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Synthesis and characterization of zinc-silibinin complexes: A potential bioactive compound with angiogenic, and antibacterial activity for bone tissue engineering

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ABSTRACT

Zinc silibinin complex [Zn(sil)(H_2O)$_2$] and mixed ligand zinc complexes such as Zn(silibinin)(phenanthroline) [Zn(sil)(phen)], and Zn(silibinin)(neocuproine) [Zn(sil)(neo)] have been synthesized and characterized. The UV–vis spectra of the Zn(II) complexes showed a considerable shift in the intra-ligand transition. From the IR spectra, it is clear that carbonyl group in the C-ring is involved in the metal chelation besides A/C-ring hydroxyl group. Thermal gravimetric analysis showed that [Zn(sil)(neo)] has higher thermal stability compared to the other two Zn(II) complexes. The potential biological activities of the synthesized complexes were studied systematically. In osteoblast differentiation, silibinin and Zn-silibinin complexes enhanced osteoblast differentiation at the cellular level by increasing calcium deposition and ALP activity, and at molecular level increased osteoblast markers include Runx2, type 1 col, ALP and OC mRNAs expression. Additionally, Zn-silibinin complexes showed promising effects on osteoblast differentiation by regulating miR-590/Smad7 signaling pathway. Among the complexes, Zn(sil)(phen) showed more stimulatory effect on osteoblastic differentiation. These complexes also exhibited angiogenic property by increasing VEGF and Ang 1 expression in mouse MSCs and antibacterial activity against *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) strains. Thus, the present study demonstrated that the Zn-silibinin complexes exhibit great potential as a pharmacological agent for bone tissue engineering.

1. Introduction

Flavonoids are an important class of secondary metabolites in plants, which further divided into subgroups by based on their chemical radical activity. Owing to several attractive health-benefits the flavonoids have been widely studied with respect to their chemistry and biological function [1]. Silibinin is the first member of natural product called flavonolignans and it is the primary ingredient in silymarin which is extracted from the medicinal plant Silybum marianum (milk thistle). Flavonoids are generally represented with ring structures A, B and C- rings where the reactive hydroxyl groups along with carbonyl group are present [2]. The presence of reactive carbonyl and hydroxyl groups the flavonoid has huge biological significance and it is more facile for metal chelation [3]. The literature showed that the metal flavonoids are represented as potential pharmaceutical and therapeutic agents when compared to their parent ligand(flavonoid alone) [2]. For instance, metal flavonoid complexes with varying hydroxyl substitutions have also attracted attention in bone formation [4]. More significantly, silibinin can act as an antioxidant against oxidative stress-related neuropathy [5]. Silibinin has been reviewed as a potential chemopreventive drug against many cancer types including breast, skin, lung, prostate, kidney etc., and silibinin based anti-cancer therapies are under clinical trials [6].

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Silibinin is reported to be an efficient bioactive antioxidant, protecting glutathione in cells and stabilizing the membranes from oxidative stress [7]. It has traditionally been used to treat liver diseases on account of its hepatoprotective properties [8]. In addition, silibinin possesses antitumor [9], anti-bacterial [10], anti-inflammatory [11] activities. Apart from these bioactivities, there are reports emphasized that the Silibinin stimulates human bone marrow stem cells differentiation towards osteoblast [12]. It regulates bone remodeling by promoting osteoblast formation and inhibiting osteoclast function [13]. Silibinin is hydrophobic in nature with multi-ring structure. The major disadvantage of using silibinin is their very poor solubility in solvent or aqueous medium which reduces its water solubility and bioavailability, thus limiting its clinical applications [14]. Hence, there is an immediate need for an alternative approach to overcome the limitations of hydrophobic drugs, to increase their bioavailability, and improve their efficacy. Considering all these, we have rationally designed mixed ligand zinc(II) complexes with silibinin as a ligand and phenanthroline and neocuproine as co-ligands. The synthesized complexes were checked for their potential activity against osteoblast differentiation and angiogenesis.

2. Materials and methods

Typically, silibinin, phenanthroline, neocuproine, zinc(II) sulfate purchased from Sigma-Aldrich (St. Louis, MO, USA). Minimum Essential Medium Eagle (MEM) was purchased from Hi -Media Laboratories and fetal bovine serum (FBS) was purchased from Cistron laboratories. Fetal bovine serum (FBS) was purchased from GIBCO. Both the cells were maintained in Minimal Essential Media (MEM) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 50 μg/ml CO2 at 37 °C. All the reagents used in the study were of analytical grade. Antibody to β-actin and Smad7 were purchased from Santa Cruz Biotechnology, CA, USA and Neo Markers, USA. Mill – Q triply deionized water was employed for further studies.

2.1. Synthesis of zinc complexes

2.1.1. Synthesis of [Zn(sil)(H_2O)$_2$]

Zinc complex was synthesized by dissolving silibinin (0.12 g, 0.5 mmol) in methanol solution followed by the addition of zinc sulfate (0.072 g, 0.5 mmol) Rajalakshmi et al., 2011. A yellow solution was allowed to reflux for 15 min and the color of the solution turned faint yellow. After 30 min it was filtered and the faint yellow solution was allowed to dry at room temperature. The complex was recrystallized from acetonitrile solution. Yield: 86%. Found: C, 43.23; H, 3.36; S, 4.79. Anal Calcd for $C_{24}H_{23}SZnO_{16}$: C, 43.36; H, 3.49; S, 4.82.

2.1.2. Synthesis of [Zn(sil)(phen)]

[Zn(sil)(phen)] was synthesized by refluxing methanolic solution of $Zn(SO_4)_2{\cdot}7H_2O$ (0.072 g, 0.5 mmol) with silibinin (0.12 g, 0.5 mmol) for 15 min. Subsequently, phenanthroline (0.045 g, 0.5 mmol) was added to the above solution and refluxed continuously for another 30 min [15]. A pale turbid yellow precipitate obtained after 30 min was filtered, and washed with chloroform and dried in vacuum. The yellow tint white solid was recrystallized from acetonitrile solution. Yield: 78%. Found: C, 53.31; H, 3.39; N, 3.28; S, 3.88. Anal Calcd for $C_{36}H_{27}ZnN_2O_{14}S$: C, 53.44; H, 3.36; N, 3.46; S, 3.96.

2.1.3. Synthesis of [Zn(sil)(neo)]

Complex [Zn(sil)(neo)] was synthesized by the same procedure as followed for [Zn(sil)(phen)] instead of phen, neocuproine (0.052 g, 0.5 mmol) was added [16]. A pale turbid yellow precipitate obtained immediately after the addition of neocuproine. The solid was filtered and allowed to evaporate at room temperature. The complex was recrystallized from acetonitrile solution. Yield: 69%. Found C, 54.39; H, 3.68; N, 3.28; S, 3.91. Anal Calcd for $C_{38}H_{31}ZnN_2O_{14}S$: C, 54.52; H, 3.73; N, 3.35; S, 3.83.

2.2. Instrumentation

Absorption spectral measurements for the ligands and the zinc(II) complexes were carried out on Schimadzu UV-1800 spectrophotometer using 1 mL quartz cuvettes of 1 cm pathlength. The electronic spectra were recorded in methanol/acetonitrile. The IR spectra for the ligands and the corresponding complexes were recorded using Perkin-Elmer RX I FT-IR spectrophotometer. The samples were prepared as pellets using KBr and the spectrum were monitored in the region of 4000–400 cm^{-1}. Thermogravimetric analysis was carried out using TGA Q50 TA instruments. Scanning was carried out from ambient temperature to 800 °C in the presence of nitrogen gas with the sample purge flow of 40 mL min^{-1} and balance purge flow of 60 mL min^{-1}. The electrobalance calibration was done using a reference material like calcium oxalate. The sample was loaded in a platinum pan and the analysis was carried out at the heating rate of 20 °C min^{-1}. It measures the amount and rate of weight change in a material, either as a function of increasing temperature or isothermally as a function of time, in a controlled atmosphere. Percentage of weight loss verses temperature was examined.

2.3. Cytotoxicity and cell morphology

Mouse mesenchymal stem cells, MSCs (C3H10T1/2) and human osteoblast-like cells (MG-63) were obtained from the National Center for Cell Sciences (NCCS), Pune, India. It was cultured under standard culture conditions, 5% CO_2, 37 °C, 10% FBS containing DMEM. MTT assay was used to assess the cellular cytotoxicity. The MG-63 cells were platted at the concentration of $3 \times 10^4/cm^2$ in 24 well plates and used for MTT assay. DMSO was used to dissolve the silibinin and Zn-silibinin derivatives (Zn(sil)(H_2O)$_2$, Zn(sil)(phen) and Zn(sil)(neo)) to get 100 mM stock solution. Different concentration (20–120 μM) of silibinin and Zn-silibinin derivatives were administrated with monolayer cells and MTT assay was performed as described previously [17]. For cell morphology analysis the mouse MSCs were seeded at the concentration of $3 \times 10^4/cm^2$ in 24 well plates and 60 μM concentration of silibinin and Zn-silibinin derivatives were treated for 48 h. At the end of treatment period, the cells were washed with 1X PBS twice and fluorescein diacetate (FDA) solution (30 μg/ml) was added. Lastly, the cell morphological analysis was done under a fluorescent microscope with a 20 × objective [18].

2.4. Alkaline phosphatase activity

MG63 cells were seeded in 24-well plates with the concentration of $3 \times 10^4/cm^2$ and treated with the 60 μM concentration of silibinin and Zn-silibinin derivatives. Alkaline phosphatase (ALP) activity was measured as previously described [19]. Briefly, the cells were washed with ice-cold PBS and fixed with 10% formalin and 1:1 ratio of ethanol and acetone. The ALP solution was prepared as described elsewhere [19] and the solution was incubated with the fixed cells at 37 °C for 20 min. ALP substrate, p-nitrophenyl phosphate (4 mg/ml) was added and read at 405 nm by spectrophotometer. The OD was plotted as a bar diagram.