

Investigation of aromatase inhibitory activity of metal complexes of 8-hydroxyquinoline and uracil derivatives

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Purpose: Estrogens play important roles in the pathogenesis and progression of breast cancer as well as estrogen-related diseases. Aromatase is a key enzyme in the rate-limiting step of estrogen production, in which its inhibition is one strategy for controlling estrogen levels to improve prognosis of estrogen-related cancers and diseases. Herein, a series of metal (Mn, Cu, and Ni) complexes of 8-hydroxyquinoline (8HQ) and uracil derivatives (4–9) were investigated for their aromatase inhibitory and cytotoxic activities.

Methods: The aromatase inhibition assay was performed according to a Gentest™ kit using CYP19 enzyme, wherein ketoconazole and letrozole were used as reference drugs. The cytotoxicity was tested on normal embryonic lung cells (MRC-5) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Results: Only Cu complexes (6 and 9) exhibited aromatase inhibitory effect with IC₅₀ 0.30 and 1.7 μM, respectively. Cytotoxicity test against MRC-5 cells showed that Mn and Cu complexes (5 and 6), as well as free ligand 8HQ, exhibited activity with IC₅₀ range 0.74–6.27 μM.

Conclusion: Cu complexes (6 and 9) were found to act as a novel class of aromatase inhibitor. Our findings suggest that these 8HQ–Cu–uracil complexes are promising agents that could be potentially developed as a selective anticancer agent for breast cancer and other estrogen-related diseases.

Keywords: aromatase inhibitor, anticancer, metal-based compound

Introduction

Estrogens are well known as steroidal sex hormones. They not only play roles in the development and maintenance of the female reproductive system,¹ but also take part in the regulation of many physiological processes.^{2–7}

Estrogens are synthesized from cholesterol precursors⁸ in a multistep process facilitated by different enzymes.⁸ The last conversion process of C19 steroid (ie, androstenedione or testosterone) into the C18 steroid (ie, estrone or estradiol) by aromatization is considered to be the rate-limiting step of estrogen production.⁸ This key step is facilitated by the cytochrome P450 complex.^{9–13} Abnormal estrogen productions have been found to give rise to diseases and complications. Decreased level of estrogens was found to be involved with age-related conditions.^{2,14} Moreover, estrogens facilitate the growth of certain estrogen-dependent cancers such as breast^{15,16} and endometrial cancer.^{17–19}

The treatment of breast cancer is multimodal,²⁰ in which endocrine therapy is one of the possible approaches.⁹ Aromatase inhibition is considered a promising approach to decreasing the amount of estrogen production.¹ Recently, aromatase inhibitors have

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been developed and used as anti-breast cancer agents with preferable treatment outcomes in postmenopausal women.²¹

Aromatase inhibitors can be classified into two classes based on their chemical structures and mechanism of action.⁹ Steroidal aromatase inhibitors competitively and covalently bind to aromatase enzyme in an irreversible fashion.²² Non-steroidal aromatase inhibitors contain azoles as the privileged structure¹ for coordinating with heme iron (Fe) atom of aromatase enzyme leading to reversible enzyme inhibition.²³ Moreover, antifungal agents containing the aza-heterocycle have been shown to afford aromatase inhibitory activity.^{24–27} Such a finding indicates the significance of a heterocyclic compound bearing the N-atom as a potential aromatase inhibitor.⁹

On the basis of existing literature, other classes of N-heterocyclic compounds, such as quinoline and pyrimidine, have drawn considerable interest. For example, 8-hydroxyquinoline (8HQ) is a quinoline derivative with potent molecular recognition and coordinating properties to metals.²⁸ It is widely used as a metal chelator as well as for analytical and separation purposes.²⁸ Recently, the anticancer activity of 8HQ-based compounds has been reported.^{29–33} Moreover, 8HQ and its derivatives exhibit a diverse range of biological activities, such as anti-neurodegenerative,^{34,35} antimicrobial,^{36–39} antimalarial,^{40–42} antioxidant,^{30,34} anti-inflammatory,⁴³ and anticancer activities.⁴⁴

Uracil derivatives are widely known for their anticancer properties. For example, 5-fluorouracil is a fluorinated uracil analog with anticancer activity against a wide range of cancers,^{45,46} such as head and neck,^{47,48} gastrointestinal,^{49,50} and breast cancer.^{51–53} Recently, 5-fluorouracil has been reported to be one of the most prescribed anticancer drugs.⁵⁴

Transition metal ions play vital roles in many biological processes. They exist in multiple valence and oxidation states,⁵⁵ which allows them to readily participate in electron transfer reactions and interact with negatively charged molecules.⁵⁵ Transition metal complexes have been reported and used as anticancer agents.^{56,57} In this regard, many beneficial effects of metal complexation have been achieved. For example, metal ions can act as chaperones in order to deliver active ligands to target sites in a selective manner. Metal complexation has been shown to improve pharmacokinetic properties such as size, charge, and lipophilicity as well as minimize toxicity.⁵⁸ It has also been reported that metal complexes can mimic the activity of enzymes such as superoxide dismutase.^{58–61}

In efforts to increase the cancer survival rate, attention has been focused in recent years on multitasking drugs.⁶² Such multitasking drugs are developed by combining drugs in order to improve their efficiency and minimize toxicity.

This strategy aims to achieve synergistic effects exerted by each drug as well as to improve drug delivery properties by controlling the lipophilicity and size of the drug.⁶²

According to these principles of searching for novel aromatase inhibitors, this study employed 8HQ (1) and uracil derivatives including 5-nitouracil (5Nu [2]) and 5-iodouracil (5Iu [3]) as ligands for the synthesis of mixed-ligand metal complexes 4–9 (Figure 1) that were subsequently tested for their aromatase inhibitory activity and cytotoxicity.

Material and methods

Reagents for cell culture and assay were the following: Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum from JR Scientific Inc., Woodland, CA, USA; penicillin–streptomycin and 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich Co (St Louis, MO, USA); and dimethyl sulfoxide (DMSO) from EMD Millipore, Billerica, MA, USA. CYP19 (a recombinant human aromatase) and *O*-benzyl fluorescein benzyl ester (DBF) were supplied with the BD Gentest™ kit from BD Biosciences-Discovery Labware, Woburn, MA, USA.

Tested compounds

Mixed-ligand metal complexes (4–9) were synthesized according to a previously described method³⁰ using 1:1:1 molar ratio of 8HQ:M:5Nu (or 5Iu), where M = Ni, Mn, and Cu. The reaction was carried out in boiling methanol. Products were collected by filtration and washed four to five times with cold methanol. The compounds were confirmed by their infrared spectra, magnetic moment, and melting points.

Infrared spectra were obtained on a Perkin Elmer Spectrum System 2000 Fourier transform infrared spectrometer (Perkin Elmer Inc., Waltham, MA, USA). Magnetic moment was obtained with an MK 1 Magnetic Susceptibility Balance (serial 15257; Sherwood Scientific, Cambridge, UK). Melting points were determined on a Griffin melting point apparatus, UK and were uncorrected.

Cytotoxicity assay

Cytotoxicity was tested using normal embryonic lung cells (MRC-5). The cells were grown in DMEM medium supplemented with 100 U/mL penicillin–streptomycin and 10% fetal bovine serum. Briefly, cell lines suspended in the culture medium were seeded in a 96-well microtiter plate (3599; Corning Incorporated, Corning, NY, USA) at a density of 5×10^3 to 2×10^4 cells/well, and incubated at 37°C under a humidified atmosphere (95% air, 5% CO₂) for 24 hours. An equal volume of additional medium containing

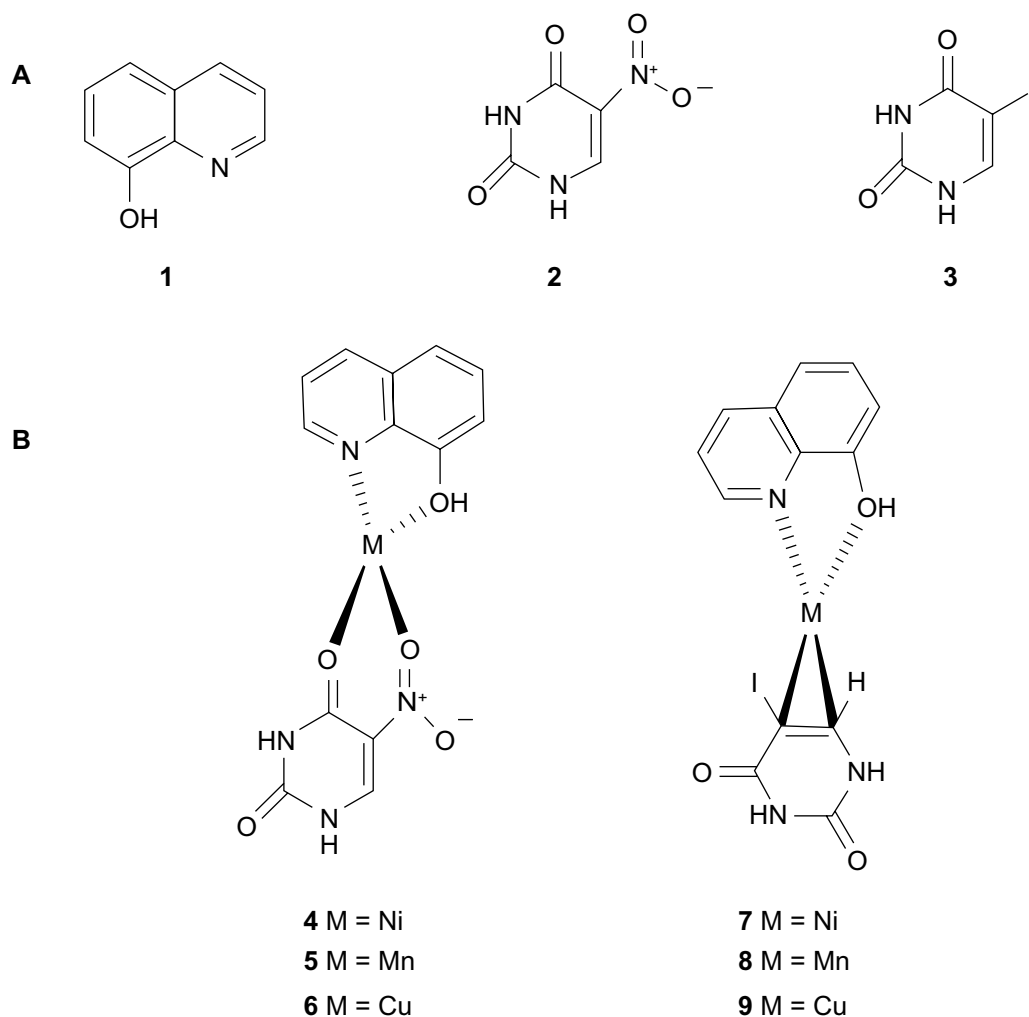


Figure 1 Chemical structures of ligands (1–3) (**A**) and metal complexes (4–9) (**B**).

either the serial dilutions of the tested compounds, positive control (doxorubicin), or negative control (DMSO) was added to the desired final concentrations. The plates were further incubated for an additional 48 hours. Cell viability was determined by staining with MTT assay.^{63–65} The MTT solution (10 μ L/100 μ L medium) was added to all wells of an assay and plates were incubated as described above for 2–4 hours. Subsequently, DMSO was added to dissolve the resulting formazan by sonication. The plates were read on a microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA) using a test wavelength of 550 nm and a reference wavelength of 650 nm. IC_{50} value is defined as the drug or the compound concentration that inhibits cell growth by 50% (relative to negative control).

Aromatase inhibition assay

Aromatase inhibitory effect of metal complexes (4–9) and free ligands (1–3) was performed using the method reported

by Stresser et al,⁶⁶ with minor modifications. This method was carried out according to the Gentest kit using CYP19 enzyme and DBF as a fluorometric substrate. DBF was dealkylated by aromatase and then hydrolyzed, providing the fluorescein product.

For the aromatase inhibition assay, 100 μ L of cofactor, containing 78.4 μ L of 50 mM phosphate buffer (pH 7.4); 20 μ L of 20 \times nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (26 mM NADP⁺, 66 mM glucose-6-phosphate, and 66 mM MgCl₂); and 1.6 μ L of 100 U/mL glucose-6-phosphate dehydrogenase, were pipetted into a 96-well black plate and preincubated in a water bath (37°C) for 10 minutes. The reaction was initiated by addition of 100 μ L of enzyme/substrate (E/S) mixture containing 77.3 μ L of 50 mM phosphate buffer (pH 7.4); 12.5 μ L of 16 pmol/mL CYP19; 0.2 μ L of 0.2 mM DBF; and 10 μ L of 0.25 mM diluted tested sample (or 10% DMSO as a negative control or ketoconazole 52 μ M or letrozole 6.6 nM

as a positive control). To exclude background fluorescence of the sample, E/S was added after the reaction was terminated. After incubation at 37°C for 30 minutes, the reaction was stopped by addition of 50 µL of 2.2 N NaOH. To develop adequate signal-to-background ratio, the plate (with lid) was then incubated for 2 hours at 37°C in an air incubator. Fluorescence signal was measured using an excitation wavelength of 490 nm and emission wavelength of 530 nm with cutoff 515 nm. Percentage of inhibition (% inhibition) was calculated by Equation 1. Samples with % inhibition greater than 50 were further diluted and assayed in triplicate. Finally, IC₅₀ values were determined by the plot of concentrations versus % inhibitions.

$$\% \text{ inhibition} = 100 - [(\text{sample} - \text{blank}) / (\text{DMSO} - \text{blank}) \times 100]. \quad (1)$$

Results

Metal complexes of 8HQ–uracils (4–9) and free ligands (1–3) were investigated for their aromatase inhibitory activity and cytotoxicity toward normal embryonic lung (MRC-5) cells.

The results (Table 1) revealed that only Cu complexes (6 and 9) exhibited aromatase inhibitory effect in a dose-dependent manner, with IC₅₀ 0.30 and 1.70 µM, respectively. Mn complexes (5 and 8), Ni complexes (4 and 7), and all free ligands (1–3) were shown to be inactive. The plots of concentration versus % inhibition of active complexes (6 and 9) are shown in Figure 2. Cytotoxicity test of compounds 1–9 (Table 2) against MRC-5 cells showed that metal complexes (5 and 6) and 8HQ exhibited the activity, with IC₅₀ range 0.74–6.27 µM. The dose–response curves of complexes (5 and 6) are shown in Figure 3.

Table 1 Aromatase inhibitory (IC₅₀) activity of metal complexes

Compound	IC ₅₀ (µM) ^a
8HQ (1)	Inactive ^b
5Nu (2)	Inactive ^b
5Iu (3)	Inactive ^b
8HQ–Ni–5Nu (4)	Inactive ^b
8HQ–Mn–5Nu (5)	Inactive ^b
8HQ–Cu–5Nu (6)	0.30±0.04
8HQ–Ni–5Iu (7)	Inactive ^b
8HQ–Mn–5Iu (8)	Inactive ^b
8HQ–Cu–5Iu (9)	1.70±0.50
Letrozole ^c	0.33±0.40 ^d
Ketoconazole ^c	2.60±0.70

Notes: ^aData are presented as mean ± standard deviation, n=3; ^binactive denotes compound with inhibition ≤50% at 12.5 µM; ^cletrozole and ketoconazole were used as reference drugs. ^dIC₅₀ is shown as nM.

Abbreviations: 5Iu, 5-iodouracil; 5Nu, 5-nitrouracil; 8HQ, 8-hydroxyquinoline.

Discussion

One of the most commonly occurring types of cancer in women is breast cancer, which is also one of the leading causes of death in women worldwide.⁶⁷ Estrogens have been found to be one of the leading factors associated with the pathogenesis and progression of estrogen-dependent diseases such as breast cancer¹ and endometriosis.^{8,68} Aromatase is the key enzyme in the rate-limiting step of estrogen production,⁸ and its inhibition by aromatase inhibitors is one of the approaches to treating and improving prognosis of breast cancer^{69–71} and estrogen-related diseases.^{72–74} Recently, aromatase inhibitors have become the drug of choice for estrogen-dependent cancers^{70,71} and estrogen-related diseases.^{8,72,73}

In this study, a series of 8HQ–uracils metal (Ni, Mn, and Cu) complexes (4–9) were synthesized and investigated for their aromatase inhibitory activity. The results showed that, among all of the tested metal complexes and free ligands, only Cu complexes (6 and 9) were found to be active. Particularly, both Cu complexes were shown to afford more potent aromatase inhibitory activity compared to the positive control, ketoconazole. This can be deduced from the IC₅₀ values of 8HQ–Cu–5Nu (6) and 8HQ–Cu–5Iu (9), which were 8.67- and 1.5-fold less than the ketoconazole, respectively. However, such Cu complexes (6 and 9) displayed weaker aromatase inhibitory activity than letrozole, which is the inhibitor currently used against breast cancer in postmenopausal women. This suggests that Cu ion plays crucial roles in aromatase inhibition. At this point in time, the aromatase inhibition by 8HQ–Cu–uracil (5Nu and 5Iu) complexes has not been previously reported.

It is well recognized that Cu ions are considered one of the risk factors predisposing to the development of cancers facilitated by tumor angiogenesis, growth, and metastasis.^{75–77} Elevated levels of Cu ions are found in tissues and serum of patients with cancers such as breast, prostate, colon, brain, and lung cancer.^{78–81} These observations suggest that Cu could serve as one of the selective targets for the treatment of cancers.⁸²

The mechanism of action of non-steroidal aromatase inhibitors can be attributed to their lone-pair electrons as carried by heteroatoms ofazole rings that interact with the Fe atom of the porphyrin ring inside the binding site of aromatase, thereby leading to the inactivation of the enzyme.²³ Complexation or coordination of metal ions and ligands occurs in a dynamic fashion, as the complexation or coordination of metal ions and ligands can bind in a reversible manner. The dynamic and reversible fashion of binding between metal ions and ligands can be used to

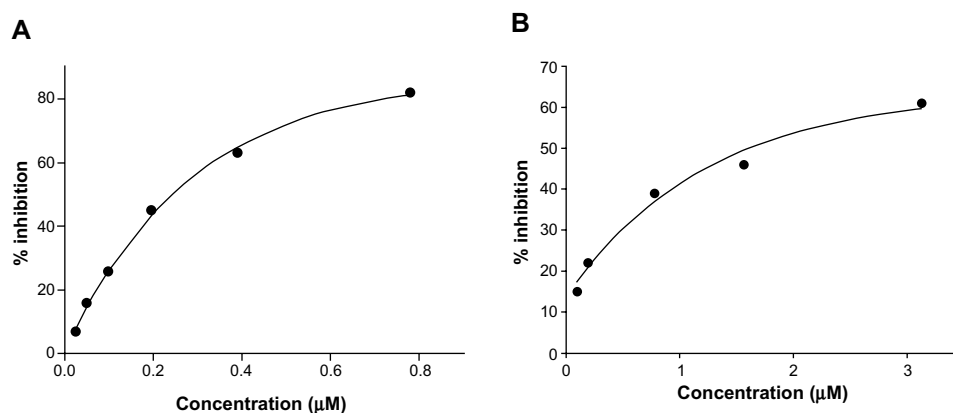


Figure 2 Aromatase inhibitory activity of metal complexes 6 (A) and 9 (B).

explain the experimental results of metal complexes. In this study, metal complexes (4–9) were formed by metal center atoms binding with two different ligands. The possible release mechanism of metal complexes is dependent on electronegativity and metal binding affinity of ligands, as well as pKa at the site of action. Owing to the inherent nature of positively charged metal ions, they mostly occur in the bound state with negatively charged or electron donor molecules.⁸³ Thus, the metal complex may possibly dissociate into one free ligand and one positively charged metal-bound ligand.^{39,84} Moreover, different sites of metal complex dissociation give rise to different products. The site of metal complex breakage is dependent on the binding affinity between metal ions and each ligand as well as the electronegativity of the ligands.

8HQ is the investigated ligand with a potent metal chelating ability.^{33,85–87} It contains lone-pair electron donors as carried by the ring's nitrogen (N) as well as oxygen (O) atom of the OH group, which can be donated upon metal

complexation. Therefore, the metal ion may possibly retain its binding with 8HQ. On the other hand, the high electronegativity property of uracil ligands (5Lu or 5Nu) can result in the release of 8HQ–metal charged complex and free uracil ligand.^{39,61} Our results suggest that Cu is required for aromatase inhibition. A plausible explanation may be attributed to the redox-reactive nature of Cu ion that gives rise to its high affinity to bind electrons and organic molecules.⁸³ Therefore, Cu ions in the released complexes (possibly 8HQ–Cu) could bind to organic compartments of aromatase enzyme, which may consequently cause conformational change of the enzyme leading to perturbation of the catalytic activity. Alternatively, the released 8HQ–Cu charged complex could interact with the target site and subsequently release 8HQ free ligand, which is a potent Fe chelator.^{84,88} Consequently, 8HQ could possibly chelate Fe contained within the aromatase enzyme, thereby leading to the inactivation of the aromatase enzyme. However, our results indicate that the free ligand 8HQ was an inactive aromatase inhibitor with 18% inhibitory

Table 2 Cytotoxicity (IC_{50}) of metal complexes against MRC-5 cell line

Compound	IC_{50} (μM) ^a
8HQ (1)	6.27±0.58
5Nu (2)	Noncytotoxic ^b
5Lu (3)	Noncytotoxic ^b
8HQ–Ni–5Nu (4)	NT ^c
8HQ–Mn–5Nu (5)	5.19±1.26
8HQ–Cu–5Nu (6)	0.74±0.04
8HQ–Ni–5Lu (7)	NT ^c
8HQ–Mn–5Lu (8)	NT ^c
8HQ–Cu–5Lu (9)	NT ^c
Doxorubicin	Noncytotoxic ^b

Notes: ^aData are presented as mean ± standard deviation, n=3; ^bnoncytotoxic denotes compounds with $IC_{50} > 50 \mu g/mL$; ^cinsoluble in testing medium.

Abbreviations: 5Lu, 5-iodouracil; 5Nu, 5-nitouracil; 8HQ, 8-hydroxyquinoline; NT, not tested.

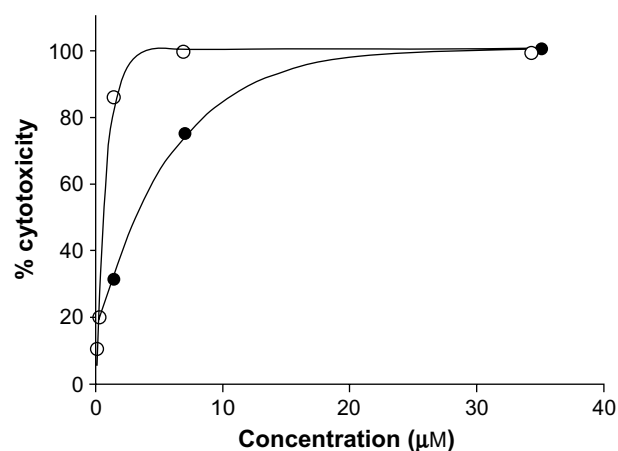


Figure 3 Cytotoxicity of metal complexes (5 and 6) against MRC-5 cell line. **Note:** Solid circles represent 5 and empty circles represent 6.

effect at 12.5 μM . It is therefore reasonable to consider that the inhibitory activity requires the compound to have high absorption to the target site of action. This is clearly observed for highly lipophilic 8HQ–Cu–5Nu (5Iu) complexes (6 and 9) as compared to the free ligand 8HQ. A closer look at the structure of 8HQ–metal–uracil complexes revealed that the electronegativity of coordinating atoms of uracil ligands influenced the dissociation of the complexes. Particularly, two O atoms were used in 5Nu complexation, while two carbon (C) atoms with double bond were used in the case of 5Iu. The electronegativity of the O atom is much higher than that of the C atom. Therefore, the electron withdrawing effect of 5Nu is much stronger than that of 5Iu, leading to a higher ability of 5Nu to dissociate from the molecule of metal complex. This phenomenon could be used in explaining why the 8HQ–Cu–5Nu complex (6) exhibited more potent aromatase inhibitory effect than that of the 8HQ–Cu–5Iu complex (9). The Cu complex 6 displayed a higher IC_{50} value (0.74 μM) in cytotoxicity, compared to its aromatase inhibition with IC_{50} of 0.30 μM . Other metal (Ni and Mn) complexes (4, 5, 7 and 8), as inactive aromatase inhibitors, exerted their weak inhibitory effects at 12.5 μM in the percentage of 8%, 33%, 13%, and 27%, respectively. This may be due to the property of metal-centered atoms affecting the compounds' interactions with the target site of action.

Selectivity is another important issue to consider in the development of anticancer drugs.⁵⁴ It was found that drugs with poor selectivity can cause undesirable toxicities.⁸⁹ Since, cancers require Cu ions for their growth and metastasis.^{75–77} For medicinal use, Cu ion could be a selective target in cancer therapy.⁸² In addition, 8HQ⁹⁰ and uracil derivatives⁴⁵ are anticancer agents.^{88,91} Particularly, 8HQ has been shown to provide substantial cytotoxic activity against the human breast cancer MCF-7 cell line.³⁵

Conclusion

Aromatase inhibitors represent one of the most promising types of agent for the treatment of estrogen-dependent cancers and diseases. A series of mixed-ligand 8HQ–uracil derivative metal complexes were synthesized and investigated for their aromatase inhibitory and cytotoxic activities. Notably, among all of the tested metal complexes, Cu complexes (6 and 9) were found to act as a novel class of aromatase inhibitor, with higher activity than the reference drug (ketoconazole) but less than the letrozole. Our findings suggest that these 8HQ–Cu–uracil complexes are promising agents that could be potentially developed as a selective anticancer agent for breast cancer and other estrogen-related diseases.

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Disclosure

The authors report no conflicts of interest in this work.

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