Synthesis, characterization and antitumor activity of benzaldehyde nitrogen mustard picolinoyl hydrazone complexes

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Summary

Cu^{II}, Ni^{II}, Co^{II}, Zn^{II} and Cd^{II} complexes of picolin-amido-4-bis(2-chloroethyl)aminobenzaldimine (PBAB) have been synthesized and characterized by elemental analysis, conductivity, i.r. u.v.-vis. and e.p.r. spectra. The results suggest a square planar structure for the Cu^{II} complex, a tetrahedral geometry for the Zn^{II} and Cd^{II} complexes and an octahedral structure for the Co^{II} and Ni^{II} complexes. The complexes have been screened for antitumor activity against SMMC-7721 human liver cancer cell line; all of them exhibit biological activity at high concentrations for the cell line studied and a synergic effect between Cu^{II} and PBAB is evident.

Introduction

Nitrogen mustards have been used as antitumor agents and it is well known that this kind of compound can exert carcinostatic action against transplanted animal tumors[1]. However, lack of selectivity towards tumor cells and frequent high toxicity have often limited their use as practical chemotherapeutic agents in the management of human neoplastic disease[1]. Seeking for higher effect-lower toxicity antitumor agents is an active research field. Benzaldehyde nitrogen mustard and picolinic acid hydrazide both have significant biological activity, and their derivatives have been widely investigated[2–3]. The antitumor activity of certain transition metal such as cisplatin is also well known[4–5]. However, reports on metal complexes of nitrogen mustards are few[6–7]. It has been shown[7] that the stability of nitrogen mustard in water can be enhanced by coordination to a metal. This might also allow for the development of slow release drugs, whilst the synergic effect between metals and drugs is also worthy of study. Based on this, we have modified a literature procedure[8–9] to synthesize a picolinoyl hydrazone of benzaldehyde nitrogen mustard. New transition metal complexes of this ligand have been prepared and investigated for antitumor activity.

Experimental

All the chemicals used were AR grade reagents. Benzaldehyde nitrogen mustard was made by a three-step synthesis. In the first step, cold ethylene oxide (88 g, 2 mol) was added to a cold solution of freshly distilled aniline (93 g, 1 mol) and 50 g of 1% aqueous AcOH solution. The mixture was heated slowly to 70 °C during 2 h and kept at this temperature for 20 h. The white solid product, *N,N*-bis(hydroxyethyl)aniline, was iso-

lated under reduced pressure (206–208 °C, 15 mmHg); yield 162 g, 90%. The next two reactions were carried out according to references[8–10]. Picolinic acid hydrazide was made by a two-step synthesis. In the first step, 5 cm^3 of concentrated H_2SO_4 was added slowly to a cold suspension of picolinic acid (12.3 g, 0.1 mol) in 40 cm^3 absolute EtOH, and the mixture was refluxed for 26 h. The cold solution was poured into ice-water and extracted with Et_2O. The white product was obtained by removing solvent, yield is 7.0 g, 46%. In the second reaction, $N_2H_4 \cdot H_2O$ (85%, 4 ml, 0.09 mol) was added to a solution of ethyl picolinate (7 g, 0.046 mol) in EtOH (40 ml), the mixture was refluxed for 2 h and cooled to room temperature. White needle crystals of picolinic acid hydrazide were isolated by filtration and washed with EtOH; yield 2.5 g, 40%. The hydrazone (PBAB) was prepared by the following procedure; a solution of benzaldehyde nitrogen mustard (2.46 g, 0.01 mol) in absolute EtOH (30 cm^3) was added to a solution of picolinic acid hydrazide (1.37 g, 0.01 mol) in absolute EtOH (20 cm^3). The mixture was refluxed under acid catalysis (one drop AcOH) for 0.5 h; the yellow precipitate which formed on cooling the mixture to room temperature was collected by filtration, washed with absolute EtOH and recrystallized from MeCN; yield 3.1 g, 85%.

Preparation of the complexes

In a typical preparation, a solution of hydrated copper (II) nitrate (48 mg, 0.2 mmol) in THF (5 cm^3) was added dropwise to a solution of PBAB (146 mg, 0.4 mmol) in THF (10 cm^3) with stirring, whereupon a yellow-green precipitate formed immediately. The mixture was continuously stirred for 8 h to ensure complete precipitation, then the product was filtered off, washed with THF and dried *in vacuo*; yield 88 mg, 90%.

MTT assay

SMMC7721 human liver cancer cells were propagated continuously in culture on Eagles MEM medium supplemented with 10% inactived fetal calf serum (FCS) and antibiotics. Monolayer cells harvested from exponential phase were treated using trypsin (0.05%) and then diluted with medium and counted using a hemocytometer, single-cell suspension solutions (1×10^5/ml) were prepared, and then inoculated into 96 well plates to a volume of 100 μl/well (1×10^4 cell/well). The plates were incubated in a humidified atmosphere of 90% N_2-5% O_2-5% CO_2 for 24 h, then the compound to be studied was added in concentration gradient, and the final concentration was maintained at 125, 100, 75 and 50 μmol$\cdot l^{-1}$. The plates were incubated at 37 °C

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for 48 h. After the culture medium was removed from the wells, 100 μl (1 mg/cm^3) MTT solution of (3-[4,5-dimethyl(thiazol-2-yl)-2,5-diphenyl] tetrazolium bromide was added to each well and the plates were incubated for 4 h. The remaining MTT solution was then removed and 150 μl DMSO was added to each well to dissolve the formazan crystals[11].

Absorbance readings on each well were measured on an ELISA spectrophotometer at 570 nm. Blank wells whose absorbances were zero contained no cells or medium, but MTT solution was added and removed after incubation, and 150 μl DMSO was then added. The control wells were consistent with the experimental wells except that they did not contain the compounds to be studied.

The survival ratio (SR) is expressed as $A/A_0 \times 100\%$, where A is average absorbance of the experimental wells and A_0 the average absorbance of the control wells. The logarithm of concentration of the compounds and SR were treated by linear regression analysis and ID$_{50}$ values were obtained from the equations. ID$_{50}$ was defined as the drug concentration required to reduce the number of live cells by 50%.

The elemental analyses were performed on an Elementar Vario. EL instrument (Elementar Vario. EL, Germany). I.r. spectra were recorded in the range 4000–200 cm^{-1} using KBr pellets with a 170SX-FTIR spectrometer (Nicolet, U.S.A.). ^{1}H n.m.r. spectra were recorded on an AM-400 instrument (Brucker, Switzerland) using DMSO-d$_6$ as solvent. Molar conductances of the complexes in DMSO were measured at room temperature with a DDS-11A type conductivity bridge (Shanghai analytical instrument factory, China). E.p.r. spectra were recorded on an ER200-SRC instrument (Brucker) in the polycrystalline state at room temperature. Electronic spectra were obtained with a Shimadzu u.v.-240 spectrophotometer.

Results and discussion

Elemental analyses for free PBAB and its complexes are presented in Table 1. All of the complexes are stable in air. They are slightly soluble in ethanol and acetone, and also easily soluble in DMF and DMSO. The molar conductivity data in DMSO show that the copper, cobalt and nickel complexes are non-electrolytes, whilst the zinc and cadmium complexes are 1:2 electrolytes[12].

The ^{1}H n.m.r. spectrum of free PBAB shows signals at 11.91 and 8.52 ppm for the NH and CH=N protons respectively. Signals due to the pyridine ring protons occur as multiplets between 8.08–8.70 ppm (4H; $J_{3,4} = 3.4$ Hz; $J_{4,5} = 5.84$ Hz; $J_{5,6} = 6.28$ Hz). The benzene ring protons appear in the range 6.6–7.6 ppm (dd; 4H; $J = 6.6$ Hz) and the signals due to the CH$_2$CH$_2$ protons are at 3.72 and 3.81 ppm. The i.r. absorbances (Table 2) at 1687 and 1598 cm^{-1} can be assigned to v(C=O) and v(C=N) (azomethine), which differ from the benzaldehyde nitrogen mustard and picolinic acid hydrazide; in the spectrum of the latter, v(C=O) appears at 1660–1670 cm^{-1}, hence this is evidence for the formation of a hydrazone[13], with PBAB in mainly the keto form[14].

I.r. spectra

The i.r. absorbances of free PBAB and its complexes are presented in Table 2. Free PBAB shows a band assigned to v(N—H) at 3268 cm^{-1}. For the other complexes apart from the copper species, this band remains almost unchanged after complexation, precluding the possibility of coordination through the imine nitrogen atom[3]. The negative shift of the amide-I ($\Delta v = 19$–54 cm^{-1}), positive shifts of the amide-II ($\Delta v = 10$ cm^{-1}) and the amide-III ($\Delta v = 6$–9 cm^{-1}) bands compared to the free ligand indicate involvement of the carbonyl oxygen atom in coordination[15]. Ring breathing[15], in-plane bending and out-of-plane bending[16] vibrations of the pyridine are shifted to appreciably higher frequencies in the spectra of the complexes, consistent with coordination through the ring nitrogen[15]. This is further supported by the presence of a v(M—N) band in the 270–280 cm^{-1} region[17].

In the spectrum of the copper(II) complex, the absence of v(NH), amide-I, amide-II and amide-III bands and appearance of new bands due to v(N=C—O) at 1542 and 1387 cm^{-1}[18], indicate enolization and

Table 1. The elemental analytic data and molar conductivities

| Compound | Found (Calcd.) % | | | | Λm |
	M	C	N	H	(Ω^1 cm^2 mol^{-1})
PBAB	–	55.9(56.0)	15.3(15.2)	5.0(5.2)	–
[Cu(PBAB-H)NO$_3$]	13.4(13.2)	41.7(41.4)	14.3(14.4)	3.5(3.9)	25
[Cd(PBAB)$_2$](NO$_3$)$_2$	11.6(11.9)	42.2(42.4)	14.5(14.7)	3.8(4.0)	69
[Ni(PBAB)$_2$(NO$_3$)$_2$]	6.4(6.6)	44.7(44.9)	15.3(14.9)	4.0(4.4)	20
[Zn(PBAB)$_2$](NO$_3$)$_2$	7.1(7.1)	44.4(44.1)	15.2(15.4)	3.9(4.2)	64
[Co(PBAB)$_2$(NO$_3$)$_2$]	6.5(6.2)	44.8(44.9)	15.3(15.0)	4.0(4.3)	23

Table 2. Important i.r. bands of ligand and complexes (cm^{-1})

Compound	amide-I	amide-II + δ_{NH}	amide-III	v(C=N) azomethine	pyridine breathing	in-plane bending	out-plane bending	v(MO/MN)
PBAB	1687	1508	1236	1598	966	633	401	–
Cu(PBAB-H)NO$_3$	–	–	–	1593	1000	658	410	398/271
Cd(PBAB)$_2$(NO$_3$)$_2$	1658	1518	1241	1594	1014	658	409	393/280
Ni(PBAB)$_2$(NO$_3$)$_2$	1634	1518	1244	1597	1019	647	421	390/273
Zn(PBAB)$_2$(NO$_3$)$_2$	1637	1518	1243	1598	1018	677	416	393/280
Co(PBAB)$_2$(NO$_3$)$_2$	1633	1518	1244	1596	1018	642	413	393/273

deprotonation of the keto group upon coordination[14]. Positive shifts of v(N—N) (33 cm^{-1}), ring-breathing (44–53 cm^{-1}), in-plane bending (9–44 cm^{-1}) and out-of-plane bending (8–20 cm^{-1}) vibrations of the pyridine ring suggest coordination through the pyridine ring nitrogen[19]. The v(C=N) (azomethine) band remains unaffected after complexation, indicating non-involvement of the azomethine nitrogen atom in coordination. Bands at 1474, 1030, 810, 1284 and 729 cm^{-1} can be assigned to v_1–v_5 of NO_3^-; the combination band $v_2 + v_5$ appears at 1760 cm^{-1}, suggesting that the NO_3^- ions acts as a bidentate ligand[20] in the copper(II) complex. The unidentate nature of NO_3^- in the cobalt and nickel complexes is demonstrated by the appearance of i.r. bands at 1474–1475, 1243–1244, 1018–1019, 858–868 and 753–754 cm^{-1} [21].

E.p.r. spectrum of the copper complex

The e.p.r. spectrum of the polycrystalline copper complex recorded at room temperature shows two peaks (Figure 1); the weak signal at low field and the strong one at high field can be assigned to $g_\parallel$ and $g_\perp$ respectively. The values of these parameters were calculated as 2.360 and 2.072 respectively. $^2B_1$ is the ground state for most copper complexes[22] when the unpaired electron is in a $d_{x^2-y^2}$ orbital, while $^2A_1$ is the ground state when an unpaired electron is in a d_{z^2} orbital ($g_\parallel > g_\perp > 2.00$). In the present case, the $g_\parallel > g_\perp$, therefore, the unpaired electron is likely to be in the $d_{x^2-y^2}$ orbital. The observation ($g_\parallel > g_\perp > 2.04$) indicates a square planar geometry around the copper(II) ion. Also observation that $g_\parallel > 2.3$ is indicative of the ionic nature of the complex[22]. The G parameter obtained from the expression $(g_\parallel - 2)/(g_\perp - 2)$ is greater than 4, suggesting that exchang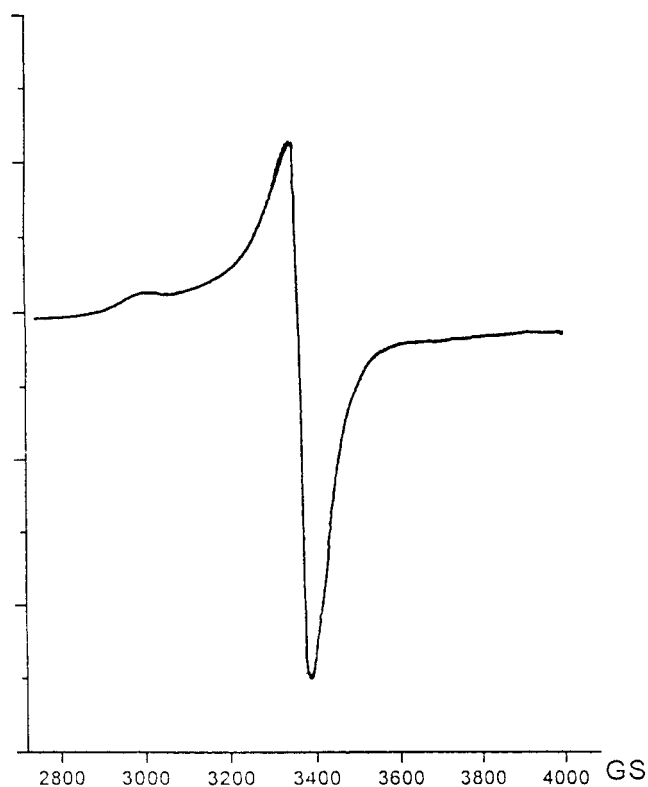e interactions between copper(II) centers in the solid state are negligible. No signal at half field was observed in the spectrum, ruling out the possibility of a dimeric form[24].

Electronic spectra

The electronic spectrum of free PBAB in DMSO solution shows bands at 278 and 333 nm, attributed to $\pi - \pi^*$ transitions of the side chain imine group and to the pyridine ring in resonance with the C=O group respectively[25]. In the complexes, the considerable increase in intensity of the pyridine ring band compared to free PBAB indicates that the ring nitrogen is involved in coordination[26]. Red shifts (8–10 nm) are observed for all the complexes. Absorption bands (in nujol) for the cobalt complex at 520–500 nm and 480–440 nm may be tentatively assigned to $^4T_{1g}(F) \rightarrow {}^4A_{2g}(v_2)$ and $^4T_{1g}(F) \rightarrow {}^4T_{1g}(P)(v_3)$ transitions characteristic of octahedral cobalt[15,22]. The electronic spectrum of the nickel complex shows only a strong broad peak at 440 nm, which can be assigned to the $^3A_{2g} \rightarrow {}^3T_{1g}(F)$ transition. The v_1 band[25] expected at 943–930 nm would lie outside the range of the spectrophotometer, however an octahedral nickel geometry may be tentatively proposed. The band at 740–720 nm for the copper complex may be assigned to the $^2B_{1g} \rightarrow {}^2A_{1g}$ and $^2B_{1g} \rightarrow {}^2E_g$ transitions. The position of this band indicates a square planar stereochemistry[27]. A high energy band at 480–400 nm may be assigned to ligand-metal charge transfer.

Based on the above mentioned data, an octahedral geometry for the cobalt(II) and nickel(II) complexes, a square planar structure for the copper(II) complex and a tetrahedral geometry for the zinc(II) and cadmium(II) complexes can be tentatively proposed (Figure 2).

Antitumor activity

MTT assay is a novel method for quantifying metabolically viable cells through their ability to reduce a soluble yellow tetrazolium salt to blue-purple formazan crystals[28]. The crystals are thought to be produced by the mitochondrial enzyme succinate dehydrogenase[29] and can be quantified by redissolving and measuring the absorbance of the resultant solution correlating with the number of live cells. The results of studies of the biological activities of the complexes are given in Table 3. The results show that all the complexes have a definite antitumor activity. Although the parent metal nitrates have some cytotoxicity effect at high concentration, the effect is not evident at their ID_{50} dosage (SR > 90%). The ID_{50} values of the copper complexes are smaller than the ID_{50} of free PBAB, so it is believed that there is a synergic effect between copper and PBAB, although
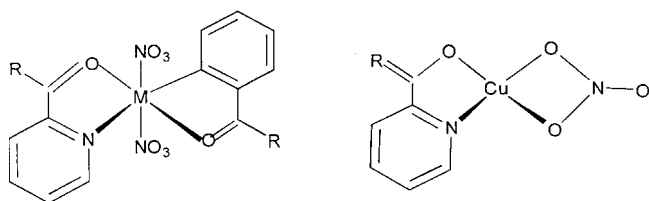

Figure 1. E.p.r. spectrum of the copper complex at room temperature.

Figure 2. Proposed structures of the complexes. R = NHN=CHC$_6$H$_4$N(CH$_2$CH$_2$Cl)$_2$, M = Co or Ni.

Table 3. *In vitro* cytotoxicities of the compounds (ID values in μmol L^{-1})

Compound	ID_{50}
PBAB	54.6
[Cu(PBAB-H)NO$_3$]	32.5
[Ni(PBAB)$_2$(NO$_3$)$_2$]	71.2
[Co(PBAB)$_2$(NO$_3$)$_2$]	65.7
[Zn(PBAB)$_2$](NO$_3$)$_2$	94.1
[Co(PBAB)$_2$(NO$_3$)$_2$]	30.4

this effect however the situation is not evident in the other complexes.

Acknowledgement

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