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Anti-cancer effects of honokiol via mitochondrial dysfunction are strongly enhanced by the mitochondria-targeting carrier berberine

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ABSTRACT. Mitochondrion is a favorable therapeutic target in cancer given its regulation of bioenergetics and cell death. Honokiol exhibits antiproliferative effects through mitochondriamediated death signaling. To enhance its anticancer potential and selectivity, we conjugated honokiol to berberine, a mitochondria-targeting carrier. All designed derivatives displayed one order of magnitude increased cytotoxicity compared with the parent compounds, especially with massive cytoplasmic vacuoles. Biological evaluation demonstrated the representative compound **6b** localized within the mitochondria and mitochondrial dilation resulted in vacuolization. **6b** induced vacuolation-associated cell death and apoptosis with obvious mitochondrial dysfunction

as demonstrated by booming reactive oxygen species generation, opening mitochondrial permeability transition pore and reducing mitochondrial membrane potential. The targeting property also conferred **6b** selectivity for tumor cells compared to normal cells. **6b** inhibited cancer cell proliferation in zebrafish xenograft model. These results demonstrate that berberine-linked honokiol derivatives open up a direction for novel mitochondrial targeting antitumor agents.

KEYWORDS. Honokiol, Berberine, Mitochondria-targeting derivatives, Vacuoles, Mitochondrial dysfunction.

INTRODUCTION

Cancer cells have developed various biological capabilities to adapt to extraordinary microenvironments, sustain survival and evade cell death. Among these hallmarks of cancer, resisting cell death remains a great challenge to chemotherapy by reducing the accumulation of cellular drugs and escaping apoptotic execution¹⁻². Mitochondria are pivotal regulators in the processes of programmed cell death, including apoptosis, mitochondrial permeability transition (MPT)-driven necrosis, and paraptosis etc²⁻⁴. Direct targeting of therapeutic agents to mitochondria improves antiproliferative effects and cell selectivity and decreases cancer invasion in *vitro* and *vivo*⁵⁻⁷.

Honokiol (HN), a biphenyl component extracted from *Magnolia officinalis*, exhibits multiple antineoplastic activities by regulating mitochondria-mediated death signaling. HN treatment (from 20 to 60 μ M) inhibits the growth and proliferation of cancer cells via caspase-dependent and - independent apoptosis⁸⁻¹⁰, paraptosis with swelling of endoplasmic reticulum and/or mitochondria¹¹ or cyclophilin D-dependent mitochondrial permeability transition pore-driven necrosis³. To improve the anticancer effect and cell selectivity of honokiol, we attempted to design

mitochondria-targeted honokiol derivatives. Currently, the most common mitochondria-targeting delivery system is delocalized lipophilic cations (DLCs), such as triphenylphosphonium, rhodamine and pyridinium, which selectively accumulate in mitochondria of cancer cells based on the increased mitochondrial membrane potential ($\Delta \psi_m$) of cancer cells compared with normal cells¹². The natural isoquinoline alkaloid berberine (BBR) also serves as a DLC and exhibits several advantages, such as antiproliferative effects, safety and fluorescent properties¹³⁻¹⁷. In this work, we synthesized ten mitochondria-targeted derivatives by conjugating honokiol to a berberine moiety and studied the anti-cancer activity and mechanisms of action using compound **6b** as a representative agent.

RESULTS

Design and synthesis. Ten berberine-linked honokiol derivatives were designed and synthesized according to the Scheme 1. Briefly, the key intermediate compound **3** was synthesized from condensation of BBR (1) with acetone, followed by reaction with various iodides to give compounds **4a** ~ **4c**, which are berberine analogues bearing linkers of different length in the C-13 position¹⁸. Etherification of hydroxyl groups of HN (**2a**) with iodoalkanes yielded compounds **2b** ~ **2e**. Finally, in the present of excessive K_2CO_3 , reactions of compounds **4a**/**4b**/**4c** with **2a** in refluxing MeCN yielded **5a** ~ **5c** and **6a** ~ **6c** after isolation using column chromatography. The condensation of compound **4b** with **2b** ~ **2e** produced **5d**, **6d**, **5e** and **6e**. The structure of these novel derivatives were confirmed via ¹H-NMR, ¹³C-NMR and HR-MS with the aid of HSQC and HMBC. The purity of key compounds for biological evaluation was established as ≥95% by high performance liquid chromatography (HPLC).

Scheme 1. Synthesis of target derivatives Series 5 and 6^{*a*}.



^{*a*}Reagents and conditions: (a) 5 N NaOH, acetone, rt, stir 30 min. (b) MeCN, N₂, various iodides, avoid light, reflux $5 \sim 8$ h. (c) K₂CO₃, DMF, various iodides, rt, stir overnight. (d) K₂CO₃, MeCN, reflux $2 \sim 6$ h.

In *vitro* biological activity. MTT assays were employed to test the cytotoxicity of target derivatives in six human cancer cell lines and one normal hepatocyte cell line HL7702. Doxorubicin was selected as the positive control drug. The biological activity results are listed in Table 1 and graphs of MTT data with concentration range against all the cell lines are shown in Figure S1. The antiproliferative activity of compounds $5a \sim 5e$ and $6a \sim 6e$ increased by one order of magnitude in the tested cancer cell lines compared with HN or BBR (Table 1). Structure-activity analysis suggested that the length of the linker between the moiety of BBR and HN influences cytotoxicity. In general, a longer linker exhibits a lower IC₅₀ value. For example, as the length of the linker increased from 3C to 8C, the IC₅₀ value of $5a \sim 5c$ against A2780 ranged from 3.03 to 1.97 μ M. To study the potency of bare hydroxyls in HN, we synthesized the etherification products of 5b and 6b bearing methyl and propyl groups, i.e., compound 5d and 5e as well as 6d

and **6e**, respectively. These ethers exhibited slightly increased cytotoxic effects compared with **5b** and **6b**, and the type of and position of alkyl group had no obvious effect on antiproliferative activity. 50% inhibitory concentration of intermediate $2b \sim 2e$ against cancer cells decreased slightly compared with parent compound HN, but their cytotoxic effects are still much inferior to series 5 and 6 and their cell selectivity are not obvious.

Compound	IC ₅₀ (μM)						
	A2780	A549	HepG2	HT29	PANC1	PC3	HL7702
BBR	> 50	> 50	> 50	> 50	> 50	31.72±0.51	> 50
HN	31.58±4.20	38.04±0.08	23.85±2.06	31.58±4.20	31.24±4.10	29.03±6.98	25.49±1.87
5a	3.03 ± 0.23^{b}	> 5	3.71 ± 0.68^{b}	> 5	> 5	2.26 ± 0.54^{b}	5.21 ± 0.89^{b}
5b	$1.59{\pm}0.18^{b}$	2.90 ± 0.24^{b}	2.32 ± 0.17^{b}	> 5	3.24 ± 0.37^{b}	$1.59{\pm}0.04^{b}$	3.04 ± 1.05^{b}
5c	1.97 ± 0.61^{b}	$2.09{\pm}0.08^b$	1.27 ± 0.22^{b}	$2.54{\pm}1.07^{b}$	2.48 ± 0.39^{b}	2.00 ± 0.09^{b}	5.77 ± 0.27^{b}
5d	1.36 ± 0.48^{b}	1.71 ± 0.03^{b}	$1.04 \pm 0.23^{b,c,}$	1.56 ± 0.63^{b}	2.96 ± 0.48^{b}	1.12 ± 0.35^{b}	2.97 ± 0.30^{b}
5e	1.68 ± 0.35^{b}	1.66 ± 0.23^{b}	$0.92 \pm 0.12^{b,c}$	2.39 ± 1.40^{b}	2.56 ± 0.62^{b}	1.43 ± 0.47^{b}	3.62 ± 0.02^{b}
6a	1.56 ± 0.33^{b}	3.25 ± 1.04^{b}	$2.19{\pm}0.16^{b}$	> 5	6.02 ± 1.24^{b}	1.17 ± 0.23^{b}	3.56 ± 0.42^{b}
6b	2.22 ± 0.76^{b}	$3.00{\pm}0.93^{b}$	1.63 ± 0.33^{b}	> 5	$3.34{\pm}0.62^{b}$	2.25 ± 0.03^{b}	4.61 ± 0.07^{b}
6c	1.52 ± 0.41^{b}	$1.84{\pm}0.83^{b}$	0.77 ± 0.04^{b}	2.89 ± 1.08^{b}	2.09 ± 0.62^{b}	1.74 ± 0.50^{b}	4.82 ± 0.02^{b}
6d	1.17 ± 0.03^{b}	1.33 ± 0.37^{b}	$1.05 \pm 0.15^{b,d}$	1.64 ± 0.80^{b}	$1.73 \pm 0.33^{b,d}$	0.99 ± 0.50^{b}	3.43 ± 0.34^{b}
6e	1.41 ± 0.23^{b}	$1.25 \pm 0.11^{b,d}$	$0.95 \pm 0.17^{b,e}$	1.95 ± 0.64^{b}	$1.69 \pm 0.10^{b,e}$	1.22 ± 0.23^{b}	3.05 ± 0.18^{b}
2b	14.67±2.56	21.97±2.03	17.47±3.58	21.07±0.51	16.73±0.08	17.87±0.03	23.94±2.10
2c	24.66±3.90	> 30	29.65±0.66	35.37±4.86	16.42±0.09	24.84±0.66	35.27±2.37
2d	14.73±0.08	20.97±0.56	19.11±2.90	26.47±0.52	17.79±0.52	21.08±0.55	27.83±3.08
2e	14.69±0.64	14.72±0.09	15.48±2.45	26.41±0.50	15.90±0.02	17.01±0.78	22.35±0.83

Table 1. The antiproliferative activity of tested derivatives against various cell lines^{*a*}.

doxorubicin 0.71 ± 0.08 3.08 ± 0.15 0.67 ± 0.24 5.92 ± 1.57 1.73 ± 0.25 0.93 ± 0.01 2.24 ± 0.39 a The values were presented as the mean \pm standard deviations (SD), and cell viability was assessedafter incubation for 48 h. $^{b}P < 0.0001$ vs. HN. $^{c}P < 0.0001$ vs. 5b. $^{d}P < 0.05$ vs. 6b. $^{e}P < 0.01$ vs.6b. f Doxorubicin was used as a positive control.

Morphologically, we found a quick and impressive accumulation of cytoplasmic vacuoles in series 5/6 treated cells as assessed by optical microscopy (Figure S2, S3), especially compound **6b**. It is an interesting and extraordinary event in cell death. We need to explore the vacuolization deeply. The parent agent BBR had no effect on vacuolization at the tested dose (40 μ M), and greater than 30 μ M HN induced obvious masssive vacuoles (Figure S4), which indicated the HN moiety was important for vacuolization.

For subsequent investigation, a representative compound and cell line need to be selected. Among all cancer cell lines tested, the hepatoma cell line HepG2 was relatively more sensitive to these compounds regarding cytotoxicity or vacuolization (Figure S3). In terms of compound, since any variation in chemical structure might alter the mechanism of action, we tended to choose a derivative bearing similar structure with parent agents, so that their mechanism could be compared. Compound **6b** was chosen as a representative compound given its structural similarity, higher vacuole inducing potency, relatively good cytotoxicity and cell selectivity. It possesses a moderate-length linker and a bare hydroxyl structurally, suppressed HepG2 cell viability with an IC_{50,48h} of 1.63 μ M, 14.6-fold reduced compared with HN, and initiated striking cytoplasmic vacuoles in a time- and dose-dependent manner (Figure 1A, 1B). Moreover, **6b** exhibited some preferable cell selectivity between HepG2 cells and the normal cell line HL7702, the IC₅₀ ratio in HL7702 to HepG2 cells is 2.8, while there is no selectivity for HN (Table 1). Thus, the subsequent experiments were performed using **6b** in HepG2 cells.

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Figure 1. Effects of 6b on cell viability and vacuolation. (A) Cells were treated with increasing doses of 6b for indicated time. The values were determined using MTT assays. (B) Vacuolization induced by 6b. Cells were treated with 4 μ M 6b for indicated time (upper images) or with increasing concentrations for 10 h (lower images), and morphological changes were imaged by optical microscopy. Scar bar 10 μ m.

To study the importance of the whole skeleton of BBR-linked HN derivatives on their biological performance and the contribution of the synergy effects of HN and BBR moiety, we further synthesized some structural fragments of **6b** such as compound **2f** and **4d** (Figure 2A, synthetic method see supporting Scheme S1), and then designed some drug combinations of HN moiety with BBR moiety to detect their antiproliferative effects and compare with **6b**. The degree of drug interaction in terms of synergism and antagonism were analyzed by CompuSyn software. In all cases where combination index (CI) can be determined, following diagnostic rule apply: CI < 1 indicates synergism, CI=1 indicates additive effect and CI > 1 indicates antagonism. Fa-CI point, a plot of CI on y-axis as a function of effect level (Fa) on the x-axis, exhibited the drug interaction visually¹⁹. As shown in Figure 2B, for combination effect, all data points yielded from combination of BBR with HN are above the CI=1 horizontal line (Table S1), indicating an

antagonistic effect. Other drug combinations, **2f** with BBR, **4d** with HN, produced partially CI < 1 (values of 0.79 to 0.98), indicating weak synergism at different doses case (Table S2, S3). The combination of **2f** with **4d** yielded CI value of 0.269 to 0.921 revealed a moderate synergistic effect (Table S4). But the cytotoxicity in HepG2 of all drug combinations is lower than **6b** at the same dose (Figure 2C). These results suggested that the entire molecular skeleton of **6b** is essential to its cytotoxicity. For morphological changes, the effect on vacuolization of intermediate **2f** is weaker than that of its parent compound HN, while BBR derivative **4d** exhibited some ability to induce vacuoles at 40 μ M as well (Figure S4). In drug combinations, more obvious vacuolation were observed than that in single compound group (Figure S5), which displayed the synergism relationship between BBR and HN scaffold in terms of morphological manifestation.



Figure 2. The degree of drug combinations in HepG2. (A) Chemical structures and IC_{50} against HepG2 of **2f**, **4d** and **6b**. (B) Fa-CI point yielded from various drug combinations. The CI and Fa values were calculated by CompuSyn software. (C) Growing inhibition of designed drug combinations and **6b** in HepG2 cells. Cells were treated with indicated drugs for 48 h and viability were tested by MTT assays.

6b mediates cytoplasmic vacuolation via dilation of mitochondria. Upon treatment with different doses of **6b** for indicated time, decreasing cell viability and increasing remarkable cytoplasmic vacuoles are completely synchronized (Figure 1), suggesting that formation and development of vacuoles were pivotal for **6b**-induced cytotoxicity. Thus, it is important and

necessary to explore the formation of cytoplasmic vacuolation. The mitochondria-specific fluorescent probe mito-tracker red CMXRos was used to study whether cytoplasmic vacuoles originated from mitochondria. As shown in Figure 3A, mitochondria labeled with red fluorescence in untreated cells displayed a compact, rod-like and reticulate shape, whereas appeared swollen and turgid in **6b**-treated cells. The merged red fluorescence and phase-contrast images demonstrated that mito-tracker was incorporated into the small vacuoles, suggesting these vacuoles originated from swelling of mitochondria. Dilated mitochondria after treatment with **6b** were observed by transmission electron microscopy as well (Figure 3B). These findings indicate that mitochondria was demolished by **6b** and it played a crucial role in vacuolation-associated cell death.



Figure 3. Cytoplasmic vacuoles originated from dilation of mitochondria. (A) Cells were stained with 150 nM mito-tracker probe for 15 min after treatment with 3 μ M **6b** for 12 h. Fluorescent

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and phase-contrast images were obtained by laser confocal microscopy. Scar bar, 10 μ m. (B) Transmission electron microscopy images of microstructural changes of mitochondria in HepG2 cells incubated with or without 3 μ M **6b**. Black arrowheads point mitochondria. Scar bar in the original and enlarged images indicates 1.2 μ m and 0.6 μ m respectivily.

Cellular uptake and localization of 6b. To confirm that the increasing cytotoxicity was related to mitochondrial targeting, intracellular drug content and subcellular localization were measured by HPLC assays and confocal microscopy, respectively. The total cellular uptake of **6b** was 2.5-fold increased compared with honokiol after treatment with 5 μ M drugs for 12 h in HepG2 cells (Figure 4A). The concentration of **6b** and HN in isolated mitochondria was 66.3 μ M and 17.8 μ M respectively, revealing that mitochondrial accumulation of **6b** was 3.7-fold increased compared with HN (Figure 4A). Since **6b** can emit blue fluorescence according to its emission spectrum (Figure S6), we employed laser confocal microscopy to examine its potential subcellular distribution. As shown in Figure 4B, we observed clusters of blue fluorescence in the **6b**-treated group but not the blank control. Here, **6b** (blue) colocalized with mitochondria (red) stained with the mito-tracker probe. These data indicate that BBR facilitated HN entry into cells and locate in mitochondria.



Figure 4. Cellular intake and subcellular localization of **6b**. (A) Standard curve of HN and HPLC chromatograms of extracted HN from HepG2 cells (a). Standard curve of **6b** and HPLC

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chromatograms of extracted **6b** from HepG2 cells (b). Histogram of mitochondrial, nonmitochondrial and total cellular accumulation of HN and **6b** *P < 0.5, *** P < 0.001(c). (B) Colocalization of **6b** with mito-tracker in HepG2 cells was imaged by confocal microscopy. Scar bar, 10 μ m.

6b induces apoptosis and vacuolation-associated cell death. Based on the mitochondrial targeting feature of **6b** and swelling of mitochondria in treated cells, we hypothesized that this compound may induce mitochondria-driven cell death. Flow cytometry detected the number of apoptotic cells by annexin V/PI double staining. HepG2 cells treated for 24 h revealed that 6b induced significantly apoptosis in a dose-dependent manner and 1 μ M, 2 μ M and 4 μ M **6b** induced apoptosis in 9.0%, 8.9% and 42.9% of cells, respectively (Figure 5A). Western blot indicated cleaved caspase 3, 8 and 9 were upregulated, together with decreasing product of procaspase 12 and increasing expression of cleaved poly (ADP-ribose) polymerase (PARP) (Figure 5B). Next, we detected the cellular localization of apoptosis-inducing factor (AIF), a protein that induces apoptosis in the presence of irreversible caspase inhibitor z-VAD-fmk²⁰. The result obtained via immunofluorescence staining showed that AIF was not translocated to the nucleus but distributed widely in cytoplasm in the presence of **6b** (Figure 5C). These demonstrate caspasedependent extrinsic and intrinsic apoptosis invovled in **6b**-induced cell death. Pretreatment with the pan-caspase inhibitor z-VAD-fmk slightly affected apoptosis and cell death initiated by 4 μ M **6b** (Figure 5D (a, b)), but had no obvious impact on the size and number of vacuoles (Figure 5D) (c)), indicating that alternative non-apoptotic programs may exist. Then, we examined whether necroptosis or autophagy is involved in the process of vacuolization and cell proliferation inhibition. Necrostatin-1 (Nec-1), a small molecule inhibitor of necroptosis²¹, partially prevented cell death in approximately 12 h, but no significant difference was noted between the 6b and 6b

plus Nec-1 group, and couldn't rescue cell after treatment for 12 h. Morphologically Nec-1 didn't block **6b**-induced vacuolization (Figure 6A). The autophagy inhibitors chloroquine (CQ) and 3methyladenine (3-MA) couldn't averted vacuolization or cell death as well (Figure 6B), and the expression of LC3 II and P62, the key indicator protein of autophagy, shown no significant changes in **6b**-treated cells compared with control. Invalid response of Nec-1, CQ and 3-MA on viability and vacuolization, with undifferentiated expression of autophagy marker, highlight necroptosis and autophagy are not indispensable in **6b**-induced antineoplastic process. These results suggest that mitochondrial caspase-dependent apoptosis is involved in cell death but not vacuolization, whereas the necroptosis and autophagy pathways have no effect on either. Therefore, we focused more attention on mitochondrial dysfunction to assess the crucial role of **6b** in cell death.



Figure 5. Compound **6b** induced apoptosis. (A) Apoptosis initiated by **6b**. HepG2 cells were treated with 1, 2 and 4 μ M **6b** for 24 h. Apoptotic levels were assessed using annexin V/PI double staining and flow cytometry. Related histogram for living and apoptotic cells are expressed. ** P < 0.01 vs. control. (B) Western blot measured caspase family proteins and PARP levels in HepG2

cells treated with $0 \sim 4 \ \mu M$ **6b**. (C) Immunofluorescence assays detected the position of AIF following treatment with 4 μ M **6b** for 24 h. Red fluorescence indicates AIF, whereas blue represents the nucleus. Scar bar, 50 μ m. (D) The effects of z-VAD-fmk on vacuolization and cell death induced by **6b**. Cells were pretreated with 20 μ M z-VAD-fmk for 2 h and then incubated with 4 μ M **6b** for 24 h. Flow cytometry analysis of cell distribution and quantification of the number of surviving and apoptotic levels (a). MTT assays tested cell viability after exposure to different doses of **6b** in the presence or absence of z-VAD-fmk (b). Optical microscopy images of HepG2 cells preincubated with or without z-VAD-fmk before treatment with 3 μ M **6b** (c). Scar bar, 10 μ m.



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Figure 6. Cell death and cytoplasmic vacuoles induced by **6b** were not associated with necrosis and autophagy. (A) The effects of Nec-1 (30 μ M, pretreated for 2 h) on cell viability and vacuolization in HepG2 cells treated with 3 μ M **6b**. MTT assays tested survival rates. The green curve indicates viability after treatment with **6b**, blue demonstrates viability in cells treated with **6b** and Nec-1. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. control. Morphological changes were imaged using optical microscopy after treatment for 24 h. Scale bar, 10 μ m. (B) Cell viability following treatment with 3 μ M **6b** for 24 h in the presence of CQ (10 μ M) or 3-MA (1 mM). Growing inhibition analysis examined by MTT assays, *** P < 0.001 vs. control. Phase-contrast images of cells treated with indicated agents were performed. Scar bar, 10 μ m. Western blot was performed to detect LC3 and P62 expression.

6b initiates mitochondrial dysfunction via interference with reactive oxygen species homeostasis and mitochondrial permeability. To obtain a general sense of the role of **6b** in mitochondrial dysfunction, we assessed the level of reactive oxygen species (ROS), changes of mitochondrial permeability transition pore (MPTP) and $\Delta \psi_m$ following **6b** treatment. Mitochondria are major contributors of endogenous ROS in tumors. ROS generated from mitochondria control cellular redox regulation and render cells vulnerable to chemotherapeutic drugs²². We detected the ROS generation by 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA). ROS levels were indeed upregulated in a dose-dependent manner (Figure 7A (a)) beginning 2 h after treatment with 2 μ M **6b** and persisting up to 24 h (Figure 7A (b)). Oxidants are essential for the induction of mitochondrial permeability transition pore, resulting in mitochondrial degradation and cell death²³. ²⁴. We then detected the status of the permeability transition pore using the probe calcein-AM and the quencher CoCl₂. Calcein-AM can enter cells freely and be hydrolyzed to the fluorescent form of calcein, which is only able to escape mitochondria when the permeability transition pore is open.

Given that the residual calcein present in cytosol and nucleus is quenched by $CoCl_2$, the fluorescence localized to mitochondria is imaged against dark background²⁵. As shown in Figure 7B, green fluorescence in mitochondria is lost following treatment with 2 μ M **6b** for 12 h, and dispersive puncta were observed upon exposure to 4 μ M **6b**. The collapse of $\Delta \psi_m$ caused by **6b** was obtained by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) staining (Figure 7C). These results demonstrate that **6b** disturbed redox homeostasis, promoted MPTP opening, reduced mitochondrial membrane permeability and gradually resulted in distinct mitochondrial dysfunction in HepG2 cells.



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Figure 7. Mitochondrial dysfunction induced by **6b**. (A) ROS generation in HepG2 cells exposed to **6b**. Cells were treated with **6b** at indicated concentrations for 24 h or treated with 2 μ M **6b** for different times. Samples were loaded with 10 μ M DCFH-DA for 30 min and tested by flow cytometry. Ros-up was the positive agent provided in the test kit, *** P < 0.001 vs. Control. (B) Mitochondria leakage induced by **6b**. Cells were exposed to **6b** at indicated doses for 12 h and then stained with calcein-AM in the presence of CoCl₂. Images were captured by fluorescence microscopy. (C) Changes in $\Delta \psi_m$ were analyzed using JC-1 following treatment for 24 h. Histogram of JC-1 aggregates are expressed. *** P < 0.001 vs. control.

In *vivo* anti-tumor effects of 6b. The antiproliferative efficiency of 6b against HepG2 cells in *vivo* was assessed in zebrafish xenograft model, an adopted animal model for drug screening popularly^{5, 26}. Generally, embryos were obtained from pairwise mathing of sexually mature zebrafish and then microinjected of HepG2 cells labeled with cell tracker CM-Dil to enstablish xenograft model. Zebrafish bearing hepatoma cancer cells were incubated with different concentrations of 6b for 48 h. A group of zebrafish administered by doxorubicin seved as a positive control. Based on the reduced red fluorescence intensity in xenotransplantation, 6b inhibited significantly the growth of HepG2 cells in *vivo* compared with the control group (Figure 8).



Figure 8. Compound **6b** inhibited the proliferation of HepG2 in zabrafish xenograft model. (A) Tumor growth in every group was detected using confocal microscopy after drugs administration for 48 h. (B) Histogram of fluorescence intensities in different groups of xenograft zebrafish. *** P < 0.001 vs. control.

CONCLUSION AND DISCUSSION

Current literatures about the antitumor activity and mechanism of honokiol derivatives have uncovered its potential in chemotherapy. In an attempt to improve its therapeutic profile, there has been interest in obtaining derivatives with increased anti-cancer effects and cell selectivity and identifying the precise mechanisms of action in cell death. The mitochondrion is a most studied target in cancer therapy given its indispensable role in energy metabolism, redox signaling and programmed cell death². Mitochondria-targeted cytotoxic conjugates formed by linking parent drugs to mitochondria-directing moieties are major approaches in targeted therapeutics driven by the increased mitochondrial membrane potential in cancer cells (-220 mV) compared with normal

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cells (-160 mV)^{27, 28.} Recent studies repurposed isoquinoline alkaloid BBR as a mitochondriatargeting carrier based on its delocalized positive charge and amphiphilicity^{14, 17}. Our efforts to improve cell selectivity and enhance the cytotoxicity of the parent drug HN focused on developing mitochondria-targeting BBR-linked HN derivatives and assessing its overall mechanisms of action in cell death.

In the present work, we designed and synthesized series of novel derivatives based on the natural product honokiol conjugated with BBR, a natural DLC that serves as a mitochondriatargeting carrier. Compound **6b** was chosen as a representative for deep study based on its potential inducing vacuoles, as well as its increased anticancer effect and selectivity compared with its parent compounds HN and BBR. For increasing cytotoxicity and cell selectivity of these novel derivatives, enhanced endocytosis and specific mitochondrial targeting feature could illustrate (Figure 4). Researches that study alkyl-substituted BBR derivatives summarized the enhanced lipophilicy and improving tissue penetration^{29, 30}, which allowed BBR moiety mediate HN to penetrate biomembranes. In addition, cytoplasmaic membrane potential enhanced endocytosis of cations dependent on potential difference outside and inside the cell membrane. Nernst equation $[\Delta \psi = 61.5 * \log_{10} (C_{in}/C_{out})]$ describes cellular uptake of cationic drugs. C_{in} and C_{out} are the concentration of ions in the inner and outer of the biomembrane, such as cytoplasmic and mitochondrial membrane. The equation indicated that every 61.5 mV membrane potential will induce 10 times increasing of concentration in inner membrane space¹². In the process of drugs accumulation, cytoplasmaic membrane potential of -30~40 mV typically leads to a 3~4-fold increasing in the cytosolic domain compared with extracellular area in principle. In fact, the total cellular accumulation of 6b was about 2.5-fold increasing than that of HN (Figure 4A). Next, cationic **6b** loaded in cytoplasm can be concentrated in mitochondria with the help of higher

mitochondrial membrane potential in cancer cells (Figure 4). Thus, more BBR-linked HN derivatives penetrated cell membrane and targeted selectively to mitochondria to exert the anticancer efficiency though these force.

The detail mechanisms of action of **6b** was explored in this study. Here, **6b** induced massive dilation of mitochondria and marked mitochondria-mediated cell death, including vacuolation-associated cell death and apoptosis. ROS play a dual role in survival. Elevated ROS production contributes to oncogenic transformation, but excessive toxic levels increased oxidative stress and induced tumor cell death²². In our mechanistic study, we found that the ROS balance was rapidly disrupted in treated cells approximately 2 h after treatment (Figure 7A) and followed by massive cytoplasmic vacuoles that formed from dilated mitochondria (Figure 3). Oxidant stress elicits an abrupt increase in inner mitochondrial membrane permeability via induction of the mitochondria permeability transition pore, which results in mitochondrial depolarization.^{23, 24}In **6b**-treated cells, the MPTP began to open in succession with reducing of $\Delta \psi_m$ (Figure 7B, 7C). Here, **6b** disrupts the integrity of the mitochondrial inner and outer membranes upon entry into mitochondria possibly via ROS-driven formation of the permeability transition pore.

In this context, BBR serves as a mitochondrial-targeting carrier, and the BBR-linked HN derivative **6b** exhibits some differences in its ability to induce cell death, compared with its parent compound HN. Although exposure of HN resulted in ROS-mediated apoptosis and autophagy in human osteosarcoma cells or hepatocellular carcinoma^{9, 31}, the cell death initiated by **6b** was associated with vacuolization as early as apoptosis, without autophagy and necroptosis in HepG2 cells. Consistently, conjugating the "war head" of HN to the mitochondrial-targeting carrier BBR contributes to the alternative mechanisms of action and increased antitumor effects. In addition **6b**

treatment inhibited the growth of HepG2 cells in xenograft zebrafish, that confirming anti-tumor efficiency in *vivo* of **6b** (Figure 8).

Taken together, we demonstrate that BBR serves as an effective mitochondrial-targeting cation carrier, and the antitumor efficacy and potency of honokiol can be increased upon conjugation to BBR.

EXPERIMENTAL SECTION

General Procedures. NMR spectra were obtained using a Bruker Avance DRX-600 spectrometer operating at 600 (¹H) and 150 (¹³C) MHz. The chemical shifts are reported as δ (ppm), and the coupling constants (*J*) are given in Hz for NMR. HR-ESI-MS was conducted using an LTQ Orbitrap XL instrument. Column chromatography was performed with silica gel (200~300 mesh; Qingdao Haiyang Chemical Co. Ltd., Qingdao, China). Thin-layer chromatography (TLC) was performed with precoated silica gel GF-254 glass plates (Qingdao Haiyang Chemical Co. Ltd., Qingdao, China) and polyamide plates (Sinopharm Chemical Regent Co. Ltd., Taizhou, China). For biological evaluation, the purity of key compounds was established as ≥95% by HPLC using an Agilent 1100 series with a ZORBAX SB-C18 column (250 mm × 4.6 mm, 5 μ m). The eluent used for HPLC was methanol-0.1% acetic acid aqueous solution (gradient elution, 0 ~ 30 min , 70% ~ 100% methanol), and the detection wavelength was 265 nm. Berberine chloride and honokiol possessed a purity of at least 95% were purchased from Dibo Bio-tech Co. Ltd., Shanghai, China and Ailan Chemical Technology Co. Ltd., Shanghai, China, respectively.

Preparation of intermediate 2b ~ **2f**, **3 and 4a** ~ **4d**. To a solution of honokiol (**2a**) (2 mmol) and excessive K_2CO_3 (12 mmol) in DMF, various iodides (4 mmol) was added dropwise. After stiring overnight at room temperature and monitored by TLC, the mixture was taken into 10-fold

water and extracted with ethyl acetate three times. The layer of ethyl acetate was distilled under reduced pressure and the residue was purified via column chromatography on silica gel (petroleum ether/ethly acetate, $50/1 \sim 30/1$, v/v) to obtain compound $2b \sim 2f$. Berberine chloride (1) (5.0 g, 13.5 mmol) was dissolved in 5 N NaOH (25 mL) and acetone (5 mL) was added dropwise while stirring. After stirring for 30 min at room temperature, the reaction mixture was filtered and washed with 80% MeOH to give intermediate **3**, directly used for next reaction without purify by chromatography. Treatment of compound **3** (5.08 mmol) with various iodide (25.5 mmol) in acetonitrile at 80°C for $6 \sim 10$ h. Intermediates $4a \sim 4d$ were yielded by column chromatography on silica gel (DCM/MeOH, $100/1 \sim 50/1$, v/v).

Synthesis of target compounds of series 5 and 6. Compound 2a (1 mmol) and K_2CO_3 (10 mmol) was dissolved in 10 mL MeCN and stirred until the mixture turned yellow. Then, the intermediate 4a/4b/4c (1 mmol) diluted in 5 mL acetonitrile was added dropwise and stirred at 80°C for 2 ~ 6 h until the reaction was complete according to the TLC detection using polyamide plates. The reaction mixture was filtered to remove excessive K_2CO_3 and distilled under reduced pressure. The residue was purified by column chromatography with silica gel using DCM/MeOH 120/1 as the eluent to give compounds 5a/6a and 5b/6b and 5c/6c as yellow solids. Products 5d, 6d, 5e and 6e were obtained via the reaction of intermediate 4b (0.3 mmol) with 2b ~ 2e (0.5 mmol) respectively, in the presence of excessive K_2CO_3 (2 mmol) in refluxing acetonitrile.

3',5-diallyl-4'-methoxy-[1,1'-biphenyl]-2-ol (2b). Light yellowish oil. 41% yield. ¹H NMR (600 MHz, CDCl₃) δ 7.27 (dd, *J* = 8.3, 1.9 Hz, 1H, H-6'), 7.23 (d, *J* = 2.4 Hz, 1H, H-2'), 7.03 (dd, *J* = 6.5, 2.3 Hz, 2H, H-4, H-6), 6.92 (d, *J* = 8.4 Hz, 1H, H-5'), 6.87 (d, *J* = 9.0 Hz, 1H, H-3), 6.04 - 5.91 (m, 2H, H-8, H-8'), 5.10 - 5.02 (m, 4H, H-9, H-9'), 3.84 (s, 3H, OCH₃-1"), 3.42 (d, *J* = 6.7 Hz, 2H, H-7). ¹³C NMR (150 MHz, CDCl₃) δ 157.1, 150.9, 137.9,

136.6, 132.2, 130.6, 130.3, 129.8, 129.2, 128.8, 128.0, 128.0, 115.9, 115.7, 115.6, 111.0, 55.6, 39.5, 34.4 . ESI-MS m/z calculated for $C_{19}H_{19}O_2^-$ [M-H]⁻ 279.1391, found 279.1391.

3,5'-diallyl-2'-methoxy-[1,1'-biphenyl]-4-ol (2c). Light yellowish oil. 24% yield. ¹H NMR (600 MHz, CDCl₃) δ 7.19 (2H, H-2', H-6'), 7.01 (s, 1H, H-6), 6.98 (d, J = 8.1 Hz, 1H, H-4), 6.80 - 6.77 (m, 1H, H-3), 6.68 (d, J = 7.9 Hz, 1H, H-5'), 5.97 - 5.84 (m, 2H, H-8, H-8'), 5.12 - 4.94 (m, 4H, H-9, H-9'), 3.68 (s, 3H, OCH₃-1"), 3.33 (d, J = 5.7 Hz, 2H, H-7'), 3.26 (d, J = 6.1 Hz, 2H, H-7). ¹³C NMR (150 MHz, CDCl₃) δ 153.8, 152.2, 136.8, 135.5, 131.3, 130.4, 130.1, 129.9, 129.3, 127.9, 126.9, 124.0, 115.37, 114.52, 114.35, 110.39, 54.69, 38.34, 34.09. ESI-MS m/z calculated for C₁₉H₁₉O₂- [M-H]⁻ 279.1391, found 279.1378.

3',5-diallyl-4'-propoxy-[1,1'-biphenyl]-2-ol (2d). Light yellowish oil. 32% yield. ¹H NMR (600 MHz, CDCl₃) δ 7.24 (dd, *J* = 8.1, 2.3 Hz, 1H, H-6'), 7.23 (d, *J* = 2.5 Hz, 1H, H-2'), 7.05 - 7.00 (m, 2H, H-4, H-6), 6.91 (d, *J* = 8.3 Hz, 1H, H-5'), 6.88 (d, *J* = 8.8 Hz, 1H, H-3), 5.98 (m, 2H, H-8, H-8'), 5.13-5.01 (m, 4H, H-9, H-9'), 3.96 (t, *J* = 6.4 Hz, 2H, OCH₂-1"), 3.44 (d, *J* = 7.3 Hz, 2H, H-7'), 3.33 (d, *J* = 6.9 Hz, 2H, H-7), 1.84 (h, *J* = 7.0 Hz, 2H, CH₂-2"), 1.09-1.04 (t, 3H, CH₃-3"). ¹³C NMR (150 MHz, CDCl₃) δ 156.5, 150.9, 137.8, 136.7, 132.1, 130.5, 130.2, 129.9, 128.8, 128.7, 127.94, 127.86, 115.8, 115.6, 115.5, 111.7, 69.6, 39.4, 34.5, 22.7, 10.7. ESI-MS m/z calculated for C₂₁H₂₃O₂- [M-H]⁻ 307.1704, found 307.1687.

3,5'-diallyl-2'-propoxy-[1,1'-biphenyl]-4-ol (2e). Light yellowish oil. 21% yield. ¹H NMR (600 MHz, CDCl₃) δ 7.34 (d, *J* = 2.3 Hz, 1H, H-2'), 7.30 (dd, *J* = 8.2, 2.3 Hz, 1H, H-6'), 7.12 (d, *J* = 2.4 Hz, 1H, H-6), 7.05 (dd, *J* = 8.3, 2.4 Hz, 1H, H-4), 6.87 (d, *J* = 8.3 Hz, 1H, H-3), 6.79 (d, *J* = 8.3 Hz, 1H, H-5'), 6.00 (dddt, *J* = 40.3, 16.8, 10.0, 6.6 Hz, 2H, H-8, H-8'), 5.20 - 5.11 (m, 4H, H-9, H-9'), 3.87 (t, *J* = 6.5 Hz, 2H, OCH₂-1"), 3.43 (dt, *J* = 6.7, 1.6 Hz, 2H, H-7'), 3.35 (d, *J* = 6.9 Hz, 2H,

H-7), 1.76 - 1.65 (m, 2H, CH₂-2"), 0.95 (t, J = 7.5 Hz, 3H, CH₃-3"). ¹³C NMR (150 MHz, CDCl₃) δ 154.4, 153.0, 137.9, 136.6, 132.2, 131.7, 131.3, 130.9, 130.5, 128.9, 127.9, 124.7, 116.3, 115.5, 115.3, 112.7, 70.2, 39.4, 35.1, 22.6, 10.8. ESI-MS m/z calculated for C₂₁H₂₃O₂⁻ [M-H]⁻ 307.1704, found 307.1700.

3',5-diallyl-4'-(pentyloxy)-[1,1'-biphenyl]-2-ol (2f). Light yellowish oil. 43 % yield. ¹H NMR (600 MHz, CDCl₃) δ 7.25 (dd, J = 8.3, 2.4 Hz, 1H), 7.22 (d, J = 2.2 Hz, 1H), 7.06 - 7.00 (m, 2H), 6.92 (d, J = 8.3 Hz, 1H), 6.89 (d, J = 8.0 Hz, 1H), 5.98 (dddt, J = 22.1, 16.8, 10.0, 6.7 Hz, 2H, H-8, H-8'), 5.13 - 5.01 (m, 4H, H-9, H-9'), 4.00 (t, J = 6.4 Hz, 2H, OCH₂-1"), 3.43 (dt, J = 6.8, 1.5 Hz, 2H, H-7'), 3.34 (dt, J = 6.7, 1.6 Hz, 2H, H-7), 1.82 (m, 2H, CH₂-2"/3"/4'), 1.48 (m, 2H, CH₂-2"/3"/4'), 1.40 (m, 2H, CH₂-2"/3"/4"), 0.95 (t, J = 7.3 Hz, 3H, CH₃-5"). ¹³C NMR (150 MHz, CDCl₃) δ 156.6, 150.9, 137.9, 136.8, 132.2, 130.5, 130.3, 129.98, 128.83, 128.81, 128.02, 127.94, 115.9, 115.65, 115.62, 111.8, 68.2, 39.5, 34.6, 29.1, 28.4, 22.6, 14.2. ESI-MS m/z calculated for C₂₃H₂₇O₂- [M-H]⁻ 335.2017, found 335.2017.

13-(3-iodopropyl)-9,10-dimethoxy-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-

a]isoquinolin-7-ium iodide (4a). Yellow powder. 47% yield. ¹H NMR (600 MHz, CD₃OD) δ 9.81 (s, 1H, H-8), 8.22 (d, *J* = 9.4 Hz, 1H, H-11/12), 8.16 (d, *J* = 9.4 Hz, 1H, H-11/12), 7.34 (s, 1H, H-1), 7.04 (s, 1H, H-4), 6.12 (s, 2H, OCH₂O-15), 4.79 (t, *J* = 5.8 Hz, 2H, NH₂-6), 4.22 (s, 3H, OCH₃-16), 4.13 (s, 3H, OCH₃-17), 3.66-3.63 (m, 2H, linker-CH₂), 3.17-3.11 (m, 2H, H-5), 2.28 (p, *J* = 6.9 Hz, 2H, linker-CH₂). ¹³C NMR (150 MHz, CD₃OD) δ 151.9, 151.6, 149.0, 146.5, 145.6, 138.4, 135.3, 134.50, 134.47, 127.2, 123.2, 122.1, 110.8, 109.4, 103.7, 62.6, 58.8, 57.5, 49.6, 35.1, 31.5, 29.2, 4.9. ESI-MS m/z calculated for C₂₃H₂₃INO₄⁺ [M-I]⁺ 504.0666, found 504.0657.

13-(5-iodopentyl)-9,10-dimethoxy-5,6-dihydro-[1,3]dioxolo[4,5-*g***]isoquinolino[3,2***a***]isoquinolin-7-ium iodide (4b).** Yellow powder. 45% yield. ¹H NMR (600 MHz, CD₃OD) δ 9.80 (s, 1H, H-8), 8.19 (d, *J* = 9.4 Hz, 1H, H-11/12), 8.16 (d, *J* = 9.2 Hz, 1H, H-11/12), 7.30 (d, *J* = 5.7 Hz, 1H, H-1), 7.04 (s, 1H, H-4), 6.13 (d, *J* = 2.8 Hz, OCH₂O-15), 4.80 (t, *J* = 5.7 Hz, 2H, NH₂-6), 4.21 (s, 3H, OCH₃-16), 4.13 (s, 2H, OCH₃-17), 3.56 (t, *J* = 6.5 Hz, 1H, H-5'), 3.47 (q, *J* = 8.0, 7.0 Hz, 2H, H-1'), 3.21 (t, *J* = 6.9 Hz, 1H, H-5'), 3.14 (q, *J* = 5.7 Hz, 2H, H-5), 1.88 (p, *J* = 7.3 Hz, 2H, linker -CH₂-), 1.81 (h, *J* = 7.0 Hz, 2H, linker -CH₂-), 1.56(m, 2H, linker -CH₂-). ¹³C NMR (150 MHz, CD₃OD) δ 151.8, 151.4, 146.4, 145.3, 136.1, 135.2, 134.4, 127.2, 123.2, 122.3, 121.8, 110.5, 109.4, 103.7, 62.7, 58.9, 57.5, 45.5, 33.9, 33.1, 31.2, 30.8, 30.5, 29.3, 27.5, 6.5. ESI-MS m/z calculated for C₂₅H₂₇INO₄⁺ [M-I]⁺ 532.0979, found 532.0981.

13-(8-iodooctyl)-9,10-dimethoxy-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-

a]isoquinolin-7-ium iodide (4c). Yellow powder. 41% yield.¹H NMR (600 MHz, CD₃OD) δ 9.79 (s, 1H, H-8), 8.19 (d, *J* = 9.5 Hz, 1H, H-11/12), 8.15 (d, *J* = 9.4 Hz, 1H, H-11/12), 7.30 (s, 1H, H-1), 7.05 (s, 1H, H-4), 6.14 (s, 2H, OCH₂O-15), 4.80 (t, *J* = 5.8 Hz, 2H, NH₂-6), 4.21 (s, 3H, OCH₃-16), 4.13 (s, 3H, OCH₃-17), 3.44 (t, *J* = 8.0 Hz, 2H, H-1'), 3.23 (t, *J* = 6.9 Hz, 2H, H-8'), 3.13 (t, *J* = 5.8 Hz, 2H, H-5), 1.88(2H, linker-CH₂-), 1.82-1.76 (m, 2H, linker-CH₂-), 1.47 (m, 2H, linker-CH₂-), 1.42-1.32 (m, 6H, linker-CH₂-). ¹³C NMR (150 MHz, CDCl₃) δ 150.6, 149.9, 147.4, 146.3, 145.6, 136.2, 134.5, 133.8, 133.3, 125.5, 122.3, 120.5, 120.4, 109.3, 108.7, 102.3, 63.3, 58.0, 57.1, 33.5, 31.2, 30.5, 30.1, 29.6, 29.0, 28.8, 28.5, 7.6. ESI-MS m/z calculated for C₂₈H₃₃INO₄⁺ [M-I]⁺ 574.1449, found 574.1444.

9,10-dimethoxy-13-pentyl-5,6-dihydro-[1,3]dioxolo[4,5-*g*]isoquinolino[3,2-*a*]isoquinolin-7ium iodide (4d). Yellow solid. 20% yield. ¹H NMR (600 MHz, CD₃OD). ¹H NMR (600 MHz, CD₃OD) δ 9.78 (s, 1H, H-8), 8.18 (d, *J* = 9.5 Hz, 1H, H-11/12), 8.15 (d, *J* = 9.4 Hz, 1H, H-11/12), 7.29 (s, 1H, H-1), 7.04 (s, 1H, H-4), 6.13 (s, 2H, OCH₂O-15), 4.80 (t, J = 5.9 Hz, 2H, H-6), 4.21 (s, 3H, OCH₃-16), 4.13 (s, 3H, OCH₃-17), 3.44 – 3.39 (m, 2H, H-5/1'), 3.15 – 3.10 (m, 2H, H-5/1'), 1.92 – 1.85 (m, 2H, CH₂-2'/3'/4'), 1.47 (q, J = 7.4 Hz, 2H, CH₂-2'/3'/4'), 1.40 (dt, J = 8.9, 7.0 Hz, 2H, CH₂-2'/3'/4'), 0.94 (t, J = 7.2 Hz, 3H, CH₃-3'). ¹³C NMR (150 MHz, CD₃OD) δ 151.8, 151.3, 148.7, 146.3, 145.3, 137.8, 136.3, 135.1, 134.5, 127.1, 123.1, 122.3, 121.8, 110.4, 109.3, 103.8, 62.6, 58.9, 57.5, 32.7, 31.8, 30.7, 29.2, 23.6, 14.4. ESI-MS m/z calculated for C₂₅H₂₈NO₄⁺ [M-I]⁺ 406.2013, found 406.2006.

13-(3-((3',5-diallyl-4'-hydroxy-[1,1'-biphenyl]-2-yl)oxy)propyl)-9,10-dimethoxy-5,6-

dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-*a***]isoquinolin-7-ium iodide (5a).** Yellow solid. 20% yield. ¹H NMR (600 MHz, CD₃OD). δ 9.68 (s, 1H, H-8), 7.90 (d, *J* = 9.3 Hz, 1H, H-11/12), 7.79 (d, *J* = 9.4 Hz, 1H, H-11/12), 7.28 (s, 1H, H-1), 7.18 (d, *J* = 2.3 Hz, 1H, H-6'), 7.15 (dd, *J* = 8.2, 2.3 Hz, 1H, H-6''), 7.06-7.03 (2H, H-4', H-2''), 6.91 (s, 1H, H-4), 6.87 (d, *J* = 8.1 Hz, 1H, H-5''), 6.78 (d, *J* = 8.1 Hz, 1H, H-3'), 6.05 (s, 2H, OCH₂O-15), 5.99 (ddt, *J* = 16.8, 10.1, 6.7 Hz, 1H, H-8'/8''), 5.86 (ddt, *J* = 16.8, 10.1, 6.7 Hz, 1H, H-8'/8''), 5.86 (ddt, *J* = 16.8, 10.1, 6.7 Hz, 1H, H-8'/8''), 5.10-5.02 (m, 2H, H-9'/9''), 4.91 (dt, *J* = 17.1, 1.8 Hz, 1H, H-9'/9''), 4.84-4.80 (m, 1H, H-9'/9''), 4.70 (t, *J* = 5.7 Hz, 2H, NCH₂-6), 4.20 (s, 3H, OCH₃-16), 4.12 (s, 3H, OCH₃-17), 3.99 (t, *J* = 5.0 Hz, 2H, OCH₂-3'''), 3.60 (t, *J* = 7.9 Hz, 2H, H-1'''), 3.35 (d, *J* = 6.7 Hz, 2H, H-7'), 3.23 (d, *J* = 6.8 Hz, 2H, H-7''), 2.97 (t, *J* = 5.8 Hz, 2H, H-5), 2.20 (q, *J* = 7.0 Hz, 2H, H-2'''). ¹³C NMR (150 MHz, CD₃OD) δ 155.4, 155.3, 151.6, 151.4, 148.8, 146.2, 145.2, 139.3, 138.3, 138.1, 135.5, 135.0, 134.3, 133.9, 132.3, 131.9, 131.4, 129.2, 128.9, 127.1, 127.0, 123.0, 122.3, 121.6, 115.7, 115.6, 115.5, 113.6, 110.5, 109.3, 103.6, 68.5, 62.6, 58.8, 57.5, 40.4, 35.3, 31.9, 29.2, 28.0. HR-ESI-MS *m/z* calculated for C ₄₁H₄₀NO₆⁺ [M-I]⁺ 642.2850, found 642.2852.

13-(5-((3',5-diallyl-4'-hydroxy-[1,1'-biphenyl]-2-yl)oxy)pentyl)-9,10-dimethoxy-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-*a***]isoquinolin-7-ium iodide (5b). Yellow solid. 18% yield. ¹H NMR (600 MHz, CD₃OD) \delta 9.73 (s, 1H, H-8), 8.04 (s, 2H, H-11, H-12), 7.18 (d,** *J* **= 2.3 Hz, 1H, H-2"), 7.08 (s, 1H, H-1), 7.03 (dd,** *J* **= 8.3, 2.3 Hz, 1H, H-4'), 7.00 (d,** *J* **= 2.2 Hz, 1H, H-6'), 6.98 (dd,** *J* **= 8.2, 2.3 Hz, 1H, H-6"), 6.95 (s, 1H, H-4), 6.90 (d,** *J* **= 8.3 Hz, 1H, H-3'), 6.56 (d,** *J* **= 8.2 Hz, 1H, H-5"), 6.00 (s, 2H, OCH₂O-15), 5.99-5.93 (m, 1H, H-8'), 5.80 (ddt,** *J* **= 16.7, 10.0, 6.6 Hz, 1H, H-8"), 5.06-4.86 (m, 4H, H-9', 9"), 4.75 (t,** *J* **= 5.8 Hz, 2H, NCH₂-6), 4.23 (s, 3H, OCH₃-16), 4.11 (s, 3H, OCH₃-17), 3.95 (t,** *J* **= 5.7 Hz, 2H, H-5"), 3.33 (d,** *J* **= 7.0 Hz, 2H, H-7'), 3.27 (t,** *J* **= 8.1 Hz, OCH₂-1"'), 3.06 (d,** *J* **= 6.6 Hz, 2H, H-7"), 3.01 (t,** *J* **= 5.9 Hz, 2H, H-5), 1.86, 1.75, 1.54 (each, 2H, linker-CH₂-2"'/3"'/4"'). ¹³C NMR (150 MHz, CD₃OD) \delta 155.6, 154.9, 151.7, 151.3, 148.7, 146.4, 145.1, 139.4, 138.3, 137.7, 136.1, 134.9, 134.4, 133.6, 132.4, 131.9, 131.6, 131.4, 129.2, 128.8, 127.1, 126.6, 123.2, 122.2, 121.7, 115.62, 115.55, 115.2, 113.7, 110.6, 109.2, 103.6, 69.9, 62.7, 58.9, 57.6, 40.4, 35.1, 31.9, 30.7, 29.6, 29.2, 27.8. HR-ESI-MS** *m/z* **calculated for C₄₃H₄₄NO₆+ [M-I]⁺ 670.3163, found 670.3171.**

13-(8-((3',5-diallyl-4'-hydroxy-[1,1'-biphenyl]-2-yl)oxy)octyl)-9,10-dimethoxy-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-*a***]isoquinolin-7-ium iodide (5c). Yellow solid. 15% yield. ¹H NMR (600 MHz, CD₃OD) \delta 9.75 (s, 1H, H-8), 8.11 (d,** *J* **= 9.4 Hz, 1H, H-11/12), 8.08 (d,** *J* **= 9.4 Hz, 1H, H-11/12), 7.23 (s, 1H, H-1), 7.21 (d,** *J* **= 2.2 Hz, 1H, H-2"), 7.10 (dd,** *J* **= 8.2, 2.3 Hz, 1H, H-6"), 7.02 (m, 2H, H-4', H-6'), 6.97 (s, 1H, H-4), 6.91(d,** *J***=9.0 Hz, 1H, H-3'), 6.70 (d,** *J* **= 8.3 Hz, 1H, H-5"), 6.00 (s, 2H, OCH₂O-15), 5.99-5.91 (m, 2H, H-8', H-8"), 5.08-4.92 (m, 4H, H-9', H-9"), 4.75 (t,** *J* **= 5.9 Hz, 2H, NCH₂-6), 4.20 (s, 3H, OCH₃-16), 4.09 (s, 3H, OCH₃-17), 3.91 (t,** *J* **= 6.1 Hz, 2H, H-8"), 3.36 (3H, H-1", H-7'), 3.32 (1H, H-7'), 3.29 (dt,** *J* **= 6.5, 1.6 Hz, 2H, H-7"), 3.07 (t,** *J* **= 5.9 Hz, 2H, H-5), 1.82 (m, 2H, linker-CH₂), 1.64 (m, 2H, linker-CH₂), 1.47-**

1.37 (m, 6H, linker-CH₂), 1.31 (m, 2H, linker-CH₂). ¹³C NMR (150 MHz, CD₃OD) δ 155.8, 155.1, 151.8, 151.3, 148.7, 146.3, 145.1, 139.4, 138.5, 137.8, 136.6, 135.0, 134.4, 133.6, 132.38, 132.26, 131.6, 131.3, 129.3, 128.8, 127.1, 126.8, 123.1, 122.3, 121.8, 115.6, 115.4, 115.3, 114.2, 110.4, 109.3, 103.7, 69.7, 66.7, 62.6, 58.9, 57.5, 40.4, 35.4, 31.8, 30.5, 30.3, 30.0, 29.7, 29.2, 27.1. HR-ESI-MS *m/z* calculated for C₄₆H₅₀NO₆⁺ [M-I]⁺ 712.3633, found 712.3636.

13-(5-((3',5-diallyl-4'-methoxy-[1,1'-biphenyl]-2-yl)oxy)pentyl)-9,10-dimethoxy-5,6-dihydro -[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium iodide (5d). Yellow solid. 48% yield. ¹H NMR (600 MHz, CDCl₃) δ 10.45 (s, 1H, H-8), 7.80 (d, J = 9.3 Hz, 1H, H-11/12), 7.75 (d, J =9.3 Hz, 1H, H-11/12), 7.36 (d, J = 2.3 Hz, 1H, H-2"), 7.32 (dd, J = 8.4, 2.3 Hz, 1H, H-6"), 7.13 (d, J = 2.3 Hz, 1H, H-6'), 7.08 (dd, J = 8.3, 2.3 Hz, 1H, H-4'), 7.05 (s, 1H, H-1), 6.89 (d, J = 8.4 Hz, 1H, H-3¹/5¹), 6.87 (s, 1H, H-4), 6.82 (d, J = 8.4 Hz, 1H, H-3¹/5¹), 6.05 (d, J = 4.3 Hz, 2H, OCH₂O-15), 5.97 (dddt, J = 16.8, 10.1, 8.4, 6.7 Hz, 2H, H-8', H-8''), 5.16 (s, 2H, NCH₂-6), 5.13-4.95 (m, 4H, H-9', H-9''), 4.37 (s, 3H, OCH₃-16), 4.07 (s, 3H, OCH₃-17), 3.97 (t, J = 6.1 Hz, 2H, OCH₂-5"), 3.80 (s, 3H, OCH₂-1""), 3.37 (dt, J = 6.6, 1.5 Hz, 2H, H-7'/7"), 3.34 (dt, J = 6.6, 1.6 Hz, 2H, H-7'/7'', 3.23 (t, J = 8.4 Hz, 2H, H-1'''), 3.16 (t, J = 5.5 Hz, 2H, H-5), 1.81, 1.66, 1.28-1.23 (each, m, 2H, linker-CH₂-). ¹³C NMR (150 MHz, CDCl₃) δ 156.4, 154.4, 150.6, 149.9, 147.4, 146.5, 146.0, 137.9, 137.1, 136.2, 134.2, 133.9, 133.2, 132.7, 131.3, 131.1, 130.9, 128.4, 128.1, 127.8, 125.5, 122.4, 120.4, 120.2, 115.8, 115.5, 113.0, 110.0, 109.2, 108.7, 102.3, 68.4, 63.3, 57.8, 57.1, 55.7, 39.6, 34.4, 30.9, 30.1, 28.83, 28.79, 26.6. HR-ESI-MS m/z calculated for C₄₄H₄₆NO₆⁺[M-I]⁺ 684.3320, found 684.3315.

13-(5-((3',5-diallyl-4'-propoxy-[1,1'-biphenyl]-2-yl)oxy)pentyl)-9,10-dimethoxy-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium iodide (5e). Yellow solid. 38% yield. ¹H NMR (600 MHz, CDCl₃) δ 10.39 (s, 1H, H-8), 7.79 (d, *J* = 9.3 Hz, 1H, H-11/12), 7.73 (d, *J* =

9.3 Hz, 1H, H-11/12), 7.36 (d, J = 2.3 Hz, 1H, H-2"), 7.30 (dd, J = 8.4, 2.3 Hz, 1H, H-6"), 7.13 (d, J = 2.3 Hz, 1H, H-6'), 7.07 (dd, J = 8.4, 2.3 Hz, 1H, H-4'), 7.05 (s, 1H, H-1), 6.89 (d, J = 8.3 Hz, 1H, H-3'/5"), 6.86 (s, 1H, H-4), 6.80 (d, J = 8.4 Hz, 1H, H-3'/5"), 6.04 (s, 2H, OCH₂O-15), 5.97 (dddt, J = 16.9, 10.2, 6.7, 3.6 Hz, 2H, H-8', H-8"), 5.14(2H, NCH₂-6), 5.09-5.49 (m, 4H, H-9', H-9"), 4.38 (s, 3H, OCH₃-16), 4.05 (s, 3H, OCH₃-17), 3.97 (t, J = 6.0 Hz, 2H, OCH₂-5""/1""), 3.90 (t, J = 6.3 Hz, 2H, OCH₂-5"'/1""), 3.37 (d, J = 6.7 Hz, 4H, H-7', H-7"), 3.22 (m, 2H, H-1"), 3.19 (t, J = 5.9 Hz, 2H, H-5), 1.87 (m, 2H, linker-CH₂/H-2""), 1.81(m, 4H, linker-CH₂/H-2""), 1.65 (m, 2H, linker-CH₂/H-2""), 1.03 (t, J = 7.4 Hz, 3H, H-3""). ¹³C NMR (150 MHz, CDCl₃) δ 155.9, 154.4, 150.5, 149.9, 147.3, 146.5, 145.9, 137.9, 137.3, 136.2, 134.1, 134.0, 133.2, 132.7, 131.3, 131.1, 130.8, 130.7, 128.3, 128.0, 125.5, 122.3, 120.4, 120.2, 115.7, 115.5, 113.0, 110.9, 109.1, 108.7, 102.2, 69.8, 68.4, 63.4, 58.0, 57.0, 39.6, 34.7, 30.9, 30.2, 28.8, 28.7, 26.5, 22.9, 10.9. HR-ESI-MS m/z calculated for C₄₆H₅₀NO₆+ [M-I]⁺ 712.3633, found 712.3632.

13-(3-((3,5'-diallyl-2'-hydroxy-[1,1'-biphenyl]-4-yl)oxy)propyl)-9,10-dimethoxy-5,6-

dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-*a***]isoquinolin-7-ium iodide (6a). Yellow solid. 25% yield. ¹H-NMR(600 MHz, CD₃OD): \delta 9.78 (s, 1H, H-8), 8.23 (d,** *J* **= 9.3 Hz, 1H, H-12), 8.11 (d,** *J* **= 9.4 Hz, 1H, H-11), 7.36 (s, 1H, H-1), 7.32 (dd,** *J* **= 8.4, 2.3 Hz, 1H, H-6"), 7.28 (d,** *J* **= 2.3 Hz, 1H, H-2"), 7.03 (d,** *J* **= 2.2 Hz, 1H, H-6'), 6.94 (dd,** *J* **= 8.2, 2.2 Hz, 1H, H-4'), 6.86 (s, 1H, H-4), 6.80 (d,** *J* **= 8.4 Hz, 1H, H-3'), 6.78 (d,** *J* **= 8.4 Hz, 1H, H-5"), 6.09 (s, 2H, OCH₂O-15), 6.01-5.91 (m, 2H, H-8', 8"), 5.06-4,99 (m, 4H, H-9', 9"), 4.77 (s, 2H, NCH₂-6), 4.21 (s, 3H, OCH₃-16), 4.12 (s, 3H, OCH₃-17), 3.95 (m, 2H, OCH₂-3"'), 3.80 (t,** *J* **= 6.8 Hz, 2H, H-1"'), 3.33 (m, 2H, H-7'), 3.28 (d,** *J* **= 6.7 Hz, 2H, H-7"), 2.97 (t,** *J* **= 5.8 Hz, 2H, H-5), 2.28 (m, 2H, H-2"'). ¹³C NMR (150 MHz, CD₃OD) \delta 156.4, 153.6, 151.8, 151.3, 148.8, 146.5, 145.3, 139.6, 138.52, 138.45, 135.5, 135.0, 134.5, 132.8, 132.5, 131.9, 131.6, 129.5, 129.3, 129.0, 128.9, 127.2, 123.2, 122.3,**

116.9, 115.7, 115.5, 111.5, 110.7, 109.3, 103.6, 67.2, 62.6, 58.7, 57.5, 40.5, 35.5, 31.7, 29.2, 27.4,
23.3. HR-ESI-MS *m/z* calculated for C₄₁H₄₀NO₆⁺ [M-I]⁺ 642.2850, found 642.2860.

13-(5-((3,5'-diallyl-2'-hydroxy-[1,1'-biphenyl]-4-yl)oxy)pentyl)-9,10-dimethoxy-5,6-dihydro-[**1,3]dioxolo[4,5-***g*]isoquinolino[**3,2-***a*]isoquinolin-7-ium iodide (6b). Yellow solid. 23% yield. ¹H NMR (600 MHz, CD₃OD) δ 9.77 (s, 1H, H-8), 8.15 (d, *J* = 9.4 Hz, 1H, H-12), 8.04 (d, *J* = 9.5 Hz, 1H, H-11), 7.34 (dd, *J* = 8.3, 1.8 Hz, 1H, H-6'), 7.30 (d, *J* = 2.3 Hz, 1H, H-2''), 7.29 (d, *J* = 1.9 Hz, 1H, H-1), 7.01 (s, 1H, H-4), 7.00 (d, *J* = 2.3 Hz, 1H, H-6'), 6.92 (dd, *J* = 8.1, 2.2 Hz, 1H, H-4'), 6.89 (dd, *J* = 8.5, 1.5 Hz, 1H, H-5''), 6.79 (d, *J* = 8.2 Hz, 1H, H-3'), 6.08 (s, 2H, OCH₂O-15), 5.99–5.90 (m, 2H, H-8', 8''), 5.08-4.94 (m, 4H, H-9', 9''), 4.77(t, *J* = 5.9 Hz, 2H, NCH₂-6), 4.20 (s, 3H, OCH₃-16), 4.07 (s, 3H, OCH₃-17), 4.01 (t, *J* = 6.0 Hz, 2H, OCH₂-5'''), 3.46 (dd, *J* = 9.4, 6.4 Hz, 2H, H-1'''), 3.33 (s, 2H, H-7''), 3.30 (s, 2H, H-7'), 3.08 (q, *J* = 4.7, 3.6 Hz, 2H, H-5), 1.95, 1.86, 1.68 (each, 2H, linker-CH₂-2'''/3'''/4'''). ¹³C NMR (150 MHz, CD₃OD) δ 156.9, 153.5, 151.8, 151.4, 148.8, 146.3, 145.2, 139.6, 138.6, 137.9, 136.2, 135.1, 134.4, 132.5, 131.9, 131.6, 129.5, 129.3, 129.1, 129.0, 127.1, 123.1, 122.3, 121.8, 116.9, 115.62, 115.55, 115.45, 112.0, 110.5, 109.4, 103.7, 68.6, 62.7, 58.9, 57.5, 40.4, 35.6, 31.5, 30.7, 29.8, 29.2, 27.1. HR-ESI-MS *m/z* calculated for C₄₃H₄₄NO₆+ [M-I]⁺ 670.3163, found 670.3168.

13-(8-((3,5'-diallyl-2'-hydroxy-[1,1'-biphenyl]-4-yl)oxy)octyl)-9,10-dimethoxy-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-*a*]isoquinolin-7-ium iodide (6c). Yellow solid. 18% yield. ¹H NMR (600 MHz, CD₃OD) δ 9.74 (s, 1H, H-8), 8.15 (d, *J* = 9.3 Hz, 1H, H-11/12), 8.11 (d, 1H, H-11/12), 7.32 (dd, *J* = 8.5, 2.2 Hz, 1H, H-6"), 7.27 (m, 2H, H-1, H-2"), 7.01 (s, 1H, H-4), 6.97 (d, *J* = 2.2 Hz, 1H, H-6'), 6.91 (dd, *J* = 9.0, 2.1 Hz, 1H, H-4'), 6.89 (m, 1H, H-5"), 6.77 (d, *J* = 8.2 Hz, 1H, H-3'), 6.08 (s, 2H, OCH₂O-15), 5.95 (m, 2H, H-8', 8"), 5.09-4.92 (m, 4H, H-9', 9"), 4.74 (t, *J*

= 5.7 Hz, 2H, NCH₂-6), 4.19 (s, 3H, OCH₃-16), 4.10 (s, 3H, OCH₃-17), 4.01 (t, J = 5.1 Hz, 2H, OCH₂-8^{III}), 3.39 (m, 2H, H-1^{III}), 3.35 (m, 2H, H-7^{II}), 3.29 (m, 2H, H-7^{II}), 3.07 (t, J = 5.8 Hz, 2H, H-5), 1.90-1.84 (m, 2H, linker-CH₂), 1.84-1.78 (m, 2H, linker-CH₂), 1.51 (m, 4H, linker-CH₂), 1.41 (m, 4H, linker-CH₂). ¹³C NMR (150 MHz, CD₃OD) δ 157.0, 153.5, 151.8, 151.4, 148.8, 146.3, 145.1, 139.6, 138.6, 137.8, 136.3, 135.1, 134.4, 132.5, 132.4, 131.9, 131.5, 129.5, 129.3, 129.2, 129.0, 127.2, 123.1, 122.2, 121.8, 116.9, 115.5, 112.0, 110.5, 109.4, 103.7, 69.0, 62.6, 58.8, 57.5, 40.4, 35.6, 31.9, 30.5, 30.3, 30.1, 29.9, 29.2, 27.1, 23.3. HR-ESI-MS *m/z* calculated for C₄₆H₅₀NO₆⁺ [M-I]⁺ 712.3633, found 712.3631.

13-(5-((3,5'-diallyl-2'-methoxy-[1,1'-biphenyl]-4-yl)oxy)pentyl)-9,10-dimethoxy-5,6-dihydro -**[1,3]dioxolo[4,5-g]isoquinolino[3,2-***a***]isoquinolin-7-ium iodide (6d). Yellow solid. 48% yield. ¹H NMR (600 MHz, CDCl₃) \delta 10.38 (s, 1H, H-8), 7.91 (d,** *J* **= 9.4 Hz, 1H, H-11/12), 7.81 (d,** *J* **= 9.3 Hz, 1H, H-11/12), 7.35 (dd,** *J* **= 8.3, 2.3 Hz, 1H, H-6"), 7.31 (d,** *J* **= 2.2 Hz, 1H, H-2"), 7.13 (s, 1H, H-1/4'/6'), 7.10 (s, 1H, H-1/4'/6'), 7.10-7.09 (1H, H-1/4'/6'), 6.91-6.89 (m, 2H, H-4, H-3'/5"), 6.87 (d,** *J* **= 8.4 Hz, 1H, H-3'/5"), 6.09 (s, 2H, OCH₂O-15), 5.99 (dddt,** *J* **= 18.4, 16.8, 10.1, 6.8 Hz, 2H, H-8', H-8"), 5.10-5.02 (m, 4H, H-9', H-9"), 4.36 (s, 3H, OCH₃-16), 4.05 (s, 3H, OCH₃-17), 4.04 (d,** *J* **= 6.0 Hz, 2H, NCH₂-6), 3.78 (s, 3H, OCH₃-1""), 3.48 (s, 2H, H-1""/5""), 3.42(d,** *J***=6.8 Hz, 2H, H-7'/7"), 3.36 (d,** *J* **= 6.7 Hz, H-7'/7"), 3.34 (d,** *J* **= 7.2 Hz, 2H, H-1""/5""), 3.19 (t,** *J* **= 5.7 Hz, 2H, H-5),1.92, 1.77, 1.27-1.23 (each, m, 2H, linker-CH₂-). ¹³C NMR (150 MHz, CDCl₃) \delta 155.8, 155.0, 150.6, 150.0, 147.5, 146.4, 145.8, 137.9, 137.3, 136.3, 134.3, 133.9, 133.3, 132.4, 131.1, 131.0, 130.9, 130.4, 128.5, 128.3, 128.1, 125.7, 122.4, 120.5, 120.3, 115.7, 115.6, 111.4, 110.9, 109.2, 108.7, 102.3, 67.5, 63.2, 58.0, 57.1, 55.84, 39.5, 34.7, 31.0, 30.2, 29.0, 28.9, 26.5. HR-ESI-MS** *m/z* **calculated for C₄₄H₄₆NO₆+ [M-I]+ 684.3320, found 684.3324.**

13-(5-((3,5'-diallyl-2'-propoxy-[1,1'-biphenyl]-4-yl)oxy)pentyl)-9,10-dimethoxy-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium iodide (6e). Yellow solid. 40% yield. ¹H NMR (600 MHz, CDCl₃) δ 10.41 (s, 1H, H-8), 7.91 (d, J = 9.3 Hz, 1H, H-11/12), 7.79 (d, J = 9.3 Hz, 1H, H-11/12), 7.41 (d, J = 2.3 Hz, H-2"), 7.36 (dd, J = 8.3, 2.3 Hz, 1H, H-6"), 7.13 (s, 1H, H-1), 7.12 (d, J = 2.3 Hz, 1H, H-6'), 7.07 (dd, J = 8.3, 2.3 Hz, 1H, H-4'), 6.89 (s, 1H, H-4), 6.88 (d, J = 8.3 Hz, 1H, H-3'/5''), 6.87 (d, J = 8.3 Hz, 1H, H-3'/5''), 6.08 (s, 2H, OCH₂-15), 5.99 (dddt, J)J = 20.7, 16.9, 10.1, 6.8 Hz, 2H, H-8', H-8"), 5.11 - 5.00 (m, 4H, H-9', H-9"), 4.36 (s, 3H, OCH₃-16), 4.06 (d, J = 6.9 Hz, 2H, NCH₂-6), 4.04 (s, 3H, OCH₃-17), 3.89 (t, J = 6.4 Hz, 2H, OCH₂-5"'/1""), 3.43 (d, J = 6.7 Hz, 2H, H-7'/7"), 3.37 (d, J = 6.7 Hz, 2H, H-7'/7"), 3.19 (t, J = 5.9 Hz, 2H, H-5), 1.97 (2H, linker-CH₂/H-2""), 1.95 (2H, linker-CH₂/H-2""), 1.80 - 1.75 (m, 2H, linker- $CH_2/H-2''''$), 1.75 – 1.69 (m, 4H), 0.97 (t, J = 7.4 Hz, 3H, H-3''''). ¹³C NMR (150 MHz, CDCl₃) δ 155.6, 154.5, 150.6, 149.9, 147.4, 146.3, 145.8, 138.0, 137.3, 136.2, 134.3, 133.8, 133.2, 132.2, 131.6, 131.0, 130.4, 128.4, 128.1, 127.9, 125.6, 122.3, 120.41, 120.36, 115.63, 115.59, 112.6, 110.8, 109.2, 108.8, 102.3, 70.1, 67.5, 63.3, 57.9, 57.1, 39.6, 34.7, 30.9, 30.3, 29.0, 28.8, 26.5, 22.7, 11.0. HR-ESI-MS m/z calculated for C₄₆H₅₀NO₆⁺ [M-I]⁺ 712.3633, found 712.3632.

Biological materials. Human lung adenocarcinoma cells A549, human ovarian cancer cells A2780, human normal liver cells HL7702, and human prostate carcinoma cells PC3 were cultured in RPMI 1640 medium (Macgene, Beijing, China). Human colorectal carcinoma cells HT29 were maintained in Mycoy'5A medium (Gibco, Grand Island, USA). The human hepatoblastoma cell line HepG2 and human pancreatic cancer cell line PANC-1 were grown in Dulbecco's Modified Eagle Medium (DMEM) (Macgene, Beijing, China). The medium was supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Solarbio, Beijing, China). Cells were incubated in 5%

CO₂ at 37 °C. As achieved approximately 80% confluency, cells were harvested to seed into appropriate plates for different experiments. Wild-type AB of strains zebrafish were from the Biology Institute of the Shandong Academy of Sciences. The probe mito-tracker red CMXRos, JC-1, DAPI, Calcein-AM, z-VAD-fmk, ROS assay kit, BCA assay kit and cell mitochondria isolation kit were purchased from Beyotime (Nanjing, China). The annexin V/PI apoptotic detection kit was purchased from Bestbio (Shanghai, China). The 24-well glass bottom plates was obtained from Cellvis (Hangzhou, China). Primary antibodies against β -actin, PARP, AIF, Caspase 9, Caspase 12, P62, LC3 I/II and HRP-conjugated secondary antibody was purchased from Proteintech (Wuhan, China). Caspase 3 and Caspase 8 antibody were from Affinity Biosciences (HO, USA). Alexa fluor 594-linked secondary antibody was from ZSJQ-BIO (Beijing, China). Finally, the inhibitor nectrostatin-1 was obtained from Targetmol (MA, USA), chloroquine, taxol were obtained from Sigma (MO, USA), 3-Methyladenine was from Aladdine (Shanghai, China).

MTT assays. Cells were seeded into 96-well plates and incubated overnight. Following drugs treatment, 10 μ L of MTT solution (5 mg/mL) was added into each well, and cells were incubated for 4 h at 37 °C. After incubation, the solution containing MTT was removed, and 100 μ L DMSO was added per well to detect the absorbance of the soluble MTT produced at 570 nm using a microplate reader (Bio-Rad, CA, USA). The cytotoxic effect of compound was expressed as IC₅₀ (50% inhibiting concentration). Each cell viability experiment was repeated in triplicate.

Cell mitochondria isolation. Mitochondria were extracted according to the kit instruction. HepG2 cells seeded into 100-mm dishes were treated with 5 μ M **6b** or HN for 12 h, and then homogenized with mitochondrial extraction reagent. Resulting cell homogenate were centrifuged at 600g and 4

°C for 10 min, and supernatants were collected for additional centrifugation (11000g, 4 °C, 10 min). Sediments after centrifugation of 11000g were isolated mitochondria.

Cellular uptake of 6b. HPLC is a method used to detect the quantity of compounds in cell. Mitochondrial or non-mitochondrial sediments were lysed using an ultrasonic crusher. Then, MeCN was added to whole cell lysates to precipitate proteins, and samples were centrifuged for 15 min at 10000 rpm. After drying of supernatant, the residue was redissolved with MeOH and detected by HPLC (Agilent Technologies, CA, USA). We injected drug solution into the HPLC system, which employed a C18 column (250 mm×4.6 mm, 10 μ m) and methanol-0.1% acetic acid (80:20) as the mobile phase. The peaks of **6b** and HN were analyzed at 265 nm and 210 nm, respectively. This test was repeated thrice.

Colocalization with mitochondria of 6b. HepG2 cells were seeded into 20-mm glass bottom dishes to observe the localization of **6b**. Mitochondria were stained with 150 nM mito-tracker red CMXRos for 20 min after exposure to **6b**. Mito-tracker colocalization with **6b** was captured by confocal microscopy (Carl Zeiss, Germany).

Transmission electron microscopy. Cells plated in 100-mm dishes were treated with 3 μ M **6b** for 15 h or 30 h, and then collected to fix in 2.5% glutaraldehyde at 4°C overnight. The samples were dehydrated by a series of graded alcohols and embedded in resin. Transmission electron microscopy was performed to capture the microstructural images of cell samples.

Western blot. Cells treated with drugs were washed twice with PBS, lysed in RIPA lysis buffer on ice for 20 min and centrifuged to yield supernatant. The protein concentration in the supernatant was examined using the BCA assay kit. The protein samples were loaded onto 10% SDS-PAGE gel to extract the mixture of proteins and transferred to PVDF membranes by electrophoresis. The

version).

membranes were blocked with 5% nonfat milk in TBST prepared freshly for 2 h at room temperature and then washed thrice with TBST. After blocking, the membranes containing target protein were incubated with appropriate primary antibodies overnight at 4°C. HRP-conjugated secondary antibodies and the ECL system (Millipore, Germany) were used to detected immunoblot bands. The bands were analyzed using Image lab software. **Immunofluorescent Staining.** Cells were seeded in 20-mm glass bottom dishes. After drug treatment for 24 h, the cells were fixed with methanol:acetone (1:1) for 10 min and washed thrice with phosphate buffer saline (PBS) at 4°C. Then, 5% bovine serum albumin was added to block proteins at room temperature for 1 h. After washing with PBS, the primary antibody against AIF was added to wells and incubated at 4°C overnight, followed by staining with Alexa fluor 594conjugated secondary antibody. Next, the nucleus was stained with DAPI for 10 min. Samples were assessed using laser confocal microscopy and images were processed using ZEN (blue

Analysis of Cell Apoptosis. Cell apoptosis was examined using annexin V/PI staining. Cells were seeded into 6-well plates and incubated overnight to allow for attachment. Cells were treated with **6b** for 24 h and harvested. These samples were stained with binding buffer containing 3 μ L annexin V and 3 μ L PI for 30 min in the dark. Measurements were performed using flow cytometry (Becton Dickinson, USA).

Detection of Intracellular Reactive Oxygen Species. The fluorescent probe DCFH-DA was used to examine intracellular ROS levels. Cells were treated for indicated time. The solution was removed, and cells were stained with 1 mL FBS-free medium containing 10 μ M DCFH-DA for 30 min in the dark and then washed twice with PBS. Cells were detached from wells by trypsin

digestion, harvested at 1200 rpm for 5 min, and resuspended in 400 μ L PBS. The samples were analyzed by flow cytometry.

Measurement of Mitochondria Membrane Potential. Mitochondria membrane potential was monitored by staining with JC-1. Cells were seeded into 6-well plates, incubated with **6b** for 24 h at 37°C and then trypsinized to collect treated cells. Cells were stained with the fluorescent probe JC-1 at a final concentration of 2.5 μ g/mL for 30 min. Cells were washed thrice and centrifuged to remove the supernatant containing JC-1. Finally, the cells were resuspended with PBS for flow cytometric measurement.

Monitoring Mitochondrial Permeability Transition Pore Opening. Cells were plated in 24well glass bottom plates (Cellvis, China). After drugs treatment, cells were coloaded with 1.5 μ M Calcein-AM and 5 mM CoCl₂ in DMEM medium at 37°C for 20 min. CoCl₂ was added to quench the cytoplasmic fluorescence, so the calcein signal from the mitochondria was exclusively imaged²⁵. Cells were washed thrice using PBS and then detected with a 525-nm bandpass filter using fluorescence microscopy (Olympus, Japan).

Inhibition of Cancer Growth in *Vivo*. Wild-type AB of strains zebrafish were obtained from the Biology Institute of the Shandong Academy of Sciences. Embryos were generated by mathing of heterosexually mature zabrafish in breeding tank and incubated in special embryonic medium (5.0 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl₂, and 0.16 mM MgSO₄) at 28 °C. HepG2 cells were labled with cell tracker CM-Dil in *vitro* and microinjected into normally developed 2-day old zebrafish larvae under the stereomicroscopy. After 4 hours post injection, zebrafish with same injection spot were selected and planted into 6-well plate. These xenograft zebrafish seeded in well plates were sorted into different groups (approximately $15 \sim 20$ zebrafish per group) and administered by 0.8,

1.6, 2.4 μ M **6b** or 1.0 μ M doxorubicin for 48 h. Each group was established three holes. After drugs administration, the inhibition of tumor growth defined as fluorescence intensity were examined with laser confocal microscopy (Olympus, Japan). All experimental procedures were approved by the Animal Care and Use Committee of Shandong Academy of Sciences.

ASSOCIATED CONTENT

Supporting Information

Cell viability curves of derivatives in different cell lines; Synthetic routes of **2f** and **4d**; Morphological changes of cells treated with parent and target compounds and different drug combinations; Combination index values analyzed by CompuSyn software; 3D emission spectrum of **6b**; NMR spectra and ESI-MS of all derivatives; HPLC chromatograms for purity analysis of all derivatives (PDF). SMILES molecular strings and IC₅₀ data (CSV).

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The manuscript was written using contributions of all authors.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AIF, apoptosis-inducing factor; BBR, berberine; CO, chloroquine; CvsA, cvclosporin A; DCFH-DA, 2',7'-Dichlorodihydrofluorescein diacetate; DCM, dichloromethane; DMEM, Dulbecco's modified Eagle medium; DLCs, delocalized lipophilic cations; DMF, N,N-Dimethylformamide; ECL, enhanced chemiluminescence; HMBC, Heteronuclear multiple bond coherence; DMSO, dimethyl sulfoxide; HN, honokiol; HR-ESI-MS, high resolution electrospray ionization mass spectrometry; HSQC, Heteronuclear Single Quantum Coherence; IC₅₀, 50% inhibition concentration; J_{λ} coupling constant; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzimidazolocarbocyanine iodide; MeCN, acetonitrile; MeOH, methanol; $\Delta \psi_m$, mitochondria membrane potential; MPTP, mitochondrial permeability transition pore; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide: Nec-1, necrostatin-1; NMR, nuclear magnetic resonance; PARP, poly (ADP-ribose) polymerase; PBS, phosphate buffer saline; PI, propidium iodide; ppm, parts per million; ROS, reactive oxygen species; HPLC, high performance liquid chromatography; rpm, revolutions per minute; TLC, thin-layer chromatography; TMS, tetramethylsilane.

REFERENCES

(1) Hanahan, D.; Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* 2011, *144*, 646-674.

(2) Indran, I. R.; Tufo, G.; Pervaiz, S.; Brenner, C. Recent Advances in Apoptosis, Mitochondria and Drug Resistance in Cancer Cells. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 2011, *1807*, 735-745.

Li, L.; Han, W.; Gu, Y.; Qiu, S.; Lu, Q.; Jin, J.; Luo, J.; Hu, X. Honokiol Induces a Necrotic
 Cell Death through the Mitochondrial Permeability Transition Pore. *Cancer Res.* 2007, *67*, 4894-4903

(4) Yoon, M. J.; Kim, E. H.; Lim, J. H.; Kwon, T. K.; Choi, K. S. Superoxide Anion and Proteasomal Dysfunction Contribute to Curcumin-induced Paraptosis of Malignant Breast Cancer Cells. *Free Radical Bio. Med.* **2010**, *48*, 713-726.

(5) Ye, Y.; Zhang, T.; Yuan, H.; Li, D.; Lou, H.; Fan, P. Mitochondria-Targeted Lupane Triterpenoid Derivatives and Their Selective Apoptosis-Inducing Anticancer Mechanisms. *J. Med. Chem.* **2017**, *60*, 6353-6363.

(6) Pan, J.; Lee, Y.; Cheng, G.; Zielonka, J.; Zhang, Q.; Bajzikova, M.; Xiong, D.; Tsaih, S.; Hardy, M.; Flister, M.; Olsen, C. M.; Wang, Y.; Vang, O.; Neuzil, J.; Myers, C. R.; Kalyanaraman, B.; You, M. Mitochondria-Targeted Honokiol Confers a Striking Inhibitory Effect on Lung Cancer via Inhibiting Complex I Activity. *iScience* **2018**, *3*, 192-207.

(7) Millard, M.; Gallagher, J. D.; Olenyuk, B. Z.; Neamati, N. A Selective Mitochondrial-Targeted Chlorambucil with Remarkable Cytotoxicity in Breast and Pancreatic Cancers. *J. Med. Chem.* **2013**, *56*, 9170-9179.

(8) Ishitsuka, K.; Hideshima, T.; Hamasaki, M.; Raje, N.; Kumar, S.; Hideshima, H.; Shiraishi,
N.; Yasui, H.; Roccaro, A. M.; Richardson, P.et al. Honokiol Overcomes Conventional Drug
Resistance in Human Multiple Myeloma by Induction of Caspase-Dependent and -Independent
Apoptosis. *Blood* 2005, *106*, 1794-1800.

(9) Huang, K.; Chen, Y.; Zhang, R.; Wu, Y.; Ma, Y.; Fang, X.; Shen, S. Honokiol Induces Apoptosis and Autophagy via the ROS/ERK1/2 Signaling Pathway in Human Osteosarcoma Cells in *Vitro* and in *Vivo*. *Cell Death Dis*. **2018**, *9*, 157-173.

(10) Deng, J.; Qian, Y.; Geng, L.; Chen, J.; Wang, X.; Xie, H.; Yan, S.; Jiang, G.; Zhou, L.;
Zheng, S. Involvement of p38 Mitogen-Activated Protein Kinase Pathway in Honokiol-Induced
Apoptosis in a Human Hepatoma Cell Line (hepG2). *Liver Int.* 2008, *28*, 1458-1464.

(11) Wang, Y.; Zhu, X.; Yang, Z.; Zhao, X. Honokiol Induces Caspase-independent Paraptosis via Reactive Oxygen Species Production that is Accompanied by Apoptosis in Leukemia Cells. *Biochem. Bioph. Res. Co.* **2013**, *430*, 876-882.

(12) Zielonka, J.; Joseph, J.; Sikora, A.; Hardy, M.; Ouari, O.; Vasquez-Vivar, J.; Cheng, G.; Lopez, M.; Kalyanaraman, B. Mitochondria-Targeted Triphenylphosphonium-Based Compounds:

Syntheses, Mechanisms of Action, and Therapeutic and Diagnostic Applications. *Chem. Rev.* **2017**, *117*, 10043-10120.

(13) Mantena, S. K. Berberine, a Natural Product, Induces G₁-phase Cell Cycle Arrest and Caspase-3-Dependent Apoptosis in Human Prostate Carcinoma Cells. *Mol. Cancer Ther.* 2006, *5*, 296-308.

(14) Song, J.; Lin, C.; Yang, X.; Xie, Y.; Hu, P.; Li, H.; Zhu, W.; Hu, H. Mitochondrial Targeting Nanodrugs Self-Assembled from 9-O-octadecyl Substituted Berberine Derivative for Cancer Treatment by Inducing Mitochondrial Apoptosis Pathways. *J. Control. Release* 2019, *294*, 27-42.

(15) Li, Q.; Zhou, T.; Liu, C.; Wang, X.; Zhang, J.; Wu, F.; Lin, G.; Ma, Y.; Ma, B. Mitochondrial Membrane Potential Played Crucial Roles in the Accumulation of Berberine in HepG2 cells. *Bioscience Rep.* **2019**, *39*, R20190477.

(16) Pereira, G. C.; Branco, A. F.; Matos, J. A. C.; Pereira, S. L.; Parke, D.; Perkins, E. L.;
Serafim, T. L.; Sardão, V. A.; Santos, M. S.; Moreno, A. J. M.; Holy J.; Oliveira P. J..
Mitochondrially Targeted Effects of Berberine [Natural Yellow 18, 5,6-Dihydro-9,10Dimethoxybenzo(g)-1,3-Benzodioxolo(5,6-a) Quinolizinium] on K1735-M2 Mouse Melanoma
Cells: Comparison with Direct Effects on Isolated Mitochondrial Fractions. *J. Pharmacol. Exp. Ther.* 2007, *323*, 636-649.

(17) Lyamzaev, K. G.; Pustovidko, A. V.; Simonyan, R. A.; Rokitskaya, T. I.; Domnina, L. V.;
Ivanova, O. Y.; Severina, I. I.; Sumbatyan, N. V.; Korshunova, G. A.; Tashlitsky, V. N.; Roginsky
V. A.; Antonenko Y. N.; Skulachev M. V.; Chernyak B. V.; Skulachev V. P.. Novel Mitochondria-

Targeted Antioxidants: Plastoquinone Conjugated with Cationic Plant Alkaloids Berberine and Palmatine. *Pharm. Res.-Dordr.* **2011**, *28*, 2883-2895.

(18) Wang, J.; Yang, T.; Chen, H.; Xu, Y.; Yu, L.; Liu, T.; Tang, J.; Yi, Z.; Yang, C.; Xue, W.;
Yang, F. The Synthesis and Antistaphylococcal Activity of 9, 13-Disubstituted Berberine Derivatives. *Eur. J. Med. Chem.* 2017, *127*, 424-433.

(19) Chou, T. C. Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. *Cancer Res* **2010**, *70*, 440-446.

(20) Susin, S. A.; Lorenzo, H. K.; Zamzami, N.; Marzo, I.; Snow, B. E.; Brothers, G. M.;
 Mangion, J.; Jacotot, E.; Costantini, P.; Loeffler, M. Molecular Characterization of Mitochondrial
 Apoptosis-Inducing Factor. *Nature* 1999, *397*, 441-446.

(21) Degterev, A.; Huang, Z.; Boyce, M.; Li, Y.; Jagtap, P.; Mizushima, N.; Cuny, G. D.; Mitchison, T. J.; Moskowitz, M. A.; Yuan, J. Chemical Inhibitor of Nonapoptotic Cell Death with Therapeutic Potential for Ischemic Brain Injury. *Nat. Chem. Biol.* **2005**, *1*, 112-119.

(22) Sabharwal, S. S.; Schumacker, P. T. Mitochondrial ROS in Cancer: Initiators, Amplifiers or an Achilles' heel? *Nat. Rev. Cancer* **2014**, *14*, 709-721.

(23) Hseu, Y.; Thiyagarajan, V.; Ou, T.; Yang, H. CoQ₀-induced Mitochondrial PTP Opening Triggers Apoptosis via ROS-mediated VDAC1 Upregulation in HL-60 Leukemia Cells and Suppresses Tumor Growth in Athymic Nude Mice/Xenografted Nude Mice. *Arch. Toxicol.* **2018**, *92*, 301-322.

(24) Schriewer, J. M.; Peek, C. B.; Bass, J.; Schumacker, P. T. ROS-Mediated PARP Activity Undermines Mitochondrial Function After Permeability Transition Pore Opening During Myocardial Ischemia-Reperfusion. J. Am. *Heart Assoc.* **2013**, *2*, e000159.

(25) Petronilli, V.; Miotto, G.; Canton, M.; Brini, M.; Colonna, R.; Bernardi, P.; Di Lisa, F.
Transient and Long-Lasting Openings of the Mitochondrial Permeability Transition Pore can be
Monitored Directly in Intact Cells by Changes in Mitochondrial Calcein Fluorescence. *Biophys. J.* **1999**, *76*, 725-734.

(26) Teng, Y.; Xie, X.; Walker, S.; White, D. T.; Mumm, J. S.; Cowell, J. K. Evaluating Human Cancer Cell Metastasis in Zebrafish. *BMC Cancer* **2013**, *13*, 453-464.

(27) Zorova, L. D.; Popkov, V. A.; Plotnikov, E. Y.; Silachev, D. N.; Pevzner, I. B.; Jankauskas,
S. S.; Babenko, V. A.; Zorov, S. D.; Balakireva, A. V.; Juhaszova, M.; Sollott, S. J.; Zorov D. B.
Mitochondrial Membrane Potential. *Anal. Biochem.* 2018, *552*, 50-59.

(28) Jeena, M. T.; Kim, S.; Jin, S.; Ryu, J. Recent Progress in Mitochondria-Targeted Drug and Drug-Free Agents for Cancer Therapy. *Cancers* **2020**, *12*, 4-23.

(29) Iwasa, K.; Moriyasu, M.; Yamori, T.; Turuo, T.; Lee, D.; Wiegrebe, W. In *Vitro* Cytotoxicity of the Protoberberine-Type Alkaloids. *J Nat Prod* **2001**, *64*, 896-898.

(30) Fu, S.; Xie, Y.; Tuo, J.; Wang, Y.; Zhu, W.; Wu, S.; Yan, G.; Hu, H., Discovery of Mitochondria-Targeting Berberine Derivatives as the Inhibitors of Proliferation, Invasion and Migration Against Rat C6 and Human U87 Glioma Cells. *MedChemComm* **2015**, *6*, 164-173.

(31) Han, L.; Xie, L.; Li, L.; Zhang, X.; Zhang, R.; Wang, H. Reactive Oxygen Species Production and Bax/Bcl-2 Regulation in Honokiol-Induced Apoptosis in Human Hepatocellular Carcinoma SMMC-7721 cells. *Environ. Toxicol. Phar.* **2009**, *28*, 97-103.

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