The Botanical Glycoside Oleandrin Inhibits Human T-cell Leukemia Virus Type-1 Infectivity and Env-Dependent Virological Synapse Formation

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ABSTRACT
At present, there are no antiretroviral drugs that inhibit incorporation of the envelope glycoprotein into newly-synthesized virus particles. The botanical glycoside, oleandrin, derived from extracts of Nerium oleander, has previously been shown to reduce the levels of the gp120 envelope glycoprotein on human immunodeficiency virus type-1 (HIV-1) particles and inhibit HIV-1 infectivity in vitro. We therefore tested whether oleandrin or an extract from N. oleander could also inhibit the infectivity of the human T-cell leukemia virus type-1 (HTLV-1): A related enveloped retrovirus and emerging tropical infectious agent. The treatment of HTLV-1+ lymphoma T-cells with either oleandrin or a N. oleander extract did not significantly inhibit viral replication or the release of p19Gag-containing particles into the culture supernatants. However, the collected virus particles from treated cells exhibited reduced infectivity on primary human peripheral blood mononuclear cells (huPBMCs). Unlike HIV-1, extracellular HTLV-1 particles are poorly infectious and viral transmission typically occurs via direct intercellular interactions across a virological synapse. We therefore investigated whether oleandrin or a N. oleander extract could inhibit virus transmission from a GFP-expressing HTLV-1+ lymphoma T-cell-line to huPBMCs in co-culture assays. These results demonstrated that both oleandrin and the crude phytoextract inhibited the formation of virological synapses and the transmission of HTLV-1 in vitro. Importantly, these findings suggest oleandrin may have broad antiviral activity against enveloped viruses by reducing the incorporation of the envelope glycoprotein into mature particles, a stage of the infection cycle not targeted by modern HAART.

Keywords: HTLV-1; HIV-1; Envelope glycoprotein; Oleandrin; Nerium oleander; Antiviral; HAART; Apoptosis; HAM/TSP

INTRODUCTION
The botanical glycoside, oleandrin, and an extract of Nerium oleander have been shown to prevent the incorporation of the gp120 envelope glycoprotein of HIV-1 into mature virus particles and inhibit viral infectivity in vitro [1]. These findings prompted us to investigate whether oleandrin could similarly inhibit another related retrovirus, the HTLV-1. Growing evidence suggests that certain plant species, including N. oleander and N. indicum, may contain pharmacological compounds with beneficial medicinal properties [2-12]. Although N. oleander is considered poisonous and many of its chemical byproducts elicit cardiotoxicities [13-16], the cardenolides derived from N. oleander extracts, including oleandrin, 8-hydroxy-oleandrigenin-3-O-β-D-digitoside, 5α-oleaside A, 14-carboxyl-neriaside, 21-hydroxy-
neriaside, among others, have been shown to have anti-
proliferative, anti-inflammatory, and anti-tumorigenic effects
[3,4,6,7,17-20]. Indeed, a N. oleander-derived drug, PBI-05204,
which contains oleandrin as a bioactive agent, has completed
both Phase I and initial Phase II clinical trials to test its efficacy
and safety in patients with advanced solid cancers [21]. Pan et al.
[22] have shown that PBI-05204 inhibited pancreatic tumor
growth by targeting the phosphatidylinositol-3-kinase
(PI3K)/Akt signaling pathway. Oleandrin has also been shown
to accumulate in the central nervous system (CNS) and
penetrate the blood-brain-barrier (BBB) following injection [23];
and Garofalo et al. [3] have further demonstrated that oleandrin
inhibited tumor growth and disease progression in a murine
xenograft model of malignant glioma. Moreover, oleandrin
exhibited neuroprotective effects and protected neuronal tissues
from damage, due to glucose and oxygen deprivation,
dependent on the induction of brain-derived neurotrophic
factor (BDNF), in brain slices and in vivo models of ischemic stroke [10,24]. It is an intriguing notion that the ability of
oleandrin to cross the BBB and inhibit HTLV-1 infectivity and
the expression of viral antigens could have potential therapeutic
implications for the treatment of HTLV-1-associated neuro-
inflammatory diseases, including HTLV-1-associated
myelopathy/tropical spastic paraparesis (HAM/TSP) [25,26].

The HTLV-1 is a delta oncoretrovirus and blood-borne pathogen
that is endemic to tropical equatorial regions, including
Southeast Asia (e.g., Japan, China, Taiwan, and Malaysia),
Australia and Melanesia, Northern and Central Africa, the
Middle East, Central and South America, and certain islands of
the Caribbean (e.g., the FWI). It is estimated there are 10-15
million HTLV-1-infected individuals worldwide; and this virus is
considered an emerging health threat, as was described in a 2018
open letter to the World Health Organization (WHO) [27]. Indeed,
HTLV-1 has been detected with high incidences of
seropositivity in remote indigenous populations in central
Australia and South America [28,29]. The HTLV-1 infects CD4+
T-lymphocytes and causes adult T-cell leukaemia/lymphoma
(ATLL)-a rare, yet aggressive haematological malignancy with high
rates of therapy-resistance and generally poor clinical outcomes
[30-32], in addition to several autoimmune/inflammatory
conditions, including infectious dermatitis [33,34], rheumatoid arthriti
[35,36], uveitis [37,38], keratoconjunctivitis [39], sicca syndrome [40,41], Sjögren’s syndrome [42,43], and HAM/TSP,
among others [25,26,44-46]. The replication of HTLV-1 and
the expression of viral antigens is positively and negatively regulated
by products encoded by the conserved 3’ pX region, including
the viral transactivator protein Tax, the latency-maintenance
factors: p30[II], HBZ, and p13[II], and the hbz antisense mRNA
which can modulate the expression of p30[II] [47-63]. While
ATLL is etiologically linked to viral latency associated with
oncogenic transformation and the clonal expansion of HTLV-1-
infected cells [30-32], the inflammatory diseases, such as HAM/
TSP, are caused by autoimmune and/or immunopathological
responses to proviral replication and the expression of viral
antigens [25,26,44-46,64-67]. HAM/TSP is a progressive
neuroinflammatory disease that results in the deterioration and
demyelination of the lower spinal cord. The persistence of
proviral replication and the proliferation of HTLV-1-infected
cells in the CNS leads to a cytotoxic T-cell response targeted
against viral antigens, and which may be responsible for the
autoimmune destruction of nervous tissues [25,68-70].

Here we demonstrate that purified oleandrin and a N. oleander
extract inhibit the infectivity of HTLV-1 particles released into
the culture supernatants of treated cells, and also reduce the
intercellular transmission of HTLV-1 by inhibiting the Env-
dependent formation of virological syncapses.

MATERIALS AND METHODS

Cell-lines and isolation of primary huPBMCs

The virus-producing HTLV-1-transformed (HTLV-1+) SLB1
lymphoma T-cell-line [71] (kindly provided by P. Green, The
Ohio State University-Comprehensive Cancer Center) was
cultured in a humidified incubator at 37°C under 10% CO_{2} in
Iscove’s Modified Dulbecco’s Medium (IMDM; ATCC No.
30-2005), supplemented with 10% heat-inactivated fetal bovine
serum (FBS; Biowest), 100 U/ml penicillin, 100 µg/ml streptomycin-sulfate, and 20 µg/ml gentamycin-sulfate (Life
Technologies). Primary human peripheral blood mononuclear
cells (huPBMCs) were isolated from whole blood samples,
without identifiers by the SMU Memorial Health Center under a protocol approved by the SMU Institutional
Review Board and consistent with Declaration of Helsinki
principles. In brief, 2 ml of whole blood was mixed with an
equal volume of sterile phosphate-buffered saline (PBS), pH 7.4,
in polypropylene conical tubes (Corning) and then the samples
were gently layered over 3 ml of Lymphocyte Separation
Medium (MP Biomedicals). The samples were centrifuged for 30
min at 400 × g in a swinging bucket rotor at room temp. The
buffy-coat huPBMCs were subsequently aspirated, washed 2X in
RPMI-1640 medium (ATCC No. 30-2001), and pelleted by
centrifugation for 7 min at 260 × g. The cells were resuspended
in RPMI-1640 medium, supplemented with 20% FBS, 100
U/ml penicillin, 100 µg/ml streptomycin-sulfate, 20 µg/ml gentamycin-sulfate, and 50 U/ml recombinant human
 interleukin-2 (hu-IL-2; Roche Applied Science), and stimulated
for 24 h with 10 ng/ml phytohemagglutinin (PHA; Sigma-
Aldrich) and grown at 37°C under 10% CO_{2} in a humidified
incubator. On the following day, the cells were pelleted by
centrifugation for 7 min at 260 × g and washed 2X with
RPMI-1640 medium to remove the PHA, and then resuspended
and cultured in complete medium, supplemented with
antibiotics and 50 U/ml hu-IL-2, as described.

Generation of GFP-expressing HTLV-1+ SLB1/pLenti-
GFP T-cell clones

To generate the GFP-expressing HTLV-1+ SLB1 T-cell clones, 2 ×
10^{6} SLB1 cells were plated in 60 mm² tissue-culture dishes
(Corning) in IMDM, supplemented with 10% heat-inactivated
FBS and antibiotics, and then transduced with lentiviral
particles containing a pLenti-6.2/V5-DEST- protein expression
vector which also carries a blasticidin-resistance gene [53]. After
6 h, the transduced cells were pelleted by centrifugation for 7
min at 260 × g at room temperature, washed 2X with serum-free
IMDM, and resuspended in complete medium supplemented
with 5 µg/ml blasticidin (Life Technologies) and aliquoted into 96-well microtiter plates (Corning). The cultures were maintained with blasticidin-selection for two weeks in a humidified incubator at 37°C and 10% CO₂. The GFP-expressing lymphoblasts were screened by fluorescence-microscopy, and then plated by limiting-dilution in 96-well microtiter plates to obtain homogenous GFP-expressing cell clones. The resulting HTLV-1+ SLB1/pLenti-GFP T-lymphocyte clones were expanded and repeatedly passaged; and the expression of GFP was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using a rabbit polyclonal Anti-GFP (FL) antibody (Santa Cruz Biotechnology).

**Quantitation of virus production and particle infectivity by Anti-HTLV-1 p19GaG ELISAs**

To determine the effects of oleandrin or an extract of *N. oleander* upon HTLV-1 proviral replication and the release of newly-synthesized extracellular virus particles, the HTLV-1+ SLB1 lymphoma T-cell-line was plated at 2 × 10⁴ cells per well in 300 µl of complete medium, supplemented with antibiotics, in 96-well microtiter plates and incubated at 37°C under 10% CO₂. The purified oleandrin compound and extract of *N. oleander* (Phoenix Biotechnology) [1] were resuspended in the Vehicle solution (20% v/v dimethyl sulfoxide, DMSO, in MilliQ distilled/deionized H₂O) at a stock concentration of 2 mg/ml and then sterilized using a luer-lock 0.2 µm syringe filter (Millipore). The HTLV-1+ SLB1 cells were treated with oleandrin or the *N. oleander* extract at concentrations of 10, 50, and 100 µg/ml, or with increasing amounts (1.5, 7.5, and 15 µl) of the Vehicle control for 72 h. The 96-well microtiter plates were then centrifuged for 7 min at 260 × g at room temp using an Eppendorf A-2-DWP swinging plate rotor to pellet the cells, and the levels of extracellular p19GaG-containing HTLV-1 particles released into the culture supernatants were quantified relative to a p19GaG protein standard by performing colorimetric Anti-p19GaG enzyme-linked immunosorbent assays (ELISAs; Zeptometrix). The samples were analyzed with triplicate replicates on a Berthold Tristar LB 941 multimode microplate-reader at 450 nm in absorbance mode.

To assess the infectivity of newly-synthesized extracellular HTLV-1 particles collected from oleandrin-treated cells, 2 × 10⁴ HTLV-1+ SLB1 T-lymphoblasts were plated in 300 µl of complete medium, supplemented with antibiotics, and the cultures were treated for 72 h with increasing concentrations (10, 50, and 100 µg/ml) of oleandrin or a *N. oleander* extract, or the Vehicle control (1.5, 7.5, and 15 µl). Then, 50 µl of the virus-containing supernatants were used to directly infect huPBMCs plated at a density of 2 × 10⁴ cells per well on 96-well microtiter plates in complete medium, supplemented with antibiotics and hu-IL-2. The oleandrin compound, *N. oleander* extract, or Vehicle control were maintained in the huPBMCs culture medium to control for possible re-infection events by newly-produced particles. After 72 h, the relative levels of extracellular p19GaG-containing HTLV-1 virions released into the culture supernatants by the infected huPBMCs were quantified through Anti-HTLV-1 p19GaG ELISAs as described.

**Measuring cellular apoptosis**

To assess the relative cytotoxicity of the oleandrin compound, extract of *N. oleander*, or the Vehicle control in treated cell cultures, 2 × 10⁴ HTLV-1+ SLB1 lymphoma T-cells or activated/cultured huPBMCs were plated in 300 µl of complete medium, supplemented with antibiotics, and maintained at 37°C under 10% CO₂ in a humidified incubator. The cultures were treated with either increasing concentrations (10, 50 and 100 µg/ml) of oleandrin or *N. oleander* extract, or the Vehicle control (1.5, 7.5, 15 µl) and incubated for 72 h. Cyclophosphamide (50 µM; Sigma-Aldrich)-treated cells were included as a positive control for apoptosis. The cells were then aspirated and plated on Permanox 8-chamber tissue-culture slides (Nalge) that had been pre-treated with a sterile 0.01% solution of Poly-L-Lysine and Concanavalin A (1 mg/ml; Sigma-Aldrich). The samples were subsequently stained using a microscopy apoptosis detection kit with Annexin V conjugated to fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI; BD-Pharmingen), and the relative percentages of apoptotic (i.e., Annexin V-FITC and/or PI-positive) cells per field were quantified in-triplicate by confocal fluorescence-microscopy using a 20x objective lens. The total numbers of cells per field were quantified by microscopy using a DIC phase-contrast filter.

**HTLV-1 transmission and virological synapse formation in co-culture assays**

As the transmission of HTLV-1 typically occurs through direct contact between an infected cell and uninfected target cell across a virological synapse [72-76], we tested whether oleandrin, a *N. oleander* extract, or the Vehicle control might influence the formation of virological synapses and/or the transmission of infectious HTLV-1 particles via intercellular interactions in vitro. For these experiments, 2 × 10⁴ virus-producing HTLV-1+ SLB1 T-cells were plated in 96-well microtiter plates and treated with mitomycin C (100 µg/ml) in 300 µl of complete medium for 2 h at 37°C under 10% CO₂ [77]. The culture media was then removed, the cells were washed 2X with serum-free IMDM, and the cells were treated for either 15 min or 3 h with increasing amounts (10, 50, and 100 µg/ml) of oleandrin or *N. oleander* extract, or the Vehicle control (1.5, 7.5, and 15 µl). Alternatively, 2 × 10⁴ of the GFP-expressing HTLV-1+ SLB1/pLenti-GFP T-cells were plated on 8-chamber tissue-culture slides in 300 µl of complete medium and treated with mitomycin C, washed 2X with serum-free IMDM, and then treated with oleandrin, *N. oleander* extract, or the Vehicle control as described for confocal microscopy experiments. We next aspirated the medium, washed the HTLV-1+ SLB1 cells 2X with serum-free medium, and added 2 × 10⁴ huPBMCs to each well in 300 µl of RPMI-1640 medium, supplemented with 20% FBS, antibiotics and 50U/ml hu-IL-2, and then cocultured the cells for another 72 h (the cells were co-cultured for 6 h to visualize virological synapse formation and viral transmission by confocal microscopy using the SLB1/pLenti-GFP lymphoblasts) at 37°C under 10% CO₂ in a humidified incubator. As a negative control, huPBMCs were cultured alone in the absence of virus-producing cells. The oleandrin, *N. oleander* extract, and Vehicle were maintained in the co-culture medium. The relative levels of extracellular p19GaG-containing HTLV-1 particles released into
the co-culture supernatants as a result of intercellular viral transmission were quantified by performing Anti-HTLV-1 p19Gag ELISAs. Virological synapses formed between the GFP-positive HTLV-1+ SLB/pLenti-GFP cells and huPBMCs were visualized using immunofluorescence-confocal microscopy by staining the fixed samples with an Anti-HTLV-1 gp21Env primary antibody and a rhodamine red-conjugated secondary antibody. Diamidino-2-phenyl-indole, dihydrochloride (DAPI; Molecular Probes) nuclear-staining was included for comparison and to visualize uninfected (i.e., HTLV-1-negative) cells. The intercellular transmission of HTLV-1 to the huPBMCs in co-culture assays was quantified by counting the relative percentages of HTLV-1 gp21Env-positive (and GFP-negative) huPBMCs in 20 visual fields using a 20x objective lens.

Microscopy

The Annexin V-FITC/PI-stained samples to quantify cellular apoptosis and cytotoxicity were visualized by confocal fluorescence-microscopy on a Zeiss LSM800 instrument equipped with an Airyscan detector and stage CO₂ incubator, using a Plan-Apochromat 20 × 0.8 objective lens and Zeiss ZEN system software (Carl Zeiss Microscopy). The formation of virological synapses and viral transmission (i.e., determined by quantifying the relative percentages of Anti-HTLV-1 gp21Env-positive huPBMCs) between the mitomycin C-treated HTLV-1+ SLB1/pLenti-GFP lymphoblasts and cultured huPBMCs were visualized by immunofluorescence-confocal microscopy using a Plan-Apochromat 20 × 0.8 objective lens. The relative fluorescence-intensities of the DAPI, Anti-HTLV-1 gp21Env-specific (rhodamine red-positive), and GFP signals were graphically quantified using the Zen 2.5D analysis tool (Carl Zeiss Microscopy). The GFP-expressing HTLV-1+ SLB1/pLenti-GFP T-cell clones were screened by confocal fluorescence-microscopy on a Nikon Eclipse TE2000-U inverted microscope and D-Eclipse confocal imaging system, equipped with 633 nm and 543 nm He/Ne and 488 nm Ar lasers, using a Plan Fluor 10 × 0.30 objective lens and DIC phase-contrast filter (Nikon Instruments).

Data analysis

The statistical significance of experimental data sets was determined using unpaired two-tailed Student’s t-tests (alpha = 0.05) and calculated p values using the Shapiro-Wilk normality test and Graphpad Prism 7.03 software. The p values were defined as: 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***) <0.0001 (****). Unless otherwise noted, error bars represent the SEM from at least three independent experiments.

RESULTS

The botanical glycoside oleandrin does not inhibit viral replication or the release of p19Gag-containing HTLV-1 particles

Oleandrin is a lipid-soluble, botanical cardiac glycoside comprised of: (1) the steroid aglycone, oleandrigenin, and (2) a sugar moiety (e.g., D-diginosyl) and its chemical structure is shown in Figure 1a [1,4,78]. To determine whether the purified oleandrin compound, or an extract of N. oleander, could inhibit HTLV-1 proviral replication and/or the production and release of p19Gag-containing virus particles, the virus-producing HTLV-1-transformed SLB1 lymphoma T-cell-line [71] was treated with increasing concentrations of oleandrin or a N. oleander extract, or the sterile Vehicle control (20% DMSO in MilliQ-treated ddH₂O) and then incubated for 72 h at 37°C under 10% CO₂. The cells were later pelleted by centrifugation and the relative levels of extracellular p19Gag-containing virus particles released into the culture supernatants were quantified by performing Anti-HTLV-1 p19Gag ELISAs (Zephtometric). The results in Figure 1b demonstrate that neither oleandrin, nor the N. oleander extract, significantly inhibited viral replication or the production and release of p19Gag-containing virions relative to untreated cells or the Vehicle negative control (note: the data are presented on an abbreviated scale and the concentrations of HTLV-1 p19Gag in the culture supernatants were determined relative to a p19Gag protein standard).

Figure 1a: Diagram of the chemical structure of oleandrin.

Figure 1b: The HTLV-1+ SLB1 lymphoma T-cell-line was treated for 72 h with the Vehicle control (1.5 ml, 7.5 ml, or 15 ml), or increasing concentrations (10 mg/ml, 50 mg/ml, and 100 mg/ml) of the oleandrin compound or an extract of N. oleander. Viral replication and the release of extracellular particles into the culture supernatants were quantified by performing Anti-HTLV-1 p19Gag ELISAs (Zephtometric). Untreated (UT) cells are shown for comparison.

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We next assessed the cytotoxicity of the different dilutions of the purified oleandrin compound and N. oleander extract in treated HTLV-1+ SLB1 lymphoblast cultures. For these experiments, SLB1 T-cells were treated with increasing concentrations (10, 50, and 100 µg/ml) of oleandrin or a N. oleander extract for 72 h as described above. As a negative control, the cells were also treated with increasing amounts (1.5, 7.5, and 15 µl) of the Vehicle solution which corresponded to the volumes used in the drug-treated cultures. Cyclophosphamide-treated SLB1 cells were included as a positive control for apoptosis. Then, the samples were washed and stained with Annexin V-FITC and propidium iodide (PI) and analyzed by confocal fluorescence-microscopy. The relative percentages of apoptotic (i.e., Annexin V-FITC and/or PI-positive) cells per field were quantified in triplicate using a 20x objective lens, as compared to the total numbers of cells visualized using a DIC phase-contrast filter. These results (Figures 1c and 1d) revealed that the lowest concentration (10 µg/ml) of oleandrin and the N. oleander extract did not induce significant cytotoxicity/apoptosis. However, the higher concentrations (50 and 100 µg/ml) of the crude phytoextract induced notably more apoptosis than did the oleandrin compound (Figures 1c and 1d). This is consistent with the fact that oleandrin represents only 1.23% of the N. oleander extract [1] which also contains other toxic cardenolides, including folinerin and digitoxigenin [13]. The cytotoxicity caused by oleandrin was not significantly higher than the Vehicle control in treated HTLV-1+ SLB1 cells (Figures 1c and 1d).

We therefore tested whether these agents could inhibit the infectivity of newly-synthesized extracellular p19Gag-containing HTLV-1 particles obtained from treated SLB1 T-cells. For these studies, HTLV-1+ SLB1 lymphoma T-cells were treated with increasing concentrations of either the oleandrin compound or N. oleander extract, or the Vehicle control for 72 h in 96-well microtiter plates, and then the virus-containing supernatants were collected and used to directly infect primary cultured, human peripheral blood mononuclear cells (huPBMCs) in vitro. Following 72 h, the relative levels of extracellular p19Gag-containing virus particles released into the culture supernatants, as a result of direct infection, were quantified by performing Anti-HTLV-1 p19Gag ELISAs. Interestingly, the lowest concentration (10 µg/ml) of both oleandrin and the N. oleander extract inhibited the infectivity of newly-synthesized p19Gag-containing virus particles released into the culture supernatants of treated cells, relative to a comparable amount of the Vehicle control (Figure 2a). The levels of inhibition, however, seemed to plateau and did not continue to increase with higher concentrations, which suggests their molecular target(s) could be limiting and/or saturated at the concentrations tested (Figure 2a).
The HTLV-1+ SLB1 lymphoma T-cell-line was treated with the Vehicle control, or increasing concentrations (10 mg/ml, 50 mg/ml, and 100 mg/ml) of the N. oleander extract or oleandrin compound for 72 h and then the virus-containing supernatants were collected and used to directly infect primary huPBMCs. The Vehicle control, N. oleander extract, or oleandrin were also included in the culture media for the huPBMCs. After 72 h, the culture supernatants were collected and the relative amounts of extracellular virus particles produced were quantified by performing Anti-HTLV-1 p19Gag ELISAs.

We also assayed the cytotoxicity of purified oleandrin and the N. oleander extract, compared to the Vehicle negative control, in treated huPBMCs. Primary buffy-coat huPBMCs were isolated and stimulated with phytohemagglutinin (PHA) and cultured in the presence of recombinant human interleukin-2 (hIL-2). The cells were then treated for 72 h with increasing concentrations of oleandrin or a N. oleander extract, or with increasing volumes of the Vehicle. The samples were subsequently stained with Annexin V-FITC and PI and the relative percentages of apoptotic (i.e., Annexin V-FITC and/or PI-positive) cells per field were quantified by confocal fluorescence-microscopy and counting in-triplicate. As shown in Figure 2b, the oleandrin compound exhibited moderate cytotoxicity (e.g., 35-37% at the lowest concentration) in huPBMCs as compared to the Vehicle control. By contrast, the N. oleander extract was significantly cytotoxic and induced high levels of programmed cell-death even at the lowest concentration (Figure 2b). The huPBMCs were somewhat more sensitive to purified oleandrin than the HTLV-1+ SLB1 lymphoblasts; however, the huPBMCs were drastically more sensitive to the crude N. oleander extract which also contains other cytotoxic compounds [13].

Unlike HIV-1, HTLV-1 particles are poorly infectious and viral transmission typically occurs via intercellular interactions across a virological synapse [72-76]. Therefore, we next investigated whether oleandrin or the N. oleander extract could interfere with the transmission of HTLV-1 particles to target huPBMCs in co-culture experiments. For these studies, the virus-producing HTLV-1+ SLB1 T-cell-line was treated with mitomycin C [77] and then with increasing amounts of oleandrin, N. oleander extract, or the Vehicle control for either 15 min or 3 h. The SLB1 cells were washed 2X with serum-free medium and equivalent numbers of huPBMCs were then added to each well, and the samples were co-cultured for 72 h in complete medium at 37°C under 10% CO2 in a humidified incubator. The relative intercellular transmission of HTLV-1 was assessed by performing Anti-HTLV-1 p19Gag ELISAs to measure the levels of extracellular virus released into the culture supernatants. Interestingly, the results in Figure 3a demonstrate that both oleandrin and the N. oleander extract inhibited the transmission of HTLV-1 as compared to the Vehicle control –although there were no differences observed between the 15 min and 3 h of pre-treatment of the HTLV-1+ SLB1 cells.
Figure 3a: Primary huPBMCs were co-cultured with mitomycin C-treated HTLV-1+ SLB1 lymphoma T-cells which were pre-treated for either 15 min or 3 h with the Vehicle control, or increasing concentrations (10 mg/ml, 50 mg/ml, and 100 mg/ml) of the N. oleander extract or oleandrin compound. The Vehicle control, extract, and compound were also present in the co-culture media. After 72 h, the supernatants were collected and the amounts of extracellular virus particles released were quantified by performing Anti-HTLV-1 p19Gag ELISAs. The data represent the mean ± standard deviation (error bars) from three independent experiments.

To directly visualize the formation of virological synapses and the interaction of target huPBMCs with the virus-producing cells, we generated HTLV-1+ SLB1 T-cell clones that stably express the Green fluorescent protein (GFP; Figure 3b). The HTLV-1+ SLB1 lymphoma T-cell-line was transduced with lentiviral-GFP particles and the GFP-positive cell clones were selected on blasticidin, and subsequently confirmed by direct fluorescence-microscopy and immunoblotting (Figure 3b). The formation of virological synapses between the huPBMCs and mitomycin C-treated HTLV-1+ GFP-expressing SLB1 cells was visualized by staining the samples with an Anti-HTLV-1 gp21Env primary antibody and rhodamine-red-conjugated secondary antibody, together with DAPI nuclear-staining, and then performing confocal immunofluorescence-microscopy. The arrows in the top panels of Figure 3c indicate virological synapses between the huPBMCs (i.e., gp21Env-positive; GFP-negative) and HTLV-1+ SLB1 cells treated with the Vehicle control. There were markedly fewer synapses observed in the samples treated with oleandrin or the N. oleander extract (Figures 3c). This was further evidenced by the reduced percentages of HTLV-1 gp21Env-positive huPBMCs in these treated samples as compared to the Vehicle control (Figure 3c).

Figure 3b: A GFP-expressing HTLV-1+ SLB1 T-cell-line was generated by transducing SLB1 lymphoma T-cells with a pLenti-6.2/V5-DEST-GFP vector with selection on blasticidin (5 mg/ml; Life Technologies) for two weeks. The GFP-positive clones were screened by fluorescence-microscopy (top panels) and immunoblotting (lower panels), and expanded and repeatedly passaged. The DIC phase-contrast image is provided for comparison.

Figure 3c: The formation of virological synapses between huPBMCs and the mitomycin C-treated HTLV-1+ SLB1/pLenti-GFP lymphoblasts (green cells) that had been pre-treated for 3 h with the Vehicle control or increasing amounts (10 µg/ml, 50 µg/ml, and 100 µg/ml) of the N. oleander extract or oleandrin compound were visualized by staining the samples with an Anti-HTLV-1 gp21Env primary antibody and rhodamine-red-conjugated secondary antibody, together with DAPI nuclear-staining, and then performing confocal immunofluorescence-microscopy. The arrows in the top panels of Figure 3c indicate virological synapses between the huPBMCs (i.e., gp21Env-positive; GFP-negative) and HTLV-1+ SLB1 cells treated with the Vehicle control. There were markedly fewer synapses observed in the samples treated with oleandrin or the N. oleander extract (Figures 3c). This was further evidenced by the reduced percentages of HTLV-1 gp21Env-positive huPBMCs in these treated samples as compared to the Vehicle control (Figure 3c).
DISCUSSION

Oleandrin and other structurally similar digoxin-related cardenolides exert most of their toxic effects by inhibiting sodium/potassium pump ATPase catalytic activity [5-7,79]. Conversely, Yang et al. [80] have shown that the inhibition of sodium/potassium pump ATPase activity by oleandrin reduced the sensitivity of swine testicular cells to infection by the porcine transmissible gastroenteritis virus in vitro. Oleandrin has also been demonstrated to inhibit nuclear factor-kappa B (NF-B) and activator protein-1 (AP-1)-dependent transcriptional signaling through the c-Jun NH2-terminal kinase (JNK) and mitogen-activated/extracellular signal-regulated kinase (MEK) pathways, and thus could have anti-inflammatory and anti-tumorigenic properties [81,82]. Indeed, Afaq et al. [19] have shown that topicaly-applied oleandrin inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation and tumor promotion in a murine model of skin cancer. While the mechanism(s) by which oleandrin interferes with NF-KB-mediated inflammatory signaling remains to be fully determined, Manna et al. [83] have suggested oleandrin could inhibit interleukin-8 (IL-8)-receptor functions by altering plasma membrane fluidics and downregulating this cytokine receptor from the cell surface. Oleandrin also induces the production and accumulation of damaging reactive oxygen species (ROS) which could contribute to its overall cytotoxicity and anti-tumorigenic effects [20]. Turan et al. [8] have also demonstrated that extracts of N. oleander resulted in decreased levels of the P-glycoprotein on the surfaces of treated leukemic cell-lines. Though speculative, it is likely that oleandrin’s ability to inhibit the incorporation of envelope glycoproteins into budding virions could reflect oleandrin-induced alterations to the plasma membrane and vesicle fluidics in a manner analogous to the downregulation of Pglycoprotein and the IL-8-receptor [8,83].

It is possible the inhibition of HTLV-1-infectivity, viral transmission and virological synapse formation by oleandrin could have implications for the suppression of viral antigen into the CNS [64-66]. HTLV-1-associated myelopathy/tropical spastic paraparesis as a stage of the infection cycle that is not targeted by modern HAART. Jejcic et al. [89] have previously demonstrated that a synthetic peptide mimetic, glycyrl-prolyl-glycine amide (GPG-NH(2)), prevented the maturation of the HIV-1 envelope glycoprotein into gp120/gp41 and caused it to become degraded in the endoplasmic reticulum, and resulted in the production of defective virus particles with reduced envelope-incorporation and decreased infectivity. Intriguingly, Rajbhandari et al. [11] have shown that extracts from a related botanical, Nerium indicum, possessed antiviral activity against influenza virus (A/WSN/33 (H1N1) London isolate) and herpes simplex virus type-1 (HSV-1, KOS strain) in vitro. James et al. [90] have further reported that oleandrin, as well as an extract of N. oleander, inhibited virus production in treated Vero cells infected with either the Marburg or Ebola filoviruses, or Venezuelan equine encephalitis virus.

CONCLUSION

It is therefore possible that phytomedicinal compounds, such as the cardenolide oleandrin, may one day represent a cost-effective therapeutic strategy to help combat enveloped virus infections in developing countries with limited access to modern antivirals.

CONFLICTS OF INTEREST

R.A.N. is the President and Chief Scientific Officer of Phoenix Biotechnology, Inc. which is the source of the N. oleander extract used in this study. This company did not fund the research and only provided test materials.

ACKNOWLEDGEMENT

The authors thank P. Green for kindly providing the HTLV-1-transformed SLB1 lymphoma T-cell-line. T.H. is a recipient of a 2015 American Society for Microbiology-Eugene & Millicent Goldschmidt Graduate Student Fellowship.

FUNDING SOURCE

This work was supported by a grant from the National Cancer Institute/National Institutes of Health 1R15CA202265-01A1 to R.H.

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