Cytotoxic Activity of Halogenated Monoterpenes from *Plocamium cartilagineum*

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Nine halogenated monoterpenes isolated from the red alga *Plocamium cartilagineum* have been evaluated for their cytotoxic effects on the tumor cell lines CT26 (murine colon adenocarcinoma), SW480 (human colon adenocarcinoma), HeLa (human cervical adenocarcinoma) and SkMel28 (human malignant melanoma) with several multidrug resistance mechanisms and the mammalian non-tumor cell line CHO (Chinese hamster ovary cells). The activities of these compounds were compared with those of the insecticide γ-hexachlorocyclohexane (lindane) due to chemical structure similarities. Compounds **1**, **2**, **3**, and **5** exhibited selective cytotoxicity against colon and cervical adenocarcinoma cells. Interestingly, the effect of compound **3** was specific and irreversible to human colon adenocarcinoma SW480 cells, which overexpress the transmembrane P-glycoprotein often related to chemoresistance. None of the anti-tumor doses of these compounds was cytotoxic against CHO cells. Furthermore, analysis of cellular extracts after incubation with the test compounds and rotenone (positive uptake control) demonstrated the intracellular accumulation of **1**, **2**, **3**, and **5**.

Key words: Halogenated Monoterpenes, Cytotoxicity, Tumor Cells

Introduction

Generally, cancer cells express resistance mechanisms to a variety of chemical products with different structures and mechanisms of action. The phenomenon known as multidrug resistance-phenotype (MDR) is mainly due to the overexpression of any of the transmembrane proteins, P-glycoprotein (Pgp) and the multidrug-resistance protein (MRP1) (Ling, 1997; Cole and Deeley, 1998). These proteins act as energy-dependent drug efflux pumps that couple ATP hydrolysis to the transport of diverse molecules across the cell membrane (Higgins, 1992; Klein *et al.*, 1999). On the other hand, cancer cells can overexpress the intracellular glutathione/glutathione S-transferase detoxification system (GSH/GST). The major components of this system include glutathione (GSH), GSH-related enzymes and glutathione conjugate export pump (GS-x pump) (Zhang *et al.*, 1998). On the other hand, the GSH peroxidase (GSHpx) catalyses the oxidation of reduced GSH to oxidized GSH (GSSG) protecting cells from the damage of highly reactive free radicals. GSHpx detoxifies cells from organic peroxides and metabolizes xenobiotics. All these mechanisms prevent product accumulation in cells and reduce their toxicity.

Many pharmaceutical agents have been discovered by screening natural products from plants, animals, marine organisms and microorganisms (da Rocha *et al.*, 2001; Fricker, 2001). Marine algae represent a significant proportion of the world's biodiversity and are a source of biologically active natural products including antibacterial, antiviral, insecticidal and antitumor activities (Harada and Kamei, 1998; Apt and Behrens, 1999; Harada *et al.*, 2000). Red algae of the family Plocamiaceae are known to contain a wide variety of natural polyhalogenated terpenes which exhibit diverse biological activities (San-Martín *et al.*, 1991; Rovirosa *et al.*, 1990; König *et al.*, 1999a, 1999b). Moreover, there are recent reports on the insect antifeedant and cytotoxic activity on insect Sf9 and mammalian CHO cells of compounds isolated from *Plocamium cartilagineum* (Argandoña *et al.*, 2002). In this work we have studied the cytotoxic activity of some of these compounds against mammalian CHO cells and the tumor cell lines CT26 (murine

colon adenocarcinoma), SW480 (human colon adenocarcinoma), HeLa (human cervical adenocarcinoma) and SkMel28 (human malignant melanoma). These cancer cell lines represent different resistance mechanisms. Human HeLa cells neither exhibit Pgp nor MRP1 (Zhao *et al.*, 1994; Daoud *et al.*, 2000) but they express intermediate levels of GSH-conjugate export activity (Paulo *et al.*, 1998). CT26 cells express low levels of Pgp and MRP1 (Dong *et al.*, 1994). Moreover, there are reports on the existence of murine GSTs (Xiao *et al.*, 1999). Human melanoma SkMel28 cells do not exhibit Pgp but express GSHpx and GSTs (Alvino *et al.*, 1997) and low levels of MRP (Berger *et al.*, 1997). The human colon adenocarcinoma SW480 cells have elevated levels of Pgp (Iwahashi *et al.*, 1993) with low levels of MRP1 and the γ-glutamylcysteine synthetase (γ-GCS) (Lin-Lee *et al.*, 2001), responsible for *de novo* synthesis of GSH. Additionally, in order to gain insight into the mechanism of action of compounds we have also studied their cellular accumulation.

Experimental

Materials

The compounds furoplocamioid C (**1**), prefuroplocamioid (**2**), pirene (**3**), and the cyclohexanes **4**–**9**, including mertensene (**7**) and violacene (**8**), were isolated from *Plocamium cartilagineum* L. (Dixon) as previously described (Argandoña *et al.*, 2002). γ-Hexachlorocyclohexane (lindane) was from Dr. Ehrenstorfer GmbH, Germany, RPMI 1640, fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin were from GIBCO-BRL (United Kingdom). Rotenone and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. The compounds were dissolved freshly and diluted in culture medium before addition to the cell cultures.

Cell lines and culture conditions

Mammalian Chinese hamster ovary cells (CHO) (from Dr. Pajares C. Biomédicas, CSIC, Spain), murine colon adenocarcinoma (CT26), human colon adenocarcinoma (SW480), human cervical adenocarcinoma (HeLa) and human malignant melanoma (SkMel28) (from Deutsches Krebsforschungszentrum, DKFZ, Heidelberg, Germany) were grown as previously described (Argandoña *et al.*, 2002).

Cytotoxicity assay

Cell viability was analyzed by means of an adaptation of the MTT colorimetric assay method (Mossman, 1983). In brief, cells in the logarithmic growth phase were added to 96-well flat-bottom microtiter plates at a density of 2.5×10^3 cells/well for CHO, CT26, SW480 and SkMel28 cells and 5×10^3 cells/well for HeLa cells and incubated for 6 d with different concentrations of the compounds **2**–**9** dissolved in absolute ethanol or dimethyl sulfoxide (DMSO) for compound **1** and lindane. This prolonged time of incubation was used to predict possible adverse cytotoxic effects of compounds on CHO cells. In all cases, the viability of the cells treated under the same conditions with the residual concentration of solvents was $\geq 95\%$. The relative potency of the active compounds (IC$_{50}$, effective inhibitory concentration to give 50% cell viability) was determined as described (Argandoña *et al.*, 2002). For reversibility experiments, cells were incubated with the minimal cytotoxic concentration of each compound, washed three times with fresh culture medium and cultured in compound-free medium for different periods of time. Three independent experiments were carried out in duplicate.

Determination of compound uptake by the cells

The uptake of compounds by cells was determined as previously described with some modifications (Savaraj *et al.*, 1994). In brief, exponential growing cells in 24-well plates were treated by triplicate with the desired cytotoxic concentration of each compound for different days. Untreated cells as well as cells incubated with the respective dose of rotenone (dissolved in DMSO) and with the residual concentration of DMSO (v/v) were used as controls. At the end of incubation, cells from each triplicate were collected, counted by trypan blue staining, pelleted by centrifugation, washed with phosphate-buffered saline (PBS), and resuspended in chloroform/isopropanol (1:1, v/v). The cell extracts were dried and resuspended in methanol. The absorbance of the cell extracts was measured at 300 nm in a Perkin-Elmer Lambda 1A spectrophotometer. The absorbance was substracted for that of cell-free wells incubated under the same conditions and corrected by 10^6 cells.

Results and Discussion

Table I shows the minimal inhibitory concentration (MIC) of the test compounds required to produce a cytotoxic effect on the different cell lines. Compounds **1**, **2**, **3**, and **5** (Fig. 1) were the most selective against cancer cells versus CHO cells. Compounds **1** and **3** had selective effects to CT26 and SW480 cell lines, respectively, with MIC values of 63 μM (IC$_{50}$ 30 μM) and 131 μM (IC$_{50}$ 73 μM). In addition, at 48 h of incubation compound **1** showed selective Sf9 cytotoxicity when compared to CHO (Argandoña *et al.*, 2002). Compound **2** had a selective cytotoxic effect on CT26 and

SW480 cells with MIC value of 66 μM (IC$_{50}$ 45 μM and 32 μM, respectively). Compound **5** was the most active and it was cytotoxic against SW480 and HeLa cells with MIC value of 5.70 μM (IC$_{50}$ 0.080 μM and 0.066 μM, respectively). The structurally-related neurotoxic and cytotoxic insecticide lindane (Woolley *et al.*, 1985) was not cytotoxic to any of the tumor cell lines studied here. It has been recently reported that lindane did not affect CHO cells but it was cytotoxic to insect Sf9 cells (Argandoña *et al.*, 2002).

Table II shows the time course from 1 to 6 d of cellular viability after incubation with pre-determined MICs of compounds **1**, **2**, **3**, and **5** with their corresponding sensitive cell lines. Compound **3** showed moderate cytotoxicity to SW480 after 1 d of incubation. After 3 d of treatment, the most significant effect was observed for compounds **3** and **5**. Compounds **1** and **2** were not cytotoxic subsequent to 3 d of treatment.

To determine whether the cytotoxic effect was reversible, cells were incubated for 6 d with compounds **1** and **2** and for 3 d with compounds **3** and **5**. Then, the treated and the respective untreated cells were washed and their recovery was studied (Table II). All the cell lines were able to grow after removal of compounds **1**, **2**, and **5**. Compound **5** had the slowest reversion effect on SW480 and HeLa. Compounds **1** and **2** were less active than **5**, cell viability was practically recovering after 3 d. In summary, the effect of the compounds on cell viability was found to be reversible except for compound **3** that showed an interesting specific and irreversible effect on human adenocarcinoma SW480 cells.

To determine whether the active compounds entered the cell, we incubated the sensitive lines with the active compounds for different days. Table III
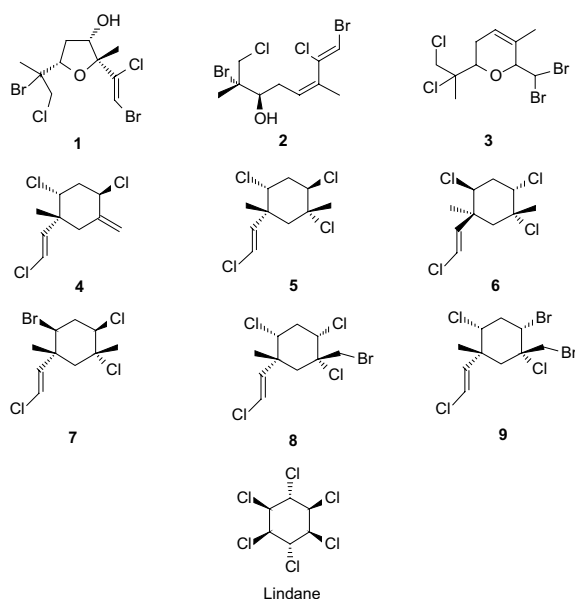

Fig. 1. Chemical structures of furoplocamioid C (**1**), prefuroplocamioid (**2**), pirene (**3**), cyclohexanes (**4**–**9**), including mertensene (**7**), violacene (**8**), and lindane.

Table I. Minimal inhibitory concentration (MIC) of the test compounds on several mammalian cell lines.

Compound	CHO	CT26	SW480	HeLa	SkMel28
			MIC [μM]		
1	126	63	126	126	126
2	132	66	66	132	132
3	262	262	131	262	262
4	3.30	6.52	3.30	13.05	6.52
5	23	181	5.70	5.70	23
6	362	362	362	362	362
7	39	78	78	312	> 312
8	141	141	141	282	282
9	63	125	125	125	250
Lindane	> 344	> 344	> 344	> 344	> 344

Table II. Time course and reversibility of the effect of compounds on cell viability.

Compound	Time [d]	Viability (%)[a]			Reversibility (%)[a]			
		CT26	SW480	HeLa	CHO	CT26	SW480	HeLa
1 (63)[b]	1	80 ± 12	–	–	95 ± 5	20 ± 0	–	–
	3	63 ± 6	–	–	111 ± 9	98 ± 1	–	–
	6	2 ± 0	79 ± 2	75 ± 11	–	–	–	–
2 (66)	1	97 ± 5	99 ± 5	–	85 ± 7	57 ± 10	32 ± 0	–
	3	106 ± 7	35 ± 4	–	106 ± 7	93 ± 0	87 ± 18	–
	6	5 ± 1	0	34 ± 0	–	–	–	–
3 (131)	1	–	50 ± 1	–	85 ± 0	–	36 ± 1	–
	3	–	8 ± 0	–	119 ± 6	–	0	–
	6	32 ± 0	3 ± 0	54 ± 4	–	–	–	–
5 (5.70)	1	–	74 ± 3	80 ± 4	105 ± 4	–	14 ± 3	5 ± 0
	3	–	16 ± 4	21 ± 1	144 ± 25	–	67 ± 1	34 ± 3
	6	90 ± 1	3 ± 0	3 ± 0	–	–	99 ± 7	80 ± 1

[a] Percentage of cell viability (percent absorbance of the respective untreated control cells). In reversibility assays, the percentage of cell viability was calculated following treatment with compounds for 6 d (compounds **1** and **2**) and for 3 d (compounds **3** and **5**) and then removal of the compounds (time days).
[b] MIC of each compound [μM].

Table III. Relative cellular uptake of compounds by CT26 and SW480 cells.

Compound	Uptake (%)[a]	
	CT26[b]	SW480[c]
1	240	–
Rotenone	275	–
DMSO	64	–
2	–	355
Rotenone	–	289
DMSO	–	0
3	–	440
Rotenone	–	280
DMSO	–	0
5	–	500
Rotenone	–	350
DMSO	–	50

[a] The absorbance of untreated control cells (incubated in culture medium alone) was considered to be 100%.
[b] CT26 cells were incubated with compound **1** (88 μM) for one day.
[c] SW480 cells were incubated with compounds **2** (92 μM), **3** (131 μM), and **5** (127 μM) for 3 d, 3 d, and 1 d, respectively. The experiment was performed independently at least two times, and a representative result is shown.

shows the time of maximal uptake. Rotenone was used as a positive uptake control since it is well known that this compound acts intracellularly by interrupting mitochondrial electron transfer at the NADH dehydrogenase-ubiquinone junction of the respiratory chain (Palmer *et al.*, 1968). CT26 and SW480 cells accumulated rotenone at a rate of 3-times higher than the respective untreated control cells. None of the cells showed background absorbance after incubation with DMSO. The uptake of compound **1** by CT26 cells was 2.5-times higher and the intracellular uptake of compounds **2**, **3**, and **5** by SW480 cells was found to be about 4-, 4-, and 5-times that of respective untreated cells, respectively.

Taking into account that cervical cancer is the second most common cancer among women world-wide in developing countries (Parkin *et al.*, 1988; Leminen *et al.*, 1990) and colon cancer is a leading cause of death in the Western world and one of the most untreatable and therapy resistant cancers (Gryfe *et al.*, 1997), compounds **5** and **3** could be considered as candidates for combating multidrug resistant human adenocarcinoma colon and cervical cancers.

In summary, the present results indicate that furoplocamioid C (**1**), prefuroplocamioid (**2**), pirene (**3**), and the cyclohexane **5** have greater cytotoxic activity to cancerous versus noncancerous cells. Moreover, their cytotoxic effects have proven to be selective. Other halogenated monoterpenes from the red alga *Portieria hornemannii* have also shown *in vitro* differential cytotoxicity against a panel of human tumor cell lines. Halomon exhibited selectivity of action being most cytotoxic against brain, renal and colon tumor cell lines while the leukemia and melanoma lines are

least affected (Fuller *et al.*, 1992, 1994). To our knowledge, this is the first report on the potential anti-tumor activity of halogenated monoterpenes from *Plocamium cartilagineum*. Finally, chemotherapy and/or radiotherapy are essential strategies for the treatment of cancer. However, the anti-tumor activity of drugs is often restricted or ineffective due to resistance mechanisms in cancer cells and moreover most of the anti-cancer drugs are not very specific and tend to have serious side-effects. It is therefore necessary to study new effective chemical compounds for the successful treatment of all forms of cancer.

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