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Silver(I) tenoxicam complex: In vitro valuation of the antiproliferative activity over B16 melanoma cell line using electrochemotherapy

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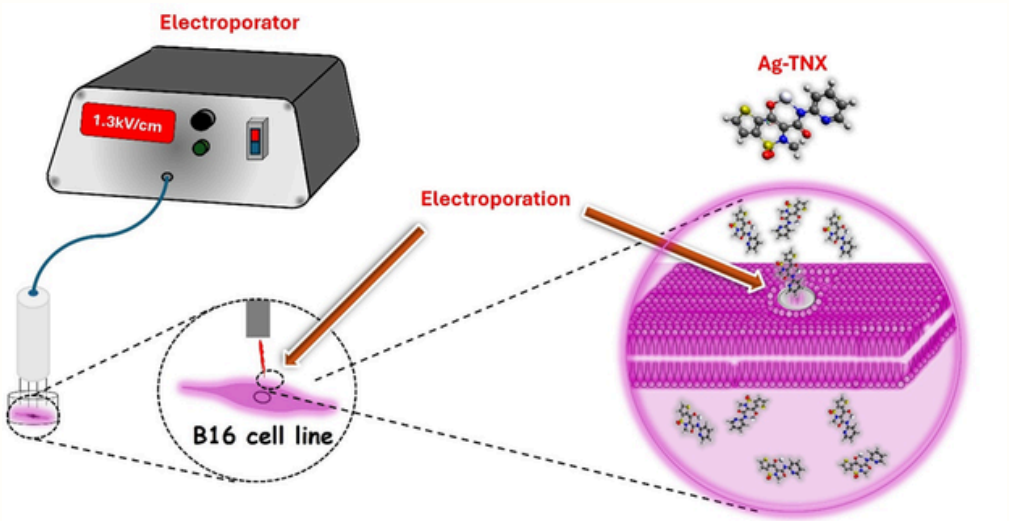

Abstract: Cancer is one of the leading causes of death, and melanoma is one of the deadliest types of skin cancers. However, if diagnosed and treated early, it is potentially curable. Electrochemotherapy is a therapeutic technique for local treatment against various types of neoplasia. It uses a combination of chemotherapeutic drugs and electroporation, which allows molecules that are generally not permeable to the plasma membrane to enter cells via electrical pulses. The objective of this work is the synthesis and characterization of a silver metal complex, using tenoxicam (Ag-TNX) as a ligand, and to evaluate its antiproliferative effects on melanoma tumor cells *in vitro*, replacing conventional chemotherapy drugs used in electrochemotherapy. Tests were carried out to characterize the complex, including FTIR, thermogravimetric analysis, and elemental analysis, as well as cytotoxicity tests. *In vitro* electroporation associated with the Ag-TNX complex was applied, and the absorption of the agent was determined by using a Franz cell. The Ag-TNX agent showed antibacterial activity over *S. aureus* ATCC 25924, *E. coli* EC 958, *E. coli* BR43, and *P. aeruginosa* ATCC 27853 and a superior antiproliferative effect when the electroporation was applied compared to the isolated Ag-TNX, demonstrating promising results for future expansion of the therapeutic arsenal of antineoplastic and antibacterial agents.

Keywords: Tenoxicam; Silver complex; Antitumoral activity; Antibacterial activity Electroporation; Electrochemotherapy

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Introduction

Melanoma skin cancer, one of the most lethal skin cancers in humans[1], is a neoplasm that develops from melanocytes and can progress to metastatic stage[2], a potentially curable condition if diagnosis and treatment occur early, is caused, usually, by periodic ultraviolet light esposure[1], considerate 17th in global prevalence and 22th in terms of mortality, based on Global Cancer Statistics in 2022[3].

Apart from being a pathology of significant severity, skin cancers are prone to bacterial infections[4], which makes the simultaneous treatment of skin cancer and bacterial infection a great challenge, apart from the increased risk of disseminated infections[5].

Currently, the most widely used treatment strategies for skin cancer include surgical excision, chemotherapy, external and internal radiotherapy, immunotherapy, photodynamic therapy and cryotherapy. However, conventional approaches to the treatment of skin cancer have therapeutic limitations due to systemic adverse effects, drug resistance and subtherapeutic concentrations of antitumor drugs at the tumor site[6].

Compared to systemic administration, topical administration of pharmaceuticals offers several benefits, including targeted delivery, regulated active agent release, ease of application, and fewer systemic side effects[7].

Electroporation is a minimally invasive drug delivery technique that allows the entry of molecules, which are generally non-permeable, into cells through the plasma membrane by applying electrical pulses through microneedles[8], promoting a transient change in membrane stability[9]. The first experiments using *in vitro* electroporation date back to the 80s and 90s[10].

In a study in which electroporation was applied to mammalian cells, cells in suspension were electroporated using 10 pulses of 100 µs duration and it was observed that cellular permeabilization of the dye (triptan blue) occurred in field intensities between 0.7 kV/cm to close to 1.5 kV/cm[11].

The use of the electroporation method associated with chemotherapy is called electrochemotherapy (EQT) and is a local treatment modality against cancer[12]. EQT consists of allowing substances with low or no ability to cross the plasma membrane through electroporation and retaining the chemotherapy at its target site[13,14].

A variety of drugs were tested in order to observe the potentiation of these agents with the use of EQT. However, bleomycin and cisplatin were the two antineoplastics that showed a substantial increase with the use of this technique[15].

In the last two decades, the applicability of EQT for the treatment of solid and cutaneous tumors has rapidly become popular among veterinary oncologists[16]. It is a therapeutic modality that has been widely carried out in dog and cat oncology treatment centers[17,18,19].

This technique is widely used to treat primary melanomas in dogs and cats, achieving complete remission in stage I melanomas[20]. In another study, a complete remission rate of 41.4% was observed in twelve dogs that received EQT as local therapy for oral melanoma at varying stages of the disease[21].

In addition to its wide use in veterinary medicine, EQT has also been applied in human medicine. This technique is mainly aimed at primary and metastatic cutaneous and subcutaneous tumors of the head and neck, such as basal cell carcinoma and melanomas[22,23,24,25,26].

Following the discovery and clinical use of cisplatin for the treatment of cancer, a number of studies have focused on the development of antitumor metallodrugs. These include carboplatin and other metal complexes that can produce reactive oxygen species through redox reactions to mediate their anti-cancer properties, such as arsenic trioxide, copper complexes, gold compounds, and platinum derivatives. Some of these also have antiangiogenic properties, inhibiting the production of vascular endothelial growth factor (VEGF) or targeting particular signaling pathways related to angiogenesis[27].

Silver complexes, especially silver sulfadiazine, are already used in bacterial infections in humans and animals. It is a compound capable of treating bacterial infections, burns, injuries and preventing infectious diseases[28]. Furthermore, it is known that silver has anti-inflammatory properties[29,-31].

The antineoplastic action of silver is based on different mechanisms when compared to platinum derivatives in terms of its interaction with DNA. In silver complexes, the interaction is preferentially directed towards nitrogenous bases rather than phosphate groups[32,33].

The results obtained by Aquaroni et al.[34] demonstrated a promising *in vitro* antiproliferative activity of the silver(I) with the ligand 4-aminobenzoic acid on nine tumor cell lines, including the multidrug resistant lineage NCIADR/RES (multidrug resistant ovary).

Another study by Candido et al.[35] demonstrated that the silver(I) complex with the non-steroidal anti-inflammatory nimesulide showed *in vivo* antiproliferative activity using a skin squamous cell carcinoma model induced in mice.

The *in vitro* anticancer activity of a silver(I) with imidazolium salts in dichloromethane, developed by Salah et al.[36], demonstrated cytotoxicity on three human cancer cell lines including breast adenocarcinoma (MCF7), hepatocellular carcinoma (HePG2), and lung adenocarcinoma (A549).

Tenoxicam (TNX), an another non-steroidal anti-inflammatory drug (NSAID), commonly used to treat chronic inflammatory diseases such as rheumatoid arthritis[37], as well as acute inflammatory diseases such as fever and pain, and to prevent blood clots[36] where pro-inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α) play an important role in acute inflammatory reactions[37]. Several *in vitro* studies and animal model studies suggest that NSAIDs have promising antineoplastic activity[37-41]

Muslu et al.[37] described the TNX metal complex with transition metals as $[Cu(TNX)_2]$, $[Zn(TNX)_2]$, $[Pt(TNX)_2]$ and $[Pd(TNX)Cl_2]$ demonstrated efficient reduction of pro-inflammatory tumor necrosis factor (TNF)-alpha production when compared to TNX alone.

Study by Mohamed et al.[38] described the anticancer activity of the TNX and 2,2-bipyridine complexes with the transition metals Cr(III), Fe(III), Co(II), Ni(II), Cu(II) and Y(III) on human colorectal carcinoma (HCT-116), human hepatocellular carcinoma (HepG2), human breast adenocarcinoma (MCF-7) cell lines.

Although several studies have described metal complexes with TNX with antibacterial and anticancer activity, there are no studies in the literature relating silver(I) complexes with tenoxicam with antibacterial and anticancer activity associated with the application of electroporation. In this work, we described the synthesis and evaluation of the *in vitro* antiproliferative and antibacterial activities of the Ag-TNX complex over four different tumorigenic human cell lines, and two non-tumor lines, as well as against bacterial species. In this work we also evaluated the potential use of electroporation as a topical delivery system of Ag-TNX for the treatment of skin cancer and bacterial infections. The aim is to increase the therapeutic arsenal for the treatment of skin cancer, a pathology involving an active inflammatory process, which can promote tumor invasion and metastasis.

Experimental

Materials and equipaments

The tenoxicam binder was purchased by Fagron Personalizin Medicine® laboratory $AgNO_3$, acquired by the Labsynth® laboratory. KOH was purchased by Sigma Aldrich®. Streptomycin and trypsin were purchased by the company Inlab®. DMEM – Low Glucose culture media and fetal bovine serum, purchased by Nova Biotecnologia®. Dimethyl sulfoxide (DMSO) purchased by Synthy®. Sodium chloride, bibasic potassium phosphate and potassium chloride, acquired by Synth®, as well as dibasic sodium phosphate, acquired by Vetec® for the production

of phosphate-saline buffer (PBS).

Agilent Cary 630 spectrometer, operating in the total attenuated reflectance mode, from 4000-600 cm^{-1}, was used to obtain the spectra of fourier transform infrared (FTIR). Elemental analyses were performed in a Perkin Elmer 2400 CHNS/O Analyzer, while nuclear magnetic resonance spectra of the compounds dissolved in deuterated dimethylsulfoxide were obtained solutions by using Bruker Avance III 400 and 500 MHz spectrometers. Thermogravimetric analysis (TGA) results were obtained using a TGA SDT Q600 thermoanalyzer from TA Instruments with air flow of 50 cm^3 min^{-1} in an oxygen atmosphere and a heating rate of 10ºC min^{-1} in a range of 25 ºC to 900 ºC. Semi-automatic Dry Heat Vertical Diffusion Cell Apparatus Logan was used for *in vitro* skin permeation studies. An inverted microscope, model EU 10 convs, resolution, 10 mpixel, power 2.0, made in PRC, was aquired by BEL to determinated IC_{50}. The VetCP 125 (Implastic®) device with electrode made up of six thin 2.5 cm long metallic needles, forming three equidistant pairs and separated by 0.5 cm was used in *in vitro* electrochemotherapy tests. UV/visible scanning spectrophotometer (Cole-parmer).

Methods

Synthesis of the Ag-TNX complex

A mass of 0.3374 g (1 mmol) of the TNX ligand was diluted in 20 mL of CH_3OH. Afterwards, the pH was adjusted to 8 with KOH (0.5M) for total solubilization in the solvent and deprotonation. Next, the reaction was initiatedby adding 0.1698 g (1mmol) of the Ag-NO_3 starting salt, diluted in 20 mL of H_2Od. The complex synthesis reactions were carried out at room temperature, by adding the ligand dropwise to the respective aqueous solution of the deprotonated ligand. The synthesis was carried out under constant stirring with the aid of a magnetic stirrer. After 30 minutes of reaction, a greenish precipitate was obtained and separated by filtration using filter paper and stored for 24 hours in an oven with a temperature between 35 ºC and 37 ºC and protected from light. The complex obtained was evaluated for synthesis yield, then stored in a sterile microcentrifuge tube, wrapped in aluminum foil, away from light until use. Elemental analysis results confirmed the composition of the $[Ag(C_{13}H_{10}N_3O_4S_2)].H_2O$, Anal. Calc. (%): C, 33.78; H, 2.62; N, 9.09. Found (%) C, 34.74; H, 1.93; N, 9.37.

Determination of minimum bactericidal concentration (MBC)

MBC's determination was performed using bacterial strains *Staphylococcus aureus* (ATCC-25923), *Escherichia coli* EC958 and *E. coli* BR43 that produce ESBL, and *Pseudomonas aeruginosa* PA01 biofilm producers, following CLSI recommendations (CLSI, 2022). After being added to tubes holding 10.0 mL of BHI, the chosen bacterial strains were cultured for 18 hours at 35–37°C. The new sterile BHI tubes were filled with enough bacterial suspension inoculum to reach a turbidity of 1.0 on the McFarland nephelometric scale (~$3.0x10^8$ $CFU{\cdot}mL^{-1}$). The MBC was then determined by collecting 10 µL from each well and applying to the MH agar surface using a multichannel pipette. The plates were maintained at 37°C in a bacteriological oven for 18 hours. Following incubation, the lowest concentration required to reduce the bacterial population by 100% was identified. All assays were performed in triplicate.

Test of the antiproliferative and cytotoxic activity of the Ag-TNX complex

To study the antiproliferative and cytotoxic activity of the Ag-TNX complex, tumor cells from the cutaneous melanoma lineage (B16) were used. The cells were cultured in 5 mL of DMEM – Low Glucose medium, supplemented with 20% fetal bovine serum and 1% streptomycin. The cells were added to 96-well plates (100 µL/

well, at an inoculation density of 6 x 10^4 cells mL) in triplicate, incubated for 48 h at 37 ºC in an oven with 5% CO_2. A mass of 50 mg of the Ag-TNX complex was suspended in a solution containing 2 mL of DMSO and 13 mL of H_2O_d. After incubation and total cell confluence, the agent was added to the wells of a 96-well microplate at the following concentrations: 3.3, 1.6, 0.83, 0.41, 0.20, 0.10 and 0.05 mg·mL^{-1}. After 24 h, 20 µL of 0.02% resazurin was added to the wells for subsequent reading of the results and completion of the antiproliferative activity test.

Study of the antiproliferative activity on tumor cells exposed to the metal complex through the application of electroporation

The study of antiproliferative activity from the application of electroporation was conducted after culturing tumor cells of the B16 lineage in 5 mL of DMEM – Low Glucose medium, supplemented with 20% fetal bovine serum and 1% streptomycin. The cells were then inoculated into cell culture plates in wells and incubated for 48 h at 37 ºC in an oven with 5% CO_2, until they reached total confluence.

The tests were carried out in triplicates and occurred as follows: all wells were inoculated with tumor cells. Three wells were our control line, therefore, only tumor cells were present in their contents. Three of the wells received only the synthesized agent at twice the minimum IC_{50} concentration found in previously performed tests, by optical microscopy (400x). A further three wells received the agent at twice the minimum concentration of IC_{50}, associated with electroporation, in which a voltage of 1.3 kV/cm was applied covering the entire length of the well, at a frequency of 5,000 Hz, which promoted a charge of 8 pulses per second and, finally, three wells received only electroporation at the voltage, frequency and number of pulses described above.

After carrying out the test, it was analyzed whether there was an increase in the antiproliferative activity of cells exposed to the metal complex in association with electroporation in comparison to those that received only the synthesized agent, or only electroporation, at the following moments: immediately after the application of electroporation; 90 minutes after electroporation; after washing and cleaning the wells with PBS and, finally, 24 hours after carrying out the experiment with the wells supplied with 500 µL of DMEM - Low Glucose culture medium and 100 µL of resazurin in each well.

Study of *in vitro* permeation, using Franz cells after exposure to synthesized agents and application of electroporation

The *in vitro* permeation study was carried out using the transdermal diffuser to study skin permeabilization using Franz cells. Porcine ear dermis from an animal recently slaughtered in a slaughterhouse was used in this experiment. This biological material will be treated in the following way: removal of the cartilage with the aid of tweezers and a scalpel, followed by the removal of the adjacent subcutaneous tissue, which will then be sectioned, processed and frozen for later use.

The Ag-MLX was administered to the previously treated biological material, performing topical application of the agent in a gel form. The complex was diluted to a concentration of 50 mg in 100 µL of $C_3H_8O_3$ (glycerol) and 0.5 mL of ultrasound gel. After this process, electroporation was applied at a voltage of 1.3 kV/cm, at a frequency of 5,000 Hz, which promoted a charge of 8 pulses per second. The materials were then coupled to the transdermal diffuser.

For 6 hours, every 90 minutes, 3mL samples of the solution contained in the cell's receptor compartment were collected and subjected to OD determination in UV-Vis at 340nm and 298nm. After each analysis, the contents removed from the receiving compartment were returned to the receiving compartment.

To determine these results, two processes were carried out: (1) the absorbance averages of the control group in the samples that received electroporation containing only the ultrasound gel and $C_3H_8O_3$ on the surface of the porcine ear skin, used to dilute the complexes; (2) the standard curve of the synthesized metal complex, in serial dilutions and starting from 50 $mg \cdot mL^{-1}$, for subsequent evaluation of samples analyzed by spectrophotometer after applying the electroporation technique on porcine skin, exposed to Ag-TNX at a concentration of 50 $mg \cdot mL^{-1}$.

Results

Vibrational absorption spectroscopy in the infrared region (FTIR) of the Ag-TNX complex

Reading the band patterns in the graph below makes it possible to observe that there was probably a compromise of the metal to the structure of the bioactive ligand. The line pattern shown in blue color refers to isolated TNX. The purple line pattern refers to the Ag-TNX complex. The stability conferred in the region of 3102,246 cm^{-1} of the Ag-TNX complex in comparison to the displacement observed in isolated tenoxicam, indicates possible coordination of the complex in the region of the carboxylic group (O-H) or the amine group (N-H) (Figure 1).

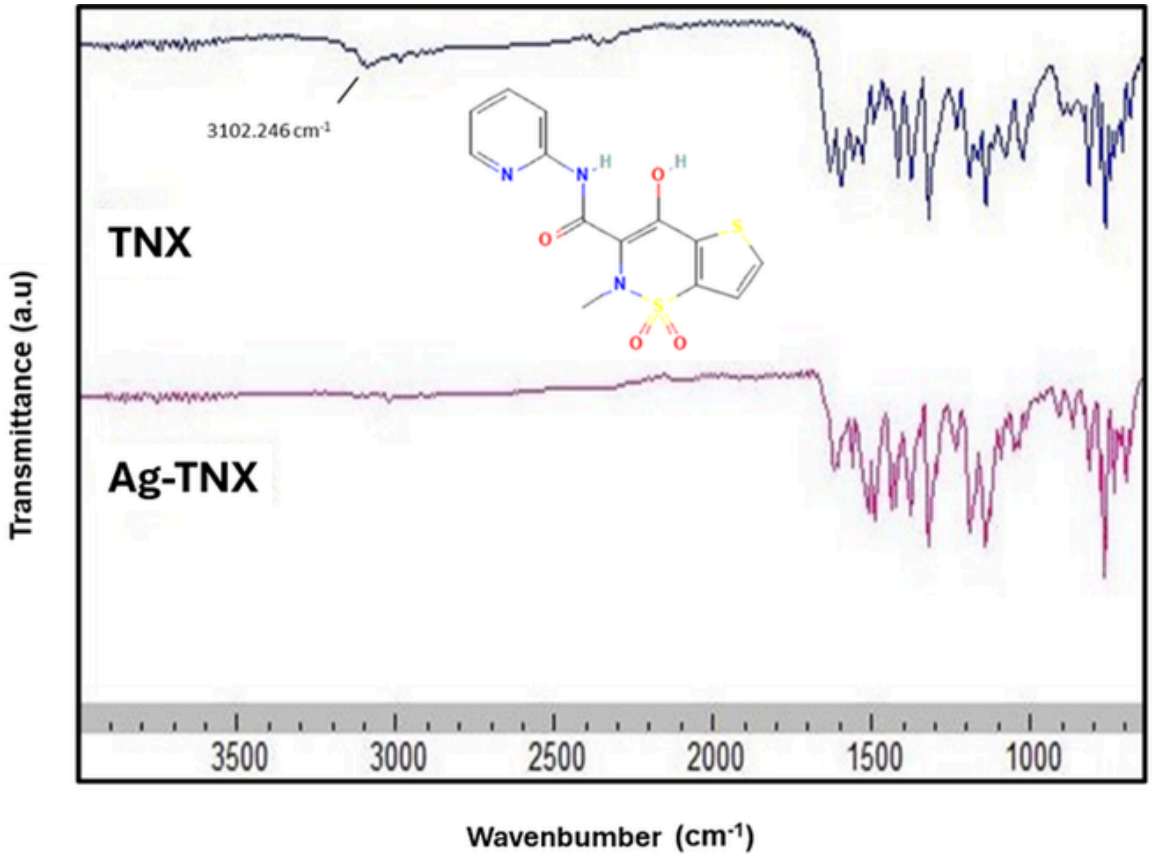

Figure 1 - FTIR analysis of tenoxicam ligand and Ag-TNX metal complex

Thermogravimetric analysis of the Ag-TNX complex

The thermogravimetric curve is shown in Figure 2. According to the experimental data, the composition of the complex, formulated as $[Ag(C_{13}H_{10}N_3O_4S_2)].H_2O$ was confirmed. The ligand is lost at temperatures from 165 ºC to 613 ºC. The calculated percentage for ligand loss was 65.12%. The final residue is consistent with the formation of metallic silver calculated for Agº (%): 34.88%.

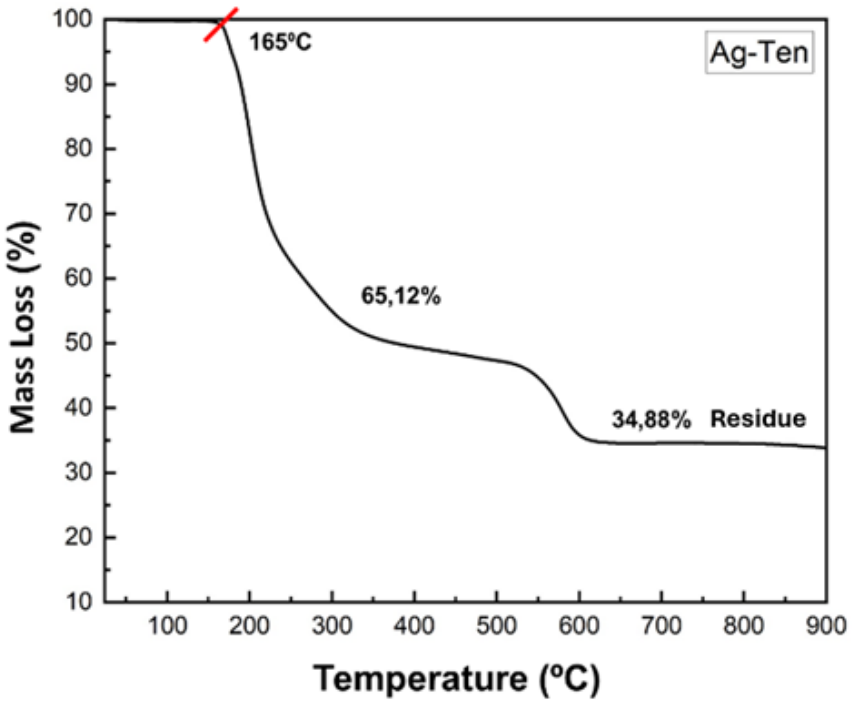

Figure 2 - Thermogravimetric curve of the Ag-TNX complex

MBC determination of the Ag-TNX Complex

Table 1 presents the results of MBC of the Ag-TNX complex compared to the free TNX ligand and $AgNO_3$

Table 1 - MBC values of $AgNO_3$, Ag-TNX metal complex and TNX free ligand (mmol-L^{-1})

	MBC (mmol·L^{-1})			
Material	*S. aureus* ATCC 25924	*E. coli* EC 958	*E. coli* BR43	*P. aeruginosa* PAO1
$AgNO_3$,	0,114*	0,114*	0,114*	0,114*
Ag-TNX	0,132*	0,132*	0,263*	0,132*
TNX	R	R	R	R

R - There was no bacterial growth inhibition activity
*Student T test is used to determine statistical significance for bacterial strains tests. The results were expressed on average ($p < 0.005$).

Tests of the antiproliferative and cytotoxic activity of the Ag-TNX metal complex

The result obtained after reading the plate containing the B16 lineage cells showed that the concentration of 0.2 mg·mL^{-1} was the minimum concentration to reach the IC_{50} of the Ag-TNX complex, resulting in a minimum concentration of 0.45 mmol- L^{-1} (Figure 3).

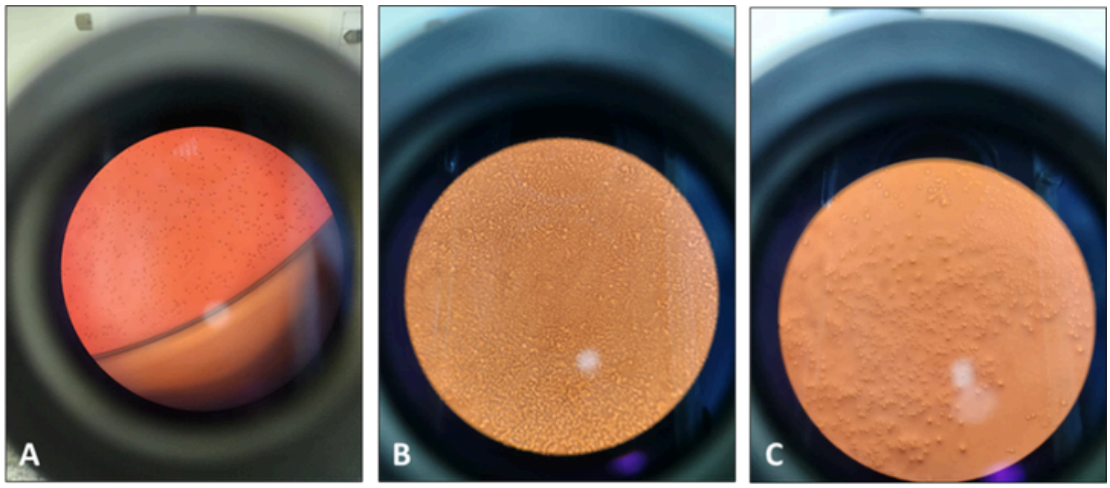

Figure 3 - Photomicrograph after inoculation of B16 cells. B) Photomicrography of cell confluence after 24h. C) Photomicrograph of the IC_{50} of Ag-TNX

Test of antiproliferative activity on tumor cells exposed to the Ag-TNX silver metal complex through the application of electroporation

The antiproliferative activity tests were carried out on cutaneous melanoma tumor cells (B16) and exposed to the silver Ag-TNX complex at twice the minimum concentration of the IC_{50} found in the tests previously carried out, i.e., 0.4 mg·mL^{-1}, associated, or no, to electroporation. The evaluation of the response to the proposed treatment was based on the analysis of the wells through microscopic observation and recording of photomicrographs at the times described in the methodology.

The image below shows the photomicrographs of the control group, that is, the wells that received only the inoculation of tumor cells of the B16 lineage. From microscopic observation, there was no significant difference in cell morphology, cell quantity, or the arrangement of these cells in the observed wells at any stage of the experiment (Figure 4).

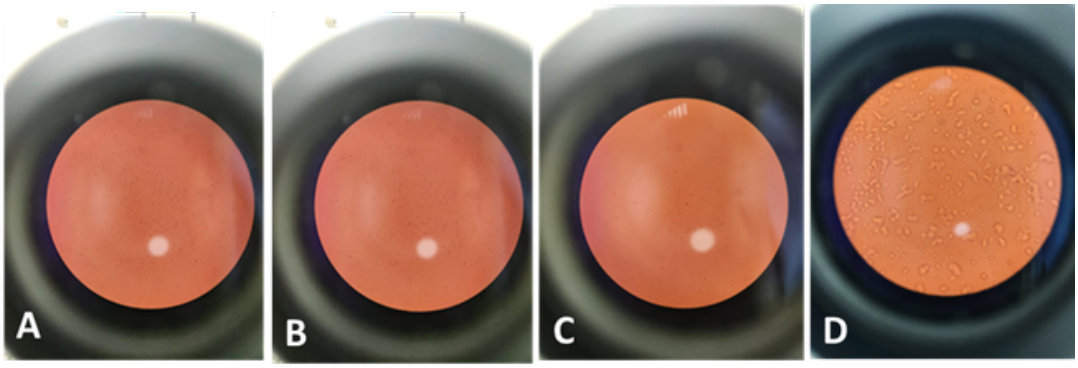

Figure 4 - Photomicrographs of the control group. Cell confluence and adherence observerd in each culture plate: A) at the beginning of the experiment. B) Immediately after the start of the experiment. C) 90 minutes after the start of the experiment. D) After washing and cleaning the wells with PBS.

In the second observation, ninety minutes after the complex was introduced, it was no longer possible to observe the cell mantle adhered to the bottom of the plate, showing that only the isolated agent was effective in causing the death of tumor cells. After washing the wells, the presence of some isolated cells added on the plate showed that this complex promoted the death of more than 90% of the cells. However, there was no cellular activity 24 hours after the start of the experiment (Figure 5).

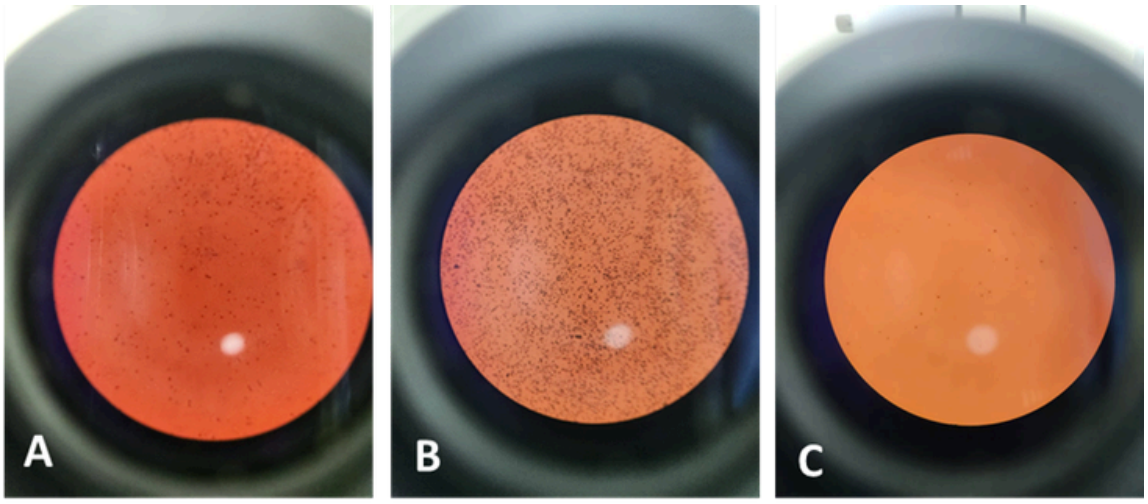

Figure 5 - Photomicrographs of the metal complex group. A) Immediately after the introduction of the Ag-TNX complex. B) After 90 minutes from the beginning of the experiment. C) After washing and cleaning the wells with PBS.

As demonstrated in the microscopic evaluation of the Ag-TNX group associated with electroporation, a significant difference was observed in the immediate phase after the start of the experiment compared to the metal complex group, demonstrating that, after the application of electroporation, there was a substantial reduction in the cell mantle adhered to the bottom of the culture plate. Cell death occurred in more than 90% of the cells present immediately after the application of the technique, showing superior antitumor activity compared to the isolated metal complex (Figure 6).

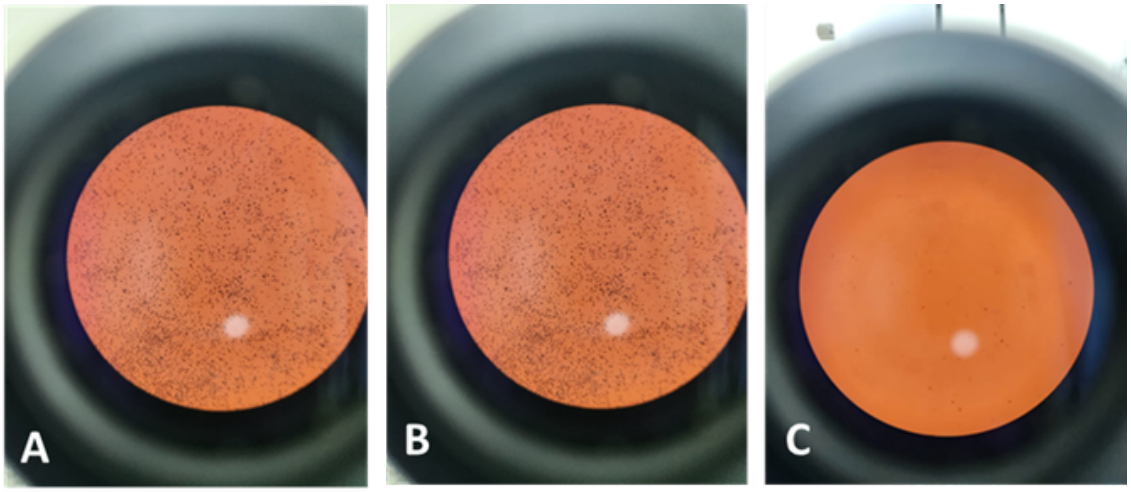

Figure 6 - Photomicrographs of the metal complex group associated with electroporation. A) Immediately after the introduction of Ag-TNX complex and application of electroporation. B) 90 minutes after the start of the experiment. C) After washing and cleaning the wells with PBS.

When evaluating the group subjected to isolated electroporation, it was observed that the cells underwent changes in their morphology immediately after the application of electroporation resulting in a process of cytomegaly (increase in cell size). Probably, the depermeabilization of the plasma membrane allowed the passage of solutes and solvents contained in the culture medium to the intracytoplasmic medium. After 90 minutes, the cells recovered their initial morphology. After washing the wells with PBS, it was noted that the cells were still present in the wells, showing that the isolated application of electroporation is sufficient to cause the death of the tumor cells, without reducing their capacity for cell proliferation (Figure 7).

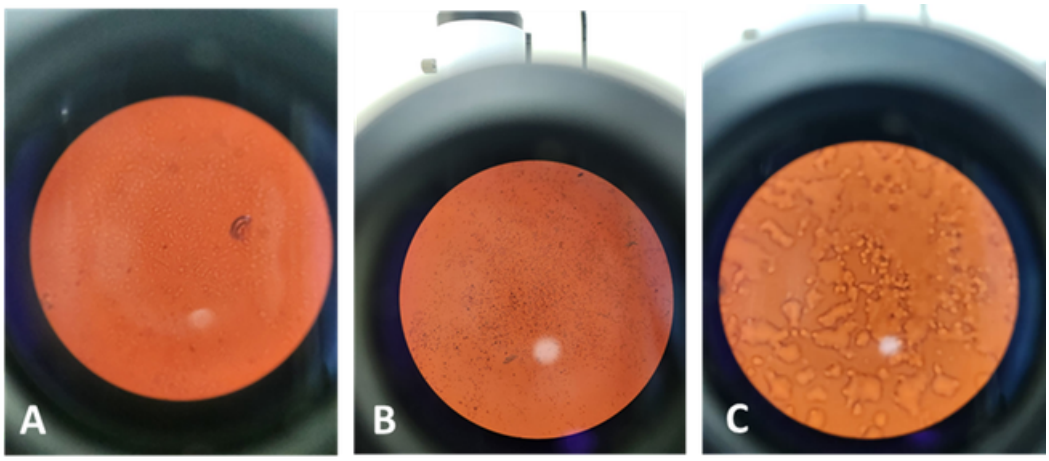

Figure 7 - Photomicrographs of the isolated electroporation group. A) Immediately after electroporation. B) 90 minutes after the star of the experiment. C) After washing and cleaning the well with PBS

After 24 hours, the experiment was terminated and the culture plates were analysed after the wells were supplied with 500 µL of DMEM Low – Glucose medium and 100 µL of resazurin. Visual evaluation of the plates showed that the wells of the control group and isolated electroporation exhibited cellular activity, meaning that the cells maintained their active metabolism. As for the evaluation of the wells in the metal complex and metal complex groups associated with electroporation, no cellular activity was observed, since there was no change in the color of the culture medium supplied with resazurin, demonstrating the total effectiveness of the treatment.

In vitro permeation test, using Franz cells after exposure to the silver metallic complex Ag-TNX and application of electroporation

In vitro Franz cell permeation tests using the Ag-MLX complex revealed that there was no permeation of the agents after electroporation exposure. The average absorbance concentrations of the control group, performed and collected in triplicate, were as follows: 0.005 in the first 90 minutes; 0.005 after 180 minutes; 0.006 after 270 minutes and, finally, 0.012 after 360 minutes. The results obtained from the samples that received the synthesized agents associated with electroporation were subjected to Lambert Beer's Law to determine the final concentration.

The standard curve for the Ag-TNX metal complex was performed using a UV-Vis spectrophotometer Cole-parmer. The initial concentration evaluated was the same as that used in the topical form impregnated on porcine skin prior to electroporation: 50 $mg \cdot mL^{-1}$. However, it was only possible to determine the absorbance concentration in solutions with dilutions ranging from 0.39 $mg \cdot mL^{-1}$ to 0.02 $mg \cdot mL^{-1}$ (Figure 8).

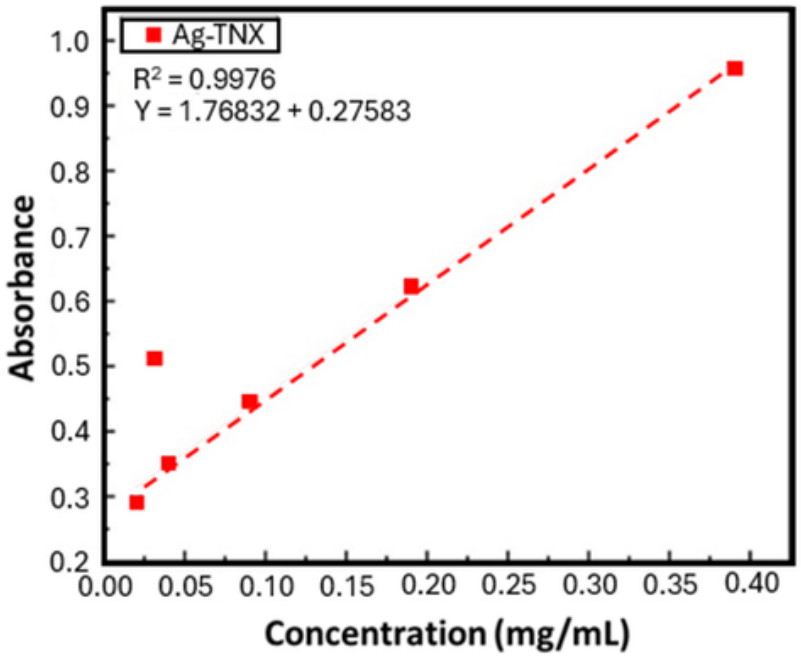

Figure 8 - Standard curve of the Ag-TNX complex analyzed by UV-Vis spectrophotometer

The linear regression coefficient of determination (R^2) was 0.9976. The angular coefficient was found to be 1.76832, while the linear coefficient was found to be 0.27583. By plotting the standard curve of the Ag-TNX complex and measuring the absorbance of the solutions evaluated in the Franz cell in triplicate, the average absorbance concentrations of the Ag-TNX group after electroporation were found to be: 0.007 in the first 90 minutes; 0.033 after 180 minutes; 0.078 after 270 minutes and, finally, 0.11 after 360 minutes.

After applying Beer-Lambert's law to these results, it was found that the final concentration obtained from the Ag-TNX complex was 0. This indicates that there was no absorbance of the agent after the application of electroporation, which demonstrates a reduction in the risk of systemic adverse reactions.

Conclusions

The Ag-TNX complex obtained showed stoichiometry 1: 1. FTIR characterization of the Ag-TNX complex compared to TNX ligand suggests that the displacement of the TNX ligand bands is due to the coordination of the complex through the carboxylic group (O-H) or the amine group (N-H). The Ag-TNX complex presented antiproliferative activity in human tumor cells of lineage B16 (mouse cutaneous melanoma) and inhibitory growth activity of bacterial strains, *S. aureus ATCC 25924*, clinical isolates *E. coli EC 958*, *E. coli BR43* extended-spectrum β-lactamase producers, and multi-resistant Gram-negative pathogen *P. aeruginosa PAO1*, demonstrating promising results for future expansion of therapeutic arsenal of antineoplastic and antibacterial agents. The Ag-TNX complex associated with *in vitro* electroporation has demonstrated antiproliferative activity in Tumor B16 tumor cells at a shorter period compared to antiproliferative activity observed in cultures of the same cells lines, in the presence of Ag-TNX, without electroporation. This demonstrates that electroporation, when associated with synthesized antitumor agent, promotes a superior response to the isolated agent. In B16 melanoma cell lines exposed to the isolated electroporation technique, no antiproliferative response has been observed, demonstrating that the technique applied in isolation is not able to promote antiproliferative effects or induce tumor cell apoptosis. *In vitro* permeation tests performed with Franz cells have shown that the Ag-TNX silver complex absorption after electroporation was significantly low, suggesting a reduced risk of systemic adverse effects and providing greater security for *in vivo* studies in an animal model.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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