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Design, synthesis, molecular modeling, and bioactivity evaluation of 1,10-phenanthroline and prodigiosin (Ps) derivatives and their Copper(I) complexes against mTOR and HDAC enzymes as highly potent and effective new anticancer therapeutic drugs

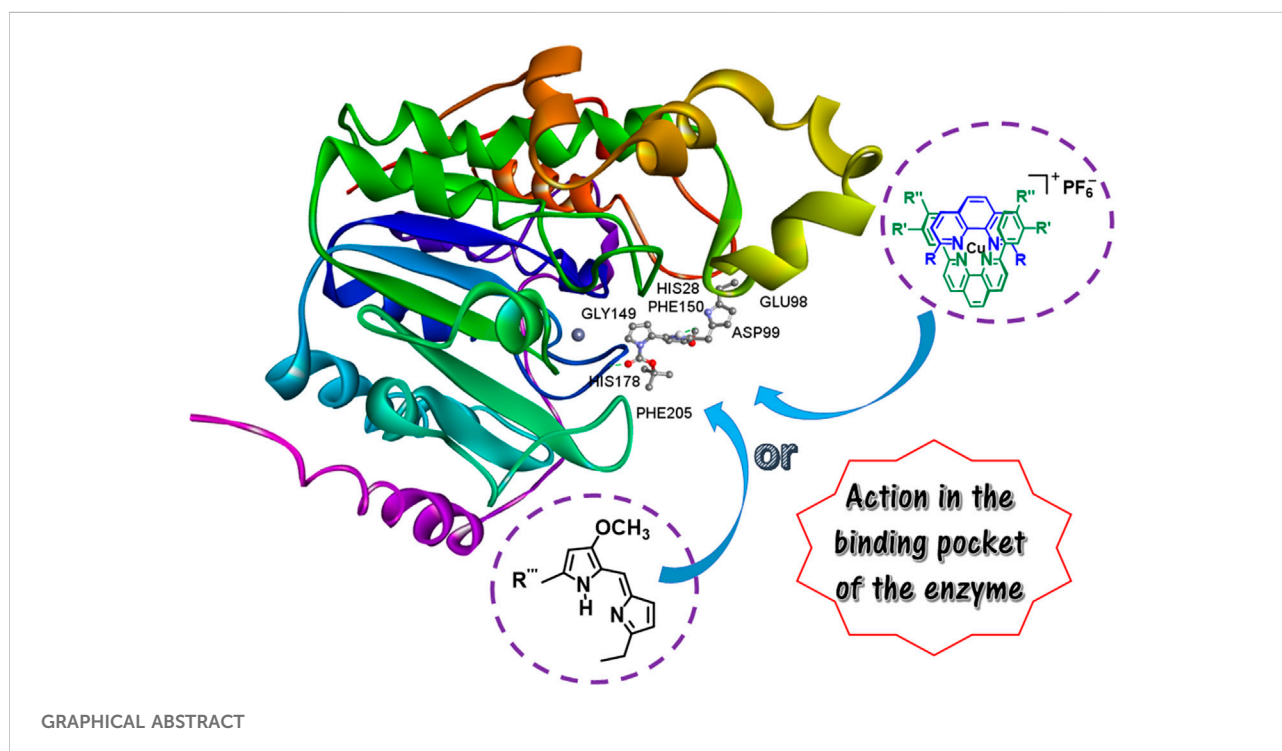
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Breast cancer is the second type of cancer with a high probability of brain metastasis and has always been one of the main problems of breast cancer research due to the lack of effective treatment methods. Demand for developing an effective drug against breast cancer brain metastasis and finding molecular mechanisms that play a role in effective treatment are gradually increasing. However, there is no effective anticancer therapeutic drug or treatment method specific to breast cancer, in particular, for patients with a high risk of brain metastases. It is known that mTOR and HDAC enzymes play essential roles in the development of breast cancer brain metastasis. Therefore, it is vital to develop some new drugs and conduct studies toward the inhibition of these enzymes that might be a possible solution to treat breast cancer brain metastasis. In this study, a series of 1,10-phenanthroline and Prodigiosin derivatives consisting of their copper(I) complexes have been synthesized and characterized. Their biological activities were tested *in vitro* on six different cell lines (including the normal cell line). To obtain additional parallel validations of the experimental data, some *in silico* modeling studies were carried out with mTOR and HDAC1 enzymes, which are very crucial drug targets, to discover novel and potent drugs for breast cancer and related brain metastases disease.

KEYWORDS

breast cancer brain metastases, 1,10-phenanthroline, anticancer therapeutic drugs, mTOR, HDAC, HDAC1, molecular modeling, prodigiosin



Introduction

Cancer is the second leading cause of death for people, and about one in six attributed deaths is due to cancer (WHO, 2022). Breast cancer has been considered as the leading type of cancer within females and counts for 25% of all cases (IARC, 2014). This cancer type also has the secondary highest possibility of brain metastasis (Leone and Leone, 2015) following lung cancer. In 2012, 1.68 million breast cancer cases were diagnosed with more than 500,000 deaths (IARC, 2014). Approximately 10%–20% of breast cancer patients suffer from malignant brain metastasis (Pangeni et al., 2015). Due to the development of prognostic and diagnostic methods and the longer survival rate of primary breast cancer patients, this percentage is continuously increasing in recent years (Rostami et al., 2016). The mortality of breast cancer brain metastasis is currently viewed as one of the central themes in breast cancer research. Many efforts have been directed to the development of breast cancer therapeutics and prognostic tools (Lin et al., 2002; Kölbl et al., 2015). However, there is no efficient anticancer therapeutic/treatment specific for breast cancer, especially for those patients who have a high brain metastasis risk. Thus, there is an increasing demand for

the development of effective breast cancer drug to inhibit the enzymes that play an important role on modulating breast cancer progression.

In the last decades, inhibition of histone deacetylases (HDAC) and mammalian target of rapamycin (mTOR) enzymes has emerged as a potential strategy (Yao et al., 2021) to reverse abnormal epigenetic changes associated with cancer, and several classes of HDAC and mTOR inhibitors have been found to have potent and specific anticancer activities in preclinical studies (Kawai et al., 2003; Fasolo and Sessa, 2012; Min et al., 2012; Tang et al., 2017; Bian et al., 2018; Guo et al., 2020; Yao et al., 2021). The targeted inhibition of HDAC as a potent anticancer therapy approach (Tang et al., 2017; Bian et al., 2018; Guo et al., 2020) and mTOR as a promising target for anticancer therapies due to its central role in the control of cancer cellular growth, division, survival, and angiogenesis and its frequent dysregulation in cancer cells are two of the very important potent anticancer therapy approaches. In particular, the better understanding of the mTOR chemical structure and pathway in return has led to developing mTOR inhibitors with different targets (Fasolo and Sessa, 2012). Therefore, most researchers in the field have focused their studies on developing new and different inhibitors targeting such

enzymes, and actively been studying to elucidate the precise mechanism of action of such inhibition processes. For instance, Yao et al. (2021) have studied the biological evaluation of dual mTOR/HDAC6 inhibitors in MDA-MB-231 cells proposing that dual targeting mTOR and HDAC inhibitors is a promising strategy for triple negative breast cancer (TNBC) treatment. They have designed and synthesized a series of dual mTOR/HDAC6 inhibitors by structure-based strategy and found that one of the compounds was a potent dual mTOR/HDAC6 inhibitor with IC₅₀ value of 133.7 nM against mTOR and 56 nM against HDAC6, presenting mediate antiproliferative activity in TNBC cells. They have also found to induce significant autophagy, apoptosis and suppress migration in MDA-MB-231 cells. Fasolo and Sessa (2012) have also shown that the mTOR is a protein kinase involved in the phosphatidylinositol 3-Kinase (PI3K)/AKT signalling pathway with a central role in the control of cell growth, survival, and angiogenesis, and multiple and frequent dysregulations of this pathway in human tumors make it a central target in the development of new anticancer treatments. Another study (Bian et al., 2018) has shown that HDAC inhibitor suppresses proliferation and invasion of breast cancer cells through regulation of miR-200c targeting CRKL, in which miR-200c was significantly downregulated in breast cancer cell lines compared to normal cell lines and inversely correlated with the levels of class IIa HDACs and CRKL, suggesting that the HDAC-miR200c-CRKL signaling axis could be a novel diagnostic marker and potential therapeutic target in breast cancer. Guo et al. (2020) also found that expression of HDAC1 and retinoblastoma binding protein 4 (RBBP4, playing an important role in transcription, cell cycle, and proliferation) correlate with clinicopathologic characteristics and prognosis in breast cancer *via* the performed immunohistochemistry on 240 breast cancer patients to assess such expression. It was found that HDAC1 and RBBP4 expression in breast cancer was significantly higher than that in normal tissues and claimed that the mechanism may be regulated transcription or translation of estrogen receptor (ER) and progesterone receptor (PR) by HDAC1. Kawai et al. (2003) have also worked on the overexpression of HDAC1 modulating breast cancer progression by negative regulation of estrogen receptor α (ER- α), which is a critical growth regulatory gene in breast cancer and its expression level is tightly linked to the prognosis and treatment outcomes of breast cancer patients (loss of ER- α expression in breast epithelial cells is critical for breast cancer progression). They have shown that HDAC1 interacts with ER- α *in vitro* and *in vivo* and suppresses its transcription activity, in which their findings strongly suggest that HDAC1 affects breast cancer progression by promoting cellular proliferation in

association with a reduction in both ER- α protein expression and transcriptional activity. HDAC1 may thus be a potential target for therapeutic intervention in the treatment of a subset of ER-negative breast cancers, and inhibition of HDAC1 expression or activity may provide a new strategy for breast cancer therapy. In another study (Lu and Liu, 2020), Lu and Liu have reviewed the functions of ER- α and the mechanism of resistance to endocrine therapy as well as the recently reported potent selective ER Degraders (SERDs) that are promising strategy for ER positive endocrine-resistant breast cancer. With the emphasis on their diverse chemical structures and pharmacological profiles, they have categorized oral SERDs into five subtypes, such as steroidal, acrylic acid, basic amino, PROTAC, and long alkyl chain subtypes, which may provide new insights into the new treatment approaches for ER positive endocrine-resistant breast cancer. Similarly, Tang et al. has currently shown in their work (Tang et al., 2017) that HDAC1 triggers the proliferation and migration of breast cancer cells *via* upregulation of interleukin-8 (IL-8). Their data has shown that mRNA and protein levels of HDAC1 in 75% of the breast cancer cells are greater than that in their corresponding adjacent normal cells (fibroblast 3T3 and epithelial breast MCF10A). They have claimed that knockdown of HDAC1 by specific siRNAs can suppress the proliferation and migration of breast cancer cells and inhibit the expression of IL-8, suggesting that HDAC1 may be a potential therapy target for breast cancer treatment.

Along with the abovementioned literature studies, it can clearly be seen that there are many studies that show novel synthetic inhibitors for HDAC and mTOR enzyme inhibition that have been designed, synthesized, and examined for their antitumor and anticancer activities as well as their mechanisms of action. In one of these studies, inhibitors against HDAC for its antitumor activity and the underlying molecular mechanisms of such activity on MDA-MB-231 (231) human breast cancer cells have been examined, and the data from the study suggested that the HDAC inhibitor, namely IN-2001, is a novel promising therapeutic agent with potent antitumor effects against human breast cancers (Min et al., 2012). With the better acknowledgement and understanding of molecular and cellular mechanisms of HDAC and mTOR inhibition, studies in the field have been directed towards novel potent anticancer agents (Macleod, 1952; Butler et al., 1969; Dwyer et al., 1969; Ranford et al., 1993; Fricker, 1994; Zoroddu et al., 1996; Erkkila et al., 1999; Choi et al., 2001; Marzano et al., 2002; de Ruijter et al., 2003; Zhang and Lippard, 2003; Halkidou et al., 2004; Saha et al., 2004; Krusche et al., 2005; Heffeter et al., 2006; Walsh et al., 2006; Senese et al., 2007; Weichert, 2009; Espona-Fiedler et al., 2012; Müller et al., 2013; Danevčič et al., 2016; Wang et al., 2016; Uba and Yelekçi, 2017; Shouksmith et al., 2019).

In recent years, there have been a few reports highlighting the use of transition metal complexes as anti-cancer agents

and their possible anti-cancer chemotherapeutic potential (Fricker, 1994; Lippard and Berg, 1994; Solomon et al., 1996; Zhang and Lippard, 2003; Heffeter et al., 2006; Que et al., 2008; Hanahan and Weinberg, 2011; Brady et al., 2014; Chang, 2015; Denoyer et al., 2015; Sun et al., 2022; Ge et al., 2022; Lu et al., 2022). Of these transition metals, copper in particular plays very important role in mammalian cells, e.g., copper homeostasis (Denoyer et al., 2015; Li, 2020; Ge et al., 2022), inhibition of colorectal cancer progression with diethyldithiocarbamate-copper complex (Huang et al., 2021), *in vitro* and *in vivo* use as potential anticancer and nonsteroidal anti-inflammatory (NSAIDS) therapeutics or agents (Hussain et al., 2019), and use as antitumor (Lumme et al., 1984) and anticancer agents (Marzano et al., 2009) targeting topoisomerases I and II (Molinaro et al., 2020). Transition metal (e.g., copper) complexes of 1,10-phenanthroline (PHEN) and its derivatives have been widely used in the treatment of a variety of cancers, such as testicular, breast cancers and brain metastasis following lung cancer (Marzano et al., 2002). Because PHEN with its planar structure is an important metal chelator, and various metal complexes containing PHEN and Schiff bases with a functional group with a C=N bond, e.g., the taurine Schiff base copper complex—potently inhibits the activity of the proteasome and induces apoptosis in MDA-MB-231 human breast cancer (Zhang et al., 2008; Hindo et al., 2009; Zhang et al., 2012; Zuo et al., 2013; Zhang et al., 2015), possesses anticancer activity (Zhang et al., 2017). Copper complexes of these ligands are of great interest since they exhibit numerous biological activities, such as antitumor (Ranford et al., 1993), antimycobacterial (Saha et al., 2004), antimicrobial (Zoroddu et al., 1996), and intercalating agents of DNA (Erkkila et al., 1999). Because copper is a highly essential mineral nutrient, that is, both beneficial and toxic to the cell due to its redox properties [the two physiologically relevant oxidation states, Cu(II) and Cu(I)—the predominant form in the reducing environment of the cell cytosol]. This precious metal is also increasingly implicated in death pathways and cell proliferation, that is, a fundamental process for the exponential growth of tissue and the development and homeostasis of multicellular organisms. Therefore, copper is a required cofactor for enzymes that mediate a host of essential cellular functions, such as mitochondrial respiration, biosynthesis of hormones, pigments, neurotransmitters, and antioxidant defence. However, oxidative stress and cytotoxicity can also be induced by dysregulation of copper stores (Lippard and Berg, 1994; Solomon et al., 1996; Que et al., 2008; Hanahan and Weinberg, 2011). From the active site cofactor perspective, copper is also a dynamic signalling metal and metalloallosteric regulator (Brady et al., 2014; Chang, 2015). Recent studies have shown that transition metal signaling has forged new links between chemists and biologists, and thus

this linkage helps translating the basic sciences of copper into clinical therapies and diagnostics to exploit vulnerabilities of copper-dependent diseases (Denoyer et al., 2015; Li, 2020; Ge et al., 2022). Another obvious role of copper in metastasis is the regulation of angiogenesis, that is, a fundamental process required for metastatic potential, and many clinical and preclinical studies have provided enough evidence regarding copper coordination compounds to use both as single agents and in combination with other treatments (Denoyer et al., 2015). Combining with some other drugs, such anti-cancer agents with their redox activity have been successfully used to treat brain, ovarian, bladder and breast cancer (Macleod, 1952; Butler et al., 1969; Dwyer et al., 1969). On the other hand, Prodigiosin (Ps), known as a natural medicine, is a secondary and natural metabolite produced by various microorganisms. Ps consists of three pyrrole rings which are active in many reactions (Walsh et al., 2006) with its multiple biological functions, including antibacteria, immunosuppression, anti-inflammatory and anticancer activity (Danevčič et al., 2016). In addition, its anticancer function has attracted immense interests because Ps is a dual mTOR inhibitor. The mTOR pathway necessary for cancer development has two essential components: mTORC1 and mTORC2. Of these, mTORC1 is responsible for tumor cell proliferation and survival while mTORC2 mediates cell migration and invasion (Espona-Fiedler et al., 2012). Typical mTOR inhibitors such as Rapamycin can only inhibit mTORC1 while Ps can inhibit both components (mTORC1 and mTORC2). In addition, a recent study (Wang et al., 2016) has shown that in addition to mTOR inhibition, Ps could also block Wnt/ β -catenin signaling pathway which contributes to the cancer initiation and progression. These unique inhibition mechanisms of Ps are strongly suggesting that this compound is a novel and potent candidate of next generation anticancer drug. Currently, research efforts are focusing on the synthesis of Ps derivatives which are expected to provide better anticancer activity and lower cytotoxicity. On the other hand, studying the inhibition of HDAC, a family of proteins whose main function is the removal of acetyl groups from lysine residues on histone and non-histone substrates, thereby leaving an acetate group, will be at least as important as mTORs. Because deregulation of Class I HDAC (HDAC1, 2, 3 and 8) activity from HDACs (of the 18 isoforms) has been associated with cancer (de Ruijter et al., 2003; Weichert, 2009; Müller et al., 2013; Shouksmith et al., 2019) and its involvement has been suggested (Senese et al., 2007) to be crucial in controlling mammalian cell proliferation. In particular, the HDAC1 isoform has been associated with various types of cancer and its overexpression in gastric and prostate cancers has been reported (Choi et al., 2001; Halkidou et al., 2004). It has also been shown (Krusche et al., 2005) that HDAC1 inhibits ER- α protein expression and

transcriptional activity. Thus, HDAC1 may regulate the progression of breast cancer and associated metastasis, and inhibition of HDAC1 emerges as a promising therapeutic target for cancer therapy (Senese et al., 2007; Uba and Yelekçi, 2017).

Considering breast cancer, molecular, and cellular mechanisms of HDAC and mTOR inhibition, and the abovementioned proved enzyme-inhibitor-breast cancer correlations, the overarching goal of this study is the development of effective anticancer agents to combat with one of the major health problems in the world, i.e., breast cancer brain metastasis. Since this health issue is suffering 10%–20% of breast cancer patients and considered as a serious issue due to its mortality and lack of efficient therapeutic intervention in the treatment of breast cancer brain metastasis, new anticancer drug(s) need to be exploited. In this context, 1,10-phenanthroline and Ps derivatives including their copper(I) complexes have been selected as target drug candidates owing to their anticancer activities stated in different literatures. Including the supporting experimental evidence (enzymatic assays at either biochemical or cellular levels) for the mechanism of action of inhibitors acted against mTOR and HDAC from those abovementioned relevant literature studies, both mTORs (mTORC1 and mTORC2) and HDACs (HDAC1, 2, 3, and 8—particularly HDAC1) have been aimed on purpose for computational calculations and modeling studies with the selected compounds and their relevant metal complexes. Upon synthesis, purification and characterization of such derivatives and their copper(I) complexes, they have been tested on five breast cancer cell lines MDA-MB-231 (231), MDA-MB-361 (361), HTB131, HTB22, and MDA-MB-231BR (231BR) and one brain cancer cell line (CRL-1620 (CRL)) for the novel anticancer agent screening (and involving one additional normal (non-cancer) cell line MCF10A—a breast epithelial cell collected from patient with fibrocystic disease).

Materials and methods

Some experimental details have been presented below. All other materials, methods, additional figures and tables, and instrumentation have been described in the [Supplementary Material](#).

Molecular modeling studies

Enzyme preparation

The crystal structure of mTOR and HDAC1 were retrieved from protein data bank and used for protein setup. [<http://www.rcsb.org>, (for mTOR pdb code: 4jsv; resolution 3.5 Å) and (for HDAC1, pdb code: 4BKX; resolution 3.0 Å)] (Morris et al., 1998).

Each structure was cleaned of all water molecules and inhibitors as well as all non-interacting ions before being used in the docking studies. For mTOR and HDAC1, one of the two subunits was taken as the target structure. Using a fast Dreiding-like force field, each protein's geometry was first optimized and then submitted to the “Clean Geometry” toolkit of Discovery Studio (BIOVIA, 2016) for a more complete check. Missing hydrogen atoms were added based on the protonation state of the titratable residues at a pH of 7.4. Ionic strength was set to 0.145 and the dielectric constant was set to 10. The AutoDock Tools (v. 1.5.7) (ADT) (Morris et al., 2009) graphical user interface program was employed to setup the enzymes for docking.

Ligand setup

The 3D structures of ligand molecules were built, in SPARTAN20 (<https://www.wavefun.com/>) and optimized at (PM3) level and saved in pdb format. The ADT package was also used here to generate the docking input files of ligands. Autodock4.2.6 (Morris et al., 1998) docking program was used for all docking processes. The detailed procedure for docking methods was reported in our earlier work (Akdoğan et al., 2011).

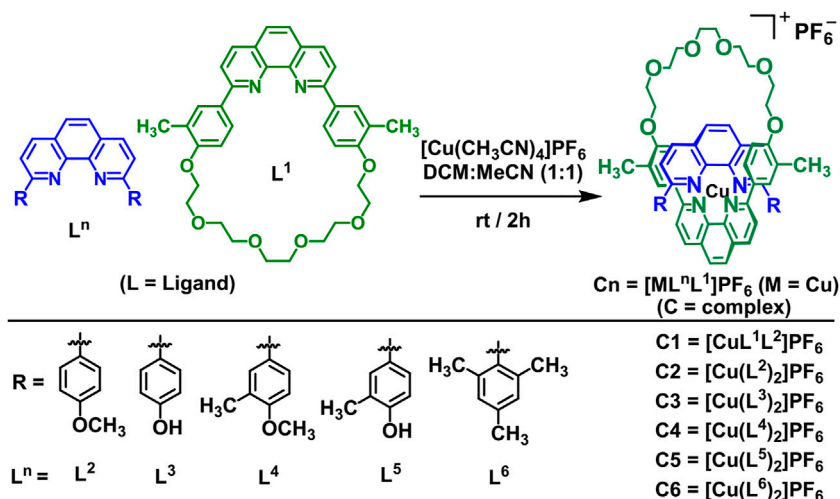
Chemistry

Materials and methods

Anhydrous dichloromethane and acetonitrile were distilled over CaH₂ under nitrogen and stored over molecular sieves prior to use. Ligands (L² and L³) (Dietrich-Buchecker and Sauvage, 1990) and ligands (L⁴ and L⁵) (Zhong et al., 2010; Kang et al., 2014) were prepared using known literature procedures. [Cu(CH₃CN)₄]PF₆ was purchased from Aldrich. All other starting materials were either purchased from commercial sources and used without any further purification, or they were prepared according to the procedures reported in literature. ¹H NMR and ¹³C NMR (decoupled mode) spectra were recorded on a JEOL ECS-400 or Varian Unity Inova 500 MHz spectrometer. DMSO-*d*₆ (99.9% D with 0.05% v/v TMS) was used as the NMR sample solvent.

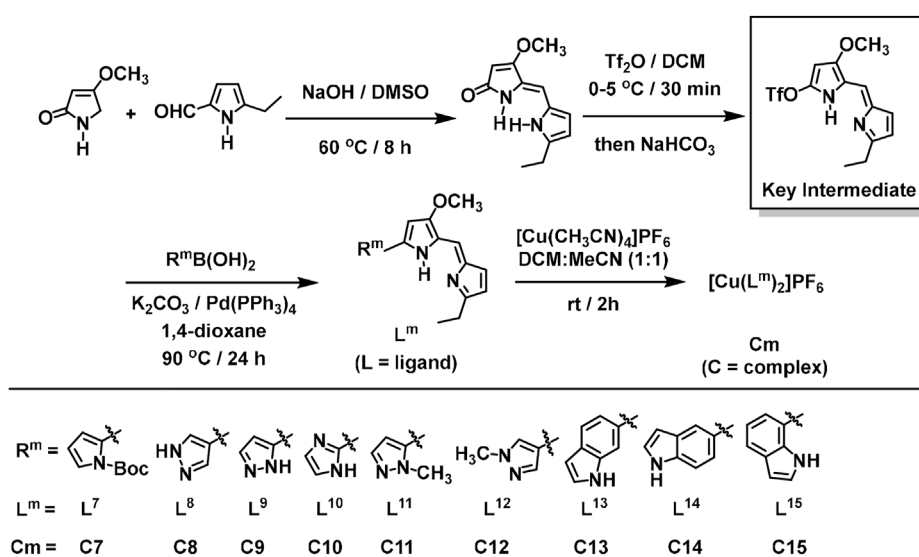
Preparation of ligands and complexes

The new anticancer drug candidates were synthesized by the routine organic approaches. Precursors of synthetic compounds can be purchased from commercial companies. All synthetic steps are well-established organic reactions. Chemical purification and identification through MS, NMR, and X-ray are the classic methods used for many years. Novel Prodigiosin (Ps) and 1,10-phenanthroline derivatives are synthesized and tested. The syntheses of the ligands (L²–L⁵) have been previously reported (Dietrich-Buchecker and Sauvage, 1990; Zhong et al., 2010). The detailed synthetic procedures for the complexes and the molecular structural characterization data for the target



SCHEME 1

General schematic diagram of the synthesis of the bis(homo- and/or hetero-) copper(I) complexes (C1–C6) — 2:1 ligand-to-metal complexes, as PF_6^- salts — from their respective 1,10-phenanthroline-based ligand derivatives (L^1 – L^6).



SCHEME 2

Synthesis of the key intermediate, and general schematic diagram of the synthesis of the Prodigiosin (Ps) derivatives (L^7 – L^{15}) and their respective 2:1 ligand-to-metal copper(I) complexes (C7–C15), as PF_6^- salts.

compounds are presented in the [Supplementary Material](#) (Sections A–C and F).

Crystallography

Diffraction data ([Supplementary Tables S5–S10](#) in the Section F of the [Supplementary Material](#)) for the 2-trifluoromethansulfonyloxy-4-methoxy-5-[(5-ethyl-2H-pyrrol-

2-ylidene)methyl]-1H-pyrrole (Key Intermediate) structure was obtained on a Bruker Kappa APEX-II CCD diffractometer [operated at 1500 W (50 kV, 30 mA) to generate (graphite monochromated) Mo K α radiation ($\lambda = 0.71073 \text{ \AA}$)]. A suitable crystal was selected (a Zeiss Stemi 305 microscope was used to identify a suitable specimen) and the crystal was mounted on a MiTeGen holder in Paratone oil. The crystal was

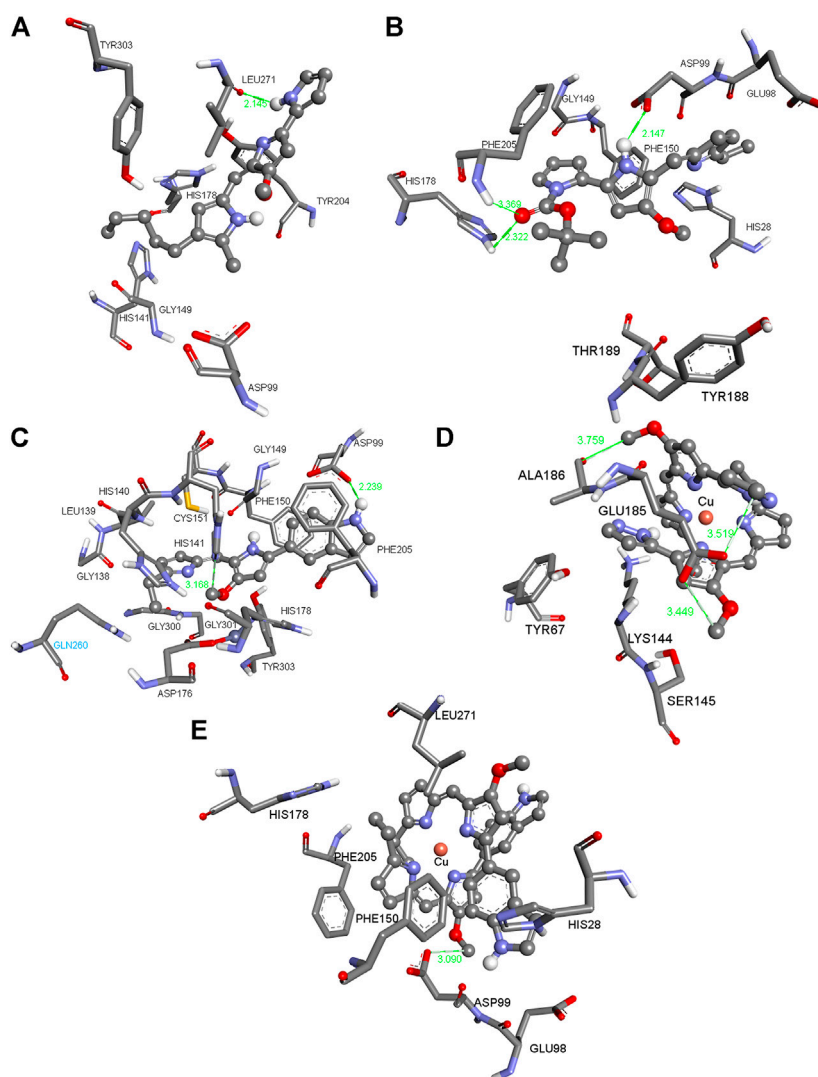


FIGURE 1

Three-dimensional (3D) images with the displayed hydrogen bonding distances (in green) generated via molecular docking of (A) Ps, ligands (B) L⁷ and (C) L¹⁴ and copper (I) complexes (D) C₉ and (E) C₁₄ into HDAC1 enzyme. Amino acid side chains are shown as sticks, the ligands as ball and sticks, and the chelating atom coppers are depicted as round copper tone.

kept at 100 K during data collection. Using Olex2 (Dolomanov et al., 2009), the structure was solved with the XT (Sheldrick, 2015) structure solution program using Intrinsic Phasing and refined with the XL (Sheldrick, 2007) refinement package using Least Squares minimization.

Biology

Cell lines MDA-MB-231 (231), MDA-MB-361 (361), CRL-1620 (CRL), HTB131, and HTB22 were purchased from American Type Culture Collection (ATCC, Manassas, VA), and MDA-MB-231BR (231BR) was a generous gift from Dr.

Paul Lockman (West Virginia University, School of Pharmacy, Morgantown, WV). Cell media, penicillin-streptomycin solution, fetal bovine serum, and phosphate-buffered saline (PBS) were purchased from ATCC. Trypsin-EDTA solution (Corning), cell culture flasks, and pipettes were purchased from VWR.

Cell culturing and cytotoxicity tests

MDA-MB-231 (231) and HTB131 were grown with RPMI-1640 medium (with 10% Fetal Bovine Serum and 2% Penicillin-Streptomycin Solution). MDA-MB-231BR (231BR), CRL-1620 (CRL), and MCF10A were grown with DMEM (with 10% Fetal Bovine Serum and 2% Penicillin-Streptomycin Solution). HTB22 was grown with EMEM (with 10% Fetal Bovine

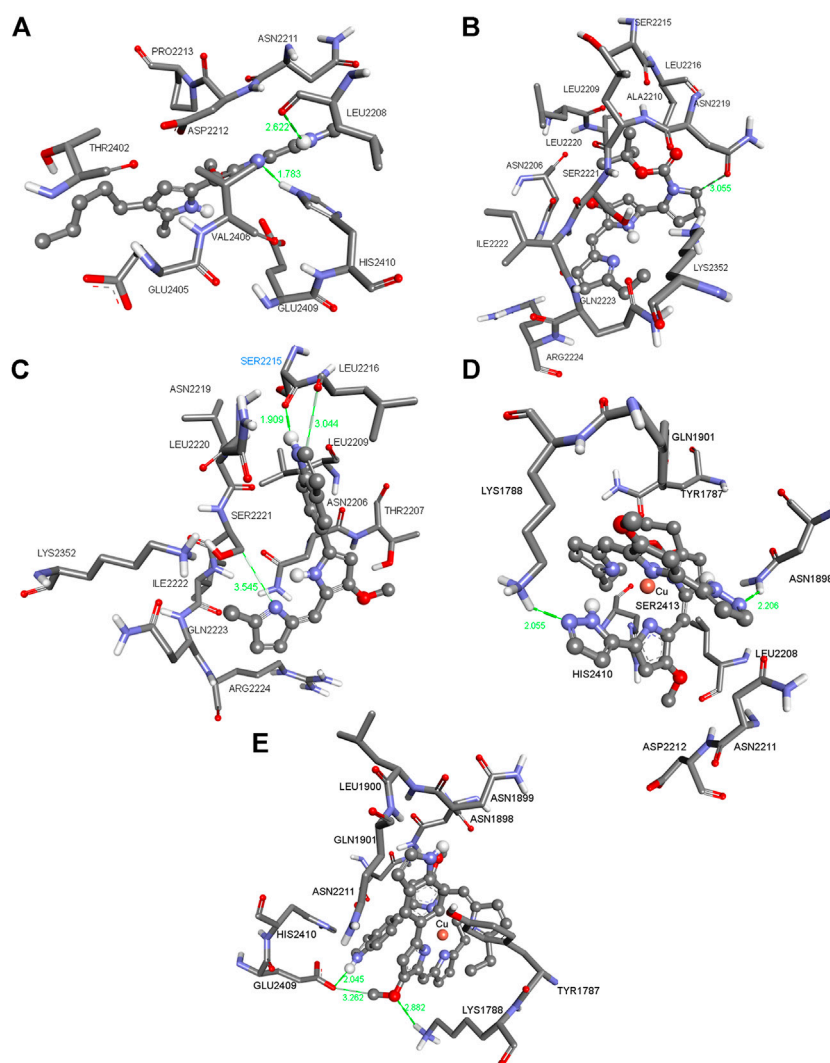


FIGURE 2

Three-dimensional (3D) images with the displayed hydrogen bonding distances (in green) generated via molecular docking of (A) Ps, (B) ligands L⁷ (C) and L¹⁴ and copper(I) complexes (D) C⁹ and (E) C¹⁴ into mTOR enzyme. Amino acid side chains are shown as sticks, the ligands as ball and sticks, and the chelating atom coppers are depicted as round copper tone.

Serum and 2% Penicillin-Streptomycin Solution). Cell lines were taken out from liquid nitrogen and thawed in a 37°C water bath. After thawing, cell cultures were transferred to 75 cm² flasks and incubated at 37°C with 5% CO₂ for 4–7 days. After reaching 80% of cell confluence, cells were ready for subculturing or testing.

Anticancer activity tests were performed using a Cell Counting Kit-8 (CCK8, SigmaAldrich) following manufacturer's instruction (Shen et al., 2016). Briefly, 100 µl of diluted cell suspension (approximately 10000 cells) was transferred to 96-well plate, incubated at 37°C with 5% CO₂ for 24 h. Then, the compounds were diluted into a serial of concentrations in DMSO. Next, 0.5 µl of diluted compounds were added to 100 µl of cell medium and gently mixed, resulting

in the final concentration of DMSO at 0.5%. 100 µl of the compound contained medium were then used to replace the original medium and incubated at 37°C for 48 h. After incubation with compounds, 10 µl of CCK8 reagent was added to each well and mixed by gently tapping the plate. Then, the plate was incubated at 37°C for 2–4 h until the orange color was dark enough to measure. The plate was measured using a Multiskan microplate reader (Fisher Scientific) at 450 nm. The IC₅₀ was calculated by SPSS statistics (IBM). Three controls were used to estimate the analysis: 1) positive control, contained cells and medium (0.5%DMSO) without compounds, used to calculated IC₅₀; 2) negative control, contained medium without compounds and cells, used to

TABLE 1 Calculated binding energies (in kcal/mol) and inhibition constants (in μM) for Ps, some ligands (L^7 , L^9 , and L^{14}) and copper(I) complexes (C1, C9, and C14). The binding energies and inhibition constants of the other ligands and complexes can be found in the [Supplementary Material \(Supplementary Table S1\)](#).

| Compound ID | mTOR | | HDAC1 | |
|-----------------|-----------------------------|--|-----------------------------|--|
| | Binding energies (kcal/mol) | Inhibition constants (μM) | Binding energies (kcal/mol) | Inhibition constants (μM) |
| Ps | -4.89 | 258.68 | -6.99 | 7.53 |
| L^7 | -5.04 | 202.51 | -7.06 | 6.64 |
| L^9 | -4.84 | 281.77 | -6.35 | 22.27 |
| L^{14} | -6.62 | 14.15 | 9.08 | 0.220 |
| C1 | -8.31 | 0.808 | -6.88 | 9.10 |
| C9 | -5.84 | 52.19 | -4.16 | 885.39 |
| C14 | -5.90 | 47.11 | -6.69 | 12.52 |

estimate the influence of medium color to the CCK8 results; 3) chemical control, contained medium and compounds without cells, used to estimate the influence of chemical color to the CCK8 results. All cytotoxicity tests were performed in six biological replicates. Each compound was performed two cycles of screening: primary screening using 10 times serial dilution to determine the approximate concentration and then secondary screening using a narrow concentration range to acquire the accurate IC_{50} .

Results and discussion

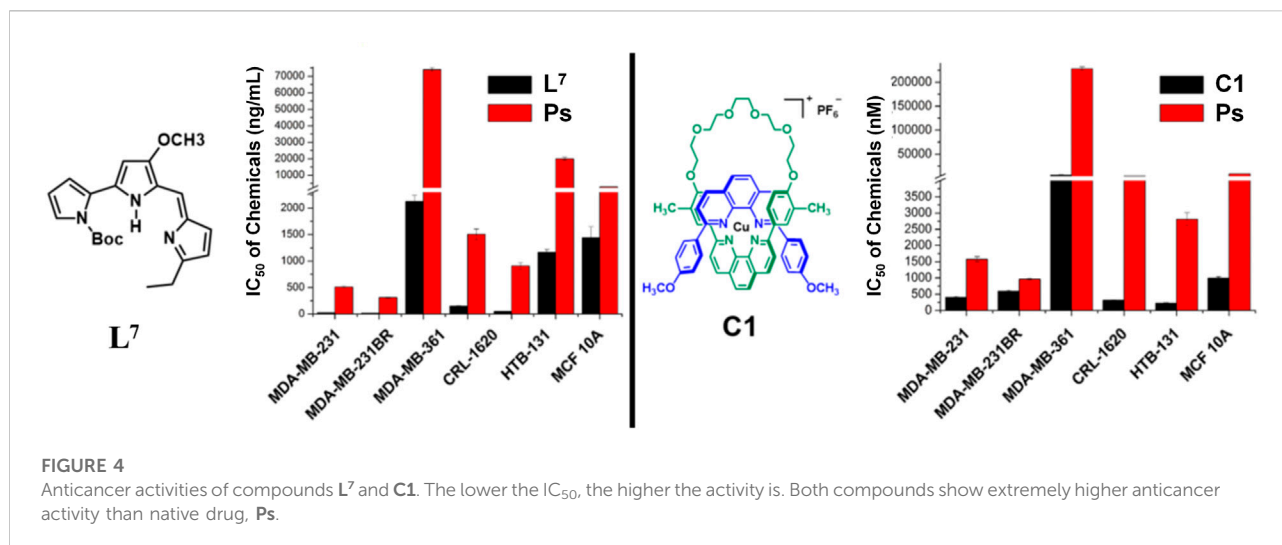
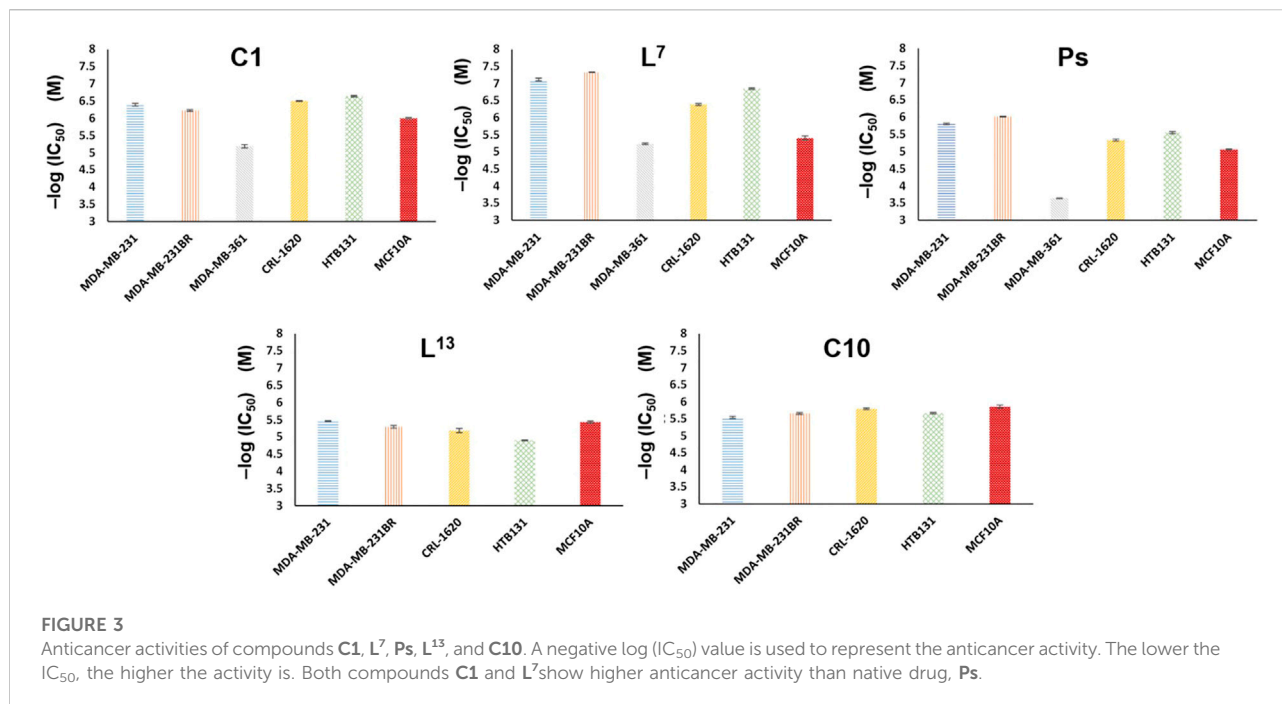
Molecular design, synthesis, and structural characterization

The ligands (L^1 – L^5) and complexes (C1–C5) were synthesized (Scheme 1) in high yield by adapting the exact and/or slightly modified literature procedures (our earlier publications) (Kang et al., 2008; Cetin, 2017; Cetin et al., 2017; Cetin et al., 2020). The synthesis of ligand L^1 involves in syntheses (Kang et al., 2008; Kang et al., 2014; Cetin, 2017) of both L^4 and L^5 prior to the final step for the desired product. The methyl groups directly bonded to the phenyl rings in the ligand L^1 (and L^4 as well) helped serve as additional spectroscopic markers for identification of the ligand as well as the corresponding copper(I) complexes C1 and C4 that were synthesized in high yields as shown in Scheme 1 (Kang et al., 2008; Kang et al., 2014; Cetin, 2017), and characterized by a variety of spectroscopic techniques. The C1 is a copper(I) complex of 2,9-di (4-methoxyphenyl)-1,10-phenanthroline (L^2) and the green macrocycle (L^1 in Scheme 1) with the counterion PF_6^- anion as the only bis(hetero-) complex. The synthesis of 2,9-dimesityl-1,10-phenanthroline (dmesp) ligand (L^6) was adapted from the literature procedures (Schmittl et al., 1997; Kohler et al., 2016; Cetin

et al., 2017; Kohler et al., 2017; Hayes D et al., 2018; Hayes L et al., 2018). The dmesp ligand was readily prepared by the Suzuki coupling of 2,4,6-trimethyl-phenylboronic acid and 2,9-dichloro-1,10-phenanthroline in good yield.

The syntheses of 2:1 ligand-to-metal complexes (C1–C6), as PF_6^- salts, were accomplished by addition of tetrakis (acetonitrile)copper(I) hexafluorophosphate to the solutions of ligands in a dry solvent mixture of acetonitrile: dichloromethane (50:50). The adopted synthetic procedures (Schmittl et al., 1997; Kohler et al., 2016; Cetin et al., 2017; Kohler et al., 2017; Hayes D et al., 2018; Hayes L et al., 2018) for complexes are summarized below (Scheme 1). The ligands and complexes were purified and characterized by spectroscopic analysis and the spectra matched that reported in the literature (Schmittl et al., 1997; Kang et al., 2008; Kang et al., 2014; Kohler et al., 2016; Cetin, 2017; Cetin et al., 2017; Kohler et al., 2017; Hayes D et al., 2018; Hayes L et al., 2018; Cetin et al., 2020).

The ligands (L^7 – L^{15}) and complexes (C7–C15) were synthesized (Scheme 2) in high yield by use of the modified literature procedures (Melvin et al., 2002). The key intermediate and prior precursors shown in Scheme 2 were synthesized and characterized by spectroscopic analysis (Sections B, C, and F in the Supplementary Material) and the spectra matched that reported in the literature (Melvin et al., 2002). Upon synthesis of the key intermediate, the ligands (L^7 – L^{15}) were synthesized by using different commercially available boronic acid precursors and characterized by spectroscopic analysis. Similar to the abovementioned complex synthesis (Kang et al., 2008; Kang et al., 2014; Cetin, 2017), the 2:1 ligand-to-metal complexes (C7–C15), as PF_6^- salts, were prepared, purified and characterized by spectroscopic analysis (Scheme 2). All the ligands (L^1 – L^{15}) and their corresponding complexes (C1–C15) are air-stable and capable of being dissolved in most common organic solvents.

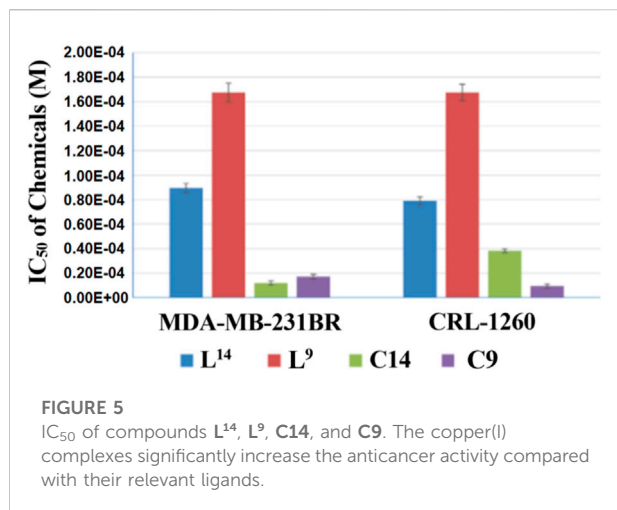


Biology and computational modeling

Both mTOR and HDAC1 enzymes, which are implicated in the biological activities of cancerous cells, were specifically chosen *in silico* works. The level of these enzymes drastically increases in the cancerous state of the cells in comparison to normal cells (Millard et al., 2013; Yang et al., 2013). In order to obtain additional validations to support the experimental results, molecular modeling studies (Table 1, Supplementary Tables S1, S2, and Figures 1, 2) were carried out using newly

synthesized compounds against mTOR and HDAC1 enzymes targets.

The compounds in Figures 1, 2 (for the other compounds, see Supplementary Tables S1, S2 in the Supplementary Material) were docked into the active sites of mTOR and HDAC1 enzymes to predict their binding affinities and binding modes employing AutoDock 4.2 docking software. Although the experimental studies were carried out using whole cells *in silico* studies on these crucial enzymes generated consistent results agreeing with the experimental inhibition values. All the compounds were

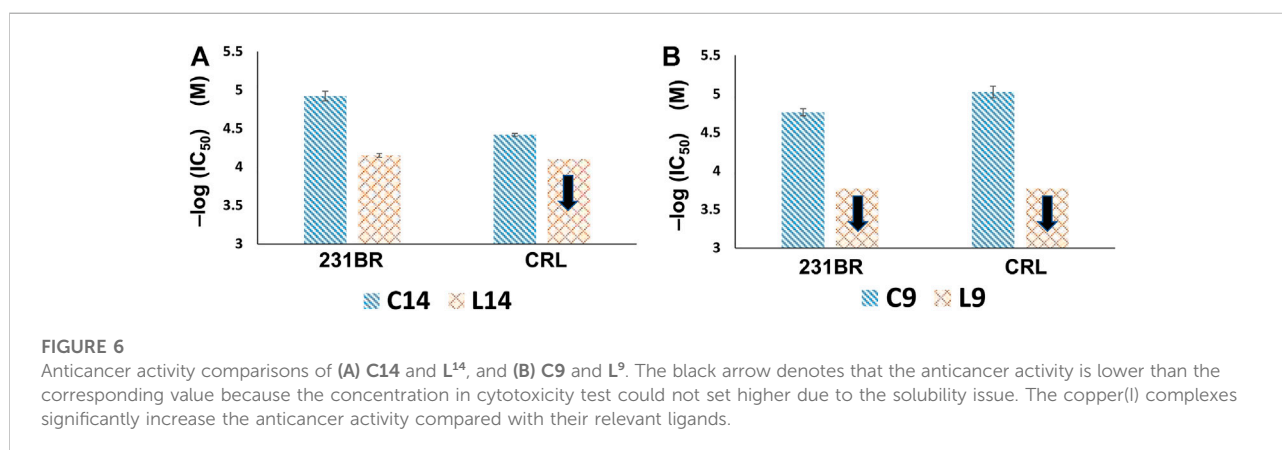


more active than that of reference compound **Ps** (405 μ M for mTOR and 348 μ M for HDAC1). Compounds **C1** and **L¹⁴** showed excellent binding affinity against mTOR (0.808 μ M) and HDAC1 (0.220 μ M) enzymes, supporting the experimental data (Table 1).

Figure 1A shows the pose of the **Ps** in the binding pocket of HDAC1 enzyme. A strong hydrogen bond occurs between the amide group of the ILE271 and the N-H bond of the **Ps**. The other interacting side chain residues with **Ps** are TYR303, TYR204, HIS178, GLY149, HIS141 and ASP99 which make various van der Waals and hydrophobic interactions with **Ps**. Figure 1B shows the pose of **L⁷** in the binding pocket of HDAC1 enzyme. Three strong hydrogen bonds occur between the ASP99, PHE205, and HIS178 and the ligand **L⁷** and such strong interactions make this compound one of the best inhibitors among this series. Figure 1C indicates the pose of **L14** in the binding pocket of the HDAC1 enzyme. With varying distances, three significant hydrogen bonds occur between the SER2221, LEU2216, and SER2215. Figures 1D,E show the copper complexes **C9** and **C14**. These complexes interact with the

nearby amino acids and make strong hydrogen bonds with the amino acids lining the binding pockets. These strong hydrogen bonds and the other hydrophobic and van der Waals interactions make these **C9** and **C14** complexes potential inhibitors against HDAC1 enzyme. Similarly, Figures 2A–E show the pose of **Ps**, **L⁷**, **L¹⁴**, **C9**, and **C14** in the binding pocket of mTOR enzyme, respectively.

The newly synthesized molecules in which their binding affinities were predicted *in silico* were tested on five different breast cancer lines, MDA-MB-231 (231), MDA-MB-361 (361), HTB131, HTB22, and MDA-MB-231BR (231BR), and one brain cancer cell line, CRL-1620 (CRL). The cell lines have been treated with these synthetic molecules to estimate their IC₅₀ anticancer capacities (Section E in the Supplementary Material). Among these cell lines, 231BR attracted most of our interest because it is a brain metastatic specific breast cancer cell line that has 100% rate of brain metastasis and cannot migrate to any other organs (Yoneda et al., 2001). The anticancer capacities of each compound were first screened by 10 times dilution to generate different molar concentrations ranging in 5 orders of magnitudes. This wide concentration range was applied for primary screening (Supplementary Table S3) to narrow down the targets. The approximate IC₅₀ was estimated in the primary screening step and used to design the concentration range of the secondary cytotoxicity tests (Supplementary Table S4). Any compounds showed IC₅₀ less than 10⁻⁵ M in primary screening were selected for the secondary cytotoxicity tests that had a molar concentration range within two orders of magnitude to acquire the accurate IC₅₀. Anticancer drug taxol (paclitaxel) was used as the reference to estimate the efficacy of each compound. Among these cell lines, 231BR was used in the primary screening because this cell grows the fastest and most sensitive to all compounds. The anticancer activity against 231BR is of highest interest. Meanwhile, the same treatments were performed against the normal breast cell line, MCF10A, to test their cytotoxicity. After the primary screening, five compounds, **L⁷**, **C1**, **Ps**, **L¹³**, and **C10** exhibited relatively promising



anticancer activities against 231BR with an $IC_{50} < 10^{-5}$ M (Supplementary Table S3). Then, a secondary cytotoxicity test was performed on each of these five compounds using a narrower concentration range (at least three biological replicates) to determine their IC_{50} against different cancer cell lines (Supplementary Table S4). Figure 3 depicts the anticancer activities of these compounds. A negative logarithm of IC_{50} was applied to represent the corresponding anticancer activity. Two of the abovementioned compounds (**C1** and **L7**) have demonstrated high anticancer activities and relatively low (Figure 4) cytotoxicity (on normal cell line MCF10A). A prodigiosin (**Ps**) derivative **L7** exhibited lowest IC_{50} (highest activities) against brain-seeking cell line 231BR ($4.63E-08 \pm 6.50E-10$ M) and 231 ($7.58E-08 \pm 7.20E-09$ M) that are considered as most invasion breast cancer cell lines while showed more than 80 times lower cytotoxicity on normal cell line ($3.39E-06 \pm 5.57E-07$ M). Additionally, **L7** also exhibited an overwhelming anticancer activity when compared with the precursor prodigiosin (**Ps**), the natural drug. The anti-breast cancer activity of the compound **L7** is more than 20 times better than the precursor drug, **Ps**.

Compound **C1**, on the other hand, is a novel compound. Figure 3 depicts that the compound **C1** that we have thus far synthesized has the different selectivity among cancer cell lines from compound **L7**. The copper(I) complex, **C1**, has better anticancer activities on breast cancer cell line HTB131 ($2.28E-07 \pm 1.11E-08$ M) and brain cancer cell line CRL ($3.15E-07 \pm 9.77E-09$ M). This new compound may provide us a novel anticancer mechanism and target, with some possible structural modifications and changes to decrease cytotoxicity on normal cells. However, all compounds showed lower anticancer activity against 361, demonstrating the selectivities need to be considered cautiously. Moreover, as a reference, commercialized anticancer reagent Taxol (paclitaxel) achieved more than 700 times lower cytotoxicity on normal cell line (IC_{50} of $3.00E-05$ M) relative to 231BR (IC_{50} $3.90E-08$ M), making it an efficient anticancer drug. When compared to commercialized anticancer reagent paclitaxel, the relatively lower anticancer activity on cancer cell lines and higher cytotoxicity on normal cell line of all compounds demand future efforts to enhance the anticancer activity while decreasing the cytotoxicity.

In addition, copper(I) complexes of the ligands were tested on the same cell lines as well. Figures 5, 6 showed the results that when coupled with copper(I), the anticancer activity increased. Compounds **C14** and **C9** are copper(I) complex of compounds **L14** and **L9**, respectively. IC_{50} of complexes **C14** and **C9** significantly decreased which meant the increasing of anticancer activity when compared with their ligands **L14** and **L9**. When compared **C14** and **L14**, the anticancer activity increased 5.9 times on 231BR from IC_{50} $7.08E-05 \pm 3.89E-06$ M to $1.20E-05 \pm 1.75E-06$ M (Figure 6A). The copper effect was even more significant for **C9**. When compared **C9** and **L9**, the anticancer activity increased more than 9.8 times on CRL

from $IC_{50} > 1.68E-04$ M to $1.72E-05 \pm 1.86E-06$ M (Figure 6B). Although the ligands showed less activity than ligand **L7**, this result provided a possible way for further improvement of the anticancer activity.

Among all the breast cancer cell lines, MDA-MB-231BR (231BR) is unique. This cell line has a 100% tendency to brain metastasis and cannot migrate to any other organs (Yoneda et al., 2001; Gupta et al., 2013). This brain-seeker cell line can be substantial in the breast cancer brain metastasis studies. We have reported the differential gene expressions in 231BR compared with other cell lines (Peng et al., 2019). Through anticancer agent screening, we can acquire compounds which have extremely high activity on this cell line compared to others. This may provide a potential treatment specific for the breast cancer patients who are at a large brain metastasis risk.

Conclusion

In this study, a series of 1,10-phenanthroline and Prodigiosin (**Ps**) derivatives consisting of their 2:1 ligand-to-metal copper(I) complexes, as PF_6^- salts, have been isolated and characterized. Their biological activities were tested *in vitro* on six different cell lines, and several compounds with good activity were preliminarily verified. Of these compounds, **L7**, **C1**, **Ps**, **L13**, and **C10** exhibited relatively promising anticancer activities against our highest interest cell line, 231BR, with an $IC_{50} < 10^{-5}$ M after the primary screenings. **C1** and **L7** have also demonstrated higher anticancer activities and relatively lower cytotoxicities on normal cell line MCF10A in the performance of the secondary cytotoxicity test using a narrower concentration range against different cancer cell lines. **L7**, in particular, has exhibited lowest IC_{50} (highest activity) against brain-seeking cell line 231BR and 231 that are considered as most invasion breast cancer cell lines while also showing more than 80 times lower cytotoxicity on MCF10A. In addition, **L7** has an overwhelming anticancer activity (more than 20 times better) than **Ps**, the natural drug. **C1**, on the other hand, has better anticancer activities on HTB131 and CRL compared to **L7**. The IC_{50} of **C14** and **C9** also showed increased anticancer activities compared to their relevant ligands **L14** and **L9**, respectively. When compared **C14** and **L14**, the anticancer activity of **C14** increased by 5.9 times on 231BR, whilst the anticancer activity of another copper(I) complex **C9** on CRL also rised by 9.8 times. These increases showed that the copper(I) complexes of the ligands were even more significant role on anticancer activities of such complexes. To obtain additional validations of the experimental data, some *in silico* modeling studies were carried out with mTOR and HDAC1 enzymes to predict their binding affinities and binding modes. Such studies also supported the experimental data that **C1** and **L14** showed excellent binding affinity against mTOR and

HDAC1 enzymes. Through anticancer agent screening, we can acquire the compounds which have very high activity on 231BR compared to other cell lines and may provide great therapeutical potential for the breast cancer patients who are at a large brain metastasis risk. Further syntheses of some of the above listed compounds as well as the structural optimization and modification of the synthesized molecules are still under study in our laboratories.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding and co-corresponding authors.

Author contributions

MC, KY, WP, MM, YM, and DU designed the experiments; MC, KY, WP, MM, YM, and DU wrote, reviewed and edited the main text; MC, WP, MM, and YM investigated the experiments; MC, MM, and YM designed and synthesized the compounds; MC, WP, and YM conducted the biological studies, cell culturing and cytotoxicity tests, and YM supervised these experiments; KY run the bioactivity evaluation against mTOR and HDAC Enzymes *via* molecular modeling and computational studies; and DU conducted the crystallography experiments. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.980479/full#supplementary-material>

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