

Zinc chelation: a metallothionein 2A's mechanism of action involved in osteosarcoma cell death and chemotherapy resistance

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Osteosarcoma is the most common primary tumor of bone occurring in children and adolescents. The histological response to chemotherapy represents a key clinical factor related to survival. We previously showed that statins exhibit antitumor effects *in vitro*, inducing apoptotic cell death, reducing cell migration and invasion capacities and strengthening cytotoxic effects in combination with standard drugs. Comparative transcriptomic analysis between control and statin-treated cells revealed strong expression of several genes, including metallothionein (MT) 2A. MT2A overexpression by lentiviral transduction reduced bioavailable zinc levels, an effect associated with reduced osteosarcoma cell viability and enhanced cell differentiation. In contrast, MT2A silencing did not modify cell viability but strongly inhibited expression of osteoblastic markers and differentiation process. MT2A overexpression induced chemoresistance to cytotoxic drugs through direct chelation of platinum-containing drugs and indirect action on p53 zinc-dependent activity. In contrast, abrogation of MT2A enhanced cytotoxic action of chemotherapeutic drugs on osteosarcoma cells. Finally, clinical samples derived from chemo-naïve biopsies revealed that tumor cells expressing low MT2A levels correspond to good prognostic (good responder patients with longer survival rate), whereas high MT2A levels were associated with adverse prognosis (poor responder patients). Taken together, these data show that MT2A contributes to chemotherapy resistance in osteosarcoma, an effect partially mediated by zinc chelation. The data also suggest that MT2A may be a potential new prognostic marker for osteosarcoma sensitivity to chemotherapy.

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Osteosarcoma is the most common primary tumor of bone. The rapid development of metastatic lesions and resistance to chemotherapy remain the two major challenges responsible for the failure of treatments and poor survival rates. The standard treatment for osteosarcoma includes neoadjuvant chemotherapy, which includes a combination of cisplatin, doxorubicin and vincristine, followed by surgical resection and postoperative chemotherapy. The clinical factors influencing survival include the presence of metastasis at diagnosis and the histological response to preoperative chemotherapy. Thus, the search for novel combinatorial strategies increasing the efficacy of standard treatments represents an area of paramount clinical impact.

We previously showed that the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors statins exhibit anti-tumor effects on osteosarcoma cells.^{1–3} Our transcriptome and molecular analyses revealed that statins modulate the expression of several genes. One of them, *Cyr61* conferred enhanced metastatic potential to human and murine models.⁴ Another statin-regulated gene identified in osteosarcoma was metallothionein (MT) 2A that belongs to MTs family.

These low-molecular weight proteins (3.5–14 kDa) characterized by a very high percentage of cysteine residues (up to 30%)⁵ exhibit a high affinity for heavy metal ions and can modulate concentrations of the free physiological essential trace elements zinc and copper (reviewed in Ruttkay-Nedecký *et al.*⁶). In humans, 18 isoforms have been identified^{7,8} and classified in four classes that are referred as MT-I to MT-IV. The classes MT-I/II are ubiquitously expressed, whereas MT-III and IV are expressed in a tissue-restricted manner.^{9,10}

MTs present high affinity for biological essential intracellular metals such as zinc or copper and participate in the homeostasis of these ions.^{11–13} They can also chelate divalent heavy metals such as cadmium, lead, mercury or platinum via the thiol groups of cysteine residues and thus help to cell detoxification and protection.¹⁴ MTs overexpression has been linked to chemoresistance in carcinoma cells,¹⁵ non-small cell lung cancer cell lines,¹⁶ tongue squamous cell carcinoma cell lines,¹⁷ gastric tumor cell lines¹⁸ or ovarian carcinoma cell lines.¹⁹ In bone tumors, although MT levels were found to be increased in sera from patients with osteosarcoma compared with healthy patients²⁰ and in tumor biopsies compared with

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Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; FCS, fetal calf serum; MT, metallothionein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; TPEN, N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylene-diamine; ZIP, Zrt- and Irt-like protein; ZnT, zinc transporter

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non-malignant bone biopsies,²¹ its use as a potential marker of tumor differentiation or cell proliferation, or as a predictor of poor prognosis, remains unclear.

In this study, we therefore evaluated the role of MT isoform 2A in osteosarcoma cell behavior, investigated the cell response to cytotoxic agents according to various levels of MT2A and evaluated its prognostic value for patient outcome.

Results

Atorvastatin increases the expression level of MT2A in osteosarcoma cells. We previously showed that atorvastatin modified the expression level of several genes in human osteosarcoma cells (SaOS2) as revealed by comparative transcriptomic analysis.⁴ The expression of MT2A was upregulated at early time point (6 h) and sustained up to 24 h (Table 1). The expression of the other isoforms of MT was not significantly modified by statin treatment at any time points. To confirm these results, RT-qPCR was performed on SaOS2 and U2OS incubated with or without 10 μ M atorvastatin for 24 h (Figure 1a). Statin supplementation induced a twofold increase in MT2A mRNA expression level compared with untreated cells. Atorvastatin supplementation also induced an increase in MT2A expression at the protein level (Figure 1b). We then evaluated the modulation of intracellular zinc concentrations in SaOS2 and U2OS cells incubated with statin. We observed a dose-dependent decrease in bioavailable zinc under atorvastatin incubation (Figure 1c).

Altogether, these results indicate that atorvastatin rapidly upregulates MT2A expression in osteosarcoma cells and reduces intracellular zinc level.

MT2A overexpression modulates osteosarcoma cell viability. We generated new cell lines by transduction with an integrative lentiviral vector encoding the complete coding sequence of the MT2A (LV-MT2A). As expected, MT2A mRNA and protein levels were significantly higher in transduced cells as compared with parental cells (Figures 2a and b).

Cell viability, assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test, was slightly reduced in MT2A-overexpressing cells compared with parental cells at all tested time points (– 15 to – 20%; Figure 2c). We then evaluated the percentage of viable, apoptotic and necrotic cells by cell staining with ethidium bromide and acridine orange. The number of viable cells was slightly decreased in MT2A-overexpressing cells, in both SaOS2 and U2OS cell lines (Figure 2d). We found no difference in necrotic cell number, but we observed an increase in the apoptotic cell number in MT2A-overexpressing cells as compared with parental cells (Figure 2d). Cell proliferation rate, estimated by a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay, was decreased in MT2A-overexpressing cells as compared with parental cells (Figure 2e). Estimation of DNA content by flow cytometry using propidium iodide staining revealed a significant increase in sub-G0/G1 cell populations in MT2A-overexpressing cells as compared with parental cells (Figure 2f).

Among the different signaling pathways, pERK1/2, pJNK and pAKT levels were decreased in MT2A-overexpressing cells compared with parental cells, whereas pP38 levels remained unchanged (Supplementary data 1A). A gene reporter assay confirmed that MT2A overexpression reduced transcriptional activity through AP1 complex (Supplementary data 1B). Finally, the pro-apoptotic ratio bax/bcl-2 was increased in MT2A-overexpressing cells compared with parental cells (Figure 2g).

Overall, these results suggest that the overexpression of MT2A in osteosarcoma cells leads to a reduction in cell proliferation rate and an induction of apoptosis, resulting in reduced cell viability.

MT2A expression level influences osteoblastic differentiation in osteosarcoma cells. As proliferation and differentiation processes are usually interdependent, we evaluated the osteogenic potential of osteosarcoma cells modified for MT2A. We found that MT2A overexpression induced an increase in mRNA levels of osteoblastic markers such as runx2, osterix, type I collagen and alkaline phosphatase in both SaOS2 and U2OS cell lines (Supplementary data 2A). Alkaline phosphatase enzymatic activity was also upregulated in MT2A-overexpressing cells compared with parental cells, as evaluated by colorimetric assay or cytochemical staining (Supplementary data 2B and C). Matrix mineralization was increased more than twofold in MT2A-overexpressing cells compared with parental cells in both cell lines despite their different basal differentiation stage (Supplementary data 2D and E).

These data indicate that MT2A level of expression influences osteosarcoma cell differentiation toward the osteogenic lineage.

MT2A overexpression modulates osteosarcoma cell viability through zinc chelation. We then wanted to evaluate the part of zinc concentration on osteosarcoma cell viability. SaOS2 and U2OS parental cells were incubated for 48 h in the presence of increasing concentrations of the intracellular chelator of zinc TPEN (N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylene-diamine). As expected, TPEN dose-dependently decreased SaOS2 and U2OS cell viability, assessed by the MTT test (Figure 3a). These results indicate that the levels of available intracellular zinc are required for osteosarcoma cell viability.

The comparative transcriptome analyses revealed no significant modulation of mRNA expression level of zinc transporters, either zinc transporter (ZnT)/SLC30 or Zrt- and Irt-like protein (ZIP)/SLC39 families (Table 1). These data reinforce the specific role of the isoform MT2A alone.

To estimate the relative concentration of free intracellular zinc in parental and MT2A-overexpressing cells, we used a zinc-specific fluorophore (zincquin). Overexpressing MT2A resulted in a reduction of free intracellular zinc concentration (Figure 3b). Medium supplementation with ZnCl₂ led to a dose-dependent increase in intracellular zinc in both parental and MT2A-overexpressing cells, but the difference observed between MT2A-overexpressing cells and parental cells was preserved regardless of extracellular zinc concentration (Figure 3b).

Table 1 Transcripts expression level in human (SaOS2) osteosarcoma cells

	Fold change after 6 h	Fold change after 15 h	Fold change after 24 h
<i>Metallothioneins family</i>			
MT1A	0.97 ($P > 0.10$)	0.96 ($P > 0.10$)	1.10 ($P > 0.10$)
MT1B	0.97 ($P > 0.10$)	0.99 ($P > 0.10$)	1.48 ($P > 0.10$)
MT1E	0.97 ($P > 0.10$)	1.04 ($P > 0.10$)	0.89 ($P > 0.10$)
MT1F	0.97 ($P > 0.10$)	1.00 ($P > 0.10$)	1.14 ($P > 0.10$)
MT1G	0.97 ($P > 0.10$)	1.28 ($P > 0.10$)	1.42 ($P > 0.10$)
MT1J	0.97 ($P > 0.10$)	0.99 ($P > 0.10$)	1.04 ($P > 0.10$)
MT1K	0.97 ($P > 0.10$)	1.00 ($P > 0.10$)	1.05 ($P > 0.10$)
MT1L	0.97 ($P > 0.10$)	1.00 ($P > 0.10$)	1.07 ($P > 0.10$)
MT2A	3.25 ($P = 0.03$)**	2.90 ($P = 0.05$)**	2.85 ($P = 0.02$)**
MT4	0.97 ($P > 0.10$)	0.92 ($P > 0.10$)	0.94 ($P > 0.10$)
<i>ZnT family</i>			
SLC30A1	1.07 ($P > 0.10$)	1.07 ($P > 0.10$)	1.02 ($P > 0.10$)
SLC30A2	1.02 ($P > 0.10$)	1.03 ($P > 0.10$)	1.03 ($P > 0.10$)
SLC30A3	0.93 ($P > 0.10$)	0.98 ($P > 0.10$)	0.93 ($P > 0.10$)
SLC30A4	0.99 ($P > 0.10$)	1.08 ($P > 0.10$)	1.01 ($P > 0.10$)
SLC30A5	0.95 ($P > 0.10$)	0.98 ($P > 0.10$)	0.97 ($P > 0.10$)
SLC30A6	0.97 ($P > 0.10$)	1.20 ($P > 0.10$)	1.05 ($P > 0.10$)
SLC30A7	0.97 ($P > 0.10$)	1.01 ($P > 0.10$)	0.91 ($P > 0.10$)
SLC30A8	0.93 ($P > 0.10$)	1.07 ($P > 0.10$)	0.94 ($P > 0.10$)
SLC30A9	1.07 ($P > 0.10$)	0.96 ($P > 0.10$)	1.05 ($P > 0.10$)
SLC30A10	1.11 ($P > 0.10$)	0.92 ($P > 0.10$)	1.02 ($P > 0.10$)
<i>ZIP family</i>			
SLC39A1	0.97 ($P > 0.10$)	1.07 ($P > 0.10$)	0.77 ($P > 0.50$)
SLC39A2	1.01 ($P > 0.10$)	1.07 ($P > 0.10$)	1.06 ($P > 0.10$)
SLC39A3	1.00 ($P > 0.10$)	0.89 ($P > 0.10$)	0.93 ($P > 0.10$)
SLC39A4	0.97 ($P > 0.10$)	1.08 ($P > 0.10$)	0.97 ($P > 0.10$)
SLC39A5	1.01 ($P > 0.10$)	1.01 ($P > 0.10$)	1.02 ($P > 0.10$)
SLC39A6	1.23 ($P > 0.10$)	1.05 ($P > 0.10$)	0.86 ($P > 0.10$)
SLC39A7	0.81 ($P > 0.10$)	0.93 ($P > 0.10$)	1.24 ($P > 0.10$)
SLC39A8	1.01 ($P > 0.10$)	0.99 ($P > 0.10$)	0.88 ($P > 0.10$)
SLC39A9	1.03 ($P > 0.10$)	1.30 ($P > 0.10$)	0.92 ($P > 0.10$)
SLC39A10	0.93 ($P > 0.10$)	0.90 ($P > 0.10$)	0.98 ($P > 0.10$)
SLC39A11	0.97 ($P > 0.10$)	1.07 ($P > 0.10$)	1.18 ($P > 0.10$)
SLC39A12	1.05 ($P > 0.10$)	0.94 ($P > 0.10$)	1.00 ($P > 0.10$)
SLC39A14	0.98 ($P > 0.10$)	1.09 ($P > 0.10$)	1.16 ($P > 0.10$)

ZIP, Zrt- and Irt-like protein; ZnT, zinc transporter.

Cells were incubated for 6, 15 or 24 h with/without 10 μ M atorvastatin. The mRNA expression level of the main families of molecules involved in zinc homeostasis was evaluated by quantitative RT-PCR. Normalized intensities of each transcript were determined for two independent experiments. Cutoffs for selection are equal to 1 for the absolute value of log2 (ratio), and to 0.05 for the P -value. Fold change of the ratio of atorvastatin/control is indicated. ** indicates P -value < 0.05 versus untreated cells.

The inhibitory effect of MT2A overexpression on cell replication was dose-dependently compensated by extracellular zinc addition (Figure 3c). A supplementation with $> 5 \mu$ M $ZnCl_2$ fully offset the decrease in intracellular zinc content induced by MT2A overexpression in both SaOS2 and U2OS cells. Conversely, a culture medium containing FCS pre-treated with a chelating resin (chelex) led to a decrease in parental cell proliferation rate compared with normal medium (Figure 3d). The supplementation of this chelex medium with increasing concentrations of $ZnCl_2$ gradually restored a cell proliferation rate comparable to normal medium.

Taken together, these results indicate that intracellular zinc has a major role in osteosarcoma cell viability and proliferation. These results also indicate that an overexpression of MT2A reduces intracellular zinc availability, contributing to reduced growth kinetics of these cells.

MT2A overexpression modulates cell response to cytotoxic agents. As MTs are involved in cell detoxification from heavy metals, we compared response with platinum-containing cytotoxic agents in MT2A-overexpressing cells to

parental cells. As expected, cisplatin and transplatin (less effective isomer) reduced cell viability. The overexpression of MT2A reduced the inhibitory effect of both compounds in both cell lines (Figures 4a and b). In contrast, MT2A did not affect SaOS2z cell response to doxorubicin or vincristine (Figure 4c). Surprisingly, overexpression of MT2A reduced the inhibitory effect of these compounds in U2OS cells (Figure 4d).

These results suggest that MT2A overexpression reduces the cellular response to anti-proliferative agents depending on the nature of the drug, such as the presence or absence of a metal ion, and depending on the cell line genotype. Indeed, the U2OS cell line encodes wild-type p53, whereas the p53 gene is entirely deleted in the SaOS2 cell line. Such mutations can induce resistance to p53-mediated apoptosis, specially in response to cytotoxic drugs.²² We thus further evaluated p53 implication in MT2A mechanism of action, using the U2OS cell line only.

MT2A modulates p53 conformation-dependent activity. The presence of a zinc ion is required for DNA binding and

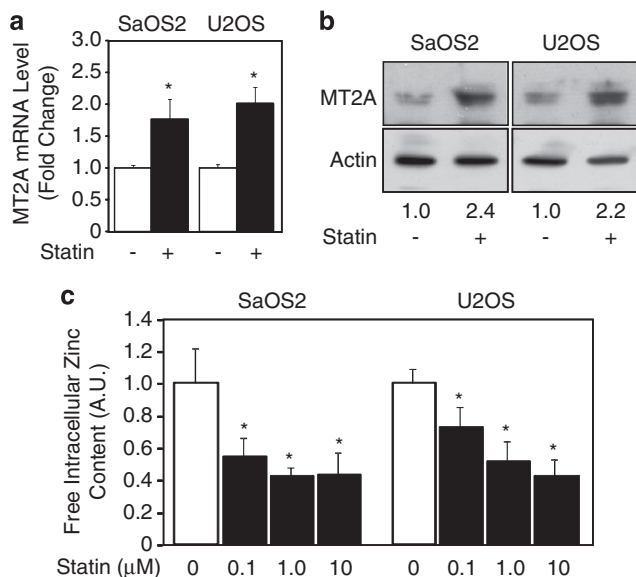


Figure 1 Atorvastatin increases MT2A expression in osteosarcoma cells. SaOS2 and U2OS cells were incubated in the presence or absence of atorvastatin (10 μ M) for 24 h. (a) MT2A mRNA levels were evaluated by RT-qPCR. (b) MT2A and housekeeping gene β -actin protein levels were assessed by western blot analysis. (c) Free intracellular zinc content was evaluated by fluorimetry. Results are expressed as mean \pm S.D. * $P < 0.05$ versus untreated cells

maintaining of an active conformation of p53 protein. We observed a lower proportion of the active form of p53 in MT2A-overexpressing U2OS cells compared with parental cells, whereas the total p53 protein level was not affected (Figure 5a). Overexpression of MT2A also reduced p21 protein level in osteosarcoma cells as compared with parental cells (Figure 5b). Overexpression of MT2A did not affect TAp73 and p63 protein levels (Figures 5c and d). Supplementation of parental cells with doxorubicin led to an increase in MT2A protein level (Figure 5e) and to a reduced proportion of active p53 (Figure 5f).

These results suggest that MT2A overexpression modifies p53 protein conformation, reducing its antitumor activity and then reducing cytotoxic action of doxorubicin.

MT2A silencing amplifies osteosarcoma cell response to cytotoxic agents. Next, we evaluated the effect of MT2A silencing on U2OS cells. Cell transduction with lentiviral vector led to reduced MT2A mRNA and protein levels (Figures 6a and b). In contrast to MT2A overexpression, we did not detect changes in cell viability, apoptosis or proliferation rate between MT2A-silenced cells and control cells (data not shown). However, MT2A silencing increased free intracellular zinc concentration compared with U2OS control cells (Figure 6c), confirming that MT2A modulates the intracellular levels of bioavailable zinc.

We also found that MT2A silencing reduced mRNA levels of the osteoblastic markers runx2, osterix, type I collagen and alkaline phosphatase in both SaOS2 and U2OS cell lines (Supplementary data 3A). Alkaline phosphatase enzymatic activity and matrix mineralization were decreased in MT2A-silenced cells compared with control cells (Supplementary

data 3B–E, respectively), confirming that MT2A influences osteoblastic cell differentiation.

We then evaluated the cell response to chemotherapy drugs containing platinum or not. MT2A silencing amplifies the inhibitory effect of all tested cytotoxic compounds on U2OS cell viability (Figure 6d). Cytotoxic activity of doxorubicin correlates with the amount of drug incorporated into DNA. MT2A silencing results in an increased level of doxorubicin combined with DNA, whereas MT2A overexpression markedly reduced the amount of DNA-incorporated doxorubicin (Figure 6e). An indirect evaluation was also performed by the measure of Hoechst 33342 amount, a nuclear stain used as a post treatment to doxorubicin. It confirmed that MT2A level influences doxorubicin amount incorporated into DNA (Figure 6f).

Altogether, these results suggest that MT2A silencing in osteosarcoma cells does not significantly affect cell viability but can enhance sensitivity to drugs such as doxorubicin by facilitating its interaction with DNA.

Prognostic value of MT2A mRNA expression in osteosarcoma. Finally, we evaluated MT2A mRNA level in biopsies collected from osteosarcoma patients before any chemotherapy treatment. Post-treatment responses were scored and were linked to RT-qPCR results. We observed that MT2A mRNA level was significantly lower in good responder group than in poor/non-responder group ($P < 0.0001$; statistical test power = 0.9991; Figure 7a). Estimated mean disease-free survival rates were calculated using Kaplan–Meier's method (Figure 7b). For the low MT2A group, the mean survival time was > 120 months and for the high MT2A responder group, the mean survival time was 37 months ($P < 0.05$).

These data suggest that MT2A mRNA level evaluated at diagnostic could be of prognostic value for chemotherapy response in osteosarcoma, discriminating between good and poor responders.

Discussion

MTs are known to be involved in detoxification process, masking or neutralizing drugs and promoting cell survival.²³ Our present data show that the MT2A protein level influences free intracellular zinc ion content in osteosarcoma cells. Importantly, we provide evidence that metal chelation by MT2A controls cell proliferation, survival and differentiation. More importantly, MT2A expression levels also modulate chemoresistance to some cytotoxic drugs. Our data indicate that the mechanism of action is dependent not only on the presence or absence of a metal ion in the cytotoxic compound but also on p53 status.

The implication of MT in cancer is controversial. An increase in MT level was reported in serum from patients with lymphoid leukemia, lung carcinoma, thyroid carcinoma, head and neck cancer or melanoma,²⁴ whereas a decrease in MT level was reported in renal cancer, prostate or liver adenocarcinoma.^{25–27} These discrepancies may result from the attributed or the sought role of MT in carcinogenesis, promoting tumor growth, tumor incidence or chemotherapy resistance. Given the high-sequence homology between

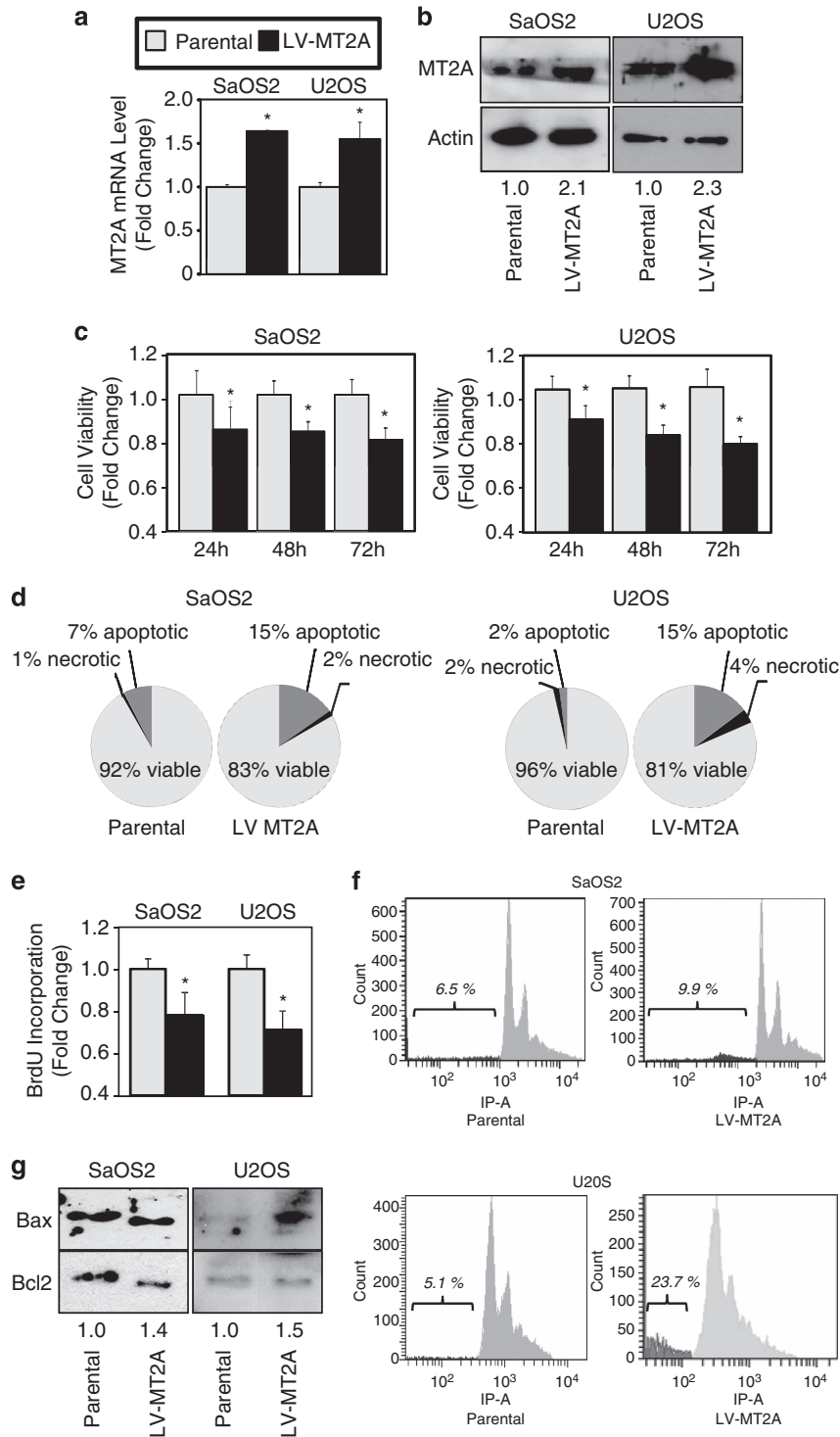


Figure 2 MT2A overexpression reduced osteosarcoma cell viability. SaOS2 and U2OS cell lines were transduced with an integrative lentiviral vector encoding the complete coding sequence of MT2A (LV-MT2A). (a) MT2A mRNA levels were evaluated by RT-qPCR. (b) MT2A protein levels were evaluated by western blot analysis. (c) Cell viability was evaluated using the MTT test. (d) Relative percentage of viable, necrotic and apoptotic cells were evaluated by acridine orange/ethidium bromide staining. (e) Cell replication was evaluated using a BrdU incorporation assay. (f) Apoptotic cell proportion (sub-G0/G1) was evaluated by flow cytometry. (g) Bax and Bcl-2 protein levels were evaluated by western blot analysis. Results are expressed as mean \pm S.D. and arbitrary units relative to parental cells. * $P < 0.05$ versus untreated cells

different MT isoforms, none of previous studies have discriminated and estimated the expression levels among different MT family members or at least among MT I/II. In our

model of bone tumor osteosarcoma, among all MTs, only the 2A isoform has its expression level significantly increased by atorvastatin or simvastatin supplementation in osteosarcoma

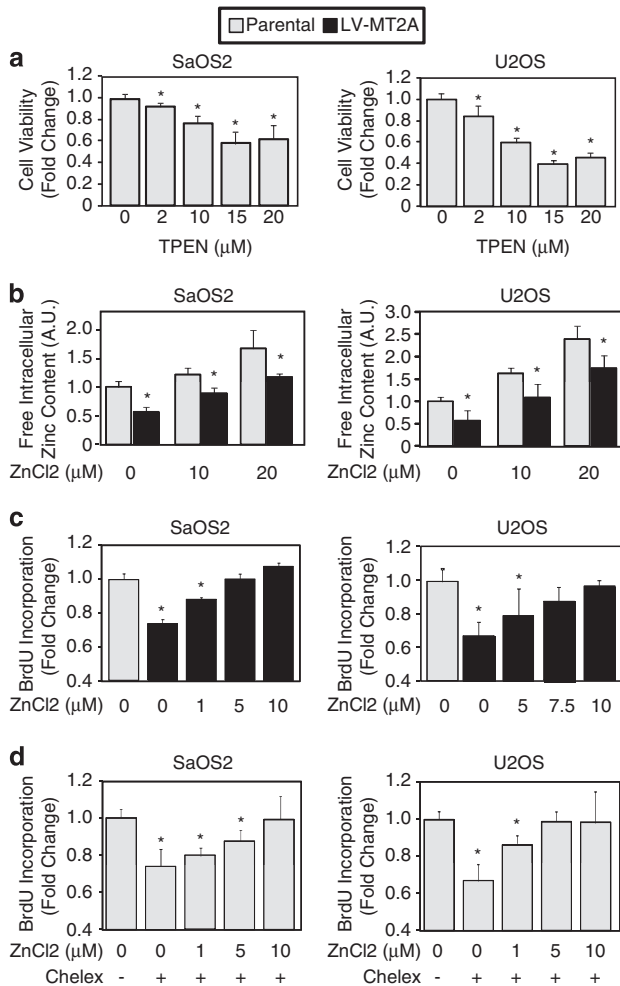


Figure 3 MT2A overexpression modulates osteosarcoma cell viability through zinc chelation. SaOS2 and U2OS parental cells were incubated with increasing doses of Zn chelator TPEN. (a) Cell viability was evaluated using the MTT test. (b) SaOS2 and U2OS parental and MT2A-overexpressing cells were incubated with increasing doses of ZnCl₂. Free intracellular zinc content was evaluated by fluorimetry. (c) SaOS2 and U2OS MT2A-overexpressing cells were incubated with increasing doses of ZnCl₂. Parental cells were maintained in normal medium. Cell replication was evaluated using a BrdU incorporation assay. (d) SaOS2 and U2OS parental cells were incubated in chelex-treated medium supplemented with increasing doses of ZnCl₂. Cell replication was evaluated using a BrdU incorporation assay. Results are expressed as mean \pm S.D. * $P < 0.05$ versus untreated cells

cells. We provide evidence of a direct stimulatory effect of statin supplementation on MT mRNA and protein levels. This suggests that osteosarcoma cells may rather use MT2A isoform in response to toxic agents such as statin. It would be interesting to evaluate the profile of MT and expression of isoforms rather than global MTI and/or MTII classes for more accurate discriminations for use as a marker of tumor differentiation, cell proliferation and invasiveness or prognosis predictor (reviewed in Costarelli *et al.*³⁵).

Zinc has a physiologically important role in bone homeostasis and has been demonstrated to have a stimulatory effect on osteoblastic bone formation and mineralization (reviewed in Yamaguchi²⁸). Regulation of zinc ion homeostasis by MTs is well known.^{29–31} We showed here that statin

dose-dependently reduce bioavailable intracellular zinc content in osteosarcoma cells, which is in agreement with different studies that report a decrease in zinc plasma levels from patients treated with statins.^{32–35} In the present study, we detected a rapid induction in MT2A expression during statin treatment and modulations of intracellular zinc content upon modulations of MT2A protein level. No significant modulation of expression was detected for zinc transporters consisting in importer (ZIP family) and exporter (ZnT family) proteins under statin treatment. It can be thus suggested that statins modulate zinc level through MT induction, but further experiments are needed to fully confirm this hypothesis.

Zinc supplementation has been shown to stimulate osteoblastic cell proliferation and differentiation.^{36,37} It has also been reported that MT2A level increased during rat mesenchymal stromal cell osteogenic differentiation.³⁸ As expected, we found a modulation of both cell proliferation and differentiation in our models of osteosarcoma cells modified for MT2A compared with parental cells, and especially a strong inhibition of differentiation process without increased cell viability under MT2A silencing. Such inhibition of osteoblastic maturation or even a de-differentiation process could be highly beneficial and exploited as an adjuvant therapy, as current chemotherapies mostly target the proliferative cells. A possible effect of zinc chelation on cancer stem cells behavior or differentiation reprogramming may be considered but need much further investigations.

According to metastatic feature, we previously reported that statin supplementation inhibited osteosarcoma cell migration and invasion capacities.² In the present study, we did not observe modification in cell motility or invasiveness of MT2A-overexpressing cells or MT2A-silenced cells compared with osteosarcoma parental cells. We neither observed modulation in matrix metalloproteinase 2 activity, a zinc-dependent metalloproteinase involved in osteosarcoma cell invasion process.² Taken together, these results reinforce the specificity of action of the different MT isoforms and suggest that MT2A isoform rather control cell viability than invasiveness or motility, at least in our osteosarcoma models.

Chemotherapy is the main treatment for osteosarcoma. Platinum-containing compounds such as cisplatin can be chelated by MT, which may render this drug ineffective in cancer cells (reviewed in Knipp³⁹). We evaluated the response of our modified cells in term of viability and indeed observed a reduction in osteosarcoma cell sensitivity to cisplatin when MT2A was overexpressed. In contrast, silencing MT2A led to increased cisplatin toxicity. Even if the *cis* configuration is described as being required for its antitumor activity,⁴⁰ we found that MT2A silencing can favor cytotoxic activity of both cisplatin and transplatin, thereby reinforcing the role of metal chelation in MT2A mechanism of action. These results have the limitations related to the *in vitro* approach but are promising for next *in vivo* validations. Platinum-based anticancer drugs are effective pharmaceuticals and are still the mostly used agents against malignancies. In addition, standard clinical protocols for osteosarcoma tumors combine cisplatin to other cytotoxic compounds, such as doxorubicin or vincristine, that do not contain metal ion and act through other molecular mechanisms. We found that MT2A overexpression or silencing can

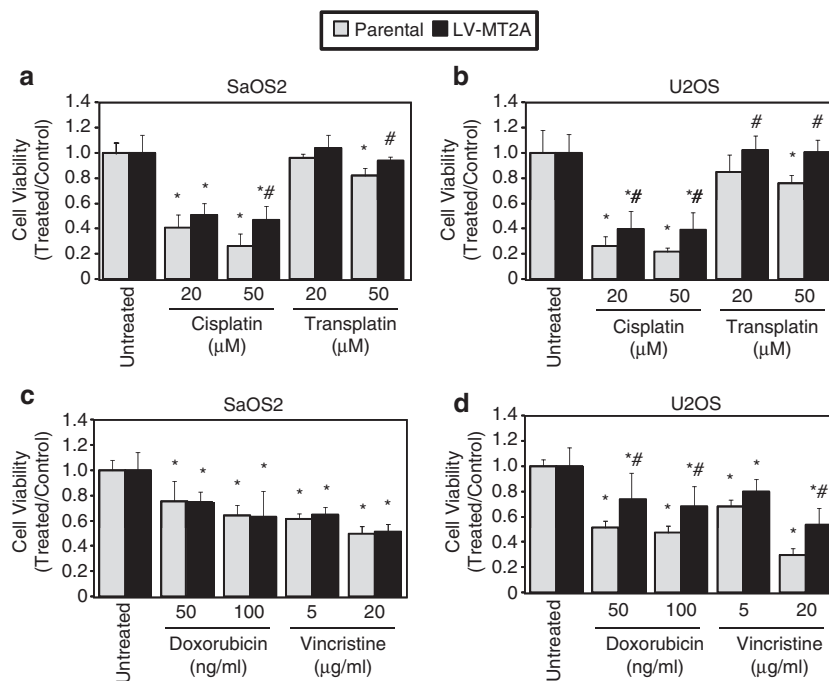


Figure 4 MT2A overexpression modulates cell response to cytotoxic agents. (a) SaOS2 parental and MT2A-overexpressing cells were incubated with or without cisplatin or transplatin for 48 h. Cell viability was evaluated using the MTT test. (b) U2OS parental and MT2A-overexpressing cells were incubated with or without cisplatin or transplatin for 48 h. Cell viability was evaluated using the MTT test. (c) SaOS2 parental and MT2A-overexpressing cells were incubated with or without doxorubicin or vincristine. Cell viability was evaluated using the MTT test. (d) U2OS parental and MT2A-overexpressing cells were incubated with or without doxorubicin or vincristine. Cell viability was evaluated using the MTT test. Results are expressed as mean \pm S.D. and as arbitrary units relative to corresponding untreated cells. * $P < 0.05$ versus untreated cells; # $P < 0.05$ versus parental cells

also modulate osteosarcoma cell sensitivity to these compounds. These observations agree with results of other reports.^{41–43} A direct action through metal chelation cannot be considered, as no metal ion is present in these compounds. We rather suggest an indirect action through a key metal-dependent factor. It has been described that zinc supplementation results in an increase of DNA damage induced by doxorubicin in an acute lymphoblastic leukemia cell line.⁴⁴ The zinc-binding transcription factor p53 is essential for cell cycle regulation and apoptosis induced by genotoxic DNA damage.^{45,46} It has even been described as predictive factor of chemoresistance in breast cancer.⁴⁷ In intact cells, p53 protein activity is crucially dependent on the availability of zinc ions, but MT can form a complex with p53^{48,49} causing the removal of Zn²⁺ from p53 by their sulfhydryl groups. This leads p53 to adopt a ‘mutant-like’ form with inhibition of its binding to DNA^{50,51} and proteins.⁵² In our model, we found that overexpression of MT2A indeed reduces the pool of active form of p53. Alternative or additive mechanisms involving zinc-dependent factors other than p53 may be likely involved and they merit further investigation in future.

Some studies have reported that different isoforms of MT were upregulated in osteosarcoma versus non-malignant bone,^{20,21} but no data are available according to patient outcome or chemoresistance. In the present study, we determined the MT2A mRNA level in 17 patients suffering from osteosarcoma before neoadjuvant chemotherapy and found the highest levels of MT2A in biopsies from patients that will be poor responders compared with patients that are

considered as good responders to chemotherapy. A marked difference was also observed in survival rate between the two groups, reinforcing the relation between treatment efficacy or response and patient outcome. These results strengthen the concept that MT2A level evaluation at diagnosis could help to predict tumor response to chemotherapy and thus patient outcome. The identification of individual isoforms seems to be a promising way in searching new prognostic markers in cancer at least for osteosarcoma. A previous study on biopsies of high-grade malignant osteosarcoma reports the absence of correlation between the expression level of MTs before chemotherapy and patient survival.⁵³ However, in this study, quantification was conducted by immunohistochemistry on paraffin-embedded samples using an antibody recognizing all isoforms of MT-I and MT-II subgroups, which may had reduce sensitivity of the measurement. Determining the expression of individual isoforms of human MT proteins is problematic because of the high-sequence homology that is present among those family members (up to over 90%).⁵⁴ To overcome this issue, we proposed a detection of MTs isoforms by quantitative RT-PCR using specific primers, which was based on the isolation of RNA from clinical samples.

In our model, we showed that the MT2A expression level is correlated with chemotherapy resistance in osteosarcoma cells. We can thus hypothesize that besides their high homology, each isoform may have a specific cellular role. This also led us to hypothesize that targeting specific isoform could be possibly helpful to improve chemo-sensitivity.

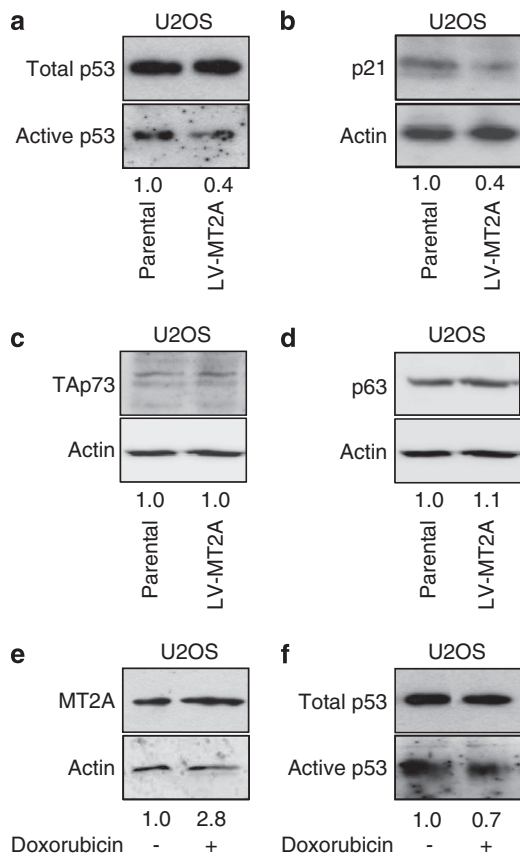


Figure 5 MT2A modulates p53 conformation-dependent activity. (a) Relative content of active form of P53 was evaluated by immunoprecipitation using a conformational-dependent antibody. (b) P21 protein levels were evaluated in U2OS parental and MT2A-overexpressing cells by western blot analysis. (c,d) P73 and P63 protein levels were evaluated in U2OS parental and MT2A-overexpressing cells by western blot analysis. (e) MT2A protein levels were evaluated in U2OS cells incubated in the presence or absence of doxorubicin (100 ng/ml). (f) Relative content of active form of P53 was evaluated by immunoprecipitation using a conformational-dependent antibody

Although no specific MT inhibitor has yet been described, MT2A might be considered as a biomarker for chemosensitivity of osteosarcoma tumors.

Materials and Methods

Cell culture. Human osteosarcoma cell lines SaOS2 and U2OS (American Type Culture Collection, Rockville, MD, USA) were routinely maintained in Dulbecco's-modified Eagle's medium (PAA Laboratories, Velizy-Villacoublay, France) supplemented with 10% heat-inactivated fetal calf serum (FCS; PAA Laboratories), 10 000 U/ml L-glutamine and 10 000 μ g/ml penicillin/streptomycin, at 37 °C under 5% CO₂/95% air in an humidified incubator.

Reagents. All reagents were obtained from Sigma-Aldrich (Lyon, France). Zinc-depleted medium was prepared by incubating FCS with 10% Chelex-100 (under overnight agitation) before addition to medium. Atorvastatin and transplatin were solubilized in dimethyl sulfoxide at concentrations of 10 and 15 mM, respectively. Doxorubicin, cisplatin and vincristine were solubilized in H₂O at concentrations of 10 mg/ml; 25 mM and 1 mg/ml, respectively.

Expression microarrays. RNA from SaOS2 cells incubated with or without atorvastatin (10 μ M) for 6, 15 or 24 h were labeled and hybridized on pan-genomic microarrays containing the human RING/MRC oligonucleotide collection.⁴

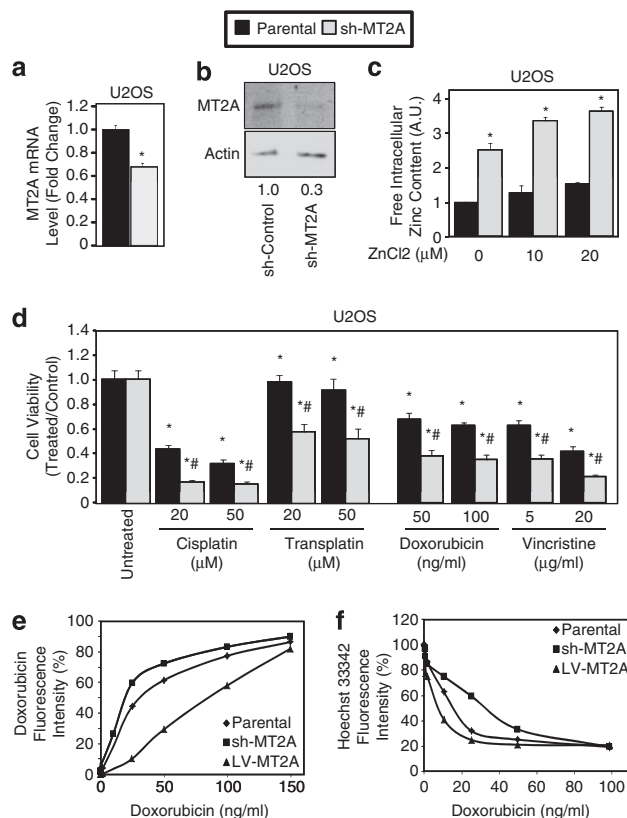


Figure 6 MT2A silencing amplifies osteosarcoma cell response to cytotoxic agents. U2OS cell line was transduced with an integrative lentiviral vector encoding a specific shRNA sequence targeting MT2A (sh-MT2A). (a) MT2A mRNA levels were evaluated by RT-qPCR in U2OS parental and MT2A-silenced cells. (b) MT2A protein levels were evaluated by western blot analysis in U2OS parental and MT2A-silenced cells. (c) U2OS parental and MT2A-silenced cells were incubated with increasing doses of ZnCl₂. Free intracellular zinc content was evaluated by fluorimetry. (d) U2OS parental and MT2A-silenced cells were incubated with or without cisplatin, transplatin, doxorubicin or vincristine for 48 h. Cell viability was evaluated using the MTT test. (e) Relative amounts of doxorubicin combined to DNA were evaluated by flow cytometry into parental, silenced and overexpressing U2OS cells. (f) Relative amounts of Hoechst 33342 bound to DNA were evaluated by flow cytometry into parental, silenced and overexpressing U2OS cells. Results are expressed as mean \pm S.D. * P < 0.05 versus untreated cells; # P < 0.05 versus parental cells

Lentiviral constructs and cell transduction. The pIRESneo2 plasmid containing the human MT2A complete coding sequence was kindly provided by Dr. VanderWesthuizen (North West University, Potchefstroom, South Africa). This coding sequence was cloned into HIV1-derived vector TRIP between the BamH1 and Xho1 sites. Virion particles were produced by transfection of HEK293T cells.⁵⁵ Ready-to-use lentiviral particles containing vector-encoding human MT2A shRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell proliferation. Cell proliferation rate was evaluated by BrdU assay using the Kit Biotrak ELISA System (GE Healthcare, Orsay, France). Cells were seeded at 15 000 cells/cm² in complete medium. After 48 h, BrdU (10 μ M) was added to the medium for the last 2 h of culture. Six replicates were used for each condition and experiments were repeated twice.

Cell viability. Cell viability was assessed after 48 h by the MTT assay as previously described.⁵⁶ Experiments were repeated three times with six replicates for each condition.

Acridine orange/ethidium bromide staining. A double staining with ethidium bromide and acridine orange was performed as previously described.⁵⁶

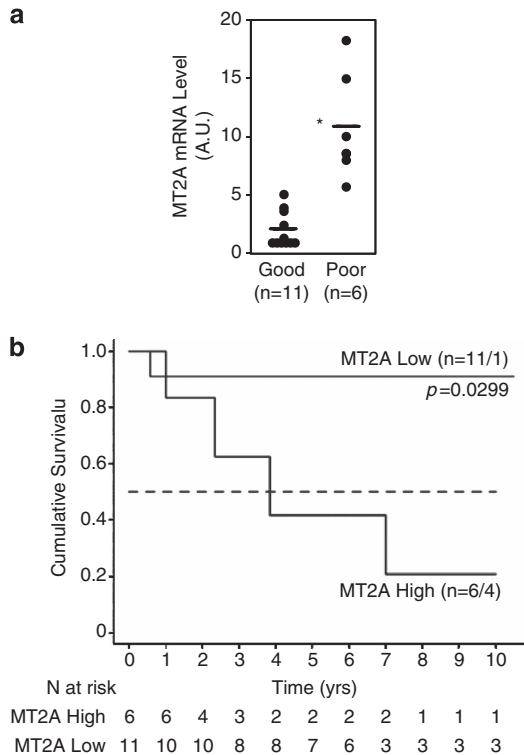


Figure 7 Prognostic value of MT2A mRNA expression in osteosarcoma. (a) MT2A mRNA levels were evaluated by RT-qPCR, in biopsies from good and poor responders. Results were expressed as arbitrary unit (AU). Mean values of each group are indicated as black bars. * $P < 0.05$ versus untreated cells. (b) Kaplan–Meier survival curves from patients with low MT2A mRNA level or patients with highest MT2A mRNA level

Cell cycle analysis by flow cytometry. Cell cycle phases were assessed by propidium iodide staining and flow cytometric analysis was carried out with FACS Canto II cytometer (Becton Dickinson, Le Pont-De-Claix, France) using standard methods. Briefly, cells were collected with trypsin-EDTA and fixed in cold 70% ethanol. Cells were then incubated in PBS supplemented with 0.1% Triton-X 100, 40 mg/ml RNase A and 8 μ g/ml propidium iodide for 60 min at RT. The experiment was repeated twice.

Competitive fluorochrome staining analysis by flow cytometry. Doxorubicin uptake in DNA was evaluated directly and indirectly through competitive bis-benzimide staining. Cells were incubated with or without increasing concentrations of doxorubicin for 24 h and then with 5 μ g/ml Hoechst 33342 for 90 min. After PBS washes, cells were harvested by trypsin, suspended to a concentration of 3×10^5 cells/ml in ice-cold PBS and analyzed by flow cytometry. Both doxorubicin (λ_{ex} 488 nm, λ_{em} 606 nm) and DNA complexed Hoechst (λ_{ex} 405 nm, λ_{em} 465 nm) were detected. Experiments were repeated three times.

Intracellular zinc concentration. The zinc concentration was evaluated by intracellular fluorimetry using the specific probe: zinquin (ethyl-[2-methyl-8-p-toluenesulphonamido-6-quinolyl]oxy]-acetate; Sigma-Aldrich). Cells were rinsed once with PBS and then incubated in PBS/1% BSA/25 μ M zinquin for 45 min at 37 °C in the dark. Cells were then centrifuged at 2000 $\times g$ for 5 min and suspended in PBS. Fluorescence was measured using λ_{ex} 368 nm and λ_{em} 490 nm. The maximal fluorescence, reflecting the total amount of intracellular zinc, was determined after the addition of saponin (0.25%). Assay was performed three times with 5–6 replicates for each condition.

RNA extraction and reverse transcription. Total RNA were isolated from cell cultures by phenol/chloroform extraction using Trizol Reagent (Invitrogen, Cergy-Pontoise, France) and solubilized in H₂O supplemented with RNase inhibitor (Ambion, Courtabouef, France). Three micrograms of RNA were

incubated in a buffer containing reverse transcriptase MMLV (Moloney Murine Leukemia Virus, 10 U), DTT (3 mM), oligodT primers (0.05 mg/ml) and dNTPs (1 mM) for 90 min at 37 °C. A final step of inactivation of the enzyme is carried out at 95 °C for 5 min.

Chemonaive cell subpopulations were isolated as previously described from newly diagnosed patients⁵⁷ and total RNA was extracted as previously described. Among 17 patients available for follow-up, the tumors were located in the proximal tibia in 10 patients, in the distal femur in 7 patients and in clavicle in 1 patient. After surgical resection, the percentage of histologically viable tumor was scored.⁵⁸ If the percentage of necrosis was $< 90\%$, the patient was considered to be a poor responder, and if $> 90\%$ of necrosis was found, the patient was considered as a good responder.

Quantitative PCR amplification. The expression level of mRNA encoding the MT2A was evaluated and normalized relative to that of GAPDH. The amplification was performed as previously described⁴ using SYBR Green PCR kit (ABgene, Courtabouef Cedex, France) and pairs of specific primers: GAPDH sense 5'-CGAGATCCCTCCAAAATCAA-3' forward 5'-TGTGGTCAT GAGTCCTTCCA-3'; MT2A sense 5'-TGCACCTCTGCAAGAAA-3' forward 5'-CAGCAGCTGCACTTGCC-3'.

Western blot and immunoprecipitation. Proteins from total cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described.⁴ Anti- β -actin was purchased from Sigma-Aldrich, and anti-p21, anti-p53 and anti-p63 were purchased from Santa Cruz Biotechnology. Anti-MT2A was purchased from Abcam (Cambridge, UK) and anti-p73 from Calbiochem (Nottingham, UK).

For immunoprecipitation, aliquots of clarified total cell lysates (250 μ g) were incubated overnight under weak agitation at 4 °C with 2 μ g specific antibody and 20 μ l Adembeads Protein AG (Ademtech, Pessac, France). Components of the bound immune complex were eluted according to the manufacturer's recommendations and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as above. Anti-p53 antibodies PAb1620 and PAb240 were purchased from Abcam.

Statistical analysis. Comparisons between *in vitro* data were performed using the two-factor analysis of variance with a minimal level of $P < 0.05$ considered to be significant. Clinical samples data were analyzed using non-parametric Mann–Whitney test with $P < 0.05$ considered to be significant.

Conflict of Interest

The authors declare no conflict of interest.

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