Zn(II)-curc targets p53 in thyroid cancer cells

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Abstract. TP53 mutation is a common event in many cancers, including thyroid carcinoma. Defective p53 activity promotes cancer resistance to therapies and a more malignant phenotype, acquiring oncogenic functions. Rescuing the function of mutant p53 (mutp53) protein is an attractive anticancer therapeutic strategy. Zn(II)-curc is a novel small molecule that has been shown to target mutp53 protein in several cancer cells, but its effect in thyroid cancer cells remains unclear. Here, we investigated whether Zn(II)-curc could affect p53 in thyroid cancer cells with both p53 mutation (R273H) and wild-type p53. Zn(II)-curc induced mutp53H273 downregulation and reactivation of wild-type functions, such as binding to canonical target promoters and target gene transactivation. This latter effect was similar to that induced by PRIMA-1.

In addition, Zn(II)-curc triggered p53 target gene expression in wild-type p53-carrying cells. In combination treatments, Zn(II)-curc enhanced the antitumor activity of chemotherapeutic drugs, in both mutant and wild-type-carrying cancer cells. Taken together, our data indicate that Zn(II)-curc promotes the reactivation of p53 in thyroid cancer cells, providing in vitro evidence for a potential therapeutic approach in thyroid cancers.

Introduction

Thyroid carcinoma is a common malignant tumor of endocrine glands and the most common cancer in head and neck; it exhibits the full range of malignant behaviors from the relatively indolent occult differentiated type to uniformly aggressive and lethal anaplastic variety (1). Thyroid tumor formation is associated with many genetic and environmental causes. In addition, numerous factors are also required to promote thyroid cancer progression (2). Although well differentiated thyroid carcinomas are efficiently cured by surgery and radioiodine, undifferentiated thyroid carcinomas (10-20% of total) are very resistant to chemotherapy (3-6). Therefore, novel and more efficacious therapies are urgently needed for these tumors.

TP53 is the most commonly mutated gene in human cancers, thus, >50% of all human cancer cases carry mutations within the TP53 locus (7). Unlike other oncosuppressors, p53 gene always carries a single monoallelic missense mutation that mainly resides in its DNA-binding domain (DBD) that abolishes its ability to bind to specific DNA sequences recognized by wild-type (wt) p53 (8). Loss of wt53 activity leads to inhibition of its oncosuppressor function promoting resistance to chemo- and radio-therapies (9). Mutant p53 (mutp53) proteins frequently exhibit a dominant-negative activity over the wt53 allele by interacting with wt53 and reducing cellular concentration of functional wt53 (10). Recent studies have shown that mutations of the TP53 gene can confer oncogenic functions (gain of function, GOF) that are exerted in a variety of ways, ranging from enhanced proliferation in culture, increased tumorigenicity in vivo, and enhanced resistance to a variety of commonly used anticancer drugs (11-13). Indeed, tumors emerging from mutant-p53 knock-in mice display aggressive and metastatic traits that are never detected in tumors developing in a p53-deficient mice (14,15). Mutations of p53 are detectable in 15% of malignant thyroid tumors and are associated with the progression from differentiated to anaplastic carcinoma (16). Thus, p53 mutations have an effect on infiltration, lymphatic metastasis and prognosis of thyroid carcinoma (17).

Several efforts are in progress to discover efficient agents, such as small molecules, that target mutp53 in cancers, including thyroid cancers (18), both to abolish mutp53 function or to reactivate the wt53 counterparts (19). The final aim is to restore p53 oncosuppressor functions and improve tumor response to therapies. We recently showed that Zn(II) supplementation in several cancer cells carrying mutp53, including colon, breast, and glioblastoma, restores a folded conformation from mutp53 misfolding, rescuing wt53/DNA-binding and transactivation (20-22). The rationale to use Zn(II) comes from the fact that p53 protein includes one zinc ion (Zn2+) as an important cofactor for the proper protein folding to efficiently bind the DNA of sequence-specific target genes (23). Interestingly, mutp53 proteins are prone to the loss of the Zn(II) bound to the DNA binding domain (DBD) with the
consequence of protein unfolding and aggregation with loss of wild-type activity (24), therefore, zinc supplementation has been shown to be a useful strategy to target mutp53 proteins (25,26).

In this study we asked whether zinc supplementation might affect p53 in two different thyroid cancer cell lines, one carrying p53-R273H (Arg to His at codon 273) mutation (FTC-133) that is one of the p53 mutation responsible of resistance to antitumor drugs (11) and another with wtp53 (WRO). We used a novel curcumin-based zinc compound [Zn(II)-curc] that we previously showed to reactivate mutp53 proteins (27) and to induce mutp53 degradation through autophagy (28). Here, we found that Zn(II)-curc reduced mutp53 protein levels in FTC-133 cells, reactivating the wtp53 functions with increased response to therapies. Of note, Zn(II)-curc also induced wtp53 transcriptional activity in wtp53-carrying cell line. These findings suggest that Zn(II)-curc may be a useful compound in therapeutic regimens of thyroid cancers, carrying both mutant and wtp53, that will be worth further testing for translational purpose in clinical practice.

Materials and methods

Cell culture and reagents. Human thyroid FTC-133 cell line was obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK). It derives from a lymph node metastasis of a follicular thyroid carcinoma and carries p53R273H mutation and EGFR overexpression. FTC-133 and WRO (wtp53) (ECACC) cancer cell lines were routinely maintained in DMEM-Ham's-F12 (Life Technology-Invitrogen), and 2 mmol/l L-glutamine in a humidified atmosphere with 5% CO\textsubscript{2} and 95% air at 37˚C.

For treatments the following reagents were used: Zn(II)-curc (29,30) dissolved in DMSO was added to the culture medium to a final concentration ranging between 40 and 120 µM; chemotherapeutic agent adriamycin (ADR) (Sigma), dissolved in PBS was added to the culture medium to a final concentration ranging between 0.2 and 2 µg/ml; cisplatin (Teva Pharma-Italia, Italy) was added to the culture medium at subconfluence in triplicate in 60-mm Petri dishes and 24 h later treated with Zn(II)-curc alone or combination with chemotherapeutic agents for 24 h. Both floating and adherent cells were collected and cell viability was determined by trypsin blue exclusion by direct counting with a hemocytometer, as reported (31). The percentage of cell viability, as blue/total cells, was assayed by scoring 200 cells per well three times.

Western blot analysis. Total cell extracts were prepared by incubation at 4°C for 30 min in lysis buffer [50 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 150 mmol/l KCl, 1 mmol/l diithio-
reitol, 5 mmol/l EDTA, pH 8.0, 1% Nonidet P-40] plus a mix of protease and phosphatase inhibitors (Roche Diagnostic). Cell lysates were spun at 15,000 g for 15 min to remove debris and collect the supernatant (total cell extracts). Protein concentration was then determined using BCA Protein Assay kit (Bio-Rad). Equal amount of total cell extracts were resolved according to molecular weight on 10-18% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (PVDF, Millipore). Unspecific binding sites were blocked by incubating membranes for 1 h in 0.05% Tween-20 (v/v in TBS) supplemented with 5% non-fat powdered milk or bovine serum albumin, followed by overnight incubation with the following primary antibodies: LC3B (Sigma-Aldrich), rabbit polyclonal anti-p53 (FL393), rabbit polyclonal anti-p21 and anti-p62 (Santa Cruz Biotechnology, Dallas, TX, USA), (p)-Akt (Ser473), tot-Akt (Cell Signaling Technologies, Danvers, MA, USA), polyclonal anti-EGFR antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and purified mouse anti-phospho-histone H2AX (Ser139, γH2AX) (Millipore, clone JBW301). Equal lane loading was monitored by probing membranes with antibodies specific for β-actin (Calbiochem, San Diego, CA, USA) or tubulin (Immunological Sciences, Rome, Italy). Primary antibodies were detected with appropriate horseradish peroxidase-labeled secondary antibodies (Bio-Rad, Hercules, CA, USA). Enzymatic signals were visualized using chemiluminescence (ECL Detection system, GE Healthcare, Milan, Italy).

DNA extraction and semi-quantitative RT-PCR. Cells were harvested in TRIzol reagent (Invitrogen) and total RNA was isolated following the manufacturer's instructions. The first strand cDNA was synthesized from 2 µg of total RNA with MuLV reverse transcriptase kit (Applied Biosystems, Foster City, CA, USA). Semi-quantitative RT-PCR was performed essentially as previously described (20). cDNA was synthesized from 2 µg of total RNA with MuLV reverse transcriptase kit (Applied Biosystems). Semi-quantitative reverse-transcribed (RT)-PCR was carried out by using Hot-Master Taq polymerase (Eppendorf S.r.l., Milan, Italy) with 2 µl cDNA reaction and gene-specific oligonucleotides under conditions of linear amplification. PCR products were resolved on 2% agarose gel and visualized with ethidium bromide staining using UV light. The 28S mRNA sequence was used as control for efficiency of RNA extraction and transcription. Densitometric analysis was applied to quantify mRNA levels compared to control gene expression.

Chromatin immunoprecipitation (ChIP) assay. ChIP analysis was carried out essentially as previously described (20). Briefly, cells were crosslinked with 1% formaldehyde for 10 min at room temperature and then inactivated by the addition of 125 mM glycine. Chromatin extracts containing DNA fragments with an average size of 500 bp were incubated overnight at 4°C with milk, shaking using polyclonal anti-p53 antibody (FL393, Santa Cruz Biotechnology). Before use, protein G (Pierce) was blocked with 1 µg/µl sheared herring sperm DNA and 1 µg/µl BSA for 3 h at 4°C and then incubated with chromatin and antibody for 2 h at 4°C. PCR was performed with Hot-Master Taq (Eppendorf) using 2 µl of immunoprecipitated DNA and promoter-specific primers spanning p53 binding sites.
Immunoprecipitation with non-specific immunoglobulins (IgG, Santa Cruz Biotechnology) was performed as negative controls. PCR products were resolved on 2% agarose gel and visualized by ethidium bromide staining using UV light.

Statistical analysis. Each experiment, unless otherwise specified, was performed at least three times, and data are presented as the mean ± SD. Statistical significance was determined using Student's t-test and a P-value of ≤0.05 was considered statistically significant.

Results

Zn(II)-curc induces autophagy and mutp53 downregulation in FTC-133 cells. We previously reported that Zn(II)-curc induces mutp53 degradation through autophagy in breast cancer cells (28). As defects in autophagy may be found in cancer, we evaluated whether Zn-curc could induce autophagy in FTC-133 thyroid cancer cells. We evaluated the expression of microtubule-associated protein light chain 3 (LC3) protein that, after conversion from LC3-I to its autophagosome membrane-associated lipidated form LC3-II, is considered a cellular readout of autophagy, and the expression levels of p62, a bona fide autophagic substrate (32). As shown in Fig. 1, Zn(II)-curc increased LC3-II expression and reduced p62 levels; parallel to induction of autophagy, Zn(II)-curc triggered mutp53 downregulation in FTC-133 cells (Fig. 1). The use of chemical inhibitors of autophagic/lysosomal degradation cloroquine (CQ) (32) prevented Zn(II)-curc-induced mutp53 degradation (Fig. 1). These findings indicate that Zn(II)-curc induced mutp53 degradation in FTC-133 cells, carrying p52R273H mutation, likely through autophagy.

Zn(II)-curc restores wtp53/DNA binding and target gene transcription in FTC-133 thyroid cancer cells. We then evaluated whether Zn(II)-curc was promoting reactivation of wtp53 function in FTC-133 cells. We first performed p53/DNA binding by ChIP analyses and found that Zn(II)-curc treatment indeed restored the wtp53 binding to several specific target promoters, including Puma, p53AIP1, MDM2, and p21, while abolished the p53 binding to the mutant p53 target promoter MDR1 (multi-drug resistance 1) (Fig. 2A). As a consequence, the expression of the canonical wtp53 target genes was evaluated by reverse transcription polymerase chain reaction (RT-PCR) analyses. FTC-133 cells were exposed to increasing doses of Zn-curc (40, 80 and 120 µM for 24 h) that...
induced expression of the typical p53 target genes already at the lowest dose used, while reduced the expression of mutp53 target MDR1 expression starting from 80 µM dose (Fig. 2B). These findings indicate that Zn(II)-curc produced an imbalance between misfolded/folded p53 proteins in FTC-133 cells that favoured wild-type over mutant p53 functions.

Comparison between Zn(II)-curc and PRIMA-1 on p53 activity. PRIMA-1 is a low molecular weight compound with antitumor activity that has been shown to be more effective in inducing apoptosis in mutp53 cells than in wtp53 cells (33). We then compared the p53 reactivating effect of Zn(II)-curc with that of PRIMA-1 in both FTC-133 (mutp53) and WRO (wt53) cells. In vivo transcription of endogenous p53 target genes was examined by employing RT-PCR analysis. The results show that the induction of wt53 target genes such as Bax, Noxa, Puma and downregulation of the mutp53 target gene MDR1 was comparably and efficiently achieved by both treatments, that is, Zn(II)-curc and PRIMA-1 (Fig. 3A); of note, Zn(II)-curc induced also the wt53 target, cell cycle-related p21 gene, unlike PRIMA-1 (Fig. 3A). This latter result is in agreement with PRIMA-1 pro-apoptotic specific function (33). Interestingly, Zn(II)-curc, differently from PRIMA-1, induced p53 target genes in WRO cells, carrying wt53 (Fig. 3B), likely due to the DNA intercalating ability of Zn(II)-curc compound sensing the DNA damage response (29). Thus, Zn-curc induced H2AX phosphorylation (γH2AX) (Fig. 3C) that is a marker to detect the genotoxic effect of different anticancer agents (34).

Figure 3. Comparison between Zn(II)-curc and PRIMA-1 on p53 activity. (A) FTC-133 and (B) WRO cells were treated with Zn(II)-curc (80 µM) and PRIMA-1 (10 µM) for 24 h before total mRNAs were reverse transcribed for analysis of p53 target genes, using RT-PCR. 28S was used as a control for efficiency of RNA extraction and transcription. One representative experiment, out of two independent experiments with similar results, is shown. (C) WRO cells were treated with Zn(II)-curc (80 µM) for 24 h. Equal amount of total cell extracts were subjected to immunoblot analysis with anti-γH2AX (phospho-Ser139) antibody. Anti-β-actin was used as protein loading control.

That is a marker to detect the genotoxic effect of different anticancer agents (34).

Zn(II)-curc modifies prosurvival molecular pathways in FTC-133 cells. P53H273 mutation has been shown to increase epidermal growth factor receptor (EGFR) levels as well as EGFR/PI3K/Akt signaling pathway, facilitating cell proliferation, tumor growth and migration (35). EGFR overexpression is described in several thyroid carcinomas, including follicular thyroid carcinomas (36), and recent evidence implicates EGFR overexpression in advanced thyroid carcinoma progression (37). Therefore, we evaluated the effect of Zn(II)-curc on EGFR and Akt expression levels in FTC-133 cells. Western blot analyses show that the strong expression of EGFR was markedly reduced by Zn(II)-curc (Fig. 4A); similarly, Akt phosphorylation was noticeably inhibited by Zn(II)-curc treatment (Fig. 4B). Moreover, p53 mutants have been shown to regulate E-cadherin expression (38). Downregulation of the E-cadherin cell-cell adhesion molecule is a key event for epithelial to mesenchymal transition (EMT) in tumor progression and it is associated with the development of invasive carcinoma, metastatic dissemination, and poor clinical prognosis (39). We found that Zn(II)-curc induced re-expression of E-cadherin mRNA levels in FTC-133 cells, that strongly correlated with downregulation of N-cadherin expression, a mesenchymal marker (Fig. 4C), as evidenced by densitometric analysis (Fig. 4C, lower panel) indicative of a reversion of EMT
Figure 4. Zn(II)-curc modifies prosurvival molecular pathways in FTC-133 cells. (A) Subconfluent FTC-133 cells were treated with Zn(II)-curc (80 µM) for 16 and 24 h. Equal amount of total cell lysates were subjected to immunoblot analysis to detect p-Akt and total-Akt. Anti-β-actin was used as protein loading control. (B) Cells treated as in (A) for 24 h were subjected to immunoblot analysis to detect EGFR expression levels. Anti-β-actin was used as protein loading control. One representative experiment, out of two independent experiments with similar results, is shown. (C) FTC-133 cells were treated with Zn(II)-curc (80 µM) for 24 h before total mRNAs were reverse transcribed for analysis of E-cadherin and N-cadherin gene expression, using RT-PCR. 28S was used as a control for efficiency of RNA extraction and transcription. The lower panel represents densitometric analysis of gene expression, plotted as expression ratio to 28S. The data represent the mean of two independent experiments ± SD.

Figure 5. Zn(II)-curc increases the cytotoxic effect of drugs. FTC-133 (A) and WRO (B) cells (2x10^5) were plated at subconfluence in 60-mm Petri dish and the day after treated with ADR (0.2 and 2 µg/ml) and cisplatin (cis, 0.5 and 5 µg/ml) alone or in combination with Zn(II)-curc (80 µM) for 24 h. Cell death was measured by trypan blue exclusion assay and expressed as percentage ± SD of three independent experiments performed in duplicate. Paired Student's t-test was used for statistical analysis of comparison between the values of drugs alone with that of drugs in combination with ZnCl₂ treatments. *p<0.01. (C) FTC-133 and WRO cells were plated as in (A) and (B) and treated with ADR (0.2 µg/ml) and cisplatin (cis, 0.5 µg/ml) alone or in combination with PRIMA-1 (10 µM) for 24 h. Cell death was measured by trypan blue exclusion assay and expressed as percentage ± SD. *p<0.01.
phenotype. Altogether, these findings indicate that Zn(II)-curc could modify prosurvival pathways and counteract mutp53-regulated pathways responsible of GOF.

**Zn(II)-curc increases the cytotoxic effect of drugs.** In order to study the biological effect of Zn(II)-curc we treated FTC-133 and WRO cells with Zn(II)-curc alone or combination with cisplatin (cis) or adriamycin (ADR) and assessed viability using trypan blue staining. We found that the weak antitumor effect achieved by two different doses (0.2 and 2 µg/ml) of ADR in FTC-133 cells was significantly increased by Zn(II)-curc co-treatment (Fig. 5A). Similarly, cell death induced by both low (0.5 µg/ml) or high (5 µg/ml) dose of cisplatin was increased by Zn(II)-curc co-treatment, although FTC-133 resulted more sensitive to high dose of cisplatin (5 µg/ml) (Fig. 5A); in agreement with the activation of wtp53 target genes, seen in Fig. 3B, Zn(II)-curc increased the cytotoxic effect of cisplatin and ADR also in this wtp53-carrying cell line (Fig. 5B). In comparison, PRIMA-1 increased chemosensitivity of mutp53 FTC133 cells, while did not affect the response to drugs of wtp53 WRO cells (Fig. 5C). Altogether, these results show that Zn(II)-curc enhanced the cytotoxic effect of chemotherapeutic drugs in both mutant and wild-type-carrying cancer cells, unlike PRIMA-1 that was effective only in mutp53 cells.

**Discussion**

In this study we found that Zn(II)-curc, a mutant p53 reactivator, was able to downregulate mutp53 in FTC-133 (p53H273) thyroid cancer cells restoring the wtp53 functions and improving cancer cell response to antitumor drugs. In addition, Zn(II)-curc was also able to activate endogenous wtp53 in wtp53 cells WRO.

Several studies have shown that restoration of wild-type p53 activity in vitro (40) and in mice (41-43) induces rapid tumor regression, demonstrating that reconstitution of p53 is indeed sufficient for elimination of tumors even in the presence of other tumor-associated genetic alterations (41-43). Over the past years several strategies have been approached to develop p53-targeted drugs, and in particular to develop small molecule compounds that activate wtp53 and restore wtp53 function of mutant p53 proteins (44), as abrogation of mutp53 has been shown to reduce tumor malignancy (45). Thus, some mutp53 proteins such as p53H273 have been shown to acquire gain of function (GOF) that actively contributes to cancer development and progression and chemoresistance (11). At the molecular levels GOF effects were shown to be linked to the ability of mutp53 to increase the expression of several genes including **MDR1** (multi-drug resistance protein). Moreover, p53H273 mutation has been shown to increase epidermal growth factor receptor (EGFR) levels as well as EGFR/Pi3K/Akt signaling pathway, facilitating cell proliferation and tumor growth (35). Here, we found that Zn(II)-curc was able to downregulate mutp53 protein levels. In agreement with the loss of mutant p53 protein, Zn(II)-curc reduced EGFR expression and Akt phosphorylation as well as induced reversion of EMT, suggesting modulation of mutp53-dependent gain of function pathways. The reversion of EMT by Zn(II)-curc is particularly intriguing because EMT induction has been shown to promote cancer stem cell (CSC) traits in the thyroid cancer cells, with the potential to induce cancer stem cell generation and tumor progression in thyroid cancers (46). The effect of Zn(II)-curc on mutp53 downregulation depended in part on induction of autophagy, as previously reported (28). Autophagy plays a dual role during cancer development, progression and treatment, mainly depending on the type and stage of the tumor. The induction of autophagy by Zn(II)-curc is interesting in light of studies showing that, in thyroid cancer, autophagy activation increases chemosensitivity, enhances the iodine uptake ability, and reverses the dedifferentiation phenotype (47). Parallel to mutp53 downregulation, Zn(II)-curc reactivated the wild-type p53 functions, likely through the imbalance of misfolded/folded protein conformation. These molecular changes correlated with improvement, at biological level, of the cytotoxic effect of chemotherapeutic drugs, increasing thyroid cancer cell death.

The presence of p53 mutations is a late event in thyroid cancer progression. The mutations of p53 or increased expression of p53 protein is associated with the progression from differentiated to anaplastic carcinoma and is implicated in chemoresistance (17). Recently, some attention has been focused on Zinc compounds for p53 reactivation (48). In this regard, compounds that can both promote mutp53 degradation and reactivation of wtp53 functions may have therapeutic advantages, as is the case with Zn(II)-curc. To strengthen the role of Zn(II)-curc as mutp53 reactivator, the effect of Zn(II)-curc was compared to that of PRIMA-1, a low molecular weight compound that reactivates mutp53 and that has been shown to be more effective in inducing apoptosis in mutp53 cells than in wtp53 cells (33). Such compound has been shown to induce cell death in thyroid cancer cells carrying p53 mutations and particularly p53H273 mutation (18). Of note, Zn(II)-curc restored wtp53 activity similarly to PRIMA-1 in FTC-133 cells and increased the cytotoxic effect of drugs. In addition, Zn(II)-curc was able to activate endogenous wtp53 in WRO cells, unlike PRIMA-1 that did not have such effect on wtp53 protein.

The classic treatment of thyroid cancer is total thyroidectomy, followed by, in some cases, radioiodine treatment. Surgically inoperable and radioiodine-refractory differentiated thyroid cancers, poorly differentiated thyroid cancer, and anaplastic thyroid cancer are currently the major causes of deaths related to thyroid cancer and do not have effective treatments (3-6). In this regard, molecular-based management strategies for thyroid nodules and thyroid cancer are a novel and intense area of development in molecular thyroid-cancer medicine (49). In line with this strategy, the present findings, although preliminary due to the small number of cell lines analyzed, suggest that Zn(II)-curc may be a useful adjuvant in the treatment of thyroid cancer cells carrying both both mutant and wild-type p53 proteins. Thus, although well differentiated thyroid carcinomas are efficiently cured by surgery and radioiodine, undifferentiated thyroid carcinomas (10-20% of total) are very resistant to chemotherapy and the restoration of chemosensitivity, through p53 reactivation for instance, may be an efficient antitumor strategy. The use of Zn(II)-curc in clinical practice is strengthen also by our previous studies showing other biological effects of zinc supplementation such as restoration of immune response (50) and inhibition of hypoxia-induced molecular pathways (51,52). This is...
particularly relevant because hypoxia-inducible factor is often overexpressed in thyroid cancer contributing to chemoresistance and tumor progression (53). In earlier observations Zn(II) has been found to have cytotoxic effect on thyroid cancer cell lines (55), although the molecular mechanisms were not fully elucidated. Here we propose the use of Zn(II)-curc as p53 (re) activator in therapeutic regimens of thyroid cancers, carrying both mutant and wt p53, that will be worth further testing for translational purpose in clinical practice.

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