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Antiproliferative evaluation of tall-oil docosanol and tetracosanol over CHO-K1 and human melanoma cells

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ABSTRACT

Background: Polycosanols derived from plant species have traditionally been used in medicine as antiproliferative agents for treating various viruses (primarily the herpes simplex virus). However, few studies have studied their effects on hyperproliferative cell lines. In this work, the antiproliferative capacity of polycosanols from tall-oil pitch, obtained from black liquor soaps in the kraft pulping process of cellulose (specifically from *Pinus radiata*, *Pinus taede*, and *Eucalyptus globulus*), was evaluated on CHO-K1 and CRL-1974 human melanoma cell lines.
Results: The proliferative capacities and cell viabilities were measured for 72 and 140 h, respectively. Treatment with docosanol produced differential effects on the CHO-K1 and human melanoma cells and significantly affected their proliferation rates, but not their cell viabilities. Tetracosanol produced a significant negative effect on the proliferation of human melanoma cells, and this effect was less than that caused by docosanol. However, it had no effect on the proliferation of CHO-K1 cells and did not induce any significant effect on the viability of the studied cell lines.
Conclusion: Docosanol and tetracosanol induced antiproliferative effects on the studied cell lines and exhibited significantly greater effects on the oncogenic cell lines. Prior to this study, the capacity of these polycosanols has never been investigated. Future studies will be necessary to determine their mechanisms of action on these cell systems.

1. Introduction

Long-chain aliphatic alcohols (polycosanols) and fatty acids with more than 20 carbon atoms are of great interest as medical, personal care, and pharmaceutical products. Polycosanols have primarily been used to treat the herpes simplex virus (HSV-1) [1,2,3,4,5,6] and the respiratory syncytial virus [3]. Hyperproliferative skin lesions [7], which can be benign or malignant (keloids, skin cancer), can be treated and cured with these alcohols. Additionally, polycosanols can be used as anti-inflammatory agents [8] in epithelial prostate cells [9, 10] and in the treatment of certain conditions caused by enveloped viruses, such as Kaposi sarcomas [11]. However, this type of alcohol is extremely rare in nature and is only found in small quantities in sugarcane, spinach, evening primrose oil [12], beeswax [13,14], the native Chilean plant *Myoschilos oblongum* (Orocoipo, Codocoipo) [10], the Brazilian shrub *Gallesia gorazema* (Phytolaccaceae) [15], and some Oriental medicinal plants [16,17,18].

The discovery of new utilities for polycosanols has created new opportunities to exploit the sources that contain these alcohols. One of these sources is the black liquor soap, from the kraft pulping process of cellulose, which is a byproduct resulting from the processing of these soaps in the recovery of other compounds, such as wood sterols [19].

Tall-oil is a fraction obtained from black liquor soap in the kraft pulping process. After further fractionation to isolate a heavy component called pitch [20,21], a light fraction rich in polycosanols can be recovered by distillation. Subsequently, a short-path distillation yields three additional fractions rich in docosanol (C22), tetracosanol (C24) and higher fatty alcohols (C > 24), respectively [19] (Fig. 1). Because of these compounds, black liquor soap has been considered one of the most suitable raw materials for the commercial production of docosanol (D-nol) and, in particular, tetracosanol (T-nol).

There have been several studies conducted on the therapeutic uses of polycosanols. Extensive investigations on the herpes simplex virus *in vivo* and *in vitro* have shown that docosanol exerts potent inhibitory effects on the ability of the virus to infect target cells. Current evidence suggests that docosanol inhibits viral replication by interfering with early intracellular events surrounding viral entry into target cells [3]. However, for a few cell lines, the effects of docosanol are scarce. In mammalian cells, there is no information about the cytotoxic effects of these alcohols. Recent studies have reported the antiproliferative properties of natural extracts that are known to contain molecules

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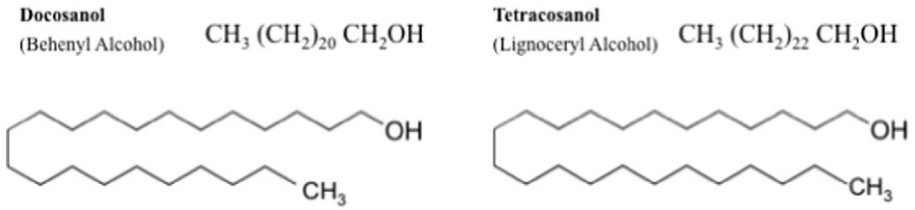

Fig. 1. Structure of long-chain aliphatic alcohol (polycosanols): docosanol and tetracosanol.

with high numbers of carbon atoms. However, few of these molecules are polycosanols as most are flavonoids, terpenoids or quinones [22, 23,16,24,25,26].

The objective of this work was to determine the antiproliferative effects of polycosanols on the growth of CHO-K1 mammalian cells and melanoma human cells. These polycosanols were obtained from the tall-oil pitch of black liquor soaps byproducts of the kraft pulping process of cellulose, derived from plants of *Pinus radiata*, *Pinus taede*, and *Eucalyptus globulus* in Chile.

2. Materials and methods

2.1. Raw materials

Polycosanols in the light fraction of tall oil (Härting S.A. Santiago, Chile) were concentrated by short path distillation (KDL5, UIC GmbH, Alzenau-Hoerstein, Germany) from 7 to 21.8%. Then, a mixture with over 90% of polycosanols was obtained by crystallization. This mixture was subjected to fractional distillation in a packed glass column (stainless steel), and three high purity fractions were obtained (*i.e.*, docosanol > 98%; tetracosanol > 99% and hexacosanol > 95%) [27].

2.2. Formulation of long-chain alcohols in Pluronic® F-68

Long-chain alcohols (docosanol > 98% and tetracosanol > 99%) were obtained by a previously described protocol and were suspended in Pluronic® F-68 (Poloxamer 188; Mr 8400; BASF, Parsippany, NJ) as described by Katz et al. [3]. Pluronic® F-68 (Plu) was added to a Dulbecco's modified Eagle's medium (DMEM) with 8.3 mg/mL of warm (37°C) high-glucose. The solution was then heated to 50°C and filtered using a filter with 0.2-μm cutoff. Long-chain aliphatic alcohols were added to 33 mg/mL of DMEM and autoclaved at 121°C for 30 m. Later, the stock solution of long-chain aliphatic alcohols was added to the stock solution of Plu. The mixture was heated to 86°C, while sonicating (Branson 450 sonifier) for 21 min at an initial output of 65 W. The final stock solution of long-chain aliphatic alcohols and Plu was added to cell cultures for a final dilution factor of 0.15. The corresponding Plu control solutions were identically prepared without the addition of the long-chain alcohols.

2.3. Cell culture assays

Chinese hamster ovary cells K1 (CHO-K1) obtained from Sigma-Aldrich (Sigma, 98070106), and human melanoma cells (CRL-1974™) obtained from ATCC (Manassas, USA), were grown to assess the antiproliferative effect of the long-chain aliphatic alcohols. The cells were grown in high-glucose DMEM supplemented, with 10% heat-inactivated fetal bovine serum (Gibco, 26140-079), 2 g/L D-glucose (Sigma, G7021) and 2 mM L-glutamine (Sigma, G8540). Cells were grown in a CO_2 incubator (Forma Scientific, USA) at 37°C in a 5% CO_2 atmosphere with 95% relative humidity. All cell lines were seeded at 1.5×10^5 cell/mL and cultured in 12-well plates (Orange Scientific, 4430400) for 12 h to allow complete adherence. To assess its antiproliferative capability, adherent cells were incubated with 15 mM of long-chain aliphatic alcohols for 72 and 144 h with CHO-K1 and melanoma cells, respectively, all measurements were done in triplicate. To perform cell counts, the culture medium was aspirated, and the cells were detached from the surface using trypsin (0.25%) (Sigma, T4049). Once the cells detached, culture medium was added to inactivate the trypsin and to resuspend the cells. Cells were counted using a hemocytometer (Neubauer, Germany), and cell viability was determined *via* trypan blue exclusion staining (T8154, Sigma, USA) (1:1 mixture of 0.2% trypan blue in saline and cell sample).

2.4. Statistical analysis

Each measured experimental condition was performed in triplicate, and two independent samples were taken at each time point for every culture. Values were expressed as the means ± the standard error. An analysis of variance was used to compare the results using the Design-Expert 7 software.

3. Results and discussion

3.1. Effect of long-chain aliphatic alcohols on CHO-K1 cell growth

The effects of long-chain aliphatic alcohols (docosanol and tetracosanol) on the growth of CHO-K1 cells were investigated (Fig. 2). The maximum cell density achieved by the control culture was 8.1×10^5 cell/mL, which was not significantly affected by the presence of Plu surfactant (1.25 mg/mL). However, at higher concentrations of Plu, a negative effect was observed on cellular growth (data not shown). A dose of 15 mM of docosanol was previously tested for the virus; the tests results can be found in a previous publication [3]. Higher concentrations of docosanol could not be tested due to the instability of the Plu suspension.

The presence of docosanol (15 mM) had a negative effect on cell growth population and reduced it by 15% relative to that of the control culture after 72 h of culture. However, the presence of tetracosanol did not trigger a cytotoxic effect on the growth of CHO-K1 cells.

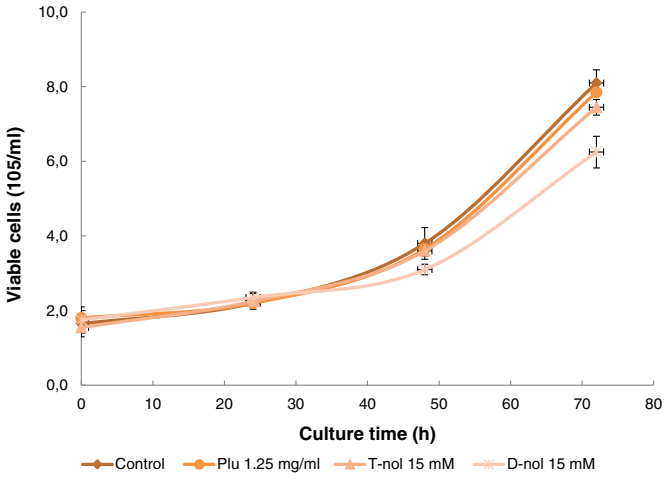

Fig. 2. Effect of long-chain aliphatic alcohol type on CHO-K1 cell growth.

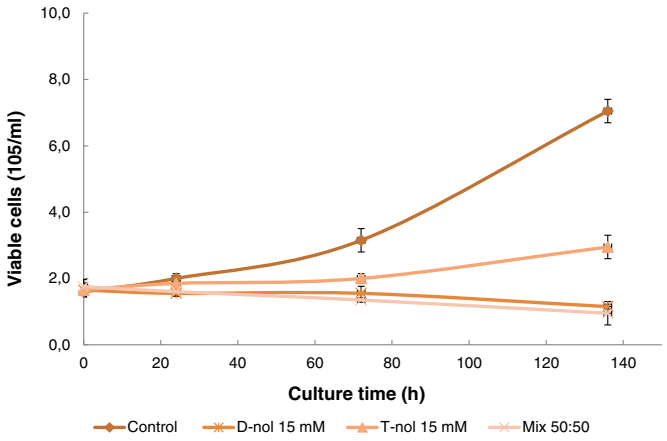

Fig. 3. Effect of long-chain aliphatic alcohol type on melanoma cell growth.

A cytotoxic effect due to the presence of docosanol has not been previously described in the growth of CHO-K1 cells, established cell lines or any oncogenic cell line. The literature has only reported a negative effect on the propagation of various viruses (*i.e.*, the herpes simplex and Kaposi viruses). We further studied this response in a human oncogenic cell line.

3.2. Effect of long-chain aliphatic alcohols on melanoma cell growth

The effects of different long-chain aliphatic alcohols (docosanol and tetracosanol) and their mixture were investigated on the growth of human melanoma cells (Fig. 3). The maximum cell density was significantly reduced as a result of the treatment with these long-chain aliphatic alcohols. The use of tetracosanol affected the cell density and inhibited growth by 58%. Cell growth was more acutely affected when cultures were treated with docosanol or a mixture of the alcohols, as evidenced by the 86% growth inhibition. This response was of greater severity than those observed in the CHO-K1 cultures, which exhibited a relatively robust growth. As shown in Fig. 2 and Fig. 3, the response to these alcohols was cell line dependent. Although the alcohols significantly arrested cell growth, no adverse effects were observed on the cell viabilities (Table 1).

Similar effects have been observed for other molecules with high carbon numbers, like terpenes, diterpenes, and triterpenes against oncogenic cell lines. A few molecular mechanisms responsible for growth inhibition action have been reported. For example, the inhibition of matrix metalloproteinases [28] was observed in prostate tumor cells, which are involved in metastasis; and lipid peroxidation and oxidative stress were observed in HL-60 or CEM cells [29]. The long-chain aliphatic alcohols are the less reactive type of alcohols, as their reactivity decreases as their carbon chain length increases. In this regard, the information about the oxidation capability of this type of alcohol to aldehydes, acids, and esters (widely used in cosmetic and as emulsifiers) is scarce [30]. Therefore, it is still difficult to determine the molecular mechanisms underlying their antiproliferative properties.

Table 1
Percentage of viability of CHO and melanoma cell cultures in the presence of long-chain aliphatic alcohols.

	Viability (%)	
	CHO-K1 cells	Melanoma cells
Control	98 ± 4	96 ± 3
Tetracosanol	96 ± 6	94 ± 4
Docosanol	95 ± 4	97 ± 5
Mixture (50:50)	–	96 ± 4
Plu	97 ± 5	–

Values shown are the mean ± SEM of triplicates.

In conclusion, long-chain aliphatic alcohols (docosanol and tetracosanol) exhibited inhibitory effects on the growth of CHO-K1 and human melanoma oncogenic cell lines. The antiproliferative capacity of these molecules is promising. A deeper understanding of their mechanisms would require additional research on the cell lines investigated and other oncogenic cell lines.

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Authors' contribution

Proposed the theoretical frame: CA, AO, MV. Conceived and designed the experiments: CA, MV. Contributed reagents, materials, analysis tools: CA, AO. Wrote the paper: MV, AO, CA. Performed the experiments: MV, AO. Analyzed the data: CA, AO, MV.

Conflict of interest

There is no conflict of interest.

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