Translational Downregulation of HSP90 Expression by Iron Chelators in Neuroblastoma Cells

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ABSTRACT

Iron is an essential cellular nutrient, being a critical cofactor of several proteins involved in cell growth and replication. Compared with normal cells, neoplastic cells have been shown to require a greater amount of iron, thus laying the basis for the promising anticancer activity of iron chelators. In this work, we evaluated the effects of molecules with iron chelation activity on neuroblastoma (NB) cell lines. Of the 17 iron chelators tested, six reduced cell viability of two NB cell lines with an inhibition of growth of 50% below 10 μM; four of the six molecules—ciclopiroxamine (CPX), piroctone, 8-hydroxyquinoline, and deferasirox—were also shown to efficiently chelate intracellular iron within minutes after addition. Effects on cell viability of one of the compounds, CPX, were indeed dependent on chelation of intracellular iron and mediated by both G0/G1 cell cycle block and induction of apoptosis. By combined transcriptome and translatome profiling we identified early translational downregulation of several members of the heat shock protein group as a specific effect of CPX treatment. We functionally confirmed iron-dependent depletion of HSP90 and its client proteins at pharmacologically achievable concentrations of CPX, and we extended this effect to piroctone, 8-hydroxyquinoline, and deferasirox. Given the documented sensitivity of NB cells to HSP90 inhibition, we propose CPX and other iron chelators as investigative antitumor agents in NB therapy.

Introduction

Neuroblastoma (NB) is the most common extracranial solid childhood cancer, derived from undifferentiated neural crest cells (Brodeur, 2003). It is a complex and heterogeneous disease, whose outcome—from spontaneous regression to rapid progression—is determined by many clinical and biologic features, such as age at diagnosis, disease stage, numerical and structural chromosomal alterations, etc. (Cheung and Dyer, 2013). Recent advances in understanding these molecular, cellular, and genetic features of NB have allowed refining the classification of patients into risk groups, followed by more accurate therapy stratification. On the one hand, this has led to reduction of cytotoxic treatments and thus adverse treatment-related late effects in patients with more favorable outcomes. However, on the other hand, treatment of patients who currently have a poor prognosis is still a challenge, emphasizing the need of alternative approaches and calling for a shift from broad-spectrum chemotherapy to personalized molecularly targeted treatments (Cole and Maris, 2012).

Currently, amplification of MYCN remains the best-characterized genetic marker correlated with high-risk disease and poor prognosis (Huang and Weiss, 2013). Despite deregulation of MYCN as a hallmark of high-risk NB, being a transcription factor this protein is of very difficult drugability. Alternative pharmacological approaches have focused on MYCN mRNA, with the aim of either suppressing its production (Thiele et al., 1985) or favoring its degradation (Burkhart et al., 2003). Recently, we conducted a screen of Food and Drug Administration–approved drugs on a high-risk MYCN-amplified NB cell line targeting yet another level of MYCN regulation (Sidarovich et al., 2014). Specifically, we were looking for compounds able to modulate MYCN protein levels via its 3′UTR-dependent post-transcriptional control mechanisms. Among the four molecules selected as truly dependent on MYCN 3′UTR, three (doxorubicin, daunorubicin, and epirubicin) were anthracyclines, a class of drugs used in the first line treatment of NBs. The fourth molecule was an off-patent synthetic antimicrobial agent, ciclopirox olamine (CPX). Although originally developed for the topical treatment of cutaneous fungal infections, CPX has recently been reported to display preclinical efficacy as an effective antitumor agent in the treatment of various cancers (Clement et al., 2002; Eberhard et al., 2009; Zhou et al., 2010; Song et al., 2011; Ma

ABBREVIATIONS: 17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; calcein-AM, acetoxymethyl ester of calcein; CPX, ciclopirox olamine; DEGs, differentially expressed genes; DFO, deferoxamine mesylate salt; DOHH, deoxyhypusine hydroxylase; HT, holo-transferrin; 8HQ, 8-hydroxyquinoline; HSP, heat shock protein; IPA, ingenuity pathway analysis; NB, neuroblastoma; qPCR, quantitative polymerase chain reaction.
et al., 2013; Sen et al., 2013). Moreover, its efficacy in the treatment of hematologic malignancies is currently being evaluated in a phase I clinical study (Weir et al., 2011; Minden et al., 2014).

Interestingly, both anthracyclines and CPX share a unique feature—the ability to chelate iron. This observation made us willing to investigate more generally the chelation of iron as a potential therapeutic strategy in the treatment of NB. In fact, iron chelators have received considerable attention as potential antitumor agents over the last decade, owing to the avidity of cancer cells for iron (Richardson et al., 2009; Torti and Torti, 2013). Many proteins involved in intracellular iron regulation have been shown to contribute to the malignant phenotype, thus making cancer cells more susceptible to iron deprivation compared with their normal counterparts. The effect of iron chelators on the cellular iron metabolism is complex, since they act on multiple molecular targets. Iron chelators have been referred to as regulators of iron-regulatory proteins, inhibitors of iron-dependent enzymes, and compounds facilitating the redox cycling of iron to generate cytotoxic reactive oxygen species within tumors (Richardson et al., 2009; Torti and Torti, 2013). In addition, a clear link between iron and various signaling pathways has been established, even though the detailed mechanism of iron dependency needs to be clarified. Thus, it is not surprising that there is continuing effort being made to improve the potency and selectivity of iron chelators against cancer cells.

We initiated this study to evaluate if compounds with reported iron-chelating properties are effective against NB. Our results show that a subset of tested molecules potently reduced viability of NB cells and at the same time efficiently chelated intracellular iron. We further focused on a single iron chelator, CPX, and were able to show that CPX-mediated effects on cell viability were indeed iron dependent and resulted from both deregulated cell cycle progression and induction of apoptosis. mRNA profiling performed on CPX-treated cells revealed translational downregulation of the heat shock protein (HSP) family of genes. The main chaperone, HSP90, a known tumor target, was confirmed to be reduced at the protein level by CPX, and the effect was extended to the subset of iron chelators effective on NB cells. Therefore, we found that in NB cells HSP90 is inhibited by iron chelators of different chemical structures, opening these molecules or their derivatives to further evaluation as potential NB drugs.

Materials and Methods

Chemicals. CPX; deferoxamine mesylate salt (DFO); piricetone alamine; 8-hydroxyquinoline (8HQ); deferiprone; 2-[(L)-tagatose (7); 2,2'-bipyridyl; omadine; 2,3-dihydroxybenzoic acid; diethylenetriaminepentaacetic acid; o-phenantroline; 2-picolinic acid; L-mimosine; ferrichrome; deoxraxzone; ferrozine; holo-transferrin (HT); and cycloheximide were obtained from Enzo Life Sciences (Farmingdale, NY), and acetoxymethyl ester (allylamino)-17-demethoxygeldanamycin (17-AAG) was purchased from Sequoia Research Products (Pangbourne, UK) and 17-picolinic acid; o-phenantroline; 2-picolinic acid; L-mimosine; ferrichrome; deoxraxzone; ferrozine; holo-transferrin (HT); and cycloheximide were obtained from Sigma-Aldrich (St. Louis, MO). Deferasirox was purchased from Novartis (Basel, Switzerland). Deferiprone was purchased from Chem-Lab (St Louis, MO). 8-Hydroxyquinoline (8HQ); deferiprone; D-(+)-tagatose; 2,2'-bipyridyl; omadine; 2,3-dihydroxybenzoic acid; diethylenetriaminepentaacetic acid; o-phenantroline; 2-picolinic acid; L-mimosine; ferrichrome; deoxraxzone; ferrozine; holo-transferrin (HT); and cycloheximide were obtained from Sigma-Aldrich (St Louis, MO).

Cell Lines. The NB cell lines CHP134, KELLY, LA-N-2, NB69, SK-N-AS, SK-N-BE(2), and SK-N-SH were purchased from the European Collection of Cell Cultures (Salisbury, UK); CHP-212, HeLa, and HEK-293 cell lines were purchased from American Type Culture Collection (Manassas, VA); and SiMa was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The cell line MCP7 was received from the Banca Biologica e Cell Factory (Genoa, Italy). All cell lines were cultured in a humidified (37°C, 5% CO2) incubator in media, prepared following the instructions of the suppliers.

Viability Assays. The cell viability was measured either by the AlamarBlue viability assay (Life Technologies, Carlsbad, CA) or the xCELLigence real-time cell-analysis dual-plate system (Roche, Basel, Switzerland). Cells were plated in transparent 96-well plates at predefined concentrations. For each cell line a control plate for time-zero measurement was included along with experimental plates. The AlamarBlue assay was typically performed on the day of compound addition for control plates and at 24–48 hours of treatment of experimental plates. The AlamarBlue reagent was added in an amount equal to 10% of the cell culture volume, and the plates were returned to an incubator. Seven hours later fluorescence was detected with an excitation wavelength of 570 nm and emission wavelength of 600 nm using the Tecan Infinite M200 instrument (Männedorf, Switzerland). The percentage of viable cells was calculated using the following equations:

\[
\frac{(T_i - T_0)/(C - T_0)}{100}
\]

if \(T_i \geq T_0\), and

\[
\frac{(T_i - T_0)/T_0}{100}
\]

if \(T_i < T_0\), where \(T_i\) (time zero) is a fluorescence measurement at the time of drug addition (performed in control plates), while control growth \(C\) and test growth \(T_i\) are fluorescence measurements in the presence of a vehicle and a compound of interest, respectively. To evaluate in real time the effect of CPX on viability of CHP134 cells, 8000 cells were plated in 16-well E-plates, and after 24 hours they were treated with increasing concentrations of CPX ± HT. Cell viability was estimated based on the cell index recorded on the real-time cell-analysis system every 15 minutes. The cell index is derived from fluctuations in electrical impedance, reflecting interaction of cells with microelectrodes integrated on the bottom of each well of the E-plate.

Three-Dimensional Multicellular Spheroid Model. CHP134 cells were plated in a round bottom ultralow attachment 96-well plate (Corning, Corning, NY) at a concentration of 1000 cells per well in 100 μl complete media. After centrifugation at 300 g for 5 minutes cells were plated into an incubator. After 3 days spheroids were imaged using the high-content imaging system Operetta (PerkinElmer, Waltham, MA) and then treated with 0-20 μM CPX ± HT following addition of 100 μl fresh media to each well. Images were acquired at 48, 72, and 96 hours of treatment and analyzed using the Harmony software (PerkinElmer), which quantitates the spheroids area and roundness. The high-content output value was expressed as area × roundness.

Analysis of Cell Cycle by Flow Cytometry. Cell cycle analysis was performed on CHP134 cells treated with various concentrations of CPX for 24 hours using the Click-iT EdU Flow Cytometry Assay kit according to the protocol of the supplier (Life Technologies). Briefly, before harvesting, the samples were incubated with 15 μM Click-iT EdU for 50 minutes. The samples containing 1 × 10^5 cells were fixed in 70% ethanol at ~20°C overnight. The samples were permeabilized using Click-iT saponin-based permeabilization and wash reagent followed by EdU labeling with AlexaFluor 488 azide. The DNA content was measured using the 7-aminoactinomycin D stain (Life Technologies) at 2 μg/ml concentration. Finally, the samples were analyzed by flow cytometry on the BD FACSCanto II instrument (BD Biosciences). EdU-Alexa Fluor 488 azide and DNA content were detected using 488 nm excitation with the band-pass filter 520/20 and the long-pass filter 570, respectively.

High-Content Imaging Apoptosis Assay. CHP134 cells growing in 96-well plates (CellCarrier, PerkinElmer) were treated with CPX (0–20 μM) for 24 and 48 hours. Cells were fixed by adding an

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equal volume of 8% formaldehyde to the culture media. Fixed cells were incubated with a blocking/permeabilization solution (3% bovine serum albumin, 0.3% Triton X-100 in phosphate-buffered saline). The samples were stained using anti-actinive caspase 3 (Abcam, Cambridge, UK) as the primary antibody and Alexa Fluor 488 F(ab)’2 fragment of goat anti-rabbit IgG (Life Technologies, Carlsbad, CA) as the secondary antibody. Finally, nuclei were stained with Hoechst 33422 (Molecular Probes). Images were acquired using the Operetta high-content system with a 20× LWD objective (PerkinElmer). Analysis was performed using the Harmony software (PerkinElmer).

**Calecin-AM Assay.** Intracellular iron measurements were performed as described elsewhere (Eberhard et al., 2009). Briefly, the culture media of cells growing in 6-well plates was aspirated and substituted with a phosphate-buffered saline supplemented with 20 mM HEPES, 1 mg/ml bovine serum albumin, and 250 mM calecin-AM (pH 7.3). After 5 minutes incubation at 37°C, the cells were detached, washed, and resuspended in phosphate-buffered saline supplemented with 20 mM HEPES and 1 mg/ml bovine serum albumin. The cells loaded with calecin were treated with iron chelators for 10 minutes. Changes in intracellular calecin fluorescence were measured by flow cytometry (BD FACSCanto). The increase in fluorescence upon addition of iron chelators was calculated as the percentage of the difference between the median fluorescence measured in treated and untreated samples.

**Extraction of Polysomal and Total RNA.** CHP134 cells growing in 10 cm dishes were treated with a vehicle or 5 μM CPX for 24 hours. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Venlo, The Netherlands) following the instructions of the supplier and eluted in 50 μl of RNase-free water. To extract polysomal RNA, the following procedure was applied. The dishes with CHP134 cells were supplemented with 100 μg/ml cycloheximide and then returned to an incubator for 4 minutes. After removal of growing media, the cell-culture dishes were placed on ice and rinsed with phosphate buffer saline supplemented with cycloheximide. Cells were lysed directly in the dishes by adding an ice-cold lysis buffer (10 mM NaCl, 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, 100 μg/ml cycloheximide, 0.2 U/μl RNase inhibitor, 1 mM diithiothreitol). The lysates were collected with a cell scraper, pipetted into a microcentrifuge tube, and after 5 minutes of incubation on ice centrifuged for 10 minutes at 12,000 rpm. The supernatants were fractionated by ultracentrifugation (Sorvall rotor, 100 minutes at 180,000g; Thermo-Fisher, Waltham, MA) through a 15–50% linear sucrose gradient containing 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM MgCl₂. Polysomal fractions were collected monitoring the absorbance at 254 nm on the Density Gradient Fractionation System (Teledyne Isco, Lincoln, NE). Polysomal RNA was isolated after proteinase K treatment, phenol-chloroform extraction, and isopropanol precipitation, and finally resuspended in 50 μl of RNase-free water. RNA quality was assessed using the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Santa Clara, CA).

**mRNA Profiling.** All of the RNA samples were submitted to microarray analysis using the Agilent-014850 Whole Human Genome Microarray 4× 44K G4112F chip from Agilent Technologies. Three biologic replicates were done for each condition (control and treated with CPX at 90 minutes for total and polysomal RNA) and 12 hybridization arrays were obtained. cRNA probe generation along with array hybridization, washing, and staining were carried out according to the standard One-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling) protocol (Agilent Technologies). Hybridized microarray slides were scanned with an Agilent DNA Microarray Scanner G2505C at 5 μm resolution with the manufacturer’s software (Agilent ScanControl 8.1.3). The scanned images were analyzed numerically and the background corrected using the Agilent Feature Extraction Software, version 10.7.7.1, according to the Agilent standard protocol GE1_107_Sep09. The output of Feature Extraction was analyzed with the R software environment for statistical computing (http://www.r-project.org/) and the Bioconductor library of biostatistical packages (http://www.bioconductor.org/). Low signal Agilent probes, distinguished by a repeated absent detection call across the majority of the arrays in every condition, were filtered out from the analysis. Signal intensities across arrays were normalized with the quantile normalization algorithm. Differentially expressed genes (DEGs) were determined with the Translatome Bioconductor package, adopting a threshold based on the statistical significance of the change measured with a moderated t test (P < 0.01) implemented in the Limma Bioconductor package. Translation efficiencies were calculated as the ratio of polysomal RNA and total RNA signals. All microarray data are available through the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) using the accession number GSE60675.

**Gene Enrichment Analysis.** The DAVID resource was used for enrichment analysis of the transcriptome and the translatome DEGs lists, using annotations from Gene Ontology (http://geneontology.org), Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg), PFAM (http://pfam.xfam.org/), and INTERPRO (http://www.ebi.ac.uk/interpro/). The significance of over-representation was determined using a P value threshold of 0.05.

The ingenuity pathway analysis (IPA) software (IngenuitySystems, http://www.ingenuity.com) was used to assess the involvement in known pathways and networks of transcriptome and translatome DEGs. IPA uses the Fisher exact test to determine the enrichment of genes in canonical pathways. IPA also generates gene networks by using experimentally validated direct interactions stored in the Ingenuity Knowledge Base. The networks generated by IPA have a maximum size of 140 genes, and they receive a score indicating the likelihood of the genes to be found together in the same network due to chance. IPA networks were generated separately from transcriptome and translatome DEGs. A score of 4 was used as a threshold for identifying significant gene networks, indicating that there was only a 1/10,000 probability that the presence of genes in the same network was due to chance.

**Real-Time Quantitative Polymerase Chain Reaction.** To produce cDNA, 1 μg of total or polysomal RNA was reverse transcribed according to the protocol of the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Polysomal chain reactions were performed in a 10 μl reaction volume containing 5 ng template cDNA, 1× Kapa Probe Fast quantitative polymerase chain reaction (qPCR) Universal Master Mix (2×) and 1× TaqMan Gene Expression assay of interest (Life Technologies) (Supplemental Table 1). All reactions were carried out in 96-well plates (Hard-Shell Low-Profile 96-Well Skirted PCR plates; Bio-Rad Laboratories) on the CFX96 real-time PCR detection system (Bio-Rad Laboratories), followed by a regression Cq value determination method. The cycling conditions comprised 9-minute polymerase activation at 95°C, and 40 cycles at 95°C for 3 seconds, 60°C for 20 seconds, and 72°C for 1 second qPCR amplification efficiency was calculated for each gene using a relative standard curve derived from a cDNA mixture of samples (a 5-fold dilution series with five measuring points). The relative mRNA expression level of each gene was determined in Excel as described elsewhere (Hellemans et al., 2007). The geometric mean of three reference genes (HPRT1, SDHA, and TBP) was used for normalization of the expression level of target genes.

**Western Blotting.** Total proteins were extracted from pelleted cells in RIPA buffer followed by three freezing/thawing cycles. Following quantification with the Bradford reagent (Sigma-Aldrich), 10 μg total proteins were loaded and electrophoresed using SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories). After the blocking step, membranes were incubated with a primary antibody at +4°C overnight. Primary antibodies used were against HSP90 (Abcam), HSP72 (Abcam), HSC70 (Abcam), RAP1 (Abcam), TrkA (Millipore), glyceraldehyde-3-phosphate dehydroge- nase (Santa Cruz Biotechnology, Dallas, TX), and tubulin (Santa Cruz Biotechnology). The secondary antibodies used were goat anti-mouse, anti-rat, and anti-rabbit IgG-HRP (Santa Cruz Biotechnology). Blots were imaged with the ChemiDoc XRS+ imaging system (Bio-Rad Laboratories) after addition of the ECL Prime detection reagent (GE Healthcare, Buckinghamshire, UK).
Results
Compounds with Intracellular Iron Chelation Properties Decrease Cell Viability of NB Cells. To assess the general anticancer activity of iron chelators in NB, we selected 17 commercially available compounds with known iron chelating properties (Supplemental Fig. 1). The initial set of iron chelating compounds was tested for their effects on viability of CHP134 cells at two concentrations of 1 and 10 μM to cover a wider range of potential efficacy. After incubation for 48 hours cell viability was evaluated using the AlamarBlue assay. As shown in Fig. 1A, this experiment identified nine compounds that at 10 μM inhibited proliferation of CHP134 cells by at least 90% compared with a vehicle-treated control. The efficacy of seven out of nine compounds in reducing cell viability was confirmed in dose-response studies extended to two NB cell lines, CHP134 and SiMa (Fig. 1B). Piroctone, CPX, 8HQ, and omadine resulted in lower inhibition of growth of 50% (IC50) values, while deferiprone, deferasirox and DFO were less effective. We then measured the ability of the selected compounds to bind intracellular iron in the calcein-AM assay (Breuer et al., 1995b) to verify if this feature could be correlated with the suppression of cell viability. Calcein-AM is a non-fluorescent cell permeant compound hydrolyzed by intracellular esterases into the impermeable fluorescent anion calcein. Calcein chelates intracellular iron, and thus its fluorescence is quenched; therefore, compounds able to displace intracellular iron from its complex with calcein result in increased fluorescence signal. The ability of the compounds to chelate intracellular iron was assessed by flow cytometry after 10 minutes of treatment at the final concentration of 10 μM with the exception of the poorly permeating chelator DFO, which was tested at 100 μM as the negative control. As shown in Fig. 1C, piroctone, 8HQ, CPX, and deferasirox demonstrated the highest chelation ability, whereas deferiprone increased intracellular calcein fluorescence to a lesser extent. In contrast, DFO had no effect on calcein fluorescence even at 10 times higher concentration, which is in agreement with the fact that it penetrates the membrane and binds intracellular iron only after prolonged incubations (Breuer et al., 1995a).

Of note, the extent of antiproliferative effects of individual iron chelators cannot always be linked purely to their capacity to chelate iron (compare Fig. 1, B and C). Indeed, the efficiency of iron chelators acting via iron sequestration mechanisms is determined by multiple factors. It depends on membrane permeability of the compounds; different iron complex formation constants, and thus on different capacities to sequester intracellular iron; the presence of other metal ions competing with iron; and the capacity to access the active-site metal atom of cellular target enzymes (Clement et al., 2002). For example, two iron chelators, DFO and deferiprone, have comparable iron complex formation constants (Clement et al., 2002). However, deferiprone, a lipophilic compound that penetrates cellular membranes well, was able to sequester intracellular iron much more effectively than DFO (Fig. 1C), which is hydrophilic and hence poorly permeable (Kalinski and Richardson, 2005). Still, DFO inhibited viability of NB cells more efficiently than deferiprone (Fig. 1B), indicating that the compounds affect distinct iron-dependent proteins/pathways with different

Fig. 1. Identification of iron chelators with antiproliferative activity in NB cells. (A) CHP134 cells were treated for 48 hours with 1 μM and 10 μM of CPX (1); DFO (2); deferasirox (3); piroctone olamine (4); 8HQ (5); deferiprone (6); n-(-)-tagatose (7); 2,2′-bipyridyl (8); omadine (9); 2,3-dihydroxybenzoic acid (10); diethylenetriaminepentaacetic acid (11); o-phenantroline (12); 2-picolinic acid (13); t-mimosine (14); ferrichrome (15); dexrazoxane (16); and ferrozine (17). Cell viability was measured by AlamarBlue assay. The data represent the mean ± S.D. of three measurements. (B) CHP134 and SiMa cells were treated with 0.33–100 μM of CPX, DFO, deferasirox, piroctone olamine, 8HQ, deferiprone, and omadine for 48 hours. Cell viability was measured by AlamarBlue assay. Data represent the mean percentage of viable cells ± S.D. from three measurements. (C) SiMa cells were loaded with the intracellular iron-chelating fluorescent dye calcein-AM. Cells were treated with the compounds indicated in (B) for 10 minutes (all at 10 μM, but DFO at 100 μM). Intracellular iron bound by calcein was measured by flow cytometry. Percentage increase ± S.D. in the median intracellular calcein fluorescence of three replicates is shown.
efficiencies. In fact, DFO was reported to inhibit deoxypseudine hydroxylase (DOHH), an essential enzyme for maturation of the translation factor eIF5A, and DNA synthesis about 10 times more efficiently than deferiprone (Clement et al., 2002).

A peculiar behavior observed in the calcein-AM assay for omadine resembled the decrease in fluorescence detected in cells loaded with excess of iron (data not shown), and thus could be interpreted as an increase of the intracellular iron pool. This observation, together with the high sensitivity of both CHP134 and SiMa cells to omadine, suggests a mechanism of action not related to its property of binding intracellular iron. Indeed, the cytotoxic effects of omadine are almost immediate, and not reversed upon withdrawal of the compound from the culture media or addition of iron, confirming a mechanism unrelated to iron binding (Kontoghiorghes et al., 1986; Blatt et al., 1989).

Taken together, three compounds—piroctone, CPX, and 8HQ—demonstrated prominent effects on NB cell viability that could be correlated to their marked avidity for intracellular iron. Two of them, piroctone and CPX, belong to the same class of hydroxypryridones, sharing very similar chemical structures. Of note, CPX is currently evaluated in a phase I clinical trial study in patients with relapsed and refractory hematologic malignancy (Minden et al., 2014). Thus, CPX could potentially be rapidly repositioned as a new investigational antitumor agent in treatment of NB. Therefore, CPX was evaluated in secondary assays.

**CPX is Cytotoxic to NB Cells in an Iron-Dependent Manner.** To further unveil the mechanism by which CPX inhibits cell viability, the studies were extended in a panel of cell lines, including MYCN-amplified and non–MYCN-amplified NB as well as non-NB cell lines (Fig. 2, A–C). CPX inhibited cell viability with an IC50 value in the range of 1–10 μM in eight out of nine NB cell lines, suggesting that this effect is not dependent on a specific NB cell type. In a previous publication we demonstrated that CPX results in upregulation of MYCN protein within the first hours of treatment with a concomitant increase in MYCN mRNA levels (Sidarovich et al., 2014). Therefore, a question arose if CPX-mediated effects on cell viability could be linked to MYCN levels in tested NB cell lines. Correlation analysis did not reveal any significant association between CPX IC50 values and MYCN copy number, mRNA expression, and protein levels (Supplemental Fig. 2). The effect of CPX on viability of non-NB cell lines was less marked: in MCF7 and HEK-293 cells IC50 values were around 10 μM, while HeLa cells did not reach the threshold of 50% inhibition at any concentration of CPX tested.

Next, we evaluated if the antiproliferative activity of CPX on NB cells could be reversed either by removal of the compound from the culture media or by its supplementation with iron. First, CHP134 and SiMa cells were cultured in CPX-containing media for 6, 12, 24, 30, 36, and 48 hours and then in CPX-free media for up to 48 hours, when the number of viable cells was counted. As can be appreciated from Supplemental Fig. 3, 6 hours of treatment did not affect cell viability at any concentration, indicating that there is a minimum time of cells’ exposure to CPX treatment. Instead, the effects of 20 μM CPX exerted within the first 12 hours were not recovered in the subsequent 36 hours. Similarly, the impact of 6.6 μM CPX treatment of 24 hours was stable for the next 24 hours. These data indicate that CPX effects are persistent in vitro in a time- and concentration-dependent manner.

To determine if supplementation of media with iron was able to prevent CPX-induced cytotoxicity, we first evaluated in calcein-AM assay whether CPX was able to bind intracellular iron in selected NB cellular models, and then if the phenotype could be rescued by adding an excess of iron. Indeed, 10 μM CPX significantly increased intracellular calcein fluorescence in eight of the nine tested NB cell lines, whereas the potent extracellular chelator DFO had a significantly lower effect on calcein fluorescence even at 10 times higher concentration (Supplemental Fig. 4). Then, the tumor cells were treated with aerial dilutions of CPX with and without iron supplementation to see if excess iron could abrogate CPX-induced toxicity. To increase iron levels specifically within the cells, iron was added in the form of HT, the different transferrin that releases iron intracellularly after it is bound to transferrin receptors and internalized into cells. The addition of excess iron delayed the cytotoxic effects of CPX in CHP134 cells (Fig. 2, A and D), indicating that CPX-induced cytotoxicity was mostly dependent on iron chelation. However, the fact that CPX-mediated effects on viability of CHP134 and other NB cell lines were not completely abrogated by the addition of HT (Supplemental Fig. 5) pointed out the presence of iron-independent mechanisms of CPX action (Sen et al., 2013). Finally, the antiproliferative effects of CPX on CHP134 cells grown as monolayers could be reproduced in a multicellular three-dimensional spheroid model, and similar to what happens in two-dimensional cultures, these effects could be partially prevented by supplementing media with iron (Fig. 2E; Supplemental Fig. 6). These data also indicate a potential therapeutic effect of CPX in vivo.

**CPX Inhibits Cell Proliferation and Induces Apoptosis.** We further evaluated whether CPX decreased cell viability through inhibition of proliferation and/or induction of cell apoptosis. The cell cycle distribution of CHP134 cells upon CPX treatment was assessed by flow cytometry using the Click-iT EdU chemistry multiplexed with a 7-aminoactinomycin D DNA stain. At the two highest concentrations CPX effectively blocked CHP134 cells in the G2/M phase (Fig. 3, A and B). Treatment with 2 and 0.6 μM CPX resulted in a modest accumulation of the cells in the S-phase and a contemporary decrease in the median fluorescent intensity of the EdU+ positive population (S-phase cells; Fig. 3, A–C). In other words, within the same period of time CPX-treated cells incorporated less EdU compared with control cells, which might be attributable to an inhibition of DNA synthesis. The effect seems to be concentration dependent and not time dependent, since a similar cell cycle distribution was observed for 24- and 48-hour treatments with 2 or 0.6 μM CPX (Supplemental Fig. 7). The data of the cell cycle analysis were augmented by continuous long-term single-cell observations (Schroeder, 2011), demonstrating that upon treatment with higher concentrations of CPX cells do not undergo doubling and massive cell death is observed, while the lower concentrations of CPX resulted in significantly prolonged doubling time (data not shown).

To evaluate the ability of CPX to cause apoptosis, a high-content imaging apoptosis assay was performed. As shown in Fig. 3D, a strong increase in fluorescence intensity reflecting the activation of caspase-3 was observed upon CPX treatment in a concentration- and time-dependent manner. The activation of caspase-3 was accompanied by a reduction in cell number. The concentration- and time-dependent increase of caspase-3 activity upon the CPX treatment was confirmed using the Caspase-Glo 3/7 assay, in which the luminescence
produced was proportional to the caspase-3/7 enzymatic activity (data not shown). Altogether, analysis of the data on cell cycle distribution and caspase-3 activation indicate that CPX-mediated inhibition of cell viability is associated with deregulated cell cycle progression and induction of apoptosis.

**CPX Induces Translational Downregulation of Members of the HSP group.** To enable a comprehensive overview of the gene expression response upon CPX treatment, a genome-wide multilevel transcriptome and translatome analysis was performed (Arava et al., 2003; Tebaldi et al., 2012). The polysomal mRNA (i.e., the mRNAs engaged in translation—the translatome) and the total mRNA (the total amount of transcribed mRNAs—the transcriptome) from CHP134 control cells and cells treated with 5 μM CPX for 90 minutes were extracted and quantified with gene expression microarrays. A total of 14,196 genes resulted after data preprocessing, normalization, and quality check with bioconductor packages (see Materials and Methods). A scatter plot containing the whole set of translatome and transcriptome variations is displayed in Supplemental Fig. 8A. The translatome response to CPX was poorly concordant with the transcriptome one, as resumed by the low Spearman correlation value (0.33). This may be partially due to the short lapse of time at which cells were sampled. DEGs as an effect of the CPX treatment were determined with the rTranslatome package (Tebaldi et al., 2014) adopting a threshold based on the statistical significance of the change (the complete lists of DEGs are available in Supplemental Tables 2–5). At this stage translational regulation, which is supposed to act with faster dynamics than transcriptional regulation (Schwanhäusser et al., 2011), encompasses the majority of observed changes, while transcriptional regulation requires more time to become effective and influence the cellular phenotype. Indeed, 1270 and 520 DEGs were registered in the translatome and transcriptome, respectively. Classes of DEGs are summarized in Fig. 4A. Considering genes with significant variations only in one of the two levels, 68% of DEGs (in yellow and orange in Fig. 4A) had significant changes only at the transcriptome level and 24% of DEGs (in blue in Fig. 4A) changed significantly only at the translatome level. Comparing the two lists of genes, only 132 genes (8% of DEGs, in green in Fig. 4A) showed significant homodirectional changes, reflecting the low concordance between transcriptional and translational movements. Therefore, the early response of CHP134 cells to CPX was mostly...
directed at the translational level, changing the loading on polysomes of several mRNAs. Finally, the microarray data were validated for 10 selected genes with disparate translational variations by qPCR, giving a high level of concordance (Supplemental Fig. 8B).

To better understand the meaning of the cell response to the CPX treatment, we switched the focus of the analysis from movements of single genes to their clustered biologic annotations. To this end, ontological enrichment analysis was performed on the lists of the transcriptome and translatome DEGs. Enrichments were calculated using annotations and statistical tests provided by the DAVID resource (Huang et al., 2009) (see Materials and Methods; the complete lists of significantly enriched terms are available in Supplemental Tables 6–8). From this analysis, HSPs of the HSP70 family emerged as significantly and specifically enriched among translationally downregulated DEGs (for the HGNC annotation of HSP protein families, see Fig. 4B; Supplemental Fig. 8C; Supplemental Table 9). An alternative approach, provided by the IPA, was employed to detect the presence of interaction networks among the sets of DEGs. The whole set of IPA networks is available in Supplemental Table 10. This analysis confirmed the presence of a cluster of interconnected HSPs, including members of the HSP90 family, among translationally downregulated genes. All this evidence suggests that CPX treatment consistently reduces the translational efficiency (defined by the ratio of translatome and transcriptome signals) of HSP members. This phenomenon is displayed in Fig. 4C, where the distributions of translational efficiency changes of HSP families are compared with the distribution of all microarray genes: HSP90 proteins show the most pronounced decrease in translation efficiency.

Fig. 3. CPX induces cell cycle arrest and apoptosis in CHP134 cells. (A–C) Dose-dependent effects of CPX (0–20 μM, 24 hours) on cell cycle phase distribution. The cells were processed for cell cycle analysis using the Click-IT EdU flow cytometry assay kit. (A) Representative Alexa-Fluor 488 EdU (exponential axis) and DNA 7-aminoactinomycin D (7-AAD) histograms (linear axis) are displayed. (B) Dose-response curve demonstrates the percentage of cells in G0/G1, S, and G2/M phases from the data displayed in panel (A). (C) Dose-response curve showing the median fluorescence intensity (MFI) of the EdU+ population from the data displayed in panel (A). (D) CHP134 cells growing in 96-well plates were treated with CPX (0–20 μM) for 24 and 48 hours. A high-content imaging apoptosis assay was performed on fixed cells immunofluorescently labeled for activated caspase-3 (Casp).
Iron Chelators Downregulate Expression of the HSP90 Protein. Mammalian HSPs include several major families of molecular chaperones classified by their molecular weights: HSP90, HSP70, and HSP60, and the small HSPs (Jego et al., 2013). Family members of HSPs are expressed either constitutively or induced by many different stimuli. For example, the HSPA1A and HSPA8 transcripts, which were found to be translationally downregulated upon CPX treatment (Supplemental Fig. 9), encode for two major cytoplasmic isoforms of the HSP70 protein: a ubiquitously expressed HSC70 and an inducible HSP72. Likewise, the most prominent members of the HSP90 family are the constitutively expressed HSP90α (encoded by HSP90AA1) and the stress-inducible HSP90β (encoded by HSP90AB1). However, in cancer cells this differential expression pattern is commonly lost and increased constitutive expression of inducible isoforms is observed (Jego et al., 2013).

To understand whether the effects of CPX on HSP group members evidenced by gene expression analysis as early as at 90 minutes of treatment are persistent in time, CHP134 cells were treated with 5 mM CPX for 24 hours followed by purification of total and polysomal mRNA and qPCR analysis. Downregulation of HSP expression was detected also after 24 hours of treatment (Supplemental Fig. 9). Although the degree of the polysomal mRNA downregulation of the measured HSPs remained approximately the same at 24 hours compared with 90 minutes of treatment, upon prolonged treatment the transcriptome changes came up with the translatome level. At the same time, transcriptional targets of hypoxia inducible factor-1, in which the stability is increased upon iron depletion (Richardson et al., 2009), were upregulated at the transcriptome and translatome levels with high concordance (Supplemental Fig. 10). This observation highlights that CPX effects on the group of HSPs follow a different pattern compared with the well documented hypoxia inducible factor-1-mediated pathway.

HSP90 and HSP70 proteins play a crucial role in cancer cell survival. Together with additional proteins they form the chaperone machine used by cancer cells to ensure the correct conformation and stability of mutated and/or overexpressed oncogenic proteins, which otherwise would undergo degradation. Thus, cancer cells often become dependent on these HSPs, which are dispensable for the survival of normal cells (Trepel et al., 2010; Jego et al., 2013). Therefore, we focused our further attention on HSP90, HSC70, and HSP72, encoded by HSP90AA1, HSPA8, and HSPA1A, respectively. First, we tested if the expression variations observed for these genes at 24 hours are iron dependent. To this purpose CHP134 cells were treated either with 5 μM CPX alone or with 5 μM CPX plus HT, or with HT only. Subsequent qPCR analysis of total and polysomal mRNA revealed that CPX effectively reduced HSP90, HSP72, and HSC70 mRNA abundance in an iron-dependent manner (Fig. 5A). Interestingly, CPX treatment resulted in marked fluctuations of the inducible HSP72 mRNA at the transcriptome level, provided by PFAM and INTERPRO. Enrichment false discovery rate values are displayed, with significant values (<0.05) colored in blue shades. (C) Box whisker plot of log 2 translational efficiency changes induced by CPX. Translational efficiencies are calculated as the ratio of translatome to transcriptome signals. The distribution of the whole set of human genes with microarray probes (in dark gray) is compared with the distribution of HSP protein families: HSP70, HSP40, and HSP90, and the union of all HSP members. Poly, polysomal; tot, total.
while the changes at the level of translatome were somewhat buffered, again exemplifying the general uncoupling between transcriptome and translatome responses (Tebaldi et al., 2012). At the protein level, CPX caused a robust reduction of HSP90 expression (Fig. 5B), while the concomitant reduction of HSP72 and HSC70 (Supplemental Fig. 11) was not observed, possibly because of post-translational compensatory mechanisms (Qian et al., 2006; Mao et al., 2013). Inhibition of the HSP90 chaperone activity was further supported by depletion of its client proteins, RAF1 and TrkA (Fig. 5B).

In an attempt to perform a rescue experiment over-expressing HSP90, we realized that the extremely high level of HSP90 in these NB cells was making it impossible. As estimated from infection of CHP134 cells with lentiviral particles carrying either HSP90 cDNA or enhanced green fluorescent protein and luciferase reporters, the average expression level of ectopic HSP90 transcripts is not more than one-thirtieth of the endogenous one, rendering it challenging to change the level of this protein exogenously (Supplemental Fig. 12). To overcome this technical hindrance, we followed an alternative approach to investigate the impact of HSP90 targeting by CPX. We evaluated whether the combination of CPX treatment, affecting HSP90 translational efficiency and finally protein abundance, and of pharmacological inhibition of the HSP90 ATPase activity could synergize in terms of their effects on viability. Therefore, CHP134 cells were treated with CPX in combination with 17-allylamino,17-demethoxygeldanamycin (17AAG), a potent inhibitor of HSP90 activity. A synergism of the two molecules was observed for cell viability at the concentrations below EC50, as shown by the combination index versus fraction affected plot (Fig. 5C). Thus, CPX treatment sensitized CHP134 cells to pharmacological HSP90 inhibition, indicating the active role of CPX in HSP90 downregulation.

Overall, our data demonstrate the ability of CPX to translationally reduce HSP90 protein levels. Based on the iron-dependent mechanism of this inhibition, we hypothesized that this effect might be attributable to iron chelators in general. Therefore, we investigated if the other iron chelators would result in HSP90 downregulation. CHP134 cells were treated for 24 and 48 hours with piroctone, 8HQ, and deferasirox, the three most potent and specific intracellular iron chelators resulting from our limited screen (Fig. 1, B and C). For each compound the effective concentrations for treatment were selected slightly below IG50 values based on the viability curves in Fig. 1B and corresponded to 2 μM for piroctone, 3 μM for 8HQ, and 20 μM for deferasirox. As shown in Fig. 5D, treatment of CHP134 cells with these iron chelators reduced the levels of both HSP90 and its client proteins. Thus, not only CPX but certain other iron chelators evoked a cellular response that downregulated HSP90 protein levels.

In summary, our preclinical results qualify CPX and a few other iron chelators as candidates for further drug development.
and exploration in NB therapy. One potential drawback could result from the fact that the effect of iron chelators on cancer cells is multifactorial, and therefore could potentially interfere with conventional chemotherapeutics. In our cell model a combined treatment of CPX and topotecan or cisplatin, two chemotherapeutic drugs used for second-line treatment of NB, resulted in no antagonism for the first and slight synergism for the second (Supplemental Fig. 13). The obvious advantage, instead, is that some iron chelators, including CPX, are already approved by the Food and Drug Administration and thus do not need further preclinical trials to evaluate toxicity, thus providing a potential shortcut for drug reposition.

Discussion

Iron is a crucial component of a variety of cellular pathways that are essential for cell replication, metabolism, and growth (Richardson et al., 2009; Torti and Torti, 2013). Iron enables the function of vital iron- and heme-containing enzymes including mitochondrial and detoxifying enzymes, enzymes involved in DNA synthesis, cell cycle, etc. Cancer cells have higher iron requirements possibly due to their rapid rate of proliferation. Hence, pathways of iron metabolism are often perturbed in cancer cells, making them different from their nonmalignant counterparts (Torti and Torti, 2013). Therefore, targeting iron-dependent pathways/proteins may selectively kill cancer cells with minimal damage to normal cells.

Iron chelators are agents that bind iron with high affinity (Richardson et al., 2009). They may act by withdrawing iron leading to tumor growth inhibition. Alternatively or additionally, they may form redox-active iron complexes resulting in the formation of reactive oxygen species, damaging critical intracellular targets, and thereby eliciting a cytotoxic response. Iron chelators have demonstrated potent antineoplastic properties in a number of cancers in vitro, and some of them, including CPX, DFO, deferasirox, are currently under preclinical or early clinical investigation as anticancer therapeutics (Torti and Torti, 2013). Among various tumors, the use of iron chelators as a possible adjunct to NB therapy has been also investigated. In particular, DFO, the first and most frequently studied chelator in NB, was reported to exert antiproliferative effects on NB cells, with minimal effects on viability of noncancerous cells and non-NB tumor cells (Blatt and Stitely, 1987; Becton and Bryles, 1988). With these promising preclinical results and owing to its safety profile, DFO antitumor activity has been investigated in clinical trials. DFO has been used in patients with NB either as monotherapy (Donfrancesco et al., 1990) or as part of combination chemotherapy (Donfrancesco et al., 1995), showing some promising preliminary results. However, in a different clinical study DFO failed to produce partial or complete response in children with recurrent NB (Blatt, 1994). Moreover, DFO treatment was ineffective in inhibiting the growth of human NB xenografts (Selig et al., 1998). This discrepancy in results could be due to a number of factors, including the DFO intravenous route of administration combined with a short plasma half-life, raising the need for long infusions to exert its effect (Kalinowski and Richardson, 2005). In addition, the highly hydrophilic nature of DFO severely limits its membrane permeability, and hence its efficiency (Breuer et al., 1995a). These limitations of DFO prompted an extended search for more effective iron chelators. Experiments with newer synthetic iron chelators, Triapine and 311, corroborated the data observed upon DFO treatment, namely, the greater susceptibility to iron chelation of NB cells compared with normal cell types (Chaston et al., 2003). In comparison with DFO, both Triapine and 311 demonstrated a greater antiproliferative effect on the NB cells tested.

In this study, we evaluated the potential of 17 compounds with reported iron chelation properties to inhibit the growth of NB in cell culture. Among the compounds tested, seven demonstrated concentration-dependent effects on the viability of two NB cell lines with IC_{50} ranging over a 10-fold spectrum (from approximately 2 \mu M for piroctone to about 70 \mu M for deferoxamine); five of the seven compounds were shown to efficiently chelate intracellular iron. Among the iron chelators tested, CPX was of special interest because it has an acceptable toxicity profile and is currently being evaluated as an anticancer agent in a phase I study in patients with advanced hematologic malignancies (Weir et al., 2011; Minden et al., 2014). As indicated by our data, CPX exerted strong antiproliferative activity on NB cells that could be reversed, at least partially, by loading the cells with iron, suggesting that the primary mechanism of CPX action is via iron sequestration. Of note, IC_{50} values of about 1–10 \mu M detected in CPX-treated NB cells are pharmacologically achievable (Weir et al., 2011; Minden et al., 2014). The CPX-mediated effect on viability of NB cells resulted from a combination of cell cycle deregulation and induction of apoptosis, corroborating previously published data on other tumor cell types (Zhou et al., 2010).

Interestingly, the effects of CPX on cell cycle distribution of CHP134 cells suggest that the CPX mode of action has a dose threshold in the concentration region between 2 and 6 \mu M. At concentrations below the threshold CPX slows down cell cycle progression, while at concentrations above the threshold CPX efficiently blocks the cells in the G0/G1 phase. This could be due to the capacity to target only a subset of iron-dependent proteins at lower concentrations, while for inhibiting other proteins higher concentrations of CPX could be needed. Several enzymes have been reported to be cellular targets for CPX, including ribonucleotide reductase (Eberhard et al., 2009) and DOHH (Clement et al., 2002; Mémin et al., 2014). Ribonucleotide reductase is a rate-limiting enzyme in DNA synthesis responsible for converting nucleoside diphosphates to deoxynucleoside diphosphates. Therefore, the augmentation of NB cell number in the EdU threshold region of CPX effects observed in CHP134 cells. In addition to DOHH, the G1 arrest induced by CPX could be
mediated by multiple mechanisms involving cyclin D1, CDK2, RB1, and CDK inhibitor p21CIP/WAF1 (Zhou et al., 2010). However, the link between these proteins and upstream iron-dependent events remains unclear.

We involved the HSP family as a potential CPX target through a two-level mRNA profiling study. HSPs are essential for cellular homeostasis by mediating correct protein folding, preventing protein aggregation, contributing to cell survival via inhibition of apoptosis, etc. Given the cytoprotective function of HSPs, it is not surprising that cancer cells often become addicted to HSPs, which is reflected by abnormal high expression and/or activity of some family members (Jego et al., 2013). As a consequence of deficient hypusyl-eIF5A. Moreover, HSP90 has a putative therapeutic effect for a class of HSP90 inhibitors; a functional activity of the representative compound, 17-AAG, was subsequently confirmed in viability assays. Our data corroborate the reported findings showing that 17-AAG indeed significantly reduced viability of NB cells. Most importantly, we were able to demonstrate that CPX treatment sensitized NB cells to 17-AAG–mediated inhibition of HSP90 activity.

One of the hallmarks of HSP90 inhibition, along with client protein degradation, is the subsequent induction of HSP70 (Maloney et al., 2007). This upregulation hinders effective action of HSP90 inhibitors, protecting the cells from it. Differently, upon treatment with CPX no induction of HSP70 proteins could be observed along with inhibition of HSP90, pointing out a mechanism of CPX action different from conventional HSP90 inhibitors and rendering it particularly interesting for therapy. In line with this, the recent work of Mémim et al. (2014) described that CPX resulted in hypusyl-eIF5A–mediated translational downregulation of yet another member of the HSP group, HSP27. Our data corroborate these findings, although at 24 hours we detected CPX-mediated inhibition of HSP27 both at the transcriptome and translatome levels (data not shown). It is appealing to assume that HSP90 downregulation could also be a consequence of deficient hypusyl-eIF5A. Moreover, HSP90 has been identified as a potential interaction partner of eIF5A and its modifying enzymes (Sievert et al., 2012).

The activity of CPX on HSP90 is shared by the other three iron chelators demonstrated to induce relevant cytotoxicity in NB cells. Of these, piroctone belongs to the same group of chelators. Therefore, the chemical property of iron chelation exerted intracellularly is the key determinant to reduction of translational efficiency of HSP90 and NB cell toxicity in this study.

In summary, we identify a potential mechanism by which CPX and other iron chelators act in NB cells to suppress viability—downregulation of HSP90 translation. Future detailed dissection of this and other key molecular targets of the active compounds we investigated could pave the way for efficient drug combinations in the treatment of high-risk NB patients.

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Authorship Contributions

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Conducted experiments: Sidarovich, Adami, Gatto, Greco.

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