Cryptomoschatone D2 from *Cryptocarya mandiocanna*: cytotoxicity against human cervical carcinoma cell lines


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ABSTRACT

Among the substances isolated from *Cryptocarya sp.*, some styrylpyrones, such as goniothalamin, demonstrate antiproliferative activity in a broad range of human cell lines. In the present study, we assessed the cytotoxicity of a styrylpyrone (cryptomoschatone D2), isolated from *Cryptocarya mandiocanna*, in HPV-infected (HeLa and SiHa) and uninfected (C33A) human cervical carcinoma cell lines and a human lung fibroblast line (MRC-5). The cytotoxicity was tested by the MTT assay. In this assay, cells were treated with cryptomoschatone D2 at 15, 30, 60 or 90 µM for 6, 24 or 48 hours, as well as for 6 hours followed by a post-treatment recovery period of 24, 48 or 72 hours. High cytotoxicity (dose- and time-dependent) was observed in HeLa, SiHa, C33A and MRC-5 cell lines. Although in general the styrylpyrone cytotoxicity was not significantly different among the cell lines tested, it was apparently stronger in HeLa and C33A than in MRC-5 and SiHa in the 24 or 48-hour treatments. Moreover, HeLa and SiHa were able to recover their ability to proliferate, in direct proportion to the post-treatment recovery time. On the other hand, C33A did not demonstrate a similar post-treatment recovery. We can conclude that cryptomoschatone D2 possesses high dose-dependent or time-dependent cytotoxicity.

Keywords: Cell culture. Antiproliferative activity. Styrylpyrone, Cryptomoschatone D2.

INTRODUCTION

Many treatments are available against human papilloma virus (HPV)-associated disease, but the current therapeutic strategies are generally inadequate, with high rates of recurrence (Stanley, 2003). A variety of natural products promote cytotoxicity in several human cancer cell lines and these could also be considered for use in a therapeutic strategy to treat cervical cancer associated with HPV infection (Mavar-Manga et al., 2006). Although cytotoxic chemotherapy is largely associated with nonspecific growth inhibition, narrow therapeutic indices and undesirable side-effects (Divya & Pillai, 2006), natural product cytotoxicity could be an effective and selective method of inducing cell death in HPV-immortalized cervical cell lines (Divya & Pillai, 2006).

Plants extracts are commonly used in Brazilian popular medicine, based on popular knowledge accumulated over centuries, for treatment of many diseases (Pimenta & Nepomuceno, 2005; Verschaeve et al., 2004). While an enormous variety of Brazilian plant species are known, many species have still neither been analysed chemically nor tested for biological activity (Albuquerque et al., 2007). In addition, substances isolated from plants and properly characterized could turn out to be important for the anticarcinogenic activity of those plants. *Cryptocarya* is one of the largest pantropical genera in Lauraceae, which is present in South America, South Africa, Madagascar, Asia, Australia and Oceania (Van der Werff, 1991). The genus includes about 350 species, mostly found in South America, especially in the southern part of Brazil (Van der Werff, 1991; Rohwer, 2003; Moraes, 2005). Moreover, Cryptocarya species are located in the two main biomes in São Paulo State.
Previous studies on the biological activity of Cryptocarya species have shown their anti-inflammatory and antiproliferative activity (Drewes et al., 1997; Zschocke & Van Staden, 2000). In addition, phytochemical studies on the bark and leaves of the Atlantic Rainforest tree Cryptocarya moschata “Brazilian nutmeg” show the presence of styrylpyrones and flavonoid glycosides in this species (Cavalheiro & Yoshida 2000; Nehme et al., 2002).

Styrylpyrones are a class of compounds with potential anti-tumor activity, although the mechanisms of action by which they exert this activity are currently unknown (Zhong et al., 2005; Zhang et al., 2005). Some of the pyrones and styrylpyrones isolated from this genus have been shown to inhibit the growth of breast cancer cell lines (Hawaiarah & Stanslas, 1998; Phieie et al., 1998; Ee et al., 1999). Previous studies on styrylpyrone derivatives suggest that they may be bioactive compounds with antiproliferative and selective cytotoxic activity (Lee et al., 2003). However, to our knowledge, no study has been carried out to investigate the cytotoxic activity of pure substances isolated from Cryptocarya mandiocanna, a tree similar to C. moschata found in the same region.

Thus, the aim of the present study was to assess the cytotoxic activity of cryptomoschatone D2, isolated from Cryptocarya mandiocanna, in cervical carcinoma cell lines (HeLa, SiHa and C33A).

MATERIAL AND METHODS

Plant Material

Leaves were collected from Cryptocarya mandiocanna Meissner (Lauraceae) trees in the Carlos Botelho State Park (São Paulo State, Brazil) in February 2002. Voucher specimens of this material (Cavalheiro CB 353) were deposited at the State Herbarium “Maria Eneida P. Kaufmann” of the Botanical Institute in São Paulo.

Extraction, purification and identification of cryptomoschatone D2

Dried and powdered leaves of C. mandiocanna (3.6 kg) were extracted by maceration with methylene chloride (3x), yielding 265 g of crude extract, after concentration. The crude extract was initially fractionated by liquid-liquid extraction between MeOH/water 9:1 (v/v) and hexane and the hydroalcoholic phase was subsequently fractionated between MeOH/Water 6:4 (v/v) and EtOAc. The EtOAc phase (55 g) was fractionated by solid phase extraction (SPE) on silica gel (63-200 μm, Merck), eluted with a series of mixtures of hexane and EtOAc. The fraction released with 8:2 (v/v) hex/EtOAc yielded 5.0 g after drying and was subsequently subjected to low pressure liquid chromatography (LPLC) on a silica-gel column (30 x 4 cm, 40-63 μm) eluted in gradient step mode with hexane/EtOAc/MEOH, from 60:32:8 to 40:48:12 (v/v), yielding 30 subfractions. Subfraction 7 was subjected to reversed-phase preparative HPLC on a C18 column (Phenomenex Luna 10 Phenylhexyl, 250 x 21.2 mm, 10 μm) eluted with MeOH/Water 43:57 (v/v), at a flow rate of 12 mL min-1, yielding 169 mg of cryptomoschatone D2 (figure 1), identified by UV, 1H and 13C NMR data (Cavalheiro & Yoshida, 2000; Nehme et al., 2005).

Figure 1. Chemical structure of styrylpyrones (cryptomoschatone D2) isolated from Cryptocarya mandiocanna. Source: (Telascrêa, 2006).

Cell Culture

The human cervical carcinoma cell lines, SiHa (HPV 16), HeLa (HPV 18), C33A (not infected with HPV) were kindly provided by Drª Luiza Lina Villa of the Ludwig Institute for Cancer Research, Brazil. The cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and containing 100 U/mL penicillin/streptomycin, 25 ng/mL amphotericin B, 10 ng/mL kanamycin, 200 ng/mL ciprofloxacin. The cells were plated into 25 cm² flasks and incubated in a microenvironment at 37°C in a humidified atmosphere with 5% CO₂, until they reached 90 to 100% confluence.

Cytotoxicity Assay

Cytotoxicity was determined from the fraction of live cells surviving the treatment, measured by their ability to reduce 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a violet-colored formazan. Cells were seeded in 96-well plates at 2.5 × 10³ cells per well and cultured in DMEM containing 10% FBS until the cells attached for 24 hours. The cells were then treated with cryptomoschatone D2 (isolated from Cryptocarya mandiocanna) at various concentrations (15, 30, 60 and 90 μM), for 6, 24 and 48 hours, in serum-free medium. In addition, the cell lines were treated with the same concentrations of cryptomoschatone D2, for 6 hours, followed by 24, 48 and 72 hours of recovery in the serum-free medium after treatment. After both treatments, the cells were incubated with MTT (5 mg/mL) for 2-4 hours at 37°C, the medium was removed and the formazan dye crystals were solubilized in 100 μL dimethyl sulfoxide (DMSO). The viability index (VI%) was calculated by measuring the optical density of each well at 540 nm, using of a Bio-Tek Powerwave X microplate reader (BioTek Instruments, Inc., USA). The viability index (VI%) was calculated by the equation proposed by Zhang et al. (2005), where T and C represent the mean optical density of the treatment and negative control, respectively:

\[ VI (%) = \left( \frac{T}{C} \right) \times 100\% \]
The MTT assay was performed in three independent experiments and each assay plate carried a positive control (doxorubicin 15 µg/mL), negative control (untreated cells) and vehicle control (DMSO 1%).

Statistical analysis

To determine the dose and time dependence of the cytotoxicity of cryptomoschatone D2 in HeLa, SiHa, C33A and MRC-5 cells, one-way Anova with the post-hoc Tukey test was applied. P values were two-sided and significance was declared when P ≤ 0.05. All data were analyzed with the aid of BioEstat 4.0 software (Belém, PA, Brazil).

RESULTS

Cytotoxicity Assay

Since there is evidence that drug scheduling may lead to distinct sensitivities and responses to drugs, we tested two schedules in this study: a) continuous exposure for three different times (6, 24 and 48 hours), without a recovery time, and (b) a single time of exposure (6 hours) followed by three recovery times (24, 48 and 72 hours).

Initially, the cell lines were incubated continuously with various concentrations of cryptomoschatone D2 (15-90 µM) for 6, 24 and 48 hours. Cryptomoscatone D2 reduced cell viability in a time-dependent manner, especially at the higher tested concentrations (60 and 90 µM) (Figure 2). The sensitivities of each specific cell line to the different exposure times and concentrations were compared. It was found that the cell viability of HeLa cells fell significantly more in response to 24h and 48h continuous exposure than after 6h of treatment, at all tested concentrations (Figure 2a). SiHa cells showed a similar trend, though not statistically significant. On the other hand, for the C33A cell line, the 24h continuous treatment was effective in reducing the cell viability only at the two higher tested concentrations (60 and 90 µM), while the 48h continuous treatment reduced cell viability at all tested concentrations (Figure 2c).

A similar result was obtained with the MRC-5 cell line, whose cell viability was reduced only after 48h continuous treatment (but not significantly). These data suggest that the 4 cell lines show time-dependent sensitivity to continuous cryptomoschatone D2 treatment.

When the cell lines were compared with each other regarding their sensitivities to the various treatments, no differences were detected in the 6 h and 48 h treatment assays, but in the 24 h treatment assay, cryptomoschatone D2 significantly reduced the viability of HeLa cells relative to the other cell lines at all tested concentrations ($p<0.05$) (Figure 2a). On the other hand, the non-tumor MRC-5 cell line was the most resistant to the 24h treatment with cryptomoschatone D (Figure 2d), suggesting that HPV 18-infected cancer cells may be more sensitive to 24 h of continuous exposure to cryptomoschatone D2 than uninfected, non-tumor cells. Lastly, the effects of the treatment on SiHa cells, at all concentrations, but especially at the highest concentration (90µM), were quite similar at 24h to those at 48h of treatment (Figure 2b).

Next, we tested the ability of the cells to recover viability at 24, 48 and 72 hours after the end of 6 hours of treatment with cryptomoschatone D2. The HPV18-infected HeLa cells showed a reduction in cell viability 24 h after treatment with the higher concentrations (60 and 90 µM); the cell viability was recovered at 48 and 72 h after the treatment (90 µM). No loss of viability was detected in response to 15 µM and, at 30 µM, some loss was sensed, but only at 72 h after the treatment with cryptomoschatone D2 (Figure 3a). A similar, but lesser prominent, behavior was shown by SiHa (Figure 2b). The non-HPV-infected C33A showed a greater reduction in % viability 24h after the treatment, at all tested concentrations (including 15 and 30 µM), but cell viability was recovered at the subsequent times (48 and 72h) (Figure 3c). MRC-5 cells suffered a significant reduction in viability 48h after the treatment, recovering almost totally at 72h (Figure 3d). These results suggest that the four cell lines exhibited different recovery kinetics, which varied with the concentration of the test compound.

When the cell lines were compared with each other regarding the various times of recovery, it was noted that they react differently (Figure 3). The % viability was higher in HPV-infected cell lines HeLa and SiHa, 24 h after the treatment with 15 and 30 µM of cryptomoschatone D2, than in C33A and MRC-5 ($p<0.05$). Forty-eight hours after the treatment, HPV-infected cells showed fuller recovery of cell viability than MRC-5 (at concentrations of 15 to 60 µM; 30 µM, $p<0.05$) or C33A (60 µM, not significant); although the difference was not statistically significant, C33A recovered less efficiently from the effects of the 60 and 90 µM concentrations, 72 h after the treatment, than the other cell lines. These results suggest that the genetic background (presence or absence of HPV, tumor or non-tumor cells) may influence the recovery of cell viability of the cell lines used in this study.

DISCUSSION

Carcinoma of the uterine cervix in women remains a major problem worldwide, with about 400,000 new cases per year and almost 250,000 deaths, accounting for about 15% of all neoplasms. It is now clear that infection with genital Human Papillomavirus (HPV) is the cause of this lesion (Stanley, 2003). HPV is the etiological agent of cervical cancer (Zur Hausen, 1996). The high risk HPV types, HPV-16 and –18, are frequently detected in malignant tumors and are associated with 70% of all cervical carcinomas (Um et al., 2002). Current modes of treatment, including surgical ablation, radiotherapy, and adjuvant chemotherapy, have been less than satisfactory, particularly for locally advanced disease.

Chemotherapy is the greatest challenge in cancer treatment and there is an urgent need to find new chemical compounds that possess at least some selectivity among different types of cancer (Tian et al., 2007). Styrylpyrones have been shown to exert a strong cytotoxicity, especially the goniothalamins, which have become a new highlight in oncopharmacology, following taxol (Tian et al., 2006). Several compounds derived from styrylpyrones were able to inhibit the proliferation of MCF-7 breast cancer cells (Lee et al., 2003; Chien & Pilhe, 2003), while Z-cryptofolione
Figure 2: Cytotoxic effects of various times of continuous treatment with cryptomoschatone D2 in HPV-infected malignant HeLa (A) and SiHa (B), non-HPV infected malignant C33A (C) and non-malignant MRC-5 cells (D). Cells were treated with various cryptomoschatone D concentrations for 6, 24 and 48 hours. Results are mean ± SEM (n=3); % cell survival is relative to vehicle control. P<0.05: (*) compared to 24 h and 48h; (**) compared to 6 and 24 h.
Figure 3: Cytotoxic effects at various times of recovery (24, 48 and 72 h) after 6 hours of treatment with cryptomoschatone D2 on HPV-infected malignant HeLa (A) and SiHa (B), non-HPV infected malignant C33A (C) and non-malignant MRC-5 cells (D). Results are mean ± SEM (n=3); % cell survival is relative to vehicle control. P<0.05: (*) compared to 24 h and 48 h (90 µM); (**) compared to 24 and 72 h (15, 30 and 90 µM).
and cryptomoschatone D2 have been shown to promote G (2) cell-cycle arrest and inhibition of nuclear transport (Sturgeon et al., 2007). This last substance, subject of the present study, has a quite similar chemical structure to goniotalamin, with an additional 1,3-butanediol chain. This additional carbon chain can apparently interfere with cell viability, resulting in the antiproliferative activity of cryptomoschatone D2.

In the present study, the styrylpyrone (cryptomoschatone D2) isolated from Cryptocarya mandioccana, showed dose- and time-dependent cytotoxicity to HeLa, SiHa and C33A cells. Although no significant difference was observed among the cell lines, higher cytotoxicity to HeLa and C33A was observed after 24 and 48 hours of treatment, while the treatment was less cytotoxic to SiHa. In contrast, treatment of human cervical cancer cell lines with ursoic acid showed significant growth inhibition in cell lines infected with HPV (HeLa, SiHa, CaSki), but was less effective in cell lines not infected with HPV(C33A) (Yim et al., 2006). A recent study demonstrated that natural products that inhibit cell proliferation show selective cytotoxicity to HPV-infected cervical carcinoma cell lines, compared to non-HPV infected cells (Divya & Pillai, 2006). Curcumin induces cell death in HeLa, SiHa and C33A cells in a concentration- and time-dependent manner, although HeLa cells were found to be more sensitive to curcumin (Divya & Pillai, 2006). Curcumin selectively inhibited expression of viral onogenesis E6 and E7 (Divya & Pillai, 2006) and downregulated both HPV18 transcription factor (Prusty & Das, 2005) and anti-apoptotic Bcl-2 protein (Singh & Bhat, 2004).

Curcumin (diferuloyl methane) has Michael reaction acceptor activity, is cytotoxic to cervical cancer cells and induced apoptosis in HPV-infected cells (Divya & Pillai, 2006; Prusty & Das, 2005; Singh & Bhat, 2004). In fact, the Michael reaction acceptor present in the cryptomoschatone D2 molecule could be associated with the high cytotoxicity observed in the present study. The Michael reaction acceptor in curcumin is considered to function as a pharmacophore, responsible for its ability to promote ErbB2 degradation (Jung et al., 2007). This acceptor seems to be a requirement for the antiproliferative effect of cryptomoschatone D2, when it interacts with target proteins and nuclear export factors (Sturgeon et al., 2007). The styrylpyrones have, at least, three pharmacophore groups, including the Michael acceptor, which is also related to their antiproliferative activity (Pilli et al., 2006). Carbon-6 of styrylpyrones, with its (R) or (S) configurations, also interferes in this activity. An increase in the number of hydroxyls in the side chain of the styrylpyrones may increase or reduce their effects on the capacity of different cell lines to grow (Narayanan et al., 1998; Lee et al., 2005).

When the cytotoxicity of HeLa, SiHa and C33A were analyzed 24, 48 and 72 hours after treatment ended, we found that HeLa and SiHa were able to reestablish their proliferation rate steadily, in accordance with the post-treatment recovery time. In contrast, under the same conditions of treatment, C33A cells were unable to restore the same proliferation ratio as the HPV-infected cell lines. The capacity to reestablish the proliferation ratio could be associated with the presence of HPV, suggesting that this styrylpyrone may have selective activity on the expression of HPV oncoproteins E6 and E7. The different responses to cryptomoschatone D2 by HeLa, SiHa and C33A cells might be explained as follows: 1) The HPV-positive cell lines have different viral loads per cell, SiHa (HPV-16 positive) having 1 to 10 copies per cell while HeLa (HPV-18 positive) has 10 to 50 copies per cell (Oh et al., 2004); 2) HPV-16 appears to possess a different mechanism to promote deregulation of cell proliferation from that of HPV-18. We conclude that the styrylpyrone cryptomoschatone D2 possesses a potent dose- and time-dependent antiproliferative activity in HeLa, SiHa, C33A and MRC-5 cell lines.

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