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# The Impact of CV247 Component on Human Cancer Cell Lines



This study is the first to investigate the effect of CV247, a potent cytotoxic-cytostatic agent, in cell lines from several types of human cancer. CV247 was originally developed for use in veterinary practice to treat different types of cancer in animals, primarily in dogs. Currently, CV247 is being evaluated for efficacy and safety in pilot studies in humans. This supportive *in vitro* study was designed to measure the effect of CV247 on four different types of cancer cell lines (breast, colon, prostate and lung) using chemosensitivity (viability and cytotoxic-cytostatic) assays after different exposure time periods (0h, 24h and 48h). The results were then compared with flow cytometry-based assays. All of the above categorised assays showed that CV247 can markedly decrease the initial number of viable cancer cells, especially in breast and colon cancer lines, when compared with untreated cells.

**Key words:** viability assays, CV247 agent, colon cancer, breast cancer

## Introduction

CV247 is a patented combination of four known substances, with properties that may be of benefit in cancer treatment. Preliminary studies confirmed that CV247 improved the quality of life in animals, particularly in dogs, with a wide variety of cancer types. In addition, cancer regression was observed in NOD/SCID mice with engrafted tumours. Observations from these studies suggested that CV247 did not treat the tumour *per se* but reduced the final tumour mass. Recent pilot studies in man have shown that CV247 has improved the quality of life in some patients with terminal stage of cancer and particularly those with progressive prostate cancer.

CV247 consists of manganese and copper gluconates, ascorbic acid (vitamin C) and sodium salicylate (SS).

In this study, cytotoxicity tests, such as MTT (methyl-tetrazolium dye), SRB (Sulforodhamine B assay) and CV (Crystal Violet elution dye assay), as well as flow cytometry-based assays (PI, 7-AAD) have been used in order to investigate the effect of CV247 on cancer cell line (breast, colon, prostate and lung) viability.

## Materials and Methods

In order to test the effect of CV247 in several types of cancerous cells, a number of cancer cell lines obtained from the European Collection of Cell Culture (ECACC) have been used. All the cell lines supplied by ECACC undergo comprehensive quality control and authentication procedures. These include testing for mycoplasma by culture isolation, Hoechst DNA staining and PCR, together with culture testing for contaminant bacteria, yeast and fungi. Authentication procedures used include species verification by isoenzyme analysis and identity verification by DNA profiling. Classical DNA fingerprinting using multi-locus probes is carried out for non-human cell lines. Human cell lines are analysed by PCR for short tandem repeat sequences within

chromosomal satellite DNA (STR-PCR).

The studied cancer cell lines represented breast carcinomas (MDA-MB231, MFM-223, T47D), colon carcinomas (LoVo, HCT-8, HT55), prostate carcinomas (22Rv1, LNCaP, VCaP) and lung carcinomas (H69V, Lc-2/ad, COLO699N). In order for cells to proliferate, they were incubated at 37°C, in 5% CO<sub>2</sub> environment, in 75cm<sup>2</sup> flasks (Orange Scientific, 5520200) containing the growth medium essential for each cell line together with the appropriate amount of heat-inactivated foetal bovine serum (FBS) (Invitrogen, 10106-169), 1% Penicillin/Streptomycin (Sigma, P0781) and 2mM L-Glutamine (Sigma, G5792).

## Chemosensitivity Assays

The first panel of the experiments included assays which were based on the quantification analysis of living cells using indirect parameters (chemosensitivity – colorimetric assays)

Cells were detached by trypsinisation (Trypsin-0.25% EDTA, Invitrogen, 25200-072) during logarithmic phase and were plated in triplicates in 96-well plates (Corning, Costar 3599) in four different densities (2,000 cells/well, 10,000 cells/well, 18,000 cells/well, 26,000 cells/well) in a final volume of 200µl of culture medium per well. After 70-80% confluence of the culture, the medium was removed and CV247 agent was added in seven graduated concentrations, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml, diluted in water (the maximum equivalent of 800 µg of ascorbic acid, 700 µg of sodium salicylate, 5.6 µg of copper gluconate and 5 µg of manganese gluconate per well). The optical absorbance resulting from the number of living cells was measured at different exposure times (0h, 24h and 48h).

Three different types of chemosensitivity-cytotoxicity assays were used, and at the end of the incubation periods the number of living cells, measured at an optical density of 570nm using a µQuant™ Biomolecular Spectrophotometer MQX200 and Gen5™ Microplate Data Collection & Analysis software (BioTek® Instruments, Inc, April 2008, ©2006-2008, Revision E). Since absorbance measurements are influenced by many factors such as sample turbidity, dust particles and bubbles, dirty microplates, well geometry and absorption to well surfaces, a second wavelength of absorbance for all the individual assays was studied in order to subtract the noise and deviations. In MTT assay, the absorbance value at A570nm was corrected by a second measurement at A630nm. The same method was used for SRB and CV assay with an additional measurement at 690nm<sup>1,2,3,4</sup>.

For the MTT protocol, 20 µl of 5mg/ml MTT [bromide 3-(4,5-dimethylthio-azo-2)-2,5-diphenyl-tetrazole] (Sigma, M2128) was added to each well of plated cells which were then incubated for 3h at 37°C. At the end of the incubation period, the medium was removed and cells were rinsed with PBS (Sigma, P3813). The formazan crystals were dissolved with 100 µl of dimethylsulphoxide (DMSO) (Sigma, D4540)<sup>5,6,7,8,9,10,11</sup>.

For the SRB assay, cells were fixed by layering 50 µl of 10% trichloroacetic acid (Fluka, 91228) and the plates

incubated at 40C for 1h. Afterwards, cells were rinsed with water and stained with 100 µl of 0.4 % SRB (Sigma, 341738), dissolved in 1 % acetic acid (Carlo Erba, 401422), for 15 min. The unbound stain was removed, by washing twice with 1 % acetic acid followed by the addition of 200 µl of 10mM Tris Buffer pH 10.5 (Sigma, T6791) in order to release the bound dye<sup>12,13,14</sup>. Finally, for the CV assay, the medium was removed from the 96-well plates and each well was rinsed with PBS. Cells were fixed by adding 100 µl of 10 % formalin (MERCK, 1.04003.2500) for 20 min. The formalin was removed and then 100µl of 0.25 % aqueous crystal violet (Sigma, HT901) was added for 10 min. Finally, by addition of 100µl of 33 % acetic acid, the stain or dye was dissolved<sup>13,15,16,17</sup>.

## Flow Cytometry-Based Assay

The second panel of the experiments included a flow cytometry-based assay. In order to check each cell line's behaviour after treatment with CV247 agent, 3x10<sup>6</sup> cells were plated in culture flasks and a comparison between treated and untreated cells was made at different exposure times (0h, 24h, 48h). For this reason, two different fixing protocols were used. Firstly, cells were fixed by using 70 % methanol (Carlo Erba, 414816) in PBS (1h in 40C) and secondly by using 10 % paraformaldehyde (Sigma, 15,812-7) followed by an incubation period (30 minutes at room temperature), in order to obtain similarities or differences on staining. The staining with 7-aminoactinomycin (Beckman Coulter, A07704) and propidium iodide/ RNase staining buffer (Beckton Dickinson, 550825) was made according to the manufacturer's instructions<sup>18,19,20,21,22,23,24</sup>.

## Statistical Analysis

All treatments for every cell line were conducted in triplicate, three times. The statistical significance of all effects was evaluated by "difference of the means" test (p < 0.05).

## Results

The results were different in each cell line. However, it was observed that CV247 at concentrations of 50µg/ml and 100µg/ml had a greater effect. The results were also time-dependent. The effectiveness of the drug was found to be better after 24h and 48h of incubation. CV247 appeared to have a more pronounced cytotoxic-cytostatic effect in breast and colon cancer cell lines, notably in HCT-8 and HT55 colon cancer cell lines, and T47D and MDA-MB 231 breast cancer cell lines. CV247 was only observed to have an effect on a single prostate cancer cell line (VCaP).

In order to calculate the decrease fold, the absorbance measurements have been used. The absorbance is given by the Beer-Lambert law where the formula is  $A = \epsilon \cdot c \cdot l$ , where A is absorbance,  $\epsilon$  is the extinction coefficient, l is the distance the light travels through the material, and c is the concentration of the absorbing species within the material (28). See Figs 1-2 and Tables 1-4.

## Discussion

It is well known that cancer has the highest mortality rate on a global level. Many studies have been made in order to discover the mechanisms of carcinogenesis and cancer progression. Researchers suggest that DNA cross-linking and damage, and the accumulation of mutations in enzymatic function or in

Figure 1: CVE assay results for MDA-MB 231 cell line, Toloudi M.

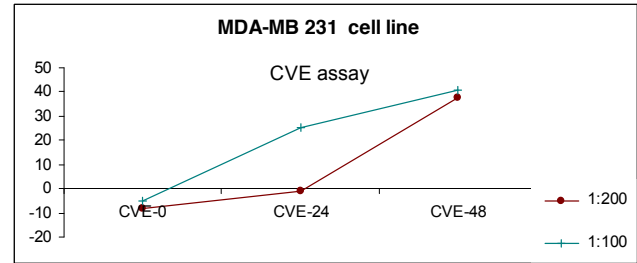


Figure 2: SRB assay results for MDA-MB 231 cell line, Toloudi M.

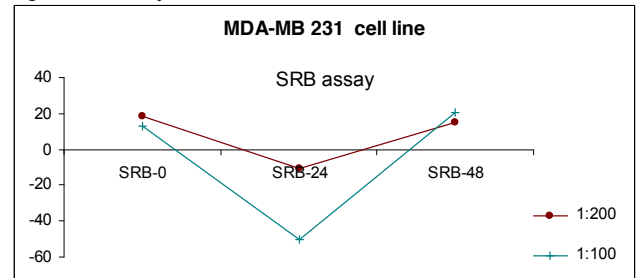


Figure 3: MTT assay results for MDA-MB 231 cell line, Toloudi M.

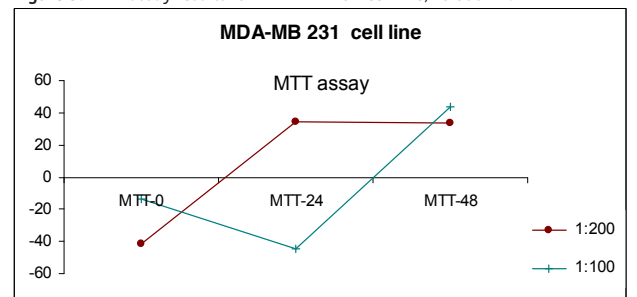


Figure 4: CVE assay results for HT55 cell line, Toloudi M.

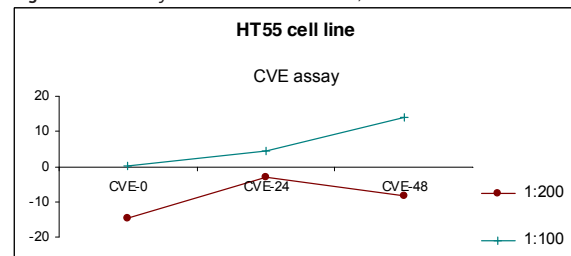


Figure 5: SRB assay results for HT55 cell line, Toloudi M.

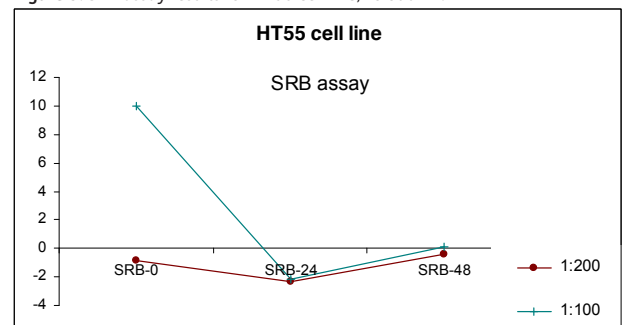
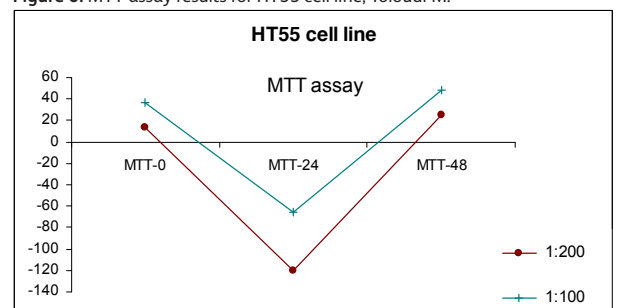


Figure 6: MTT assay results for HT55 cell line, Toloudi M.



enzymatic cascades such as signal transduction pathway, are some of the causes for the development of the disease. With regard to all of these, many substances have been tested in order to ascertain their suitability as candidate anti-cancer drugs.

These substances interfere with mechanisms that inhibit carcinogenesis and T cell proliferation in response to mitogens, enhance the immune system and activate the enzymatic pathways in order to cause tumour cell death<sup>29,30</sup>. During cancer progression the interaction between malignant cells and stroma passes through three sequential phases: elimination or immune surveillance; equilibrium; and immune escape. Through cancer progression, the cancerous cells overpass the immune surveillance, and the dynamic equilibrium between cancer cell's grow rate and cell's killing due to immunity goes towards the cancer growth. At this point, cells evade immune checking and a tumour development is present. All the three phases are called immune editing and cancer immune surveillance is considered to be an important host protection process to inhibit carcinogenesis. Several types of cells and cytokines play critical roles to this response. Initially, the immune system identifies cancerous and premalignant cells and depletes them. Natural killer group 2D protein (NKG2D) which is a c-lectin-like receptor on the surface of human natural killer (NK) cells, T cells, and CD8+ T cells, when boned with their ligands, can activate a killer algorithm against tumour attack. Although, in some cases, tumour-derived soluble factors facilitate the escape from immune surveillance allowing progression of the tumour and in fact cause malignancies and metastasis<sup>31,32,33</sup>.

In particular it has been discovered that SS inhibits, non-selectively, cyclo-oxygenase enzymes (COX), in particular COX2, and this has probably a pathological role connected with carcinogenesis<sup>34,35,36</sup>. COX-2 and COX-1 are enzymes that catalyse the conversion of arachidonic acid to prostaglandins and thromboxane. The COX-2/Prostaglandin E2 (PGE2) pathway seem to be particularly implicated in colorectal tumorigenesis through a variety of different mechanisms, such as promoting tumour maintenance and progression, encouraging metastatic spread and participating in tumour initiation. Finally, COX-2 and nitric oxide synthase (iNOS), appears to promote tumour cell proliferation, inhibit apoptosis and stimulate angiogenesis through activation of HIF1 $\alpha$ <sup>37,38,39,40</sup>.

As previously mentioned, CV247 is composed of manganese and copper gluconates, vitamin C and sodium salicylate. All four components have properties of potential benefit in the treatment of cancer and cancer progression. Collectively they have been shown to improve symptoms and prolong life in some patients with terminal cancer, possibly via stimulation of the immune system or down-regulation of the production of cytokines such as IL-18.

From the evidence of the current study, and from its known properties, it can be assumed that SS contributes to the apparent anti-proliferate effect of CV247 through inhibition of COX2. Additionally, it is known that many enzymes are inactive in cancer cells due to the lack of essential redox metals. Manganese and copper gluconates may restore the balance of these essential metals which may lead to cancer cell apoptosis, and yet leave healthy cells unaffected. Finally these essential metals lead to increased activity of enzymes such as superoxide dismutase which neutralise free radicals by virtue of the metal cation's oscillating oxidation state. By restoring the activity of

**Table 1:** Statistic evaluation of absorbance values in MDA-MB 231 cell line, Toloudi M.

<b>MDA-MB 231 Breast Cancer Cell Line</b>						
ABSORBANCE	CVE assay					
	0hrs		24hrs		48hrs	
	MEAN	STD. DEV.	MEAN	STD. DEV.	MEAN	STD. DEV.
Unstimulated cells	0.079	±0.014	0.098	±10.02	0.1	±10.028
1:200	0.112	±0.044	0.064	±0.003	0.066	±0.004
1:100	0.090	±0.011	0.142	±0.084	0.056	±0.011
ABSORBANCE	SRB assay					
	0hrs		24hrs		48hrs	
	MEAN	STD. DEV.	MEAN	STD. DEV.	MEAN	STD. DEV.
Unstimulated cells	1.899	±0.261	1.793	±0.239	1.601	±0.154
1:200	1.550	±0.06	1.982	±0.113	1.358	±0.154
1:100	1.659	±0.138	2.700	±0.017	1.269	±0.042
ABSORBANCE	MTT assay					
	0hrs		24hrs		48hrs	
	MEAN	STD. DEV.	MEAN	STD. DEV.	MEAN	STD. DEV.
Unstimulated cells	0.305	±0.006	0.468	±0.025	0.646	±0.064
1:200	0.33	±0.011	0.472	±0.116	0.402	±0.049
1:100	0.321	±0.011	0.350	±0.039	0.383	±0.062

**Table 2:** Statistic evaluation of decrease fold values, Toloudi M.

DECREASE FOLD	<b>MDA-MB 231 Breast Cancer Cell Line</b>								
	CVE assay			SRB assay			MTT assay		
	0hrs	24hrs	48hrs	0hrs	24hrs	48hrs	0hrs	24hrs	48hrs
1:200	-8.2	-0.9	37.8	18.4	-10.5	15.2	-41.8	34.7	34.0
1:100	-5.2	25.2	40.7	12.6	-50.6	20.7	-13.9	-44.9	44.0

**Table 3:** Statistic evaluation of absorbance values in HT55 cell line, Toloudi M.

<b>HT55 Colon Cancer Cell Line</b>						
ABSORBANCE	CVE assay					
	0hrs		24hrs		48hrs	
	MEAN	STD. DEV.	MEAN	STD. DEV.	MEAN	STD. DEV.
Unstimulated cells	1.246	±0.170	2.019	±0.106	3.101	±0.109
1:200	1.428	±0.024	2.081	±0.281	3.358	±0.242
1:100	1.243	±0.096	1.929	±0.085	2.663	±0.219
ABSORBANCE	SRB assay					
	0hrs		24hrs		48hrs	
	MEAN	STD. DEV.	MEAN	STD. DEV.	MEAN	STD. DEV.
Unstimulated cells	2.061	±0.101	2.798	±0.009	2.944	±0.035
1:200	2.079	±0.091	2.863	±0.023	2.957	±0.004
1:100	1.856	±0.170	2.859	±0.022	2.941	±0.009
ABSORBANCE	MTT assay					
	0hrs		24hrs		48hrs	
	MEAN	STD. DEV.	MEAN	STD. DEV.	MEAN	STD. DEV.
Unstimulated cells	2.448	±0.708	0.526	±0.085	0.891	±0.071
1:200	2.120	±0.145	1.161	±0.668	0.67	±0.032
1:100	1.539	±1.077	0.874	±0.706	0.459	±0.047

**Table 4:** Statistic evaluation of decrease fold values in HT55 cell line, Toloudi M.

DECREASE FOLD	<b>HT55 Colon Cancer Cell Line</b>								
	CVE assay			SRB assay			MTT assay		
	0hrs	24hrs	48hrs	0hrs	24hrs	48hrs	0hrs	24hrs	48hrs
1:200	-14.6	-3.1	-8.3	-0.9	-2.3	-0.4	13.4	-120.7	24.8
1:100	0.2	4.5	14.1	9.9	-2.2	0.1	37.1	-68.2	48.5

the enzymes such as GST and GS intracellular free radical and consequential DNA damage decrease.

This study provides for an interesting comparison with the studies conducted in man. An early pilot study of orally administered CV247 in 37 patients with progressive malignancies

(prostate, ovarian, colorectal, breast and miscellaneous cancer) found that favourable responses were only apparent in patients with ovarian and prostate cancers. A follow-up Phase II study in 120 patients with early progressive prostate cancer found that CV247 was of benefit by slowing disease progression, as measured by PSA doubling time, especially in treatment-naïve patients. There were no serious side-effects in this study and only minimal cases of nausea and dyspepsia. However, there was no significant difference between CV247 and one of its components, sodium salicylate<sup>41</sup>.

The present in vitro study was designed to evaluate the cytotoxic-cytostatic effects of CV247, and though there is clear evidence that the number of cancer cells decreased, of the four different cancer types that were tested the most efficient (positive) results arose mostly from breast and colon cancer cell lines, and not prostate. This might suggest cancer specificity and explain why in patients with prostate cancer, equal benefit is derived from CV247's anti-inflammatory component. The results from this study are most encouraging and further studies are underway to evaluate the modus operandi of CV247, the longevity of effect and possible benefits when in combination with established cytotoxic agents in both breast and colon cancer cell lines.

### Conclusion

In conclusion, this is probably the first study to investigate CV247 as a cytotoxic-cytostatic in a large spectrum of cancer cell types. It was found that CV247 was able to decrease the number of viable cancer cells by up to 30 % of the total cell population, particularly in colon and breast cancer cell lines when compared to untreated cell lines. Clearly the study only revealed a trend with regard to the capabilities of CV247 as a candidate cytotoxic agent, and further studies are underway in larger numbers of human cancer cell lines with different periods of exposure, with and without established cytotoxic agents.

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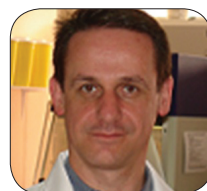
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**Ioannis Papisotiriou**

I was born in Germany in 1973 and after years I return in childhood in Greece where I studied in Medical school of Thessaloniki and I specialised in Human Genetics in Switzerland. Two master degree rewards have been obtained in molecular biology in Medicine from the Westminster University (UK) and in oncology from the University of Nottingham (UK). A promotion have been performed (MD) in MLU in Germany under the field of evaluation of TKIs in human cancer cell lines. Since 2004 I am the director and founder of RGCC Ltd which is activated in both areas of services (Research and Clinical) Email: [papisotiriou.ioannis@rgcc-genlab.com](mailto:papisotiriou.ioannis@rgcc-genlab.com)