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In vitro effects of benzimidazole/thioether-copper complexes with antitumor activity on human erythrocytes

Mario Suwalsky[a,*], Ivan Castillo[b], Brenda N. Sánchez-Eguía[b], María José Gallardo[c], Nathan Dukes[d], Edelmiro Santiago-Osorio[e], Itzen Aguiñiga[e], Ana R. Rivera-Martínez[e]

[a] Faculty of Chemical Sciences, University of Concepción, Chile
[b] Instituto de Química, Universidad Nacional Autónoma de México, Circuito Exterior, CU, 04510, Mexico
[c] Center for Optics and Photonics, University of Concepción, Chile
[d] Faculty of Medicine, Pontifical Catholic University of Chile, Santiago, Chile
[e] Facultad de Estudios Superiores-Zaragoza, Universidad Nacional Autónoma de México, Batalla del 5 de Mayo y Fuerte de Loreto, Ciudad de México 09230, Mexico

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ABSTRACT

Two cytotoxic copper(II) complexes with N-H and *N*-methylated benzimidazole-derived ligands (Cu-$\mathbf{L^1}$ and Cu-$\mathbf{L^{1Me}}$; $\mathbf{L^1}$ = bis(2-methylbenzimidazolyl)(2-methylthioethyl)amine, $\mathbf{L^{1Me}}$ = bis(1-methyl-2-methylbenzimidazolyl)(2-methylthioethyl)amine) were synthesized and exposed to human erythrocytes and molecular models of its membrane. The latter were bilayers built-up of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), classes of lipids present in the external and internal moieties of the human red cell membrane, respectively. Scanning electron microscopy (SEM) of erythrocytes incubated with solutions of both Cu(II) complexes showed that they induced morphological changes to the normal cells to echinocytes, and hemolysis at higher concentrations. Real-time observation of the dose-dependent effects of the complexes on live erythrocytes by defocusing microscopy (DM) confirmed SEM results. The formation of echinocytes implied that complex molecules inserted into the outer moiety of the red cell membrane. X-ray diffraction studies on DMPC and DMPE showed that none of these complexes interacted with DMPE and only Cu-$\mathbf{L^1}$ interacted with DMPC. This difference was explained by the fact that Cu-$\mathbf{L^{1Me}}$ complex is more voluminous than Cu-$\mathbf{L^1}$ because it has two additional methyl groups; on the other hand, DMPC molecule has three methyl groups in its bulky terminal amino end. Thus, by steric hindrance Cu-$\mathbf{L^{1Me}}$ molecules cannot intercalate into DMPC bilayer, which besides is present in the gel phase. These results, together with the increased antiproliferative capacity of the *N*-methylated complex Cu-$\mathbf{L^{1Me}}$ over that of Cu-$\mathbf{L^1}$ are rationalized mainly based on its higher lipophilicity.

1. Introduction

Metal-based compounds have been used as anticancer drugs since 1960 with the development and application of cisplatin, which is prescribed for almost half of cancer patients. Although it is one of the most used anticancer drugs, especially in solid tumors [1,2] severe side effects, toxicity and different resistance pathways have led to efforts to find better alternatives. Copper has become an appealing candidate to replace platinum drugs based on its role in biological systems as an essential element that participates in different biochemical processes [3,4]. Recent studies have shown that human cancer cells take up greater amounts and have higher copper levels than normal cells [5,6]. On the other hand, many copper based complexes have shown anticancer activity both *in vivo* and *in vitro*, with well documented antitumor activity [7,8,9]; this has stimulated the synthesis of copper complexes with organic ligands with antiproliferative properties [10,11]. Despite the numerous cytotoxic copper complexes so far reported, the mechanisms underlying their activity are not fully established. Former hypotheses contemplated a similarity between Cu and Pt in DNA binding, intercalation, and cleavage. However, new proof of proteasome inhibition [12], oxidative damage by production of reactive oxygen species [13,14,15], non-apoptotic programmed cell death [5,16], and caspase inhibition leading to apoptotic cell death [17] have been considered as viable cytotoxic routes. Benzimidazoles are recognized to have antitumor properties [18], showing DNA binding capacity. In this context, we have synthesized copper complexes with the chelating tetradentate ligands $\mathbf{L^1}$ and $\mathbf{L^{1Me}}$ ($\mathbf{L^1}$ = bis(2-methylbenzimidazolyl)(2-methylthioethyl)amine, $\mathbf{L^{1Me}}$ = bis(1-methyl-2-

* Corresponding author.
E-mail address: msuwalsk@udec.cl (M. Suwalsky).

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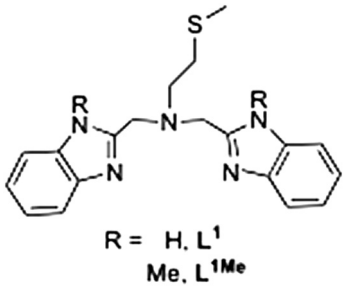

Fig. 1. Structural formulas of $\mathbf{L^1}$ and of its methylated analog $\mathbf{L^{1Me}}$.

methylbenzimidazolyl)(2-methylthioethyl)amine) [19,20] featuring a thioether group and benzimidazole-based donors (Fig. 1), for which we herein report their *in vitro* antitumor activity.

In order to have a better understanding of the mechanism of the interaction of these complexes with cells at their access places, *i.e.* cell membranes, the copper complexes Cu-$\mathbf{L^1}$ and Cu-$\mathbf{L^{1Me}}$ were assayed in two systems: human erythrocytes and molecular models of its membrane. The molecular models of the erythrocyte membrane consisted of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) bilayers, illustrative of phospholipids located in the outer and inner moieties of the red cell membrane, respectively [21,22]. The capability of the complexes to interact with DMPC and DMPE was evaluated by X-ray diffraction, while intact human erythrocytes were observed by scanning electron (SEM) and defocusing microscopy (DM). These systems and techniques have previously been applied by us in order to determine the interactions and structural effects on cell membranes of other metal compounds with therapeutic properties [23,24]. The results mentioned above were also pondered to determine their potential relation with the antiproliferative activity determined for complexes $\mathbf{L^1}$ and Cu-$\mathbf{L^{1Me}}$ against several human cell lines.

2. Experimental

2.1. Reagents and techniques

Solvents and reagents were obtained from commercial suppliers, and were used without further purification. Ligand Bis(1-methyl-2-methylbenzimidazolyl)(2-methylthioethyl)amine($\mathbf{L^{1Me}}$) and the complex{[Bis(1-methyl-2-methylbenzimidazolyl)(2-methylthioethyl) amine]copper[bis(perhclorate)]} ([$\mathbf{L^{1Me}}$Cu$(ClO_4)_2$]·$2H_2O$, Cu-$\mathbf{L^{1Me}}$) were prepared according to the literature procedures [19,20]. NMR spectra were recorded in a JEOL Eclipse 300 spectrometer in $CDCl_3$ with tetramethylsilane as an internal standard at 300 (^{1}H) or 75 MHz (^{13}C). Infrared spectra were obtained with a Perkin Elmer 203-B spectrometer in the range 4000–400 cm^{-1} as KBr disks. Mass spectra were obtained on a JEOL JMS-SX-102A mass spectrometer at an accelerating voltage of 10 kV, with a nitrobenzyl alcohol matrix and Xenon atoms at 6 keV (FAB^+), or a Bruker Daltonics Esquire 6000 spectrometer with ion trap (Electrospray). Melting points were determined on an Electrothermal Mel-Temp apparatus and were uncorrected.

2.2. Synthesis

2.2.1. Bis(2-methylbenzimidazolyl)(2-methylthioethyl)amine (L^1)

1-*tert*-butoxycarbonyl-2-cloromethylbenzimidazole (533 mg, 2 mmol), 2-methylthioethylamine (91 mg, 1 mmol), potassium carbonate (495 mg, 5 mmol), and sodium iodide (20 mg, 0.14 mmol) were placed in 15 mL CH_3CN in a round bottom flask, and the mixture was heated to reflux for 5 h. The solution was then filtered through celite, concentrated to dryness, and extracted with 3 × 10 mL of dichloromethane. The solution was concentrated to dryness, and dissolved in the minimum amount of acetone; trifluoromethylsulfonic acid was added (0.30 mL, 3.3 mmol), and the mixture was stirred overnight. Slow evaporation of volatiles afforded an off-white solid that was dissolved in 20 mL of dichloromethane and washed with 0.1 M NaOH to afford 225 mg of L^1 after separation and evaporation of volatiles as a colorless solid (64%). Mp: 108–111 °C. IR (KBr): 3336, 3051, 2919, 2870, 1658, 1617, 1524, 1448, 1414, 1330, 1305, 1267, 1221, 1202,1136, 1046, 1015, 993, 959, 933, 898, 849, 828, 676, 651, 589, 460, 438. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.58 (s, 2H, N*H*), 7.57 (m, 2H, BzIm), 7.23 (m, 6H, BzIm), 4.16 (s, 4H, BzImCH_2), 2.91 (t, 2H, ^3J = 6.0 Hz,NCH_2), 2.68 (t, 2H, ^3J = 6.0 Hz, SCH_2), 2.10 (s, 3H, SCH_3) ppm. FAB^+ MS: *m/z* 352 $[L^1 + H]^+$.

2.2.2. Bis(1-methyl-2-methylbenzimidazolyl)(2-methylthioethyl)amine (L^{1Me})

Compound $\mathbf{L^{1Me}}$ was prepared in an analogous fashion to $\mathbf{L^1}$, with 1-methyl-2-cloromethylbenzimidazole instead of 1-*tert*-butoxycarbonyl-2-cloromethylbenzimidazole, as reported previously [19]. This procedure affords $\mathbf{L^{1Me}}$ as a colorless solid in 55% yield. Mp: 175–177 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.83 (m, 2H, BzIm), 7.20 (m, 6H, BzIm), 3.94 (s, 4H, BzImCH_2), 3.60 (s, 6H, NCH_3), 2.87 (t, 2H, ^3J = 6.9 Hz,NCH_2), 2.54 (t, 2H, ^3J = 6.9 Hz, SCH_2), 1.76 (s, 3H, SCH_3) ppm. FAB^+ MS: *m/z* 307 [$\mathbf{L^{1Me}}$ - (CH_2CH_2SMe) + H]$^+$.

2.2.3. {[Bis(2-methylbenzimidazolyl)(2-methylthioethyl)amine]copper[bis(perhclorate)]} hydrate [$L^1Cu(ClO_4)_2$]H_2O (Cu-L^1)

To a solution of $\mathbf{L^1}$ (50 mg, 0.14 mmol) in 10 mL ethanol was added $Cu(ClO_4)_2$·$6H_2O$ (53 mg, 0.14 mmol), and the mixture was heated to reflux for 4 h. After cooling to room temperature a green solid started to deposit, volatiles were evaporated under reduced pressure, and the product was washed with 2 × 10 mL of diethyl ether to afford Cu-$\mathbf{L^1}$ as a turquoise solid in 64% yield (50 mg). Mp. 183–185 °C (dec); IR (KBr): 3251, 2922, 1622, 1543, 1496, 1476, 1448, 1381, 1322, 1276, 1219, 1066, 1042, 1000, 956, 927, 844, 777, 746, 704, 619, 494, 454, 432, 410. ESI MS: *m/z*449 [***CuCl-L^1***]$^+$.UV–vis (THF): 257 (4000), 298 (2030), 688 (106). Anal. Calcd. for $C_{19}H_{31}Cl_2CuN_5O_{13}S$[***$L^1Cu(ClO_4)_2$***]·$4H_2O$ (%): C, 32.42; H, 4.44; N, 9.95; S, 4.55. Found: C, 31.95; H, 4.37; N 9.37; S, 4.26.

2.2.4. {[Bis(1-methyl-2-methylbenzimidazolyl)(2-methylthioethyl)amine]copper[bis(perhclorate)]}hydrate[$L^{1Me}Cu(ClO_4)_2$]H_2O(Cu-L^{1Me})

The complex was prepared similarly to Cu-L^1, as previously informed [20]. This procedure affords Cu-L^{1Me} as a turquoise solid in 89% yield. Mp. 223–225 °C; IR (KBr): 3467, 3356, 3099, 3035, 2975, 1614, 1504, 1454, 1362, 1323, 1296, 1251, 1079, 932, 911, 778, 749, 706, 620, 544, 506, 464, 430. ESI MS: *m/z* 441.8 $[L^{1Me}Cu]^+$, 476.8 $[L^{1Me}CuCl]^+$, 540.8 $[L^{1Me}Cu(ClO_4)]^+$.UV–vis (CH_3CN): 326 (1200), 665 (92).Anal. Calcd. for $C_{21}H_{27}Cl_2CuN_5O_5S$ ([$L^{1Me}Cu(ClO_4)_2$]H_2O): C, 42.32; H, 4.57; N, 11.75. Found: C, 42.74; H, 3.89; N, 12.04.

2.3. X-ray diffraction of phospholipid bilayers

Synthetic DMPC (lot 140PC-224, MW 677.9), and DMPE (lot 140PE-54, MW 635.9) were from Avanti Polar Lipids (AL, USA). About 2 mg of each phospholipid were received in Eppendorf tubes and then added 200 μL of (a) distilled water (control), and (b) aqueous solutions of the copper complexes in a range of concentrations. The samples were then incubated for 1 h at 30 °C and 60 °C with DMPC and DMPE, respectively, and relocated into 1.5 mm diameter glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany), centrifuged for 10 min at 2000 rpm and diffracted with Ni-filtered CuKα radiation from a Bruker Kristalloflex 760 (Karlsruhe, Germany) X-ray generator. The relative reflection intensities were obtained in an MBraun PSD-50 M linear position sensitive detector system (Garching, Germany); no correction factors were applied. The experiments were performed at 18 °C ± 1 °C, which is below the main phase transition temperature of both DMPC (24.3 °C) and DMPE (50.2 °C). Higher temperatures would have