Journal of Inorganic Biochemistry 170 (2017) 8–16

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

The dissociation of the Hsp60/pro-Caspase-3 complex by bis(pyridyl)oxadiazole copper complex (*CubipyOXA*) leads to cell death in NCI-H292 cancer cells

Celeste Caruso Bavisotto [a,b], Dragana Nikolic [b,c], Antonella Marino Gammazza [a,b], Rosario Barone [a,b], Filippa Lo Cascio [a], Emanuele Mocciaro [a], Giovanni Zummo [a], Everly Conway de Macario [d], Alberto JL Macario [b,d], Francesco Cappello [a,b], Valentina Giacalone [e], Andrea Pace [b,e], Giampaolo Barone [b,e], Antonio Palumbo Piccionello [b,e,1], Claudia Campanella [a,b,*,1]

[a] Department of Experimental Biomedicine and Clinical Neurosciences, Section of Human Anatomy "Emerico Luna", University of Palermo, Palermo, Italy
[b] Euro-Mediterranean Institute of Science and Technology, Palermo, Italy
[c] Biomedical Department of Internal Medicine and Medical Specialties, University of Palermo, Palermo, Italy
[d] Department of Microbiology and Immunology, School of Medicine, University of Maryland at Baltimore; and IMET, Columbus Center, Baltimore, MD, USA
[e] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo, Italy

ARTICLE INFO

Article history:
Received 24 September 2016
Received in revised form 16 January 2017
Accepted 9 February 2017
Available online 10 February 2017

Keywords:
CubipyOXA
Cancer
Apoptosis
Hsp60
Pro-caspase-3 (pC3)
Hsp60/pC3 complex

ABSTRACT

Cell survival and proliferation are central to carcinogenesis, involving various mechanisms among which those that impede apoptosis are important. In this, the role of the molecular chaperone Hsp60 is unclear since it has been reported that it can be both, pro- or anti-apoptotic. A solution to this riddle is crucial to the development of anti-cancer therapies targeting Hsp60. We addressed this question using a tumor cell line, NCI-H292, and $[Cu(3,5\text{-bis}(2'\text{-pyridyl})\text{-}1,2,4\text{-oxadiazole})_2(H_2O)_2](ClO_4)_2$, *CubipyOXA*, a copper-containing compound with cytotoxic properties. We treated cells with various doses of the compound and measured cell viability; apoptosis indicators; and levels of Hsp60, pro-Caspase-3 (pC3), Caspase-3 (C3), and complex Hsp60/pC3, with complementary methods. The quantitative dose-response curves of the levels of Hsp60, activated C3, inactivated pC3, Hsp60/pC3 complex and indicators of cell apoptosis, and cell death, all coincided to show that *CubipyOXA* has pro-apoptotic activity and promotes cell death. The curves also indicate that the pro-apoptotic effects of *CubipyOXA* could likely be due to a lowering of Hsp60 levels and to its blocking the formation of the Hsp60/pC3 complex and/or its dissociating the complex when already formed, thus, interfering with the anti-apoptotic action of Hsp60. These findings shed some light on how a tumor cell may avert apoptosis using Hsp60 and point to the anti-cancer potential of drugs, such as *CubipyOXA*, which interfere with Hsp60/pC3 complex formation, and thus allow the apoptotic cascade to proceed. In view of these findings it becomes clear that the novel compound *CubipyOXA* should be considered a potential, high-efficiency antitumor agent deserving further testing.

1. Introduction

Cancer cells live longer and proliferate more than normal counterparts because they overexpress specific proteins and receptors needed for tumor growth. Among other mechanisms that also favor carcinogenesis, cancer cells undergo less apoptosis. Therefore, one objective in the development of therapies for cancer is to find ways to favor apoptosis, for example by blocking the mechanism which, in cancer cells, interferes with it.

Inorganic compounds have attracted increasing attention for biomedical applications, such as detection and treatment of cancer and other diseases, drug delivery, and in vitro bio-sensing applications [1]. These compounds are taken into consideration because of their

Abbreviations: CubipyOXA, $[Cu(3,5\text{-bis}(2'\text{-pyridyl})\text{-}1,2,4\text{-oxadiazole})_2(H_2O)_2](ClO_4)_2$; *ZnbipyOXA*, $[Zn(bipyOXA)_2(H_2O)_2]^{2+}(ClO_4)_2$; Hsp60, heat shock protein 60; pC3, procaspase-3; NCI-H292, mucoepidermoid carcinoma cell line; HepG2, liver hepatocellular cell line; HT29, human colon adenocarcinoma cell line; RPMI medium, Roswell Park Memorial Institute medium; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RIPA, radioimmunoprecipitation assay; TRITC, tetramethylrhodamine; DTT, dithiothreitol; BSA, Bovine Serum Albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

* Corresponding author at: Department of Experimental Biomedicine and Clinical Neurosciences, Section of Human Anatomy "Emerico Luna", University of Palermo, Palermo, Italy. Euro-Mediterranean Institute of Science and Technology, Palermo, Italy.
E-mail addresses: antonio.palumbopiccionello@unipa.it (A. Palumbo Piccionello), claudiettacam@hotmail.com (C. Campanella).
[1] These authors contributed equally to the present work.

http://dx.doi.org/10.1016/j.jinorgbio.2017.02.004
0162-0134/

cytotoxic activity toward human cancer-cell lines [2], for example, by disrupting protein homeostasis [3].

The molecular chaperone Hsp60 assists protein folding in prokaryotes and in eukaryotic cells in order to maintain protein homeostasis and tissue physiology [4]. Hsp60 has been implicated in carcinogenesis via its interaction with components of the Caspase cascade that leads to apoptosis. However, opinions about the role of this chaperone in carcinogenesis, and in apoptosis in particular, differ and it is not yet clear when and how Hsp60 is pro-apoptotic (i.e., it acts as an anti-cancer factor) or the contrary, namely it works in favor of the tumor by interfering with apoptosis [5–10].

Our recent studies have shown that the induction of stress on cancer cells affects the Hsp60 levels [6,11,12] and it is known that this chaperone plays a crucial role in apoptosis. Furthermore, we found that Hsp60 interacts with Pro-Caspase-3 (pC3) in the mucoepidermoid carcinoma cell line NCI-H292 and this association persists after the induction of oxidative stress [5]. In a previous work, we investigated the antitumor activity of a new copper complex, capable to reduce the vitality of two cancer-cell lines: HepG2 and HT29 [13], probably by interacting with the DNA [14]. These and other similar results lead us to postulate that while oxidative stress induces apoptosis in NCI-H292 cells, Hsp60 might have an anti-apoptotic effect in the same cells. The aim of the work reported here was to investigate the effect of two new pro-apoptotic compounds, *CubipyOXA* ([Cu(3,5-bis(2′-pyridyl)-1,2,4-oxadiazole)$_2$(H_2O)$_2$](ClO_4)$_2$) and *ZnbipyOXA* ([Zn(bipyOXA)$_2$(H_2O)$_2$]$^{2+}$(ClO_4)$_2$) [15], in NCI-H292 cells, and to examine the levels of Hsp60 and Hsp60/pC3 complex.

CubipyOXA induced apoptosis in HepG2 and HT29 cells in a dose- and time-dependent manner [13]. As of today, very few copper complexes have been described that induce apoptosis through the involvement of the Caspase 3 (C3) [16–17]. The mechanism of C3 activation in copper-mediated cell death has not been fully elucidated [18]. It was found that copper-containing molecules and molecular complexes that trigger apoptosis have an impact on the levels of proteins involved in the apoptotic cascade, determining either an increase of pro-apoptotic or a decrease of anti-apoptotic molecules [18–21]. In view of these contrasting data and considering the similarly contrasting views on whether Hsp60 has a pro- or anti-apoptotic role in tumor cells, we set out to do experiments aiming at clarifying the connections between Hsp60, pC3, C3, and apoptosis. We examined the effects of the *CubipyOXA* and *ZnbipyOXA* on the tumor cell line NCI-H292 and monitored cell viability and apoptosis indicators, and the levels of Hsp60, pC3, C3, and their interactions.

2. Experimental section

2.1. Chemicals and synthesis

All chemicals and solvents were commercial and used as received, without further purification. Proton and carbon nuclear magnetic resonance spectra were recorded with a Bruker AC300 spectrometer, and solvent residual peaks were used as reference. Flash chromatography was performed by using silica gel (0.040–0.063 mm, Merck, Darmstadt, Germany) and mixtures of ethyl acetate and petroleum ether (fraction boiling in the range of 40–60 °C) in various ratios. Melting points were determined with a Reichart-Thermovar hot-stage apparatus and are shown uncorrected. Synthesis was performed according to published procedures [13].

2.1.1. 3,5-bis(2′-pyridyl)-1,2,4-oxadiazole (bipyOXA)

1.00 g of 2-cyanopyridine (8.14 mmol) and 0.56 g of 2-picolinamidoxime (4.07 mmol) were mixed in a sealed tube and heated at 120 °C for 8 h. The residue was chromatographed, yielding 0.59 g of 3,5-bis(2′-pyridyl)-1,2,4-oxadiazole (65%): mp 173–176 °C (from EtOH) (lit. 173–175 °C [13]); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.44–7.58 (m, 2H); 7.87–7.98 (m, 2H); 8.28–8.31 (d, 1H); 8.42–8.44 (d, 1H); 8.84–8.89 (m, 2H). Elemental analysis: Anal. Calcd for $C_{12}H_8N_4O$: C, 64.28; H, 3.60; N, 24.99. Found: C, 64.36; H, 3.53; N, 25.02.

2.1.2. Complex CubipyOXA [Cu(bipyOXA)$_2$(H_2O)$_2$]$^{2+}$(ClO_4)$_2$

A light blue solution of $Cu(ClO_4)_2$ 6H_2O (0.09 g, 0.24 mmol) in absolute ethanol was added dropwise and under constant stirring to a colorless solution of 3,5-bis(2′-pyridyl)-1,2,4-oxadiazole (0.11 g, 0.50 mmol) in absolute ethanol and at 23 °C. The mixture was let under stirring for 12 h, the precipitate filtered, washed by cold absolute ethanol and dried under vacuum (0.152 g; 85%). The solid was recrystallized from acetonitrile. Blue crystals of *CubipyOXA*, suitable for biological tests, were obtained. Identity of the obtained complex was confirmed through comparison of the IR spectrum with that reported in the literature [13]. Elemental analysis: Anal. Calcd for $C_{24}H_{20}Cl_2CuN_8O_{12}$: C, 38.59; H, 2.70; N, 15.00. Found: C, 38.53; H, 2.67; N, 15.09.

2.1.3. Complex ZnbipyOXA [Zn(bipyOXA)$_2$(H_2O)$_2$]$^{2+}$(ClO_4)$_2$

A solution of $Zn(ClO_4)_2$ 6H_2O (0.14 g, 0.4 mmol) in absolute ethanol was added dropwise and under constant stirring to a colorless solution of 3,5-bis(2′-pyridyl)-1,2,4-oxadiazole (0.179 g, 0.8 mmol) in absolute ethanol and at 23 °C. The mixture was let under stirring for 12 h in the dark, the white precipitate filtered, washed by cold absolute ethanol and dried under vacuum (0.215 g; 72%). The solid was recrystallized from acetonitrile giving colorless crystals of *ZnbipyOXA*. Identity of the obtained complex was confirmed through comparison of the IR spectrum with that reported in the literature [15]. Elemental analysis: Anal. Calcd for $C_{24}H_{20}Cl_2N_8O_{12}Zn$: C, 38.50; H, 2.69; N, 14.97. Found: C, 38.46; H, 2.71; N, 14.86.

2.2. Cell culture and treatment protocols

The NCI-H292 (human mucoepidermoid bronchial carcinoma) cell line was obtained from the American Type Culture Collection and maintained in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS) and supplemented with 2 mM glutamine, 50 U/ml penicillin, and 50 mg/streptomycin. Cells were grown as monolayers attached to the culture vessel and cultured at 37 °C, 5% CO_2 in a humidified incubator. The passage number of cells used in this study ranged from 12 to 35. Unless otherwise stated, cell culture reagents were purchased from GIBCO BRL LIFE Technologies (Invitrogen, Milan, Italy). Before starting each experiment, confluent cell monolayers were incubated in serum-free medium for 24 h. Cells were treated for 24 h with various concentrations of *CubipyOXA* or *ZnbipyOXA* dissolved in 1 mM Tris-HCl pH 7.4.

2.3. MTT test

Cytotoxicity was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell viability test. MTT was obtained from Sigma (Milan, Italy), and the assay was performed as described [22]. Briefly, 5×10^3 cells were plated in 200 μl of complete (with FCS, fetal calf serum) medium per well in 96-well plates. After 24 h, cells were treated with various concentrations of *Cubipy*OXA or *Znbipy*OXA (0 to 320 μM) for 24 h. At the end of the treatment period, the medium containing the compound was replaced by MTT dissolved in fresh medium and then it was added to the cell cultures at a final concentration of 0.5 mg/ml. After 4 h incubation, cells were solubilized in 200 μl DMSO/well and the optical density (OD) was measured with a plate reader (Titertek Multiskan MCC/340, Flow Laboratories, Basel, Switzerland) at 570 nm (630 nm as reference). Cell viability was expressed as the percentage of the OD value of treated cells compared with untreated controls, according to the following equation: Viability = (OD SAMPLE/OD CONTROL) × 100. Each experiment was carried out in duplicate and a total of three experiments were performed in every case. GI50 values were calculated at 24 h of treatment as follows: $GI50 = 100 \times (T - T0)/(C - T0) = 50$.

2.4. Cell lysate preparation and protein quantification

Treated and untreated cells were lysed by submersion into ice-cold lysis solution containing radioimmunoprecipitation assay (RIPA) buffer, as previously described [23]. Cell lysates were then incubated for 30 min on ice and then centrifuged at 16,000 ×*g* for 30 min at 4 °C. Proteins were quantified using the Bio-Rad DC assay kit (Bio-Rad Laboratories, Milan, Italy) according to the manufacturer's instructions. Briefly, protein concentrations were determined by comparing the absorbance value with a known value based on a calibration curve for Bovine Serum Albumin (BSA). The absorbance was measured at 750 nm using a plate reader.

2.5. Antibodies

Anti-Hsp60 (clone LK1) monoclonal antibody was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and used diluted 1:1000; anti pro-Caspase-3/Caspase-3 (CPP32) polyclonal antibody was purchased from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA) and used diluted 1:1000; anti β-actin (sc-47,778) monoclonal antibody was purchased from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and used diluted 1:500. Horseradish peroxidase (HRP)-conjugated sheep anti mouse antibody and anti-rabbit antibody and protein A sepharose were purchased from Amersham Biosciences (Ge Healthcare Life Science, Milan, Italy). Mouse IgG antibody conjugated with tetramethyirhodamine (TRITC) was from Sigma-Aldrich.

2.6. Immunofluorescence

Immunofluorescence was performed as described [24]. Briefly, NCI-H292 cells were deposited on an 8-well microscope-chamber slide at the density of ten thousand cells/well, cultured for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 and then treated with various doses (within the range 0–160 μM) of *CubipyOXA* for 24 h. Cells were fixed with ice cold methanol for 30 min, washed in phosphate buffered saline (PBS) pH 7.4 and then were incubated with unmasking solution (tri-sodium citrate 10 mM, 0.05% Tween 20, pH 6) for 10 min at 23 °C. After rinsing twice with PBS, the cells were blocked with 3% (*w*/*v*) Bovine Serum Albumin (BSA, Sigma Aldrich) for 30 min at 23 °C and incubated with the primary antibody, anti-HSP60, overnight at 4 °C. The day after, the cells were incubated with the TRITC-conjugated fluorescent secondary antibody for 1 h at 23 °C in a moist chamber. The nuclei were counterstained with Hoechst (Sigma-Aldrich) for 15 min at 23 °C. Finally, the slides were covered with drops of PBS and mounted with coverslips. Imaging was immediately performed with a Leica DM5000 upright fluorescence microscope (Leica Microsystems, Heidelberg, Germany).

2.7. Immunoprecipitation

Immunoprecipitation was performed as previously described [14]: 500 μg of proteins were incubated with 5 μg of anti-Hsp60 at 24 °C for 2 h, followed by incubation with 20 μl protein-A Sepharose (Amersham Biosciences, Milan, Italy) at 4 °C for 12 h. Subsequently, the incubation mixture was centrifuged in a microcentrifuge at 14,000 ×*g* for 30 s at 4 °C and the collected pellet was resuspended in lysis buffer and centrifuged again: this procedure was repeated three times. The last pellet was solubilized by boiling into 2× sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris-HCl [pH 6.8] and 0.001% bromophenol blue) and used for SDS-PAGE as described under Western Blotting. The same protocol was used for the anti-pro-Caspase-3 antibody.

2.8. Western blotting

Western Blotting analyses of protein cell lysates and immunoprecipitates were performed as previously described [25]. Briefly, 40 μg of proteins from cell lysates or 500 μg from immunoprecipitates were added to 4× Laemmli buffer and heated for 5 min at 95 °C. Proteins were resolved by 12% SDS-PAGE along with molecular weight marker (Bio-Rad). Then proteins were transferred to nitrocellulose membranes and blocked with 5% fat milk, and probed for 12 h at 4 °C. Then membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Blots were detected using the Supersignal West Femto, according to the manufacturer's instructions (Pierce, Milan, Italy) and chemiluminescent signals were recorded with a ChemiDoc XRS imager (Bio-Rad). Densitometric analysis of blots was performed using the NIH Image J 1.40 analysis program (National Institutes of Health, Bethesda, MD, USA).

2.9. Determination of apoptosis by flow cytometry

Fifty thousand NCI-H292 cells/well were plated into 24-well plates and grown until 80% confluence. Growth medium was replaced with FCS-free medium for 24 h and then cells were treated with *CubipyOXA* (various doses within the range 0 to160 μM) for 24 h. These times and doses were chosen based on the information given by the MTT assay on cell viability. Then NCI-H292 cells were harvested for apoptosis analysis according to a previously published technique [26], in which binding of Annexin V (AxV) is used to detect phosphatidylserine that is externalized on the outer leaflet of the plasma membrane of apoptotic cells. AxV-FITC (1 μg/ml) and propidium iodide (PI, 2.5 μg/ml) were added to tubes containing 1×10^5 cells/100 μl binding buffer. Cells were incubated in the dark for 15 min at 4 °C prior to flow cytometry analysis and then analyzed using a FACScan flow cytometer (Becton Dickinson, Oxford, UK). Control tubes lacking either AxV-FITC or PI, or both, were included to complete the controls. Analysis of dot-plots of fluorescence detector FL1 (AxV-FITC) versus FL2 (PI) was performed using Win MDI 2.8 (Flow Cytometry Software, University of Massachusetts, MA, USA). The degree of early apoptosis was expressed as the number of AxV+/PI - cells shown as the percentage of total cells. The late apoptotic cells were characterized as AxV+/PI+, and necrotic cells as PI+. Each experiment was carried out in duplicate and three experiments were performed in every instance.

2.10. Statistical analysis

Each experiment was ran in duplicate and was repeated three times. One-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons were used for statistical evaluation. All statistical analyses were performed using the program GraphPad PrismTM 4.0 (GraphPad Software Inc., San Diego, CA, USA). All data are presented as arithmetic mean ± SD, and the threshold level of statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Synthesis

The synthesis of the copper and zinc complexes [11] was performed by mixing a solution of the metal as perchlorate salt, with the bipyOXA ligand in ethanol (Fig. 1A). In turn, the ligand bipyOXA was synthesized through a solvent-free reaction between 2-cyanopyridine and 2-picolinamidoxime, by following the amidoxime-route (Fig. 1B) [27,28].

3.2. CubipyOXA is cytotoxic at various doses

The cytotoxicity of *Cubipy*OXA and *Znbipy*OXA on NCI-H292 cells was determined by MTT assay. Exposure for 24 h to increasing

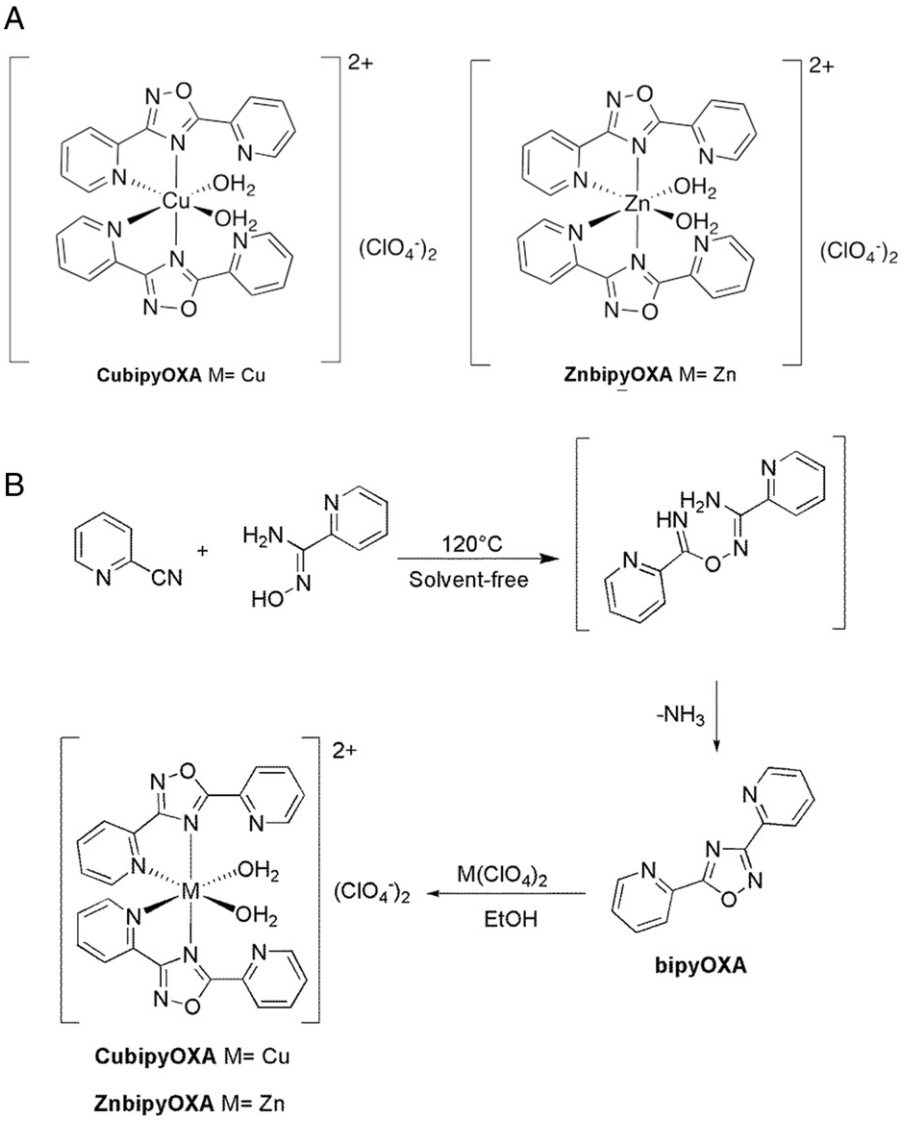

Fig. 1. A. Chemical structure of *CubipyOXA* and *ZnbipyOXA*. B. In the upper panel it is shown the ligand bipyOXA synthesis using a solvent-free reaction between 2-cyanopyridine and 2-picolinamidoxime, by following the amidoxime-route. In the lower panel it is shown the synthesis of bipyOXA and its Cu and Zn complexes, performed by mixing a solution of the metal as perchlorate salt, with the bipyOXA ligand in ethanol.

concentrations within the range 0–320 μM of *CubipyOXA* caused a significant diminution of cell viability, particularly at doses of 30 μM or higher (Fig. 2). GI50 was calculated at 30 μM as follows: GI50 = 100 × (T − T0)/(C − T0) = 50. From the data obtained, we chose for further experiments the following doses: 0, 30, 80, and 160 μM of *Cubipy*OXA. *Znbipy*OXA did not show significant cytotoxicity (data not shown) and was not studied further.

3.3. Hsp60 and C3 levels after CubipyOXA treatment

To evaluate the effect of *CubipyOXA* on Hsp60 and C3 levels, we performed Western blotting analyses and immunofluorescence. Exposure of the cells for 24 h to *CubipyOXA* diminished the levels of Hsp60 in a dose-dependent manner as shown by Western blotting data (Fig. 3A and B) and immunofluorescence (Fig. 3C and D). A significant decrease of Hsp60 levels began after treating cells with 80 μM of *CubipyOXA* (Fig. 3B). By contrast the levels of activated C3 were increased in comparison to the inactivated form (pC3) (Fig. 4A and B). Western blotting showed

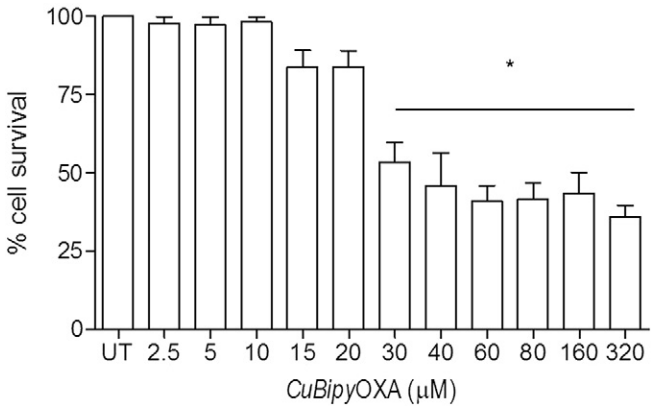

Fig. 2. *CubipyOXA* effect on cell viability. MTT Assay: the NCI-H292 cell line was treated for 24 h with *CubipyOXA* at various concentrations as indicated in the horizontal axis. Cell viability (vertical axis; arithmetic mean ± SD (N = 3)) significantly decreased with the higher doses. (*, significantly different from untreated (UT) cells; $p < 0.001$).

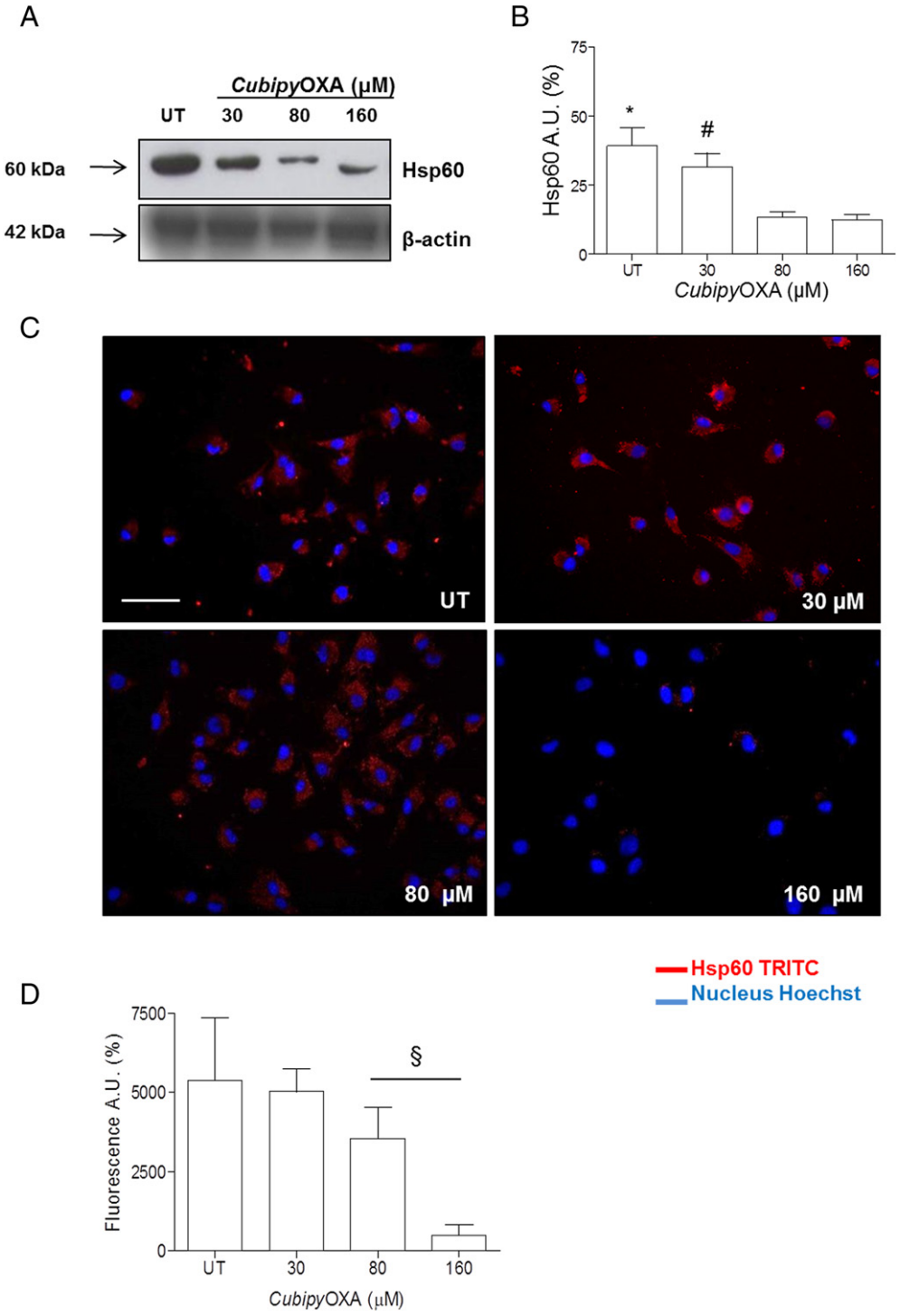

Fig. 3. *CubipyOXA* effect on Hsp60 levels. A. Representative Western blot showing a dose-dependent decrease in the levels of Hsp60 in cells treated with *CubipyOXA*. B. Densitometric quantification of Western blot bands showing the dose-dependent decrease in the levels of Hsp60 in cells treated with *CubipyOXA*. (*, significantly different from 80, and 160 μM; $p < 0.001$; # significantly different from 80, and 160 μM; $p < 0.001$). C. Representatives images and semi-quantitative evaluation of immunofluorescence, showing a distribution of the chaperonin and its decrease of Hsp60 after *CubipyOXA* treatment in a dose-dependent manner (Bar = 30 μm). D. Histogram showing the decrease of Hsp60 level after the treatment (§, significantly different from untreated (UT) cells; $p < 0.001$) (magnification 40×).

a dose-dependent decrease of pC3 accompanied by a simultaneous increase of activated C3 levels after *CubipyOXA* treatment (Fig. 4A).

3.4. Hsp60/pC3 complex decreases in cells treated with CubipyOXA

In order to investigate the possible interaction between Hsp60 and C3, we tested for the presence of the Hsp60/pC3 complex before and after treatment with *CubipyOXA* (Fig. 4C and D). The Hsp60/pC3 complex was present in untreated cells but its levels decreased in cells treated with *CubipyOXA*. Western blotting showed a significant diminution in the levels of the Hsp60/pC3 complex when cells were treated with 160 μM of *CubipyOXA*. Thus, a parallelism became evident between decreased levels of Hsp60 and apoptotic activation by the C3 pathway.

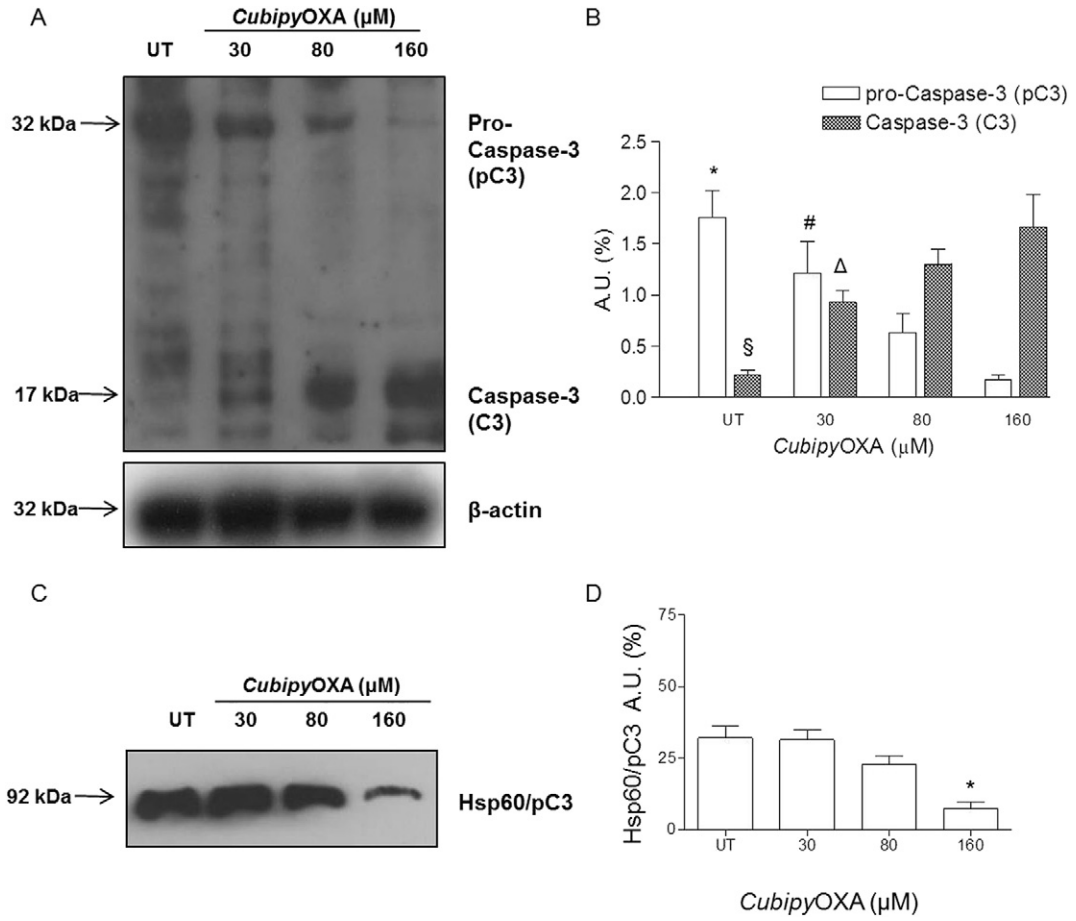

Fig. 4. Hsp60/pC3 interaction and its dissociation after *CubipyOXA* treatment. A. *CubipyOXA* affects the levels of pro-Caspase-3 and Caspase-3. Representative Western blots showing a dose-dependent decrease of pro-Caspase-3 (pC3; 32 kDa) and increase of Caspase-3 (C3; 17 kDa) in cells treated with various doses of *CubipyOXA*, as indicated on top. β-actin (42 kDa) is shown as the basal reference. B. Histogram showing the densitometric evaluation of the Western-blot bands for pro-Caspase-3 and Caspase-3. (*, significantly different from 30, 80, and 160 μM; $p < 0.001$; #, significantly different from 160 μM; $p < 0.05$; §, significantly different from 80, and 160 μM; $p < 0.001$; Δ, significantly different from 80, and 160 μM; $p < 0.001$). C. The complex Hsp60/pro-Caspase-3 (Hsp60/pC3) decreases in cells treated with *CubipyOXA* in a dose-dependent manner. Representative Western blots showing a dose-dependent decrease of complex Hsp60/pC3 (32 kDa) in cells treated with various doses of *CubipyOXA*, as indicated on top. D. Histogram showing the densitometric evaluation of the Western-blot bands for the complex Hsp60/pC3. (*, significantly different from untreated cells (UT) or cells treated with 30 or 80 μM; $p < 0.01$).

3.5. CubipyOXA induces apoptosis

We measured the effects on viability/apoptosis of *CubipyOXA* in NCI-H292 cells. After 24 h of exposure to increasing concentrations within the range 0–160 μM of *CubipyOXA*, there was an increase of apoptotic events with diminution of surviving cells. The percentage of early and late apoptotic cells (PI^- AnV^+ and PI^+ AnV^+) began to increase significantly at 30 μM (17%) with respect to untreated cells (UT) and this percentage increased at 80 and 160 μM to 40% and 50%, respectively (Fig. 5). The MTT test revealed that the percentage of cell death increased with increasing does of *CubipyOXA* and cell death correlated with apoptosis activation (Fig. 5). In addition, *CubipyOXA* induced the appearance of highly condensed nuclei which represents a typical apoptotic hallmark, as demonstrated by morphological analyses performed with fluorescence microscopy after staining with Hoechst (Supplementary Fig. S1).

4. Discussion

Data obtained in the last decade indicate that Heat shock proteins (Hsps) have anti-apoptotic and, consequently, pro-tumor properties [29,30,31]. Apoptosis contributes to tumor demise following chemotherapy but, in patient management, it is still unclear how to achieve a healthy balance between cell proliferation and death [32]. In some instances, elevated levels of Hsps protect tumor cells against therapy-induced apoptosis [33]. Hsps can block both the intrinsic and the extrinsic apoptotic pathways through the interaction with proteins involved in the apoptotic process [30,31,33]. For example, high levels of Hsp27, or Hsp70, or Hsp90 inhibit apoptosis by preventing Caspase activation in a variety of cellular models [34–36]. However, the molecular mechanism of this anti-apoptotic effect mediated by Hsps is still unclear and it is possible that, under certain circumstances, may be due by a variety of different modulators [34]. It has been shown that Hsp70 can inhibit apoptosis by interacting with a member of the Fas death-inducing signaling upstream of Caspase-8 [37,38]. Hsp70, during oxidative stress, can stabilize Bcl-2, protecting the cell against apoptosis [39]. Also other Hsps, such as Hsp27 and Hsp72, are involved in protection against stress-induced apoptosis, depending on the cellular context, by interaction with components of the apoptotic cascade [40,41].

Current strategies for the development of anticancer drugs include the identification of molecules that are crucial for tumor progression and can be used as targets for blocking agents. Along this line of thought, inhibition of certain Hsps known to be involved in carcinogenesis has been proposed as a potential *anti*-cancer treatment strategy worth testing [42–44,35]. Furthermore, monitoring the levels of Hsps can be a useful way of following up the response to therapy [6].

In the search for new pharmaceuticals and bioactive compounds, oxadiazoles are useful scaffolds [45,46]. For instance, the biological activity of a copper complex with oxadiazoles has been studied in two

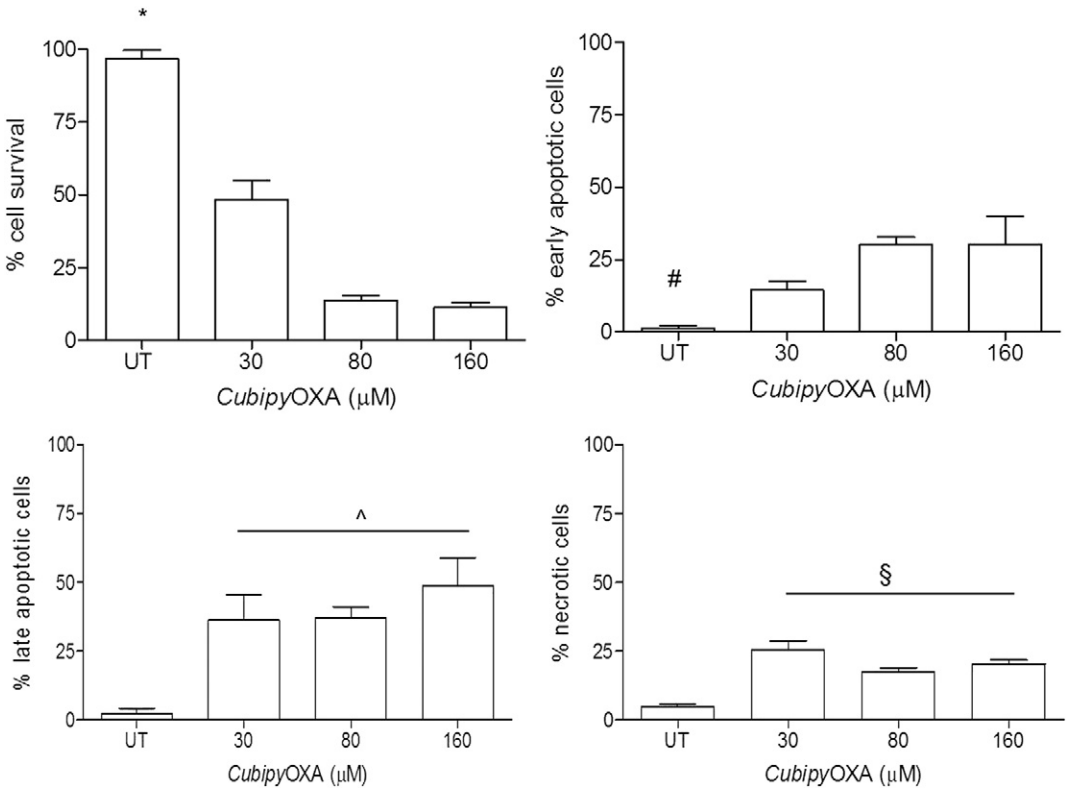

Fig. 5. Evaluation of apoptosis indicators by flow cytometry. After 24 h exposure to increasing concentrations (indicated in the horizontal axis) of *CubipyOXA* there was an increase of apoptotic indicators (vertical axis) which correlated with the decrease in cell viability. The percentage of apoptotic cells (PI^- AnV^+ and PI^+ AnV^+) significantly increased at 30 μM by comparison with the untreated (UT) cells. (*, significantly different from 30, 80, and 160 μM; $p < 0.001$; #, significantly different from 30, 80, and 160 μM; $p < 0.001$; ^, significantly different from 30, 80, and 160 μM; $p < 0.001$; §, significantly different from 30, 80, and 160 μM; $p < 0.001$).

cancer-cell models [11]. A copper complex reduced the viability of HepG2 and HT29 cells in a dose- and time-dependent manner and the cells showed signs of apoptosis. In the work reported here, the copper complex *CubipyOXA* was tested to assess its chemotherapeutic potential on NCI-H292 cells. *CubipyOXA* lowered the cells viability in a dose- and time-dependent manner. Moreover, after 24 h of exposure to increasing concentrations of *CubipyOXA*, there was an increase in the apoptotic features that paralleled the drop in cell viability. The percentage of apoptotic cells (PI-AnV + and PI + AnV+) significantly increased in cells exposed to the compound in comparison with untreated controls. *CubipyOXA* most likely acts as a DNA-condensing agent as indicated by its spectroscopic and hydrodynamic features that are compatible with a DNA groove binder [13] and coincide with the nuclei condensation we found at high doses of *CubipyOXA*, all of which is directly correlated with the antiproliferative effect of the compound.

It has already been reported that Hsp60 has a pro-apoptotic role favoring activation of Caspase-3 (C3) [10,47] but others claim that Hsp60 is cytoprotective because it stabilizes the levels of survivin and inhibits p53, thus keeping the cell alive [9]. It is also known that Hsp60 forms a stable complex with pro-Caspase-3 (pC3) with the consequent anti-apoptotic effect [5]. Accordingly, we tried to answer the question whether *CubipyOXA*, capable of inducing death in cancer cells, would have an effect on the Hsp60/pC3 complex and on Hsp60 levels: the aim was to develop an effective drug for anticancer therapy [48]. We found that in *CubipyOXA*-treated cells, Hsp60 levels decreased by comparison with untreated cells, in a dose-dependent manner as demonstrated by Western blotting and immunofluorescence. Concomitantly, the levels of activated C3 (17 kDa) increased while the levels of inactivated pC3 (32 kDa) decreased. The complex Hsp60/pC3 was present in untreated cells but it decreased progressively with increasing doses of *CubipyOXA* to finally become undetectable in cells exposed to 160 μM of the copper compound.

Various data support the hypothesis that Hsp60 favors carcinogenesis and show a close correlation between high levels of Hsp60 and different types of cancer, thus the immunopositivity for Hsp60 can be considered a biomarker useful for the diagnosis and monitoring these types of malignancies [33,49–50]. Frequently, the immunopositivity for Hsp60, in cancerous tissue or circulating cancer cells, correlates with a poor prognosis [49,51–53]. In contrast to these findings, we previously found that the loss of Hsp60 immunopositivity can be related to development and progression of bronchial cancer [54], which reflects the variety of roles that Hsp60 can play.

In our experimental model, the high levels of Hsp60 likely cause the inhibition of the pC3 activation and the resistance to apoptosis. The compound *CubipyOXA* determines the decrease of the Hsp60 levels, probably due to DNA binding by *CubipyOXA* [13], and the separation of the complex Hsp60/pC3 and consequently the C3 activation of the caspase cascade, associated with a tumor-cell growth arrest. These findings suggest that the reduction of Hsp60 levels might have various causes, as it has been already demonstrated, in which Hsp60 could be tagged by post-translational modifications, such as hyperacetylation and ubiquitination. This could regulate the degradation of the chaperone by the ubiquitin-proteosome system [12,55]. Otherwise, the post-translational modifications could constitute a signal for extracellular secretion through the lipid raft pathway [11,49,56]. The function of extracellular Hsp60 is still under discussion. For instance, it may mediate interactions with the immune cells and other body tissues and alter the tumor microenvironment [11,12,55,57].

As far as we know, no previous studies have shown the activity of a compound able to interfere with the interaction between Hsp60 and pC3 as *CubipyOXA* seems to do. Therefore, *CubipyOXA* represents a

new candidate for developing novel anti-tumor treatment strategies directed against the pro-cancer activity of Hsp60.

5. Conclusion

The quantitative dose-response curves of the levels of Hsp60, activated C3, inactivated pC3, Hsp60/pC3 complex and indicators of cell apoptosis, and cell death, all coincided to show that *CubipyOXA* has pro-apoptotic activity and promotes death of NCI-H292 cells. We have proposed the possible mechanism underlying this effect. Most likely, the pro-apoptotic effects of *CubipyOXA* are due to its lowering the Hsp60 levels and to its blocking the formation of the Hsp60/pC3 complex, and/or its dissociating the complex when already formed, thus, interfering with the anti-apoptotic action of Hsp60, without affecting the Hsp60 functions in normal cells. Therefore, *CubipyOXA* represents a novel candidate drug for cancer treatment targeting Hsp60 that merits further studies in vivo.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2017.02.004.

Acknowledgements

Financial support from the Italian MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca) within the "FIRB-Futuro in Ricerca 2008" Program - Project n. RBFR08A9V1 and the "FIRB-Futuro in Ricerca 2012" Program - Project RBFR12SIPT is gratefully acknowledged. A.J.L.M. and E. C.de M. were partially supported by IMET; A.J.L.M. and F.C. were partially supported by IEMEST. This work was done under the umbrella of the agreement between the Euro-Mediterranean Institute of Science and Technology (Italy) and Institute of Marine and Environmental Technology (USA) signed in March 2012 (this is IMET contribution number 17-194).

References

[1] S. Goel, F. Chen, W. Cai, Synthesis and biomedical applications of copper sulfide nanoparticles: from sensors to theranostics, Small 10 (2014) 631–645, http://dx.doi.org/10.1002/smll.201301174.
[2] B. Żołnowska, J. Sławiński, A. Pogorzelska, K. Szafrański, A. Kawiak, G. Stasiłojć, M. Belka, S. Ulenberg, T. Bączek, J. Chojnacki, Novel 5-Substituted 2-(Aylmethylthio)-4-chloro-N-(5-aryl-1,2,4-triazin-3-yl)benzenesulfonamides: Synthesis, molecular structure, anticancer activity, apoptosis-inducing activity and metabolic stability, Molecules 21 (2016)http://dx.doi.org/10.3390/molecules21060808.
[3] S. Santagata, Y.M. Xu, E.M. Wijeratne, R. Kontnik, C. Rooney, C.C. Perley, H. Kwon, J. Clardy, S. Kesari, L. Whitesell, S. Lindquist, A.A. Gunatilaka, Using the heat-shock response to discover anticancer compounds that target protein homeostasis, ACS Chem. Biol. 7 (2012) 340–349, http://dx.doi.org/10.1021/cb200353m.
[4] F. Cappello, E. Conway de Macario, L. Marasà, G. Zummo, A.J.L. Macario, Hsp60 expression, new locations, functions and perspectives for cancer diagnosis and therapy, Cancer Biol Ther. 801-9 (2008).
[5] C. Campanella, F. Bucchieri, N.M. Ardizzone, A. Marino Gammazza, A. Montalbano, A. Ribbene, V. Di Felice, M. Bellafiore, S. David, F. Rappa, M. Marasà, G. Peri, F. Farina, A.M. Czarnecka, E. Conway de Macario, A.J.L. Macario, G. Zummo, F. Cappello, Upon oxidative stress, the antiapoptotic Hsp60/pro-caspase-3 complex persists in mucoepidermoid carcinoma cells, Eur. J. Histochem. 4 (2008) 221–228.
[6] F. Cappello, A. Marino Gammazza, A. Palumbo Piccionello, C. Campanella, A. Pace, E. Conway de Macario, A.J.L. Macario, Hsp60 chaperonopathies and chaperonotherapy: targets and agents, Expert Opin. Ther. Targets 2 (2014) 185–208, http://dx.doi.org/10.1517/14728222.2014.856417.
[7] D. Chandra, G. Choy, D.G. Tang, Cytosolic accumulation of HSP60 during apoptosis with or without apparent mitochondrial release: evidence that its pro-apoptotic or pro-survival functions involve differential interactions with caspase-3, J. Biol. Chem. 43 (2007) 31289–31301, http://dx.doi.org/10.1074/jbc.M702777200.
[8] S.J. Charette, J.N. Lavoie, H. Lambert, J. Landry, Inhibition of Daxx-mediated apoptosis by heat shock protein 27, Mol. Cell. Biol. 20 (2000) 7602–7612.
[9] J.C. Ghosh, T. Dohi, B.H. Kang, D.C. Altieri, Hsp60 regulation of tumor cell apoptosis, J. Biol. Chem. 8 (2008) 5188–5194, http://dx.doi.org/10.1074/jbc.M705904200.
[10] A. Samali, J. Cai, B. Zhivotovsky, D.P. Jones, S. Orrenius, Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of jurkat cells, EMBO J. 8 (1999) 2040–2048, http://dx.doi.org/10.1093/emboj/18.8.2040.
[11] C. Campanella, A. D'Anneo, A. Marino Gammazza, C. Caruso Bavisotto, R. Barone, S. Emanuele, F. Lo Cascio, E. Mocciaro, S. Fais, E. Conway de Macario, A.J.L. Macario, F. Cappello, M. Lauricella, The histone deacetylase inhibitor SAHA induces HSP60 nitration and its extracellular release by exosomal vesicles in human lung-derived carcinoma cells, Oncotarget (2015)http://dx.doi.org/10.18632/oncotarget.6680.
[12] M. Gorska, A. Marino Gammazza, M.A. Zmijewski, C. Campanella, F. Cappello, T. Wasiewicz, A. Kuban-Jankowska, A. Daca, A. Sielicka, U. Popowska, N. Knap, J. Antoniewicz, T. Wakabayashi, M. Wozniak, Geldanamycin-induced osteosarcoma cell death is associated with hyperacetylation and loss of mitochondrial pool of heat shock protein 60 (hsp60), PLoS One 8 (2013), e71135. http://dx.doi.org/10.1371/journal.pone.0071135.
[13] A. Terenzi, G. Barone, A. Palumbo Piccionello, G. Giorgi, A. Guarcello, P. Portanova, G. Calvaruso, S. Buscemi, N. Vivona, A. Pace, Synthesis, characterization, cellular uptake and interaction with native DNA of a bis(pyridyl)-1,2,4-oxadiazole copper(II) complex, Dalton Trans. 38 (2010) 9140–9145, http://dx.doi.org/10.1039/c0dt00266f.
[14] G. Facchin, N. Veiga, M.G. Kramer, A.A. Batista, K. Várnagy, E. Farkas, V. Moreno, M.H. Torre, Experimental and theoretical studies of copper complexes with isomeric dipeptides as novel candidates against breast cancer, J Inorg Biochem. (2016) (pii: S0162-0134(16)30156-8. doi: 10.1016/j.jinorgbio.2016.06.005).
[15] A. Terenzi, G. Barone, A. Palumbo Piccionello, G. Giorgi, A. Guarcello, A. Pace, Synthesis and chemical characterization of Cu^{II}, Ni^{II} and Zn^{II} complexes of 3,5-bis(2′-pyridyl)-1,2,4-oxadiazole and 3-(2′-pyridyl)5-(phenyl)-1,2,4-oxadiazole ligands, Inorg. Chim. Acta 1 (2011) 62–67, http://dx.doi.org/10.1016/j.ica.2011.03.057.
[16] J. Tan, B. Wang, L. Zhu, DNA binding and oxidative DNA damage induced by a quercetin copper(II) complex: potential mechanism of its antitumor properties, J. Biol. Inorg. Chem. 5 (2009) 727–739, http://dx.doi.org/10.1007/s00775-009-0486-8.
[17] A. Chakraborty, P. Kumar, K. Ghosh, P. Roy, Evaluation of a Schiff base copper complex compound as potent anticancer molecule with multiple targets of action, Eur. J. Pharmacol. 1–3 (2010) 1–12 (doi:0.1016/j.ejphar.2010.08.003).
[18] C. Santini, M. Pellei, V. Gandin, M. Porchia, F. Tisato, C. Marzano, Advances in copper complexes as anticancer agents, Chem. Rev. 1 (2014) 815–862, http://dx.doi.org/10.1021/cr400135x.
[19] J. Shao, Z.Y. Ma, A. Li, Y.H. Liu, C.Z. Xie, Z.Y. Qiang, J.Y. Xu, Thiosemicarbazone Cu(II) and Zn(II) complexes as potential anticancer agents: syntheses, crystal structure, DNA cleavage, cytotoxicity and apoptosis induction activity, J. Inorg. Biochem. 136 (2014) 13–23, http://dx.doi.org/10.1016/j.jinorgbio.2014.03.004.
[20] J.L. García-Giménez, M. González-Alvarez, M. Liu-González, B. Macías, J. Borrás, G. Alzuet, Toward the development of metal-based synthetic nucleases: DNA binding and oxidative DNA cleavage of a mixed copper(II) complex with N-(9H-purin-6-yl)benzenesulfonamide and 1,10-phenantroline. Antitumor activity in human Caco-2 cells and Jurkat T lymphocytes. Evaluation of p53 and Bcl-2 proteins in the apoptotic mechanism, J. Inorg. Biochem. 103 (2009) 923–934, http://dx.doi.org/10.1016/j.jinorgbio.2009.04.003.
[21] G. Cerchiaro, K. Aquilano, G. Filomeni, G. Rotilio, M.R. Ciriolo, A.M. Ferreira, Isatin-Schiff base copper(II) complexes and their influence on cellular viability, J. Inorg. Biochem. 99 (2005) 1433–1440, http://dx.doi.org/10.1016/j.jinorgbio.2005.03.013.
[22] A.M. Merendino, F. Bucchieri, C. Campanella, V. Marcianò, A. Ribbene, S. David, G. Zummo, G. Burgio, D.F. Corona, E. Conway de Macario, A.J.L. Macario, F. Cappello, Hsp60 is actively secreted by human tumor cells, PLoS One 5 (2010), e9247. http://dx.doi.org/10.1371/journal.pone.0009247.
[23] A.M. Merendino, F. Bucchieri, R. Gagliardo, A. Daryadel, F. Pompeo, G. Chiappara, R. Santagata, V. Bellia, S. David, F. Farina, D.E. Davies, H.U. Simon, A.M. Vignola, CD40 ligation protects bronchial epithelium against oxidant-induced caspase-independent cell death, Am. J. Respir. Cell Mol. Biol. 2 (2006) 155–164, http://dx.doi.org/10.1165/rcmb.2005-0433OC.
[24] A. Marino Gammazza, M. Rizzo, R. Citarrella, F. Rappa, C. Campanella, F. Bucchieri, A. Patti, D. Nikolic, D. Cabibi, G. Amico, P.G. Conaldi, P.L. San Biagio, G. Montalto, F. Farina, G. Zummo, E. Conway de Macario, A.J.L. Macario, F. Cappello, Elevated blood Hsp60, its structural similarities and cross-reactivity with thyroid molecules, and its presence on the plasma membrane of oncocytes point to the chaperonin as an immunopathogenic factor in Hashimoto's thyroiditis, Cell Stress Chaperones 3 (2014) 343–353, http://dx.doi.org/10.1007/s12192-013-0460-9.
[25] R. Barone, F. Macaluso, P. Catanese, A. Marino Gammazza, L. Rizzuto, P. Marozzi, G. Lo Giudice, T. Stampone, F. Cappello, G. Morici, G. Zummo, F. Farina, V. Di Felice, Endurance exercise and conjugated linoleic acid (CLA) supplementation up-regulate CYP17A1 and stimulate testosterone biosynthesis, PLoS One 8 (2013), e79686. http://dx.doi.org/10.1371/journal.pone.0079686.
[26] I. Vermes, C. Haanen, H. Steffens-Nakken, C. Reutelingsperger, A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V, J. Immunol. Methods 1 (1995) 39–51.
[27] C.G. Fortuna, C. Bonaccorso, A. Bulbarelli, G. Caltabiano, L. Rizzi, L. Goracci, G. Musumarra, A. Pace, A. Palumbo Piccionello, A. Guarcello, P. Pierro, C.E. Cocuzza, R. Musumeci, New linezolid-like 1,2,4-oxadiazoles active against gram-positive multiresistant pathogens, Eur. J. Med. Chem. 65 (2013) 533–545, http://dx.doi.org/10.1016/j.ejmech.2013.03.069.
[28] C.G. Fortuna, R. Berardozzi, C. Bonaccorso, G. Caltabiano, L. Di Bari, L. Goracci, A. Guarcello, A. Pace, A. Palumbo Piccionello, G. Pescitelli, P. Pierro, E. Lonati, A. Bulbarelli, C.E. Cocuzza, G. Musumarra, R. Musumeci, New potent antibacterials against gram-positive multiresistant pathogens: effects of side chain modification and chirality in linezolid-like 1,2,4-oxadiazoles, Bioorg. Med. Chem. 24 (2014) 6814–6825, http://dx.doi.org/10.1016/j.bmc.2014.10.037.
[29] X. Gao, W. Liu, L. Huang, T. Zhang, Z. Mei, X. Wang, J. Gong, Y. Zhao, F. Xie, J. Ma, L. Qian, HSP70 inhibits stress-induced cardiomyocyte apoptosis by competitively binding to FAF1, Cell Stress Chaperones 4 (2015) 653–661, http://dx.doi.org/10.1007/s12192-015-0589-9.
[30] A.L. Joly, G. Wettstein, G. Mignot, F. Ghiringhelli, C. Garrido, Dual role of heat shock proteins as regulators of apoptosis and innate immunity, J. Innate Immun. 3 (2010) 238–247, http://dx.doi.org/10.1159/000296508.

[31] H. Mjahed, F. Girodon, M. Fontenay, C. Garrido, Heat shock proteins in hematopoietic malignancies, Exp. Cell Res. 318 (2012) 1946–1958, http://dx.doi.org/10.1016/j.yexcr.2012.05.012.
[32] R. Kim, M. Emi, K. Tanabe, The role of apoptosis in cancer cell survival and therapeutic outcome, Cancer Biol. Ther. 11 (2006) 1429–1442.
[33] F. Rappa, F. Farina, G. Zummo, S. David, C. Campanella, F. Carini, G. Tomasello, P. Damiani, F. Cappello, E. Conway de Macario, A.J.L. Macario, HSP-molecular chaperones in cancer biogenesis and tumor therapy: an overview, Anticancer Res. 12 (2012) 5139–5150.
[34] C. Garrido, M. Brunet, C. Didelot, Y. Zermati, E. Schmitt, G. Kroemer, Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties, Cell Cycle 22 (2006) 2592–2601, http://dx.doi.org/10.4161/cc.5.22.3448.
[35] A.J. Massey, D.S. Williamson, H. Browne, J.B. Murray, P. Dokurno, T. Shaw, A.T. Macias, Z. Daniels, S. Geoffroy, M. Dopson, P. Lavan, N. Matassova, G.L. Francis, C.J. Graham, R. Parsons, Y. Wang, A. Padfield, M. Comer, M.J. Drysdale, M. Wood, A novel, small molecule inhibitor of Hsc70/Hsp70 potentiates Hsp90 inhibitor induced apoptosis in HCT116 colon carcinoma cells, Cancer Chemother. Pharmacol. 3 (2010) 535–545, http://dx.doi.org/10.1007/s00280-009-1194-3.
[36] D.D. Mosser, R.I. Morimoto, Molecular chaperones and the stress of oncogenesis, Oncogene 16 (2004) 2907–2918, http://dx.doi.org/10.1038/sj.onc.1207529.
[37] X. Gao, W. Liu, L. Huang, T. Zhang, Z. Mei, X. Wang, J. Gong, Y. Zhao, F. Xie, J. Ma, L. Qian, HSP70 inhibits stress-induced cardiomyocyte apoptosis by competitively binding to FAF1, Cell Stress Chaperones 4 (2015) 653–661, http://dx.doi.org/10.1007/s12192-015-0589-9.
[38] Y. Zhao, W. Wang, L. Qian, Hsp70 may protect cardiomyocytes from stress-induced injury by inhibiting Fas-mediated apoptosis, Cell Stress Chaperones 1 (2007) 83–95.
[39] B. Jiang, P. Liang, G. Deng, Z. Tu, M. Liu, X. Xiao, Increased stability of Bcl-2 in HSP70-mediated protection against apoptosis induced by oxidative stress, Cell Stress Chaperones 2 (2011) 143–152, http://dx.doi.org/10.1007/s12192-010-0226-6.
[40] A.M. Chow, R. Steel, R.L. Anderson, Hsp72 chaperone function is dispensable for protection against stress-induced apoptosis, Cell Stress Chaperones 3 (2009) 253–263, http://dx.doi.org/10.1007/s12192-008-0079-4.
[41] A. Vasconsuelo, L. Milanesi, R. Boland, Participation of HSP27 in the antiapoptotic action of 17beta-estradiol in skeletal muscle cells, Cell Stress Chaperones 2 (2010) 183–192, http://dx.doi.org/10.1007/s12192-009-0132-y.
[42] F. Cappello, E. Conway de Macario, G. Zummo, A.J.L. Macario, Immunohistochemistry of human Hsp60 in health and disease: from autoimmunity to cancer, Methods Mol. Biol. 787 (2011) 245–254, http://dx.doi.org/10.1007/978-1-61779-295-3_18.
[43] G.N. Castro, N. Cayado-Gutiérrez, F.C. Zoppino, M.A. Fanelli, F.D. Cuello-Carrión, M. Sottile, S.B. Nadin, D.R. Ciocca, Effects of temozolomide (TMZ) on the expression and interaction of heat shock proteins (HSPs) and DNA repair proteins in human malignant glioma cells, Cell Stress Chaperones 2 (2015) 253–265, http://dx.doi.org/10.1007/s12192-014-0537-0.
[44] C.G. Evans, L. Chang, J.E. Gestwicki, Heat shock protein 70 (hsp70) as an emerging drug target, J. Med. Chem. 12 (2010) 4585–4602, http://dx.doi.org/10.1021/jm100054f.
[45] L. Lentini, R. Melfi, A. Di Leonardo, A. Spinello, G. Barone, A. Pace, A. Palumbo Piccionello, I. Pibiri, Toward a rationale for the PTC124 (ataluren) promoted readthrough of premature stop codons: a computational approach and GFP-reporter cell-based assay, Mol. Pharm. 3 (2014) 653–664, http://dx.doi.org/10.1021/mp400230s.
[46] F.S. Palumbo, M. Di Stefano, A. Palumbo Piccionello, C. Fiorica, G. Pitarresi, I. Pibiri, S. Buscemi, G. Giammona, Perfluorocarbon functionalized hyaluronic acid derivatives as oxygenating systems for cell culture, RSC Adv. 44 (2014) 22894–22901, http://dx.doi.org/10.1039/C4RA01502A.
[47] S. Xanthoudakis, S. Roy, D. Rasper, T. Hennessey, Y. Aubin, R. Cassady, P. Tawa, R. Ruel, A. Rosen, D.W. Nicholson, Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis, EMBO J. 8 (1999) 2049–2056, http://dx.doi.org/10.1093/emboj/18.8.2049.
[48] A. Pace, G. Barone, A. Lauria, A. Martorana, A.P. Piccionello, P. Pierro, A. Terenzi, A.M. Almerico, S. Buscemi, C. Campanella, F. Angileri, F. Carini, G. Zummo, E. Conway de Macario, F. Cappello, A.J.L. Macario, Hsp60, a novel target for antitumor therapy: structure-function features and prospective drugs design, Curr. Pharm. Des. 19 (2013) 2757–2764.
[49] C. Campanella, F. Rappa, C. Sciumè, A. Marino Gammazza, R. Barone, F. Bucchieri, S. David, G. Curcurù, C. Caruso Bavisotto, A. Pitruzzella, G. Geraci, G. Modica, F. Farina, G. Zummo, S. Fais, E. Conway de Macario, A.J.L. Macario, F. Cappello, Heat shock protein 60 levels in tissue and circulating exosomes in human large bowel cancer before and after ablative surgery, Cancer 121 (2015) 3230–3239, http://dx.doi.org/10.1002/cncr.29499.
[50] C. Hamelin, E. Cornut, F. Poirier, S. Pons, C. Beaulieu, J. Charrier, H. Haïdous, E. Cotte, C. Lambert, F. Piard, Y. Ataman-Önal, G. Choquet-Kastylevsky, Identification and verification of heat shock protein 60 as a potential serum marker for colorectal cancer, FEBS J. 278 (2011) 4845–4859, http://dx.doi.org/10.1111/j.1742-4658.2011.08385.x.
[51] W.W. Tong, G.H. Tong, H. Kong, Y. Liu, The tumor promoting roles of HSP60 and HIF2α in gastric cancer cells, Tumour Biol. 37 (2016) 9849–9854, http://dx.doi.org/10.1007/s13277-015-4783-2.
[52] X. Xu, W. Wang, W. Shao, W. Yin, H. Chen, Y. Qiu, M. Mo, J. Zhao, Q. Deng, J. He, Heat shock protein-60 expression was significantly correlated with the prognosis of lung adenocarcinoma, J. Surg. Oncol. 104 (2011) 598–603, http://dx.doi.org/10.1002/jso.21992.
[53] A. Glaessgen, S. Jonmarker, A. Lindberg, B. Nilsson, R. Lewensohn, P. Ekman, A. Valdman, L. Egevad, Heat shock proteins 27, 60 and 70 as prognostic markers of prostate cancer, APMIS 116 (2008) 888–895, http://dx.doi.org/10.1111/j.1600-0463.2008.01051.x.
[54] F. Cappello, A. Di Stefano, S.E. D'Anna, C.F. Donner, G. Zummo, Immunopositivity of heat shock protein 60 as a biomarker of bronchial carcinogenesis, Lancet Oncol. 10 (2005) 816, http://dx.doi.org/10.1016/S1470-2045(05)70393-4.
[55] A. Marino Gammazza, C. Campanella, R. Barone, C. Caruso Bavisotto, M. Gorska, M. Wozniak, F. Carini, F. Cappello, A. D'Anneo, M. Lauricella, G. Zummo, E. Conway de Macario, A.J.L. Macario, V. Di Felice, Doxorubicin anti-tumor mechanisms include Hsp60 post-translational modifications leading to the Hsp60/p53 complex dissociation and instauration of replicative senescence, Cancer Lett. 385 (2017) 75–86.
[56] C. Campanella, C. Caruso Bavisotto, A. Marino Gammazza, D. Nikolic, F. Rappa, S. David, F. Cappello, F. Bucchieri, S. Fais, Exosomal heat shock proteins as new players in tumour cell-to-cell communication, J. Circ. Biomark. 3 (2014) 5, http://dx.doi.org/10.5772/58721.
[57] D. Hayoun, T. Kapp, M. Edri-Brami, T. Ventura, M. Cohen, A. Avidan, R.G. Lichtenstein, HSP60 is transported through the secretory pathway of 3-MCA-induced fibrosarcoma tumour cells and undergoes N-glycosylation, FEBS J. 279 (2012) 2083–2095, http://dx.doi.org/10.1111/j.1742-4658.2012.08594.x (4).