Resveratrol and *Viscum album* anticancer effect in canine mammary tumor cell lines

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Abstract

Mammary gland tumors are the most common neoplasms in female intact dogs and share some biological and histopathological aspects with those in women with breast cancer, making them a good model in comparative oncology. Resveratrol is a polyphenol found in several plants, and some studies have indicated that it acts in the neoplastic process as an anticancer drug. *Viscum album* is a hemiparasitic plant widely used as an adjuvant treatment for cancer in some countries. Thus, this study aimed to evaluate the antitumor potential of resveratrol and homeopathic *Viscum album* together and separately using two previously characterized canine mammary tumor cell lines (UNESP-CM9 and UNESP-CM60). The cell viability test (MTT) was performed, which revealed an IC50 of 3.11 μl/100 ml for UNESP-CM9 and 2.993 μl/100 ml for UNESP-CM60 for *Viscum album*, and for resveratrol, the IC50 was 281.6 μM for UNESP-CM9 and 105.5 μM for UNESP-CM60. The combination of both natural compounds led to tumor cell death at a lower IC50. The cell migration assay demonstrated an increase in cell migration time with both treatments. UNESP-CM9 closed 35.66% of the wounds in the control group and 15.51% of the wounds in the *viscum* group, while UNESP-CM60 closed 39.46% of the wounds in the control group and 19.95% of the wounds in the *viscum* group and 2.41% of the wounds in the resveratrol group. Thus, these two compounds have antitumor potential, making them possible alternatives to conventional treatments.

Keywords: comparative oncology, natural compounds, dogs, biocompounds.

Introduction

In the last 10 years, there has been a significant increase in comparative oncology investigations, given that canine and human mammary tumors present several similarities at both the pathological and molecular levels (14), such as spontaneous occurrence, age of onset, histopathology, and response to therapy (1, 13). Mammary gland tumors are the most commonly diagnosed type of cancer worldwide and are the main cause of cancer-related death in women (1, 10). It is the most common neoplasm in female intact dogs, with 30–54% malignant tumors (15), and some subtypes are highly metastatic, with the possibility of dissemination to regional lymph nodes reaching 50% (7).

Multidrug resistance is considered a major impediment to cancer treatment, as most cancer-related deaths are from metastasis or resistance to chemotherapy (17). One of the causes of resistance to chemotherapy is tumor stem cells, and current research has shown that natural compounds, mainly phytochemicals, have the ability to reach these cells (5). Natural products, several of which originate from natural sources, such as plant-derived vincristine and paclitaxel, have played a key role in the history of anticancer drug discovery (9).

Resveratrol is a polyphenol that can be found in many plants, such as peanuts, grapes, and berries. It is known to have important pharmacological effects and is considered a potent anti-inflammatory and antioxidant agent (2). It acts in four stages of carcinogenesis—initiation, promotion, progression
and metastasis—through the modulation of pathways that control cell division and growth, apoptosis, inflammation and angiogenesis (2, 19). It has already been successfully used to treat cancer both in vitro and in vivo, and its mechanisms of action are related to the inhibition of migration and invasion through the PI3K/Akt and Wnt/β-catenin pathways, inhibition of the epithelial–mesenchymal transition through TGF-β1 and increased sensitivity to chemotherapy (25).

Viscum album is a hemiparasitic plant of several tree species, and the components of its extract include mainly flavonoids, triterpenes, lectins, polysaccharides, amino acids, viscotoxins and various alkaloids (18, 23). The use of V. album in the treatment of cancer is well known, and the effects attributed to mistletoe extracts are based on stimulation of the immune system and direct cytotoxicity (16). Lectins and viscotoxins are identified as the main antitumor agents responsible for inducing apoptosis in these cells (18, 23).

These compounds have been studied in vitro, in vivo, and in clinical trials, alone or in combination, to develop new therapies for humans. To the best of our knowledge, these promising agents have not yet been reported in canine models. The aim of this study was to describe the cytotoxic potential of V. album and resveratrol in two canine mammary carcinoma cell lines.

Materials and Methods

Materials and chemicals


Cell lines and cell culture

The UNESP-CM9 and UNESP-CM60 cell lines were previously established and characterized by Lainetti et al. (6), and cell growth was evaluated according to (6). The cells used were in the logarithmic growth phase in all experiments.

In vitro cytotoxicity assay

A colorimetric MTT assay was used to determine the cytotoxic effects of resveratrol and homeopathic V. album. For this purpose, 1x10⁴ UNESP-CM9 and UNESP-CM60 cells/well were seeded in a 96-well plate at different drug concentrations. After 24 hours of seeding in DMEM supplemented with 10% FBS, the media was replaced with DMEM without BFS plus four concentrations of the tested drugs under the same conditions and for the same period of time (24 hours). For V. album at UNESP-CM9 and UNESP-CM60, concentrations of 1, 2, 3 and 4 µL/100 µL were tested. For resveratrol at UNESP-CM9, concentrations of 200, 230, 260 and 300 µM were tested. For UNESP-CM60, concentrations of 50, 100, 125 and 200 µM were tested. The combination of Viscum and resveratrol was tested using the isolated IC50 of each natural compound. Homeopathic V. album was used at different dilutions in distilled water. Resveratrol was diluted in DMSO.

The MTT assay (Invitrogen™, Thermo Fisher Scientific, USA) was performed according to the manufacturer’s instructions, and Spectro colorimetric analysis was performed in a microplate reader (570 nm range). All the compounds were tested in triplicate at four concentrations made in a serial dilution, guided by control groups.

Cellular migration

Cellular migration was tested by a wound healing test after treatment with the IC₅₀ doses of resveratrol and V. album. The cells were seeded in a 6-well plate (1x10⁵ cells/well) until they reached 80% confluence. After that, a 100 µL pipette tip was used to generate a linear wound. To remove the detached cells, the plates were double washed with DPBS (500 µL) and agitated for 5 min.

For reference, each well of the plate was photographed at the time the wound was made and after 7 hours. During this time, the cells were incubated in fresh DMEM (without FBS), and the test compound was added. Wound healing was measured in three different regions using the GIMP 2.10.14 program. The mean distance for each cell line was calculated as the mean and standard deviation. All compounds and the control group were tested in triplicate.

Data analysis

The IC₅₀ values of all the tested drugs were obtained by plotting the spectrum colorimetric data in a dose–response curve after the application of data normalization and a nonlinear regression method in GraphPad Prism 8.0.1 software. The values presented are the means and standard deviations of triplicate tests, and statistical significance (p< 0.05) was determined by a comparison of each tested group with the control (vehicle) group by independent t tests and ANOVA.

For the cell migration analysis, the mean and standard deviation were calculated. Wound healing (difference) was measured by the formula D₀-D₁, where D₀ was the first measurement and D₁ was the initial measurement. The treated groups and controls were compared using the Mann–Whitney test.
considering p<0.05 as an indicator of statistical significance, which was performed with GraphPad Prism 8.0.1 software.

**Results**

**Cellular viability**

In both canine mammary carcinoma cell lines (UNESP-CM9 and UNESP-CM60), resveratrol and homeopathic *V. album* decreased cellular metabolic activity in a dose-dependent manner (Table 1 and Fig. 1). When *Viscum album* was combined with resveratrol, the concentrations of both compounds were lower than the IC<sub>50</sub> values in both cell lines, and the concentration of *V. album* was maintained at a fixed value of 1 μl/100 μl. The treated groups and the control were compared by individual t tests, with all groups compared to the control having p < 0.05 considered significant.

**Cellular migration**

*Viscum album* and resveratrol in both canine mammary carcinoma cell lines (UNESP-CM9 and UNESP-CM60) increased the time to wound closure in a cell migration assay (Table 2 and Figs. 2 and 3). Both treatments were significantly different from DMEN, delaying wound closure and cell proliferation.

**Discussion**

Resveratrol and *V. album* have already been widely tested as antitumor drugs in several tumor cell lines, including

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**Table 1.** IC<sub>50</sub> of the drugs tested in UNESP-CM9 and UNESP-CM60, two canine mammary carcinoma cell lines:

<table>
<thead>
<tr>
<th>DRUG</th>
<th>UNESP-CM9</th>
<th>UNESP-CM60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>281.60 μM</td>
<td>105.50 μM</td>
</tr>
<tr>
<td><em>Viscum album</em></td>
<td>3.11μl/100μl</td>
<td>2.99μl/100μl</td>
</tr>
</tbody>
</table>

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**Table 2.** Wound healing test measurements. The mean size of the wound at 0 and 7 hours and the percentage of wound closure at 7-h intervals. The groups were compared using the Mann–Whitney test.

<table>
<thead>
<tr>
<th>CELL</th>
<th>GROUP</th>
<th>Wound size(μm)</th>
<th>Healing 0 h</th>
<th>Healing 7 h</th>
<th>%</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 h</td>
<td>7 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM9</td>
<td>DMEM</td>
<td>47.83</td>
<td>17.06</td>
<td>35.66%</td>
<td></td>
<td>0.667</td>
</tr>
<tr>
<td></td>
<td><em>Viscum album</em></td>
<td>45.13</td>
<td>7.00</td>
<td>15.51%</td>
<td></td>
<td>0.0051</td>
</tr>
<tr>
<td></td>
<td>Resveratrol</td>
<td>43.90</td>
<td>3.83</td>
<td>8.72%</td>
<td></td>
<td>0.0007</td>
</tr>
<tr>
<td>CM60</td>
<td>DMEM</td>
<td>43.66</td>
<td>17.23</td>
<td>39.46%</td>
<td></td>
<td>0.0112</td>
</tr>
<tr>
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<td><em>Viscum album</em></td>
<td>44.76</td>
<td>8.93</td>
<td>19.95%</td>
<td></td>
<td>0.0023</td>
</tr>
<tr>
<td></td>
<td>Resveratrol</td>
<td>64.70</td>
<td>1.56</td>
<td>2.41%</td>
<td></td>
<td>0.0034</td>
</tr>
</tbody>
</table>

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**Figure 1.** Cytotoxicity assay (MTT - 24 h) of the canine mammary carcinoma cell lines UNESP-CM9 (left) and UNESP-CM60 (right). A- Note the decrease in cell viability as the *Viscum album* concentration increased for both cell lines. B- Note the toxic effect of resveratrol on both cell lines in a dose-dependent manner. C- Note the synergistic effect of *Viscum album* and resveratrol on both cell lines, at the highest dose, all cells died, with 0% viability.

**Figure 2.** Wound healing test in the tested and control groups at 0 and 7 h in the UNESP-CM9 canine mammary gland carcinoma cell line.
mammary tumors, but they have not yet been tested in canine mammary carcinoma cells. Considering that dogs are valuable natural models for comparative oncology, we tested these two compounds in two canine mammary carcinoma cell lines.

Resveratrol, a polyphenol found in several foods, such as grapes, blueberries and peanuts, showed cytotoxic activity in both cell lines, with an IC$_{50}$ ranging from 105.5-381.6 μM, similar to the findings of Behroozaghdam et al. (2), who reported that resveratrol prevented the development of skin tumors in mice by inducing apoptosis and inhibiting the growth of metastatic melanoma cells.

In our study, in contrast to our results, in a study carried out by Sun et al. (25) demonstrated the inhibitory effect on human mammary gland cells (MCF-10A). The antiproliferative effect of $V. ~album$ on other tumor types, such as osteosarcoma, was demonstrated at different concentrations ranging from 1.5 ng/mL to 50 μg/mL through the induction of apoptosis by activating CASP8, CASP9 and CASP3 (11).

In conclusion, the agents $V. ~album$ and resveratrol proved to be effective in vitro in canine mammary cell lines with antitumoral effects, as they decreased cell viability and migration, suggesting that these natural compounds are promising agents for further in vivo studies using dogs as a natural model for mammary carcinoma clinical trials.
Funding

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Ethical approval

Approval by CEUAFMVZ UNESP Botucatu 0134/2018.

References


