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Anticancer activity and DNA-binding investigations of the Cu(II) and Ni(II) complexes with Coumarin Derivative

Taofeng Zhu[1†], Yuan Wang[2†], Weiliang Ding[3†], Jun Xu[2], Ruhua Chen[1], Jing Xie[4], Wenjiao Zhu[3], Lei Jia[2*], Tieliang Ma[3*]

1. Department of Respiratory, the Affiliated Yixing Hospital of Jiangsu University, Yixing, Jiangsu Province, China

2. Department of Physics and Chemistry, Henan Polytechnic University, Jiaozuo, Henan Province, China

3. Central Laboratory, the Affiliated Yixing Hospital of Jiangsu University, Yixing, Jiangsu Province, China

4. Department of Geriatrics, the Affiliated Yixing Hospital of Jiangsu University, Yixing, Jiangsu Province, China

†: Contributed equally.

*: Corresponding Author: Tieliang Ma, E-mail: matieliang@foxmail.com. The Affiliated Yixing Hospital of Jiangsu University, #75 Tongzhenguan Road, Yixing, Jiangsu, 214200, China.

Tel: +86-510-87921196; Fax: +86-510-87921110

Lei Jia, Email: jlxj@hpu.edu.cn. Henan Polytechnic University, Jiaozuo, Henan, 454000, China . Tel.: +86-391-3987818.

Abstract

Two new copper(II) (**2**) and nickel(II) (**3**) complexes with a new coumarin derivative have been synthesized and structurally characterized. The DNA-binding activities of the two complexes have been investigated by spectrometric titrations, ethidium bromide displacement experiments, CD (circular dichroism) spectral analysis and viscosity measurements. The results indicate that the two complexes, especially the complex **2**, can strongly bind to calf-thymus DNA (CT–DNA). The intrinsic binding constants K_b of the complexes with CT–DNA are 2.99×10^5 and 0.61×10^5 for **2** and **3**, respectively. Comparative cytotoxic activities of the two complexes are also determined by MTT assay. The results show that the drugs designed here have significant cytotoxic activity against the human hepatic (HepG2), human promyelocytic leukemia (HL60) and human prostate (PC3) cell lines. Cell apoptosis was detected by AnnexinV/PI flow cytometry and the results show that the two copper complexes can induce apoptosis of the three human tumor cells. In conclusions, the two complexes show considerable cytotoxic activity against the three human cancer and induce apoptosis of the threes.

Keywords: anticancer activity; coumarin derivative; DNA-binding properties; human tumor cell lines; cell apoptosis

Introduction

Currently, cancer is the second cause of death, accounting for about a quarter of all deaths [1]. During past decades, the synthesis and studies of metal complexes with active drugs as

ligands increase the interest for inorganic, pharmaceutical and medicinal chemistry and concentrate much attention as an approach to new drug development [2-4]. Transition metals appear more appealing for this purpose because they can support a multitude of coordination numbers and geometries that go far beyond sp, sp^2 and sp^3 hybridization of carbon [5] and the metal complexes have been found to be more active and desirable drugs than the ligands themselves which may be due to the in vivo formation of metallic complexes [6].

Among the ligands employed for drug design, the compounds known as coumarin (1,2-benzopyrone) serves as an important pharmacophore in drug discovery [7]. As their ability to inhibit human immunodeficiency virus integrase, coumarin derivatives have also been evaluated in the treatment of human immunodeficiency virus [8,9]. Further it is evident from the literature [10-13] that, the transition and rare earth complexes of hydroxycoumarin derivatives are also subjects of increasing interest in bioinorganic and coordination chemistry [14].

In this paper, we focused our interests in synthesizing and evaluating the key DNA-binding interactions of a new coumarin derivative containing a large planar aromatic ring systems and its novel copper(II) and nickel(II) complexes. In our studies, the interaction of the two complexes with calf-thymus (CT) DNA was investigated using different kinds of spectrophotometric methods and viscosity measurements. The studies suggest that intercalative binding mode appears to be acceptable. The work represents the first assessment of the potential application of the two coumarin-metal complexes as novel therapeutic agents for the treatment of cancer.

1. Materials and Methods

1.1 Materials

All starting materials were obtained commercially and used as received. Calf thymus DNA (CT-DNA) and ethidium bromide (EB) were obtained from Sigma Chemical Co. All the measurements involving the interactions of the three metal complexes with CT DNA were carried

out in doubly distilled water buffer containing 5 mM Tris and 50 mM NaCl, and adjusted to pH 7.1 with hydrochloric acid. UV-vis spectrometer was employed to check the solution of CT-DNA purity (A_{260}: A_{280} > 1.80) and the concentration (ε = 6600 M^{-1} cm^{-1} at 260 nm) in the buffer . The ternary copper(II) complexes were dissolved in a mixture solvent of 1 % CH_3OH or 1 % DMF and 99 % Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, pH 7.1) at concentration 1×10^{-3}M. The A549 human lung cancer, SGC7901 human Gastric cancer and ECA109 human Esophageal cancer cell lines were obtained from the Cell Culture Center of the Basic Institute of Medical Sciences, Peking Union Medical College. Cell culture reagents were purchased from Gibco (CA, USA). Annexin V- FITC and PI double staining was purchased from BD Biosciences (NJ, USA). 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (MO, USA).

1.2 Methods

1.2.1 General Experimental

The UV-vis absorption spectra were recorded using a Varian Cary 100 spectrophotometer and fluorescence emission spectra were recorded using a Hitachi F-4500 spectrofluorophotometer. The elemental analyses were performed in the microanalytical laboratory, Department of Chemistry, LanZhou University. The ^{1}H NMR spectra were recorded with a Bruker ACF300 FT-NMR instrument using TMS as an internal reference in DMSO-d_6 (dimethyl sulfoxide) for the ligand. The infrared spectra (KBr pellet) were recorded using an FTS165 Bio-Rad FTIR spectrophotometer in the range of 4000-400 cm^{-1}. Conductivity measurements were performed in DMF (*N*,*N*-dimethylformamide) with a DDS-11A conductometer at 25.0 °C. Viscosity experiments were carried out on an Ubbelodhe viscometer. The CD spectra were recorded on a Jasco J-810 spectropolarimeter. Absorption titration experiments were performed by fixing concentrations of **2** and **3** as constant at 10 μM while varying the concentration of ct DNA. While measuring the absorption spectra, equal quantity of CT-DNA was added to the complexes and the

reference solution to eliminate the absorbance of DNA itself. Fluorescence spectra of the competitive binding experiments were carried out by maintaining the EB and ct DNA concentration at 3 μM and 30 μM, respectively, while increasing the concentrations of the compounds. Fitting was completed using an Origin 6.0 spreadsheet, where values of the binding constants K_b were calculated. Viscosity experiments were carried out on an Ubbelodhe viscometer, immersed in a thermostated water-bath maintained at 25.0±0.1 °C. Titrations were performed for the compounds (1-6 μM), and each compound was introduced into DNA solution (50 μM) present in the viscometer. Flow time was measured with a digital stopwatch and each sample was measured three times and an average flow time was calculated. Data were presented as $(\eta/ \eta_0)^{1/3}$ versus the ratio of the concentration of the compound and DNA, where η is the viscosity of DNA in the presence of compound, and η_0 is the viscosity of DNA alone. Viscosity values were calculated from the observed flow time of DNA-containing solution corrected from the flow time of buffer alone (t_0), $\eta = t\text{-}t_0$. The CD spectra of DNA were recorded on a Jasco J-810 spectropolarimeter at 25.0±0.1 ℃. Calf thymus DNA used were 200 μM in concentration and compounds solutions was added to a ratio of 1:1 (DNA/compound). Each sample solution was scanned in the range of 200–350 nm. CD spectrum was generated which represented the average of three scans from which the buffer background had been subtracted.

1.2.2 Cytotoxic activity

To evaluate their cancer chemotherapeutic potential, the ability of the DMSO soluble copper complexes **2** and **3** to kill human derived cancer cells were investigated using the human hepatocarcinoma cancer (HepG2), human myeloid leukemia cancer (HL-60), human prostate (PC3) cell lines and were determined by calculation of IC_{50}.The IC_{50} was measured by MTT assay. Briefly, the three kinds of tumor cells (HepG2. HL-60. PC3) were plated at a density of 1×10^4 cells per well in 96 -well plates overnight and then treated with different concentrations of copper complexes **2** and **3** after 72h. Twenty microliters of MTT solution were added to each well and the cells were cultured for another 4 h at 37℃. The medium

was completely removed and 100 μL DMSO was added to solubilize MTT formazan crystals. The plates were then agitated and the optical density was determined at 570 nm (A570) using an ELISA plate reader. At least three independent experiments were performed.

1. 2.3 Quantification of apoptosis by Annexin V and PI double staining

Apoptotic rates were determined by flow cytometry using an Annexin V/PI apoptosis kit. Briefly, the three kinds of tumor cells were seeded at a density of 1×10^6 cells per well in 6-well plates overnight and then treated by copper complexes **2** and **3** with each corresponding IC_{50} dose for 24 h. Cells (1×10^6) were collected by centrifugation and washed twice with cold PBS. Staining was performed according to the manufacturer's instructions and the cells were analyzed using a FACS can flow cytometer and analyzed using CellQuest software. At least three independent experiments were performed.

1.2.4 The synthesis of the compounds

Synthesis of the compound **1** and the Ligand H_2L shown in scheme 1: The compound **1** was prepared according to the literature.[15] An ethanol solution (30 ml) containing salicylaldehyde (1.22 g, 10 mmol) was added dropwise to another ethanol solution (30 ml) containing the compound **1** (2.54 g, 10 mmol). After refluxing for 12 h, the mixture was cooled to room temperature, the yellow precipitate solid was collected by filtration and washed with cool ethanol. Recrystallization from anhydrous methanol gave the ligand H_2L, which was dried in vacuum. Yield, 82.7%. ^{1}H-NMR (DMSO-d_6, ppm) d : 12.18 (1H, s, NH), 11.20 (1H, s, OH), 8.78 (1H, s, CH=N), 8.72 (1H, s,), 6.95-7.70 (10H, ph-H). IR (KBr, cm^{-1}): ν(OH) 3435, ν (N-N) 2991, ν (C=O) 1632, ν(C=N) 1578, ν (C-N) 1317.

Synthesis of the complexes: The complex **2** was prepared as follows. The ligand H_2L (1 mmol, 0.358 g) containing (1 mmol, 0.04g) NaOH was dissolved in 10mL anhydrous ethanol, $CuClO_4\cdot6H_2O$ (0.5 mmol, 0.185g) in anhydrous ethanol (10 ml) was then added dropwise with stirring. After stirring for 8 h at room temperature, a large amount of blue precipitate

appeared. They were separated from the solution by suction filtration, purified by washing several times with ethanol, and dried for 24 h in vacuum. Yield: 88.5%. Anal. Calcd for **2** $C_{42}H_{28}CuN_4O_{10}$: C, 62.11; H, 3.47; N, 6.90. Found: C, 61.52; H, 3.69; N, 7.11. IR (KBr, cm-1): ν(OH) 3430, ν (N-N) 2987, ν (C=O) 1612, ν(C=N) 1536, ν (C-N) 1302. ESI-MS Calc. for $C_{42}H_{24}CuN_4O_8$: 776.21. Found: 775.1 (Figure S1).

The preparation of the complex **3** is similar to that of complex **2**, by using $NiClO_4 \cdot 6H_2O$ in place of CuClO4·6H2O. Anal. Calcd for **3** $C_{42}H_{28}CuN_4O_{10}$: C, 62.48; H, 3.50; N, 6.94. Found: C, 62.86; H, 3.83; N, 7.25. IR (KBr, cm-1): ν(OH) 3428, ν (N-N) 2992, ν (C=O) 1617, ν(C=N) 1538, ν (C-N) 1306. ESI-MS Calc. for $C_{42}H_{24}NiN_4O_8$: 771.36. Found: 770.0 (Figure S2).

1. 2.5 Statistical analysis

All data were analyzed by SAS 6.12 software and the results were expressed by mean–SD. To compare the differences between the groups, statistical significance was analyzed using a one-way analysis of variance followed by post hoc comparisons. Statistical significance was defined as p-values < 0.05.

2. Results

2.1 Chemical Characterization

The synthetic route of the ligands is show in scheme 1. The ligand is soluble in methanol and ethanol, while the complexes are slightly soluble in methanol, insoluble in ethanol. The two complexes are air stable for extended periods. Since the crystal structure of the complexes have not been obtained yet, we characterized the complex and determined its possible structure by elemental analyses, molar conductivities, and IR data. The elemental analyses show that the formulas of the complexes conform to $M(HL)_2 \cdot 2H_2O$ (M= Cu, Ni). The likely structure of the metal-complex is shown in Figure. 1. The tested metal-complexes were prepared in DMF and freshly diluted in tris buffer system (at pH 7.4, 7.8). These complexes

are quite stable, showing ignorable dissociation in the tris buffer, as the UV-visible absorption spectra have no obvious change for the solutions very freshly prepared and the stored minutes or days.

2.2 biological activities

To evaluate their cancer chemotherapeutic potential, the ability of the DMSO soluble copper complexes **2** and **3** to kill human derived cancer cells was investigated using the human hepatic (HepG2), human promyelocytic leukemia (HL60) and human prostate (PC3) cell lines and was determined by calculation of IC_{50} (the drug concentration causing a 50% reduction in cellular viability). Cells were continuously exposed to test agent for 72 h, and their effects on cellular viability was evaluated. It was intended that the results from these studies would allow the identification of those copper and nickel derivatives with cancer chemotherapeutic potential. Comparison of IC_{50} values, allowed the relative potency of each of the test compounds to be determined and ranked. The IC_{50} values obtained for all tested compunds are presented in Table 1. The morphology examination also show that the proliferation of the cells are significant inhibited and the cells exhibit morphological change such as cell shrinkage and cell detachment (Figure 5).

Apoptotic rates were determined by flow cytometry using an Annexin V/PI apoptosis kit, as shown in Figure 6. The number of apoptotic cells induced by complexs 2 and 3 were more than control cell lines（$p<0.05$, especially by complex 2.

2.3 DNA-Binding

The absorption spectra of **2** and **3** in the absence and presence of ct DNA are given in Figure 2. In the absence of ct DNA. Upon increasing DNA concentrations, the hypochromisms increased up to 29.3 % at 299 nm and 34.5 % at 423 nm for complex **2**; 21.4 % at 299 nm and 23.3 % at 423 nm for complex **3**.

In order to further investigate the interaction mode between the two complexes and ct DNA, the fluorescence titration experiments were performed. The intrinsic fluorescence intensities of DNA and that of EB in Tris-HCl buffer are low, while the fluorescence intensity of EB will be enhanced on addition of DNA owing to its intercalation into the DNA. If the complexes can intercalate into DNA, the binding sites of DNA available for EB will be decreased, and hence the fluorescence intensity of EB will be quenched [16]. In our experiments, as depicted in Figure 3a for complex **2** and Figure 3b for complex **3**, the fluorescence intensity of EB at 584 nm shows a remarkable decreasing trend with the increasing concentration of the complex **2** or **3**.

Circular dichroic spectral technique is useful in diagnosing changes in DNA morphology during drug-DNA interactions, as the band due to base stacking (275 nm) and that due to right-handed helicity (248 nm) are quite sensitive to the mode of DNA interactions with small molecules. The circular dichroic spectrum of CT DNA (Figure 4) exhibits a positive band at 275 nm due to base stacking and a negative band at 246 nm due to helicity of B DNA.

3. Discussion

3.1 Chemical Characterization

The elemental analyses show that the formulas of the complexes conform to $M(HL)_2 \cdot 2H_2O$ (M= Cu, Ni). The likely structure of the metal-complex is shown in Figure 1. The tested metal-complexes were prepared in DMF and freshly diluted in tris buffer system (at pH 7.4, 7.8). These complexes are quite stable, showing ignorable dissociation in the tris buffer, as the UV-visible absorption spectra have no obvious change for the solutions very freshly prepared and the stored minutes or days. The IR spectra of the two complexes are very similar. The $\nu_{C=O}$ of the free ligand are at 1632 cm^{-1}, for complex **2** and **3** this peak shifted to 1612 and 1617 cm^{-1}, $\nu_{(ligand-complexes)}$ is equal to 20 and 15 cm-1, respectively. The band at 617 and 599 cm^{-1} for **2** and **3** is assigned to ν_{M-O}. This data strongly indicates that the oxygen of the carbonyl has formed a coordinative bond with the metal ions. The band at 1578 cm^{-1} for

the free ligand is assigned to the $\nu_{C=N}$ stretch [17], which shifts to 1536 and 1538 cm^{-1} for the complexes **2** and **3**. These further confirm that the nitrogen of the imino group bonds to the metal ions.

3.2 DNA-Binding Mode and Affinity

The observed hyperchromism in Figure 2 for complexes **2** and **3** unambiguously revealed the active participation of coumarine moieties in associating with the DNA[18]. The hypochromicity, characteristic of intercalation [19] has been usually attributed to the interaction between the electronic states of the compound chromophores and those of the DNA bases [20], while the red shift has been associated with the decrease in the energy gap between the highest and the lowest molecular orbitals (HUMO and LUMO) after binding of the complex to DNA [13]. However, the lack of red shift suggests that the binding mode of both **2** and **3** was not classical intercalation. The binding constants [21], K_b for the complexes **2** and **3** have been determined from the plot of [DNA]/ (ε_a - ε_f) *vs.* [DNA] and found to be 2.99 $\times 10^5$ M^{-1} and 0.61 $\times 10^5$ M^{-1} respectively. The results indicate that the binding strength of complex **2** is stronger than that of **3**.

The fluorescence titration experiments indicated that some EB molecules are released from EB-DNA after an exchange with the complex **2** or **3** which result in the fluorescence quenching of EB. This observation is often the characteristic of intercalation. And he quenching plots (inset) followed the classical Stern-Volmer equation, $F_0/F=K_q[Q]+1$, where F_0 is the emission intensity in the absence of quencher, F is the emission intensity in the presence of quencher, K_q is the quenching constant, and [Q] is the quencher concentration. The shape of Stern-Volmer plots can be used to characterize the quenching as being predominantly dynamic or static. Plots of F_0/F versus [Q] appear to be linear and K_q depends on temperature. The quenching plots illustrate that the quenching of EB bound to DNA by the compounds is in good agreement with the linear Stern-Volmer equation (Fig. 3(a) and

3(b), inset). In the plots of F_0/F versus [Q], K_q is given by the ratio of the slope to the intercept. The K_q value for **2** is 1.9×10^4 M^{-1} while the compound **3** is 0.85×10^4 M^{-1}, which shows that **2** is more able than **3** in replacing the strong DNA intercalators EB, in consistent with the higher value of K_b spectrophotometrically determined.

Incubation of the DNA with the present compounds induced changes in the CD spectrum. On addition of all the complexes to CT-DNA, faint red shift with intensity increase in the positive or negative bands are observed. When addition of **2** to CT-DNA, it is observed that the negative-band position was shifted to 246 nm with more evident increase than **2** in molar ellipticity, while the intensity of the positive band in the CD spectrum of DNA is perturbed remarkably with no shift. This phenomenon may be due to the intercalation of the complex through π-stacking which stabilizes the right-handed B form DNA [22,23].

3.3 Cytotoxic activity and cell apoptosis

The two metal complexes displayed a concentration-dependent cytotoxic profile in all cell lines. Since the IC_{50} values for complexes **2** and **3** are statistically lower than that for metal-free ligand in all of the tested cells, it suggests that coordinated metal ions play a major role in mediating potency of the complexes.

The IC_{50} values for complex **3** on all the cells are statistically different, it displayed a greater effect against HL60 and PC3 cell lines, which means **3** is sensitive for the HepG2 cell line. While the complex **2** show good cytotoxic activities for all of the tested cell lines and the results also suggest that **2** had more significant cytotoxic activities than **3** against the three human cancer cell lines, which is consistent with the result of DNA binding studies above. This may be due to the complexes inducing DNA damage in cancer cells and the nature of the compound itself.

And, in this detection, cells stained single-positive Annexin V- FITC were considered mostly early apoptotic cells and cells stained single positive for PI were considered mostly necrotic cells, while cells that were stained double-positive could be either necrotic or apoptotic

cells. As shown in Fig. 6, The number of apoptotic cells induced by complex 2 was more than the other tested cell lines, which is consistent with the result of DNA binding studies and cytotoxic activity.

4. Conclusions

In conclusions, a new coumarin derivative and two traditional metal complexes are prepared and characterized; the DNA-binding properties of the complexes are investigated by absorption, fluorescence, circular dichroic spectral and viscosity measurements. The results clearly indicate that the two complexes can bind to DNA through intercalation mode. The reported two complexes also show considerable cytotoxic activity against three cell lines (human hepatocarcinoma cancer HepG2, human myeloid leukemia cancer HL-60, human prostate PC3), and the IC_{50} values of all the metal complexes are lower than that of the ligand, which also show many potential practical applications, such as the development of nucleic acid molecular probes and new therapeutic reagents for cancers. Further experiments are necessary to analyze the action of copper complexes *in vivo*.

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The authors declare that they have no conflict of interest.

Figure legand:

Scheme 1. The synthetic route of the ligands.

Figure 1. The estimated structure of the metal complexes.

Figure 2. Electronic spectra of the complex 2 (a), complex 3 (b) in Tris-HCl buffer upon addition of calf-thymus DNA. [Compound] = 10 M, [DNA] = 0-20 M. Arrow shows the absorbance changes upon increasing DNA concentration. Inset: plots of [DNA]/ (εa-εf) vs. [DNA] for the titration of complex 2 and 3 with CT-DNA.

Figure 3. The emission spectra of DNA-EB system (15 M and 1.5 M EB), ex =500nm, em = 520-720 nm, in the presence of the complex 2 (a), complex 3 (b). [DNA]=10 M, [Compound] = 0-35 M. Arrow shows the emission intensity changes upon increasing complex concentration. Inset: Stern-Volmer plot of the fluorescence titration data of the complexes.

Figure 4. CD spectra of CT-DNA (120 M) in the absence and presence of the complexes 2 and 3 (60 M).

Figure 5. Phase-contrast micrographs of cells treated with complexes 2 and 3. Inhibition rate under different concentration of complexes 2 and 3.

Figure 6. Annexin V-FITC/PI staining detected apoptosis in three cancer cells after treatment of the tested compounds

Figure. S1 The mass spectroscopy of the complex 2.

Figure. S2 The mass spectroscopy of the complex 3.

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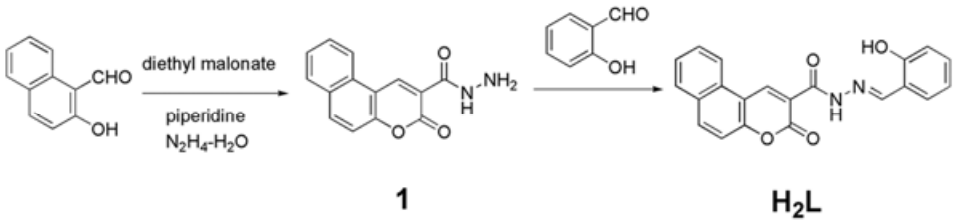
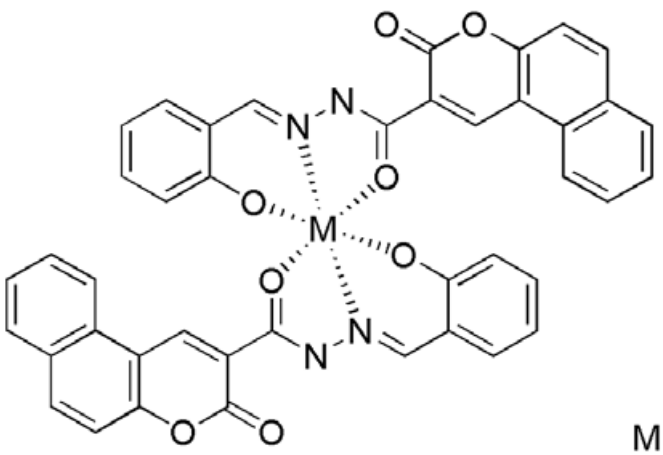
Table 1. IC_{50} values of the tested compounds

cell line	IC_{50} (μM)				
	$Cu(ClO4)_2·6H_2O$	$Ni(ClO4)_2·6H_2O$	H_2L	**2**	**3**
PC3	>300	>300	>160	43	>100
HepG2	>300	>300	>160	38	69
HL60	>300	>300	>160	15	>100





M =Cu or Ni