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Morphological effects induced by Cucurbitacin E on ovarian cancer cells *in vitro*

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Abstract

Objective: To study the effects of Cucurbitacin E, extracted from *Ecballium elaterium* L. A.Rich. on ovarian cancer cell lines *in vitro*. Materials and methods: Human ovarian cancer cells (OV_95_CC3) and human lymphocytes were treated with Cucurbitacin E (CuE), a tetracyclic triterpenoid. Morphological changes were examined under the microscope, using the Papanicolau staining procedure, after one and twenty-four hours incubation. Results: Marked effects were observed in treated ovarian cancer cells. Reversible budding of cells and thread formation were observed in previous studies. However, in the present study, cells treated with CuE demonstrated more dramatic changes, which were irreversible and more pronounced after twenty-four hours. These changes were not observed in untreated ovarian cells and normal lymphocytes treated with the same compound. Conclusion: The results obtained indicate that Cucurbitacin E is toxic to ovarian cancer cells but not to normal peripheral lymphocytes.

Key words: *Ecballium elaterium* L., Cucurbitacin E, ovarian cancer, lymphocytes, morphology.

1. Introduction

In our preliminary investigation [1], ovarian and stomach cancer cell viability was observed with various concentrations of Cucurbitacin E (CuE). CuE was isolated from *Ecballium elaterium* L. (squirting cucumber). A series of experiments were initiated to determine the potential cytotoxic effects of CuE *in vitro* on the two mentioned cancer cell lines. It was highly effective against ovarian cancer cells while busulphan was more effective against stomach cancer cells. Therefore the results obtained prompted further analysis.

Several Cucurbitacins have been previously tested. In their studies Gitter and co-workers [2] reported that the Cucurbitacins (i.e. D, I, E and CuE methylether) produced blistering and

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thread formation of Ehrlich ascites and sarcoma tumour cells. However, no other changes such as swelling or vacuolisation were observed.

Further morphological studies were carried out on CuD and CuI [3], using Ehrlich ascites and Sarcoma Black tumour cells. The results were in accordance with the previous study [2].

Shohat and co-workers [4] worked on the effects of CuI on human leukaemic and normal lymphocytes. It was observed that blister formation occurred in response to CuI. These three studies claimed that CuI was the most active compound among the Cucurbitacins. No other morphological investigations have been carried out involving Cucurbitacins, since the investigation of Shohat and co-workers [4].

The cytotoxicity of CuE is related to its structure. There are two sites of interest in the molecule; the 5α-cucurbitane hydrocarbon skeleton and the aliphatic side chain at C-17. The 23 - 24 double bond and the 25-acetyl [5, 6] moiety on the aliphatic side chain is are related to the alkylating activity on the DNA [7]. The 5α-cucurbitane hydrocarbon skeleton is related to the receptor binding at the cell membrane [8]. As a consequence, it may be postulated that the Cucurbitacins are effective against hormone-responsive tumours, which include ovarian, breast, prostate and others.

More recent investigations were carried out on Cucurbitacins, one of which was the NCI protocol for CuE [9]. CuE was also tested on prostate cancer PC3 *in vitro* [10], and other Cucurbitacins were tested on the two-stage carcinogenesis of skin tumour [11, 12]. Despite all these investigations, no morphological studies were conducted. In the present study, morphological investigations were pursued to determine the effects of CuE on a hormone-responsive cell line (OV_95_CC3).

2. Materials and methods

2.1 Chemicals

CuE was isolated from *Ecballium elaterium* fruit by solvent extraction and tested for its purity by various analytical methods against a known standard [1].

2.2 Cell Cultures

Human ovarian (OV_95_CC3) cell line and human lymphocytes were obtained from the Department of Anatomy, University of Malta. The ovarian cells were cultured and subcultured to propagate the cell line. The lymphocytes were isolated from the blood of a healthy human male volunteer, using Histopaque-1077 (Sigma, U.S.A).

2.3 Application of the cytotoxic agent

CuE serial dilutions ranging from 0.18 to 180 µM were used as treatments for the two cell types cultured. Aliquots of cell suspension (2 ml) were pipetted in the wells of a six-well plate, containing a sterile Thermanox (Nunc, Denmark) coverslip at the base. The cells were allowed to adhere to the coverslips by incubation at 37°C and 5% CO_2 for 2 days.

Meanwhile, the separated lymphocyte fraction was resuspended in RPMI with PHA and 2 ml aliquots were transferred to universal containers (Nunc, Denmark). The cells were incubated for 2 days to determine any lymphocyte activation. Both ovarian cells and lymphocytes were exposed to 2 ml of the four different CuE concentrations, leaving only one sample of each cell line untreated, as a control of cell viability. One set of cells was incubated for 1 h while the other set for 24 h at 37°C and 5% CO_2. This was performed in triplicate containers.

2.4 Morphological observations

After the specified time interval, lymphocytes were precipitated onto the slides using the Cytospin 2 (Shandon, UK) and fixed in alcohol. All fixed specimen were then stained by the

polychromic Papanicolau staining technique [13]. After staining, the slides were mounted in D.P.X. and examined under high power microscope (oil-immersion) using a Zeiss compound microscope.

Differential counts were performed by taking several photomicrographs under the low power. At least four replicates were prepared. Cells with abnormal morphology were counted as a group and the values obtained were inserted in the following equation to obtain percentage counts:

$$\text{Abnormal cell morphology (\%)} = \frac{\text{Number of abnormal cells}}{\text{Total number of cells}} \times 100$$

2.5 Statistical analysis

The values are expressed in mean±SEM (n=10, lymphocytes; n=4, ovarian cancer cells). The data was analysed by using the Kruskal-Wallis one way analysis of variance (ANOVA) to determine non parametric statistical significance ($P \leq 0.05$).

Table 1.
Percentage abnormal morphological changes, and ovarian cancer cells observed after 1 h and 24 h, at the four concentrations of CuE used.

	Percentage Abnormal Morphological Charges [Mean ± SEM]	
Concentration (µM)	**Ovarian cancer cells 1h**	**24 h**
0	0.000±0.000	0.000±0.000
0.18	4.355±0.802	†27.913±2.135
1.8	†18.102±2.769	†19.957±0.883
18	†24.900±1.063	‡31.710±1.083*
180	†23.730±1.666	‡35.378±0.889*

Each point is the mean ± SEM (ANOVA: *p<0.05, v=9 against control for each cell type and at each separate time interval) : † denotes budding and threading while ‡ denotes formation of apoptotic bodies.

3. Results and discussion

The untreated lymphocytes (Fig. 1a) remained unaltered and viable after 1 and 24 h time intervals. Untreated ovarian cancer cells (Fig. 1b) showed normal cell morphology, with no abnormal alterations in the cytoplasm, nucleus or cell membrane. The cells were viable at 1 and 24 h. CuE did not induce lymphocyte damage (Fig. 1c).

In fact, the morphology remained unaltered and the cells were viable even after 24 h. Very low CuE concentrations (i.e. 0.18 µM), produced significant alterations in the ovarian cancer cells as opposed to lymphocytes and untreated ovarian cancer cells. Abnormal morphology characterised by budding was observed after 1 h (Fig. 1d) and irreversible damage, which involved the formation of apoptotic bodies was noted after 24 h (Fig. 1e). The cells, which remained intact but showed altered morphology, took up trypan blue, confirming their death. The percentage abnormal morphological changes are tabulated below (Table 1).

The untreated lymphocytes had an irregular morphology, which is typical of activated T-lymphocytes. The treated lymphocytes were not affected by the drug as cell viability was still high after 1 and 24 h (Attard, 1996 unpublished observations). The finding that the lymphocytes were unaffected by the drug is in accordance with the study of Shohat and co-workers [4].

In the latter case, lymphocytes were treated with CuI (0.4 and 1.2 µM). These observations are in accordance with the postulations made by Dougherty and co-workers [14]. These stated that a steroid-like compound with no hydroxyl group at C_{17}, no ketone group at C_{20} and a ketone group at C_{11} only, is practically non-toxic to lymphocytes. This configuration fits with that of CuE.

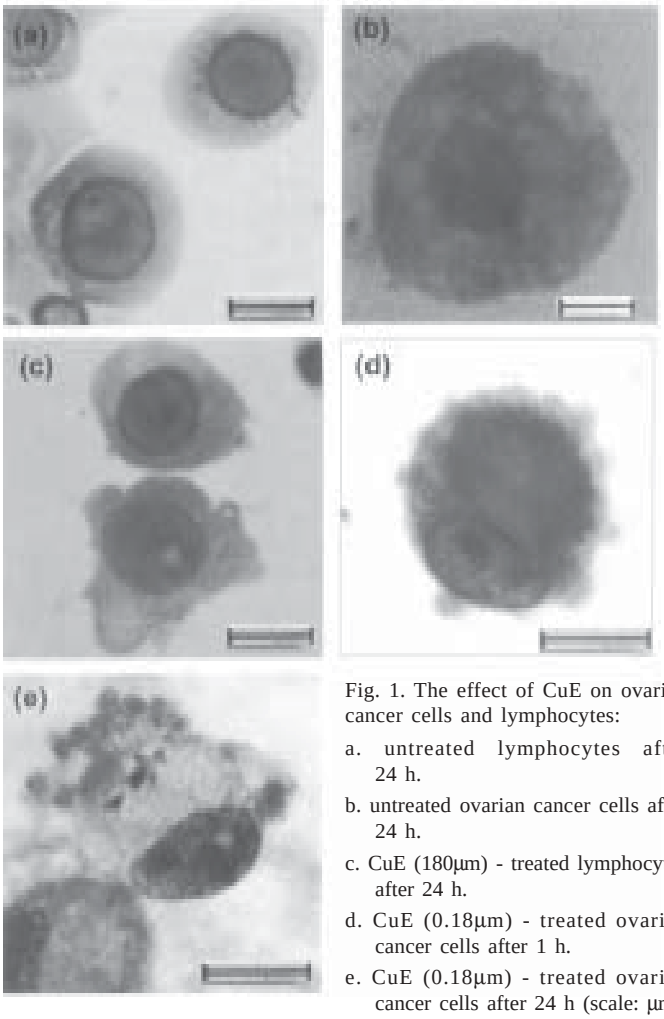

Fig. 1. The effect of CuE on ovarian cancer cells and lymphocytes:

a. untreated lymphocytes after 24 h.

b. untreated ovarian cancer cells after 24 h.

c. CuE (180μm) - treated lymphocytes after 24 h.

d. CuE (0.18μm) - treated ovarian cancer cells after 1 h.

e. CuE (0.18μm) - treated ovarian cancer cells after 24 h (scale: μm).

The untreated ovarian cancer cells had a regular morphology, i.e. round cells with an oval nucleus. However, with drug treatment, there were unusual morphological changes, which appeared after 1 h, for all concentrations except the 0.18 μM concentration. The cells manifested budding, which consisted of cytoplasmic protrusions. These buds detach from the cell to form isolated apoptotic bodies.

At the same time, the nucleus shrunk with occasional cells showing disintegration of the nucleoli. Cell disintegration occurred only with the high concentrations (18 μM and 180 μM) after 24 h ($P<0.05$). This is the late phase of apoptosis. These changes were irreversible, with blebbing showing early signs of cell death. No blistering was observed as described by the research teams of Gitter [2], Gallily [3] and Shohat [4].

Moreover, the morphological changes described in previous studies were reversible after 24 h. This indicates, that in these studies, the effects of the Cucurbitacins was only temporary while in the present study with ovarian cancer cells, the effects were more pronounced after 24 h, suggesting, the induction of cell death through apoptosis, by Cucurbitacins on this cell line.

On the other hand, the budding effect, on lymphocytes, was described by Doughtery and co-workers [14] when treated with corticosteroids. King and co-workers [15] described the presence of cytoplasmic blebs in Ehrlich ascites cells, when treated with a mercurial metabolic inhibitor (salyrgin). These cytoplasmic blebs presumably correspond to cell budding.

The freeing of the nuclei, in the present investigation is in accordance with the findings of Palmer and co-workers [16], who described this effect when sodium lauryl sulphate was applied to Ehrlich ascites cells at concentrations of 0.6 and 0.7 mM.

This study shows that Cucurbitacins may have a potential effect on cells, that are hormone-responsive, and hence prompts further studies in this direction.

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