Cancer Biology and Signal Transduction

Metallothionein 1G and Zinc Sensitize Human Colorectal Cancer Cells to Chemotherapy

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Abstract

Metallothioneins (MT) are a family of low molecular weight proteins that are silenced during colorectal cancer progression, mainly through epigenetic mechanisms, and this loss is associated with poor survival. In this article, we show that overexpression of the MT1G isoform sensitizes colorectal cell lines to the chemotherapeutic agents oxaliplatin (OXA) and 5-fluorouracil (5-FU), in part through enhancing p53 and repressing NF- κ B activity. Despite being silenced, MTs can be reinduced by histone deacetylase inhibitors such as trichostatin A and sodium butyrate. In fact, this induction contributes to the cytotoxicity of these agents, given that silencing of MTs by siRNAs reduces their growth-inhibitory activities. Zinc ions also potently enhance MT expression and are cytotoxic to cancer cells. We show for the first time that OXA and 5-FU induce higher levels of intracellular labile zinc, as measured using the fluorescent probe FLUOZIN-3, and that such zinc contributes to the activation of p53 and repression of NF- κ B. Addition of zinc enhanced growth inhibition by OXA and 5-FU, and was also capable of resensitizing 5-FU–resistant cell lines to levels comparable with sensitive cell lines. This effect was MT independent because silencing MTs did not affect zinc cytotoxicity. In conclusion, we show that MT induction and zinc administration are novel strategies to sensitize colorectal cancer cells to presently utilized chemotherapeutic agents. *Mol Cancer Ther;* 13(5); 1369–81. ©2014 AACR.

Introduction

Colorectal cancer is the third most frequent cancer worldwide, having a mortality rate near 50% (1). Current therapeutic strategies rely heavily on complete surgical removal of the tumor, despite which 40% of patients recur. Chemotherapeutic adjuvant treatment in stage II disease is controversial and improves overall survival by 22% in stage III. In the metastatic setting, overall 5-year survival rates for stage IV patients are less than 10% (2). Therapeutic regimens are mainly based on 5-fluorouracil (5-FU), oxaliplatin (OXA), and irinotecan, all of which produce considerable side effects. Thus, it is of paramount importance to develop new therapies or to improve currently available agents.

Metallothioneins (MT) are a family of low molecular weight proteins that share significant sequence homology,

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and are involved in zinc and redox metabolism (3) as well as in many aspects of cancer biology (4, 5). The human genome contains at least 11 functional MT genes that may be divided into four subgroups (MT1-4). There are several MT1 isoforms each encoded by its own gene and along with MT2A are ubiquitously expressed. Given their stress-inducible nature and their capacity to chelate toxic metals and electrophiles, many studies have proposed MT expression to confer resistance to many toxic drugs (6, 7). On the other hand, given their capacity to influence zinc metabolism and this metal's availability to many zinc-dependent proteins and transcription factors, other studies have associated them with chemosensitivity (8, 9). Indeed, MTs either donate or take away zinc ions from several zinc-dependent proteins, including p53 (10, 11), thereby regulating their function. We and others have previously demonstrated that these proteins are progressively silenced during colorectal cancer progression, and that this is associated to poorer patient survival. (12–14).

Zinc is a required nutrient for proliferation, but elevated concentrations are known to promote cell death by many different mechanisms (15). Free zinc ions exist in the picomolar range and may be considered negligible due to tight regulation by zinc transporters, MTs, and organelle sequestration (16). Intracellular zinc pools consist mainly of tightly bound, unexchangeable zinc bound to proteins (the "immobile" pool), and of the exchangeable, loosely bound zinc termed the "labile" pool, which is complexed to low molecular weight ligands and MTs (17). The latter represents about 5% of the total

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intracellular zinc and participates in zinc transfer reactions and signaling. (18). This metal is also a potent inducer of MT synthesis and has been proposed to enhance chemosensitivity by restoring wild-type p53 conformation (19, 20).

In this paper, we studied the effects of MT overexpression in colorectal cell lines on the efficacy of 5-FU and OXA treatment. We also explored the effects of chemotherapy on zinc metabolism and the potential addition of zinc to resensitize chemoresistant cell lines.

Materials and Methods

Reagents and cell lines

The *MT1G* cDNA was cloned into the pcDNA3.1/myc-His(-)A expression vector, resulting in a MT1G–myc fusion protein as previously described (21). OXA and 5-FU were obtained in the pharmacy of the Alexander Fleming Institute (Buenos Aires, Argentina). Zinc chloride, sodium butyrate (BUT), EDTA, N,N,N',N'-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), and pifithrin-alpha (PFT- α) were all purchased from Sigma-Aldrich Inc and trichostatin A (TSA) from InvivoGen.

The human colorectal cancer cell lines HCT116 and HT-29 were obtained from the American Tissue Culture Collection (ATCC) and maintained as previously described (13). After all experiments were finalized, both cell lines and their derivatives were subjected to short tandem repeat profiling and compared with the ATCC's database for authentication. Both cell lines were stably transfected with the MT1G or empty vector using Lipofectamine 2000 (Invitrogen) as described by the manufacturer and selecting at least three stable clones with G-418 (Invitrogen) at 800 and 500 μ g/mL for HCT116 and HT-29 cells, respectively. The resulting cell lines expressing or not MT1G were called MT1G⁺ or MOCK cell lines, respectively, and tested by Western blot analysis using the antimyc antibody (Invitrogen). OXA- and 5-FU-resistant derivatives of both HCT116 and HT-29 cell lines were generated by successive passaging in increasing concentrations of these agents up to 2 and 15 µmol/L, respectively (Supplementary Fig. S1). For use in experiments, cells were previously cultured for two passages in drug-free medium.

Proliferation and dose-response curves

Proliferation curves were done by plating 4,000 cells in triplicate 96-well plates and measuring cell viability at the indicated time points by the MTT assay (Sigma) using 1 mg/mL MTT for 90 minutes at 37°C followed by incubation in 200 μ L isopropanol (Merck) for 1 hour at 37°C and reading the resulting absorbance at 570 nm. For dose–response curves, the same method was applied, except that cells were incubated in the presence of different doses of OXA or 5-FU for 72 hours. IC₅₀ was calculated using GraphPad Prism 5.0 software. For experiments evaluating the effect of TPEN, different siRNA's and PFT on chemotherapy outcome, 24 hours after, plating cells were pretreated for 5 hours with 5 μ mol/L TPEN or 125 nmol/L

siRNA's, and then exposed for 24 hours to OXA ($2 \mu mol/L$), 5-FU (15 $\mu mol/L$), BUT (2 mmol/L), or TSA (30 ng/mL), depending on the experiment. PFT treatment was done concomitantly with OXA at 20 $\mu mol/L$ for 24 hours. Cells were then left in drug-free medium for another 48 hours, before MTT analysis.

Clonogenic assays, cell-cycle analysis, and apoptosis assay

Clonogenic assays were performed by plating 800 cells in 35 mm dishes (in duplicate) and treating them 24 hours later with $1 \mu mol/L OXA$ or $3 \mu mol/L 5$ -FU, with or without the addition of $100 \,\mu mol/L ZnCl_2$, for 3 days. Fourteen days after plating, the resulting clones were Giemsa stained and counted manually under a microscope. Cell-cycle analysis after treating cells with 3 µmol/L 5-FU or 2 µmol/L OXA for 72 hours was performed by detaching cells with EDTA, fixing in 70% ethanol for 2 hours on ice and staining with propidium iodide solution (2 mg% with 200 μ g/mL RNAse A, and 0.1% Tritón X-100 in PBS) for 15 minutes at 37°C. Cells were analyzed on a FACSCalibur flow cytometer using the CellQuest software (BD Biosciences) for data analysis. Apoptosis was estimated after treating cells with 10 µmol/L OXA for 48 hours, using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences), following the manufacturer's recommendations.

In vivo xenograft studies

Eight- to 10-week-old male nude mice were subcutaneously injected with 2×10^6 HCT116 MOCK or MT1G⁻ cells. When tumors reached 100 mm³, mice were randomized and intraperitoneally treated with 10 mg/kg OXA, 40 mg/kg 5-FU, or 100 µl PBS (5 mice per group) once a week during 4 weeks, and tumor size was measured with a caliper to calculate tumor volume using the formula: tumor volume (mm³) = [length (mm)] × [width (mm)]2 $\times \pi/6$. In another experiment, following the same protocol, we evaluated the effect of the addition of zinc chloride to 5-FU treatment on HCT-5-FU-resistant cells. Zinc was administered orally (by oral gavage) at 10 mg/kg thrice weekly: the first time concomitantly to 5-FU treatment and the rest in the next 2 consecutive days. HCT-5-FU-resistant cells were thus separated into four groups (PBS, zinc only, 5-FU only, and 5-FU + zinc), whereas HCT116 cells were treated with 5-FU only or PBS, as a measure of 5-FU sensitivity. All animal procedures were approved by the Institutional Animal Care Board of the Leloir Institute (Buenos Aires, Argentina). Mice weight was measured twice weekly and remained unaltered compared with PBS controls in both experiments.

Quantitative reverse transcription PCR, Western blotting, and immunofluorescence

Quantitative reverse-transcription PCR (qRT-PCR) was used to quantify mRNA levels as previously described (13). The primers used are listed in Supplementary Table S1. For experiments measuring induction of genes after OXA treatment, $50 \ \mu mol/L$ OXA was used for the times indicated. Detection of MTs by Western blotting and immunofluorescence was done using the anti-MT clone E9 antibody (Dako Corporation) that recognizes all MT1 and 2 isoforms, as described in (13). For Western blotting, anti-p53 clone DO-7 (Sigma) and anti- β actin clone C-74 (Sigma) were used. Cytoplasmic extracts were prepared by lysing cells in hypotonic buffer (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, NP40 0.5%) and remaining nuclear proteins extracted with radioimmunoprecipitation assay buffer.

siRNA transfection

Two siRNA's targeting the MT1G isoform (si1G.1 and si1G.2) and one targeting all functional MT1+2 isoforms were designed and sequences shown in Supplementary Table S1. Two siRNA's targeting RELA-p65 were taken from (22). siRNA's were produced with Silencer siRNA Construction Kit (Ambion Inc.) and transfected at 125 nmol/L using LF2000 as described by the manufacturer.

Measurement of intracellular labile zinc

For this purpose, we used the cell-permeable zincspecific fluorophore FluoZin-3-AM (FZ; Invitrogen). Cells were incubated for 30 minutes at room temperature with 2 µmol/L FZ in PBS, washed in PBS, and incubated a further 30 minutes in PBS at room temperature to allow for the intracellular cleavage and activation of the fluorophore. For flow-cytometric analysis, 2×10^5 cells were detached, washed, and resuspended in 100 µL FZ. For fluorescence microscopy, cells were plated in sterile plastic cover-slips and observed without fixation, using DP2-BSW software (Olympus Corporation). For fluorimetric analysis, 20,000 cells were plated in triplicate in 96-well plates and incubated as described. Fluorescence was measured using 485/10 nm excitation and 535/25 nm emission filters, 400 ms acquisition. To control for plating differences of different cell lines, we incubated cells with propidium iodide solution (as described in the cell-cycle analysis section) and measured fluorescence intensity with 535/ 25 nm excitation and 595/35 nm emission filters. Fluorescence intensities (F) were taken as the quotient between fluozin and propidium iodide values. Data were expressed as normalized fluorescence FZ = (F - F) F_{TPEN} /(F_{Zn} – F_{TPEN}), so as to get values relative to a "maximum" intensity given by pretreatment with zinc 400 μ mol/L for 8 hours (F_{Zn}, resulting in FZ = 1) and a "minimum" intensity given by 20 µmol/L TPEN treatment during the final 30 minutes incubation of fluozin $(F_{TPEN}, resulting in FZ = 0)$. This score allowed us to better compare results of different experiments.

Statistical analysis

Data are expressed as mean \pm SEM and *P* values less than 0.05 were considered significant, denoted by one asterisk, whereas ^{**} means *P* < 0.01 and ^{***} means *P* < 0.001. Comparison of means was made with the Student *t* test,

with one-way ANOVA followed by the Dunnett posttest for three or more groups, or with two-way ANOVA followed by the Bonferroni posttest for two variables. GraphPad Prism 5.0 software was used for analysis.

Results

MT1G overexpression in HT-29 and HCT116 cell lines sensitizes them to chemotherapy

We expressed the MT1G isoform as an MT1G-myc fusion protein in HT-29 and HCT116 cell lines ("MT1G⁺" cell lines; Supplementary Fig. S2A). Using cells transfected with the empty vector ("Mock" cell lines) as controls, we found no differences in their in vitro proliferation rates, as measured by the MTT assay (Supplementary Fig. S2B). Given the proposed roles for MTs in apoptosis and drug detoxification, we studied whether MT1G⁺ lines differed in their susceptibility to two of the most widely used chemotherapeutic agents in colorectal cancer, OXA and 5-FU (5-FU). As shown in Fig. 1, both HCT116 and HT-29 MT1G⁺ cells were more sensitive to growth inhibition by both agents, as measured by dose-response curves (Fig. 1A and B and Supplementary Fig. S2C and S2D) and clonogenic assays (Fig. 1C). In fact, IC_{50} values on average were around twice as low in both cell lines, for both treatments (for 5-FU, 1.74 and 1.90 times lower in HCT116 and HT-29, and for OXA, 1.60 and 2.23 times lower, respectively). Apoptotic death after OXA treatment, as determined by flow-cytometric Annexin⁺/propidium iodide⁻ staining, was also greater in these lines (Fig. 1D and E) rising from 11.67 \pm 1.04% to 23.42 \pm 8.43% in HCT116 and from 13.24 \pm 1.78% to 30.82 \pm 1.21% in HT-29. Cell-cycle analysis in HCT116 revealed that MT1G⁺ cells have a significantly higher percentage of cells arrested at the G_0 - G_1 phase after 5-FU treatment (Fig. 1F and Supplementary Fig. S2E) and at the G₂-M phase after OXA treatment (Fig. 1G and Supplementary Fig. S2E). For both treatments, MT1G⁺ cells showed higher levels of sub-G₀ cells (Supplementary Fig. S2F) in agreement with the apoptosis assay. Given the greater in vitro cytotoxicity of MT1G⁺ cells to these chemotherapeutic agents, we performed studies using nude mice xenografts of HCT116-derived cell lines to validate these findings in the *in vivo* setting. To our surprise, MT1G⁺ cells grew at a lower rate than MOCK cells (Fig. 1H), in contrast with the in vitro proliferation rates described above (Supplementary Fig. S2B). MT1G⁺ cells also grew slower than controls when mice were treated intraperitoneally with OXA or 5-FU (Fig. 1I and J), implying that MT1G expression confers a better response to both chemotherapeutic agents.

We also used siRNAs to inhibit the endogenous expression of MT1G (si1G.1 and si1G.2) or of all MTs (siMTs). Figure 2A shows that siMTs can effectively inhibit MTs protein levels by 70% (using an antibody that recognizes all MT1+2 isoforms), whereas Fig. 2B demonstrates the specificity of siMTs and both si1G's in inhibiting most MTs or only MT1G, respectively.

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Figure 1. MT1G overexpression sensitizes cells to chemotherapy. A and B, IC₅₀ values for individual MOCK and MT1G⁺ clones of HCT116 and HT-29 cells after 5-FU (A) and OXA (B) treatments. C. clonogenic assays showing less colony formation in MT1G⁺ cells. D and E, apoptosis measured by Annexin V/propidium iodide (An⁺PI⁻) in HCT116 (D) and HT-29 (E) cells after OXA treatment. F and G, cell-cycle distribution of HCT116 cells after 5-FU (F) and OXA (G) treatments. H-J, xenograft studies of nude mice inoculated with HCT116 MOCK or MT1G⁺ cells and treated with vehicle (H), OXA (I), or 5-FU (J). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

OXA treatment after silencing of MTs showed that cells were more resistant to this treatment, indicating that endogenous MTs are also involved in chemosensitivity (Fig. 2C).

HDAC inhibitors mediate cell death in part by stimulating MT expression

Given that forced expression of MT1G sensitizes cells to chemotherapy, we explored whether pharmacologic

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Figure 2. Effect of siRNA-mediated inhibition of MT1G (si1G.1 and 1G.2) or all functional MT1+2 isoforms (siMTs) on growth inhibition mediated by OXA or HDACi. A, Western blotting for MT1+2 protein expression showing 70% knockdown of MTs by siMTs in HCT116. B, specificity of each siRNA expressed as percentage of mRNA inhibition of each MT isoform after siRNA treatment, compared with siNEG, and measured by qRT-PCR, in HT-29. C-E, effect of inhibition of MTs in HCT116 on cell viability after OXA (C) TSA (D) or butyrate (E) treatment, measured by the MTT assay. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

induction of endogenous MTs might also increase cvtotoxicity. We have previously reported that histone deacetvlase inhibitors (HDACi) such as TSA and BUT can stimulate MT expression in colon cancer cells (13). These agents are also cytotoxic and are being evaluated in clinical trials as possible new therapeutic drugs (23). Therefore, we evaluated whether MT induction was necessary for their cytotoxic action using siRNAs against MT1G or against all MTs, and measuring viability with the MTT assay. Interestingly, as shown in Fig. 2D and E, silencing MT1G or all MTs abrogated butyrate's and significantly reduced TSA's ability to inhibit cell proliferation. This suggests that induction of MTs by HDACi is at least partially responsible for their cytotoxic action, and sustains the hypothesis that MT induction is a novel viable therapeutic strategy.

Zn induces MT expression and relocalization

Zinc supplementation is another way to stimulate MTs expression in a dose-dependent manner (Supplementary Fig. S3A). This effect also occurs in p53-mutated HT-29 cells, and at higher levels in OXA-resistant HT-29 derivatives (HT29-OXAR) generated in our laboratory (Supplementary Fig. S3B). Given that extracellular zinc does not freely permeate cell membranes, high zinc concentrations are needed to increase intracellular labile zinc levels, as shown in Supplementary Fig. S3C using the zincspecific fluorophore FluoZin-3-AM (FZ). Interestingly, MTs are induced at about the same zinc concentrations that increase intracellular labile zinc. Conversely, labile zinc chelation by TPEN significantly reduced MT1G and MT2A levels, demonstrating that MT expression is dependent on intracellular zinc levels (Supplementary Fig. S3D). Immunofluorescence staining of MTs shows that although HT-29 cells express MTs only in the cytoplasm (Supplementary Fig. S4A-S4D), HCT116 cells show both nuclear and cytoplasmic staining which shifts to mainly cytoplasmic upon zinc treatment (Supplementary Fig. S4E and S4F).

Chemotherapy treatment modulates zinc metabolism

Little is known about whether chemotherapy treatment modulates zinc metabolism. Twenty-four hours after OXA treatment, MTs protein levels were significantly reduced in HCT116 cell lines, paralleling p53 induction (Fig. 3A). HT-29 cells, unlike HCT116, express *MT1G* mRNA, and this was significantly reduced after both OXA and 5-FU treatment, as well as in OXA- and 5-FU–resistant cell lines (Fig. 3B). *MT2A* mRNA levels showed a similar tendency to decrease after treatment, but this was not statistically significant (Fig. 3C). Interestingly, HT-29 and HCT116-resistant cell lines show higher basal *MT2A* mRNA (Fig. 3C) and MTs protein levels (Fig. 3D), suggesting the possibility that different MT isoforms may have different effects on chemoresistance.

Given the relationship between MTs, chemosensitivity, and zinc levels, we measured labile intracellular zinc



Figure 3. MT expression after chemotherapy treatment and in chemoresistant cells. A, Western blotting for MT1+2 and p53 expression after 8, 24, and 48 hours of 2 µmol/L OXA treatment showing MT downregulation paralleling the induction of p53 in HCT116 cells. The p53-mutant HT-29 cell line was used as a positive control for p53 overexpression. B, *MT1G* mRNA levels are also downregulated 24 hours after OXA and 5-FU treatments, and in OXA- and 5FU-resistant cells. Note that HCT116 expresses barely detectable *MT1G* mRNA levels. C, *MT2A* mRNA is slightly diminished with OXA treatment, but is basally higher in chemoresistant cells. D, Western blot analysis showing that MT expression is higher in chemoresistant cells, despite MT1G downregulation. **, *P* < 0.01; ***, *P* < 0.001.

upon OXA and 5-FU exposure using the FZ probe. Interestingly, both chemotherapeutic agents induce FZ fluorescence, as measured by fluorescence microscopy (Fig. 4A and B), flow cytometry (not shown), and fluorimetry (Fig. 4C-H), which was evident at 6 hours after OXA exposure (Fig. 4C). Pretreatment of cells with nontoxic doses (5 µmol/L) of TPEN for 5 hours was able to prevent this increase after 6 hours of OXA exposure but not after 24 hours (Fig. 4D). MT1G⁺ cells showed higher induction of labile zinc (Fig. 4E and F), but this was not different between HCT116-sensitive and 5-FU-resistant cell lines (Fig. 4G). As shown in Fig. 4H, after knockdown of MTs basal zinc fluorescence was unchanged, suggesting that FZ does not measure MTs-bound zinc ions. OXA-mediated increase in FZ fluorescence was also unchanged after silencing MT expression, suggesting that MTs are not the source of the released zinc. Neither was extracellular zinc, because chelation by nontoxic doses (data not shown) of the non-cell-permeable agent EDTA did not modify FZ increase (Fig. 4D). Immunofluorescence staining of HCT116 cells revealed that both OXA and 5-FU stimulate cytoplasmic localization of MTs (Supplementary Fig. S4G). This was also confirmed by Western blot analysis of nuclear and cytoplasmic fractions (Supplementary Fig. S5). This effect occurs in response to intracellular zinc release given that pretreatment with TPEN abrogated the shift in subcellular localization, as shown in Supplementary Fig. S4H. In HT-29 cells, MTs stay in the cytoplasm before and after chemotherapy treatment. Therefore, chemotherapy agents induce labile zinc liberation from non-MT stores, and cytoplasmic relocalization of MTs in cells that have basal nuclear MT expression. This suggests that MTs respond to chemotherapy-induced zinc release much in the same way as exogenous zinc administration.

To explore the possibility that alterations in the expression of zinc transporters may account for the observed alterations in labile zinc, we measured mRNA levels of several transporters from both the ZIP [*SLC39A1* (ZIP1), *SLC39A4* (ZIP4), *SLC39A5* (ZIP5), *SLC39A7* (ZIP7), *SLC39A8* (ZIP8), *SLC39A13* (ZIP13), and *SLC39A14* (ZIP14)] and ZnT [*SLC30A1* (ZnT1), *SLC30A4* (ZnT4), *SLC30A5* (ZnT5), *SLC30A6* (ZnT6), *SLC30A7* (ZnT7), *SLC30A8* (ZnT8), and *SLC30A9* (ZnT9)] families (16) known to be expressed in intestinal cells. As shown in Supplementary Fig. S6, MT1G⁺ HCT116 cells expressed slightly lower levels of some ZIP family members (*ZIP1*, *ZIP7*, *ZIP13*, and *ZIP14*), whereas 6 hours after OXA treatment, only *ZIP1* mRNA was significantly reduced.

Enhanced chemosensitivity of MT1G⁺ cells is due to p53 activation or NF-KB repression

To gain insight into the possible mechanisms by which MT1G sensitizes tumor cells to chemotherapy, we studied the relevance of the p53 and NF-κB pathways, given previous reports showing crosstalk with MTs (8, 11, 19, 24). First, we measured viability after treating Figure 4. Chemotherapy induces an increase in intracellular labile zinc levels, measured with fluozin 3-AM (FZ). A and B, fluorescence micrographs showing FZ fluorescence before (A) and 24 hours after 5-FU treatment (B). C-H. FZ fluorescence quantified by fluorimetric analysis. C, FZ levels rise after 6 hours of OXA treatment. D, five hours of 5 µmol/L TPEN pretreatment abrogates FZ increase after 6 hours of OXA (50 µmol/L) treatment, but this effect is lost at 24 hours. Extracellular zinc chelation by EDTA 100 µmol/L concomitantly with OXA has no effect. Both HT-29 (E) and HCT116 (F) MT1G⁺ cells show higher induction levels of labile zinc after OXA treatment, whereas HCT-5FU-resistant cells show no change compared with the sensitive HCT116 cell line (G). H, knockdown of MT1G or of all MTs does not modify basal or OXA-induced FZ levels. *, *P* < 0.05; ***, *P* < 0.001.



HCT116 cells with PFT (a p53 activity inhibitor) concomitantly with OXA. Figure 5A shows that PFT treatment reduced growth inhibition by OXA only in $MT1G^+$ cells, suggesting that enhancement of p53 activity in the p53 wild-type HCT116 cell line is a possible mechanism of action of MT1G. To confirm this, we used three wellknown targets of p53 [involved in cell-cycle arrest: *CDKN1A* (*P21*) and *GADD45A*, or *apoptosis PMAIP1* (*NOXA*)] as a measure of its transcriptional activity, and found that MT1G⁺ cells showed a stronger induction in all three genes at 6 and 12 hours after treatment (Supplementary Fig. S7A–S7C). To confirm that this induction is

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Figure 5. MT1G enhances p53 and represses NF-kB activity. A, p53 inactivation by PFT treatment attenuates OXA-mediated growth inhibition in MT1G⁺ HCT116 cells, but has no effect on HT-29 p53mutant cells (B). C, the p53 target genes P21, GADD45A, and NOXA are induced at higher levels in MT1G⁺ cells in a p53-dependent manner. 6 hours after OXA 50 µmol/L exposure. D and E, knockdown of p65 by siRNA enhances growth inhibition by OXA and 5-FU in HT-29 (D), but less so in HCT116 cells (E). F, the p65 target gene CXCL1 is induced in HT-29 MOCK cells after OXA treatment in a p65-dependent manner, but not in MT1G⁺ cells. G, IL-8 is another p65 target gene that was not induced in MOCK, but was repressed in MT1G⁺ cells after OXA treatment, to levels comparable with those obtained by silencing p65. H and I, labile zinc chelation by nontoxic doses of TPEN blunted P21 activation (H) and enhanced CXCL1 induction (I) after 6 hours of OXA treatment. *. *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

due to p53 activation, we again treated these cells with PFT. As control, PFT neither altered growth inhibition by OXA (Fig. 5B) nor induction of p53 target genes in p53mutant HT-29 cells (Supplementary Fig. S7D). As shown in Fig. 5C, after PFT treatment *P21*, *GADD45A* and *NOXA* levels were reduced compared with OXA alone, only in MT1G⁺ but not in MOCK cell lines. This indicates that MT1G enhances p53 transcriptional activity, although other mechanisms may exist given the remaining levels of *P21* induction.

Next, we used two siRNAs directed against the p65 subunit of NF- κ B (sip65.1 and sip65.2 (22), to study the

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involvement of this pathway in chemoresistance. When p65 was silenced, the growth-inhibitory activity of OXA and 5-FU was enhanced in HT-29 and to a lower extent in HCT116 cells, (Fig. 5D and E) indicating that p65 expression contributes to chemoresistance. To see whether MT1G expression altered this signaling pathway, we measured mRNA levels of two known NF-KB target genes involved in chemoresistance [interleukin (IL)-8 and CXCL1; ref. 25] after OXA treatment of MOCK and MT1G⁺ cells. As shown in Fig. 5F, although CXCL1 was induced in HT-29 MOCK cells in a p65-dependent manner, this effect was blunted in $MT1\overline{G}^+$ cells. *IL-8* was not induced in MOCK cells, although its levels were reduced in MT1G⁺ cells after OXA treatment (Fig. 5G). Neither gene was altered in HCT116 cells (Supplementary Fig. S7E and S7F). Taken together, these results suggest that the enhanced chemosensitivity of MT1G⁺ cells in the HT-29 cell line is due to suppression of NF-KB signaling.

Finally, we evaluated whether the observed rise in labile zinc after chemotherapy treatment was responsible for p53 activation or p65 repression by MT1G. For this purpose, we measured p53 and NF- κ B target genes after OXA exposure, with or without TPEN pretreatment at nontoxic doses. Figure 5H shows that chelating labile zinc totally abrogated the induction of *P21* by OXA, both in MOCK and MT1G⁺ HCT116 cells. Conversely, Fig. 5I shows that TPEN pretreatment enhanced *CXCL1* induction by OXA in HT-29 MOCK cells, and abrogated MT1G's ability to repress this induction. Altogether, these data suggest that labile zinc induction by OXA contributes to p53 activation and p65 repression, thereby contributing to cell death.

Zinc enhances the cytotoxicity of chemotherapy, including chemoresistant cells

Zinc ions are toxic to cancer cells in a concentrationdependent manner, especially as from 200 µmol/L (Supplementary Fig. S8A and S8B), coincident with the rise in intracellular zinc (Supplementary Fig. S3C). We evaluated whether zinc treatment would enhance the cytotoxicity of chemotherapy, as previously reported (19, 20). Indeed, both dose–response curves with a fixed concentration of zinc and clonogenic assays (Fig. 6A and B) confirmed that zinc cotreatment was more effective in cell growth inhibition than OXA alone, both in MOCK and MT1G⁺ lines. We thus studied the possibility that MTs may underlie zinc's cytotoxic capacity by using siRNAs to inhibit MT induction after toxic doses of zinc. However, knockdown of MTs did not affect zinc's ability to inhibit proliferation (Fig. 6C), suggesting that other mechanisms are responsible. Quite the contrary, silencing of all MTs enhanced zinc toxicity, therefore suggesting that MT induction and relocalization act as a protective response to quelate or redistribute intracellular zinc ions to avoid its toxicity.

In order for zinc addition to be therapeutically significant, it would be desirable that it could sensitize chemoresistant cell lines as well. Proliferation assays (Fig. 6D) of HT-29 OXA-sensitive and -resistant (HT29-OXAR) cells, in the presence of 2 µmol/L OXA with or without 100 μ mol/L zinc, show that although the sensitive cells completely die at the end of the assay, resistant cells continue to grow in the presence of OXA alone, but do so at a significantly lower rate with the addition of zinc (P < 0.01). Clonogenic assays for all four resistant cell lines were used to evaluate growth up to 14 days of treatment. As shown in Supplementary Fig. S8C-S8F, although addition of zinc tended to diminish colony formation in all cell lines, this was significant only for HT29-OXAR cells. Zinc alone also had a small but significant growthinhibitory effect only on this cell line. We therefore decided to use the HCT-FUR line to test our hypothesis in the in vivo setting using nude mice xenografts. As shown in Fig. 6E, although 5-FU treatment effectively inhibited the growth of the HCT116 cell line, it was much less effective in the 5-FU-resistant cell line. The zinc-only treatment had a significant growth-inhibitory effect on resistant cells, but importantly, was not toxic to mice, as there was no significant weight loss (Fig. 6F) nor noticeable behavioral alterations, in any of the treatment groups. Strikingly, the 5-FU + Zn treatment slowed resistant tumor growth to rates resembling the sensitive cell line treated with 5-FU alone, indicating that the combination treatment was able to resensitize this chemoresistant cell line to 5-FU therapy.

Discussion

In the present paper, we have shown that MT1G expression sensitizes colorectal cancer cell lines to OXA and 5-FU treatments. This was mediated at least in part through p53 activation in the p53-wild-type HCT116 cell line, and through NF-κB (p65) signaling in HT-29 p53-mutant cells. Induction of MTs can be accomplished by HDACi, and this induction was shown to contribute to their antitumoral properties. Given the fact that MTs are progressively silenced during colorectal cancer progression (13), the possibility of reinducing their expression might thus represent a novel strategy to improve responses to standard-of-care as well as novel therapeutic agents. HDAC inhibitors have been shown to synergize with agents such as OXA and 5-FU (26, 27) and are promising therapeutic agents being evaluated in clinical trials (23). This study also raises the possibility that MTs might prove to be predictive markers for the efficacy of HDACi, and this should be addressed in future studies. Indeed, MTs are part of a transcriptional signature induced in HDACisensitive colon cancer cell lines (28). Moreover, MT1G expression has been correlated with the synergistic effect of HDACi and taxane treatment in breast cancer (29).

It is noteworthy that chemoresistant cells showed downregulation of MT1G expression, suggesting that this may contribute to the chemoresistant phenotype. Consistent with this, MT1G has previously been reported to be hypermethylated in cisplatin-resistant cell lines (30). Given that both HCT116 and HT-29 cell lines have similar

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Figure 6. Zinc enhances growth inhibition by chemotherapeutic agents and resensitizes chemoresistant cell lines. IC50 values (A) and clonogenic growth (B) are diminished after addition of 100 µmol/L zinc chloride to OXA treatment, in both for MOCK and MT1G⁺ cells. C, knockdown of MTs does not abrogate growth inhibition by 300 μ mol/L zinc in HT-29 cells. D, zinc chloride potentiates in vitro growth inhibition of HT29-OXAR cells by 2 µmol/L OXA, as measured by proliferation at different time points. E, in vivo mouse xenograft studies of HCT116 5-FUR cells show that zinc alone attenuates tumor growth and zinc addition to 5-FU sensitizes resistant cells to levels comparable with those of HCT116-sensitive cells. F, weight of mice was unaltered by any treatment scheme, indicating no appreciable toxicity to the mice. G, hypothetical model depicting the proposed mechanism by which MT1G overexpression and labile zinc (Zn;) increase potentiate tumor killing by chemotherapeutic agents. Complete arrows indicate stimulation and dashed arrows indicate inhibition. *, P < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

sensitivity to OXA and 5-FU, but the latter has higher expression of *MT1G* mRNA, chemoresistance is surely not solely dependent on the level of MT1G expression. On the other hand, *MT2A* and total MTs protein levels were higher in chemoresistant cell lines. Many reports have suggested MT expression to be associated with chemoresistance rather than chemosensitivity, although a causal

relationship has not been conclusively established (4, 5). Our study suggests that different MT isoforms might be differently associated with either chemoresistance or sensitivity, and that this should be studied in further detail to unravel their possible predictive value. MT biology is certainly different in different tumor types (4), but at least the MT1G isoform is uniformly reported as having tumor

suppressor phenotypes, and therefore its reinduction in tumor cells is a very interesting therapeutic strategy.

Another way to reinduce MTs synthesis in tumor cells is by zinc treatment, which is also cytotoxic to tumor cells at high doses. A very important finding of this study is that zinc supplementation was able to resensitize chemoresistant cell lines. Compared with other metals, zinc excess is relatively well tolerated (31) and might therefore be an attractive agent to complement chemotherapeutic regimens. Moreover, zinc may be selectively toxic to tumoral rather than normal cells, as evidenced in (32) and (33), where zinc was preferentially accumulated and promoted DNA damage only in the former. In our study, although zinc alone significantly delayed tumor growth, it showed no signs of toxicity to mice. In support of this, zinc administration has been shown to enhance the therapeutic indices of various antineoplastic agents (34, 35). Interestingly, zinc by itself has been shown in xenograft models to be an effective antitumoral agent (36, 37), as well as being implicated in the mechanism of action of novel therapies (38–41). There are multiple studies suggesting that zinc addition to chemotherapeutic agents improves their antitumoral capacity, in xenograft models (20, 42) as wells as in humans (43, 44), and against chemoresistant cells (45).

Although many mechanisms have been proposed to explain zinc's cytotoxicity, there are no known chaperones responsible for its incorporation into proteins, with MTs being the closest known possibility. This suggested to us that MTs might be required to deliver zinc to its many targets and therefore mediate its cytotoxic effects. However, silencing MTs did not alter its growth-inhibitory effects, therefore rejecting our hypothesis. It should be borne in mind that the consequences of MT induction may differ according to the zinc content of cells; MTs induction in a low zinc environment may serve to transmit zinc signals induced by agents such as OXA and 5-FU, whereas their induction in a high zinc environment may serve to buffer zinc increase. In support of this idea, MT knockout mice are more sensitive both to zinc excess and deficiency (46). Whatever the case, this study suggests that MTs and extracellular zinc are independent ways to sensitize tumor cells to chemotherapy.

Conversely, chemotherapeutic agents modulate zinc metabolism, raising labile intracellular zinc levels, localizing MTs to the cytoplasm, and diminishing total MTs levels. Zinc ions released intracellularly from the zinc/ thiolate clusters of MTs or secreted from specialized organelles are potent effectors of proteins and are considered zinc signals (47, 48). The observed increase in labile zinc was unaltered after silencing MTs, suggesting that they are not the source of this zinc. Neither was it extracellular zinc because chelation with the non-cellpermeable agent EDTA did not prevent its induction. Possible sources may be cytoplasmic organelles (mitochondria, lysosomes, endoplasmic reticulum) or other labile zinc pools such as low molecular weight ligands. Zinc levels may also be altered by regulation of zinc transporter activities, which move zinc toward the cytosol

(ZIP family) or toward intracellular organelles or the extracellular space (ZnT family; ref. 16). After 6 hours OXA treatment, however, of the transporters studied only ZIP1 mRNA was slightly but significantly downregulated in MOCK but not MT1G⁺ cells. This transporter has been reported to be localized in the membrane of the endoplasmic reticulum, and its mRNA levels shown to be reduced after zinc treatment (49), which would be consistent with zinc being accumulated in intracytoplasmic stores. However, this should be taken as an exploratory study to identify possible candidates modulated by MT1G expression or chemotherapy treatment, and future studies should be designed to evaluate their protein levels, zinc transport activity, and colocalization with labile zinc, to fully evaluate whether they are indeed responsible for the observed zinc changes.

After zinc induction by chemotherapeutic agents, cells with basal nuclear MTs like HCT116 respond by localizing MTs to the cytoplasm. This is indeed in response to labile zinc increase because it is inhibited by TPEN pretreatment. This also suggests that the cytoplasm is the main site of action of both zinc and MTs. MTs are downregulated following chemotherapy treatment and although we did not attempt to explain the reason for this, one possibility is that apo-MT (i.e., zinc-free MT), is generated after the transfer of zinc from MTs to cell-death promoting targets. Apo-MT is degraded by proteases in vitro much faster than metal-bound forms (50) and although the validity of this has been questioned in vivo (51), apo-MT generation might explain the observed downregulation of MT expression. We were not able to use proliferation assays to evaluate whether this rise in labile zinc contributes to growth inhibition by OXA or 5-FU. This is because treatment with nontoxic doses of the zinc chelator TPEN was not enough to prevent zinc rise at 24 hours of OXA treatment, time needed to observe growth inhibition in our proliferation assays. However, we were able to show that at 6 hours after treatment, TPEN does prevent labile zinc induction by OXA, inhibiting p53 and enhancing NF-κB activation. This strongly suggests, as depicted in Fig. 6G, that zinc signals evoked by OXA or 5-FU treatment contribute to cell death by activating p53 and repressing NF-KB signaling pathways, and that enhancing these signals by MT1G induction or zinc administration might prove to be novel strategies to enhance the efficacy of chemotherapy.

In conclusion, our study states the proposal that MT1G reexpression in colorectal cancer may be a viable strategy to sensitize tumor cells to chemotherapy, and that it may be brought about by HDACi. Zinc supplementation to chemotherapy regimens was able to resensitize chemoresistant tumor cells independently of MT induction and should be considered in future clinical studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.M. Arriaga, M. Bianchini **Development of methodology:** J.M. Arriaga

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Arriaga, A. Greco Analysis and interpretation of data (e.g., statistical analysis, biostatis-

tics, computational analysis): J.M. Arriaga, M. Bianchini

Writing, review, and/or revision of the manuscript: J.M. Arriaga, M. Bianchini

Study supervision: J. Mordoh, M. Bianchini

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