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Evaluation of the cytotoxic effect of the monoterpene indole alkaloid echitamine in-vitro and in tumour-bearing mice

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Abstract

The cytotoxic effect of various concentrations of echitamine chloride was studied in HeLa, $HepG_2$, HL60, KB and MCF-7 cell lines in-vitro and in mice bearing Ehrlich ascites carcinoma (EAC). Exposure of various cells to different concentrations of echitamine chloride resulted in a concentration-dependent cell killing, and KB cells were found to be most sensitive amongst all the cells evaluated. EAC mice treated with 1, 2, 4, 6, 8, 12 or 16 mg kg^{-1} echitamine chloride showed a dose-dependent elevation in the anti-tumour activity, as evident by increased number of survivors in comparison with the non-drug treated controls. The highest dose of echitamine chloride (16 mg kg^{-1}) caused toxicity in the recipient mice, therefore 12 mg kg^{-1} was considered the best cytotoxic dose for its anti-tumour effect. Administration of 12 mg kg^{-1} echitamine chloride resulted in an increase in the median survival time (MST) up to 30.5 days, which was 11.5 days higher than the non-drug treated control (19 days). Administration of 16 mg kg^{-1} echitamine chloride to EAC mice resulted in a time dependent elevation in lipid peroxidation that reached a peak at 6 h post-treatment, whereas glutathione concentration declined in a time dependent manner and a maximum decline was reported at 3 h post-treatment. Our study demonstrated that echitamine chloride possessed anti-tumour activity in-vitro and in-vivo.

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Introduction

Chemotherapy has been the mainstay of cancer treatment for the past 50 years. It is a major treatment modality used for the control of advanced stages of malignancies and also as a prophylactic against possible metastases in combination with radiotherapy. However, most of the chemotherapeutic drugs exhibit severe normal toxicity, resulting in undesirable side effects. Moreover, many of the potent drugs used for cancer treatment are very expensive, mutagenic, carcinogenic and teratogenic. Hence, there is a need to find alternative drugs, which are highly effective, inexpensive and affordable.

Terrestrial plants have played a dominant therapeutic role in the treatment of human ailments. Different civilizations have used their own unique herbal-based knowledge practiced by herbal healers or practitioners. The American Indians used the extracts from the roots of May apple (*Podophyllum peltatum*) as an effective treatment for skin cancers and venereal warts. The main constituent, podophyllotoxin was the forerunner of the group of anticancer agents known as the podophyllins, which includes etoposide and teniposide (Mann 2002). Similarly, the plant *Catharanthus rosea* or the rosy periwinkle was used as a hypoglycaemic agent in many parts of Asia, but it was not until 1958 that the main constituents, vinblastine and vincristine, were found to be potent cytotoxics (Pezzuto 1997). The Ayurvedic practitioners and several Native American tribes used Taxus species (*Taxus brevefolia*, *T. canadensis* and *T. baccata*) to treat some non-cancerous and cancerous conditions (Cragg et al 1997). Paclitaxel, the compound initially isolated from *Taxus brevifolia*, has become the most widely used drug in cancer treatment today. Other related taxoids (baccatins) have also gained importance as precursors to synthesize paclitaxel

analogues such as docetaxel, which is also an effective clinical antineoplastic agent (Cragg et al 1997; Mann 2002).

Alstonia scholaris R. Br. (Devil's tree), a tree belonging to the family Apocynaceae, has been used since time immemorial in the folklore and traditional systems of medicine in India, because of its vast range of therapeutic applications including cancer treatment. The ripe fruits are used in syphilis insanity and epilepsy. It is also used as a tonic, antiperiodic and antihelmentic. The milky juice of *Alstonia scholaris* has been applied to treat ulcers. The bark is the most extensively used part of the plant and is used in many compound herbal formulations (Nadkarni 1976). It is a bitter tonic, alternative and febrifuge and is reported to be useful in the treatment of malaria, diarrhoea and dysentery (Nadkarni 1976; Satyavati et al 1987; Chemexcil 1992; Warrier et al 1996).

Echitamine, one of the major alkaloids present in *Alstonia*, has been reported to be cytotoxic in-vivo and in-vitro against fibrosarcoma in rats and sarcoma-180 in mice (Mohana et al 1985; Kamarajan et al 1991, 1995; Saraswathi et al 1998a, Ramirez & Garcia-Rubio 2003). It has also been reported to increase the cytotoxic effect of doxorubicin, cyclophosphamide and berberine in-vivo (Saraswathi et al 1997; Jagetia & Baliga 2003, 2004). The anticancer activity should be tested in various systems to establish the efficacy of the compound. As far as we know, the effect of echitamine has not been evaluated in cultured human tumour cells and in the mice bearing Ehrlich ascities carcinoma. Therefore, this study was undertaken to evaluate the anticancer activity of echitamine in various cultured neoplastic cancer cells of human origin and in mice bearing Ehrlich ascities carcinoma.

Materials and Methods

Isolation of echitamine

Echitamine (99.9% purity) was isolated from the bark of *Alstonia scholaris* (Saraswathi et al 1997). The purity of the sample was confirmed by co-chromatography, UV spectra and IR spectra.

Preparation of the drug

An appropriate amount of echitamine chloride was dissolved in sterile physiological saline (SPS) diluted to the required concentrations in Eagle's minimum essential medium and filter-sterilized immediately before use. The volume of drug inoculation was always kept constant at $50\,\mu L\,mL^{-1}$ irrespective of the variation in the drug concentration.

Cytotoxic effect in-vitro

Cell line and culture

HeLa, $HepG_2$, HL60, KB and MCF-7 cells procured from the National Center for Cell Science, Pune, India, were grown in Eagle's minimum essential medium or RPMI medium (HL60), supplemented with 10% fetal calf serum, 1% L-glutamine and $50\,\mu g\,mL^{-1}$ gentamicin sulfate. The cells were routinely grown in $75\,cm^2$ flasks (Nunc, Rosklide, Denmark) with loosened caps, and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Pratt and Willis test

The cytotoxic effect of echitamine chloride was evaluated by the Pratt and Willis test (Pratt & Willis 1985) in HeLa, $HePG_2$, KB and MCF-7 cells. Briefly, a fixed number of exponentially growing cells (1×10^4) were inoculated into several individual culture dishes and were allowed to grow for 24 h, thereafter the different cell cultures were treated with echitamine chloride for a specified time. After the elapse of stipulated time, media from the cultures were replaced with fresh drug-free medium with care, so as to avoid disturbances to the attached cells. The cells were dislodged by trypsin-EDTA treatment 72 h after the initiation of cultures, and the viability of the cells was determined by trypan blue dye exclusion test, using a haemocytometer under an inverted transmitted light microscope (Ernst Leitz Wetzlar GmbH, Wetzlar, Germany).

As HL60 cells grow in suspension, the cytotoxicity studies were carried out as follows. The cells along with the drug-containing medium from the culture dishes were transferred to sterile tubes, centrifuged, washed thrice with phosphate-buffered saline (PBS) and resuspended in the drug-free RPMI medium. The cells were then transferred to culture dishes and placed into a CO_2 incubator at 37°C until the completion of the experiment. The viability of cells was determined using the trypan blue dye exclusion test as described above.

Evaluation of optimum duration

The optimum duration for drug exposure was evaluated by the Pratt and Willis test (Pratt & Willis 1985) in HeLa cells as described above. Briefly, 1×10^4 viable cells were inoculated into several individual culture dishes and allowed to grow for 24 h, and the cultures were divided into different groups. The cultures of the PBS group were exposed to PBS for 0, 2, 4, 6, 8, 12, 18 or 24 h. The cultures of the echitamine chloride group were treated with $25\,\mu g\,mL^{-1}$ echitamine chloride for 0, 2, 4, 6, 8, 12, 18 or 24 h. The cultures of the doxorubicin group were treated with $25\,\mu g\,mL^{-1}$ doxorubicin as a positive control for 0, 2, 4, 6, 8, 12, 18 or 24 h. After the elapse of stipulated time, media from the cultures of all groups were replaced carefully with fresh drug-free medium. The cells were dislodged by trypsin-EDTA treatment 72 h after initiation of cultures, and viability of the cells was determined by the trypan blue dye exclusion test using a haemocytometer.

Evaluation of cytotoxicity in HeLa, $HepG_2$, HL60, KB and MCF-7 cells

The cytotoxicity of various concentrations of echitamine chloride was evaluated in HeLa, HepG2, HL60, KB and MCF-7 cells and separate experiments were carried out for each cell line. The detailed procedure has been described above.

Briefly, 1×10^4 viable cells from each cell line were seeded into several culture dishes and allowed to grow for 24 h. Thereafter, the cells were treated with 0, 1.25, 2.5, 5, 10, 25, 50, 75, 100, 150 or $200\,\mu g\,mL^{-1}$ echitamine

chloride and the drug-containing media were replaced with the fresh drug-free medium 6 h after echitamine chloride or doxorubicin treatment. The cells were left undisturbed and allowed to grow for another 42 h. The cells were dislodged by trypsin-EDTA treatment 72 h after initiation of the cultures and the viability of the cells was determined using the trypan blue dye exclusion test.

All the above experiments were repeated five times and triplicate cultures were used for each drug concentration for each cell line. The mean of all experiments for individual cell lines was expressed as the result. The test of homogeneity was carried out to find if there were any differences among the various repeat experiments. The results did not show statistically significant differences among the repeat experiments, therefore the values of all the experiments were combined and presented in the tables and figures.

Anti-tumour activity in-vivo

Animals and tumour model

Animal care and handling were according to the guidelines set by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Ten- to twelve-week-old male Swiss albino mice (30–36 g) were selected from an inbred colony maintained under the controlled conditions of temperature ($23 \pm 2^{\circ}$C), humidity ($50 \pm 5\%$) and light (10 and 14 h of light and dark, respectively). The animals had free access to sterile food and water. Throughout the experiment, four animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding. The study was approved by the Animal Ethical Committee of Kasturba Medical College, Manipal, India.

Ehrlich ascites carcinoma (EAC) procured from the Cancer Research Institute (ACTREC), (Mumbai, India) was maintained and propagated by intraperitoneal serial transplantation in an aseptic environment. No spontaneous regression of EAC tumour was observed throughout the study. The experiments were carried out by injecting 10^6 viable EAC cells intraperitoneally in sterile conditions into each animal. The day of tumour inoculation was considered as day 0.

Determination of the optimum dose

The study was carried out in accordance with the standard protocol recommended by the National Service Center of Cancer Chemotherapy (Geran et al 1972). The animals were divided into three groups 24 h after tumour inoculation. The sterile physiological saline (SPS) group received a single administration of 0.3–0.36 mL sterile physiological saline. The doxorubicin group received a single injection of $5\,mg\,kg^{-1}$ doxorubicin 24 h after tumour inoculation. The echitamine chloride group received a single injection of 1, 2, 4, 8, 12 or $16\,mg\,kg^{-1}$ echitamine chloride 24 h after tumour inoculation (Krishnaswamy & Purushothaman 1980).

The animals were monitored regularly for weight changes, signs of toxicity and mortality. The weight of animals in all groups was recorded every third day up to 30 days after tumour inoculation. A 33% of drug related deaths or a weight loss of 5 g per mouse was considered as an index of toxicity. The animal survival was monitored daily up to 120 days, since the survival of animals up to 120 days was roughly equivalent to 5 years survival in man (Nias 1990). The tumour response was assessed on the basis of median survival time and tumour free survival. The median survival time (MST) was calculated as follows:

$$\text{MST} = (\text{First death} + \text{last death in the group})/2 \quad (1)$$

The increase in median life span (% IMLS) was calculated as follows:

$$\text{IMLS} = ((\text{MST of treated mice} - \text{MST of control})/\text{MST of control}) \times 100 \quad (2)$$

Biochemical estimations

The animals were inoculated with the tumour cells as described above and the tumour was allowed to grow for six days. On the seventh day, the tumour-bearing animals were administered a single dose of $16\,mg\,kg^{-1}$ echitamine chloride intraperitoneally. A group of four animals were killed at 1.5, 3, 6, 9 or 12 h after the echitamine chloride administration. The tumour cells were aspirated in sterile conditions and were washed with sterile saline thrice. The cells were counted under an inverted microscope and 10^7 cells were sonicated (Virsonic, USA) and processed for the estimation of glutathione and lipid peroxidation studies.

Glutathione (GSH)

GSH content was measured by the method of Moron et al (1979). Briefly, proteins were precipitated by 25% trichloroacetic acid (TCA), centrifuged and the supernatant was collected. The supernatant was mixed with 0.2 M sodium phosphate buffer pH 8.0 and 0.06 mM 5,5′dithio 2-nitrobenzoic acid (DTNB) and incubated for 10 min at room temperature. The absorbance of the sample was read against the blank at 412 nm in a UV-visible double beam spectrophotometer (Shimadzu UV-260, Shimadzu Corp, Japan). The GSH concentration was calculated from the standard curve.

Lipid peroxidation

Lipid peroxidation was measured by the method of Buege & Aust (1978). Briefly, the cell homogenate was mixed with TCA–thiobarbituric acid (TBA)–HCl and was heated for 15 min in a boiling water bath. After centrifugation the absorbance was recorded at 535 nm using a UV-visible double beam spectrophotometer. Lipid peroxidation was expressed as malondialdehyde (MDA) in nmol/1×10^7 cells. The concentration of lipid peroxidation in the sample was determined against the standard curve of 1,1,3,3-tetramethoxy propane (TMP).

Statistical analysis

Statistical analyses were carried out using BMDP Statistical Software (BMDP Statistical Software Inc, Los Angles, CA) on an IBM PC. One-way analysis of variance test was used with application of post-hoc test for comparisons in the in-vitro studies. Significance of treatment for survival studies was carried out using the 'Z' test (Abramowitz & Stegun 1972) and Student's *t*-test was used for biochemical estimations.

Results

Evaluation of optimum duration for echitamine chloride

Exposure of HeLa cells to PBS did not have any adverse effect on the cell viability and a maximum number of viable cells were observed in this group. Treatment of HeLa cells with 25 $\mu g\,mL^{-1}$ echitamine chloride resulted in a time-dependent decline in the number of viable cells and a nadir was reached at 24-h post-treatment (Figure 1). However, the echitamine chloride exposure up to 6 h caused a 55% decrease in the cell viability and this period was used for all further studies (Figure 1).

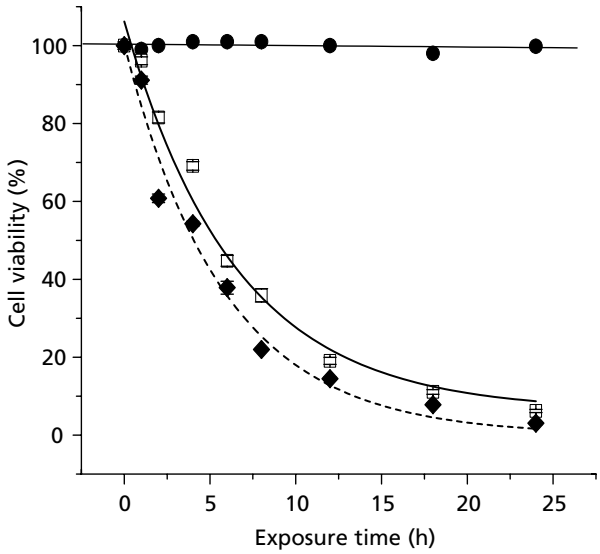

Figure 1 Effect of various exposure times on the cytotoxicity in HeLa cells treated with 25 $\mu g\,mL^{-1}$ echitamine chloride. Upper curve, PBS; middle curve, echitamine chloride; and lower curve, 25 $\mu g\,mL^{-1}$ doxorubicin.

Evaluation of cytotoxicity in HeLa, $HepG_2$, HL60, KB and MCF-7 cells

Exposure of HeLa cells to different concentrations of echitamine chloride resulted in a concentration-dependent reduction in the cell viability up to 50 $\mu g\,mL^{-1}$, thereafter the increase in the drug concentration caused an abrupt decline in the cell viability and a nadir was reached at 200 $\mu g\,mL^{-1}$ echitamine chloride (Table 1). The IC50 was found to be 30 $\mu g\,mL^{-1}$.

The exposure of $HepG_2$ cells to 1.25–50 $\mu g\,mL^{-1}$ caused a gradual decrease in the cell viability and a further increase to 75 $\mu g\,mL^{-1}$ echitamine chloride caused an abrupt decline in the cell viability, where only 36.8% viable cells were observed. The increasing concentration of echitamine chloride caused a concentration-dependent depletion in the cell viability and a nadir was reached at 200 $\mu g\,mL^{-1}$ echitamine chloride (Table 1). The IC50 was found to be 50 $\mu g\,mL^{-1}$.

The exposure of HL60 cells to various concentrations of echitamine chloride resulted in a gradual and concentration-dependent depletion in the cell viability up to 25 $\mu g\,mL^{-1}$, where 64.7% viable cells were observed. A further increase in the drug concentration to 50 $\mu g\,mL^{-1}$ caused a sudden decline in the number of viable cells. The greatest cytotoxic effect was observed at 200 $\mu g\,mL^{-1}$ echitamine chloride, where 4.8% viable cells could be reported (Table 1). The IC50 was found to be 51 $\mu g\,mL^{-1}$.

The exposure of KB cells to different concentrations of echitamine chloride resulted in a steady and concentration-dependent reduction in the number of viable cells up to 50 $\mu g\,mL^{-1}$. A further increase in the drug concentration caused a sudden decline (50% of 50 $\mu g\,mL^{-1}$) in the number of viable cells. A nadir in cell survival was observed at 200 $\mu g\,mL^{-1}$ echitamine chloride, where it was lowest (1.33%) among all cell lines studied (Table 1). The IC50 was calculated and found to be approximately 10.5 $\mu g\,mL^{-1}$.

The exposure of MCF-7 cells to various concentrations of echitamine chloride resulted in a steady and concentration-dependent reduction in the number of viable cells up

Table 1 Alteration in the viability of various human cell lines exposed to different concentrations of echitamine chloride in-vitro

Echitamine chloride ($\mu g\,mL^{-1}$)	Percent cell viability ± s.e.m.				
	HeLa	HepG2	HL60	KB	MCF-7
0	99.40 ± 0.56	100.2 ± 0.72	99.8 ± 0.86	99.8 ± 0.86	100.4 ± 0.51
1.25	93.22 ± 0.72[a]	98.8 ± 0.67	94.6 ± 1.12[a]	90.8 ± 0.96[b]	100.2 ± 0.79
2.5	86.88 ± 1.07[b]	97.2 ± 0.54[a]	88.8 ± 1.39[b]	80.6 ± 1.07[b]	98.8 ± 0.37[a]
5	68.78 ± 1.24[b]	84.8 ± 1.06[b]	80.8 ± 1.46[b]	67.8 ± 1.24[b]	93.4 ± 0.51[a]
10	54.31 ± 1.06[b]	74.6 ± 1.42[b]	74.2 ± 0.86[b]	52.4 ± 1.71[b]	84.2 ± 0.86[b]
25	45.71 ± 1.59[b]	66.4 ± 1.37[b]	65.2 ± 0.73[b]	37.6 ± 1.02[b]	70.7 ± 1.71[b]
50	34.85 ± 1.01[b]	53.1 ± 2.11[b]	49.2 ± 0.86[b]	26.4 ± 1.74[b]	52.6 ± 1.25[b]
75	17.65 ± 1.89[b]	36.8 ± 1.24[b]	38.8 ± 0.96[b]	13.4 ± 1.44[b]	37.2 ± 2.16[b]
100	10.41 ± 1.03[b]	26.4 ± 1.07[b]	24.1 ± 0.71[b]	6.82 ± 1.69[b]	25.2 ± 1.73[b]
150	6.78 ± 0.24[b]	11.2 ± 1.13[b]	19.2 ± 1.06[b]	1.61 ± 0.82[b]	13.8 ± 1.06[b]
200	4.93 ± 0.21[b]	5.1 ± 0.83[b]	4.82 ± 0.37[b]	1.33 ± 0.33[b]	6.76 ± 0.49[b]

[a] $P < 0.01$, [b] $P < 0.001$, compared with PBS control (0 $\mu g\,mL^{-1}$).

to 25 $\mu g\,mL^{-1}$. A further increase in the drug concentration to 50 $\mu g\,mL^{-1}$ caused an abrupt decline (52.6%) in the number of viable cells and a nadir was reached at 200 $\mu g\,mL^{-1}$ echitamine chloride (Table 1). The IC50 was calculated and found to be 52 $\mu g\,mL^{-1}$.

Anti-tumour activity in-vivo

No spontaneous regression was observed in the mice injected with EAC cells and the animals exhibited a constant increase in weight due to tumour cell multiplication and growth. The first death was observed on day 16 and all the control animals died by day 21 post-tumour inoculation (Figure 2). The median survival time (MST) was found to be 19 days (Table 2). Treatment of 24-h-old tumours with 2, 4, 8, 12 or 16 $mg\,kg^{-1}$ echitamine chloride inhibited weight gain in animals, indicating arrest of tumour cell proliferation and growth.

Administration of 2 or 4 $mg\,kg^{-1}$ echitamine chloride did not significantly alter the MST (19.5 days) when compared with the physiological saline group. A dose-dependent increase was observed for MST in the tumour-bearing mice treated with different doses of echitamine chloride. However, a maximum effect was observed for 12 $mg\,kg^{-1}$ echitamine chloride, where the MST increased by 11.5 days when compared with the sterile physiological saline treatment, while it was 16 days for doxorubicin, which was used as a positive control. The IMLS increased up to 59% (Table 2). The animals treated with 16 $mg\,kg^{-1}$ echitamine chloride (the highest drug-dose), exhibited toxic side effects in the form of ruffling of hairs, sluggishness, lacrimation and weight loss.

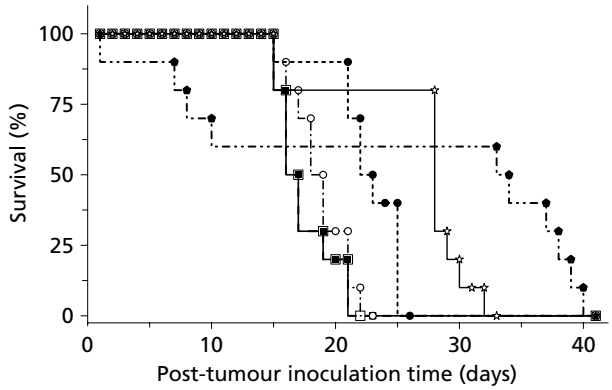

Figure 2 Kaplan Meir's estimate of survival of tumour-bearing mice treated with different doses of echitamine chloride. Open squares, 0; solid squares, 2; open circles, 4; solid circles, 8; open star, 12; and solid pentagon, 16 $mg\,kg^{-1}$ echitamine chloride.

Table 2 Effect of different doses of echitamine chloride on the median survival time (MST) of EAC mice

Drug dose	MST (days)	IMLS (%)	Percent survivors on day			
			20	30	40	50
Sterile physiological saline	19.0	–	20	0	0	0
Echitamine chloride 2 $mg\,kg^{-1}$	19.5	2.63	20	0	0	0
Echitamine chloride 4 $mg\,kg^{-1}$	19.5	2.63	30[a]	0	0	0
Echitamine chloride 8 $mg\,kg^{-1}$	24.0[a]	29.72	100[b]	0	0	0
Echitamine chloride 12 $mg\,kg^{-1}$	30.5[b]	58.97	100[b]	20	0	0
Echitamine chloride 16 $mg\,kg^{-1}$	24.0[a]	26.3	70[b]	70[b]	10	0
Doxorubicin 5 $mg\,kg^{-1}$	34.0[b]	78.94	100[b]	80[b]	20	0

[a] $P < 0.05$; [b] $P < 0.001$, compared with SPS.

Biochemical studies

Glutathione

Administration of 16 $mg\,kg^{-1}$ echitamine chloride caused a time-dependent decline in GSH content up to 3 h post–treatment, where the GSH content was minimal (Figure 3). Thereafter, an elevation in GSH was observed up to 12 h post-treatment, without restoration to normal level (Table 3).

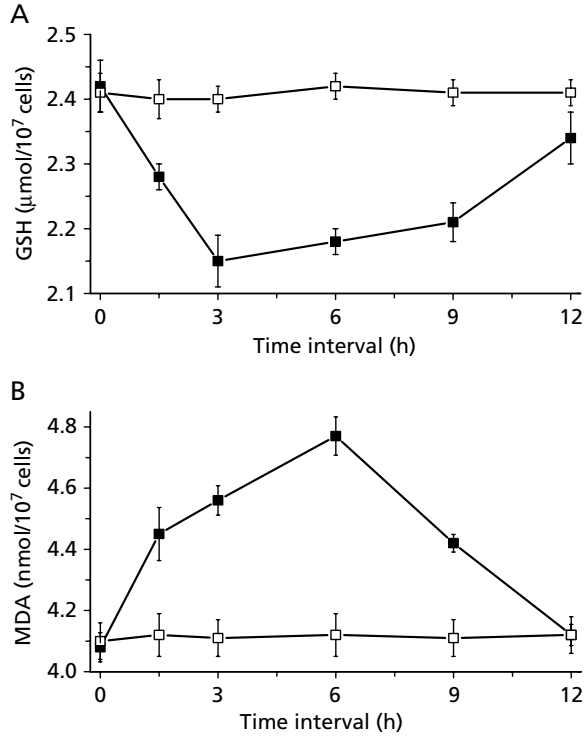

Figure 3 Alterations in the GSH concentration (A) and lipid peroxidation (B) in EAC mice treated with 16 $mg\,kg^{-1}$ echitamine chloride at different post-treatment times. Open squares, sterile physiological saline; solid squares, echitamine chloride.

Table 3 Alterations in the GSH concentration and lipid peroxidation in EAC mice treated with 16 mg kg^{-1} echitamine chloride at different post-treatment times

Time (h)	GSH (μmol/10^7 cells)		MDA (nmol/10^7 cells)	
	Sterile physiological saline	Echitamine chloride	Sterile physiological saline	Echitamine chloride
0	2.41 ± 0.03	2.42 ± 0.05	4.10 ± 0.06	4.13 ± 0.03
1.5	2.40 ± 0.03	2.26 ± 0.06[a]	4.12 ± 0.07	4.39 ± 0.03[b]
3	2.40 ± 0.02	2.19 ± 0.02[b]	4.11 ± 0.06	4.48 ± 0.04[b]
6	2.42 ± 0.02	2.21 ± 0.02[b]	4.12 ± 0.07	4.67 ± 0.02[c]
9	2.41 ± 0.02	2.25 ± 0.03[a]	4.11 ± 0.06	4.32 ± 0.05[a]
12	2.41 ± 0.02	2.39 ± 0.07	4.12 ± 0.06	4.18 ± 0.06

[a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, compared with sterile physiological saline.

Lipid peroxidation

Lipid peroxidation in EAC cell increased in a time-dependent manner up to 6 h post-treatment and declined thereafter (Figure 3). However, normal levels could not be restored even up to 12 h post-treatment (Table 3). The decline in GSH was associated with the elevation in lipid peroxidation, indicating an inverse relationship between GSH and lipid peroxidation.

Discussion

Exposure time is of cardinal importance for the screening of anticancer activity of any drug, since the anticancer activity of the drug may vary with drug exposure time. Therefore, studies were carried out in HeLa cells to select the optimum drug exposure time using 25 μg mL^{-1} echitamine chloride. The results showed that increasing drug exposure time caused a significant decline in the population of viable HeLa cells that reached a nadir at 24 h after drug exposure. A 55% decrease in the viable cell population was observed when the echitamine chloride was available for 6 h and further studies were carried out using this time. The cytotoxic effect of echitamine chloride increased with the increase in the drug concentration and a nadir was reached at 200 μg mL^{-1}. The KB cells were found to be most sensitive. The IC50 of 10.5, 30, 50, 51 and 52 μg mL^{-1} was observed for KB, HeLa, HepG$_2$, HL60 and MCF-7 cells, respectively. An earlier study had reported an IC50 of 200 μM for Vero cells exposed to echitamine chloride (Kamarajan et al 1995). Reports regarding the effect of echitamine chloride on various cultured human cell lines were scanty. However, a similar observation had been reported in the EAC cells in-vitro (Saraswathi et al 1998a). A concentration-dependent elevation in the cytotoxicity had been reported in HeLa cells treated with various fractions of stem bark extract of *Alstonia scholaris*, which contained echitamine chloride as one of the chemical constituents (Jagetia & Baliga 2005). Similarly, many other active constituents of *Alstonia scholaris* such as alstonine, bisindole, villalstonine, pleiocarpamine, *O*-methylmacralstonine and macralstonine have been reported to possess pronounced cytotoxic activity in different cell lines (Beljanski & Beljanski 1986; Keawpradub et al 1997, 1999).

Administration of different doses of echitamine chloride (2, 4, 8, 12 or 16 mg kg^{-1}) caused inhibition of tumour development, which was evident by the reduction in body weight gain and increased tumour free survival. This indicated the effectiveness of echitamine chloride in restricting tumour cell multiplication. The best antitumour effect was seen for 8 and 12 mg kg^{-1} echitamine chloride, where the MST increased by 1.3-fold when compared with the concurrent saline group. Similarly, treatment of EAC mice with different doses of stem bark extract of *Alstonia scholaris* had been reported to increase the MST in a dose-dependent manner (Jagetia & Baliga 2003). Among all the echitamine chloride doses screened, 12 mg kg^{-1} (3/5 of the LD50 dose; unpublished observation) was considered to be the best dose, since it increased the MST (30.5) by 11.5 days over the concurrent non-drug treated controls and it was not associated with any toxic side effects such as debility, loss of body weight and death. These observations were in agreement with earlier reports on fibrosarcoma and sarcoma-180 in rats and mice (Mohana et al 1985; Kamarajan et al 1991, 1995; Saraswathi et al 1998a). Other phytochemical compounds like RC-18, fostriecin, ukrain, strychnopentamine, withafarin and plumbagin have also been reported to enhance the life span of tumour-bearing mice (Adwankar & Chitnis 1982; Boritzki et al 1988; Bruller 1992; Quetin-Leclercq et al 1993; Sharada et al 1996; Uma Devi et al 1999).

The exact mechanism of action for echitamine chloride is not known. The cytotoxic effect of echitamine chloride may not be due to a single mechanism but due to the operation of several mechanisms in concert with one another. The inhibition of DNA synthesis, serine proteases and PKA by echitamine chloride may have been responsible for the cytotoxic effect (Rajic et al 2000). Induction of DNA fragmentation and apoptosis may be another important mechanism by which echitamine chloride induced cytotoxic effect. Inhibition of the glycolysis pathway by echitamine chloride may have deprived tumour cells of their main energy source, thus killing the cells effectively. Increase in oxidant status by echitamine chloride may also have played a role in cell killing. Echitamine chloride has been reported to reduce DNA synthesis and inhibit glycolysis and glycoprotein synthesis in-vivo and in-vitro (Kamarajan et al 1995; Saraswathi et al 1997, 1998a, b). Echitamine chloride increased lipid peroxidation and reduced glutathione concentration in tumour cells in this study. A similar effect had been observed earlier (Kamarajan et al 1995; Saraswathi et al 1997, 1998a).

This study demonstrated that the cytotoxic effect of echitamine chloride increased in a concentration-dependent manner in various cultured human cell lines. The IC50 was between 10.5 and 52 μg mL^{-1}, depending upon the cell line.

The KB cells were the most sensitive. These findings were corroborated by in-vivo study, where MST of EAC mice increased in a dose-dependent manner up to $12\,mg\,kg^{-1}$ echitamine chloride. The mechanisms responsible for the cell killing effect may be related to DNA damage, inhibition of glycolysis and increased oxidative stress by echitamine chloride.

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