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Synthesis and evaluation of a class of 1,4,7-triazacyclononane derivatives as iron depletion antitumor agents

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ABSTRACT

Iron depletion has been confirmed as an efficient strategy for cancer treatment. In the current study, a series of 1,4,7-triazacyclononane derivatives HE-NO2A, HP-NO2A and NE2P2A, as well as the bifunctional chelators *p*-NO_2-PhPr-NE3TA and *p*-NH_2-PhPr-NE3TA were synthesized and evaluated as iron-depleting agents for the potential anti-cancer therapy against human hepatocellular carcinoma. The cytotoxicity of these chelators was measured using hepatocellular cancer cells and compared with the clinically available iron depletion agent DFO and the universal metal chelator DTPA. All these 1,4,7-triazacyclononane-based chelators exhibited much stronger antiproliferative activity than DFO and DTPA. Among them, chelators with phenylpropyl side chains, represented by *p*-NO_2-PhPr-NE3TA and *p*-NH_2-PhPr-NE3TA, displayed the highest antiproliferative activity against HepG2 cells. Hence, these compounds are attractive candidates for the advanced study as iron depletion agents for the potential anti-cancer therapy, and could be further in conjugation with a targeting moiety for the future development in targeted iron depletion therapy.

Iron is essential in many important cellular processes, such as oxygen delivery, electron transport, and DNA repair.[1] However, excessive or misplaced tissue iron may donate electrons to oxygen, thus giving rise to the production of cytotoxic reactive oxygen species (ROS). The formation of excessive ROS was known to cause serious tissue damage leading to iron overloading diseases and cancers.[2,3] Previous studies have shown that the overloaded iron in the body is correlated to the generation and the development of cancers.[4] Cancerous cells require larger quantities of iron than normal cells, which is reflected by the marked overexpression of transferrin receptor (TfR) for the uptake of iron into cells.[5] The overexpressed TfR on the cell surface was found in several types of cancers, including breast cancer, prostate cancer, liver cancer, leukemia and lymphoma.[6] The increased iron uptake played a pivotal role during the intensive DNA synthesis in neoplastic cells.[7] As the rate-limiting step in DNA synthesis, ribonucleotide reductase (RR) catalyzed the reduction of ribonucleotides to deoxyribonucleotides only in the presence of iron in its active site.[8]

The increased iron dependence of cancer cells suggested that iron depletion may be an effective strategy to inhibit the rapid proliferation of cancer cells.[9] Indeed, clinically available iron chelator desferrioxamine (DFO) for the treatment of iron overloading disease β-thalassemia and universal metal chelator diethylene triamine pentaacetic acid (DTPA) were shown to lead cellular iron deprivation and suppress the growth of aggressive cancer cells (Fig. 1).[1,6,10] Meanwhile, it was confirmed in many studies that DFO displayed both antiproliferative and pro-apoptotic effects on various cancer cell lines, including melanoma, breast carcinoma, prostate carcinoma, leukemia, lymphoma and hepatocellular carcinoma.[1,6,10] And among these cells, the antiproliferative effect of DFO on hepatocellular carcinoma cells were more pronounced.[11] On the contrary, normal hepatocytes exhibited the resistance to the antiproliferative activity of DFO when compared to various hepatoma cell lines.[12] The selectivity between hepatoma cells and normal hepatocytes suggests that DFO may be a potentially useful agent for treating hepatoma.[12] Moreover, mechanism studies confirmed that DFO inhibited the RR activity via the chelation of intracellular iron pool, thus preventing the iron from incorporating into the enzyme active site.[11] In addition, polyaminocarboxylate chelator DTPA was also extensively explored for the anticancer activity against neuroblastoma and ovarian carcinoma cell lines.[13] Unlike DFO, DTPA is a membrane impermeable iron chelator, thus

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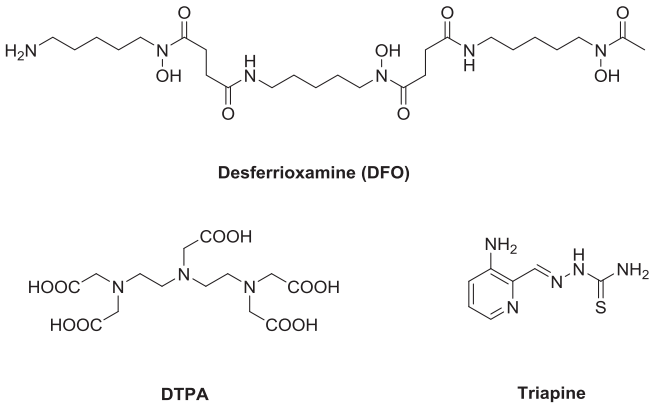


Fig. 1. Structures of iron chelators for clinical anticancer treatment.

the antiproliferative activity was probably produced by the chelating effect on the extracellular pool of iron.[10] More recently, a promising iron depletion agent, triapine (Fig. 1) has shown remarkable antitumor activity on several types of cancers in clinical trials.[14] And combined with a range of chemotherapeutics, triapine has also demonstrated promising results in several phase II clinical trials.[15]

During the past few years, 1,4,7-triazacyclononane (TACN) derivatives have attracted our attention, such as NETA, and its bifunctional version C-NETA (Fig. 2).[16] The origin of interest in the macrocycle TACN was from the property that TACN can coordinate facially to Fe^{3+} with the metal lying out of the plane defined by three nitrogen atoms to form exclusively five-membered chelate rings. Therefore, TACN appears to be an excellent basic platform to start with for the development of novel iron chelators as antitumor agents. In this study, a class of TACN-based chelators, HE-NO2A, HP-NO2A and NE2P2A, as well as the bifunctional versions of C-NETA, *p*-NO_2-PhPr-NE3TA and *p*-NH_2-PhPr-NE3TA (Fig. 2) were synthesized. Antiproliferative activity of the above chelators together with reported chelators C-NETA was evaluated against HepG2 cancer cells in vitro.

The structures of TACN-based chelators HE-NO2A, HP-NO2A, NE2P2A, C-NETA, *p*-NO_2-PhPr-NE3TA and *p*-NH_2-PhPr-NE3TA are shown in Fig. 2. Previously, we reported the synthesis of HE-NO2A and its analogue HP-NO2A, both of which featured a TACN platform combining with one hydroxypyridinonate and two carboxylic acid pendant arms.[17] Recently, a new bifunctional version of C-NETA, denoted as *p*-NO_2-PhPr-NE3TA, was designed and synthesized in our laboratory.[18] Both C-NETA and *p*-NO_2-PhPr-NE3TA possess a nitro group which can be further converted to an amino (NH_2) or isothiocyanate (NCS) group for conjugation with a receptor-targeting molecule. In particular, *p*-NO_2-PhPr-NE3TA contains a *p*-nitro-phenylpropyl group on nitrogen in the pendant arm, and the long propyl chain in the structure was designed to reduce potential steric hindrance during the formation of iron complex. And *p*-NO_2-PhPr-NE3TA possesses seven coordinating groups, which may be more effective in binding to the hexacoordinate iron than eight coordination groups in C-NETA. In current study, ligand *p*-NO_2-PhPr-NE3TA was synthesized according to a reported method with slight modification.[18] In the meantime, the reduction version of *p*-NO_2-PhPr-NE3TA, denoted as *p*-NH_2-PhPr-NE3TA, was also prepared.

As shown in Scheme 1, the key step for the preparation of the target ligands *p*-NO_2-PhPr-NE3TA and *p*-NH_2-PhPr-NE3TA is the coupling reaction of fragment **4** with **5** in the presence of anhydrous K_2CO_3 in acetonitrile (MeCN).[17] Specifically, fragment **4** was prepared starting from commercially available material 1-(3-bromopropyl)-4-nitrobenzene (**1**). Reaction of **1** with excess

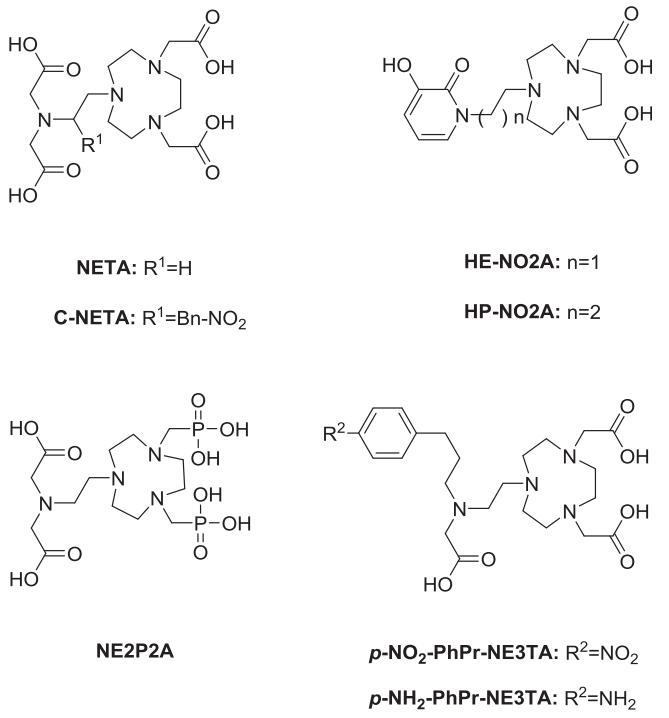
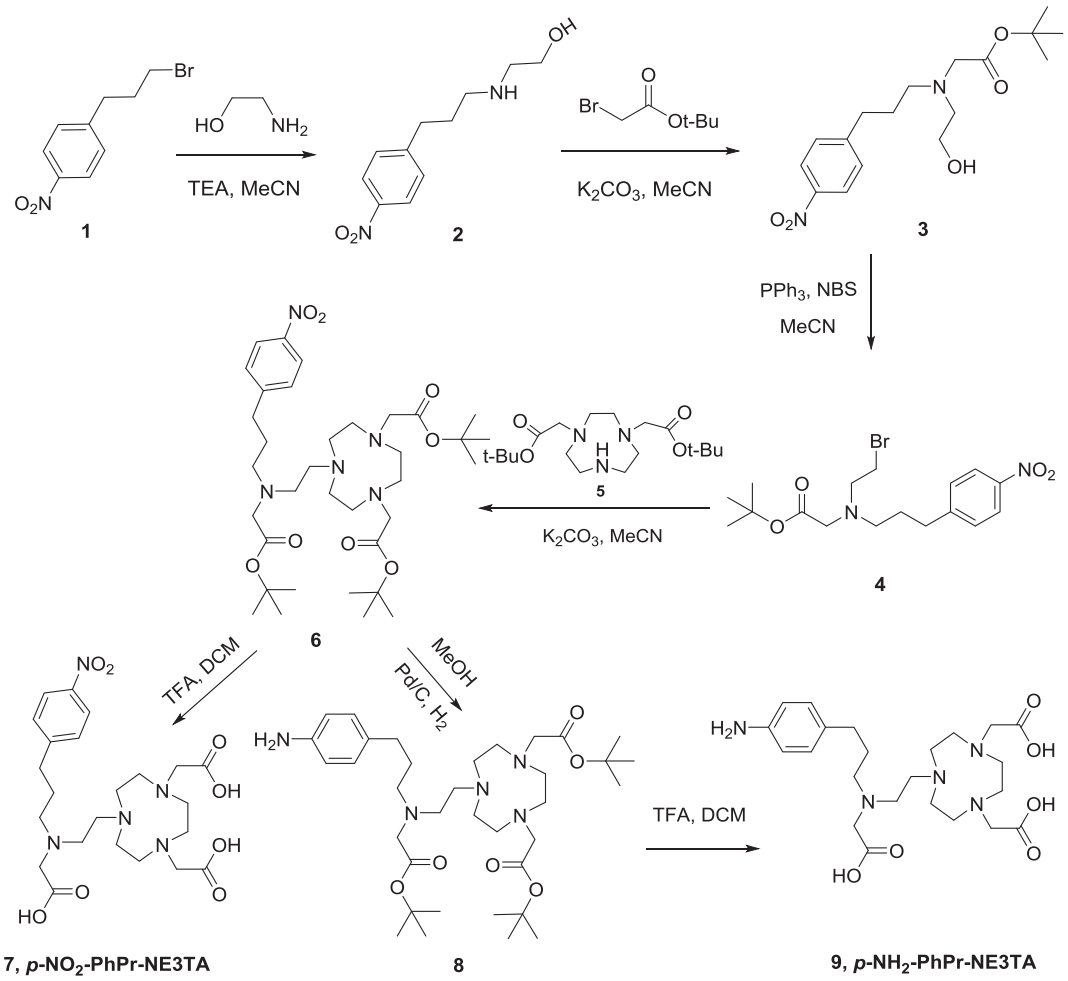


Fig. 2. Structures of TACN-based iron-depleting antitumor agents.

amount of 2-aminoethanol in the presence of triethylamine (TEA) in MeCN gave *N*-*p*-nitro-phenylpropyl ethanol amine **2**. Subsequently, compound **2** was alkylated by *tert*-butyl bromoacetate to provide **3**, which was further reacted with *N*-bromosuccinimide (NBS) and triphenylphosphine (PPh_3) to yield corresponding bromide **4**. The crucial intermediate **6** was obtained in the coupling reaction of **4** and **5**, which was isolated by column chromatography in good yield (63.2%). The *tert*-butyl groups in precursor **6** were then successfully removed by treatment with trifluoroacetic acid (TFA) in methylene dichloride (DCM) to afford desired chelator *p*-NO_2-PhPr-NE3TA (**7**) in an excellent yield (99.0%). Reaction of intermediate **6** in 10% Pd/C in methanol under H_2 gas at room temperature provided the aniline **8**, which was then subjected to deprotection of *tert*-butyl groups, thereby affording the desired chelator *p*-NH_2-PhPr-NE3TA (**9**) in 93.5% yield.

Chelators synthesized above are based on the TACN derivatives with acetate pendant arms. However, little has been explored for other suitable pendent donor groups which is one of the decisive factors determining the effectiveness of iron chelator.[19] Recently, many reports indicate that replacing carboxylate pendant arms in chelators with phosphonate pendant arms can accelerate the metal-binding kinetics.[20] In this context, the di-methylphosphonate pendant armed TACN derivative NE2P2A was designed and synthesized. Particularly, NE2P2A contains an additional carboxylate pendant arm in combination with TACN platform which is similar with the coordination groups in DTPA, thus promoting to form stable complexes with iron in fast thermodynamic kinetics. An efficient and convenient synthetic route of NE2P2A was developed as illustrated in Scheme 2. The key step is the coupling reaction of the fragment **11** with **12** in the presence of anhydrous K_2CO_3 in MeCN to yield crucial intermediate **13**.[21] Fragment **11** was synthesized according to a known procedure as reported previously.[22] Briefly, reaction of 2-aminoethanol with excess amount of benzyl bromoacetate in the presence of anhydrous $KHCO_3$ in dimethyl formamide (DMF) gave the dialkylated product **10**, which was subsequently converted to bromide **11**. A coupling reaction of **11** with **12** gave intermediate **13** in 39.1% yield. The ethyl and ben-

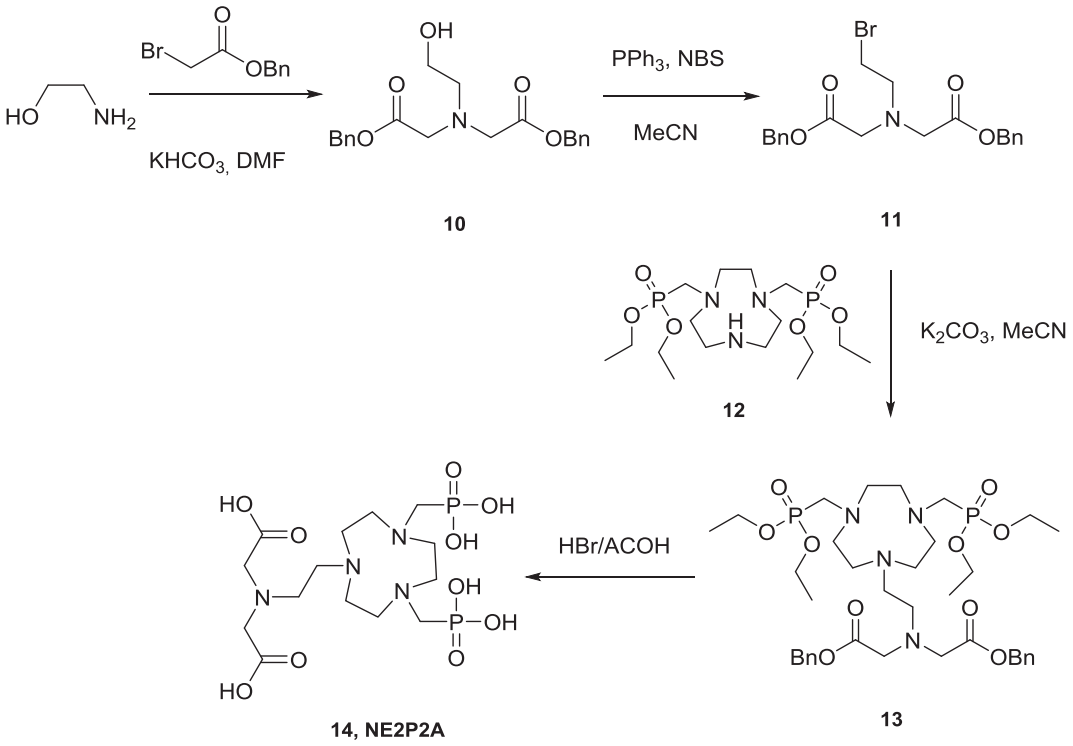


Scheme 1. Synthesis of *p*-NO_2-PhPr-NE3TA and *p*-NH_2-PhPr-NE3TA.

zyl protecting groups in **13** were simultaneously removed with the hydrolysis agent HBr/AcOH to obtain the target compound NE2P2A (**14**) in 93% yield (see Supplementary Material for further details).

Owing to the unique design of integrating both hydroxypyridinonate and carboxylic acid pendant arms on TACN platform, HE-NO2A and HP-NO2A were supposed to have high potential capability on iron chelation, thus cell viability study was initially applied using HepG2 cells to explore their cell cytotoxicity. As shown in Fig. 3, both HE-NO2A and HP-NO2A produced about 80% inhibiting effect at the concentration of 50 μM, while DFO and DTPA displayed 40% and 60% inhibitory effect under the same condition, respectively. To investigate the chelating effect of these agents on cell viability, the HepG2 cells were incubated with equal amount of iron chloride (50 μM) to saturate each chelators. Under the conditions of this investigation, almost no inhibitory activity of HE-NO2A, HP-NO2A, DFO or DTPA could be observed (Fig. 3). The result suggests that these chelators may exhibit antiproliferative activity as a result of iron chelation. To further evaluate the antitumor activity of HE-NO2A and HP-NO2A, antiproliferative activity of the chelators in a series of concentrations was measured using HepG2 cells and compared to clinical iron chelators DFO and DTPA. As shown in Fig. 4, HE-NO2A and HP-NO2A displayed remarkably higher inhibitory activity than DFO or DTPA within the tested concentration range. Inspection IC_{50} values of these chelators in the Table 1, HE-NO2A, HP-NO2A possess the respective IC_{50} of 6.9 ± 0.9 μM and 4.5 ± 0.2 μM, both of which are much lower than that of DTPA (38.3 ± 1.4 μM) and DFO (>100 μM). The capability of HE-NO2A and HP-NO2A on iron chelation may originate from the TACN platform with two acetic acid pendant arms which has been confirmed to possess strong coordinating ability towards iron.[17] In addition, the pendent oxygen-containing donor hydroxypyridinonate demonstrated high affinities to hard Lewis acid, which may contribute to these chelators binding to iron more effectively. HP-NO2A possesses a slightly more potent antiproliferative activity compared to HE-NO2A. It seems that a long propyl chain between TACN platform and hydroxypyridinonate group may reduce potential steric hindrance during the iron chelating process, thus leading to enhanced cytotoxicity.

As shown in Fig. 3, the antiproliferative activity of *p*-NO_2-PhPr-NE3TA, *p*-NH_2-PhPr-NE3TA and NE2P2A is associated with iron chelation. The cytotoxicity of *p*-NO_2-PhPr-NE3TA, *p*-NH_2-PhPr-NE3TA, and NE2P2A together with C-NETA were further evaluated using HepG2 cells (Fig. 4). As shown in Table 1, *p*-NO_2-PhPr-NE3TA and *p*-NH_2-PhPr-NE3TA produced the strongest antiproliferative activity with respective IC_{50} value of 1.4 ± 0.1 μM and 1.5 ± 0.1 μM, both of which were lower than that of C-NETA (8.2 ± 0.3 μM). These results support our proposed hypothesis that increased distance between nitro functional unit and TACN platform is conducive to the formation of iron complex, leading to enhanced cytotoxicity. And it should be noted that *p*-NH_2-PhPr-NE3TA possesses almost the same cytotoxicity as its parent ligand *p*-NO_2-PhPr-NE3TA in HepG2 cells, suggesting that a *p*-amino-phenylpropyl group in *p*-NH_2-PhPr-NE3TA may not affect the complexation with iron. This may potentially contribute to the further conjugation of



Scheme 2. Synthesis of NE2P2A.

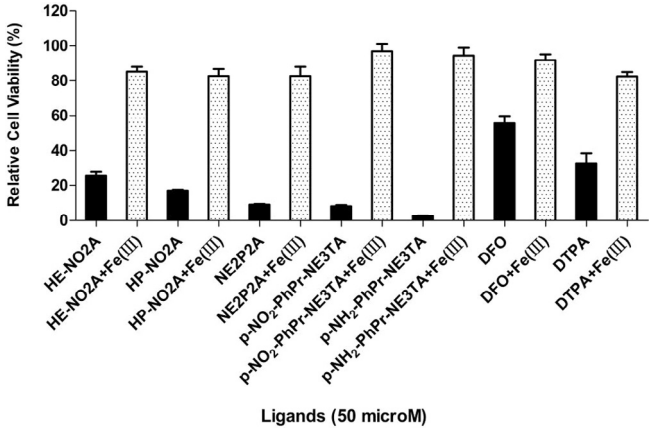


Fig. 3. Effects of chelating agents and iron saturation on HepG2 cancer cells growth.

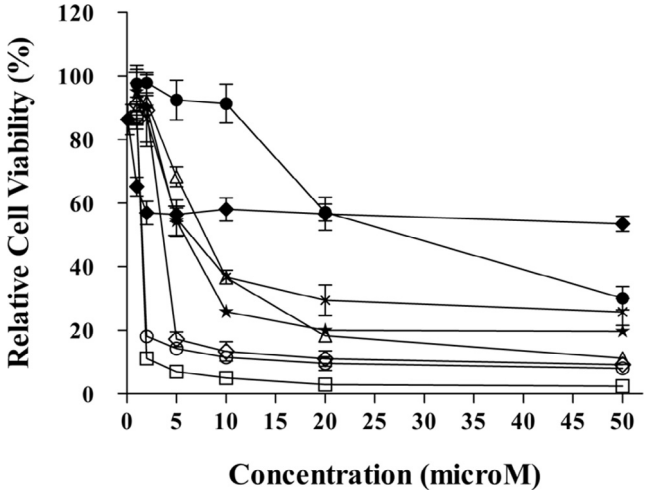


Fig. 4. Effects of chelators HE-NO2A (×), HP-NO2A (★), NE2P2A (◇), C-NETA (△), *p*-NO_2-PhPr-NE3TA (□), *p*-NH_2-PhPr-NE3TA (○), DFO (◆) and DTPA (●) on viability of HepG2 cancer cells.

this chelator with various tumor-targeting peptides or antibodies for targeted iron depletion therapy. On the other hand, NE2P2A displayed higher inhibiting activity compared to HE-NO2A, HP-NO2A and C-NETA, and possessed significantly decreased IC_{50} value of 3.8 ± 0.6 μM (Table 1). However, NE2P2A showed a slight increase in IC_{50} value as compared with *p*-NO_2-PhPr-NE3TA or *p*-NH_2-PhPr-NE3TA, which might due to the more negative charges and the lack of hydrophobic groups in its structure. In summary, all TACN-based chelators studied herein exhibited much stronger inhibitory activity than clinical iron chelators DFO and DTPA.

With the promising antiproliferative activity of TACN-based chelators, we attempted to evaluate the cytotoxicity of these chelators (50 μM) in normal hepatocytes cell line (LO2). As shown in the Fig. 5, minimal growth inhibitory effects were observed in LO2 cells which were cultivated with DFO or DTPA. When treated with TACN-based chelators, LO2 cells displayed at least 3∼8 times higher cell viability as compared to HepG2 cells under the same condition. In other words, TACN-based chelators exhibited outstanding higher antiproliferative activity in cancerous cells than in normal cells. These results suggest that the rapid proliferation of cancerous cells can be efficiently suppressed by using iron depletion chelators with no or slight influence to normal cells, supporting the proposed hypothesis that cancerous cells require larger quantities of iron than normal cells.

In the present study, we have prepared a class of TACN-based chelators and evaluated their cytotoxicity using HepG2 cancer cell line. Our results suggest that the non-functionalized chelators HE-NO2A, HP-NO2A and NE2P2A exhibited notably higher antiproliferative activity than clinical iron-chelating agents DFO and DTPA. The introduction of hydroxypyridinonate or phosphonate pendent

Table 1
IC_{50} values of chelators in HepG2 cancer cells.[a]

IC_{50} (μM)	
Ligands	HepG2
HE-NO2A	6.9 ± 0.9
HP-NO2A	4.5 ± 0.2
NE2P2A	3.8 ± 0.6
C-NETA	8.2 ± 0.3
p-NO_2-PhPr-NE3TA	1.4 ± 0.1
p-NH_2-PhPr-NE3TA	1.5 ± 0.1
DFO	>100
DTPA	38.3 ± 1.4

[a] The HepG2 cells were incubated for 72 h with chelators at various concentration, and the cell relative viability was measured using MTT methods (see Supplementary Material for further details).

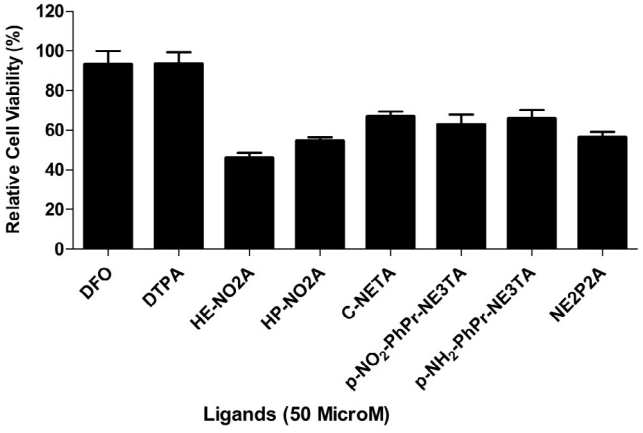


Fig. 5. Cytotoxicity of chelating agents against LO2 cells.

donor groups resulted in significantly increased antiproliferative activity (HE-NO2A, HP-NO2A and NE2P2A, respectively), providing a new orientation for developing novel iron depletion antitumor agents. Meanwhile, reported chelator C-NETA displayed a slightly decreased inhibitory effect on HepG2 cells as compared to non-functionalized chelators. The promising bifunctional chelator C-NETA was optimized via modifying the nitro group in the structure. The result of cytotoxicity measurements demonstrated that novel bifunctional versions of C-NETA, *p*-NO_2-PhPr-NE3TA and *p*-NH_2-PhPr-NE3TA displayed the highest inhibitory activity against cancerous cells. Both the non-functionalized chelators (HE-NO2A, HP-NO2A and NE2P2A) and the bifunctional chelators (C-NETA, *p*-NO_2-PhPr-NE3TA and *p*-NH_2-PhPr-NE3TA) are promising anticancer therapeutic agents. It is noteworthy that two novel bifunctional ligands, *p*-NO_2-PhPr-NE3TA and *p*-NH_2-PhPr-NE3TA demonstrated the most potentials since they can be further modified to link with various peptides and monoclonal antibodies for targeted iron depletion therapy.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.11.048.

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