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Genotoxic assessment of the copper chelated compounds Casiopeinas: Clues about their mechanisms of action

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ABSTRACT

Casiopeinas is the generic name of a group of copper chelated complexes designed to be used as antineoplastic. Some of these compounds have shown promising results, and in fact, one of them named Casiopeina III-ia has completed preclinical trials and is ready to start clinical phase I in Mexico. As part of the tests that have to be done to every molecule intended to be used in humans, bacterial assays are required because of their sensitivity, speed and reproducibility and among them, Ames test and the SOS Chromotest are widely used to evaluate DNA damage. With the aim to contribute to complete safety information related to genotoxicity and support the hypothesis about their mode of action, four different Casiopeinas (Cas II-gly, Cas III-Ea, Cas III-ia and Cas III-Ha) were tested for genotoxicity with these assays, as well as differential cytotoxicity upon *Escherichia coli* mutants defectives in some DNA repair mechanisms. However, although it is well known that these molecules produce DNA breakage, the results of the Chromotest and Ames test were negative. Despite this is controversial, a possible explanation is that there is a direct interaction between DNA and the Casiopeinas tested.

1. Introduction

Chemotherapy is widely employed to treat cancer, yet the capacity of many cancer cells to acquire resistance to antineoplastic drugs is an important issue that limits the effectiveness of many treatments currently used. The need for new antitumor agents, along with the hypothesis that molecules designed with essential metal atoms may be effective and less toxic than other frequently used metal based antineoplastics such as cisplatin, stimulated several groups to develop copper-based drugs with potential use as therapeutic agents [1].

Copper exhibits considerable biochemical action either as an essential trace metal or as a constituent of various exogenously administered compounds with potential clinical uses [2]. In its former role, it is bound to ceruloplasmin, superoxidismutases and other proteins as albumin or endogen ligands, while in its latter, it is part of complexes that interact with biomolecules, mainly proteins and nucleic acids [2–4]. Copper is incorporated into a number of metalloenzymes involved in drug/xenobiotic metabolism, carbohydrate metabolism, catecholamine biosynthesis and the cross-linking of collagen, elastin, and hair keratin as well as in the antioxidant defense mechanism [5]. Moreover, copper-dependent enzymes are usually involved in redox reactions. Enzymes such as cytochrome C oxidase, superoxide dismutase, ferroxidases, monoamine oxidase, and dopamine β-monooxygenase, interact with reactive oxygen species (ROS) or molecular oxygen [5] to yield the corresponding reduced species. However, because of the capability of this essential metal to participate in redox reactions, it is able to produce large amounts of ROS through a Fenton-like reaction [6]. An excess of copper could result in health disorders related to oxidative stress.

Several biological activities of copper complexes have been studied by several research groups, demonstrating the antiviral, antibacterial and antitumor activities of some Cu(II) complexes [1,3,7–9]. The behavior of a metal complex and its biological activity as a result is dependent on both, peculiar features of the metal and the ligand environment that can have a marked effect on the overall reactivity of the complex. Even though the mechanism of these activities is still unclear, some recent results indicate that cytotoxic copper complexes promote the degradation of nucleic acids [10].

The main objective of our research group is to design novel copper complexes as potential antitumor agents [9]. In order to achieve this, a novel family of mixed copper-chelating compounds registered with the generic name of Casiopeinas has been designed, which has the following basic formula: $[Cu(N—N)(\alpha\text{-L-amino acidato})]NO_3$ and $[Cu(N—N)(O—O)]NO_3$. The N—N donor is an aromatic substituted diimine (1,10-phenanthroline (phen) or 2,2-bipyridine (bpy)) and the O—O donor is acetylacetonate (acac) or salicylaldehydate (salal) [11–13]. It has been demonstrated that some copper-based compounds of this family may exhibit a higher antiproliferative potency in vitro than cisplatin for human ovarian carcinoma (CH1) [14], murine leukemia (L1210) [14], various cervico-uterine carcinomas and other cell lines.

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In vivo assays have also shown promising results, revealing activities comparable to reference drugs, in some cases at lesser molar doses, with acceptable toxicity [15–17].

Although the mechanism of action is not completely understood, studies point toward several possibilities that are not mutually exclusive and could even act cooperatively. The whole genetic expression in cells exposed to some Casiopeinas has been studied, showing the selectivity of these molecules in expressing apoptosis-related genes, while some of those involved in reproduction and/or cell growth are turned off [18]. These compounds inhibit cell proliferation and produce dose-dependent cell death by apoptosis [14,19] through mechanisms dependent and independent of caspase activation [17,18]. Some data also suggest that Cas III-ia induces cell death by autophagy and apoptosis, due in part to the activation of ROS dependent JNK signaling [20]. In general, cell growth inhibition and DNA degradation are enhanced when reducing agents are presented simultaneously with ROS increase [17,21–23], suggesting that DNA oxidation may be triggering the cell death. Nevertheless, the final effect might be the result of several processes acting alone or in concomitance. Overall, the evidence supports three main targets: a) generation of reactive oxygen species [22–24] with DNA oxidation and degradation [10,23], plus depletion of antioxidant defenses like GSH as consequence [24–26]; b) mitochondrial toxicity [27–29]; and c) DNA damage through direct interaction with complex by an intercalative or non-intercalative mechanism along with oxidative damage [22,30–32]. DNA interaction has been one of the most studied targets of Casiopeinas and several experiments have been conducted to support this hypothesis [33]. In order to understand the interaction with DNA, a complete theoretical and experimental study of a series of the Casiopeinas, containing glycine as a secondary ligand, indicates the importance of the π-back bonding in the chemical properties of these compounds as well as some parameters including electronic paramagnetic resonance (EPR), structural and electrochemical ones [34–36]. A recently published work [33] showed that copper complexes interact with DNA by means of different binding modes; the mode of binding and intensity of these interactions are influenced by both ligands coordinated to copper. According with this work, at least four interactions are possible: intercalative, coordination bond between metal and the phosphate group, minor groove binding, and partly substitution of ligands with some coordinating groups in DNA. The proportion in which each interaction occurs will depend on the features conferred by both ligands and its substituents. Findings are in agreement with DNA-fiber EPR studies [31] and molecular dynamics simulations [34, 35].

Structure–activity relationship studies have revealed how toxicity and activity are influenced by the structure [15,37,38] allowing the judicious selection of the most promising compounds to go through preclinical studies. One of these compounds, known as Casiopeina III-ia, has completed preclinical trials and it is ready to start clinical phase I in Mexico.

On the other hand, it is required to test every new molecule intended to be used in humans, to assess any plausible adverse effect. In this sense, a biological test system to identify these effects should be simple, fast, reproducible and highly sensitive and specific. Despite the genotoxicity studies are not considered essential to support clinical trials for therapeutics intended to treat patients with advanced cancer [39], genotoxicity tests should be performed to support marketing [40]. Since these studies have been used mainly for the prediction of carcinogenicity, the International Conference on Harmonization (ICH) guidelines propose a battery of genotoxicity test approach arguing that a single test is not enough to detect all genotoxic mechanisms relevant in tumorigenesis [40]. This approach suggests two options for the standard battery that are considered equally suitable. In both of them, the assessment of mutagenicity in a bacterial reverse gene mutation test is required (Ames test). The suggested standard set of test does not imply that other genotoxicity test is considered inadequate or inappropriate, and in fact, the guidelines promote the use of additional test for further investigation of genotoxicity test results and underlying mechanisms [40].

In summary, bacterial systems for mutagenicity or genotoxicity tests are widely accepted, since they have been found to be predictive of potential carcinogenicity in humans, meet several of the previously mentioned characteristics, the cost is relatively low and results are obtained rapidly. As a result, the Food and Drug Administration (FDA) and the ICH guidelines recommend the use of such systems to test the mutagenic potentials of new synthesized compounds. Microbial genotoxicity assays can be divided into two main groups: those that detect point mutations, such as the *Salmonella* mutagenicity test (Ames test), or the arabinose resistance test [41], and those designed to reveal the induction of the SOS response — a set of genes that become derepressed by DNA damage to cope with stress situations [42–44]. In order to contribute with more information about genotoxicity and its possible underlying mechanism, in the present work we used the bacterial SOS Chromotest and the Ames test, as well as the comet assay with four representative complexes of the already synthesized Casiopeinas. This paper presents complementary techniques and results that support previous hypothesis.

2. Materials and methods

2.1. Chemicals

Casiopeinas used in this work were synthesized by Dr. Maria Elena Bravo-Gómez at the School of Chemistry, UNAM employing methods previously reported [11–13] and the purity was evaluated by elemental analysis (Table 1); structures are shown in Fig. 1. All the other compounds, unless otherwise indicated, were purchased from Sigma-Aldrich.

2.2. Bacterial strains

The bacterial strains used in these experiments are listed in Table 2. Strains PQ30, PQ33 and PQ37 were kindly donated by Dr. Quillardet. Strains with the suffix IN were constructed in our laboratory as reported earlier [45,46]. All of them bear the genetic fusion *sulA*::Mud(*Ap*, *lacZ*), which makes the synthesis of the enzyme β-galactosidase to be controlled by the SOS response.

2.3. SOS Chromotest

The Chromotest is a bacterial assay designed to evaluate DNA damage based on the inducible property of the SOS response, a group of approximately 60 genes that increase their expression when DNA damage occurs. In the strains used in this assay, the *lacZ* gene that codes for β-galactosidase is fused to *sulA*, which belongs to the SOS pathway, thus the synthesis of the enzyme is associated to SOS and will transcribe only when this response is induced, indicating genetic damage. Since the compounds tested could inhibit the protein synthesis or be toxic, which might lead to an incorrect estimation of the induction of β-galactosidase and cause false negative results, the constitutive transcription of alkaline phosphatase was included in this strain, serving as an overall indicator of protein synthesis and indirectly as an indicator of toxicity. Briefly, liquid cultures were grown overnight, diluted 50 fold in fresh LB broth and incubated in a water bath shaker at 37 °C until mid-logarithmic growth (2×10^8 cells/ml) was reached. Cultures were centrifuged and resuspended in the same volume of HEPES buffer and then 1 ml aliquots of the cell suspensions were treated with different concentrations of the Casiopeinas for 30 min at 37 °C (0.1, 1, 10 and 100 μM for CasI-gly, Cas III-Ea and Cas III-ia and 0.1, 1, 10 and 16 mM in the case of Cas III-Ha). Cells were then washed twice with HEPES buffer to eliminate residual Casiopeinas and either diluted in phosphate saline buffer and plated in LB for viable counts or diluted in LB broth for SOS activity evaluation. After treatments, bacteria were diluted 10-fold in LB and