# Evaluation of the in vitro cytogenotoxic profile of the silver(I) metal complex with furosemide (Ag-FSE)

Avaliação do perfil citogenotóxico in vitro do complexo metálico de prata(I) com furosemida (Ag-FSE)

Evaluación del perfil citogenotóxico in vitro del complejo metálico de prata (I) con furosemida (Ag-FSE)

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**Abstract**
The aim of the present study was to evaluate the cytotoxic potential of a new complex of silver(I) with furosemide (Ag-FSE) over a panel of tumor and non-tumor human cells, as well as its genotoxicological safety. According to the results obtained, cell line derived from colorectal adenocarcinoma (Caco-2) was the most sensitive to the Ag-FSE complex, and among the non-tumor cells was the non-transforming fibroblasts known as GM07492A, which stimulated the characterization of cytotoxic properties against these cells. In addition to interfering with the cellular metabolic profile (evidenced by the resazurin method), Ag-FSE alters the integrity of cell membranes (results obtained by the Trypan blue assay) regardless of the cell line evaluated. However, interference with clonogenic capacity is dependent on the cell being tested; the cytotoxic activity evaluated by the clonogenic survival assay was evidenced against Caco-2 cells, but not against GM07492A cells. Furthermore, Ag-FSE did not induce an increase in the extent of DNA damage detectable by the comet assay, nor in the frequency of micronuclei compared to the negative control, demonstrating the absence of genotoxicity under the experimental conditions used. The data of the present study provide information on various biological aspects of the Ag-FSE complex and help in the generation of new drug candidates with potential to impact the health area, targeting cancer treatment.
**Keywords:** Genomic instability; Silver complex; Toxicogenetics; Cytotoxicity; Mutagenicity.

**Resumo**
O objetivo do presente estudo foi avaliar o potencial citotóxico de um novo complexo de prata(I) com furosemida (Ag-FSE) sobre um painel de células humanas tumorais e não tumorais, bem como sua segurança genotoxicológica. De acordo com os resultados obtidos, a linhagem celular derivada do adenocarcinoma colorretal (Caco-2) foi a mais

sensível ao complexo Ag-FSE, e dentre as células não tumorais foram fibroblastos não transformantes conhecidos como GM07492A, o que estimulou a caracterização das propriedades citotóxicas contra essas células. Além de interferir no perfil metabólico celular (evidenciado pelo método da resazurina), Ag-FSE altera a integridade das membranas celulares (resultados obtidos pelo ensaio do azul de Trypan) independentemente da linhagem celular avaliada. No entanto, a interferência na capacidade clonogênica depende da célula que está sendo testada; a atividade citotóxica avaliada pelo ensaio de sobrevivência clonogênica foi evidenciada contra células Caco-2, mas não contra células GM07492A. Além disso, Ag-FSE não induziu aumento na extensão do dano ao DNA detectável pelo ensaio Cometa, nem na frequência de micronúcleos em relação ao controle negativo, demonstrando ausência de genotoxicidade nas condições experimentais utilizadas. Os dados do presente estudo fornecem informações sobre diversos aspectos biológicos do complexo Ag-FSE e auxiliam na geração de novos candidatos a fármacos com potencial de impacto na área da saúde, visando o tratamento do câncer.

**Palavras-chave:** Instabilidade genômica; Complexo de prata; Toxicogenética; Citotoxicidade; Mutagenicidade.

**Resumen**
El objetivo del presente estudio fue evaluar el potencial citotóxico de un nuevo complejo de plata(I) con furosemida (Ag-FSE) en un panel de células tumorales y no tumorales humanas, así como su seguridad genotoxicológica. De acuerdo con los resultados obtenidos, la línea celular derivada del adenocarcinoma colorrectal (Caco-2) fue la más sensible al complejo Ag-FSE, y entre las células no tumorales se encontraban los fibroblastos no transformantes conocidos como GM07492A, lo que estimuló la caracterización. de las propiedades citotóxicas contra estas células. Además de interferir con el perfil metabólico celular (evidenciado por el método de resazurina), Ag-FSE altera la integridad de las membranas celulares (resultados obtenidos por el ensayo de azul de tripano) independientemente del linaje celular evaluado. Sin embargo, la interferencia con la capacidad clonogénica depende de la célula que se esté analizando; la actividad citotóxica evaluada por el ensayo de supervivencia clonogénica se evidenció frente a las células Caco-2, pero no frente a las células GM07492A. Además, Ag-FSE no indujo un aumento en la extensión del daño del ADN detectable por el ensayo Comet, ni en la frecuencia de micronúcleos en relación con el control negativo, lo que demuestra la ausencia de genotoxicidad en las condiciones experimentales utilizadas. Los datos del presente estudio brindan información sobre varios aspectos biológicos del complejo Ag-FSE y ayudan en la generación de nuevos candidatos a fármacos con potencial impacto en el área de la salud, con miras al tratamiento del cáncer.

**Palabras clave:** Inestabilidad genômica; Complejo de plata; Toxicogenética; Citotoxicidad; Mutagenicidad.

## 1. Introduction

Metal ions associated with suitable ligands originate metal complexes that can have different biological and/or pharmacological properties (Yan et al., 2017), due to the numerous characteristics of coordination geometry, oxidation state, redox, kinetic and thermodynamic state of an inorganic molecule (Thompson & Orvig, 2006; Cohen, 2007).

Scientific interest in this line of research intensified due to the accidental discovery of cisplatin around 1960; one of the first metallic drugs, completely inorganic, with chemotherapy activity used until today in the therapy of testicular, ovarian, cervical, bladder and lung cancer (Cirri et al., 2019; Ghosh, 2019), which despite the benefits, it has several undesirable effects and therefore serves as an inspiration for the search for new compounds for the treatment of cancer (Cohen, 2007; Cirri et al., 2019; Ghosh, 2019).

As a result, it promoted the development of several metal-based drugs, essential and non-essential, with the potential to be used in imaging diagnosis, in addition to having different pharmacological activities (Barry & Sadler, 2013; Mjos & Orvig, 2014; Mahapatra et al., 2019).

The antibacterial potential is of particular interest and has been widely explored due to bacterial multi-resistance acquired by the excessive use of antibiotics (Mosconi et al., 2014).

According to Lustri et al. (2017), the complex of silver with furosemide (Ag-FSE) has shown a significant in vitro antibacterial activity against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and yeast *Candida albicans* (ATCC 90028), and the absence of mutagenic activity by Ames assay.

Silver complexes have the ability to interfere with bacterial metabolism, break the cell wall and bind to its DNA, preventing bacterial replication and the possibility of resistance development (Rocha et al., 2011).

Currently, with new technologies, it has been possible to adopt other forms of this metal, such as refined silver salts, colloidal silver and nanoparticles, further expanding its application, making it present in food packaging coatings, fabrics, dressings, ointments, purifiers. air and water (Möhler et al., 2018). Studies also show that silver has antiparasitic, antifungal and antitumor activity, which compared to cisplatin has less toxicity and more cytotoxicity depending on the ligand (Medici et al., 2015; Ciol et al., 2018; Liang et al., 2018; Rocha et al., 2019; Esteban-Parra et al., 2019; Kizrak et al., 2019; Favarin et al., 2019).

Therefore, in the perspective of the discovery of new drugs, in this study we explore the in vitro antitumor activity of Ag-FSE, as well as its genotoxic potential by the Comet and micronucleus assays.

## 2. Methodology

This is an experimental research, carried out in a laboratory, with a quantitative approach (Pereira et al., 2018) that aimed to evaluate the cytotoxic potential of a new complex of silver(I) with furosemide (Ag-FSE), through assays that assess cell viability (resazurin redox assay, trypan blue exclusion test and clonogenic survival assay) in a panel of tumor and non-tumor human cells and its genotoxicological profile by the comet and micronucleus assays.

The procedures for synthesis and characterization of Ag-FSE (Figure 1) are described in Lustri et al. (2017).

**Figure 1 -** Proposed structure of the Ag-FSE complex based on theoretical calculations.
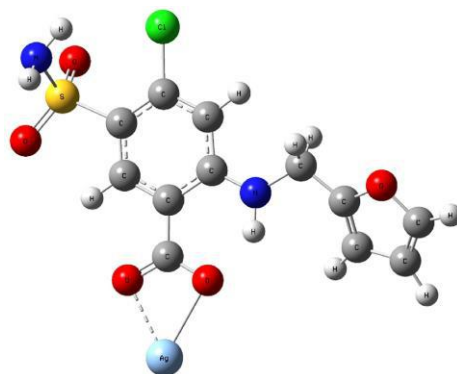


Source: Authors.

### 2.1. Cytotoxicity

#### 2.1.1 Resazurin redox assay

Cell lines evaluated by resazurin redox assay were non-transforming fibroblastos (GM07492A), human lung fibroblasts (MRC-5, ATCC® CCl-171™), human umbilical vein endothelial cells (HUVEC, ATCC - CRL-1730™), human colorectal adenocarcinoma (Caco-2, Rio de Janeiro Cell Bank no. 0059), human hepatocellular carcinoma (HepG2, ATCC-HB-8065™), lung adenocarcinoma cells (A549, ATCC-CCL-185™), human cervical adenocarcinoma (HeLa, ATCC® CCl-2™) and mammary gland adenocarcinoma (MCF-7, ATCC® HTM-22™). The cells were maintained as monolayer in plastic culture flasks in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented according to the needs of each lineage with 10% or 20% fetal bovine serum (FBS, Nutricell), at 37 °C in an atmosphere of 5% $CO_2$ and 95% air under saturating humidity.

In 96-well microplates, the cells ($1.5 \times 10^4$ cells/well) were exposed to different concentrations of Ag-FSE, silver nitrate ($AgNO_3$, metallic salt) and furosemide (FSE, ligand) that ranged from 2.2 to 285 µmol·L$^{-1}$. In addition, methyl methanesulfonate (MMS, 150 µmol·L$^{-1}$) was used as positive control (cytotoxic drug) and dimethyl sulfoxide (DMSO 0.1% (v/v)) as vehicle control.

After 24 h treatment, 0.01% w/v of resazurin solution (50 µL) was added to each well and the plates were incubated for 2h. The reading was performed in a microplate Synergy H1 (BioTek®) reader using excitation and emission filters at wavelengths of 560 and 590 nm, respectively. The assay was performed in three individual experiments in triplicate.

### 2.1.2 Trypan blue exclusion test

The protocol was performed in 6-well plates. Caco-2 and GM 07492A cells ($1 \times 10^5$ cells/well) were exposed to different concentrations of Ag-FSE, silver nitrate ($AgNO_3$, metallic salt) and furosemide (FSE, ligand) that ranged from 1.4 a 22.8 µmol·L$^{-1}$. Experimental controls constituted of negative (without any treatment), vehicle (DMSO 0.1% (v/v)) and positive (MMS, 50 µmol·L$^{-1}$) controls were also included in the treatment. After 24 h treatment, 10 µL of the cell suspension was homogenized with trypan blue dye (0.2%), and then quantified on TC20 Automated Cell Counter (Bio-RAD). The experiments were performed in triplicate.

### 2.1.3 Clonogenic survival assay

The colony-forming assay in Caco-2 and GM 07492A cells was conducted under the same experimental conditions as the trypan blue assay. After 24 h treatment, 200 cells were seeded per well in 6-well plates. The experiments were carried out for 7 days. At the end of the growth period, the culture medium was removed and cells were washed with phosphate-buffered saline (PBS, pH 7.4), fixed in methanol/acetic acid/distilled water (1:1:8) for 30 min, and stained with 3% Giemsa for 30 min. After staining, the colonies were washed with distilled water, and then counted. The experiments were performed in triplicate.

### 2.1.4 Cytotoxicity (IC$_{50}$)

The inhibitory concentration to 50% (IC$_{50}$), which represents the required concentration of the test sample capable of inhibiting 50% of cell growth, was calculated by plotting the viability or survival of the cells in relation to the respective concentrations of the test substances by the GraphPad Prism 9.0 program (GraphPad Software, San Diego, CA, USA). This parameter was calculated for all cytotoxicity assays.

## 2.2 Genotoxicity

### 2.2.1 Comet assay

In this assay, also in 6-well plates, HepG2 cells ($1 \times 10^5$ cells/well) were exposed to non-cytotoxic concentrations of Ag-FSE (0.7, 1.4 and 2.8 µmol·L$^{-1}$), cell viability greater than 70% assessed by trypan blue exclusion assay, in addition to untreated, vehicle (DMSO 0.0025%) and positive (hydrogen peroxide, 50 µmol·L$^{-1}$) controls.

After 24 h of treatment, the mixture of 20 µL of cell suspension of each treatment group and 120 µL of 0.5% low melting point agarose (dissolved in PBS) was added to the microscope slides coated with 1.5% normal-melting agarose.

The microscope slides were immersed in a lysis buffer with 1% Triton X for 18 h and then placed in an electrophoretic tank and dipped into a cool electrophoresis solution (NaOH 300 mmol·L$^{-1}$ and EDTA 1 mmol·L$^{-1}$, pH 13) for 20 min. The settings of electrophoresis were 25 V and 350 mA for 20 min. The slides were then submerged in a neutralization buffer (0.4 mol·L$^{-1}$ Tris–HCl, pH 7.5) for 15 min, dried at room temperature, and fixed in 100% ethanol for 5 min.

The samples were then stained by GelRed™ (Biotium Inc., Fremont, CA, EUA) (GelRed/PBS; 1:10.000 µL v/v) and measured on a fluorescence microscope (Carl Zeiss, Axiostar Plus; Jena, Germany) with an excitation filter of 515–560 nm and a suppression filter of 590 nm.

The percentage of DNA in the tail was established visually. A total of 300 cells (in three independent assays) were examined per treatment and the total score was calculated by multiplying the number of damaged cells by the value of the respective comet class (0, 1, 2 or 3) and then taking the sum for each treatment.

### 2.2.2 Cytokinesis-block micronucleus assay

For genotoxicity assessment by micronucleus (MN) assay, in 6-well plates, $1 \times 10^5$ cells/well were exposed to four concentrations of Ag-FSE (1.4, 2.8, 5.7 and 11.4 $\mu mol \cdot L^{-1}$) for 3 hr in Chinese hamster ovary (CHO) cells (OECD, 2016) and 24 h in HepG2 cells, in addition to untreated, vehicle (DMSO 0.0025%) and positive (hydrogen peroxide, 100 $\mu mol \cdot L^{-1}$, for CHO and aflatoxin $B_1$, 5 $\mu mol \cdot L^{-1}$, for HepG2) controls.

After the incubation period, 6.25 $\mu mol \cdot L^{-1}$ of pre-prepared cytochalasin B (Sigma-Aldrich) solution in culture medium were added for blocking cytokinesis, and the plates were placed in the $CO_2$ incubator for 1.5 normal cell cycle lengths.

At harvest time, the cells were rinsed with PBS, trypsinized, and the pellet was hypotonized in 1% sodium citrate at 37°C and homogenized. After a new centrifuge, the pellet was resuspended in methanol:acetic acid (3:1) and again homogenized. Fixed cells were then transferred to slides, stained with 3% Giemsa, and analyzed under a light microscope.

Interphase cells were harvested and stained to analyze the presence of MN. The criteria established by Fenech (2007) were employed for the analysis of MN and binucleated cells. A total of 3000 binucleated cells were scored per treatment, corresponding to 1000 cells/treatment/repetition.

In addition, 500 viable cells per repetition between mononucleated, binucleated and multinucleated (three or more nuclei) were counted to calculate the Cytokinesis-Block Proliferation Index (CBPI), which allows the assessment of cell toxicity or delay in the cell cycle by determination of cell proliferation in cultures, according to the formula (OECD, 2016):

$$CBPI = \frac{(\text{No. mononucleate cells}) + (2 \times \text{No. binucleate cells}) + (3 \times \text{No. multinucleate cells})}{(\text{Total number of cells})}$$

The percentage of inhibition was calculated according to the formula:

$$\% \text{ inhibition of cell proliferation} = 100 - 100\{(CBPI_T - 1)/(CBPI_C - 1)\}$$

where, $CBPI_T$ represents the CBPI of the cells that received different concentrations of the Ag-FSE treatment and $CBPI_C$ represents the CBPI of the negative control cells.

### 2.3 Statistical analysis

The data were analyzed statistically by the Graph Pad Prism 9.0 software (Graph-Pad Software Inc., San Diego, CA, USA) using analysis of variance one way ANOVA to determine the statistical significance, followed by the Tukey test considering $p < 0.05$. The tests were performed in triplicate and the experimental criterion was the significance of the response to treatment in relation to the negative control.

## 3 Results and Discussion

In order to investigate new molecules in the search for potential therapeutic agents, the synthesis, spectroscopic characterization, in addition to the antimicrobial and mutagenic activities of the Ag-FSE complex were evaluated and the results described by Lustri et al. (2017).

Due to the promising antimicrobial potential found, and the absence of mutagenic effect by the Ames test (Lustri et al., 2017), the present study focuses on evaluating cytotoxicity over a panel of tumoral and non tumoral human cells, and mutagenicity by the micronucleus and comet assay, in order to complement the data already obtained from this complex and provide reliable data to support future clinical research.

The effectiveness of silver complexes against bacteria and cancer cells depends on several factors: lipophilicity, redox propensity, solubility and stability in water, and rate of release of silver ions. These factors are strictly controlled by the characteristics of the ligands and their requirements on steric and electronic properties (Medici et al., 2019).

FSE is a sulfonylamide that belongs to the loop diuretics group. These compounds produce an increase in sodium excretion and urinary flow of about 30 times when compared to normal excretion transporters, blocking Na+, K+ and Cl in the ascending limb of the loop of Henle. They are drugs used clinically mainly in the treatment of kidney diseases, liver cirrhosis, hypertension and edema (Barroso et al., 1996; Malode et al., 2011).

The FSE ligand was not cytotoxic under the conditions of this study, demonstrating that the cytotoxicity profile of the complex is related to the metallic salt, $AgNO_3$, which can be considered a source of free Ag(I) ions.

Tables 1 and 2 presents the inhibitory concentration to 50% ($IC_{50}$) values observed through the evaluation of the cytotoxic activity of Ag-FSE and $AgNO_3$ against the tumor and non-tumor cells obtained by resazurin redox assay, trypan blue exclusion and clonogenic survival assay.

The results showed varying degrees of sensitivity of the cell lines after treatment with the test substances, being that Caco-2 was the tumor cell most sensitive to the inhibitory activity of the Ag-FSE complex. Among the non-tumor cell lines, the GM 07492A cells were the most sensitive to this complex, which encouraged us to further studies with these two cells to investigate the mechanisms that induce cytotoxicity.

Ag-FSE induced cytotoxicity in GM 07492A cells by the resazurin assay, with $IC_{50}$ close to that of the the metallic salt ($IC_{50}$ of $19.2 \pm 1.1$ µmol·$L^{-1}$ for Ag-FSE and $18.8 \pm 1.2$ µmol·$L^{-1}$ for $AgNO_3$) (Table 1). Caco-2 cells were more sensitive to treatment with the complex, which proved to be 15X more cytotoxic than $AgNO_3$. These results show that the coordination of Ag ions by biologically relevant ligands can potentiate the inhibition of Caco-2 cells.

**Table 1.** Inhibitory concentration to 50% ($IC_{50}$, µmol·$L^{-1}$) values of the Ag-FSE complex and metallic salt (Ag-$NO_3$), in the tumor and non tumor cells obtained by resazurin redox assay.

| | $IC_{50}$ (µmol·$L^{-1}$ ± SD) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Treatments** | **GM 07492A** | **MRC-5** | **HU-VE-C** | **HepG2** | **A-549** | **Caco-2** | **HeLa** | **MCF-7** |
| **$AgNO_3$** | $18.8 \pm 1.2$ | $39.4 \pm 2.0$ | $12.9 \pm 0.9$ | $71.23 \pm 12.0$ | $20.0 \pm 2.4$ | $101.8 \pm 3.5$ | $38.8 \pm 4.2$ | $35.2 \pm 7.0$ |
| **Ag-FSE** | $19.2 \pm 1.1$ | $108.7 \pm 6.5$ | $25.7 \pm 0.8$ | $9.2 \pm 0.5$ | $128.7 \pm 14.0$ | $6.6 \pm 0.5$ | $26.2 \pm 0.4$ | $29.2 \pm 0.03$ |

$IC_{50}$ values were determined from three independent experiments using concentration (µmol·$L^{-1}$) in triplicate (mean ± standard deviation, SD). The ligand (furosemide) did not show cytotoxic activity, under the conditions used in this study ($IC_{50}$ >3023 µmol·$l^{-1}$)

By trypan blue method, we observed that the starting salt and the complex induce changes in cell membrane integrity at the highest concentrations evaluated; the cytotoxic activity of Ag-FSE and $AgNO_3$ was evidenced in both cells. The complex was approximately 3X more cytotoxic than metallic salt against GM 07492A cells ($IC_{50}$ of $9.3 \pm 1.1$ µmol·$L^{-1}$ for Ag-FSE and

27.2 ± 3.0 μmol·L$^{-1}$ for AgNO$_3$) and 1.6X against Caco- 2 (IC$_{50}$ of 4.5 ± 1.1 μmol·L$^{-1}$ for Ag-FSE and 7.3 ± 0.5 μmol·L$^{-1}$ for AgNO$_3$) (Table 2).

The clonogenic survival assay was performed under the same experimental conditions as the trypan blue assay. Ag-FSE and AgNO$_3$ induced similar effects; both did not interfere in the capacity of GM 07492A cells to divide and proliferate, and thus form colonies, at all concentrations evaluated, while in Caco-2 cells, IC$_{50}$ values were 5.4 ± 0.5 μmol·L$^{-1}$ for Ag-FSE and 5.1 ± 1.2 μmol·L$^{-1}$ for AgNO$_3$ (Table 2).

The results of these assays suggest that Ag-FSE and AgNO$_3$ interfere with the cellular metabolic profile (evidenced by the resazurin method) and alter the membrane integrity (results obtained by the trypan blue assay) regardless of the cell lineage evaluated. However, interference in clonogenic capacity is dependent on the cell being tested.

Furthermore, this study also showed the greater sensitivity of Caco-2 cells (tumor cell line) compared to the non-tumor cell line GM 07492A to different treatments, which corroborates other studies in the literature that show a special sensitization of Caco-2 cells by silver nanoparticles and silver ions (Iqbal et al., 2013; Oberemma et al., 2016; Zande et al., 2016; Vila et al., 2017).

Ciol et al. (2018) described the synthesis, characterization and biological activities of the complex of Ag(I) with cycloserine (AgCIC). Among the results obtained, the authors showed that the complex was able to inhibit the growth of all tumor cells tested, being particularly more active in vitro against breast cancer cells and leukemia cells than doxorubicin, in addition to its remarkable selectivity in relation to cancer cells when compared to the non-tumor cell line, suggesting less toxicity than commercial chemotherapeutics. Manzano et al. (2020) synthesized and characterized three Ag(I) complexes with fluoroanthranilic acid (fa) isomers, which were named Ag4fa, Ag5fa and Ag6fa, in addition to analyzing their antibacterial, antiproliferative and mutagenic activities. Cytotoxic activity assays revealed that the Ag4fa complex was more selective against Caco-2 cells when compared to non-tumor cell lines (GM 07492A, MRC-5 and HUVEC). Aquaroni et al. (2020) carried out a study demonstrating the antibacterial and antiproliferative activity of the complex of Ag with 4-aminobenzoic acid (Ag-pABA). The complex exhibited antiproliferative activity in a panel of eight types of tumor cell lines and no mutagenic activity. By analysis of the selectivity index, the concentration of Ag-pABA needed to inhibit the growth of leukemic cells (K562) was 6X lower than that needed to inhibit non-tumor human keratinocytes. As can be seen, several studies show promising results considering the search for new bioactive Ag compounds for the treatment of cancer.

**Table 2.** Inhibitory concentration to 50% (IC$_{50}$, μmol·L$^{-1}$) values of the Ag-FSE complex and metallic salt (AgNO$_3$), in the Caco-2 and GM 07492A cells obtained by the trypan blue exclusion and clonogenic survival assays

| | IC$_{50}$ (μmol·L$^{-1}$ ± SD) | | | |
| --- | --- | --- | --- | --- |
| **Treatments** | **Trypan blue** | | **Clonogenic survival** | |
| | **Caco-2** | **GM 07492A** | **Caco-2** | **GM 07492A** |
| **AgNO$_3$** | 7.3 ± 0.5 | 27.2 ± 3.0 | 5.1 ± 1.2 | >58.9 |
| **Ag-FSE** | 4.5 ± 1.1 | 9.3 ± 1.1 | 5.4 ± 0.5 | >22.8 |

IC$_{50}$ values were determined from three independent experiments using concentration (μmol·L$^{-1}$) in triplicate (mean ± standard deviation, SD). The ligand (furosemide) did not show cytotoxic activity, under the conditions used in this study (IC$_{50}$ >30.2 μmol·l$^{-1}$)

Regarding genotoxicity, the comet assay detects DNA damage before the repair mechanism. The basic principle of this test is the migration of DNA in an agarose matrix under electrophoretic conditions. When observed under a microscope,

the cells have the appearance of a comet, with a head (the nuclear region) and a tail containing the DNA fragments that migrate towards the positive pole (Lu et al., 2017; Singh et al., 1988; Tice et al., 2000). The MN test, which assesses the induction of chromosomal mutations, is currently one of the best-established cytogenetic tests in the field of toxicological genetics, with international validation and applicable to any nucleated cell population (Fenech, 2007; Kirsch-Volders et al., 2014).

Table 3 details the data obtained by the comet assay, where it is observed that Ag-FSE, at concentrations tested for a period of 24 h in HepG2 cells, does not present genotoxic potential, as it did not induce a statistically significant difference in DNA damage when compared to the negative control, under the conditions used in this study.

In the different treatments, most of the cells analyzed are in classes 0 and 1 and the calculated score values ranged from 25.7 to 34.2 for the groups treated with Ag-FSE and $19.7 \pm 3.5$ for the negative control, while in the positive control this value was $162 \pm 8.6$.

Ag-FSE also did not induce a statistically significant increase in the frequency of MNs compared to the negative control, regardless of treatment time (3 or 24 hours) and cell used (CHO or HepG2), showing that Ag-FSE or possible metabolites are not mutagenic (Table 4).

About cytostasis, another parameter evaluated in this study, there was a statistically significant reduction in CBPI values only at the highest concentration evaluated, which made the counting of MNs unfeasible. In CHO, treatment with 11.4 $\mu mol \cdot L^{-1}$ of FSE-Ag caused 63.2% inhibition of cell proliferation (CBPI: $1.32 \pm 0.10$) and in HepG2 86.6% inhibition ($1.14 \pm 0.06$) (Table 4). These data corroborate the toxicity profile of Ag-FSE and helped in choosing the doses for the experiments.

**Table 3.** DNA migration in the comet assay observed for HepG2 cultures treated for 24 h with different Ag-FSE concentrations.

| Treatments | Classes | | | | Score |
|---|---|---|---|---|---|
| | **0** | **1** | **2** | **3** | |
| **NC** | $81.0 \pm 6.7$ | $18.3 \pm 4.8$ | $0.7 \pm 0.5$ | 0 | $19.7 \pm 3.5$ |
| **SC** | $86.5 \pm 7.8$ | $9.7 \pm 4.0$ | $2.5 \pm 0.5$ | $1.3 \pm 0.5$ | $18.6 \pm 1.2$ |
| **PC** | $19.5 \pm 1.1*$ | $23.5 \pm 4.0$ | $32.5 \pm 7.8*$ | $24.5 \pm 2.6*$ | $162 \pm 8.6*$ |
| **Ag-FSE** | | | | | |
| **0.7 $\mu mol \cdot L^{-1}$** | $78.3 \pm 6.5$ | $17.7 \pm 3.2$ | $4.0 \pm 1.7$ | 0 | $25.7 \pm 8.6$ |
| **1.4 $\mu mol \cdot L^{-1}$** | $72.7 \pm 2.1$ | $21.7 \pm 4.0$ | $4.3 \pm 1.0$ | $1.3 \pm 0.5$ | $34.2 \pm 5.4$ |
| **2.8 $\mu mol \cdot L^{-1}$** | $69.0 \pm 7.5$ | $29.0 \pm 1.7$ | $2.0 \pm 0.5$ | 0 | $33.0 \pm 2.3$ |

Values are presented as mean ± SD, based on three independent experiments. A total of 300 cells were examined per treatment group and the total score was calculated by multiplying the number of cells scored by the respective comet class value (0, 1, 2 or 3) and then calculating the sum of each treatment. NC: negative control (complete culture medium), SC: solvent control (DMSO, 0.0025%), PC: positive control (hydrogen peroxide, 50 $\mu mol \cdot L^{-1}$).
* $p < 0.05$ statistically different from the negative control.

**Table 4.** Frequency of micronuclei (MNs) and cytokinesis-block proliferation index (CBPI) in CHO cells after 3 h of treatment and HepG2 after 24 h of treatment with the Ag-FSE complex.

| Treatments | CHO | | HepG2 | |
|---|---|---|---|---|
| | MNs[a] | CBPI[b] | MNs[a] | CBPI[b] |
| NC | $3.0 \pm 1.0$ | $1.87 \pm 0.05$ | $5.6 \pm 4.2$ | $2.04 \pm 0.01$ |
| SC | $4.3 \pm 1.5$ | $1.86 \pm 0.18$ | $6.1 \pm 2.4$ | $1.99 \pm 0.06$ |
| PC | $38 \pm 6.0$* | $1.90 \pm 0.04$ | $35 \pm 15.2$* | $2.00 \pm 0.03$ |
| Ag-FSE | | | | |
| 1.4 $\mu$mol·L$^{-1}$ | $2.7 \pm 0.6$ | $1.87 \pm 0.11$ | $7.7 \pm 3.3$ | $1.97 \pm 0.05$ |
| 2.8 $\mu$mol·L$^{-1}$ | $4.7 \pm 1.5$ | $1.81 \pm 0.14$ | $5.7 \pm 2.0$ | $1.92 \pm 0.03$ |
| 5.7 $\mu$mol·L$^{-1}$ | $3.0 \pm 0.6$ | $1.86 \pm 0.13$ | $8.2 \pm 2.6$ | $1.84 \pm 0.11$ |
| 11.4 $\mu$mol·L$^{-1}$ | - | $1.32 \pm 0.10$* | - | $1.14 \pm 0.06$* |

Values are presented as mean $\pm$ SD of the number of micronuclei (MNs) and cytokinesis-block proliferation index (CBPI) in CHO cells (3 h of treatment) and HepG2 (24 h of treatment). NC: negative control (complete culture medium), SC: solvent control (DMSO, 0.0025%), PC: positive control (hydrogen peroxide, 100 $\mu$mol·L$^{-1}$, in the experiments with CHO and aflatoxin B$_1$, 5 $\mu$mol·L$^{-1}$, in the experiments with HepG2).
[a]A total of 3000 binucleated cells were analyzed per treatment group.
[b]A total of 1500 cells were analyzed per treatment group.
* $p < 0.05$ statistically different from the negative control.

Furthermore, previously evaluated by our group, Ag-FSE was also not able to induce gene mutations in the Ames test (Lustri et al., 2017).

The systematic discovery and development of Ag complexes have produced a large number of promising antibacterial, antifungal and anticancer compounds (Liang et al., 2018). Despite the versatility and promising activities of metal complexes, studies investigating the safety profile of these complexes are essential, as characteristics such as mutagenicity, carcinogenicity and toxicity may limit their use in therapy, since, for example, mutagenicity can lead to the development of secondary tumors (Pracharova et al., 2018).

Given this context, the absence of genotoxicity of Ag-FSE is highly relevant, as it is extremely important to have a balance between the therapeutic and toxicological effects of a pharmacological compound.

Milionis et al. (2018) evaluated the genotoxicity by the MN test of a new Ag compound (I) obtained by the reaction of AgNO$_3$ with the antibiotic ciprofloxacin by the MN test, in addition to its antibacterial potential. The results showed low genotoxicity in cultures of normal human corneal epithelial cells (HCET).

Plotnikov et al. (2016) did not detect DNA damage by comet assay and chromosomal aberrations in blood cells of an Ag-based compound of general formula $C_6H_{19}Ag_2N_4LiO_6S_2$, even at the highest concentrations.

Overall, the literature shows that genotoxicity data for Ag+ are limited. Ag+ is proven to be cytotoxic but did not induce genotoxicity in several different assays (Eliopoulos & Mourelatos, 1998; Demir et al., 2011). Ag iodide, for example, was not mutagenic in the Ames test and in the sister chromatid exchange assay (Eliopoulo & Mourelatos, 1998). AgNO$_3$ did not induce mutations, while Ag nanoparticles were positive in the *Drosophila* wing cell somatic mutation assay (Demir et al., 2011). Thus, in vitro toxicity studies with Ag exposed to cell lines or by other methodologies are useful to confirm its actual mutagenic effects or carcinogenic alterations.

## 4. Conclusion

In conclusion, this study allowed us to explore various biological aspects of the Ag-FSE complex. The data from the present study, in addition to contributing to the search for new metal complexes with promising biological activities, provide an understanding of their interaction with genetic material. We demonstrate that the cytotoxicity of Ag-FSE is not related to genomic instability with direct damage or its metabolites to DNA, as Ag-FSE was not genotoxic when evaluated by the comet and MN assays. Further studies are needed to confirm this hypothesis, and to clarify their mechanisms of action, besides many more biological and physicalchemical studies to develop it into new antitumor drug or with other pharmacological application.

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