Hyperthermia enhances cytotoxicity of amine oxidase and spermine on drug-resistant LoVo colon adenocarcinoma cells

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Abstract. Hyperthermia is currently receiving widespread attention when associated with other therapeutic modalities, such as irradiation or chemotherapy, in the treatment of cancer. The occurrence of resistance to cytotoxic pharmacological agents in tumor cells, associated with several phenotypic alterations, is one of the major obstacles to successful anticancer chemotherapy. We investigated a new strategy to overcome multidrug resistance (MDR) cancer cells, using bovine serum amine oxidase (BSAO), which forms toxic products from spermine (H_2O_2 and aldehydes). The cytotoxicity of the products was evaluated in drug-sensitive (LoVo WT) and multidrug-resistant (LoVo DX) colon adenocarcinoma cells at 37 and 42˚C, using a clonogenic cell survival assay. Cytotoxicity was considerably enhanced at 42˚C. Both toxic species contributed to the thermal enhancement of cytotoxicity induced by BSAO and spermine. Cytotoxicity was eliminated in the presence of catalase and aldehyde dehydrogenase (ALDH). An interesting finding was that BSAO and spermine at <1 μM, which were non toxic at 37˚C, became cytotoxic at 42˚C and resemble thermosensitizers. Cell survival results and electron microscopy investigations suggest that, at 42˚C, LoVo DX cells are not resistant to the cytotoxic enzymatic oxidation products of spermine, as was already demonstrated in these cells at 37˚C. Moreover, microscopy modifications caused by both toxic products were more pronounced in LoVo DX than in LoVo WT cells, where morphological cytoplasmatic alterations were shown. Our findings suggest that hyperthermia combined with the enzymatic toxic oxidation products of spermine might be a promising anticancer strategy, mainly against MDR tumor cells.

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Introduction

Hyperthermia has been used in cancer treatment for many years and it represents the treatment of tumor cells by high temperatures, usually in the range of 42-45˚C (1). The effect of hyperthermia is particularly beneficial against tumors when used with radiotherapy and chemotherapy (2). The combination of hyperthermia with chemotherapy appears to be useful because localized heating may provide an increase in drug cytotoxicity within a defined target region. The magnitude of the enhancement of drug cytotoxicity by hyperthermia varies with different drugs and with the temperature used (1). In fact, heat induces irreversible biological damage and drug interactions are probably due to several possible targets in cells, such as cellular membrane systems, enzymes-proteins and nucleic acids.

Several *in vitro* and *in vivo* studies have shown the benefits of hyperthermia in association with radiochemotherapy against various cancer types. Furthermore, hyperthermia is showing promising results in clinical oncology, particularly in Europe (2,3). Considerable progress has been made in recent years in the development of improved methods of heat delivery (3).

Multiple drug resistance has been investigated extensively because it is one of the major obstacles to successful chemotherapy of metastatic diseases (4,5). The development of resistance to one anticancer drug frequently gives rise to resistance to numerous other structurally and functionally unrelated chemotherapeutic compounds (6). This phenomenon, called multidrug resistance (MDR), is associated with a number of phenotypic alterations. Cancer cells with classical MDR usually display decreased intracellular drug accumulation and/or drug distribution (6-8). These modifications are generally due to overexpression of genes which encode membrane-bound transporter proteins, such as a 170-kDa P-glycoprotein (P-gp), multidrug resistance associated protein (MRP) and lung resistance protein (LRP) (9,10). These membrane glycoproteins behave as energy-dependent efflux pumps which are capable of expelling a large variety of xenobiotics, including several anticancer drugs, from cells.

In recent years, numerous *in vitro* and *in vivo* studies have been aimed at the development of chemosensitizers to overcome the drug resistance phenotype. Several chemical

compounds, such as verapamil, etoposide, quinidine, trifluoroperazine and the immunosuppressive agent cyclosporine A, can partially reverse MDR by reducing drug efflux from cells. Various clinical attempts using cyclosporine A (11), verapamil and etoposide have described their roles in reversing or modulating P-gp activity (12). Unfortunately, the high amounts of these substances required to overcome MDR in patients are very toxic, causing undesirable side effects such as hypotension, heart block and myelosuppression. Thus, continued research is required to find more effective clinical applications for overcoming MDR due to P-gp.

Among these methods, a biological strategy to enhance the therapeutic effects of hyperthermia is to use heat together with pharmacological agents that become much more cytotoxic at high temperatures. These compounds, such as cysteamine and aminothiol N-(2-mercaptoethyl)-1,3-propanediamine (WR-1065), defined as thermosensitizers, are not toxic at 37˚C but become potent cell inactivators at elevated temperatures (13). Another group of drugs, all of which were considered to be heat sensitizers, are the naturally occurring polyamines, putrescine, spermine and spermidine (14).

Our study suggests a new anticancer strategy using bovine serum amine oxidase (BSAO, EC 1.4.3.6), a copper enzyme of 170 kDa MW, which oxidatively deaminates polyamines containing primary amino groups, such as spermidine and spermine. The enzymatic reaction, following a transaminase-type mechanism, produces an aminoaldehyde or an aminodialdehyde respectively, H_2O_2 and ammonia (15,16). It also involves dioxygen and water as substrates. In the case of spermine, there is controversy concerning the chemical nature of the aldehyde(s) formed in this reaction; it has been demonstrated that both monoaldehyde and dialdehyde are generated (15), the dialdehyde is unstable, and a further breakdown product appears to be acrolein (CH_2=CHCHO) (17). Acrolein was found to be a major toxic product formed from spermidine and spermine by amine oxidase and it accumulated in the plasma of patients with chronic renal failure (18).

Enzymatic oxidation products of polyamines (aldehyde(s) and H_2O_2) have been implicated in programmed cell death (19), inhibition of DNA and protein synthesis (20,21), apoptosis (22) and inhibition of mammalian cell proliferation (23-25). We previously demonstrated that both H_2O_2 and aldehyde(s), formed by purified BSAO in the presence of exogenous spermine, were involved in cytotoxicity on several cultured cell lines (23-25). An interesting result is that the BSAO/spermine enzymatic system was able to induce higher cytotoxicity in multidrug-resistant (MDR) cells with overexpression of P-glycoprotein than in their wild-type counterparts (25-27).

In this study, we demonstrate the cytotoxicity induced at 42˚C by the enzymatic oxidation products of exogenous spermine in drug-sensitive human colon adenocarcinoma (LoVo WT) cells and in multidrug-resistant (LoVo DX) cells that overexpress P-glycoprotein. Since promising results are appearing from clinical investigations involving heat associated with chemotherapy (28), useful effects could also be obtained using heat together with bovine serum amine oxidase in the presence of biogenic amines. The results observed in LoVo DX cells, following this combined strategy, are compared to those obtained in drug-sensitive LoVo WT cells, in order to evaluate whether there are differential effects in cytotoxic responses and whether H_2O_2 and aldehyde(s) play a role in the modulation of multidrug resistance. Moreover, cell survival data and both scanning and transmission electron microscopy observations suggest that, at 42˚C, the LoVo multidrug-resistant phenotype is not resistant to the cytotoxic enzymatic oxidation products of spermine, as was already demonstrated in these cells at 37˚C (25,27) and also in multidrug-resistant Chinese hamster ovary (CHO) cells (CH^RC5) (26). These findings suggest that the purified copper-enzyme, in the presence of spermine and hyperthermia, could prove to be useful in cancer treatment.

Materials and methods

Purification of BSAO. Bovine blood was withdrawn at a slaughterhouse, mixed with 3.8% sodium citrate solution (an anticoagulant) and then treated according to Turini *et al* (29) to purify the copper enzyme amine oxidase. Some modifications were added to the method: a CM-Cellulose column, equilibrated with phosphate-buffer (0.01 M) at pH 5.8, to remove haemoglobin, followed by an AE-Agarose column, in phosphate-buffer (0.01 M) at pH 7.2 to eliminate the blue copper enzyme ceruloplasmin and then two ionic exchange chromatographies were performed using a Q-Sepharose column equilibrated with phosphate-buffer (0.025 M) at pH 6.8 and a Q-Sepharose column, in phosphate-buffer (0.02 M) at pH 8.0. The enzyme was eluted highly purified with an NaCl gradient. All purification steps were carried out in a cold room, at 4°C.

The BSAO purification factor was approximately 1,600-fold and a single band was obtained on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Enzymatic activity was assayed spectrophotometrically at 25˚C by monitoring the formation of benzaldehyde at 250 nm (ε = 12,500 $M^{-1} \cdot cm^{-1}$). The specific activity of BSAO was 0.38 IU/mg, with international unit (IU) defined as micromoles of benzylamine oxidized per minute. The protein concentration was determined by absorbance at 280 nm, using an absorption coefficient of 1.74 $l \cdot g^{-1} \cdot cm^{-1}$.

Cell cultures. In this study, a human colon adenocarcinoma cell line (LoVo WT), isolated from a metastatic nodule, and its MDR variant (LoVo DX) were used. Both cell lines were grown in monolayer in Ham's F12 medium (Gibco BRL/Life Technologies Ltd., Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Cramlington, UK), 1% L-glutamine (Gibco BRL/Life Technologies Ltd.), 1% penicillin (50 U/ml)-streptomycin (50 μg/ml) (Gibco BRL/Life Technologies Ltd.), 1% vitamins (Gibco BRL/Life Technologies Ltd.) in a humidified atmosphere of 5% CO_2 in a water-jacketed incubator at 37˚C. The pleiotropic MDR cell line, LoVo DX, was selected for resistance to doxorubicin (DOX) (Adriblastina; Pharmacia & Upjohn, Milan, Italy) from its drug-sensitive parental LoVo cell line (30). LoVo DX cells are also resistant to other chemotherapeutic agents, such as etoposide and vincristine (31).

Treatments and cell survival experiments. Cell survival experiments were carried out using semiconfluent cells that

had been incubated for 24 h at 37˚C with fresh culture medium. Cells were harvested with 10 mM EDTA in phosphate-buffered saline (PBS) and then by addition of 0.25% trypsin solution in PBS, washed by centrifugation (2 min, 1,500 g) and resuspended in PBS supplemented with 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO). Freshly harvested LoVo cells (10^5/ml) were incubated at 42˚C for different time intervals in the presence of the following reagents, used alone or in association: BSAO (17.06 μg/ml corresponding to 1.00×10^{-4} μmoles/ml or 6.98×10^{-3} U/ml), spermine (6 and 12 μM), catalase (240 U/ml) from bovine liver (Sigma), ALDH (EC 1.2.1.5) from yeast (0.4 U/ml) and NAD^+ (1.8 μg/ml; Boehringer-Mannheim, Mannheim, Germany). Spermine (Fluka, Buchs, Switzerland) was freshly prepared before each experiment and, if present, added last. Cells were then centrifuged, washed twice in PBS-BSA and finally resuspended in 1 ml of PBS-BSA.

Cell suspensions were then plated in tissue culture-coated Petri dishes and incubated at 37˚C. The sensitive colony survival assay that determines the ability of the cells to reproduce and form macroscopic colonies in culture (>50 cells), was chosen as the index of cytotoxicity. After 20 days, colonies were fixed with 96% ethanol, stained with methylene blue and counted manually. Control plating efficiencies at 42˚C were higher than 82% and 78% for LoVo WT and LoVo DX cells, respectively. Percent cell survival was determined as the ratio between the mean number of colonies in treated and control sample.

Scanning electron microscopy. For scanning electron microscopy (SEM), cells were grown to near confluence on glass coverslips. The experiments were carried out by incubating the cells in Ham's F12 medium containing 1% BSA, without serum, at 42˚C for different times with BSAO alone (6.98×10^{-3} U/ml), spermine alone (6 or 12 μM), and BSAO and spermine together. Untreated cells grown at 37˚C were used as controls. After incubation, cells were washed with F12 medium and then processed for SEM observation as previously described (32). The samples were examined using a Cambridge Stereoscan 360 scanning electron microscope (Cambridge Instruments, Cambridge, UK).

Transmission electron microscopy. For transmission electron microscopy (TEM), cells were grown to near confluence and harvested as above described, then washed, centrifuged and resuspended in 2 ml of F12 medium-1% BSA, without serum. The cells were incubated for 60 min at 42˚C in the presence of BSAO alone (6.98×10^{-3} U/ml), with exogenous spermine alone (6 or 12 μM) or with both BSAO and spermine. Untreated cells grown at 37˚C were used as controls. After incubation, cells were washed with F12 medium and then processed for TEM observation as previously described (32). TEM observations were performed using a Philips 2085 electron microscope (FEI Company, Eindhoven, The Netherlands).

Results

It has been demonstrated that polyamines, such as spermine and spermidine, inhibit cell growth in medium containing fetal calf serum. This finding suggested that a toxic effect

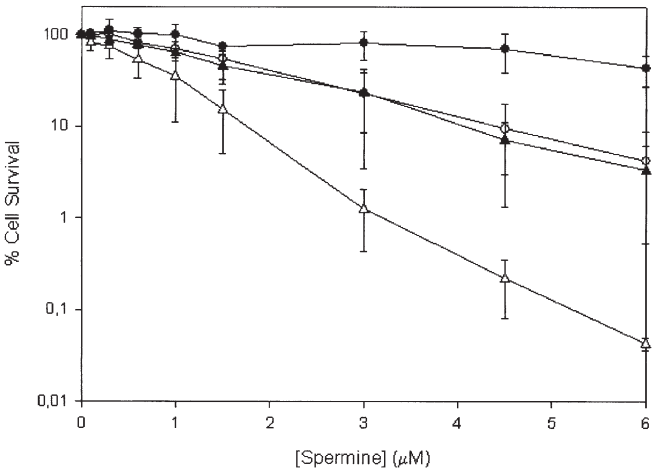

Figure 1. Effect of exogenous spermine concentration on percentage cell survival in the presence of purified BSAO (6.98×10^{-3} U/ml) in LoVo WT (circles) and LoVo DX (triangles) cells during 60 min at 37˚C (solid symbols) and 42˚C (open symbols). Means and standard deviations (SDs) are shown for 2-5 estimations from 4-6 experiments. When not shown, SDs lie within symbols.

was mediated by H_2O_2 and aldehyde(s) formed from a copper amine oxidase activity normally present in mammalian serum (21,33). In this study we evaluated by a plating efficiency test and by scanning and transmission electron microscopy, the cytotoxic responses of P-glycoprotein-overexpressing human colon adenocarcinoma LoVo DX cells to the hydrogen peroxide and aldehyde(s) products generated during the enzymatic oxidation reaction of spermine. Fetal bovine serum containing a low copper amine oxidase activity was omitted from the incubation medium in these experiments. In fact, the cytotoxicity induced by purified BSAO, in the presence of exogenous spermine, was assayed in PBS-1% bovine serum albumin, and it was detected *in vitro* in MDR cells and compared to that observed in their drug-sensitive LoVo WT counterparts, as a function of spermine concentration as well as exposure time. In addition, the cell survival and morphological changes of sensitive and resistant LoVo cells were investigated by carrying out the treatment in both normo (37˚C) and hyperthermic (42˚C) conditions.

The pleiotropic MDR colon adenocarcinoma LoVo DX cells and the drug-sensitive parental LoVo WT cells were previously characterized for their sensitivity to DOX and for expression of the transporter molecule, P-gp (34). Briefly, LoVo DX cells were approximately 100 times more resistant to DOX than LoVo WT cells. Moreover, LoVo WT cells were negative as concerns the surface P-gp expression analysed by flow cytometry, while MDR variants gave a high level of P-gp labelling. In fact, the intensity of their fluorescent signal increased approximately 2-log as compared to the sensitive cells.

Fig. 1 shows the percent cell survival of both LoVo WT and LoVo DX cells incubated for 60 min with exogenous spermine (0-6 μM) in the presence of BSAO (6.98×10^{-3} U/ml), at 37˚C or 42˚C. In both cell lines, cytotoxicity increased as a function of spermine concentration up to 6 μM. There was considerable enhancement of cytotoxicity at 42˚C, compared to 37˚C. At both temperatures, at the highest spermine

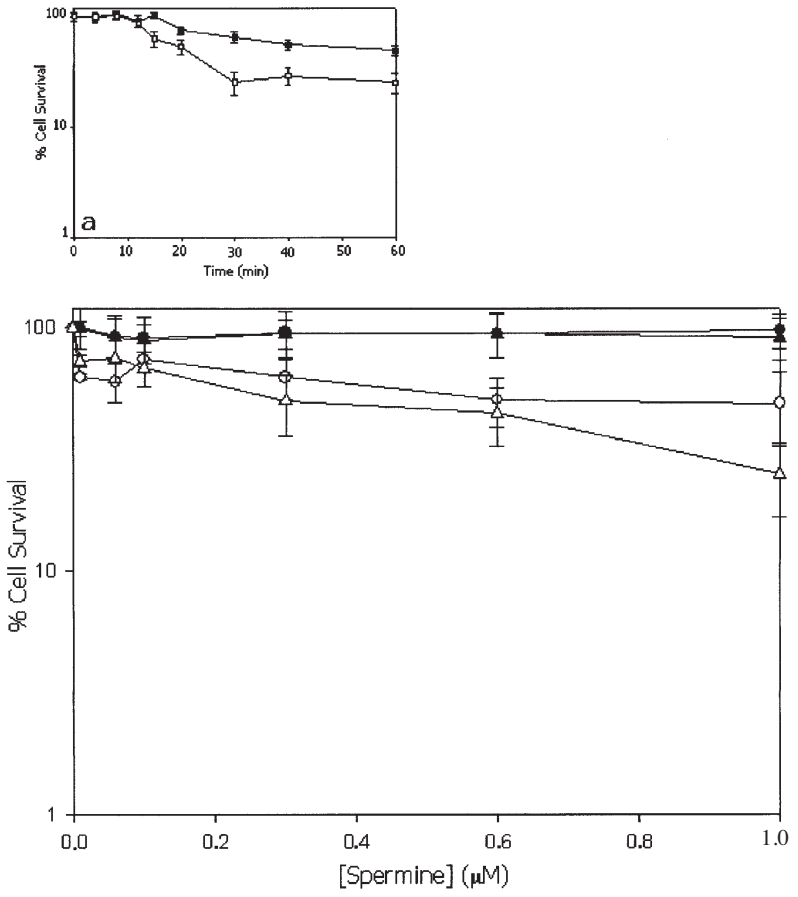

Figure 2. Temperature dependence for cytotoxicity of purified BSAO ($6.98x10^{-3}$ U/ml) as a function of exogenous spermine concentration, up to 1 μM, on percentage cell survival in LoVo WT (circles) and LoVo DX (triangles) cells during 60 min at 37˚C (solid symbols) and 42˚C (open symbols). Insert (a) indicates cytotoxicity in the presence of spermine 1 μM during 60 min of incubation in LoVo WT (solid square) and LoVo DX (open square). Means and SDs are shown for 2-5 estimations from 4-6 experiments. When not shown, SDs lie within symbols.

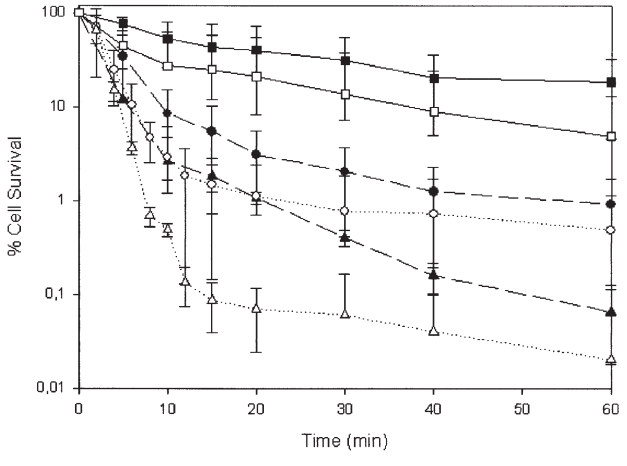

Figure 3. Time course for temperature and spermine concentration dependence on cytotoxicity of BSAO ($6.98x10^{-3}$ U/ml) in the presence of spermine 12 μM on percentage cell survival in LoVo WT (circles) and LoVo DX (triangles) cells during 60 min at 37˚C (solid symbols) and 42˚C (open symbols); and at 37˚C with spermine 6 μM in LoVo WT (solid square) and LoVo DX (open square). Means and SDs are shown for 2-5 estimations from 4-6 experiments. When not shown, SDs lie within symbols.

concentration, greater cytotoxicity was observed in MDR cells than in sensitive cells. At 42˚C, the survival of LoVo WT cells was approximately 4%, while only a very low percentage (<1%) of LoVo DX cells maintained their viability. At 37˚C, lower cytotoxicity was observed in both LoVo WT and LoVo DX cells. No cytotoxicity was revealed in controls of both cell types, exposed for 60 min at 37˚C to either BSAO alone or spermine alone (data not shown). The effect of 42˚C hyperthermia on the percentage cell survival versus the time of exposure to BSAO in the presence of exogenous spermine (6 μM) was also determined. The percentage cell survival was approximately 2.34% in LoVo WT and approximately 0.38% in LoVo DX cells after 60 min of incubation.

Spermine concentrations lower than 1 μM in the presence of BSAO were not cytotoxic after 60 min of incubation at 37˚C but induced considerable cell killing at 42˚C (Fig. 2). Consequently, 50% cell survival occurred at 42˚C in LoVo WT and 25% in LoVo DX cells for the spermine concentration of 1 μM, which was non-toxic at 37˚C. Heat alone, in the absence of the BSAO/spermine enzymatic system, caused a small decrease (<20%) in cell survival (data not shown). However, this small contribution of heat alone to the cytotoxic effect did not account for the magnitude of enhancement of cytotoxicity induced by heat in association with spermine and BSAO. The thermal enhancement increased as a function of spermine concentration, up to 6 μM as shown in Fig. 1. Cytotoxicity that was attributed to all of the oxidation products of spermine, H_2O_2 and aldehyde(s), was therefore enhanced at 42˚C.

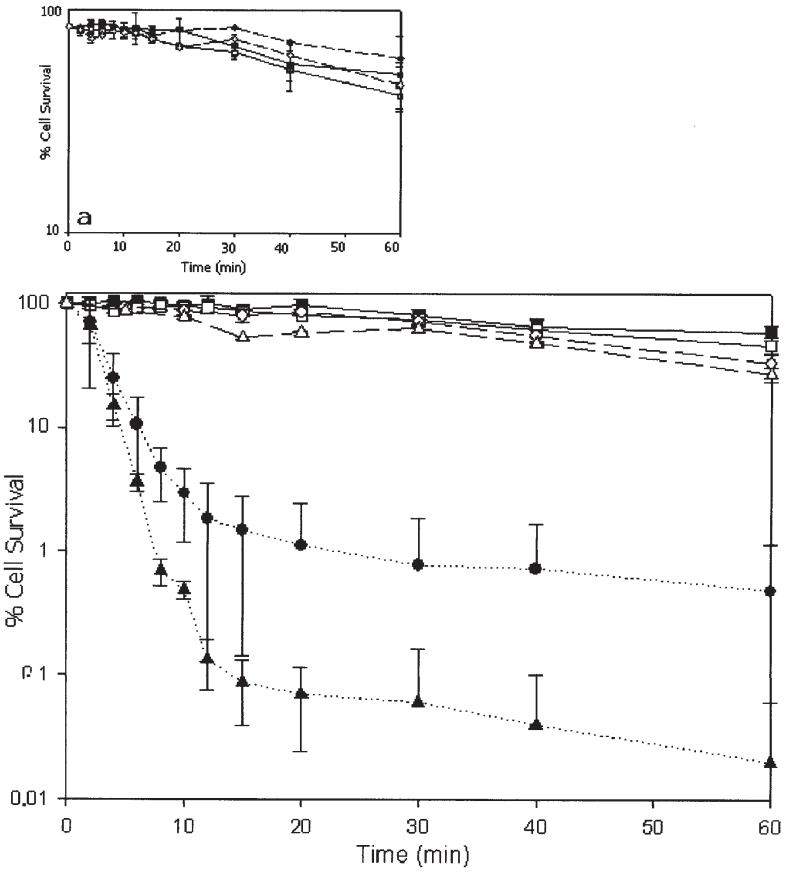

Figure 4. Effect of catalase and ALDH on cytotoxicity induced by BSAO in the presence of spermine. LoVo WT (circles) and LoVo DX (triangles) cells were incubated at 37˚C with purified BSAO ($6.98x10^{-3}$ U/ml) and exogenous spermine (12 μM) without (solid symbols) or with (open symbols with dot) catalase (240 U/ml); or with both (square symbols) ALDH (0.4 U/ml) and catalase. The insert (a) reports an extra addition of ALDH (0.4 U/ml, dotted line) in LoVo WT (solid rhombic) and LoVo DX (open rhombic), compared to the treatment with catalase and ALDH (square symbols). Means and SDs are shown for 2-6 estimations from 4-6 experiments. When not shown, SDs lie within symbols.

Heat enhancement of cytotoxicity induced by BSAO ($6.98x10^{-3}$ U/ml), as a function of exposure time, was demonstrated by adding to the incubation mixture two different exogenous spermine concentrations of 6 and 12 μM at 37˚C and 12 μM at 42˚C (Fig. 3). As expected, it may be seen that the cytotoxic effect in LoVo WT and LoVo DX cells was more marked in the presence of 12 μM spermine than 6 μM spermine, at 37˚C. Moreover, an increased cytotoxicity was observed in the presence of spermine 12 μM after 60 min of incubation at 42˚C. The higher cytotoxicity in the presence of spermine 12 μM was attributed to the formation of an increased amount of H_2O_2 and aldehyde(s), during the enzymatic reaction.

A previous study has shown that the cytotoxicity observed during the first 10 min of incubation was mainly attributed to H_2O_2 which rapidly crossed the plasma membrane (25). Therefore, to further explain the contribution of each of the enzymatic oxidation products involved in the enhancement by heat, we studied the effect of both exogenous catalase and NAD-dependent ALDH on cytotoxicity caused by 12 μM spermine and BSAO, during 60 min of incubation at 42˚C (Fig. 4).

As already demonstrated at 37˚C in CHO cells (23,24), LoVo cells (25,27) and by other authors (21), we also studied the effect of exogenous catalase (an enzyme which decomposes H_2O_2) on the cytotoxicity in LoVo cells at 42˚C hyperthermia (Fig. 4). The graph shows the percentage cell survival versus the time of exposure to BSAO in the presence of exogenous spermine 12 μM, without and with 240 EU/ml of catalase. With purified BSAO and spermine alone, the time-dependent decrease in LoVo cell survival reached the previously detected values after 60 min of exposure, as also indicated in Fig. 3. On the other hand, in the presence of catalase, a marked reduction of the cytotoxic effect, approximately 65% in LoVo WT and 55% in LoVo DX, occurred in both cell lines. These findings suggest that H_2O_2, in these experimental conditions, was the enzymatic product mainly responsible for the cell killing in both cell lines (Fig. 4). Moreover, these results also suggested that at 42˚C, H_2O_2 was not the sole toxic agent and that other molecules formed by the enzymatic oxidation of spermine, such as aldehyde(s), dialdehyde and acrolein, were more involved in causing cytotoxicity than at 37˚C (25). Hyperthermia probably activated both enzymatic oxidation products, enhancing substantially their cytotoxic effect in MDR cells.

The aldehyde(s) formed during the enzymatic reaction caused cytotoxicity in LoVo DX cells, but after longer incubation times. The toxic effect of the aldehyde(s) was more evident in MDR cells compared to the sensitive ones, for longer times (≥ 30 min after treatment with spermine 6 or

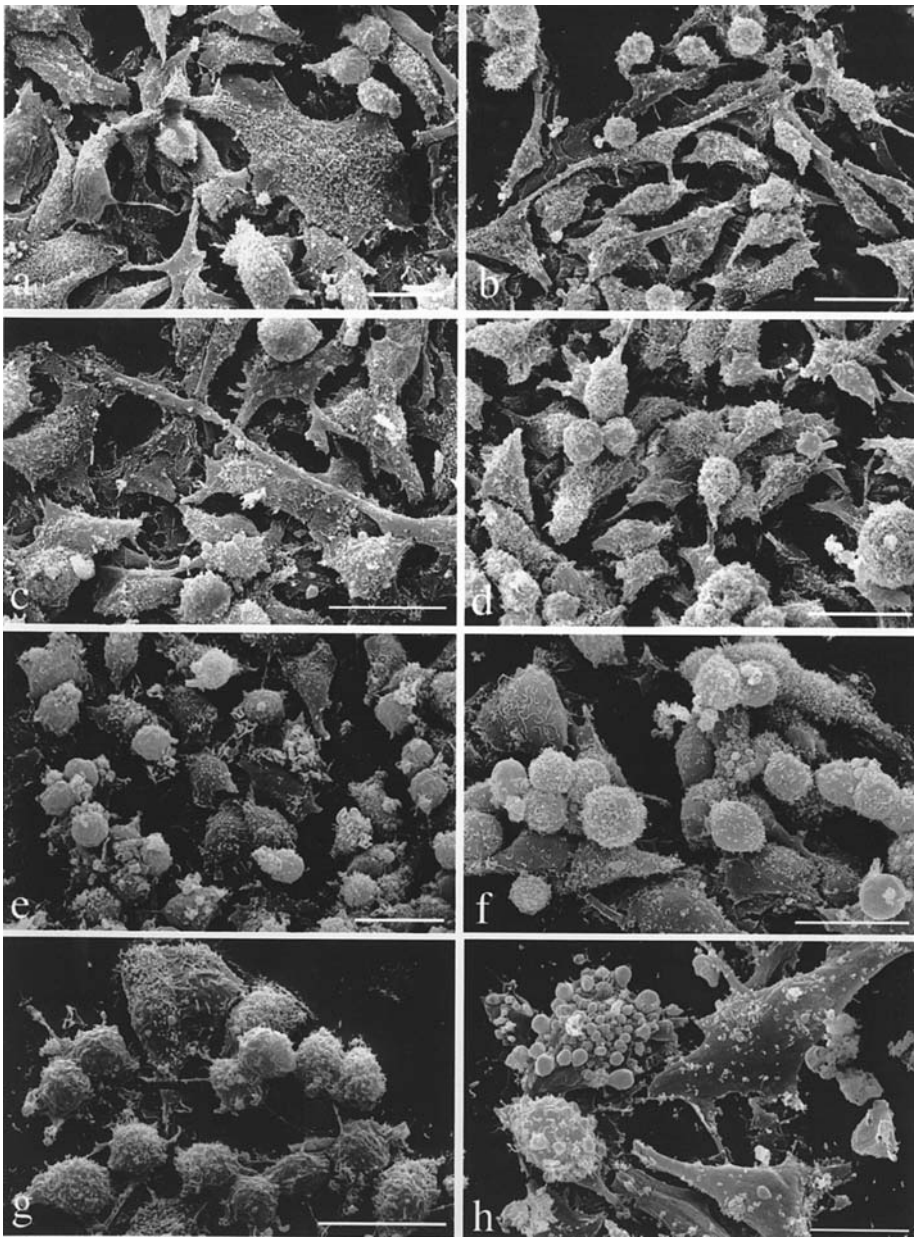

Figure 5. Scanning electron microscopic observations. (a) Control LoVo WT and (b) control LoVo DX cells; (c) LoVo WT and (d) LoVo DX cells after incubation for 1 h at 42˚C; (e) LoVo WT and (f) LoVo DX cells treated with BSAO and 6 μM spermine at 42˚C; (g) LoVo WT and (h) LoVo DX cells treated with BSAO and 12 μM spermine at 42˚C. LoVo DX cells showed more pronounced alterations, consisting of rounding, detachment and surface blebbing, than their sensitive counterparts, either after heat treatment alone (cfr c with d) or after the combined hyperthermia/BSAO-spermine treatment (cfr e with f and g with h). Scale bars, 20 μm.

12 μM at 37˚C and <20 min after treatment with spermine 12 μM at 42˚C, Fig. 3). Therefore, a relevant difference in cytotoxicity between MDR and sensitive cells occurred after longer incubation times, under experimental conditions where higher concentrations of H_2O_2 and spermine-derived aldehyde(s) had been formed. However, heat also enhanced cell killing in LoVo WT cells but the cytotoxic effect was less pronounced than for LoVo DX cells.

In order to evaluate the contribution of spermine-derived aldehyde(s) in causing cytotoxicity on LoVo cells, the effect of exogenous NAD-dependent ALDH (0.4 U/ml), in the presence of catalase, on the cytotoxicity induced by 12 μM spermine and purified BSAO, was investigated. After addition of both enzymes, cytotoxicity was considerably decreased throughout the 60 min of incubation. However, there was still a gradual decrease in percentage cell survival. This could not be explained by heat inactivation of ALDH at 42˚C. The addition of another 240 U/ml of catalase did not prevent this cytotoxic effect (data not shown), while an extra addition of 0.4 U/ml of NAD-ALDH further decreased cytotoxicity. Therefore, the addition of 0.4 U/ml of NAD-ALDH was necessary to convert the high amounts of aldehydes or acrolein in acid which had accumulated during the enzymatic oxidation of spermine after longer times.

Scanning (SEM) and transmission (TEM) electron microscopy observations, performed on both wild-type sensitive and multidrug-resistant LoVo cells after treatment with the combination of BSAO and spermine at 37˚C and 42˚C, to

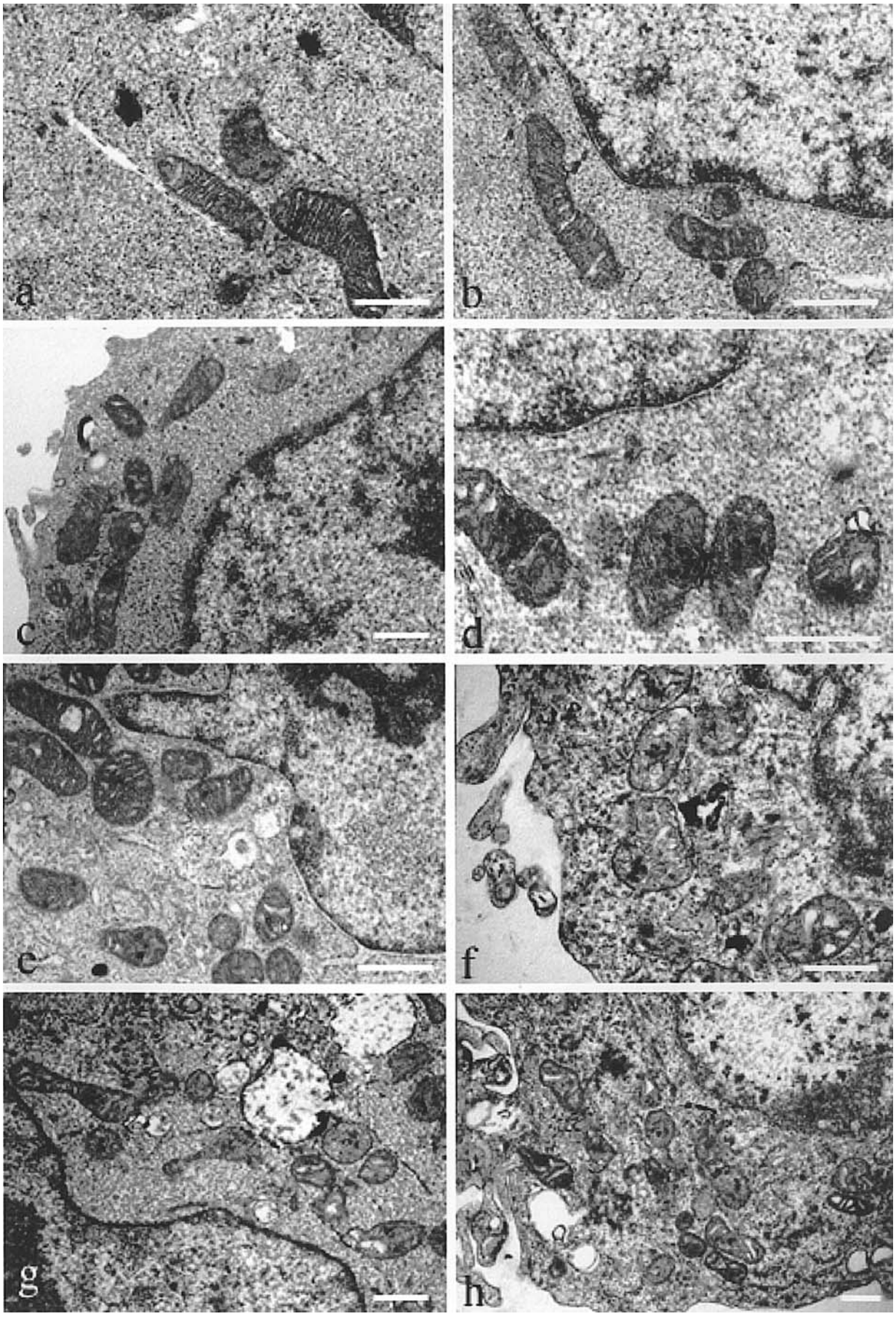

Figure 6. Transmission electron microscopic observations. (a) Control LoVo WT and (b) control LoVo DX cell; (c) LoVo WT and (d) LoVo DX cell after incubation for 1 h at 42˚C; (e) LoVo WT and (f) LoVo DX cell treated with BSAO and 6 μM spermine at 42˚C; (g) LoVo WT and (h) LoVo DX cell treated with BSAO and 12 μM spermine at 42˚C. Alterations of the mitochondrial structure were more evident in treated resistant cells. Moreover, the treatment with BSAO-spermine under hyperthermic conditions produced very severe structural damage, particularly at mitochondrial level (f and h). Scale bars, 0.5 μm.

confirm the previously reported (25) higher sensitivity of MDR cells to the oxidation products of spermine, allowed the verification of a close relationship between the extending of morphological and ultrastructural changes and the level of the induced cytotoxic effect. Moreover, TEM analysis indicated the mitochondria as the main intracellular target involved in the enhancement of the cytotoxic action induced by hyperthermia.

Fig. 5a and b shows control untreated LoVo WT and LoVo DX cells, respectively, observed by SEM. No significant morphological difference between the two cell types was detected. Most of the cells showed adherence to the substrate with a typical polygonal shape and the surface covered by randomly distributed microvilli. The treatment with BSAO alone or spermine alone did not induce any appreciable modification (data not shown). After 60 min of incubation at 42˚C, LoVo WT cells appeared to be substantially unmodified (Fig. 5c) when compared to control cells whereas, interestingly, LoVo DX cells appeared to be more sensitive to the hyperthermic treatment, many cells appearing roundish and tending to detach from the substrate (Fig. 5d).

SEM observations of sensitive and resistant LoVo cells (Figs. 5e and f, respectively) treated with BSAO and 6 μM spermine at 42°C confirmed the synergic effect of the exposition under hyperthermic conditions (Fig. 1); in both cell lines, the morphological alterations were well evident and particularly LoVo DX cells appeared to be roundish with numerous surface blebs and tended to detach from the substrate. These morphological signs of cell damage were even more evident when using 12 μM spermine concentrations and, in this case, LoVo DX cells (Fig. 5h) also appeared to be more affected by the treatment than their wild-type counterparts (Fig. 5g).

Observed by TEM, both control LoVo WT (Fig. 6a) and LoVo DX cells (Fig. 6b) showed a well preserved ultrastructure with the cytoplasm characterized by the presence of numerous mitochondria with an elongated shape and parallel cristae in a dense and uniform matrix. Similar to that observed by SEM analysis, treatment with BSAO or spermine alone did not induce any detectable alteration in the mitochondrial structure in both cell lines (data not shown). The mitochondria of wild-type cells did not show any significant structural change after incubation for 60 min at 42˚C (Fig. 6c). Also, the other ultrastructural features appeared to be unmodified after the hyperthermic treatment. Instead, in heat-treated MDR LoVo cells, mitochondria showed a less regular profile with the beginning of intracristal swelling (Fig. 6d). When the hyperthermic treatment was associated with BSAO-spermine administration, an evident differential response between sensitive and drug-resistant cells was observed, according to clonogenic cell survival determinations. In fact, LoVo WT cells treated with BSAO and 6 μM spermine at 42˚C still presented quite a good general ultrastructure (Fig. 6e), even though some mitochondria showed condensed matrix and altered cristae. These modest mitochondrial alterations in LoVo WT cells appear to be very similar to those previously observed in the same cells treated with the same spermine concentration at 37˚C (27). On the contrary, multidrug-resistant cells exhibited marked modifications (Fig. 6f); in particular, all mitochondria showed altered shape with vacuolized cristae in a rarefied matrix. Finally, treatment with BSAO and 12 μM spermine under hyperthermic conditions induced severe alterations in both wild-type (Fig. 6g) and MDR (Fig. 6h) cells, even if such changes appeared to be much more pronounced in resistant cells.

Discussion

It has been demonstrated that cancer cells are selectively killed by hyperthermia alone (35). Numerous studies evidenced a beneficial effect of hyperthermia when associated with other therapeutic modalities, such as irradiation or chemotherapy, in the treatment of human cancers (36,37). This led researchers to evaluate the clinical potential of hyperthermia using several temperatures (ranging from 40˚C to 43˚C) (1). Localized hyperthermia enhances the cytotoxic process of several antitumoral drugs and has considerable potential in cancer therapy (38,39). This has been explained by a favourable influence on blood flow, cell membrane permeability and drug uptake (40). Hyperthermia could act at the initial stage of the treatment, probably by accelerating the kinetics of the membrane molecular interactions and by favouring drug delivery into the tumor mass (41).

The present study deals with the possible effects of both hydrogen peroxide and aldehyde (produced by the BSAO/polyamine-spermine enzymatic system) in inducing higher cytotoxicity, at 42˚C, in MDR human colon adenocarcinoma cells compared to the sensitive ones. Rapidly dividing tumor cells contain increased levels of polyamines such as spermine, spermidine and putrescine (42). These high concentrations can be explained by both enhanced putrescine synthesis by ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis (43), and increased uptake of these polycations (44). Pharmacological molecules with an antiproliferative activity against cancer cells (45), such as α-difluoromethylornithine (DFMO) (46) and methylglyoxal-bis-guanidylhydrazone (47,48), can inhibit the enzymes involved in the biosynthesis of polyamines (ODC and S-adenosyl-methionine decarboxylase, respectively) (49), reducing their levels in cells and resulting in a cytostatic effect (50,51).

The results described in the present report suggest that, in the presence of spermine 6 μM for 60 min of incubation at 42˚C, hydrogen peroxide is the enzymatic product mainly involved in causing cytotoxicity in both LoVo WT and LoVo DX cells (Fig. 2), as already demonstrated at 37˚C (25). However, both the enzymatic reaction products, H_2O_2 and aldehyde(s), were responsible for cytotoxicity, as the addition of catalase alone did not show complete protection. In the presence of both exogenous catalase and NAD-dependent ALDH, cytotoxicity was completely inhibited throughout the incubation at 37˚C (25,27). At 42˚C, under the same experimental conditions of BSAO/spermine 6 μM with both exogenous enzymes for 60 min of incubation and in the presence of an extra addition of ALDH (0.4 U/ml), a high protection against the cytotoxic products was observed. The residual low cytotoxicity (<15%) was attributed to heat (Fig. 4). The data obtained suggest that aldehyde(s) formed during the enzymatic oxidation of spermine at 42˚C were responsible for cytotoxicity that cannot be explained by hydrogen peroxide. Our results clearly show that over-

expression of P-glycoprotein in LoVo DX cells did not confer any resistance to both cytotoxic products, H_2O_2 and spermine-derived aldehyde(s). Rather and interestingly, MDR cells appeared to be more sensitive to the enzymatic oxidation products in the presence of higher spermine concentrations (12 μM), where a higher amount of H_2O_2 and aldehyde(s) was produced, and in hyperthermic conditions. Probably, the enhancement of BSAO-mediated cytotoxicity at 42˚C is not the result of an increase in enzyme activity at the higher temperature. It has been demonstrated that BSAO shows a characteristic dependence of enzyme activity on temperatures between 35˚C and 45˚C with Q_{10} value of 1.17 (52). This value is lower than that for other enzymes that usually show an approximate Q_{10} value of 2. Therefore, in this study, 42˚C induced only a minor increase in specific activity of BSAO from 0.38 IU/mg to 0.41 IU/mg, corresponding to only a 1.1-fold increase. Thus, we hypothesize that the great enhancement of the cytotoxic effect observed at 42˚C can not be explained by an increase only in specific enzyme activity. It could be interpreted by an increased rate of interaction of the enzymatic reaction products with specific target sites, such as DNA or cell plasma membranes, involved in cell death. Cell membranes also represent important sites of cell damage by heat (53). Furthermore, 42˚C hyperthermia enhances membrane fluidity and, consequently, can increase the transport functions of the plasma membrane (54). Spermine is able to enter the cells by energy-dependent transport across the plasma membrane (55). Thus, exogenous spermine could contribute to the enhancement of heat-induced cytotoxicity (14). However, very low spermine concentrations alone, as used in our experimental conditions, did not induce any cytotoxic effect in either cell line. In fact, the findings clearly showed the essential role of enzymatic oxidation products of polyamines (24,25,27,33,56), rather than polyamines themselves, in causing cytotoxicity (57).

It was observed that the concentrations of spermine necessary to induce cytotoxicity are different in cell lines of various histotype (23-25). An interesting result was that spermine concentrations ≤ 1 μM (Fig. 2), in the presence of BSAO, which were not toxic at 37˚C on LoVo WT and DX cells, became cytotoxic at 42˚C. A similar phenomenon was also observed in CHO cells (24). BSAO and concentrations of spermine less than 1 μM were only cytotoxic at 42˚C and induced, after 60 min of incubation, approximately 50% and 25% of cell killing on LoVo WT and LoVo DX cells, respectively. The findings suggest a marked enhancement of cytotoxicity on LoVo cells induced by heat, attributed to both enzymatic oxidation products of spermine, H_2O_2 and aldehydes. The enzymatic oxidation products of spermine behaved similarly to other thermosensitizers, as aminothiol WR-1065 or cysteamine (58,59).

Hyperthermia alters the membrane permeability to several molecules, including polyamines and certain ions (60). In our study, morphological and ultrastructural modifications were only revealed in MDR cells after mild hyperthermic treatment. In fact, heat-treated LoVo DX cells at 42˚C for 1 h exhibited slight alteration of their cellular shape including mitochondrial changes. This could be due to the effect of hyperthermia on certain cytoskeletal protein components and mitochondrial membranes, respectively (41). Therefore, the findings of cell survival studies were supported by scanning and transmission electron microscopy. Both, aldehyde(s) and H_2O_2, formed during the enzymatic oxidation of spermine, were able to enter the cells where they caused the cytotoxic effect. This was more pronounced in multidrug-resistant cells, where morphological alterations in the cytoplasmatic organization and ultrastructural modifications were shown; in particular, the structure of mitochondria of LoVo DX cells treated with BSAO and 6 μM spermine at 42˚C was changed dramatically, showing dilated cristae and intracristal swelling. In the presence of BSAO and 12 μM spermine, these changes were much more evident and all mitochondria in MDR cells showed a drastically damaged ultrastructure. These structural alterations may be associated with functional modifications as already demonstrated by flow cytometry studies, on LoVo DX cells treated at 37˚C (25,61). In these experimental conditions, mitochondrial membrane potential (MMP) studies showed that, after treatment with BSAO/spermine enzymatic system, earlier and higher mitochondrial membrane depolarization was found in LoVo DX than in LoVo WT cells. On the basis of these results, it could be hypothesized that LoVo DX cells were more sensitive to H_2O_2 and aldehyde(s) because of their higher mitochondrial electron transport chain activity (25). Moreover, it was shown that persistent reactive oxygen species (ROS) overproduction, as a direct consequence of a more active respiratory chain, and membrane hyperpolarization lead to collapsed MMP and hypopolarization, impairment of the mitochondrial function and apoptosis (62). Considering the motives described, it could be hypothesized that LoVo DX cells also produced a high amount of ROS in hyperthermic conditions, which could not be completely removed by endogenous cellular and mitochondrial antioxidant defences, as in normal conditions (63). When LoVo DX cells are treated with BSAO and spermine, they might no longer be able to remove additional reactive oxygen species and lose mitochondrial functionality earlier and to a greater extent than LoVo WT cells. Therefore, the effects of the enzymatic oxidation products of spermine (hydrogen peroxide and aldehydes) were able to induce severe changes in the mitochondrial structure that may not be reflected in the damage of DNA, as already observed (i.e. nuclear condensation and fragmentation), but the alterations, such as dilation of the cristae and disruption of membranes, that are common features of necrosis were mainly observed in MDR cells (64). These findings suggest that such modifications may also have consequences at the level of the cellular membrane and several intracellular proteins.

In view of these results, the use of the amine oxidase enzyme in cancer therapy may be considered. By delivering purified BSAO into cancer cells, cytotoxic products of polyamines oxidation could be produced *in situ* for their selective killing. The main challenge is how the enzyme can be delivered *in vivo* to oxidize endogenous polyamines present at high concentrations in cancer cells, to form H_2O_2 and aldehyde(s), for possible clinical applications. Bachrach *et al* were able to obtain enrichment in amine oxidase activity of cells using a purified enzyme preparation entrapped in reconstituted envelopes of Sendai viruses (21). We have obtained the incorporation of bovine serum amine oxidase into liposomal vesicles on the bases of previous experience with liposomal entrapping of superoxide dismutase (65). Besides, macro-

molecular anticancer drugs can be conjugated with biocompatible polymers which function as carriers and stabilizers, resulting in decreased drug toxicity and increased therapeutic efficacy (66). The results have shown that, by conjugating BSAO with polyethylene glycol hydrogel (PEG), the yield of immobilization was approximately 40% (67).

Therefore, in the context of therapeutic application, our results demonstrate that the cellular sensitivity of LoVo cells to the toxic products is significantly increased by hyperthermia, mainly in cells expressing the multidrug resistance (MDR) phenotype. Localized hyperthermia could enhance the cytotoxic effects of the enzymatic oxidation products of spermine, within the tumor region, without increasing normal tissue damage. To take advantage of the higher levels of polyamines in cancer cells versus normal tissue, cytotoxic products such as H_2O_2 and aldehyde(s) could be generated *in situ* to induce cytotoxicity by delivering copper amine oxidases into the tumor mass (68). It is our hope that this approach will open new avenues in combating cancer and in treating MDR cancer patients.

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