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DNA intercalation and cleavage studies of plumbagin and phenanthroline-based Cu(II) complex, [Cu(PLN)(PHEN)]NO_3

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Abstract
Plumbago indica L. is a plant belonging to the family Plumbaginaceae and is used in traditional system of medicine worldwide including Sri Lanka. The roots of these plants are rich in plumbagin with antioxidant, anti-inflammatory, antibacterial, antifungal and also anticancer activities. Although plumbagin has been shown to be more toxic to cancer cells, it has also been shown to be toxic to normal cells at high concentrations. Therefore, design of a “hybrid drug molecule” of plumbagin with an appropriate transition metal may provide an opportunity to reduce the toxicity and enhance the therapeutic potential of plumbagin in its use for cancer treatment. The metal complex [Cu(PLN)(PHEN)]NO_3 was synthesized and characterized by IR and UV-Visible spectroscopy and ESMS. The synthesized metal complex is able to intercalate to DNA replacing ethidium bromide and is also able to cleave plasmid pBR322 DNA successfully. These findings may lead to the possible use of plumbagin as a “hybrid drug molecule” for the treatment of human cancers.

Keywords: *Plumbago indica* L., plumbagin, DNA intercalation, DNA cleavage, anticancer

1. Introduction

Since ancient times humans have relied on plant materials for the treatment of various diseases including cancer [1]. Although, a plethora of natural compounds isolated from plants have been found to possess anticancer activity and several such compounds have shown to be remarkably effective in preclinical settings, the usage of these compounds to diagnose and treat cancer in humans still remains a challenge [2]. The reasons behind this may be attributed to inefficient systemic delivery, poor bioavailability and reduced oxidative stability associated with these natural compounds. Thus, novel strategies are needed to overcome these problems to enable natural compounds to be effective therapeutic agents in modern medicine [3].

Since the success of cisplatin and its related platinum complexes as anticancer agents, a large number of metal complexes containing synthetic ligands have been synthesized as anticancer agents [4, 5]. In recent years, enormous attention has been turned towards the design of “hybrid drug molecules” with the coordination of natural compounds with transition metals to augment the outcome of natural compounds for cancer treatment [6]. Copper is a bio essential element that plays a key role in the endogenous oxidative DNA damage associated with aging and cancer [7]. Copper is less toxic than nonessential transition metals such as platinum and its anticancer properties are also well documented [8]. Thus, copper has become the transition metal of choice for coordination of natural compounds.

Plumbago indica L., called “Rathnitul” in Sinhala is a plant belonging to the family Plumbaginaceae that possess many medicinal properties and are extensively used in the traditional system of medicine worldwide including Sri Lanka [9, 10]. The root of these plants are a rich source of plumbagin (PLN), a hydroxy-naphthoquinone (5-hydroxy-2-methyl-1, 4-naphthoquinone), which is one of the simplest secondary metabolites found in plants [10]. Research in the last several decades has shown that plumbagin is associated with a wide range of potential therapeutic effects. These are attributed to the highly potent biological activities exhibited by plumbagin, which include antioxidant, anti-inflammatory, antibacterial and antifungal activities [9]. In addition, plumbagin has also been reported to possess anticancer activity [9]. Although plumbagin has been shown to be more toxic to cancer cells, it has also been shown to be toxic to normal cells at high concentrations [9]. Plumbagin, in addition to possessing the common problems associated with natural compounds, is also volatile.

Therefore, design of a "hybrid drug molecule" of plumbagin with an appropriate transition metal may provide an opportunity to reduce the toxicity and enhance the therapeutic potential of plumbagin in its use for cancer treatment. Anticancer properties of plumbagin, [Cu $(PLN)_2$] and [Cu (bipy) $(PLN)]^+$ have already been reported [9,11]. Thus, the present work was undertaken to explore the feasibility of the coordination of Cu(II) with plumbagin and anticancer ligand, phenanthroline (PHEN) for the generation of a novel plumbagin-based "hybrid drug molecule" with pleiotropic action against human cancers.
Here, we report the synthesis and characterization of plumbagin and phenanthroline-based copper (II) metal complex, [Cu (PLN) (PHEN)] NO_3 and its DNA intercalation and cleavage studies against human DNA and plasmid pBR322 DNA respectively.

2. Materials and Methods

2.1 Plant material

Juvenile plants of *Plumbago indica* L. were obtained from the Botanical Garden of the Bandaranaike Memorial Ayurveda Research Institute (BMARI), Nawinna, Sri Lanka (April, 2014) and grown in 4:1, pot soil : sand medium. After 6 months plants were removed from soil, roots were separated, washed, wiped and cut in to small pieces and was used for the study. A voucher specimen of *Plumbago indica* L. was deposited in the herbarium of the Botany division of the BMARI (reference number 638 e).

2.2 Solvents, chemicals and reagents

Solvents, chemicals and reagents were purchased from Sigma-Aldrich Chemie (Germany) unless otherwise stated. Ethidium bromide, plasmid pBR322 DNA and agarose (molecular grade) were purchased from Promega cooperation (USA). Water was used after distillation through GFL distillation apparatus. Tris-HCl-NaCl buffer solution (5 mM Tris, 50 mM NaCl, pH was digitally adjusted to 7.4 using HCl with a Sartorius professional meter, Tris = tri (hydroxymethyl) aminomethane) was prepared using double distilled water. TBE buffer solution (2 mM EDTA, pH was digitally adjusted to 8 using NaOH with a Sartorius professional meter, 89 mM boric acid, 89 mM Tris, EDTA = ethylenediaminetetraacetic acid) was prepared using double distilled water. Genetech Molecular Diagnostics and School of Gene Technology, Colombo 08, Sri Lanka provided human DNA.

2.3 Instrumentation

UV-Visible spectra were obtained using Perkin Elmer Lambda 35, double beam scanning spectrophotometer equipped with optical cells photo diode detector. IR spectra were obtained using a Thermo Nicolet iS10 FT-IR spectrometer. Mass spectrometry was carried out using Micromass LCT (ESMS) instrument. Fluorescence was measured using E-gel imager UV Transilluminator with E-gel R imager software.

2.4 Extraction of plumbagin

The extraction of plumbagin was carried out according to a previously published method [12]. Fresh roots of *P. indica* (20 g) were refluxed with hexane (100 mL) for 2 hours. The hexane layer was extracted with limewater (25 mL x 3). The aqueous layer was neutralized with 2 M HCl and extracted with hexane (25 mL x 3). The hexane layer was evaporated under *vacuum* and the crude plumbagin obtained was purified by recrystallization using hexane as the solvent. Recrystallized plumbagin was obtained as orange colour needles (0.36 g, 0.72 % on fresh weight basis) and was characterized by GC-MS, IR and UV-Visible spectroscopy and melting point determination.

2.5 Synthesis of [Cu (PLN) (PHEN)] NO_3 complex

The synthesis of plumbagin and phenanthroline-based Cu (II) complex was carried out according to a previously published method [11]. A mixture of plumbagin (PLN) (0.113 g, 0.6 mmol) containing CH_3ONa (0.032 g, 0.6 mmol) and $Cu(NO_3)_2 \cdot 3H_2O$ (0.290 g, 1.2 mmol) in methanol (20 mL) was refluxed for 2 hours. To this 1, 10-phenanthroline (PHEN) (0.119 g, 0.6 mmol) in methanol (15 mL) was added and refluxed further for 2 hours. The resulting solution after filtration and slow evaporation produced a dark-red crystalline product. This was purified by recrystallization using methanol as the solvent. Recrystallized [Cu (PLN) (PHEN)] NO_3 complex was obtained as dark-red crystals (0.232 g, 69%) and was characterized by IR and UV-Visible spectroscopy and mass spectrometry.

2.6 DNA intercalation studies

DNA intercalation property of [Cu (PLN) (PHEN)] NO_3 was carried out using a developed method. A 10 µg/mL human DNA solution and 2 µg/mL ethidium bromide solution were prepared using the Tris-HCl-NaCl buffer. A concentration series of the metal complex (25, 50, 100, 150, 200, 300, 400, 500, 600, 800, and 1000 µM) was also prepared using the Tris-HCl-NaCl buffer. Experimental solutions were prepared in eppendorf tubes by mixing 5 µL of human DNA solution with 10 µL of ethidium bromide solution. Negative control 1 was prepared by mixing 5 µL of human DNA solution with 10 µL of ethidium bromide solution and 5 µL of buffer. Negative control 2 was prepared by mixing 10 µL of ethidium bromide solution and 10 µL of buffer. All the solutions were incubated in the dark for 15 minutes. After which, to each experimental solution 5 µL from each of the metal complex solution was added and mixed well. Then 20 µL from each solution was pipetted out on to a parafilm as droplets and were visualized by using the E-gel imager UV transilluminator.

2.7 DNA cleavage studies

Gel electrophoretic mobility shift assay was carried out according to a previously published method to investigate the ability of [Cu (PLN) (PHEN)] NO_3 to cleave DNA [11, 13]. A 0.1 µg/mL supercoiled plasmid pBR322 DNA solution was prepared using the Tris-HCl-NaCl buffer. Two stock solutions of the metal complex (200 and 500 µM) were also prepared using the Tris-HCl-NaCl buffer. Experimental solutions were prepared in eppendorf tubes by mixing 5 µL of DNA solution and correct volumes of the metal complex stock solution and buffer to maintain the final volume as 20 µL and concentration of the metal complex as 10, 50, 100, 150, 200 and 250 µM respectively in the final solution. The negative control was prepared in an eppendorf tube by mixing 5 µL of DNA solution and 15 µL of buffer. All the solutions were incubated in the dark for 1 hour at 37 ^{0}C. The solutions upon loading were electrophoresed for 2 hours at 50 V on 0.8% agarose gel in TBE buffer containing 0.5 µg/mL ethidium bromide and then visualized using E-gel imager UV transilluminator.

3. Results and Discussion

Plumbagin was extracted from *P. indica* roots using hexane, which is the most suitable solvent according to previously published reports [12]. The crude plumbagin thus obtained was recrystallized also using hexane to produce pure plumbagin as orange colour needles (0.36 g, 0.72 %). This was characterized by GC-MS, UV-Visible and IR spectroscopy. The purity of plumbagin was verified by melting point determination. In GC-MS studies the gas chromatogram showed a single peak and corresponding mass spectrum exhibited a peak at m/z 188 which tallied with the molecular weight of plumbagin. In the IR spectrum of plumbagin in methanol the two carbonyl groups; the hydrogen bonded one and the other appeared at 1640 cm^{-1} and 1660 cm^{-1} respectively. The UV-Visible spectrum of plumbagin in methanol showed an intense band at λ_{max} 210 nm and an less intense band at λ_{max} 265 nm due to π-π^* transitions and a weak band at λ_{max} 412 nm due to a n-π^* transition. The melting point of plumbagin was 76-78 ^{0}C. GC-MS, IR and UV-Visible spectroscopic results and melting point were in accordance with previously published data [14, 15] and confirmed that plumbagin has been successfully isolated and is in pure form to be used for metal complexation studies.

The metal complex [Cu (PLN) (PHEN)] NO_3 (Figure 1) was synthesized by refluxing a mixture of PLN, CH_3ONa and Cu $(NO_3)_2 \cdot 3H_2O$ in methanol followed by the addition of PHEN and further refluxation [11]. The crude obtained after the slow evaporation of the reaction mixture was recrystallized using methanol to yield the pure metal complex as dark-red crystals (0.25 g, ≈ 69 %). This was characterized by UV-Visible and IR spectroscopy and ESMS. IR spectroscopic studies of the metal complex were carried out in methanol. Of the two C=O groups of plumbagin only the hydrogen bonded C=O group should be affected by metal complexation. Accordingly the C=O stretching of the hydrogen bonded C=O group was observed at 1620 cm^{-1} shifted from 1640 cm^{-1} while no change was observed for the C=O stretching of the free C=O group. The C-H stretchings of PHEN were observed at 845 and 785 cm^{-1} shifted from 720 and 625 cm^{-1} due to coordination of PHEN nitrogen atoms with the metal. The shifts observed for C=O stretching of PLN and C-H stretchings of PHEN are in accordance with published reports for metal complexation [13, 14]. The UV-Visible spectrum of PLN in methanol showed an intense band at λ_{max} 210 nm and an less intense band at λ_{max} 265 nm due to π-π^* transitions and a weak band at λ_{max} 412 nm due to a n-π^* transition. The UV-Visible spectrum of PHEN in methanol showed an intense band at λ_{max} 230 nm due to a π-π^* transition and a less intense band at λ_{max} 262 nm due to a n-π^* transition. The UV-Visible spectrum of the metal complex had shifted absorption bands that of both PLN and PHEN indicating the presence of both PLN and PHEN ligands in the metal complex. Most importantly, ESMS showed a single peak at m/z 430 corresponding to the molecular weight of $[Cu (PLN) (PHEN)]^+$ confirming that [Cu (PLN) (PHEN)] NO_3 has been successfully synthesized.

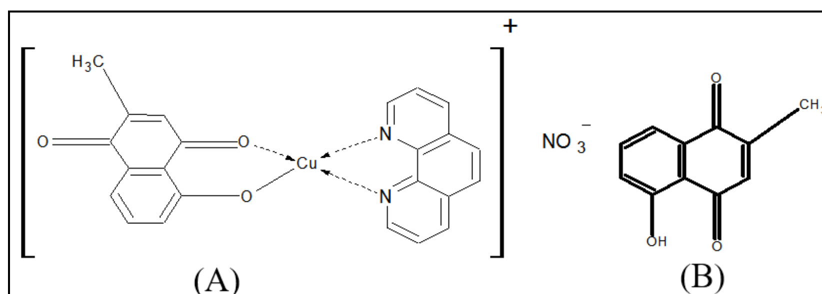

Fig 1: Structures of (A) - [Cu (PLN) (PHEN)] NO_3 complex and (B) - Plumbagin

Ethidium bromide is a cationic dye that intercalates between the base pairs of nucleic acids producing a fluorescent emissive complex [16]. This was first reported by Le-Pecq *et al.* in 1967. When non-emissive molecules intercalate to DNA replacing ethidium bromide in the ethidium bromide bound DNA complex a reduction in the fluorescence emission is observed. This reduction in the fluorescence emission is useful to investigate the ability of non-emissive molecules to intercalate to DNA. In the present study aliquots of human DNA and ethidium bromide giving the maximum fluorescence emission was mixed with increasing concentrations of the synthesized metal complex separately. The fluorescence emission produced by ethidium bromide bound DNA complex gradually decreased in the presence of increasing concentrations of the metal complex and was not observed at and beyond 400 µM of the metal complex when observed using E-gel imager UV transilluminator (Figure 2). Negative control 1 which had human DNA and ethidium bromide produced the maximum fluorescence emission while negative control 2 which had only ethidium bromide did not produce any fluorescence emission when observed using E-gel imager UV transillumintor. According to the results obtained the synthesized metal complex, [Cu (PLN) (PHEN)] NO_3 is able to successfully intercalate to DNA replacing ethidium bromide.

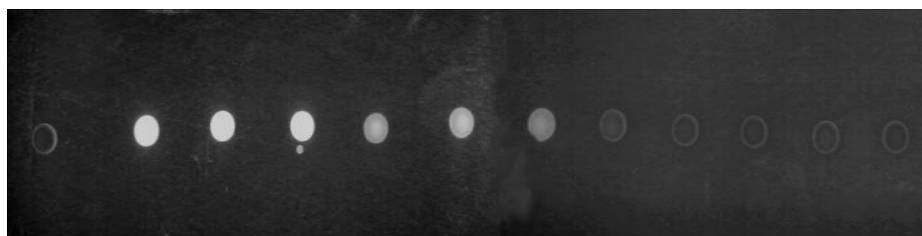

Fig 2: Effect on fluorescence emission of ethidium bromide bound human DNA on varying concentrations of [Cu (PLN) (PHEN)] NO_3 (Left to right: negative control 2, negative control 1 and ethidium bromide bound human DNA in the presence of 25µM, 50µM, 100µM, 150µM, 200µM, 300 µM, 400 µM, 500 µM, 600 µM, and 700 µM metal complex)

Plasmid pBR322 DNA has a superhelical circular structure. In gel electrophoresis superhelical circuler DNA (Form I) has the highest migration rate while nicked circular DNA (Form II) formed when one DNA strand of superhelical circuler DNA is cleaved has the slowest migration rate. When both strands of superhelical circuler DNA are cleaved linear DNA (Form III) is generated which has a migration rate in between Form I and II in gel electrophoresis [13]. The ability of the synthesized metal complex, $[Cu(PLN)(PHEN)]NO_3$ to cleave DNA was studied by agarose gel electrophoretic mobility shift assay using plasmid pBR322 DNA. Ethidium bromide was used to visualize DNA in agarose gel under E-gel imager UV transilluminator. According to the results obtained the synthesized metal complex cleaved DNA into Form II and III simultaneously, however, more of Form II was observed (Figure 3). This was more pronounced at higher concentrations of the metal complex and superhelical circuler DNA was hardly observed at 250 µM of the metal complex. These results confirm that the synthesized metal complex is able to cleave DNA successfully.

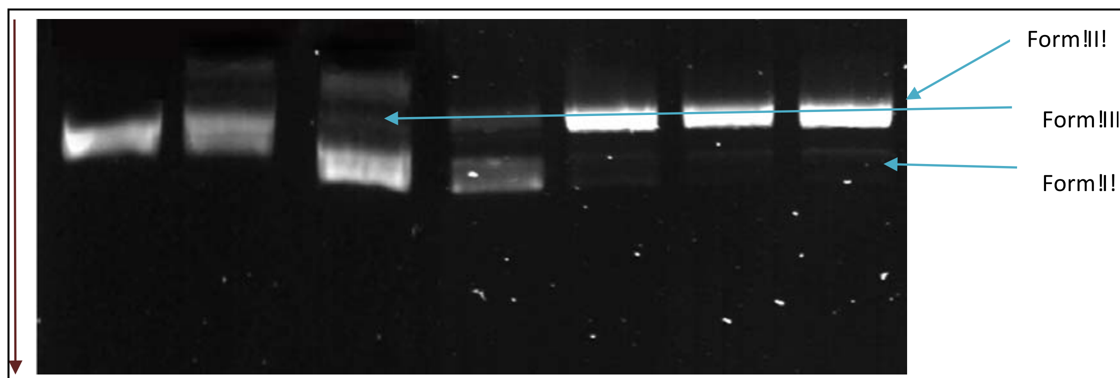

Fig 3: Electrophoretic behaviour of plasmid pBR322 DNA by [Cu (PLN) (PHEN)] NO_3 complex. (Left to right: lane 1 - plasmid pBR322 DNA alone, lanes 2-7 – plasmid pBR322 DNA in the presence of 10 µM, 50 µM, 100 µM, 150 µM, 200 µM and 250 µM metal complex)

4. Conclusion

A plumbagin and phenanthroline-based copper (II) metal complex, [Cu (PLN) (PHEN)] NO_3 has been synthesized and characterized. The synthesized metal complex is able to successfully intercalate and cleave DNA. These findings may lead to the possible use of plumbagin as a “hybrid drug molecule” for the treatment of human cancers.

5. Acknowledgement

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