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Casiopeina III-ia induces apoptosis in HCT-15 cells in vitro through caspase-dependent mechanisms and has antitumor effect in vivo

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Abstract The aim of this study was to evaluate the in vitro and in vivo effects of the new chemotherapy agent Casiopeina III-ia [(4,4′-dimethyl-2,2′-bipiridine)(acetylacetonate) Copper (II) nitrate] on HCT-15 (p53–/-) colon cellular line. In vitro, the drug reduced the viability and induced necrosis and apoptosis in a dose dependent manner, without affecting cell cycle phases. Apoptosis was related to Bax increasing levels, suggesting a caspase-dependent mechanism of death, as verified by nucleosomal fragmentation of DNA. In vivo, the antitumor activity of Casiopeina III-ia was tested in HCT-15 cells transplanted to nude mice. In this study we will show that the novel antineoplastic agent Casiopeina III-ia is active on this colon tumor line, setting out as a good candidate for the treatment of colon tumors refractory to chemotherapy.

Keywords Casiopeina · Apoptosis · Chemotherapy · p53(-/-) · Bax

Introduction

Cancer chemotherapy, has as a main goal to activate the selective cellular death of the neoplastic cells, without damaging the normal cells (Johnstone et al. 2002). Among the involved mechanisms of cell death, apoptosis plays a

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central role in the death induced by drugs on tumor cells. The resistance to multiple chemotherapeutic agents has been associated to this kind of death (Kaufmann and Earnshaw 2000; Kim et al. 2003).

Most of the anticancerous drugs seem to initiate apoptosis directly through the mitocondrial pathway and activate caspases (Trejo-Solis et al. 2005). Two mechanisms of apoptosis have been identified. The first mechanism is mediated by death receptors, like Fas and TNF-α (Nagata 1997) that activate specific sequences of initiators caspases 2, 8 and 10 (Martin et al. 1998; Muzio et al. 1998). These initiators caspases can break and activate effector caspases, such as caspases 3, 6 and 7 (Muzio et al. 1997). The second mechanism, cytochrome c is released from mitochondria to the cytoplasm that interacts with Apaf-1 (apoptotic protease-activating factor-1) and promotes its oligomerization (Liu et al. 1996; Srinivasula et al. 1998). The recruitment of procaspase 9 to this active apoptosome finishes in its auto activation and the later activation of caspase 3 (Li et al. 1997). In both pathways, the activation of effector caspases amplifies the apoptotic signal to assure the cellular death in an irreversible way. Caspases are responsible for the morphologic changes because they interact with microfilaments of the cytoskeleton, such as actin, gelsoline, fodrine and others (Kothakota et al. 1997; Mashima et al. 1999). Also, caspase 3 is involved in the Poly (ADP-ribose) polymerase inhibition and in the breaking of nuclear DNA into fragments of nucleosomal size, which presumably represents the most severe damage to the cell (Zhang and Xu 2001).

Oxaliplatine and 5-fluorouracile in combination with other drugs have been used as treatments of choice for advanced colon cancer, with response rates of 20–30% of the treated patients (IMPACT investigators 1995; Cvitkovic and Bekradda 1999; Saunders and Iveson 2006). In the search of new agents, the use of drugs with essential metal-base, such as copper, was conceived as an antineoplastic compound. Thus, Casiopeinas® are created, whose general formula is: $Cu[(N–N)(A–A)]NO_3$ where A–A = N–O, O–O) (Ruiz-Azuara 1993). Within this drug family, Casiopeina III-ia® (Fig. 1) is one of the most widely studied (Gracia-Mora et al. 2001). Its mechanism of action is through the inhibition of α-cetoglutarate dehydrogenase and cytochrome c release, which can compromise the energy dependent processes, such as cellular duplication (Marín-Hernández et al. 2003). Moreover, the presence of Cu^{+2} in the Casiopeinas center can be associated to the production of reactive oxygen species (ROS) through the Fenton reaction (Shackelford et al. 2000). It has been demonstrated that some Casiopeina compounds show a great antiproliferative activity in vitro on ovarian carcinoma (CH1), murine leukemia (L1210) (De Vizcaya-Ruiz et al. 2000). It also has showed several cervical uterine carcinomas (Gracia-Mora et al. 2001) and on rat glioma in vitro and in vivo (C6) (Trejo-Solís et al. 2005). Therefore, we decided to study the antiproliferative and apoptotic effect in vitro and antitumor and systemic effects in vivo of Casiopeina III-ia on the HCT-15 cancer colorectal cell line. Our data show that Cas III-ia induces apoptosis in vitro through caspase-dependent mechanisms and exerts antiproliferative effects in a xenotransplantation model.

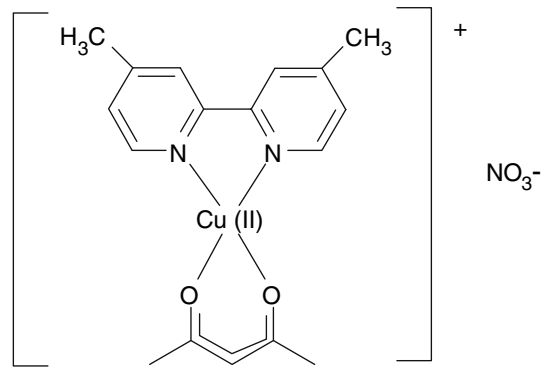

Fig. 1 Casiopeina III-ia chemical structure

Experimental procedures

Reagents

Casiopeina® III-ia (Cas III-ia) [(4,4′-dimethyl-2,2′-bipiridine)(acetylacetonate) Copper (II)] nitrate; molecular weight: 444.93 g/mol, was provided by Dr. M.E. Bravo (Mexico city, Mexico) as previously described (Ruiz Azuara 1993). Cas III-

ia was dissolved in sterile water 1 mg/ml, keeping it away from light and refrigerated during one week. Cis–diamine–dichlorine platinum (II) (CDDP); molecular weight: 299 g/mol (Alpha Aesar, Ward Hill, MA) was dissolved in sterile water 1 mg/ml and used immediately after prepared. The other reagents were obtained from commercial sources.

In vitro experiments

Cells: HCT-15 cell line was obtained from ATCC (American Tissue Culture Collection, Rockville, MD) and maintained at 37°C in 5% CO_2 atmosphere under sterile conditions in Dulbecco's Modified Eagle's Medium (Sigma Chemical Co., St. Louis, MO), supplemented with 5% fetal bovine serum (Sigma Chemical Co.). Cells were treated in all the in vitro experiments separately for 24 h with 0, 2.5, 5.0, 10, 15 and 20 μg/ml of Cas III-ia.

Cell viability: HCT-15 cells seeded in 96 well plates were treated separately with increasing concentration of Cas III-ia for 24 h and measured as described by Skehan et al. (1990). Cultures were fixed with trichloroacetic acid and stained for 30 minutes with 0.4% (wt/vol) sulforhodamine B (SRB) (Sigma Chemical Co.) dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base [tris (hydroxymethyl) amino methane] to determine optical density in a 96-well microtiter plate reader at 564 nm (Multiskan System, Finland).

Cell cycle: After the cell exposition to 0, 2.5, 5.0, 10, 15 and 20 μg/ml of Cas III-ia for 24 h, cells were harvested and centrifuged twice (1200 rpm/5 min) in cold PBS and resuspended in CycleTest Plus DNA (Becton and Dickinson, San Jose, CA) reactive, following the manufacturer indications. Briefly, the cell suspension was added with 250 μl trypsin buffer solution and incubated for 10 min, then incubated with 200 μl of trypsin inhibitor plus RNAse for 10 min. Finally, 200 μl of Propidium Iodide were added and incubated for 10 min at 4°C. The samples were measured immediately using a FACSCalibur flow cytometer (Beckton Dickinson, San Jose, CA) and analyzed in a CellFit software.

Western Blot: Control and treated HCT-15 cells were harvested, washed once with ice cold PBS, resuspended in 200 μl of cold Cell Lysis-M buffer (Sigma Chemical Co.) plus protease inhibitors (10 mg/ml Leupeptin, 1.0 μg/ml aprotinin and 0.1 nM phenymethylsulfonyl fluoride (Sigma Chemical Co.) and then stirred gently for 30 min in refrigeration. Cells were centrifuged for 15 min at 12500 rpm and the supernatants were removed and frozen. Protein concentration was determined with BCA Protein assay kit (Pierce Rockford, IL). The samples containing 40 μg of proteins were mixed with equal volume of loading buffer (Tris–HCL 125 mm, pH 6.8, 20% glycerol, SDS 4%, 0.02% of bromophenol blue and 10% 2-mercaptoethanol) and were warmed up to 67°C for 5 min. Later the samples were centrifuged at 5,000 rpm for 1 min and migrated in 12% SDS polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a nitrocellulose membrane for two hours at 70 V with 25 mM Tris–HCl, pH 8.0, 125 mM glycine and 10% methanol. The membranes were blocked with 5% skimmed milk in 1 × PBS for 1 h, and the PCNA (Proliferating cell nuclear antigen) DAKO antibody (Carpinteria, CA) was added to the membrane overnight at 4°C. Followed by three consecutive washes with PBS, the membrane was incubated with a sheep anti mouse IgG-horseradish peroxidase complex (Santa Cruz Biotechnologies) for 1 h at room temperature, followed by three washes with PBS. The blot was observed with the system of enhanced chemiluminiscence (Santa Cruz Biotechnologies). Later, the blot was exposed to Kodak XAR-5-ray film (Sigma Chemical Co.) for 1 min, and then developed. A similar procedure with BAX, and ß-Actine was applied (Santa Cruz Biotechnologies). The intensities of the resulting bands were evaluated in a Molecular Dynamics computing densitometer using ImageQuant software version 3.2.2.

Morphologic alterations: For this aim, the cells treated under the different schemes were harvested mechanically, washed twice in PBS, and separated in two groups. In one of them, cells were extended on a slide and air fixed at room temperature. Then the slides were stained with Diff Quick (Grupo Hyesa, Mexico City). The other group was

fixed with 2.5% glutaraldehyde (diluted with cacodylates buffer) for 4 h at 4°C. Later on, the cells were fixed with osmium tetraoxide (OsO_4) for 1 h, dehydrated and deposited in Epon resin. Semi fine sections of 1 μm were stained with toluidine blue and observed by light microscope. Subsequently, ultra fine sections were mounted on copper grids and stained with uranyl acetate and counterstained with lead citrate ($Pb_3(C_6H_5O_7)_2$) and then examined with Zeiss EM 900 transmission microscope (Stuttgart, Germany). More than 50 cells were examined by treatment, describing the most frequent morphologic alterations.

Apoptosis determination: Apoptosis was determined with Annexine V and propidium iodide by flow cytometry as was previously described (Vermes et al. 1995). The method is based on the union of Annexine V to phosphatidilserine that translocates from the internal to the external surface of the membrane of cells in early apoptosis. Following the treatment of the cells for 24 h, they were harvested with trypsin-EDTA (1×) (GIBCO) and centrifuged twice at 200 g/5 min in cold PBS and resuspended in 100 μl of incubation buffer (10 mM HEPES/NaOH pH 7.4, NaCl 140 mM, 5 mM $CaCl_2$). Then the cellular suspensions were treated with 5 μl of Annexin-V-Fluos and 10 μl of Propidium Iodide stock solution (50 μg/ml). After 20 min of incubation, 400 μl of HEPES buffer was added and the samples were analyzed immediately in a FACSCalibur flow cytometer (Beckton Dickinson, San Jose, CA) using an excitation of 488 nm, a Bandpass filter 515 nm for fluoresceine detection and a >560 nm filter for the IP detection.

Apoptosis was also assayed by another method, in which the fragmented DNA was isolated and analyzed by agarose 2% (w/v) gel electrophoresis and stained with ethidium bromide, as described in the DNA extraction method *Quantum PrepR AquaPure Genomic DNA kit* (Bio-Rad Laboratories, CA). DNA ladders were documented by photography.

In vivo experiments

Antitumor activity: 25 Male, 6-week-old nude mice (nu/nu) (Harlan Inc) were kept under specific pathogen-free conditions with free access to autoclaved food and water. All mice were injected subcutaneously with 1×10^6 HCT-15 cells in the low left flank. When the tumor reached a 0.3 cm diameter, the mice were randomly allocated in one of the following groups: Control ($n = 10$) distilled water every 24 h for 21 days; CDDP ($n = 5$) 4 mg/kg (13.5 μmol/kg) each 7 days,4 doses; Cas III-ia ($n = 5$) 3.0 mg/kg (6.74 μmol/kg) each 4 days, 6 doses; Cas III-ia ($n = 5$) 6.0 mg/kg (13.5 μmol/kg) each 4 days, 6 doses. The drug application was intraperitoneal. The animals were weighed and the tumors measured in length and width using Vernier calibrators every day. Using the established formula [length (cm) × width^2 (cm) × π]/6, tumor sizes were converted in tumor volume. Then, the relative tumor volume (RTV) was calculated at day 21 as follows:

$$\text{RTV} = (\text{tumor volume at day X} / \text{tumor volume at Day 0}) \times 100$$

Also, the days in doubling size for each tumor were assessed. The nude mouse research was approved by the Chemistry Faculty (UNAM) ethical committee.

The percentage of weight loss, as a toxicity indicator, was calculated for each animal as follows:

$$[(\text{weight at day 21}/\text{weight at day 0}) - 1] \times 100$$

The experiment finished 24 h after the last drug application in each group and the animals were killed in a CO_2 chamber. Necropsies were carried out, removing samples of liver, kidney, lungs, spleen, intestine, brain, and half of tumor and immediately immersed into 10% formalin (pH 7.4) for 24 hours. The samples were processed with routine histological methods and stained with hematoxyline-eosine (H-E). All the histological sections were evaluated assigning a random number to each slide. The tumor mitotic index was determined with the number of atypical mitoses in 10 high magnification fields (40×). The apoptotic index was determined at tumor periphery, where chromatin condensation, pycnotic cell separation and the formation of chromatin vesicles were counted in 10 high magnification fields (40×).

Statistical analysis: All in vitro studies were made in triplicate and data were analyzed with non-parametric Kruskal Wallis test. Data from in vivo experiments was analyzed with the non-parametric Kruskal Wallis test except the percentage of weight loss, which was analyzed with ANOVA followed by Tukey. The accepted statistically significant difference was $P < 0.05$.

Results

Cas III-ia inhibits cell proliferation in a dose-dependent manner and induces morphological changes related to apoptosis

To characterize the effect of Cas III-ia on cell viability and cell cycle, HCT-15 cells were treated separately with increasing concentrations (0, 2.5, 5.0, 10, 15 and 20 μg/ml) for 24 h. Figure 2A depicts the dose-dependent effect of Cas III-ia on cell viability; most cells died after treatment with 15 μg/ml and 20 μg/ml of Cas III-ia for 24 h ($P < 0.05$). At the cytological evaluation, starting with the 5 μg/ml dose, morphological changes related to apoptosis were observed. The cells were round, small and chromatin condensed (Fig. 2B). In addition, cell cycle analysis was performed, and it was found that the drug addition did not change the duration of the cell cycle phases (Fig. 3A) and the western blot analysis revealed that the levels of PCNA did not change in any of the concentrations of Cas III-ia (Fig. 3B). Cas III-ia induced cell viability reduction and morphological changes in a dose dependent manner without altering the cell cycle phases, so these results suggest that Cas III-ia exerts a cytotoxic effect on HCT-15 through a mechanism involving the induction of apoptosis.

Cas III-ia induces morphological changes related to apoptosis and oncosis

The formation of chromatin aggregates at the nuclear membrane, and the apoptotic bodies formation are the most relevant characteristics of apoptosis induction. The observation of swollen organelles and cells are characteristic of oncosis (Ziegler and Gloscurth 2004). To

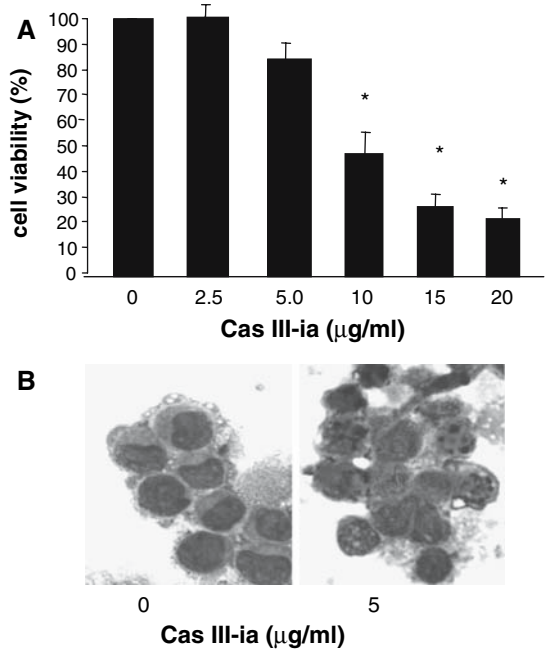

Fig. 2 Viability effects of Cas III-ia. (**A**) Dose dependent effect on viability through sulforhodamine test. The data represents the media ± standard error. *$P < 0.05$ (compared to control) of 3 independent experiments. (**B**) Representative photomicrography of cytological alterations in control and treated cells

investigate whether the Cas III-ia death induction was the result of apoptosis, ultra structural analyses were performed. Untreated cells were round, with moderate cytoplasm and the nucleus showed anisokaryosis and laxus chromatin (Fig. 4A). At the lower doses (2.5 and 5.0 μg/ml) HCT-15 cells revealed prominent mitochondrial swelling, with discrete vacuolated cytoplasm and in some cases, swollen mitochondria with loss of crest structures (Fig. 4B). From 5.0 μg/ml. Two kinds of dead cells are identified: Some cells have severe hydropic degeneration with swollen organelles, mainly mitochondria and rupture of plasmatic membrane (Fig. 4C). In other cells, the nucleus showed chromatin adhered to the internal nucleus membrane forming crescents, without severe cytoplasmatic variations (Fig. 4D). These alterations were more evident as Cas III-ia was more concentrated. It is important to denote that the mitochondrial alteration in the lowest dose (2.5 μg/ml) occurred at the time when the cytoplasm and cellular membrane were intact and the

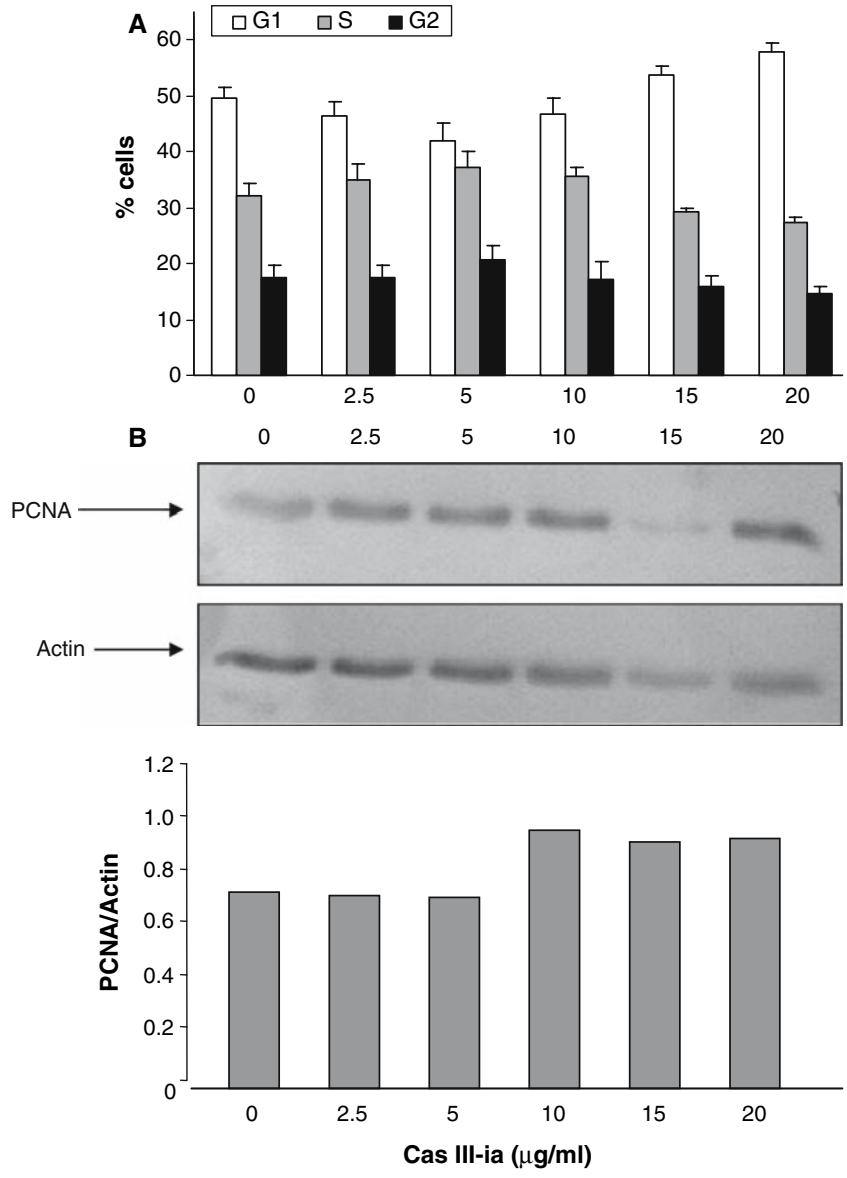

Fig. 3 (**A**) Cell cycle analysis of HCT-15 cells treated with 0, 2.5, 5, 10, 15, and 20 μg/ml of Cas III-ia. The data represents the media ± standard error of 3 independent experiments. (**B**) PCNA expression on total lysate of control and treated cells. The bars graphic shows the relative units of PCNA normalized with actine

chromatin was not condensed. This data showes that apoptosis and oncosis are mechanisms related to death induction by Cas III-ia, being the mechanism of death related to mitochondria.

Apoptosis induced by Cas III-ia is dose-dependent and involves caspase dependent mechanisms

One of the most relevant characteristics of the apoptosis mechanism is the phosphatidilserine exposure on cell surface; this can be detected by the selective union of Annexin V. The treatment with Cas III-ia showed early apoptosis induction in a dose dependent manner, being statistically different ($P = 0.02$) at the dose of 10, 15 and 20 μg/ml (3.5 ± 1.2%, 11.3 ± 4.6%, 17.7 ± 2.1%, respectively), compared to the control group (1.4 ± 0.1%) (Fig. 5A). It's important to denote that this data correlates with the findings on cell viability (Fig. 2A).

To determine the apoptosis mechanism, Bax (proapoptotic member of Bcl-2 family) was determined by western blot from total cellular

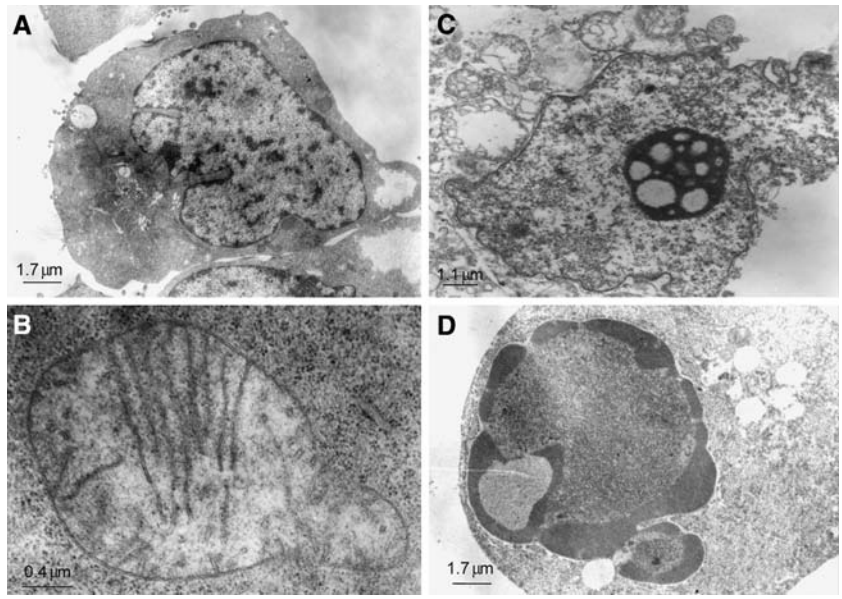

Fig. 4 Ultrastructural findings. (**A**) Control colon carcinoma HCT-15 cell. (**B**) Swollen mitochondria from a cell treated with 2.5 µg/ml of Cas III-ia, with moderate loss of crests architecture. (**C**) Swollen cell with loss of organelles structure, characteristic feature of a necrotic cell. (**D**) Chromatin condensation at nuclear membrane, with slight changes in the cytoplasm, characteristic of apoptosis

extracts. Bax demonstrated to increase its levels in a dose dependent manner (Fig. 5B). Moreover, oligonucleosomal DNA fragmentation was induced starting with the dose of 10 µg/ml, showing the typical ladder pattern of apoptosis (Fig. 5C). These results indicate that apoptosis induction is related to proapoptotic Bax protein increase. Moreover, the internucleosomal DNA fragmentation indicates a possible involvement of caspase-3.

In vivo antitumor effects of Cas III-ia

To determine if Cas III-ia exerts antitumor functions, RTV, days in doubling size, mitotic, and apoptosis index were assessed (Table 1). The RTV of tumors in animals treated with Casiopeína showed slow growth, compared with control animals. The RTV at day 21 in control animals was 3527.88 ± 819.8 whereas in animals treated with Cas III-ia, RTV was decreasing with dose increments. With 6.0 mg/kg the RTV was statistically significant compared with the control group, with 661.3 ± 241.5 ($P = 0.02$). Regarding the days that the tumor doubles its size, the control took 5.1 ± 0.9 days, whereas CDDP took 5.4 ± 2.4 days. For Cas III-ia, the 6 mg/kg dose showed statistical difference ($P = 0.02$) compared to the control group, reaching values of 12.2 ± 3.7 days.

When the mitotic index of the Cas III-ia treated group was compared to the control group, reductions of 43.5% and 58% were observed in animals treated with 3.0 mg/kg and 6.0 mg/kg, respectively. For the control group, mitotic index was 8.5 ± 0.7, whereas in animals treated with CDDP was 8.4 ± 2.1. In animals treated with Cas III-ia using the 6.0 mg/kg dose, the index was 3.5 ± 1.1 mg/kg ($P = 0.03$). Additionally, all treatment modalities resulted in induction of apoptotic cell death morphology ($P = 0.04$) at tumor periphery (Table 1). These data suggest that Cas III-ia exerts antitumor in vivo function through proliferation inhibition and apoptosis induction.

Collateral effects of Cas III-ia

Regarding to in vivo toxicity produced by Cas III-ia, weight loss percentage of the mice was calculated (Fig. 6A). When weight loss percentage of treated animals were compared to the control group, reductions of 8.2 ± 3.9% and 5.7 ± 3.5% were observed for animals treated with CDDP and 6.0 mg/kg of Cas III-ia, respectively. Only CDDP is statistically different when compared to the control group ($P = 0.005$). For the control group and the one treated with 3.0 mg/kg Cas III-ia, weight gains of 3.8 ± 1.2% and 1.7 ± 2.7% were observed, respectively.

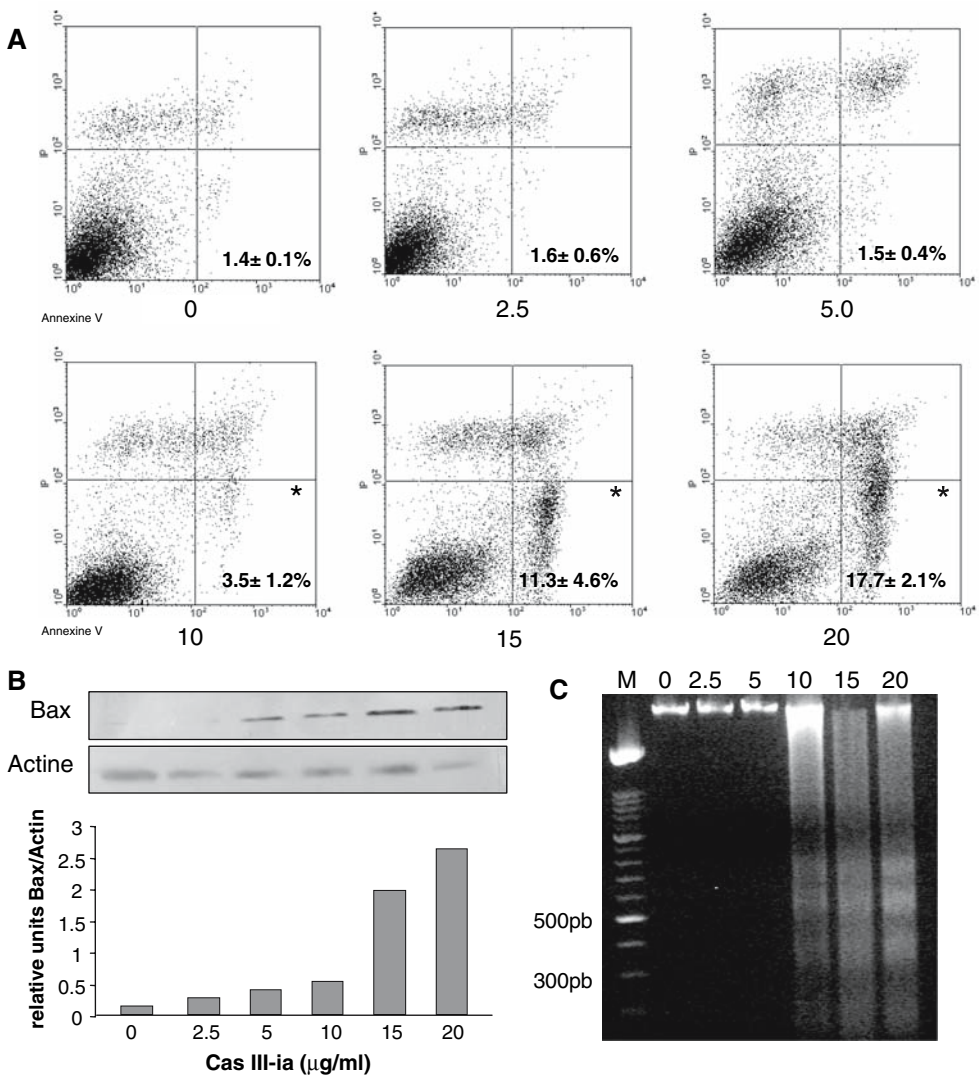

Fig. 5 (**A**) Plot cytograms of Annexine V and Propidium Iodide from HCT-15 cells treated for 24 h with 0, 2.5, 5, 10, 15, and 20 μg/ml of Cas III-ia. *$P < 0.05$ (compared to control) of 3 independent experiments. (**B**) Bax expression in total cellular lysate from control and 24 h treated cells: The bar graphic shows the relative units of Bax normalized with actine. (**C**) DNA electrophoresis in 2% agarose gel. The molecular weight marker is a ladder of 100 base pair (left lane). The DNA was isolated from cells treated with ascending doses of Cas III-ia during 24 h

Table 1 In vivo effects of Cas III-ia on HCT-15 cells transplanted into nude mice

Treatment groups	*n*	TRV	*D*	MI	AI
Destilled water (Vehicle control)	10	3527.88 ± 819.8	5.1 ± 1.0	8.5 ± 0.7	4.2 ± 1.1
CDDP (Positive control)	5	2755.2 ± 342.9	5.4 ± 0.5	8.4 ± 2.1	17.6 ± 1.9*
Casiopeina III-ia 3.0 mg/kg	5	2709.5 ± 1324.2	9.0 ± 3.5	4.8 ± 2.2	18.91 ± 06*
Casiopeina III-ia 6.0 mg/kg	5	661.3 ± 241.5*	12.2 ± 3.6*	3.5 ± 1.1*	20.26 ± 6.4*

Tumor Relative Volume (TRV), Days in doubling size (*D*), Mitotic Index (MI) and Apoptotic index at tumor periphery (AI) determined in CDDP and Cas III-ia treated nude mice xenotransplanted with HCT-15. Data represents media ± standard error

* $P < 0.05$ (compared to control group)

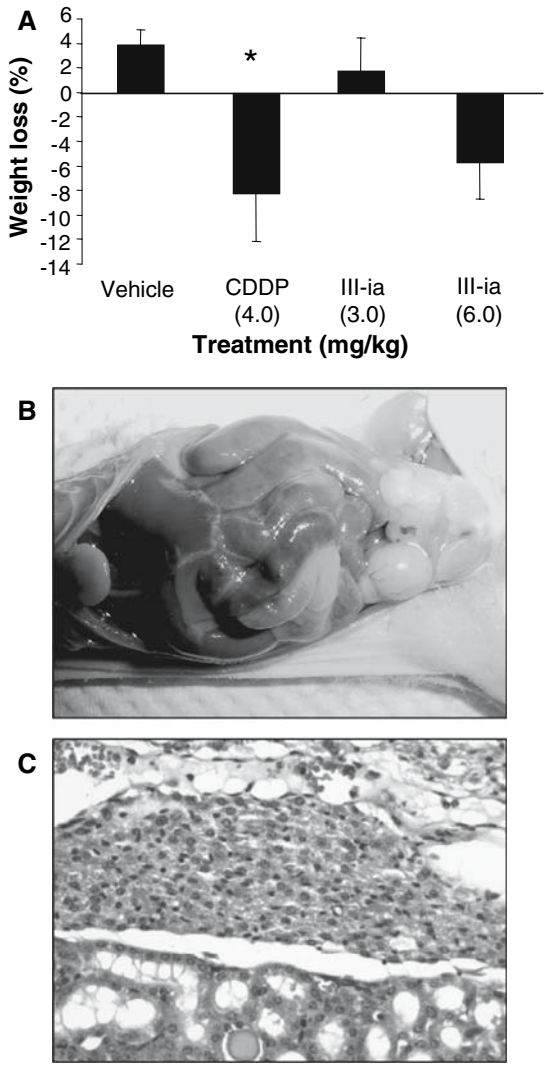

Fig. 6 Collateral effects of Cas III-ia. (**A**) In vivo percentage of weight loss of nude mice under different schemes and dosage. $*P < 0.05$ (compared to control group). (**B**) Intestinal adherences in a nude mouse treated with 6.0 mg/kg of Cas III-ia. (**C**) Kidney histological section, showing abundant fibrin and mononuclear infiltrate on the kidney surface

At necropsy, ascites and adherences between the intestine serose and liver with intestine serose were observed in animals treated with 3.0 mg/kg and 6.0 mg/kg Cas III-ia. The severity of these lesions was dose dependent. One dead animal was observed when treated with the fifth dose of 6.0 mg/kg Cas III-ia (Fig. 6B). At the histological analysis of the visceral peritoneum, abundant fibrin and moderate infiltrate of lymphocytes and macrophages was observed (Fig. 6C), which corresponds to adherences. These results indicate that instead of good anti neoplastic activity, Cas III-ia exerts a chronic irritative effect to the peritoneal serose in a dose dependent manner.

Discussion

Among the most important apoptosis inducing antineoplastic drugs are those with a metallic center, such as CDDP. The present study shows the activity of the new antineoplastic copper based drug Casiopeina III-ia on colon adenocarcinoma HCT-15 cell line. The findings supporting this are: (a) Diminution on cell viability in a dose dependent manner (Fig. 2A) without affecting PCNA levels or cell cycle stages (Fig. 3) and with morphological and ultra structural changes related to apoptosis (Figs. 2B, 4), plus (b) phosphatidilserine exposure (Table 1, Fig. 5A); (c) increased levels of Bax and internucleosomal DNA fragmentation, the latter related to caspase dependent apoptosis (Fig. 5B,C). (d) Also, Cas III-ia exerts antitumor effects on HCT-15 xenotransplanted to nude mice (Table 1) with chronic peritoneal inflammatory effect associated to the drug application (Fig. 6).

It is well known that apoptosis induction by chemotherapy agents is an established way to induce cell death in transformed cells. A large number of stress induces apoptosis by the intrinsic pathway, which involves cytochrome c liberation from mitochondria and its union to Apoptotic protease activating factor 1 (Apaf-1) and caspase 9 activation (Budihardjo et al. 1999). The tumor suppressor p53 has an essential role in apoptosis signaling, because it mediates the expression of multiple apoptosis inductor proteins such as Bax, puma, noxa, and p53AIP1 (Fridman and Lowe 2003). It is known that Bax plays an essential role conferring chemosensitivity to tumor epithelial cells, being its integrity essential for the apoptosis maintenance through different stimuli (Theodorakis et al. 2002). Nevertheless, the p53 status in HCT-15 is defective (Mashima et al. 2005), so we postulate that Bax activation could be mediated by another transcriptional factor, as p73 (Irwin et al. 2003; Ramadan et al. 2005). Regarding this, it was

demonstrated that DNA damage induced by certain chemotherapy agents potentiates the apoptotic function of p73 by enhancing the ability of p73 to selectively activate the transcription of pro apoptotic target genes of p53 (Costanzo et al. 2002).

Furthermore, it is well known that compounds derived from other transition metals (such as ruthenium) are capable of activating p53 and p73, which is related to increases in expression of p21 and Bax (Gaiddon et al. 2005). Nevertheless, cells presenting mutations on p53 domain fail to activate p21 and have reduced ability to induce G1 arrest, which explains the unaltered levels of PCNA and the maintenance of the cell cycle phases (Fan et al. 1995; Wahl et al. 1996).

Likewise, it has been shown that the major membrane potential of neoplastic cells facilitates the accumulation of cationic lipophilic solutes smaller than 1500 D at the internal mitochondrial membrane (Fantin et al. 2002), activating the cytochrome c release and the execution of the intrinsic pathway of apoptosis. This coincides with the ultra structural findings, where mitochondrial swelling is observed (Fig. 4B) without alteration of viability with the 2.5 μg/ml dose. Additionally, previous studies suggest that Casiopeinas are capable to induce ROS (Trejo-Solís et al. 2005), to bind DNA through interaction with Adenine and Thymine (Tovar-Tovar et al. 2004) and to block oxidative phosphorylation (Marín-Hernández et al. 2003). Altogether, these data postulate more than one mechanism of action for Cas III-ia.

Beside, the xenotransplantation studies demonstrate that Cas III-ia diminishes the proliferation of tumor cells, so it is capable to delay the days in doubling size and to reduce the mitotic index at the major dose administered, with evidences of apoptosis as part of its in vivo mechanism (Table 1). Altogether, these data indicate that Cas III-ia exerts cytotoxic activity in vitro and cytotoxic and cytostatic effects in vivo. These differences can be explained by the existence of a metabolite only present with the ADME (Administration, distribution, metabolism, excretion) system. This is supported by previous studies using hepatocytes, where we can not notice Unscheduled DNA Synthesis (UDS) in 24 h cultures once the cytochrome p450 disappears (Brouns et al. 1979). However the UDS was observed in fresh hepatocyte cultures in a dose dependent manner with the application of Cas III-ia. (MSc Gracia Mora, UNAM).

The macroscopic and histological study showed adhesions and inflammation on the peritoneal surface. The latter is product of a chronic irritative effect caused directly by ischemia and inflammation over the peritoneal surface, which interferes with fibrinolysis and leads to organization rather than resolution of the fibrin-cellular matrix (di Zerega 1994; Monk et al. 1994). Nevertheless, no statistical differences were observed between lost of weight (as a toxicity indicator) by Cas III-ia treated groups and control group.

It is known that cancerous cells with inactive p53 are resistant to apoptosis inducing drugs mediated by the mitochondria (Bunz et al. 1999), so the search of new drugs with antineoplastic activity is a reasonable quest; our findings postulate Cas III-ia is a potential candidate for the therapy of cancer with resistant phenotype. As observed, Cas III-ia: (1) Induces apoptosis in p53 altered cells, (2) Increases Bax levels, related to apoptosis induction and (3) Has antitumor activity in vivo. Taken together all theses events, Cas III-ia is a potential candidate to be used in colon cancer therapy. Nevertheless, in vivo toxicity during prolonged exposure times still has to be investigated.

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