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Inducible expression of maize polyamine oxidase in the nucleus of MCF-7 human breast cancer cells confers sensitivity to etoposide

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Summary. In this study, polyamine oxidase from maize (MPAO), which is involved in the terminal catabolism of spermidine and spermine to produce an aminoaldehyde, 1,3-diaminopropane and H_2O_2, has been conditionally expressed at high levels in the nucleus of MCF-7 human breast cancer cells, with the aim to interfere with polyamine homeostasis and cell proliferation. Recombinant MPAO expression induced accumulation of a high amount of 1,3-diaminopropane, an increase of putrescine levels and no alteration in the cellular content of spermine and spermidine. Furthermore, recombinant MPAO expression did not interfere with cell growth of MCF-7 cells under normal conditions but it did confer higher growth sensitivity to etoposide, a DNA topoisomerase II inhibitor widely used as antineoplastic drug. These data suggest polyamine oxidases as a potential tool to improve the efficiency of antiproliferative agents despite the difficulty to interfere with cellular homeostasis of spermine and spermidine.

Keywords: Polyamines – Polyamine oxidase – Hydrogen peroxide – Aminoaldehydes – Etoposide – Human breast cancer cells – Terminal catabolism

Abbreviations: BSA, Bovine serum albumin; CuAO, copper-dependent amine oxidases; Dah, 1,6-diaminohexane; Dap, 1,3-diaminopropane; DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle's medium; Dox, doxycycline; etoposide, 4′-demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucopyranoside); FITC, fluoroscein-isothiocyanate; MDL72527, N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine; MPAO, maize polyamine oxidase; NLS, nuclear localization signal; PAO, polyamine oxidases; PMSF, phenylmethylsulfonylfluoride; Put, putrescine; ROS, reactive oxygen species; SMO, spermine oxidases; Spd, spermidine; Spm, spermine; SSAT, spermine/spermidine acetyl transferase; Tet, tetracycline; XTT, sodium 3′-(1-(phenylamino-carbonyl)-3,4-tetrazolium)-bis (4-methoxy-6-nitro) benzene sulfonic acid

Introduction

The polyamines spermine (Spm), spermidine (Spd) and putrescine (Put) are important cellular effectors playing key roles in DNA, RNA and membrane stabilization, DNA replication, transcription, protein synthesis, ion channel modulation and protection against reactive oxygen species (ROS) (Cohen, 1998; Childs et al., 2003; Thomas and Thomas, 2003; Wallace et al., 2003; Huang et al., 2005).

A positive link has been recognized between cellular polyamine concentration and cell growth. In particular, neither mammalian cells lacking polyamine biosynthetic enzymes nor cells depleted of polyamines are able to replicate, while insufficient polyamine levels result in suboptimal growth (Thomas and Thomas, 2003). Furthermore, several types of cancer cells have been reported to have an aberrant polyamine metabolism and a high intracellular polyamine content (Heby and Persson, 1990; Bachrach, 2004; Huang et al., 2005; Seiler and Raul, 2005). Altered levels of intracellular polyamines have also been reported in Alzheimer's disease (Morrison and Kish, 1995) and cystic fibrosis (Russell et al., 1979), and a correlation between cell death and polyamine metabolism has also been observed in various cellular systems (Schipper et al., 2000; Pignatti et al., 2004).

The modulation of polyamine levels has been an important therapeutic target for many years. Several studies on the induction, inhibition, over-expression or gene knock-out and -down of enzymes involved in polyamine synthesis, such as ornithine decarboxylase, S-adenosylmethionine decarboxylase, spermine synthase (Wallace and Fraser, 2004 for a review; Mackintosh and Pegg,

2000; Korhonen et al., 2001; Stefanelli et al., 2001; Ikeguchi et al., 2004) and spermine/spermidine acetyl transferase (SSAT) (Vujcic et al., 2000; Niiranen et al., 2002; Chen et al., 2003) have indicated these enzymes as crucial points in controlling the intracellular polyamine levels and cell proliferation. These studies also have revealed the difficulty in changing the intracellular polyamine content due to the complex homeostatic mechanisms which involve polyamine biosynthesis, catabolism and transport across cell membranes.

The copper-dependent amine oxidases (CuAO) and the FAD-dependent polyamine oxidases (PAO) and spermine oxidases (SMO), enzymes involved in polyamine catabolism, have also been indicated as possible modulators of cell growth (Bachrach et al., 1987a, b; Mondovì et al., 1982; Amendola et al., 2005; Toninello et al., 2006). Animal CuAO oxidise the polyamines Put, Spd and Spm mainly at the primary amino groups to produce ammonia, H_2O_2 and an aminoaldehyde in a terminal catabolic pathway (Seiler, 2004), while animal PAO oxidise Spm and Spd (or their acetylated derivatives) at the secondary amino groups to produce Spd and Put, respectively, in addition to 3-aminopropanal (or 3-acetamidopropanal) and H_2O_2 (Wu et al., 2003; Vujcic et al., 2003). Furthermore, SMO oxidise only Spm to produce Spd, 3-aminopropanal and H_2O_2 (Wang et al., 2001; Vujcic et al., 2002; Cervelli et al., 2003). Thus, both animal PAO and SMO are involved in a polyamine back-conversion pathway (Seiler, 2004).

In animals CuAO, PAO and SMO might be involved in important cellular processes not only through regulation of cellular polyamine levels but also through their reaction products. In particular, H_2O_2 is considered to be both a cytotoxic and a regulatory effector. Indeed, depending on the concentration and the cell type, it can generate either severe oxidative damage to cellular components or a mild oxidative imbalance that can modulate numerous cellular signal transduction pathways as well as regulate gene expression (Sun and Oberley, 1996; Suzuki et al., 1997; Ha et al., 2000; Filomeni et al., 2005). Cytotoxicity of H_2O_2 produced by exogenously added purified CuAO and Spm has been described in several cell lines (Averill-Bates et al., 1994; Agostinelli et al., 2006a) and in a mouse melanoma model (Averill-Bates et al., 2005). Furthermore, SMO have been indicated as a primary source of cytotoxic H_2O_2 in polyamine analogue-treated human breast cancer cells (Pledgie et al., 2005) and direct oxidative damage to DNA has been reported to occur in a neuroblastoma cell line over-expressing murine SMO (Amendola et al., 2005). Regulatory and toxic effects have also been reported for aminoaldehydes (Yu et al., 2003; O'Brien et al., 2005). In particular, 3-aminopropanal has been recently shown to participate as a cytotoxin in human cerebral ischemia (Ivanova et al., 2002) and acrolein, generated spontaneously from 3-aminopropanal or 3-acetamidopropanal, has been reported to induce apoptotic cell death in microglial cells (Takano et al., 2005). Aminoaldehydes or acrolein generated by CuAO have also been described to have toxic effects (Averill-Bates et al., 1994; Agostinelli et al., 2006a). Furthermore, levels of plasma acrolein produced by PAO and/or SMO activity have been correlated to the degree of severity of chronic renal failure and have been indicated as novel biochemical markers for diagnosis of cerebral stroke (Sakata et al., 2003; Tomitori et al., 2005). In addition, 3-aminopropanal and 3-acetamidopropanal can be further metabolised to form β-alanine which in turn is involved in the biosynthesis of pantothenic acid, a metabolic precursor to important cofactors of several metabolic enzymes (White et al., 2001).

In the present study, we have investigated the possibility of interfering with polyamine homeostasis and cell proliferation by conditional expression of maize PAO (MPAO) (Tavladoraki et al., 1998) in the nucleus of MCF-7 human breast cancer cells using a tetracycline-regulated expression system (Tet-off) (Gossen and Bujard, 1992). MPAO has been chosen for this study because it is characterised by a higher turnover rate and substrate affinity than animal PAO, SMO and CuAO (Elmore et al., 2002; Cervelli et al., 2003; Wu et al., 2003; Polticelli et al., 2005). In addition, MPAO could be more efficient in altering intracellular polyamine levels, since it is involved in the terminal catabolism of Spd and Spm producing 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, respectively, in addition to 1,3-diaminopropane (Dap) and H_2O_2 (Cona et al., 2006). Recombinant protein expression has been targeted to the nucleus because of the important role polyamines play in several nuclear processes (Cohen, 1998; Childs et al., 2003; Thomas and Thomas, 2003). The findings demonstrate that the conditional expression of recombinant MPAO in the nucleus of MCF-7 cells increased Put levels and induced Dap production but it did not alter the cellular content of Spd and Spm. Furthermore, recombinant MPAO conferred growth sensitivity to treatment with etoposide, a potent topoisomerase II inhibitor (Baldwin and Osheroff, 2005), thus confirming that strategies aiming to increase the intracellular activity of amine oxidases may strengthen the antiproliferative efficacy of antineoplastic treatments.

Materials and methods

Chemical products

Spd, horseradish peroxidase, 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid have been all purchased from Sigma-Aldrich. Restriction and DNA-modifying enzymes have been purchased from New England Biolabs, Invitrogen, Stratagene and Promega. Other chemicals have been obtained from Bio-Rad and J. T. Baker. All oligonucleotides have been synthesised by Invitrogen. Etoposide (4′-demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucopyranoside)) has been obtained from Bristol-Myers Squibb.

Cell cultures

The MCF-7 human breast cancer cell line stably transfected with pTet-off plasmid for constitutive expression of the tetracycline (Tet)-controlled transactivator (tTA) (Gossen and Bujard, 1992) has been purchased from Clontech. Cells have been cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 4 mM L-glutamine, 10% (v/v) fetal bovine serum (Tet System Approved; Clontech), penicillin (Sigma-Aldrich) at 100 units/ml, streptomycin (Sigma-Aldrich) at 100 units/ml and the antibiotic G418 (Invitrogen) at 100 μg/ml. The cells have been maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells have been harvested with trypsinization, washed and stained with 0.4% (w/v) Trypan Blue (Sigma-Aldrich) for identification of dead cells. Viable cells have been counted using a Neubauer hemacytometer.

Plasmid construction

The coding region of the mature MPAO (without the sequence encoding for the signal peptide determining extracellular localization in plants) has been amplified from the whole *MPAO* cDNA (Tavladoraki et al., 1998) using the oligonucleotides *MPAOnucfor1* (5′-GTGTCAGGATCC*CGC CACC*ATGgcaaccgtcggccccagggtcatcg-3′) and *MPAOnucrev1* (5′-GACA CTATCGATActa*TACCTTTCTCTTCTTTTTTGGATCTACCTTTCTCTTCT TTTTTGGATCTACCTTTCTCTTCTTTTTTGGATC*gtcatacttccctggacatggta cttgca). The *MPAOnucfor1* oligonucleotide has been designed in such a way to substitute the MPAO 5′-*UTR* with the Kozak consensus ribosome binding site (*in italics*) in order to increase translation efficiency in animal cells (Kozak, 1999). After the first methionine codon following the Kozak sequence, a short sequence encoding for the first amino acids of the mature MPAO (*in small letters*) is present in the *MPAOnucfor1* oligonucleotide. The *MPAOnucrev1* oligonucleotide has been designed to insert, at the 3′-terminus of the MPAO coding region (*in small letters*) and before the stop codon (*in small letters*), three repetitions of a sequence (TACCTTTCTCTTCT TTTTTGGATC; *in italics*) encoding for a nuclear localization signal (NLS) derived from the simian virus 40 large T-antigen (octapeptide DPK KKRKV) (Lanford et al., 1986). The *MPAOnucfor1* and *MPAOnucrev1* oligonucleotides have also been designed to insert restriction sites *Bam*HI and *Cla*I (underlined regions), respectively, necessary for cloning of the *MPAOnuc* cDNA into the pTRE2hyg expression vector containing a Tet-responsive element (*TR*E) (Clontech). PCR amplification has been done using the *Pfu Turbo*® DNA polymerase (Stratagene) in a DNA GeneAmp PCR System 2400 (Perkin Elmer) with the following cycling parameters: 5 min of denaturation at 94 °C; 30 cycles of 94 °C for 1 min, 58 °C for 2 min and 72 °C for 2 min; 10 min at 72 °C for final extension. The PCR product has then been purified using the QIAquick™ gel extraction kit (Qiagen) and cloned in the pTRE2hyg vector to obtain the MPAO expression construct named MPAOnuc-pTRE2hyg.

Transfection

MCF-7 cells already stably transfected with the pTet-off plasmid have been transfected additionally with the MPAOnuc-pTRE2hyg plasmid by using CLONfectin™ (Clontech) according to the manufacturers' recommendations. Stably transfected clones, selected in medium containing 100 μg/ml antibiotic G418, 100 μg/ml hygromycin B (Clontech) and 1 μg/ml doxycycline hydrochloride (Dox; a tetracycline derivative) (Clontech), have been tested for MPAO expression in the presence or in the absence of Dox by RT-PCR, Western blot analysis and MPAO enzyme activity assays. Clones that expressed low basal levels of MPAO under +Dox conditions and high induced levels of MPAO under −Dox conditions have been selected for further study. The selected clones have been maintained continuously in medium containing 0.2 μg/ml Dox until experiments have been initiated.

RNA isolation and RT-PCR analysis

Total cellular RNA has been extracted from MCF-7 cultures by using the TRIZOL reagent (Invitrogen) according to manufacturer's instructions. To eliminate genomic DNA contaminants, total RNA has been treated with DNase I (Invitrogen). The first-cDNA strand has been synthesized from total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and an oligo-dT primer. PCR amplification has been performed with the EurobioTaq® DNA polymerase (Eurobio) using gene-specific oligonucleotides in a DNA GeneAmp PCR System 2400 (Perkin Elmer) with the following cycling parameters: 2 min of denaturation at 95 °C; 30 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 2 min; 10 min at 72 °C for final extension. The gene-specific oligonucleotides used have been *MPAOnucfor2* (5′-GGCGTCACCGTCAAGACAGAG-3′) and *MPAOnucrev2* (5′-TCGTCCGACTGCTGCTCGATG-3′) which amplify a fragment of 300 base pairs from the MPAO cDNA. Negative controls have been included consisting of RT-PCR reactions performed in the absence of reverse transcriptase during first-strand synthesis.

Protein extraction from transfected MCF-7 cells

To obtain total cellular extracts, cell pellets, after washing with PBS (68 mM NaCl, 17 mM NaH_2PO_4, 58 mM Na_2HPO_4, pH 6.0), have been resuspended in 0.2 M sodium phosphate buffer pH 6.0 containing 1 mM phenylmethylsulfonylfluoride (PMSF) and disrupted by sonication. After centrifugation at 17000 g for 10 min, the cleared supernatant containing the total soluble proteins has been analyzed for recombinant protein accumulation by Western blot analysis and/or enzyme activity assays. To obtain nuclear extracts, detached cells have been resuspended in 10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.1% (v/v) NP-40, 1 mM PMSF (800 μl/10^7 cells). After incubation at 4 °C for 10 min and centrifugation at 1400 g at 4 °C for 10 min, the cleared supernatant (cytoplasmic extract) has been separated from the pellet (nuclei). Nuclei have been disrupted by sonication in 0.2 M sodium phosphate buffer, pH 6.0 (400 μl/10^7 cells) and centrifuged to obtain the soluble nuclear extracts. Protein quantification in the various fractions has been performed using a protein assay kit (Bio-Rad) and bovine serum albumin (BSA) as a standard.

Western blot analysis

Western blot analysis has been performed utilizing a rabbit anti-MPAO polyclonal antibody (Rea et al., 2004) and a mouse anti-α-tubulin monoclonal antibody (Santa Cruz Biotechnology). An anti-rabbit (Sigma-Aldrich) and an anti-mouse (Amersham Biosciences) antibody coupled to horseradish peroxidase have been used as secondary antibodies and the detection of the labelled proteins has been done by chemiluminescence (Boehringer-Mannheim).

MPAO activity assays

MPAO enzyme activity has been determined by recording the formation of a pink adduct ($\varepsilon_{515} = 2.6 \times 10^4\ M^{-1}\ cm^{-1}$) resulting from the H_2O_2-

dependent oxidation of 0.1 mM 4-aminoantipyrine and of 1.0 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid in the presence of 4 mM Spd in 0.2 M sodium phosphate buffer, pH 6.5 containing 0.08 mg ml^{-1} of horseradish peroxidase.

Determination of polyamine levels

Polyamines have been extracted from cellular or nuclear pellets with 0.6 M perchloric acid containing 0.03 mM 1,6-diaminohexane (Dah) as an polyamine internal standard. Polyamines have been then quantified after derivatization with dansyl chloride and separation by HPLC (THERMO FINNIGAN) on a reverse-phase C18 column (Spherisorb S5 ODS2, 5 µm particle diameter, 4.6 mm × 250 mm) using a discontinued methanol to water gradient (40–60% methanol in 2 min, 60–95% methanol in 20 min, 95–100% in 2.5 min, 100% for 1.5 min, 100–40% in 6 min at a flow rate of 1.5 ml/min). Eluted peaks have been detected by a spectrofluorometer (Spectra SYSTEM FL 3000; excitation 365 nm, emission 510 nm), recorded and integrated by an attached computer using the Thermo Finnigan Chrom-Card 32 bit software. Polyamine concentration in the nuclei or in the total cellular homogenates has been referred to the corresponding protein content and expressed as nmol/mg of proteins.

Immunofluorescence microscopy

Cells have been cultured on glass coverslips and fixed in methanol-acetone 1:1 (v/v) for 2 min at −20 °C. After washing with cold PBS, the cells have been incubated overnight at 4 °C with rabbit anti-MPAO polyclonal antibody in PBS containing 2% (w/v) BSA (PBSB). Subsequently, cells have been extensively washed with PBSB and have been incubated with fluoroscein-isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (Vector Laboratories) in PBSB for 1 h at 37 °C. After extensive washing, cells have been counterstained with 40 µg/ml 4′,6′-diamidino-2-phenylindole dihydrocloride (DAPI; Sigma-Aldrich) and examined under a fluorescent microscope (Zeiss). A minimum of 500–1000 cells have been examined for each case.

Cell proliferation assay

Cell proliferation has been evaluated using XTT solution (sodium 3′-(1-(phenylamino-carbonyl)-3,4-tetrazolium)-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) from the "Cell Proliferation Kit II" (Roche Molecular Biochemicals). Cells have been incubated with 0.3 mg/ml XTT in DMEM supplemented with L-glutamine and fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 for 4 h after which the absorbance at 450 nm has been recorded using a plate reader with a reference wavelength of 690 nm. Data obtained have been elaborated using GraphPad Prism.

Statistical analysis

Statistical significance has been evaluated with data from at least three independent experiments by using Student's *t*-test or one-way ANOVA test. A $P < 0.05$ has been considered statistically significant.

Results

Selection of transfectants with a Dox-dependent recombinant MPAO expression

With the aim of interfering with polyamine homeostasis and cellular proliferation by altering polyamine catabolism, a MCF-7 cell line stably transfected with the pTet-off plasmid has been furthermore transfected with the MPAOnuc-pTRE2hyg construct allowing tetracycline-dependent MPAO expression in the nucleus. Stably transfected clones have been selected in hygromycin and grown as clones in the presence of Dox (MPAOnuc-MCF7 clones). Of the 30 MPAOnuc-MCF7 clones, one clone (4.31 clone) has been selected for further study based on RT-PCR analysis (data not shown) which has showed high levels of MPAO-specific mRNA when cells have been grown in the absence of Dox and non-detectable MPAO-specific mRNA levels when grown in the presence of Dox. MPAO-specific mRNA has not been

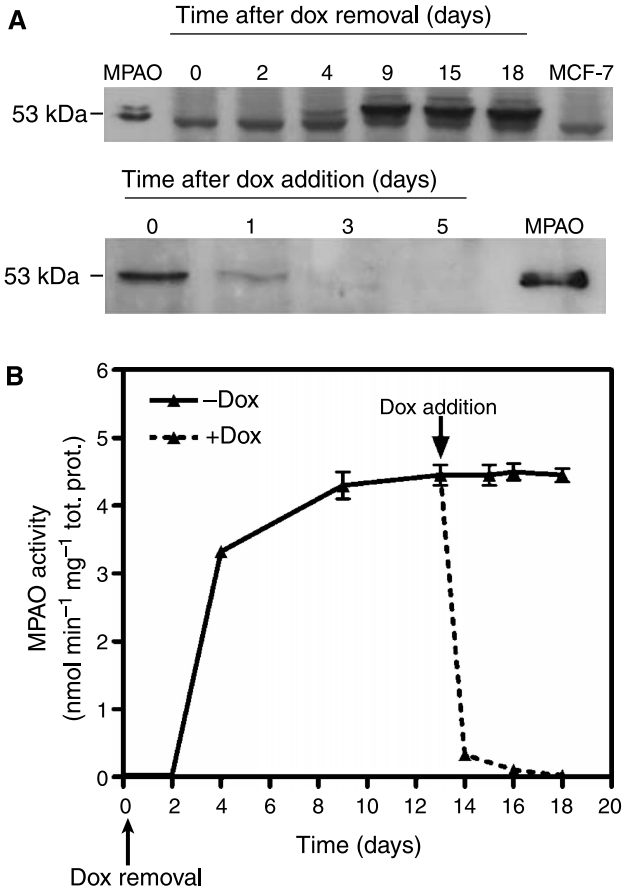

Fig. 1. Time-course of recombinant MPAO accumulation in the MPAOnuc-MCF7 cells (4.31 clone) after removal or addition of Dox. (**A**) Recombinant MPAO protein accumulation in the MPAOnuc-MCF7 cells at various time intervals after removal or addition of Dox has been determined by Western blot analysis of total cellular extracts using an anti-MPAO polyclonal antibody. MPAO, purified native MPAO used as a positive control. MCF-7, extract from MCF-7 cells non transfected with the MPAOnuc-pTRE2hyg plasmid. Cellular extracts have been normalized for the amount of the total soluble proteins before analysis. (**B**) Recombinant MPAO protein accumulation in the MPAOnuc-MCF7 cells at various time intervals after removal or addition of Dox has been determined by enzyme activity assay using Spd as a substrate. Values are the means ± SE from three replicates

present also in the non transfected MCF-7 cells, which is in agreement with the low homology between MPAO and animal PAO/SMO.

To verify whether the Dox-regulated production of MPAO-specific mRNA in the 4.31 MPAOnuc-MCF7 clone is accompanied by accumulation of the recombinant protein, total cellular protein extracts have been analysed by Western blot at various time intervals after Dox removal (Fig. 1A). This analysis revealed the accumulation of a detectable amount of recombinant MPAO as soon as 4 days after Dox removal. Later, MPAO accumulation levels increased with time, reaching a plateau at about 10 days following removal of Dox, after which MPAO accumulation levels remained constant for at least 8 days (Fig. 1A). The induction of MPAO protein accumulation following Dox removal (−Dox) has been closely paralleled by an increase in MPAO enzyme activity levels (Fig. 1B), reaching a maximum level of 4.4 nmol min^{-1} mg^{-1} total proteins. The addition of Dox (+Dox) to the MPAOnuc-MCF7 cells which had been grown for 18 days in the absence of Dox inhibited MPAO expression and resulted in a rapid decrease in MPAO protein and enzyme activity levels within one day (Fig. 1). These results confirm that the Tet-Off expression system is highly responsive and indicate that recombinant MPAO has a short half-life in the MCF-7 cells. These data also demonstrate that recombinant MPAO is functionally expressed in the 4.31 stably transfected clone. Interestingly, the maximum amounts of MPAO enzyme activity observed is about 150-fold higher than those of endogenous SSAT (Vujcic et al., 2000; Kee et al., 2004; Pledgie et al., 2005), PAO and SMO (Pledgie et al., 2005) and about 15-fold higher than those of the recombinant SSAT expressed using the Tet-off expression system in MCF-7 human breast carcinoma cells and LNCaP prostate carcinoma cells (Vujcic et al., 2000; Kee et al., 2004).

Subcellular localization of MPAO in the MPAOnuc-MCF7 cells

To confirm the sub-cellular localization of recombinant MPAO, 4.31 MPAOnuc-MCF7 cells grown in the absence or in the presence of Dox have been analysed by immunofluorescence microscopy. Results have showed a complete overlap between the green fluorescence associated with the anti-MPAO antibody and the blue fluorescence corresponding to the DAPI-stained nuclei in the −Dox cells (Fig. 2). This proves a nuclear localization for the recombinant protein which is consistent with the presence of a sequence encoding for three NLS at the 3′-terminus of the MPAO cDNA in MPAOnuc-pTRE2hyg construct.

Nuclear localization of recombinant MPAO in the −Dox MPAOnuc-MCF7 cells has been further shown by analysis of nuclear and cytoplasmic extracts for the presence of the recombinant protein. Western blot analysis using the anti-MPAO polyclonal antibody (Fig. 3A) and enzyme activity assays (Fig. 3B) demonstrated the presence of a high amount of MPAO in the nuclear extracts of the −Dox MPAOnuc-MCF7 cells. A small amount of MPAO has also been reported in the cytoplasmic extracts of the −Dox MPAOnuc-MCF7 cells, which could represent either the newly synthesised protein prior to translocation to the nucleus or the contamination of the cytoplasmic extracts with nuclear proteins. Conversely,

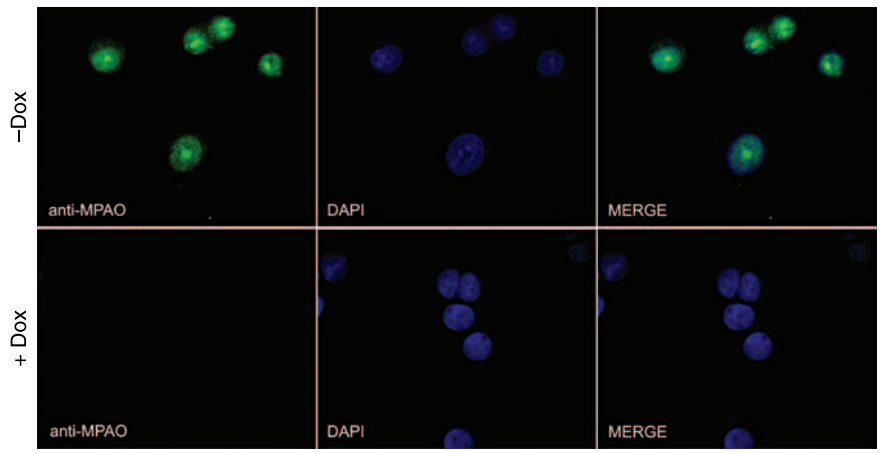

Fig. 2. Sub-cellular localization of recombinant MPAO in the stably transfected MPAOnuc-MCF7 cells by fluorescence microscopy. Cells grown in the presence (+Dox) or in the absence (−Dox) of Dox for 10 days have been immunostained (green fluorescence) by a rabbit anti-MPAO polyclonal antibody and FITC-conjugated anti-rabbit IgG antibody. Nuclei have been also counterstained by DA PI. *Merge*: overlapping images from immunostaining and DAPI staining (For a color reproduction, the reader is referred to the web version of this article)

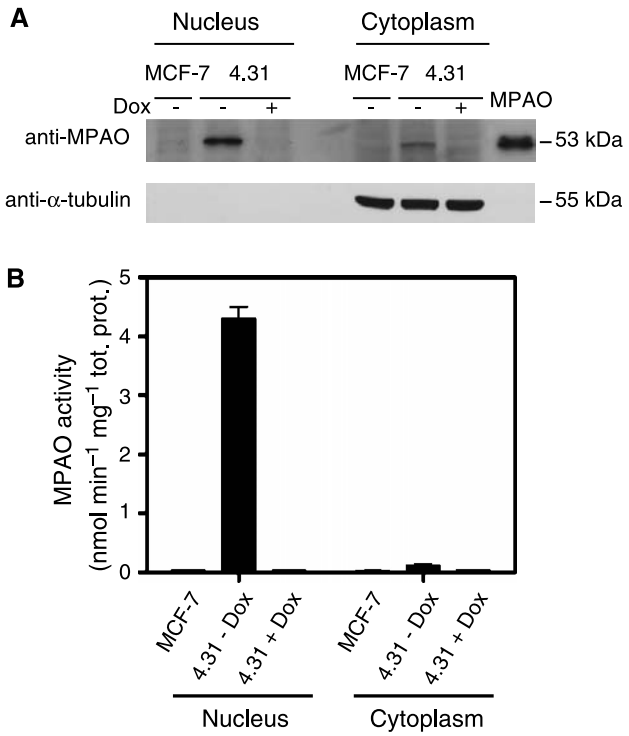

Fig. 3. Sub-cellular localization of recombinant MPAO in the stably transfected MPAOnuc-MCF7 cells. MPAOnuc-MCF7 cells grown in the presence (+) or in the absence (−) of Dox for 10 days have been analysed for the presence of MPAO. **A** Western blot analysis of nuclear and cytoplasmic extracts using an anti-MPAO polyclonal antibody or anti-α-tubulin antibody. Extracts have been normalised for the amount of the total soluble proteins. MPAO, purified native MPAO used as a positive control. **B** Analysis by enzyme activity assays of nuclear and cytoplasmic extracts using Spd as a substrate. MCF-7, extract from MCF-7 cells non transfected with the MPAOnuc-pTRE2hyg plasmid. Results are shown as means ± SE of three replicates

Western blot analysis with an antibody recognizing the cytoplasmic protein α-tubulin showed the presence of this protein only in the cytoplasmic extracts and not in nuclear extracts (Fig. 3A), thus excluding contamination of the nuclear extracts by cytoplasmic proteins.

Determination of polyamine levels in the 4.31 MPAOnuc-MCF7 cells

To determine the effect of MPAO expression on the polyamine levels, nuclear and total cellular polyamine pools have been analysed (Table 1). This analysis has been performed both early (5 days) after Dox removal to avoid induction of polyamine homeostatic mechanisms and late (10 days) after Dox removal to have maximal expression levels of recombinant MPAO obtaining similar results in both cases. Interestingly, a high amount of Dap, which is one of the MPAO reaction products, has been observed in the −Dox MPAOnuc-MCF7 cells while it was undetectable in the +Dox MPAOnuc-MCF7 cells. In particular, the amount of Dap present in the −Dox MPAOnuc-MCF7 cells has been equimolar to that of Spd and Spm. These data suggest that recombinant MPAO is indeed able to metabolize polyamines in the −Dox MPAOnuc-MCF7 cells. However, only a small, not statistically significant, decrease in the levels of the MPAO specific substrates Spd and Spm in both the nuclear and total cellular extracts of the −Dox MPAOnuc-MCF7 cells compared to those of the +Dox MPAOnuc-MCF7 cells has been obtained. On the other hand, an almost 3-fold increase in the amount of Put, which is neither substrate nor product of MPAO, has been shown in the −Dox MPAOnuc-MCF7 cells with respect to the +Dox MPAOnuc-MCF7 cells. This may be due either to enhanced activity of the polyamine biosynthetic enzymes or to changes in polyamine cellular transport to compensate for changes in polyamine levels following recombinant MPAO expression. These in turn could explain the lack of changes in overall Spd and Spm levels despite the accumulation of high amounts of Dap.

Table 1. Effect of conditional MPAO expression on polyamine levels in the MPAOnuc-McF7 cells

		Polyamine pools			
		Put	Spd	Spm	Dap
		nmol mg^{-1} prot.[a]			
Total extracts	+Dox	7.5 ± 0.8	42.4 ± 3.2	24.2 ± 1.9	ND
	−Dox	27.9 ± 2.1*	38.2 ± 2.7	18.5 ± 1.1	21.9 ± 1.7
Nuclear extracts	+Dox	5.4 ± 0.7	30.3 ± 2.6	17.5 ± 1.5	ND
	−Dox	17.3 ± 1.1*	28.1 ± 2.0	14.8 ± 0.8	15.1 ± 0.8

MPAOnuc-McF7 cells (4.31 clone) grown in the presence (+Dox) or in the absence (−Dox) of Dox for 5 days have been analysed for nuclear and total cellular polyamine levels. Data are expressed as mean ± SE of four independent experiments

[a] Data are expressed as nmol per mg of nuclear or total cellular proteins

* Indicate values significantly different from those of the control +Dox cells by Student's *t*-test ($P < 0.01$)

Effect of recombinant MPAO expression on cell growth

The presence of a high amount of Dap in the −Dox MPAOnuc-MCF7 cells suggests an equimolar production of H_2O_2 and aminoaldehydes which could have a cytotoxic effect (Ha et al., 2000; Ivanova et al., 2002; Yu et al., 2003; Amendola et al., 2005; O'Brien et al., 2005; Takano et al., 2005). In consequence, the effect of recombinant MPAO expression on cell growth has been assessed. Cell growth has been determined by using a cell-proliferation assay (Fig. 4). The results have shown that recombinant MPAO expression in the 4.31 MPAOnuc-MCF7 cells does

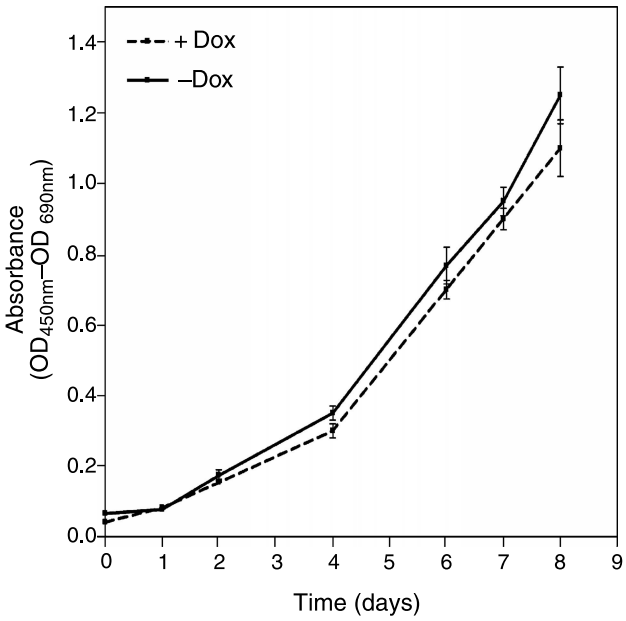

Fig. 4. Growth of MPAOnuc-MCF7 cells. MPAOnuc-MCF7 cells have been grown in the presence (+Dox) or in the absence (−Dox) of Dox. Cell growth has been determined using the XTT-based cell proliferation assay method described under Materials and methods. A representative experiment, which has been repeated three times, is reported. Results are shown as means ± SE of six replicates

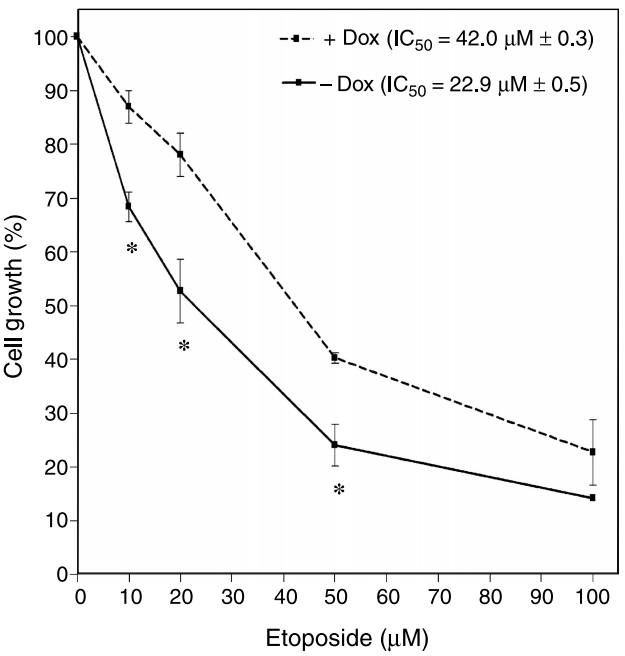

Fig. 5. Sensitivity of MPAO expressing MPAOnuc-MCF7 cells to etoposide. 4.31 MPAOnuc-MCF7 cells grown in the presence (+Dox) or in the absence (−Dox) of Dox for 5 days have been treated with the indicated concentrations of etoposide for 24 h. Cell growth has been measured using the XTT assay method described in Materials and methods and expressed as % of values from control cells not treated with etoposide. Results are shown as means ± SE ($n = 4$ with six replicates per experiment). Asterisks indicate values significantly different from those of the control +Dox cells at each concentration of etoposide by one-way ANOVA test ($P < 0.05$)

not affect cell growth and proliferation. Furthermore, it has also been observed by flow-cytometry analysis that recombinant MPAO expression in the MPAOnuc-MCF7 cells has no effect on the cell cycle.

Instead, recombinant MPAO expression in the 4.31 MPAOnuc-MCF7 cells conferred higher growth sensitivity to 24 h treatment with etoposide, a widely used antineoplastic drug which inhibits topoisomerase II at the strand rejoining step resulting in single and double strand breaks in DNA. Data from a dose-response analysis (Fig. 5) have indeed showed that the −Dox MPAOnuc-MCF7 cells are characterised by an IC_{50} value (etoposide concentration at which 50% inhibition of cell growth is observed) for etoposide of 22.9 ± 0.5 µM which is significantly lower than that of the +Dox MPAOnuc-MCF7 cells ($IC_{50} = 42.0 \pm 0.3$ µM) ($P < 0.05$) (Fig. 5).

Discussion

In recent years, it has been hypothesised that alteration of intracellular polyamine content and production of H_2O_2 and aminoaldehydes through manipulation of polyamine catabolic enzymes could contribute to cell-growth inhibition. In the present study, we have conditionally expressed MPAO in the nucleus of MCF-7 human breast cancer cells using a tetracycline-regulated expression system. MPAO has been chosen for this study because it is characterised by a higher turnover rate and substrate affinity (50 sec^{-1} and 2 µM, respectively, with Spd as best substrate) (Polticelli et al., 2005) compared to animal PAO (4.8 sec^{-1} and 36.8 µM, respectively, with N^1-acetyl-Spd as best substrate) (Wu et al., 2003), SMO (4.5 sec^{-1} and 90 µM, respectively, with Spm as best substrate) (Cervelli et al., 2003) and CuAO (7.9 sec^{-1} and 20 µM, respectively, with Put as best substrate) (Elmore et al., 2002). Furthermore, MPAO could be more efficient in altering intracellular polyamine levels, since it is involved in the terminal catabolism of Spd and Spm, at variance with the animal PAO and SMO which are both involved in a polyamine back-conversion pathway. Indeed, the so far characterised PAO from monocotyledonous plants, such as MPAO and barley PAO, oxidise Spd and Spm producing an aminoaldehyde, Dap and H_2O_2 (Cona et al., 2006) and only recently, a PAO from the dicotyledonous *Arabidopsis thaliana* plant has been shown to oxidise Spm with a similar mode to that of animal PAO and SMO (Tavladoraki et al., 2006). Recombinant protein expression has been targeted to the nucleus because reduction of polyamine content and accumulation of the MPAO reaction products (mainly H_2O_2 and aminoaldehydes) in the nucleus could interfere with DNA

stability and thus with cell proliferation under physiological conditions and/or in the presence of antiproliferative drugs.

Our data show that it is possible to obtain high expression levels of recombinant MPAO in the nucleus of the MCF-7 cells, although MPAO is a plant enzyme with a native extracellular localization. Indeed, a high amount of MPAO enzyme activity has been recorded in the −Dox MPAOnuc-MCF7 cells (4 nmol min^{-1} mg^{-1} tot. prot.), which is much higher than that of the endogenous catabolic enzymes. In particular, compared to the levels of endogenous SSAT (20 pmol min^{-1} mg^{-1} tot. prot.) (Vujcic et al., 2000), SMO (30 pmol min^{-1} mg^{-1} tot. prot.) and PAO (17 pmol min^{-1} mg^{-1} tot. prot) (Pledgie et al., 2005) in the same cell type, the amount of recombinant MPAO enzyme activity in the −Dox MPAOnuc-MCF7 is approximately two orders of magnitude higher. Furthermore, the amount of recombinant MPAO enzyme activity is approximately one order of magnitude higher than that of recombinant SSAT conditionally over-expressed in the MCF-7 cells stably transfected with tetracycline-regulated SSAT human cDNA or murine gene (270 pmol min^{-1} mg^{-1} tot. prot.) (Vujcic et al., 2000) and two orders of magnitude higher than the murine SMO over-expressed in mouse neuroblastoma cells (Amendola et al., 2005).

The accumulation of an elevated amount of Dap in the −Dox MPAOnuc-MCF7 cells (Table 1), necessarily resulting from the terminal catabolism of Spd and Spm by MPAO, suggests that MPAO is not only highly expressed in these cells, but it is also functional. However, despite the accumulation of a high amount of Dap, recombinant MPAO expression in the −Dox MPAOnuc-MCF7 cells apparently did not interfere with intracellular Spm and Spd levels. The lack of changes in the overall quantity of intracellular Spd and Spm could be due to compensatory metabolic adjustments as suggested by the increase in the amount of Put in the −Dox MPAOnuc-MCF7 cells. Similar results have also been obtained in LNCaP prostate carcinoma cells conditionally over-expressing SSAT, in which, despite the accumulation of an elevated amount of acetylated polyamines, intracellular levels of Spd and Spm failed to decrease (Kee et al., 2004). Indeed, in this case, the levels of Put, Spd and Spm increased substantially during the first 24 h following SSAT induction, after which they declined to levels that were near basal levels. Similarly, SSAT conditional over-expression in MCF-7 cells failed to decrease Spm levels, although Spd levels were reduced after 4 days of SSAT induction (Vujcic et al., 2000). Over-expression of murine SMO in the murine neuroblastoma cells also resulted in only a small decrease in the amount of its substrate Spm, unchanged amounts of Spd and increased amounts of Put (Amendola et al., 2005).

The accumulation of Dap (about 20 nmol mg^{-1} tot. prot.) in the −Dox MPAOnuc-MCF7 cells also suggests the production of an equimolar amount of H_2O_2 and aminoaldehydes which, however, seems not to be sufficient to affect MPAOnuc-MCF7 cell growth. Indeed, H_2O_2 and aldehyde(s) have been shown to have a cytotoxic effect at concentrations above a threshold level, such as 10 μM and 50 μM, respectively, when added exogenously to Chinese hamster ovary cells (Averill-Bates et al., 1994).

The lack of cytotoxicity of the MPAO toxic reaction products in the MPAOnuc-MCF7 cells may be due to the higher efficiency of the detoxification and/or damage-repairing systems in respect to the rate of their production. In relation to this hypothesis, we cannot exclude the possibility that recombinant MPAO activity in the nucleus is limited by the presence of only a small amount of free polyamines, the rest of them forming complexes with macromolecules, such as DNA (D'Agostino and Di Luccia, 2002; D'Agostino et al., 2005), in which they may not be oxidised by MPAO. The aggregated polyamines may, however, be slowly released from these complexes and the H_2O_2/aminoaldehydes derived from their oxidation by MPAO may be gradually detoxified before being accumulated to toxic levels.

SSAT over-expression in MCF-7 or LNCaP cells greatly altered cell proliferation despite the much lower SSAT enzyme activity levels compared to recombinant MPAO (Vujcic et al., 2000; Kee et al., 2004). In this case, cell growth inhibition by SSAT over-expression was not due to oxidative stress or to aminoaldehyde accumulation, but probably either to the accumulation of an elevated amount of acetylated polyamines, which could exert a toxic effect, or to the decrease of intracellular acetyl-CoA levels which, apart from being a SSAT cofactor, is involved in several pathways including fatty acid synthesis, histone acetylation and other processes fundamental for cell growth.

The ectopic expression of MPAO in the MPAOnuc-MCF7 cells (−Dox MPAOnuc-MCF7 cells) conferred higher growth sensitivity to etoposide treatment compared to MPAOnuc-MCF7 cells not expressing the recombinant protein (+Dox MPAOnuc-MCF7 cells). It is possible that, when topoisomerase II (a key enzyme involved in the DNA repairing system) is inactivated following etoposide treatment, the amount of the toxic MPAO reaction products formed in the −Dox MPAOnuc-MCF7 cells may be enough to generate a relevant cellular damage due to a

decreased efficiency of the detoxification and/or damage-repairing systems. In agreement with our data, murine SMO over-expression in mouse neuroblastoma cells conferred higher sensitivity to radiation exposure (Amendola et al., 2005), while SMO knock-down reduced sensitivity of human breast cancer cells to the polyamine analogue N^1,N^1-bis(ethyl)norspermine (BENSpm) (Pledgie et al., 2005). Furthermore, in multidrug-resistant human adenocarcinoma and melanoma cells it has been demonstrated that hyperthermia or treatment with the lysosomotropic compound MDL72527 increased the toxicity of bovine serum CuAO and spermine added extracellularly (Agostinelli et al., 2006a, b and c).

In summary, expression of MPAO, an enzyme involved in the terminal catabolism of Spd and Spm, in the nucleus of MCF-7 cells caused an increase in Put and Dap intracellular levels but it did not interfere with Spd and Spm levels, probably due to compensatory metabolic adjustments, thus confirming the tight regulation of cellular polyamines. Furthermore, even though recombinant MPAO expression at high levels in MCF-7 cells did not lead to sufficient amounts of H_2O_2 or cytotoxic aldehydes to inhibit cell growth under normal growth conditions, it conferred higher sensitivity to treatment with the anticancer drug etoposide. Since these results could be cell-line dependent, it would be interesting to determine the effect of MPAO expression on cell growth also in other cell lines with the long-term aim of modulating the mechanisms through which the various anticancer agents exert their antiproliferative effects. It would be also interesting to analyse the effect of recombinant MPAO targeting to a different intracellular compartment. These studies could permit a deeper understanding of the dynamics of polyamine homeostasis and offer alternative strategies for the development of antitumour treatments.

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