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Toxicity of enzymatic oxidation products of spermine to human melanoma cells (M14): Sensitization by heat and MDL 72527

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Abstract

In situ formation of cytotoxic metabolites by an enzyme-catalyzed reaction is a recent approach in cancer chemotherapy. We demonstrate that multidrug resistant human melanoma cells (M14 ADR) are more sensitive than the corresponding wild type cells (M14 WT) to hydrogen peroxide and aldehydes, the products of bovine serum amine oxidase (BSAO)-catalyzed oxidation of spermine. Hydrogen peroxide was mainly responsible for the loss of cell viability. With about 20%, the aldehydes formed from spermine contribute also to cytotoxicity. Elevation of temperature from 37 °C to 42 °C decreased survival of both cell lines by about one log unit. Pre-treatment with N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine (MDL 72527), a lysosomotropic compound, sensitized cells to toxic spermine metabolites. MDL 72527 (at 300 μM) produced in M14 cells numerous cytoplasmic vacuoles which, however, disappeared by 24 h, even in the presence of the drug. Mitochondrial damage, as observed by transmission electron microscopy, correlated better with the cytotoxic effects of the treatment than vacuole formation. Since the release of lysosomal enzymes causes oxidative stress and apoptosis, we suggest that the lysosomotropic effect of MDL 72527 is the major reason for its sensitizing effect.

Keywords: Bovine serum amine oxidase; Melanoma cell; Spermine; MDL 72527; Hyperthermia

1. Introduction

The polyamines spermidine and spermine and their precursor putrescine are ubiquitous in the living world. Their numerous biological functions, which include cell growth and proliferation, have extensively been documented [1], and the attempts to exploit polyamine metabolic enzymes as targets, as well as to utilize the polyamine backbone as pharmacophore for the design of anticancer drugs have been reviewed [2,3]. Polyamines may also become a source of cytotoxic metabolites. For the generation of cytotoxic metabolites bovine serum amine oxidase (BSAO; E.C. 1.4.3.6), a copper-containing enzyme was used. In Fig. 1 the major reactions of spermine with bovine serum amine oxidase (BSAO) are shown. It has previously been demonstrated that Chinese hamster ovary cells are sensitive to hydrogen peroxide and the aldehydes that are formed from spermine by BSAO catalysis [4], and the growth of a mouse melanoma was inhibited by exposure to BSAO and spermine [5]. Toxic polyamine metabolites are currently explored as a strategy in tumor therapy [6,7].

In agreement with extensive experience in thermochemotherapy [8] an increase of the incubation temperature from 37 °C to 42 °C enhances cell death of cultured cells exposed to spermine metabolites [9,10]. Other experiments demonstrated that multidrug resistant (MDR) cells (as obtained by exposure to doxorubicin [11]) are more sensitive to spermine metabolites than their normal counterparts [12,13], an aspect of particular

Abbreviations: BSA, bovine serum albumin; BSAO, bovine serum amine oxidase; CHO, Chinese hamster ovary; DX, doxorubicin; ADR, adriamicin resistent; IU, international unit; MDL 72527, N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine; MDR, multidrug resistance; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SD, standard deviation

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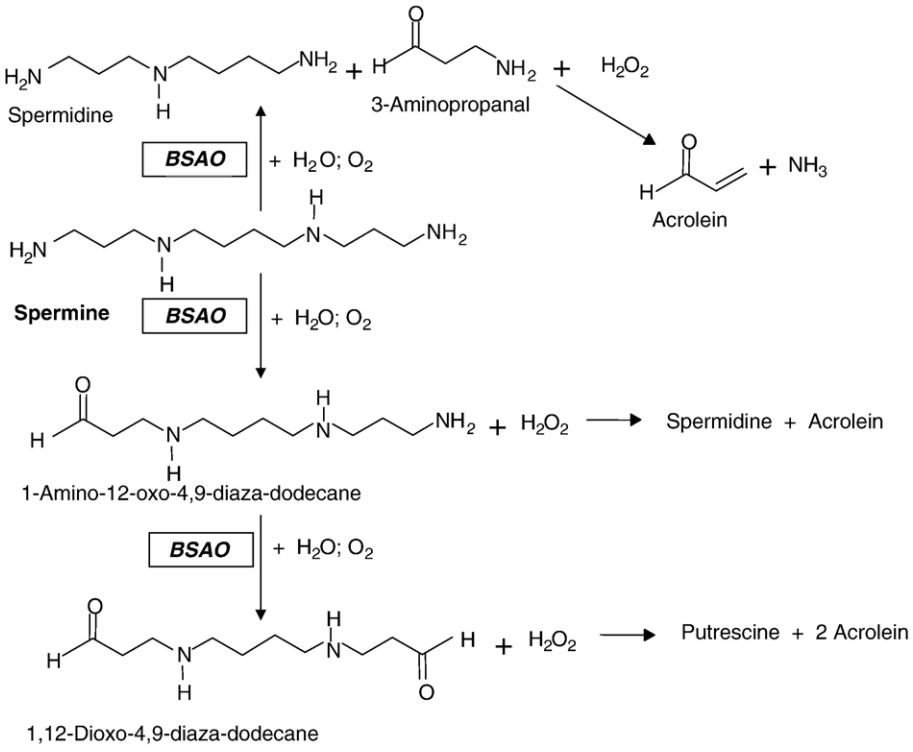

Fig. 1. Scheme of bovine serum amine oxidase (BSAO) catalyzed reactions of spermine. The direct formation of spermidine and 3-aminopropanal from spermine by BSAO catalysis was reported by Houen et al. [37]. Spermidine is also a substrate of BSAO and may contribute to hydrogen peroxide and aldehyde formation (not shown). It has not been established, which aldehydes are preferentially formed under the reaction conditions (see Materials and methods) by BSAO-catalysis. Acrolein is the product of spontaneous β-elimination from the aldehydes. The factors favoring this reaction are not known.

interest, since one of the problems of conventional anticancer therapy is the development of drug resistance.

In the present work wild type human melanoma M14 cells (M14 WT), and the doxorubicin-resistant line (M14 ADR) were investigated with the aim to explore in a first step the therapeutic potential of in situ formation of cytotoxic spermine metabolites. Elevation of incubation temperature enhanced cytotoxicity of spermine metabolites in Chinese hamster ovary cells and LoVo cells [12,14]. The present experiments were carried out in order to investigate, whether this phenomenon was reproduced in an other cell line. In addition the effect of pre-treatment with a lysosomotropic compound, MDL 72527, on the cytotoxic effect of the spermine metabolites was explored.

2. Materials and methods

2.1. Chemicals

If not stated otherwise, chemicals were from Sigma Chemical Co. (St. Louis, USA). Spermine tetrahydrochloride was from FLUKA (Buchs, Switzerland), and NAD^+ from Boehringer (Mannheim, Germany). Cell culture materials were from Gibco Life Technologies Ltd. (Paisley, Scotland). Doxorubicin was from Pharmacia & Upjohn (Milan, Italy). MDL 72527 was synthesized as described earlier [15].

2.2. Cell culture

The parental human melanoma cell line M14, isolated from an epidermal melanoma, and the corresponding MDR variant M14 ADR, selected for resistance to adriamicin [16] have been used in the present investigation. In addition to doxorubicin, M14 ADR cells are also resistant to other chemotherapeutic agents such as etoposide and vincristin.

Cells were grown in monolayer in RPMI 1640 (with glutamine), 10% foetal bovine serum (Hyclone, Europe Ltd., Cramlington, UK), penicillin (50 U/ml), streptomycin (50 μg/ml) and non-essential amino acids in the same concentration as streptomycin, in a humidified atmosphere of 5% CO_2 in a water-jacketed incubator at 37 °C.

2.3. Treatments and clonogenic cytotoxicity assay

Cell survival experiments were carried out using confluent cells that had been incubated during 24 h in RPMI 1640 medium containing MDL 72527 at 200 to 400 μM, in the presence of serum, at 37 °C. Cells were detected by addition of 10 mM EDTA in phosphate-buffered saline (PBS), washed with PBS supplemented with 1% bovine serum albumin (PBS/BSA) and pelleted by centrifugation (2 min, 1500×*g*). The cells were re-suspended in PBS/BSA. Aliquots of freshly harvested M14 cells were incubated at 37 °C for different time intervals in the presence or absence of BSAO (6.5×10^{-3} IU/ml), spermine, catalase (240 U/ml) (from bovine liver), aldehyde dehydrogenase (EC 1.2.1.5) (from yeast) (0.4 U/ml) and NAD^+ (1.8 μg/ml). The spermine solution was freshly prepared before each experiment and, was added last. After incubation the cells were centrifuged, washed twice in PBS–BSA, and re-suspended in 1 ml PBS–BSA.

Potential antitumor effect was evaluated by a plating efficiency assay, which determines the ability of the cells to form macroscopic colonies (>50 cells). Aliquots of cell suspensions containing 10^5 cells per ml were plated in tissue culture dishes (50 mm ∅) containing 5 ml complete culture medium, and were incubated at 37 °C. After 14 days, the colonies were fixed with 96% ethanol, stained with methylene blue and counted. Control plating efficiencies were higher than 85% and 80%, for M14 WT and M14 ADR cells, corresponding to $9.0\times10^4\pm1.0\times10^4$ and $8.5\times10^4\pm1.0\times10^4$ number of cells, respectively. Percent cell survival was determined as the ratio between the mean number of colonies in treated and control samples.

2.4. Scanning electron microscopy

For scanning electron microscopy cells were grown to near confluence on glass coverslips in RPMI 1640 medium containing MDL 72527 at 200 to 400 μM, in the absence of serum, at 37 °C for different times in the presence or absence of BSAO (6.5×10^{-3} IU/ml), and spermine (6 μM). After incubation, cells were washed with PBS/BSA, and were then processed for scanning electron microscopy as previously described [17]. The samples were examined by a Cambridge Stereoscan 360 scanning electron microscope (Cambridge Instruments, Cambridge, UK).

2.5. Transmission electron microscopy

For transmission electron microscopy (TEM) cells were grown to near confluence and harvested as described above, washed with PBS/BSA, centrifuged and re-suspended in 2 ml of RPMI 1640 medium. After incubation for 60 min at 37 °C in the presence or absence of BSAO (6.5×10^{-3} IU/ml), or with spermine alone (6 μM) cells were washed with RPMI 1640 medium and then processed for TEM as described [17]. Ultrathin sections were examined with a Philips EM 208S electron microscope (FEI Company, Eindhoven, The Netherlands).

2.6. Flow cytometry

All fluorescence were analyzed using a FACScan flow cytometer equipped with 15 mV, 488 nm air-cooled argon ion laser and analyzed using CellQuest software (Becton Dickinson). The fluorescence emission were collected through a 530 nm band pass filter for FITC and a 575 nm band pass filter for PI, acquired in *log* mode. At least 10000 events were analyzed.

2.7. Determination of apoptotic cell death by Annexin V-FITC staining

To detect phosphatidylserine translocated from the inner face to the outer surface of plasma membrane in the initialing step of apoptosis, an Annexin V-FITC kit (MBL, Medical & Biological Laboratories Co., Ltd., Japan) was used [18]. About 50 phosphatidylserine monomers are estimated to be bounded per Annexin V molecule.

M14 WT and MDR cells (1×10^{6}/ml) were pre-treated with 300 μM MDL 72527 for 24 h and then incubated with BSAO (6.5×10^{-3} IU/ml) and spermine (12 μM) for 1 h at 37 °C. After treatment cells were centrifuged and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.5, 140 mM NaCl, and 2.5 mM $CaCl_2$). Cell suspension (of about 5×10^{5}) were then incubated with 1 μg/ml of Annexin V-FITC and with 1 μg/ml of propidium iodide (PI) for 10 min at room temperature in the dark. The populations of Annexin V-positive/PI-negative cells (early apoptosis) and Annexin V-positive/PI-positive cells (late apoptosis) were evaluated by flow cytometry [18]. The reported values are the mean of three independent experiments.

2.8. Determination of intracellular ROS content

The intracellular reactive oxygen species (ROS) content was determined by using the non-fluorescent dye dihydrorhodamine 123 (DHR123, Molecular Probes). This compound represents a chemically reduced form of rhodamine 123 (R123) and freely diffuses into the cells. After oxidation by ROS, DHR123 is converted to green fluorescent R123 [19]. Both M14 cell lines, WT and MDR, either control or after treatment with BSAO/spermine, by previous pre-treatment with MDL 72527, were harvested with EDTA, then washed, centrifuged and resuspended in complete RPMI medium. Cells (5×10^{5}/ml) were labelled with 1 μM DHR123 for 20 min at 37 °C. After washing with cold PBS, cells were analyzed by flow cytometry. Values are expressed in arbitrary units (A.U.) calculated by the ratio of the mean fluorescence channel (MFC) of labelled samples on the MFC of negative controls. The reported values are the mean of three independent experiments.

2.9. Purification of BSAO

BSAO was purified to homogeneity essentially as described by Turini et al. [20] with additional ion exchange chromatographic steps [21]. The specific activity of the enzyme preparation was 0.38 IU/mg. (1 IU = 1 μmole benzylamine oxidized per min at 25 °C).

3. Results

3.1. Loss of viability of M14 WT and M14 ADR cells due to exposure to BSAO and spermine. Effect of catalase and aldehyde dehydrogenase on the cytotoxicity of spermine metabolites

The dose–response curve for 60 min incubations with 6.5×10^{-3} IU/ml BSAO and 0–21 μM spermine is shown in Fig. 2. The MDR human melanoma cells (called M14 ADR or M14 DX) were more sensitive to the treatment at all concentrations of spermine than the corresponding wild type cells. At the highest spermine concentration the survival of M14 WT cells was about 0.6%, while only 0.2% of the M14 ADR cells remained viable. BSAO alone or spermine at 21 μM alone was not toxic to either cell line. FBS containing low amine oxidase activity, usually present in the incubation medium, was omitted in these experiments. The absence of serum did not induce any alterations of cells as observed in cell survival experiments when samples in PBS/BSA were compared with cells incubated in medium containing serum [12].

The sensitivity of numerous cancer cell lines to an increase of the temperature above 37 °C is the basis of clinical hyperthermia. Fig. 3 shows the percent survival of M14 WT and M14 ADR cells incubated for 60 min with 6 μM spermine in the presence of 6.5×10^{-3} IU/ml BSAO at 37 °C or 42 °C. The cytotoxic effect on both cell lines increased as a function of incubation time. M14 ADR cells were more sensitive to the treatment at both temperatures, and there was a considerable enhancement of cytotoxicity due to elevation of the incubation temperature. At 42 °C about 0.8% M14 WT cells survived the treatment, but only 0.1% of the M14 ADR cells.

In order to determine the contribution of hydrogen peroxide to cytotoxicity under our experimental conditions, 240 U/ml of catalase were added to the culture medium. This amount is

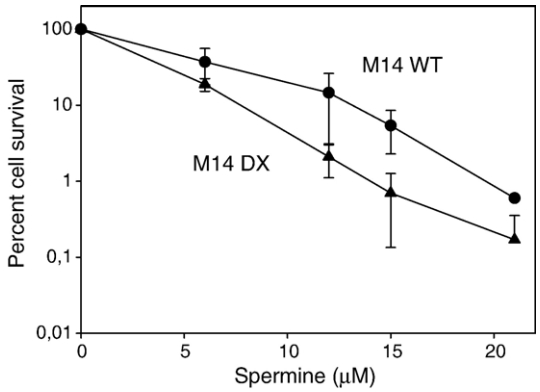

Fig. 2. Effect of BSAO and spermine on the survival of M14 melanoma cells. M14 WT and M14 ADR cells were incubated in the presence of 6.5×10^{-3} IU/ml BSAO and increasing concentrations of spermine for 60 min at 37 °C. Subsequently plating efficiency was determined. Each point represents the mean value of the results of 2 to 5 plates of three or four experiments. The error bars indicate ± S.D. When not shown, S.D. was smaller than the symbol.

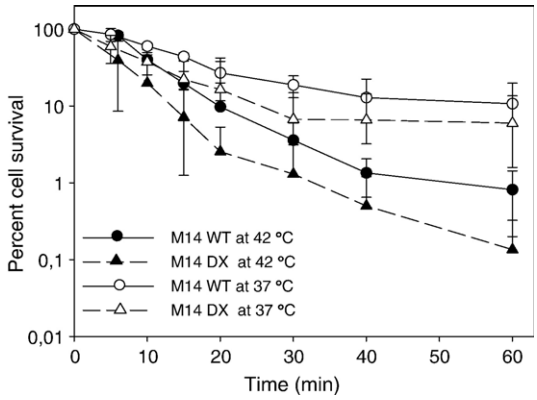

Fig. 3. Effect of BSAO and spermine on the survival of M14 melanoma cells. Time–effect relationship and effect of the incubation temperature on cell survival. M14 WT and M14 ADR cells were incubated in the presence of 6.5×10^{-3} IU/ml BSAO and 6 μM spermine for up to 60 min at 37 °C or 42 °C, followed by the determination of the percentage of surviving cells by a plating efficiency assay. Each point represents the mean value of the results of 2 to 5 plates of three or four experiments. The error bars indicate±S.D. When not shown, S.D. was smaller than the symbol.

sufficient to prevent the accumulation of hydrogen peroxide to cytotoxic concentrations. As appears from Fig. 4b, the cytotoxic effect of BSAO/spermine-treatment (Fig. 4a) was reduced by about 80%, both in wild type and MDR cells. In an analogous experiment the toxic effect of spermine derived aldehydes (Fig. 4c) was suppressed by addition of 0.4 U/ml aldehyde dehydrogenase and NAD^+ (1.8 μg/ml). The results of this experiment show that cytotoxic effect of BSAO/spermine is reduced, if the aldehydes formed from spermine are rapidly converted into amino acids. These observations support previous reports on Chinese hamster ovary cells [22].

3.2. Sensitization of M14 cells to BSAO/spermine-induced toxicity by MDL 72527

Combinations of drugs with different cytotoxic mechanisms may produce additive or even synergistic effects. In this work we tested MDL 72527 in combination with enzymatically generated toxic spermine metabolites. MDL 72527 is known as a highly potent inactivator of polyamine oxidase, but also as a weak cytotoxic agent with lysosomotropic activity [23]. M14 cells were quite insensitive to MDL 72527. Incubation with 300 μM of this drug for 24 h at 37 °C had no significant effect on cell survival of melanoma cells (Fig. 5), as already demonstrated in colon adenocarcinoma cells [24]. Since MDL 72527 is a P-gp substrate, 300 μM concentration was therefore chosen for all experiments, which were performed to show sensitization of the melanoma cells to spermine metabolites. Melanoma cells during 1 h of incubation with MDL 72527 alone (control) or cells pre-treated for 24 h with 300 μM MDL 72527 and then with 6 μM of spermine alone did not show any cytotoxic effect. Instead, if pre-incubation with MDL 72527 was followed by exposure to BSAO and spermine, the survival of both cell lines, M14 WT

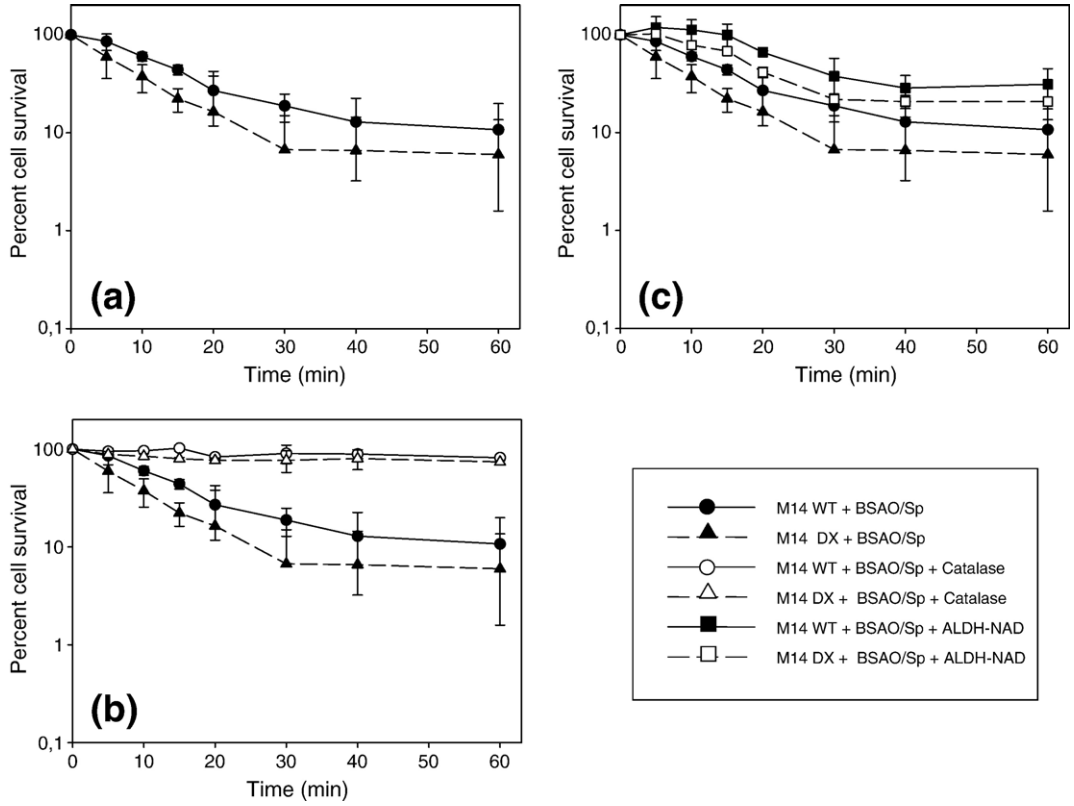

Fig. 4. The effect of catalase and aldehyde dehydrogenase on the toxicity of spermine metabolites. (a) Effect of exposure of M14 WT and M14 ADR cells to 6.5×10^{-3} IU/ml BSAO and 6 μM spermine at 37 °C. (b) Effect of addition of BSAO/6 μM spermine and 240 U/ml catalase to the cell suspension. (c) Effect of addition of BSAO/6 μM spermine and 0.4 U/ml aldehyde dehydrogenase and NAD^+ (1.8 μg/ml) to the cell suspension. Each point represents the mean value of the results of 2 to 5 plates of three or four experiments. The error bars indicate ± S.D. When not shown, S.D. was smaller than the symbol.

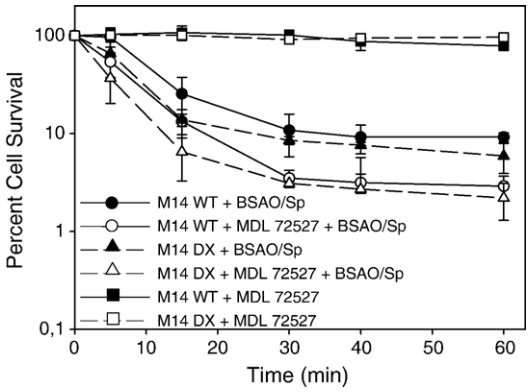

Fig. 5. Effect of pre-incubation with MDL 72527 on the sensitivity of M14 melanoma cells to cytotoxic polyamine metabolites. Cells were pre-incubated with 300 μM MDL 72527 for 24 h. After washing they were exposed for up to 60 min to BSAO (6.5×10^{-3} IU/ml) and spermine (6 μM) at 37 °C (white circles and triangles), followed by the clonogenic assay. The points represent the mean value of 2 to 5 plates of three to four experiments. The error bars indicate ± S.D. When not shown, S.D. was smaller than the symbol.

and M14 ADR, decreased considerably. In Fig. 5 the results of 24 h pre-incubation with 300 μM MDL 72527 are shown. It is evident from this figure that both cell lines are highly sensitized by MDL 72527. Increasing the spermine concentration increases the toxic effect on both cell lines by about the same factor (Fig. 6). Only about 0.1% of the sensitized M14 cells were viable after 60 min of incubation with BSAO and 15 μM spermine, while about 5% survived without pre-treatment with MDL 72527.

3.3. Flow cytometry analyses of Annexin V and ROS content

Apoptosis induction in melanoma cells, after treatment with BSAO/spermine, was evaluated by using Annexin V labeling. The fraction of Annexin V positive/PI negative cells in early apoptosis was higher in MDR cells (13.02%±3.13) than in the WT ones (3.25%±0.20). While the fraction of Annexin V positive/PI positive cells in late apoptosis was 44.13%±1.92 in MDR cells and 34.98%±8.77 in the WT ones. In both cells lines the pre-treatment with MDL 72527 did not significantly modified the two apoptotic fractions. The MDL 72527 alone showed similar effects to untreated controls.

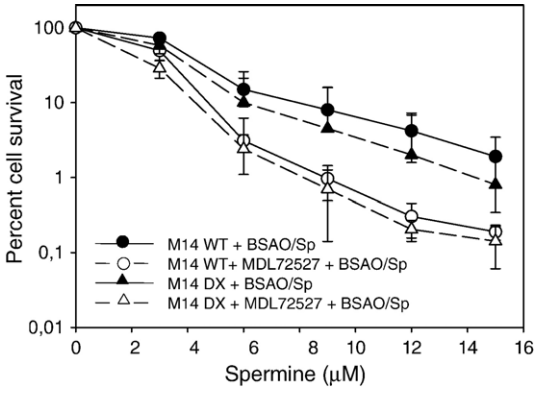

Fig. 6. Effect of pre-incubation with MDL 72527, and of spermine concentration on the survival of M14 melanoma cells. M14 WT and M14 ADR cells were pre-incubated with 300 μM MDL 72527 for 24 h. After washing they were exposed for 60 min (at 37 °C) to BSAO (6.5×10^{-3} IU/ml) and spermine at concentrations ranging from 0 to 15 μM (white circles and triangles), followed by the determination of plating efficiency. Each point represents the mean value of the results of 2 to 5 plates of three or four experiments. The error bars ± S.D. When not shown, S.D. was smaller than the symbol.

The intracellular ROS contents, evaluated by DHR 123 are reported in Table 1. The fluorescent signal values was slightly increased in both cell lines after treatment with MDL 72527 whereas a noticeable increase was revealed after the combined treatment (MDL 72527+BSAO/spermine).

The lower fluorescent signal in MDR cells may be attributed to the loss of the fluorescent dye due to the greater cell damage induced by BSAO/spermine on resistant cells, as shown in the microscopic analysis.

3.4. Electron microscopic analysis

Electron microscopy may reveal cellular targets of the cytotoxic polyamine metabolites. In Figs. 7a and b control M14 WT and M14 ADR cells, grown at 37 °C are shown. They are typically elongated and their surface is covered by randomly disseminated microvilli. After incubation for 60 min with BSAO (6.5×10^{-3} IU/ml) and spermine (6 μM) at 37 °C, the cells of both cell lines were less elongated than the untreated controls; some assumed a polygonal shape and some others tended to become rounded with blebs on their surface (Figs. 7c and d). These had a tendency to detach from the substrate. M14 WT and M14 ADR cells incubated for 60 min at 42 °C in the absence of BSAO and spermine (Figs. 8a and b) were polygonal, and their surface was covered by microvilli. After treatment with BSAO and 6 μM spermine at 42 °C the morphology of wild type cells appeared to be slightly modified, some rounded cells being observable, while the M14 ADR cells showed an irregular shape, or were round, with numerous surface blebs they tended to detach (Figs. 8c and d). Spermine or BSAO alone had no effect on cell morphology.

Both M14 WT and M14 ADR cells grown at 37 °C had a well-preserved ultrastructure (Figs. 9a and b). After exposure to BSAO and spermine at 37 °C M14 WT cells did not show consistent aberrations (Fig. 9c). In contrast, the mitochondria of MDR cells showed a highly condensed matrix and vacuolized cristae (Fig. 9d). Control M14 WT cells exhibited no significant ultrastructural changes after 60 min incubation at 42 °C. (Fig.

Table 1
Intracellular ROS content of M14 cells exposed to MDL 72527 alone or in association with the enzymatic reaction products of spermine

	M14 WT	M14 ADR
Control	18.74	14.97
MDL 72527, 300 μM	20.01	16.85
MDL 72527, 300 μM+(BSAO+sp12 μM)	146.25	29.40

ROS content was evaluated by flow cytometry after DHR123 staining. Values are expressed in arbitrary units (A.U.) calculated by the ratio of the mean fluorescence channel (MFC) of labelled samples on the MFC of negative controls. The results shown in the table are representative of one of three experiments carried out in the same experimental conditions, which gave very similar results.

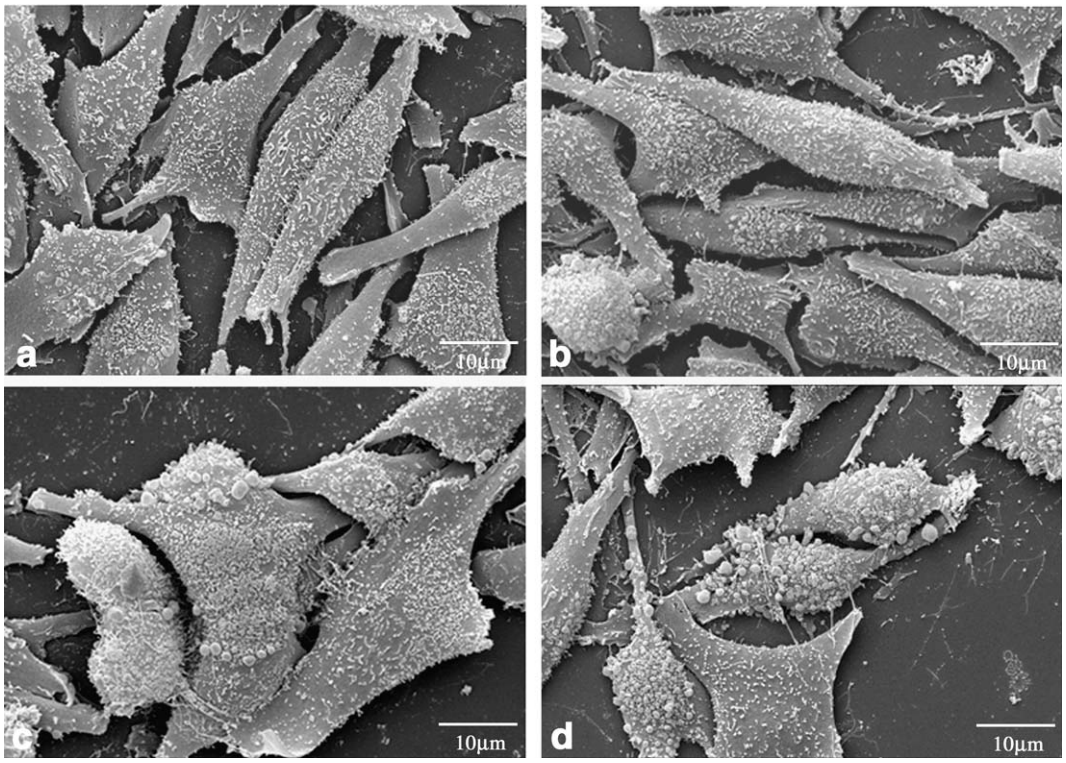

Fig. 7. Effect of exposure to BSAO and spermine on the morphology of M14 WT and M14 ADR cells (scanning electron micrographs). (a) Untreated M14 WT cells. (b) Untreated M14 ADR cells. (c) M14 WT cells exposed for 60 min to 6.5×10^{-3} IU/ml BSAO and 6 μM spermine at 37 °C. (d) M14 ADR cells, same treatment as in panel c.

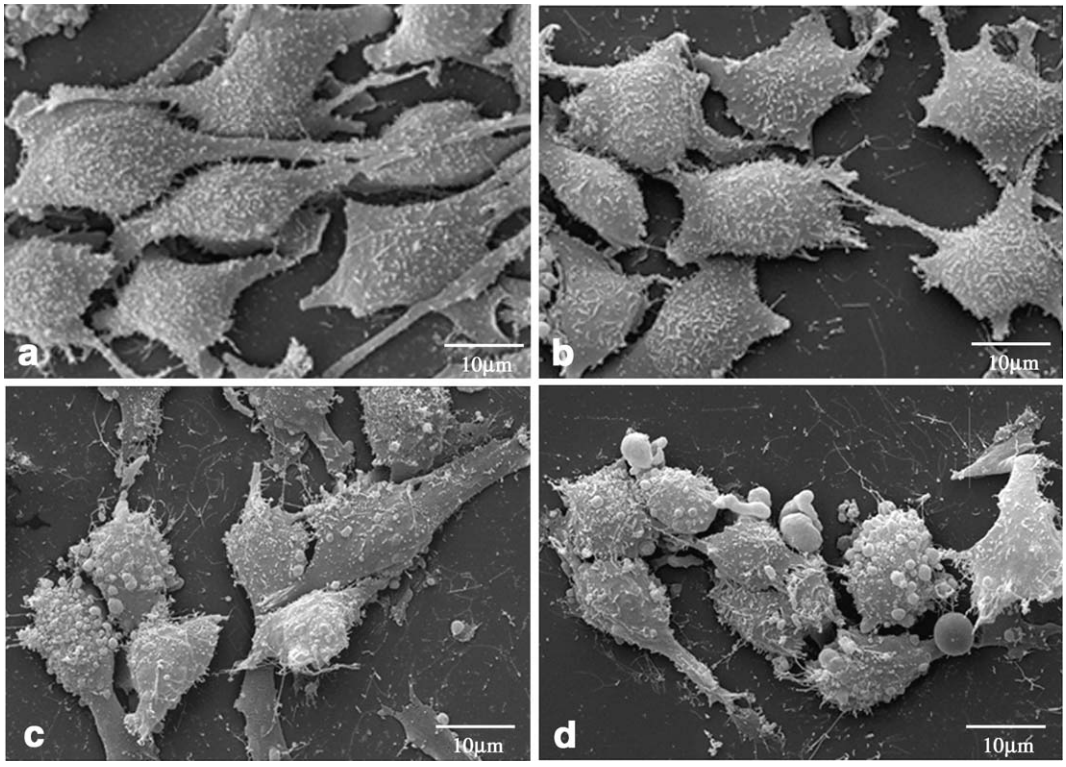

Fig. 8. Effect of exposure to 42 °C on the morphology of M14 WT and M14 ADR cells (scanning electron micrographs). (a) M14 WT cells exposed for 60 min to 42 °C. (b) M14 ADR cells, same treatment as in panel a. (c) M14 WT cells incubated in the presence of 6.5×10^{-3} IU/ml BSAO and 6 μM spermine for 60 min at 42 °C. (d) M14 ADR cells, same treatment as in panel c.

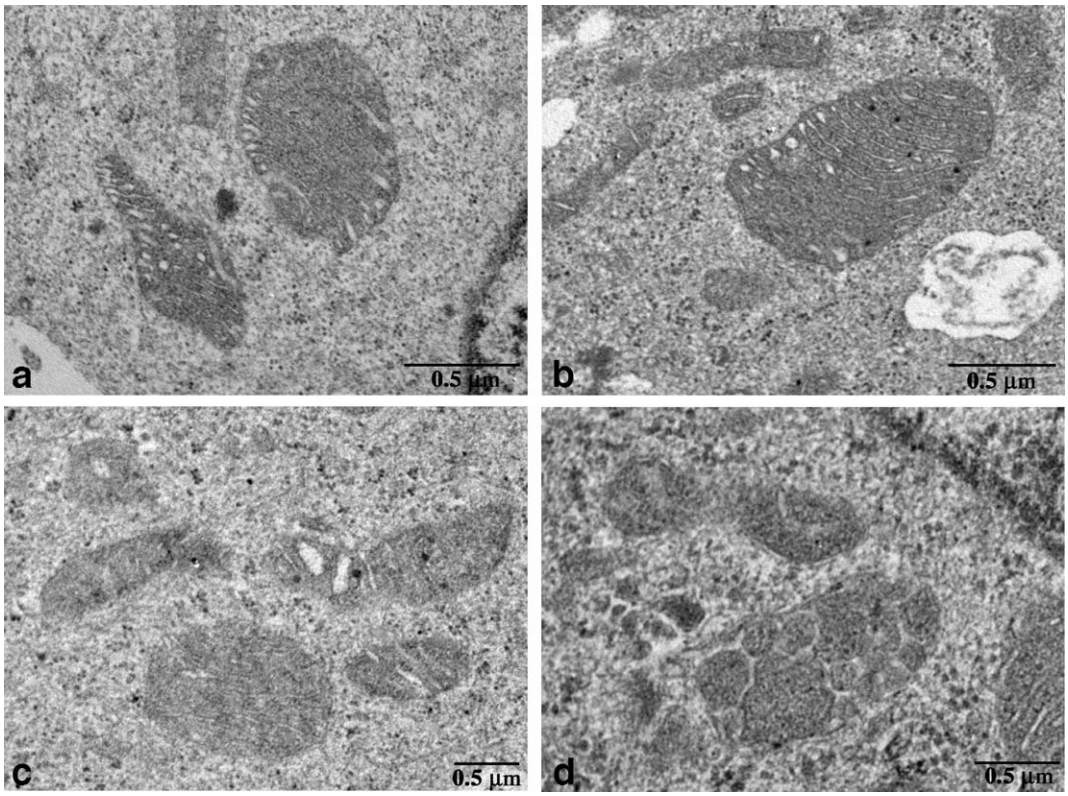

Fig. 9. Ultrastructural morphology of M14 WT and M14 ADR cells (transmission electron micrographs). (a) Untreated M14 WT cells. (b) Untreated M14 ADR cells. (c) M14 WT cells exposed for 60 min to 6.5×10^{-3} IU/ml BSAO and 6 μM spermine at 37 °C. (d) M14 ADR cells, same treatment as in panel c.

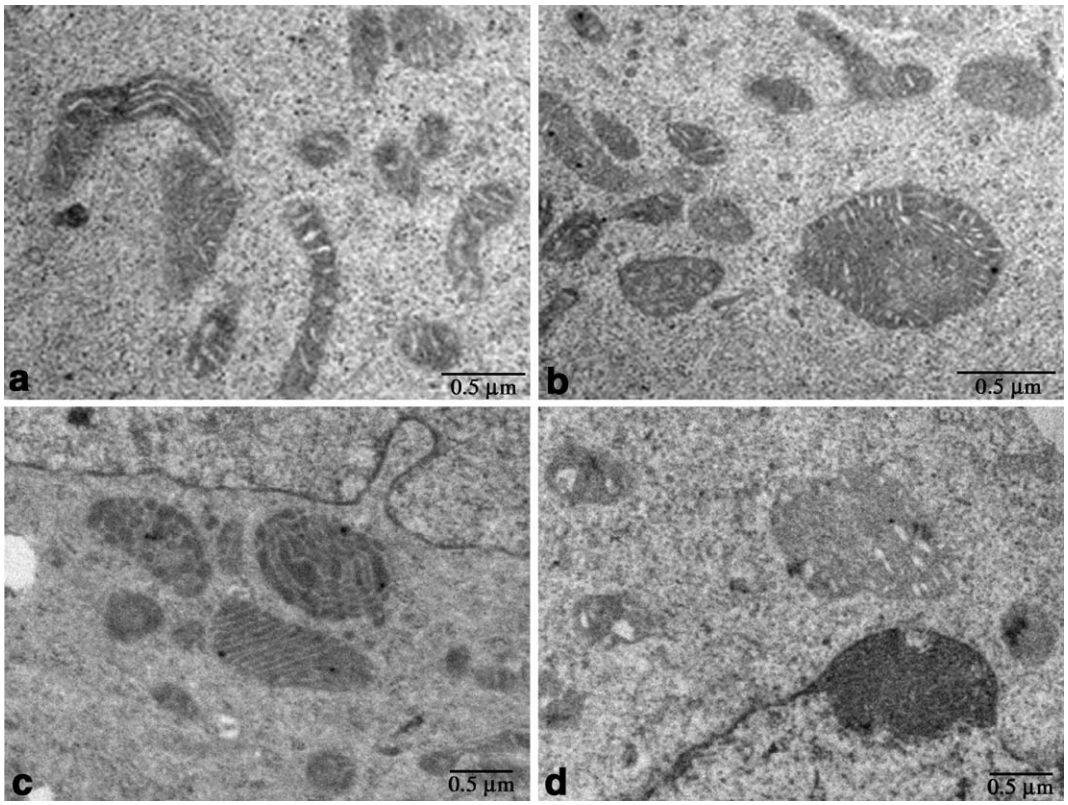

Fig. 10. Effect of exposure to 42 °C on the ultrastructural morphology of M14 WT and M14 ADR cells (transmission electron micrographs). (a) M14 WT cells exposed for 60 min to 42 °C. (b) M14 ADR cells, same treatment as in panel a. (c) M14 WT cells incubated in the presence of 6.5×10^{-3} IU/ml BSAO and 6 μM spermine for 60 min at 42 °C. (d) M14 ADR cells, same treatment as in panel c.

10a), M14 ADR cells, however, showed slight mitochondrial changes under the same conditions (Fig. 10b). After treatment with BSAO and spermine at 42 °C, the ultrastructure of M14 WT cells exhibited detectable modifications: most mitochondria showed a highly condensed matrix with vacuolized cristae (Fig. 10c), but no increase in the overall size of the mitochondria was observed. These modifications appeared much more evident in MDR cells, which displayed damaged mitochondria (Fig. 10d). Many of these cells also exhibited condensed nuclear chromatin, suggesting the onset of the apoptotic process. The ultrastructural comparison between treated M14 WT and M14 ADR cells clearly shows marked differences in their response to exposure to toxic polyamine metabolites at 37 °C and 42 °C, in agreement with the differences in cell survival observed in the clonogenic assays.

In Fig. 11. the effect of pre-treatment with 300 μM MDL 72527 alone for 24 h and then followed by exposure to BSAO and 6 μM spermine for 60 min at 37 °C is shown. The electron micrographs of these cells revealed a significant sensitizing effect of the polyamine oxidase inhibitor. Treatment with MDL 72527 alone produced in M14 WT cells evident morphological changes, mainly consisting in the appearance of a large number of lysosomes (arrows) and numerous cytoplasmic vacuoles (arrow heads) in Fig. 11a. The other subcellular structures appeared to be well preserved. The morphology of M14 ADR cells was less affected by MDL 72527, the only finding being the presence of numerous small lysosomes (Fig. 11b). In M14 WT cells treated for 24 h with MDL 72527, and then with BSAO and spermine (Fig. 11c) numerous lysosomes and cytoplasmic vacuoles were observable, and mitochondria were heavily damaged with rarefied matrices and fragmented cristae (arrows in Fig. 11c). After the same treatment resistant (M14 ADR) cells, showed very marked ultrastructural alterations. Particularly evident was the formation of a number of small roundish mitochondria with swollen cristae, likely a consequence of mitochondrial fragmentation (Fig. 11d).

4. Discussion

In conventional cancer chemotherapy numerous problems hamper successful treatment. Among these the lacking tumor specificity of the cytotoxic drugs, and the development of MDR of cancer cells belong to the most difficult problems to solve. Hence there is a demand for alternative therapeutic strategies. Although still at a beginning, the in situ formation of toxic compounds or radicals by enzyme catalyzed reactions is a promising start. For the slow release of toxic spermine metabolites into the tumor, the use of BSAO conjugated to biocompatible polymers is considered [5,25]. The administration of spermine may be superfluous in a therapeutic setting, since tumor cells have high polyamine concentrations, and the polyamines are released if cells were damaged. In view of the

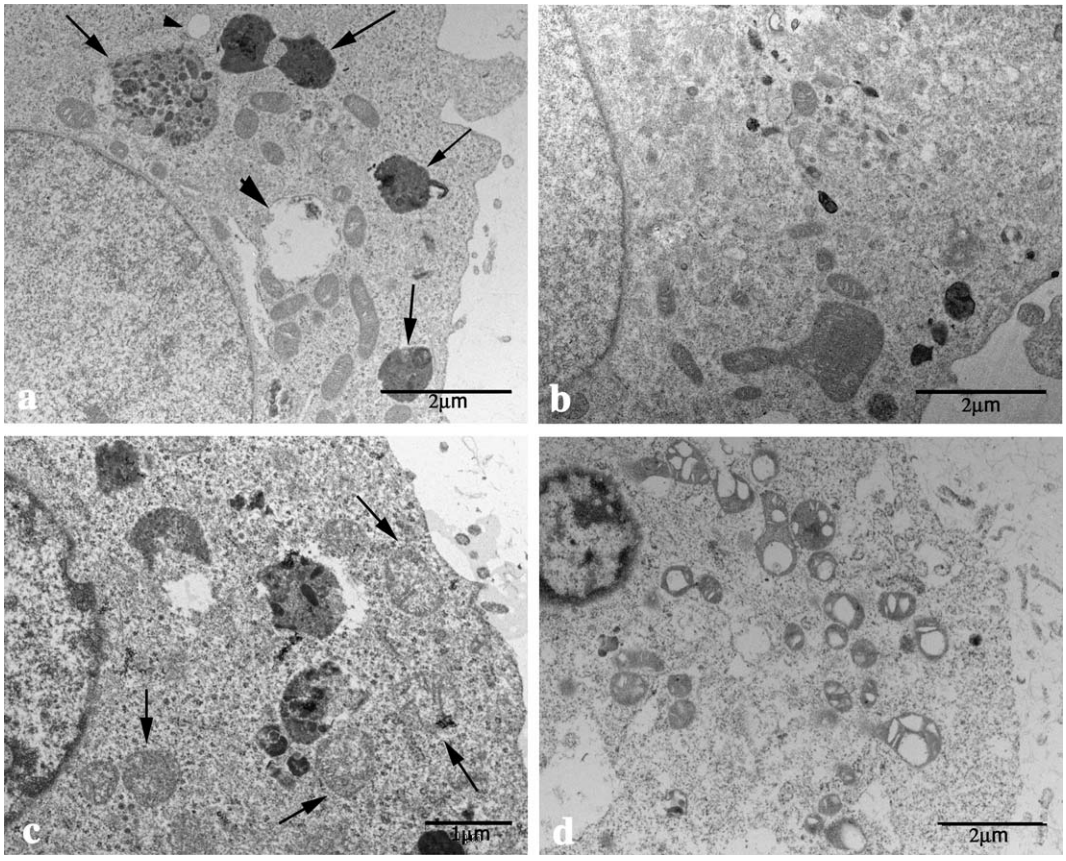

Fig. 11. Effect of pre-treatment with MDL 72527 on the ultrastructural morphology of M14 WT and M14 ADR cells (transmission electron micrographs). (a) M14 WT cells exposed to 300 μM MDL 72527 for 24 h at 37 °C. (b) M14 ADR cells, same treatment as in panel a. (c) M14 WT cells were first exposed for 24 h to 300 μM MDL 72527, and then for 60 min to 6.5×10^{-3} IU/ml BSAO and 6 μM spermine at 37 °C. (d) M14 ADR cells, same treatment as in panel c.

sensitivity of human cancer cell lines to hydrogen peroxide, as opposed to CHO cells [22], and in order to allow observing intermediary stages of cell damage, an experimental protocol was chosen that produces a slow release of metabolites over a limited period of time: 6 μM spermine is well below 25.5 μM, the K_m value of BSAO [4]. As is obvious from the biphasic time–effect relationship of cytotoxicity, (Fig. 3A) the impairment of cell viability by formation of toxic metabolites from spermine is not proportional to the time of exposure. During the first 30–40 min loss of viability per min was greatest because hydrogen peroxide is formed rapidly. However, the rate of its formation decreases, as spermine is metabolized. Since damaged cells release catalase, polyamines, and numerous other cell constituents, which may support or antagonize the formation and life span of hydrogen peroxide and aldehydes, the reaction conditions are not the same during the experimental period. However, because the experiments were carried out under strictly identical conditions (constant enzyme activity, constant cell number etc.) the results of different experiments (even with different cell lines) are comparable.

If formed in the vicinity of tumor cells the cytotoxic products of BSAO-catalyzed spermine oxidation, hydrogen peroxide and aldehydes (Fig. 1) are able to reduce the viability of various cells [9,12], including mouse melanoma [5]. The development of MDR is associated with phenotypic alterations. MDR cancer cells usually display a decrease in intracellular drug accumulation and/or drug distribution due to the over-expression of genes, which encode membrane-bound transporter proteins, such as a 170 kDa P-glycoprotein [26,27]. P-glycoprotein functions as energy-dependent pump, that expels drugs out of cells [28]. Prevention of accumulation by MDR cells of the toxic products of spermine oxidation appears to play no, or a minor role, because M14 ADR cells were not less, but significantly more sensitive to exposure to BSAO and spermine than M14 WT cells. The higher sensitivity to cytotoxic spermine derivatives of LoVo DX cells as compared with wild type cells has previously been attributed to an earlier and higher mitochondrial membrane depolarisation, and a higher basal production of reactive oxygen species [12].

There is an ongoing debate about the question, whether hydrogen peroxide or the spermine-derived aldehydes are the major effectors. Under the reaction conditions described in the methods section, catalase in the culture medium prevented the loss of about 80% viable cells (Fig. 4b), advocating for hydrogen peroxide as the most important cytotoxic metabolite of spermine in our experimental setting. Previous experiments support this view. If formed by the glucose oxidase reaction, or added as such to the cell suspension, hydrogen peroxide was cytotoxic at lower concentrations than acrolein [22]. Moreover, hydrogen peroxide generated in situ from hypoxanthine by reaction with xanthine oxidase has anti-tumor effects in vivo [29]. A contribution of the aldehydes is undoubted. However it is an open question, which spermine-derived aldehyde (Fig. 1) is most important. According to Sharmin et al. [30] acrolein is among the spermine metabolites nearly exclusively responsible for the induction of cell death. However, a role of acrolein in the early, most active phase of cell damage is under our conditions unlikely, because its spontaneous formation from the aldehydes deriving from spermine is a time-dependent process of unknown velocity, while in contrast hydrogen peroxide is produced immediately.

From a therapeutic point of view the improvement of the efficacy of in situ formation of cytotoxic polyamine metabolites is essential. This may be achieved by combinations of the treatment with cytotoxic drugs, or by heat. Hyperthermia in combination with cytotoxic drugs, preferentially with drugs that enhance cytotoxicity at elevated temperature [8], and the administration of drug combinations are clinically accepted methods. As appears from this work, the metabolites of spermine appear to behave as thermosensitizers. Likewise sensitizing cells by a lysosomotropic compound improved cell damage by hydrogen peroxide and other spermine metabolites. An extension of these experiments with the aim to further improve the cytotoxic effect of spermine metabolites appears important.

Treatment of M14 melanoma cells with BSAO and spermine was paralleled by characteristic morphological changes. In general the morphological alterations of M14 WT and M14 ADR cells mirrored the results of the cell survival experiments. We presume that the phenotypic changes (transformation of the elongated cells into polygonal and rounded shapes) is the result of the impairment of the cytoskeleton due to reaction with hydrogen peroxide and aldehydes. Severe changes of the mitochondrial structure, such as dilatation of the cristae and disruption of membranes were mainly observed in multidrug-resistant cells. Phosphatidylserine exposure on the outer surface of the cytoplasmic membrane clearly showed the onset of the apoptotic process. The formation of cytoplasmic vacuoles by treatment with MDL 72527 has previously been described for several cell lines [31,32]. However, in contrast with the present observations, vacuole formation in the published works was not reversible in the presence of MDL 72527. It should however be pointed out that the time of exposure to MDL 72527 in this work was limited to 48 h. It is not excluded that at longer exposure times vacuoles re-appear in melanoma cells. In contrast with vacuole formation, the ultrastructural irregularities of the mitochondria appear to correlate with the cytotoxic effects of the treatment. In agreement with this finding is the fact that mitochondriotoxic effects of exposure to spermine metabolites are among the early events [13].

The ultrastructural alterations observed in this work (Fig. 11) is evidence in favour of the lysosomotropic properties of MDL 72527, that were first described by Dai et al. [31]. We believe that the sensitization of M14 melanoma cells to the treatment with BSAO and spermine is mainly due to the effect of MDL 72527 on the endosomal–lysosomal system. A major role of lysosomes in cell death is presently not doubted [33], and it is known that the release of lysosomal enzymes causes oxidative stress [34,35]. The amplification of oxidative stress by the subsequent exposure of cells to hydrogen peroxide, and the toxic effects of the highly reactive spermine-derived aldehydes, explains the increase of cytotoxicity by an order of magnitude, as also demonstrated by the ROS content determinations (Table 1). According to Zdolsek et al. [36] even the cytotoxicity of hydrogen peroxide is linked with the

damage of lysosomal membranes. This observation may be considered as an additional argument in favour of a major role of lysosomes in the cytotoxicity of spermine-derived metabolites. In conclusion, toxic polyamine metabolites combined with either hyperthermia or sensitization by lysosomotropic compounds appear to be a promising strategy particularly against MDR resistant cancer cells.

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