer in MCF-7 cells (14).

This study was designed to investigate the influence of iCR and its two main fractions TTG and CAE on the invasiveness of highly metastatic MDA-MB 231 breast cancer cells.

Materials and Methods

An isopropanolic-aqueous extract of *Actaea (=Cimicifugae) racemosa rhizoma* (iCR, B.Nr. 231050) was provided by Schaper and Brümmer GmbH and Co. KG, Salzgitter, Germany. The concentration of the extract was 77.4 mg/ml in relation to the dry matter. The concentration of the total triterpene glycosides (TTG) in iCR, calculated as standard triterpene glycoside 23-epi-26-deoxyactein (formerly called 27-deoxyactein) amounted to 27 mg/ml. The concentration of polyphenolic substances, *i.e.* cinnamic acid esters (CAE), was measured according to the calibration of isoferulic acid. The total concentration of CAE was 3.7 mg/ml. The alcohol concentration was 40% (v/v). TTG and CAE fractions were obtained from the iCR batch (B.Nr. 231050) by freeze-drying and chromatographic separation. The TTG and CAE compound groups resuspended in ethyl alcohol 60% (v/v) were supplied by Schaper and Brümmer GmbH and Co. KG (Salzgitter, Germany). Paclitaxel and doxycycline were purchased from Sigma (Buchs, Switzerland).

Cell lines and culture conditions. The breast-adenocarcinoma cell lines, estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB 231, were from ATCC (Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and 2 mM L-glutamine, 1% penicillin/streptomycin (PenStrep) under a fully humidified atmosphere and 5% CO₂ at 37°C. The studies were carried out using cells from passages 3-7, which were preincubated 24 h in DMEM without phenol red, containing 5% charcoal stripped fetal calf serum (CCS) to remove exogenous estrogens. In all experiments, untreated and vehicle-treated cells were included as controls. The final concentration of ethanol and isopropanol in culture media during treatment did not exceed 0.5% (v/v). All cell culture reagents were obtained from Sigma (Buchs, Switzerland).

Cell viability assay. The effect of iCR (38.7-154.8 µg/ml), TTG (0.1-5 µg/ml) and CAE (0.1-5 µg/ml) on the viability of cells, *i.e.* live-dead discrimination, was investigated by flow cytometry. Cells (3x10⁴/well) treated for 24 h were stained with propidium iodide (PI; Sigma) at a final concentration of 5 µg/ml. After incubation for 15 min at room temperature, red fluorescence, indicative of dead cells, was measured on a FACScalibur (BD, Mountain View, CA, USA). Bivariate dot-plots of forward scatter *versus* red fluorescence intensity were performed to discriminate nonviable (PI+ events) and viable (PI- events) cells.

Cell growth assay. Crystal violet (CV) staining is a colorimetric determination of cell numbers. Cells at a density of 3000/well, that had been treated for 24 h with iCR (19.3-154.8 µg/ml), TTG (1-100 µg/ml) or CAE (1-25 µg/ml) were centrifuged and supernatants were removed. Cells were stained with 0.5% CV (in

20% methanol; Sigma) for 10 min at room temperature. In a second step, cell-bound dye was dissolved in citrate-buffered ethanol (0.05 M in 50% ethanol) and quantified at 550 nm in an ELISA reader (BMG LABTECH, Offenburg, Germany) (14). Each treatment was measured in triplicate in two independent experiments.

Invasion and migration assays. The invasive capability of breast cancer cells was quantified in a membrane invasion culture system, based on a bioassay for the digestion of extracellular matrix (ECM) proteins (Matrigel) and the migration through a filter barrier (19). Cell motility was assessed by a migration-only assay, without Matrigel. Thus, invasion and migration assays were carried out likewise differing only in the membrane coating process. The BD Biocoat™ Tumor Invasion System (BD, Biosciences, Bedford, MA, USA) was used. In invasion chambers, filters of 8-µm pore size were coated with BD Matrigel™ and reconstituted at 37°C with DMEM before use. Matrigel is a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma. Tumor cell suspensions (3x10⁴ cells/filter) with or without iCR (38.7-154.8 µg/ml), TTG (0.1-5 µg/ml) or CAE (0.1-5 µg/ml) were added to the upper compartment whereas the bottom wells were immediately filled with fibroblast-conditioned medium as chemoattractant. As controls in parallel we tested human breast cancer cells MCF-7 which are characterized by their low invasive potential. As a positive control, the anti-metastatic agents paclitaxel (70 nM) and doxycycline (80 µM) were used. After 24 h of incubation, the non-migrated and non-invasive cells in the upper chamber were carefully removed, and the adherent cells present on the lower surface of the insert were fixed with methanol and stained with Giemsa dye or with 0.5% crystal violet in 20% methanol. Migrated and invaded cells were quantified by light microscopy and/or after extraction of adhesive cells with 30% acetic acid the absorbance of the cell lysate was measured in a microplate reader at 600 nm. Cells were always counted microscopically in three randomly chosen fields (magnification x 200) of the membrane (upper left, centre, lower right). Each invasion and migration experiment was carried out in duplicate and repeated at least twice. Data are expressed as the percentage of invasion through the Matrigel matrix or of migration through an uncoated membrane relative to the untreated controls.

Statistical analysis. All data are expressed as means±S.D. Statistical differences were evaluated by Student's two-tailed *t*-test and *p*<0.05 was considered significant. IC₅₀ values were obtained by linear or exponential regression analysis of data using the Microsoft Excel software.

Results

Influence of iCR, TTG and CAE on viability and cell growth of MDA-MB 231 and MCF-7 breast cancer cells. To exclude putative cytotoxic effects of substances, the viability of MDA-MB 231 and MCF-7 cells was determined after 24 h treatment. Live and dead cells were discriminated by flow cytometry using a PI exclusion assay. We estimated the influence on cell survival according to the following criteria: >90% viable cells, unaffected; 80-90% as modestly affected; and values <80% viable cells were ascribed to cytotoxic effects

Table I. Effect of iCR, TTG and CAE on cell survival and their half maximal inhibitory concentrations IC₅₀ after 24 h exposure.

Treatment	Dose [µg/ml]	Survival [%]		IC ₅₀ [µg/ml]	
		MCF-7 mean±SD	IC ₅₀ mean±SD	MDA-MB231 mean±SD	IC ₅₀ mean±SD
Control	0	100.0±3.8		100.0±6.5	
iCR	38.7	97.5±2.3	128.5±4.9	104.3±1.2	92.5±3.5*
	77.4	95.2±3.4		89.0±2.7	
	154.8	89.0±4.2		90.9±4.1	
TTG	0.1	96.9±1.9		103.9±0.8	
	1.0	93.5±1.5	>100	102.6±1.1	96.3±5.3
	5.0	86.7±2.7		102.5±2.6	
CAE	0.1	94.3±1.9		104.6±1.4	
	1.0	90.9±2.4	~25 ^a	103.1±2.3	26.2±1.4
	5.0	83.3±3.7		96.7±3.9	

*p<0.05 vs. IC₅₀ of MCF-7 cells.

^ainhibition of cell growth at highest concentration of CAE (25 µg/ml) reached about 40%.

of the compound. Accordingly, all investigated concentrations of iCR, TTG and CAE exerted no cytotoxic effect, as shown in Table I. In untreated MDA-MB 231 and MCF-7 cells we observed 91.7% and 89.6% viable cells. Viability of treated cells was thus normalized to these untreated control cells. The viability of MDA-MB 231 cells after exposure to the paclitaxel (70 nM) and doxycycline (80 µM) was 85.7% and 89.3%, respectively.

As shown in Figure 1, treatment of MCF-7 and MDA-MB 231 cells (3000/well) with either iCR (19.3-154.8 µg/ml), TTG (1-100 µg/ml) or CAE (1-25 µg/ml) dose-dependently reduced the number of cells and prevented their exponential growth. In MCF-7 cells, the above doses of iCR, TTG and CAE caused 3.5-73.5%, 0.7-35.2% and 0.3-37.5% growth inhibition after 24 h. A stronger inhibitory effect of iCR on cell growth was observed in MDA-MB 231 cells with reductions of 3.5-85.8%. However, the inhibition of MDA-MB 231 cell growth by TTG (1.1-51.5%) and CAE (1.0-46.3%) was comparable to that in MCF-7 cells (Figure 1). The 50% growth inhibitory concentration (IC₅₀) of iCR differed significantly (p=0.01) in the cancer cell lines, whereas these for TTG and CAE fractions were closely related as shown in Table I.

Suppression of invasive activity of MDA-MB 231 cells by iCR, TTG and CAE. We have demonstrated that iCR and its two main fractions expressed antiproliferative effect on ER-positive and-negative breast cancer cells. To further these observations, anti-invasive effects of iCR, TTG and CAE were examined on highly metastatic MDA-MB 231 breast cells and poorly invasive control cells. iCR, TTG and CAE were applied at concentrations which exhibited no or only a marginal effect on tumor cell viability. In orientating

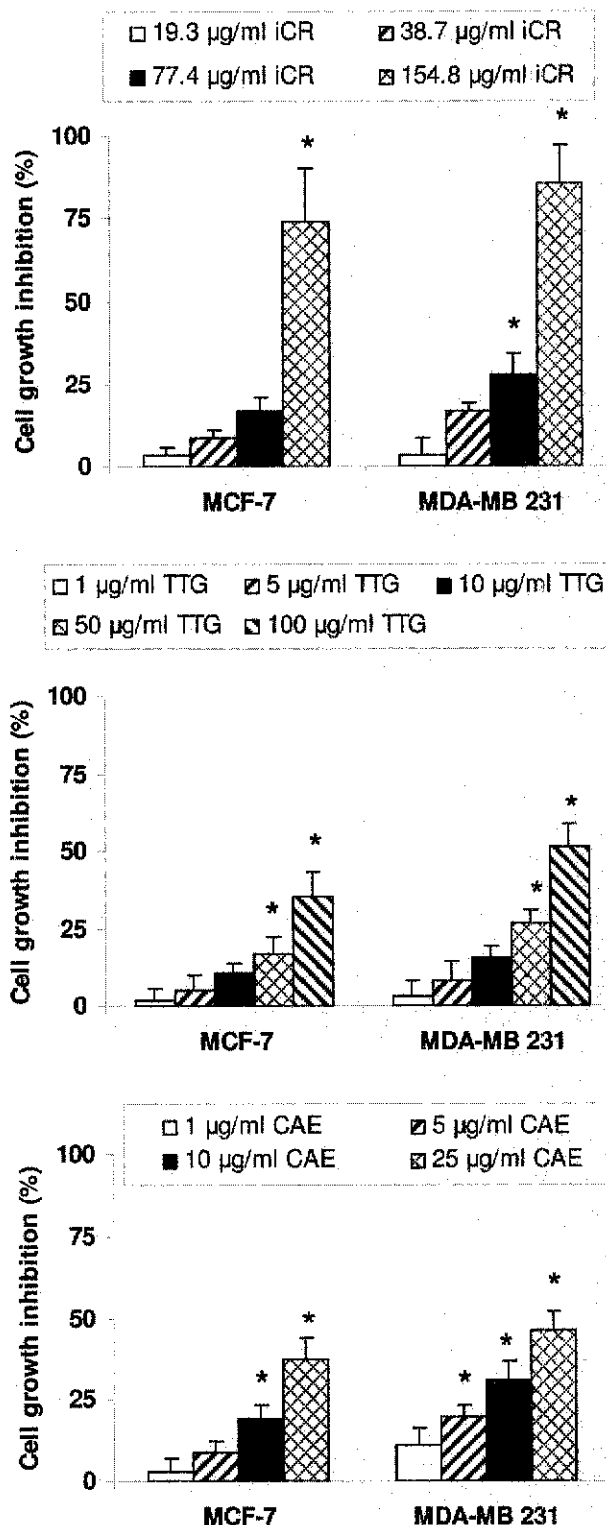


Figure 1. Effect of iCR, TTG and CAE on tumor cell growth. MCF-7 and MDA-MB 231 cells were treated for 24 h with or without substances at the indicated concentrations. Attached cells were stained with crystal violet and the absorbance of the cell lysate was measured at 540 nm. Data are expressed as mean±SD of triplicate wells from two independent experiments. *p<0.05 vs. untreated control.

comparative experiments, only 4.3% or 5.0% of MCF-7 cells invaded through the Matrigel coated membrane as determined using light microscopy or *via* cell lysates respectively, whereas 41.5% or 58.7% of MDA-MB 231 cells proved invasive. All test compounds showed an inhibitory effect on cell invasion through the Matrigel membrane. As shown in Figure 2, the invasive activity of MDA-MB 231 cells was effectively suppressed by 20%, 51% and 35% after incubation with iCR at concentrations of 38.7, 77.4 and 154.8 $\mu\text{g/ml}$, respectively. At a dose of 5 $\mu\text{g/ml}$, TTG and CAE reduced cell invasion by 34% and 25.5%, respectively. In the positive controls, paclitaxel (70 nM) and doxycycline (80 $\mu\text{g/ml}$) reduced invasion of cancer cells by 46.5% and 47.5%, respectively. The quantification of invasive cells by light microscopy, which was routinely performed before the colorimetric determination of the cell lysates, showed good correlation with the latter method. Spectrophotometrically as well as microscopically, at a concentration of 77.4 $\mu\text{g/ml}$, iCR exerted the strongest inhibitory effect of iCR namely 51.8%. TTG and CAE (5 $\mu\text{g/ml}$) fractions inhibited the invasion of MDA-MB 231 cells to a lesser extent of 26.6% and 19.5%, respectively (Figure 2).

Cell migration. Cell migration of MDA-MB 231 cells was tested at the same concentrations of iCR, TTG and CAE under culture conditions parallel to the invasion assays, only on uncoated chamber filters. The motility of cells was moderately inhibited. As shown in Figure 2, iCR at the highest concentration of 154.8 $\mu\text{g/ml}$ reduced the cell migration by about 20%. Concentrations of 5 $\mu\text{g/ml}$ of TTG and CAE caused only about 10% inhibition in cell migration. In contrast to these modest effects, paclitaxel (70 nM) and doxycycline (80 μM), inhibited the motility of MDA-MB 231 cells by 49.5% and 46.5%, respectively.

Discussion

Most metastatic and estrogen-independent breast cancers are associated with poor prognosis (20). Metastasis is a complex multi-step process, involving cell adhesion, invasion and motility. Hence, interruption of one or more of these steps is one approach of anti-metastatic therapy. To separate the anti-metastatic activity of a compound from its cytotoxic effect, the inhibition of invasiveness must be observed at non-cytotoxic concentrations of the compound (19). Therefore we first investigated the cytotoxicity of substances (Table I) and used only non toxic concentrations for further experiments on the invasive behaviour of MDA-MB 231 cells. ER-negative MDA-MB 231 cells showed a higher sensitivity to the cytotoxic effects of iCR than the ER-positive MCF-7 (Table I). These results are in accordance with our previous findings (8). However, there were no differences in the sensitivity of MCF-7 and MDA-

MB231 cells to either TTG or CAE as expressed in their IC_{50} values. These results suggest that the plant extracts contain a mixture of components which obviously interact with each other. As these are additive, synergistic or antagonistic interactions, the effect of any total extract could differ from those of its single constituents (21).

Migration and invasion are key functional activities in the progression of early stage breast cancer into a more aggressive state. Anchorage-independent growth, *i.e.* cellular growth independent from basement membrane, as a prerequisite for migration and invasion is one of the hallmarks of the metastatic phenotype. In contrast to synthetic estrogens, black cohosh did not induce the growth of breast cancer cells in an anchorage-independent soft agar assay, but moreover caused an inhibition of colony formation of MCF-7 and T47D cells (22). The nature of the applied assay complements cellular transformation *in vivo* and thus mirrors the possible progression of early stage breast cancer into a more aggressive state. To further elucidate these findings, refined *in vitro* assays were applied that mimic the *in vivo* processes. The most commonly used *in vitro* assay is a modified Boyden chamber assay using a basement membrane matrix preparation, Matrigel (19). This Matrigel matrix serves as a reconstituted authentic basement membrane *in vitro* providing a true barrier to non-invasive cells. Our experiments evidenced a 10-fold higher potential of invasiveness for MDA-MB 231 cell in comparison to ER-positive MCF-7 cells. An increased invasiveness of breast cancer cells is frequently associated with absence of ER, the characteristic expressed by MDA-MB 231 cells (23).

iCR achieved its strongest effect, *i.e.* an approximately 50% inhibition of MDA-MB 231 cell invasion, at a concentration of 77.4 $\mu\text{g/ml}$ (corresponding to 27 $\mu\text{g/ml}$ TTG and 3.7 $\mu\text{g/ml}$ CAE). This effect was comparable with the effect of paclitaxel and doxycycline used as positive controls. Interestingly, higher concentration of iCR did not further augment the anti-metastatic activity. At a concentration range between 1-5 $\mu\text{g/ml}$, both TTG and CAE fractions exerted only modest anti-invasive activity of about 25% and 15%, respectively (Figure 2), whereas a further increase in the dose of TTG and CAE (10 $\mu\text{g/ml}$) had no effect on cell invasion (data not shown). Different doses of the compounds may influence the cellular membrane properties, which may be the reason for the loss of effect of substances. Inhibition of cell invasion occurred in the absence of cytotoxicity (iCR, TTG and CAE) and with only moderate antiproliferative effect of 27%, 8.2% and 11% (iCR, TTG and CAE) suppression of cell growth. Previously we showed that the antiproliferative activity of iCR on tumor cells was strongly associated with programmed cell death, executed *via* caspase activation by the cleavage of CK 18, another very important antitumor process (8). The two TTG and CAE fractions also acted as inducers of apoptosis (14).

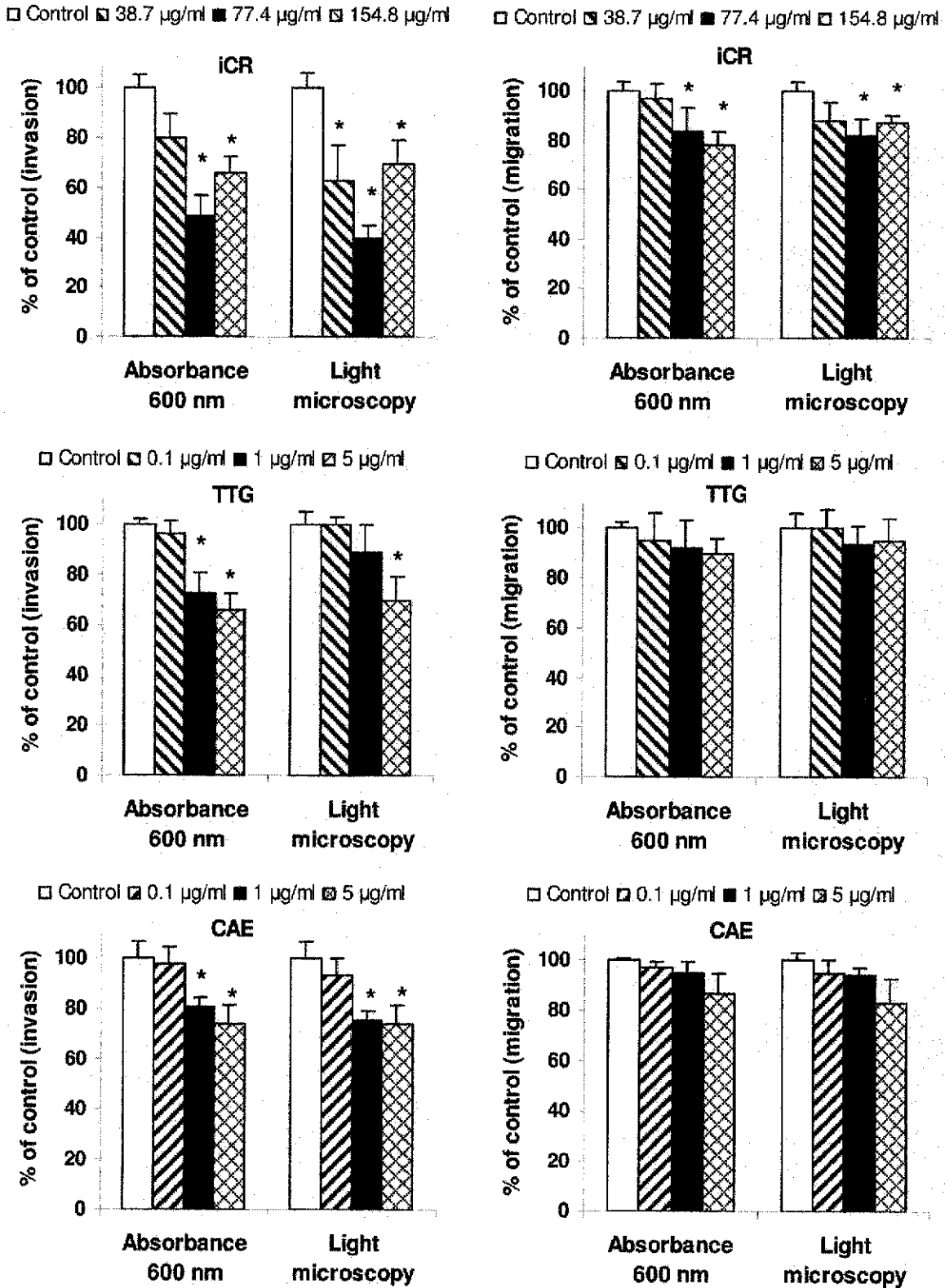


Figure 2. Effect of iCR, TTG and CAE on tumor cell invasion and migration. Highly metastatic MDA-MB231 breast cancer cells were incubated for 24 h with the indicated concentrations of substances in Matrigel coated (invasion) or uncoated (migration) transwell chambers. Cells that had invaded or migrated were stained with crystal violet and the absorbance of the cell lysate was measured at 600 nm (left) or stained with Giemsa for enumeration by light microscopy (right). The data are expressed as mean \pm SD of duplicate chambers from two experiments. * $p < 0.05$ vs. control.

Motility is another property of malignant cells needed for them to migrate from the primary site to a secondary organ. Any alteration of this property would interrupt the metastatic cascade. The current study also showed that iCR-treated cells had a moderately reduced motility, as demonstrated in a matrigel-free migration system. Low doses of TTG and CAE did not affect cell migration (Figure 2). In the positive controls, paclitaxel and doxycycline equally inhibited the motility and invasiveness of MDA-MB 231 cells at the tested concentrations. These results were in accordance with the data previously reported (24-26).

The mechanisms by which iCR (TTG and CAE) inhibit cell invasion is not clear and needs further investigations. Cyclooxygenase (COX)-2 enzyme plays a pivotal role in the metastatic process of cancer (27). Previous studies demonstrated that the inhibition of COX-2 decreased breast cancer cell motility and invasion as well as matrix metalloproteinase (MMP) expression (28, 29). It was reported that in the vena cava of iCR-treated female rats, COX-2 mRNA was downregulated and the metastatic protein vascular endothelial growth factor (VEGF) did not show any change in comparison to untreated control rats (30). Experiments performed in our laboratory in a cell free COX-2 inhibitor screening assay (Cayman Chemical Company, Ann Arbor, USA) showed that iCR at 1:500 dilution, corresponding to 154.8 µg/ml slowly suppressed the COX-2 activity (data not published).

Conclusion

Our findings indicate that iCR inhibits tumor cell invasiveness in non-cytotoxic doses. Together with its antiproliferative and apoptosis inducing properties iCR appears to be a safe and promising herbal medication, e.g. for the treatment of cancer in combination with other therapies. Its suitability for the treatment of e.g. menopause-like complaints induced by anti-hormonal therapy with tamoxifen has been shown (31). Its further potential for the treatment of cancer in combination with other therapies require additional experiments to elucidate the mechanism involved in its anti-invasive effect.

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