Studying the Effect of Anvirzel™, Carboplatin and Docetaxel Combination in NSCLC Cell Lines

Lung cancer is the leading cause of cancer-related mortality in both men and women. NSCLC accounts for approximately 85 % of all lung cancers. The aim of this study is to determine whether or not there is efficacy combining Anvirzel™, Carboplatin and Docetaxel in non-small cell lung cancer cell lines. Chemosensitivity-viability assays were performed in established human NSCLC cell lines for different incubation times and different densities of the three formulations. The results were time- and density-dependent. In 48h and 72h, optimal efficiency has been observed. The combination of the above compounds was more effective in the CALU-1 cancer cell line, and especially at lower densities. The combination of Anvirzel™, Carboplatin and Docetaxel seems to be more effective than monotherapy; while requiring low densities avoids dose-dependent toxicity.

Key words: Anvirzel™, Carboplatin, Docetaxel, viability assays, NSCLC

Nerium oleander (family Apocynaceae) is one of the most poisonous shrubs, because of its toxicity in all its parts[1,2]. Oleandrin, which is a cardiac glycoside, is the most significant toxin of the plant, however it has been used in treatment of cardiac insufficiency, and many studies have demonstrated the potential effects on different types of cancer[3,4,5]. Anvirzel™ is an extract of *Nerium oleander* that is mainly consist of Oleandrin and its derivative Oleandrigenin, and recent studies have pointed out its anti-cancer properties[6]. Carboplatin is a platinum-based chemotherapy drug, which is widely used against many carcinomas, due to its reduced side-effects compared with the primary cisplatin[7]. The combination with gemcitabine (GemCarbo chemotherapy), is commonly used in the treatment of lung cancer[8,9]. Docetaxel is an anti-mitotic chemotherapy drug, mainly for the treatment of carcinomas after the failure of anthacycline-based chemotherapy. A lot of studies have proved that treatment with Carboplatin plus Docetaxel combination is safe and effective in patients with NSCLC[10,11]. The present study aims to determine if there is efficacy in using an Anvirzel™-Carboplatin-Docetaxel combination, unlike single use of the above compounds, in human established non-small cell lung cancer cell lines. In order to determine the efficacy, chemosensitivity-viability assays, and especially methyl-tetrazolium dye (MTT), sulforhodamine B (SRB) and crystal violet staining (CVE) assays, have been used. MTT measures the activity of enzymes in mitochondria, as it is taken up through endocytocis by cells, and is metabolised by enzymes to formazan, giving a purple colour. Its main disadvantage is that it measures only the mitochondrial activity, thus is not a good factor to distinguish dead and alive cells. The measurements are time-dependent and are affected by pH and glucose concentrations[12,13,14,15]. The measurement of proteins has been used to overlap the deficiencies of the above assay. For the quantification of cellular proteins, SRB and CVE assays have been used. The first one is more sensitive to the detection of small numbers of cells with a linear relationship between SRB staining and cell number, and the absorption is not affected by pH[16,17,18,19,20,21]. CVE binds electrostatically to proteins and stains DNA, and it may constitute an improving extension of MTT[22,23]. All the assays are reliable, simple and reproducible.

Materials and Methods

Cell Lines

The study was performed in three established human non-small cell lung cancer cell lines, which were obtained by the ECACC (European Collection of Cell Cultures) from HPA (Health Protection Agency, UK). CALU-1, COLO699N and COR-L 105 cell lines have been tested. Cells were cultured as a monolayer in 75 cm2 flasks (Orange Scientific, 5520200, Belgium) in the medium indicated for each line with the appropriate amount for each cell line of heat-inactivated fetal bovine serum (FBS, Invitrogen, 10106-169, California) and 2 mM L-glutamine (Sigma, G5792, Germany), and incubated at 37oC, in a 5 % CO2 atmosphere. In CALU-1's medium non-essential amino acids (1 %) (Sigma, M7145, Germany) and sodium pyruvate (1 %) (Sigma, S8636, Germany) were also added.

Drugs

Anvirzel™ (Salud Integral, Honduras) was diluted in water, Docetaxel (Sigma, 01885, Germany) was diluted in N,N-Dimethylformamide (Fluka, 40255, Germany) and Carboplatin (Sigma, C2538, Germany) was diluted in deionised water. The densities that have been studied were from 0.01 ng/ml to 10 ng/ml for Anvirzel™, and from 0.01 ug/ml to 1 ug/ml for both Docetaxel and Carboplatin.

Viability Assays

Cells were detached by trypsinisation (Trypsin-0.25 % EDTA, Invitrogen, 25200-072, California) during the logarithmic phase of culture growth, and plated in 96-well plates (18,000 cells/well) (Corning, Costar 359, USA) in a final volume of 200µl of medium per well. After 70-80 % confluence of the culture, the medium was removed and drugs individually and in all possible combinations were added to cells. The absorbance was measured after 24h, 48h and 72h of incubation.

The 96-well plates were fixed by 10 % TCA-trichloroacetic acid (Fluka, 91228, Germany), and were incubated at 4^{0}C for 1h for an SRB assay. Afterwards, plates were rinsed with water and cells were stained with 0.4 % SRB (Sigma, 341738,

Germany), dissolved in 1 % acetic acid (Carlo Ebra, 401422, Italy) for 15 min at room temperature (RT). The unbound stain was washed twice with 1 % acetic acid. Ultimately, 10mM Tris Buffer pH 10.5 (Sigma, T6791, Germany) was added, in order to dissolve the dye.

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

Finally, for the MTT assay, methyl-tetrazolium dye (Sigma, M2128, Germany), in a density of 5mg/ml (diluted in PBS) was added to each well, and plates were incubated for 3h at 37^{0}C. After the incubation, the medium was discarded and the cells were rinsed with PBS. Ultimately, the formazan crystals were dissolved in dimethylsulphoxide (Sigma, D4540, Germany). The calculation of absorbance measurements was given in the Beer-Lambert law, where the formula is A=Ɛcl. “A” is the absorbance, “Ɛ” 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 material.

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, and a second wavelength was measured in order to subtract the noise. For the MTT assay, the value of additional wavelength was 630nm and for SRB and CVE assay was 690nm.

Statistical Analysis

All treatments for each cell line were performed in triplicate, three times. The statistical significance of all effects was evaluated by “difference of the means” test ($p < 0.05$).

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

CALU-1 Cell Line ABSORBANCE		
CVE Assay	48h	72h
Unstimulated cells	1,052	2,097
0.01 ng/ml Anvirzel	0,948	2,005
0,1ug/ml Carboplatin	1,019	2,27
0,1ug/ml Docetaxel	0,386	0,386
0.01 ng/ml Anvirzel + 0,1ug/ml Carboplatin + 0,1ug/ml Docetaxel	0,297 (p=0,0004<0,05)	0,114 (p=0,0001<0,05)
MTT Assay	48h	72h
Unstimulated cells	0,549	1,131
0.01 ng/ml Anvirzel	0,626	1,025
0,1ug/ml Carboplatin	0,518	1,047
0,1ug/ml Docetaxel	0,137	0,084
0.01 ng/ml Anvirzel + 0,1ug/ml Carboplatin + 0,1ug/ml Docetaxel	0,108 (p=0,008<0,05)	0,056 (p=0,001<0,05)
SRB Assay	48h	72h
Unstimulated cells	2,587	2,843
0.01 ng/ml Anvirzel	2,59	2,934
0,1ug/ml Carboplatin	2,624	2,938
0,1ug/ml Docetaxel	1,564	1,727
0.01 ng/ml Anvirzel + 0,1ug/ml Carboplatin + 0,1ug/ml Docetaxel	1,516 (p=0,00004<0,05)	1,003 (p=0,0009<0,05)

Results

The results that were obtained were different for each cancer cell line, demonstrating once again the heterogeneity of carcinomas, even in the same type of cancer. The combination of Anvirzel™-Carboplatin-Docetaxel, affected the CALU-1 cell line more, and the rest less. The results were time- and density-dependent. After 24h of incubation, efficacy was observed almost nowhere due to the above combination, as well as due to the single use of each compound. The optimum results were obtained at lower densities of Anvirzel™ and at densities less than 1 ug/ml for the other drugs at 48 and 72 hours of incubation. Specifically, in CALU-1 cell line the combination of 0.01 ng/ml of Anvirzel™ with 0.1 ug/ml of Carboplatin and 0.1 ug/ml of Docetaxel decreased the cells null up to 95 %. There was also an observed reduction of the population in higher densities of Carboplatin and

Chart 1: Reduction percentage in CALU-1 cancer cell line for CVE Assay

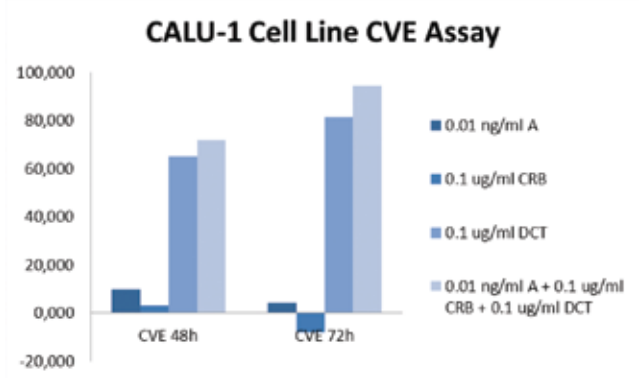


Chart 2: Reduction percentage in CALU-1 cancer cell line for SRB Assay

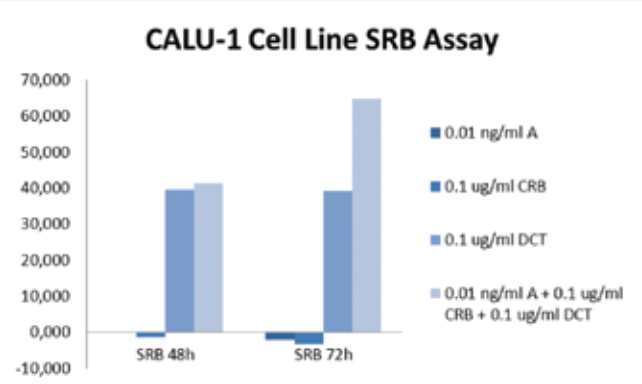
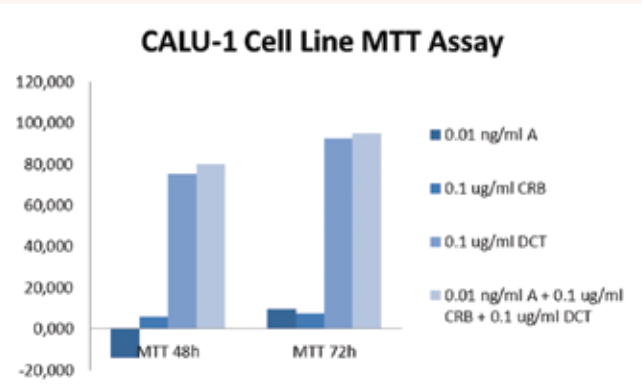


Chart 3: Reduction percentage in CALU-1 cancer cell line for MTT Assay



Docetaxel; however the percentage was up to 85 %. In the COLO699N cell line it has been observed that the more effective combination is the one with the lower densities (0.01 ng/ml Anvirzel™, 0.01 ug/ml Carboplatin and 0.01 ug/ml Docetaxel). The lowest efficacy, both of combination and individual use, was observed in the COR-L 105 cancer cell line. Also in this cell line, the combination of 0.01 ng/ml of Anvirzel™ with 0.1 ug/ml of Carboplatin and 0.1 ug/ml of Docetaxel was optimal, and decreased the cells null up to 65 %. The schedule (Table 1) and following diagrams (Diagrams 1-3) illustrate the chemosensitivity-viability assays results.

Discussion

Anvirzel™ is a compound extracted from *Nerium oleander*, and mainly consists of polysaccharides, proteins and two cardiac glycosides, Oleandrin and its derivative Oleandrigenin. As cardiac glycosides, they inhibit the Na^+/K^+-ATPase and are able to inhibit proliferation of tumour cells through high concentrations of intracellular calcium[5,25]. Other studies have demonstrated that Oleandrin suppresses the activation of many transcription factors and also enhances the radio-sensitivity of tumours[26,27,28]. Cardiac glycosides initiate Apo2L/TRAIL-induced apoptosis in non-small cell lung cancer cells by up-regulation of death receptors 4 and 5[29]. Oleandrin induces cell death through activation of caspases in a variety of human tumour cells, but also activates calcineurin and NF-AT via FasL[30]. Anvirzel™ interacts with the membrane Na^+/K^+-ATPase pump in prostate cell lines and thus inhibits the FGF-2 export[31].

Carboplatin as an alkylating agent interacts with DNA. Although it is less potent than Cisplatin, it has the main advantage of reduced side-effects. It is used in combination with Paclitaxel, or with Docetaxel for treatment of NSCLC[32,33].

Docetaxel belong to the chemotherapy drug class taxane, and is an anti-mitotic drug which binds to microtubules and stabilises them[34]. It is an analogue of paclitaxel, having a hydroxyl functional group on carbon 10 and a tert-butyl carbamate ester, whereas paclitaxel has an acetate ester and a benzyl amide respectively[10]. Docetaxel plus Carboplatin is effective as a second-line treatment in patients with advanced non-small cell lung cancer[32].

Methyl-tetrazolium dye, sulforhodamine B and crystal violet staining assays were performed to evaluate the combination of the three above formulations in established NSCLC cell lines. The panel of the three different assays has been used to ensure reliable and repeatable results.

The CALU-1 cell line was the one in which the combination was more effective, even at low densities. It is well known that Anvirzel™ has better efficacy at densities from 0.01 ng/ml to 1 ng/ml. In the particular cell line, the optimal densities were 0.01-1 ng/ml of Anvirzel™, 0.1 ug/ml of Carboplatin and 0.1 ug/ml of Docetaxel. This combination reduces the cell number up to 95 % after 72 hours of incubations, whereas the monotherapy reached a lower rate. In the COLO699N and COR-L105 cell lines, lower reduction percentages were observed, which, however, were observed at the same density range. The effect was time-dependent, as its effects have not been observed after 24 hours of incubation, but only after 48, and especially 72, hours of incubation.

Conclusion

The present study has proved that the combination of Anvirzel™-Carboplatin-Docetaxel is more effective than monotherapy in different NSCLC cell lines. The remarkable thing is that the above combination has better efficacy at low densities of the three formulations, thus preventing the toxicity of chemotherapy. There is an urgent need for further studies to be done that will reveal and assess the interaction of extracted substances with established chemotherapy drugs.

Acknowledgements

The authors thank two anonymous referees who made useful comments that improved the manuscript.

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