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Cytotoxicity of the β-carboline alkaloids harmine and harmaline in human cell assays in vitro

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Abstract

β-Carboline alkaloids are natural products widely distributed in plants and also found in alcoholic beverages, well-cooked foods and tobacco smoke. Various authors have reported genotoxic activities of several carboline in prokaryotic and eukaryotic cells that have been attributed to their abilities to intercalate into DNA. But studies on the genotoxic and on the cytotoxic potencies in human cells in vitro are not found in the literature. In the present study the toxicities of one full aromatic β-carboline alkaloid (harmine) and one dihydro-β-carboline alkaloid (harmaline) were evaluated by means of two in vitro human cell assays: the cytochalasin-B blocked micronucleus (CBMN) assay and the viability/colony formation assay with four different human cultured non-transformed (CCD18Lu) and transformed (HeLa, C33A and SW480) cells. Neither alkaloid was able to induce micronuclei levels above that of control levels in a wide range of doses tested; although, harmine at the highest concentrations assayed induced apoptotic as well as necrotic cells. Harmine produced a good viability of all cell lines assayed (control and tumor) while harmaline significantly reduced the viability of transformed and non-transformed cell lines in a dose-dependent manner. Harmine displayed a dose-dependent inhibitory effect on cell proliferation against all human carcinoma cells, but the SW480 transformed cell line showed a higher sensitivity. These results suggested that harmine was identified as a useful inhibitor of tumor development.

Keywords: β-Carboline alkaloids; Harmine; Harmaline; Cytochalasin-B blocked micronucleus (CBMN) assay; Viability and cell colony formation assay

Introduction

β-Carboline alkaloids, also known as harmala's alkaloids, because they were first isolated from *Peganum harmala* (Zygophillaceae), are natural products widely distributed in plants. Several β-carbolines, like harman, are formed from tryptophan and pyruvate or acetate

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precursors in alkaloid biosynthesis in plants, thus they are detected in several plant species from a wide range of plant families such as: *Anadenanthera* spp. (Leguminoseae), *Banisteriopsis* spp., *Terapteris* spp. (Malpighiaceae) and *Passiflora* spp. (Pasifloreaceae) (Hashimoto et al., 1988; Bruneton, 1992; Picada et al., 1997). These plants are used in folk medicine in Brazil for their antispasmodic and sedative properties. It has been shown that human beings are not only exposed to β-carboline alkaloids through medicinal plants but also through alcoholic beverages, cigarette smoking and well-cooked foods such as fried meat and fish (De Meester, 1995). Pharmacological, neurophysiologic and biochemical activities of β-carboline alkaloids have been reported: inhibition of cytochrome P450 (Tweedie et al., 1988); inhibition of monoamine oxidase (Kim et al., 1997); binding to several serotonin, benzodiazepines and dopamine receptors (Glennon et al., 2000); and inhibition of DNA topoisomerase activities (Funayama et al., 1996). Some of the mutagenic and carcinogenic effects of various carboline alkaloids have been related to their ability to intercalate into DNA (De Meester, 1995; Taira et al., 1997) leading to altered DNA replication fidelity and enzymatic activities in DNA-repair processes (Funayama et al., 1996; Remsen and Cerruti, 1979). Alkaloid extracts of *P. harmala* seeds tested in vitro on mice skin carcinoma and sarcoma cell lines significantly reduced cell proliferation (Lamchouri et al., 1999). In human neuroblastoma SH-SY5Y cells harman and norharman induced apoptosis as well as necrosis (Uezono et al., 2001).

Harmine and harmaline have been found in human plasma after the ingestion of "ayashuasca" (Callaway et al., 1996), a hallucinogenic beverage that is used in Brazil for religious purposes (Boeira et al., 2002). Toxic and genotoxic effects of both β-carboline alkaloids have been reported in prokaryotic and eukaryotic cells: signs of a weak induction of frameshift mutations of harmine in *Salmonella typhimurium* TA97 with metabolic activation; UV-mediated toxic bioactivity of harmine in the meristematic cells of *Allium cepa* (Hazen and Gutierrez-Gonzálvez, 1988); chromosome aberrations in Chinese hamster ovary (CHO) cells after treatment with the clastogens mitomycin C and UV light in the presence of S9-mix (Sasaki et al., 1992).

Due to the varied ways of exposure to β-carbolines in human beings and to the lack of information of their toxicity in short-term human cell assays in vitro it is important to assess the genotoxicity and cytotoxicity of two structurally-related β-carboline alkaloids: harmine and harmaline. The cytochalasin-B blocked micronucleus (CBMN) assay was used because of its sensitivity and simplicity in measuring potential genotoxic damage of xenobiotic compounds through clastogenicity and aneugenicity, as well as through necrosis and apoptosis (Fenech, 1997; Fenech et al., 1999a). The viability and cell colony formation assay was employed to assess the potential cytotoxic inhibitory activity of the compounds against different non-transformed and transformed human cell lines (Abdullaev and Frenkel, 1992a, b).

Materials and methods

Chemical products and concentrations assayed

The aromatic β-carboline alkaloid harmine (CAS 343-27-1, approx. 98% purity) and the dihydro-β-carboline alkaloid harmaline (CAS 6027-98-1, approx. 95% purity) were obtained from Sigma (www.sigma-aldrich.com). For the micronucleus assay the concentrations chosen were 1, 10, 20 and 40 μM for harmine and 0.858, 8.58, 17.167 and 34.33 μM for harmaline. These concentrations were obtained from a stock solution of 1 mg of each alkaloid dissolved in 1 ml of distilled water. Distilled water was used as negative control, mitomycin C (MMC, Sigma) was used as positive control. Dulbecco's modified Eagle's medium (D-MEM) was purchased from Gibco/BRL Products (Grand Island, NY). Fetal bovine serum (FBS) was obtained from HyClone (Road Logan, UT). Cell proliferation kit II was obtained from Roche. For the colony formation assay the concentrations employed for harmine were 10, 20 and 40 μM.

Cytochalasin-B blocked micronucleus (CBMN) assay

Two healthy, non-smoking donors (one female and one male) aged between 25 and 35 years were recruited after giving informed consent. Between 5 and 7 ml of blood was collected by venipuncture into Vacutainer® tubes containing 0.3 ml of heparin as an anticoagulant. The cultures of blood, taken early in the morning before breakfast, were prepared in duplicate. Two experiments were performed for each compound tested. The blood samples were centrifuged at $340 \times g$ in a bench-top centrifuge (Solbat J-600) for 30 min at room temperature. Lymphocytes (T and B cells) were isolated by density centrifugation from the interface between the light plasma fraction and the dense fraction which contained red blood cells and polymorphonuclear leukocytes or granulocytes. Approximately 1 ml of the isolated lymphocytes was added to 5 ml of McCoy's 5A culture medium supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 2.8% (w/v) phytohaemagglutinin (Gibco) and 1% (w/v) penicillin/streptomycin (Gibco). Cultures were incubated at 37 °C with 5% CO_2 concentration for 24 h. The different concentrations of the two alkaloids were added, as well as 0.2 ml (80 ng) of mitomycin C (MMC) as positive control. Negative

control tubes were treated with the solvent (water) alone. Cultures were incubated at 37 °C for another 20 h then 0.3 ml of cytochalasin B (3 mg/ml) was added to each tube. Cultures were incubated at 37 °C for another 28 h. Cells were harvested 72 h after the initiation of the culture. The cell suspensions were centrifuged at 340 × *g* for 10 min. The supernatant was aspirated to a minimal level, cells were resuspended, and a hypotonic solution (potassium chloride, KCl 0.075 M) at 37 °C was added. After 5 min, the cell suspensions were centrifuged at 340 × *g* for 10 min. The supernatant was aspirated to a minimal level, and fixed in 1 ml of methanol/glacial acetic acid (3:1, v/v). Resuspended fixed cells were centrifuged for 5 min at 113, 3 × *g*. One additional change of the fixative was done. The cells were resuspended by drawing and expelling with a Pasteur pipette, dropped onto wet clean glass slides and allowed to dry at room temperature. After 24 h the air-dried slides were stained for 10 min with 2.5% (w/v) Giemsa (45 ml of Sorensen solution and 5 ml of Giemsa), rinsed, coded and analyzed. Scoring was carried out under a light microscope at a magnification of 1000 × (Fenech, 1997; Fenech et al., 1999b, 2003; Kirsh-Volders et al., 2003). At least 1000 binucleated cells/culture of each of the two parallel cultures and two different experiments (4000 binucleated cells/dose) were analyzed. Other observations were made for cells with multiple micronuclei and nucleoplasmic bridges. Cells showing chromatin condensation as well as exhibiting nuclear fragmentation into smaller nuclear bodies within intact cytoplasmic and nuclear boundaries were classified as apoptotic. Cells exhibiting a pale cytoplasm with numerous vacuoles and damaged cytoplasmic membrane were classified as necrotic (Fenech et al., 1999a).

Statistical analysis

The statistical significance of differences between concentrations was determined using the χ^2-test at the level of significance of $P<0.05$. Determination of cell proliferation was done by calculating the cytokinesis-block proliferation index with cytochalasin-B (CBPI) with the formula:

$$\mathrm{CBPI} = \frac{M_1 + 2M_2 + 3M_3 + 4M_4 + 5M_5}{N}$$

where M is the number of cells with one (M_1, monucleated), two (M_2, binucleated), three (M_3, trinucleated), four (M_4, tetranucleated), five (M_5, pentanucleated) nucleus; and N is the total number of cells scored. For the evaluation of genotoxicity (MN and bridges) the Kruskall Wallis test and the difference from proportions (Z statistics) were used (statistical Program version 6.0). Student's t-test was used for cytotoxicity (CBPI). The level of significance was set at $P<0.05$.

Cell culture and viability assay

For the experiments, one non-transformed and three types of human tumor cell lines were chosen: CCD18Lu (lung epithelial non-transformed fibroblast cells), HeLa (cervix epitheloid carcinoma), C33A (cervix carcinoma) and SW480 (colon carcinoma). Cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). We choose C33A and HeLa cells for these experiments, since cancer of the cervix is the second most common cancer in women worldwide; furthermore, cervical cancer is a major health issue affecting a great number of Mexican women (21%) (www.inegi.gob.mx). SW480 tumor line was included due to the incidence of colorectal cancer is highest in developed countries such as United States and Japan and lowest in developing countries in Africa and Asia. According to the American Cancer Society it is the third most common type of cancer in both men and women in the United States (www.oncologychannel.com/coloncancer). Cells were grown in D-MEM with 10% FBS in a CO_2 water-jacketed incubator (Nuaire, Plymouth, MN) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air during 3 5 days. Then the cells were washed twice with phosphate-buffered saline (PBS) at pH 7, 1 ml trypsin (0.05%) dissolved in phosphate buffer at pH 6, incubated during 3 5 min; 9 ml D-MEM medium with 10% FBS was added, the cells were resuspended and quantified in a Neubauer chamber. The cells were seeded at a density of 2×10^3 cells/well in a 96-well microplate, and then exposed to the different concentrations of the alkaloids. After 24 h of incubation at 37 °C tetrazolium salt (XTT) was added (50 µl/well of 5 mg/ml solution in PBS) for 24 h. Solubilization of the converted orange dye was accomplished by adding 50 µl/well of N HCl/isopropyl alcohol. The reaction product was quantified by measuring the absorbance at 480 nm using a Bio-Rad microplate reader (Denton et al., 1995). Results are expressed as the mean ± S.D. of quadruplicate plates. Data were analyzed using the Statistical Analysis System, software version 6.02 values were considered significant when $P<0.05$.

Colony formation assay

For the colony formation assay tested cells (2×10^5) were seeded into 100-mm Petri dishes with 10 ml D-MEM medium. After 3 5 days, different concentrations of harmine were added and incubation continued for 3 h. The cells were then trypsinized (0.05% trypsin), counted and 200 cells were seeded in 35-mm Petri dishes for colony formation estimation. Three replicate colony determinations were carried out for each culture. After 10 days of incubation, the resulting colonies were rinsed with 5 mM phosphate buffer, pH 7.6, containing

150 mM NaCl, fixed with methanol, stained with Giemsa (Sigma) and the number of colonies (with diameter > 0.05 mm) per dish were determined as described by Abdullaev and Frenkel (1992b). Results are expressed as the mean ± S.D. of triplicate plates. Inhibition is defined as the ratio of the colony numbers in the treated group to that in the control group. Data were analyzed using the Statistical Analysis System, software version 6.02 values were considered significant when $P < 0.05$.

Results

Genotoxicity studies

The results obtained for the micronucleus assay are shown in Table 1. As the data obtained from two parallel cultures and two different experiments were not statistically different, according to non-parametric 2 × 2 chi-square tables ($P < 0.05$), they were pooled. A total of at least 4000 binucleated cells per concentration were analyzed for each donor. Also, the numbers of cells with one, two, three, four or five nuclei were recorded. Only the lowest concentrations of the compounds assayed yielded a survival rate of 100%. Interestingly the higher concentrations of harmine (20 and 40 μM) induced apoptotic as well as necrotic cells, and the highest inhibited cell proliferation. The frequencies of micronuclei in binucleated cells demonstrated that the results are negative at all concentrations tested when compared to the concurrent negative control. The percentage of binucleated cells obtained was between 19% and 83% for harmine and between 59% and 83% for harmaline. The CPBI varied between 1.94 and 1.75 for the latter and from 2 to 1 for the former, thus harmine was shown to be cytotoxic (Fig. 1).

Cytotoxic studies on malignant cells

Cellular viability in control and in different human malignant cell lines in vitro was assessed with a microassay for quantitation of anchorage-independent growth of cells with tetrazolium salt (XTT). In four independent assays the reduction of tumor cell viability was consistent. Harmine produced a good viability of all cell lines assayed (control and tumor) while harmaline significantly reduced the viability of control and malignant cell lines in a dose-dependent manner (Fig. 2). Due to these results we decided to examine the effect on colony formation of harmine exclusively.

In order to gain information about the extent of cytotoxicity induced by harmine, transformed and non-transformed human cell lines were cultivated to determine the percentage of growth in the assay. The effect of different concentrations (from 10 to 40 μM) of harmine on colony formation of control (CCD18Lu) and human tumor cells (HeLa, C33A and SW480) were examined. Results obtained from three experiments demonstrated that the planar full N-aromatic β-carboline harmine inhibited colony formation of all tumor cell lines in a dose-dependent way and appears to be less effective for non-transformed epithelial cells (fibroblasts). All tested malignant cells showed a good response to the effect induced by harmine, but the SW480 malignant cell line showed a higher sensitivity to the alkaloid. The C33A and HeLa carcinoma cell lines were least sensitive to the inhibitory effects induced by harmine (Fig. 3).

Discussion

The aim of this study was to investigate harmine and harmaline toxicity on human cell assays in vitro. The methods chosen to assess their possible genotoxicity and cytotoxicity included the micronucleus assay and the method of viability/colony formation in normal and malignant cell lines. The CBMN assay has become one of the most commonly used methods for assessing chromosome breakage and loss in human lymphocytes, either in vitro or in vivo. The use of cytochalasin B, an inhibitor of polymerization which blocks mitotic cytokinesis, ensure discrimination between cells which have undergone one division after exposure to the chemicals (binucleated cells) and undivided cells (mononucleated cells) and it also enables the frequency of dividing cells to be quantified rapidly (Fenech, 1997). Also the extent of observed DNA damage induced by a chemical or physical agent may depend on whether the agent induces or inhibits necrosis and/or apoptosis. Induction of necrosis could result in the intracellular release of degradative enzymes from subcellular particles such as lysosomes, which may cause partial digestion of DNA. Inhibition of apoptosis may allow cells that have experienced a significant level of DNA damage to proceed through the cell cycle and survive as mutated micronuclated cells (Fenech et al., 1999a).

Results obtained in the present study in the micronucleus experiments clearly showed that the β-carbolines assayed were not able to significantly induce double-strand breakage-related events. But, necrotic and apoptotic cells were observed at the highest concentrations assayed for harmine, which means that toxic induction of DNA damage took place. Necrosis was more prevalent by a factor of between 2- and 25-fold. Thus harmine was much more cytotoxic than harmaline. Similar results were observed in V79 Chinese hamster lung fibroblasts in vitro (Boeira et al., 2001). Since β-carboline alkaloids inhibit cytochrome P450 enzymes and in particular harmine showed to inactivate

Table 1. Number and distribution of micronuclei in binucleated cells, frequency of MN, distribution and frequency of cells according to the number of nuclei, percentage of binucleated cells and cytochlasin-block proliferation index of binucleated cells obtained in the in vitro micronucleus assay with different concentrations of two β-carboline alkaloids. Results of two parallel cultures and two independent experiments with each donor

Donor/ sex[a]	Compound/ concentration (μM)	Binucleated cells Distribution of MN			Total of bi-nucleated cells	Frequency of MN and Stat.Diag.[b]	Distribution of cells according to the number of nuclei					Total number of cells	Percentage of binucleated cells	CBPI
		0	1	2			Mononucl.	Binucleat.	Trinucleat.	Tetranucl.	Pentanucl.			
	Harmine													
F	Control	4141	12	2	4155	0.0034	1127	4155	212	298	3	5795	72	1.95
	MMC (3 μg/ml)	4028	56	0	4084	0.014	1198	4084	187	200	0	5669	72	1.89
	1	4085	23	2	4110	0.0061	1020	4110	271	357	5	5763	71	1.9
	10	4049	8	0	4057	0.002	762	4057	65	52	0	4936	83	1.87
	20	714	5	0	719	0.0069	1636	719	0	0	0	2355[c]	30	1.3
	40	1	0	0	1		537	1	0	0	0	538[d]	0.2	1
M	Control	4121	7	0	4128	0.0017	1003	4128	263	558	1	5953	69	2
	MMC (3 μg/ml)	4003	65	0	4068	0.016	1229	4068	169	121	0	5587	73	1.84
	1	4025	6	3	4034	0.0022	1123	4034	350	442	1	5949	68	1.95
	10	4145	15	0	4160	0.0036	1098	4160	245	228	5	5736	72	1.88
	20	1866	5	0	1871	0.0027	1258	1871	0	0	0	3129[e]	60	1.45
	40	7	0	0	7		1594	7	0	0	0	1601[f]	0.4	1
	Harmaline													
F	Control	3974	26	0	4000	0.0065	971	4000	906	727	0	6604	61	2.2
	MMC (3 μg/ml)	3936	64	0	4000	0.016	1246	4000	1332	1000	0	7578	53	2.3
	0.858	4045	16	0	4061	0.0039	932	4061	305	222	0	5520	74	1.93
	8.58	2138	22	2	2162	0.011	1028	2162	41	45	0	3276	66	1.73
	17.17	1784	15	0	1799	0.0083	857	1799	33	21	0	2170	83	2.14
	34.33	1292	3	0	1295	0.0023	532	1295	19	42	0	1888	69	1.66
M	Control	3979	21	0	4000	0.0052	1247	4000	287	179	0	5713	70	1.89
	MMC (3 μg/ml)	3941	59	0	4000	0.015	755	4000	467	197	0	5419	74	2.02
	0.858	4137	10	0	4147	0.0024	900	4147	152	134	0	5333	78	1.91
	8.58	2568	16	0	2584	0.0062	1646	2584	57	72	0	4359	59	1.67
	17.17	1511	12	0	1523	0.0079	646	1523	48	17	0	2234	68	1.75
	34.33	1152	9	0	1161	0.0077	591	1161	64	18	0	1834	63	1.73

[a]Sex: F = female, M = male.
[b]Statistical diagnoses according to a non-parametric 2 × 2 chi-square tables, Fisher's exact test.
[c]Apoptotic cells = 57 (0.024); necrotic cells = 582 (0.25).
[d]Apoptotic cells = 86 (0.16); necrotic cells = 298 (0.55).
[e]Apoptotic cells = 62 (0.019); necrotic cells = 1588 (0.51).
[f]Apoptotic cells = 210 (0.13); necrotic cells = 588 (0.37).

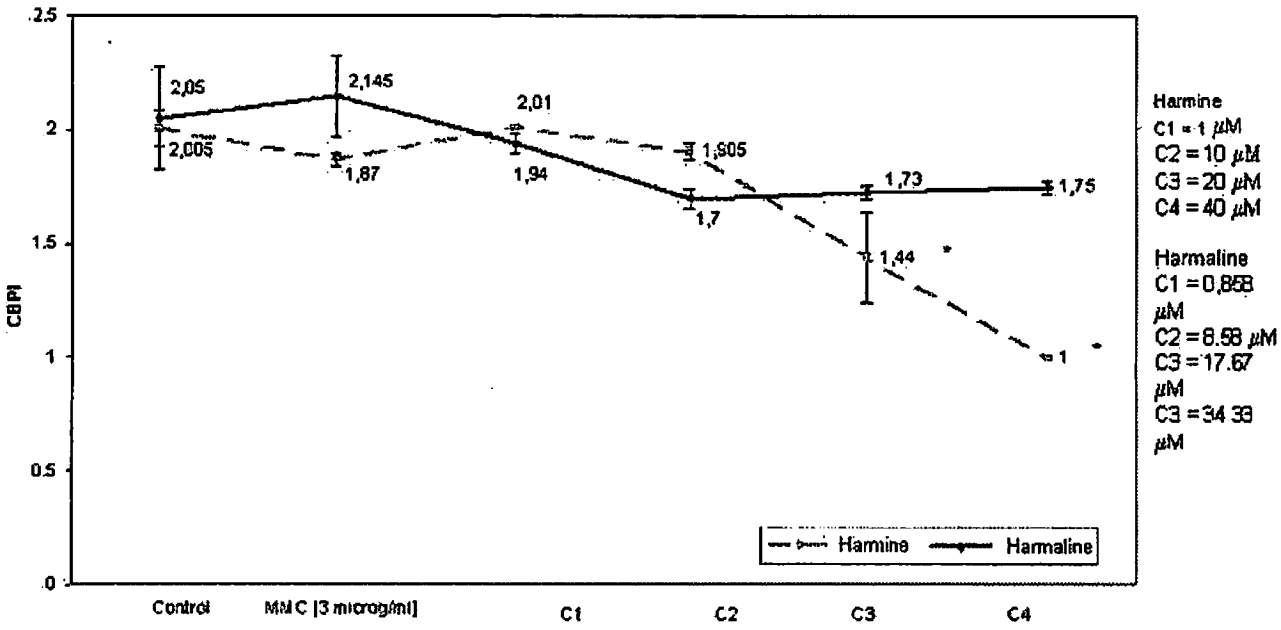

Fig. 1. CPBI obtained in the in vitro MN assay with the β-carbolines harmine and harmaline.

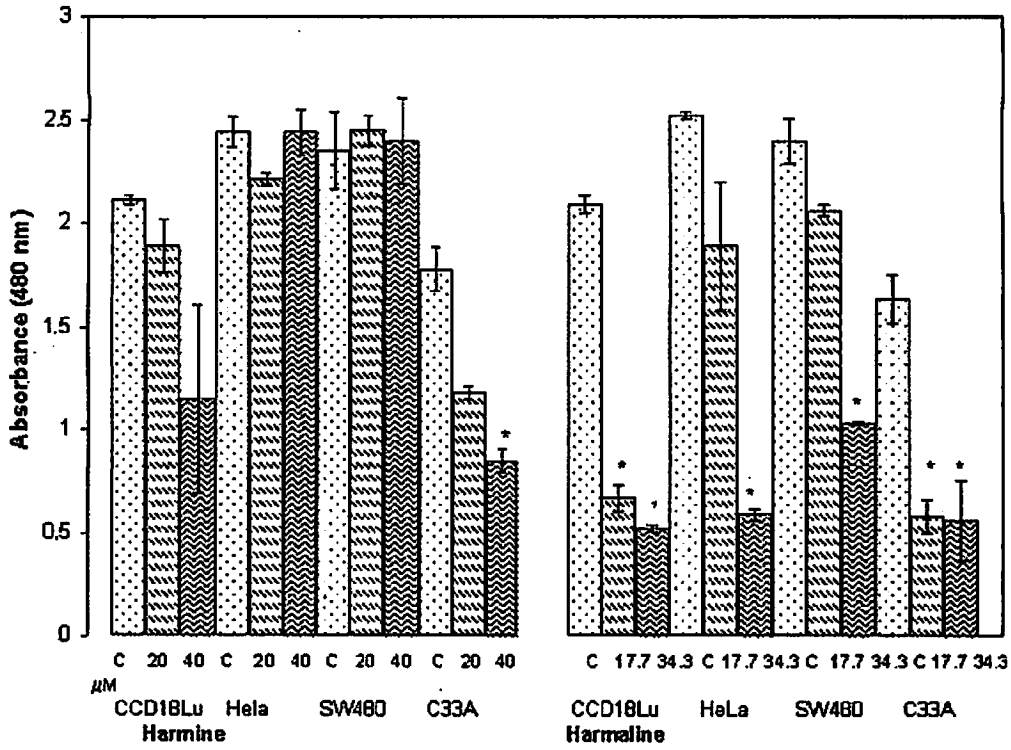

Fig. 2. Viability of different human cell lines exposed to harmine and harmaline. Each value represents the mean ± S.D. of quadruplicate plates. *$P<0.05$.

metabolization in the SOS chromotest (Tweedie et al., 1988; Picada et al., 1997) the compounds were not tested in the presence of S9 mix in the CBMN assay. Moreover harmine caused weak positivity to induce frameshift mutations in strains of *S. thyphimurium* in the presence of metabolic activation (Wehner et al., 1979) but did not

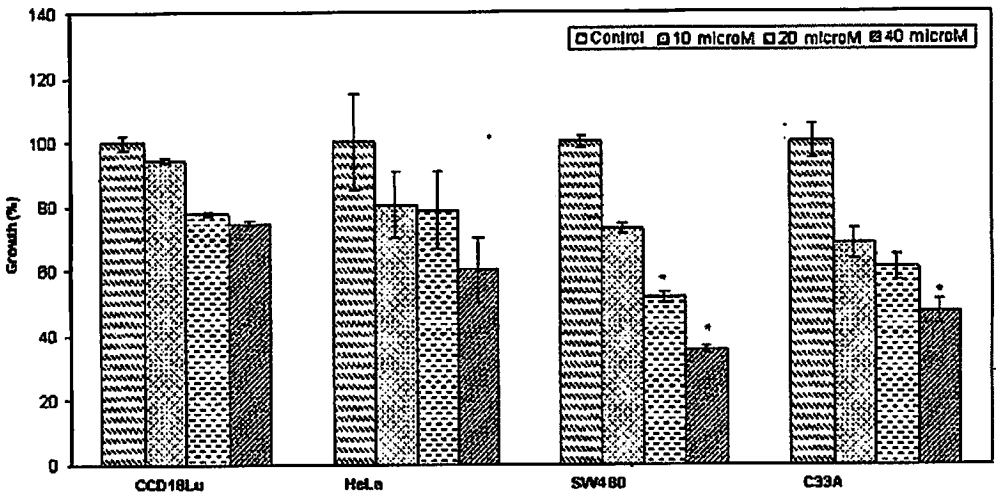

Fig. 3. Growth of colonies of different human cell lines in the presence of harmine (colonies/dish formed by untreated cells accepted as 100%). Each value represents the mean ± S.D. of triplicate plates. $^{*}P<0.05$.

show any mutagenic activity in the base-substitution strain TA100 with as well without metabolic activation (Picada et al., 1997). Harmaline was not mutagenic in TA97, TA98 and TA100 strains of *S. thyphimurium* neither in presence nor in absence of metabolic activation and could not induce chromosomal aberrations in CHO cells (Sasaki et al., 1992). In our own experiments, non-significant results were obtained for both alkaloids.

Harmine can be demethylized in vitro yielding harmol, which in turn is conjugated in vivo with glucuronic acid and/or sulfate, but in vitro these reactions do not take place or are poorly operative resulting in the free form (Mulder and Bleeker, 1975; Tweedie and Burke, 1986). Both tricyclic aromatic compounds, harmine and harmaline, have a methoxyl ligand at C-7 position and a methyl group at C-1 (De Meester, 1995), it has been shown that these β-carbolines interact with DNA by intercalation through the methoxyl group (Taira et al., 1997) or the methyl ligand (Cao et al., 2005). The only structural difference between the β-carbolines studied is the presence of full aromaticity in harmine and partial aromaticity in harmaline. In our study the dihydro-β-carboline harmaline was less cytotoxic in the MN assay than the full aromatic harmine. Thus a structure activity relationship was observed: the polycyclic planar full N-heteroaromatic structure showed to be more reactive than the partial aromatic one. Result that is in agreement with the study performed by Duportail and Lami (1975) that showed that the hydrogenation of a double bond of the pyridine ring converting the full planar aromatic β-carboline harmine to the partial planar aromatic dihydro-β-carboline harmaline alters the binding properties of the molecule with DNA.

Malignant cell lines represent important model systems to study neoplasm, and also, as we have shown, for the study of the potency of chemical compounds to inhibit cell proliferation. The XTT colorimetric assay used showed to be reproducible with low variability and a good alternative for the quantitation of cell line response to the alkaloids tested under anchorage-independent growth conditions. By the method of colony formation, it was demonstrated that harmine have an effect upon the type of human malignant cells but had less effect on control fibroblast cells. Furthermore harmine displayed an inhibitory effect of all tumor cell lines in a dose-dependent way, although the SW480 (colon carcinoma) malignant cell line showed a higher sensitivity to the alkaloid. The cervix carcinoma (C33A and HeLa) tumor cell lines were least responsive to the effects induced by harmine. The activity observed in the inhibition of cell proliferation in the present study could be related to the DNA binding properties of this chemical and to its ability to interact with DNA topoisomerases, enzymes that are the molecular target for many antitumor drugs. It has been shown recently that harmine and its derivatives inhibit the DNA relaxation activities of topoisomerase I, but not of topoisomerase II, and that the most intercalating compound corresponds to the most potent topo I poison, suggesting that DNA binding is well correlated with topo I inhibition and cytotoxicity in vitro (Cao et al., 2005).

Finally due to the fact that human beings are sufficiently exposed to these alkaloids through plants used for hallucinogenic beverages, medicinal drugs, well-cooked food and tobacco smoke, it is important to conduct additional studies to determine their role as potential anticancer drugs. In particular in vivo studies

of β-carboline alkaloids are needed to determine their interference with initiation or progression steps of carcinogenesis.

In conclusion, the present results reveal that harmine and harmaline were not able to induce double-strand breakage-related effects, but harmine was a potent inducer of cytotoxicity through necrosis and apoptosis. Furthermore a specific inhibitory effect on cell proliferation of tumor cell-lines was exerted by the full planar aromatic alkaloid, thus our results suggest that harmine was identified as a useful inhibitor of tumor development.

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