Cold Plant Extract Mixture from the Plants Solanaceae Are Cytotoxic for Cancer Cells

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Abstract: Through the years several insights showed that the cold plant extracts from certain plants can trigger apoptosis or necrosis of cancer cells. The cold plant mixture from the plants *Capsicum chinense* and *Alium sativum*, triggered DEVD-ase activity, apoptosis and necrosis in 10–1000 mg/mL concentrations. When the cells were pretreated with z-VAD-fmk, it was determined that the caspase activity was not blocked, when the cells were treated with the crude plant extract. By these means it was shown that the above mentioned mixture of plant extracts is cytotoxic for several types of cancer cell lines.

Key words: *Allium sativum*, apoptosis, cancer, *Capsicum chinense*, cytotoxicity, necrosis.

1. Introduction

Chemical companies, keenly interested in finding new drugs, have scoured the world seeking new sources of plant materials, which have been used by the indigenous people or as cottage industry (domestic/herbal) remedies, in the treatment of cancer. Over the past centuries there have been hundreds of indigenous plants used in traditional therapies in various countries throughout the world. Some of them have been tested and are still being trialled, in current efforts, to find a way of curing cancer, but are being recognized as a difficult exercise, because of the complex nature of the disease. Modern research proves the efficacy of some plants, such as astragalus, eleutherococcus, shisandra and shiitake mushrooms and many of the plants used are traditional among the herbs. However, there are many used by traditional herbalists, which could be included in the range of plant materials, offering value in therapy; some being used as a herbal extract, some as food and some as

homoeopathic medicine. There are some specific plants, which have an effect on certain types of cancer by improving their specific physiological functions. So far, no magical overall one formula has been found, which could be applicable for all types of cancer. However, medical and herbal research continues [1].

In recent years, a significant emphasis has been placed on fighting cancer through the combination of chemotherapy (using cytotoxic agents) and naturally occurring chemo-preventive agents. Plant extracts have been used as chemo-preventive agents, because their mechanism of action on cancer cells is different from cytotoxic agents, while some clinical work has found their combination to be effective [2]. Many of the plant extracts were the basis for identifying new active molecules, which are cytotoxic [2, 3].

In order to address the health issue of cancer, we have focused our efforts in finding a new plant extract mixture, which could have a cytotoxic effect on cancer cells. As one can see nowadays, the ultimate goal of cytotoxic therapy is to induce the death of tumour cells. Most cytotoxic and hormonal treatments, as well as radiation, ultimately kill cancer cells by causing

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irreparable cellular damage, because they trigger necrosis.

In order to address the health issue of cancer, I have focused my research on finding a new natural source of plants, which extracts would have apoptotic and/or cytotoxic effect on cancer cells.

2. Materials and Methods

2.1 Preparation of Plant Extract

The selected plants were grown in a greenhouse, where the soil constituted of 30% sand. Plants *Capsicum chinense* and *Allium neapolitanum* fresh or dry were imported from Asia or Middle East. Once the plants grown in the greenhouse produced blossoms and ripe fruits, they were collected and dried in the sun. Once dry, they were grinded into a powder. The powder was suspended in DMEM to which penicillium and streptomycin were added together with glutamax (1% v/v of each antibiotic and glutamax in the final mixture). The mixture was left at room temperature for 3 days and shaken several times in between. After 3 days the extract was filtered and was by these means ready for *in vitro* experiments to test its effect on cancer cells.

2.2 Materials

In my research I have been using the following materials: MTS from Promega (USA), Bradford reagent from Bio-Rad (Germany), z-VAD-fmk, Ac-DEVD-AFC, DTT from Bachem (Switzerland), FBS from Gibco (USA), antibiotics penicillium, streptomycin and glutamax from Gibco (USA), annexin V-PE and 7-AAD for flow cytometry were purchased from BD Biosciences (USA). Mitotracker CMXRos was from Molecular Probes (Eugene, OR, USA). All other reagents (RIPA buffer, caspase buffer, KDMEM, Tryple Select, PBS) were prepared at the Institute according to standard procedures [4, 5].

2.3 Cell Lines

For the experiments I used the following cell lines:

hepatoma cell line HepG2, neohuman diploid fibroblasts (NHDF) and neuroblastoma cell line SH-SY5Y (all ATCC).

Cells were grown in Dulbecco's modified Eagle's medium (DMEM purchased from Sigma) supplemented with 20% fetal calf serum (FBS), 1% penicillium/streptomycin and 1% glutamax, while grown to obtain confluence growth. The cells used in the experiment were grown in DMEM, free of any kind of supplement of animal origin and with the only addition of penicillium/streptomycin (1% v/v in the final solution in the medium) and glutamax (1% v/v in the final solution in the medium).

2.4 Cell Death

All cells were grown in 10 cm plates to obtain 70–90% confluence growth and afterwards washed with PBS and titrated with Tryple Select, 5–15 minutes (depending on the cell line). In the next step the cells were transferred into 96-well and 6-well plates at a ratio of 10 000 cells/well (HepG2, NHDF, SH-SY5Y) and then grown overnight. The next day the cells were treated with the plant extract in the range of 0.001 mg/ml–10 mg/ml (increasing the concentrations by a factor of 10) and incubated for 24–48 hours (depending on the cell line and the onset of apoptosis). I used two types of control. The first control represented the cells grown only in DMEM and the other control the cells were grown in DMEM, which penicillium/streptomycin in 1% v/v of the final concentration were added. Since there was no statistical difference between two types of the controls, only the results with the control cells grown in DMEM with penicillium/streptomycin are shown.

2.5 Characterisation of Cell Death/Apoptosis

Cells, treated with the plant extract, were at the beginning photographed with an Olmypyus IX71 light emission microscope before being subjected to analysis with MTS. Only those samples were taken into account, where visible changes in cells were noticed.

First signs of apoptosis were measured by the addition of 20 μ L of MTS into 100 μ L samples in the 96-well plate. After 45 minutes of incubation, I measured the absorbance at 490 nm with a 96-well plate reader (TECAN). For further analysis I had chosen concentrations of the plant extract which induced 20–50% cell death and furtherly measured the caspase activity in these samples. I gathered 40 μ g of untreated proteins and treated the cells with the plant extract in the presence or absence of the inhibitor z-VAD-fmk to determine the caspase-3 activity by measuring the proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AFC (Bachem), as is also described in Cirman et al. (2004) [6]. Apoptosis was quantified with flow cytometry measurements of phosphatidylserine exposure and 7-AAD incorporation and by the measurement of the DEVD-ase activity of the caspase-3. Briefly, 100 μ l aliquots of cells were labelled with annexin V-PE and 7-AAD, according to the manufacturer's instructions. The cells were then subjected to flow cytometry analysis using FACS calibur flow cytometer (BD Biosciences) and analyzed with the CellQuest software.

To determine by which apoptotic pathway (intrinsic, extrinsic) the plant extract triggers apoptosis, I used Mitotracker Red CMXRos to assess and monitor the integrity of mitochondria. Mitotracker CMXRos was added to the cells at a final concentration of 20 nM. Following 30 minutes of incubation, at 37°C, the cells were washed in PBS and again re-suspended in PBS for measurements by flow cytometer. Here I measured the red fluorescence of 10000 cells per sample, corresponding to mitochondria using the FL3 channel.

Cells were cultured at 1×10^6 in 6-well plates overnight, before treatment with the plant extract at final concentration, depending on the cell line. In control experiments cells were incubated overnight only in DMEM. After incubation with the plant extract, specific for each cell line, cells were observed by light microscopy (Olympus IX71, Japan, magnification 40 and 60). To prepare the extracts the cells were collected

and pelleted by centrifugation at 1000 rpm for 5 min and washed twice with $1 \times$ PBS. Whole-cell extracts were prepared in RIPA buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40 0.5% w/v deoxycholic acid, 1 mM EDTA). Following 10 min of incubation on ice insoluble materials were removed, with centrifugation at 14 000 rpm for 10 min. Cytosolic extracts were prepared, as previously described [6]. Total protein concentration was determined by using the Bradford assay.

3. Results and Discussion

3.1 General Toxicity of the Plant Extract

In order to determine, whether the plant extract mixture is cytotoxic for cancer cells, we had chosen several cancer and immortalized cell lines, for the above mentioned studies. The cells were used from different tissues to ensure wide coverage. Here the results in human liver cancer cells HepG2, immortalized human neuroblastoma cells SH-SY5Y and immortalized human fibroblasts NHDF are shown.

The plant extract was shown to be extremely effective to induce apoptosis and cytotoxicity in the cancer cell line (HepG2) as well as in the immortalized cell line SH-SY5Y. The best results were achieved in human cancer cell line HepG2, where especially higher concentrations of the plant extract of 0.1–10 mg/mL induced apoptosis and necrotic cell death (Fig. 1). The immortalized cell line NHDF was resistant to the action of the plant extract, since there was no rounding, detachment or other visible cellular changes. It was determined that while treating the cells with the concentration 10 mg/ml, they stopped to multiply. In the immortalized cell line SH-SY5Y it was shown, that this cell line was more susceptible to the plant extract, since optimal concentration for the onset of cell death was 0.01 mg/mL, while concentrations 1 mg/mL and 10 mg/mL produced total swelling and bursting of the cells, what ended in cell debris.

Fig. 1 Plant extract triggers apoptosis in HepG2 cancer cell line and in an immortalized cell line SH-SY5Y. Typical apoptotic morphology with features such as cell shrinkage, rounding and detachment from the surface (see arrows) was observed usually after 24–48 hours of incubation with the plant extract at various concentrations (from upper left to bottom right PE 0.001 mg/ml, 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml and PE 10 mg/ml). However no such morphology occurred in the NHDF immortalized cell line regardless of the concentration.

3.2 Activation of Apoptosis/Necrosis by the Plant Extract

Regarding the optimal time of incubation for apoptosis and necrosis to occur, there were similarities in all cancer cell lines and some immortalized cell lines. It was observed that apoptosis occurred already within 24 hours (SH-SY5Y immortalized cell line), but the optimal effects were reached in 48 hours, especially in HepG2 cell line (Figs. 2–3). As determined from the experiments, the plant extract had cytotoxic effect in cancer cell lines and in the immortalized cell line

Fig. 2 Plant extract induces DEVD-ase activity as well. DEVD-ase activity was measured after 48 hours of incubation, with the plant extract, at various concentrations. With different concentrations of the plant extract, we observed different DEVD-ase activity, which were measured and expressed in arbitrary units. All values represent means of triplicate determination $\pm$ SD, statistical difference ± 10 –15%. It is seen there were different changes in a certain cell line. The maximum DEVD-ase activity was in HepG2 cancer cell line determined when treating the cells with the highest concentration of the plant extract, which was 10 mg/mL in contrast to the SH-SY5Y cells, where this concentration was 0.01 mg/mL.

Fig. 3 Flow cytometry analysis and the number of apoptotic/necrotic cells in HepG2 cancer cell line, NHDF and SH-SY5Y immortalized cell line after treatment with the plant extract. Grey shade of the bar represents apoptotic, while black necrotic cells. Statistical difference ± 10 –15%.

SY-SY5Y, where had also activated caspase-3. My further researches are now focused on determination of the active molecule or molecules in the plant extract (part of the next article to be published soon).

Fig. 4 Analysis of the integrity of mitochondria with the intracellular dye MitoTracker Red CMXRos. It is shown the loss of mitochondrial membrane potential gradually increased while increasing the concentration of the plant extract. In order the cell to enter apoptosis, we need at least 30% of the mitochondrial potential loss. This was in HepG2 as well as in SH-SY5Y achieved with different concentrations. In HepG2 the maximum concentration of 10 mg/ml produced 33% of mitochondrial membrane potential loss, while in SH-SY5Y 30% of loss was achieved with the concentration 0.1 mg/mL. Statistical difference ± 5 –10%.

The best optimal response in cancer cell lines I obtained, was with the fraction concentration of 10 mg/mL in HepG2 and with the concentrations 0.001–0.01 mg/mL in SH-SY5Y. There was no effect in the immortalized cell line NHDF, while treating the cells with all selected concentrations. It is visible from the analysis (bar marked PE 1–plant extract concentration 1 mg/mL), that I have obtained 38% apoptotic cells in HepG2, which was enough to confirm the onset of apoptosis. Nevertheless, there were also necrotic cells present, in the range of 30–50%, with concentrations above 1 mg/ml, of the plant extract. In SH-SY5Y I have obtained 30% of necrotic cells and

33% apoptotic cells with the concentrations 0.001–0.01 mg/mL. As it is shown the inhibitor z-VAD-fmk did not prevent HepG2 cancer cells nor SH-SY5Y immortalized cells from entering apoptosis/necrosis. Therefore we did not use the highest concentration of the plant extract in HepG2 for further experiments as well as in SH-SY5Y.

3.3 Possible Molecular Pathways

In order to determine at least general pathway of apoptosis/necrosis, we used certain dyes to research the integrity of cellular organelles. In the beginning we focused on mitochondria. As it was clearly seen the plant extract did have effect on mitochondria, which lost their integrity, when treating the cells with the certain concentration of the plant extract. In HepG2 we again needed higher concentration to induce the loss of mitochondria potential, which was 10 mg/mL. When we performed experiments with MitoTracker Red CMXRos in SH-SY5Y immortalized cell line, we discovered the concentration was much lower (0.1 mg/mL), which induced approximately the same quantitative damage of mitochondria as in HepG2.

4. Conclusion

The aim of our research was to show that there are still plants, and their extracts, to be studied in order to find suitable natural mixtures, which would consequently induce apoptosis and necrosis in cancer cells, which was clearly shown in the case of Taxol and plants from the family *Berberidaceae* [7].

One example similar to my case are the extracts of *Astragalus membranaceus* are marketed as life prolonging extracts in humans. A proprietary extract of the dried root of *Astragalus membranaceus*, called TA-65, was associated with a significant age-reversal effect in the immune system, in that it led to decline in the percentage of senescent cytotoxic T cells and natural killer cells after six to twelve months of use. In October 2010, Intertek/AAC Labs, an ISO 17025 internationally recognized lab, found the largest

component of TA-65 to be Cycloastragenol. Telomerase activation was feared to pose an increased risk of cancer because telomere shortening is a mechanism that limits cell proliferation. However, short telomeres result in chromosome instability, hence there is also a potential mechanism for telomere lengthening to protect against cancer (as distinct from mutation-induced activation). In our case however we have not focused our research on the telomere lengthening by our plant extract, but the main discovery was that the plant extract does trigger cell death of cancer cells, with its target to be the mitochondria [8].

In our experiments we have determined that the cold plant extract mixture from the plants *Capsicum chinense* and *Allium neapolitanum* showed high cytotoxic activity in HepG2 cancer line and immortalized cell line SH-SY5Y. It was most important to choose fresh plants grown in the Middle East or Asia, before they were dried and grinded into powder.

While observing the effect of the prepared extracts, it was interesting to see that concentrations above 1 mg/ml also triggered necrosis, beside apoptosis. It is due to the rich composition of the plant extract that beside apoptosis also necrosis was triggered in greater or lesser extent depending on the concentration of the plant extract. This is an interesting turning point towards identifying the molecule or synergistic molecules together with their optimal concentrations, which are responsible for triggering programmed cell death in HepG2 cancer cells and immortalized cells SH-SY5Y. Since it is known that each type of cancer is different from the other, would this mixture be effective in other cancer cells in different concentrations?

Similar case was studied by Soviet medical researchers for extensive use of *Eleutherococcus spp.* in the treatment of cancer, and the results of these studies are promising the same as in my case. The herb's diverse pharmacological properties make it a

useful adjunct to conventional cancer treatments, enhancing their efficacy, reducing their side-effects and strengthening the body's own defences, and thus increasing the chances of the patient's survival and successful recovery.

It is known that the trauma of surgery causes immunosuppression owing to the degree of stress response that occurs [9]. In the case of surgery for removal of malignant tumours this may be disastrous, as metastases can spread and establish themselves virtually unimpeded. *Eleutherococcus spp.* has been demonstrated to be of value here for two reasons. First, as an immunomodulator it prevents glucocorticoid immunosuppression by restricting excess production of glucocorticoids (though high levels have a suppressant effect, increased levels of glucocorticoids are in fact essential in the inductive stage of immunogenesis [10]. Second, as an immunostimulant it directly stimulates the immune system via the activation of T cells, of γ -interferon production by lymphocytes and of anti-neoplastic natural killer T cells and macrophages [11]. To conclude my first researches I can state that the plant extract from the plants *Solanaceae* itself is a very potent mixture for triggering cytotoxicity in HepG2 cancer cells and immortalized cells SH-SY5Y. We must emphasize that it is very important, the plants were fresh before dried and grinded into powder for the preparation of the extract suitable for the in vitro experiments. Furthermore the plant extract mixture should be prepared and mixed in a proper combination and concentration. Since we are obviously dealing with the mixture of molecules, which are not very stable, we have therefore designed further experiments, which revealed the effective molecules in the plant extract.

As we have seen there are many plants rich in its composition which offer new insights in treating cancer in more favourable way for the patient and with greater chance of his/her survival. Currently, we are proceeding to solve this puzzle to identify the active molecules responsible for the cytotoxic effect, of our plant extract mixture. According to known data on

these plants we have proven the extract offers rich composition of different molecules as well as undisputed effect of cell death in cancer cells. Some molecules were already determined to have beneficial health effect, but we have found different molecules, which were proven to trigger programmed cell death. Since this was part of a larger project with the Slovenian Scientists from the Chemical Institute of Ljubljana, the article with further data on this theme is going to be published soon.

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