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Casiopeína IIgly induced cytotoxicity to HeLa cells depletes the levels of reduced glutathione and is prevented by dimethyl sulfoxide

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Abstract

The newly synthesized copper coordination compound Casiopeína IIgly (Cas IIgly) is a promising alternative drug in the treatment of cancer, since it has shown cytotoxicity and genotoxicity in different tumour models. Given its enhanced effects after ascorbic acid-mediated copper reduction, Cas IIgly's activity is thought to be related to oxidative damage. In the present work, oxidized Cas IIgly failed to induce cytosolic oxidative damage in HeLa cells (only 0.9% of the cell population), and in 2.3% of the treated cells when previously reduced, as evaluated through the oxidation of dihydrorhodamine 123 (DHR 123). However, it showed cytotoxicity, since HeLa cells treated with 10–80 μg/mL Cas IIgly proliferated only at 30% of their normal rate, and at 15% when treated with reduced Cas IIgly. This cytotoxicity is strongly abolished in the presence of the hydroxyl scavenger dimethyl sulfoxide. The decrease, from 3994 to 530 nanograms of reduced glutathione (GSH) per million cells after treatment with 80 μg/mL Casiopeína IIgly, indicates that this drug causes the expenditure of this naturally occurring antioxidant. These results altogether suggest that, albeit Cas IIgly induced cytotoxicity is not related to cytosolic DHR 123 oxidation, it may be related to oxidative damage.

Keywords: Casiopeína IIgly; Copper; Oxidative damage; Dihydrorhodamine 123 (DHR 123); Reduced glutathione (GSH)

1. Introduction

The novel drug Casiopeína IIgly (Cas IIgly) (Fig. 1) is a copper (Cu(II)) chelate with dimethyl phenanthroline and the aminoacid glycine as bidentate ligands (Ruiz-Ramírez et al., 1993a,b; Gracia-Mora et al., 2001). Its promising use as a chemotherapeutic agent in the treatment of cancer arises from various studies conducted *in vitro* and *in vivo*, in which its ability to reduce the size and volume of cultured and implanted tumours (De Vizcaya et al., 2000; Gracia-Mora et al., 2001), its low toxicity to healthy tissues and, furthermore, its capacity to induce apoptosis in glioma C6 cells (Trejo-Solís et al., 2005) have been shown.

Regarding Cas IIgly mode of action, it has been demonstrated that this compound interferes both the electron transfer chain and oxidative phosphorylation in mitochondria isolated from cardiac muscle and glioma cells (Marín-Hernández et al., 2003; Trejo-Solís et al., 2005), and its ability to promote DNA fragmentation and apoptosis is as well documented previously (Rivero-Müller et al., 1998). Cas IIgly failed to induce lipid peroxidation to plasma membrane in HeLa cells and in human lymphocytes (Alemón-Medina et al., 2007), but produced DNA damage and cell death, which were promoted after the

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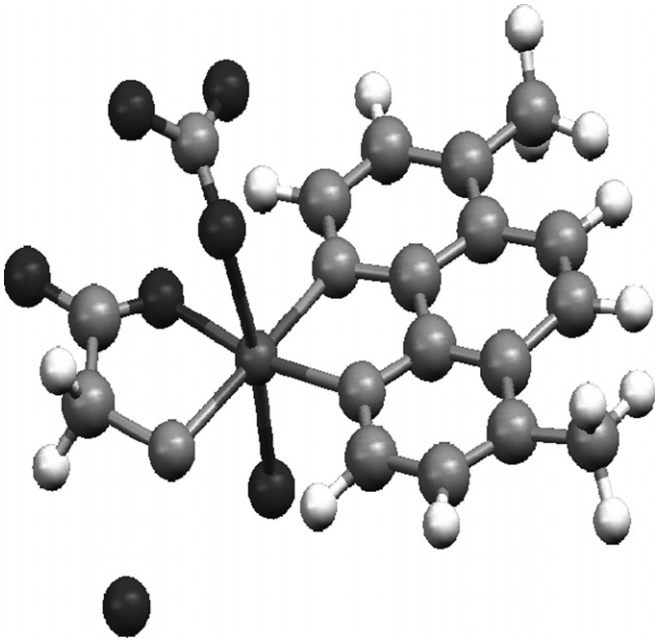

Fig. 1. Cas IIgly chemical structure.

copper atom reduction in the presence of ascorbic acid (Asc) extracellularly.

Since Cas IIgly induced genotoxicity and cytotoxicity has correlated to oxidative damage to the nucleus and mitochondria, it is also possible that this drug could induce this kind of damage to another cell compartment such as the cytosol. That is why the aim of the present work is to find out if Cas IIgly is able to provoke cytosolic oxidative damage, by assaying the oxidation of the probe dihydrorhodamine 123, the depletion of reduced endogenous antioxidant defences, such as reduced glutathione (GSH), and finally the protective role of an exogenous antioxidant, such as dimethyl sulfoxide.

2. Materials and methods

2.1. Reagents

Cas IIgly was synthesized and prepared by our group as previously reported (Patent) and structure reported (Ruiz-Ramírez et al., 1993a, 1993b). Dihydrorhodamine 123 (DHR 123), sulforhodamine, propidium iodide (PI) and ascorbic acid (Asc) were purchased from Sigma Chemical Company; dimethyl sulfoxide (DMSO) from Aldrich; copper nitrate ($Cu(NO_3)_2{\cdot}2.5\ H_2O$) and hydrogen peroxide were from J.T. Baker.

2.2. Cell culture

HeLa cells were grown in DMEM culture medium containing 10% foetal calf serum, 1% antibiotics (penicillin, streptomycin and amphotericin) and 1% antimycotic solution, in an incubator at 37 °C with a 5% CO_2 atmosphere. Cells were harvested by trypsinization with a trypsin–EDTA solution (0.01%) and incubated in the same conditions during a period of 24 h prior to the treatment with Cas IIgly.

2.3. Cells treatment

Cas IIgly was dissolved in sterile double-distilled water at different concentrations in the range from 10 to 80 μg/mL to treat the cells, according to our previous working solutions (Alemón-Medina et al., 2007) that corresponded to 10-fold its IC_{50} (Gracia-Mora et al., 2001). Hydrogen peroxide and the system of copper nitrate/Asc were used as oxidative damage inducing agents (positive controls), so that the possible oxidant activity of Cas IIgly could be compared. Cells were exposed to both oxidized Cas IIgly and reduced Cas IIgly extracellularly in the presence of ascorbic acid, and to the other agents for 30 min at 37 °C and a 5% CO_2 atmosphere; then washed with fresh culture medium and PBS for the cytotoxicity and oxidative damage tests, respectively.

2.4. Cytosolic oxidative damage test

Oxidative damage was evaluated in HeLa cells, according to the protocol developed by Goossens and co-workers (1995), using the non-fluorescent probe dihydrorhodamine 123 (DHR 123) coupled to propidium iodide staining. Through its oxidation in the presence of reactive oxygen species, such as the hydroxyl radical and the superoxide anion ($^{\cdot}OH$ and $O_2^{\cdot-}$), DHR 123 changes to its fluorescent form rhodamine 123 (Emmendörffer et al., 1990; Rothe et al., 1991) in the cytosol and, given its positive charge and hydrosolubility, it remains solubilized mainly in the mitochondria, being therefore an adequate indicator of oxidative damage. Green fluorescence of the cells with such oxidative damage can be observed under a fluorescence microscope (Hund Wetzlar) at 488 nm, and dead cells can be distinguished among them by means of their orange fluorescence due to the intercalation of the probe propidium iodide in their nuclear DNA (Tanke et al., 1982).

A volume of 5 μL of DHR 123 (1 μM in DMSO) was administered to the cells at the beginning of the treatment with Cas IIgly or the positive controls, and propidium iodide was added 20 min later, at a final concentration of 30 μM. Cells were centrifuged at 720*g*, the medium aspirated with a micropipette tip and the cell pellet resuspended in 50 μL of new culture medium and 20 μL of glycerol; then put in a microscope slide and observed immediately in the fluorescence microscope.

2.5. Determination of cell death

Cytotoxicity (cell death) test was carried out based on the sulforhodamine B (SRB) assay (Skehan et al., 1990; Papazisis et al., 1997), as the inhibition of proliferation in HeLa cells. Directly after harvesting, cells were put in 96-well

microtiter plates, each well containing 2×10^4 cells in 100 μL (not all of the wells were filled with cells), and left during 24 h at the incubation conditions in order for the cells to recuperate. Testing compounds were administered to the cells as described above, for 30 min. After treatment, cells were washed with drug-free culture medium and incubated for 12 h. The medium was removed and cells were fixed with 100 μL of ice-cold trichloroacetic acid (10%, w/v in distilled water), maintained for 1 h at 4 °C and washed four times with distilled water at room temperature. The plate was left upside down over a soft paper towel at room temperature for 24 h till the cells got dried. Cells were stained with a 0.4% solution of sulforhodamine B (w/v in 1% acetic acid) for 5 min and washed three times with 1% acetic acid. Cells were dried again as pointed out. Hundred microliters of unbuffered TRIS base (10 mM, pH 10.5) was poured to each well containing the dried and stained cells, and the optical density (OD) was read at 564 nm in an ELISA microtiter plate reader. The OD of the treated cells was normalized to that corresponding to the control cells, and the percentage of inhibition of proliferation was calculated.

2.6. Intracellular reduced glutathione

Reduced glutathione levels were determined through its reduction by Ellman's reagent (DTNB) (Sedlack and Lindsay, 1968), which is then concomitantly reduced, passing from a colourless to a yellowish appearance, which can be evaluated spectrophotometrically; so that, the greater optical density of the sample, reflects a greater amount of reduced glutathione. One mL of culture medium containing one million HeLa cells was used for each treatment set. After treatment with Cas IIgly, hydrogen peroxide or copper nitrate/ascorbic acid, cells were centrifuged at 2000*g* for 2 min, then resuspended in a 0.5 mL PBS buffered solution containing EDTA (5 mM), sodium cholate (0.25%) and digitonin (0.01%). To release endogenous glutathione, cells were thermally disrupted, by storing them first at −70 °C for 2 min and in a water bath at 37 °C immediately afterwards. Cells were centrifuged at 10,000*g* for 20 min and the 500 μL supernatant was recovered in another tube containing 500 μL of 10% (w/v) trichloroacetic acid. This mixture was stored in an ice bath for 30 min, centrifuged at 10,000*g* for 20 min to obtain the acid-soluble fraction. The pellet was resuspended in 50 μL of 0.02 M EDTA, then 150 μL of 0.2 M Tris–EDTA (pH 8.2), 10 μL of (0.01 M) Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)) and 790 μL of absolute methanol were added. Absorbance was read at 412 nm and the amount of GSH was obtained from a standard curve. Results were expressed as GSH nanograms per million (1×10^6) cells.

2.7. Statistical analysis

Cytosolic oxidative damage results are expressed as the mean of six independent experiments plus standard deviation; eight different repeats were performed in the assay of inhibition of proliferation in HeLa cells, and five independent experiments were carried out in determining the intracellular reduced glutathione levels. Data were statistically evaluated by the one way ANOVA test ($P \leqslant 0.05$).

3. Results

3.1. Oxidative damage

Cells treated with oxidative damaging agents and loaded with the probes DHR 123 and PI were observed in the fluorescence microscope under the 40X magnifying objective, and four different cell appearances were distinguished: non-fluorescent, healthy cells, which were alive and had an intact cytoplasm; green fluorescent cells, alive with oxidative damage; orange fluorescent or dead cells; green and orange fluorescent cells, which had oxidative damage and died because of it. Thousand cells per slide were counted, this amount was taken as the cell population's hundred percentage and the percentages of living, oxidative damaged or dead cells were calculated from this number.

As expected, hydrogen peroxide (50 μg/mL) promoted DHR 123 oxidation in HeLa cells, by approximately half of the cell population, that is, 46% of cells with oxidative damage, but did not induce an appreciable amount of dead cells, which suggests that the oxidative damage induced by this peroxide is not strong enough to kill the cells, at least in the present experimental conditions.

Since the present assay is a fast method of evaluating cell culture oxidative damage, taking place in about 30 min, Cas IIgly was administered at 40 μg/mL (100 μM), a concentration corresponding to a value of 10-fold its IC_{50} (Gracia-Mora et al., 2001), which is evaluated in 12-h studies. Even at this concentration, this drug did not induce an appreciable amount of cells with oxidative damage, since the number of green fluorescent cells was in the same order of magnitude as in the control set. This result suggests that the number of cells with this kind of damage, after treatment with Cas IIgly, corresponds to that of spontaneous damage that occurs without any treatment. After Cas IIgly's copper atom reduction in the presence of ascorbic acid, and thus promoting redox-caused damage, the number of cells with oxidative damage increased to a little extent, different from that of the untreated set, but significantly lesser than the oxidant effect induced by hydrogen peroxide (Table 1).

However, unlike hydrogen peroxide, Cas IIgly promoted cell death, as indicated by the high number of orange fluorescent cells, being 23.8% and 39.7% of dead cells when Cas IIgly was added in its oxidized and reduced states, respectively (see Table 1). These data suggest that Cas IIgly may cause cell death by a mechanism not related to cytosolic DHR 123 oxidation.

Copper nitrate showed an oxidant activity in both cell types, in its oxidized Cu(II) as well as in its reduced Cu(I) form. This inorganic copper salt, unlike the copper

Table 1
Effect of Cas IIgly on HeLa cells, expressed as percentage of cell survival and oxidative damage, compared to the positive controls of copper nitrate, and hydrogen peroxide

Treatment	Cell survival (%)	Dead cells (%)	Cells with oxidative damage (%)
Control	94.2 ± 3.02	4.92 ± 2.5	0.88 ± 1.02
Cas IIgly (40 μg/mL)	76.4 ± 0.5	22.7 ± 0.4	0.9 ± 1.8
$Cu(NO_3)_2$ (25 μg/mL)	71.6 ± 10.1	19.7 ± 3.3	8.7 ± 2.7
Asc (200 μg/mL)	88.4 ± 2.6	11 ± 2.1	0.6 ± 1.2
Cas IIgly + Asc	60.1 ± 7.2	37.6 ± 6.99	2.3 ± 0.26
$Cu(NO_3)_2$ + Asc	73.02 ± 1.6	10.98 ± 0.6	16 ± 1.3
H_2O_2 (50 μg/mL)	48.98 ± 5.8	5.02 ± 2.8	46 ± 4.9

Results are expressed as the mean ± SD from six independent experiments, 1000 cells counted on each.

chelate (Cas IIgly) was able to induce cytosolic DHR 123 oxidation (oxidative damage), and such effect increased when it was reduced in the presence of ascorbic acid (see Table 1). Ascorbic acid alone did not induce appreciable cell death or oxidative damage.

3.2. Inhibition of proliferation in HeLa cells

The assay of trichloroacetic acid fixation followed by staining the cells with sulforhodamine B, employed here to detect the inhibition of proliferation, indicates binding of this stain to the cell surface proteins in living cells (Skehan et al., 1990), so the higher the absorbance of the stained sample indicates the higher amount of protein-bound stain and, therefore, the greater number of living cells in the sample. That is why the absorbance of the non-treated cells must be taken as 100% of cell proliferation, and any decrease in absorbance due to the treatment of Cas IIgly or the other agents corresponds to a decrease in cell proliferation percentage or cytotoxicity rate.

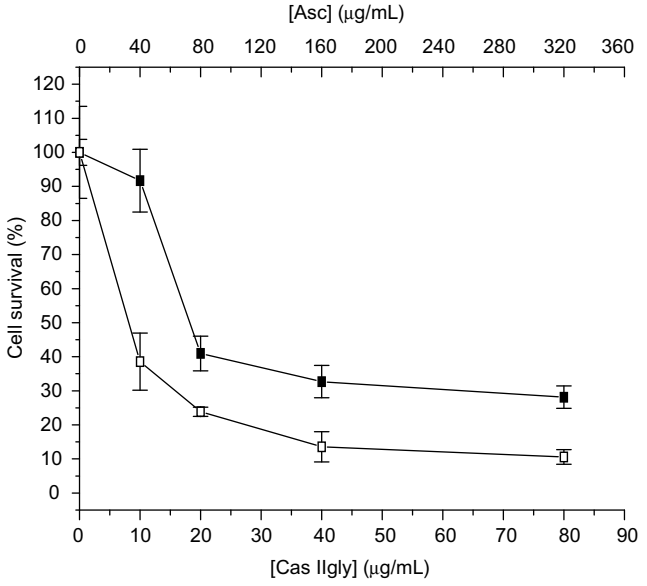

Fig. 2. Inhibition of proliferation in HeLa cells treated with increasing concentrations of Cas IIgly (from 10 to 80 μg/mL; 30 min interval of exposure); Cas IIgly oxidized (–■–) and reduced by ascorbic acid (–□–). (–▲–) Ascorbic acid alone. Results expressed as the mean of six independent experiments and their corresponding standard deviation.

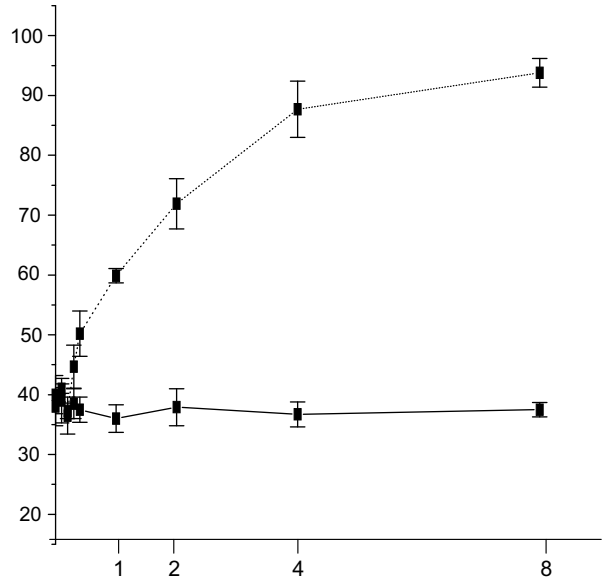

Fig. 3. DMSO, from 1 to 8 % (v/v), inhibited the cytotoxicity caused by Casiopeína IIgly. Solid line (–■–) represents the percentage of cell proliferation induced by 20 μg/mL Cas IIgly during 30 min; dotted line (--■--) shows the recovered proliferation rate after DMSO addition for another 30 min of incubation time.

Cas IIgly induced cytotoxicity on HeLa cells, as it interfered with the growth extent of this neoplastic cell line (Fig. 2). Such effect increased proportionally with Casiopeína concentration in the range of 10–80 μg/mL, and was enhanced when the drug was reduced prior to administering it to the cells, showing that cell death caused by this compound is augmented through this redox reaction, probably involving Cas IIgly's copper atom.

To support the finding that Cas IIgly may lead to cell death via an oxidation reaction, an antioxidant supplementation trial, basically consisting of the free radical scavenger DMSO, was carried out prior to the cytotoxicity test. DMSO was chosen as the exogenous antioxidant due to its ability to react with hydroxyl radicals and also due to the high solubility of Cas IIgly in it. Although DMSO is a cytotoxic agent, it was used at very small and non-lethal concentrations, from 1% to 8%. The data depicted in Fig. 3 shows that the inhibition of proliferation caused by oxidized Cas IIgly (20 μg/mL) is almost completely abolished as increasing concentrations of DMSO are added to the cells.

3.3. Depletion of reduced glutathione

As shown in Fig. 4 (and Table 2), Cas IIgly induced a depletion of endogenously produced GSH in HeLa cells,

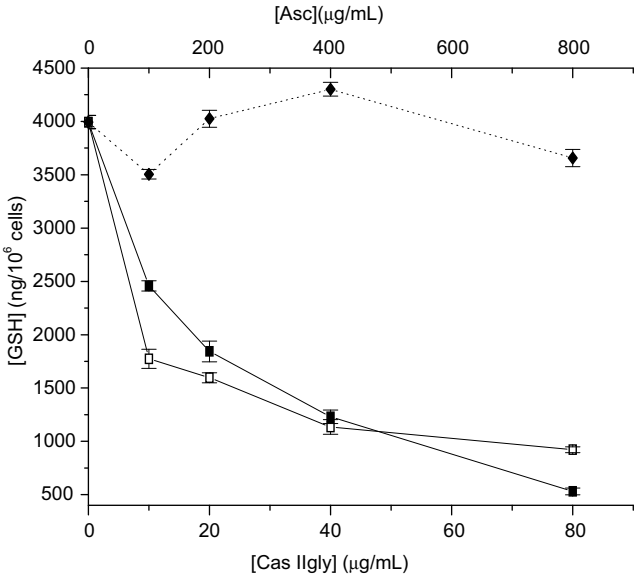

Fig. 4. Reduced glutathione (GSH) depletion by Cas IIgly. Oxidized (–■–) and reduced (–□–) Cas IIgly; ascorbic acid (--◆--). Data represent the mean ± SD from five independent experiments.

Table 2
Cas IIgly induced depletion of reduced glutathione (GSH) at the same rate, regardless of the oxidation state

[Cas IIgly] (μg/mL)	[Asc] (μg/mL)	[GSH] (ng/10^6 cells)
0	0	3994 ± 63
10	0	2458 ± 68
20	0	1843 ± 97
40	0	1230 ± 61
80	0	530 ± 34
0	160	3505 ± 45
0	320	4025 ± 79
0	640	4302 ± 64
0	1280	3656 ± 81
10	160	1774 ± 89
20	320	1596 ± 47
40	640	1135 ± 68
80	1280	921 ± 28
$Cu(NO_3)_2$ + Asc		1751 ± 43

Control consisting of copper nitrate/ascorbic acid. Exposure time to Cas IIgly, Asc and copper nitrate was 30 min.

from 3994 to only 534 nanograms of GSH per 1 x 10^6 cells, at the highest Casiopeína concentration (80 μg/mL). This effect was not enhanced with ascorbic acid-reduced Cas IIgly. This effect was not enhanced with Cas IIgly previously reduced with ascorbic acid.

4. Discussion

Dihydrorhodamine 123 (DHR 123) is a partially water-soluble non-fluorescent dye probe, which can be converted to its fluorescent form rhodamine 123 (R 123) through its oxidation in the presence of hydroxyl or superoxide radicals (Qin et al., 2005; Chang et al., 2005). As evaluated in several reports with confocal microscopy and flow cytometry, the oxidation of DHR 123 in cellular systems takes place mainly in the cytosol, and the distribution of the fluorescent resulting form (R 123) within the cell occurs in the mitochondria, given its liposolubility and cationic properties (Sakata et al., 1998; Wrona et al., 2005; Walrand et al., 2003); so that, the fluorescence of R 123 after DHR 123 oxidation is an adequate index of cellular oxidative damage.

To evaluate Cas IIgly's capacity to induce oxidative damage, this drug was administered to the cells in both oxidized (Cu(II)) and reduced (Cu(I)) forms, the latter in the presence of ascorbic acid, so that an oxidation–reduction reaction similar to the Fenton and Haber–Weiss system was developed by Cas IIgly. Under these conditions, Cas IIgly induced only spontaneous amounts of cells with R 123 fluorescence, and this sort of damage was scarcely promoted when the drug was reduced extracellularly. However, as stated above, Cas IIgly was able to induce the expenditure of endogenous reduced glutathione.

These results may be related to one of two different situations. First, Cas IIgly is able to provoke oxidative damage, but it is successfully abrogated by the cell's naturally occurring antioxidant defences, such as GSH, so its effect within the cell is not visible by means of DHR 123 oxidation, as in the present work. Second, the effect of this drug may be restricted to another cell compartment, such as the nucleus or mitochondria, which is probably of oxidative nature too, given the GSH depletion.

There is still inadequate information concerning Casiopeínas' redox chemistry and, consequently, it is not possible to be sure if these compounds are really able to promote the generation of hydroxyl or superoxide radicals *in vivo*. However, the assays carried out in this work have shown results which suggest that Cas IIgly induced cytotoxicity occurs by a mechanism related to reactive oxygen species generation. One of those results is that Cas IIgly strongly inhibited the proliferation of HeLa cells, and this effect was enhanced through Cas IIgly's copper atom reduction mediated by ascorbic acid. On the other hand, since the presence of an exogenous antioxidant such as DMSO, whose free radical scavenger property resides on its ability to react with the hydroxyl (·OH) radical, by producing carbon-centered organic radicals (Santos et al., 2003; Qian et al., 2005), contributed to prevent Cas IIgly induced cytotoxicity, we propose that this drug provoked cell death through a mechanism involving hydroxyl radical production. These results are in agreement with those obtained by Rivero-Müller et al. (2007), in which also ·OH generation is suggested since the reaction between Cas IIgly and DNA is oxygen-dependent, as well as the production of 8-oxodG in cells exposed to Cas IIgly suggests the generation of ROS as the major cause of cytotoxicity.

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