The Thioxotriazole Copper(II) Complex A0 Induces Endoplasmic Reticulum Stress and Paraptotic Death in Human Cancer Cells

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The copper(II) complex A0 induces a type of non-apoptotic cell death also known as paraptosis. Paraptosis involves extensive endoplasmic reticulum vacuolization in the absence of caspase activation. A wide panel of human cancer cell lines was used to demonstrate differences in cytotoxicity by the parapto-

sis-inducing drug A0 and the metal-based pro-apoptotic drug cisplatin. Gene expression profiling of the human fibrosarcoma HT1080 cells showed that, while cisplatin induced p53 targets, A0 up-regulated genes involved in the unfolded protein response (UPR) and response to heavy metals. The cytotoxic effects of A0 were associated with inhibition of the ubiquitin-proteasome system and accumulation of ubiquitylated pro-

teins, in a manner dependent on protein synthesis. Cyclohexi-
mide inhibited the accumulation of ubiquitylated proteins and hampered A0-induced cell death process. The occurrence of the UPR during A0-induced death process was shown by the increased abundance of spliced XBP1 mRNA, transient eIF2α phos-

phorylation, and a series of downstream events, including attenuation of global protein synthesis and increased expression of ATF4, CHOP, BIP, and GADD34. Mouse embryonic fibro-

blasts expressing a mutant eIF2α, which could not be phospho-

rylated, were more resistant to A0 than wild type cells, pointing to a pro-death role of eIF2α phosphorylation. A0 may thus rep-

resent the prototypical member of a new class of compounds that cause paraptotic cell death via mechanisms involving eIF2α phosphorylation and the UPR.

Advancement in cancer therapy requires a better understanding of why and how cancer cells are induced to die. Although in the past apoptosis was considered the only way to kill cancer cells, the role of other types of cell death has been increasingly recognized in the tumor response to therapy (see for reviews, Refs. 1, 2). Cisplatin is the most widely used anti-
cancer drug and causes cell death by inducing apoptosis. Nev-

ertheless, the high rate of resistance observed during therapy with cisplatin, as well as the occurrence of non-sensitive cancer cells, prompt the quest for agents endowed with apoptosis-independent mechanisms of action. Moreover, therapies based on the induction of non-apoptotic cell deaths may represent a promising approach to suppress the multidrug-resistant phe-

notype often associated with resistance to apoptosis.

A non-apoptotic cell death, characterized by a specific cellu-

lar morphology, was observed during embryo development or neuronal degeneration (3, 4). This death process is hallmarked by massive cytoplasmic vacuolization and is known as type III cell death (4) or paraptosis (5). Several studies have described paraptotic-like cell death processes in various models (6–14), but the underlying molecular mechanisms have remained elu-

usive thus far.

Studies from our laboratory have identified A0 as the most active thioxotriazole copper(II) complex among a number of triazole-metal based compounds screened for their cytotoxicity in human cancer cells (15–17). Although A0 and cisplatin have comparable potencies in HT1080 human fibrosarcoma cells, the latter induces typical caspase-dependent apoptosis, while A0 inhibits caspase-3 activity and elicits a death process lacking the typical features of apoptosis (18). In contrast, huge vacuoles, derived from the dilatation of the endoplasmic reticulum (ER), are the most evident features of A0-induced cell death, consist-

ently with the induction of a paraptotic process. A possible explanation is that A0, by inhibiting caspase-3 (18), impairs the execution of the apoptotic program, thus addressing the cells to alternative death pathways. Interestingly, recent evidence sug-

gests that paraptosis becomes preponderant when apoptosis executors are somehow inhibited (19).

A0 induces copper overload leading to an increase of the cellular oxidized glutathione (16). Moreover, it has been re-

cently demonstrated that other copper complexes inhibit pro-

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teasome activity in cancer cells both in vivo and in vitro, indicating that the metal may work as a novel anticancer agent through the accumulation of misfolded proteins (20–22). Despite these phenotypic data, very few molecular studies have been aimed to a better comprehension of copper-induced cell death in cancer cells (23). However, it has been recently shown that copper induces genes involved in the ubiquitin-proteasome system and in the oxidative stress response (24). In another study, copper complexes, other than A0, have been demonstrated to cause a cell death process with paraptotic features (25).

ER-derived cytoplasmic vacuolization, the hallmark of paraptotic-like cell death, is increasingly recognized as a phenotype indicating a perturbed functional link between ER and the proteasome. Indeed, excluding those of clearly autophagic origin, giant vacuoles derived from the ER are described under conditions such as (i) treatment with proteasome inhibitors (26, 27) or inhibitors of HSP90 (28); (ii) silencing of crucial components of the endoplasmic reticulum-associated degradation (ERAD) system, such as the protein VCP/p97 (29, 30); and (iii) the expression of mutant oncoproteins that induce an ER-associated unfolding protein response (31).

In this work, we have compared the differences in sensitivity of cancer cells to A0 and cisplatin, and investigated the mechanism of action of A0, showing a specific transcriptional and translational cellular response that underlie paraptotic cell death.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—The cell human cancer cell lines Caco-2, Calu-3, HOS, HT1080, HT29, PANC-1, RD, A375, A431, A549, CAPAN-1, CFPAC-1, HeLa, HepG2, Hep2, U2-OS, SAOS-2, SW872, MCF-7, MCF-7/DX, SH-SY5Y, PNT1A, PC3, 2008, C13*, and the mouse embryonal fibroblasts S/S and A/A were obtained and cultured as described under supplemental methods. HT1080PTR were established in our laboratory treating continuously HT1080 parental cells with increasing doses of cisplatin until a cell population able to grow in the presence of 10 μM of the drug was obtained. The procedure for HT1080PTR selection is detailed under supplemental methods.

Drugs and Compounds—The A0 (cis- [CuCl2(H2L)]Cl) copper(II) complex of HL (4-amino-1,4-dihydro-3-(2-pyridyl)-5-thiooxo-1,2,4-triazole) was synthesized as described previously (15). A0 was freshly dissolved in dimethyl sulfoxide (DMSO) at a concentration of 25 mM before each experiment. Cisplatin was dissolved at a concentration of 1 mM in 0.154 M NaCl solution and frozen at −20 °C. To complex metals with HL ligand, a 50 mM solution in DMSO was prepared for the ligand and for each metal. An equal volume of the ligand and metal solution were mixed to obtain a 1000 μM of the drug was obtained. The procedure for HT1080PTR selection is detailed under supplemental methods.

Caspase inhibitors (Sample Pack from R&D Systems) were dissolved 10 mM in DMSO and used at a final concentration of 50 μM. The inhibitors are abbreviated as followed: Z-VAD-FMK, general caspase inhibitor; pan: Z-WEHD-FMK, caspase-1 inhibitor: 1; Z-VDVAD-FMK, caspase-2 inhibitor: 2; Z-DEVD-FMK, caspase-3 inhibitor: 3; Z-YVAD-FMK, caspase-4 inhibitor: 4; Z-VEID-FMK, caspase-6 inhibitor: 6; Z-IETD-FMK, caspase-8 inhibitor: 8; Z-LEHD-FMK, caspase-9 inhibitor: 9; Z-AEVDFMK, caspase-10 inhibitor: 10; Z-LEED-FMK, caspase-13 inhibitor: 13. The fluorogenic proteasome substrate Suc-Leu-Leu-Val-Tyr-AMC was purchased by Alexis.

Cell Viability Assay—Cell viability was assessed with the resazurin assay as previously reported (18). See supplemental methods for the detailed procedure. Briefly, cells were seeded in multiwell plates 24 h before treatment. Cells were treated with drugs at the selected concentration for 48 h. At the end of the treatment cells were incubated in a resazurin solution and fluorescence recorded. Cytotoxicity was expressed as percent of control, and the value calculated according to the equation reported in supplemental methods. Dose response curves were fitted with nonlinear regression. IC50 values were calculated with GraphPad Prism 4.0TM.

Colony-forming Assay—HT1080 and HT1080PTR cells were seeded in 6-well plates at a density of 150 cells/cm2 and immediately treated with the indicated concentration of cisplatin. Cells were incubated for 8 days renewing medium every 3 days. Colonies were then washed with phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde, and stained with a 0.5% solution (w/v) of methylene blue in PBS supplemented with 1% ethanol. Digital images of colonies were acquired with a Nikon DS5MC digital camera and colonies greater than 100 μm were visually counted.

RNA Extraction for Transcriptional Profiling—1 × 106 cells were seeded in 100-mm culture dishes. After 24 h, the cultures were incubated in normal growth medium (12 h) or in the presence of 25 μM A0 (1, 3, 6, 12 h) or 25 μM cisplatin (3, 6, 12 h). At the end of the treatments, cells and culture medium were collected, washed with PBS, and total RNA was extracted and processed as reported in supplemental methods.

Microarray Data Analysis—Raw microarray data were processed for summarization with the BeadStudio software (v. 1.5.1, 3), with Rank-Invariant Normalization and filtering by Detection. If the detection value for both samples in at least one replicate was higher than 0.95, the probe was kept for further analysis. Statistical analysis of the processed data were conducted by performing a Dunnett’s t test (32) for multiple comparisons between each treatment condition and the untreated control. 749 probes, corresponding to 734 genes, emerged for being regulated by A0 and/or cisplatin at least one time point with a false discovery rate below 5%, a minimum log2 ratio of 0.7 and an α of 1.5 (supplemental Table S2). The statistical search for genes differentially regulated genes by A0 and cisplatin (supplemental Table S3) is described under supplemental methods. Gene displaying similar expression patterns were clustered using the FLAME algorithm implemented in the GEDAS software (33).

To interrogate the connectivity map data base, gene symbols from gene expression were mapped to HGU-133A probes IDs according to Biomart Ensembl release 47 and redundant probes were filtered out. Profiles for up-regulated
and down-regulated genes were analyzed with the connectivity map resource.

**qRT Polymerase Chain Reaction**—Total RNA, obtained from cells treated as described above for microarray analysis, were purified with the RNeasy Mini Kit (Qiagen S.p.a., Milano, Italy). Reverse transcription and real time quantitative PCR were performed as described under supplemental methods. The primers used are reported in supplemental Table S4. Relative quantitative evaluation of transcript levels was calculated by comparing ΔCt normalized on GAPDH transcript levels.

**Western Blot**—Cells grown to subconfluence were incubated in the absence or in the presence of A0 or cisplatin at the indicated concentrations. Culture medium was collected together with the cells and samples processed as described under supplemental methods. Briefly, 30 μg of protein were loaded on 10% or 15% gels for SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane in a 10% blocking solution (Roche Applied Science). Blots were exposed overnight at 4 °C to the antibodies diluted in the blocking solution. See supplemental methods for antibodies suppliers and concentration used. Membranes were exposed to anti-mouse or anti-rabbit horseradish peroxidase-conjugated Western blotting detection reagent (Exacta-Cruz, Santa Cruz Biotechnology). The blots were visualized using enhanced chemiluminescence (Amersham Biosciences).

**Fluorometric Proteasome Activity Assay**—HT1080 cells were collected in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM dithiothreitol). The lysates were centrifuged at 14,000 x g for 10 min, and protein content was determined in the supernatant. For the evaluation of the chymotrypsin-like activity, aliquots of 30 μg were incubated for 15 min at 37 °C in 100 μl of 50 mM Tris-HCl, pH 7.5, with 20 μM fluorogenic substrate (Suc-Leu-Leu-Val-Tyr-AMC) in the absence or in the presence of A0, CuCl2, or MG132. After incubation, the AMC release was measured (excitation 355 nm, emission 460 nm) using a Wallac 1420 Victor2 Multilabel Counter.

**Protein Synthesis**—On the day before the experiment, 1 x 10^5 cells/well were seeded in a 6-well plate. After treatments, stand-
ard culture medium was replaced with MEM without serum, leucine, methionine, and lysine, supplemented with 1-[4,5-3H]leucine (5 μCi/ml, Amersham Biosciences). After 30 min, cells were harvested by trypsinization, added to the respective collected medium, centrifuged, and incubated in ice cold 5% trichloroacetic acid. Pellets were washed twice with trichloroacetic acid and proteins suspended in 5% sodium-deoxycholate in 1 N NaOH. Scintillation fluid was added to the protein solution and the incorporated radioactivity counted with a scintillation spectrometer (Wallac Microbeta Trilux counter). In parallel, aliquots were used for Lowry protein determination. Two-tailed Student’s t test for unpaired samples was used for statistical analysis.

**Immunofluorescence**—8 × 10^4 cells were seeded on chamber slides and grown to subconfluence. Cells were left untreated or treated with 25 μM A0 for 12 h. Cells were then fixed in 3.7% paraformaldehyde, permeabilized with methanol, and blocked with 5% anti-goat serum. The incubation with rabbit anti-calnexin antibody (1:50, Cell Signaling) was performed at 4 °C overnight. After washing, cells were incubated with 4g/μL Alexa fluor anti-rabbit antibody (1:800) and then visualized by means of Nikon Eclipse 300 inverted fluorescence microscope.

**LDH Release Assay**—HT1080 cells were seeded in a 12-well plate at a density of 2 × 10^5 cells/well. The day after cells were treated with A0 in the absence or in the presence of cycloheximide, as detailed in the legend of Fig. 4. At the end of the treatment the culture medium was collected from each well and brieﬂy centrifuged to pellet floating cells and debris. The assay was performed as described in supplemental methods.

**Cell Transfection**—See supplemental methods for detailed transfection procedure. Cells were transfected with a mixture containing FuGENE6 (Roche Applied Science) and DNA vectors encoding for wild type, S51A eIF2α, or green fluorescent protein. eIF2α S51A and WT vector were kindly provided by Dr. David Ron (NYU School of Medicine, Skirball Institute, NY). After transfection, cells were treated with A0 (20 μM) for 16 h, and viability recorded determined using the resazurin method or propidium iodide to stain dead cells. Images in phase contrast and fluorescence of 10 randomly chosen microscopic ﬁelds were taken for each condition. Fluorescent GFP- or PI-positive cells were counted on the digital images.

**RESULTS**

**Differential Sensitivities of Human Cancer Cells to A0 and Cisplatin**—Previous results from our laboratory have demonstrated that the copper(II) complex A0 causes non-apoptotic death of cancer cells (18). As shown in Fig. 1a, the cytotoxic effect of A0 in HT1080 cells is selectively mediated by copper, since other bivalent metals of the first transition series failed to induce comparable toxic effects when complexed with the A0 ligand. To assess the involvement of caspases in the A0-induced cell death, we used specific inhibitors of caspase-3 as well as of other caspases. Consistently with our previous results (18), the cytotoxic effect of A0 was not affected by any of the caspase inhibitors tested (Fig. 1b), whereas the cisplatin-induced decrease of viability was signiﬁcantly decreased. Moreover, a Western blot analysis indicated that A0 activated neither caspase-9 (Fig. 1c, upper panel) nor caspase-3 (Fig. 1c, lower panel). In contrast, both enzymes underwent activation cleavage during cisplatin treatment.

We used a panel of 23 human cancer cell lines to determine the half-maximal inhibitory concentrations (IC50) of A0 and cisplatin (Fig. 1d and supplemental Table S1). In all the cell lines tested, the cytotoxic effect of A0 was associated with the appearance of massive vacuoles, typical of type III cell death or paraptosis (4, 18) (data not shown). Neither correlation nor linear regression between A0 and cisplatin IC50 values were significant (Spearman test r = 0.29, n = 14, and r2 = 0.08), indicating that the sensitivities to A0 and cisplatin are uncorrelated. Moreover, A0 caused a comparable, dose-dependent response in the ovarian cell line 2008 in 2008 and in its subline C13*, a well-known cisplatin-resistant cell model (34) (IC50 values of 9 and 12 μM, respectively, Fig. 1e).

To conﬁrm the absence of cross-resistance to the two metal-based drugs, we used the human ﬁbrosarcoma HT1080 cells that are very sensitive to both A0 and cisplatin (18). Given that no cisplatin-resistant HT1080 cells are commercially available, we generated a cisplatin-resistant population from this cell line. After eight months of selection, we obtained a population (HT1080Ptr), which formed colonies in the presence of high cisplatin concentrations (Fig. 1, f and g). HT1080Ptr were less clonogenic than the parental cells in control medium (Fig. 1f), but were able to form colonies even in the presence of 10 μM cisplatin. The quantitative results of the assay (Fig. 1g) indicated
that the colony-forming competence of HT1080PTR cells was not significantly affected by cisplatin concentrations as high as 5 μM. Consistently, HT1080PTR cells grew more slowly than parental cells under control conditions but actively proliferated even in the presence of 10 μM cisplatin, when parental cells did not survive (Fig. 1b). The dose response curves, obtained for A0 and cisplatin in the two cell lines (Fig. 1i), yielded comparable IC₅₀ values for A0 (14 and 12 μM, for HT1080PTR and HT1080, respectively), while confirmed the difference in sensitivity to cisplatin (IC₅₀ values of 39 μM and 9 μM for HT1080PTR and HT1080 cells, respectively). HT1080PTR cells when incubated with cytotoxic concentrations of A0, exhibited the typical massive cytoplasmic vacuolization with a substantial maintenance of nuclear integrity (Fig. 1j). These data show that acquisition of resistance to cisplatin does not affect the ability of HT1080PTR cells to undergo paraptotic death when treated with A0.

A0 and Cisplatin Induce Distinct Transcriptional Responses—To characterize better the differences between the cytotoxic effects of A0 and cisplatin, we performed gene expression profiling on HT1080 cells. Cells were treated with the two drugs for the times indicated in Fig. 2, and two biological replicates were obtained for each condition. Biotinylated cRNA probes, generated from total RNA, extracted from control and treated cells, were hybridized to microarrays covering 24,000 transcripts. Statistical analysis of the microarray data highlighted 749 probes, corresponding to 734 genes, the expression of which was either increased or decreased by A0 and/or cisplatin in at least one time point (detailed under “Experimental Procedures” and supplemental methods). Notably, expression of only 74 genes was significantly changed by both drugs, suggesting distinct transcriptional effects of A0 and cisplatin. A0 produced more changes in gene expression than cisplatin (487 versus 321 significantly regulated genes, respectively). Clustering of the regulated genes using the FLAME algorithm (33) led to the definition of sixteen clusters (Fig. 2a), plus one cluster of outliers, i.e. genes that cannot be reliably assigned to any cluster. Seven clusters showed responses with opposite signs (Fig. 2a, clusters 1, 6, 7, 11, 14, 15, 17), whereas only clusters 10 and 13 displayed genes exhibiting similar responses to A0 and cisplatin. The statistical search for genes with a differential response to A0 and cisplatin yielded 386 transcripts, corresponding to 359 genes, grouped by FLAME in 5 clusters (Fig. 2b). Cluster 1 was the largest and contained 150 genes induced by A0 and not by cisplatin, while cluster 2 contained genes the expression of which was selectively decreased by A0. Clusters 3 and 4 contained genes up- or down-regulated respectively by cisplatin only. Taken together, these data indicate that the A0-driven transcriptional response was clearly different from that driven by cisplatin.

A0 but Not Cisplatin Induces ER Stress-related Genes—To evaluate the biological significance of the clusters of genes regulated by A0 or cisplatin, we performed a functional keyword enrichment analysis using the DAVID web-tool (see Ref. 35). The analysis identified clusters significantly enriched in specific functional categories (Table 1). In particular, five clusters shown in Fig. 2a (1, 5, 6, 10, 13) were found enriched in sixteen categories (Table 1, upper). Table 1, lower, reports seven categories that enrich clusters differentially regulated by A0 and cisplatin (see Fig. 2b). These categories involved “nucleolus-related genes,” which were significantly more abundant in cluster 2 (down-regulated by A0 but not by cisplatin), “negative regulators of cell proliferation” in cluster 3 (up-regulated by cisplatin but not by A0) and “regulators of GTPases” in cluster 4 (down-regulated by cisplatin but not by A0). The remaining four categories (“chaperone,” “unfolded protein binding,” “heavy metal binding,” and “copper ion binding”) were overrepresented in the cluster of genes up-regulated by A0 and not by cisplatin (Fig. 2b, cluster 1). Two categories, chaperone and unfolded protein binding, exhibited extremely high levels of significance (p < 1.0E-010).

The categories chaperone and unfolded protein binding comprehend many genes functionally related to ER stress. We validated the results obtained with microarray analysis assessing by qRT-PCR the expression of ten ER stress-related, six metal-responsive, and four p53 target genes. The data obtained by the microarray analysis on the expression of these genes were confirmed with qRT-PCR (Fig. 3a). The analyses of the single genes are shown in Fig. 3b. A0-induced genes related to endoplasmic reticulum (ER) stress, such as those for
the pro-death protein CHOP, its target TRB-3 (36), GADD34, caspase-4, but also for the pro-survival BIP and HERP proteins. All the metal responsive genes tested, representative of transcripts with known metal or antioxidant response elements in their promoters, were markedly induced during A0 treatment (HSPA6, FOS, HMOX-1, and MTX-1 were 300-, 60-, 30-, and 17-fold increased, respectively). The expression of their protein products was assessed with Western blotting (Fig. 4) and, as expected, was increased upon cell incubation with A0 but not with cisplatin. GADD34 increased progressively reaching the highest expression at 12 h of incuba-
FIGURE 4. A0 modulation of UPR in HT1080 cells. Cell extracts were obtained under the same conditions used for microarray unless otherwise indicated. a, immunoblot analysis of the expression of GADD34, CHOP, and BIP. GAPDH was used as loading control. A typical experiment repeated three times with comparable results was shown. b, analysis of XBP-1 mRNA splicing. qRT-PCR analysis was performed with primers designed to recognize selectively the spliced form of XBP-1 mRNA. Points are means ± S.D. of three independent experiments. c, detection of ubiquitylated proteins. The immunoblot analysis was performed with an anti-ubiquitin antibody. Molecular weights (kDa) were indicated, and α-tubulin was used as loading control. A typical experiment, repeated three times with comparable results, is shown. d, effect of A0 on proteasome activity. Dose response curves for the inhibitory effect of A0 and CuCl2 on the chymotrypsin-like activity were obtained incubating HT1080 cell extracts with the fluorogenic proteasome substrate in the absence (control) or in the presence of the compounds at indicated concentrations. The effect of 2.5 μM MG132 is shown for comparison. Data are means ± S.D. of two independent experiments with six replicates each. e, influence of A0 on changes in cell viability induced by the proteasome inhibitor MG132. Cells were incubated for 24 h with the indicated concentrations of MG132 in the presence (black bars) or absence (white bars) of 20 μM A0. Data are means ± S.D. of two independent experiments with six replicates each. Two-tailed Student’s t test for unpaired samples was used for statistical analysis, *, p ≤ 0.05; **, p ≤ 0.01; and ***, p ≤ 0.001. f, immunofluorescence analysis of A0 and MG132 induced vacuolization. Cells were treated for 24 h with 20 μM A0 (C and D), 1 μM MG132 (E and F), or left untreated (A and B). A, C, and E, representative phase contrast images; B, D, and F, calnexin immunostaining of the same fields. g, effect of protein synthesis inhibition on the A0-induced cell death. HT1080 cells were left untreated or treated for 24 h with A0 25 μM, cycloheximide (2 μg/ml), or both. After treatment, the activity of lactate dehydrogenase released in the medium was assessed and reported as arbitrary units. Data are means ± S.D. in a representative experiment with four replicates. The experiment was repeated three times with comparable results. Significance was assessed with a two-tailed Student’s t test for unpaired samples, ***, p ≤ 0.001. h, effect of protein synthesis inhibition on the A0-induced accumulation of ubiquitylated proteins. Cells were incubated under the same condition adopted for LDH assay. Molecular weights (kDa) are indicated and α-tubulin reported as loading control. A typical experiment, repeated three times with comparable results, is shown.
Copper Complex Induces ER Stress and Paraptotic Cell Death

...tion while CHOP and BIP increased at 1 h and decreased thereafter.

Another marker of UPR is the splicing of XBP-1 mRNA, executed by the ER-resident transmembrane protein IRE1. The spliced mRNA is then translated into an active transcription factor, qRT-PCR, performed with primers able to amplify selectively the spliced form of XBP-1 mRNA, demonstrated that during A0 treatment there was a rapid and progressive increase (Fig. 4b) of the level of the spliced XBP-1 mRNA, 8-fold higher than control at 12 h. Taken together, the analysis of transcripts and proteins expressed in A0-treated cells indicated the induction of UPR. UPR was also consistent with the time-dependent increase in the amount of polyubiquitinylated proteins observed in A0-treated (Fig. 4e) but not in cisplatin-treated cells (not shown).

To assess the hypothesis that A0 directly inhibits the proteasome, cell extracts were incubated with increasing concentrations of A0 and the chymotrypsin-like activity was measured. As shown in Fig. 4d, A0 decreased the activity in a dose-dependent way, with an IC50 of 20 μM. Consistently with the observations of other groups (20, 22), we confirmed that copper per se (CuCl2) acted as a potent inhibitor of the proteasome chymotrypsin-like activity (IC50 5 μM).

We next studied cell viability and morphology of cells treated with the inhibitor of the proteasome MG132, which causes accumulation of polyubiquitinylated proteins similarly to A0 treatment. HT1080 cells were incubated with increasing concentrations of MG132 in the absence or in the presence of 20 μM A0. The results indicated that the two drugs have an additive effect (Fig. 4e) in decreasing cell viability. Moreover the morphology of cells treated with MG132 was strikingly similar to that described for A0 and was hallmarkled by the presence of paraptotic-like cytoplasmic vacuolization (Fig. 4f, panels C and E). Interestingly, other authors observed massive vacuolization in different cancer cell models treated with MG132 (19). The vacuoles induced by either A0 or MG132 were positive for calnexin, an ER-resident protein, clearly indicating their origin from the ER (Fig. 4f, panels D and F).

To assess the role of protein synthesis in the cell death process triggered by the copper complex, we performed A0 treatment in the presence of the protein synthesis inhibitor cycloheximide. Cycloheximide effectively protected cells from the cytotoxic effect of A0 (Fig. 4g) and completely prevented the appearance of the cytoplasmic vacuoles (result not shown). Consistently, the A0-induced accumulation of polyubiquitinylated proteins was inhibited by the concomitant treatment with cycloheximide (Fig. 4h). These results, while indicated that the mechanisms of action of A0 and MG132 are, at least in part, overlapping, demonstrated that protein synthesis is required for the induction of cell death by the copper complex.

eIF2α Phosphorylation Plays a Critical Role in the A0-induced Cell Death Process—UPR induction is known to activate the eukaryotic translation initiation factor 2α kinase 3, also known as PERK (PKR (double-stranded RNA-dependent protein kinase)-like ER kinase). The translation initiation factor eIF2 catalyzes the first regulated step of protein synthesis, by delivering the initiator tRNA to the translation pre-initiation complex (37). During the UPR the phosphorylation of the α-subunit of eIF2 (eIF2α) causes the attenuation of general translation, a defensive mechanism acted by the cell during the UPR to prevent further overload of the already engulfed endoplasmic reticulum. At the same time, specific UPR mRNAs, such as that of ATF4, are preferentially increased (38). Western blot of phosphorylated and total eIF2α showed a rapid increase of phospho-eIF2α after 1 h of treatment of HT1080 cells with A0 (Fig. 5a). The amount of the phosphorylated form progressively decreased to basal values after 6 h of incubation. In contrast, the treatment with cisplatin had no effect on the level of phosphorylated eIF2α. Consistently, the protein level of the transcription factor ATF4 markedly increased during the incubation with A0, but not with cisplatin.

We next determined if A0 caused PERK activation. During A0 treatment the active phospho-PERK increased from 1 to 6 h and decreased thereafter (Fig. 5a). As a control, we confirmed that treatment of cells with dithiothreitol increased P-PERK, as previously shown (39). These data suggest the involvement of PERK in the A0-induced phosphorylation of eIF2α.

The pattern of phosphorylation of eIF2α in A0-treated cells would suggest that the initial inhibition of protein synthesis (phosphorylation of eIF2α) is followed by a translational recovery (dephosphorylation), thus allowing translation of mRNAs, which are important for the cellular response to the drug. However, in contrast to our expectations, protein synthesis, evaluated from the incorporation of L-[3H]leucine, followed a trend irrespective of the status of eIF2α and was progressively reduced during the incubation with A0 reaching the 50% of control value at 12 h (Fig. 5b). These data suggest that the decrease in protein synthesis in A0-treated cells involves also mechanisms other than eIF2α phosphorylation.

To assess directly the role of eIF2α phosphorylation in the paraptotic-like process activated by A0, we determined the effect of A0 on HT1080 cells transfected with a vector expressing a mutant form of eIF2α (S51A) that prevents the phosphorylation of the protein. As a control, HT1080 cells were transfected with a vector expressing wild-type (WT) eIF2α. To evaluate the transfection efficiency, cells were co-transfected with a plasmid encoding for GFP. After 16 h of treatment with A0 the viability of cells transfected with the S51A eIF2α was significantly higher than that recorded for cells transfected with WT eIF2α (Fig. 5c, left). Consistently, the percentage of rounded, dying GFP+ cells was higher in WT than in S51A eIF2α-transfected cells (Fig. 5c, right). These data suggest that eIF2α phosphorylation promotes cell death of A0-treated cells.

To dissect the actual contribution of eIF2α phosphorylation to the A0-induced UPR mouse embryonic fibroblasts (MEFs) homozygous for the mutation S51A eIF2α (A/A) and the wild-type counterpart (S/S) were used. We first evaluated if A0 treatment was associated with phosphorylation of eIF2α in wild-type S/S MEFs. The results documented that A0 elicited a transient increase in the level of phospho-eIF2α (Fig. 5d). To clarify the dependence on eIF2α status of the rate of protein synthesis, 1-leucine incorporation was monitored in S/S and A/A cells incubated for different times with 80 μM A0 (Fig. 5e). A0 progressively inhibited protein synthesis in both cell models with S/S cells exhibiting a faster and larger effect. At 6 h of treatment, protein synthesis was inhibited by more than 70% in
S/S phosphorylation-competent cells but substantially unaffected in A/A eIF2α-mutant cells. At 24h of treatment, leucine incorporation was inhibited by 78% in S/S cells but only by 46% in A/A cells. Thus, as observed in HT1080 cells, eIF2α dephosphorylation was not mirrored by resumption in the general translation rating. To assess if the phosphorylation could modulate the abundance of pro-death proteins, a Western blot for CHOP was performed in S/S and A/A MEFs. As shown in Fig. 5f, a marked induction of CHOP was found in S/S cells but not in the counterpart unable to phosphorylate eIF2α. A dose
Copper Complex Induces ER Stress and Paraptotic Cell Death

response experiment (Fig. 5g) showed that A0 produced 100% cytotoxicity in eIF2α-wild type S/S cells (IC₅₀ of 70 µM), while, in eIF2α-mutant A/A cells, the maximal effect was lower than 60% and the IC₅₀ rose to 105 µM. The different sensitivity to A0 of A/A and S/S cells was confirmed by the observation of cultures in phase contrast microscopy. When incubated with 80 µM A0, A/A cell culture was substantially spared (Fig. 5h, left), while a marked cytotoxic effect was evident in S/S cells (Fig. 5h, middle), which, moreover, showed the typical paraptotic vacuolization (Fig. 5h, right).

**DISCUSSION**

In the present study we demonstrate that the thioxotriazole copper(II) complex A0, but not cisplatin, induces paraptotic-like cell death with the concomitant induction of the UPR. We reached this conclusion comparing the toxicity of the two drugs in a panel of human cancer cells. In contrast with the caspase-dependent apoptosis induced by cisplatin, the present results confirm that the death process triggered by A0 was caspase-independent and characterized by a massive vacuolization of the endoplasmic reticulum. Gene expression profiles and morphological characteristics of the treated cells indicated that the responses to the two drugs were different, thus supporting the hypothesis of distinct mechanisms of action. Moreover, no cross-resistance between A0 and cisplatin was found in two pairs of cisplatin-resistant/sensitive cell lines, the C132/2008 cells (34) and the newly established HT1080PTRA/H1080 lines.

Evidence that A0 and cisplatin act through different mechanisms was obtained with microarray analysis performed on HT1080 cells treated with either drug. While the analysis confirmed that cisplatin induces the expression of p53 target genes to block the cell cycle (i.e. CDKN1A, BTG2) and to trigger apoptosis (i.e. p5313) (40), A0 was found to induce a distinct and much broader transcriptional response. The two major categories which were up-regulated by A0, and unaffected by cisplatin, were ER stress-related genes and genes regulated by metals. The induction of metal-response genes paralleled the accumulation of large amounts of copper upon incubation with the complex (16). On the other hand, the induction of ER stress-related genes is a novel finding of this study and could represent the molecular basis of the observed paraptotic-like morphology.

ER dilatation and vacuolization are processes that define type III or paraptotic-like cell death and, recently, a relationship has been observed between this type of cell death and the ER stress induced by proteasome inhibitors (19). Consistently, we show here that in HT1080 cells A0 and MG132 induce very similar morphological alterations, hallmarked by ER-derived vacuolization, and have an additive cytotoxic effect. The ER dilatation induced by A0 was paralleled by a strong increase in the levels of cytoplasmic heat shock proteins together with ER-resident chaperones (i.e. BIP, DNAJB9) and other markers of ER stress such as CHOP, GADD34, and HERP. A further link between A0-induced paraptotic-like cell death, and the UPR was obtained from the connectivity map resource (41). Indeed, the sets of genes which were up or down regulated only by A0 and not by cisplatin (extracted from clusters 1 and 2 of the differential response set, see Fig. 2h) showed high similarity scores with the responses to well known UPR inducers and proteasome inhibitors such as, geldanamycin, 17-allylamino-geldanamycin, and MG-132 (29, 42, 43). Interestingly the highest similarity score was obtained for 15-α prostaglandin J2 that is most recently been demonstrated to induce a paraptotic-like cell death (44), strikingly similar to that triggered by A0. Moreover, a microarray analysis, performed on cancer cells treated with a putative proteasome inhibitor (45), identified a set of 28 up-regulated genes, 21 of which were also up-regulated by A0. These observations, together with the ability of A0 to increase the amount of polyubiquitinated proteins, prompted us to assess if A0 directly inhibits the proteasome activity. The results (Fig. 4d) demonstrate that the copper complex significantly inhibits the chymotrypsin-like activity of proteasomes. However, despite the partially overlapping activities of A0 and proteasome inhibitors, only the latter execute cell death activating the caspases (46) while the copper complex inhibits caspase-3 (18) and triggers a caspase-independent death program (Fig. 1, b and c). Therefore, the paraptotic-like cell death may be a backup cell death pathway which takes place when a critical amount of unfolded proteins is reached and, concomitantly, the
apoptotic machinery is hindered. This hypothesis is consistent with information presented in other reports (19).

Several of the results presented here point to the involvement of the UPR in A0-induced cell death: (i) A0 treatment increased the amount of unfolded poly-ubiquitinated proteins; (ii) the suppression of protein synthesis by the translation inhibitor cycloheximide prevented the accumulation of misfolded proteins and decreased the cytotoxicity of the copper complex; (iii) the spliced form of XBP1 mRNA remained overexpressed throughout the treatment with A0, pointing to the persistent activation of the IRE-1 branch of the UPR; (iv) the PERK branch of the UPR pathway was transiently activated in agreement with the rapid increase in the phosphorylated forms of PERK and eIF2α.

Through the attenuation of global protein synthesis and the simultaneous, selective translation of downstream proteins in the UPR pathway, eIF2α phosphorylation may play either pro-death or pro-life roles, depending upon the cell model and the experimental conditions adopted (47). For example, as far as cells undergoing ER stress are concerned, a phosphorylation-incompetent eIF2α protects cells from cell death induced by proteasome inhibitors (48), while sensitizes cells to tunicamycin-dependent ER stress (49). In A0-treated cells, both effects were observed, with a persistent inhibition of protein synthesis and the induction of ATF4, BIP, the pro-death protein CHOP and GADD34. In particular, the progressive decrease of P-eIF2α levels mirrored the induction of GADD34, a protein that promotes the dephosphorylation of eIF2α and, hence, constitutes a feedback loop for the translational recovery after ER stress (50). Unexpectedly, in A0-treated cells eIF2α-dephosphorylation was not associated with the rescue of protein synthesis (compare panels a versus b and panels d versus e of Fig. 5). Moreover, the attenuation of protein synthesis is also observed in A/A cells (Fig. 5e). These results demonstrate that A0-dependent inhibition of global protein synthesis cannot be entirely attributed to eIF2α phosphorylation.

To address the role of eIF2α dephosphorylation in A0-induced cell death we used salubrinal, an inhibitor of the PP1/GADD34 complex (51). The inhibitor delayed eIF2α dephosphorylation in A0-treated cells (not shown) but, unfortunately, also lowered the viability of HT1080 cells in the absence of A0.
Copper Complex Induces ER Stress and Paraptotic Cell Death

(data not shown) and, hence, could not be used to assess the role of eIF2α phosphorylation in the cell death process induced by the copper complex. Instead, the role of eIF2α phosphorylation was directly addressed transfecting HT1080 cells with a vector encoding for non-phosphorylatable (S51A) eIF2α mutant. The results showed that the mutant eIF2α exerts a partial protection on the cytotoxic effects of A0. An additional proof of the pro-death role of eIF2α phosphorylation was obtained using A/A MEFs, which are homozygous for the eIF2α S51A mutation and, hence, unable to phosphorylate eIF2α (49). The results demonstrated that the sensitivity of A/A MEFs to the copper complex is reduced, thus indicating that eIF2α phosphorylation plays a pro-death role in A0-treated cells. Although the mechanism of this action is not known, it is likely to involve the translation of mRNAs encoding for pro-death proteins.

We propose a model for the mechanism of action of A0 (Fig. 6), based on both the experimental evidence presented here and data from the literature. According to this model, the treatment of sensitive cancer cells with the copper complex induces ER stress and, at the same time, to inhibit caspase-3 (18) which, in turn, exerts a partial pro-death arm of the UPR. The ability of A0 to induce a dramatic ER stress and, at the same time, to inhibit caspase-3 (18) forces the cell toward a paraptotic-like death.

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