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Diaryl sulfide analogs of combretastatin A-4: Toxicogenetic, immunomodulatory and apoptotic evaluations and prospects for use as a new chemotherapeutic drug

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ABSTRACT

Combretastatin A-4 exhibits efficient anti-cancer potential in human tumors, including multidrug-resistant tumors. We evaluated the mutagenic, apoptotic and immunomodulatory potential of two diaryl sulfide analogs of combretastatin A-4, 1,2,3-trimethoxy-5-([4-methoxy-3-nitrophenyl]thio)benzene (analog **1**) and 1,2,3-trimethoxy-5-([3-amino-4-methoxyphenyl]thio)benzene (analog **2**), as well as their association with the anti-tumor agent cyclophosphamide, in Swiss mice. Such evaluation was achieved using the comet assay, peripheral blood micronucleus test, splenic phagocytosis assay, and apoptosis assay. Both analogs were found to be genotoxic, mutagenic and to induce apoptosis. They also increased splenic phagocytosis, although this increase was more pronounced for analog **2**. When combined with cyclophosphamide, analog **1** enhanced the mutagenic and apoptotic effects of this anti-tumor agent. In contrast, analog **2** did not enhance the effects of cyclophosphamide and prevented apoptosis at lower doses. These data suggest that analog **1** could be an adjuvant chemotherapeutic agent and possibly improve the anti-neoplastic effect of cyclophosphamide. Additionally, this compound could be a candidate chemotherapeutic agent and/or an adjuvant for use in combined anti-cancer therapy.

1. Introduction

Combretastatin A-4 (CA-4) is isolated from the bark of the African tree *Combretum caffrum* (Combretaceae). CA-4 is composed of two aromatic rings linked by a double bond in a *cis* configuration at the C-5 position of ring A (Fig. 1A). Trimethoxy substitutions in ring A have been reported to be prerequisites for the maintenance of activity, whereas ring B is tolerant of structural modifications, mostly at position C-3′ (Li et al., 2011).

CA-4 shows efficient anti-cancer potential in human tumors (including multidrug-resistant tumors). Studies have also reported CA-4 activity in leukemia, colon tumors, glioblastoma, melanoma, prostate carcinoma, ovarian adenocarcinoma, and bronchoalveolar lung carcinoma (Dark et al., 1997; Li et al., 1998; Siemann et al., 2009).

The cytotoxic activity of CA-4 is related to its high affinity for the tubulin binding site, which leads to blockade of cell division and, consequently, cell death. If acting on endothelial cells present

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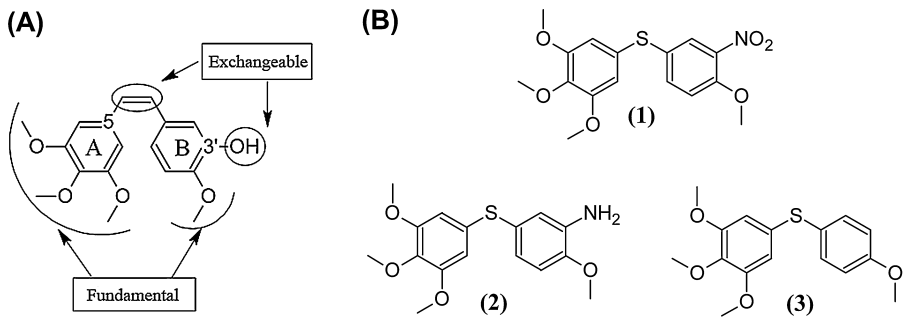

Fig. 1. (A) Molecular structure of CA-4; (B) structures of diaryl sulfides **1**, **2** and **3**. Compounds **1** and **2** were employed in this study. Compound **3** was modified to generate analogs **1** and **2**.

in tumors, CA-4 leads to their death. It is, therefore, also regarded to be an anti-angiogenic agent, reducing the vascularization of, as well as the supply of oxygen and nutrients to tumors (Kanthou et al., 2004).

CA-4 is used in anti-cancer therapies, but it exhibits side effects such as cardiovascular disorders, hypertension, hypotension, lymphopenia, and pain. However, it does not exhibit the common cytotoxic effects of other agents, such as myelosuppression and alopecia (Brandão et al., 2010).

In the present study, two analogs of CA-4 were used: 1,2,3-trimethoxy-5-([4-methoxy-3-nitrophenyl]thio)benzene (**1**) and 1,2,3-trimethoxy-5-([3-amino-4-methoxyphenyl]thio)benzene (**2**) (Fig. 1B). Through *in vitro* biological tests, Santos et al. (2013) demonstrated that the nitro group in analog **1** decreases cytotoxic activity and increases anti-tubulin activity compared with analog 1,2,3-trimethoxy-5[(4-methoxyphenyl)sulfonyl]benzene (**3**) (Barbosa et al., 2009); the amino group of analog **2** increases cytotoxicity and anti-tubulin activity (Santos et al., 2013).

Therefore, in the present study, toxicogenetic, immunomodulatory and apoptotic assessment of **1** and **2** was undertaken *in vivo*, in association with the chemotherapeutic agent cyclophosphamide. The latter is used widely to treat many types of tumors: lymphomas, multiple myeloma, leukemia, ovarian adenocarcinoma, breast carcinoma, and malignant lung tumors. The aim of this study was to investigate the mode of action of these compounds with or without cyclophosphamide.

2. Material and methods

The study was conducted according to the guidelines of the Universal Declaration of Animal Rights. The study protocol was approved by the Ethics Committee on Animal Use of the Federal University of Mato Grosso do Sul UFMS (protocol numbers 399/2012 and 523/2013).

2.1. Chemical agents and experimental design

Diaryl sulfides **1** and **2** were synthesized, purified and identified by Santos et al. (2013). Both compounds were first diluted in Tween 80 (4%) and then in ethanol (1%), which was employed as treatments control. Diaryl sulfides were administered at 5, 7.5 and 10 mg/kg of body weight (bw) intraperitoneally (i.p.). Cyclophosphamide (Fosfaseron®, Laboratórios Ítaca, Rio de Janeiro, Brazil) was prepared in physiologic (0.9% NaCl) solution (pH 7.4) and administered at dose of 100 mg/kg bw (i.p.) in a single dose as the positive control. Experimental groups and applied doses of compounds are shown in Table 1.

Seventy sexually mature, male Swiss mice (Mus musculus) were obtained from the Central Animal Facility of the Center of Biological and Health Sciences of the Federal University of Mato Grosso do Sul (CCBS/UFMS). Animals were subdivided into 14 experimental groups of five mice. Animals were kept in propylene cages with the floor covered with sawdust, and were given commercial feed (Nuvital®, Nuvital Nutrientes, Colombo – PR, Brazil) and filtered water *ad libitum*. A 12-h light-dark cycle as well as a temperature of 22 ± 2 °C and humidity of 55% ± 10% was employed in ventilated racks (ALESCO®, Rio de Janeiro, Brazil).

Table 1
Experimental groups and doses of agents employed in this study.

Treatments	Analog (**1**)	Analog (**2**)	CP
Control	–	–	–
CP 100 mg/kg	–	–	+
D1 5 mg/kg	+	–	–
D1 7.5 mg/kg	+	–	–
D1 10 mg/kg	+	–	–
CP + D1 5 mg/kg	+	–	+
CP + D1 7.5 mg/kg	+	–	+
CP + D1 10 mg/kg	+	–	+
D2 5 mg/kg	–	+	–
D2 7.5 mg/kg	–	+	–
D2 10 mg/kg	–	+	–
CP + D2 5 mg/kg	–	+	+
CP + D2 7.5 mg/kg	–	+	+
CP + D2 10 mg/kg	–	+	+

CP: cyclophosphamide; D1: Assessment of analog 1 alone 5, 7.5 and 10 mg/kg of body weight,. CP + D1: Assessment of analog 1 (5, 7.5 and 10 mg/kg of body weight) in combination with Cyclophosphamide (100 mg/kg of body weight). D2: Assessment of analog 2 alone 5, 7.5 and 10 mg/kg of body weight. CP + D2: Assessment of analog 2 (5, 7.5 and 10 mg/kg of body weight) in combination with Cyclophosphamide (100 mg/kg of body weight). Different letters indicate statistically significant differences ($p < 0.05$; ANOVA/Tukey).

Samples of peripheral blood (20-μL) were collected for the micronucleus test at 24 (T1), 48 (T2) and 72 h (T3) after administration of the analogs. At T1, 20 μL of peripheral blood was collected for the comet assay. Another 20-μL aliquot was collected at T3 for differential counting of blood cells. After T3, the mice were euthanized. Their organs were collected for splenic phagocytosis and apoptosis assays.

2.2. Biological assays

2.2.1. Comet assay

The comet assay was conducted according to the protocol of Singh et al. (1988). Samples were analyzed using a L 2000A Epifluorescent Microscope (Bioval®, Rio de Janeiro, Brazil) at 40× magnification using an excitation filter of 420–490 nm and a barrier filter of 520 nm. One-hundred cells were analyzed visually per animal, as described by Kobayashi et al. (1995).