Cancer Chemother Pharmacol (2003) 51: 474–482
DOI 10.1007/s00280-003-0609-9

ORIGINAL ARTICLE

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Sanguinarine-induced apoptosis is associated with an early and severe cellular glutathione depletion

Received: 6 January 2003 / Accepted: 27 February 2003 / Published online: 17 April 2003

Abstract *Purpose*: The quaternary benzophenanthridine alkaloid sanguinarine exhibits a broad range of activity, including cytotoxicity against various human tumour and normal cell lines. Here, we examined its potency as an anticancer drug. *Methods*: The differential cytotoxicity against cancer versus normal cells was assessed in vitro by two fluorimetric assays (RRT and Hoechst 33342 dye DNA assays, respectively) in a panel of human solid cancer cell lines and a human fibroblast primary culture. The ability to induce apoptosis was demonstrated in PC3 human prostatic adenocarcinoma cells by analysis of morphological changes, internucleosomal DNA fragmentation, cellular poly(ADP-ribose) polymerase cleavage and caspase 3/7 activation. Production of reactive oxygen species was evaluated by the 2′,7′-dichlorofluorescin diacetate assay. Depletion of cellular glutathione content was assessed with the monochlorobimane assay. *Results*: Sanguinarine markedly inhibited the growth of all tested cells (IC_{50} 0.9–3.3 μ*M*) without differential cytotoxicity against normal versus cancer cells. In PC3 cells, continuous treatment with 5 μ*M* sanguinarine induced an early (within 10 min) cellular reduced glutathione depletion insensitive to dithiothreitol or *N*-acetylcysteine treatment, followed by a caspase 3/7-dependent apoptotic response within 2 h. Complementary assays suggested that the glutathione depletion was initiated by direct reactivity of sanguinarine with reduced glutathione. *Conclusions*: Taken together, these results show that (1) sanguinarine exhibits no specificity for cancer cells, and (2) its strong cytotoxicity is probably due to a rapid apoptotic response induced by an early and severe glutathione-depleting effect. They also suggest that the clinical usefulness of this alkaloid as an anticancer drug is limited.

Keywords Sanguinarine · Apoptosis · Caspases Glutathione depletion · ROS production · Anticancer candidate

Abbreviations *DCFH-DA* 2′,7′-dichlorofluorescin diacetate · *DEM* Diethylmaleate · *DTT* Dithiothreitol · *GSH* Reduced glutathione · *HBSS/Hep* Hank's balanced salt solution/Hepes 15 m*M*, pH 7.4 · *NAC* *N*-acetyl cysteine · *PARP* Poly (ADP-ribose) polymerase · *PBS* Phosphate-buffered saline · *PBSMT* 1×PBS, pH 7.4, 5% m/v non-fat powdered milk, 0.1% v/v Tween 20 · *ROS* Reactive oxygen species · *RP-HPLC* Reversed-phase high pressure liquid chromatography · *RRT* Resazurin reduction test · *SDS* Sodium dodecyl sulphate

Introduction

Chemotherapy is an essential strategy for the treatment of disseminated cancer, but its efficacy is restricted by both intrinsic and acquired resistance to drugs. To circumvent chemoresistance, intense research has focused on drugs targeting new mechanisms involved in tumour growth and metastasis. During the last 20 years, various active drugs meeting these criteria have been discovered. Most are of natural origin. Some have been extracted from plants (e.g. paclitaxel from *Taxus* species). Others resulted from hemisynthetic modifications of natural precursors present in large amounts in plants (docetaxel from deacetylbaccatine III) or from the synthesis of structural analogues of highly active natural compounds whose toxicity prevents therapeutic use (camptothecin derivatives, topotecan and irinotecan).

Our strategy has consisted of identifying drugs inducing apoptosis via unusual mechanisms then eliciting their biosynthesis or that of analogues by encoding

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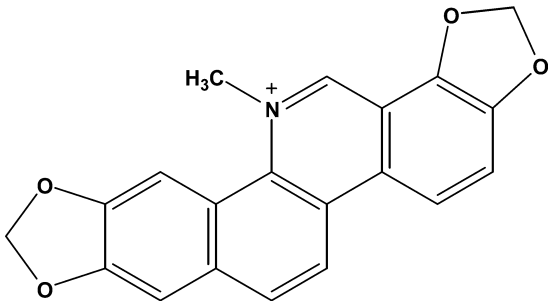

Fig. 1 Chemical structure of sanguinarine

or modifying genes involved in the biosynthetic pathway. This work has focused on sanguinarine (13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i] phenanthridinium; Fig. 1), a benzophenanthridine alkaloid used for years as a dental product for the treatment of gingivitis and plaque [1], which has been the subject of great interest as an anticancer drug. This alkaloid is known to display a broad range of activities (e.g. antimicrobial, antioxidant and antiinflammatory effects; for a review see http://www.ars-grin.gov/cgi-bin/duke/chemical.pl), including cytotoxicity at micromolar concentrations against various human tumour and normal cell lines [2, 3, 4, 5, 6]. Recently, Weerasinghe et al. have reported that sanguinarine induces bimodal cell death in leukaemia cells. Apoptosis was observed at low concentrations and blister cell death at high concentrations [7, 8].

The biochemical mechanisms involved in the different biological activities are not completely understood, but many cellular targets have been implicated, including some involved in cell death machinery. Sanguinarine has been reported to inhibit Na^+/K^+ ATPase [9] and protein kinases A [10], to inhibit NF-κB activation [11], and to open the sarcoplasmic reticulum calcium release channel, probably via an interaction with ryanodine receptors [12]. It has also been reported to interact directly with and to intercalate DNA [13, 14] and to inhibit microtubule polymerization [15, 16]. Apoptotic responses have recently been reported to be Bcl-2 family protein-dependent. Bax appears to be involved in apoptosis induction via a caspase 3-dependent program that can be blocked by Bcl-2 overexpression [7, 8].

Based on all these observations, we considered, following others [2, 4], that sanguinarine itself or some of its derivatives might be good candidates as proapoptotic drugs for cancer therapy. Various derivatives have been obtained by encoding in plant cells c-DNA for modified enzymes involved in benzophenanthridine pathways, coupled with inducible cytochrome P-450-dependent oxidase of bisbenzylisoquinoline alkaloid biosynthesis (Barthomeuf, personal communication). Some are under investigation for their anticancer potency. We report here the effects of sanguinarine on the proliferation of cells from various solid cancers chosen from the most frequent cancers in Western countries versus normal cells, and the results of investigations on the mechanisms involved in cytotoxicity.

Materials and methods

Materials

C-2-10 mouse monoclonal anti-poly(ADP-ribose) polymerase (PARP) antibody was obtained from Novus-Biologicals (Littleton, Colo.) and antimouse immunoglobulin peroxidase conjugate from Dako (Dako, Trappes, France). A DNeasy tissue kit was obtained from Qiagen (Courtaboeuf, France). All other chemicals were purchased from Sigma Aldrich (L'Isle d'Abeau Chesnes, France).

Production and purification of sanguinarine

Sanguinarine was obtained from transgenic root cultures of *Eschscholzia californica* Cham (Papaveraceae) in which cDNA encoding a new methyl jasmonate-inducible cytochrome P-450-dependent mono-oxygenase (*S*)-(*cis*)-*N*-methylstylopine-14-hydroxylase has been cloned using *Agrobacterium rhizogenes* strain C58C1 containing the binary vector pBI121 [17]. Sanguinarine was purified by RP-HPLC on a C18 Ultrasphere column (7.8×250 mm; Interchim, Montluçon, France) using an isocratic gradient of methanol/water (8:2) containing 0.1% (v/v) triethylamine at a flow rate of 1.25 ml/min, then unambiguously identified by ^{1}H and ^{13}C-NMR analysis. The purity assayed by RP-HPLC according to Guedon et al. [18] was higher than 98%.

Cell culture

Normal human fibroblasts were purchased from Biopredic International (Rennes, France) as a frozen culture. The cells were obtained from a 36-year-old female undergoing abdominal surgery, and the cells used in this work were from the 7th to 12th passage of the culture. M4Beu, a human melanoma cell line, was established in the laboratory of Dr. J.F. Doré (INSERM, Unit 218, Lyon, France) from metastatic biopsy specimens and had been maintained in cell culture for almost 15 years [19]. Breast cancer adenocarcinoma MCF7, prostatic adenocarcinoma PC3, colon adenocarcinoma DLD-1 and lung non-small-cell carcinoma A 549 human cell lines, and L-929 murine cell line were purchased from the European Collection of Cell Cultures (ECACC; Salisbury, UK). Stock cell cultures were maintained as monolayers in 75-cm^2 culture flasks in Glutamax Eagle's minimum essential medium (MEM) with Earle's salts (Gibco-BRL, Paisley, UK) supplemented with 10% fetal calf serum (Sigma), and 5 ml of a 100× solution of vitamins (Gibco), 5 ml 100 m*M* sodium pyruvate (Gibco), 5 ml of 100× non-essential amino acids (Gibco) and 2 mg gentamicin base (Gibco). Cells were grown at 37°C in a humidified incubator under an atmosphere containing 5% CO_2.

Survival assays

Cells were plated at a density of 5×10^3 cells in 190 µl culture medium in each well of 96-well microplates (Nunclon; Nunc, Roskilde, Denmark) and were allowed to adhere for 16 h before treatment with sanguinarine. A stock solution of sanguinarine was prepared in sterile 0.15 *M* NaCl (Eurobio, Les Ulis, France) and kept at −20°C until use. Then 10 µl of a 20× sanguinarine solution in 0.15 *M* NaCl was added to the cultures. A 48-h continuous drug exposure protocol was used. The antiproliferative effect of sanguinarine was assessed by both the resazurin reduction test (RRT) and determination of DNA cellular content after cell lysis.

Resazurin reduction test

Resazurin was recently identified as alamar blue dye [20]. The RRT was carried out as follows. Briefly, plates were rinsed with 200 µl PBS (Gibco) at 37°C using an automatic microplate washer (Cell Wash; Labsystems, Helsinki, Finland) emptied by overturning on absorbent towelling. Then 150 µl of a 25 µg/ml solution of resazurin in MEM without phenol red was added to each well using an automatic microvolume dispenser (Multidrop 384; Labsystems). Plates were incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. Fluorescence was then measured on an automated 96-well plate reader (Fluoroskan Ascent FL; Labsystems) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Under the conditions used, fluorescence was proportional to the number of living cells in the well. The IC_{50}, defined as the drug concentration required to inhibit cell proliferation by 50%, was calculated from the curve of concentration-dependent survival percentage, defined as fluorescence in experimental wells compared with fluorescence in control wells, after subtraction of blank values.

After reading, cells were prepared for cellular DNA quantitation with Hoechst dye 33342. They were rinsed with PBS, resazurin solution was then eliminated using an automatic microplate washer and plates were stored at −80°C until the Hoechst assay.

Hoechst dye 33342 assay

The Hoechst dye 33342 assay was carried out according to the method of Rago et al. [21] with minor modifications. On the day of assay, plates were thawed at room temperature for 10 min. A volume of 100 µl 0.01% (m/v) SDS solution in sterile distilled water was then distributed into each well with an automatic dispenser, and the plates were incubated for 1 h at room temperature and frozen again at −80°C for 1 h. After thawing (approximately 15 min), 100 µl per well of Hoechst dye 33342 solution (Sigma) at 30 µg/ml in a hypersaline buffer (10 m*M* Tris HCl, pH 7.4, 1 m*M* EDTA and 2 *M* NaCl) was added to each well. The plates were incubated in this solution for 1 h protected from light at room temperature on a plate shaker. Fluorescence was then measured at 360/460 nm on a microplate fluorescence reader.

Under the conditions used, fluorescence was proportional to the amount of biomass, and the IC_{50} was calculated as above.

Characterization of apoptotic morphological changes

Cells were plated in 10-cm diameter plates (Nunclon) in 10 ml culture medium, then allowed to grow until 70% confluence was reached. The culture medium was removed and new complete medium containing sanguinarine plus test compounds (assay), sanguinarine alone (control) or solvent (blank) was added and the cells incubated for the duration of the assay. At the end of each test period, cells were harvested and assayed for viability using trypan blue. Cell morphology was assessed by phase-contrast microscopy or by traditional microscopy after Giemsa staining.

Caspase 3/7 activity

Caspase 3/7 activity was assayed using an EnzChek Caspase 3 assay kit (Molecular Probes, Leiden, The Netherlands) according to the recommendations of the supplier. The cleavage of the rhodamine 110-derived *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp peptide Z-DEVD-R110 was detected by measurement of fluorescence at 485/530 nm. To confirm that the fluorescence signal was due to caspase-3-like proteases, one subset of extracts was pretreated with the specific reversible inhibitor Ac-DEVD-CHO at 20 µ*M* for 10 min.

Analysis of apoptosis-related PARP cleavage by Western blotting

PARP cleavage of the 85 kDa fragment characteristic of apoptosis was examined according to the method of Sallman et al. [22]. Briefly, cells were harvested by trypsinization. The suspension was centrifuged at 1600 *g* for 8 min and the pellet was suspended in PBS. After centrifugation, the supernatant was removed and the cells lysed and sonicated on ice in a solution of reducing loading buffer comprising 62.5 m*M* Tris HCl, pH 6.8, 6 *M* urea, 10% (v/v) glycerol, 2% (m/v) SDS, 0.003% (m/v) bromophenol blue, a cocktail of protease inhibitors (Complete EDTA-free proteases inhibitors; Roche Diagnostic, Meylan, France) 1% (v/v), and freshly added 5% (v/v) β-mercaptoethanol. All samples were stored at −20°C until use. Before loading on SDS-polyacrylamide gel, 20 µl of each sample was incubated for 15 min at 65°C.

Samples were loaded on a SDS 10% polyacrylamide gel, then run for 2 h at 100 V in a Mini Protean II electrophoresis apparatus (Bio-Rad, Hercules, Calif.) using 25 m*M* Tris, 192 m*M* glycine and 0.1% SDS (m/v) as running buffer. Proteins were transferred at 4°C with stirring to an Immobilon nitrocellulose membrane (Millipore, Bedford, Mass.) in a Mini Trans-Blot transfer cell (Bio-Rad) using 25 m*M* Tris, 192 m*M* glycine and 20% (v/v) methanol (SDS, Peypin, France) as transfer buffer. Electrotransfer was carried out at 100 V for 1 h. After transfer and checking of protein profiles by staining with ponceau S, the nitrocellulose membrane was washed with PBSMT for 5 min before Western blot analysis.

All the following steps were done with gentle shaking at room temperature. The membrane was saturated in PBSMT overnight, then incubated with C2-10 antibody in PBSMT for 2 h (final dilution 1/200). After three washes of 10 min each in PBSMT, the membrane was incubated for 30 min with anti-IgG antibody in PBSMT (anti-mouse IgG antibody conjugated to peroxidase; Dako; final dilution 1/10,000). Lastly, the blot was washed three times for 10 min in PBS with Tween 20 0.1% (v/v) before application of the chemiluminescent reagent (ECL; Amersham Pharmacia Biotech, Little Chalfont, UK). Chemiluminescence was detected by exposure to Biomax MR-1 film (Kodak, Rochester, N.Y.).

Analysis of DNA fragmentation by agarose gel electrophoresis

Cellular DNA was extracted from adherent and floating treated cells using a DNeasy tissue kit according to the recommendations of the supplier with minor modifications. The main differences consisted in treatment of the cell pellet with 1 µg/ml DNAse-free RNAses (Eurogentec, Seraing, Belgium) before extraction of DNA, and ethanol precipitation of DNA before solubilization in buffer AE. Electrophoresis was performed in a Horizon 11:14 apparatus (Gibco-BRL, Gaithersburg, Md.) containing 1.8% agarose (Bioprobe, Montreuil, France) gel in TBE buffer (Tris-borate 89 m*M*, pH 8.3, EDTA 2 m*M*) at 40 V for 5 h. The DNA ladder was visualized by ethidium bromide staining.

Detection of cellular ROS

Production of ROS was evaluated by the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay as described by Wang and Joseph [23] with some modifications. Briefly, L-929 or PC3 cells were plated at a density of 1×10^4 cells per well in 100 µl culture medium and incubated overnight at 37°C. Cells were then washed once and incubated in 100 µl 1× HBSS/HEPES 15 m*M*, pH 7.4 (HBSS/Hep) containing 20 µ*M* DCFH-DA (Sigma-Aldrich) for 1 h at 37°C. After washing, the cells were incubated again at 37°C in HBSS/Hep alone or containing either 100 µ*M* *t*-butyl hydroperoxide, or the stated concentration of sanguinarine. Fluorescence was measured after 30 min at 485/530 nm.

Evaluation of cellular GSH content

Assay of GSH content was adapted from the method of Hedley and Chow [24]. Briefly, L-929 cells were plated at a density of 1×10^4 cells per well in 100 µl culture medium and incubated overnight at 37°C. Cells were washed with HBSS/Hep and incubated for the stated time with 100 µ*M* diethylmaleate (DEM, positive control) or 0–5 µ*M* test compounds in HBSS/Hep.

The cells were washed with 200 µl HBSS/Hep and incubated again for 15 min in 150 µl HBSS/Hep containing 7.5 µ*M* monochlorobimane (Molecular Probes). Fluorescence was measured at 393/460 nm using an automated 96-well plate reader. The results are expressed as percentage of control after subtraction of blank values.

Evaluation of reactivity with GSH

The ability of sanguinarine to react in vitro with GSH was assayed by the monochlorobimane assay. Briefly, 80 µl GSH at 625 µ*M* in 100 m*M* phosphate buffer, pH 7.4, was distributed to each well. A volume of 20 µl of 0–2.5 m*M* sanguinarine in distilled water was then added and the microplates were incubated at 37°C for 30, 60, 90, or 120 min. To quantify GSH, 10 µl phosphate buffer containing 25 µ*M* monochlorobimane was added before incubation for 30 min at 37°C. Fluorescence at 393/460 nm was measured as previously described, and the results were expressed as percentage of control after subtraction of blank values.

Statistical analysis

Statistical analysis was carried out by analysis of variance with the Bonferroni *t*-test. Significance was assumed for P values <0.05.

Results

Growth-inhibitory activity of sanguinarine in normal human fibroblasts versus human tumour cell lines

Sanguinarine was assayed for its ability to inhibit the growth of both normal human fibroblasts and a panel of solid tumour cell lines by two complementary methods: the RRT and the Hoechst assay. The RRT measured the residual metabolic activity of cultures, and the Hoechst assay, the DNA content in each well and, hence, the corresponding biomass. Sanguinarine showed a broad antiproliferative spectrum but no differential cytotoxicity (cytotoxicity against normal versus cancer cells) (Fig. 2). A narrow range of IC_{50} values were observed (0.9–3.3 µ*M*). The strongest effect was on PC3 prostatic adenocarcinoma cells, the weakest on MCF7 breast cancer adenocarcinoma cells.

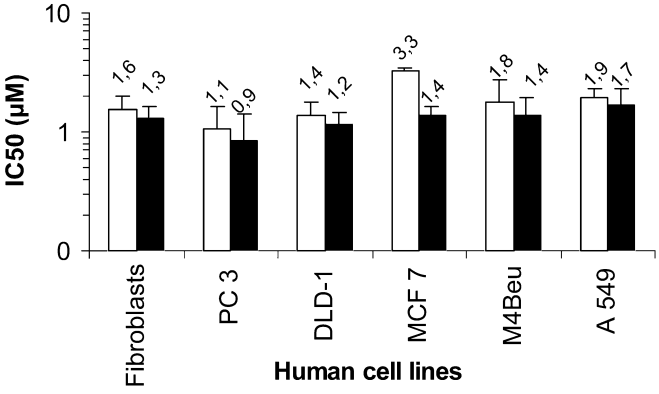

Fig. 2 In vitro growth-inhibitory activity of sanguinarine in various normal and solid tumour cell lines. Cells were treated with various concentrations of sanguinarine (0.15–5 µ*M*) for 48 h. The growth-inhibitory activity of sanguinarine was determined both by the RRT (*open bars*) and in terms of cellular DNA content determined with Hoechst 33342 dye assay (*black bars*) and the IC_{50} values were calculated. Values are the means + SD of three independent determinations

Sanguinarine induces apoptosis in solid tumour cell lines

To investigate whether the growth inhibitory activity was the result of cytolytic or cytostatic activity, morphological changes induced by 48-h continuous treatment with 5 µ*M* sanguinarine were analysed at regular intervals by reversed-phase microscopy. More than 75% of round and shrunken cells were clearly visible after 1 to 4 h in all tested cell lines except A549 lung adenocarcinoma cells, in which no morphological evolution was observed up to 48 h (data not shown). To unambiguously demonstrate that cell death was related to apoptosis, we investigated both the morphology of adherent cells fixed and stained with Giemsa and internucleosomal DNA fragmentation. Assays were carried out on PC3 cells treated with 5 µ*M* sanguinarine. After 2 h, cells showed clear apoptotic characteristics such as cell shrinkage, nuclear material condensation and in some cases nuclear fragmentation (Fig. 3a). The DNA ladder was also clearly observed (Fig. 3b). To determine the kinetics of the apoptotic response, PC3 cells were then investigated for two events recognized as specific to apoptosis: induction of caspases 3 and/or 7 and cleavage of PARP 116 kDa nuclear enzyme in its 85 KDa fragment. Induction of caspases 3/7 was enzymatically assayed using the rhodamine 110-derived substrate Z-DEVD-R110 and PARP cleavage by Western blotting.

Caspase activity was clearly induced 60 min after continuous treatment with 5 µ*M* sanguinarine (Fig. 4a) whereas the fragment related to PARP cleavage was detectable until 30 min after treatment and clearly visible 30 min later (Fig. 3b). PARP cleavage was also observed in DLD-1, M4Beu and MCF7 cell lines (data not shown) 2 h after treatment with 5 µ*M* sanguinarine. These results unambiguously demonstrate that the antiproliferative activity of sanguinarine was related to apoptosis. Dithiothreitol (DTT) has previously been reported to block different sanguinarine-induced activities [11, 12]. To verify whether thiol derivatives interact with apoptotic response, new assays were performed by coincubating PC3 cells with100 µ*M* DTT or 1 m*M* *N*-acetyl cysteine (NAC) before evaluation of apoptosis by phase-contrast microscopy and the PARP cleavage assay. NAC and DTT failed to block the apoptotic response. The more effective compound, DTT, delayed by only 30 min the appearance of the morphological and

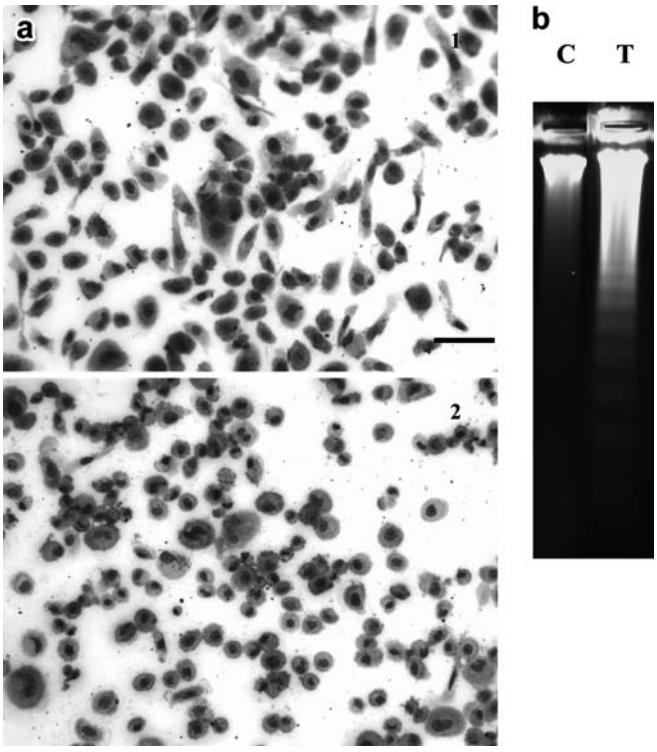

Fig. 3a, b Sanguinarine induces apoptotic features within 2 h in prostatic carcinoma cells PC3. **a** Cells were plated in Petri dishes 40 h before treatment with 5 μ*M* sanguinarine for 0 h (*1*) or 2 h (*2*). Adherent cells were fixed with ethanol and stained with Giemsa solution (*bar* 100 μm). **b** Cells were treated with vehicle (*C*) or 5 μ*M* sanguinarine (*T*), and 2 h later adherent and floating cells were collected by trypsinization. Cellular DNA was isolated and subjected to agarose gel (1.8%) electrophoresis. The DNA ladder was visualized by ethidium bromide staining

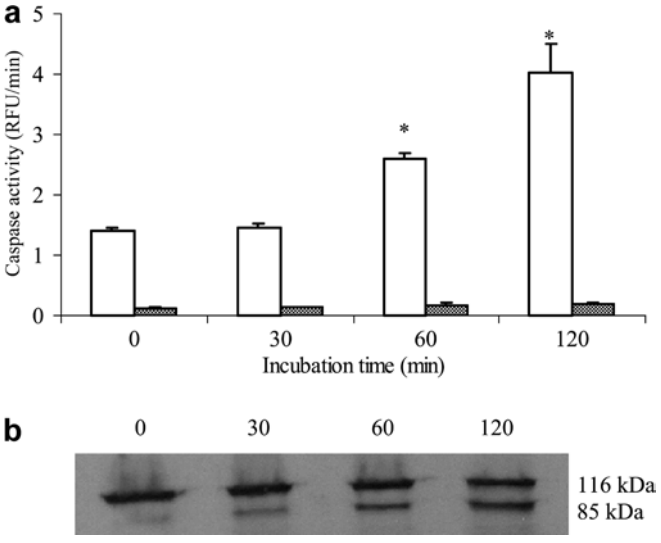

Fig. 4a, b Sanguinarine treatment induces caspase 3- or 7-dependent apoptosis in PC3 cells with PARP cleavage. Cells were plated in Petri dishes 40 h before treatment with 5 μ*M* sanguinarine for 0, 30, 60 or 120 min. Adherent and floating cells were harvested and half of each suspension was pelleted by centrifugation. Caspase 3/7 activity (**a**) with (*grey bars*) or without (*white bars*) specific inhibitor ac-DEVD-CHO (each condition in sextuplicate) and PARP cleavage (**b**) were investigated as described in Materials and methods on one pellet each. The results presented are the means + SD of six determinations and are representative of two independent experiments. $*P < 0.05$ versus time 0

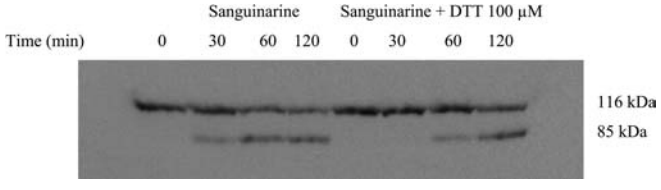

Fig. 5 Sanguinarine-induced apoptosis in PC3 cells is independent of cotreatment with thiol-containing compounds. PC3 cells were plated in Petri dishes 40 h before treatment. Cells were treated with 5 μ*M* sanguinarine alone or in combination with 100 μ*M* dithiothreitol for 0, 30, 60 or 120 min. Adherent and floating cells were harvested and PARP cleavage was investigated as described in Materials and methods. The results presented are representative of two independent experiments

biochemical markers of apoptosis. PARP cleavage was delayed by 30 min (Fig. 5) and the first round cells were observed 60 min after treatment instead of 30 min when sanguinarine was used alone (data not shown). These results show that the apoptotic response induced by sanguinarine was only partially sensitive to DTT.

Sanguinarine initiates GSH depletion by direct chemical interaction rather than by inducing ROS generation. Sanguinarine has been reported to be responsible for "epidemic dropsy", a fatal syndrome observed in India resulting from ingestion of edible mustard oil contaminated by argemone seeds [25]. Banerjee et al. have demonstrated a dose-dependent relationship between two markers of oxidative stress (especially GSH depletion) in erythrocytes and the presence of measured sanguinarine levels in serum of patients poisoned by argemone oil [26]. As a chemically induced GSH depletion has recently been reported to be apoptogenic [27, 28], we hypothesized that GSH depletion was responsible for apoptosis induced by sanguinarine. To test this hypothesis, both concentration- and time-dependent changes in intracellular GSH were assayed using monochlorobimane as fluorescent dye. It is well known that the monochlorobimane assay is more specific for GSH in rodent than in human cells [29]. We therefore performed all assays in the L-929 immortalized murine cell line in which the L-buthionine sulphoximine-dependent cellular thiol content represents more than 90% of the monochlorobimane-reacting species (Debiton E, personal communication). It was verified that this cell line exhibits similar sensitivity to sanguinarine treatment (IC_{50} 1.2 ± 0.4 and 0.9 ± 0.3 μ*M* by RRT and Hoechst assay, respectively) and similar kinetics for PARP cleavage to PC3 cells (data not shown).

A clear drop in GSH content was observed during a 30-min incubation with cytotoxic concentrations of sanguinarine (2.5 and 5 μ*M*), whereas no modification in cellular GSH was found after treatment at a safe concentration (0.25 μ*M*; Fig. 6a). DEM was used as a positive control at 100 μ*M* because previous data had

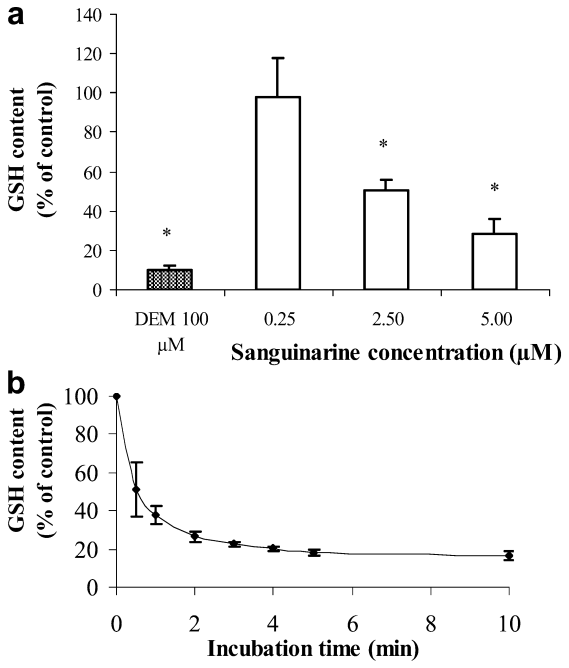

Fig. 6a, b Sanguinarine treatment induces a rapid concentration-dependent depletion of cellular GSH in L-929 cells. **a** Cells were treated for 30 min with 100 µ*M* diethylmaleate (grey bar) or the indicated concentrations of sanguinarine (*white bars*). The protocol was the same as above. The results presented are the means + SD of six determinations and are representative of two independent experiments. **P* < 0.05 versus vehicle treatment. **b** L-929 cells were plated in 96-well microplates 40 h before treatment with 5 µ*M* sanguinarine for the indicated times. Treatment solutions were removed and the cell monolayer was rinsed. Monochlorobimane solution was then added and the plates were incubated for a further 15 min before measurement of fluorescence on a microplate reader

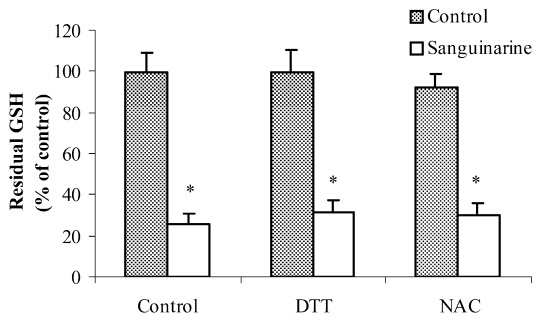

Fig. 7 Sanguinarine-induced depletion of cellular GSH is independent of thiol-containing compound in L-929 cells. L-929 cells were plated in 96-well microplates 16 h before treatment. Cells were treated with vehicle (*Control*), dithiothreitol 100 µ*M* (*DTT*) or 1 mM *N*-acetyl cysteine (*NAC*) with (*white bars*) or without (*grey bars*) 5 µ*M* sanguinarine for 30 min. Treating solutions were removed and the cell monolayer was rinsed. Monochlorobimane solution was then added and the plates were incubated for a further 15 min before measurement of fluorescence on a microplate reader. The results presented are the means + SD of six determinations and are representative of two independent experiments. **P* < 0.05 versus vehicle treatment

shown that at this concentration, DEM induces GSH depletion followed by apoptotic cell death in PC3 cells [27]. When sanguinarine was used at a concentration of 5 µ*M*, less than 50% of initial GSH content remained 1 min after the addition of sanguinarine, and levels of 20–30% were maintained after 5 min (Fig. 6b). Co-treatment with thiol compounds (DTT at 100 µ*M* or NAC at 1 m*M*) failed to prevent GSH depletion (Fig. 7). These results demonstrate that at cytotoxic concentrations, sanguinarine induced an early and severe depletion of cellular GSH which was not counteracted by thiol-reducing compounds. To verify whether this depletion was not a result of damage to the cell membrane, the viability of cells treated for 5 min with sanguinarine were compared with that of control cells using trypan blue. No difference in trypan blue uptake was observed between treated and control cells, indicating that at this time-point, neither loss in cell viability nor modification in membrane integrity had occurred (data not shown).

New experiments were done to investigate the mechanism(s) involved in the GSH decrease. Whether this loss in redox potential was related to an induction of oxidative stress was first evaluated. Experiments were done first in L-929 cells and then in PC3 cells submitted to continuous treatment with 5 µ*M* sanguinarine with 100 µ*M* *t*-butyl hydroperoxide, a well-known peroxide inducer, as a reference treatment. ROS were assayed by the DCFH-DA assay. No significant increase in peroxides was observed during 30 min incubation with sanguinarine (data not shown), demonstrating that neither apoptosis nor GSH depletion were due to ROS generation. These results suggest that the early GSH depletion observed after sanguinarine treatment might be related to direct interaction of sanguinarine with GSH. To test this hypothesis, we used an adapted monochlorobimane assay that permitted direct determination of residual GSH out of the cell environment. Sanguinarine was found to induce a profound depletion in monochlorobimane-reactive GSH comparable to that induced by DEM under these experimental conditions. The decrease was time- and concentration-dependent (Fig. 8). After the addition of 0.25 m*M* sanguinarine, a gradual decrease in free GSH was observed. Approximately 35% of the initial GSH content remained after 120 min versus 20% with 2 m*M* DEM (Fig. 8a). No decrease in free GSH was observed with L-buthionine sulphoximine, that depletes nonreactive GSH, in cells used in this assay as a negative control. An isomolar mixture of sanguinarine/GSH (0.5 m*M*) showed 20% residual monochlorobimane-reactive species at 120 min of incubation (Fig. 8b). Taken together these results are in accordance with our hypothesis of a direct interaction between sanguinarine and GSH.

Discussion

This study demonstrated that: (1) at low concentrations, sanguinarine strongly inhibited the growth of all tested tumour and normal cell lines, and (2) this effect was

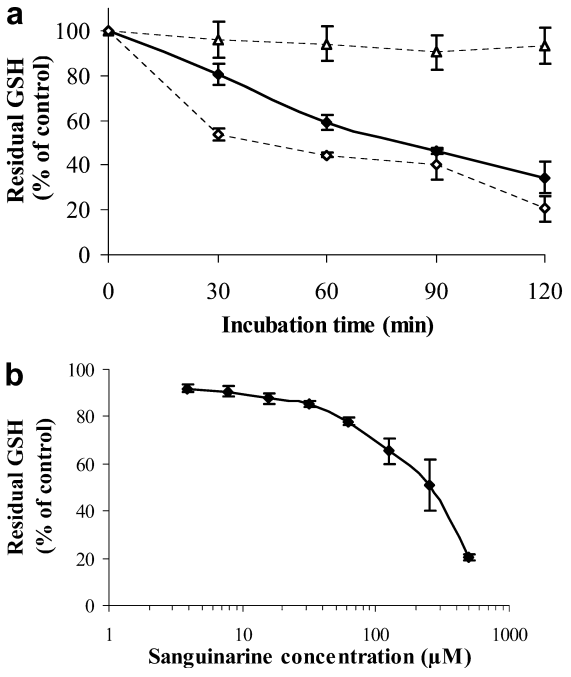

Fig. 8a, b Sanguinarine reacts directly with GSH in a time- and concentration-dependent manner. GSH solution (final concentration 500 μ*M*) and vehicle (*control*), sanguinarine (final concentration 0.25 m*M* (♦), 2 m*M* L-buthionine sulphoximine (△) or 2 m*M* diethylmaleate (◇) were subsequently added to each well. Microplates were incubated for the indicated times at 37°C. Monochlorobimane solution (final concentration 25 μ*M*) was then added and the plates were incubated for a further 30 min before measurement of fluorescence on a microplate reader. **b** GSH and vehicle (*control*) or sanguinarine (2.5–500 μ*M*) were subsequently added to each well. The protocol was the same as above. The results presented are means ± SD of six determinations and are representative of two independent experiments

related to a rapid apoptotic response subsequent to GSH depletion. We observed a concentration-dependent cytotoxicity in a narrow range of concentrations (0.5–3 μ*M*) with IC_{50} values for growth inhibition (Hoechst assay) and residual metabolic activity (RRT) within 1–2 μ*M* in each cellular model. These values are in agreement with those recently reported in cervix adenocarcinoma HeLa cells [15]. The higher IC_{50} value observed in MCF7 after RRT might be due to specific behaviour of this cell line in this assay (similar results have been observed with other drugs), which is still unexplained. Recently, Ahmad et al. have reported a more pronounced cytotoxic effect in cancer versus normal cells [2]. In our experiments, normal human fibroblasts showed sensitivity similar to immortalized and tumour cell lines, and no differential cytotoxicity was observed.

Our work also demonstrated that sanguinarine induces a caspase 3- or 7-dependent apoptotic response in PC3 cells. Weerasinghe et al. have reported a concentration-dependent bimodal cell death in K562 leukaemia cells after a 2-h sanguinarine treatment [7, 8]. In the concentration range used, we observed only apoptotic cells. No cells with the single blister morphology were found. This is consistent with reports of this specific cellular morphology in cells treated with high concentrations of sanguinarine [30].

To date, the underlying mechanisms in sanguinarine-induced apoptosis have not been elucidated. In PC3 cells, both induction of caspases 3/7 and PARP cleavage were observed with up to 1 h of treatment, followed by DNA ladder formation 1 h later. Such kinetics of the apoptotic response are unusual in non-haematological cells treated with chemicals, and to our knowledge have been previously observed only after hyperthermia [31] or after microinjection of Bax [32]. As Bax activation has been reported to be involved in the sanguinarine-induced apoptotic cascade, sanguinarine should target a very efficient upstream Bax activation system [8]. We observed that the thiol-reducing agents DTT and NAC failed to prevent cell death induced by sanguinarine, even when they were used at high concentrations. This was unexpected because many (if not all) of the known biochemical effects of sanguinarine treatment are counteracted by DTT.

We also demonstrated that sanguinarine treatment depleted GSH content in L-929 murine cells (treated with 5 μ*M* sanguinarine) in less than 10 min. GSH depletion has been reported previously only in cultured hepatocytes treated with high concentrations of this drug (25–100 μ*M*) [5]. In this latter study, GSH depletion appeared to be correlated with an increased membrane permeability (identified by lactate dehydrogenase enzyme leakage). In our experiments, a drastic drop in monochlorobimane-reacting species was observed at low concentrations (2.5 and 5 μ*M*), and no modification in membrane permeability was detected. Cells treated with 5 μ*M* sanguinarine showed, after a 5-min treatment, a residual GSH content which represented approximately 15–20% of the control content. As mitochondrial GSH represents approximately 15% of total cellular GSH [28], our results suggest that sanguinarine completely depletes the cytosolic GSH pool but spares the mitochondrial one. This hypothesis is strengthened by the fact that sanguinarine, a quaternary ammonium alkaloid, acts as a pseudobase and is probably completely ionized at acidic pH in the mitochondrial intermembrane space, and is therefore not capable of reaching the GSH-rich matrix.

The failure to reverse this depletion by cotreatment with the thiol-reducing compounds DTT and NAC at high concentrations showed that GSH depletion is induced by another mechanism than most of its biological actions previously described. Complementary experiments in L-929 and PC3 cells indicated that the mechanism is not generation of ROS. This result is in agreement with the observations of Ulrichova et al. who reported that no lipid peroxidation (a well-known consequence of ROS production) occurs in treated hepatocytes [5]. Our results clearly demonstrate that sanguinarine is able to react directly with GSH at physiological pH in a concentration- and time-dependent manner. In this cell-free assay, the concentrations

of sanguinarine used to induce a significant inhibition of monochlorobimane-reactive GSH are approximately ten times those used in the cell environment. In our opinion, this could be interpreted as an enzyme-catalysed process in the cellular assay. Glutathione peroxidase or transferase, or both, probably play a role in this process.

It has previously been reported that in aqueous solutions around pH 7.4, sanguinarine is mostly present in a reactive quaternary cation form [15]. This iminium form is known to be highly sensitive to nucleophilic attack by thiols. In our opinion, such an attack could be the main mechanism involved in the ability of sanguinarine to inhibit certain thiol-containing protein activities. We also hypothesized that sanguinarine in its iminium form can react with GSH, and that this reaction is (at least partly) responsible for both the early step of intracellular GSH depletion induced by treatment and the subsequent promotion of apoptotic cell death via caspase 3/7 activation. This is in accordance with recent data indicating (1) depletion of intracellular thiols, especially GSH, as a mechanism involved in the apoptotic response of tumour cells treated in vitro with certain chemicals or naturally occurring substances [27, 33], or (2) that a thiol-reducing agent independent of GSH depletion may be a possible initial stimulus for hyperthermia-induced apoptosis in rat histiocytoma [34].

The complete signalling cascade by which sanguinarine treatment induces apoptotic cell death is still unknown. However, we hypothesized that the signalling pathway involved in GSH depletion-induced apoptotic cell death could be a new target for anticancer agents. Indeed, our results suggest that a drug inducing strong GSH depletion may be of interest for human cancer therapy. However, we consider that only drugs exhibiting a clear specificity for cancer cells are good candidates. Others, such as sanguinarine, may induce marked lung toxicity, as does BCNU a well-known glutathione reductase inhibitor, and have to be discarded.

References

1. Eley BM (1999) Antibacterial agents in the control of supragingival plaque—a review. Br Dent J 186:286
2. Ahmad N, Gupta S, Husain MM, Heiskanen KM, Mukhtar H (2000) Differential antiproliferative and apoptotic response of sanguinarine for cancer cells versus normal cells. Clin Cancer Res 6:1524
3. Babich H, Zuckerbraun HL, Barber IB, Babich SB, Borenfreund E (1996) Cytotoxicity of sanguinarine chloride to cultured human cells from oral tissue. Pharmacol Toxicol 78:397
4. Nakanishi T, Suzuki M, Saimoto A, Kabasawa T (1999) Structural consideration of NK109, an antitumor benzo[c]phenanthridine alkaloid. J Nat Prod 62:864
5. Ulrichova J, Dvorak Z, Vicar J, Lata J, Smrzova J, Sedo A, Simanek V (2001) Cytotoxicity of natural compounds in hepatocyte cell culture models. The case of quaternary benzo[c]phenanthridine alkaloids. Toxicol Lett 125:125
6. Vavreckova C, Gawlik I, Muller K (1996) Benzophenanthridine alkaloids of *Chelidonium majus*; II. Potent inhibitory action against the growth of human keratinocytes. Planta Med 62:491
7. Weerasinghe P, Hallock S, Liepins A (2001) Bax, Bcl-2 and NF-$\varkappa$B expression in sanguinarine induced bimodal cell death. Exp Mol Pathol 71:89
8. Weerasinghe P, Hallock S, Tang SC, Liepins A (2001) Role of Bcl-2 family proteins and caspase-3 in sanguinarine-induced bimodal cell death. Cell Biol Toxicol 17:371
9. Seifen E, Adams RJ, Riemer RK (1979) Sanguinarine: a positive inotropic alkaloid which inhibits cardiac Na^+, K^+-ATPase. Eur J Pharmacol 60:373
10. Wang BH, Lu ZX, Polya GM (1997) Inhibition of eukaryote protein kinases by isoquinoline and oxazine alkaloids. Planta Med 63:494
11. Chaturvedi MM, Kumar A, Darnay BG, Chainy GB, Agarwal S, Aggarwal BB (1997) Sanguinarine (pseudochelerythrine) is a potent inhibitor of NF-kappaB activation, IkappaBalpha phosphorylation, and degradation. J Biol Chem 272:30129
12. Schmeller T, Latz-Bruning B, Wink M (1997) Biochemical activities of berberine, palmatine and sanguinarine mediating chemical defence against microorganisms and herbivores. Phytochemistry 44:257
13. Hu CM, Cheng HW, Cheng YW, Kang JJ (2000) Induction of skeletal muscle contracture and calcium release from isolated sarcoplasmic reticulum vesicles by sanguinarine. Br J Pharmacol 130:299
14. Nandi R, Maiti M (1985) Binding of sanguinarine to deoxyribonucleic acids of differing base composition. Biochem Pharmacol 34:321
15. Slaninova I, Taborska E, Bochorakova H, Slanina J (2001) Interaction of benzo[c]phenanthridine and protoberberine alkaloids with animal and yeast cells. Cell Biol Toxicol 17:51
16. Wolff J, Knipling L (1993) Antimicrotubule properties of benzophenanthridine alkaloids. Biochemistry 32:13334
17. Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901
18. Guedon D, Cappelaere N, Simanek V (1990) HPLC analysis of the main alkaloids from *Eschscholzia californica* Cham. Phytochem Anal 1:77
19. Jacubovich R, Cabrillat H, Gerlier D, Bailly M, Doré JF (1985) Tumourigenic phenotypes of human melanoma cell lines in nude mice determined by an active antitumour mechanism. Br J Cancer 51:335
20. O'Brien J, Wilson I, Orton T, Pognan F (2000) Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 267:5421
21. Rago R, Mitchen J, Wilding G (1990) DNA fluorometric assay in 96-well tissue culture plates using Hoechst 33258 after cell lysis by freezing in distilled water. Anal Biochem 191:31
22. Sallman FR, Bourassa S, Saint-Cyr J, Poirier GG (1997) Characterisation of antibodies specific for the caspase cleavage site on poly(ADP-ribose) polymerase: specific detection of apoptotic fragments and mapping of the necrotic fragments of poly(ADP-ribose) polymerase. Biochem Cell Biol 75:451
23. Wang H, Joseph JA (1999) Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 27:612
24. Hedley DW, Chow S (1994) Evaluation of methods for measuring cellular glutathione content using flow cytometry. Cytometry 15:349
25. Thatte U, Dahanukar S (1999) The Mexican poppy poisons the Indian mustard facts and figures. J Assoc Physicians India 47:332
26. Banerjee BD, Seth V, Koner BC, Ahmed RS, Sharma M, Grover SS, Rautola RS, Avasthi R, Pasha ST (2000) Evaluation of oxidative stress in some cases of argimone oil poisoning during a recent outbreak of epidemic dropsy in India. Int J Environ Health Res 10:341
27. Coffey RNT, Watson RWG, Hegarty NJ, O'Neill A, Gibbons N, Brady HR, Fitzpatrick JM (2000) Thiol-mediated apoptosis in prostate carcinoma cells. Cancer 88:2092

28. Hall AG (1999) The role of glutathione in the regulation of apoptosis. Eur J Clin Invest 29:238
29. Ublacker GA, Johnson JA, Siegel FL, Mulcahy RT (1991) Influence of glutathione S-transferases on cellular glutathione: determination by flow cytometry using monochlorobimane. Cancer Res 51:1783
30. Liepins A, Nowicky JW, Bustamante JO, Lam E (1996) Induction of bimodal programmed cell death in malignant cells by the derivative Ukrain. Drugs Exp Clin Res 22:73
31. Barry MA, Behnke C, Eastman A (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol 40:2353
32. Shimizu S, Matsuoka Y, Shinoara Y, Yoneda Y, Tsujimoto Y (2001) Essential role of voltage-dependent anion channel in various forms of apoptosis in mammalian cells. J Cell Biol 152:237
33. Liu J, Shen HM, Ong CN (2001) Role of intracellular thiol-depletion, mitochondrial dysfunction and reactive oxygen pecies in *Salvia miltiorrhiza*-induced apoptosis in human hepatoma HepG2 cells. Life Sci 69:1833
34. Sreedhar AS, Pardhasaradhi BVV, Khar A, Srinivas UK (2002) A cross talk between cellular signalling and cellular redox state during heat-induced apoptosis in rat histiocytoma. Free Radic Biol Med 32:221