Copper(II) and manganese(III) complexes of N'-[(2-hydroxy phenyl) carbonothioyl] pyridine-2-carbohydrazide: novel therapeutic agents for cancer

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Abstract

c-Src is a non-receptor tyrosine kinase which plays a significant role in the growth mediated signaling pathway impacting cellular proliferation, differentiation, mobility, survival and transformation. Myristoylation of pp60^{c-src} leads to its membrane association and activation, a process catalyzed by N-myristoyltransferase (NMT). We have shown earlier increased NMT activity in the early stages of colon cancer. A novel sulfur nitrogen donor ligand and its Cu(II) and Mn(III) complexes have been prepared and characterized using various physicochemical analyses. These Cu(II) and Mn(III) complexes showed cytotoxicity against the colon cancer cell line HT29. The IC₅₀ for Cu(II) and Mn(III) complexes were 12.2 and 16.1 μM, respectively. HT29 cells treated with Cu(II) and Mn(III) complexes induced apoptosis and inhibited endogenous NMT activity. Furthermore, they induced higher levels of hsc70 and inhibited the expression of c-Src. Inhibition of endogenous NMT activity by metal complexes was demonstrated for the first time. This study also suggested that NMT activity is crucial for cell survival and demonstrated that cessation in activity results in apoptosis. These metal complexes may prove to be novel therapeutic agents for cancer targeting NMT.

Keywords

Metal complexes; Antitumor; Apoptosis; N-myristoyltransferase; hsc70; src

1. Introduction

Cancer is one of the leading causes of premature death worldwide. Colorectal cancer is one of the major causes of death, particularly in the western world [1,2]. A giant leap in

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understanding the genetic basis of cancer was made possible by the sequencing of the human genome.

The worldwide success of cisplatin, *cis*-diamminedichloroplatinum(II) [3–6], as a best selling anticancer drug has led to the discovery of many transition metal complexes [7]. Other than cisplatin and its derivative carboplatin which are routinely used as anticancer drugs [8] a new platinum drug called oxali-platinum is in clinical use for colorectal cancer [9]. The toxicity associated with platinum drugs limits its extensive use in the treatment of cancer. Earlier transition metals are less toxic and have shown promising antitumor activity [10–14].

Protein modification, translocation and/or abnormal expressions are associated with the onset of cancer. Recently, there has been a major thrust on understanding the lipidic modification of proteins. Protein N-myristoylation is a lipidic modification which involves the covalent attachment of myristate, a 14 carbon saturated fatty acid, to the N-terminal glycine residue of a number of mammalian, viral and fungal proteins [15–17]. In general, N-myristoylation is an irreversible co-translational protein modification. N-myristoylation ensures the proper functioning and intracellular trafficking of proteins. Many proteins involved in a wide variety of a signal cascade, cellular transformations and oncogenesis are myristoylated [15–17].

Myristoylation of proteins is catalyzed by the ubiquitously distributed eukaryotic enzyme N-myristoyltransferase (NMT) [16,17]. The cDNAs encoding NMT from humans, yeast, bovine spleen, malaria parasite and higher plants have been characterized [15–18]. NMT in lower eukaryotes is encoded by a single copy gene whereas higher eukaryotes such as bovine, human and plant NMTs are encoded by two gene copies. The second genetically distinct mammalian NMT cDNA (NMT-2) has been cloned from a human liver library and the respective mouse homologues for the two human NMTs have also been cloned [19].

NMT has been implicated in various diseases and studies from various laboratories including ours have established NMT as an emerging therapeutic and drug target [20–24]. Using a rat model we have demonstrated for the first time that NMT is more active in colonic epithelial neoplasms than in the corresponding normal appearing colonic tissue. This increase in NMT activity appears at an early stage in colonic carcinogenesis [22]. Increased NMT activity was also observed in human colonic tumors and was predominantly cytosolic [24]. Furthermore, colorectal tumors showed increased immunohistochemical staining for NMT compared to normal mucosa [24]. In addition, gallbladder carcinoma displayed strong cytoplasmic positivity for NMT with an increased intensity in the invasive component. Normal gallbladder mucosa showed weak to negative cytoplasmic staining [25]. Attenuation of NMT activity may prove to be a novel therapeutic protocol for cancer. A large number of compounds of diverse structures and proteins are capable of inhibiting NMT [26–30]. For example NMT activity can be regulated by NMT inhibitor proteins (NIP71) [31] which is homologous to heat shock cognate protein 70 (hsc70) [32]. Furthermore, we have recently shown that enolase is a potent inhibitor of NMT [30]. We have shown that early transition metal complexes of thiohydrazides have potent antitumor activity in vitro and in vivo [13]. While there have been no reports demonstrating metal complexes as potent inhibitors of

NMT activity, we report in this paper two novel metal complexes possessing antitumor and anti-NMT activity. A new nitrogen–sulfur donor ligand N'–[(2-hydroxyphenyl) carbonothioyl] pyridine-2-carbohydrazide (H₂L) and its Cu(II) and Mn(III) complexes have been prepared and characterized by element analyses, magnetic susceptibility measurements, ¹H and ¹³C NMR, IR and UV–visible spectroscopy. The physicochemical studies indicate that the complexes [Cu(HL)Cl] and [Mn(HL)(L)] are square planar and octahedral, respectively. The modes of bonding of the ligand in these complexes have been studied through IR spectroscopy. FAB mass spectra of the ligand as well as [Mn(HL)(L)] have been obtained in order to determine the molecular ion peak and various other fragments. Various biochemical assays were employed to assess the bioactivity of the ligand and metal complexes.

2. Materials and methods

[9,10-³H] Myristic acid (39.3 Ci mMol⁻¹) was purchased from Perkin Elmer (USA). *Pseudomonas* acyl CoA synthetase was purchased from ICN Biochemicals (USA). PVDF membrane and powdered milk were purchased from BioRad Laboratories (Canada). The monoclonal antibody to NMT-1, NMT-2 and HRP-conjugated goat anti-mouse antibody was obtained from BD-Biosciences, Canada. Monoclonal anti-c-Src antibody was purchased from Santa Cruz Biotechnology, Inc. (USA) and monoclonal anti-hsc70 was bought from Ce-darlane Laboratories (Canada). Cell culture reagents, DMEM, and fetal bovine serum were purchased from Invitrogen/GIB-CO (Canada). Disposable plastic tissue culture plates and other supplies were from VWR Canlab. (Canada). The chemiluminescence reagent plus was obtained from NEN Life Science products, USA. The peptide substrate based on the N-terminal ends of pp60^{c-src} (GSSKSKPKR) was synthesized by the Alberta Peptide Institute, Canada. Recombinant human NMT (hNMT) was purified as described previously in [33]. β-actin monoclonal antibody and general laboratory reagents of analytical grade were obtained from Sigma Chemical Co. (Canada and USA). Picolinic acid hydrazide (PAH) [34], ammonium polysulfide and (2-hydroxyphenyl) carbonothioyl thioacetic acid were prepared by literature methods [35].

2.1. Synthesis of ligand (H₂L)

N'–[(2-hydroxyphenyl) carbonothioyl] pyridine-2-carbohydrazide (H₂L) was synthesized by reacting (2-hydroxyphenyl) carbonothioyl thioacetic acid (2.28 g, 10 mM) and PAH (1.38 g, 10 mM), each dissolved separately in 1 equiv. of an aqueous solution of NaOH (1 N, 10 ml). The above reaction mixture was allowed to stand at 25 °C for 2 h, ice cooled and then 20% acetic acid was added drop wise to precipitate the product. The addition of acetic acid was terminated when pH became slightly acidic. The creamish yellow product was filtered, washed with water, dried and recrystallized from a 50:50 (v/v) mixture of methanol and chloroform in the presence of charcoal, finally washed with diethyl ether and dried under vacuum. Yield 1.08 g (40%). M. Wt. 273, m.p. 192 °C, elemental analyses, found (calculated) (%): C; 56.6 (57.1), H; 3.9 (4.1), N; 14.8 (15.3), *m/z* 274 (M + 1).

2.2. Preparation of metal complexes

[Cu(HL)Cl] was obtained by adding a methanolic solution of CuCl₂·2H₂O (2 mM) drop wise to a solution of H₂L (0.55 g, 2 mM) in a MeOH/CHCl₃ mixture (50% v/v) with stirring, whereupon precipitation of the complexes commenced. After 2 h of stirring the solid was filtered and washed with water, methanol and dried in vacuo. [Mn(HL)(L)] was synthesized as above except Mn(CH₃COO)₂·4H₂O (1.0 mM) was added to H₂L (2.0 mM).

[Cu(HL)Cl]: Green, M. Wt. 370.9, m.p. > 300 °C, yield 72%, ϵ_{eff} (BM) 1.79, elemental analyses (%); found (calculated) – C; 42.6 (42.4), N; 11.5 (11.4), Cu; 17.6 (17.1), Cl; 9.8 (9.6).

[Mn(HL)(L)]: Dark green, M. Wt. 597.98, m.p. > 300 °C, yield 70%, ϵ_{eff} (BM) 4.87, elemental analyses (%); found (calculated) – C; 52.4 (52.1), N; 13.9 (14.0), Mn; 9.1 (9.2), m/z 599 (M + 1).

2.3. Physical measurements

The metal content was estimated using standard procedures after decomposing the complexes with aquaregia followed by conc. H₂SO₄. The chloride was estimated as AgCl. Carbon, hydrogen and nitrogen were estimated on a Carlo Erba 1108 model microanalyzer. Magnetic susceptibility measurements were made at 25 °C on a Cahn–Faraday balance using Hg[Co (NCS)₄] as calibrant. Electronic spectra were recorded on a Cary 100 Varian EL01055314 UV–visible spectrophotometer in DMSO. IR spectra were recorded in the 4000–400 cm^{−1} region as KBr pellets on a JASCO FT/IR-5300 spectrophotometer. ¹H and ¹³C NMR spectra were recorded in DMSO-d₆ on a JEOL AL300 FT NMR spectrometer using TMS as an internal reference. ESR spectra were recorded on an X-band spectrometer model EPR-112 using DPPH as a <g> marker. The FAB mass spectra were obtained on a JEOL SX 102 mass spectrometer using nitrobenzyl alcohol as a matrix.

2.4. Cell culture

The HT29 colon cancer cell line was obtained from ATCC. Cells were grown in DMEM supplemented with 10% fetal calf serum, 1% antibiotic–antimycotic solution (GIBCO) containing penicillin G sodium, streptomycin sulfate and amphotericin B in humidified air and 5% CO₂ at 37 °C. Cells were harvested after 48 h upon treatment with various concentrations of H₂L, [Cu(HL)Cl] or [Mn(HL)(L)]. Cells were lysed in RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 10 mM DTT, 1 mM PMSF and 1% protease inhibitor cocktail (Sigma).

2.5. NMT assay

NMT activity was assayed as described earlier in [34]. Briefly, [³H]myristoyl-CoA was synthesized according to the literature [34]. The reaction mixture contained 40 mM Tris–HCl, pH 7.4, 0.1 mM EGTA, 10 mM MgCl₂, 5 mM ATP, 1 mM LiCoA, 1 μ M [³H]myristic acid (7.5 μ Ci) and 0.3 unit ml^{−1} *Pseudomonas* acyl-CoA synthetase in a total volume of 200 μ l. The reaction was carried out for 30 min at 30 °C. The conversion to [³H]myristoyl-CoA was generally greater than 95%. The assay mixture contained 40 mM Tris–HCl, pH 7.4, 0.5 mM EGTA, 0.45 mM 2-mercaptoethanol, 1% Triton X-100, pp60^{src} derived peptide (500

μM) and NMT in a total volume of 25 μl. The transferase reaction was initiated by the addition of freshly generated [³H]myristoyl-CoA and was incubated at 30 °C for 30 min. The reaction was terminated by spotting 15 μl aliquots of incubation mixture onto P81 phosphocellulose paper discs and drying under a stream of warm air. The P81 phosphocellulose paper discs were washed in two changes of 40 mM Tris-HCl, pH 7.3 for 60 min. The radioactivity was quantified in 7.5 ml of Beckman Ready Safe Liquid Scintillation mixture in a Beckman Liquid Scintillation Counter. One unit of NMT activity was expressed as 1 pmol of myristoyl peptide formed per min.

2.6. NMT inhibition assay

[Cu(HL)Cl] and [Mn(HL)(L)] were assayed for their inhibitory activity against standard hNMT. Purified recombinant hNMT (0.2 μg per assay) was assayed in the presence of pp60^{src} derived peptide substrate and metal complexes. A control experiment was performed in the absence of metal complexes and the hNMT activity was considered as 100%. All other conditions were as described above.

2.7. Western analysis

Western blot analysis was performed essentially as described previously in [35]. Samples were electrophoresed on a SDS-PAGE and transferred to PVDF membrane. Trans-blotted PVDF membrane was incubated with blocking buffer (PBS-Tween 20 plus 5% powdered milk) for 1 h at room temperature to block non-specific binding. After washing, the blot was incubated at 4 °C overnight with monoclonal antibody against NMT-1, NMT-2, hsc70, pp60^{c-src} or β-actin. After washing, the blot was incubated with HRP-conjugated goat anti-mouse secondary antibody and the bands for NMT-1, NMT-2, hsc70, pp60^{c-src} and β-actin were detected using the chemiluminescence reagent plus and exposed to X-ray films.

2.8. Cytotoxic assay

The MTT assay was employed to determine the cytotoxicity of the ligand and the metal complexes. The MTT assay was performed using a standard procedure [36]. HT29 cells were grown in a 96 well plate and treated with various concentrations of H₂L, [Cu(HL)Cl] or [Mn(HL)(L)]. IC₅₀ values were calculated by plotting concentration against % cell proliferation.

2.9. Apoptosis assay

HT29 cells were cultured in chambered slides in the presence of [Cu(HL)Cl] or [Mn(HL)(L)] at different concentrations. Control experiments included HT29 cells treated with or without DMSO or DMSO-DMF. After 48 h, treatment cells were fixed and permeabilized. Apoptotic cells were detected by the TUNEL assay. The apoptosis assay was performed using the Apoptosis Detection System, Fluorescein (Promega) following the manufacturer's protocol. Apoptotic cells having fluorescently labeled DNA were visualized under a fluorescence microscope. The percentage of apoptotic cells were calculated as the ratio of green fluorescent cells to total cells. The experiments were done twice in triplicates and five fields were counted for each experiment.

2.10. Other methods

Proteins were estimated by the Bradford method [37]. GraphPad Prism® software was used for statistical analysis.

3. Results and discussion

3.1. Chemistry

The ^1H NMR spectrum of H_2L in $\text{DMSO}-d_6$ shows two signals at δ 11.40 and 12.80 ppm due to the presence of two $-\text{NH}-\text{NH}-$ protons. Another signal at δ 4.15 ppm is due to the phenolic proton. The assignment of the OH proton has been made by comparing the ^1H NMR spectrum of H_2L with that of $[\text{N}'(2\text{-methoxyphenyl})\text{carbonothioyl}]$ pyridine-2-carbohydrazide which lacks the above signal. On comparing the spectrum of H_2L with that of its precursor, PAH, signals at δ 7.06 (t, 1H), 7.14 (d, 1H), 7.46 (t, 1H) and 8.74 (d, 1H) ppm were assigned to the pyridine ring protons. ^{13}C NMR spectrum displays 13 signals due to the presence of 13 carbon atoms. Two peaks at δ 169.46 and 163.8 ppm are due to the presence of $\text{C}=\text{S}$ and $\text{C}=\text{O}$ carbons, respectively. Significant chemical shifts for pyridine and phenyl ring carbons were observed in the H_2L spectrum when compared with that of PAH suggesting that H_2L is a new entity. The IR spectrum of the ligand reveals that it remains in thione form (Fig. 1a) wherein in solution and in the presence of metal ion it tautomerized to thiol form (Fig. 1b). Thione form in solid state is attributed to the absence of $\nu(\text{SH})$ band at 2700 cm^{-1} and the presence of a band at 3216 cm^{-1} due to $\nu(\text{NH})$.

$[\text{Cu}(\text{HL})\text{Cl}]$ was formed by loss of one proton from the ligand via enolization/thioenolization whereas $[\text{Mn}(\text{HL})(\text{L})]$ was obtained by loss of one and two protons from each ligand yielding Mn(III) complex by aerial oxidation. Complexes are soluble in coordinating solvents such as DMSO and DMF. Copper complex shows a magnetic moment of 1.79 BM revealing the presence of one unpaired electron and the electronic spectrum shows a band at $20,620\text{ cm}^{-1}$ (ϵ 165) due to the envelope of $^2\text{B}_{1g} \rightarrow ^2\text{A}_{1g}, ^2\text{B}_{2g}, ^2\text{E}_g$ transitions suggesting a square planar geometry around Cu(II). To understand the coordination mode of the ligand and its binding with metal ion, IR spectral studies were carried out. IR spectrum of $[\text{Cu}(\text{HL})\text{Cl}]$ displays a band at 3543 cm^{-1} assigned to $\nu(\text{OH})$ of the ligand. There is a significant change in the bands due to $\nu(\text{C}=\text{O})$, $\nu(\text{C}=\text{S})$, thioamide I, thioamide II and $\nu(\text{N}-\text{N})$ as compared to those of the free ligand. A negative shift of $62\text{--}64\text{ cm}^{-1}$ in $\nu(\text{C}=\text{O})$ and the presence of new bands at 828 cm^{-1} for $\nu(\text{C}-\text{S})$ and at 437 cm^{-1} for $\nu(\text{M}-\text{N})$ suggest bonding through carbonyl oxygen, thiolato sulfur and hydrazinic nitrogen. Square planar geometry of $[\text{Cu}(\text{HL})\text{Cl}]$ is further supported by its solid state ESR spectrum at 25°C which shows a broad signal with $\langle g \rangle$ value of 2.068. This is characteristic of a square planar geometry around Cu(II). Magnetic moment of 4.87 BM and the electronic spectral bands at $15,505$ (ϵ 260) and $20,450$ (ϵ 400) cm^{-1} due to the $^5\text{B}_{1g} \rightarrow ^5\text{T}_{2g}$ and $^5\text{E}_g$ transitions, respectively, indicate the presence of Mn(III) in an octahedral geometry for $[\text{Mn}(\text{HL})(\text{L})]$. IR spectrum of $[\text{Mn}(\text{HL})(\text{L})]$ shows peaks at 1665 and 1601 cm^{-1} assigned to $\nu(\text{C}=\text{O})$ and $\nu(\text{C}=\text{N})$ of NCO, respectively. This indicates that one of the two ligands is bonding in the keto form while the other is present in enolic form. Similarly, two peaks at 913 and 820 cm^{-1} for $\nu(\text{C}=\text{S})$ and $\nu(\text{C}-\text{S})$, respectively, indicate that one ligand is bonded in the thione form while the other is in the thiolato form. Similar observations were found for

$\nu(\text{OH})(\text{phenolic})$, $\nu(\text{M-Npy})$ and $\nu(\text{M-O})(\text{phenolic})$. Thus the two ligands are bonding with Mn(III) in altogether different fashion. Out of two ligands, one acts as a mononegative tridentate, bonding through thiolato sulfur, hydrazine nitrogen and pyridine nitrogen, while the other acts as dinegative tridentate, the bonding sites being the enolic oxygen, hydrazine nitrogen and phenolate oxygen. Based on above studies the tentative structure of Cu(II) and Mn(III) complexes is shown in Fig. 2.

3.2. Cytotoxic studies

Metal complexes with sulfur and nitrogen donor ligands have been shown to possess antitumor activity [12–14]. To assess whether these newly synthesized ligand and its metal complexes possess any antitumor activity we initially performed cell proliferation assay in the presence of these compounds. Recently, oxaliplatin, EloxatinTM (Sanofi) has been approved for colorectal cancer in the USA. Since Eloxatin is a transition metal complex, we wished to study the effect of H₂L and its metal complexes on the HT29 colon cancer cell line. HT29 cells were treated with various concentrations of H₂L and its metal complexes. The antiproliferative or cytotoxic properties of these compounds were evaluated by the MTT assay. The IC₅₀ values are shown in Table 1. The Cu(II) and Mn(III) complexes showed potent cytotoxic effect. Therefore, we used only metal complexes in our further experiments.

3.3. Inhibition of NMT activity

NMT has been shown to be crucial for the cell viability in lower organisms. NMT knockout in *Drosophila* resulted in various embryonic cytoskeletal and cell morphologic defects and apoptotic cell death [38]. Very recently, we demonstrated that Nmt1 is essential for early development in mammals and is the NMT employed in early embryogenesis [39]. In recent times, NMT has emerged as a potential therapeutic target for cancer especially colon cancer [21,40]. To investigate the mechanism of action of [Cu(HL)Cl] and [Mn(HL)(L)] in tumor cells, we performed a NMT inhibition assay. HT29 cells were treated with 12.2 and 16.1 μM of [Cu(HL)Cl] and [Mn(HL)(L)], respectively, for 48 h. The treatment concentration was decided on the basis of cytotoxicity data. Cell lysates were evaluated for the NMT activity. Interestingly, on treatment with metal complexes the endogenous NMT activity was inhibited significantly. Treatment with [Cu(HL)Cl] inhibited endogenous NMT activity by 48% whereas, [Mn(HL)(L)] inhibited NMT activity by 30% (Fig. 3). This is the first report demonstrating inhibition of NMT activity by metal based compounds. Further to establish that these metal complexes inhibit NMT activity in vitro, the NMT activity of hNMT was assayed in the presence of DMSO, [Cu(HL)Cl] or [Mn(HL)(L)]. Various concentration of the Cu(II) complex was used to elucidate the 50% inhibition concentration (IC₅₀). Table 1 shows the in vitro NMT inhibition by the Cu(II) complex. However, the Mn(III) complex precipitated in the reaction mixture of the NMT assay and therefore it is difficult to obtain a dose dependent curve for it. Fig. 4 shows the inhibition curve for the Cu(II) complex. The in vitro NMT inhibition by metal complexes suggests that endogenous inhibition of NMT activity in HT29 cells may be due to the direct inhibition of NMT.

3.4. Western analysis

The decreased endogenous NMT activity in HT29 cells upon treatment with [Cu(HL)Cl] and [Mn(HL)(L)] could be due to the direct interaction of these complexes at the mRNA level

resulting in decreased expression of the NMT1 and/or NMT2 or it may be due to the induction of the inhibitor proteins of NMT. To investigate the effect of these complexes on the expression of NMT, Western analysis was performed. Interestingly, NMT1 protein expression remained almost unperturbed, whereas NMT2 expression was significantly lowered by the treatment of these compounds (Fig. 5). We have already reported the functional redundancy of NMT1 and NMT2 and that NMT1 is the main NMT in the early stage of embryogenesis [39]. Also, it has been shown that dioxine selectively induces NMT2 mediated carcinogenesis [41]. The down regulation of NMT2 expression by these compounds is novel. We also observed a slight decrease in NMT1 protein expression upon [Mn(HL)(L)] treatment and NMT2 expression upon DMSO treatment. To further correlate the inhibitory effect of these novel compounds on NMT activity in view of their ability to abolish the expression of NMT2, we investigated the expression profile of its substrate and regulators. Treatment of HT29 cells with [Cu(HL)Cl] and [Mn(HL)(L)] altered the expression of c-Src and hsc70. Overexpression and/or activation of c-Src is genetically and biochemically linked to the development of several human cancers, especially those of the colon and breast [42,43]. c-Src plays a central role in numerous signaling pathways impacting cellular proliferation, transformation, differentiation, motility and survival [44–46]. Myristoylation of c-Src renders membrane association and activation [47]. c-Src is a physiological substrate of NMT and therefore we determined its expression. The expression of c-Src lowered dramatically upon treatment with these compounds (Fig. 5). Since NMT activity could be regulated by its potent inhibitor protein, hsc70 [48], we investigated the expression profile of hsc70 on treatment with the metal complexes. As shown in Fig. 4 [Cu(HL)Cl] and [Mn(HL)(L)] induced the expression of hsc70. Induction of hsc70 may be one of the factors in regulating the total endogenous NMT activity in the HT29 cell line. We also treated the HT29 cells with another cytotoxic compound E, E-3-(3,4-methylenedioxyphenylmethylene)-1-(4-nitrophenylmethylene)-2-oxo-3,4-dihydro-1H-naphthalene [49] as a positive control to study whether there is decrease in expression of NMT2 and c-Src protein in general during apoptosis. As seen in Fig. 4E, E-3-(3,4-methylenedioxyphenylmethylene)-1-(4-nitrophenylmethylene)-2-oxo-3,4-dihydro-1H-naphthalene does not decrease NMT2 and Src expression suggesting that these metal complexes specifically alter the expression of NMT2, c-Src and hsc70.

3.5. [Cu(HL)Cl] and [Mn(HL)(L)] induces apoptosis in HT29 cells

NMT has been shown to be crucial for the cell viability in lower organisms [38]. The antiproliferative and NMT inhibitory effects of these complexes led us to investigate the mode of action of these compounds. The decreased numbers of cells were due to cell death. We employed the TUNEL assay to assess the apoptotic cells (Fig. 6). DNA fragmentation is a hallmark feature of apoptotic cells. Cells having fragmented DNA can be labeled with fluorescein tagged dUTP by terminal deoxynucleotidyl transferase. These apoptotic cells can then be visualized under a fluorescent microscope. HT29 cells were treated with 12.2 and 16.1 μ M of [Cu(HL)Cl] and [Mn(HL)(L)], respectively, for 48 h. Fig. 6 shows representative images of HT29 cells treated with or without Cu(II) and Mn(III) complexes; the negative control being treatment with DMSO or DMSO-DMF alone (Fig. 6I–III). [Cu(HL)Cl] and [Mn(HL)(L)] induced 72% and 80% apoptosis, respectively (Fig. 6IV and V). Since these complexes inhibit endogenous NMT activity and induce apoptosis, it is possible that

attenuation of NMT activity and expression may contribute to the apoptotic process. Recently, Ducker et al. [50] have shown that ablation of NMT2 has a pronounced effect in inducing apoptosis. Our results corroborate these findings wherein metal complexes selectively reduced NMT2 expression and resulted in apoptosis.

Further studies are in progress to understand the substrate specificity of NMT1 and NMT2. Studies pertaining to the treatment of tumor-bearing animals for the evaluation of in vivo bioactivity of these compounds are also underway.

4. Conclusions

In this report we have described the syntheses of two novel metal complexes which inhibit endogenous NMT activity and specifically reduce NMT2 expression. The decreased expression of NMT2 resulted in the induction of apoptosis. To the best of our knowledge this is first report where metal complexes have shown anti-NMT activity. This study suggests that this is the mechanism of action of metal complexes on inducing apoptosis in a colon cancer cell line.

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Abbreviations

hsc70	heat shock cognate protein 70
NIP	N-myristoyltransferase inhibitor protein
NMT	N-myristoyltransferase

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Fig. 1.
Tentative structure of the ligand N'-[(2-hydroxyphenyl) carbonothioyl] pyridine-2-carbohydrazide (H₂L).

[Cu(HL)Cl]

[Mn(HL)(L)]

Fig. 2.
Tentative structure of [Cu(HL)Cl] and [Mn(HL)(L)].

Fig. 3.

Endogenous NMT activity. HT29 cells (I) were treated with DMSO (II), DMSO-DMF (III), 12.2 μ M [Cu(HL)Cl] (IV) or 16.1 μ M [Mn(HL)(L)] (V) for 48 h. Cells were lysed in RIPA buffer and NMT activity was carried out using pp60^{src} substrate as described under “Section 2”. Values are mean of three independent experiments carried out in duplicates $\pm$ standard deviation.

Fig. 4.

Dose dependent inhibition of NMT by [Cu(HL)Cl]. Purified recombinant human NMT (0.2 μg) was incubated in the presence of various concentration of [Cu (HL)Cl] and its activity was determined as described under “Section 2”. Values are mean of three independent experiments carried out in duplicates $\pm$ standard deviation.

Fig. 5.

Western blot analysis. Proteins (25 µg) from HT29 cells treated with or without DMSO, DMSO-DMF, [Cu(HL)Cl] or [Mn(HL)(L)] were subjected to 10% SDS-PAGE, transblotted onto PVDF membrane and was probed with monoclonal anti-NMT1, NMT2 (1:250 dilution), c-Src or hsc70 (1:1000 dilution) antibody. Lane 1: Control; Lane 2: DMSO; Lane 3: DMSO-DMF; Lane 4: [Cu(HL)Cl]; Lane 5: [Mn(HL)(L)]; Lane 6: E, E-3-(3,4-methylene-dioxyphenylmethylene)-1-(4-nitrophenylmethylene)-2-oxo-3,4-dihydro-1H-naphthalene. β-actin has been used as an internal protein loading control.

Fig. 6.

Induction of apoptosis by [Cu(HL)Cl] or [Mn(HL)(L)]. HT29 cells (I) were treated with DMSO (II), DMSO-DMF (III), 12.2 μ M [Cu(HL)Cl] (IV) or 16.1 μ M [Mn(HL)(L)] (V) for 48 h as described under “Section 2” and then evaluated for apoptosis by TUNEL assay. Cells with green fluorescent nuclei are apoptotic cells. Nucleus was stained with propidium iodide (red).

Table 1

In vitro cytotoxicity and anti-NMT activity of Cu(II) and Mn(III) complexes of N'-[(2-hydroxyphenyl) carbonothioyl] pyridine-2-carbohydrazide (H₂L)

	Compounds	IC ₅₀ (μM)
	HL ₂	50.0 ± 2.6
Cytotoxicity	[Cu(HL)Cl]	12.20 ± 0.8
	[Mn(HL)(L)]	16.10 ± 0.5
In vitro anti-NMT activity	[Cu(HL)Cl]	77.0 ± 2.5

Cytotoxicity was evaluated by MTT assay. NMT inhibition assay was carried out as described under "Section 2".