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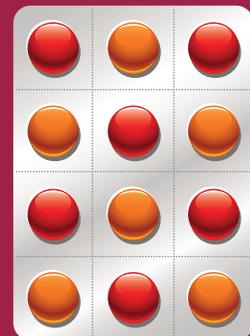
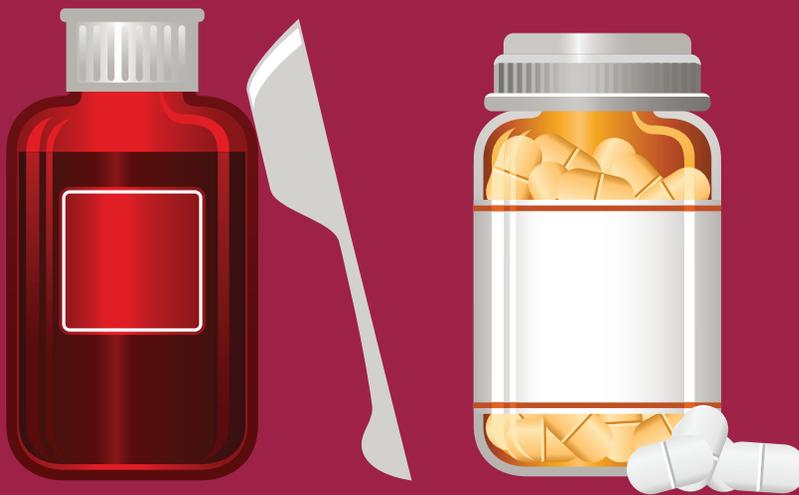
in drug development.

Determination of efficacy of Anvirzel™

in 37 established cancer cell lines.

The EU Directive Against Falsified Medicines

and The Role of Security Labels



Determination of Efficacy of Anvirzel™ in 37 Established Cancer Cell Lines



The present study investigates the efficacy of Anvirzel™, an extract of Nerium oleander, in more than 35 different human cancer cell lines, including all the types of tumours. Assays that have been used in order to measure the efficacy of Anvirzel™ in 24, 48 and 72h of incubation in 37 different cancer cell lines, were viability assays [Methyl-Tetrazolium dye (MTT), Crystal violet staining (CVE) and SulfoRodhamine B (SRB)]. In colon, lung, uterus and breast cancer cell lines, reduction of the null has been shown, up to 70% by type. All studies to date have demonstrated the efficacy of Anvirzel™ in individual cancers, such as prostate, pancreatic and myeloma cell lines. The present study demonstrates that this drug has a wider field of action, also in other cancer cell lines. It would be preferable to conduct studies concluding the interaction between Anvirzel™ and other drugs that are used already for the treatment of these cancers.

Key words: Anvirzel™, Crystal Violet, Methyl-Tetrazolium dye, SulfoRodhamine B, Cancer cell lines.

Introduction

Anvirzel™ is an extract of Nerium oleander that consists mainly of oleandrin and another cardiac glycoside, oleandrogenin. Oleandrin has been used for the treatment of patients with heart failure. Many other studies have demonstrated that it also has an anti-proliferative action, so it can be used for the treatment of tumours^{1,2,3,4}. But the major problem is the toxicity of oleandrin in normal cells and tissues^{5,6}. The compound of those glycosides, in addition to some other proteins and some polysaccharides, seems also to have an anti-proliferative action. Unfortunately the effects of this drug were slow to come to the fore because of negative results in mice^{1,7}. For this reason, Anvirzel™ studies were locked on the shelves of laboratories. Recent studies have pointed out that Anvirzel™ can decrease the viability of prostate

cancer cells^{8,9}. Up to now studies on this drug have demonstrated its efficacy in individual cancers. The most well known have to do with prostate, pancreatic and myeloma cancer cell lines. Presently there is no study to confirm or reject the efficacy of the formulation. It was very important to have an overview of the aim of Anvirzel™'s action. This is the reason that this drug has been tried on 37 different cancer cell lines. In particular, tests were done on ovarian, colon, squamous cell, lung, hepatocyte, oesophageal, cervix

epitheloid, prostate, breast, mammary, pancreatic, urinary bladder, uterus and renal carcinoma cell lines.

Materials and Methods

In our study, the effect of Anvirzel™ on 37 different human cancer cell lines was examined. Many densities of the drug were tested in 24, 48 and 72h of incubation. To consider all the above, chemosensitivity-viability assays were used. The first one was the Methyl-Tetrazolium dye assay. It is a colorimetric assay for measuring the

Table 1: Statistic evaluation of absorbance in CALU-1 cancer cell line

| MTT Assay | CALU-1 Cell Line ABSORBANCE | | |
|--------------------|-----------------------------|-------------------------|--------------------------|
| | 24h | 48h | 72h |
| Unstimulated cells | 0,257 | 0,323 | 0,887 |
| 0.01 ng/ml | 0,151 (p=0.007<0.05) | 0,113 (p=0.011<0.05) | 0,119 (p=0.0026<0.05) |

Table 2: Statistic evaluation of absorbance in LoVo cancer cell line

| CVE Assay | LoVo Cell Line ABSORBANCE | |
|--------------------|---------------------------|-----------------------|
| | 48h | 72h |
| Unstimulated cells | 0,538 | 0,611 |
| 0.01 ng/ml | 0,295 (p=0,0039<0,05) | 0,304 (p=0,02<0,05) |
| CVE Assay | LoVo Cell Line ABSORBANCE | |
| | 48h | 72h |
| Unstimulated cells | 0,538 | 0,611 |
| 0.05 ng/ml | 0,27 (p=0,0044<0,05) | 0,273 (p=0,0026<0,05) |

Table 3: Statistic evaluation of absorbance in T47D cancer cell line

| SRB Assay | T47D Cell Line ABSORBANCE | | |
|--------------------|---------------------------|--------------------------|-------------------------|
| | 24h | 48h | 72h |
| Unstimulated cells | 1,656 | 2,714 | 2,918 |
| 0,01ng/ml | 0,812 (p=0,001<0,05) | 0,805 (p=0,0001<0,05) | 0,635 (p=0,001<0,05) |

CLINICAL RESEARCH

activity of enzymes in mitochondria. These enzymes are able to reduce MTT to formazan, giving a purple colour. MTT is membrane-permeable and thus is taken up by cells through endocytosis. Because it measures only the mitochondrial activity, it is not always a good factor to distinguish whether the cells are alive or dead. Even a recently dead cell has a lower enzyme activity in mitochondria. MTT assay is affected by the intracellular concentration of glucose and pH, and the measurements are time-dependent from the metabolic status of the cell as well, since MTT can become a synergistic or antagonistic substrate for the mitochondria^{10,11,12,13,14}. To cover these deficiencies of the above method, additional assays were used which were able to give as information about protein. SRB assay is a fluorescent dye, which is used for the quantification of cellular proteins of cultured cells. This assay is more sensitive to the detection of small numbers of cells with a linear relationship between cell numbers and SRB staining intensity^{15,16,17,18,19}. The last assay is the crystal violet staining assay. Crystal violet is a dye that binds electrostatically to proteins and stains DNA. It is reliable and simple, and it may constitute an improving extension of MTT²⁰.

Cell Lines

The cell lines that have been studied were provided by the ECACC (European Collection of Cell Culture) from HPA (Health Protection Agency UK). Cells were cultured in 75cm² flasks (Orange Scientific, 5520200, Belgium) in the medium indicated for each line with the appropriate amount for each cell line of heat-inactivated foetal bovine serum (FBS, Invitrogen, 10106-169, California) and 2mM L-glutamine (Sigma, G5792, Germany), and incubated at 37°C, in a 5% CO₂ atmosphere.

Viability Assays

Cells were detached by trypsinisation (Trypsin-0.25% EDTA, Invitrogen, 25200-072) during the logarithmic phase of culture growth, and plated in 96-well plates (18,000 cells/well) (Corning Costar, 359) in a final volume of 200µl of medium per well. After 70-80% confluence of the culture, the medium was removed and Anvirzel™ (Salud Integral, Honduras) was added to cells in graduated densities (ranging from 0.01ng/ml to 200ng/ml), diluted

in water. The absorbance of Anvirzel™ was measured after 24, 48 and 72h of incubation.

For the SRB assay cells on 96-well plates were fixed by 10% TCA (Trichloroacetic acid) (Fluka, 91228), and were incubated at 40°C for 1h. Then plates were rinsed with water and cells were stained with 0.4% SRB (Sigma, 341738), and dissolved in 1% acetic acid (Carlo Erba, 401422) for 15 min at room temperature (RT). There followed flushing of the unbound stain, by washing twice with 1% acetic acid. Finally, 10mM Tris Buffer pH 10.5 (Sigma, T6791) was added, in order to release the bound dye.

For the CVE protocol the medium was removed from the 96-well plates, and then plates were rinsed with PBS (Sigma, P3813). After this, the cells were rinsed by the addition of 10% formalin (Merck, 1.04003.2500) for 20 min at RT. Formalin was removed, and 0.25% aqueous crystal violet (Sigma, HT901), dissolved in water, was added for 10 min at RT. Unbound crystal violet was rinsed by washing with water, and finally 33% acetic acid dissolved the dye.

Finally, for the MTT assay, methy-tetrazolium dye (Sigma, M2128), in a concentration of 5mg/ml was added to each well, and plates were incubated for 3h at 37°C. After the end of the incubation period, the medium was discarded and the cells were rinsed with PBS. Ultimately, the formazan crystals were dissolved in dimethylsulphoxide (Sigma, D4540).

In order to calculate the decrease, absorbance measurements have been used. The absorbance is given by the Beer-Lambert law, where the formula is $A = \epsilon \cdot c \cdot l$. "A" is the absorbance, "ε" is an extinction coefficient of the absorber, "l" is the distance the light travels through the material and "c" is the concentration of absorbing species in material²³.

The plate's optical density was measured on the µQuant spectrophotometer and the data were analysed with Gen5 software (µQuant Biomolecular Spectrophotometer MQX200 and Gen5™ Microplate Data Collection & Analysis software, BioTek® Instruments Inc., April 2008). Absorbance was measured at 570nm for all the assays. In order to subtract the noise, a second wavelength of absorbance was measured for all the assays. For the MTT assay the value of additional wavelength was 630nm, and for SRB and CVE assays, it was 690nm.

Statistical Analysis

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

Results

The results were different in each cancer cell line. Based on the data of the prostate cell lines, densities between 1 and 200ng/ml were first tested in these lines. It has been observed that in lower densities, better results are presented. After this, densities have been tested starting from 0.01ng/ml. It was found that in the densities of 0.01ng/ml and 0.05ng/ml the efficacy of Anvirzel™ was more apparent, not only in prostate cell lines, but also in other cell lines (Diagram I).

It was observed that Anvirzel™, apart from prostate cancer cell lines (VCaP prostate metastasis cell line, LNCaP prostate carcinoma cell line, PC3 prostate adenocarcinoma), decreases the viability of cancer cells in lung (COLO699N lung cancer cell line, CALU-1 lung epidemoid carcinoma cell line), colon (LoVo colon adenocarcinoma cell line, SW480 colon adenocarcinoma cell line), uterus (COLO684 uterus adenocarcinoma) and in one breast cancer cell line (T47D breast tumour cell line). The efficacy of Anvirzel™ is time-dependent, as it was found that in 72 hours the decrease was much more than that in 48 and 24 hours (Tables I-III, Diagrams II-IV).

Discussion

Anvirzel™ is an extract of Nerium oleander (family Apocynaceae), mainly containing polysaccharides, cardiac glycosides and proteins. Nerium oleander has been used in the treatment of many diseases, like hemorrhoids, ulcers, leprosy, snakebite and others. Anvirzel™ contains two very toxic glycosides, oleandrin and oleandrogenin. Oleandrin has been used to treat heart failure by inhibiting the plasma membrane Na⁺/K⁺-ATPase, leading to alterations in intracellular K⁺ and Ca²⁺^{8,22}.

Cardiac glycosides seem to have an anti-proliferative action against different types of cancers. In particular, studies with oleandrin showed autophagic death induced in the pancreatic cell line PANC-1²³. Other studies showed that this glycoside suppresses the activation of many transcription factors

Diagram 1: Effect of various densities in prostate cancer cells (22Rv1 Cancer cell line), by using Crystal Violet viability assay

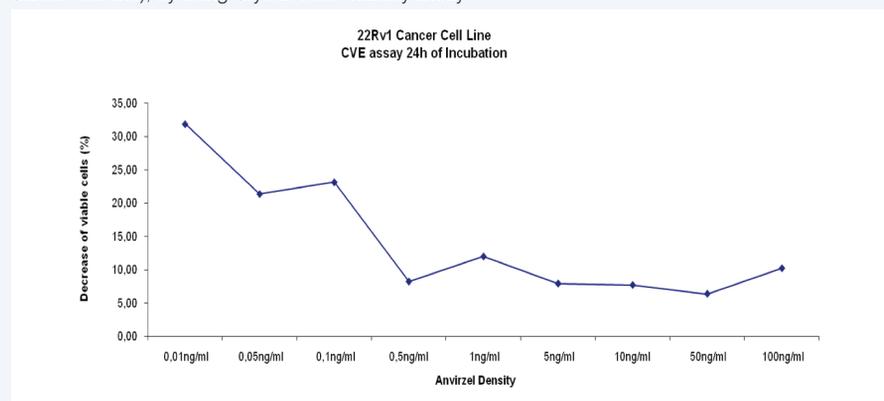


Diagram 2: Reduction percentage in CALU-1 cancer cell line

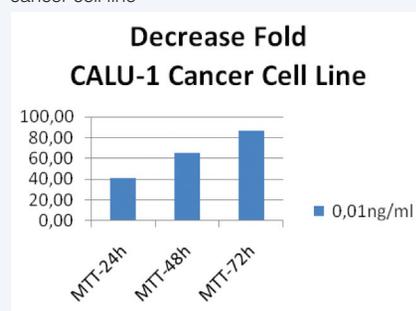


Diagram 3: Reduction percentage in Lovo cancer cell line

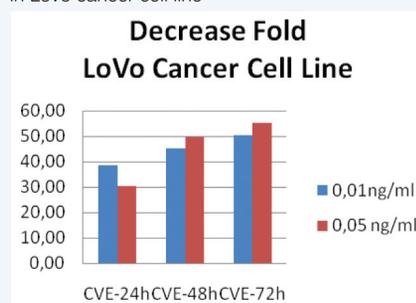
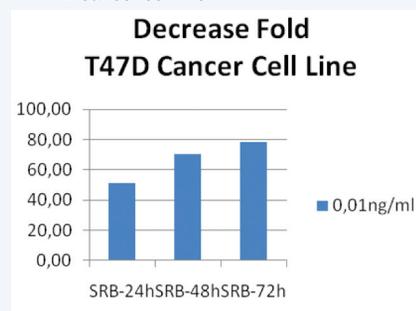


Diagram 4: Reduction percentage in T47D cancer cell line.



the existing literature and experiments that have been conducted by other laboratories, it seems essential to proceed to further studies in order to reveal and assess possible interaction between Anvizel™ and other widely used drugs with cytostatic effects that are used in cancer treatment protocols in patients. Based on the above data, Anvizel™ could become a very promising primary substance due to its broad spectrum of efficacy ■

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demonstrated that in prostate cell lines it is able to inhibit the FGF-2 (fibroblast growth factor-2) export through the interaction with the membrane Na⁺/K⁺-ATPase pump⁹. All the above studies have used different densities of Anvizel™, and they all found that the optimum density with the best efficacy is about 100ng/ml. This dose seems not to be toxic for the normal cells.

In the present study, the effect of Anvizel™ was tested on 37 different cancer cell lines. It was attempted to determine the effects on the whole range of cancer cell lines. Additionally, it was desirable to check the optimum density. Maybe the toxicity of oleandrin affects the efficiency of Anvizel™ in other cancers. Therefore, densities from 0.01ng/ml to 200ng/ml have been tested. In order to observe the viability of cells and their response to medication, colorimetric assays MTT, SRB and CVE were used. MTT (tetrazolium dye) is a yellow dye that is metabolised inside the cytosolic mitochondria of living cells to purple formazan. These reductions are taking place only if the reductase enzymes are active and therefore, can be used as a measure of viable cells. SRB (Sulforhodamine B) is an assay for the quantification of cellular proteins of cultured cells. CVE (Crystal Violet) binds electrostatically to proteins and stains DNA. The above assays have been chosen to cover the disadvantages of the one method with the benefits of the other. Apart from the effect on prostate cancer cell lines, it has been demonstrated that Anvizel™, in lower densities (0.01ng/ml-0.05ng/ml), is effective on many other types of cancers, such as lung, colon, uterus and breast.

Conclusion

Due to the previous reported and generated data and in combination of

(NF- κ B, AP-1, c-Jun NH2-terminal kinase)^{24,25}. Oleandrin can also enhance the radiosensitivity of tumours²⁶.

In an alternative study, the increased levels of intracellular Ca²⁺ leads to the increase of calcineurin. Calcineurin activates NF-AT and the receptor FasL, leading to cell death^{27,28}. More recent studies with Anvizel™ have

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