

Potential anticancer application of polyamine oxidation products formed by amine oxidase: a new therapeutic approach

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Abstract The polyamines spermine, spermidine and putrescine are ubiquitous cell components. These molecules are substrates of a class of enzymes that includes monoamine oxidases, diamine oxidases, polyamine oxidases and copper-containing amine oxidases. Amine oxidases are important because they contribute to regulate levels of mono- and polyamines. In tumors, polyamines and amine oxidases are increased 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. This study demonstrated that multidrug-resistant (MDR) cancer cells (colon adenocarcinoma and melanoma) are significantly more sensitive than the corresponding wild-type (WT) ones to H_2O_2 and aldehydes, the products of BSAO-catalyzed oxidation of spermine. Transmission electron microscopy (TEM) observations showed major ultrastructural alterations of the mitochondria. These were more pronounced in MDR than in WT cells. Increasing the incubation temperature from 37 to 42°C enhances cytotoxicity in cells exposed to spermine metabolites. The combination BSAO/spermine prevents tumor growth, particularly well if the enzyme has been conjugated to a

biocompatible hydrogel polymers. Since both wild-type and MDR cancer cells after pre-treatment with MDL 72527, a lysosomotropic compound, are sensitized to subsequent exposure to BSAO/spermine, it is conceivable that combined treatment with a lysosomotropic compound and BSAO/spermine would be effective against tumor cells. It is of interest to search for such novel compounds, which might be promising for application in a therapeutic setting.

Keywords Polyamine · Amine oxidases · Tumor cells · Multidrug resistance · Hyperthermia

Abbreviations

ADR	Adriamycin-resistant cells
ALDH	Aldehyde dehydrogenase
AO	Amine oxidase
APAO	N^1 -Acetylpolyamine oxidase
BSAO	Bovine serum amine oxidase
CHO	Chinese hamster ovary
CuAO	Copper amine oxidase
Cu/TPQ-AO	Amine oxygen oxidoreductase copper-containing
DAO	Diamine oxidase
DFMO	Difluoromethylornithine
DX	Doxorubicin-resistant cells
FAD	Flavin adenine dinucleotide
hVAP-1	Human vascular adhesion protein-1
LoVo	Colon adenocarcinoma cell
LOX	Lysyl oxidase
M14	Melanoma cell
MAO	Monoamine oxidase
MDL 72527	N^1, N^4 -bis(2,3-butadienyl)-1,4-butanediamine
MDR	Multidrug-resistant

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NSAIDS	Non-steroidal anti-inflammatory drugs
PAO	Polyamine oxidase
PEG	Polyethylene glycol
P-gp	P-glycoprotein
ROS	Reactive oxygen species
SAO	Serum amine oxidase
SH	Thiol groups
SMO	Spermine oxidase
SSAO	Semicarbazide-sensitive amine oxidase
TEM	Transmission electron microscopy
LTQ	Lysine tyrosylquinone
TNF α	Tumor-necrosis factor α
TPQ	2,4,5-Trihydroxyphenylalaninequinone
VAP-1	Vascular adhesion protein-1
WR 1065	Aminothioliol <i>N</i> -(2-mercaptoethyl)- 1,3-propanediamine
WT	Wild-type

Introduction

The polyamines spermine, spermidine and putrescine are ubiquitous cell components, which are essential for cell proliferation and differentiation (Pegg 1988). 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). Moreover, it has been demonstrated that intracellular accumulation of polyamines can induce programmed cell death (or apoptosis) in various cell types (Tobias and Kahana 1995) through activation of the caspase cascade (Stefanelli et al. 1998, 1999). The activation of caspases is triggered by the release of mitochondrial cytochrome *c* (Ghafourifar et al. 1999; Andreyev and Fiskum 1999; Hengartner 2000), which has been shown to be induced by spermine or spermidine (Stefanelli et al. 2000). The natural polyamines are substrates of several flavin adenine dinucleotide (FAD)-dependent enzymes [monoamine oxidase (MAO), polyamine oxidase (PAO), spermine oxidase (SMO)] and of copper amine oxidases (CuAOs) [diamine oxidase (DAO), serum amine oxidases (SAO), lysyl oxidase (LOX)] (A-rancia et al. 2004; Agostinelli et al. 2004). Two isoforms, termed monoamine oxidases A and B, are present in approximately the same amounts in the outer mitochondrial membrane (Edmondson et al. 2009; La Regina et al. 2007) and inhibition of either of them has been demonstrated to protect cells against apoptosis (Malorni et al. 1998; Paterson and Tatton 1998). PAO is involved in the homeostatic regulation of polyamine pools. The other oxidases are important for the terminal catabolism of polyamines, i.e.,

they catalyze the formation of metabolites (ammonia and amino acids) that can be excreted via the kidneys (Seiler 1992). Products of polyamine oxidation are toxic to cells. In serum-containing cell cultures, cytotoxicity of polyamines correlates with their property as substrates of serum amine oxidases (Morgan 1988). Oxidation represents a crucial reaction in polyamine metabolism, by means of which these polycations enter the catabolic routes for irreversible inactivation and elimination. Mono-, di- and polyamines, as well as several *N*-acyl amines, are oxidatively deaminated by amine oxidases (AOs) in a reaction consuming O₂ and H₂O, either by the removal of one primary amino group or by the cleavage of the molecule at the secondary nitrogen atoms and oxidation of the terminal carbon of the remaining molecule to an aldehyde group during the same catalytic cycle. Oxidative deamination produces aldehyde, amine and H₂O₂ in stoichiometric amounts according to the following equations (Bellelli et al. 2000; Agostinelli et al. 2009a; Edmondson et al. 2007, 2009):

1. $R-CH_2-NH_2 + H_2O + O_2 \rightarrow R-CHO + NH_3 + H_2O_2$
 2. $R-CH_2-NH-CH_2-R + H_2O + O_2 \rightarrow R-CHO + NH_2-CH_2-R + H_2O_2$
1. Cleavage at the primary amino group (operated by both copper and FAD-dependent AOs).
 2. Cleavage at the secondary amino group (operated by the FAD-dependent AOs).

This minireview deals with the fundamental structural properties of a Cu/TPQ (copper/2,4,5-trihydroxyphenylalaninequinone) AO isolated from bovine serum (BSAO) (Turini et al. 1982), involving the formation of a Schiff base between the amine substrate and the organic cofactor (Bellelli et al. 2000; Agostinelli et al. 2009a). Moreover, it describes the possibility of using purified BSAO, in the presence of exogenous spermine or endogenous polyamines, to induce cytotoxicity, on several human tumor cell lines in vitro (Calcabrini et al. 2002; Agostinelli et al. 2009b) or after injection of the enzyme into the tumor in vivo (Averill-Bates et al. 2005). Amine oxidases metabolize preferentially polyamines (spermine and spermidine) to generate the reaction products H₂O₂ and aldehyde(s) (Fig. 1) (Agostinelli and Seiler 2006). Such toxic products are able to induce stress-activated signal transduction pathways, leading to cell death, by necrosis or apoptosis (Lindsay and Wallace 1999; Seiler and Raul 2005; Agostinelli et al. 2006a).

The research for innovative therapeutic approaches, based on the use of new pharmacological agents or drug associations effective against resistant tumors and, possibly, with a low degree of undesirable side effects, is gaining interest in clinical oncology. In our study carried

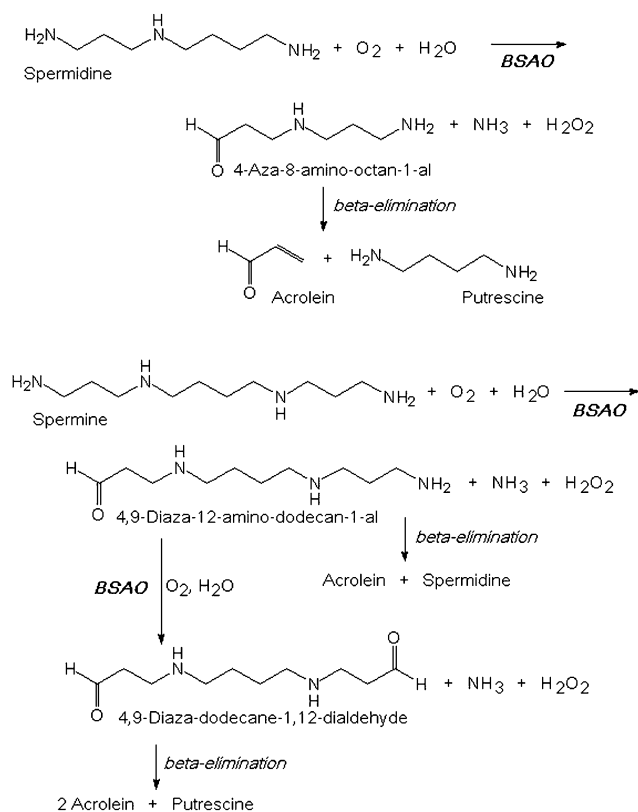


Fig. 1 Reaction scheme of the oxidative deamination of spermidine and spermine by bovine serum amine oxidase

out on melanoma (M14) (Agostinelli et al. 2006a) and colon adenocarcinoma (LoVo) (Agostinelli et al. 2006b) human cancer cells, it was observed that H₂O₂ and aldehydes, generated by subcytotoxic concentrations of BSAO and spermine, exerted higher cytotoxic effect on doxorubicin-resistant cells (M14 ADR and LoVo DX) than on their parental sensitive counterparts (M14 WT and LoVo WT). The findings showed that (a) multidrug-resistant (MDR) cells are more sensitive than wild-type cells to the cytotoxic effect of the enzymatic oxidation products of spermine, (b) cytotoxicity was mainly due to the presence of H₂O₂ and completely inhibited in the presence of both catalase and NAD-dependent aldehyde dehydrogenase (ALDH), (c) mitochondria seem to play a pivotal role in determining the differential response between sensitive and drug-resistant cells.

Classification and occurrence of amine oxidases in living organisms

The superfamily of AOs represents an important class of enzymes present in numerous living systems. These enzymes differ with respect to their molecular architecture, catalytic mechanisms, patterns of substrate specificity and

inhibitor sensitivity, and subcellular localizations (Agostinelli et al. 2004). In the most common classification, these enzymes (amine: oxygen oxidoreductases, AOs, E.C. 1.4.3.4.) are divided into two classes, based on the chemical nature of the cofactors involved (Mondovì et al. 1989). The first class is characterized by the presence of FAD-AOs, ubiquitous enzymes in the cells of most mammalian species, and the PAOs found principally in vertebrates and plants (Binda et al. 2002). The second class consists of enzymes having a tightly bound Cu²⁺ ion and a carbonyl-type group identified as either a 6-hydroxydopa quinone (2,4,5-trihydroxyphenylalaninequinone, TPQ) or a lysine tyrosylquinone (LTQ) at their active site. TPQ is easily detected, thanks to its pink absorption and its reactivity toward semicarbazide and phenylhydrazine; however, its chemical identification is relatively recent (Janes et al. 1990). Both FAD- and Cu²⁺/TPQ-amine oxidases have been isolated and characterized from numerous organisms ranging from microorganisms, plants and mammals. FAD-AOs are mainly intracellular enzymes, often associated with the outer mitochondrial membrane (Edmondson et al. 2007, 2009), whereas CuAOs are either intra- or extracellular enzymatic proteins, or in some cases integral plasma membrane proteins (Agostinelli et al. 2007a, 2009a; Floris and Mondovì 2009) (Table 1).

Mitochondrial monoamine oxidases are primarily involved in the metabolism of the biogenic monoamine neurotransmitters and exogenous aryl-alkylamines. They therefore play a role of secondary importance in the oxidation of acetylated polyamines. In mammals, two different forms of MAOs are present in most tissues, namely MAO-A and MAO-B (Abell and Kwan 2001), classified on the basis of their selective inhibition by clorgyline and selegiline (*L*-deprenyl), respectively (Binda et al. 2007; La Regina et al. 2007). Isoenzyme A occurs in catecholaminergic neurons, while isoenzyme B is present in neurons and glial cells of the human brain and also in other different cell types. This different localization suggests that the two isoforms have different physiological functions. In fact, A and B MAOs are probably related to psychiatric and neurological disorders such as depression and Parkinson's disease, respectively (Checkoway et al. 1998; Mallajosyula et al. 2008). Bianchi et al. (2005) suggested the involvement of MAO-A in cardiac cellular degeneration, since MAO-A levels have been found to dramatically increase in the heart of aged rats. The consequence of this increase in the level of MAO-A in the heart is suggested to involve increased apoptosis and necrosis of cardiac cells due to increased levels of ROS from the H₂O₂ produced. Among other roles, in humans both amine oxidases, MAO-A and MAO-B, are the major enzyme systems involved in vivo in the oxidative metabolism of xenobiotic amines, drugs, in particular (Strolin Benedetti et al. 2007).

Table 1 Classification and major characteristics of the amine oxidases are mentioned in this review

Enzyme	Classification	Cofactor occurrence	Tissue distribution	Substrate specificity	Inhibitor	Physio-pathological function
Monoamine oxidase (FAD-AO)	MAO-A	FAD	All tissues (outer mitochondrial membrane) Catecholaminergic neurons (MAO-A)	Noradrenaline, serotonin, tyramine, tryptamine	Clorgyline	Psychiatric-neurological disorders (depression), cardiac cellular degeneration
	MAO-B		Neurons and glial cells (MAO-B)	Dopamine, benzylamine, phenylethylamine, tyramine, tryptamine	Selegiline (L-deprenyl)	Psychiatric-neurological disorders (Parkinson's disease)
	PAO		All tissues (cytoplasm)	N ¹ -Acetylspermine, N ¹ , N ¹² -diacetylspermine, N ¹ -Acetylspermidine, spermine	MDL 72527	Homeostatic regulation of polyamine pools
	SMO		All tissues (peroxisomes)	Spermine		Polyamine catabolism signaling molecule)
Serum and tissues amine oxidase (Cu/TPQ-AO)	DAO	Cu, TPQ	Tissues (cytoplasm, microsomal fraction), human placenta, fetal tissues, neoplastic processes	1,3-Propanediamine, putrescine, cadaverine, agmatine, histamine, spermidine, spermine	Aminoguanidine, hydrazines, hydrazides, copper chelators	Cell growth and differentiation, detoxification, cell signaling
	BSAO		Serum	Benzylamine, spermine, spermidine		Cell growth and differentiation detoxification, cell signaling, cell adhesion Alzheimer's disease, cerebral amyloid angiopathy, diabetes mellitus, atherosclerosis
	SAO SSAO/VAP1		Biological fluids and plasmamembrane (adipocytes, placenta, kidney, liver, spleen)	Benzylamine, spermine, spermidine, aminoacetone, methylamine		
LOX		Cu, LTQ	Nuclei of fibrogenic vascular smooth muscle cells, fibroblast	Collagen, elastin (lysine residues)	β -Aminopropionitrile, α -Aminoguanidine, hydrazines, hydrazides, copper chelators	Regulation of collagen and elastin cross-linking, aortic aneurysms, lathyrism, metastatic phenotype tumors, gene regulation

Moreover, MAO-A and MAO-B also exhibit different substrate specificity. It is well known that MAO-A preferentially deaminates aromatic monoamines such as the neurotransmitters noradrenaline and serotonin, whereas MAO-B oxidizes preferentially dopamine, phenylethylamine and benzylamine (Tipton et al. 2004). Tyramine and tryptamine appear to be substrates for both isoforms.

A peroxisomal FAD-dependent enzyme N¹-acetylpolyamine oxidase (APAO) is a constitutively expressed enzyme that catalyzes the cleavage of acetylated polyamines to produce spermidine (from spermine) or

putrescine (from spermidine), 3-aceto-aminopropanal and H₂O₂. Wang et al. (2001) identified a gene for an enzyme capable of oxidizing unsubstituted spermine. They initially named the gene *PAOh1* for the first human polyamine oxidase to be cloned. Vujcic et al. (2002) subsequently confirmed that this new gene/enzyme was also involved in the polyamine catabolic pathway. The new enzyme, named SMO, is a highly inducible FAD-dependent enzyme that oxidizes spermine to produce spermidine, 3-aminopropanal and H₂O₂. Interestingly, observations indicate that SMO might represent an excellent target for chemoprevention

strategies (Casero and Marton 2007). In fact, using tumor-necrosis factor (TNF α), a pleiotropic inflammatory cytokine, it was shown that this general mediator of inflammation was able to stimulate SMO activity, resulting in a potential damage induced by reactive aldehydes and ROS. The aldehyde, 3-aminopropanal, and H₂O₂, once converted into the highly reactive hydroxyl radical (HO \cdot) through Fenton-like-catalysis, can damage RNA, DNA, membranes and proteins. A severe damage to DNA, by both enzymatic oxidation products, can lead to mutagenic changes necessary for the development and progression of multiple epithelial cancers. Moreover, H₂O₂ formed by polyamine catabolism might play a role as a signaling molecule (Babbar and Casero 2006; Casero and Pegg 2009).

Cu/TPQ-AOs [E.C. 1.4.3.6, amine: oxygen oxidoreductase (deaminating) (copper-containing)] are found in a wide variety of organisms (Mondovì et al. 1989). In microorganisms, Cu/TPQ-AOs generally have a nutritional role utilizing the primary amines as nitrogen or carbon sources. In plants, these proteins are implicated in wound healing and in cell growth by regulating the intracellular di- and polyamine levels, while the aldehyde products might have a key role in the biosynthesis of some alkaloids. In mammals, these enzymes are involved in detoxification and in metabolic and vascular diseases. Cu/TPQ-AOs are widely present in many organs and tissues, such as vascular smooth cells, adipocytes, placenta, kidney, liver, spleen and plasma showing tissue specificity in different species. Semicarbazide-sensitive amine oxidases (SSAO) belong to this class of Cu/TPQ-AOs. It was suggested that SSAOs are present in two forms: a membrane-bound form and a soluble form found in plasma (Lyles 1996). The latter, presumably, arises from the proteolytic cleavage of the membrane-bound enzyme, known also as vascular adhesion protein-1 (VAP-1) (Salminen et al. 1998; Stolen et al. 2004; Salmi and Jalkanen 2006).

The soluble Cu/TPQ-AOs, also called DAOs, occur in microorganisms (fungi and bacteria), plants, and mammals and those enzymes from bacteria (Parsons et al. 1995; Wilce et al. 1997), yeasts (Li et al. 1998; Duff et al. 2003) and from pea seedlings (Kumar et al. 1996) have been crystallized. In mammals, the best-known enzymes are those from pig kidney and intestine, and from human placenta. DAOs prefer short aliphatic diamines such as putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane) as substrates.

Some mammalian Cu/TPQ-AOs have been characterized structurally, as the human vascular adhesion protein (hVAP-1) (Airenne et al. 2005) and the BSAO (Lunelli et al. 2005). Human VAP-1 has been crystallized (Nymalm et al. 2003) but its structure has not been published yet. Models of its structure have been produced by homology modeling using the structures of the *E. coli* and *H. polymorpha* Cu/TPQ-AOs

(Salminen et al. 1998; Marti et al. 2004; Yegutkin et al. 2004) that have a sequence identity with hVAP-1 lower than 27% (Salminen et al. 1998). Conversely, hVAP-1 shows a high sequence identity (82.5%) with BSAO. Functional and mechanistic studies on BSAO have yielded limited structural information (Morpurgo et al. 1992; De Biase et al. 1996; Nakamura et al. 1997; Su and Klinman 1998; Bellelli et al. 2000), and its crystal structure has been recently determined (Calderone et al. 2003).

Cu/TPQ-AOs are involved in many physiological processes and metabolic pathways related to biological function of their natural substrates (amines) and reaction products (H₂O₂ and aldehydes) (Tipton et al. 2003). In mammals, Cu/TPQ-AOs have been associated with cell proliferation, differentiation, development of cells, apoptosis, detoxification and cell signaling. In particular, VAP-1 is involved in glucose uptake and lipolysis in adipocytes, as well as cell adhesion and granulocyte extravasation during inflammation (Lyles 1996; O'Sullivan et al. 2004; Boor et al. 2009). Moreover, the activity of human plasma Cu/TPQ-AO (the soluble form arising from VAP-1) was found to be altered in a number of disease states, such as inflammatory liver diseases, congestive heart failure, some types of cancer, diabetes mellitus (both type I and type II), and vascular disorders such as atherosclerosis (Tipton et al. 2003; Yu et al. 2003). The formaldehyde generated through the catalytic cycle of SSAO/VAP-1 can form cross links between proteins and contribute to β -amyloid misfolding and its anomalous deposition in cerebrovascular tissues. It has been hypothesized that SSAO/VAP-1 present in cerebrovascular tissues may be involved in the pathogenesis of Alzheimer's disease and cerebral amyloid angiopathy (Boor et al. 2009).

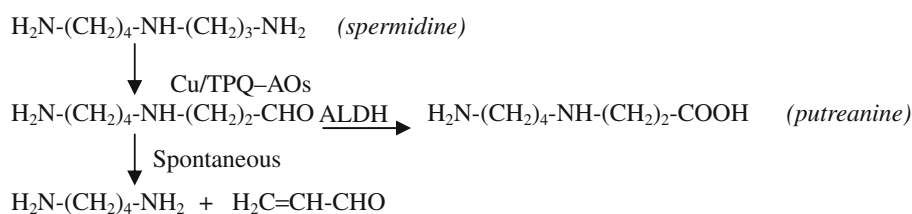
Lysine tyrosylquinone (LTQ) is present in an other copper-dependending amine oxidase, lysyl oxidase (LOX) selectively inhibited by β -aminopropionitrile. Lysyl oxidase catalyzes the oxidation of specific lysine residues within extracellular elastin and collagen thus generating residues of α -amino adipic- δ -semialdehyde within these proteins. This enzyme (LOX) has been implicated, among several other pathological conditions, in lathyrism and in a number of novel biological functions, including the regulation of the promoter activity of collagen type III, the control of cell adhesion and growth, the metastatic phenotype of certain tumors in adult animals and gene regulation (Lucero et al. 2008; Atsawasuwan et al. 2008).

Polyamine oxidation and catalytic mechanisms of Cu-amine oxidases

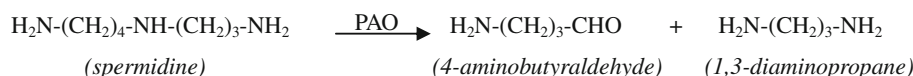
The widespread occurrence of AOs in different organisms and organs suggests their undoubtedly relevant biological

function in biogenic amine metabolism. The structural and biological functions differ depending on the source of the enzyme. AOs have a physiological role in nutrient metabolism, removing potentially toxic biogenic amines from blood plasma and regulating intracellular spermine and spermidine concentration. In prokaryotes, the AOs allow the microorganisms to use amines as carbon and nitrogen sources, whereas in plants and mammals, the AOs are involved in detoxification processes and regulation of fundamental cellular processes such as tissue differentiation, cell growth and programmed cell death are the main roles (Averill-Bates et al. 2008). In eukaryotes, it is believed that AOs influence cell growth, signaling and development (Green et al. 2002).

From a physiological point of view, two distinct catabolic pathways exist for polyamines: terminal oxidation and interconversion reactions. As a general rule, the former reaction is accomplished by the Cu/TPQ-AOs, and the latter one by the FAD-enzymes. However, this distinction is not absolute; in fact, spermine and spermidine, oxidized by Cu/TPQ-AOs, may undergo spontaneous degradation when not previously further oxidized by aldehyde dehydrogenases, forming spermidine and putrescine, respectively, with formation of the aldehyde acrolein.



Moreover, some AOs isolated from bacteria and plants, which cleave polyamines (PAO), at the secondary nitrogen to form 1,3-diaminopropane (instead of putrescine) as reaction product, which is not reutilized for re-synthesis of higher polyamines.



Disregarding these minor exceptions, this distinction of the polyamine oxidative reactions suggests a different physiological significance for the FAD- and Cu-dependent enzymes in tissues and biological fluids. FAD-dependent PAO, which is integrated into the interconversion pathway, was purified from several sources. PAO is present at high activity in nearly all cells and organs of the vertebrate

organism. It is found in both the cytosolic and peroxisome fractions. Natural substrates of PAO, in the order of their affinity to the enzyme, are N^1 -acetylspermine, N^1,N^{12} -diacetylspermine, N^1 -acetylspermidine, spermine (Seiler 2000). Homogenates of adult rat brain, with low PAO activity, catalyze the oxidative degradation of both N^1 -acetylspermine and N^1,N^{12} -diacetylspermine. The selective inactivation of PAO by N^1,N^4 -bis(2,3-butadienyl)-1,4-butanediamine (MDL 72527) inhibits the polyamine oxidation (Bey et al. 1985). In contrast, Cu/TPQ-AOs activity is present at considerably different levels in various tissues and organs. Cu/TPQ-AOs have both intracellular and extracellular localization, being present in serum and in some biological fluids.

Diamine oxidases are tissue-specific Cu/TPQ-AOs. Their preferred substrates are diamines (1,3-propanediamine, putrescine, cadaverine, agmatine, histamine), but spermidine and spermine are also substrates (Seiler 1994). Their role is in the terminal polyamine catabolism to metabolize the polyamines into products which can be excreted in the urine (Seiler 2000). Diamine oxidases oxidatively deaminate the aminopropyl moieties of spermidine and spermine and also the aminobutyl moiety of spermidine. Reaction products are aminoaldehydes, H_2O_2

and ammonia. Cells usually contain sufficient amount of aldehyde dehydrogenase activities as to allow the rapid conversion of the aminoaldehydes into the corresponding amino acids. Diamine oxidases are especially elevated in rapidly proliferating tissues, such as placenta, fetal tissues

and organs undergoing hyperplastic, hypertrophic or neoplastic processes and they are abundant in the kidney cortex of several animal species, including man. Their presence in the proximal convoluted tubules epithelium would imply a role as biogenic amine scavenger.

Under physiological conditions, Cu/TPQ-AO activity is present in the serum of swine, bovine and other animals,

but was not detected in horse and human. In man, serum amine oxidase activity rises during pregnancy or under some pathological conditions, such as cancer.

Serum Cu/TPQ-AOs oxidatively deaminate diamines and polyamines. Swine serum AO deaminates diamines and exhibits histaminase activity. Instead, in human pregnancy serum, it seems to deaminate both diamines and polyamines (Gahl and Pitot 1982a; Gahl et al. 1982b). Bovine serum amine oxidase, a copper-containing glycoprotein weighing 170 kDa, is the prototype of mammalian Cu/TPQ-AOs (Houen 1999; Agostinelli et al. 2004, 2005). Bovine serum amine oxidase is the first enzyme found to oxidatively deaminate the primary amino groups of polyamines, such as spermine and spermidine, as shown in Fig. 1 (Agostinelli et al. 2006a). The reaction of spermidine is the same to that mentioned above for DAO. However, only the aminopropyl moiety of spermidine is oxidatively deaminated by BSAO (Tabor et al. 1964). The presumed formation of acrolein has been included into Fig. 1 (Alarcon 1970).

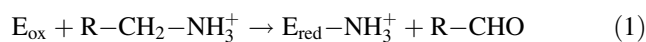
Overall, Cu/TPQ-AOs have relatively broad substrate specificities (e.g., benzylamine and heptylamine) and oxidize several other naturally occurring and synthetic amines, although with greatly varying values of K_M and k_{cat} .

The activities of Cu/TPQ-AOs with specificity for spermidine and spermine are low in humans and most mammals. Except in ruminants they seem not to play a major role in polyamine degradation. The physiological function of BSAO is presumably removal of excessive spermidine from the blood, spermidine that was liberated from bacteria (Cohen 1998).

Cu/TPQ-AOs share some fundamental structural properties. All serum amine oxidases are homodimers and have similar molecular organizations with 33 fully conserved residues close to the catalytic site. The peptide chains of the enzymes from different sources are sialylated to different degrees. Recently, the crystal structure of desialylated BSAO has been reported at 2.37 Å resolution (Lunelli et al. 2005). Each of the identical subunit (M_r 85 kDa) contains in the active site one tightly bound Cu^{2+} coordinated to three His residues, and TPQ, which is part of the consensus sequence [Asn-TPQ-Asp (or Glu)] of the peptide chain (Mu et al. 1992; Mitchell Guss et al. 2009).

The catalytic mechanism involves the formation of a Schiff base between the amine substrate and the oxidized TPQ that is followed by an intramolecular rearrangement of the double bonds. An aldehyde is released by a hydrolytic step. The oxidized form of Cu/TPQ-AOs has a distinctive pink color and absorbs in the visible region between 476 and 498 nm (Agostinelli et al. 2005). The organic cofactor in the reduced state (colorless) has at this stage an amino nitrogen covalently bound. Re-oxidation of TPQ by O_2 releases ammonia and H_2O_2 , and closes the

cycle (Janes and Klinman 1991). The order of substrate addition and product release is therefore fixed and no ternary complex is formed, as in the ping-pong type mechanisms (Hartmann and Klinman 1991; Bellelli et al. 2000). Thus, the catalytic cycle can be divided into two half-reactions: enzyme reduction by substrate at the quinone moiety (TPQ-TPQH₂) (Eq. 1) and its re-oxidation by molecular oxygen according to the reactions (Eq. 2):



In plant enzyme, the substrate-reduced colorless Cu^{II} -aminoquinol equilibrates rapidly with the Cu^I -semiquinolamine cation radical species by transferring one electron to copper, which is in turn reduced from the cupric to the cuprous state, and the solution immediately turns yellow as a result of the formation of new absorption bands centered at 464, 434 and 360 nm (Agostinelli et al. 2005, 2009a). On the contrary, mammalian Cu/TPQ-AOs, which are not formed in the radical species during the normal catalytic cycle, remain in the reduced aminoquinol form (Agostinelli et al. 2005). The second half-reaction involves the re-oxidation of the enzyme with the contemporaneous release of ammonia and hydrogen peroxide.

Therefore, Cu^{2+} appears to play an important role in both the formation of the reduced enzyme species and its re-oxidation, and it appears to control the transfer of the substrate from a hydrophobic binding site near the protein surface to the deeply buried active site (Agostinelli et al. 1997; Parsons et al. 1995; Kumar et al. 1996). Cu^{2+} has also a structural function (Agostinelli et al. 1994a).

As described above, in the case of spermidine and spermine, the primary amino group of the aminopropyl moiety is preferentially deaminated by BSAO, to form the respective mono- and the unstable di-aldehydes intermediate [*N,N*¹-bis(3-propionaldehyde)-1,4-butanediamine] (Tabor et al. 1964; Lee and Sayre 1998), but reaction of the secondary amino groups of spermine with purified BSAO has also been described (Houen 1999). In conclusion, as shown in Fig. 1, the reaction involves dioxygen and water as substrates (Tabor and Tabor 1984) while the products are H_2O_2 , aldehydes and ammonia (Tabor and Tabor 1984; Tabor et al. 1964). The aldehydes have been identified and studied for their biological properties (see, e.g., Tabor et al. 1964). The formation of acrolein from these aldehydes by spontaneous β -elimination was first postulated by Alarcon (1970), but has long been debated. It was particularly questioned by Israel et al. (1973), based on experiments with synthetic aldehydes. Recent work is in favor of the formation of acrolein as a product of BSAO-catalyzed

oxidation of spermidine and spermine in cell cultures, as will be described in the next paragraphs (Sharmin et al. 2001; Agostinelli et al. 2006a, 2009b; Averill-Bates et al. 2008). In the presence of suitable enzymes the aldehydes may be oxidized to the corresponding amino acids, or reduced to alcohols (for details of catabolism see Seiler 1992, 2004).

Polyamine enzymatic oxidation products induce cell death

The natural polyamines putrescine, spermidine and spermine are in multiple ways involved in cell growth and maintenance of cell viability. Moreover, polyamines are also involved in events inherent to genetically programmed cell death (Seiler and Raul 2005). In fact, 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 homeostatic mechanisms, polyamines can cause cytotoxic effects (Wallace and Fraser 2003). Thus, Cu/TPQ-AOs are important enzymes contributing to the regulation of the levels of polyamines catalyzing their oxidative deamination. Spermidine and spermine may so become a source of toxic metabolites (Arancia et al. 2004) and findings suggest that cytotoxic enzymatic oxidation products of spermine, formed in situ by a Cu/TPQ-AO-catalyzed reaction, might be lethal for tumors (Averill-Bates et al. 2005; Agostinelli

et al. 2006a, b, c). It was determined that polyamine concentrations are high in rapidly growing tissues such as the breast and colon cancers (Heby and Persson 1990). The oxidation of spermine by BSAO generates cytotoxic products, H_2O_2 and aldehyde(s), that have been implicated in programmed cell death, induction of cytotoxicity, inhibition of cell growth (Bachrach et al. 1987; Henle et al. 1986). These metabolites can also induce either apoptosis or necrosis, depending on both their concentrations and the cell type (Lindsay and Wallace 1999; Calcabrini et al. 2002; Agostinelli and Seiler 2006; Averill-Bates et al. 2005).

It was previously reported that purified BSAO and exogenous spermine can induce inhibition of cell proliferation, severe glutathione depletion and cell death in Chinese hamster ovary (CHO) cells (Averill-Bates et al. 1993; Agostinelli et al. 1996). Cytotoxicity was also observed in several human tumor cell lines of LoVo and M14 (Calcabrini et al. 2002; Agostinelli et al. 2006a). The cytotoxic effect induced by the enzymatic system (spermine and BSAO) appeared to be mediated by the oxidation products, H_2O_2 and aminodialdehyde or acrolein. The mechanism of cell death of DX (doxorubicin)-sensitive and -resistant human colon adenocarcinoma and melanoma cancer cells, induced by the enzymatic toxic products, was evaluated by plating efficiency assay (Fig. 2) and the ultrastructural alterations were investigated by transmission electron microscopy (TEM) (Fig. 3) (Calcabrini et al. 2002; Agostinelli et al. 2006a, b, 2009b).

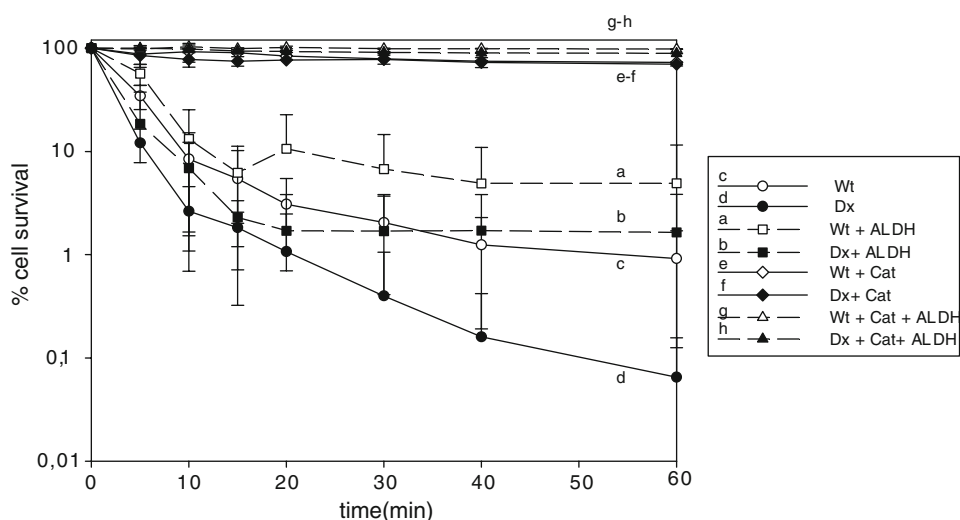
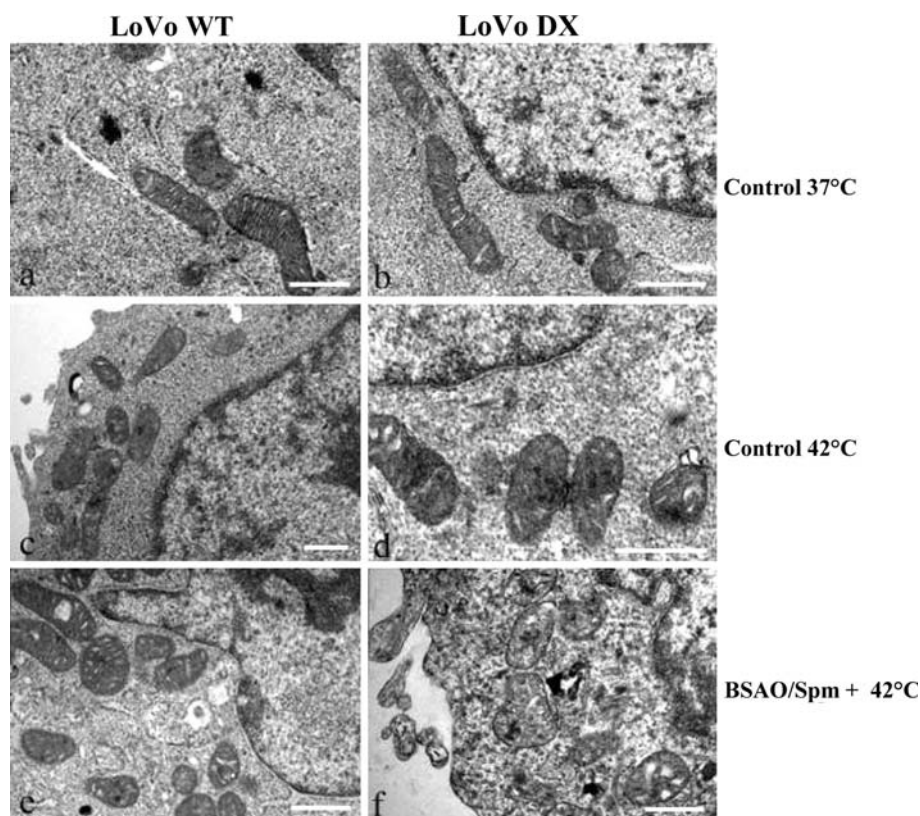


Fig. 2 Effect of catalase and aldehyde dehydrogenase on cytotoxicity induced by BSAO in the presence of spermine. LoVo WT (open symbols) and LoVo DX (solid symbols) cells were incubated at 37°C for 60 min with purified BSAO (6.50×10^{-3} U/ml) and exogenous spermine 12 μ M without (open circle; filled circle) or with catalase 240 U/ml (solid lines with open diamond; solid lines with filled

diamond), with ALDH 0.4 U/ml (dashed lines with open square; dashed lines with filled square) and with catalase and ALDH (dashed lines with open triangle; dashed lines with filled triangle). Mean \pm SD are shown for two to six estimations from four to six experiments. When not shown, SD lie within symbols

Fig. 3 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 μ M 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 damages, particularly at mitochondrial level (**f**). Scale bars 0.5 μ m



Consistent with previous observations on P-glycoprotein overexpressing MDR CHO cells (Lord-Fontaine et al. 2001), further studies demonstrated that H_2O_2 and aldehyde(s), the products of BSAO-catalyzed oxidation of spermine, were also able to overcome multidrug resistance (MDR) in both LoVo DX and M14 ADR cancer cells (Calcabrini et al. 2002; Arancia et al. 2004; Agostinelli et al. 2006a, b, c, 2009b). Interestingly, both MDR cell types are significantly more sensitive to cytotoxic spermine metabolites than their corresponding wild-type (WT) counterparts. This is of particular interest, since one of the problems of conventional anticancer therapy is development of drug resistance. This phenomenon, following exposure to cytotoxic drugs, is a major obstacle of conventional anticancer chemotherapy. 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 (P-gp) (Gervasoni et al. 1991; Arancia et al. 1998). P-Glycoprotein functions as an energy-dependent pump, that expels drugs out of cells (Gottesman and Pastan 1993).

Figure 2 shows the percentage cell survival against the time of exposure to purified BSAO (6.50×10^{-3} U/ml) in the presence of exogenous spermine (12 μ M), with catalase

240 U/ml (curves e–f), with ALDH 0.4 U/ml (curves a–b) and with both catalase and ALDH (curves g–h), at 37°C. In the presence of BSAO and spermine alone, a higher cytotoxicity was observed in LoVo DX than in LoVo WT cells. The percentage cell survival decreased in both cell lines with increasing exposure time, resulting in approx. 0.92% in LoVo WT (curve c) and approx. 0.065% in LoVo DX cells (curve d), after 60 min of incubation.

In order to evaluate the contribution of each enzymatic oxidation product in the inhibition of cell growth, experiments were performed in the presence of exogenous catalase, an enzyme that decomposes H_2O_2 or catalase and ALDH added simultaneously to the incubation mixture. Catalase (240 U/ml) afforded a marked reduction of the cytotoxic effect, corresponding to approx. 80% cell survival, on LoVo WT and LoVo DX cells (curves e and f, respectively), probably due to the degradation of H_2O_2 , formed in the catalytic reaction by the enzyme. The result suggested that H_2O_2 was not the sole toxic factor and that other products of the enzymatic oxidative deamination were involved, such as aldehyde(s), including acrolein spontaneously formed from the aminoaldehydes (Sharmin et al. 2001; Averill-Bates et al. 2008; Agostinelli et al. 2009b). The addition of exogenous NAD-dependent ALDH (0.4 U/ml) alone to metabolize the aldehyde to the corresponding carboxylic acid, had partial effects on survival

presumably by preventing the toxic effects of acrolein (curves a and b, respectively).

Fluorimetric determinations showed that about 80% of H_2O_2 formed by BSAO cross the cell membrane of LoVo cells, and the uptake reached a maximum about 10 min after the enzymatic reaction was started (Calcabrini et al. 2002). In fact, the addition of exogenous catalase to the culture medium prevented the loss of about 80% viable cells, advocating for H_2O_2 as the most important cytotoxic metabolite of spermine in our experimental setting. Several findings support this view (Calcabrini et al. 2002; Agostinelli et al. 2007b; Averill-Bates et al. 2008). Moreover, the experiments showed that the residual cytotoxicity was due to the other reaction product, aminodialdehyde or acrolein. In fact, there is an ongoing debate about the question whether H_2O_2 or the spermine-derived aldehydes are the major effectors. While a contribution of the aldehydes is undoubted, it is still an open question which spermine-derived aldehyde is most important. According to Sharmin et al. (2001), acrolein, formed by spontaneous β -elimination (Fig. 1), 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 unlikely, because its spontaneous formation from the aldehydes deriving from spermine is a time-dependent process of unknown velocity, while in contrast H_2O_2 is produced immediately at the beginning of the enzymatic reaction (Agostinelli et al. 1994b; Calcabrini et al. 2002; Agostinelli et al. 2006a). The prevention of cytotoxicity of spermine metabolites by catalase and ALDH, separately added to the incubation mixture confirmed these results (Fig. 2 curves e, f, a and b). In addition, the simultaneous presence of both exogenous enzymes, catalase and NAD-dependent ALDH, completely inhibited cytotoxicity (approx. 97%) throughout the 60 min of incubation (Fig. 2 curves g and h) (Calcabrini et al. 2002; Agostinelli et al. 2007b; Averill-Bates et al. 2008).

In order to reveal the intracellular targets involved in the mechanisms responsible for the higher cytotoxic effect of the enzymatic products on the drug-resistant cells, TEM observations and cytofluorimetric analysis were carried out. These studies revealed major pathological modifications of the mitochondria.

Both control LoVo WT (Fig. 3a) and LoVo DX cells (Fig. 3b) showed a well-preserved ultrastructure when observed by TEM, at 37°C. The nucleus was clearly defined with dispersed chromatin, while the cytoplasm was characterized by the presence of mitochondria with parallel *cristae* in a dense and uniform matrix. After treatment with BSAO and 6 μ M spermine, at 42°C, LoVo WT cells did not show any consistent ultrastructural alteration (Fig. 3e). In contrast, MDR cells exhibited marked modifications; in particular, all mitochondria showed a highly condensed

matrix and dilated *cristae* (Fig. 3f) (Calcabrini et al. 2002; Agostinelli et al. 2006b).

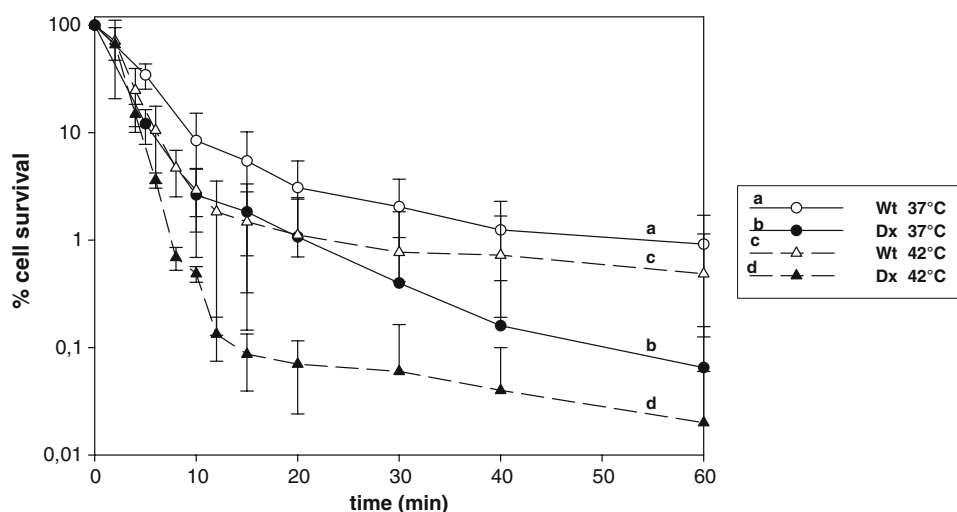
Mitochondria, therefore appear to play a pivotal role in determining the differential response between sensitive and drug-resistant cells. The higher sensitivity to cytotoxic spermine derivatives observed in LoVo DX cells, by flow cytometric analysis, was attributed to an earlier and higher mitochondrial membrane depolarization. Moreover, a higher basal production of ROS was detected in MDR cells than in the drug-sensitive cells, suggesting an increased interaction of ROS with mitochondrial electron transport chain in drug-resistant cells. In fact, H_2O_2 could directly interact with some iron of Fe/S centres located in the mitochondrial respiratory chain, raising the highly reactive hydroxyl radical (HO \cdot) by means of Fenton reaction, and thereby may induce oxidation of some thiol (SH) groups, proteins and lipids. These events lead to mitochondrial permeability transition induction and consequent bioenergetic collapse, redox catastrophe and apoptosis (Calcabrini et al. 2002; Arancia et al. 2004; Toninello et al. 2004).

Polyamine metabolites in therapeutic applications

During the past decades, considerable research has been devoted to the discovery of new and more effective pharmacological agents for clinical antitumor therapy, involving the polyamine pathway. Inhibitors of ornithine decarboxylase, such as difluoromethylornithine (DFMO), and agents that stimulate polyamine acetylation and export, such as non-steroidal anti-inflammatory drugs (NSAIDs), act at least additively to arrest growth in human cells models and suppress intestinal carcinogenesis in mice (Gerner et al. 2007; Babbar and Casero 2006). A phase III clinical trial assessed the recurrence of adenomatous polyps after treatment with DFMO plus sulindac or matched placebos (Mc Laren et al. 2008). A prospective, randomized, placebo-controlled clinical trial of combination DFMO and sulindac, a non-steroidal anti-inflammatory drug, found that the 36-month treatment was associated with an evident reduction of recurrence of all adenomas, and over a 90% reduction of recurrence of advanced and/or multiple adenomas, without evidence of serious toxicities in essentially healthy participants involved in the trials (Simoneau et al. 2008; Gerner and Meyskens 2009).

In this context, toxic polyamine metabolites are currently explored as probable candidates for a new strategy in tumor therapy (Agostinelli et al. 2004, 2007b; Agostinelli and Seiler 2006), since it has been observed that the growth of a mouse melanoma (B16-F0) was inhibited by exposure to BSAO and spermine (Averill-Bates et al. 2005, 2008). This may be achieved by combinations of the treatment

Fig. 4 Time course for temperature and spermine concentration dependence on cytotoxicity of BSAO (6.5×10^{-3} U/ml) in the presence of spermine $12 \mu\text{M}$ on percentage cell survival in LoVo WT (*open symbols*) and LoVo DX (*solid symbols*) cells during 60 min at 37°C (*solid lines with open circle*; *solid lines with filled circle*) and at 42°C (*dashed lines with open triangle*; *dashed lines with filled triangle*). Mean \pm SD are shown for two to six estimations for four to six experiments. When not shown, SD lie within the symbols



with cytotoxic drugs, or by heat. Hyperthermia in combination with cytotoxic drugs, preferentially with drugs that enhance cytotoxicity at elevated temperature (Urano et al. 1999), and the administration of drug combinations are clinically accepted methods.

The data in Fig. 4 show that H_2O_2 and aldehyde (produced by the BSAO/polyamine-spermine enzymatic system) induce greater cytotoxicity at 42°C (curves c and d), than at 37°C (curves a and b) and in MDR human cancer (LoVo and M14) cells compared to the sensitive ones. These findings were supported by TEM observations. The treatment with BSAO and $6 \mu\text{M}$ spermine under hyperthermic conditions induced severe alterations in both wild-type (Fig. 3e) and MDR (Fig. 3f) cells and the changes appeared to be much more pronounced in resistant cells (Agostinelli et al. 1994b, 2006a, b). In fact, localized hyperthermia enhances the cytotoxicity of several antitumoral drugs and has considerable potential in cancer therapy (Bates and Mackillop 1990).

Therefore, hyperthermia could act at the initial stage of the treatment, probably by accelerating the kinetics of the membrane molecular interactions and by favoring drug delivery into the tumor mass (Agostinelli et al. 1995).

Promising results are emerging from clinical studies involving hyperthermia combined with chemotherapy (Issels 1999). 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. The compounds, such as cysteamine and aminothiols *N*-(2-mercaptoethyl)-1,3-propanediamine (WR 1065), defined as thermosensitizers (Nagele et al. 1990), 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). Several findings clearly showed the essential role of enzymatic oxidation products of polyamines (Agostinelli et al. 1994b, 2006a, 2009b; Averill-Bates et al. 2008; Calcabrini et al. 2002; Arancia et al. 2004; Henle et al. 1986), rather than polyamines themselves, in causing cytotoxicity (Ben-Hur et al. 1978). Interestingly, spermine at concentrations $\leq 1 \mu\text{M}$, in the presence of BSAO, was not toxic at 37°C on both LoVo and M14 cells, but became cytotoxic at 42°C and behaved similarly to the above-reported thermosensitizers (Agostinelli et al. 2006b, 2009b). Beneficial effects could therefore be achieved using localized heating to enhance the action of toxic products generated by BSAO/spermine within the tumor region, without increasing normal tissue damage.

Currently, we are studying drug combinations with the aim of increasing cell death by toxic polyamine metabolites. The cytotoxicity of BSAO/spermine was enhanced by pre-treatment of the cells with MDL 72527. This compound is an inactivator of FAD-dependent polyamine oxidase and represents a lysosomotropic compound (Dai et al. 1999). It was demonstrated to improve the antitumor effect of DFMO (Seiler et al. 2002) which is undergoing clinical evaluation as a chemoprevention agent (Gerner et al. 2007; Wallace and Niiranen 2007; Gerner and Meyskens, 2009).

MDL 72527 has cytotoxic properties which are unrelated to its ability to inactivate PAO (Seiler et al. 2002). Cell survival experiments were therefore performed on LoVo and M14 cells. In the investigation, emphasis was placed on the ability of MDL 72527 to sensitize both cell lines to H_2O_2 and aldehyde generated from BSAO/spermine. Pre-treatment with MDL 72527 sensitized both WT and MDR LoVo and M14 cell lines to the subsequent exposure to spermine metabolites. The lowest cell survival

was observed after 24 h of pre-incubation with MDL 72527. Cytotoxicity was significantly greater by the combined treatment than by BSAO/spermine alone. An impairment of cell metabolism by this drug was indicated by the formation of numerous cytoplasmic vacuoles of lysosomal origin within 6 h. Mitochondrial damage, as observed by TEM, seemed to correlate better with the cytotoxic effects than the formation of vacuoles (Agostinelli et al. 2006a, c). These vacuoles are most probably of lysosomal origin and their formation did not correlate directly with the loss of cell viability. Instead, the results support the hypothesis that the release of lysosomal enzymes into the cytosol by MDL 72527 is responsible for its sensitizing effect. In fact, it is known that the release of lysosomal enzymes produces oxidative stress and an important role of lysosomes in both necrotic and apoptotic cell death is well founded (Guicciardi et al. 2004). Regarding the impairment of cell viability, the mitochondrial ultrastructural alterations of the cells treated with BSAO/spermine, after pre-treatment with MDL 72527, seem more relevant than the formation of cytoplasmic vacuoles, since they were more accentuated in MDR cells than in the wild-type ones. Moreover, they seem to correlate with the loss of cell viability (Agostinelli et al. 2009b). It was reported that one of the earliest signs of cell damage by BSAO and spermine is the depolarization of the mitochondrial membranes (Arancia et al. 2004).

Therefore, owing to its lysosomotropic effect, pre-treatment with MDL 72527 amplifies the ability of the metabolites formed from spermine by oxidative deamination to induce cell death.

In fact, the results suggest that enzymatically formed cytotoxic agents alone, or in combination with either the polyamine oxidase inactivator (MDL 72527) or heat treatment, activate stress signal transduction pathways, leading to apoptotic cell death. These findings suggest that lysosomotropic compounds are of interest as anticancer agents, mainly in the treatment of MDR tumors (Agostinelli and Seiler 2007).

Conclusions and perspectives

From a therapeutic point of view, the improvement of the efficacy of in situ formation of cytotoxic polyamine metabolites is essential. Therefore, the use of amine oxidase to generate enzymatic toxic products deserves to be considered in cancer therapy (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 (Calcabrini et al. 2002). This approach has the advantage that it only requires a small amount of the enzyme 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 tumor cells and growing tissues (Heby and Persson 1990), it is expected 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 for the intracellular delivery of the enzyme in vivo, for possible clinical application. In fact, in cultured normal chick fibroblasts or in fibroblasts transformed by Rous sarcoma viruses, Bachrach et al. (1987) observed an inhibition of the synthesis of proteins and nucleic acids when the cells were microinjected with amine oxidase.

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 exogenously introduced BSAO enzyme.

However, the in situ formation of toxic compounds or radicals by enzyme catalyzed reactions is a promising start. 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 antitumor potential of BSAO was therefore evaluated in vivo, using a B16 mouse melanoma model in C57BL mice. BSAO, when directly injected into the solid tumors, was able to induce tumoricidal activity by converting endogenous polyamines to toxic products in situ (Averill-Bates et al. 2005). The enzyme was administered in its native form and also immobilized in a biocompatible polymer composed of bovine serum albumin and polyethylene glycol (PEG). The immobilized enzyme showed higher operational stability and functional activities, relative to its native form (Demers et al. 2001). When immobilized BSAO was injected into the tumor, there was a marked decrease (70%) in tumor growth, compared to a decrease of 32% of tumor size when native BSAO was administered. The mechanisms of cell death were determined in vivo (Averill-Bates et al. 2005). When tumors were treated with immobilized BSAO, there was a high level of apoptotic cell death (around 70%), compared to less than 10% with the native enzyme. Native BSAO induced a high level of necrosis (around 40%), compared to less than 10% with immobilized BSAO, probably due to a burst of cytotoxic products. The advantage of the immobilized BSAO is that it allows the slow release of cytotoxic

products and thereby induces tumor cell death by apoptosis rather than necrosis, thus decreasing inflammatory damage to surrounding tissues (Averill-Bates et al. 2005, 2008; Casero and Marton 2007).

The immobilization of BSAO on other hydrogel polymers such as arginate/chitosan (less than 5 μm of diameter) will be also performed. In this regard, we are also developing new BSAO delivery systems based on the encapsulation of the enzyme in tumor-specific nano-technological envelopes (Nayvelt et al. 2007).

In conclusion, the toxic enzymatic oxidation products generated by BSAO and polyamines could be useful as a combined treatment approach with hyperthermia (42°C) or with other drugs, such as lysosomotropic compounds (Agostinelli and Seiler 2007), in the treatment of solid tumors, including melanomas and carcinomas, particularly those with MDR phenotypes, in earlier and later stages of disease.

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