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ORIGINAL ARTICLE

# Induction of oxidative damage by copper-based antineoplastic drugs (Casiopeínas®)

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**Abstract**
*Purpose* The aim of the present study is to determine in HeLa cells and in human lymphocytes, by an easy and fast method, the induction of oxidative damage to plasma membrane lipids and nuclear DNA by Casiopeínas®, which are recently synthesized coordination complexes that have been considered as a promising chemotherapeutic alternative for the treatment of cancer, since they have shown cytotoxicity and genotoxicity in several cancer cell lines and xenotransplanted tumours. The presence of an oxidized copper atom in their structure strongly suggests that their mode of action seems to be related to reactive oxygen species (ROS) generation after copper atom reduction through the Fenton and Haber–Weiss system.
*Method* Lipid peroxidation was evaluated as thiobarbituric acid reactive malondialdehyde, cytotoxicity by the fluorescein diacetate/ethidium bromide stain and genotoxicity as DNA fragmentation by the comet assay. Cells were treated with ten different Casiopeínas in a concentration range higher than their $IC_{50}$ (10–100 μM), both oxidized and reduced in the presence of ascorbic acid.
*Results* In almost all the cases, copper reduction enhanced cytotoxicity but, unlike copper nitrate used as positive control, none of them induced appreciable lipid peroxidation. Three Casiopeínas: Cas Igly, Cas-III-H-a and Cas-III-E-a, showed low, moderate and high rates of genotoxicity, respectively, and this effect was enhanced upon addition of ascorbic acid.
*Conclusion* These results suggest that ROS generation might be the cause of cytotoxicity, which seems to be related to initial genetic damage rather than to lipid peroxidation. HeLa cells showed to be more sensitive than normal cells.

**Keywords** Casiopeínas® · Copper-based drugs · ROS · Oxidative damage · Lipid peroxidation · Genotoxicity

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## Introduction

A novel series of copper-based drugs registered and patented with the name of Casiopeínas® [3, 21, 24, 25] has been developed by our group, these are mixed chelate complexes with a general condensed formula

$[Cu(N–N)(A–A)]NO_3$, where N–N represents diimine donors, either bipyridine or phenanthroline, A–A stands for N–O or O–O donors, either aminoacidates or acetylacetonate, and a central copper atom ($Cu^{2+}$) [24, 25] (Fig. 1). These drugs have been classified in different groups according to the arrangement and the combination of their organic moieties [25].

Casiopeínas (Cas) are meant as a chemotherapeutic alternative for cancer treatments and according to some preliminary experiments some of them have indeed shown antineoplastic activity both in vitro and in vivo [6, 13, 14, 26] and are apparently able to induce apoptosis in murine cancer cell lines such as L1210 and CH1 [7]. Detailed experiments in rats, employing one of the most promising Casiopeínas (Cas IIgly), showed a strong inhibition of cell proliferation against a line of glioma C6 cells, in vivo as well as in vitro. It was observed that the drug promoted an increment in reactive oxygen species (ROS) which in turn caused subsequent damage to mitochondria followed by apoptosis elicited through both, caspase-dependent as well as caspase-independent pathways [34].

However, besides mitochondria there are other potential targets where Casiopeínas might have an effect, for example, an initial screening using the microelectrophoresis test (comet assay) in peripheral blood lymphocytes and HeLa cells suggested potential DNA damage by several Casiopeínas [2] with slight differences among them apparently related to the substituents nature on the chelate structure. The potential action mechanism could be promotion of Fenton and Haber–Weiss like reactions [11, 17] due to reduction of the coordinated copper(II) to copper(I), with subsequent generation of ROS such as hydroxyl ($^{\bullet}OH$) or superoxide ($O_2^{\bullet -}$) radicals, which may react with different macromolecules such as nucleic acids, proteins or membrane lipids, causing general oxidative damage within the cell. In order to investigate some of these alternatives and at the same time to gather more knowledge as to these drugs mode of action, cyto- and genotoxicity as well as membrane lipid peroxidation induced by different Casiopeínas were examined by using an easy and fast method, including two cell types, one neoplastic and a normal counterpart, HeLa cells and human lymphocytes, respectively. Since cervix–uterine carcinoma is the main type of cancer among the female population in Mexico, HeLa cells were considered as an adequate neoplastic model, and lymphocytes were chosen for being available and easy to obtain.

## Materials and methods

### Chemicals

Ten Casiopeínas® from either group I, II or III (Table 1), synthesized and purified as previously

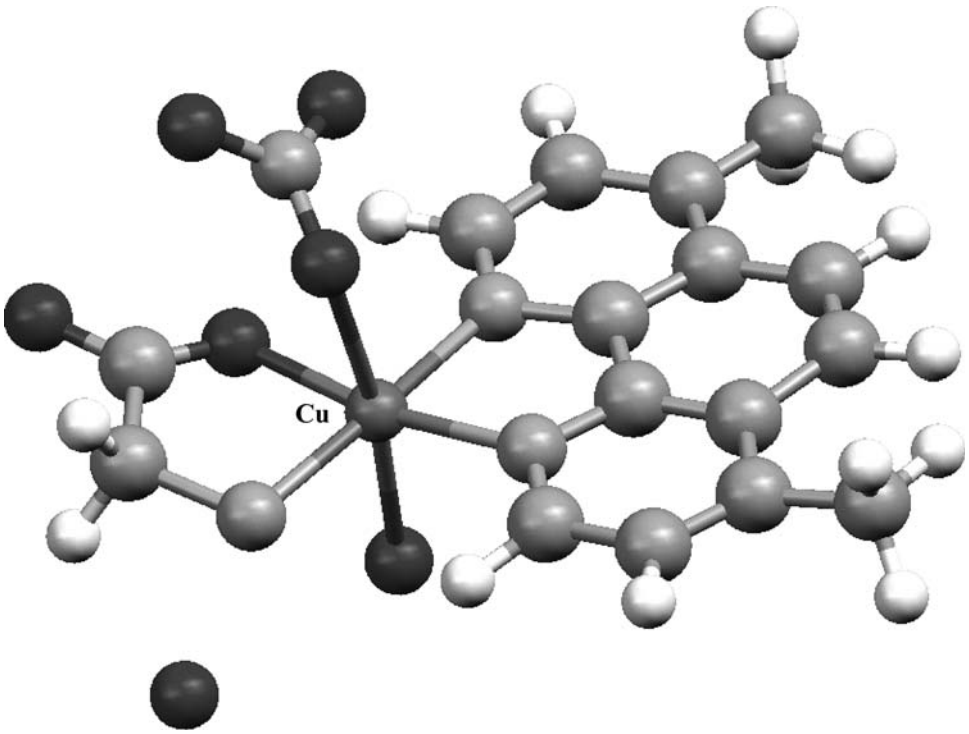



**Fig. 1** Casiopeínas general chemical structure (example Cas IIgly)

**Table 1** Casiopeínas® evaluated in this paper

| Code | Condensed formula | MW (g/mol) |
|---|---|---|
| Group I | | |
| Cas Igly | Aqua(4,7-diphenyl-1,10-phenanthroline)(glycinate) copper(II)nitrate | 531.6 |
| Cas Iser | Aqua(4,7-diphenyl-1,10-phenanthroline)(serinate) copper(II)nitrate | 560.5 |
| Cas Ival | Aqua(4,7-diphenyl-1,10-phenanthroline)(valinate) copper(II)nitrate | 572.2 |
| Cas Iile | Aqua(4,7-diphenyl-1,10-phenanthroline)(isoleucinate) copper(II)nitrate | 586.5 |
| Group II | | |
| Cas IIgly | Aqua(4,7-dimethyl-1,10-phenanthroline)(glycinate) copper(II)nitrate | 406.8 |
| Cas IIser | Aqua(4,7-dimethyl-1,10-phenanthroline)(serinate) copper(II)nitrate | 436.8 |
| Cas IIval | Aqua(4,7-dimethyl-1,10-phenanthroline)(valinate) copper(II)nitrate | 448.9 |
| Cas IIile | Aqua(4,7-dimethyl-1,10-phenanthroline)(isoleucinate) copper(II)nitrate | 462.9 |
| Group III (subgroup [Cu(N–N)(acac)]$NO_3$) | | |
| Cas-III-E-a | Aqua(4,7-dimethyl-1,10-phenanthroline)(acetylacetonate) copper(II)nitrate | 447.9 |
| Cas-III-H-a | Aqua(4,7-diphenyl-1,10-phenanthroline)(acetylacetonate) copper(II)nitrate | 570 |

reported [25], were evaluated. Group I [Cu(4,7-diphenyl-1,10-phenanthroline)(aminoacidate)]$NO_3$, group II [Cu(4,7-dimethyl-1,10-phenanthroline)(aminoacidate)] $NO_3$ and group III [Cu(N–N)(acetylacetonate)]$NO_3$. All reagents employed were purchased from Sigma Chemical Company, with the exception of copper nitrate which was obtained from J.T. Baker.

A 100-mM stock solution of each Casiopeína was prepared in ethanol, and subsequent dilutions were made with sterile double distilled water [4]. All Casiopeínas were administered at 100 μM except on the cases that are specified. An ethanol control at the same concentration employed as the Casiopeínas solvent (0.1%, w/w) was included. As positive controls, stock solutions of either copper nitrate (100 μM) or ascorbic acid (1 mM) were used, each one dissolved in sterile double distilled water and added simultaneously to the cells. Untreated cells were used as the negative control.

## Cell cultures

HeLa cells were grown in minimum essential medium supplemented with 10% FBS, 1% antibiotics (penicillin, streptomycin and amphotericin) and 0.3 mg/ml L-glutamine, at 37°C and 5% $CO_2$ atmosphere, until subconfluent phase was reached and further harvested with a 0.01% tripsin–EDTA solution.

Lymphocytes were obtained from blood samples drawn by venopuncture from a healthy adult subject and placed in heparinized tubes (Becton Dickinson Vacutainer System). Blood was mixed with an equal volume of Hanks' balanced saline solution (HBSS), and 5 ml were centrifuged in 5 ml of Ficoll Hypaque 1083 at 600 *g* for 15 min. The interphase ring of nucleated cells was collected and washed twice with HBSS, resuspended in RPMI-1640 supplemented with 10% FBS and again placed at 37°C and 5% $CO_2$ atmosphere.

## Chemical treatments

Cells of both types were treated during 30 min with different Casiopeína concentrations, with or without ascorbic acid. After treatments cells were centrifuged at 720 *g* and pellets were resuspended in phosphate buffer [33]. When using ascorbic acid, the molar rate was 10 mol of Asc per mole of Casiopeína, and as positive control Cu(NO3)2 was employed [28].

## Cytotoxicity

Cell death was evaluated according to the method of Strauss [31]. After treatments, cells were stained with a 1:1 solution of 80 μg/ml fluorescein diacetate plus 50 μg/ml ethidium bromide and observed under a Hund Wetzlar fluorescence microscope with an excitation filter of 488 nm (blue light). Living cells are stained in green while dead cells exhibit their nucleus stained in orange. Survival percentage was obtained

dividing the number of living cells by the total number of cells.

### Lipid peroxidation

Oxidative membrane damage was evaluated as micromoles of malondialdehyde (MDA), as an end product of lipid peroxidation [10]. According to the thiobarbituric acid (TBA) test, 200 µl of cell suspension from each treatment were added to 800 µl of phosphate buffer plus 500 µl of 30% trichloroacetic acid to a final volume of 1 ml [15]. Cells were vortexed, incubated on ice for 2 h, and afterwards centrifuged at 780 *g* for 15 min. After collecting the supernatant a 1-ml aliquot containing membrane lipids, was mixed with 75 µl of 0.1 M EDTA and 250 µl of 1% TBA, vortexed and placed in a water bath at 80°C during 15 min to allow reaction between TBA and end product MDA. Absorbance was read at 532 nm in a GBC UV/VIS 911A spectrophotometer and the MDA concentration was obtained from its standard curve using the extinction coefficient $1.56 \times 10^5$ per mole per centimetre.

### Genotoxicity

Genotoxicity expressed as DNA fragmentation, was assessed by means of the unicellular alkaline gel microelectrophoresis method or comet assay [4]. After treatments, cells were resuspended in RPMI-1640 and 1% low melting point agarose (w/v) to a final concentration of 0.5% and poured on top of fully frosted microscope slides previously layered with 0.5% of regular agar. Two slides per treatment were obtained and placed into cold freshly made lysis solution (pH 10) for 1 h, then gently transferred to electrophoresis buffer for 20 min to allow the DNA unwinding to occur. Electrophoresis was carried out at 20 V and 300 mA for 24 min. Slides were rinsed three times with neutralizing buffer (0.4 M Tris; pH 7.5), and each stained with 60 µl of 20 µg/ml ethidium bromide and finally observed under a Hund Wetzlar fluorescence microscope using an excitation filter of 515–560 nm (green light) at 40×. One hundred cells per slide were analysed and comet sizes were recorded by means of the *Comet Assay II Analyzer*. Genetic damage was scored as number of cells with fragmented DNA or *comets* and as tail moment, which is the result of comet tail length × DNA density. Results are expressed as the mean of six different experiments plus standard deviation, and were statistically evaluated by the one-way ANOVA test ($P \leq 0.01$).

## Results

### Cytotoxicity

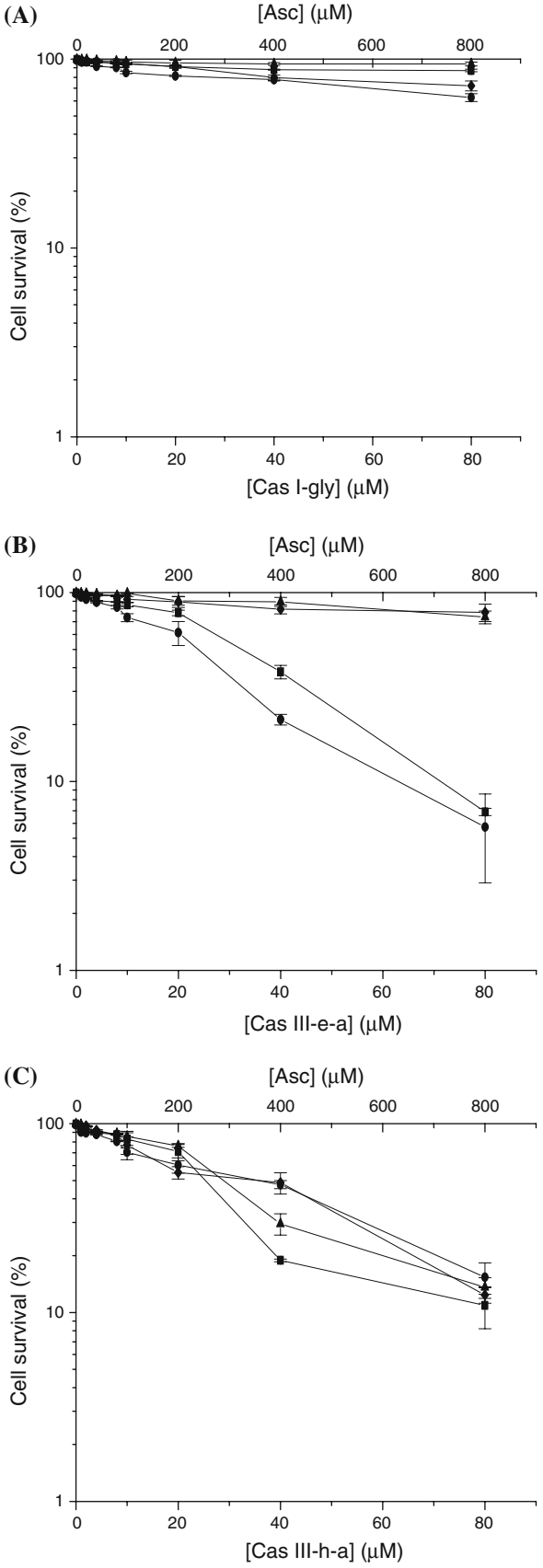
Comparative cytotoxicity at the highest concentration (100 µM) is shown in Table 2 and as can be appreciated, with the exception of the effect by Cas-III-H-a (10–12% in both cell types), survival of lymphocytes or HeLa cells is at least around 50%, though the latter are slightly more sensitive. However upon copper reduction cytotoxicity increases considerably, affecting both cell types; again the exception is Cas-III-H-a, which apart from being the most cytotoxic, showed no significant differences regardless its copper oxidation state. When relating toxicity to either cell type, or Casiopeína group, it was noticed that HeLa cells were slightly more sensitive than lymphocytes. At the same time, Casiopeínas belonging to group II were generally more cytotoxic than those from group I, with the exception of Cas Ival and Cas Iser, which when reduced were as cytotoxic as their group II counterparts (Cas IIval and Cas IIser) in HeLa cells (Table 2).

In order to determine the extent of cell death as a function of concentration, three Casiopeínas were

**Table 2** Cytotoxicity as measured by survival percentage, caused by 100 µM of different Casiopeínas in both lymphocytes and HeLa cells

Results were analysed by the *t* test ($P \leq 0.01$) and expressed as average ± standard deviation

| Casiopeína | Leukocytes | Leukocytes + Asc | HeLa | HeLa + Asc |
|---|---|---|---|---|
| Control | 99.4 ± 0.3 | 95.3 ± 0.6 | 96.6 ± 2.4 | 93 ± 0.8 |
| $Cu(NO_3)_2{\cdot}2.5H_2O$ | 99.2 ± 2.5 | 90.7 ± 2.9 | 96.1 ± 2.6 | 86.8 ± 2.6 |
| Cas Igly | 95.4 ± 1.9 | 52.7 ± 2.6 | 71.1 ± 4.0 | 57.9 ± 4.6 |
| Cas Iser | 95.2 ± 2 | 56.9 ± 3.2 | 79.8 ± 1.1 | 6.4 ± 0.3 |
| Cas Ival | 95 ± 2.2 | 72.2 ± 2.0 | 66.6 ± 3.6 | 8.3 ± 0.8 |
| Cas Iile | 97.1 ± 0.5 | 87 ± 3.5 | 84.9 ± 2.2 | 51 ± 9.4 |
| Cas IIgly | 65.9 ± 1.5 | 0.5 ± 0.1 | 78.3 ± 4.5 | 11.5 ± 0.7 |
| Cas IIser | 74.1 ± 1.2 | 0.4 ± 0.1 | 38 ± 3.5 | 1.7 ± 0.03 |
| Cas IIval | 77.1 ± 2.1 | 1.5 ± 0.04 | 69.4 ± 7.2 | 3.1 ± 0.04 |
| Cas IIile | 97.1 ± 1.3 | 17.4 ± 0.9 | 67.7 ± 8.4 | 26 ± 2.2 |
| Cas-III-E-a | 72.9 ± 1.8 | 0.4 ± 0.04 | 73.8 ± 8.0 | 1.2 ± 0.04 |
| Cas-III-H-a | 11.7 ± 1.6 | 10.2 ± 1.3 | 10.9 ± 1.4 | 10.5 ± 1.7 |




◀ **Fig. 2** Cytotoxicity expressed as percentage of cell survival as a function of Casiopeínas® increasing concentration: **a** Cas Igly; **b** Cas-III-E-a; **c** Cas-III-H-a. Every Casiopeína is represented as follows: oxidized (*filled upward triangle*) and reduced (*filled square*) in lymphocytes; oxidized (*filled diamond*) and reduced (*filled circle*) in HeLa cells. The reduction of Casiopeínas was carried out with ascorbic acid prior to releasing to cells

analysed in the range from 1 to 80 μM. These compounds, Cas Igly, Cas-III-E-a and Cas-III-H-a, showed different effects, i.e. Cas Igly with or without Asc, was less cytotoxic in both cell types (Fig. 2a); Cas-III-E-a, exerts a moderate cytotoxicity, again in both cell types, but after copper reduction by Asc becomes significantly more cytotoxic, with a survival fraction of less than 10% (Fig. 2b) and finally, Cas-III-H-a has the strongest cytotoxicity to both cell types regardless its oxidation state (Fig. 2c). Again, HeLa cells were significantly more sensitive than leukocytes (Table 2).

## Lipid peroxidation

Regardless of drug concentration or copper oxidation state, and as suggested by MDA concentrations, no appreciable oxidative damage to membrane lipids of either lymphocytes or HeLa cells, could be detected (Fig. 3, panels a and b). The cytotoxicity caused by Cas-III-E-a is not related to lipid peroxidation as we can observe in Fig. 4a. On the contrary copper nitrate (100 μM), when reduced by ascorbic acid, induced significant levels of MDA in both cell types and cytotoxicity (Fig. 4b).

## Genotoxicity

Results expressed either as number of cells showing DNA fragmentation or as tail moment support the fact that Casiopeínas induce different degrees of genetic damage as initially suggested by a preliminary screening of several Casiopeínas [2], an effect that with one exception, seems to be enhanced upon copper reduction by ascorbic acid. Accordingly when reduced, Cas-III-E-a is actually the most genotoxic (Fig. 5, panel b), followed by Cas Igly and finally by Cas-III-H-a (Fig. 5, panels a and c). Cas-III-E-a has the smallest alkyl substituent while the other two have aromatic phenyl groups. Such results suggest the possibility that the effect could be related to factors such as hydrophobicity, aromaticity or even an inductive effect by the substituents in the diimine donor (phenanthroline moiety), all of which will be further discussed. As Fig. 6 shows, Cas-III-E-a

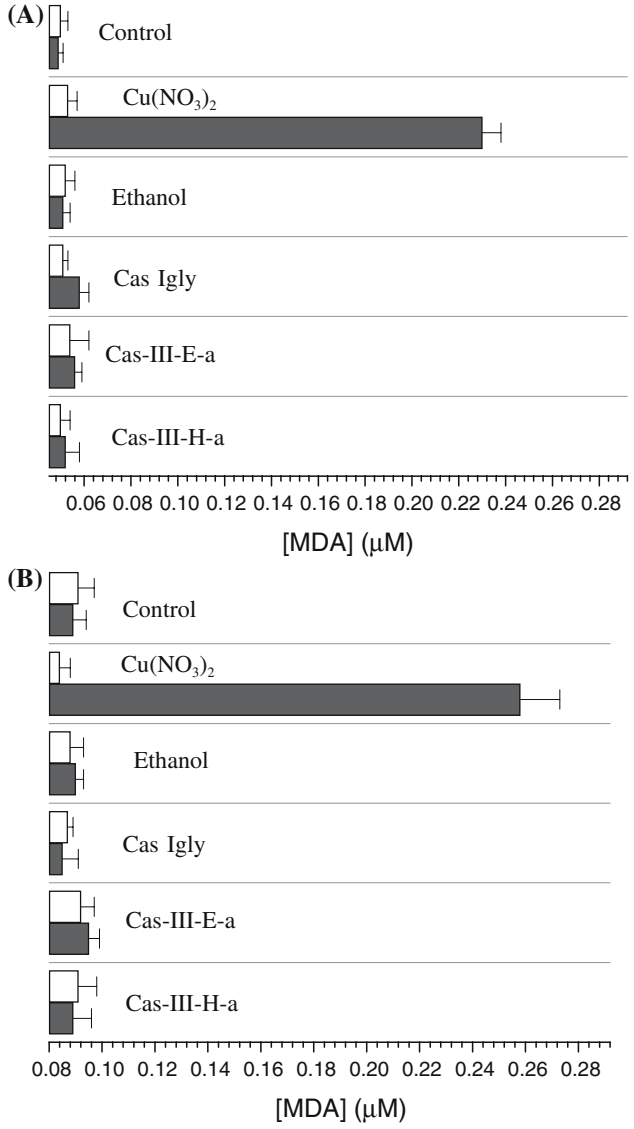



**Fig. 3** Lipid peroxidation expressed in micromoles of malondialdehyde MDA induced by 100 μM of Casiopeínas. *White bars* indicate each treatment alone and *black bars* each treatment with 1 mM ascorbic acid. Controls of 0.1% ethanol and copper nitrate (100 μM) are also included. **a** Lymphocytes and **b** HeLa cells

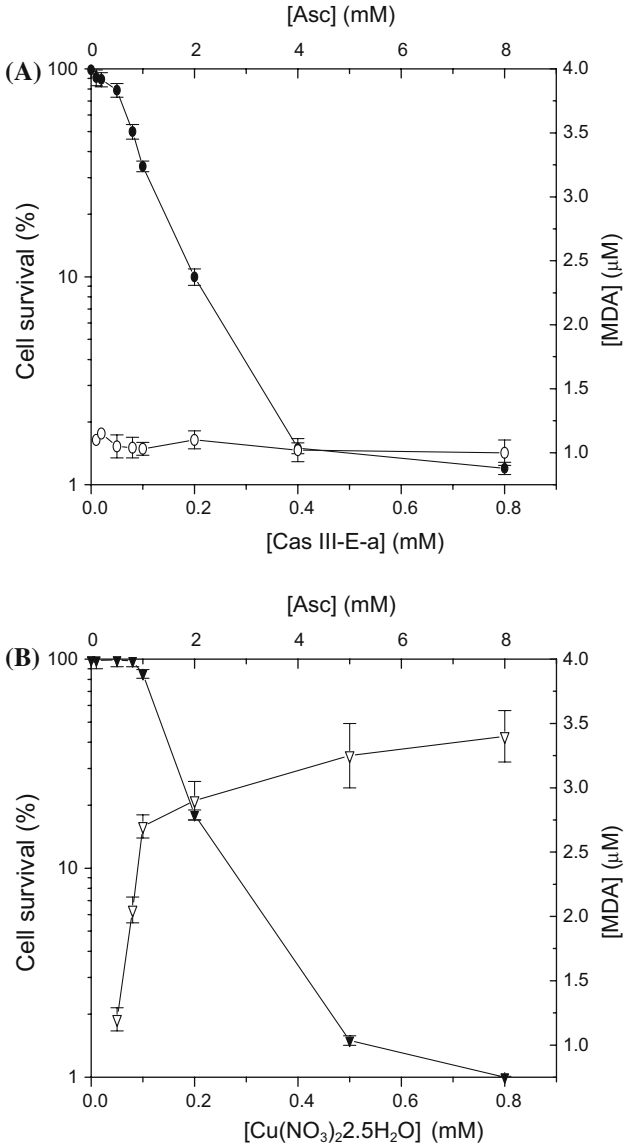
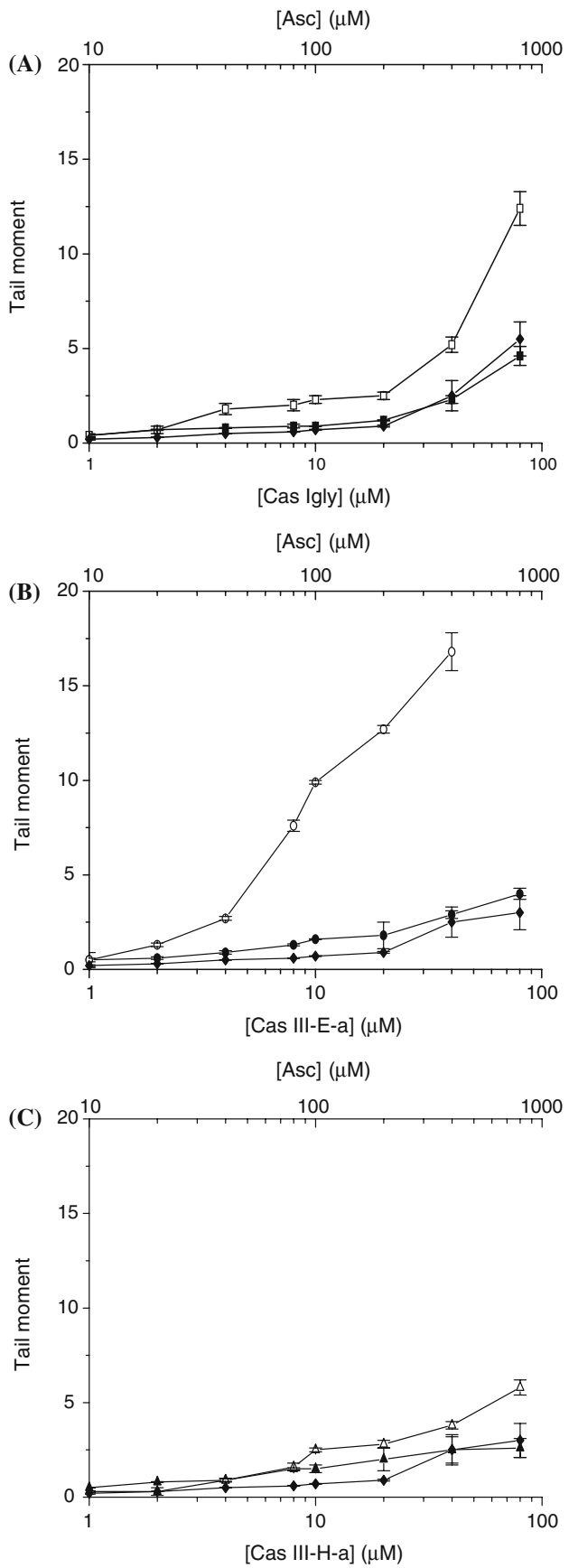



**Fig. 4** Comparative effects of Cas-III-E-a (**a**) and copper nitrate (**b**) in HeLa cells, with or without previous metal reduction by means of ascorbic acid addition. Cytotoxicity is expressed as cell survival percentage whereas lipid peroxidation is expressed as micromoles of malondialdehyde (MDA). As can be noticed in the case of Cas-III-E-a, cytotoxicity (*filled circle*) is unrelated to lipid peroxidation (*open circle*), since no MDA could be detected. On the contrary, copper nitrate when reduced, induces cell death (*filled downward triangle*) as well as lipid peroxidation (*open downward triangle*)

induced a significantly higher number of cells with damaged DNA than that of dead cells in the same population, even at the highest concentration (80 μM) a large amount of cells survived (more than 50%) but all of them showed genotoxic damage; this finding suggests that DNA fragmentation seems to occur first and cell death comes about as a consequence. Tail moment values were higher in HeLa cells than in leukocytes, indicating that this tumour cell line was significantly ($P < 0.01$) more sensitive to genetic damage induced by Casiopeínas (Fig. 7).

## Discussion

According to our results, Casiopeínas are cyto- and genotoxic to $G_0$ lymphocytes and HeLa cells. With the exception of Cas-III-H-a, both effects are enhanced whenever Casiopeínas underwent copper reduction prior cell uptake. These results suggest a mechanism of




◀ **Fig. 5** **a** Genotoxicity induced by increasing concentrations of oxidized (*solid symbols*) or reduced (*open symbols*) Casiopeínas in HeLa cells. Cas Igly (*open square*); Cas-III-E-a (*open circle*) and Cas-III-H-a (*open upward triangle*), all of them compared to ascorbic acid at concentrations of 10–800 μM (*filled diamond*). **b** Genotoxicity induced in HeLa cells by oxidized (*filled circle*) and reduced (*open circle*) Cas-III-E-a in the presence of ascorbic acid. Concentration of Casiopeína and Asc, in the same range as in Fig. 4a. **c** Genotoxicity induced in HeLa cells by Cas-III-H-a with (*filled upward triangle*) or without (*open upward triangle*) ascorbic acid addition

action involving generation of ROS. Thus we suggest the following mode of action. Casiopeínas enter the cell apparently without dissociation as suggested by the lack of membrane lipoperoxidation. Whether oxidized or reduced, intracellularly Casiopeínas would undergo further reduction by glutathione or reducing agents from the respiratory chain, as suggested by Marín et al. [18]. Preliminary results (data not shown) indicate that glutathione levels decrease in cells treated with Casiopeínas at the same experimental conditions employed in this work.

Polarity, aromaticity and the size of the substituents are all influencing factors in redox reactions where chelates may participate [19, 20], for example, a larger size or higher inductive effect of the organic ligands may interfere with electron transfer reactions. This in turn may explain differences in cyto- and genotoxicity among the Casiopeínas tested. It is important to note that Cas-III-H and Cas-III-E-a have both acetylacetonate, but due to the smaller substituent in the diimine donor of the former, it may be able to participate in redox reactions probably because of the net charge in the copper atom does not reach the level to produce a reduction reaction. An exception to this pattern is Cas-III-H-a, where copper oxidation state did not influence cytotoxicity. Then it is conceivable that intracellularly Cas-III-H-a unlike the rest, may not easily undergo redox reactions due to the presence of both, the net charge in the acetylacetonate as well as a larger size of the phenanthroline moiety.

Other factors may be the number, position and nature of the substituents in the diimine fraction. Thus, group II Casiopeínas, carrying dimethylphenanthroline as diimine donor, were generally more cytotoxic than their group I counterparts, with diphenylphenanthroline instead, so in this instance cytotoxicity appeared to be inversely related to size or bulk of the alkyl or aryl substituent (Fig. 2). Besides, O–N and O–O donors also had an influence, because Cas-III-H-a with acetylacetonate as O–O donor was more cytotoxic than Cas Igly, with an aminoacidate (glycinate) as the O–N donor. In this sense, the acetylacetonate fraction seems

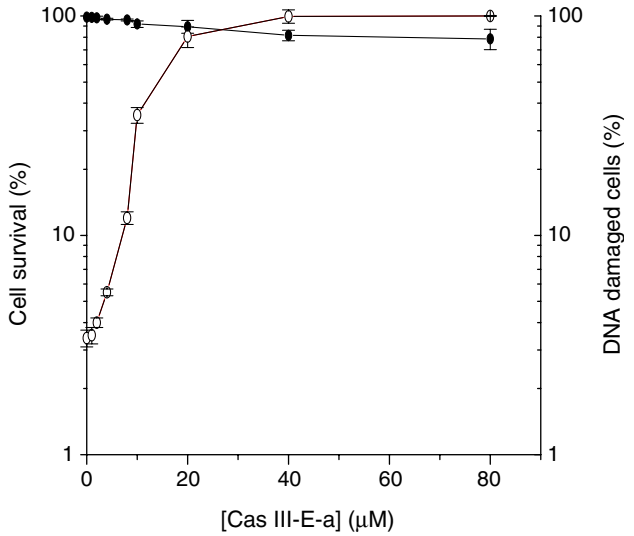



**Fig. 6** Cytotoxicity (*filled circle*) related to genetic damage (*open circle*) induced by Cas-III-E-a in HeLa cells. Albeit at the highest concentration (80 μM) cell survival reaches around 80%, all cells examined show DNA fragmentation

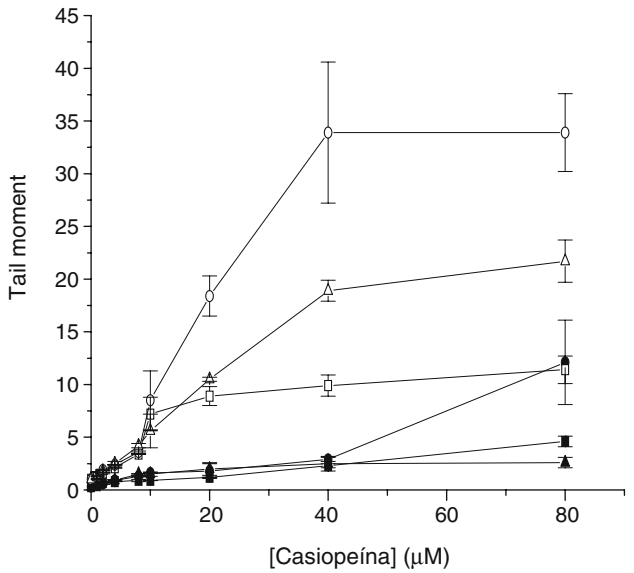



**Fig. 7** Comparative genotoxicity expressed as tail moment, at increasing Casiopeína concentrations, in HeLa cells (*open symbols*) and in lymphocytes (*solid symbols*). Cas-III-E-a (*circles*), Cas-III-H-a (*triangles*) and Cas Igly (*squares*)

to be more cytotoxic than glycine, possibly because it is not a naturally occurring molecule within the cell.

As for the difference in sensitivity between HeLa cells and leukocytes, there are some likely explanations. For example, absence of manganese superoxide dismutase (MnSOD) activity in this tumour cell line [16], could lead to superoxide ion accumulation and, therefore, a greater amount of hydrogen peroxide and hydroxyl radicals in the cytosol. Other authors have observed increased sensitivity to several oxidative agents related to alterations in the subunits of the mitochondrial electron respiratory chain [5, 22] and in this sense it has been shown that Casiopeínas indeed can interact with different electron transfers, in isolated mitochondria [18].

## Oxidative damage to membrane lipids

Lack of any detectable oxidative damage to cell membranes regardless of type, concentration or copper oxidation state could be due again to the metal availability in aqueous solutions. In all cases reduction to $Cu^{1+}$ occurred immediately after Asc addition, as corroborated by a rapid change from deep blue to a yellowish brown colour, and the fact that no oxidative damage seemed to occur in neither case suggests that Casiopeínas mode of action was different than an inorganic salt such as copper nitrate, which in aqueous solutions becomes partially dissociated [29]. When reduced the free copper moiety through free radical generation, may react with plasma membrane phospholipids causing oxidative damage as indicated by MDA presence. Casiopeínas on the contrary are metal chelates, and therefore copper may not be so easily dissociated and as is the case for other phenanthroline metal compounds [12], these drugs may enter into the cell with no apparent damage to membrane phospholipids. In fact other authors [32, 35] report that metal-promoted lipid peroxidation is prevented by chelating agents such as phenanthroline which in itself is part of several Casiopeínas coordination complexes.

## Oxidative damage to DNA

As initially observed in preliminary experiments, cytotoxicity seems to be closely related to genotoxicity. The present results confirm this observation since all complexes examined are cyto- and genotoxic to a different extent, effects that are in turn potentiated when the metal is reduced by Asc. As suggested above it seems that DNA damage, as well as cytotoxicity, tend to be related in an inverse fashion to the N–N ligand nature, since Cas-III-E-a, the smallest alkyl examined Casiopeína (dimethylphenanthroline), brought about the highest effect, while Cas Igly and Cas-III-H-a (diphenylphenanthroline) induced genetic damage to a lesser extent. In addition, and as is the case of other copper–phenanthroline complexes [8, 23, 30], Casiopeínas are able to intercalate electrostatically between the stacked DNA bases, and in due course promote

ROS oxidative attack to DNA components near the site of intercalation [9]. Following this model, it is conceivable that Casiopeínas behave in a similar fashion, that is, intercalation between DNA bases followed by ROS production through copper reduction. In turn the ability to intercalate could be influenced by the organic moieties, i.e. dimethyl- versus diphenylphenanthroline whereby Casiopeína Cas-III-E-a had a better chance to intercalate with DNA than Cas Igly or Cas-III-H-a do, with bulkier substituents.

An interesting point is the higher sensitivity of HeLa cells to genetic damage. In the first place it could be due to the fact that these tumour cells are actively dividing unlike lymphocytes which were in $G_0$, however the exposure time was for only 30 min compared to HeLa 12 h cell cycle. It has been proposed that HeLa cells lack MnSOD [20] and if Casiopeínas mode of action implies ROS generation, absence of this enzyme could potentiate the effect. As potential drugs in cancer therapy this effect is promising and indeed it has been reported elsewhere that different neoplastic cell lines died within 48 h of exposure to Cas IIgly [7].

## Relationship between genotoxicity and cell death

As an endpoint for genotoxicity, the comet assay indicates fragmented DNA, usually the result of unrepaired damage; however, it could also indicate apoptosis, since along this process DNA is cut into pieces by specific endonucleases [1]. To this respect, some authors have recently shown that one Casiopeína, Cas IIgly, is able to induce oxidative damage followed by caspase-dependent and caspase-independent apoptosis through mitochondrial derived factors [34].

In the present work, if the observed DNA fragmentation was the consequence of apoptosis, the fragments would not have increased their sizes, regardless of the concentration or oxidation state of Casiopeína; furthermore, the number of cells carrying damaged DNA should be approximately equal to that of dead cells in the same population. However, our results were far from that process, since the length of the DNA fragments increased proportionally as a function of drug concentration; moreover, the major part of the population of cells were still alive but had DNA damage, which strongly indicated that cell death came up afterwards, as a consequence of the initial genotoxic damage.

It is worth to mention that the method of staining with fluorescein diacetate/ethidium bromide, performed to evaluate Casiopeínas-induced cytotoxicity, though carried out in a short time (30 min), it indicated the percentage of cell survival in a trustful and convenient way, as compared to the assay of inhibition of proliferation in HeLa cells, which confirmed the same values of cell survival after treatment with Casiopeínas for 30 min and allowed to grow in drug-free culture medium through a period of 12 h (data not shown).

## Potential mode of action

According to our data, Casiopeínas cross the cell barrier without being dissociated, and with no apparent damage to membrane lipids. Once within the cytosol, the copper moiety becomes reduced through interaction with intracellular reducing agents such as NADH, FAD, glutathione or superoxide ion and eventually entering the nucleus. In addition and, due to their planar structure, Casiopeínas might become intercalated within the DNA double helix, and through Fenton type reactions they could generate ROS causing genetic damage in situ, that would lately be expressed as fragmentation observed by the comet assay.

Whether the cytotoxicity is the result of initial damage to the DNA or, as suggested by other works [18, 34], it is rather due to mitochondrial damage, remains to be elucidated. To solve this problem we are presently working with a modified assay [27] to assess the ability of these cells to repair the DNA damage.

As a group of potential antineoplastic drugs it would be desirable that Casiopeínas were less genotoxic without losing concomitantly their selective cytotoxicity against cancer cells. A combined protocol such as the one employed in this work may be appropriate for further screening of the different Casiopeína groups so far synthesized.

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