Triggers autophagic cell death with a di-manganese(II) developmental therapeutic

Creina Slator, Zara Molphy, Vickie McKee, Andrew Kellett

School of Chemical Sciences and National Institute for Cellular Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland

1. Introduction

Metals ions including Mn^{2+}, Mg^{2+}, Cu^{2+} and Zn^{2+} are essential in the biochemistry and physiology of living organisms as they are required cofactors for ubiquitous enzymes, transcription factors, transmembrane transporters, growth factors and receptors [1]. Deficiencies in these metal ions result in the onset of neurodegenerative diseases including Alzheimer's, Parkinson's and Menkes syndrome, while Cu^{2+} overload is associated with Wilson's disease [2]. The incorporation of metal ions into complex scaffolds containing targeting ligands, however, has resulted in the development of anti-tumoural drug candidates [3–5] capable of mediating apoptotic cell death through a combination of intrinsic and extrinsic pathways [6]. Numerous examples of metal complexes inducing cancer cytotoxicity through apoptosis have been reported in the literature [7]; this fatal mechanism relies on a class of cysteine-dependent aspartate-specific proteases (caspases) that activate and execute the apoptotic process leading to characteristic morphological changes and ultimately cell death.

While the majority of cytotoxic metallodrugs act through the induction of apoptosis, the alternative pathway of autophagy has recently emerged as an attractive process to effect cytotoxicity [8]. The phenomenon of autophagy was mechanistically unknown prior to the 1990’s until seminal work by Ohsumi revealed the systematic activation and identification of genes essential in the overall pathway [9–12]. Autophagy is considered as an evolutionary-conserved self-digestion, and quality control mechanism where cell survival and degradation processes compete in order to sustain homeostasis and regulate the longevity of proteins, nucleic acids, whole organelles and pathogenic agents [13]. Under the constraints of increased and/or external stress factors, however, elevated accumulation of autophagic vacuoles and organelle elimination renders the cell incapable of normal function.

There is an unmet need for novel metal-based chemotherapeutics with alternative modes of action compared to clinical agents such as cisplatin and metallo-bleomycin. Recent attention in this field has focused on designing intracellular ROS-mediators as powerful cytotoxins of human cancers and identifying potentially unique toxic mechanisms underpinning their utility. Herein, we report the developmental di-manganese(II) therapeutic $[\text{Mn}_2(\text{μ-oda})(\text{phen})_2(\text{H}_2\text{O})_2][\text{Mn}_2(\text{μ-oda})(\text{phen})_2(\text{oda})_2]\cdot\text{4H}_2\text{O} \ (\text{Mn-Oda})$ induces autophagy-promoted apoptosis in human ovarian cancer cells (SKOV3). The complex was initially identified to intercalate DNA by topoisomerase I unwinding and circular dichroism spectroscopy. Intracellular DNA damage, detected by γH2AX and the COMET assay, however, is not linked to direct Mn-Oda free radical generation, but is instead mediated through the promotion of intracellular reactive oxygen species (ROS) leading to autophagic vacuole formation and downstream nuclear degradation. To elucidate the cytotoxic profile of Mn-Oda, a wide range of biomarkers specific to apoptosis and autophagy including caspase release, mitochondrial membrane integrity, fluorogenic probe localisation, and cell cycle analysis were employed. Through these techniques, the activity of Mn-Oda was compared directly to i.) the pro-apoptotic clinical anticancer drug doxorubicin, ii.) the multimodal histone deacetylase inhibitor suberoyanilide hydroxamic acid, and iii.) the autophagy inducer rapamycin. In conjunction with ROS-specific trapping agents and established inhibitors of autophagy, we have identified autophagy-induction linked to mitochondrial superoxide production, with confocal image analysis of SKOV3 cells further supporting autophagosome formation.

ARTICLE INFO

Keywords:
Cancer
Manganese
Superoxide
Autophagy
Apoptosis

ABSTRACT

There is an unmet need for novel metal-based chemotherapeutics with alternative modes of action compared to clinical agents such as cisplatin and metallo-bleomycin. Recent attention in this field has focused on designing intracellular ROS-mediators as powerful cytotoxins of human cancers and identifying potentially unique toxic mechanisms underpinning their utility. Herein, we report the developmental di-manganese(II) therapeutic $[\text{Mn}_2(\text{μ-oda})(\text{phen})_2(\text{H}_2\text{O})_2][\text{Mn}_2(\text{μ-oda})(\text{phen})_2(\text{oda})_2]\cdot\text{4H}_2\text{O} \ (\text{Mn-Oda})$ induces autophagy-promoted apoptosis in human ovarian cancer cells (SKOV3). The complex was initially identified to intercalate DNA by topoisomerase I unwinding and circular dichroism spectroscopy. Intracellular DNA damage, detected by γH2AX and the COMET assay, however, is not linked to direct Mn-Oda free radical generation, but is instead mediated through the promotion of intracellular reactive oxygen species (ROS) leading to autophagic vacuole formation and downstream nuclear degradation. To elucidate the cytotoxic profile of Mn-Oda, a wide range of biomarkers specific to apoptosis and autophagy including caspase release, mitochondrial membrane integrity, fluorogenic probe localisation, and cell cycle analysis were employed. Through these techniques, the activity of Mn-Oda was compared directly to i.) the pro-apoptotic clinical anticancer drug doxorubicin, ii.) the multimodal histone deacetylase inhibitor suberoyanilide hydroxamic acid, and iii.) the autophagy inducer rapamycin. In conjunction with ROS-specific trapping agents and established inhibitors of autophagy, we have identified autophagy-induction linked to mitochondrial superoxide production, with confocal image analysis of SKOV3 cells further supporting autophagosome formation.

Received 18 October 2016; Received in revised form 7 January 2017; Accepted 11 January 2017
function and thus results in cell death [14]. Furthermore, autophagy can be induced and up-regulated in response to intracellular reactive oxygen species (ROS) [15,16], and acts as a protective antioxidant pathway for oxidative stress associated with neurodegenerative diseases [17]. The hormone therapy agent tamoxifen induces elevated levels of intracellular ROS resulting in positive feedback of Zn2+ accumulation, mediating the initiation of autophagy in breast cancer cell line, MCF-7 [18]. Metal complexes of Ru2+, Pt2+, Mn2+ and Cu2+ are also known to activate the autophagic pathway [8], however, with the exception of selected Pt2+-based complexes, the co-activation of apoptosis and autophagy occurs for almost all other metal compounds and is known to be ROS dependent. The current paradigm of autophagy research (in respect to human cancer) is considered to be multifold and somewhat contradictory: i) it acts as a suppressor toward tumorgenesis [19]; ii) it is known to promote tumour survival under starvation or hypoxic conditions of low blood supply and other stress factors attributed to tumour stroma [20,21]; and iii) the efficient induction of autophagy can be exploited as a pro-death mechanism, particularly in apoptotic defective cancer cells [22].

This group has recently reported the title compound—a Mn2+ bis-1,10-phenanthroline (phen) di-salt complex, bridged with octandioate (oda) ([Mn2(μ-oda)(phen)](H2O)).[Mn2(μ-oda)(phen)](oda)2]-4H2O (Mn-Oda)—in conjunction with a cationic Cu2+ analogue, [Cu2(μ-oda)(phen)]F3 (Cu-Oda) as potent in vitro anticancer agents with toxicity toward a panel of colorectal cancers (HT29, SW480 and the two agents revealed distinctive modes of action as both complexes were shown to powerfully act as both a superoxide dismutase Mn-Oda complex and is known to be ROS dependent. The current paradigm of autophagy research (in respect to human cancer) is considered to be ROS-mediated prior to apoptotic activation.

function and thus results in cell death [14]. Furthermore, autophagy can be induced and up-regulated in response to intracellular reactive oxygen species (ROS) [15,16], and acts as a protective antioxidant pathway for oxidative stress associated with neurodegenerative diseases [17]. The hormone therapy agent tamoxifen induces elevated levels of intracellular ROS resulting in positive feedback of Zn2+ accumulation, mediating the initiation of autophagy in breast cancer cell line, MCF-7 [18]. Metal complexes of Ru2+, Pt2+, Mn2+ and Cu2+ are also known to activate the autophagic pathway [8], however, with the exception of selected Pt2+-based complexes, the co-activation of apoptosis and autophagy occurs for almost all other metal compounds and is known to be ROS dependent. The current paradigm of autophagy research (in respect to human cancer) is considered to be multifold and somewhat contradictory: i) it acts as a suppressor toward tumorgenesis [19]; ii) it is known to promote tumour survival under starvation or hypoxic conditions of low blood supply and other stress factors attributed to tumour stroma [20,21]; and iii) the efficient induction of autophagy can be exploited as a pro-death mechanism, particularly in apoptotic defective cancer cells [22].

This group has recently reported the title compound—a Mn2+ bis-1,10-phenanthroline (phen) di-salt complex, bridged with octandioate (oda) ([Mn2(μ-oda)(phen)](H2O)).[Mn2(μ-oda)(phen)](oda)2]-4H2O (Mn-Oda)—in conjunction with a cationic Cu2+ analogue, [Cu2(μ-oda)(phen)]F3 (Cu-Oda) as potent in vitro anticancer agents with toxicity toward a panel of colorectal cancers (HT29, SW480 and the two agents revealed distinctive modes of action as both complexes were shown to powerfully act as both a superoxide dismutase Mn-Oda complex and is known to be ROS dependent. The current paradigm of autophagy research (in respect to human cancer) is considered to be ROS-mediated prior to apoptotic activation.

function and thus results in cell death [14]. Furthermore, autophagy can be induced and up-regulated in response to intracellular reactive oxygen species (ROS) [15,16], and acts as a protective antioxidant pathway for oxidative stress associated with neurodegenerative diseases [17]. The hormone therapy agent tamoxifen induces elevated levels of intracellular ROS resulting in positive feedback of Zn2+ accumulation, mediating the initiation of autophagy in breast cancer cell line, MCF-7 [18]. Metal complexes of Ru2+, Pt2+, Mn2+ and Cu2+ are also known to activate the autophagic pathway [8], however, with the exception of selected Pt2+-based complexes, the co-activation of apoptosis and autophagy occurs for almost all other metal compounds and is known to be ROS dependent. The current paradigm of autophagy research (in respect to human cancer) is considered to be multifold and somewhat contradictory: i) it acts as a suppressor toward tumorgenesis [19]; ii) it is known to promote tumour survival under starvation or hypoxic conditions of low blood supply and other stress factors attributed to tumour stroma [20,21]; and iii) the efficient induction of autophagy can be exploited as a pro-death mechanism, particularly in apoptotic defective cancer cells [22].

This group has recently reported the title compound—a Mn2+ bis-1,10-phenanthroline (phen) di-salt complex, bridged with octandioate (oda) ([Mn2(μ-oda)(phen)](H2O)).[Mn2(μ-oda)(phen)](oda)2]-4H2O (Mn-Oda)—in conjunction with a cationic Cu2+ analogue, [Cu2(μ-oda)(phen)]F3 (Cu-Oda) as potent in vitro anticancer agents with toxicity toward a panel of colorectal cancers (HT29, SW480 and the two agents revealed distinctive modes of action as both complexes were shown to powerfully act as both a superoxide dismutase Mn-Oda complex and is known to be ROS dependent. The current paradigm of autophagy research (in respect to human cancer) is considered to be ROS-mediated prior to apoptotic activation.
cycle overnight. Mn-Oda stock solutions were prepared in PBS, carbonyl cyanide m-chlorophenylhydrazone (CCCP), rapamycin (Rapa) and suberoylanilide hydroxamic acid (SAHA) solutions in DMSO and doxorubicin (Dox) in 50:50 DMSO:H2O and further diluted in culture media. Stocks containing DMSO in the mM range to ensure final incubation concentrations contained < 0.1% v/v.

2.3.2. Viability

Cells were seeded at 5×10^5 cells/well in a 96-well plate overnight and subsequently exposed to drug treatment for 24 h (1 μM Mn-Oda, 1 μM Dox, 75 μM CCCP, 50 μM Rapa and 100 μM SAHA, Fig. S3). For co-treatment experiments, antioxidants and autophagy inhibitors tiron, α-mannitol (Man), histidine (His), sodium pyruvate (Py), 3-methyladenine (3-MA) and NH4Cl, chloroquine (CQ) were treated at 1 mM (with exception of CQ at 10 μM) 2 h prior to drug exposure to facilitate intracellular accumulation. Spent media was removed, cells were detached with trypsin and neutralised with fresh media. PBS washings were kept in 1.5 ml microtubes. Subsequently, cells in culture media. Stocks containing DMSO were prepared in the mM range to ensure final incubation concentrations contained < 0.1% v/v.

2.3.3. Nexin® assay

SKOV3 cells were seeded at 3×10^4 cells/well in 24-well plates overnight and incubated with drug containing media. After the exposure period, spent media was removed, cells were washed with PBS and washings were kept in 1.5 ml microtubes. Subsequently, cells were detached with trypsin and neutralised with fresh media. PBS washings were transferred back into microtubes containing detached cells and centrifuged at 1300 rpm for 5 min and culture media was aspirated. A sufficient volume of media was added to give a concentration range of 2×10^5 to 1×10^6 cell/ml. 100 μl of sample was transferred to 96-well round bottom plate and 100 μl of Guava Nexin® Reagent was added and incubated for 20 min at room temperature. Samples were acquired on Guava EasyCyte HT using Nexin software. Intracellular accumulation. Spent media was removed, cells were detached with trypsin: EDTA (0.25%:0.02% in PBS) and whole samples (100 μl) were added to 100 μl Guava Viacount reagent and incubated for 10 min at room temperature. Viability was acquired on Guava EasyCyte HT with Viacount software.

2.3.4. Mitochondrial membrane potential

Cells were treated as previously described. Cells were resuspended in 600 μl of fresh media to give cell concentration 2×10^5 to 5×10^6 cells/ml from which 200 μl was transferred to a 96-well round bottom plate. 50X staining solution (4 μl) containing JC-1 and 7-aminoactinomycin D (7-AAD) was added to each sample, subsequently incubated at 37 °C in darkness (30 min) and acquired on Guava Nexin® Reagent and subsequently exposed to drug treatment for 24 h with compounds and fixed with 1.5% formaldehyde (15 min, room temperature) followed by ice-cold 70% ethanol and stored at −20 °C (> 12 h). Samples were stained with 200 μl PI staining solution (containing 50 μg/ml PI, 100 μg/ml DNase-free RNase A, 0.1% triton X-100 in 1X PBS) (30 min, room temperature). Samples were acquired on Guava EasyCyte HT flow cytometer and normalised to the sum of events in G0/G1, S and G2/M phases.

2.3.5. Caspase 8 FAM and 9 SR, Caspase 3/7 FAM

The following fluorescent labelled inhibitors of caspase (FLICA) were used to ascertain the activation of Caspase 3/7, 8 and 9 respectively; FAM-DEVD-FMK, FAM-LETD-FMK and SR-LEHD-FMK. Samples were prepared prior to staining in a similar manner as described in the Nexin Assay. Cells were transferred to 1.5 ml microtubes and resuspended in 100 μl media. 10 μl of 10X caspase 9 SR working solution and 10 μl of caspase 8 FAM were added and incubated for 1 h at 37 °C in the dark. Cells were resuspended in 200 μl of caspase 7-AAD working solution and transferred to 96-well round bottom plate, which was left to incubate for 10 min at room temperature. Samples were acquired on Guava EasyCyte HT flow cytometer using Guava Caspase software. Compensation to correct fluorescent overlap between filters was conducted pre-acquisition.

2.3.6. Cell cycle analysis

Cellular DNA content was conducted in a similar manner to previously reported methods [25]. 3×10^5 cell/well were seeded in 24-well culture plates, drug treated and collected as previously stated. After trypsinization, cells were fixed in 70% ice-cold ETOH and stored at −20 °C (> 12 h). Samples were stained with 200 μl PI staining solution (containing 50 μg/ml PI, 100 μg/ml DNase-free RNase A, 0.1% triton X-100 in 1X PBS) (30 min, room temperature). Samples were acquired on Guava EasyCyte HT flow cytometer and normalised to the sum of events in G0/G1, S and G2/M phases.

2.3.7. Immunodetection of γH2AX

Samples were prepared similarly to previously reported methods [26]. 6×10^4 cells/well were seeded in 12-well plates and treated for 24 h with compounds and fixed with 1.5% formaldehyde (15 min, room temperature) followed by ice-cold 70% ethanol and stored at −20 °C. Samples were resuspended in permeabilisation buffer (0.25% Triton X-100 in PBS) for 30 min on ice and blocked with 2% BSA (30 min, room temperature). Primary antibody (1:500) was incubated for 2 h at room temperature followed by secondary antibody (1:1000) for 1 h at room temperature and co-stained for 10 min with 5 μg/ml propidium iodide. Samples were acquired using ExpressPro software on Guava EasyCyte.

2.3.8. DNA degradation with COMET assay

Cells were seeded at 1.5×10^5 cells/well in 6-well plates the evening prior to drug addition. After 24 h drug incubation, cells were harvested and 50 μl was resuspended in 500 μl low melting point agarose to give a final density of 1.5×10^6 cell/ml. 50 μl was spread onto Trevigen COMET slides and allowed to solidify (1 h, 4 °C). Samples were lysed (2.5 M NaCl, 100 mM EDTA, 10 mM Tris. HCl, pH 10) overnight at 4 °C. Slides were allowed to equilibrate in cold electrophoresis buffer (300 mM NaOH, 1.0 mM EDTA, pH 13) for 30 mins at 4 °C. Electrophoresis was run at 300 mA for 30 min with buffer levels adjusted to give a consistent voltage of 25 V. Slides were then washed with water, neutralised in buffer (400 mM Tris, HCl, pH 7.5) (3×5 min), fixed with 70% ethanol (30 min) and dried for desiccated storage (15 min at 37 °C). Prior to scoring, slides where rehydrated for 15 min before staining with propidium iodide solution (10 μg/ml, 10 min room temperature) and imaged through a 10X lens on a Leica DFC 500 epi-fluorescent microscope. Images where then analysed using Open COMET plugin in Image J and plotted using GraphPad Prism software.

2.4. Superoxide detection

SKOV3 cells (6×10^5) were seeded in 12-well plates and exposed to 1.0 μM of drug for 2, 6, 18 and 24 h. After drug treatment, samples were harvested, washed with PBS, resuspended in 200 μl of 5.0 μM MitoSOX Red or dihydroethidium (DHE) and left to incubate for 15 min at 37 °C. Samples where then resuspended in PBS and transferred to 96-well round bottom plates, and acquired on Guava EasyCyte flow cytometer using ExpressPro software.

2.5. Acquisition of confocal images

2.5.1. Cell morphology

Cells (1.4×10^5) were seeded in 35 mm glass bottom petri dishes and allowed to attach overnight. Samples were then exposed to drugs for 24 h (1 μM Mn-Oda, Rapa 50 μM and SAHA 100 μM). Cells were incubated with media containing MitoTracker Deep Red (150 nM, 30 min, 37 °C) and fixed with 4% paraformaldehyde (PFA) (30 min, room temperature). Samples were permeabilised with 0.25% Triton X-100 (15 min). To avoid non-specific staining, cells were blocked with 1% BSA in PBS solution (30 min, 37 °C) and subsequently stained with Alexa Fluor 488-phalloidin (100/100ul, 30 min), followed by DAPI (4:10,000, 10 min) and mounted in ProLong Gold. Images were acquired on STED-Leica DMi8 confocal microscope equipped with CCD camera and 100X oil-immersion objective. DAPI was excited with
405 nm picocquant laser unit and emission captured at 387–474 nm. Alexa Fluor 488 was excited at 499 nm with emission captured between 490–566 nm, and MitoTracker Deep Red was excited at 653 nm where emission was captured at 658–779 nm. Images were acquired where by combinations of excitation wavelengths and emission filters for specific dyes are applied sequentially.

2.5.2. Autophagic detection with LC3

SKOV-3 cells were seeded as previously described. Samples were fixed with 4% PFA (30 min, room temperature), permeabilised with 0.25% Triton X-100 (30 mins, 4 °C) and blocked with 2% BSA (30 min, room temperature). Cells were incubated with primary antibody (1:500, overnight at 4 °C), secondary antibody goat anti-rabbit Alexa Fluor 647 (1:1000, 1 h at room temperature) and DAPI (4:10,000, 10 min at room temperature) and mounted with ProLong Gold. Images were acquired on STED-Leica DMi8 confocal microscope with 100X lens. Alex Fluor 647 was excited at 653 nm laser and emission captured between 658–783 nm, while DAPI was excited with 405 nm picocquant laser unit and emission captured between 435–560 nm.

2.5.3. Autophagic detection with MDC

Cells were seeded in glass bottom petri dishes as previously described. Following drug exposure, samples were incubated with monodansylcadaverine (MDC) (50 µM, 37 °C, 10 min), washed with PBS and confocal images were immediately acquired on STED-Leica DMi8 confocal microscope equipped with 100X objective. Samples were excited with 405 nm picocquant laser unit and emission captured 470–560 nm. Intensity profiles were analysed using Image J V2.0 on raw images in 8-bit format without further modification. MDC coloured images were enhanced in Adobe Photoshop for printing purposes only.

2.5.4. Statistical analysis

All in cellulo data are presented as mean ± standard deviation where n = 3. Unpaired t-tests with Holm-Sidak method were applied to evaluate statistical significance in GraphPad Prism V6 for all data with the exception of superoxide detection studies and viability results in the presence of autophagy inhibitors and antioxidants, where two-way ANOVAs with post-hoc analysis (Dunnnett’s test) were applied. p≤0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Preparation and structure of Mn-Oda

The complex [Mn₂(η⁴-C₅H₅)μ₂-oda](phen)₂(H₂O)₂][Mn₂(η⁴-C₅H₅)μ₂-oda](phen)(η⁴-C₅H₅-oda)]·4H₂O was prepared according to the method reported by Casey et al. [27] and contains [Mn₂(η⁴-C₅H₅)μ₂-oda](phen)(η⁴-C₅H₅-oda)]²⁺ cations and [Mn₂(η⁴-C₅H₅)μ₂-oda](phen)(η⁴-C₅H₅-oda)]²⁻ anions (both of which are centro-symmetric) along with four solvate water molecules. All of the manganese centers are 6-coordinate with two bidentate phen ligands and two oxygen donors in the cis positions. In both complex ions two manganese centers are linked by an oda⁺ ligand; the sixth site is occupied by water in the cation and by further oda⁻ groups in the anion (Fig. 1A and B). Hydrogen bonding between the coordinated water molecule and the uncoordinated carboxylate groups of the anion link the complex ions into zig-zag chains and the chains are linked together by further hydrogen bonding involving the uncoordinated water molecules, generating a 3D network (Fig. 1C).

4. In vitro Drug-DNA interactions reveal intercalation at the minor groove

4.1. Topoisomerase I mediated relaxation

Topoisomerases (Topo) are a specialised class of nuclear enzymes that catalyse the transient cleavage, passage and resealing of either a single strand (topo I) or double strands (topo II) of DNA in order to relax chain entwinement, release superhelical tension and permit change in topology during replication, transcription and recombination [28,29]. Topo I, isolated from E. coli, specifically relaxes negatively coiled superhelical plasmid DNA (scDNA) [30], such as the pUC19 substrate used in this study. Topo I mediated relaxation of pUC19 was identified with increasing Mn-Oda (Fig. 2A). The complex was found to completely relax scDNA at 20 µM with positively wound topology of intact scDNA being observed thereafter. This profile is comparable to that of classical intercalating molecules such as ethidium bromide (Fig. S1) which unwinds DNA by 26° yielding similar topoisomeric patterns [31]. Doxorubicin (Dox), a clinically used Topo II poison [32], exhibits enzymatic inhibitory effects on the Topo I with DNA relaxation occurring at 1 µM (Fig. 2B). Samples treated with Dox extending this concentration render DNA degradation and shearing most likely through ROS generation consequent to redox cycling of the quinone moiety [33–35], and these results in agreement with those described elsewhere [36]. Thus, the Mn-Oda complex is capable of unwinding dsDNA via intercalation but does not induce DNA damage or poison topoisomerase I during this process.

4.2. Circular dichroism studies

Circular dichroism (CD) spectroscopy is a powerful biophysical technique used to monitor conformational changes, drug-DNA binding interactions and structural dynamics of nucleic acids. The CD profile of classical right handed B-DNA exhibits two positive (220 nm and 268 nm) and two negative (210 nm and 246 nm) elliptical signals, while slight variations in this profile arise when the %A-T content of DNA is varied [37]. Conformations of salmon testes DNA (stDNA) and synthetic alternating copolymers poly[d(A-T)₂] and poly[d(G-C)₂] were studied with increasing r [(drug)/[DNA]] values, where r =0.010 – 0.025 (experiments containing >0.025 Mn-Oda were found to induce noise in the resulting spectra). Mn-Oda exhibits a concentration-dependent enhancement of the elliptical signals that can be attributed to hydrogen bonding and stacking interactions between nitrogenous bases and the right-handedness of DNA, irrespective of A-T content (Fig. 2C). In the case of stDNA and poly[d(G-C)₂], an increase in ellipticity associated with β-N-glycosidic linkages were also noted. Mn-Oda DNA profiles were compared to that of classical non-covalent intercalating and groove binding molecules (data not shown) with structural perturbations induced by Mn-Oda suggesting an intercalative binding mode (268 nm), particularly at the minor groove, similar to that of EtBr since elliptical signals show an increasing trend at 210, 220 and 268 nm [38]. Additional viscosity analysis further corroborated an intercalating binding motif by Mn-Oda on stDNA (Fig. S2).

5. Genotoxicity studies imply indirect DNA damage

5.1. COMET analysis

Single cell gel electrophoresis, otherwise known as the COMET assay, was employed to determine intracellular DNA damaging properties of the metal complex. Prior to analysis, viability profiles of Mn-Oda and Dox were identified over 24 h of exposure within the SKOV3 cell line using flow cytometry (Fig. S3). SKOV3 cells were then exposed to 1.0 µM concentrations of both agents, embedded onto agarose coated glass slides and lysed of cellular structure and nucleosome resulting in the nucleoid scaffold that allows DNA migration based on integrity, when subjected to alkaline gel electrophoresis. Single strand breaks (SSB) and double strand breaks (DSBs) were visualised through fluorescence microscopy (Fig. 3B). We selected the clinical anti-tumour antibiotic Dox as a positive control due to its DNA intercalating capacity, ROS generation and topoisomerase poisoning effects. The frequency distribution of the COMET tail moment induced by Mn-Oda (Fig. 3A) showed a departure from the control profile, with a higher
Fig. 1. A. The cation [Mn₂{(η¹η²-oda)(phen)₄(H₂O)₂}]²⁺, B. anion [Mn₂{(η¹η²-oda)(phen)₄(η¹-oda)₂}]²⁻, and C. hydrogen bonded chains. Hydrogen atoms omitted for clarity, hydrogen bonds indicated by black dashed lines. Redrawn from coordinates taken from reference 27. Colour key: C (grey), O (red), N (blue) and Mn (purple). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. A. Release of topological tension of supercoiled plasmid DNA by Mn-Oda and, B. Dox. C. CD profile of Mn-Oda with stDNA and alternating co-polymers poly[d(A-T)₂] and poly[d(G-C)₂] at drug loadings of 1.0 – 2.5% (respective r values of 0.010 – 0.025).
number of events occurring between tail moments of 40 and 20 (A.U.).
Dox, however, exhibited a dispersed array of tail moments with values
reaching 240 a.u. and an elongated tail, extending residual DNA
damage and reduced fluorescence intensity of the comet head. These
results are in agreement with Manjanatha et al. who reported Dox-
induced ROS could generate both direct and indirect DNA damage as
indicated by the COMET assay [39].

5.2. Cell cycle analysis

In order to investigate the toxicity mechanism of the di-Mn²⁺
complex, the effects on SKOV3 cell cycle phase distributions were
examined. All cells have an innate growth and replication cycle, the
revolution of which yields cell growth (G0/G1), replication of chromo-
somes (S) and production of daughter cells (G2/M) [40]. As shown in
Figs. 3C and 3D, Mn-Oda induces a decrease (14.3%) in the G2/M
phase and enrichment within the S (synthesis) phase (7.4%) when
compared to the untreated control. A contraction of G2/M phase was
also evident in Dox treated samples (10.4%) with escalation of S phase
(13.3%) identified. Taken together, data here indicates that at 1.0 µM
exposure over 24 h, both tested agents induce cell cycle arrest within
SKOV3 in the DNA synthesis phase.

5.3. Immunodetection of γH2AX

A primary response to dsDNA damage is the site selective phospho-
rylation of histone H2AX that is indiscriminately incorporated
during chromatin formation [41,42]. H2AX differs from other H2A
histones through a carboxyl tail containing a 139-serine residue that
becomes phosphorylated in the presence of DNA damage and denoted
as γH2AX [43]. Phosphorylated H2AX accumulate in the chromatin
surrounding the site of damage, thus creating a focus for subsequent
recruitment of DNA repair mechanisms. A proportional correlation is
observed between the extent of DNA damage and formation of γH2AX
foci thus rendering it as a pertinent method for dsDNA damage
detection. Following the advent of phosphorylation, the use of recogni-
tion antibodies for γH2AX can visualise and quantify this process
through fluorogenic conjugation. Immunodetection of γH2AX within
SKOV3 cells was quantified using flow cytometry after 24 h of exposure
to Mn-Oda and Dox (1.0 µM) (Fig. 3E). Mn-Oda was found to induce
remarkably high levels of DSBs (69.4%) in the cellular population.
The control agent Dox was also efficient in DSB generation within SKOV3,
but results here were notably lower (39.4%).

6. Mn-Oda stimulates mild caspase 9 release but does not
trigger early or late-stage apoptosis in SKOV3 cells

6.1. Annexin V

To determine whether Mn-Oda induces apoptotic cell death, a
number of critical biomarkers were investigated (Fig. 4). To probe this
potential pathway, SKOV3 cells were again exposed to Mn-Oda
and Dox (1.0 µM) over 24 h and activation of apoptosis was distinguished
from early and late stages through the detection of Annexin V and
membrane-impermeable 7-AAD. During apoptosis, membrane-bound
protein phosphatidylinerine translocates from the inner to outer surface
of the cellular membrane and renders the negatively charged phos-
pholipid as an accessible substrate for Annexin V [44,45]. Interestingly,
in these experiments, Mn-Oda induced minimal levels of Annexin V in
the early stage of apoptosis (1.5%) with none detectable in the later
stages of the 24 h time frame (Fig. 4A and B). This effect contrasts with
Dox-treated SKOV3 cells, which induced 19.7% and 45.9% at these
stages of the 24 h time frame (Fig. 4A and B). This effect contrasts with
Dox-treated SKOV3 cells, which induced 19.7% and 45.9% at these
stages.

6.2. Caspase activation

In order to confirm Mn-Oda cytotoxicity is provoked through a
non-apoptotic cytotoxic pathway, a range of essential components in
the form of caspases that contribute to the initiation and execution of
this process, were investigated (Fig. 4C-P). Stimulation of the intrinsic
apoptotic pathway results in cytosolic release of cytochrome c and thus
activation of caspase 9 within the apoptosome [46,47]. An alternative

---

**Fig. 3.** SKOV3 cells were treated with 1.0 µM Mn-Oda and Dox for 24 h and subsequently studied in the following assays. A. Comet assay analysis where the frequency of tail moment (A.U.). B. Examples of typical COMET shapes are represented below respective legends. C. Cell cycle histograms and, D. Cell cycle phase (G0/G1, S and G2/M) distributions. E. Immunodetection of γH2AX positive cells. Not significant p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001.
route of origin also exists through extracellular death factors that stimulate the activation of caspase 8 [48,49]. Both caspase 8 and 9 result in the consequent activation of executioner caspases such as 3 and 7 that commence an irreversible cascade of proteolytic degradation and membrane collapse, precipitating in programmed cell death [46,47]. Mn-Oda treated cells did not promote sufficient production of initiator caspase 8 (Fig. 4E) but did activate caspase 9 by 13.2% (Fig. 4F). However, a marginal increase only (4.4%) was observed in early apoptotic detection of executioner caspases 3/7 (Fig. 4D), but not in the latter stages (Fig. 4C). As expected, Dox had a significant effect on the activation of caspase 3/7 (Figure C and D) with activation, and subsequent apoptosis (Fig. 4A and B) originating via the intrinsic pathway (Fig. 4F).

6.3. Mitochondrial depolarisation

Due to the ability of Mn-Oda to generate intracellular ROS [4], changes in mitochondrial transmembrane potential (ΔΨm) were investigated; reduction of the redox potential across inner and outer mitochondrial membranes is also a characteristic of apoptotic induction. Depolarisation measurements were obtained using fluorogenic dye JC-1. The protonophore, and known uncoupler of ΔΨm, carbonyl cyanide m-chlorophenyl hydrazine (CCCP) [29,30], was employed as a positive control and found to depolarise 55.3% of the sample population while Mn-Oda induced 11.7% depolarisation—a marginal increase in comparison to Dox-treated and non-drug treated cells (6.0% and 4.6% respectively) (Fig. 4G). Rather than Mn-Oda directly influencing deterioration of transmembrane potential, the extent of depolarisation is most likely due to the stimulation of intrinsic apoptosis. Fluorescent quantification of these apoptotic biomarkers, in combination with cytosolic caspases and Annexin V, suggests that Mn-Oda does not directly activate apoptosis as the primary mechanism of cell death to account for the 46% decrease in cellular viability (Fig. S3).

6.4. Mn-Oda stimulates autophagy prior to apoptosis

Owing to low levels of caspase 9, the extent of mitochondrial depolarisation and lack of Annexin V and caspase 3/7 detection, there is limited evidence here to suggest that Mn-Oda directly induces cell death via apoptosis. It is likely, therefore, that apoptotic initiation is a downstream effect mediated by an alternative mechanism and this prompted us to investigate the activation of autophagy triggered by Mn-Oda. The autophagy pathway is a lysosomal degradation process, monitoring the homeostasis, longevity, turnover of biomolecules and organelles, while replenishing the nutrient pool particularly under starvation conditions [50]. This complex pathway consists of sequential stages from initiation, nucleation, elongation and finally maturation through the activation and post-transcriptional modification of autophagy related proteins (ATG) (Fig. 6A) [15,20,50]. In order to evaluate the mechanistic pathway for Mn-Oda induced toxicity, fluorescent staining with monodansylcadaverine (MDC) was primarily monitored. MDC contains a fluorogenic dansyl moiety conjugated to a terminal amine that facilities accumulation and ion trapping in low pH environments such as those found within autophagolysosomes [51]. Autophagy inducers employed in this study were: i.) rapamycin (Rapa), which inhibits mammalian target of rapamycin (mTOR) complex 1 [52,53], a downstream protein involved in the PI3K-AKT-mTOR regulation pathway, and ii.) suberoylanilide hydroxamic acid (SAHA), an inhibitor of histone deacetylase (HDAC) that induces transcriptional expression of LC3 and mTOR activation [54], independent to apoptosis (Fig. 6A). Non-treated cells demonstrate minimal fluorescence emission of MDC from innately present lysosomes (Fig. 5A). Accumulation of MDC in spherical structures was observed upon Mn-Oda and Rapa treatment with localisation observed in the perinuclear region. Upon complex treatment, quantification of fluorescent intensities (Fig. 6B) revealed enhanced emission profiles due to accumulation of MDC in autophagolysosome when compared to non-treated SKOV3 cells, indicating the induction of Mn-Oda-mediated autophagy. Given that MDC specificity for selective accumulation in autophagic vacuoles has been debated [55], further evidence of Mn-Oda triggered autophagy was identified through aggregation of the autophagic marker LC3. Cytosolic LC3 (LC3-I) is proteolytically cleaved by Atg4, converted to its lipidated form (LC3-II) upon phosphatidyethanolamine (PE) binding [56], and incorporated into the autophagosomal membrane [15]. Non-discriminate immunodetection of LC3-2 showed dispersion within the cytosol in the non-treated control (Fig. 5B). Autophagosome and consecutive autophagic vacuole formation is evident in Rapa and Mn-Oda treatments with the identification of LC3-II puncta, supporting that the cytotoxic mechanism of Mn-Oda is attributed to autophagy activation.

6.5. Cell morphology reveals Mn-Oda-promoted apoptosis

Confocal microscopy experiments were undertaken to further identify the cytotoxic effects of Mn-Oda within SKOV3 cells through the use of location-specific fluorogenic stains to visualise the nucleus, actin cytoskeleton and mitochondria (Fig. 5C). Untreated SKOV3 cells exhibited adherent epithelial morphology with an elongated dome shape. Distinct changes in cellular structure were observed in Rapa treated cells such as adherence contraction, enhanced elongation and

---

**Fig. 4.** Apoptotic investigation of Mn-Oda. A. Early and B. late apoptosis measured by the translocation of Annexin V substrate. C. Detection of caspase 3/7 in mid and D. late populations. E. Activation of initiator caspase 8 and F. caspase 9. G. Extent of mitochondrial depolarisation detected through bathochromic shift of JC-1 emission upon formation of J-aggregates. Not significant p > 0.05, *p≤0.05, **p≤0.01, ***p≤0.001.
cytoskeletal actin encapsulation absent of nuclear or mitochondrial debris. Cells exposed to SAHA exhibit similar contraction and elongation, with clear evidence of apoptotic bodies. Mn-Oda treatment, however, presents alterations in cell morphology and characteristic indicators of apoptosis, particularly with enlargement and fragmentation of the nucleus, organelle contraction, dynamic membrane blebbing and formation of apoptotic bodies[57]. In conjunction with the flow-cytometric detection of caspase 9 and the loss of mitochondrial transmembrane potential, evidence here suggests Mn-Oda induces intrinsic apoptosis as the concluding mechanism of cell death, which is preceded by the activation of autophagy.

6.6. Initiation of autophagy is superoxide-dependent

To investigate the role of Mn-Oda in the activation of autophagy, we further probed cellular viability in the presence of antioxidants and autophagy inhibitors (Fig. 6C). Inhibitors of autophagy are 3-methyladenine (3-MA), which competitively binds to class III phosphatidylinositol 3-kinase (PI3K)[58,59], and antimalarial agent chloroquine (CQ) which inhibits lysosomal protease degradation through accumulation of the weak base within acidic vacuoles, thus inhibiting autophagosome formation (Fig. 6A)[60,61]. Under physiological conditions ammonium chloride (NH₄Cl) becomes protonated, acting as a lysosomotropic agent with similar neutralisation capacity to chloroquine, and causes an increase in local pH[62]. Due to the ROS generation properties of Mn-Oda[4], antioxidants employed in this study consist of a variety of radical-specific trapping agents; tiron for superoxide (O₂•−)[63], mannitol for hydroxyl radical (‘OH)[64], histidine for singlet oxygen (‘O₂)[65,66], and sodium pyruvate for hydrogen peroxide (H₂O₂)[67]. Upon co-treatment with Mn-Oda, all autophagy inhibitors increased cellular viability with 3-MA and tiron at the highest extent (6.1% and 6.6% respectively). These results in conjunction with fluorescence detection of autophagic vacuoles, suggest that Mn-Oda promotes superoxide-mediated autophagy as hypothesised in Fig. 6A. The combination of SAHA with CQ, NH₄Cl and 3-MA significantly increased cell survival by 10.1%, 8.3% and 10.4%, respectively. Interestingly, the most substantial increase was noted for O₂•− scavenging agent tiron (15.4%) as SAHA has previously demonstrated significant ROS generation properties[68,69], which can be attributed to down-regulation of thioredoxin (TRX) a dithiol-reducing redox protein[70,71], a key response to oxidative stress. The presence of autophagy inhibitors had minimal effect on Rapa viability, which could be due to the limited exposure period of 24 h; rapamycin typically exerts toxic effects within longer time-frames with IC₅₀ concentrations of 25.3 μM in PEO1 ovarian cancer cells after 72 h[72]. Sequestering the basal levels of O₂•− enhanced live cell populations by 4.4%, most likely as O₂•− is known to inhibit the binding of TORC1 to Rapa: FKBP12 in yeast[73]. Furthermore, although rapamycin can innately generate significant levels of ROS, synergistic co-treatment with a ROS liberating co-factor, such as curcumin analogue (EF24), can lead to excessive production and enhanced cell death[74]. To further probe the subcellular site of Mn-Oda promoted superoxide generation, we employed dihydroethidium (DHE) and the mitochondrial targeting conjugate, MitoSOX Red. Both dyes specifically become oxidised by superoxide[75], resulting in fluorescence upon DNA intercalation (610 nm in chromatin and 580 nm in mitochondria). Over a time-course between 2 and 24 h at 1.0 μM Mn-Oda exposure, intracellular populations demonstrate increasing and selec-
Reactive oxygen species (ROS) play an integral role in the regulation and stimulation of autophagy [76,77]. Previous work by the Gibson group highlighted the fundamental requirement for ROS-mediated autophagic induction under specific starvation conditions.  

Fig. 6. A. Schematic of autophagy pathway [15,20,50]. Due to the number of autophagy-related genes (ATGs) and the complexity of their role in the autophagy pathway, the family is represented by ‘ATG’ (purple) for simplicity. i. Initiation begins with the formation of isolation membrane known as a phagophore, engulfing cytoplasmic material. ii. Cytosolic LC3-I is converted to the membrane-associated form LC3-II, through phosphatidylethanolamine (PE) lipidation and incorporated into autophagosomal double-membrane. iii. Docking and fusion of lysosome or late endosomes results in the formation of autophagolysosome. iv. Maturation and catabolic degradation results in recycling and restoration of nutrient stores. Autophagic inducers employed are Rapa and SAHA (purple pathway). Autophagic inhibitors are 3-methyladenine (3-MA), NH4+ and chloroquine (CQ) (orange pathway) while antioxidants utilised are tiron, mannitol (man), histidine (his) and sodium pyruvate (Py) (teal pathway). Induction was probed through immunodetection of LC3 with fluorogenically conjugated secondary antibody and monodansylcadervine (MDC). B. Intensity profiles (indicated in Fig. 5A) were analysed using Image J on raw images in 8-bit format with no further modification. C. Differential viability percentages in the presence of radical scavengers and autophagy inhibitors pre-treated at 1 mM (with the exception of CQ, 10 μM) 2 h prior to drug addition. Mn-Oda, SAHA and Rapa were treated at respective concentrations, 1 μM, 100 μM and 50 μM, over 24 h. D. Percentage of cellular population positive for MitoSOX Red and Dihydroethidium (DHE) fluorescence at 1.0 μM Mn-Oda incubated for 2, 6, 18 and 24 h. Not significant p > 0.05, *p≤0.05, **p≤0.01, ***p≤0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
leading to the activation of superoxide (O$_2^-$) production, either alone or in combination with hydrogen peroxide (H$_2$O$_2$) [78]. Furthermore, the same study revealed over-expression of the antioxidant enzyme superoxide dismutase could inhibit the activation of this pathway, resulting in catalytic depletion of O$_2^-$ to H$_2$O$_2$. The findings of the current study demonstrate clearly that Mn-Oda can intercalate dsDNA at the minor groove, however, unlike doxorubicin or Cu$^{2+}$-phenanthroline derivatives [4], direct oxidative damage of nucleic acids is aborted. Instead, there is strong evidence to suggest that DSBs, identified by immunodetection of hYH2AX, coupled with nuclear fragmentation observed in the COMET assay, are due to autophagic degradation initiated by complex-mediated intracellular ROS production. Combination of the di-Mn$^{2+}$ complex along with mediators that hinder radical generation and autophagy, indicate cell death promoted by O$_2^-$ production within the mitochondria. Metal-catalysed radical production does not directly influence nucleic acid degradation (evident from the absence of DNA shearing in topoisomerase relaxation), but rather functions as a signalling agent in the activation of autophagy, detected via MDC and immunofluorescence of LC3. Consequently, intracellular ROS insult by Mn-Oda promotes autophagy, exceeding a critical threshold of adverse conditions, and activates apoptosis via the intrinsic, mitochondrial pathway (caspase 9 and mitochondrial depolarisation) as the lethal effector of cellular death. Further sensitivity may arise from the mutational status of p53 whereby depletion or point mutations can induce autophagy; in the wider context of metallodrug development and targeting, this complex may serve as a significant milestone in the construction of small molecule therapeutic leads that promotes alternative cytotoxic mechanisms that are not dependent on traditional apoptotic initiation.

Competing interests

Authors declare there are no conflicts of interest.

Acknowledgment

This work was supported by the Irish Research Council (IRC) grants GOIPG/2014/1182 and GOIPG/2013/826. Equipment used in this study was funded under the Programme for Research in Third Level Institutions (PRTLI) Cycle 5. The PRTLI is co-funded through the European Regional Development Fund (ERDF), part of the European Union Structural Funds Programme 2011–2015. A.K. acknowledges funding from the Marie Sklodowska-Curie Innovative Training Network (ITN) ClickGene (H2020-MSCA-ITN-2014–642023). The authors thank Dr. J. Keenan and Dr. C. Gallagher for donating antibodies used in the immunodetection studies and Prof. M. Devereux and Dr. M. McCann for valuable and constructive scientific discussion.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2017.01.024.

References

[1] L.A. Finney, T.V. O’Halloran, Transition metal speciation in the cell: insights from the chemistry of metal ion receptors, Science 500 (5621) (2003) 931–936.
[2] B. Sarkar, Treatment of Wilson and menkes diseases, Chem. Rev. 99 (9) (1999) 2535–2544.
[3] Z. Molphy, A. Prizeczar, C. Slator, N. Barron, M. McCann, J. Colleran, D. Chandran, N. Gathergoood, A. Kellett, Copper phenanthroline oxidative chemical nucleases, Inorg. Chem. 53 (10) (2014) 5932–5940.
[4] A. Kellett, M. O’Connor, M. McCann, O. Howe, A. Casey, P. McCarron, K. Kavanagh, M. McNamara, S. Kennedy, D.D. May, P.S. Skell, D. O’Shea, M. Devereux, Water-soluble Bis(1,10-phenanthroline) octanedioate Cu$^{2+}$ and Mn$^{2+}$ complexes with unprecedented nano- and picomolar in vitro cytotoxicity: promising leads for chemotherapeutic drug development, Med. Chem. Commun. 2 (7) (2011) 579–584.
[5] A. Kellett, M. O’Connor, M. McCann, M. McNamara, P. Lynch, G. Rosair, Y. McKee, B. Creaven, M. Walsh, S. McClean, A. Foltyn, D. O’Shea, O. Howe, M. Devereux, Bis-phenanthroline copper(ii) phthalate complexes are potent in vitro antitumour agents with “self-activating” metallo-nuclease and DNA binding properties, Dalton Trans. 40 (5) (2011) 1024–1027.
[6] C. Slator, N. Barron, O. Howe, A. Kellett, [Cu(O-phenthalate)[phenanthroline]] exhibits unique superoxide-mediated NCI-60 chemotherapeutic action through genomic DNA damage and mitochondrial dysfunction, ACS Chem. Biol. 11 (1) (2016) 159–171.
[7] (a) N. Farrell, Metal complexes as drugs and chemotherapeutic agents, Compr. Coord. Chem. II 9 (2003) 809–840; (b) C.X. Zhang, S.J. Lippard, New Metal complexes as potential therapeutic agents, Curr. Opin. Chem. Biol. 7 (4) (2003) 481–489.
[8] (a) T. Shingu, V.C. Chumbalkar, H.-S. Gwak, K. Fujiwara, S. Kondo, N.P. Farrell, O. Bogler, The Polynuclear platinum BB3610 induces G2/M arrest and autophagy early and apoptosis late in glioma cells, Neuro-Oncol. 12 (12) (2010) 1269–1277.
[9] W.-J. Guo, Y.-M. Zhang, L. Zhao, B. Huang, F.-F. Tao, W. Chen, Z.-J. Guo, Q. Xuh. Y. Sun, Novel monofunctional platinum (II) complex mono-Pi induces apoptosis-independent autophagic cell death in human ovarian carcinoma cells, distinct from cisplatin, Autophagy 9 (7) (2013) 996–1008.
[10] C. Trejo-Solis, D. Sanchez-Farfan, S. Rodriguez-Enriquez, F. Fernandez-Valverde, A. Cruz-Salgado, L. Ruiz-Aznar, J. Sotelo, Copper compound induces autophagy and apoptosis of glioma cells by reactive oxygen species and Jak activation, BMC Cancer 12 (1) (2012) 156; (b) C. Tan, S. Lai, S. Wu, S. Hu, L. Zhou, Y. Chen, M. Wang, Y. Zhu, W. Lian, W. Peng, L. Ji, A. Xu, Nuclear permeable ruthenium(II) B-carboline complexes induce autophagy to antagonize mitochondrial-mediated apoptosis, J. Med. Chem. 53 (21) (2010) 7613–7624.
[11] W.-J. Guo, S.-S. Ye, N. Cao, J. Huang, J. Gao, Q.-Y. Chen, ROS-mediated autophagy was involved in cancer cell death induced by novel copper(II) complex, Exp. Toxicol. Pathol. 62 (5) (2010) 577–582.
[12] F. I. Paris, C. Perez-Pastene, E. Couve, P. Caviedes, S. Ledoux, J. Segura-Aguilar, Copper dopamine complex induces mitochondrial autophagy preceding caspase-independent apoptotic cell death, J. Biol. Chem. 284 (20) (2009) 13306–13315;
[13] G.R. Gorajod, A. Alaimo, S. Porta Alcom, C. Pomilis, F. Saravia, M.L. Kotler, The autophagic–lysosomal pathway determines the fate of glial cells under manganese- induced oxidative stress conditions, Free Radic. Biol. Med. 87 (2015) 237–251; (b) X. Li, K. Zhao, W. Guo, X. Liu, J. Liu, J. Gao, Q. Chen, Y. Bai, A novel manganese complex LMnAc selectively kills cancer cells by induction of ROS-triggered and mitochondrial-mediated cell death, Sci. China Life Sci. 57 (10) (2014) 998–1010; (c) J. Liu, W. Guo, J. Li, X. Li, J. Geng, Q. Chen, J. Gao, Tumor-targeting novel manganese complex induces ROS-mediated apoptotic and autophagic cancer cell death, Int J. Mol. Med. 35 (3) (2015) 607–616; (d) K. Takeshige, M. Baba, S. Tsuboi, T. Noda, Y. Ohsumi, Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction, J. B. Creaven, 119 (2) (1992) 301–311.
[14] M. Tsukada, Y. Ohsumi, Isolation and characterization of autophagy-defective mutants of saccharomyces cerevisiae, FEBS lett. 333 (1–2) (1993) 169–174.
[15] N. Mizushima, T. Noda, T. Yoshimori, Y. Tanaka, T. Ishii, M.D. George, D.J. Klionsky, M. Ohsumi, Y. Ohsumi, A protein conjugation system essential for autophagy, Nature 395 (6700) (1998) 395–398.
[16] Y. Ichimura, T. Kirisako, T. Takao, Y. Satomi, Y. Shim shimizu, N. Ishihara, N. Mizushima, I. Tanida, E. Kominnami, M. Ohsumi, T. Noda, Y. Ohsumi, A ubiquitin-like system mediates protein lipida, Nature 408 (6811) (2000)
M. McCann, J. McGinley, K. Ni, M. O’Connor, K. Kavanagh, V. McKee, J. Colleran, K. Brzozowska, M. Pinkawa, M.J. Eble, W.-U. Müller, A. Wojcik, R. Kriehuber, L. Poillet-Perez, G. Despouy, R. Delage-Mourroux, M. Boyer-Guittaut, Interplay P.K. Wu, M. Kharatishvili, Y. Qu, N. Farrell, A circular dichroism study of ethidium D. Gozuacik, A. Kimchi, Autophagy as a cell death and tumor suppressor N.R. Bachur, S.L. Gordon, M.V. Gee, Anthracycline antibiotic augmentation of D. Gewirtz, A critical evaluation of the mechanisms of action proposed for the S. Giordano, V. Darley-Usmar, J. Zhang, Autophagy as an essential cellular L. Li, G. Ishdorj, S.B. Gibson, Reactive oxygen species regulation of autophagy in N.H. Dekker, V.V. Rybenkov, M. Duguet, N.J. Crisona, N.R. Cozzarelli, C. Slator et al. In : Drug-DNA Interaction Protocols. K.R. Fox(Ed.), Methods Mol. Biol. 613 2010 In: Checkpoint Controls and Cancer. A.H. Schonthal (Ed.), Methods Mol. Biol. 281 development and response to therapy, Nat. Rev. Cancer 5 (9) (2005) 726 – 734. M. Cennacci, M. McGlinn-K. O'Connor, K. Cavanaugh, V. McKee, J. Colferan, M. Devereux, N. Gathergood, N. Barron, A. Prisecaru, A. Kellett, A new circular DNA interaction model, J. Biol. Chem. 281 (2006) 10737–10745. P. Peixoto B. Bailly M.-H. David-Cordonnier Topoisomerase I-mediated DNA relaxation as a tool to study intercalation of small molecules into supercoiled DNA. In: Binding-DNA Interaction Protocols. K.R. Fox(Ed.), Methods Mol. Biol. 613 2010 235–256. P. Pozarowski Z. Darynkiewicz Analysis of cell cycle by flow cytometry In: Checkpoint Controls and Cancer. A.H. Schonthal (Ed.), Methods Mol. Biol. 281 2006 311–314. K. Brzouwska, M. Pinkawa, M.J. Ehle, W.-U. Müller, A. Wojcik, R. Kriehuber, S. Schmitz, In vivo versus in vitro individual radiosensitivity analysed in healthy donors and in prostate cancer patients with and without severe side effects after radiotherapy, Int. J. Radiat. Biol. 85 (2009) 45–63. M.T. Casey, M. McCann, M. Devereux, M. Curran, C. Condivi, M. Convery, V. Quillet, C. Harding, Synthesis and structure of the Mn3(OH)6 complex salt [Mn(nitrat)e2(OH)(Phen)3][Mn(nitrat)e2(OH)(Phen)3][Mn(nitrat)e2(OH)(Phen)3]4H2O (nitrato)− Octahedral complex as a catalyst for H2O2 Disproportionation, J. Chem. Soc. Chem. Commun. 22 (1994) 2643–2645. N.H. Dekker, V.V. Rybenkov, M. Duguet, N.J. Crisona, N.R. Cozzarelli, D. Benjamin, M. Colombi, C. Moroni, M.N. Hall, Rapamycin passes the torch: a new mechanism of mTOR inhibition, Meth. Enzymol. 528 (2013) 217–227. D.B. Khadka, W.-J. Cho, Topoisomerase inhibitors as anticancer agents: a patent update, Expert Opin. Ther. Pat. 23 (8) (2013) 1033–1056. J.C. Wang, DNA topoisomerases, Annu. Rev. Biochem. 65 (1996) 635–692. J.J. Wang, The degree of unwinding of the DNA helix by ethidium: I. Titration of twisted PM2 DNA molecules in alkaline cesium chloride density gradients, J. Mol. Biol. 89 (1974) 783–801. G.aponcioni, K. Ahmadian, P. Pommier, Local sequence requirements for DNA cleavage by mammalian topoisomerase II in the presence of doxorubicin, Nucleic Acids Res. 18 (22) (1990) 6611–6619. N.R. Bashur, S.L. Gordon, M.V. Gee, Anthracenyl alcohol augmentation of mitochondrial electron transport and free radical formation, Mol. Pharmacol. 13 (5) (1977) 901–910. J.M. Gutteridge, G.J. Quintan, Free radical damage to deoxyribonuclease by anhydride, acrolein, and aminoquinone antitumor antibiotics: An essential requirement for iron, selenium and hydrogen peroxide, Biochim. Pharmacol. 34 (3) (1985) 4099–4103. D. Gewirtz, A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin, Biochem. Pharmacol. 57 (7) (1999) 727–741. P.D. Foglecong, C. Reckord, S. Swink, Doxorubicin inhibits human DNA Topo- isomerase I, Cancer Chemother. Pharmacol. 30 (2) (1992) 123–125. V.-M. Chang, C.K.M. Chen, M.-H. Hou, Conformational changes in DNA upon ligand binding monitored by circular dichroism, Int. J. Mol. Sci. 13 (3) (2012) 3934–3941. P.K. Wu, M. Kharatishvili, Y. Qu, N. Farrell, A circular dichroism study of ethidium bromide binding to Z-DNA induced by dinuclear platinum complexes, J. Inorg. Biochem. 96 (2002) 85–96. M.G. Manjanaathan, M.E. Bishop, M.G. Pearce, Genotoxicity of doxorubicin in P384 rats by combining the comet assay, flow-cytometric peripheral blood micronuclei test, and pathway-focused gene expression profiling, Environ. Mutagen. 55 (1) (2011) 24–34. C. Bertoli, J.M. Skothem, R.A.M. de Bruijn, Control of cell cycle transcription during G1 and S phases, Nat. Rev. Mol. Cell Biol. 14 (8) (2013) 518–528. W.M. Bonner, C.E. Redon, J.S. Dickey, A.J. Nakamura, O.A. Sedelnikova, S. Solier, D.P. Petticrew, Y. Umeda, F. Goldbusch, Nat. Rev. Cancer, (2008) 957–967. T.T. Paull, E.P. Rogakou, V. Yamazaki, C.U. Kirchgresser, M. Geller, W.M. Bonner, A Critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr. Biol. 15 (2005) 886–895. E.P. Rogakou, D.R. Flich, A.H. Orr, V.S. Ivanova, W.M. Bonner, DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139, J. Biol. Chem. 273 (10) (1998) 5858–5868. G. Koopman, C.P. Reutelingsperger, A.G. Kutken, R.M. Keenhen, S.T. Pais, M.H. Ledingham, V. for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis, Blood 84 (5) (1994) 1415–1420. B. Verhoven, R.A. Schlegel, P. Williamson, Mechanism of phosphatidylserine exposure, a phagocytic recognition signal, on apoptotic T lymphocytes, J. Exp. Med. 186 (5) (1997) 1597–1610. M.O. Enghartner, The biochemistry of apoptosis, Nature 407 (6805) (2000) 770–776. A. Destee, M. Boyce, J. Yuan, A decade of caspases, Oncogene 22 (53) (2003) 8456–8577. M. Krüdiger, G.I. Evan, Caspase-8 in apoptosis: the beginning of “the end?”, IUBMB Life 50 (2) (2000) 85–90. G.M. Cohen, Caspases: the executioners of apoptosis, Biochem. J. 326 (1) (1997) C. Slator et al. Redox Biology 12 (2017) 150–161 160
Synergistic antitumor activity of rapamycin and EF24 via increasing ROS for the treatment of gastric cancer, Redox Biol. 10 (2016) 78–89.

K.M. Robinson, M.S. Janes, M. Pehar, J.S. Monette, M.F. Ross, T.M. Hagen, M.P. Murphy, J.S. Beckman, Selective fluorescent imaging of superoxide in vivo using ethidium-based probes, Proc. Natl. Acad. Sci. USA. 103 (41) (2006) 15038–15043.

X. Wen, J. Wu, F. Wang, B. Liu, C. Huang, Y. Wei, Deconvoluting the role of reactive oxygen species and autophagy in human diseases, Free Radic. Biol. Med. 65 (2013) 402–410.

Y. Chen, S.B. Gibson, Is mitochondrial generation of reactive oxygen species a trigger for autophagy?, Autophagy 4 (2) (2008) 246–248.

Y. Chen, M.B. Azad, S.B. Gibson, Superoxide is the major reactive oxygen species regulating autophagy, Cell Death Differ. 16 (7) (2009) 1040–1052.

E. Tasdemir, M.C. Maiuri, L. Galluzzi, I. Vitale, M. Djavaheri-Mergny, M. D'Amelio, A. Criollo, E. Morselli, C. Zhu, F. Harper, U. Nannmark, C. Samara, P. Pinton, J.M. Vicencio, R. Carnaccio, U.M. Moll, F. Madeo, P. Paterlini-Brechot, R. Rizzuto, G. Szabadkai, G. Pierron, K. Blomgren, N. Tavernarakis, P. Codogno, F. Cecconi, G. Kroemer, Regulation of autophagy by cytoplasmic P53, Nat. Cell Biol 10 (2008), 2008, pp. 676–687.

Y. Yaginuma, H. Westphal, Abnormal structure and expression of the p53 gene in human ovarian carcinoma cell lines, Cancer Res. 52 (15) (1992) 4196–4199.