Nanoparticle strategies for cancer therapeutics: Nucleic acids, polyamines, bovine serum amine oxidase and iron oxide nanoparticles (Review)

ENZO AGOSTINELLI[1], FABIO VIANELLO[2], GIUSEPPE MAGLIULO[3], THRESIA THOMAS[4] and T.J. THOMAS[5]

[1]Istituto Pasteur-Fondazione Cenci Bolognetti Department of Biochemical Sciences 'A. Rossi Fanelli', Sapienza University of Rome and CNR, Institute of Biology and Molecular Pathology, 00185 Rome, Italy; [2]Department of Comparative Biomedicine and Food Science, University of Padua, 35020 Legnaro, Italy and Regional Centre of Advanced Technologies and Materials, Department of Physical Chemistry, Palacky University in Olomouc, Olomouc 77146, Czech Republic; [3]Department Organi di Senso, Sapienza University of Rome, 00185 Rome, Italy; [4]Formerly Department of Environmental and Occupational Medicine, Rutgers Robert Wood Johnson Medical School, Rutgers the State University of New Jersey, Piscataway, NJ 08854; [5]Department of Medicine, Rutgers Robert Wood Johnson Medical School, Rutgers the State University of New Jersey, New Brunswick, NJ 08901, USA

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Abstract. Nanotechnology for cancer gene therapy is an emerging field. Nucleic acids, polyamine analogues and cytotoxic products of polyamine oxidation, generated *in situ* by an enzyme-catalyzed reaction, can be developed for nanotechnology-based cancer therapeutics with reduced systemic toxicity and improved therapeutic efficacy. Nucleic acid-based gene therapy approaches depend on the compaction of DNA/RNA to nanoparticles and polyamine analogues are excellent agents for the condensation of nucleic acids to nanoparticles. Polyamines and amine oxidases are found in higher levels in tumours compared to that of normal tissues. Therefore, the metabolism of polyamines spermidine and spermine, and their diamine precursor, putrescine, can be targets for antineoplastic therapy since these naturally occurring alkylamines are essential for normal mammalian cell growth. Intracellular polyamine concentrations are maintained at a cell type-specific set point through the coordinated and highly regulated interplay between biosynthesis, transport, and catabolism. In particular, polyamine catabolism involves copper-containing amine oxidases. Several studies showed an important role of these enzymes in developmental and disease-related processes in animals through the control of polyamine homeostasis in response to normal cellular signals, drug treatment, and environmental and/or cellular stress. The production of toxic aldehydes and reactive oxygen species (ROS), H_2O_2 in particular, by these oxidases suggests a mechanism by which amine oxidases can be exploited as antineoplastic drug targets. The combination of bovine serum amine oxidase (BSAO) and polyamines prevents tumour growth, particularly well if the enzyme has been conjugated with a biocompatible hydrogel polymer. The findings described herein suggest that enzymatically formed cytotoxic agents activate stress signal transduction pathways, leading to apoptotic cell death. Consequently, superparamagnetic nanoparticles or other advanced nanosystem based on directed nucleic acid assemblies, polyamine-induced DNA condensation, and bovine serum amine oxidase may be proposed for futuristic anticancer therapy utilizing nucleic acids, polyamines and BSAO. BSAO based nanoparticles can be employed for the generation of cytotoxic polyamine metabolites.

Correspondence to: Professor Enzo Agostinelli, Department of Biochemical Sciences 'A. Rossi Fanelli', Sapienza University of Rome, Piazzale A. Moro 5, 00185 Rome, Italy
E-mail: enzo.agostinelli@uniroma1.it

Abbreviations: MDR, multidrug resistance; WT, wild-type; BSAO, bovine serum amine oxidase; SEM, scanning electron microscopy; TEM, transmission electron microscopy; P-gp, P-glycoprotein; ALDH, aldehyde dehydrogenase; ROS, reactive oxygen species; PEG, poly(ethylene glycol); ODC, ornithine decarboxylase; AOs, amine oxidases; PAOs, polyamine oxidases; ADR, doxorubicin resistant cells; FAD, flavin adenine dinucleotide; METC, mitochondrial electron transport chain; TPQ, 2,4,5-trihydroxyphenylalaninequinone; LTQ, lysine tyrosylquinone; MAO, mono amine oxidase; APAO, N^1-acetylpolyamine oxidase; *PAOh1*, first human polyamine oxidase; SMO, spermine oxidase; TNFα, tumor-necrosis factor; CLIO, cross-linked iron oxide; IU, International Unit; MRI, magnetic resonance imaging; SAMNs, superparamagnetic maghemite nanoparticles; NH, nanohydrogel; HA, hyaluronic acid; CH, cholesterol

Key words: polyamines, DNA nanoparticles, multidrug resistance, bovine serum amine oxidase, gene therapy, biomaterials, magnetic nanoparticles

Contents

1. Introduction

Nanotechnology, concerning particles and devices in the range of 1-100 nm dimension, provides new opportunities in cancer therapy. Nanoparticle based therapies have been shown to reduce systemic toxicities and improve therapeutic efficacy of drugs (1). Doxil (liposomal-polyethylene glycol doxorubicin), DanoXome (liposomal daunorubicin), Oncaspar (Polyethylene glycol-L-Asparaginase), and Abraxane (albumin-bound paclitaxel) are FDA approved therapeutic nanoparticles. There are many nanoparticle-based agents in clinical trials aimed at reducing the toxicity of chemotherapeutic drugs, such as paclitaxel, camptothecin, doxorubicin, and cisplatin (1-4). In addition, nanotechnology for cancer gene therapy is an emerging field (5). Non-viral vectors for nanoparticle-based gene therapeutics are expected to overcome limitations due to pathogenicity and immunogenicity of viral vectors. This review introduces advances in the research of novel nanoparticles based on directed nucleic acid assemblies and polyamine-induced DNA nanoparticles as well as bovine serum amine oxidase-based nanoparticles to overcome some of the problems associated with conventional anticancer therapy, including the limitations of treating drug resistant tumours.

2. Nucleic acid assemblies and their use as drug delivery vehicles

Nucleic acids are the fundamental molecules of life and the complementarity of base pairing allows them to direct information transfer and cellular functions. DNA helices are inherently nanoscale building blocks with a diameter of approximately 2 nm, a helical repeat of 3.4 nm (10.5 base pairs) and a persistence length of approximately 50 nm (4). Inspired by the Holliday junction, a four-way junction intermediate observed during recombination (6), Seeman first proposed thermodynamically stable four-way junctions with sticky ends and built double cross-over and triple cross-over motifs (7). Two-dimensional lattice, and polyhedral DNA structures such as the cube, six-connected network, pentagonal dodecahedron and truncated octahedron were also designed by Seeman and others (8,9). The tile structures further helped to build double-double crossovers and 3-, 4-, 6-, 8-, and 12-helix DNA tile complexes (10-12). Nanogrids, nano ribbons, and nanotubes were created in this manner. Three point stars, six-point stars, and T-shaped junctions increased the versatility of nanostructures based on tile-based self-assembly (12-14). T-shaped structures can give rise to orthogonal co-ordinated ladders, lattices and polar coordinated wheels (14).

The design process in tile-based DNA engineering is tedious with the assembly requiring strictly balanced stoichiometry, and the structures are limited by the length of the synthetic oligonucleotides (4). 'DNA origami' is a more recent and versatile method, first developed by Rothemund (15). The term origami refers to the Japanese folk art of folding paper into a special shape (9,15). In this method, one long strand of single-stranded DNA (7.3 kb bacteriophage M-13 DNA) is folded to produce the desired structure by the help of smaller (32 bp) staple strands. The small staple strands are complementary to at least two distinct segments of the long single stranded DNA. The long single stranded DNA and an excess of staple strands are heat-annealed to form the origami. The origami method is formulated to yield 2-dimensional and 3-dimensionl structures and use double stranded DNA. Other investigators (16,17) reported the formation of multi-domain DNA origami by using origami four-way junctions. DNA origami can also be used as a template for patterns using streptavidin molecules or enhanced green fluorescent protein (18,19). Hung *et al* (20) reported the positioning of gold nanoparticles on lithographically confined DNA origamies. Development of a molecular robot consisting of a DNA walker that moves on top of an origami in a programmed path and collects specified cargo is another advancement in this field (21-23). Strategies have been developed to make 3D structures such as three, four and six sided prisms (24) icosahedrons (25), 3D DNA box with an openable lid (26), 3D DNA box origami (27) and a tetrahedron DNA container (28).

A new route to building three dimensional nanostructures has been opened by the development of DNA bricks analogous to the LEGO building model (29). The basic building block in the structures is a 32-nucleotide single-stranded DNA which contains four regions that can hybridize to four neighbouring DNA strands. To build 3D structures, DNA bricks resembling two-stud LEGO bricks are connected with 90˚ left-handed turn, resulting in layers of bricks that are shifted 90˚ relative to each other. Whereas LEGO structures are assembled by hand, brick by brick, the DNA structures form by self-assembly. Each DNA brick is encoded with an individual sequence that determines its position and allows the structure to assemble by hybridization of complementary strands. In a one-step procedure, a cuboid structure is formed in ~72 h. By creating empty boxes and open-cavity shapes, tunnels of varying width, depth, and geometry authors allude to many types of functional nanodevices including drug-delivery vehicles (29).

Several research groups have explored the possibility of using 3-dimensional nanocontainers as delivery vehicles. Erben *et al* (30) demonstrated the encapsulation of cytochrome c within a DNA tetrahedral cage, where the protein molecule is conjugated to the 5'-end of one of the DNA strands via a surface amine. The ability of a DNA origami nanocage to encapsulate Au nanoparticles with various sizes has also been demonstrated (31). Anderson *et al* (32) created a nanoscale box with a cavity large enough to contain a ribosome and a controllable lid. Douglas and collaborators (33) engineered a DNA capsule fitted with aptamers that can be loaded with molecular cargo and unloaded upon binding to a desired target cell. DNA

icosahedron structures were also designed and constructed as smart drug delivery vehicles (34). Taken together, these studies provide the initial proof-of-principle for developing and using DNA nanocontainers for drug delivery.

3. Polyamines and their role in DNA condensation to nanoparticles

Polyamines are polycationic biogenic amines required for both eukaryotic and prokaryotic cell growth and differentiation (35,36). The natural polyamines (putrescine [$NH_2(CH_2)_4NH_2$], spermidine [$NH_2(CH_2)_4NH(CH_2)_3NH_2$] and spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$]) are formed from the decarboxylation products of ornithine and S-adenosyl-methionine in nearly all eukaryotic cells. In normal cells, polyamine concentrations are highly regulated by the action of biosynthetic and catabolising enzymes, such as ornithine decarboxylase (ODC) and spermidine/spermine acetyl transferase; however, polyamine levels are elevated in cancer cells (36). Motives for these increased levels include enhanced putrescine synthesis from ornithine by ODC, the rate-limiting enzyme, and an increased uptake of polyamines (37). However, in situations of over-accumulation or depletion of intracellular polyamine pools, cell death can occur (38,39). Polyamines attract interest because of their multiple functions in cell biology, including cell cycle regulation, gene expression and signal transduction (40-43). The primary role of polyamines in regulating cell proliferation and cell death have prompted 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 (44). 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 (45).

In addition to their biological and gene regulatory roles, polyamines provoke the condensation of DNA on nanoparticles (46,47). Condensation is an essential process for the packaging of DNA in cells. Hence, the mechanism of DNA condensation by polyamines and other multivalent cations has been an active area of research for the past forty years (48). This phenomenon has acquired further importance because condensed DNA can be transported into cells more efficiently than uncondensed DNA (47). Polycations such as polyethyleneimine, polylysine and cationic lipids have been used as DNA delivery agents in cells. These cations cause localized bending or distortion of DNA at a critical extent of charge neutralization, facilitating the formation of rods and toroid-like structures (49). Carlstedt *et al* (50) suggested that the association of DNA with polycations or surfactants might be manifested as compaction, a conformational change of single DNA chain from an extended coil to a compact globule or macroscopic phase separation, depending on the conditions. Although the general term condensation also covers compaction, condensation generally involves multiple DNA molecules. Beyond DNA condensation, an increased concentration of spermine on DNA result in phase separation. A miscibility gap has been found and the lower boundary of the phase separation region occurred at a spermine:DNA charge ratio close to unity (51). With a large excess of spermine at charge ratios >120, clear solutions are formed beyond miscibility gap. Other studies also showed similar miscibility gaps in DNA mixtures with surfactants or cyclodextrins at much lower charge ratios than spermine (50). The ability of PEI, poly-L-lysine, surfactants, and cyclodextrins to condense DNA and the hydrodynamic properties of DNA nanoparticles produced by these agents have been characterized (52,53). Structural specificity effects have been demonstrated in polyamine-mediated DNA condensation and oligonucleotide uptake in cells (54,55). Polyamines and oligonucleotides also exert a synergistic inhibition of the transcription of targeted genes (55).

4. Amine oxidases and their utilization in nanomedicine

Polyamines are substrates for a large class of enzymes, the amine oxidases (AOs), including spermine oxidase and polyamine oxidases (PAOs). AOs regulate the level of polyamines in the cell. For example, PAOs are involved in polyamine homeostasis, 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, which are excreted through the kidney (56). These enzymes operate by abstracting two electrons from primary amines and transferring them to molecular oxygen to produce the corresponding aldehyde, ammonia and hydrogen peroxide, according to the following equation:

$$R\text{-}CH_2\text{-}NH_3^+ + O_2 + H_2O \xrightarrow{\text{Amine Oxidase}} R\text{-}CHO + NH_4^+ + H_2O_2$$

The superfamily of AOs represents an important class of enzymes, which are present in numerous living systems. These enzymes differ with respect to their molecular architecture, catalytic mechanisms, and patterns of substrate specificity, inhibitor sensitivity, and subcellular localizations (57). 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 (58). The first class is characterized by the presence of flavin adenine dinucleotide (FAD; FAD-AOs) and is ubiquitous in most mammalian species, whereas PAOs are found principally in vertebrates and plants (59). The second class consists of enzymes having a tightly bound Cu^{2+} 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 due to its pink absorption in the visible region, approximately at 480 nm (60). FAD- and Cu^{2+}/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 (61,62), whereas CuAOs are either intra- or extra-cellular enzymatic proteins, or in some cases integral plasma membrane proteins (63-65).

A peroxisomal FAD-dependent enzyme, N^1-acetylpolyamine oxidase (APAO), is a constitutively expressed enzyme that catalyses the cleavage of acetylated polyamines to produce spermidine (from spermine) or putrescine (from spermidine), 3-aceto-aminopropanal and H_2O_2. Wang *et al* (66) identified

Table I. Clonogenic assay: dose response.

Spermine (μM)	% Cell survival in cell lines			
	LoVo WT	LoVo DX	M14 WT	M14 ADR
0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
3	84.0±5.09	29.0±4.30	77.1±4.5	68.8±3.53
6	45.0±6.70	7.5±1.30	37.1±1.5	18.8±0.98
12	1.7±0.62	0.2±0.02	14.6±3.1	10.4±0.56
15	0.4±0.02	0.1±0.09	5.6±0.1	2.6±0.18

Effect of exogenous spermine concentration (0-15 μM) on percentage cell survival in LoVo WT, LoVo DX, M14 WT and M14 ADR in the presence of BSAO ($6.54x10^{-3}$ U/ml), during 60 min of incubation at 37˚C. Means and SDs are shown for two to five estimations from four to six experiments.

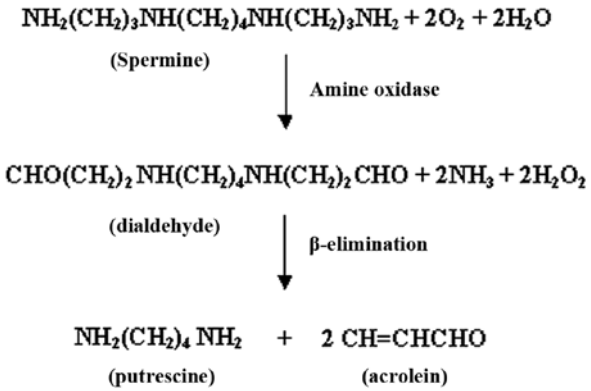

Figure 1. Reaction scheme for spermine oxidation in the presence of BSAO.

a gene *(PAOh1)* for an enzyme capable of oxidizing unsubstituted spermine. Vujcic *et al* (67) 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 H_2O_2. Subsequent studies indicated that SMO might represent a target for chemoprevention (68). In fact, using tumor-necrosis factor (TNFα), 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 H_2O_2, once converted into the highly reactive hydroxyl radical ($HO^{\bullet}$) 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. In addition, H_2O_2 formed by polyamine catabolism might play a role as a signaling molecule (69,70).

5. Cell killing by polyamines and BSAO

Attempts to exploit polyamine metabolizing enzymes as therapeutic targets, as well as to utilise the polyamine backbone as pharmacophore for the design of anticancer drugs have been investigated and reviewed (71,72). Our findings showed the possibility of using purified BSAO in the presence of exogenous spermine or endogenous polyamines to induce cytotoxicity (73). 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 multidrug resistant (MDR) (74,75). The oxidation products of polyamines, H_2O_2 and aldehyde(s), have been implicated in programmed cell death, induction of cytotoxicity and inhibition of cell division (76,77). Cytotoxic metabolites of spermine formed *in situ* by an enzyme-catalyzed reaction might be useful for the destruction of tumours (Fig. 1). An important distinction between normal and tumour cells is related to polyamine content and metabolism; in general polyamine concentrations are high in rapidly growing tissues such as tumours (78). However, AO activity has a contrasting effect on cancer cells. On the one hand it inhibits cell growth and induces cell death by necrosis (79) and/or apoptosis (74,80); on the other hand, AO activity has been correlated with cancer progression particularly when it is enhanced. Therefore, the involvement of AOs in cancer is associated with two different aspects: the direct regulation of the level of biogenic amines in cells and the formation of cytotoxic catabolites, i.e. H_2O_2 and aldehydes (74,79). Recently, several aspects of the role of AOs in cancer have been taken deeply into consideration by Toninello and collaborators (81).

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. Table I shows the percentage of cell survival as a function of exogenous spermine concentration up to 15 μM in the presence of BSAO, after 60 min of incubation. Multidrug-resistant (MDR) cells are more affected by treatment with spermine compared to their drug-sensitive counterparts. For instance, at 6 μM spermine concentration, the survival of LoVo WT cells was approx. 45%, while only a very low percentage of ~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. There was a remarkable ~80% reduction of cytotoxicity in both cell lines, apparently due to the clearance of H_2O_2 by catalase. However, this result shows that H_2O_2 is

Table II. Clonogenic assay: time course.

	% Cell survival in cell lines			
Time (min)	LoVo WT (1)	LoVo DX (2)	LoVo WT (3)	LoVo DX (4)
0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
5	76.0±12.2	45.0±3.8	104.0±7.5	106.0±11.3
10	53.0±10.4	27.0±2.4	102.0±18.3	98.2±8.1
15	43.0±3.9	24.7±2.1	99.2±2.4	98.0±16.5
20	40.2±6.8	21.0±4.9	97.0±3.7	97.0±7.9
30	31.2±5.2	13.7±8.1	97.3±5.1	96.0±8.6
40	20.2±3.3	8.9±1.6	96.8±2.3	95.3±12.9
60	18.4±1.5	4.8±1.4	96.0±6.4	94.0±2.8

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, up to 60 min, with BSAO ($6.54x10^{-3}$ U/ml) and exogenous spermine 12 μM, in absence of inhibitors (columns 1 and 2), or with catalase (240 U/ml) and ALDH (0.4 U/ml) (columns 3 and 4). Means and SDs are shown for two to five estimations from four to six experiments.

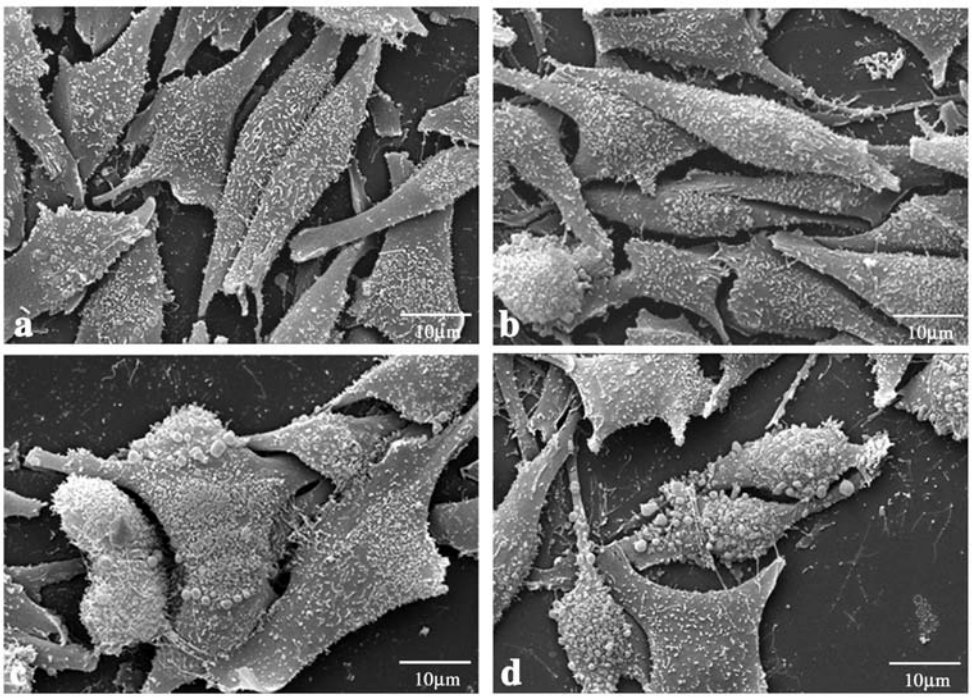

Figure 2. Effect of exposure to BSAO and spermine on the morphology of M14 WT and M14 ADR cells (scanning electron micrographs). (a) Untreated M14 WT cells. (b) Untreated M14 ADR cells. (c) M14 WT cells exposed for 60 min to $6.5x10^{-3}$ IU/ml BSAO and 6 μM spermine at 37˚C. (d) M14 ADR cells, same treatment as in (c). Bars: 10 μm. From Agostinelli *et al* (74).

not the exclusive toxic agent and that other species might be involved, such as acrolein. In order to determine the contribution of aldehydes in provoking the cytotoxicity by BSAO/spermine, catalase and NAD-dependent aldehyde dehydrogenase (ALDH) were added to the incubation mixture. In these experimental conditions, cytotoxicity was completely inhibited throughout the 60 min of incubation (Table II). MDR human melanoma cells were more sensitive than the corresponding wild-type cells at all spermine concentrations tested. At the 6 μM spermine concentration, survival of M14 WT cells was 37.1%, while only 18.8% of the M14 ADR cells remained viable (Table I). As single agents, BSAO or spermine were not toxic to either cell line up to 15 μM spermine.

The morphological and ultrastructural changes induced by treatment with BSAO/spermine were investigated by scanning (SEM) and transmission electron microscopy (TEM) to gain insight into the mechanism(s) responsible for the higher cytotoxic effect in MDR cells compared to drug-sensitive cells. Fig. 2 shows electron microscopic images of control M14 WT (panel a) and M14 ADR (panel b) cells, respectively, grown at 37˚C. These cells have elongated or polygonal shape and their surface are covered by randomly disseminated microvilli.

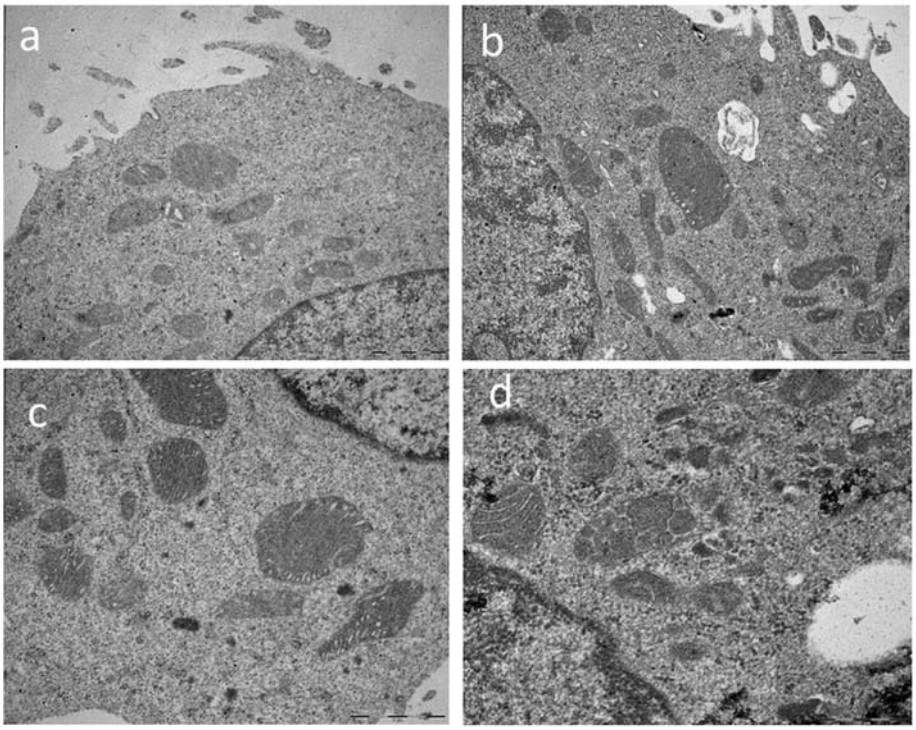

Figure 3. Ultrastructural features of mitochondria of M14 WT and M14 ADR cells (transmission electron micrographs). (a) Untreated M14 WT cells. (b) Untreated M14 ADR cells. (c) M14 WT cells exposed for 60 min to $6.5x10^{-3}$ IU/ml BSAO and 6 μM spermine at 37˚C. (d) M14 ADR cells, same treatment as in c. Bars: 0.5 μm. Panel b from Agostinelli *et al* (74).

After treatment with BSAO/spermine (6 μM) at 37˚C, cells from both cell lines (images c and d) appear less elongated than untreated control; 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 (Fig. 3a and b). After exposure to BSAO/spermine (6 μM) at 37˚C, M14 WT cells did not show any consistent aberration but some mitochondrial display dilated cristae (Fig. 3c). The alterations of mitochondria structure were much more evident in MDR cells; in particular, they showed a highly condensed matrix and vacuolised cristae (Fig. 3d). 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 to generate 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 was detected in MDR cells than that in drug-sensitive cells, suggesting an increased mitochondrial electron transport chain (METC) activity in MDR cells (79,82).

6. Hyperthermia in combination with polyamine metabolites in therapeutic applications

Hyperthermia is an alternate to cytotoxic drug therapy. Conventional cancer chemotherapy encounters several difficulties, including poor selectivity of the cytotoxic drugs and the development of MDR in the course of drug treatment. The term “hyperthermia” is generally used to imply a treatment based on the generation of heat at the tumour site (83). This approach involves raising the temperature of local environment of a tumour, resulting in a change in the physiology of diseased cells and subsequent cell death. Hyperthermia can augment the efficacy of other therapeutic modalities, such as irradiation, chemotherapy, surgery, gene therapy and immunotherapy (84-86). During hyperthermia treatment, cells undergo heat stress in the temperature range of 41-46˚C, resulting in activation and/or initiation of several intra- and extra-cellular degradation mechanisms, such as protein denaturation and aggregation, and DNA cross linking. Permanent irreversible protein damage can occur with a single heat treatment, resulting in protein aggregation and/or inhibition of many cellular functions. Histological studies showed that the heating-induced tumour cell death was mostly achieved through necrosis rather than apoptosis (87). The challenge is to heat only the tumour mass without damaging healthy tissues.

Agostinelli and colleagues studied potential effects of both H_2O_2 and aldehyde(s) (produced by the BSAO/polyamine-spermine enzymatic system) on the induction of cytotoxicity at 42˚C, rather than at 37˚C (74,79,88). In these studies, the most important cytotoxic metabolite of spermine was H_2O_2. In fact, H_2O_2, either formed by the glucose oxidase reaction, or added as such to the cell suspension, was cytotoxic

at a lower concentration than acrolein (89). In addition, H_2O_2 generated *in situ* from hypoxanthine by reaction with xanthine oxidase had anti-tumour effects *in vivo* (90). Both H_2O_2 and aldehyde(s), formed in the 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 complete protection of cells. These studies also showed that overexpression of P-glycoprotein (P-gp) in MDR cells did not confer resistance to the enzymatic oxidation products of spermine. This phenomenon might be caused by an earlier and higher mitochondrial membrane depolarization and a higher basal production of ROS (79,82).

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. A biological strategy to enhance the therapeutic effects of hyperthermia is to use heat in combination with pharmacological agents that show enhanced cytotoxicity at high temperatures. These thermosensitizer compounds, such as cysteamine and aminothiol N-(2-mercaptoethyl)-1,3-propanediamine (WR-1065), are not toxic at 37˚C, but become inactivators of cellular function at elevated temperatures (91). Another group of drugs, all of which were considered to be heat sensitizers, are the naturally occurring polyamines putrescine, spermine and spermidine (92).

The enzymatic oxidation products of spermine behave in a manner similar to that of thermosensitizers (93,94). Beneficial effects could therefore be achieved using localized heating to enhance the action of toxic products generated by BSAO/spermine within the tumour site, without increasing normal tissue damage. It was observed that the concentrations of spermine necessary to induce cytotoxicity were different in cell lines of various histotype (74,79,95,96). An interesting result was that an inactive combination of spermine (≤1 μM) and BSAO at 37˚C became cytotoxic at 42˚C for both human colon adenocarcinoma and melanoma cells, mimicking the action of thermosensitizers (74,88). These findings suggested 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 an early stage, 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 Conclusions and Future Perspectives paragraph (73,97).

7. Nanoparticle delivery of BSAO in cancer cells

A major impediment in advancing nanotechnology to the clinics is the inability of nanoparticles to undergo facile transport through the cell membrane. Several approaches have been attempted to circumvent this problem. We describe below some of the approaches used to transport BSAO in tumour cells.

Antitumoral effect in vitro and in vivo of native and immobilized BSAO on polyethylene glycol (PEG). In previous studies, H_2O_2 and aldehydes were produced outside the cells and subsequently entered inside the cells, producing cytotoxic effects. Catalytically liberated cytotoxic agents require only a few enzymatic units of the protein for toxin formation, and the cytotoxic reaction products are continuously formed over an extended period of time (57,98). Since endogenous polyamines are present at high concentrations in tumour cells and growing tissues, it is expected that toxic enzymatic oxidation products can be produced intracellularly by delivering BSAO directly into the cells, thereby achieving *in situ* killing of cells. Attempts were made to incorporate the enzyme into liposomal vesicles (99), and prepare amine oxidase-gold complexes that were bound and incorporated by hepatocytes (100). Thus, endogenous polyamines could be targeted and oxidized by the enzyme. In this context, attempts were made to produce immobilized BSAO 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 (97). Hydrogels are hydrophilic macromolecular networks that possess high water content. This feature should allow a controlled delivery of the enzyme by 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 (73). 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.

Iron oxide nanoparticles. Nanotechnological applications of iron oxides, namely maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4), have been intensely studied in different research areas, such as magnetic data storage (101,102), pigment production (103), electrochemistry (104), biosensing (105,106), drug delivery and protein purification (107). Cross-linked iron oxide (CLIO) (108), ultra-small superparamagnetic iron oxide (USPIO) (109), and mono-crystalline iron oxide nanoparticles (MIONs) (110) have been developed for diagnostic and therapeutic applications. Iron-oxide nanoparticles possess several superior properties, including magnetic properties (e.g. superparamagnetism, high values of saturation magnetization, easy control by small magnetic fields) and biochemical (e.g. non-toxicity, biodegradability, and biocompatibility) that justify their role in drug delivery. The ability of iron oxide nanoparticles to undergo cellular phagocytosis facilitates their use in contrast enhanced MRI beyond vascular and tissue morphology imaging (111), thereby enabling novel applications of iron oxide nanoparticles for MRI based diagnosis of liver diseases, cancer metastasis to lymph nodes, and *in vivo* tracking of implanted cells and grafts. Functionalized and

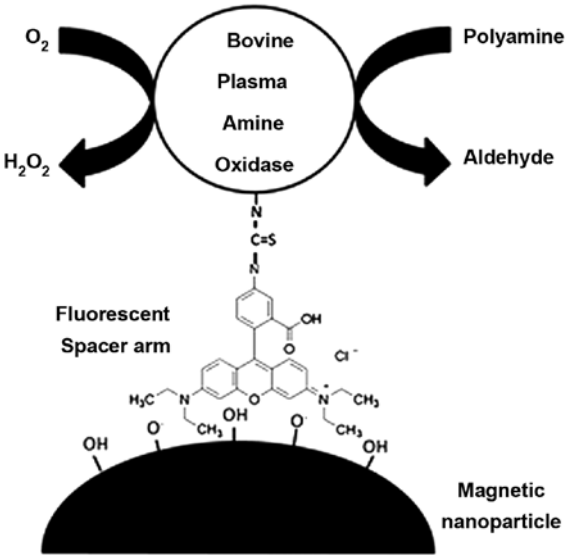

Figure 4. Schematic representation of fluorescent and magnetically drivable adduct comprising BSAO immobilized on the surface of specifically functionalized magnetic nanoparticles.

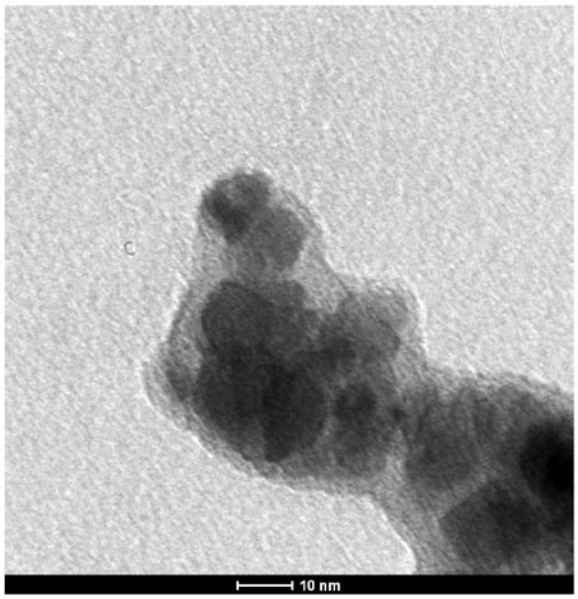

Figure 5. TEM image of maghemite nanoparticles derivatized with RITC and immobilized BSAO (SAMN@RITC-BSAO), as shown in Fig. 3. From Sinigaglia *et al* (122).

engineered magnetic nanoparticles have been developed to meet the increasing demand for non-invasive *in vivo* imaging of molecular and cellular activities, specific to a disease state. Several biomarkers, such as antibodies (32), aptamers (112), enzymes (113), peptide fragments (114) and polysaccharides (115) were used to achieve specificity and to reduce adverse side effects of iron oxide nanoparticles.

The possibility to convert dissipated magnetic energy into thermal energy led to the development and application of magnetic materials for hyperthermia treatment of cancer (83,116). Magnetic nanoparticles used for hyperthermia are only few tens of nanometer in size and therefore, allows easy passage into several tumours whose pore sizes are in 380-780 nm range. It was also found that irradiation of magnetic nanoparticles with a radio-frequency (in the range 100 kHz - 1 MHz) led to an increase in media temperature. This phenomenon can be used to increase the temperature of cells and tissue, and can be used in conjunction with hyperthermia for therapeutic purpose.

The controlled release of drugs with spatiotemporal control is the key to meeting several challenges in drug delivery applications. Controlled release of drugs from nanoparticle based delivery systems, triggered by a number of external stimuli, has been extensively studied (117). Iron oxide nanoparticles have the ability to act as chemotherapeutic agents to release drugs in a controlled manner. The most important advantage of nanoparticle-drug adducts, once that they have been released inside the cell, resides on their retention within the cell at concentrations sufficient to inhibit cell growth and functions, providing sustained drug release and improvement of treatment efficacy. Moreover, the ability of short interfering RNA (siRNA) to silence specific genes inspired the use of siRNA as a therapeutic agent for a wide spectrum of disorders including cancer, infectious diseases, and metabolic disturbances (118). In order to optimize the delivery of siRNA and enhance the efficiency of the treatment, the preparation of magnetic nanoparticles for imaging and siRNA delivery specifically to cancer cells was proposed (119). Iron oxide nanoparticles may be combined with different active agents, which could be proposed for therapy and diagnosis (120), thereby realizing their potential as theranostic agents.

BSAO bound to nanosized magnetic nanoparticles. Recently, a novel synthetic procedure for nanostructured superparamagnetic material in the size range approximately 10 nm, constituted of stoichiometric maghemite (γ-Fe_2O_3), and showing peculiar surface chemical behavior, called SAMNs (surface active maghemite nanoparticles) was developed (121). Maghemite nanoparticles and method for preparing thereof World Pat. WO/2012/010200). These superparamagnetic nanoparticles were modified with BSAO to improve the uptake, retention and therapeutic efficacy of BSAO. Fluorescent and magnetically drivable adducts comprising of BSAO and iron oxide had a diameter of 10±2 nm (SAMN@RITC-BSAO). These nanocatalyts are characterized by specific chemical behaviour and combine the advantages of immobilized enzymes with magnetic properties and a fluorescent probe (121) (Fig. 4). The multifunctional nanomaterial has been characterized by transmission electron microscopy (Fig. 5), infrared mass spectrometry and enzyme activity measurements. The immobilized enzyme partially retains its catalytic activity toward polyamine oxidation.

Further investigations showed that bare magnetic nanoparticles form stable colloidal suspension in aqueous solutions. The maximum binding capacity of BSAO was ~6.4 mg g^{-1} nanoparticles. However, the immobilization procedure reduced the catalytic activity by ~40%, compared to the activity of native enzyme. There was a 2-fold increase in the Michaelis constant of the enzyme. Taken together, these magnetically drivable nanocatalysts with a fluorescent tag and a specific activity of 0.81 IU g^{-1} are used in the presence of polyamines for selective killing of tumour cells by the *in situ* production of H_2O_2 and aldehydes (122).

8. Conclusions and future perspectives

Nanotechnology promises major advances in drug delivery, retention and efficacy as well as in diagnostic imaging. Hence these particles are theranostic agents. Gene delivery requires the transport of DNA through the cell membrane. DNA condensation to nanoparticles is a pre-requisite for facile transport of DNA. Oligonucleotide based DNA structures have been designed as drug delivery vehicles. Polyamines are excellent promoters of DNA condensation and investigations into polyamine structure-activity relationship in DNA condensation and transport continues to be an active area of research (123). Numerous studies have demonstrated that H_2O_2 and other reactive oxygen species are capable of blocking cells in G1, S or G2 phases of the cell cycle and inhibiting cell growth (124). Utilization of amine oxidase enzymes, especially BSAO can make clinical advances in the future. However, therapeutic applications of radical generating systems are still at the beginning. It is our hope that the utilization of amine oxide activity in the presence of biogenic amines will turn out to be a powerful strategy in the development of new anticancer treatments (73). 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) has been observed by elevating the temperature of tumour cell cultures from 37˚C to 42˚C (74,88,125).

This approach showed a higher sensitivity of MDR human adenocarcinoma and melanoma cells toward cytotoxic spermine metabolites, H_2O_2 and aldehydes, compared to the action of these agents on wild-type counterparts of these cells. This finding has been attributed to an early and higher mitochondrial membrane depolarization, and a higher basal production of ROS (82). 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 ($HO^{\bullet}$) by means of Fenton reaction, which induces oxidation of some thiol (SH) groups, proteins and lipids. Thus, hyperthermia combined with either toxic BSAO/polyamine metabolites or with thermosensitizing drugs is of great interest to develop a new strategy to overcome MDR of cancer cells. Iron oxide nanoparticles are important theranostic particles since they can be surface modified with reactive groups, antibodies and fluorescent molecules. BSAO immobilization was carried out on PEG and superparamagnetic nanoparticles. Moreover, a recent new strategy showed an increase in the stability of the enzyme and an improvement in releasing cytotoxic products. BSAO was conjugated on a new injectable nanohydrogel (NH), obtained by derivatizing hyaluronic acid (HA) with cholesterol (CH). The high immobilizing capacity of the HA-based anionic NHs with BSAO and retention of the catalytic activity allows the use of the immobilized enzyme BSAO as heterogeneous catalyst of a plug flow microreactor of H_2O_2 and aldehyde, inserted into a flow injection analysis system. The HA-based NH complex is a useful controlled delivery system for future therapeutic enzymes application (126). However, all the resulting systems were stable and bioactive, with respect to the free BSAO, indicating how this approach might represent a promising tool in anticancer therapy, for inducing an apoptotic effect on human cancer treatment.

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