Honokiol-Chlorambucil Co-Prodrugs Selectively Enhance the Killing Effect through STAT3 Binding on Lymphocytic Leukemia Cells In Vitro and In Vivo

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ABSTRACT: The broad-spectrum DNA alkylating therapeutic, chlorambucil (CBL), has limited safety and shows lower therapy effect because of a short half-life while used in the clinic. Therefore, it is very necessary to develop a more efficient and safer type of CBL derivative against tumors with selective targeting of cancer cells. In addition, the natural product of honokiol (HN), the novel potent chemo-preventive or therapeutic entity/carer, can target the mitochondria of cancer cells through STAT3 to prevent cancer from spreading and metastasizing. In this study, we designed and synthesized the honokiol−chlorambucil (HN−CBL) co-prodrugs through carbonate ester linkage conjugating with the targeted delivery help of the HN skeleton in cancer cells. Biological evaluation indicated that HN−CBL can remarkably enhance the antiproliferation of human leukemic cell lines CCRF-CEM, Jurkat, U937, MV4-11, and K562. Furthermore, HN−CBL can also selectively inhibit the lymphocytic leukemia (LL) cell survival compared to those mononuclear cells derived from healthy donors (PBMCs), enhance mitochondrial activity in leukemia cells, and induce LL cell apoptosis. Molecular docking and western blot study showed that HN−CBL can also bind with the STAT3 protein at some hydrophobic residues and downregulate the phosphorylation level of STAT3-like HN. Significantly, HN−CBL could dramatically delay leukemia growth in vivo with no observable physiological toxicity. Thus, HN−CBL may provide a novel and effective targeting therapeutic against LL with fewer side effects.

1. INTRODUCTION

Chlorambucil (CBL), a DNA alkylating reagent belonging to the nitrogen mustard family, is a chemotherapeutic used to treat chronic lymphocytic leukemia (CLL), lymphoma, and many other types of solid neoplasms.1,2 The N,N-bis(2-chloroethyl)amine group moiety can covalently react with proteins, nucleic acids, and phospholipids to induce inhibitory function for cell survival, while the CBL alkylation reaction with DNA is the primary form of cytotoxicity. In addition, the forms of CBL-modifying DNA cross-link include monofunctional base-pair mismatching and bifunctional double-strand DNA breaking, which cause sustained DNA damage.3,4 Because of the high reactivity of CBL with lots of biological macromolecules (nucleic acids, proteins, and phospholipids), which leads to the lower therapeutic effect with a short half-life in clinic and means a higher dose of CBL is required for the therapeutic response. However, an increased dose will increase the risk of severe side effects. In addition, these compounded consequences of instability and nonspecificity reactivity will bring down the bioaction rate of CBL. For now, although some newer drugs have been successfully developed for clinical application, the fact is that CBL remains a first-line treatment for the elderly CLL and some immune-suppressed cancer persons. Therefore, it is very important to develop the novel CBL derivatives with a higher anticancer effect and stable hypotoxicity for normal healthy tissues.5−7

Honokiol (HN, C18H18O2) is an isolated dietary biphenolic natural product from Magnolia officinalis. During the last decade, lots of studies have shown that HN exhibits a broad inhibitory effect on malignant carcinomas (e.g., myeloma and leukemia8,9) in vitro and in vivo through anticarcinogenic, proapoptotic, anti-
inflammatory, antioxidative, and antiangiogenic activities without any observable sub-toxicities. Furthermore, HN can effectively inhibit many pathways and lead to anti-proliferation of cancer cells, such as the cell signaling of NF-kB, EGFR, STAT3, cyclooxygenase, and the other cell apoptosis factor, and so forth. Meanwhile, HN can also overcome even notoriously drug-resistant neoplasms. In addition, HN was considered to be nearly as effective as an antitumor drug against colorectal cells and equal to the common chemotherapeutic adriamycin (DOX). Very importantly, HN can target cancer cells’ mitochondria through STAT3 to halt cancer progression and metastasis, which indicated that HN could be the novel potent chemo-preventive or therapeutic entity for tumor treatment.

Although clinical studies are still lacking, HN possesses so many beneficial bio-effects against cancer with high safety characteristics, which suggests that HN is a promising potential antitumor reagents from the approved therapeutics or the safe dietary natural products, rather than any other unknown compounds.  Based on the above molecular mechanism backgrounds of CBL and HN, we think that the development of the novel antitumor reagents from the approved therapeutics or the safe dietary natural products, rather than any other unknown compounds, would promote their transformation and application in cancer therapy. To complete the above proof-of-concept of enhancing the killing effect on cancer cells through co-prodrugs, and based on the biologic basis of higher esterase and low pH in the tumor tissue or cancer cells, we designed and synthesized a honokiol–chlorambucil (HN–CBL) ester co-prodrugs through carbonate ester linkage conjugation.

2. RESULTS AND DISCUSSION

2.1. Chemical Preparation and Characterization of the HN–CBL Co-Prodrugs

Based on the cleavage lysis feature of co-prodrugs HN–CBL with carbonate ester by intracellular esterase’s hydrolysis catalyzed and then released the drugs CBL and HN in the presence of EDCI. The purity of HN–CBL was >95% and measured with HPLC.

2.2. In Vitro-Targeted Release Pharmacokinetics of HN–CBL in Cancer Cells

Based on the above molecular mechanism backgrounds of CBL and HN, we think that the development of the novel antitumor reagents from the approved therapeutics or the safe dietary natural products, rather than any other unknown compounds, would promote their transformation and application in cancer therapy. To complete the above proof-of-concept of enhancing the killing effect on cancer cells through co-prodrugs, and based on the biologic basis of higher esterase and low pH in the tumor tissue or cancer cells, we designed and synthesized a honokiol–chlorambucil (HN–CBL) ester co-prodrugs through carbonate ester linkage conjugation. In our report, the releasing response mechanism of HN–CBL was that the double carbonic ester conjugated with HN and CBL, which can be simply hydrolyzed in higher intracellular esterase’s hydrolysis catalyzed environment (e.g., the cancer cells lysates) and is especially sensible to the tumor acid microenvironment (as pH = 5.5 vs pH = 7.4). When evaluating the inhibitory effect on a series of carcinoma and normal cell lines with in vitro MTT cytotoxicity methods, HN–CBL, exhibited better therapeutic potency than its parent drugs HN and CBL through directly enhancing mitochondrial activity. HN–CBL could selectively enhance the killing effect in lymphocytic leukemia (LL) cells, and no red blood cells hemolysis reaction was observed at the therapeutic concentration of HN–CBL. Moreover, HN–CBL could remarkably enhance the apoptosis in LL cells but had no damage on normal PBMCs. The computational docking and western blotting study showed that HN–CBL can also bind with the STAT3 protein at some hydrophobic residues and downregulate the phosphorylation level of STAT3-like HN. Above all, HN–CBL could dramatically delay leukemia growth in vivo with no observable physiological toxicity. Compared with free drugs, these results indicated that HN–CBL may provide a novel and selectively therapeutic co-prodrugs against LL with fewer side effects.

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In addition, the IC50 values of HN antitumor spectrum, especially in leukemia cells with selectivity. These results suggested that HN observable cytotoxicity on two normal cells LO2 and NIH3T3.

μM each treated in triplicate. (IC50 = 4.86 hypothesis of co-prodrug, HN-CBL, HPLC-MS method was used to evaluate the drug release of HN or CBL in different mediums. The hydrolysis release of HN or CBL from co-prodrug HN-CBL was measured at 37 °C in different bio mediums (as PBS in pH = 7.4 or 5.5, 10% fresh plasma and 10% cancer cells lysate.). The degradation of HN-CBL and the production of HN or CBL were measured with HPLC-MS technology. From the results of Figure 1, we found that only <10% of HN-CBL was hydrolyzed in normal isosmotic buffer PBS of pH = 7.4, but it would release more than 25% of free drugs in PBS of pH = 5.5, which proved that the co-prodrug HN-CBL has the tumor acid microenvironment sensibility response characterization. Meanwhile, when HN-CBL was incubated with 10% fresh mouse plasma including PBS (pH = 7.4), there was about 40% HN or CBL released from HN-CBL, but which would release more than 70% products in 10% CCRF-CEM cancer cell lysate with pH = 7.4 at 37 °C. The hydrolysis difference might attribute to the higher esterase expression in cancer cells. In addition, the 10% normal mouse liver tissue was used to verify the above hypothesis, and we found that HN-CBL could keep much more bio-stable than that of the CCRF-CEM cells lysate (about 50% vs 70%). Therefore, these results indicated that the HN-CBL co-prodrugs can release free drugs HN and CBL well for synergistic effects, which will improve the specificity of CBL toward cancer cells, thus decrease the risk of side effects.

2.3. HN–CBL Selectively Inhibited Leukemia Cell Proliferation. In this study, the anticancer effects of co-prodrugs HN-CBL on a total of seven cancer cell lines were investigated by the MTT assay. Our data showed that the treatment of compound HN-CBL efficiently decreased the survival of seven tested carcinoma cell lines, as lymphocytic carcinoma CCFR-CEM (IC50 = 1.09 μM), Jurkat (IC50 = 1.15 μM), U937 (IC50 = 1.29 μM), MV4-11 (IC50 = 2.78 μM), K562 (IC50 = 4.86 μM), lung cancer A549 (IC50 = 25.10 μM), and human hepatoma HepG2 (IC50 = 24.50 μM), cells, and no observable cytotoxicity on two normal cells LO2 and NIH3T3. These results suggested that HN-CBL has a relatively wide antitumor spectrum, especially in leukemia cells with selectivity. In addition, the IC50 values of HN-CBL were lower than that of CBL and HN in the seven human cancer cell lines tested, which indicated that HN-CBL’s synergistic antitumor activity was more potent than that of both HN and CBL (Table 1).

2.4. HN–CBL Selectively Inhibited Human Primary LL Cell Survival. In particular, it was notable that compound HN–CBL effectively reduced the cell survival of the carcinoma cells CCRF-CEM, U937, MV4-11, Jurkat, and K562 (IC50 = 1.09–4.86 μM). Because the co-prodrug HN–CBL decreased the survival of leukemia cells more efficiently, we continue to investigate the antiproliferation profile of co-prodrug HN–CBL and the therapeutic window in primary LL cells (Figure 2). To compare the toxicity of HN–CBL on the healthy cells, MTT colorimetric method was performed on three types of donor cells including the healthy RBCs, the healthy PBMCs, and the B cells isolated from LL patients. Compared with free drugs HN or CBL, HN-CBL did not produce RBC-hemolysis reaction at the tested concentrations (Figure S5), which implicated that HN-CBL can ablate the leukemia cells with low side effects. Compared to those derived from healthy donors, HN-CBL more preferred to target and kill the leukemia cells of LL patients than CBL, which was consistent with the release pharmacokinetic results of HN-CBL showing HN-CBL’s cancer cell specificity because of the relative higher esterase activity and lower pH value in cancer cells. 3. Discussion

3.1. CBL Selectively Inhibited Human Primary LL Cell Survival. It is well known that anticancer drug CBL has a high alkylating function, and its antileukemia bioactivity is focused on the cellular nuclear genome of cancer cells. In addition, the IC50 values of HN-CBL were lower than that of CBL and HN in the seven human cancer cell lines tested, which indicated that HN-CBL’s synergistic antitumor activity was more potent than that of both HN and CBL (Table 1).

3.2. HN–CBL Selectively Inhibited Human Primary LL Cell Survival. The natural product HN was used to co-deliver the CBL, which can target cancer cells’ mitochondria (mt) through STAT3 to halt cancer progression and metastasis. In addition, mt-DNA damage will increase ROS levels and change mt’s membrane potential. Therefore, to confirm the relation of HN–CBL-inducing cell death and the mitochondria, two mt'
bio-marks, ROS levels and mt membrane potential were detected with flow cytometry. While the leukemia cells were treated with HN−CBL, we observed that the superoxide concentration was increased and the mt membrane potential was reduced (Figure 3A,B). However, the nuclear genome cross-linking agent CBL did not statistically change mt superoxide levels and its membrane potential. These data supported the concept that HN−CBL is selectively destructive on the mitochondrial organelle.

2.6. HN−CBL-Induced LL Cell Apoptosis but No Damage on Normal PBMCs. Apoptosis is a programmed way that can induce cell death, and it has been proved that the mitochondria are involved in apoptosis through different mechanisms. Moreover, our results showed that HN−CBL can induce cancer cell death through the activity on mitochondria. Thus, the cell apoptosis experiments of Annexin V-FITC/PI staining were used to determine the inhibitory effects of HN−CBL on leukemia cells. PBMCs and LL cells were incubated with HN, CBL, and HN−CBL for 24 h. The obtained apoptosis analysis showed that HN did not indicate an statistical difference in the apoptotic percentage in LL cells as well as untreated cells (about 7.5 vs 4.8%), and CBL could remarkably induce CLL cell apoptosis by about 25%, and the significant LL cell death response was observed between HN−CBL treatment and CBL (about 40 vs 25%). However, no damage was observed on PBMCs (Figure S6). These results indicated that the cell apoptosis signal may be the main mechanism that co-prodrug HN−CBL exerts the higher bioactivity on leukemia cells than on CBL and HN (Figure 4). Our results indicated that the conjugation of HN and CBL can improve the anticancer activity of CBL and HN, and HN−CBL may be a novel chemotherapeutic for further investigation.

2.7. Molecular Docking for HN and HN−CBL Interaction with STAT3. In previous reports, HN was proved to target cancer cells’ mitochondria (mt) through STAT3 to halt cancer progression and metastasis. To explore the possible targeted delivery mechanism for HN−CBL, we further determined the interaction of HN or HN−CBL and STAT3 in silico. Molecular docking experiments were conducted for HN or HN−CBL and the crystal structure of STAT3 (PDB code: 3CWG) by Sybyl-X 2.1.1 software following the rule of the lower the energy was, the better the docking orientation. HN bound to STAT3 through alkyl and pi−alkyl interaction with a cluster of hydrophobic residues from each domain: ILE-467, HIS-332, PRO-471, and MET-470 (Figure 5A,B), which agreed with the reported STAT3 inhibitor CuB. In addition, the hydrogen or oxygen atom of HN can form a hydrogen bond with the oxygen or hydrogen atom on the amino acid of LYS-573,
ASP570, ARG-335, and ASP-566 in STAT3. The formation of the hydrogen bond enhances HN to target the STAT3 protein. Very interesting, the co-prodrug HN−CBL was also bound to STAT3 as HN through alkyl and π−alkyl interaction with a cluster of hydrophobic residues from each domain: ILE-467, HIS-332, PRO-471, MET-470, PRO-330, and MET-331 (Figure 5C,D), which indicated that HN−CBL maintains maximally the binding activity with STAT3. In addition, the hydrogen or oxygen atom of HN−CBL can form a hydrogen bond with the oxygen or hydrogen atom on the amino acid of LYS-573 and HIS-332, the hydrogen atom of HN−CBL can form a carbon hydrogen bond with the carbon atom on the

Figure 5. Molecular docking analysis for HN or HN−CBL with STAT3 and western blotting study. (A,C) Molecular binding mode of HN and HN−CBL docked into the target STAT3. (B,D) Details of binding sites of the HN and HN−CBL in STAT3 protein. (E) While leukemia cells were treated with HN or HN−CBL (10 μM) for 12 h, and the collected cell lysates were subjected to western blotting analysis.

Figure 6. In vivo xenograft anti-LL study. (A) Photo images of the harvested tumors with different treatment. (B) Antitumor volume change difference, the sacrificed tumor weight index (C) and its relative weight changes (D) between control and the other drug treatments. Note that *, p < 0.05; **, p < 0.01, compared with the solvent control group.
Figure 7. Ki-67 and TUNEL immunohistochemical staining of the harvested tumors treated with solvent control, HN, CBL, and HN–CBL co-prodrugs, respectively. In addition, the brown nucleus shows that the proliferation cells in Ki67 staining tumor tissues, the white row indicated the apoptosis tumor cells in TUNEL fluorescence analysis.

2.8. HN–CBL Anti-LL Effects In Vivo. To further demonstrate the in vivo anti-CLL efficacy of HN–CBL, the BALB/c murine CEM xenograft model was used in this section. Equimolar dosage of HN (3.5 mg/kg), CBL (8 mg/kg), HN–CBL (11 mg/kg), and solvent control was administrated via iv tail injection every two days, respectively. The subcutaneous tumor volumes (V) and nude mice weights (M) were traced for 24 days with a four-day interval. From the Figure 6, we can observe that both of HN, CBL, and HN–CBL could effectively reduce tumor growth than the control treatment, and the treatment of HN–CBL could significantly inhibit tumor volume for the HN or CBL group on the 13th day (Figure 6B). Also, the obtained tumor tissues are photographed and weighted in Figure 6A,C. Compared to HN or CBL, the photograph size and intrinsic tumor weight show that HN–CBL could enormously inhibit the leukemia cell proliferation by reducing cancer cell growth. While, Figure 6D shows that the CBL causes body weight loss, which reflect serious physio-toxicity in mice. Very interesting, the group of HN–CBL treatment can keep the mice weights stable during the time of therapy, which is like the solvent control and HN group. These interesting results suggesting that HN–CBL could lower the physio-toxicity caused by CBL in mice. Probably because of the high reactivity of CBL with lots of biological macromolecules, which leads to the lower therapeutic effect with a short half-life in clinic and means that a higher therapeutic dose of CBL will increase the risk of severe side effects.

The major organs of varied treatments were harvested for pathological analysis via H&E staining (Figure 7) on the 24th day. Major organs of the HN–CBL group showed no observed tissue injury compared with the control group, while the CBL treatment induced liver damage (Figure S7). However, the proliferation and apoptosis of the tumor tissue was further determined by immunohistochemical of Ki-67 staining and TUNEL fluorescence. As shown in Figure 7, lower proliferative cell rates were found in both drug-treated groups compared with the solvent group. In addition, the Ki-67 positive cells of HN–CBL-treated groups were reduced more than HN or CBL increased. While more apoptotic cells were observed in HN–CBL-treated groups in TUNEL fluorescence (Figure 7). Considering the anti-TUNEL fluorescence (Figure 7).

3. CONCLUSIONS

Because of the ester prodrug design has practical approaches to improve the drug bioavailability and efficiency through enhancing the bio-membrane permeability and reducing nonspecific toxicity. In our report, based on the targeted delivery help of the honokiol skeleton in cancer cells, the combination of the broad-spectrum DNA alkylating therapeutics chlorambucil and the self dietary natural product honokiol, a novel conjugated co-prodrug of HN–CBL was designed and synthesized. Biological evaluation data showed that co-prodrug HN–CBL can observably inhibit the proliferation of many carcinoma lines, particularly the LL lines, compared to the healthy PBMCs’ toxicity of HN–CBL, which can selectively kill LL cells of patients with LL. Moreover, HN–CBL can enhance mitochondrial activity in leukemia cells and induce leukemia cell apoptosis. In a proof of concept, molecular docking and western blot study showed that HN–CBL can also bind with the STAT3 protein at some hydrophobic residues and downregulate the phosphorylation level of STAT3-like HN. In vivo xenograft, HN–CLB could significantly reduce LL tumor growth. It is noteworthy that no significant physiological toxicity are observed in the HN–CBL group; however, the liver tissue injury was determined in the CBL-treated group. Together, these results showed that the co-prodrug HN–CBL could be a promising anti-LL agent with fewer side effects than CBL, while the mitochondrial dysfunction and cell apoptosis might be the main antiproliferation mechanisms.

4. MATERIALS AND METHODS

4.1. Reagents and Characterization. Chlorambucil (CBL, HPLC pure >95%), Honokiol (HN, HPLC pure >95%), N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), ethyl acetate, and sodium sulfate were purchased from Energy Chemical (Shanghai, China). Chromatographic purity acetonitrile (ACN) was from Thermofisher. MTT (98%) was from MedChemExpress (Shanghai, China). A cell apoptosis assay kit of Annexin V-FITC and the other cell experimental regents were come from Life Gibco
were isolated by Ficoll centrifugation. Red blood cells (RBCs) were separated from healthy donors’ blood and purified with cooled PBS. CBL, HN, and HN–CBL solutions were prepared in isotonic buffer, and then added to RBCs’ buffer for hemolysis analysis in 96-well plate. Simply, each well, 2 μL of RBCs were added, mixed, and then incubated for 1 h at 37 °C with 5% CO2. For 100% lysis, 50% H2O was added to wells, and for negative control (0%), cells from those wells only including PBS buffer were used. When centrifuged for 10 min at 1000 g, the supernatant was then transferred to those new tubes, PBS was added and mixed, and read at 450 nm to determine absorbances. The above cells were fresh.

4.6. Cytotoxicity Determination with MTT Method. Briefly, A549, HepG2, NIH3T3, and LO2 cells were seeded at a density of 4–6 × 103 cells/well in 96-wells plates. Leukemia cell lines (CCRF-CEM, Jurkat, U937, MV-4;11 and K562) were seeded at a density of 10–20 × 103 cells/well in 96-well flat-bottom plates. In addition, the detailed experimental processes are described as the cited paper.21 The IC50 values and statistical analysis were performed using GraphPad Prism 5.01 (GraphPad Software).

4.7. Determination of Superoxide in Mitochondria. Following the manual of the superoxide kit (Invitrogen), we have plated 4 × 104 leukemia cells in a 12-well panel for the treatment of CBL, HN, or HN–CBL. When the mixture of cells and drugs were incubated for 1.5 h, we first removed the media, and then completed the processes of trypsin digestion and centrifugation; the obtained cells were stained with MitosOX and analyzed with a BD FACSVerse flow cytometer (BD Biosciences, Ann Arbor, MI).

4.8. Membrane Potentials of Mitochondria Analysis. To compare the mitochondrial membrane potential difference between untreated and treated with HN, CBL, or HN–CBL, the Beyotime’ JC-1 kit was used to determine. In addition, the detailed experimental processes followed the protocol of the manufacturer and the cited paper.21

4.9. Apoptosis Analysis. Under the help of a BD FACSVerse flow cytometer (BD Biosciences, CA), the apoptosis in PBMC and LL cells was measured after treatment without or with HN, CBL, and HN–CBL for 24 h. When the target cells were harvested with PBS, following the manual of the apoptosis kit of Annexin V-FITC/PI from Beyotime (China), we compared the difference between untreated and treated with HN, CBL, or HN–CBL.

4.10. Molecular Docking Study of HN–CBL Co-Drugs and HN. Molecular ligand docking studies were performed with the Sybyl-X 2.1.1 software, whose energy minimized using the default parameters. The X-ray crystal structure of STAT3 (PDB code: 3CWG) was obtained from the protein data bank. The chemical structure of HN and HN–CBL is shown in Figure S1. Docking was performed with the docking box sizes large enough to include the binding sites.

4.11. Western Blotting. While the LL cells were treated with 10 μM HN or HN–CBL for 12 h, and the collected cells were lysed in 200 μL of WB&IP lysis buffer (1% Triton X-100), including 1 mM PMSF (Beyotime, China). Protein extracts (50 μg) were loaded onto an 8–15% polyacrylamide gel containing SDS, electrophoresed, and transferred to a 0.22 μm nitrocellulose membrane (PALL, USA). The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated overnight at 4 °C with the primary antibody. The blots were washed with TBST three times and then probed with HRP-conjugated.

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secondary antibodies for 2 h at room temperature. The immune complexes were visualized using a Phototope-HRP Western Blot Detection System (Pierce, USA) as previously reported.22 Actin was used to ensure equivalent loading of the whole cell protein. All data were confirmed by three individual experiments.

4.12. In Vivo Xenograft Model. Female BALB/c-Nude mice (4–6 weeks old) were obtained from the Hunan Laboratory Animal Center (Changsha, China). All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Hunan Academy of Traditional Chinese Medicine. After acclimatized to their new environment for five days, the mice were injected subcutaneously with CCRF-CEM cells (1.2 × 10^7/0.2 ml/mouse) into the flank (day 0). When the tumor volume reached about 100 mm^3, the mice were divided randomly into four groups, and then given intravenously of solvent control, HN (3.5 mg/kg), CBL (8 mg/kg), and HN−CBL co-prodrugs (11 mg/kg, is equivalent to at the HN dosage of 3.5 or 8 mg/kg of CBL) every two days, respectively. After the experiments were finished, the mice were sacrificed on day 24 of treatment, the tumors and main organs were harvested and fixed in formaldehyde for the further paraﬁn embedding.

4.13. Detection of TUNEL Apoptosis and Ki67 Proliferation Analysis in Tumor and H&E Staining of Main Organ. The tumor tissues apoptosis was detected by a TUNEL detection kit (Roche). Simply, the tumor tissues of parafﬁn-embedded specimens were dewaxed in xylene and rehydrated with decreasing concentrations of ethanol.22 In addition, the cell proliferation of tumor tissues was analyzed by an immunohistochemical staining assay of Ki-67 using the labeled streptavidin-biotin method. The morphology of main organs was evaluated by hematoxylin and eosin (H&E) staining.

4.14. Statistical Analysis. For analysis of data, values were presented as mean ± SEM for the independent experiments. Statistical differences were determined by nonpaired Student’s two-tailed t-test and p < 0.05 was considered statistically significant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02832.

Chemical structure, HPLC proﬁle, 1H NMR, UV/Vis, hemolysis assay, ﬂow cytometry, and histological determination of co-prodrug HN−CBL (PDF)

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Author Contributions

L.X., D.K., and D.W. contributed equally to this work. The paper was written through contributions of all the authors. All the authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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