Report

Anvirzel™, an extract of Nerium oleander, induces cell death in human but not murine cancer cells

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The purpose of this study was to examine the mechanism(s) and differential cell-killing effects of Anvirzel™, an extract of oleander (Nerium oleander; family: Apocynaceae), and its derivative compound Oleandrin on human, canine and murine tumor cells. Cells received different concentrations of Anvirzel (1.0 ng/ml to 500 μg/ml) or Oleandrin (0.01 ng/ml to 50 μg/ml) in both continuously treated and pulse-treated/recovery cultures. The cytotoxicity of these compounds was then determined. Both Anvirzel and Oleandrin were able to induce cell killing in human cancer cells, but not in murine cancer cells; the cell-killing potency of Oleandrin was greater than that of Anvirzel. Canine oral cancer cells treated with Anvirzel showed intermediate levels of response, with some abnormal metaphases and cell death resulting from the treatment. From these results we conclude that Anvirzel and Oleandrin act in a species-specific manner, and while testing the effectiveness of a new compound for cancer treatment, one must use not only murine but a variety of cancer cells, including those of human origin. [© 2000 Lippincott Williams & Wilkins.]

**Key words:** Anvirzel™, chromosome abnormalities, cytotoxicity, Oleandrin, species-specific effects, telomeres, TRF2.

Introduction

Although conventional chemotherapies have long been used to reduce the burden of disease in cancer patients, their side effects have been of great concern. The search is on, therefore, for alternative agents that effectively kill cancer cells, but that have minimal or no side effects on normal cells. Nerium oleander (family: Apocynaceae) is a tropical flowering plant with white, red and pink flowers. The Ayurvedic name of this plant is kanvira, and it has been used in the treatment of hemorrhoids, ulcers, leprosy and snakebite, and even in the induction of abortion. Although numerous deaths have been reported due to poisoning by means of Oleander plant ingestion, its use in folk medicine is well known.

The leaves of *N. oleander* have been used to extract cardiac glycosides, Oleandrin, adynerin, neratin, chloroside A and other compounds. Among these, Oleandrin has long been used to treat heart failure in China. In a recent article we showed that Anvirzel™ (an Oleander water extract), which contains polysaccharides and the cytotoxic compounds Oleandrin and Oleandrigenin, may have antitumor activity and may be capable of inhibiting human cancer cell growth (Newman et al., unpublished observations). Although a cellular basis for species differences in sensitivity to cardiac glycosides has been reported, these compounds' differential effects on cancer cells of different species has not been reported.

In the present studies, we have examined the *in vitro* effect of Anvirzel and its compound Oleandrin on species-specific cell killing of cancer cells of human, murine and canine origin. We have studied chromosome morphology, frequency of telomeric associations (TAs), polyploidy induction, telomeric DNA, levels of telomeric-repeat binding factors (TRF1 and TRF2), and the frequency of induced cell death in continuously and pulse-treated/recovery populations of cancer cells. Our results indicate that both Anvirzel and Oleandrin are cytotoxic to human cancer cells but are not capable of killing murine melanoma cells at even 100-fold higher drug concentrations. An oral cancer cell line of canine origin, however, showed...
intermediate susceptibility to cell killing by Anvirzel and Oleandrin.

Materials and methods

Tumor cell lines

The human prostate cancer cell lines PC-3M and LNCaP clone C4-2, a UV radiation-induced murine melanoma cell line K1735 clone X21 (kindly provided by Dr. J. Fidler, Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX) and the canine oral melanoma cell line CML-1 (a gift from Dr. L. Wolfe, Auburn University, Auburn, AL) were used in these studies. Approximately $3 \times 10^6$ cells each of these cell lines were plated in T-75 plastic culture flasks in 10 ml of RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco/BRL, Grand Island, NY) and incubated at 37°C in an atmosphere of 5% CO$_2$ and 95% air.

Treatment of cultures with Anvirzel (oleander extract) and Oleandrin

Lyophilized Anvirzel was obtained from Ozelle Pharmaceuticals (San Antonio, TX) and Oleandrin was purchased from Sigma (St Louis, MO). Anvirzel was dissolved in distilled water (10 mg/ml) and further diluted with medium. Oleandrin was dissolved in dimethyl sulfoxide (DMSO; 2 mg/ml) and further diluted with medium. Cells from the PC-3M human prostate cancer cell line were treated with Anvirzel (15, 30 and 50 µg/ml) continuously for 72 h. For recovery experiments, PC-3M cells were treated for 24 h with the above doses of Anvirzel. After treatment, cells were washed with warm phosphate-buffered saline (PBS) to remove excess drug and then fed with drug-free prewarmed medium; incubation was continued for an additional 48 h. In another set of experiments, PC-3M cells were treated with lower concentrations of Anvirzel (0.1, 0.5, 1.0, 5.0 and 10.0 µg/ml) and Oleandrin (1.0 ng/ml, 10.0 ng/ml, 0.1 µg/ml and 1.0 µg/ml) continuously for 24 h, and then harvested for cytological preparations. For TRF1 and TRF2 analysis, we also treated another human prostate cancer cell line, the LNCaP clone C4-2, with different concentrations (6.0 and 9.0 µg/ml) of Anvirzel.

For the fluorescence-activated cell sorting (FACS) analysis of the sub-diploid population, PC-3M cells were treated with Anvirzel (1, 10, 100 and 1000 ng/ml) and Oleandrin (0.01, 0.1, 1, 10, 100 and 1000 ng/ml) for 24 h, and then processed as described below. The murine melanoma cell line, K1735-X21, was treated with Anvirzel (100 and 500 µg/ml) and the dog oral melanoma cell line, CML-1, was treated with the same drug (5 and 50 µg/ml) continuously for 72 h. For recovery experiments, after 24 h of treatment, cells were washed with PBS and incubated in fresh drug-free medium for an additional 48 h. For FACS analyses, K1735-X21 cells were treated with Anvirzel (100 and 500 µg/ml) and Oleandrin (5 and 50 µg/ml) for 24 h; CML-1 cells were treated with Anvirzel (1.0, 10 µg/ml) and Oleandrin (0.1, 1 and 5 µg/ml) for 24 h, and then harvested as described. All control cultures in continuous and recovery experiments received either distilled water or DMSO.

Cell harvesting and cytological preparations

All drug-exposed and control cultures were treated with Colcemid (0.04 µg/ml) for 45 min at 37°C and then harvested for air-drying preparations following the routine procedures of this laboratory. Air-dried slides were coded and then stained in Giemsa without the induction of banding. The stained slides were evaluated for the frequency of normal, tetraploid and endoreduplicated morphologies. In addition, abnormalities (chromosome and chromatid breaks, TAs and pulverized morphology) were recorded for various cultures.

Fluorescence in situ hybridization (FISH) using human telomeric DNA probe

The human telomeric DNA probe was purchased from and used following the recommended procedure of the supplier (Oncor, Gaithersburg, MD), with slight modifications. The FISH slides were examined under a Nikon fluorescence microscope equipped with a UV-2A filter for 2,4-diamidine-2-phenylindole (DAPI) and a rhodamine filter (Omega Optical, Brattleboro, VT).

Determination of the sub-diploid population by the FACS analysis

Control and Anvirzel- and Oleandrin-treated cells were washed with cold PBS. Approximately $1 \times 10^6$ cells from each set of experiments was resuspended in 0.5 ml of propidium iodide (PI) solution (50 µg/ml PI, 0.1% Triton-X 100 and 0.1% sodium citrate in PBS). These cells were incubated at 4°C in the dark in PI working solution for 1 h and then read on the Coulter Epics XL. The percentage of hypodiploid cell populations was calculated using the multi-graph program.
Western blot analysis for TRF1 and TRF2

Control and treated cells were processed to determine the levels of TRF1 and TRF2 by Western blot analysis, as described earlier. Proteins of the cytosolic fractions were resolved by SDS-PAGE, electro-blotted and probed with TRF1 (C-19) or TRF2 (N-20) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with a secondary antibody, expected signals of TRF1 and TRF2 were detected by ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

Results

In the present work, we have studied the effects of Anvirzel and Oleandrin on two human prostate cancer cell lines (PC-3M and C4-2), a canine oral melanoma (CML-1) and a metastatic murine melanoma cell line (K1735-X21) with respect to their ability to induce endoreduplication resulting in polyploidy, formation of apoptotic bodies, metaphases with chromosome/chromatid breaks, TAs, induction of cell death, and changes in TRF1 and TRF2 levels in continuously treated and recovery cell cultures.

Figure 1. Metaphases from control (A) and Anvirzel-treated (B–D) PC-3M human prostate cancer cells showing: (A) a normal chromosome morphology, (B) an endoreduplicated metaphase spread, (C) a partial metaphase showing tri-radial configurations, and (D) selected examples of dicentrics, quadri-radials, TAs and other abnormalities.
Anvirzel and Oleandrin induce endoreduplication, TAs and cell death in PC-3M cells

Figure 1 shows examples of TAs, endomitosis and structural chromosome abnormalities in human prostate cancer PC-3M cells. As shown in Figure 1(A), a normal-looking metaphase spread of PC-3M cells from a control culture does not exhibit such anomalies. TAs that are caused by the loss of telomeric repeats are more prevalent in the tetraploid metaphases (Figure 1C and D) than in the endoreduplicated (Figure 1B) and so called normal-looking metaphases (Figure 1A). Selected examples of chromatid breaks, rings and dicentric and tri-radial configurations in human prostate cancer cells from drug-treated cultures are shown in Figure 1(D).

The frequencies of abnormal metaphases, endoreduplication and tetraploidy in Anvirzel-treated PC-3M cells are shown in Figure 2(A). PC-3M cells treated with different concentrations of Anvirzel (15, 30 and 50 μg/ml) for 24 h and then allowed to recover for 48 h showed concentration- and duration-dependent frequencies of metaphases with abnormal chromosome morphology, endomitosis and tetraploidy. Table 1 shows the frequency of such abnormalities in Anvirzel- and Oleandrin-treated PC-3M cells. Cells from the C4-2 prostate cancer line gave similar results (data not shown).

FACS analysis of PC-3M cells treated with Anvirzel showed dose-dependent increases in the percentage of hypoploid DNA-containing cells. As shown in Table 2, the percentages of hypoploid PC-3M cells were 48, 56, 59 and 62% after treatment with 1, 10, 100 and 1000 ng/ml of Anvirzel, respectively. Oleandrin induced 55% hypoploidy at a dose of 1 ng/ml, with a saturation of 84% hypoploid cells at drug doses of 10 to 100 ng/ml. These results indicate that Oleandrin is more potent than Anvirzel in inducing abnormalities in PC-3M cells and that Oleandrin has similar effects at doses from 10 to 1000 ng/ml, whereas saturation of aberration induction by Anvirzel became evident only at doses from 100 to 1000 ng/ml.

Figure 2. Histograms showing percentages of normal (N), abnormal (Abn), endoreduplicated (Endo) and tetraploid (Tera) metaphases in human prostate cancer PC-3M (A) and murine K1735-X21 melanoma (B) cell lines pulsed-treated with Anvirzel for 24 h and then allowed to recover for 48 h. Note the increased frequency of abnormal, endoreduplicated and tetraploid cells in the human prostate cancer cells (A), and their absence in the murine melanoma cells, even those treated with 100-fold higher concentrations of Anvirzel (B). From each experiment, including control, 50–100 metaphases were evaluated. Means of triplicate determinations ± SD. *p<0.01; **p<0.001.

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<th>Endoreduplication (%)</th>
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No metaphases were seen in cultures treated with Oleandrin at 0.1 and 1.0 μg/ml.
Telomeric signal intensities of PC-3M cells after treatment with Anvirzel and Oleandrin showed a reduction in the percentage of telomeric areas compared to the control interphase nuclei. However, the difference between these two groups was not very significant (data not shown).

Anvirzel and Oleandrin do not induce cell death in a metastatic murine melanoma cell line (K1735-X21)

As shown in Table 2, mouse melanoma cell line K1735-X21, treated with even 100-500 μg/ml of Anvirzel or 10-50 μg/ml of Oleandrin, did not show induction of hypodiploid cells in FACS analysis. There was no difference between control (4.7% hypodiploid cells) and K1735-X21 cells treated with the highest concentrations of Oleandrin (50 μg/ml, with 5.1% hypodiploid cells) and Anvirzel (500 μg/ml, with 2% hypodiploid cells). These results indicate that neither Anvirzel nor Oleandrin has any killing effect on mouse melanoma cells. As shown in Figure 2B, K1735-X21 cells treated with even a 100-fold higher concentration of Anvirzel did not show abnormal metaphases or higher frequencies of endoreduplicated or tetraploid metaphases. Regardless of the drug concentration (100-500 μg/ml range), the frequency of normal metaphases was greater than 90% in treated mouse melanoma cells and only 8% of metaphases were tetraploid. These results were similar to those observed in control K1735-X21 cells. Not even the chromatid/chromosome breakage frequency was increased in treated cultures. These observations were further confirmed by FISH analysis of the interphase nuclei, showing no difference in telomeric signals in control and treated murine cancer cells (data not shown).

Anvirzel and Oleandrin induce marginal cell death in a canine melanoma cell line (CML-1)

Since both Anvirzel and Oleandrin showed differential cell-killing activity against the human PC-3M cell line and the murine K1735-X21 cell line, we decided to test their effect on cancer cells of another mammalian species. As shown in Figure 3, Anvirzel induced endoreduplication in cultures of cells from a canine melanoma line (CML-1). The control metaphase spread shows a normal chromosome morphology (Figure 3A), whereas the treated metaphase (Figure 3B) shows not only endoreduplication, but also TAs. The canine
melanoma cells were treated with 1-10 μg/ml of Anvirzel and 0.1-5 μg/ml of Oleandrin for 24 h and then allowed to recover for 48 h. The frequencies of abnormal metaphases, endoreduplication and tetraploidy are shown in Figure 4. FACS analyses showed approximately 9.5% hypodiploid cells in control cultures, whereas 20.5 and 45.7% hypodiploid cells were observed after treatment with Anvirzel and Oleandrin, respectively. These results suggest that canine melanoma cells are sensitive to these drugs, but not to the extent of human PC-3M cells (Table 2). The frequencies of abnormal metaphases, endoreduplication and tetraploid chromosomes in the canine melanoma cell cultures are shown in Figure 4. Results of FACS analyses of hypodiploid cells from human prostate cancer, murine melanoma and canine oral cancer cells are summarized in Table 2. In these experiments, treating the canine oral cancer cells with 0.1-5 μg/ml Oleandrin resulted in 42-45.7% frequencies of hypodiploid cells. When these cells were treated with 1-10 μg/ml Anvirzel only 14.3-20.5% hypo-diploid cells resulted. These results further indicate that Oleandrin is more cytotoxic to these cells than Anvirzel.

TRF1 and TRF2 studies in Anvirzel-treated cells

In recent studies it has been suggested that the telomere length of chromosomes can be controlled by the telomere end-binding proteins TRF1 and TRF2. TRF1 negatively regulates telomerase activity, which regenerates lost telomere ends by replication. Therefore, an increased level of TRF1 may help prolong the survival of cancer cells. TRF2 is proposed to protect telomere ends from degradation and chromosome-end fusion. Furthermore, the loss of TRF2 may create G overhangs at the end of telomeres, which may produce DNA damage signals and hence stimulate cell cycle arrest or apoptosis. If such is the case, then drug-treated cancer cells showing reduced telomere signals and increased apoptosis may also show decreased TRF2 levels. The level of TRF1 may or may not change in such cases, since TRF1 is not directly involved in the maintenance of telomere length. In the present studies, we determined the levels of TRF1 and TRF2 in the human prostate cancer cell lines PC-3M and C4-Z and in the murine melanoma cell line K1735-XZ1 after treatment with Anvirzel. The results of the FACS analysis are given in Table 2. The levels of TRF1 in cells from these three lines was unchanged after treatment with Anvirzel. However, as expected, the levels of TRF2 in PC-3M and C4-Z cells, which were susceptible to apoptosis, were significantly decreased, in a dose-

**Figure 3.** Metaphases from a control (A) and Anvirzel-treated (B) CML-1 canine oral melanoma cells (B), showing normal chromosome morphology and endoreduplicated chromosomes with TAs, respectively. From each experiment, including control, 4C-50 metaphases were examined.

**Figure 4.** Histogram showing percentage of normal (N), abnormal (Abn), endoreduplicated (Endo) and tetraploid (Tetra) metaphases in control and recovery cultures of a canine oral melanoma cell line. Approximately 40-50 metaphases were evaluated in each control and treated culture. Means of triplicate determinations ± SD. *p < 0.01.
dependent manner, after treatment with Anvirzel (Figure 5). The level of TRF2 of the K1735-X21 cells, which were resistant to apoptosis, was unchanged after treatment with Anvirzel (Figure 5). These results thus suggest that, at least for the PC-3M and C4-2 cell lines, TRF2 may be an important cellular molecule through which Anvirzel-mediated cell killing occurs.

Discussion

Anvirzel (oleander extract) is a novel antitumor compound extracted from a flowering plant, N. oleander, belonging to the family Apocynaceae. Anvirzel consists of several compounds, including complex polysaccharides, proteins and individual sugars. It contains non-water soluble compounds, and two of these have been specifically identified by molecular weight and fragmentation characterization as Oleandrin and Oleandrigenin (Newman et al., unpublished observations). These two compounds of Anvirzel, and possibly a third one, are cytotoxic. In the present studies, we examined the cytotoxicity of Anvirzel and Oleandrin on cancer cell lines of human, murine and canine origin. Our results from tests on cultures of two human prostate cancer cell lines, PC-3M and C4-2, and a murine melanoma cell line, K1735-X21, treated with Anvirzel and Oleandrin indicate several novel features. (i) neither Anvirzel nor Oleandrin showed any cytotoxic effect on murine melanoma K1735-X21 cells, (ii) when using even low drug concentrations human prostate cancer PC-3M cells showed significant susceptibility to cell killing, (iii) the cell killing is apparently mediated through the loss of telomeric DNA, followed by the arrest of cells in G2/M phase, induction of endomitosis, extensive DNA fragmentation, reduced levels of TRF2 and finally cell death, (iv) FACS analysis revealed induction of cell death in a dose- and duration-dependent manner in the human PC-3M cell line, followed by a saturation effect, (v) Oleandrin is more cytotoxic to human prostate cancer cells than Anvirzel, and (vi) canine cancer cells also were susceptible to killing by these agents, but to a lesser extent than were human cancer cells.

In earlier studies, Anvirzel was shown to have toxic effects on cells, decreasing the level of fibroblast growth factor. Whether Anvirzel and its derivative Oleandrin exert their cytotoxic effects through inducing aberrations of chromosome morphology, polyploidy and cell death is not known. Because Anvirzel is known to have antitumor activity, we investigated the mechanism of cancer cell death in a variety of cancer cell lines of human, murine and canine origin. Our results show that both Anvirzel and Oleandrin are potent cell death inducers in two human prostate cancer cell lines, but not in a murine melanoma cell line at even 100-fold higher drug concentrations. However, cells from a canine oral cancer line showed intermediate frequencies of cell death and induced polyploidy. These observations strongly demonstrate that while testing the effectiveness of a new compound, one must use not only murine cancer cells but a variety of cancer cells originating from different mammalian species, including humans. Testing new compounds
on only murine cancer cell lines and then concluding that they would or would not work for human cancer cells could be detrimental. As a matter of fact, Oleander extract (Anvirzel) was tested several years ago on murine cells for its cytotoxic effects. Because it did not work on these cells, it was placed on a back burner, with a remark that it would not be cytotoxic on human cancer cells (Gordon Cragg, pers. commun.).

It is apparent from our results that in recovery cultures of PC-3M cells, Anvirzel had a concentration-dependent effect on the frequency of tetraploid cells, as reported previously in a murine melanoma cell line treated with Dolastatin-10 and paclitaxel. The tetraploid metaphases showed numerous structural aberrations, including TAs, dicentrics, fragments and ring formations, as shown in Figure 1(B-D). Such cells with severe DNA fragmentation subsequently undergo cell death. This phenomenon was apparent in the human cancer cells we tested, but not in the murine melanoma cell line. However, canine oral melanoma cells showed intermediate susceptibility to cell killing by Anvirzel and Oleandrin. In both human and canine cancer cells, Oleandrin was found to be more potent than Anvirzel.

Also interesting was the finding of a loss of telomere length and its correlation with TRF2 levels in the different cancer cell lines after their treatment with Anvirzel. In previous studies, it has been reported that TRF2 reduction may cause a loss of telomere length or trigger DNA damage-induced signals, resulting in cell-cycle arrest or apoptosis. One possible mechanism by which the loss of TRF2 may induce cell death is its role in protecting against end-to-end fusion of chromosomes, which is a characteristic feature of apoptotic cells. In the present studies, the Anvirzel-induced loss of telomere length and end-to-end fusion of chromosomes in PC-3M and C4-2 cells was clearly correlated with the decreased level of TRF2. However, the K1735X21 cell line, which after treatment with Anvirzel and Oleandrin did not show increased apoptosis, also did not show the loss of telomere length and end-to-end fusion of chromosomes. Furthermore, the level of TRF2 of the K1735X21 cell line remained unchanged even after treatment with an approximately 100-fold higher concentration of Anvirzel than was effective against the PC-3M and C4-2 cell lines. A role for TRF2 has been implicated in telomere loss and end-to-end fusion of chromosomes leading to apoptosis; these results thus suggest that Anvirzel-induced cell killing may be caused by the reduced levels of TRF2 in these cell lines and TRF2 reduction may be one mechanism of action whereby these drugs induce cancer cell apoptosis.

Conclusion

This study has shown that, in vitro, both Anvirzel and its derivative compound Oleandrin are highly cytotoxic to human prostate cancer cells, but not to murine melanoma cells. These agents have intermediate cytotoxic effects on a canine oral melanoma cell line. These observations strongly demonstrate that while testing the cytotoxic effects of a new compound it would be prudent to use both human and murine cancer cells, because these effects may differ in the two species as shown in this study. Finally, Anvirzel and Oleandrin both have the potential to be used in chemotherapy for a variety of human cancer types. Studies evaluating such therapies are in progress at our Cancer Center.

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References


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