Volume 3 - Issue 3

JOURNAL FOR CLINICAL STUDIES

Your Resource for Multisite Studies & Emerging Markets

PEER REVIEWED

Complying with FDA Guidance on the Prospective
Assessment of Suicidality in Clinical Trials

Singapore
Internationally recognised clinical trial hub

The impact of CV247 component
on Human Cancer Cell Lines

Thinking Outside the Box
How the pharmaceutical packaging industry is looking at its green credentials

www.jforcs.com

The Impact of CV247 Component on Human Cancer Cell Lines

PEER REVIEWED

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 salycylate (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 37oC, in 5 % CO2 enviroment, in 75cm2 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-cytotoxity 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[1,2,3,4].

For the MTT protocol, 20 µl of 5mg/ml MTT [bromide 3-(4,5-dimethyltio-azo-2)-2,5-diphanyl-tetrazole] (Sigma, M2128) was added to each well of plated cells which were then incubated for 3h at 37oC. 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 dimethyl-sulphoxide (DMSO) (Sigma, D4540)[5,6,7,8,9,10,11].

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

www.biocair.com

We have designed our website around our four areas of expertise.

We have created a Knowledge Base containing articles from our experts and useful FAQs on the following areas:

But it doesn't stop there. Biocair will be adding new articles on each of these areas on a monthly basis. So sign up today and you can be alerted when a new article in your area of interest is uploaded.

incubated at 4oC 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[12,13,14]. 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[13,15,16,17].

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, 3x106 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 4oC) 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[18,19,20,21,22,23,24].

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 $A=\varepsilon\ cl$, where A is absorbance, ε 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.

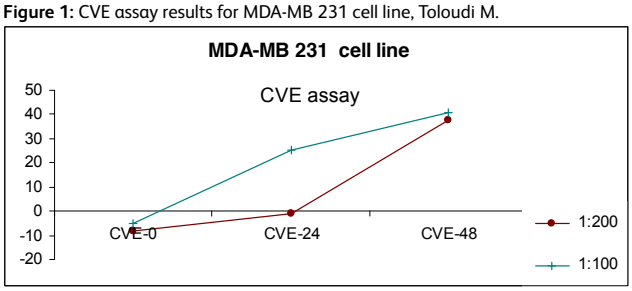


Figure 2: SRB assay results for MDA-MB 231 cell line, Toloudi M.

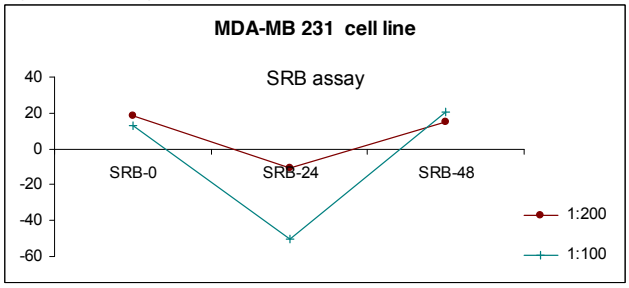


Figure 3: MTT assay results for MDA-MB 231 cell line, Toloudi M.

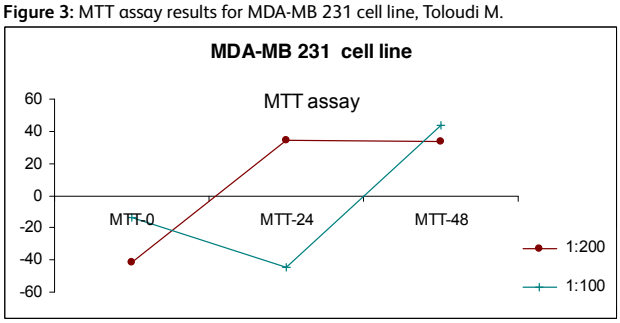


Figure 4: CVE assay results for HT55 cell line, Toloudi M.

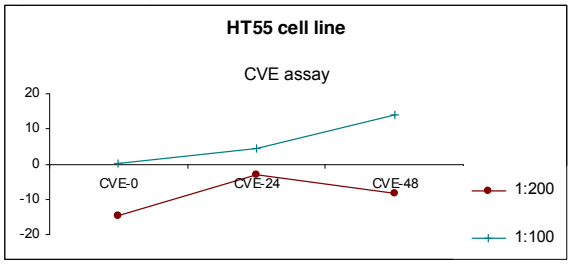


Figure 5: SRB assay results for HT55 cell line, Toloudi M.

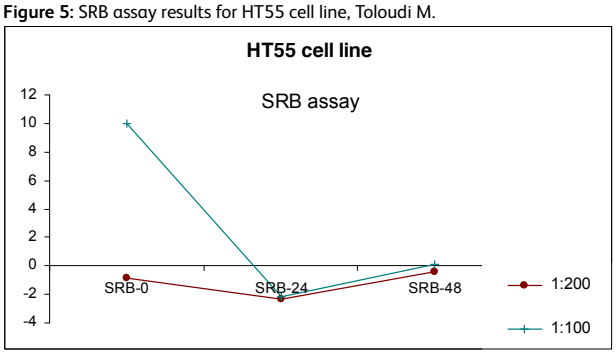
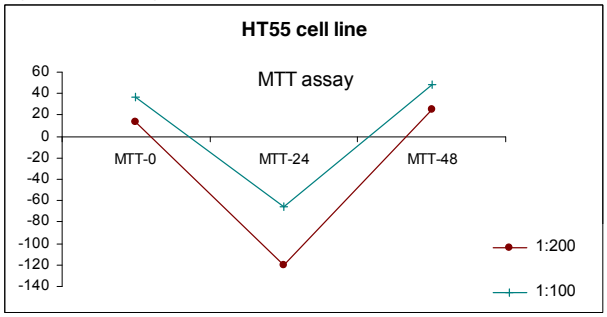


Figure 6: MTT assay results for HT55 cell line, Toloudi M.



YOU CAN'T CONTROL THE ELEMENTS...

...ZeoCool can!

ZeoCool is a revolutionary 2-8 °C active shipping system that continuously reacts to ambient temperature ensuring:

- Maximum product protection – ZeoCool uses thermostatically controlled cooling and heating.
- No system assembly required – ZeoCool uses instant push button activation.
- No need for coolant conditioning with fridges or freezers – ZeoCool is self contained.
- Maximum reliability – ZeoCool needs no internal power source.
- Reduced shipping costs – ZeoCool can reduce volumetric weight by up to 45 %.

To see how ZeoCool can help you reduce costs and ensure complete protection for your product, visit www.zeocool.co.uk

ZeoCool – where the thinking is actually done inside the box!

ZeoCool
A to B at a constant °C

ZC0110A03/1

SCA Cool Logistics, Celsius House, The Stanbridge Building, Stanbridge Rd, Leighton Buzzard, Beds LU7 4QQ UK Telephone: +44 (0)1525 243770

www.sca-cool-logistics.com

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[29,30]. 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[31,32,33].

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[34,35,36]. 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 tumorogenesis 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 HIF1α[37,38,39,40].

As previously mentioned, CV247 is composed of manganese and copper gluconates, vitamin C and sodium salycylate. 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 the enzymes such as GST and ε 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

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

MDA-MB 231 Breast Cancer Cell Line						
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	**MDA-MB 231 Breast Cancer Cell Line**								
	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.

HT55 Colon Cancer Cell Line						
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	***HT55 Colon Cancer Cell Line***								
	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	-66.2	48.5

Not everything in life is black and white...

ZEBRA

Specialising in medical and pharmaceutical translation in all language combinations, we are able to meet all your requirements from a single drug label to a complete multi-lingual marketing authorisation application. With a team of experienced and qualified in-country translators at our disposal, we ensure that projects are completed on time, every time – and you have added peace of mind, secure in the knowledge that you are dealing with an ISO accredited company that can provide declarations guaranteeing all translations.

To find out more about how we can help you, please contact
Jill Wright: Tel +44 (0) 1462 429714
jwright@zebratranslations.co.uk
www.zebratranslations.co.uk

(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[41].

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.

References

1 *Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic-cytostaticity assays. J Immunol Methods 1983; 65 (1-2):55-63.*

2 *Haselsberger K, Peterson DC, Thomas DG, Darling JL. Assay of anticancer drugs in tissue culture: comparison of a tetrazolium-based assay and a protein binding dye assay in short-term cultures derived from human malignant glioma. Anticancer Drugs 1996; 7 (3):331-8.*

3 *Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxic-cytostaticity assay for anticancer-drug screening. J Natl Cancer Inst. 1990; 82(13):1107-12.*

4 *Grady JE, Lummis WL, Smith CG. An Improved Tissue Culture Assay: III. Alternate Methods for Measuring Cell Growth. Cancer Res 1960; 20:1114-1117.*

5 *Liu Yuanbin, Peterson Daniel A, Kimura Hideo, Schubert David. Mechanism of Cellular 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Reduction. J Neurochem 1997; 69 (2):581-593.*

6 *Sargent JM. The use of the MTT assay to study drug resistance in fresh tumour samples. Recent Results Cancer Res 2003; 161:13-25.*

7 *Hayon T, Dvilansky A, Shpilberg O, Nathan I. Appraisal of the MTT-based assay as a useful tool for predicting drug chemosensitivity in leukemia. Leuk Lymphoma 2003; 44 (11):1957-62.*

8 *Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys 1993; 303 (2):474-482.*

9 *Edmondson JM, Armstrong LS, Martinez AO. A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. Methods in Cell Science 1988; 11:15-17.*

10 *Freimoser FM, Jakob CA, Aebi M, Tuor U. The MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] Assay Is a Fast and Reliable Method for Colorimetric. Determination of Fungal Cell Densities. Applied and Environmental Microbiology. 1999; 65 (8):3727–3729.*

11 *Lin ZX, Hoult JR, Raman A. Sulphorhodamine B assay for measuring proliferation of a pigmented melanocyte cell line and its application to the evaluation of crude drugs used in the treatment of vitiligo. J Ethnopharmacol 1999; 66 (2):141-150.*

12 *Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxic-cytostaticity screening. Nat Protoc 2006; 1 (3):1112-6.*

13 *Voigt W. Sulforhodamine B assay and chemosensitivity. Methods Mol Med 2005; 110:39-48.*

14 *Davey J, Lord JM. (2003). Essential Cell Biology. p:25-31, Oxford University Press.*

15 *Chiba K, Kawakami K, Tohyama K. Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells Toxicology in Vitro 1998; 12 (3):251-258.*

16 *Haselsberger K, Peterson DC, Thomas DG, Darling JL. Assay of anticancer drugs in tissue culture: comparison of a tetrazolium-based assay and a protein binding dye assay in short-term cultures derived from human malignant glioma. Anticancer Drugs 1996; 7 (3):331-8.*

17 *Keepers YP, Pizao PE, Peters GJ, van Ark-Otte J, Winograd B, Pinedo HM. Comparison of the Sulforhodamine B Protein and Tetrazolium (MTT) Assays for in vitro Chemosensitivity Testing. Eur. J. Cancer 1991; 27 (7):897-900.*

18 *Lecoeur H. Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases. Exp. Cell Res 2002; 277 (1):1–14.*

19 *Suzuki T, Fujikura K, Higashiyama T, Takata K. DNA staining for fluorescence and laser confocal microscopy. J. Histochem. Cytochem 1997; 45 (1):49–53.*

20 *Ormerod MG. Flow cytometry. 2nd ed. , âIOS Scientific Publishers 1999.*

21 *Macey MG. Flow cytometry, Principles and Applications. Humana Press 2007.*

22 *Moore A, Donahue CJ, Bauer KD, Mather JP. Simultaneous measurement of cell cycle and apoptotic cell death. Methods Cell Biol 1998; 57:265–78.*

23 *Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV. Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. Cytometry 1992; 13 (2):201-208.*

24 *Gill JE, Jotz MM, Young SG, Modest EJ, Sengupta SK. 7-Amino-*

actinomycin D as a cytochemical probe. I. Spectral properties. J. Histochem. Cytochem 1975; 23(11):793-799.
25 *Liu X, Chen H, Patel D. Solution structure of actinomycin-DNA complexes: drug intercalation at isolated G-C sites. J Biomol NMR 1991; 1 (4):323–47.*
26 *Latt S. Fluorescent probes of chromosome structure and replication. Can J Genet Cytol 1977; 19 (4):603–23.*
27 *Fetterhoff TJ, Holland SP, Wile KJ. Fluorescent detection of non-viable cells in fixed cell preparations. Cytometry 1993; 14 (Suppl. 6):27.*
28 *Wu D, Cederbaum AI. Sodium salicylate increases CYP2E1 levels and enhances arachidonic acid toxicity in HepG2 cells and cultured rat hepatocytes. Mol Pharmacol 2001; 59 (4):795-805.*
29 *Davis CD, Feng Y. Dietary copper, manganese and iron affect the formation of aberrant crypts in colon of rats administered 3, 2'-dimethyl-4-aminobiphenyl. J Nutr 1999; 129 (5):1060-7.*
30 *Michael A Caligiuri. Immune surveillance against common cancers: the great escape. Blood 2005; 106 (3):773-774.*
31 *Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology 2007; 121 (1):1-14.*
32 *Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Invest 2007; 117 (5):1137-1146.*
33 *Marshall JC, Caissie AL, Cruess SR, Cools-Lartigue J, Burnier, MNJr. The effects of a cyclooxygenase-2 (COX-2) expression and inhibition on human uveal melanoma cell proliferation and macrophage nitric oxide production. J Carcinog. 2007; 6:17.*
34 *Moore BC, Simmons DL. COX-2 inhibition, apoptosis, and chemoprevention by nonsteroidal anti-inflammatory drugs. Curr Med Chem 2000; 7 (11):1131-44.*
35 *Graham GG, Scott KF. Mechanisms of action of paracetamol and related analgesics. Inflammopharmacology 2003; 11 (4):401-13.*
36 *Greenhough A, Smartt HG, Moore AE, Roberts HR, Williams AC, Paraskeva C et al. The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. Carcinogenesis 2009; 30 (3):377-386.*
37 *Cianchi F, Perna F, Masini E. iNOS/COX-2 Pathway Interaction: A good molecular target for cancer treatment. Current Enzyme Inhibition 2005; 1 (2):97-105.*
38 *Arun B, Goss P. The role of COX-2 inhibition in breast cancer treatment and prevention. Semin Oncol. 2004; 31 (2 Suppl 7):22-9.*
39 *Hwang D, Scollard D, Byrne J, Levine E. Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. J Natl Cancer Inst 1998; 90 (6):455-60.*
40 *http://www.ivymedical.com/history-product.html*

Maria Toloudi
I studied Molecular Biology and Genetics in the University of Thrace and now I am working for about 3 years in R.G.C.C (Research Genetic Cancer Center) as member of the research and development department. I am activating especially in the field of cellular and molecular biology dealing with human cancer stem cells. **Email: toloudi.maria@rgcc-genlab.com**

Maria P. Spachidou
I am a Biologist currently working at BioAnalytica SA as a Sales and Application Specialist in the Department of Molecular Diagnostics. I am specialized in the Immunology field holding an MSc in Immunology from Imperial College, UK and a PhD in Immunology from the University of Athens, Greece. I have been previously working as a Research Assistant in the University of Athens working on autoimmunity and as a Research Associate in RGCC in the R&D Department working on cancer immunology. **Email: mspachidou@hotmail.com**

Marina Chatziioannou
I was born and grew up in Greece. I've studied Biochemistry and Biotechnology at the University of Thessaly (2001-2006). I'm working at R.G.C.C. laboratory (Research Genetic Cancer Centre, www.rgcc-genlab.com) since 2006, at the clinical department, section of Flow Cytometry. As from the company's name, I'm working on Human Circulating Tumor Cells (CTCs) mainly. **Email: chatzioannou.marina@rgcc-genlab.com**

Panagiotis Apostolou
I graduated from the Department of Molecular Biology and Genetics, Democritus University of Thrace, in 2007. Since October 2008 I'm working in the research and development department of Research Genetic Cancer Center Ltd, which activates especially in the field of cancer genetics.
Email: apostolou.panagiotis@rgcc-genlab.com

Roger Oakes
Dr Oakes is a biochemist with over 35 years experience in drug development.
Email: roger.oakes47@btinternet.com

Ioannis Papasotiriou
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: papasotiriou.ioannis@rgcc-genlab.com