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Arsenic Trioxide (ATO) Influences the Gene Expression of Metallothioneins in Human Glioblastoma Cells

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Abstract Arsenic trioxide (As_2O_3; ATO, TRISENOX®) is used to treat patients with refractory or relapsed acute promyelocytic leukaemia while its application for treatment of solid cancers like glioblastoma is still under evaluation. In the present study, we investigated the interaction of arsenic trioxide with metallothionein (MT) isoforms as a possible (protective response) resistance of glioblastoma cells to arsenic-induced cytotoxicity. Special attention was focused on MT3, the isoform expressed mainly in the brain. MT3 has low metal inducibility, fast metal binding/releasing properties and outstanding neuronal inhibitory activity. The human astrocytoma (glioblastoma) cell line U87 MG was treated with 0.6, 2 and 6–7 μM arsenic (equivalent to 0.3, 1 and 3–3.5 μM As_2O_3) for 12, 24 or 48 h and gene expression for different MT isoforms, namely *MT2A, MT1A, MT1F, MT1X, MT1E* and *MT3*, was measured by real time qPCR using SYBR Green I and Taqman® gene expression assays. *TfR*, *18S rRNA, GAPDH* and *AB* were tested as reference genes, and the last two evaluated to be appropriate in conditions of low (*GAPDH*) and high (*AB*) arsenic exposure. The gene expression of *MT3* gene was additionally tested and confirmed by restriction enzyme analysis with PvuII. In the given conditions the mRNAs of six MT isoforms were identified in human glioblastoma cell line U87 MG. Depending on arsenic exposure conditions, an increase or decrease of MT gene expression was observed for each isoform, with the highest increase for isoforms *MT1X, MT1F* and *MT2A* mRNA (up to 13-fold) and more persistent decreases for *MT1A, MT1E* and *MT3 mRNA*. Despite the common assumption of the noninducibility of MT3, the evident *MT3 mRNA* increase was observed during high As exposure (up to 4-fold). In conclusion, our results clearly demonstrate the influence of As on MT isoform gene expression. The *MT1X, MT1F* and *MT2A* increase could represent brain tumour acquired resistance to As cytotoxicity while the MT3 increase is more enigmatic, with its possible involvement in arsenic-related induction of type II cell death.

Keywords Metallothionein mRNA · MT 3 isoform · Gene expression · Glioblastoma · Astrocytoma · Arsenic trioxide · Resistance

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Introduction

Although arsenic is an environmental toxin, it has also been used as a therapeutic agent for more than 2,400 years [1]. Since year 2000, it is in regular use in treatment of acute promyelocytic leukaemia (APL) as arsenic trioxide (As_2O_3; ATO, Trisenox®—with As^{3+} as the active substance) [2]. In the form of trivalent arsenic, ATO is able to induce apoptosis and/or autophagy in various malignant cell lines. Thus, it represents a potential treatment agent for other malignancies as well. Therefore, many clinical trials have been and are currently in progress to evaluate its wider clinical usefulness in haematology and in different solid tumours like glioblastoma [3].

Glioblastoma multiforme is a brain tumour of glial cells which is extremely difficult to treat, and the 2-year survival of glioblastoma patients with therapy including surgery, radiation and chemotherapy is less than 30 % [4]. Intrinsic and acquired

drug resistance represents an important problem during treatment. The pharmacoresistance of brain tumours is clearly multifactorial, including multidrug resistance factor 1 and P-glycoprotein (MDR1/P-gp), O^6-methylguanine methyltransferase, multidrug resistance protein, metallothioneins (MTs), glutathione and glutathione-*S*-transferases, dihydrofolate reductase, protein kinase C and topoisomerase-IIα [5].

MTs are inducible low-molecular-weight (<9 kD), cysteine-rich (30 %), metal-binding proteins, identified in a wide range of phyla [6–8]. Most verterbrate species contain two distinct MT isoforms (MT1 and MT2) while mammals have four isoforms (MT1-4). They are present mainly in the cytosol, but in some circumstances, they can also be detected in the cell nucleus, lysosomes, mitochondria and in the extracellular space. It is generally accepted that they have an important role in homeostasis, transport and storage of zinc and copper. On account of their high cysteine content, MTs can bind metals and also act as radical scavengers or redox regulators inside the cell. Because of physicochemical similarities among metals, they can also bind exogenous metals and are often considered as essential biomarkers in metal-induced toxicity, facilitating metal detoxification by metal binding and protection from radicals. In brain tissue, MTs are highly expressed in star-shaped glial cells—astrocytes [9, 10] which have also been proposed as the main site of accumulation and storage of toxic metals in the brain [11]. Thus, in glioma cells they may contribute to sequestration and detoxification of metal-related anti-cancer drugs and avert irradiation-induced damage [5]. Numerous in vivo and in vitro studies have also shown the antiapoptotic action of MT 1/2 [7, 12] and MT3 [12], but the mechanisms involved are awaiting clarification.

Previous data from our laboratory already point to MTs interactions of MTs with arsenic trioxide or cadmium in the human glioblastoma cell line U87 MG [13, 14] but without data on MT isoforms and subisoforms at the mRNA level. In mammals, MTs can be found in four major MT isoforms with conserved cysteine positions, but with small differences in protein sequence and charge characteristics. MT1 and MT2 are highly inducible and present in all cells, while MT3 occurs mainly in brain tissue with special properties of lower inducibility, fast metal binding/releasing capability and additional neuronal inhibitory activity [10]. MT4 is similar to MT1/2, but typical of squamous epithelial cells. Only the MT1 isoform could be present in different subisoforms in primates and ungulates. In humans, genes encoding MTs are clustered at a single locus on chromosome 16 (16q13); multiple genes for MT1 and single genes for MT2, MT3 and MT4. Among the 17 MT genes, at least 11 can be expressed in human cells; i.e. *MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1M MT1X, MT2A, MT3* and *MT4* [6, 15]. The most widely expressed isoforms (MT1 and MT2) in mammalians are most strongly induced by metal ions (Cd, Zn, Cu, Hg), and less by other stressors like reactive oxidative species (ROS), hypoxia, cytokines, steroid hormones (glucocorticoids, progesterone, vitamin D3), lipopolysaccharides, alkylating drugs and others [9, 16]. Regulation of MT synthesis has been covered in several reviews [17, 18]. Metal-induced transcriptional activation of MT1/MT2 genes is mostly conferred by metal responsive elements (MREs) and also by antioxidant responsive elements. Regarding MREs, it is considered that increased levels of free Zn ions in cytosol interact with MRE-binding metal transcription factor (MTF-1). MTF-1 with bound Zn(II) is then translocated into nucleus where it activates the MREs region and is responsible for basal or induced expression of MTs. According to recent experimental data, MTF-1 also interacts with arsenic through thyol and selenol ligands [19]. Transcriptional regulation of MT3 [10] and MT4 genes is less clear. In general, it is believed that isoform 3 is not easily induced, as in some studies it failed to respond to various regulating agents such as Zn, Cd or dexamethasone [20], although a few studies confirmed its metal (Hg^{2+} and MeHg) inducibility in brain tissue [21, 22]. In literature surveys these data are mostly overlooked.

Following our previous observation of metal-metallothionein interactions in ATO-[13] and cadmium-treated[14] human glioblastoma cell line U87 MG, in the present work we tried to follow the interplay of arsenic and MT at the mRNA level. Thus, the aim of the present study was to investigate the influence of As on gene expression of the main MT (sub)isoforms in the human glioblastoma cell line.

Materials and Methods

Cell Culture

The human glioblastoma cell line U87 MG (astrocytoma-glioblastoma, grade IV, 44-year-old female of Caucasian origin; American Type Culture Collection -ATCC, Manassas, Virginia, USA) was grown in monolayer culture in growth medium. Minimum essential medium (MEM) with Earl's Salts without L-glutamine was used, supplemented with 10 % foetal bovine serum (FBS), 4 mM L-glutamine, 1 % penicillin/streptomycin (all from PAA Laboratories GmbH, Pasching, Austria) and 1 % nonessential amino acids (Sigma-Aldrich, Munich, Germany).

Arsenic Trioxide—Treatment Media Preparation and Arsenic Determination

Arsenic trioxide (As_2O_3) of p.a. quality was purchased from Merck (Darmstadt, Germany). As_2O_3 was dissolved in 5 mL of 1.5 M NaOH and a stock solution of 10 mM As^{3+} (arsenite) was further prepared in Milli Q water. pH was adjusted to 7 with HCl. An appropriate amount of stock solution was diluted

in culture medium directly before each treatment. The concentration of total As and the absence of oxidised arsenate (As^{5+}) in the stock solution were confirmed by high performance liquid chromatography-hydride generation atomic fluorescence spectrometry [23]. Due to the high possibility of As^{3+}/As^{5+} conversion throughout the exposure procedure [24], both species were also determined in cell growth media before and after exposure. The percentage of As^{5+} was between 3 and 7 %, once 12 % and twice below the detection limit.

Cell Viability

Cell viability and metabolic activity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay [25] with minor modification. MTT is converted to insoluble formazan by mitochondrial enzymes of living cells and the concentration of formazan crystals is directly proportional to the number of living cells. U87 MG cells were seeded on 96-well microplates at a density of 8,000 cells/well. After 24 h of growth, the medium was replaced by fresh medium containing 0, 0.6, 1, 2, 4, 7 and 10 μM As (equivalent to 0, 0.3, 0.5, 1, 2, 3.5 and 5 μM As_2O_3) and incubated. After 45 h, MTT (Sigma, St. Luis, MO, USA) was added to a final concentration of 10 % and cells were incubated for another 3 h. Formazan crystals were dissolved by dimethyl sulfoxide and the optical density (OD) measured at 570 nm (reference filter, 690 nm) with a GENios spectrofluorimeter (Tecan, Salzburg, Austria). Five individual wells were prepared for each treatment concentration. The survival was calculated as the ratio of the mean OD value of the treated (t) versus untreated (c) cells and expressed in percentages as (OD_t/OD_c) ×100

As Exposure for MT Expression

U87 MG cells (approx. 1 million; cell passages from 41 to 73) were seeded in a 5-mL cell culture flask (T25) and incubated in a 5 % CO_2 atmosphere at 37 °C and 95 % relative humidity. After 24 h of growth, the medium was replaced with fresh medium containing a final concentration of 0, 0.6, 2, 6 or 7 μM As (equivalent to 0, 0.3, 1, 3 or 3.5 μM As_2O_3) and incubated for up to 48 h. Arsenic concentrations of 7, 2 and 0.6 μM were chosen according to As blood data of APL patients during As_2O_3 therapy [26, 27]. The detailed exposure protocol is given in Table 1. Each exposure was performed in two to three experimental replicates.

Determination of MT (sub)Isoforms

RNA Extraction and cDNA Synthesis

RNA was extracted with TRIzol® Reagent (GIBCO Products, Invitrogen, Grand Island NY, USA). The concentration and purity of isolated RNA was evaluated by a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilimington, DE, USA); as usual purity was estimated from the absorbance ratio 260 nm/280 nm (mean ratio was 1.94, range 1.74–2.02). RNA integrity was evaluated with an Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) by RIN (RNA integrity number; mean RIN, 9.8, range, 8.5–10); 1.8 μg of each RNA sample was reverse transcribed to cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) and stored at −70 °C until measurement of gene expression by qPCR. All procedures were performed according to the manufacturer's instructions.

Table 1 Exposure conditions and biochemical analyses

U87 MG	As in medium (μM)	Incubation hours	Procedure
Cell viability	0, 0.6, 1, 2, 4, 7 and 10[a]	24 and 48	MTT assay
MT expression	0 and 0.6	24 and 48	qPCR (SYBR, TaqMan)
			PvuII for MT3 PCR product
	0 and 2	24 and 48	qPCR (SYBR)
	0 and 6[a]	24[b]	qPCR (SYBR)
	0 and 7	24, 48 and 24[c]	qPCR (SYBR)

[a] Differences between nominal and real concentrations of As in starting medium were mostly less than 10 %; at the marked levels, the difference was greater (measured values were 8.5 and 5.2 μM instead of 10 and 6 μM)

[b] Twelve-hour As exposure followed by 12 h nonexposure

[c] Twenty-four-hour As exposure followed by 24-h nonexposure

Real-Time Quantitative Polymerase Chain Reaction

Quantitative Polymerase Chain Reaction (qPCR), used for gene expression measurements of different MT (sub)isoforms, was performed on a LightCycler 480 II (Roche Applied Science, Madison, WI, USA) using LightCycler® 480 SYBR Green I Master (Roche Applied Science, Indianapolis, USA) or TaqMan® expression assays (Applied Biosystems, Foster City, CA, USA). Measurements were performed in three to six replicates with a negative control included for each assay. Human glyceraldehyde 3-phosphate dehydrogenase gene (*hGAPDH)*, actine beta gene (*hAB*), transferrin receptor gene (*hTfR*) and/or 18S ribosomal gene (*h18S rRNA*) were used as internal controls (all from Sigma-Aldrich, Munich, Germany). The sequences of forward primers, reverse primers and assay ID for TaqMan probes used for detecting gene-specific mRNA are given in Table 2. Primers were used at final concentrations of 0.25–0.5 μM. All PCR amplifications were performed in 20 μL reactions at two sets (procedures A and B) of PCR conditions for SYBR Green I. Procedure A consisted

Table 2 PCR primers for CYBR Green chemistry and probes for TaqMan chemistry

Gene products	Primer accession	Sequences (5′-3′) and probes	Length (bp)
MT1A	NM_005946.2	F: 5′-CTCGAAATGGACCCCAACT-3′ R: 5′-ATATCTTCGAGCAGGGCTGTC-3′	219
MT1X	NM_005952.3	F: 5′-TCTCCTTGCCTCGAAATGG-3′ R: 5′-CACAGCTGTCCTGGCATCA-3′	217
MT1F	NM_005949.3	F: 5′-AGTCTCTCCTCGGCTTGC-3′ R: 5′-ACATCTGGGAGAAAGGTTGTC-3′	234
MT1E	NM_175617.3	F: 5′-GCCCGACCTCCGTCTATAAAT-3′ R: 5′-CACTCACTCTTCTTGCAGGA-3′	215
MT2A	NM_005953.3/	F: 5′-TGCAACCTGTCCCGACTCTA-3′ R: 5′-GGAAGTCGCGTTCTTTACATCT-3′	268
		Hs01591333_g1	111
MT3	NM_005954/	F: 5′-TTCTGGTGGCTCCTGCAC-3′ R: 5′-ACCTGGCACTATCTCCACG-3′	224
		Hs00359394_g1	87
GAPDH	NM_002046/	F: 5′-CTCTGATTTGGTCGTATTGGGC-3′ R: 5′-ACTCCACGACGTACTCAGC-3′	397
		Hs02758991_g1	93
AB	NM_001101	F: 5′-GCGAGAAGATGACCCAGATC-3′ R: 5′-GGATCTTCATGAGGTAGTCAGTC-3′	231
18S rRNA	NR_003286	F: 5′-CGTGCCTACCATGGTGACCA-3′ R: 5′-TTGCCCTCCAATGGATCCT-3′	214
Tfr	NM_003234	F: 5′-GCATTCTCTAACTTGTTTGGTGGAG-3′ R: 5′-GTCCCATAGCAGATACTTCCAC-3′	188

Primers (Sigma-Aldrich, Munich, Germany); TaqMan® expression assays (Applied Biosystems, Foster City, CA, USA)

of activation at 95 °C for 5 min, followed by 45 cycles of 10 s at 95 °C for denaturation, 15 s at 59 °C (*MT1E, MT3* and *GAPDH*), 61 °C (*MT1X, MT1F, 18S rRNA* and *ACTB*), 65 °C (*MT1A*) or 15 s at 68 °C (*MT2A*) for primer annealing and 20 s at 72 °C for elongation. Procedure B consisted of activation for 5 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 15 s at 61 °C (all primers), and 20 s at 72 °C. Reactions employing SYBR Green detection were subjected to further melting curve analysis. For some samples accuracy was verified by TaqMan® assays; conditions: 15 min at 95 °C for activation, followed by 45 cycles of 30 s at 95 °C for denaturation, 60 s at 60 °C (*MT2A, MT3* and *GAPDH*) for the combined annealing and elongation phase. Finally, gene expressions were determined as relative gene expressions expressed as the fold increase in mRNA levels calculated using the Pfaffl method [28]. Efficiency was 90 to 95 % for *18S rRNA* and 80–90 % for *Tfr*, *GAPDH*, *AB* and all MT genes. The results obtained were corrected by defined calculation restrictions, first for reaction reproducibility where the C_t level difference should be below 0.5 C_t [29] and second using the criterion $\Delta C_t \leq \pm 0.5$ (or $2^{-\Delta Ct}=0.7–1.4$) [30] as a delimiter of reference gene suitability. So although for normalisation two to four human reference genes were measured (*AB*, *GAPDH*, *TfR* and/or *18S rRNA*), finally *AB* was found to be the most suitable for As exposure experiments at 2 and 6–7 μM As, and *GAPDH* at 0.6 and 2 μM As.

Confirmation of MT3 mRNA Transcripts by Restriction Enzyme Digestion (PvuII)

The specificity of the *MT3* PCR product was checked by the PvuII restriction enzyme (Promega, Madison, WI, USA). *MT3* gene contains a PvuII restriction enzyme cutting site 5′…CAG|CTG…3′ (3′…GTC|GAC…′5). In this experiment two randomly chosen samples were tested (low dose exposure control samples–0 μM As, 48 h; Table 1). The *MT3* PCR products (224 bp) were incubated with PvuII according to the manufacturer's protocol. After incubation the MT3/PvuII reaction mixture was analysed using 1.5 % agarose gel electrophoresis and an UV camera (G: box (Syngene, MD, USA). The presence of *MT3* restriction fragments of 25, 56 and 143 bp confirmed the identity of the *MT3* PCR product.

Statistical Analysis

Results for MTT test were expressed in percentages as mean values with standard deviation and for gene expression as mean values of relative gene expression expressed as the n-fold increase in mRNA levels ± standard deviation (SD). Student's *t* test was used for evaluating statistically significant differences between (1) viability of As exposed and nonexposed samples, (2) gene expression of MT (sub)isoforms of exposed and nonexposed samples and (3) gene expression of

MT (sub)isoforms of continuously and intermittently exposed samples. A value less than 0.05, 0.01 or 0.001 was used to quantify statistical significance. Normal distribution was confirmed by Shapiro–Wilks normality test.

Results

Viability of U87 MG Cells After Exposure to As in the Form of As_2O_3

Figure 1 shows the 48 h effect of arsenic in form of As_2O_3 on the survival of U87 MG cells determined by MTT assay. The IC_{50} value was calculated to be about 5.5 μM As. At the lowest As levels (0.6 μM) we also noticed a tendency to increasing survival in comparison with the control sample, an effect which is often met and recognised by some authors as adaptive response to low doses of neurotoxins [31]. Observed phenomenon was also linked to gene expression changes as can been seen below in Fig. 1. After low-dose As exposure (0.6 μM, 48 h) the gene expression of all MT (sub) isoforms decrease. It could be speculated that such universal decrease could be related to cellular changes in zinc or/and copper homeostasis [32], as arsenic compounds can influence both.

Dose and Time Dependent Gene Expression of MT (sub) isoforms in U87 MG Cells After Exposure to As

In order to estimate the influence of arsenic on MTs, the expression of different (sub)isoforms was assessed by qPCR on the basis of dose- and time-dependent exposure in U87 MG cells (Table 1). The results are given 'relative to' the expression of reference genes, among which the most appropriate were *actine beta (AB)*, already known for its use in

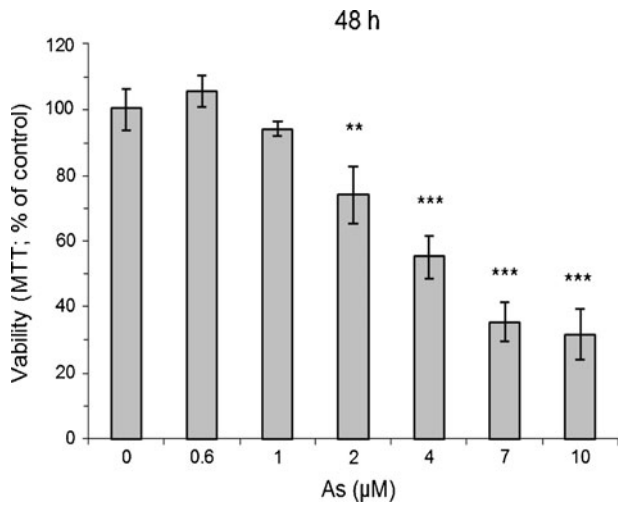

Fig. 1 Cell viability test (*MTT*; student's *t* test, *$p<0.05$; **$p<0.01$; ***$p<0.001$)

experiments with arsenic [33] or gli(om)a cells [34] and/or *GAPDH*. Due to the known influence of oxidative stress and/or metals on the GAPDH protein or its gene expression [35, 36], *GAPDH* was less reliable for high arsenic exposure experiments (As, >2 μM); the results could be underestimated. On the contrary *AB* failed in low level As experiments (0.6 μM), where the low C_t of *AB* (and 18S rRNA) can mask the results of MT genes with higher C_t. PCR amplifications were performed at two sets of PCR conditions for SYBR Green I (procedure A and/or B; given in Material and methods), which gave comparative results with a relatively lower variability in the case of procedure B.

Figure 2 shows the relative mRNA expression of six MT (sub)isoforms (*n*-fold of controls)—namely *MT1A, MT1X, MT1F, MT1E, MT2A* and *MT3*—after exposure to arsenic trioxide of 0.6, 2, 7 μM for 24 and 48 h. An unexpected decrease of expression was found for all six (sub)isoforms at the lowest and intermediate concentration levels (0.6 and 2 μM As) and for *MT1E* even at higher concentration (7 μM As), particularly at longer exposure. For *MT2A, MT1X, MT1F* a moderate increase, between 3- and 6-fold, was observed at 2 μM As after 48 h and much higher, between 10-and 14-fold at 7 μM As, regardless of the duration of exposure. One day exposure at 7 μM As also gave a 2-fold increase of *MT3*.

Differences between continuous and intermittent As exposures (6–7 uM) as described in Table 1 are given in Figs. 3 and 4.

Figure 3 shows the relative MT mRNA expression of the same (sub)isoforms after 24 h of continuous exposure in comparison with intermittent exposure where 12 h As exposure was followed by 12 h nonexposure. Figure 4 shows similar situation regarding 48 h continuous exposure in comparison with 24 h As exposure followed by 24 h nonexposure. Intermittent exposure was a rough simulation of the possible conditions during therapy, although the concentrations were rather high, 6–7 μM As. Results for continuous exposure are the same as given in Fig. 2. In comparison with continuous exposure, the intermittent situation gave lower positive responses for *MT2A*, *MTF* and *MTX* and convincingly higher positive responses for *MT1E*, *MT1A* and *MT3*.

Comparison of MT3 and MT2A mRNA Expression by Two Independent Methods

SYBR Green I was used as the main detection technique which was in some cases, particularly for *MT3* and *MT2A*, verified and confirmed by the TaqMan® expression assay (Fig. 5). TaqMan is less sensitive but more specific, as during the reaction specific amplification products are detected by specific oligonucleotides probes. The results obtained (both shown and not shown) gave good correlation

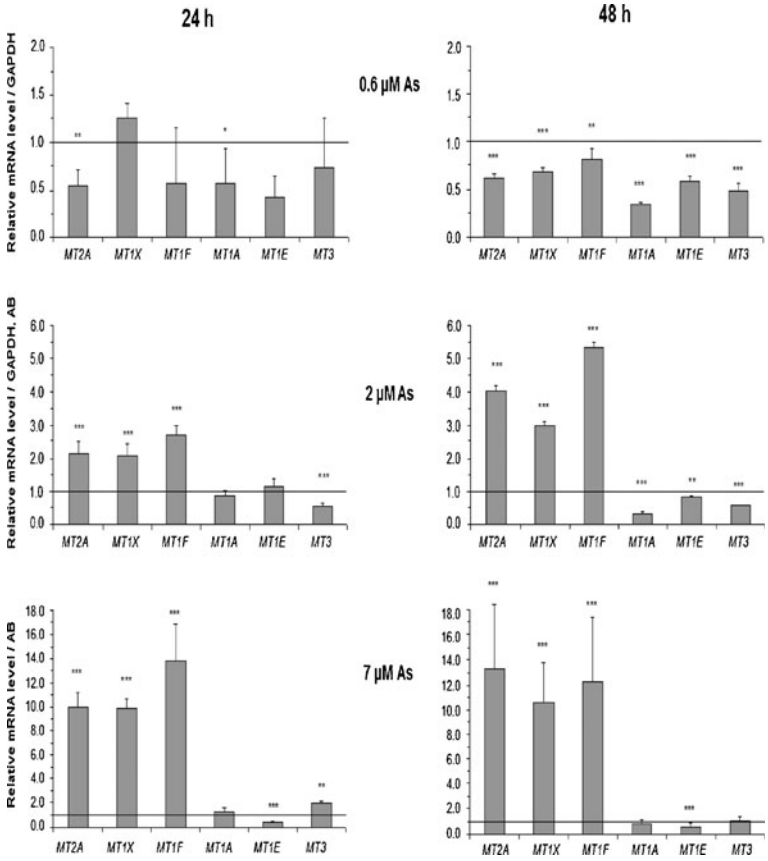

Fig. 2 Dose- and time-dependent relative MT mRNA expression in U87 MG cells treated by As_2O_3 (0.6, 2 and 7 μM As; 24 and 48 h; student's *t* test, *$p<0.05$; **$p<0.01$; ***$p<0.001$). The 1-fold increase is indicated by a *line*

between the methods. The decrease in *MT3* and *MT2A* mRNA was observed using both methods as shown in Fig. 5.

Verification of MT3 PCR Products by PvuII

MT3 is a special MT isoform and its presence in U87 MG cells has not yet been shown at gene level; therefore an additional method was utilised in order to confirm the presence of the *MT3* PCR product. The *MT3* gene possesses nucleotide sequences that can be cleaved by the restriction enzyme PvuII [37]. In our case, the *MT3* PCR product (Table 2) comprises 224 bp, with the two PvuII cutting sites resulting in cleavage of the 224 bp *MT3* PCR product into three fragments of 143, 56, 25 bp, if the product is derived from authentic *MT3* mRNA. Figure 6 shows PvuII digestion of the putative *MT3* PCR product (two randomly chosen samples; two experimental

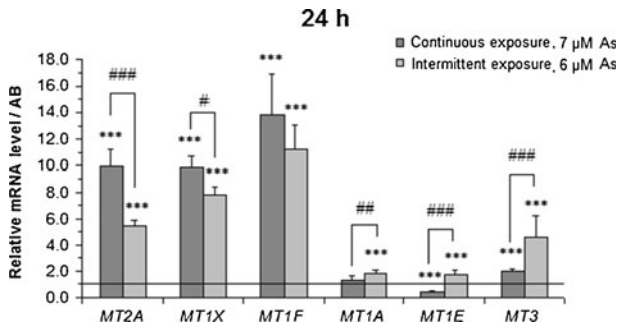

Fig. 3 Relative MT mRNA expression in U87 MG cells after continuous and intermittent As exposure during 24-h incubation (6–7 μM As; exact conditions are given in Table 1). *$p<0.05$; ** $p<0.01$; *** $p<0.001$ indicate significantly different responses between treated and untreated cells; #$p<0.05$; ##$p<0.01$; ###$p<0.001$ significantly different responses between continuous and intermittent exposure (student's *t* test). The 1-fold increase is indicated by a *line*

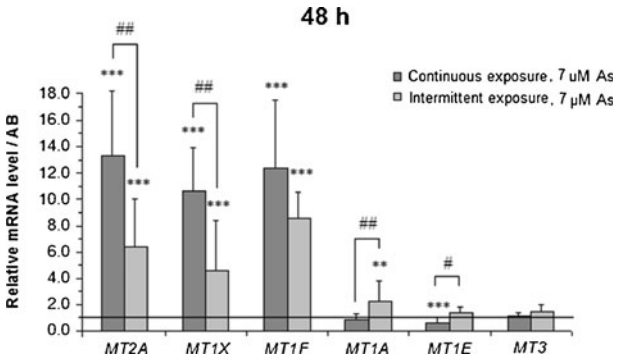

Fig. 4 Relative MT mRNA expression in U87 MG cells after continuous and intermittent As exposure during 48-h incubation (7 μM As; exact conditions are given in Table 1). *$p<0.05$; **$p<0.01$; ***$p<0.001$ indicates significantly different response between treated and untreated cells; #$p<0.05$; ##$p<0.01$; ###$p<0.001$ significantly different response between continuous and intermittent exposure (student's *t* test). The 1-fold increase is indicated by a *line*

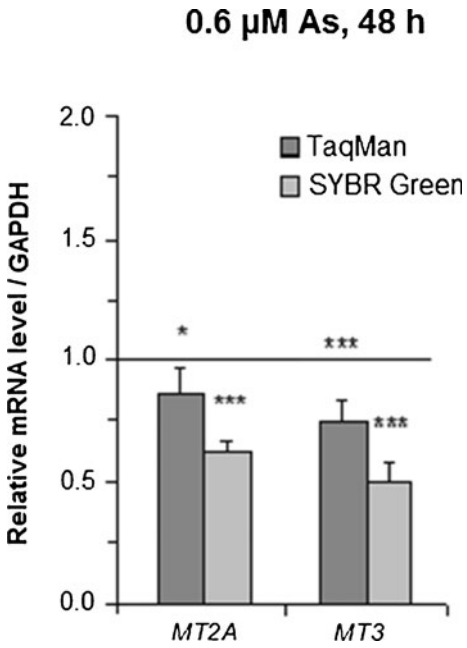

Fig. 5 Relative MT3 and MT2A mRNA expression in U87 MG cells obtained by the SYBR and TaqMan techniques (0 and 0.6 µM As, 48 h; student's *t* test, *$p<0.05$; **$p<0.01$; ***$p<0.001$). The 1-fold increase is indicated by a *line*

control of low dose experiments, 48 h incubation—Table 1) that resulted in cleavage of the original PCR band into products of approximately 143 and 56 pb while 25 bp is a very short fragment and therefore difficult to visualise on the agarose gel.

Discussion

The present study was designed to define the expression of mRNAs of 6 MT (sub)isoforms in human glioblastoma cells according to their arsenic trioxide exposure. Special interest was paid to the presence of isoform MT3 and its induction, because of its reputation as a non-easily inducible MT isoform [10, 20] and its known presence in rather low amounts in glia neoplastic cells [34]. Depending of the stage of disease its downregulation is also associated with several

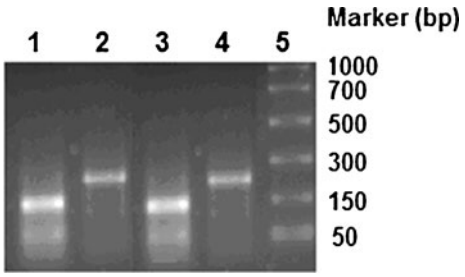

Fig. 6 PvuII cleavage of MT3 PCR product of nonexposed U87 MG cells (*lines 1 and 3* digested product 143 and 56 bp, *lines 2 and 4* undigested MT3 product 224 bp, and *line 5* marker)

neurodegenerative diseases including Alzheimer's disease [10, 38].

At the given conditions we were able to identify mRNAs of six isoforms—*MT2A, MT1X, MT1A, MT1F, MT1E* and *MT3*—out of 11 believed to have tissue and cell specific expression [6, 15]. Effort towards possible identification of the remaining isoforms was reserved for future work. Regarding the raw C_t results on control cells, it was evident that the most expressed isoform in U87 MG cells was MT2A. According to the literature the human MT2A but not MT1 is widely expressed in most cell types even in the absence of inducers. In humans MT2A is the predominant isoform, accounting for almost 50 % of total MT in neoplastic non-brain cells and MT3 can represent about 30 % of total MTs in brain tissue [7]. In nonexposed U87 MG cells the observed isoforms were expressed in the following order: *MT2A>> MT1X> MT1F, MT1A>> MT1E* and *MT3*. In U87 cells *MT3* gene expression level was lower than in normal human astrocytes (our unpublished data). Low *MT3* gene expression is in accordance with the observation of Amoureux et al. [34] for human glioblastoma cells U373 MG, while Dantes Barbosa et al. [39] and Peyre et al. [40] found similar situations for some other malignat glioma cell lines and in clinical samples of patients with relapsed ependymomas.

After exposure to higher As levels (2 and 6–7 µM), the gene expression of *MT1X, MT1F* and *MT2A* increased, while that of *MT1A, MT1E* and *MT3* mostly decreased or stayed unchanged. An exception was 1.9-fold increase of *MT3* at 24-h exposure to 7 µM As. Intermittent exposure where exposure (6–7 µM As) was combined with a recovery

Table 3 Gene expression changes of MT isoforms in U87 MG cells after As_2O_3 exposure (estimations of increase or decrease are based on average values of two to three experimental parallels)

As (µM)	Time (h)	mRNA change					
		MT2A	MT1X	MT1F	MT1A	MT1E	MT3
0.6	24	↓	o	↓	↓	↓↓	↓
	48	↓	↓	↓	↓↓	↓	↓↓
2	24	↑	↑	↑	o	o	↓
	48	↑↑	↑↑	↑↑	↓↓	↓	↓
7	24	↑↑	↑↑	↑↑	o	↓↓	↑
	48	↑↑	↑↑	↑↑	↓	↓	o
7	48[a]	↑↑	↑↑	↑↑	↑	↑	↑
6	24[b]	↑↑	↑↑	↑↑	↑	↑	↑↑

"o" no relative gene expression change, *up arrow* increase between >1- and 3-fold, *double up arrows* increase between >3- and 13-fold, *down arrow* decrease between <1- and 0.5-fold, *double down arrows* decrease of <0.5-fold

[a] Twenty-four-hour As followed by 48 h without As

[b] Twelve-hour As followed by 12 h without As

period (12-h As exposure followed by 12-h nonexposure or 24 h As exposure and 24 nonexposure)—again gave a substantial increase of *MT1X, MT1F* and *MT2A* but also—although lower—an increase of *MT1A, MT1E, MT3*. The results are summarised in Table 3.

These results show the different responses of different MT isoforms to As exposure. Nevertheless, similar and high positive increases were common to *MT1X, MT1F* and *MT2A*. The positive effect of As on gene expression of MT isoforms was shown previously by Miura and Koizumi [41] suggesting their involvement in metal-toxicity defence. They studied the responses of MT genes toward zinc, cadmium and arsenic in HeLa cells (cervical cancer). The most expressed isoform in nonexposed HeLa cells was *MT2A* followed by *MT1X* and *MT1F*. The selected metals preferentially induced *MT1X* and *MT2A*, suggesting that these isoforms may be the most important in protecting cells from cytotoxicity. In comparison, we used lower As concentrations and different malignant cells and observed strong response of the same two genes (*MT1X* and *MT12A*) and one additional gene (*MT1F*). Certainly high levels of MTs could protect cells against arsenic trioxide toxicity as MTs can bind arsenic [42] and scavenge ROS. The MT binding of arsenic was also confirmed in some samples of the present study: in cells treated by arsenic (6 μM) for 2 days, about 40 % of water soluble arsenic was found in the metallothionein protein fraction (our unpublished data).

On the other hand, the low basal levels of MT3 isoform and its convincing induction after intermittent exposure (imitating the clinical situation after *iv* infusion, Figs. 3 and 4) can be important in other processes, if not occupied by arsenic. According to recent data, MT3 isoform is needed for proper lysosomal function in astrocytes. In MT3-null astrocytes the activity of several lysosomal enzymes are significantly reduced and oxidative stress (H_2O_2) induced cell death was completely attenuated [43]. In the absence of MT3, the changes in lysosome-associated membrane protein −1 and −2 were observed together with reductions in certain lysosomal enzymes that result in decreased autophagic flux' what may result in less cell death [43]. In normal situation, lysosomal membrane permeabilisation may contribute to cell death by releasing lysosomal enzymes into cytosol, activating caspases and other toxic insults. Concerning these data it is interesting that in some tumour cell lines with downregulated MT3, its upregulation appears to be associated with reduced cell proliferation and induction of cell death by autophagy (type II cell death). Dantas Barbosa et al. [39] reported that intense upregulation of MT3 by histone deacetylase inhibitor (varinostat) triggered cell death by autophagy in some glioma and medulloblastoma cells. Since there are few studies where As_2O_3 has also been suggested to trigger activation of autophagy alone or in combination with apoptosis in glioma cells [44, 45, 46], the involvement of MT3 induction cannot be excluded.

In conclusion, our results clearly demonstrate the influence of As on the gene expression of MT isoforms which may be one of the possible mechanisms of brain tumour cell resistance to therapeutic As cytotoxicity. Despite persistent assumptions of MT3 noninducibility by nonessential metals, an evident *MT3* increase was observed during high As exposure.

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