Morpho-functional analyses reveal that changes in the chemical structure of a marine bisindole alkaloid alter the cytotoxic effect of its derivatives

Changes in the chemical structure of the marine bisindole alkaloid 2,2-bis(6-bromo-1*H*-indol-3-yl)ethanamine alter the cytotoxic effect of its derivatives

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Abstract

2,2-bis(6-bromo-1*H*-indol-3-yl)ethanamine, a marine alkaloid, appeared a potential anticancer agent against several tumor cell models thanks to the presence of a 3,3'-diindolylmethane scaffold. Here, the modifications in its chemical structure into alkaloid-like derivatives, have been evaluated, to investigate changes in its biological activities. Three derivatives have been considered and their potential apoptotic action has been evaluated through morpho-functional analyses in a human cancer cell line.

Apoptosis appears strongly decreased in the derivative compounds without the bromine units (**1**) and in those where the bromine groups have been substituted with fluorine atoms (**2**). On the contrary, the methylation of indole NH (**3**) does not alter the alkaloid apoptotic activity that occurs through the involvement of an increased oxidative stress, leading to peroxidation events and mitochondria dysfunctionality.

This manuscript highlights the alkaloid derivative cytotoxic effect, which is strictly correlated to the maintenance of bisindole structure and bromine atoms. Since molecular therapies, by targeting mitochondria pathways, have shown positive outcomes against several cancer cells, the alkaloid with bisindole scaffold and the two bromine units can be considered a promising candidate to develop new derivatives with strong anticancer property.

Key words: Marine alkaloids; bisindoles; structure-activity relationships, apoptotic activity; mitochondria, anticancer activity, U937 cell line

Introduction

Marine bisindole alkaloids showed significant cytotoxicity, antineoplastic and antimicrobial properties [1,2]. In particular, 2,2-bis(6-bromo-3-indolyl)) ethanamine (**BrDIMEA**), isolated from the Californian tunicate *Didemnum candidum* and the New Caledonian sponge *Orina* [3], is a strong cytotoxic agent in various tumor cell lines [3-5]. In our previous paper, its apoptotic mechanism of action, through caspase activation and regulated by Bcl-2 protein family, has been demonstrated in U937 cells. For this potential anticancer activity, **BrDIMEA** , a member of the large 3,3'-diindolylmethane family of alkaloids, has obtained much attention [3].

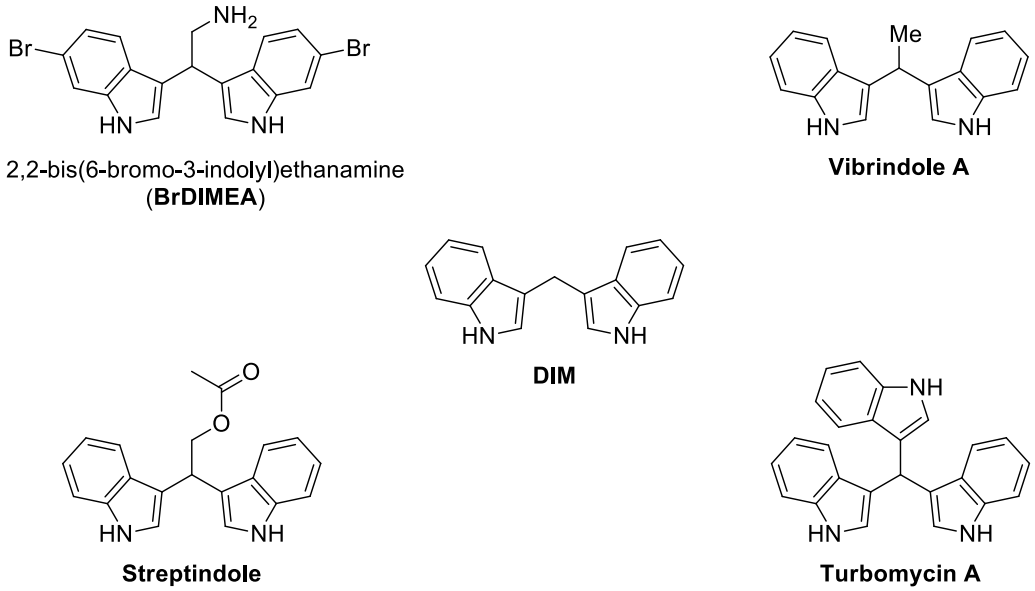


Figure 1. The marine alkaloid **BrDIMEA** and some natural antitumoral agents sharing the 3,3'-diindolylmethane (**DIM**) scaffold.

Its anticancer properties are possibly due to the common 3,3-diindolylmethane molecular unit (**DIM**), a chemical group (Figure 1) which exhibit itself antiproliferative or apoptotic activities [6-9]. The use of DIM is limited for lipophilic nature and *in vivo* chemical instability and it seems that the presence of the alkylamino side chain improves its chemical and therapeutic activities [4]. In addition, the presence of two bromoindole units could enhance the anti-cancer action. Therefore,

BrDIMEA can be considered a lead molecule for the synthesis of new compounds with a wide range of biological properties, including the antineoplastic action.

In this study, the potential apoptotic effect of three different **BrDIMEA** derivatives (**1-3**), has been evaluated through morpho-functional analyses on U937 human cancer cells by, comparing their action with that of the natural compound.

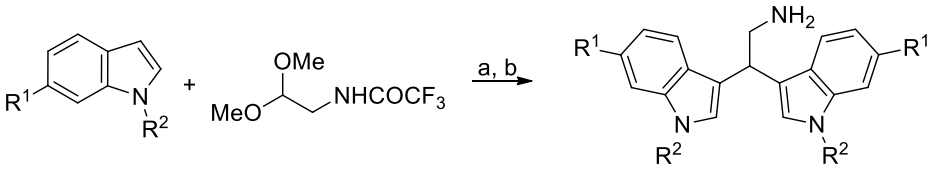
Materials and Methods

Chemicals

All organic solvents used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA), Alfa Aesar (Haverhill, MA, USA), or TCI (Tokyo, Japan). Prior to use, acetonitrile, dichloromethane and toluene were dried with molecular sieves with an effective pore diameter of 4 Å. Column chromatography purifications were performed under ‘‘flash” conditions using Merck (Darmstadt, Germany) 230–400 mesh silica gel. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel plates (silica gel 60 F254), which were visualized by exposure to ultraviolet light and an aqueous solution of cerium ammonium molybdate (CAM). ESI-MS spectra were recorded with a Waters (Milford, MA, USA) Micromass ZQ spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker (Billerica, MA, USA) AC 400 or 100, respectively, spectrometer and analyzed using the TopSpin 1.3 (2013) software package. Chemical shifts were measured by using the central peak of the solvent. The final compounds were analyzed on a ThermoQuest (Italia) FlashEA 1112 elemental analyzer for C, H, and N. The percentages found were within ±0.4% of the theoretical values. All the tested compounds were >95% pure as determined by elemental analysis.

Chemistry

Marine bisindole alkaloid **BrDIMEA** and compounds **1-3** were prepared as described in Scheme 1.



R^1	R^2	
Br	H	**BrDIMEA**
H	H	**1**
F	H	**2**
Br	Me	**3**

Scheme 1. Reaction conditions: (a) diphenyl phosphate, acetonitrile, 80°C, 24 h; (b) K_2CO_3, methanol, reflux, 2 h.

*General procedure for the synthesis of derivatives **BrDIMEA** and **1-3***

Diphenyl phosphate (0.02 mmol) was added to a solution of the appropriate indole derivative (0.4 mmol) and (trifluoroacetylamino)acetaldehyde dimethyl acetal (0.2 mmol) in anhydrous acetonitrile (0.2 mL), and the resulting mixture was stirred at 80 °C for 24 h in a sealed tube, monitoring the progress of the reaction by TLC and HPLC-MS. After cooling to room temperature, saturated aqueous $NaHCO_3$ (30 mL) and dichloromethane (30 mL) were added and the two phases were separated. The aqueous solution was extracted with dichloromethane (3 x 20 mL). After drying over dry Na_2SO_4, the combined organic phases were concentrated in vacuum and the resulting crude product was utilized without further purification. A mixture of that crude trifluoroacetamide derivative and potassium carbonate (1 mmol) in MeOH (1.87 mL) and H_2O (0.13 mL) was stirred and heated at reflux for 2 h. The MeOH was removed under reduced pressure and water was added (30 mL). The aqueous solution was extracted with dichloromethane (3 x 30

mL) and the resulting solution was dried with Na_2SO_4 and concentrated in vacuum. The crude material was purified by flash chromatography on neutral alumina.

The physico-chemical data of ***BrDIMEA*** [4,10] and **1-3** are in agreement with those reported.

Cell Culture

U937 human myelomonocytic lymphoma cell line, grown in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1% antibiotics, was maintained at 37°C in humidified air with 5% CO_2 [11]. Cell behavior was monitored with the Inverted Microscopy (IM; Eclipse TE2000-S Nikon; objective 10x).

Apoptosis induction

U937 cells have been exposed to **BrDIMEA** and its derivatives 1-**3**, at the final concentration in the cell medium of 7.5 µM. This latter is the better apoptotic dose of **BrDIMEA**, already demonstrated by Salucci et al., 2018 [3].

For apoptosis induction, cells (seeded at $1x10^6$ cells/mL) were exposed to three derivatives and to **BrDIMEA**.

Trypan Blue (TB) exclusion assay [12] has been used to evaluate living and dead cells in control condition and in treated samples.

Transmission Electron Microscopy (TEM)

U937 pellets were immediately fixed in 2.5% glutaraldehyde in 0.1 M in phosphate buffer, post-fixed in 1% OsO_4 in the same buffer, dehydrated with ethanol and embedded in araldite [13]. Semithin sections have been counterstained with Blue of Toluidine and observed at light microscope (LM). Thin sections, collected on nickel grids and stained with uranyl acetate and lead

citrate, were observed with an electron microscope (Philips CM10, 80KV, FEI Company, Italy).

Confocal Laser Scanning Microscopy (CLSM)

TUNEL

Control and treated cells were fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) pH 7.4 for 30 min., then deposited on poly-lysinated coverslips in Petri dishes overnight at 4 °C. After PBS washing, samples were permeabilized with a 2:1 mixture of ethanol and acetic acid for 5 min at −20 °C. For the TUNEL technique, all reagents were part of the Apoptag Plus kit (D.B.A., Oncor, Dallas, TX, USA) and procedures were carried out according to the manufacturer's instructions and as described in Salucci et al., 2014 [14]. Finally, slides were mounted with an antifading medium. Specimens were observed with a Leica TCS-SP5 confocal laser scanning microscope (CLSM) connected to a DMI 6000 CS inverted microscope (Leica Microsystems CMS GmbH, Mannheim, Germany); excitation was at 488 nm and emission signals were detected at 517 nm.

Mitochondrial behavior

To monitor mitochondrial behavior, fresh cells were treated with 10 nonyl-acridine orange (NAO) for evaluating mitochondria membrane integrity through peroxidation event evaluation. Fresh cells were exposed to 50 nM NAO for 10 min at room temperature and then observed through a Leica TCS-SP5 Confocal connected to a DMI 6000 CS Inverted Microscope (Leica Microsystems CMS GmbH; objectives 40x and 60x).

Excitation was at 488nm (FITC and NAO); emission signals were detected at 525 nm (FITC) and 519 nm (NAO). CLSM images are presented as single-plane images or Z-stack projections [15].

Results

In the previous paper, the best **BrDIMEA** apoptotic action has been demonstrated in U937 cells at the dose of 7.5µM.

In this work the effect of its derivatives, which show some modifications in their chemical structures respect to the original alkaloid, has been analyzed. First of all, the ability of these compounds to induce cytotoxicity in the same cell line has been evaluated trough TB assay and the number of living cells, expressed as mean value percentage ± standard deviation, has been calculated. Figure 2 shows micrographs obtained after IM and LM observations which revealed living and dead cells in all experimental conditions together with a graph on cell viability percentage. A good cell preservation confirmed by a high living cell number appears in control condition (Fig. 2A, B). Cells treated with compound **1** or **2** (in which bromide atoms are deleted and in the second compound substituted with the fluorine ones), show, at IM (Fig. 2C, E) or LM (Fig. 2D, F), a morphological behavior similar to that observed in control condition. In both situations, a reduction of cell viability about 10% has been quantified. On the other hand, the compound **3**, which maintains the DID molecular unit and the bromide groups and in which two hydrogen atoms from indole have been deleted, induces a diffuse presence of dead cells (Fig. 2G, H). Therefore, the compound 3 causes a cytotoxic effect comparable to that observed after **BrDIMEA** treatment in our previous work [3].

Morphological observations allow to distinguish the type of death and have been carried out for the compound 3 alone. Compared to control cells, which show a preserved ultrastructure (Fig. 3A, B), those exposed to 7.5 µM compound **3**, became apoptotic and, in particular, late apoptotic events can be observed (Fig. 3C, D). Therefore, an evident cell shrinkage, cytoplasmic vacuolization, mitochondria damage, chromatin condensation, micronuclei and, sometimes, secondary necrosis appear. Since compound **3** induces an apoptotic phenotype in U937 cells, the *in situ* DNA

fragmentation has been investigated, and the presence of a diffuse number of TUNEL positive nuclei (Fig. 3 inset D), absent in control condition (Fig. 3 inset A) has been observed. In particular, the fluorescent staining is localized inside micronuclei (Fig. 3 inset D).

For what concerns derivative **3**, the mitochondrial behavior has been also investigated. In particular, the presence of cardiolipin peroxidation events have been analyzed with the fluorescent probe NAO at CLSM. Respect to control cells, in which mitochondrial membrane maintains its integrity (Fig. 3E, F), those treated with compound **3** show a decline of florescence intensity (Fig. 3G, H) suggesting that the loss of mitochondrial membrane integrity could favor the release of pro-apoptotic proteins from the mitochondria. This latter result confirms the ultrastructural observations, in fact cells treated with compound **3** showed dysfunctional mitochondria which appear empty or with altered cristae, a typical condition induced by an increased oxidative stress.

Discussion

Apoptosis is a programmed cell death which maintains the healthy survival/death balance in cells [16,17]. Misregulation of apoptosis can cause cancer or autoimmunity, while enhanced apoptosis may cause degenerative diseases [11,18]. Researchers are involved in the development of some promising cancer treatment strategies which target apoptotic inhibitors including Bcl-2 family proteins and other substrates. One strong apoptotic agent in various cell models appears a marine bisindole alkaloid [19,20], which induces apoptosis in U937 cells following both extrinsic and intrinsic pathways and by involving the Bcl-2 protein family. In particular, Salucci and collaborators demonstrated the ability of this chemical alkaloid to up-regulate bax and to inactivate Bcl-2 and Bcl-xL [3], the anti-apoptotic factors, which block the release of cytochrome c from mitochondria and thus promote cell survival. Since the 2,2-bis(6-bromo-3-indolyl)-ethanamine targets anti-apoptotic Bcl-2 protein and, consequently, can have success in killing many types of

cancer, its chemical structure can be considered the basic scaffold to obtain synthetic derivatives with similar biological properties but with an improved chemical stability [21] .

For that, in this study, we have evaluated the anti-cancer potential action of three bisindole alkaloid derivatives. To identify and confirm the chemical unit responsible of apoptotic effect, a preliminary structure-activity relationships of 2,2-bis(6-bromo-3-indolyl)-ethanamine have been carried out.

Morpho-functional analyses revealed that, in the same cell model and at the same dosage, only the compound **3** maintains an apoptotic effect similar to that observed after **BrDIMEA**. Moreover, compound **3** is able to induce apoptotic cell death through the oligonucleosomic DNA cleavage and by involving the mitochondrial pathway. In fact, mitochondrial membrane integrity appears loss due to cardiolipin peroxidation, which leads to the formation of reactive aldehydes, able to react with proteins and DNA [22]. Ultrastructural analyses reveal the presence of a high number of empty mitochondria suggestive of oxidative stress increase. This data enhances that oxidative stress production can be considered one of the major causes of apoptosis in cancer cells and it could be recognized into a promising therapeutic approach for the cancer treatment [23].

These findings show that the **BrDIMEA** derivative **3** induces apoptosis via mitochondrial pathway and with a rate similar to that observed for the marine alkaloid lead compound, demonstrating that the bisindole scaffold, even N-methylated, as well as the bromine atoms, are necessary and must be maintained for developing new anticancer drug.

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Author Contributions:

227 Methodology, S.B.,M.B., S.L.,S.S; Investigation, S.S.; Data Curation, S.S. and S.L.; Writing –

228 Original Draft Preparation, S.S and S.L.; Writing – Review & Editing, S.S and S.L.; Supervision,

229 S.L. and S.S.

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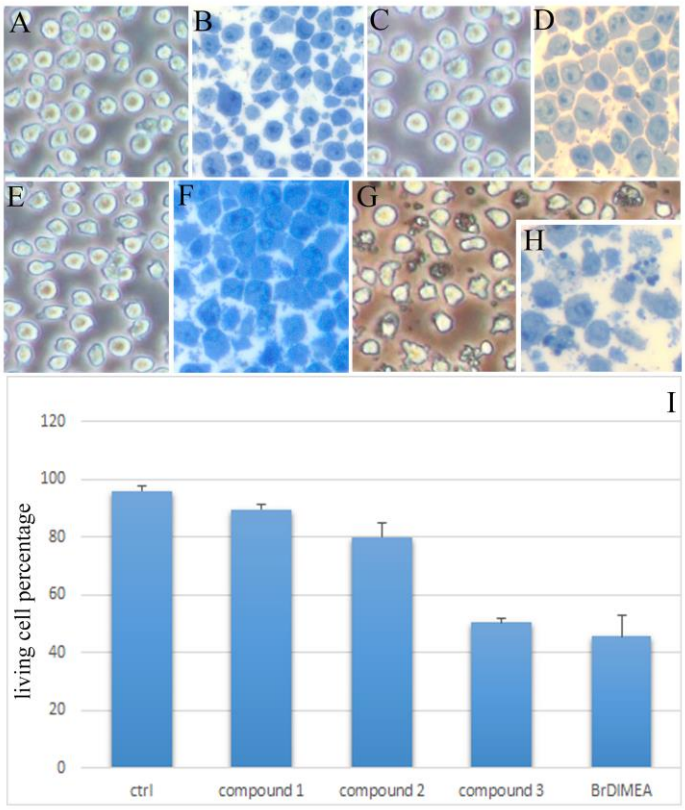
Figure legends



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299 **Figure 2. IM and LM observations of control condition (A, B) and BrDIMEA derivative 1 (C,**

300 **D), 2 (E, F) 3 (G, H) treated cells.** The graph in I shows TB exclusion assay which reveals a cell

301 viability reduction after drug administration. Data are from three separate experiments and

302 furnished as mean +/- standard deviation. Bars: 10 µm

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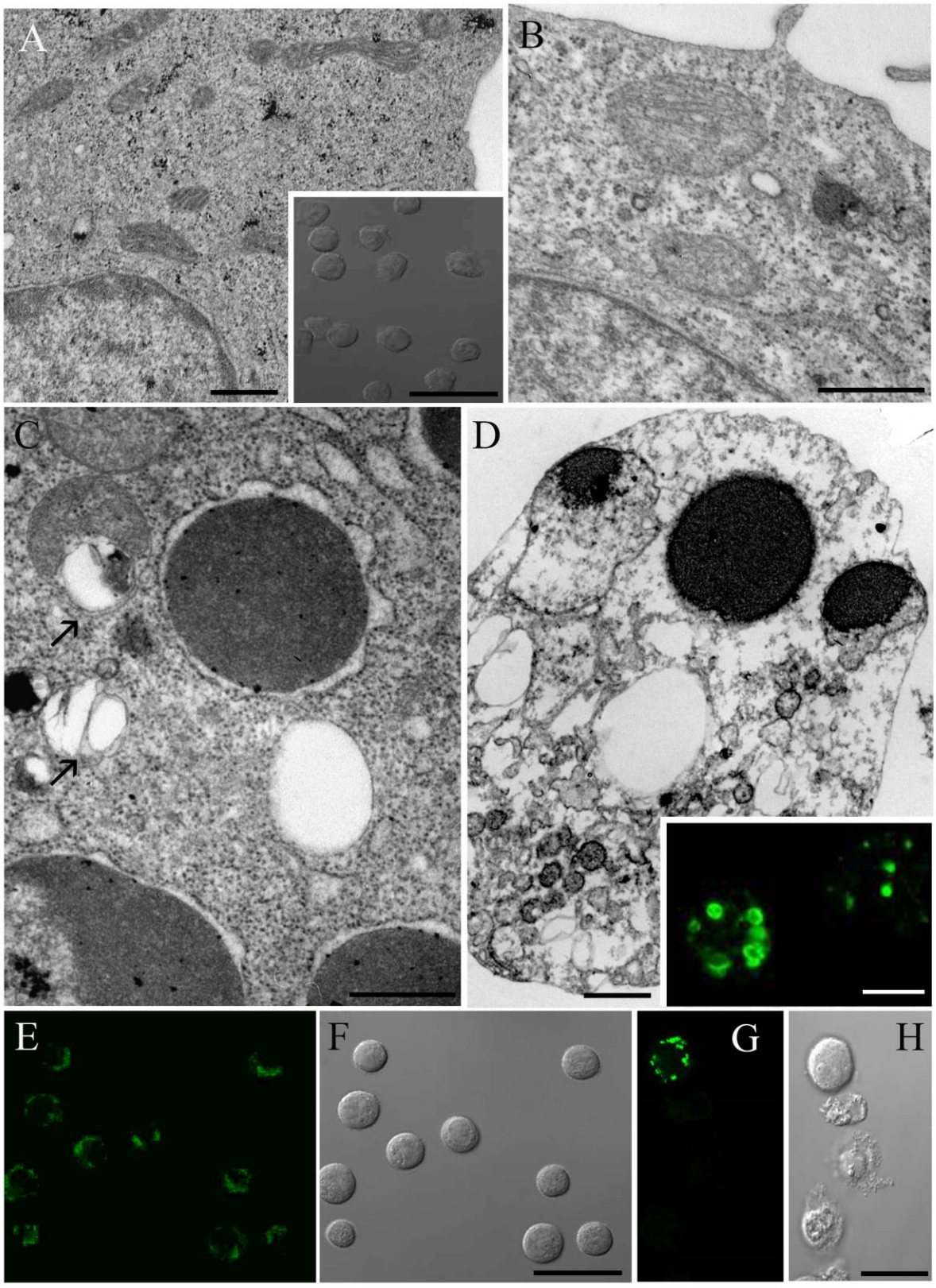

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Figure 3. TEM (A-D) and CLSM observations (inset A and C, E-H) of control cells (A, inset A, B, E, F) and those exposed to compound 3 (C, D, inset D, G, H). Apoptotic features, altered mitochondria (arrows) and TUNEL positive nuclei, absent in control condition (A, B and inset A) appears after compound 3 administration (C, D, inset D). If control cells maintain the mitochondrial membrane integrity (E, F), treated cells (G, H) show a decrease of fluorescence after NAO staining evidencing the presence of peroxidation events. Bars: 500nm for A-D; 5µm for inset D; 10 µm for inset A, E-H.

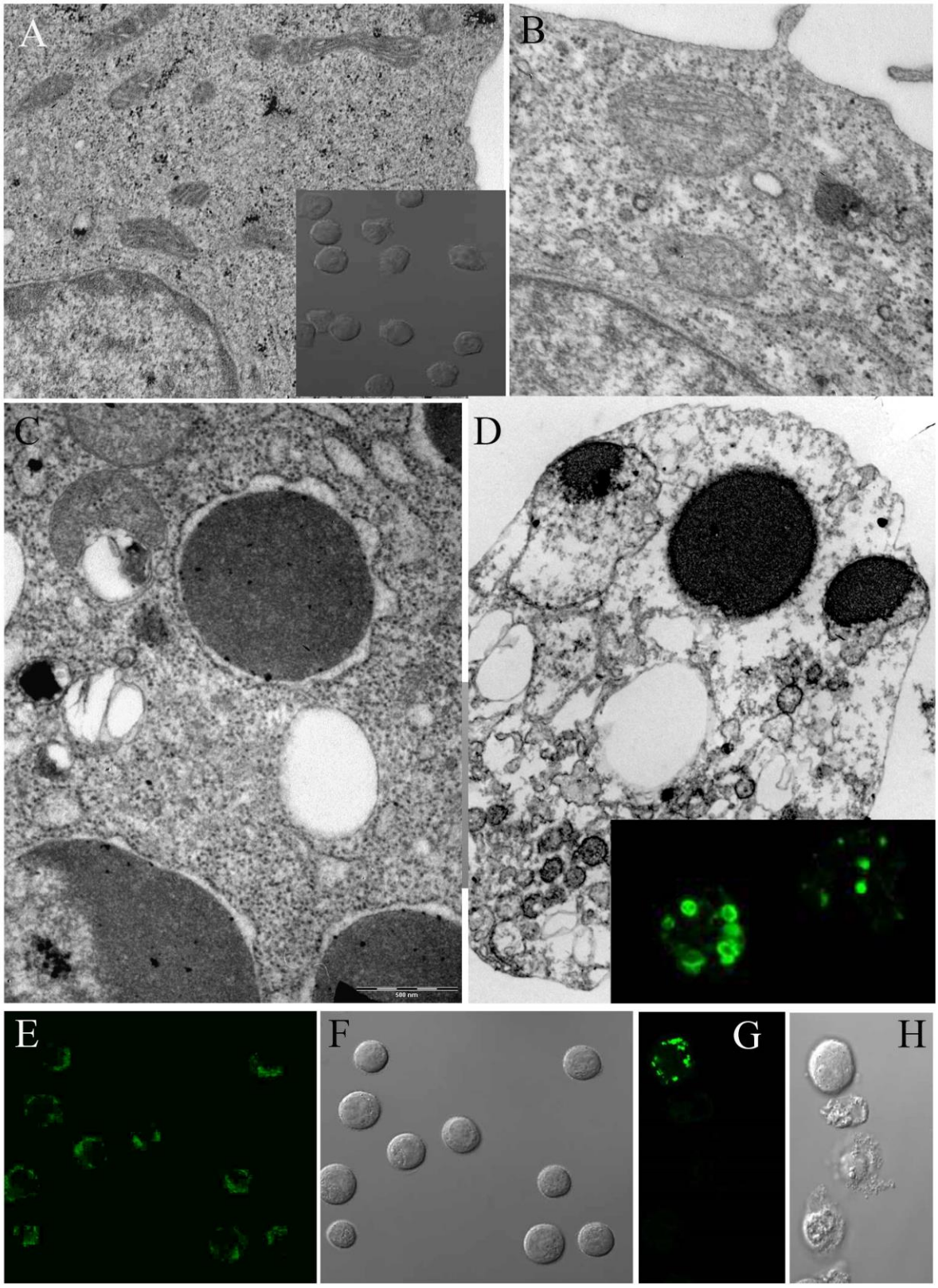

Fig. 3