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The physiological role of biogenic amines redox reactions in mitochondria. New perspectives in cancer therapy*

Review Article

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Summary. In tumours, polyamines and amine oxidases increase as compared to normal tissues. Cytotoxicity induced by bovine serum amine oxidase (BSAO) and spermine is attributed to H_2O_2 and aldehydes produced by the reaction. Increasing the incubation temperature from 37 to 42 °C enhances cytotoxicity in cells exposed to spermine metabolites. The combination BSAO/spermine prevents tumour growth, particularly well if the enzyme has been conjugated with a biocompatible hydrogel polymer. Since the tumour cells release endogenous substrates of BSAO, the administration of spermine is not required. Combination with hyperthermia improves the cytocidal effect of polyamines oxidation products. Our findings show that multidrug resistant (MDR) cells are more sensitive to spermine metabolites than their wild-type counterparts, due to an increased mitochondrial activity which induces the generation of intracellular ROS prior to the onset of mitochondrial permeability transition (MPT). It makes this new approach attractive, since the development of MDR is one of the major problems of conventional cancer therapy.

Keywords: Polyamines – Amine oxidase – Reactive oxygen species – Multidrug resistance – Mitochondria – Cancer

Abbreviations: AdNT, adenine nucleotide translocase; ADR, adriamycin resistant; ALDH, aldehyde dehydrogenase; AOs, amine oxidases; BSAO, bovine serum amine oxidase; CHO, Chinese hamster ovary; Cu-AOs, copper containing amine oxidases; DX, doxorubicin; $\Delta\Psi$, membrane potential; FAD-AO, flavin-adenin-dinucleotide dependent AO; MAO, monoamine oxidase; MDR, multidrug-resistant; METC, mitochondrial electron transport chain; MPT, mitochondrial permeability transition; N_γ, guanidine group of agmatine; ODC, ornithine decarboxylase; PAOs, FAD polyamine oxidase (not a CuAO); PEG, polyethylene glycol; P-gp, P-glycoprotein; Pi, inorganic phosphate; RLM, rat liver mitochondria; ROS, reactive oxygen species; SEM, scanning electron microscopy; SH, thiol groups; SMO, spermine oxidase; SOD, superoxide dismutase; SSAOs, semicarbazide-sensitive amine oxidases; TEM, transmission electron microscopy; TPQ, 2,4,5-trihydroxyphenylalanine quinone; VDAC, voltage-dependent anion channel; WR-1065, aminothiol N-(2-mercaptoethyl)-1,3-propanediamine; WT, wild-type

Introduction

Polyamines are polycationic biogenic amines required for both eukaryotic and prokaryotic cell growth and differentiation (Pegg, 1986). They attract interest because of their multiple functions in cell biology (Cohen, 1998) including, among many others, cell cycle regulation, gene expression and signal transduction (Bachrach et al., 2001; Childs et al., 2003).

The natural polyamines (putrescine, spermidine, spermine, and related structures) are formed from the decarboxylation products of ornithine and S-adenosyl-methionine in nearly all eukaryotic cells. The polyamine biosynthetic pathway is very active during the growth of various cancer cells. In fact, polyamines are often present at high concentrations in rapidly dividing tumour cells and growing tissues. Motives for these increased levels include enhanced putrescine synthesis from ornithine by ornithine decarboxylase (ODC), the rate-limiting enzyme, and increased uptake of polyamines (Marton and Pegg, 1995).

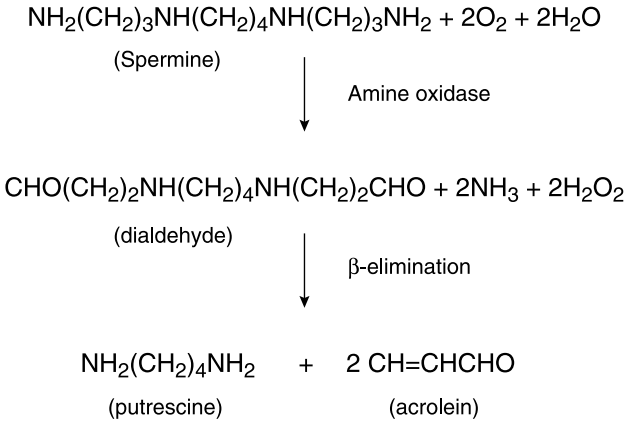
Cellular polyamine concentrations are highly regulated. However, in situations of over-accumulation or depletion

* This review is dedicated to Prof. Nikolaus Seiler.

of intracellular polyamine pools, cell death may be induced (Pignatti et al., 2004; Seiler and Raul, 2005).

The primary role of polyamines in regulating cell proliferation and death have induced scientists to investigate the role of these compounds in mitochondria, multifunctional organelles participating in a range of cellular processes such as energy production, proliferation, senescence and death (Goldenthal and Marin-Garcia, 2004). Mitochondria apparently lack a polyamine biosynthetic pathway, nevertheless substantial quantities of spermine and spermidine have been detected in the mitochondrial matrix and a specific mitochondrial polyamine transporter has been described (Toninello et al., 2004a). Moreover, very recently a specific transport system has been also described for the diamine agmatine, the new entry in polyamine family (Salvi et al., 2006). The main target of polyamines seems to be the mitochondrial permeability transition pore, a structure involved in mitochondria-mediated cell death where they exert a protective role by direct and indirect mechanisms (Toninello et al., 2004a).

Apart from being of vital importance for the viability and proliferation of most cells, the natural polyamines spermidine and spermine are also the source of cytotoxic metabolites. In fact, spermidine, spermine and other polyamines are substrates of a large class of enzymes, the amine oxidases (AOs), isolated from several sources that include spermine oxidase and polyamine oxidases (PAOs). AOs are important because they contribute to regulate the levels of these polycations; PAOs, for example, are involved in the homeostatic regulation of polyamine pools, while the other oxidases are important for the terminal catabolism of polyamines, i.e. they catalyse the formation of metabolites, like ammonia and amino acids, that can be excreted via the kidneys (Seiler, 1992).



Scheme 1. Reaction scheme for spermine oxidation in the presence of BSAO

This review deals mainly with the activity of AOs, isolated from bovine serum (BSAO), to counteract cancer growth as a new antitumour strategy. This enzyme, in the presence of spermine, generates the cytotoxic products, H_2O_2 and aldehyde(s), able to induce cytotoxicity on cancer cells (Scheme 1). In agreement with extensive experience in thermochemotherapy, an increase of the incubation temperature from 37 to 42 °C enhances the cytotoxicity on cultured cells exposed to enzymatic oxidation products of spermine (Agostinelli et al., 1994, 2006a). Other findings demonstrate that multidrug resistant (MDR) human colon adenocarcinoma (LoVo DX) and melanoma (M14 ADR) cell lines, obtained by prolonged exposure to doxorubicin, are more sensitive to spermine metabolites than their normal counterparts (Calcabrini et al., 2002; Arancia et al., 2004). This finding is of particular interest, since one of the main problems of conventional anticancer therapy is the development of drug resistance.

Binding and transport of biogenic amines

Substantial amounts of polyamines have been detected in liver, heart and brain mitochondria. The apparent lack of a biosynthetic pathway of polyamines in mitochondria suggested the presence of a transport system for these molecules in mitochondrial membranes (Toninello et al., 2004a).

Polyamine transport has been well characterized. It is mediated by an electrophoretic mechanism, dependent on membrane potential ($\Delta\Psi$) value, and exhibits a non-linear current voltage relationship. Polyamine transport is also temperature-dependent and increases with the charges number of the transported species. The polyamine transporter is common to all natural polyamines, so that they reciprocally inhibit their transport in a competitive manner. Studies of non-equilibrium binding of spermine to liver mitochondria have demonstrated the presence of two specific binding sites indicated as S_1 and S_2, both exhibiting low affinity and high binding capacity. Spermidine binds to the same two sites of spermine while putrescine binding is limited to the S_2 site. Further investigations have demonstrated that S_1 site is mainly involved in spermine and spermidine transport and in their inhibition of mitochondrial permeability transition (MPT) induction (see below). Site S_2, indeed, seems to be involved in other effects and in putrescine transport (Dalla Via et al., 1999). Flux-voltage relationships and free energy profiles derived from analyses of spermine transport provide evidence for the presence of a channel having two energy barriers, with an energy well, seat of S_1, located at $1/8$ of the length of the channel (Toninello et al., 2000).

Very recently, it has been reported that also agmatine, the biogenic amine formed by the decarboxylation of arginine, is taken up into the matrix space of mitochondria (Salvi et al., 2006). Agmatine uptake is highly dependent on mitochondrial energization and is electrophoretic in nature. It also exhibits a non-linear current voltage relationship according to the general behaviour of monovalent and polyvalent cations in mitochondria. It would seem the same for polyamines, but the observation that the divalent cations putrescine and cadaverine are ineffective in inhibiting agmatine transport, indicates the existence of distinct transport systems for agmatine and polyamines. Indeed, the lack of inhibition by basic amino acids excludes the possibility that agmatine can use their electroneutral transporter (Salvi et al., 2006). In conclusion, it has been demonstrated the existence of a specific selective transport system for agmatine, in rat liver mitochondria, which may be a channel or, alternatively, a single-binding centre-gated pore (Salvi et al., 2006). It is to be emphasized that also agmatine binds to two sites having similar binding characteristic as those of spermine and spermidine.

The role of biogenic amines in MPT

At the end of the 1970s, Hunter et al. (1976) observed that isolated mitochondria incubated with supraphysiological Ca^{2+} concentrations in combination with a wide variety of inducing agents or conditions, undergo a dramatic increase in non-specific inner-membrane permeability, a phenomenon named ''mitochondrial permeability transition'' (MPT). The existence of this event also in cultured cells and subsequently in vivo has been supported by several experimental evidences (Kim et al., 2003). The phenomenon is due to the opening of a proteinaceous pore, the transition pore, with an estimated diameter of about 3 nm, which permits a bidirectional transit of ions and solutes having molecular masses up to 1.5 kDa, located most likely at the gap junctions between the outer and the inner membranes (Kim et al., 2003). However, different diameter values for the transition pore have been experimentally demonstrated by different research groups, suggesting the existence of a pore capable of different degrees of opening or more pores of different molecular composition (Toninello et al., 2004b). The precise molecular composition is in fact not completely determined; there are evidences that it comprises components from cytosolic proteins (hexokinase, creatine kinase), outer membrane (voltage-dependent anion channel, VDAC), inner membrane (adenine nucleotide translocator, AdNT) and matrix (cyclophylin D) (Kim et al., 2003). It should be emphasized that recent genetic experiments bring serious doubts concerning the involvement of AdNT in the transition pore, but the question is greatly debated (Kokoszka et al., 2004).

Opening of the pore leads to colloid osmotic matrix swelling, collapse of the electrochemical gradient, loss of glutathione and endogenous cations and oxidation of several components including pyridine nucleotides and membrane thiols. All these events compromise cellular energy metabolism and Ca^{2+} homeostasis inducing necrotic cell death (Kim et al., 2003). Moreover, swelling of mitochondria is also directly responsible for the release of proapoptotic factors, such as cytochrome c, which induces apoptotic cell death. The type of death seems to be related to the energy balance of the cell and the duration of the opening of the transition pore (Kim et al., 2003). The possible implication of mitochondria in apoptosis by MPT induction has important consequences in cancer and degenerative diseases as well as for the development of cytoprotective drugs (Galluzzi et al., 2006).

The involvement of reactive oxygen species (ROS) in the induction of the MPT was primarily proposed by Vercesi and collaborators (1997). These authors provided experimental evidences for the presence of critical membrane thiols (probably located on AdNT) whose oxidation is responsible for pore opening. The main experimental evidences supporting this hypothesis are an enhancement in the generation of intracellular ROS prior to the opening of MPT, and its prevention by different antioxidant agents (Vercesi et al., 1997). Nowadays, the involvement of ROS in pore opening is considered of great relevance and has been confirmed in several cell lines and in different physiopathological conditions, such as in ischemia/reperfusion injury (Honda and Ping, 2006). Moreover, recent experimental evidences have shown that natural and pharmacological compounds, such as genistein, glycyrrhethinic acid, salicylic acid, could induce MPT through the increase in ROS production by the interaction with the electron transport chain (Salvi et al., 2004; Battaglia et al., 2005). All the natural occurring polyamines exert a protective effect against MPT, in isolated mitochondria, in the presence of different inducing agents (Toninello et al., 2004a).

Spermine can be considered as one of the most powerful physiological inhibitors. Peculiarly, spermine stimulates Ca^{2+} and inorganic phosphate (Pi) transport in matrix (Salvi and Toninello, 2004), the main inducers of the MPT, suggesting that its protective role does not take place through an interaction with the Ca^{2+} and Pi uptake systems. Several mechanisms have been proposed to explain this protective action. The first is a direct action

of spermine on the mitochondrial pore, such as an electrostatic interaction with the negatively charged head of cardiolipin annular domain of AdNT. This is supported by the fact that spermine is more effective than spermidine (three charges) and putrescine (two charges). A second possibility is an indirect action, involving the protein phosphorylation/dephosphorylation process. Other proposals suggest that spermine increases the affinity of ADP for an inhibitory binding site, or that the polyamine directly binds to a specific site in mitochondria responsible for MPT prevention. Taking into account that an important mechanism involved in transition pore opening is the interaction of ROS with the pore structures, a recent study on isolated rat liver mitochondria (RLM) has demonstrated that spermine directly acts against ROS like a free radical scavenger (Sava et al., 2006). In this work, it has been proved that spermine prevents oxidation of several mitochondrial components such as thiols, glutathione and pyridine nucleotides involved in the opening of the permeability transition pore. Furthermore, spermine protects also against lipid peroxidation and protein oxidation in RLM and, as these oxidations are due to the effect of a specific ROS, namely the hydroxyl radical, the protective effect of the polyamine could be a scavenging action directed against it. This confirms the protective effect of spermine on DNA structure, already demonstrated by Ha et al. (1998), in which hydroxyl radicals, or other ROS, directly react with spermine leading to the formation of spermine dialdehyde, a very low toxic compound compared with the above mentioned radicals.

These observations suggest that a primary physiological role of the polyamines can be the protection of DNA against ROS damage in the nucleus and, considering the mitochondrial transport, also of the mitochondrial DNA. While all the natural occurring polyamines exert a protective effect against MPT in isolated mitochondria, the monoamines exhibit several peculiar properties which differ from them. It has been reported that appropriate concentrations of tyramine, octopamine and benzylamine (100–500 mM) are able to trigger the MPT in the presence of Ca^{2+} (Marcocci et al., 2002). Instead, the diamine agmatine exhibits a double effect depending on its concentration. In fact, at low concentrations (10–100 μM) it acts like an inducer/amplifier of MPT induced by Ca^{2+} and Pi, while at high concentrations (1 mM and more) it behaves as an inhibitor of the phenomenon. A similar effect is exhibited by tyramine with its dose-dependent effect on MPT (Marcocci et al., 2002).

Agmatine exhibits this double effect on mitochondrial swelling and oxidation of thiol groups (SH) and glutathione, showing a dose-dependent production of H_2O_2. This can be explained in that compounds having secondary amino groups form immino radicals by interacting with Fe^{3+} ions of the iron-sulfur centers present in respiratory complexes (Dalla Via et al., 2006). In turn, by interacting with molecular oxygen, these radicals generate superoxide anions and subsequently H_2O_2 and other ROS. A nitrogen present in the guanidine group of agmatine (N_γ) may behave precisely in this way, thus explaining the prooxidant effect of this amine. The scavenging effect that agmatine shows at high concentrations can be due to a self-protection mechanism similar to that of monoamines.

The role of amine oxidases and ROS production in physiological processes

Mitochondria are the major, though not exclusive, source of endogenous ROS. The mitochondrial electron transport chain (METC) activity leads to the formation of ROS such as superoxide radical ($O_2^{\bullet -}$), H_2O_2 and the hydroxyl radical ($HO^{\bullet}$) which are usually removed by cells.

The superoxide radical is formed from molecular oxygen by reduction, in the reaction catalysed by NAD(P)H oxidases and by xanthine oxidase. Non-enzymatically, it is formed by reduction of O_2 with suitable redox-reactive compounds, such as the semi-ubiquinone/ubiquinone redox pair of the mitochondrial electron transport chain.

In mammalian organisms, H_2O_2 has a pivotal position within the ROS family. It has multiple physiological functions in signal transduction cascades and plays a role in the pathology of several disorders, including cancer and neurodegenerative diseases (Agostinelli and Seiler, 2006). Its formation by several enzymatic reactions as well as its controlled inactivation are the basis of ''redox homeostasis''. The metabolic interrelationships of H_2O_2 have been shown (Agostinelli and Seiler, 2006). $O_2^{\bullet -}$ forms H_2O_2 by a reaction that is catalysed by the superoxide dismutase (SOD), a mitochondrial Mn^{2+}-containing enzyme. Moreover, a variety of oxidases, which use molecular oxygen as substrate, also form H_2O_2: glucose oxidase, xanthine oxidase, peroxidases.

In addition, biogenic amines can also induce an increase in ROS production via the AO enzymes-mediated catabolic pathway (Agostinelli et al., 2004).

The superfamily of amine oxidases (amine: oxygen oxidoreductases, AOs) constitutes a heterogeneous class of enzymes, present in all living systems, involved in the control of the level of very active compounds, the mono-, di- and polyamines. These enzymes differ with respect to their molecular architecture, catalytic mechanisms, patterns of substrate specificity and inhibitor sensitivity as

well as subcellular localizations (Agostinelli et al., 2004). AOs are important in processes as different as bacterial growth on amines, secondary metabolism in plants and the oxidation of histamine and neurotransmitters in animals. In the most common classification, AOs are divided into two classes, based on the chemical nature of their cofactors (Mondovì et al., 1989). The first class is characterized by the presence of FAD (flavin adenine dinucleotide-AOs; EC 1.4.3.4) and includes the monoamine oxidases MAO A and MAO B, ubiquitous enzymes in the cells of most mammalian species, and the cytosolic polyamine oxidases (PAOs), found principally in vertebrates and plants. The enzymes belonging to the second class, Cu/TPQ-AOs (EC 1.4.3.6), share some fundamental structural properties: they are homodimers, each subunit containing one tightly bound Cu^{2+} ion and one carbonyl-type group which is identified as 6-hydroxydopa quinone (2,4,5-trihydroxyphenylalaninequinone, TPQ) or lysine tyrosylquinone, as cofactors (Janes et al., 1990). Both FAD- and Cu/TPQ-AOs have been isolated and characterized from microorganisms, plants and mammals. FAD-AOs are mainly intracellular enzymes, often associated with the outer mitochondrial membrane. The group of Cu/TPQ-AOs includes: (*i*) the ubiquitous intracellular AOs; (*ii*) the soluble AOs found in mammalian plasma; (*iii*) tissue-bound AOs, widely distributed in mammalian species, often indicated as semicarbazide-sensitive amine oxidases (SSAOs). Some Cu/TPQ-AOs have been highly purified and studied in detail, e.g. those from *Lens esculenta, Pisum sativum*, pig or bovine plasma and pig kidney. These enzymes operate by abstracting two electrons from amines and transferring them to molecular oxygen to form the corresponding aldehyde, ammonia and hydrogen peroxide, as described in Eqs. (1a) and (1b), even though some FAD-AOs preferentially catalyze the oxidation of secondary amino groups, releasing a primary amine instead of ammonia (Eq. (2)). The ping-pong catalytic mechanism of Cu/TPQ-AOs can be divided into two half-reactions: (1a) enzyme reduction by substrate at the quinone moiety ($TPQ \rightarrow TPQH_2$) and (1b) its reoxidation by molecular oxygen as shown below:

$$E_{ox} + R - CH_2 - NH_3^+ \rightarrow E_{red} - NH_3^+ + R - CHO \quad (1a)$$

$$E_{red} - NH_3^+ + O_2 + H_2O \rightarrow E_{ox} + NH_4^+ + H_2O_2 \quad (1b)$$

$$R_1 - CH_2 - NH - CH_2 - R_2 + O_2 + H_2O \rightarrow R_1 - CHO + NH_2 - CH_2 - R_2 + H_2O_2 \quad (2)$$

(1a, 1b) Cleavage at the primary amino group performed by both Cu/TPQ and FAD-dependent AOs; (2) Cleavage at the secondary amino group performed by the FAD-dependent AOs.

Amine oxidases, therefore, play an important role in the interconversion and oxidative deamination of physiological substrates, represented by several naturally occurring aliphatic and aromatic mono-, di- and polyamines. Acetylated amines, like N^1-acetylspermine and N^8-acetylspermidine can also be oxidatively deaminated (Agostinelli et al., 2004).

The H_2O_2 production in mitochondria by the oxidation of monoamines catalysed by MAO activity, might be extremely important in inducing mitochondria-mediated apoptosis. Malorni and collaborators (1998) demonstrated that well-known inhibitors of MAO activity, pargyline, clorgyline and deprenyl, are able to prevent the induction of apoptosis by serum starvation in human melanoma (M14) cells by maintaining mitochondrial integrity. Furthermore, inhibitors of different AOs have been reported to protect cells from apoptosis (Henle et al., 1986).

As described above, the monoamine tyramine, which is a substrate for both MAO A and MAO B, is able to induce the MPT in isolated mitochondria. The induction of MPT by tyramine is also inhibited by the MAO inhibitors clorgyline and pargyline. MPT induction has also been observed with octopamine (specific for MAO A) and benzylamine (specific for MAO B), suggesting the involvement of both MAOs (Marcocci et al., 2002).

As physiological role, BSAO (E.C. 1.4.3.6), a copper-containing enzyme, has been recognized to take part in protein post-translational modification of proteins (Mondovì et al., 2003). At the cellular level, BSAO showed to increase significantly the current of K^+ channels in N1E-115 neuronal cells, thus resulting as a modulator of the electronic properties of the ionic channels. At the organ level, BSAO shows anti-arrhythmic effect on ischemic isolated hearts at reperfusion. Furthermore, BSAO behaves as a free radical scavenger, and protects the isolated heart against dangerous effects of the reactive oxygen intermediates (ROS).

BSAO was used for the generation of cytotoxic polyamine metabolites. In Scheme 1, the major reactions of spermine with BSAO are shown. Their oxidative deamination generates H_2O_2, aldehydes and ammonia. H_2O_2 and aldehydes induce apoptotic and non-apoptotic cell death. Exposure of tumour cells to purified BSAO and spermine causes a time dependent decrease of cell viability (Calcabrini et al., 2002), and impairs the growth of a mouse melanoma (Averill-Bates et al., 2005). Toxic poly-

amine metabolites are currently explored as a possible strategy in tumour therapy, see next paragraph for details (Agostinelli et al., 2004; Agostinelli and Seiler, 2006).

Recent studies have demonstrated the existence of an inducible spermine oxidase (SMO/PAOh1), that catalyzes the reaction of conversion from spermine to spermidine and 3-aminopropane aldehyde, with the production of H_2O_2 (Pledgie et al., 2005).

The role of polyamines and their enzymatic oxidation products in cytotoxicity

The polyamines spermine, spermidine and putrescine are ubiquitous metabolites in the living word. The physiological function of these polycations in living organisms is so far not completely established but, in any case, is certainly related to the biogenic amine metabolism and therefore involved in essential processes in cell biology such as the regulation of neurotransmitters, the metabolism of histamine, the balance of the intracellular polyamine pool including, among their numerous other biological functions, cell growth and differentiation (Cohen, 1998).

Intracellular concentrations of polyamines are highly regulated. If they accumulate excessively within the cells, due to either very high extracellular concentrations or deregulation of the systems which control their homeostasis, polyamines can cause cytotoxic effects. Direct toxic effects are exerted only by high polyamine concentrations. Spermine, for example, is cytotoxic in the mM range. The cytotoxicity induced by spermidine and putrescine is lower than spermine concentration (Seiler et al., 2000). Thus, attempts to exploit polyamine metabolizing enzymes as targets, as well as to utilise the polyamine backbone as pharmacophore for the design of anticancer drugs, have been reviewed (Seiler, 2003a, b). Therefore, polyamines may also become a source of toxic metabolites. For the formation of cytotoxic metabolites, BSAO was used. The oxidation products, H_2O_2 and aldehyde(s), have been implicated in programmed cell death, induction of cytotoxicity and inhibition of cell division (Henle et al., 1986; Bachrach et al., 1987a). Cytotoxic metabolites of spermine formed in situ by an enzyme-catalyzed reaction might be useful for the destruction of tumours. The diversity between normal and tumour cells is related to polyamine content and metabolism. Polyamine concentrations are high in rapidly growing tissues such as tumours, for example, breast and colon cancer.

However, AO activity has a contrasting effect on cancer. On one hand it inhibits cell growth and induces cell death by necrosis (Calcabrini et al., 2002) and/or apoptosis (Lindsay and Wallace, 1999; Agostinelli et al., 2006b); on the other hand, AO activity has been correlated with cancer progression particularly when it is enhanced. The involvement of AOs in cancer is associated to two different aspects: the direct regulation of the level of biogenic amines in cells and the formation of catabolites, i.e. H_2O_2 and aldehydes, which have been demonstrated to be cytotoxic on several human cancer cells (Calcabrini et al., 2002; Agostinelli et al., 2006b). Recently, several aspects of the role of AOs in cancer have been taken deeply into consideration by Toninello et al. (2006).

Our findings showed the possibility of using purified BSAO in the presence of exogenous spermine or endogenous polyamines, after injection of the enzyme into the tumour, to induce cytotoxicity (Averill-Bates et al., 2005). The mechanism of cell death induced by BSAO and spermine, in the extracellular environment, was examined on human colon adenocarcinoma and melanoma cell lines, either drug-sensitive or MDR (Agostinelli et al., 2006b, c).

The cytotoxicity induced by BSAO in the presence of exogenous spermine was evaluated in both colon adenocarcinoma LoVo WT and LoVo DX cell lines as a function of spermine concentration as well as of exposure time, at 37 °C. The plating efficiency assay, a method which determines the ability of the cells to reproduce and form macroscopic colonies in culture, was applied to determine the cytotoxic effect. Figure 1 shows the percent cell sur-

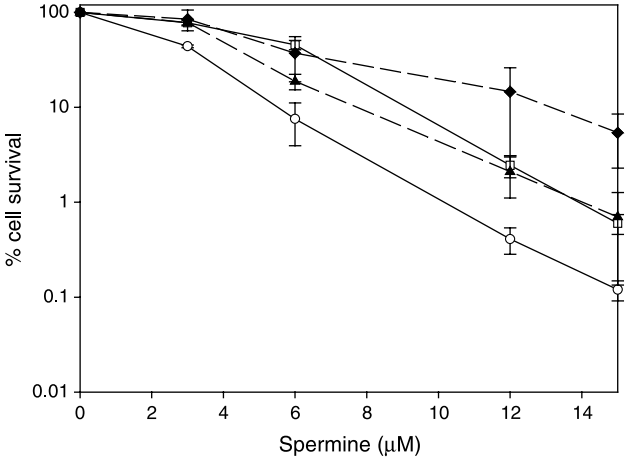


Fig. 1. Effect of exogenous spermine concentration (0–15 μM) on percentage cell survival in the presence of purified BSAO (6.54×10^{-3} U/ml) in LoVo WT (○), LoVo DX (□), M14 WT (◆) and M14 ADR (▲) cells during 60 min at 37 °C. Means and SDs are shown for two to six estimations from four to six experiments. Where not shown, SDs lie within the symbols

vival as a function of exogenous spermine concentration up to 15 μM in the presence of BSAO, after 60 min of incubation. Multidrug-resistant cells are more affected, by the treatment in the presence of different spermine concentrations, than their sensitive counterparts. For instance, at 6 μM spermine concentration, the survival of LoVo WT cells was approx. 45%, while only a very lower percentage approx. 7.5% in LoVo DX cells maintained their viability. To evaluate the contribution of H_2O_2 to cytotoxicity with respect to other enzymatic oxidation products, experiments were carried out in the presence of catalase. Catalase is a hydrogen peroxide-scavenging enzyme which converts H_2O_2 into water and oxygen. Therefore, a drastic reduction of the cytotoxic effect approx. 80% occurred in both cell lines, apparently due to the clearance of H_2O_2 by catalase. However, this result demonstrated that H_2O_2 is not the exclusive toxic agent and that other species are involved, such as aldehyde(s) including acrolein. To determine the aldehyde's contribution in inducing the cytotoxicity by BSAO/spermine, both enzymes, catalase and NAD-dependent ALDH were added to the incubation mixture. In these experimental conditions, cytotoxicity was completely inhibited throughout the 60 min of incubation (Fig. 2). Also the MDR human melanoma cells were more sensitive to the treatment at all concentrations of spermine than the corresponding wild type cells. At the 6 μM spermine concentration the survival of M14 WT cells was approx. 37.1%, while only 18.8% of the M14 ADR cells remained viable (Fig. 1).

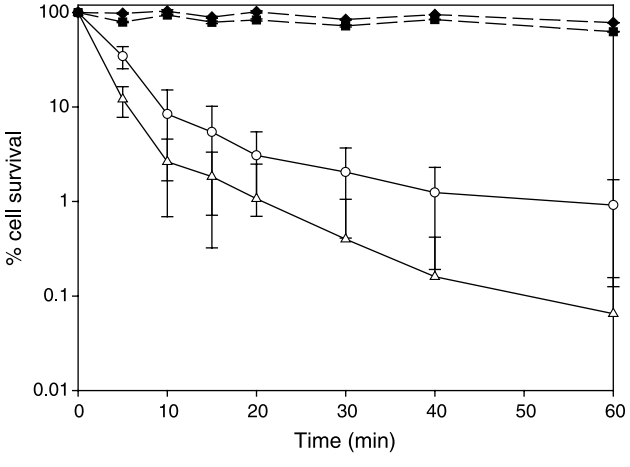


Fig. 2. Effect of catalase and ALDH on cytotoxicity induced by BSAO in the presence of spermine. LoVo WT (○) and LoVo DX (△) cells were incubated at 37 °C with purified BSAO (6.5×10^{-3} U/ml) and exogenous spermine (12 μm) without inhibitors, or with (solid symbols) catalase (240 U/ml) and ALDH 0.4 U/ml). Means and SDs are shown for two to six estimations from four to six experiments. Where not shown, SDs lie within the symbols

BSAO alone or spermine alone up to 15 μM were not toxic to either cell line.

Electron microscopy was used to reveal eventual cellular targets of the cytotoxic polyamine metabolites. To gain insight into the mechanisms responsible for the higher cytotoxic effect in MDR cells than the sensitive ones, the morphological and ultrastructural changes induced by the treatment with BSAO/spermine were investigated by scanning (SEM) and transmission electron microscopy (TEM). Figures 3a and b show control M14 WT and M14 ADR cells, respectively, grown at 37 °C. They have elongated or polygonal shape and their surface is covered by randomly disseminated microvilli. After treatment with BSAO/spermine (6 μM) at 37 °C, the cells of both lines (Figs. 3c and d) appeared less elongated than the untreated controls; some of them tended to become rounded with numerous blebs on their surface. These cells had a tendency to detach from the substrate.

Both M14 WT and M14 ADR control cells grown at 37 °C showed a well-preserved ultrastructure when observed by TEM. The cytoplasm was characterized by the presence of numerous mitochondria with parallel *cristae* in a dense and uniform matrix (Figs. 4a and b). After exposure to BSAO/spermine (6 μM) at 37 °C, M14 WT cells did not show any consistent aberration but some mitochondria display dilated *cristae* (Fig. 4c). The alterations of mitochondria structure were much more evident in MDR cells; in particular, they showed a highly condensed matrix and vacuolised *cristae* (Fig. 4d).

Similar morphological modifications and ultrastructural alterations were also observed in both LoVo colon adenocarcinoma cell lines, where MDR cells showed all the mitochondria visibly damaged.

Since mitochondria appear to play a pivotal role in determining the differential response between sensitive and drug-resistant cells, a flow cytometric study was carried out on LoVo cells in order to get information on the mitochondrial activity. The results showed a basal hyperpolarized status of the mitochondria in control MDR LoVo cells. After the treatment with BSAO/spermine, the higher sensitivity to cytotoxic spermine derivatives observed in adenocarcinoma LoVo DX cells, compared to their sensitive counterparts, has been therefore attributed to an earlier and higher mitochondrial membrane depolarisation. Moreover, a higher basal production of ROS in MDR cells than in the drug-sensitive cells was detected, suggesting an increased METC activity in MDR cells (Calcabrini et al., 2002; Arancia et al., 2004).

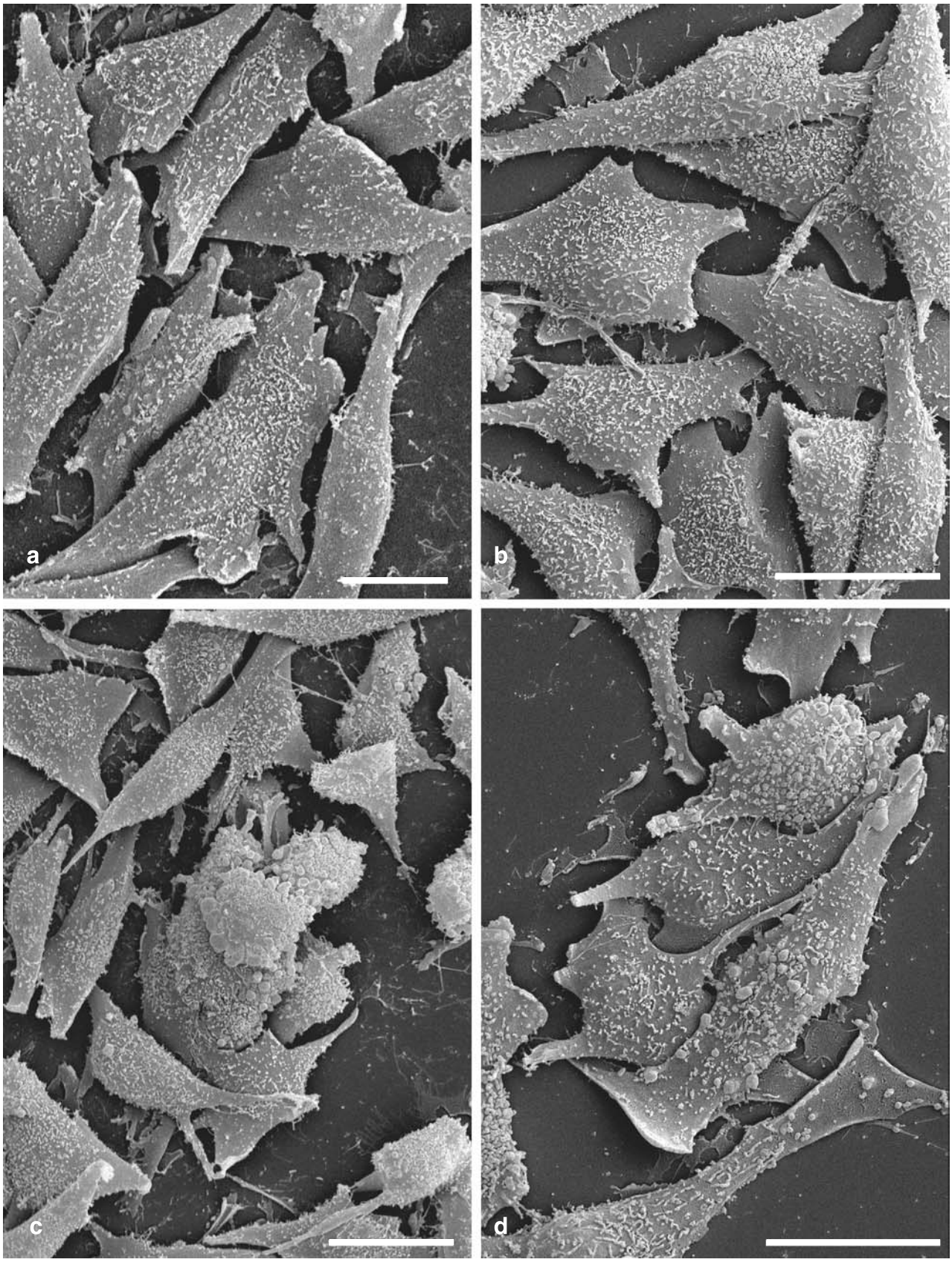


Fig. 3. Effect of exposure to BSAO and spermine on the morphology of M14 WT and M14 ADR cells (scanning electron micrographs). **a** Untreated M14 WT cells. **b** Untreated M14 ADR cells. **c** M14 WT cells exposed for 60 min to 6.5×10^{-3} IU/ml BSAO and 6 μM spermine at 37 °C. **d** M14 ADR cells,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

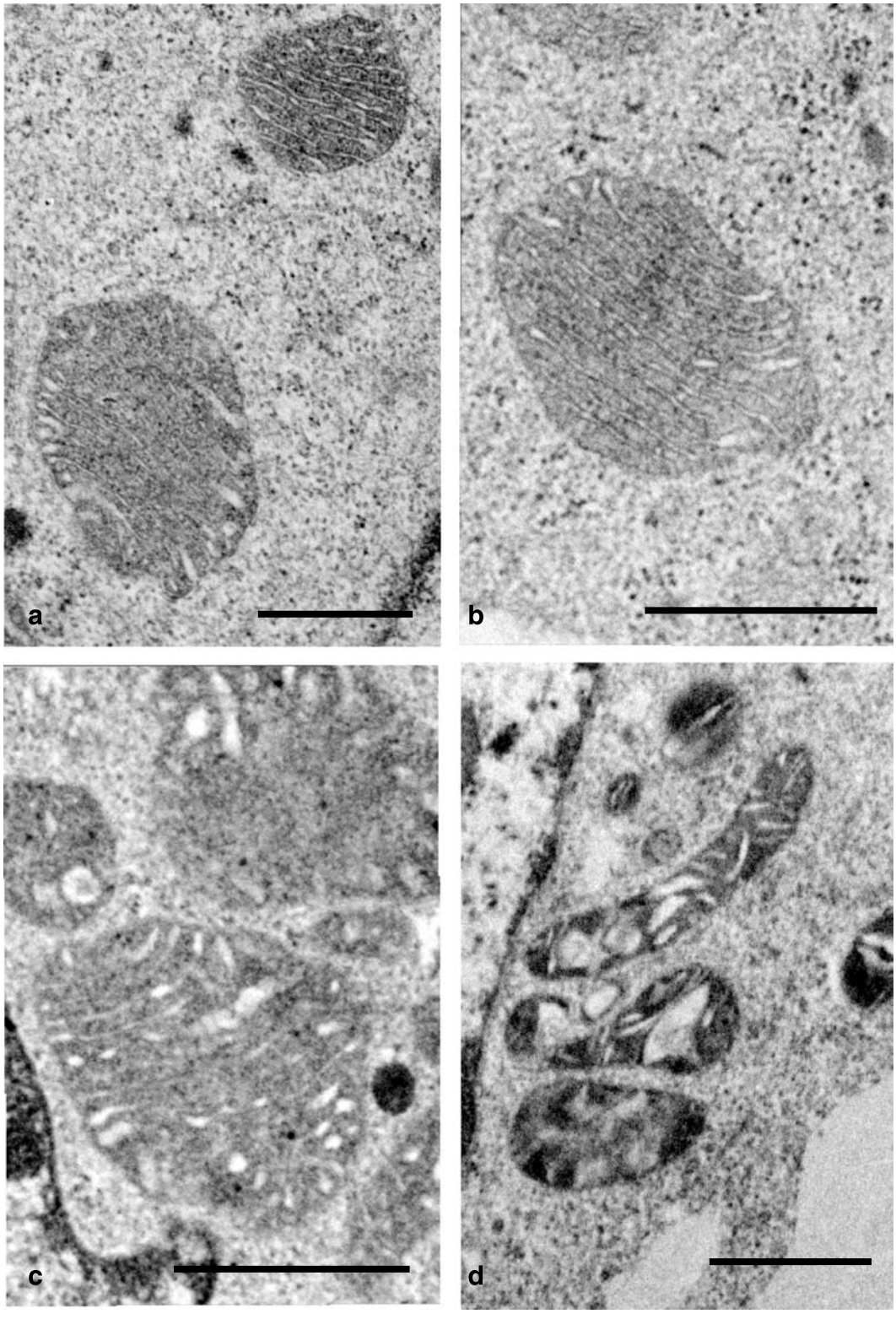


Fig. 4. Ultrastructural features of mitochondria of M14 WT and M14 ADR cells (transmission electron micrographs). **a** Untreated M14 WT cells. **b** Untreated M14 ADR cells. **c** M14 WT cells exposed for 60 min to 6.5×10^{-3} IU/ml BSAO and 6 μM spermine at 37 °C. **d** M14 ADR cells, same treatment as in **c**. Bars: 0.5μm

Amine oxidase/spermine and hyperthermia induce greater cytotoxicty in MDR cells

In conventional cancer chemotherapy, numerous difficulties obstacle successful of the treatment, such as the poor selectivity of the cytotoxic drugs for tumours and the development of MDR of cancer cells, which represent the most difficult problems to solve. Therefore, there is a requirement for alternative therapeutic strategies. Among these, it has been demonstrated that cancer cells are selectively killed by hyperthermia alone. Several studies evidenced a beneficial effect of hyperthermia when associated with other therapeutic modalities, such as irradiation or chemotherapy, in the treatment of human cancers (Vernon et al., 1996).

Our study deals with the possible effects of both H_2O_2 and aldehyde (produced by the BSAO/polyamine-spermine enzymatic system) in inducing a higher cytotoxicity at 42 °C than at 37 °C which becomes considerably greater in MDR phenotypes of both human colon adenocarcinoma and melanoma cells, compared to the sensitive ones (Calcabrini et al., 2002; Agostinelli et al., 2006a, b). The results suggest that H_2O_2, rather than aldehyde(s), is the most important cytotoxic metabolite of spermine. In fact H_2O_2, either formed by the glucose oxidase reaction, or added as such to the cell suspension, was cytotoxic at lower concentrations than acrolein (Averill-Bates et al., 1994). Moreover, H_2O_2 generated in situ from hypoxanthine by reaction with xanthine oxidase has antitumour effects in vivo (Yoshikawa et al., 1995). Anyway, in our experiments setting, both H_2O_2 and aldehyde(s), formed in presence of BSAO and spermine (6 μM) for 60 min of incubation at 42 °C, were responsible for cytotoxicity, since the addition of catalase alone did not result in a complete protection, however lower than at 37 °C. Our results clearly show that overexpression of P-glycoprotein (P-gp) in MDR cells did not confer any resistance to spermine enzymatic oxidation products. This phenomenon, as reported above, is due to an earlier and higher mitochondrial membrane depolarization and a higher basal production of ROS (Arancia et al., 2004).

Regional hyperthermia potentiates the cytotoxic action of many different anticancer drugs and has considerable potential in cancer treatment. Promising results are emerging from clinical studies involving hyperthermia combined with chemotherapy. In fact, 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 at elevated temperatures they become potent cell inactivators (Hahn, 1982). Another group of drugs, all of which were considered to be heat sensitizers, are the naturally occurring polyamines putrescine, spermine and spermidine (Gerner et al., 1980).

The enzymatic oxidation products of spermine behaved similarly to the above reported thermosensitizers (Nagele et al., 1990; Kapp and Hahn, 1979). Beneficial effects could therefore be achieved using localized heating to enhance the action of toxic products generated by BSAO/spermine within the tumour region, without increasing normal tissue damage. It was observed that the concentrations of spermine necessary to induce cytotoxicity are different in cell lines of various histotype (Averill-Bates et al., 1993; Agostinelli et al., 1994, 2006b; Calcabrini et al., 2002). Moreover, an interesting result was that spermine concentrations ≤ 1 μM, in the presence of BSAO, which were not toxic at 37 °C on both human colon adenocarcinoma and melanoma cells, became cytotoxic at 42 °C resembling thermosensitizers (Agostinelli et al., 2006a, b). A similar phenomenon was also observed in Chinese hamster ovary (CHO) cells (Agostinelli et al., 1994). The findings suggest a marked enhancement of cytotoxicity on LoVo and M14 cells induced by heat, attributed to both the enzymatic oxidation products of spermine H_2O_2 and aldehyde(s). Although still at a beginning, the in situ formation of toxic compounds or radicals by enzyme catalysed reactions is a promising start. For the slow release of toxic spermine metabolites into the tumour, the use of BSAO conjugated to biocompatible polymers is considered, as reported in the perspective paragraph (Averill-Bates et al., 2005; Demers et al., 2001).

Amine oxidases in therapeutic applications: perspectives

On the basis of the above described findings, the use of amine oxidase in cancer therapy deserves to be considered (Agostinelli et al., 2004). In the previous studies, H_2O_2 and aldehydes were produced outside the cells and subsequently they entered the cells, inducing cytotoxic effects. Catalytically liberated cytotoxic agents have the advantage to require only a few enzymatic units of the protein for toxin formation, and that the cytotoxic reaction products are continuously formed over an extended period of time (Agostinelli and Seiler, 2006).

Since endogenous polyamines are present at high concentrations in tumour cells and growing tissues, it is ex-

pected that by delivering BSAO into cancer cells, toxic enzymatic oxidation products could be produced intracellularly for selective in situ killing of the same cells. Therefore, strategies could be developed to find out how the enzyme could be delivered in vivo, for possible clinical application. In fact, in cultured normal chick fibroblasts or in fibroblasts transformed by Rous sarcoma viruses, Bachrach et al. (1987b) observed an inhibition of the synthesis of proteins and nucleic acids when the cells were enriched with amine oxidase by microinjection. Transformed cells were more sensitive than normal controls, presumably due to higher polyamine content. Moreover, attempts were made to incorporate the enzyme into liposomal vesicles (Agostinelli et al., 1988), and to prepare amine oxidase-gold complexes that are bound and incorporated by hepatocytes (Dini et al., 1991). Thus, endogenous polyamines could be targeted and oxidized by the enzyme.

In this context, our attention was particularly focused on another strategy, currently under further investigation, to produce an immobilized BSAO with the aim to increase its plasmatic half-life and therapeutic efficacy and to decrease drug toxicity. The enzyme was conjugated to a bio-compatible non-immunogenic polymer, polyethylene glycol (PEG), and then immobilized into a hydrogel-type matrix (Demers et al., 2001). Hydrogels are hydrophilic macromolecular networks that possess a high water content. This feature should allow a controlled delivery of the enzyme in crossing the cell membrane and then, also a controlled release of the enzyme in the intracellular environment to maintain a drug concentration at therapeutic levels. Therefore, the immobilized BSAO exhibited considerable advantages over the free enzyme. Both native and immobilized BSAO were then compared in vivo, in terms of their respective abilities to induce melanoma regression in mice by either apoptosis or necrosis. In fact, the growth of a mouse melanoma (B16-F0) was reduced by 70% after a single injection of the immobilized enzyme, in comparison with 32% inhibition after injection of the same amount of native BSAO. While the immobilized enzyme induced a high level (70%) of apoptosis, non-apoptotic cell death prevailed in the case of the native enzyme (Averill-Bates et al., 2005). The difference of cell death ratio was attributed to the slow, gradual release of spermine enzymatic oxidation products from the hydrogel, i.e. the long-term exposure of the tumour to ROS and aldehydes, as compared with the shorter, though more rapid release of toxic metabolites by the native enzyme.

Based on the above discussed release of the substrates of ROS formation from tumour cells, adjunct treatments suitable to enhance cell death, or impair tumour cell growth by mechanisms which synergise the effect of ROS are of obvious advantage. Combinations with conventional and new anti-tumour drugs are currently investigated, as reported in the previous paragraph.

Conclusions

Numerous studies have demonstrated that H_2O_2, as other ROS, are able to affect cell cycle progression, inducing inhibition of cell proliferation and a block in G_1, S or G_2 phases of the cell cycle (Boonstra and Post, 2004). The growth arrest can be transient or permanent and, in the latter case, the process may end in cell death by apoptosis or necrosis, depending on the entity of the oxidative stress, time of treatment and cell type.

Biogenic amines in cell redox balance may behave directly as scavengers against specific types of ROS, or may indirectly cause an increase in ROS production, via H_2O_2 generation, mediated by their oxidative deamination by amine oxidases. These enzymes are important because they contribute to regulate the levels of polyamines.

However, therapeutic applications of radical generating systems are still at the beginning. It is our hope that, if the results of further studies of these approaches will be up to expectations, the handling of amine oxidase activity, in the presence of biogenic amines, will undoubtedly turn out to be a powerful tool in the development of new anticancer treatments (Averill-Bates et al., 2005). In fact, since hyperthermia is a clinically established therapeutic method, strategies should be developed that combine hyperthermia with extracellular ROS formation. In support of this idea is the fact that a marked enhancement of cytotoxicity, attributed to H_2O_2 and spermine-derived aldehyde(s), was observed by elevating the temperature of tumour cell cultures from 37 to 42 °C (Agostinelli et al., 2006a, b).

The new approaches show a higher sensitivity, to the cytotoxic spermine metabolites H_2O_2 and aldehydes, of MDR human adenocarcinoma and melanoma cells, as compared with their wild type counterparts. This finding has been previously attributed to an earlier and higher mitochondrial membrane depolarization, and a higher basal production of ROS (Arancia et al., 2004). In fact, H_2O_2 could directly interact with some iron of Fe/S centres located in the respiratory chain, raising the highly reactive hydroxyl radical ($HO^{\cdot}$) by means of Fenton reaction, which induces some thiol (SH) groups, proteins and lipids oxidation.

In conclusion, hyperthermia combined with either toxic BSAO/polyamine metabolites or with thermosensitizing

drugs, is of great interest since it might represent a promising strategy to overcome MDR of cancer cells.

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