

Structure-Activity Relationship Study Enables the Discovery of a Novel Berberine Analogue as RXR α Activator to Inhibit Colon Cancer

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KEY WORDS: berberine analogue; RXR α activator; structure-activity analysis; B-12; colon
cancer

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3 **ABSTRACT** : We reported recently that berberine, a traditional oriental medicine to treat
4 gastroenteritis, binds and activates Retinoid X receptor α (RXR α) to suppress the growth of colon
5 cancer cells. Here, we extended our studies based on the binding mode of berberine with RXR α
6 by design, synthesis and biological evaluation of a focused library of 15 novel berberine analogues.
7 Among them, 3,9-dimethoxy-5,6-dihydroisoquinolino[3,2-a]isoquinolin-7-ium chloride (**B-12**)
8 was identified as the optimal RXR α activator. More efficiently than berberine, **B-12** bound and
9 altered the conformation of RXR α /LBD, thereby suppressing Wnt/ β -catenin pathway and colon
10 cancer cell growth via RXR α mediation. In addition, **B-12** not only preserved berberine's tumor
11 selectivity but also greatly improved its bioavailability. Remarkably, in mice, **B-12** did not show
12 obvious side effects including hypertriglyceridemia as other RXR α agonists, or induce hepatorenal
13 toxicity. Together, our study describes an approach for the rational design of berberine-derived
14 RXR α activators as novel effective antineoplastic agents for colon cancer.
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35 ■ INTRODUCTION

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38 Colon cancer is the third most common malignancy and the fourth leading cause of cancer-
39 related deaths worldwide. It is initiated in the epithelial cells lining the colon or rectum and
40 frequently results from Wnt/ β -catenin signaling pathway overactivation that is driven by mutations
41 in pathway components, such as adenomatous polyposis coli (APC) and β -catenin.¹ In the past two
42 decades, the survival rate has increased significantly profiting from multiple new therapeutic
43 methods. However, the 5-year disease-specific overall survival rate is still lower than 60% due to
44 tumor recurrences and drug resistances.^{2, 3} New agents targeting important molecules in colon
45 cancer development, with potent antitumor efficacy and low toxicity, is still urgently needed.
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3 Retinoid X receptor α (RXR α), a unique member of the nuclear receptor superfamily,
4 participates in a wide range of cellular processes under physiological and pathophysiological
5 conditions through modulating gene expression in a ligand-dependent manner. The abnormal
6 expression or post-translational modification of RXR α is closely related to the development of
7 several types of cancers, such as colon cancer.⁴⁻⁶ Hyperphosphorylation of RXR α , resulting in its
8 transactivation inhibition, was detected in a series of human colon cancer cell lines and primary
9 human colon cancer tissues.⁷ RXR α expression was also downregulated in *APC* mutant mice.⁸
10 Proteomics analyses demonstrated that more than 70% of the significantly down-regulated proteins
11 are associated with abnormal RXR α signaling pathway in colon cancer tissues.⁹ Additionally,
12 extensive crosstalk between RXR α and Wnt/ β -catenin signaling pathway was also observed in
13 colon cancer.^{10, 11} Suppression of RXR α and aberrant β -catenin expression significantly associate
14 with progression of colon cancer.^{12, 13} RXR α agonists increased β -catenin and RXR α protein
15 interaction, leading to the degradation of β -catenin and the inhibition of cell growth in colon
16 cancer.^{14, 15} Therefore, RXR α is considered to be an important target for colon cancer treatment.
17 Identifying and optimizing molecules that bind to its canonical ligand-binding pocket (LBP) has
18 long been the focus of drug discovery efforts targeting RXR α , and a large pool of such RXR α
19 ligands have been reported, such as 9-*cis*-retinoid acid (9-*cis*-RA), targretin (LGD1069) and
20 sulindac.^{16, 17} However, there are key limitations of treatment with these ligands including
21 unwanted side effects such as hypertriglyceridemia, mucosal toxicity, hypercalcemia, and
22 hepatomegaly.^{4, 16-18} The current challenge is to discover selective RXR α modulators with desired
23 pharmacological activities and minimal undesired side effects.^{4, 16, 17}

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52 Berberine (Figure 1a), an isoquinoline alkaloid isolated from several Chinese herbs such as
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Coptis chinensis, is a nonprescription medicine traditionally used to treat gastroenteritis. In recent

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3 years, pharmacological and clinical studies have displayed a broad range of benefits from
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5 berberine treatment, including antidiabetic, antihyperlipidemic, and antitumor effects.¹⁹⁻²² In
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7 several cancer models, berberine has been proven to reduce cell proliferation, induce cell apoptosis
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9 and inhibit tumor angiogenesis and metastasis, which makes berberine a promising drug for cancer
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11 therapy.^{23, 24} Notably, berberine is a radiosensitizer of tumor cells but not normal, untransformed
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13 cells, and also displays high selectivity for colon cancer cells relative to normal colon epithelial
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15 cells.²⁵ Our recent study demonstrated that berberine acted as a novel RXR α activator to suppress
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17 β -catenin signaling and cell growth in colon cancer through directly binding to a unique site (Site
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19 X) on the ligand-binding domain (LBD) of RXR α , which is likely the main reason for the tumor
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21 selectivity of berberine and thus provided new strategies for the design of new RXR α -based
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23 antitumor agents.²⁶ However, some limitations of berberine, including poor solubility, low
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25 bioavailability, and moderate tumor-suppressing activity,^{27, 28} restrict its clinical application to
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27 treat colon cancer. Structural modification of berberine would be an effective way to solve this
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29 dilemma. Some modifications of berberine have been carried out trying to improve its bioactivity
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31 and bioavailability in antimicrobial, antidiabetic, antihyperlipidemic and antitumor effects.
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33 Modification at C8 and C13 greatly increased the antimicrobial activity of berberine, which is
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35 closely related to the substituent chain length.²⁹⁻³¹ Most of the berberine derivatives with halogen
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37 and heteroaromatics on C8, C9 position displayed stronger hypoglycemic ability than berberine.^{32,}
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39 ³³ Structure-activity studies on the antitumor effect of berberine mainly focused on the C8, C9, and
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41 C13 positions; for example, 13-alkyl derivatives of berberine showed higher antitumor activities
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43 than berberine, and a moderate alkyl chain length was beneficial for its antitumor activity.³⁴⁻³⁶
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45 However, almost all these modifications are randomly made and effect-oriented, lacking a
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47 structural basis for the mechanism of berberine's effects. Most of these derivatives, by inserting
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3 either a long aliphatic chain or a heterocyclic ring, may have significant effects but lose the
4 biosafety of berberine since the structures were changed too much. Moreover, these derivatives
5 were mostly accessed by modification of natural berberine, thus significantly restricting the
6 structural diversity necessary for structure-activity relationship (SAR) studies.
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12 Our previous studies identified RXR α as an intracellular target of berberine to suppress colon
13 cancer growth and disclosed a unique binding mode of berberine with RXR α ,²⁶ which provides an
14 ideal theoretical base for SAR design of new anti-tumor berberine analogues. In the present study,
15 based on our previous findings, we designed and prepared 15 berberine analogues (nine of them
16 are new compounds) in a total synthesis manner with modifications on the C2/3/9/10/11/12
17 positions of berberine, in an attempt to find a molecule possessing similar tumor-selectivity to that
18 of berberine but with enhanced antitumor activity and bioavailability.
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31 ■ RESULTS

32 *Design and synthesis of berberine analogues targeting RXR α*

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34 We described recently how berberine (Figure 1a) acts as an RXR α activator through direct
35 binding to the Site X (non-classical ligand-binding pocket) in the ligand-binding domain (LBD)
36 of RXR α to activate its transactivating function and its regulation on Wnt/ β -catenin signaling to
37 inhibit colon cancer growth; the C2, C3 groups on the head of berberine form H-bonds with the
38 Arg371 residue of RXR α , and the C9, C10 methoxy groups on the tail region of berberine form
39 Van Der Waals contacts with RXR α (Figure 1b).²⁶ To obtain more efficient RXR α activator and
40 overcome aforementioned drawbacks residing in berberine as a promising antineoplastic agent, we
41 designed 15 berberine analogues with modifications on C2, 3, 9, 10, 11 and 12 positions based on
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the mode of berberine binding to RXR α . The structures of these analogues were shown in Table

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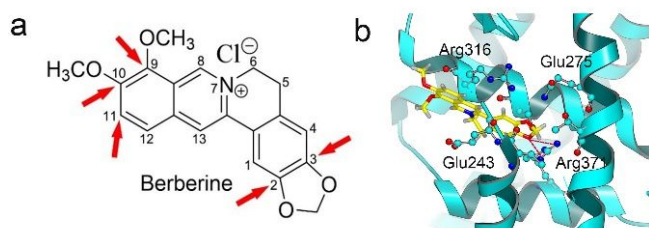


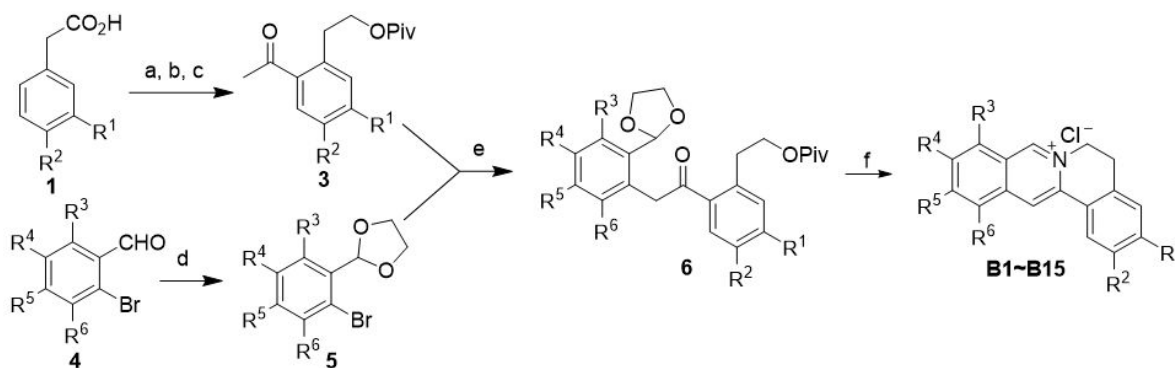
Figure 1. Chemical structure of berberine (a) and the binding site in RXR α -LBD (b)

(a) Chemical structure of berberine. Red arrows indicated the sites to be remodeled. (b) Detailed view of the interaction between berberine (yellow) and RXR α /LBD (cyan) (PDB:3FUG) in Site X. Key residues involved in berberine binding were shown in sticks; potential hydrogen-bonding interaction between berberine and RXR α -LBD residues Arg371 was shown as dashed red line.

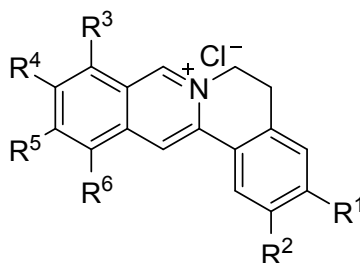
The analogues of berberine were prepared according to Donohoe's protocol³⁷ and a general synthetic sequence was illustrated in Scheme 1. The acetophenone **3** could be synthesized from the substituted phenylacetic acids through reduction followed by the protection of the resulting alcohol as a pivalate and a regioselective Friedel-Crafts acylation reaction with Ac₂O and ZnCl₂. Aryl bromide **6** was prepared from the corresponding *o*-bromobenzaldehyde through acetal protection. Then in the presence of [(Amphos)₂PdCl₂] and Cs₂CO₃, the cross-coupling between acetophenone **3** and aryl bromide **6** delivered **7**. Finally, the desired berberine analogue was formed by heating the mixture of compound **7** and NH₄Cl in EtOH/H₂O. Through this reaction sequence, 15 berberine analogues, named **B-1** to **B-15**, were synthesized smoothly (Figure 2b). Among them,

B-2 (pseudocoptisine),³⁸ **B-4** (pseudoberberine),³⁹ **B-5** (pseudopalmatine),⁴⁰ and **B-11** (palmatine)⁴¹ are natural products and **B-1**⁴² and **B-9**⁴² are known berberine analogues. The structure and purity (above 95% pure) of all berberine analogues were confirmed by qNMR analysis techniques. Berberine and its 15 analogues were screened by PAINS-remover (<http://www.cbligand.org/PAINS>) to filter out potential false-positive molecules and all these analogues were proved to be PAIN-less.

Scheme 1. Synthesis of berberine analogues



Reagents and conditions: (a) $\text{BH}_3 \cdot \text{HF}$ (2.0 equiv), THF, $0\text{ }^\circ\text{C} \rightarrow \text{rt}$; (b) PivCl (2.0 equiv), pyridine (2.0 equiv), CH_2Cl_2 , $0\text{ }^\circ\text{C} \rightarrow \text{rt}$; (c) ZnCl_2 (5.0 equiv), Ac_2O , $0\text{ }^\circ\text{C} \rightarrow \text{rt}$; (d) ethylene glycol (5.0 equiv), PTSA (0.1 equiv), HC(OEt)_3 (3.0 equiv), toluene, $90\text{ }^\circ\text{C}$; (e) $[(\text{Amphos})_2\text{PdCl}_2]$ (5 mol%), Cs_2CO_3 (2.5 equiv), THF, $90\text{ }^\circ\text{C}$; (f) NH_4Cl , EtOH/ H_2O (3:1), $90\text{ }^\circ\text{C}$ then $110\text{ }^\circ\text{C}$.

Table 1. Structures of the berberine analogues

Compound	R1	R2	R3	R4	R5	R6
B-1	OCH ₂ O		H	H	H	H
B-2	OCH ₂ O		H	OCH ₂ O		H
B-3	OCH ₃	H	H	OCH ₃	OCH ₃	H
B-4	OCH ₂ O		H	OCH ₃	OCH ₃	H
B-5	OCH ₃	OCH ₃	H	OCH ₃	OCH ₃	H
B-6	OCH ₃	OCH ₃	H	H	H	H
B-7	OCH ₃	H	H	H	H	H
B-8	OCH ₃	H	H	OCH ₂ O		H
B-9	OCH ₂ O		H	OCH ₃	H	H
B-10	OCH ₃	H	OCH ₃	OCH ₃	H	H
B-11	OCH ₃	OCH ₃	OCH ₃	OCH ₃	H	H
B-12	OCH ₃	H	OCH ₃	H	H	H
B-13	OCH ₃	OCH ₃	OCH ₃	H	H	H
B-14	OCH ₃	OCH ₃	H	H	H	OCH ₃
B-15	OCH ₂ O		H	H	H	OCH ₃

Structure-activity analyses identified **B-12** as the most effective RXR α activator among these berberine analogues

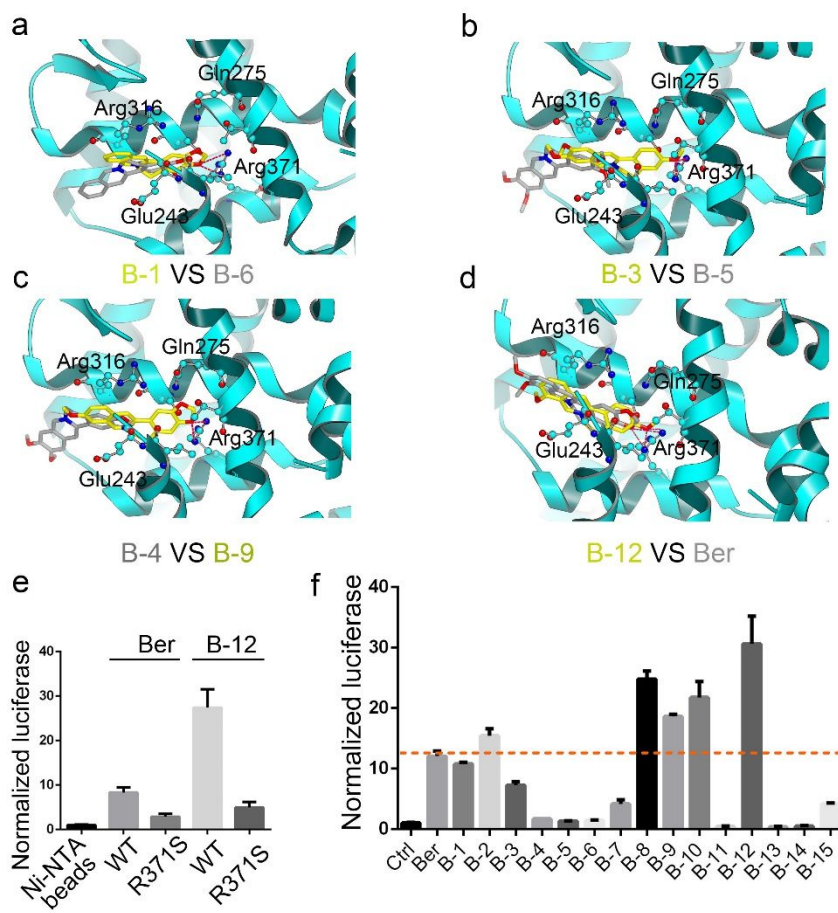


Figure 2. Structure-activity analyses identified **B-12** as the most effective RXR α activator among these berberine analogues. (**a-d**) Close-up view of the berberine analogues binding to RXR α -LBP (PDB code 3FUG). Compounds **B-1**, **B-3**, **B-9**, and **B-12** were shown in yellow; and compounds **B-4**, **B-5**, **B-6** and **Berberine (Ber)** were indicated in gray. Key residues involved in RXR α binding were shown in sticks. Potential hydrogen-bonding interaction between berberine analogues and RXR α /LBD Arg371 was shown as a dashed red line. (**e**) KM12C cells were co-transfected with pG5luc plasmid and pBIND-RXR α -LBD or its mutants. Twenty hours after

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3 transfection, cells were treated with 50 μ M berberine or **B-12**; then the activities of the reporter
4 gene were determined by luciferase assays and the basal level was normalized to 1. (f) KM12C
5 cells co-transfected with pBIND-RXR α /LBD and pG5luc were treated with berberine or its
6 analogues at 50 μ M for 15 h.
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16 With these compounds in hand, we carried out SAR studies by evaluating binding affinity to
17 RXR α through molecular docking studies and further by biological tests (Figure 2 and Table 2).
18 Firstly, the docking calculations suggested that the compounds with 2,3-methylenedioxy group
19 have much better binding conformation than that with 2,3-dimethoxy group. As shown in Figure
20 2a, the C3-oxygen of **B-1** formed two H-bonds with the backbone of Arg371 in RXR α /LBD, while
21 **B-6** could not form any effective interactions with the surrounding amino acid residues. We next
22 found that the single C3 methoxy group of **B-3** is also beneficial for the compound to bind RXR α -
23 LBD, while the C2 methoxy group in compound **B-5** seemed to hamper the compound to enter the
24 binding-pocket and form H-bond with Arg371 of RXR α by comparing **B3** and **B5** (Figure 2b),
25 which might result from the larger steric hindrance induced by the coexistence of C2 and C3
26 methoxy groups. Next, we investigated the roles of functional groups present on the C9/10/11/12
27 positions (the tail) of berberine core when the compounds have the same head structure. By
28 comparing **B-4** with **B-9**, we found 10,11-dimethoxy groups seemed to impede the binding of the
29 compounds with RXR α /LBD, because even though **B-4** has a methylenedioxy group at C2-C3
30 positions, which is more favorable for binding to RXR protein, it is still almost outside the pocket
31 (Figure 2c). Similar docking results mentioned above were obtained when comparing **B-14** and **B-**
32 **15**, **B-12** and **B-13**, **B-2** and **B-4**, respectively (Figure S1a-c). It is worth noting, when the heads
33 of compounds bear C3 single methoxy or 2,3-methylenedioxy groups, some analogues with
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3 substituents on the C9/10/11 positions of berberine, except the analogues with bismethoxy groups
4 on C10 and C11 (**B-3** and **B-4**), could well match the binding-pocket and form H-bonds with
5 Arg371, such as **B-2**, **B-8**, **B-9**, **B-10** and **B-12** (Figure S1d).
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10 For further verification, we ranked the binding potential of berberine analogues with
11 RXR α /LBD through molecular docking by four indexes, including shape matching, H-bond
12 energy, protein solvation, and small molecule solvation. As shown in Table 1, the five top-scoring
13 compounds are **B-12**, **B-8**, **B-9**, **B-10** and **B-2**, which is consistent with the above conformational
14 analysis. Next, the physiological consequences of the interactions between these compounds and
15 RXR α /LBD were validated by their abilities to transactivate RXR α (Figure 2f), as well as to inhibit
16 colon cancer cell growth, which were illustrated as IC₅₀ in Table 2. The dose-response curves of
17 representative compounds (**Ber**, **B-8**, **B-9**, **B-10**, **B-12**), along with *9-cis*-RA as positive control,
18 were shown in Figure S1e. The above results illustrated that compounds with higher binding
19 potential to RXR α as indicated in molecular docking analysis indeed had a stronger ability to
20 stimulate the transactivation activities of RXR α /LBD and inhibit the growth of colon cancer cells,
21 such as **B-8**, **B-9**, **B-10** and **B-12**; reversely, those showing poor binding potential exhibited little
22 effect on the RXR α -LBD transactivation activity and inhibition of colon cancer cell proliferation,
23 including **B-4**, **B-5**, **B-6**, **B-7**, **B-13**, and **B-14**. Among all these compounds, **B-12**, as expected,
24 exhibited the strongest transcriptional activation ability to RXR α and the greatest inhibitory effect
25 on colon cancer cell growth (Figure 2f and Table 2). **B-12** has the lowest IC₅₀ value to KM12C
26 cell growth (31.10 μ M), which was much smaller than that of berberine (76.34 μ M). Based on
27 these results, we further explored the binding conformation of compound **B-12**, and the results
28 indicated that compound **B-12** manifested a better binding conformation with RXR α /LBD (Figure
29 2d), and its H-bond lengths with Arg371 of RXR α (3.0 Å, 3.11 Å) was far shorter than that of
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berberine (3.9 Å, 3.2 Å). Subsequent mutation analysis confirmed that Arg371 is essential for the binding of RXR α /LBD with both **B-12** and berberine (Figure 2e). We further confirmed the superiority of **B-12** to berberine on cancer cell growth and RXR α -dependent transactivation in another two colon cancer cell lines HCT116 and SW620. Results showed that **B-12** exhibited excellent IC₅₀ values in the growth inhibition of HCT116 and SW620 cells (27.17 μ M and 36.19 μ M, respectively), which were less than one-third of that of berberine (83.09 μ M and 126.20 μ M, respectively) (Figure S2a). **B-12** also acted much better than berberine to suppress colon cancer cell growth indicated by EdU (5-Ethynyl-2'-deoxyuridine) assay and PCNA (proliferating cell nuclear antigen, a cell proliferation marker) expression level (Figure S2b-c), and to enhance the transactivation activity of endogenous RXR α on RXR DNA response element (RXRE) in KM12C, HCT116 and SW620 cells (Figure S2d).

Table 2. Berberine analogues' molecular docking conformational analysis score sheet and their inhibitory effects (IC₅₀) on KM12C cell proliferation

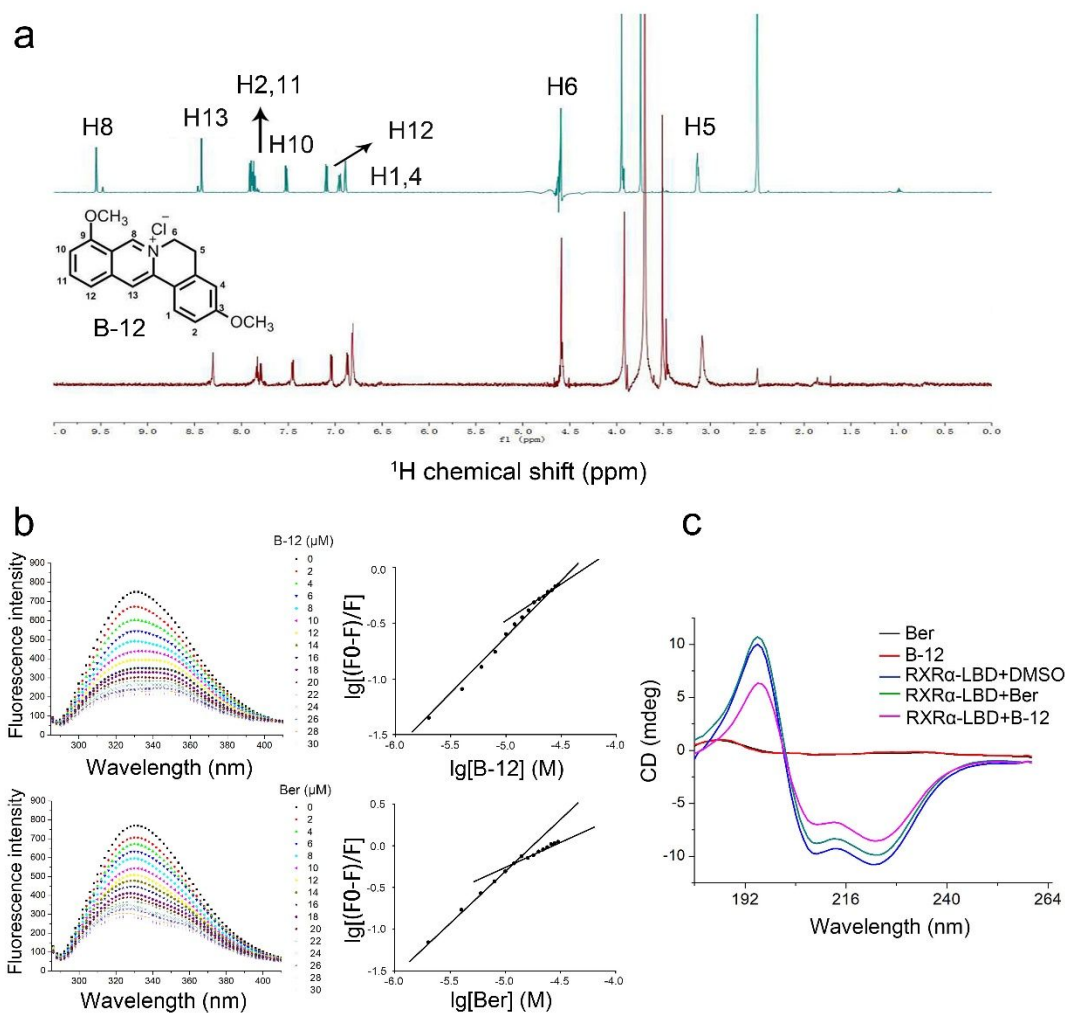
Compound	Total Score	Hydrogen Bond	Shape	Ligand Desolvation	Protein Desolvation	IC ₅₀ (μ M)
B-1	5.42	-0.92	-11.68	1.03	6.15	79.70
B-2	5.60	-0.94	-13.55	1.37	7.39	57.18
B-3	5.38	-0.98	-12.35	0.65	7.30	83.50
B-4	4.84	-0.96	-8.45	1.28	6.68	467.00
B-5	4.64	-0.21	-10.67	0.76	6.13	> 600
B-6	4.00	-0.49	-10.66	1.18	5.96	> 600
B-7	5.39	-0.83	-11.47	0.60	6.30	128.80
B-8	5.90	-0.69	-13.36	1.17	6.97	46.37
B-9	5.81	-0.81	-12.52	0.71	6.82	52.69

B-10	5.77	-0.98	-13.39	1.04	7.35	37.88
B-11	3.78	-0.89	7.83	1.31	-11.58	393.00
B-12	6.01	-0.95	-13.39	0.90	7.09	31.10
B-13	3.59	-0.11	-10.90	1.06	6.35	166.00
B-14	3.94	-0.47	-9.10	0.97	3.97	270.00
B-15	4.88	-0.81	-11.56	1.35	6.14	118.40
Ber	5.53	-0.90	-13.24	1.37	7.39	76.34

*Evaluation of **B-12** binding ability with RXR α*

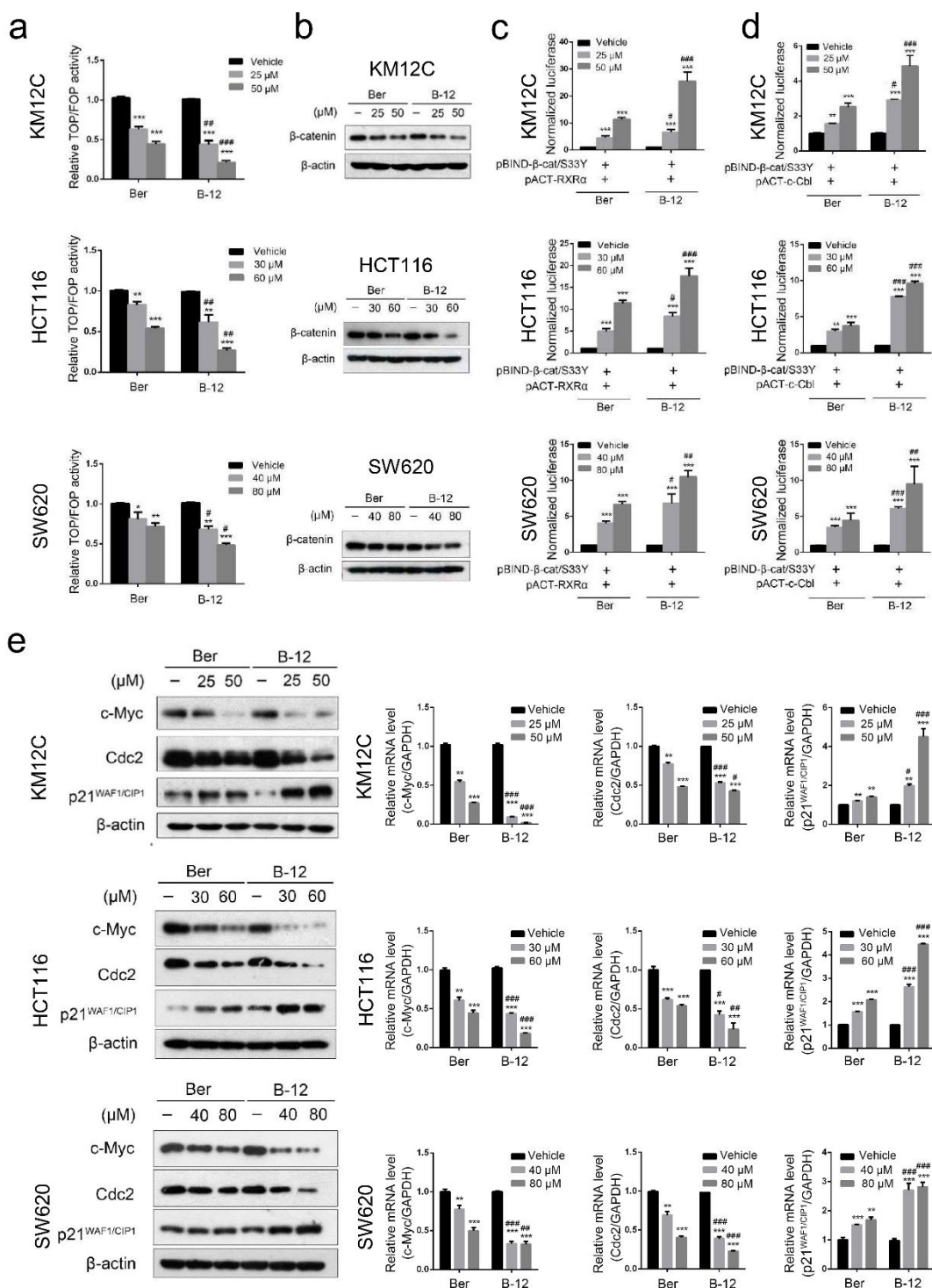
Molecular docking and bio-activity analysis above picked **B-12** out as the most distinguished activator of RXR α among these 15 newly-synthesized berberine analogues (Figure 2). Several biophysicochemical methods were then employed to examine the binding ability of **B-12** with RXR α . Firstly, we determined the physical interaction between **B-12** and RXR α /LBD by saturation transfer difference (STD)-NMR spectroscopy. As shown in Figure 3a, the ^1H NMR spectra with and without **B-12** were obtained, and most signals were observed in STD-NMR spectra showing that RXR α /LBD protein interacts with **B-12**. We then used fluorescence quenching analysis to evaluate the binding affinity of **B-12** to RXR α /LBD. His-RXR α /LBD displayed maximal fluorescence at 332 nm (Figure 3b, left panel). When the concentration of **B-12** or berberine was increased from 0 to 30 μM , the fluorescence intensity of RXR α /LBD gradually decreased, without a significant shift of the maximum fluorescence emission wavelength (Figure 3b, left panel). Plotting of the data yielded titration curves (Figure 3b, right panel), from which the apparent dissociation constants (K_d) value was calculated as 1.89×10^{-5} M and 2.48×10^{-5} M for **B-12** and berberine binding to RXR α /LBD proteins, respectively. Finally, CD spectroscopic assay was performed to test whether **B-12** could induce a change in the conformation of His-RXR α /LBD. The far-UV CD spectrum of His-RXR α /LBD exhibited two negative peaks at 208 nm and 222 nm

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3 and a positive peak at 192 nm, suggesting the presence of α -helical structure (Figure 3c). Upon **B-12**
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5 **12** binding, this conformation of His-RXR α /LBD was significantly changed, to a greater extent
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7 than that induced by berberine (Figure 3c). These results indicated that **B-12** directly interacted
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9 with RXR α /LBD and altered its conformation, and the binding affinity of **B-12** with RXR α /LBD
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11 was stronger than that of berberine.
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51 **Figure 3. B-12** directly bound to RXR α /LBD. **(a)** STD spectrum of **B-12** and RXR α /LBD. The
52 green spectra represented the ^1H NMR spectra of **B-12** alone. The red spectra represented the ^1H
53 NMR spectra of **B-12** and RXR α incubated together. **(b)** Fluorescence quenching spectra (left)
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and Stern-Volmer plot (right) for RXR α /LBD-berberine/**B-12** interaction. (c) CD spectroscopic analysis of the binding of berberine and **B-12** (5 μ M) to RXR α /LBD (1 μ M) with consequent conformational changes.



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5 **Figure 4. B-12** inhibited β -catenin signaling and cell proliferation in colon cancer cells. (**a, b**)
6 Effects of berberine and **B-12** on β -catenin signaling and β -catenin expression were respectively
7 detected by TOP/FOP luciferase system and western blot. KM12C, HCT116, and SW620 cells
8 were co-transfected with pRL-TK, TOPflash or FOPflash plasmids (for **a**). (**c, d**) Effect of
9 berberine or **B-12** on the binding ability of RXR α with β -catenin or and β -catenin with c-Cbl was
10 detected by the mammalian two-hybrid assay. KM12C, HCT116, and SW620 cells were co-
11 transfected with pBIND- β -catenin/S33Y and pACT-RXR α (for **c**) or pBIND- β -catenin/S33Y and
12 pACT-c-Cbl (for **d**) and then treated with berberine or **B-12** for 15 h. (**e, f**) Western blot analyses
13 and real-time PCR assays were performed to detect the protein/mRNA expression of Cdc2, c-Myc,
14 and p21^{WAF1/CIP1} in KM12C, HCT116, and SW620 cells. Significant differences compared with
15 the vehicle were indicated as ** $p < 0.01$ and *** $p < 0.001$. Significant differences of **B-12** vs **Ber** at
16 the same dose were indicated as # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$.
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37 ***B-12** inhibited β -catenin signaling and colon cancer cell growth via RXR α mediation*

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39 We reported recently that berberine suppressed colon cancer cell proliferation through binding
40 to RXR α and promoting its interaction with nuclear β -catenin, leading to c-Cbl-mediated
41 degradation of β -catenin.²⁶ Therefore, we examined the effect of **B-12** on Wnt/ β -catenin signaling
42 pathway in KM12C, HCT116, and SW620 colon cancer cell lines. **B-12** inhibited β -catenin-
43 dependent transcriptional activity and induced degradation of β -catenin protein more obviously
44 than berberine, as indicated by the TOP/FOP luciferase assay and western blot analysis (Figure 4a,
45 b). Mammalian two-hybrid assay further demonstrated that the interaction of RXR α with β -catenin
46 and β -catenin with c-Cbl was enhanced by **B-12** more effectively than berberine (Figure 4c, d). In
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3 addition, the expression levels of Cdc2 and c-Myc, two target genes of the β -catenin signaling
4 pathway involved in regulating the G2/M checkpoint,⁴³ and p21^{WAF1/CIP1}, a downstream effector
5 of c-Myc for G2/M checkpoint regulation,⁴⁴ were regulated by **B-12** more significantly than
6 berberine (Figure 4e, f). These results suggested that similar to berberine, **B-12** also promotes
7 RXR α - β -catenin interaction and inhibits β -catenin signaling and colon cancer cell growth, but its
8 regulatory effect is much stronger than that of berberine.
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11 We further investigated whether RXR α is essential for **B-12** to suppress the hyperactivated β -
12 catenin signaling and cell growth in KM12C cells. We stably knocked down the expression of
13 RXR α in KM12C cells with a specific shRNA against RXR α (Figure 5a). Silencing RXR α
14 expression greatly reduced the ability of **B-12** to inhibit cell growth, as detected by the MTT assay
15 (Figure 5b). Our results also showed that the expression of β -catenin, Cdc2, c-Myc, and
16 p21^{WAF1/CIP1} were regulated by **B-12** in an RXR α -dependent manner (Figure 5c and Figure S3).
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18 These data suggested that RXR α mediated the inhibitory effect of **B-12** on β -catenin signaling and
19 cell growth in colon cancer cells.
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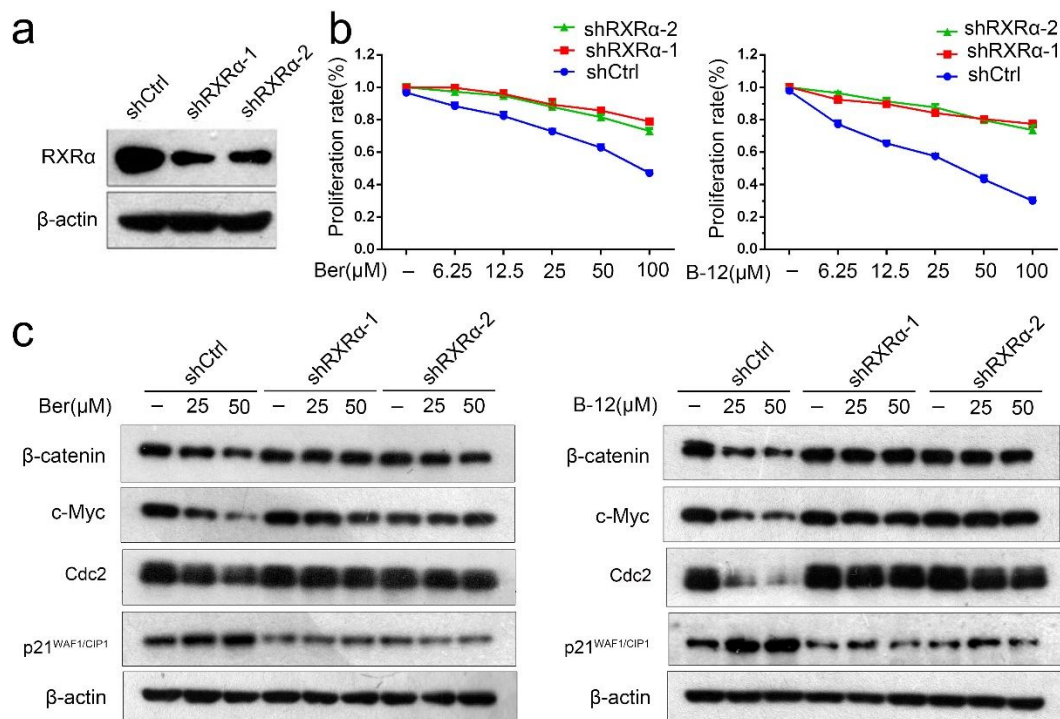


Figure 5. **B-12** suppressed β -catenin signaling and cell proliferation in an RXR α -dependent manner. **(a)** KM12C cells were transfected with control shRNA or shRNA targeting RXR α , then the expression levels of endogenous RXR α were examined by western blotting analyses. **(b, c)** KM12C cells expressing control shRNA or shRNA targeting RXR α were treated with indicated concentrations of berberine or **B-12** for 15 h, then the cell viability was measured by MTT assays **(b)** and the protein levels of Cdc2, c-Myc and p21^{WAF1/CIP1} were examined by western blot analysis **(c)**.

B-12 inhibited tumor growth in vivo

We assessed the therapeutic effects of **B-12** in a xenograft tumorigenesis model by subcutaneously injecting KM12C cells into nude mice. When the volume of xenografts had reached 80 to 200 mm³, the mice with similar tumor load were divided into three groups, and then respectively treated with vehicle, **B-12** (100 mg/kg) and berberine (100 mg/kg), each day through

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3 intragastric administration. Two weeks later, the tumors were dissected and weighed. Tumor
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5 growth was significantly reduced in **B-12**-treated and berberine-treated groups as compared to the
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7 vehicle group, and the inhibition rate was 69.1% ($P < 0.01$) and 33.6% ($P < 0.05$) for **B-12** and
8
9 berberine respectively (Figure 6a-c). **B-12** also regulated the protein and mRNA expression of
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11 PCNA, Ki67, Cdc2, c-Myc, p21^{WAF1/CIP1} and β -catenin in the xenograft tumors, more effectively
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13 than berberine, as detected by western blot, real-time PCR and immunohistochemistry (Figure 6d-f
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15 and Figure S4, 5). These results indicated that **B-12** inhibited colon cancer cell growth more
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17 efficiently than berberine *in vivo*.
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21 Unwanted side effects, such as hypertriglyceridemia, mucosal toxicity, hypercalcemia and
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23 hepatomegaly, are the major obstacles of the clinical applications of current RXR α ligands. So we
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25 also tested the possible toxicity or side effects of **B-12**. Berberine and **B-12** had no obvious
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27 hepatotoxicity and nephrotoxicity as indicated by the biochemical indexes of liver and renal
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29 function measured from the serum of mice after drug treatment (Table 3). The conclusion was
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31 further supported by the results from HE staining as no damaged hepatocellular morphology and
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33 augmented necrotic hepatocytes were observed (Figure 6g). The liver to body weight ratio also
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35 proved that berberine and **B-12** did not cause hepatomegaly (Figure S6). Moreover, unlike the
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37 classical RXR α agonists, **B-12** did not cause unwanted side effects such as hypertriglyceridemia,
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39 hypercalcemia and hypercholesterolemia (Table 3).
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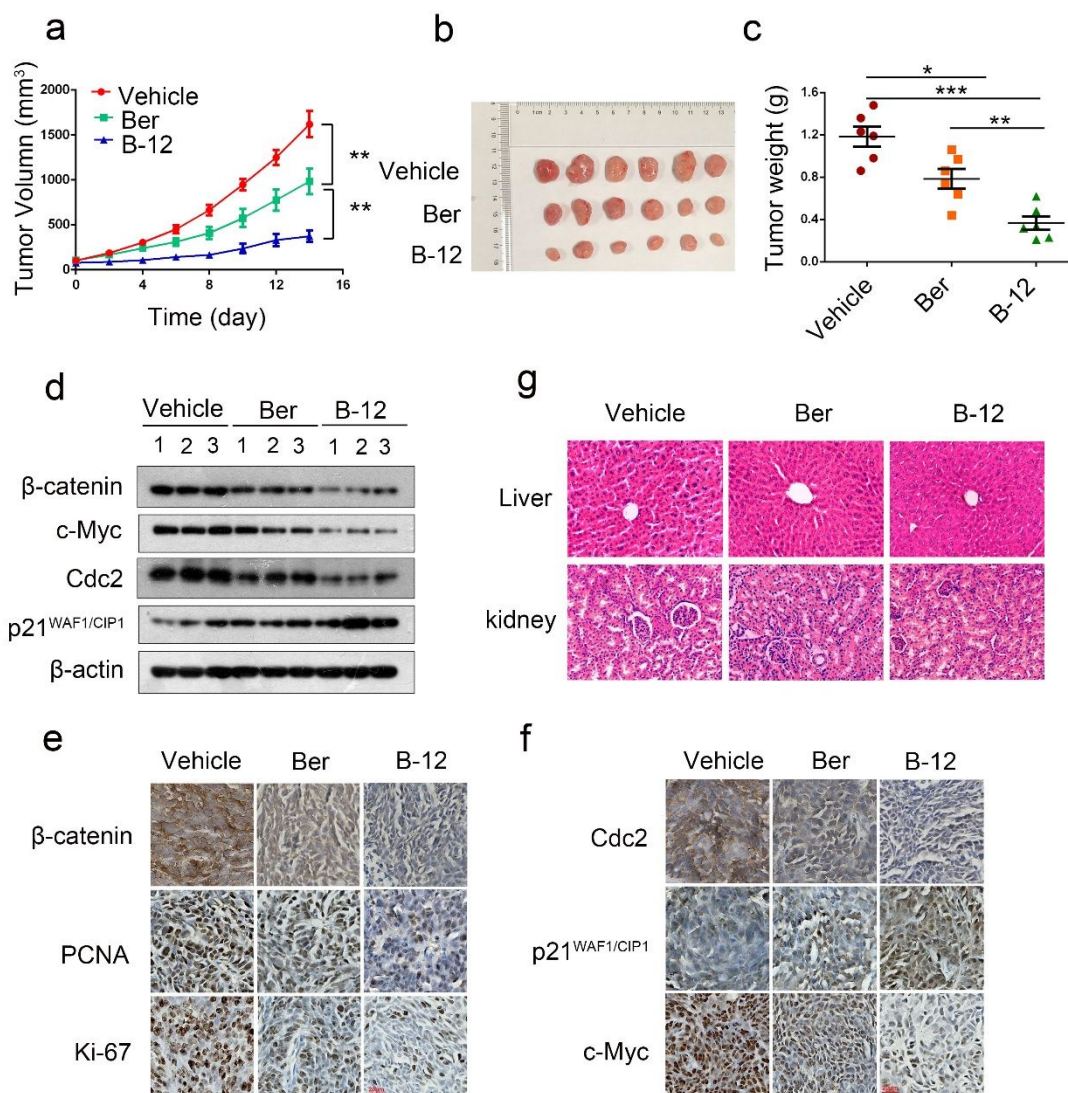


Figure 6. B-12 inhibited tumor growth in mice. Mice xenografted with KM12C cells were treated with or without berberine or B-12 as described in the method. (a) Tumor volume was measured every 2 days. (b-c) Two weeks later, mice were sacrificed and the xenograft tumors were dissected and weighed. Representative images of tumors were shown in b. (d) Expression levels of β-catenin, Cdc2, c-Myc, and p21^{WAF1/CIP1} in xenografts were analyzed by western blot. (e, f) Representative images of the expression of β-catenin, Ki-67, Cdc2, c-Myc, PCNA and p21^{WAF1/CIP1} in xenograft tumors analyzed by immunohistochemistry. (g) Liver and kidney histopathology by HE staining.

Table 3. Biochemical analyses of the serums from berberine or B-12 treated mice

Biochemical indexes	Vehicle (n=6)	Ber (n=6)	B-12 (n=6)	P values	
				Ber VS Vehicle	B12 VS Vehicle
TBIL ($\mu\text{mol/L}$)	0.28 \pm 0.024	0.26 \pm 0.032	0.21 \pm 0.029	0.4713	0.9617
IBIL ($\mu\text{mol/L}$)	0.55 \pm 0.096	0.40 \pm 0.057	0.52 \pm 0.083	0.8605	0.2423
ALT (U/L)	24.18 \pm 0.77	27.90 \pm 1.21	27.20 \pm 1.57	0.1635	0.2958
AST (U/L)	157.15 \pm 8.41	177.86 \pm 7.88	158.36 \pm 6.68	0.2239	0.9143
ALP (U/L)	76.53 \pm 4.31	71.28 \pm 3.16	78.35 \pm 5.99	0.8728	0.8739
CREA ($\mu\text{mol/L}$)	27.02 \pm 1.22	24.36 \pm 1.68	28.95 \pm 1.40	0.4813	0.6713
UREA (mmol/L)	8.21 \pm 0.26	8.08 \pm 0.62	8.39 \pm 0.41	0.8449	0.7600
Ca (mmol/L)	2.27 \pm 0.059	2.13 \pm 0.034	2.23 \pm 0.021	0.1413	0.9079
TG (mmol/L)	0.96 \pm 0.056	0.77 \pm 0.069	0.74 \pm 0.031	0.1241	0.0574
TC (mmol/L)	2.32 \pm 0.11	2.35 \pm 0.12	2.49 \pm 0.087	0.9908	0.9991

Data were presented as the mean \pm SEM. Statistical significance is analyzed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Detection of tumor selectivity and bioavailability of B-12

Berberine has a significant advantage of high tumor selectivity (killing tumor cells but not normal cells), which makes it a promising anti-tumor drug. Therefore, we tested whether **B-12** could still retain this advantage of berberine, by detecting the cell viability in response to a series of concentrations of **B-12** and berberine in human normal intestinal epithelial cell line HIEC-6 and

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2
3 mouse fibroblast epithelial cell line MEF. Treatment with **B-12** or berberine caused only a slight
4 decrease in viability of HIEC-6 and MEF cells, suggesting that **B-12** still preserved the high tumor
5 selectivity of berberine (Figure 7a-b). Poor solubility and low bioavailability are two major
6 weaknesses of berberine. Excitingly, the solubility of **B-12** was increased to 10.66 times compared
7 with berberine (Supplementary Table 1). The increasing solubility of **B-12** is probably due to the
8 decreasing intermolecular π , π -interaction caused by the more bulky C-3 methoxy group. We thus
9 test whether the bioavailability of **B-12** is also better than berberine. SD rats were treated with
10 berberine (100 mg/kg) or **B-12** (100 mg/kg) by gavage, then the blood was taken from the tail vein
11 at different periods, and the drug concentration in the blood was detected by LC-MS. The results
12 showed that both berberine and **B-12** reached the maximum drug concentration at approximately
13 2 hours, but the maximum drug concentration of **B-12** was 167 times higher than that of berberine
14 in the blood of rats (Figure 7c and Table 4). The results of the pharmacokinetic parameters of
15 berberine and **B-12** were shown in Table 4. Compared with berberine, the peak concentration
16 (C_{max}) was increased to 167.5 times; the area under the curve (AUC) was increased to 21.25
17 times; the bioavailability (F%) of **B-12** was raised to 26.10 times (Table 4), which might also
18 contribute to **B-12**'s stronger *in vivo* inhibitory effect on tumor growth. These results indicate that
19 **B-12** not only reserves the high tumor selectivity of berberine but also greatly improves its
20 bioavailability.
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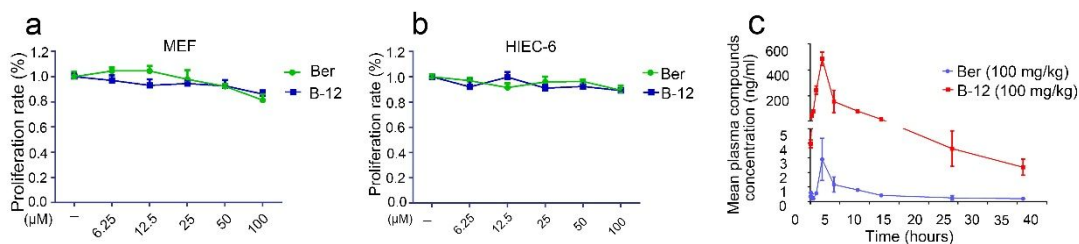


Figure 7. Detection of tumor selectivity and bioavailability of **B-12**. **(a, b)** MEF and HIEC-6 cells were treated with berberine or **B-12** for 15 h. The viability of cells was determined by MTT assay. **(c)** Berberine (100 mg/kg) or **B-12** (100 mg/kg) were given to SD rats by intragastric administration, and plasma samples were collected at 0.038, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 and 36 h for analyses of berberine and **B-12** by LC-MS.

Table 4. The pharmacokinetic parameters of berberine and B-12 (PO 100mg/kg)

Compound	Ber	B-12
$T_{1/2}$ (h)	28.98	11.59
T_{max} (h)	2.000	2.000
C_{max} (ng/mL)	2.893	486.0
AUC_{0-t} (ng·h/mL)	99.00	2104
MRT_{INF} (h)	31.51	15.67
F%	0.544	14.20

■ DISCUSSION AND CONCLUSIONS

Many studies have identified RXR α as an important target for cancer therapy, especially in colon cancer.^{5, 45-48} We recently reported that berberine inhibits colon cancer cell growth through direct binding to a unique site (non-classical ligand-binding pocket) in RXR α /LBD to activate its transactivating function and its regulation on β -catenin pathway.²⁶ To obtain more efficient berberine analogues as RXR α activators, which are very likely to retain the advantage of berberine, we designed and synthesized a series of novel berberine analogues with modifications on the C2,

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3 3, 9, 10, 11 and 12 positions of berberine core based on its binding mode with RXR α (Figure 1-
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5 2). By analyzing the structure-activity relationship of berberine analogues and RXR α , we
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7 identified **B-12** as an effective analogue with stronger anticancer activity, comparative tumor
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9 selectivity, and significantly improved bioavailability, notably with no adverse side effects caused
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11 by traditional RXR α agonists, and no obvious toxicity to mice, which made it a promising
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13 therapeutic agent for colon cancer treatment. (Figure 2-7).
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17 Through structure-bioactivity analysis, we have summarized the important relationships
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19 between the structural components of berberine analogues and their binding and activation of
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21 RXR α . First, the presence of particular groups on the C2, C3 positions (the head moiety) of
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23 berberine to form H-bonds with the Arg371 residue of RXR α may be required. Single C3 methoxy
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25 group or C2-C3 methylene-dioxy group can easily and closely interact with RXR α through
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27 formation of H-bonds with RXR α Arg371, and consequently exhibiting superior bio-activities in
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29 the activation of RXR α and the inhibition of colon cancer cell growth; moreover, single C3
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31 methoxy group is a little more favorable than the C2-C3 methylenedioxy group probably due to
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33 its less steric hindrance; however, the coexistence of C2 methoxy and C3 methoxy groups with
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35 larger steric hindrance seems to prevent the compound from approaching and forming H-bonds
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37 with RXR α Arg371, thereby failing to activate RXR α and suppress colon cancer. Second, the
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39 presence of single C9 or C10 methoxy group, C10-C11 methylenedioxy group or C9/C10
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41 bismethoxy groups on the tail region of berberine may be necessary to make maximal interaction
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43 with the hydrophobic groups in the Site X of RXR α /LBD through Van Der Waals contacts; while
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45 the presence of 10,11-dimethoxy groups or single C12 methoxy group led to poor RXR α binding
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47 potential and biological activity, which may be attributed to the unfavorable steric effect. These
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49 features may help guide the further improvement of current compounds.
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3 As we mentioned above, berberine binds RXR α in a unique mode. Unlike other RXR α ligands
4 such as 9-*cis*-RA, berberine lacks a classic carboxyl group which is crucial for 9-*cis*-RA to interact
5 with RXR α through forming a salt bridge with Arg316 in the classical ligand-binding mode of
6 RXR α ⁴⁹. These distinct structures and binding modes of berberine and 9-*cis*-RA lead to different
7 conformational changes of RXR α and consequently some diverse biological functions.
8 Furthermore, the concomitant binding of berberine and 9-*cis*-RA to RXR α /LBD is possibly the
9 main reason for their synergistic effect on RXR α -dependent transcriptional activity and colon
10 cancer cell growth, suggesting a great potential for the combination of berberine and 9-*cis*-RA to
11 treat colon cancer²⁶. Indeed, combination therapy with different drugs for cancer has long been a
12 successful way to increase drug efficacy, reduce toxicity and reverse drug resistance. For instance,
13 clinical trials have proven that the combination of all-*trans* retinoic acid and arsenic trioxide is a
14 feasible treatment in acute promyelocytic leukemia with a higher cure rate and less relapse⁵⁰. In
15 the present study, **B-12** combined with 9-*cis*-RA had a more significant synergistic effect than
16 berberine with 9-*cis*-RA (Figure S7), suggesting that **B-12** might be a better drug than berberine
17 for combined drug therapy. Besides, in mouse model we also found that, unlike other RXR α
18 ligands, **B-12** did not show side effects such as hypertriglyceridemia, hypercalcemia, and
19 hypercholesterolemia (Table 3), which are the main reasons for the limited use of the existing
20 RXR α agonists in clinical practice; moreover, B-12 did not induce obvious hepatorenal toxicity or
21 hepatomegaly as most anti-tumor drugs did (Table 3, Figure 6 and Figure S6). Further studies,
22 including *in vitro* and *in vivo* experiments, are still required to elucidate its potential.
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49 In conclusion, this study describes a novel SAR design of berberine analogues based on the
50 structural features of berberine analogues as RXR α activators, which might serve as a theoretical
51 basis for designing and evaluating the next generation of berberine-based drugs against colon
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3 cancer. Especially, we developed **B-12** as a better RXR α activator than berberine, with stronger
4 anticancer activity, higher bioavailability, and comparative tumor selectivity.
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10 ■ EXPERIMENTAL SECTION

11 *Chemistry*

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13 All reactions were carried out under an argon atmosphere with dry solvents under anhydrous
14 conditions unless otherwise stated. All the chemicals were purchased commercially and used
15 without further purification unless otherwise stated. The boiling point of petroleum ether (PE) is
16 between 60-90 °C. Tetrahydrofuran (THF) was distilled from sodium-benzophenone;
17 Dichloromethane (CH₂Cl₂) was distilled from calcium hydride. Toluene was distilled from sodium
18 under argon atmosphere. Reactions were monitored by thin-layer chromatography (TLC) carried
19 out on 0.25 mm Qingdao silica gel plates (F254) using UV lights as the visualizing agent and
20 KMnO₄. Flash column chromatography was performed over Qingdao silica gel (200-300 mesh).
21 Infrared spectra were recorded on a Nicolet AVATER FTIR330 spectrometer as thin film and are
22 reported in reciprocal centimeter (cm⁻¹). High-resolution mass spectra (HRMS) were recorded on
23 a Micromass QTOF2 Quadrupole/Time-of-Flight Tandem mass spectrometer using electron spray
24 ionization. NMR spectra were recorded on Bruker AV-400 and Bruker AV-500 instruments and
25 were calibrated using residual undeuterated solvents (CHCl₃, $\delta_{\text{H}} = 7.26$; DMSO-*d*₆, $\delta_{\text{H}} = 2.50$;
26 CD₃OD, $\delta_{\text{H}} = 3.31$) and deuterated solvents (CDCl₃, $\delta_{\text{C}} = 77.0$; CD₃OD, $\delta_{\text{C}} = 49.0$; DMSO-*d*₆, δ_{C}
27 = 39.5) as internal references. The data for ¹H NMR are reported as follows: chemical shift,
28 multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet or unresolved, brs = broad singlet),
29 coupling constants (Hz) and integration. The structure and purity of B1-B15 was determined by
30 qNMR with triphenylmethane as an internal standard. The purity of key compounds (**B-2**, **B-8**, **B-**
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3 **9, B-10, B-12**) were also analyzed by HPLC. The purity of all the compounds were determined to
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5 be > 95%.
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10 *General Synthetic Procedures for the Berberine Analogues B1-B15 (B-12 as an Example)*

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12 *2-(2-Bromo-6-methoxyphenyl)-1,3-dioxolane (5a)*. With a modified Donohoe's procedure: To a
13
14 stirred mixture of 2-bromo-6-methoxybenzaldehyde (2.15 g, 10.0 mmol) and ethylene glycol (2.78
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16 mL, 50 mmol) in toluene (200 mL) was added HC(OEt)₃ (5.0 mL, 30.0 mmol) and *p*-TSA (190
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18 mg, 1.0 mmol) in sequence. The resultant mixture was heated at 90 °C for 18 h and then cooled to
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20 room temperature and extracted with Et₂O. The combined layers were washed with saturated brine,
21
22 dried over MgSO₄ and concentrated *in vacuo*. Purification of the residue by flash chromatography
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24 (PE/EtOAc = 10:1) afforded **5a** as a colorless oil (2.56 g, 99%). ¹H NMR (500 MHz, CDCl₃) δ
25
26 7.17 – 7.14 (m, 1H), 7.10 (dd, *J* = 10.6, 5.6 Hz, 1H), 6.83 (dd, *J* = 8.2, 1.0 Hz, 1H), 6.44 (s, 1H),
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28 4.27 – 4.19 (m, 2H), 4.01 – 3.96 (m, 2H), 3.78 (s, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 159.7,
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30 130.8, 126.1, 123.7, 123.5, 110.7, 101.1, 65.7, 55.9. HRMS (ESI, *m/z*) calcd for C₁₀H₁₁BrO₃, [M]⁺:
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32 257.9892, found: 257.9898.
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38 *3-Methoxyphenethyl pivalate (2a)*. To a solution of 3-methoxyphenylacetic acid (1.66 g, 10.0
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40 mmol) in THF at 0 °C was added borane-THF complex solution (20.0 mL, 20.0 mmol). The
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42 mixture was allowed to warm to room temperature and stirred for 2 h before being quenched with
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44 1 M aqueous solution of NaOH. The resulting mixture was extracted with Et₂O. The combined
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46 organic layers were washed with saturated brine, dried over MgSO₄ and concentrated *in vacuo*.
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48 The residue was redissolved in CH₂Cl₂ (50 mL) and cooled to 0 °C. To the mixture was added
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50 pyridine (0.8 mL, 20.0 mmol) and pivaloyl chloride (2.78 mL, 20.0 mmol) sequentially. The
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52 resulting mixture was allowed to warm to room temperature and stirred for 18 h. The reaction was
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3 quenched with H₂O (5 mL) and extracted with CH₂Cl₂. The combined organic layers were washed
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5 with saturated brine, dried over MgSO₄ and concentrated *in vacuo*. Purification of the residue by
6
7 flash chromatography (PE/EtOAc = 20:1) afforded **2a** as a colorless oil (2.12 g, 90%). ¹H NMR
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9 (500 MHz, CDCl₃) δ 7.17 (t, *J* = 7.75 Hz, 1H), 6.78 (d, *J* = 7.6 Hz, 1H), 6.77 – 6.72 (m, 2H), 4.25
10
11 (t, *J* = 6.8 Hz, 2H), 3.75 (s, 3H), 2.88 (t, *J* = 6.8 Hz, 2H), 1.15 (s, 9H). ¹³C NMR (125 MHz, CDCl₃)
12
13 δ 178.1, 159.5, 139.3, 129.2, 121.1, 114.4, 111.7, 64.6, 54.8, 38.6, 35.0, 26.9. HRMS (ESI, *m/z*)
14
15 δ 178.1, 159.5, 139.3, 129.2, 121.1, 114.4, 111.7, 64.6, 54.8, 38.6, 35.0, 26.9. HRMS (ESI, *m/z*)
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17 calcd for C₁₄H₂₀O₃, [M+H]⁺: 237.1491, found: 237.1487.

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19 *2-Acetyl-5-methoxyphenethyl pivalate (3a)*. To a solution of **2a** (1.89 g, 8.0 mmol) in acetic
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21 anhydride (20 mL) at 0 °C was added ZnCl₂ (5.45 g, 40.0 mmol). The mixture was allowed to
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23 room temperature and stirred for 18 h. The solution was concentrated *in vacuo* and then the residue
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25 was redissolved in EtOAc. The mixture was washed with saturated aqueous solution of NaHCO₃,
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27 water and brine. The organic phase was dried over MgSO₄ and concentrated *in vacuo*. Purification
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29 of the residue by flash chromatography (PE/EtOAc = 10:1) afforded **3a** as a colorless oil (1.66g,
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31 75%). ¹H NMR (500 MHz, CDCl₃) δ 7.78 (d, *J* = 8.5 Hz, 1H), 6.82 – 6.76 (m, 2H), 4.30 (t, *J* = 6.6
32
33 Hz, 2H), 3.84 (s, 3H), 3.26 (t, *J* = 6.6 Hz, 2H), 2.56 (s, 3H), 1.14 (s, 9H). ¹³C NMR (125 MHz,
34
35 CDCl₃) δ 199.1, 178.3, 151.4, 146.8, 133.4, 129.3, 114.9, 113.5, 64.8, 55.8, 38.6, 33.6, 29.1, 27.1.
36
37 HRMS (ESI, *m/z*) calcd for C₁₆H₂₂O₄, [M+Na]⁺: 301.1416, found: 301.1410.

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42 *2-(2-(2-(1,3-Dioxolan-2-yl)-3-methoxyphenyl)acetyl)-5-methoxyphenethyl pivalate (6a)*. To a
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44 stirred solution of **5a** (596 mg, 2.3 mmol) and **3a** (1.28 g, 4.6 mmol) in THF (10 mL) in a 30 mL
45
46 vial was added Cs₂CO₃ (1.87 g, 5.75 mmol) and (Amphos)₂PdCl₂ (81 mg, 0.115 mmol). The
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48 resulting solution was bubbled with argon for a minimum of 10 min and then quickly equipped
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50 with a Teflon septum. The mixture was heated at 90 °C for 18 h. The mixture was then cooled to
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52 room temperature and quenched with water and extracted with Et₂O. The combined organic layers
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3 were washed with saturated brine, dried over MgSO₄ and concentrated *in vacuo*. Purification of
4 the residue by flash chromatography (PE/EtOAc = 4:1) afforded **6a** as a colorless oil (756 mg,
5 72%). ¹H NMR (500 MHz, CDCl₃) δ 7.90 – 7.85 (m, 1H), 7.34 – 7.28 (m, 1H), 6.87 – 6.77 (m,
6 4H), 6.24 (s, 1H), 4.40 (s, 2H), 4.33 (t, *J* = 6.6 Hz, 2H), 3.84 (s, 3H), 3.81 (s, 3H), 3.80 – 3.75 (m,
7 2H), 3.74 – 3.67 (m, 2H), 3.24 (t, *J* = 6.6 Hz, 2H), 1.15 (s, 9H). ¹³C NMR (125 MHz, CDCl₃) δ
8 198.7, 178.5, 161.5, 159.0, 142.2, 137.1, 131.7, 130.3, 129.9, 125.4, 121.2, 117.9, 111.5, 109.9,
9 98.7, 64.9, 64.5, 55.8, 55.3, 44.9, 38.7, 34.1, 27.3. HRMS (ESI, *m/z*) calcd for C₂₆H₃₂O₇, [M+H]⁺:
10 457.2226, found: 457.2221.
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22 *3,9-Dimethoxy-5,6-dihydroisoquinolino[3,2-*a*]isoquinolin-7-ium chloride (B-12)*. To **6a** (723
23 mg, 1.58 mmol) in a 15 mL vial was added 1 M solution of NH₄Cl in 3:1 of EtOH/H₂O (4 mL).
24 The solution was bubbled with argon for a minimum of 10 min and quickly equipped with a Teflon
25 septum. The stirred mixture was heated at 90 °C for 18 h. Then the solution was heated at 110 °C
26 for another 48 h. The mixture was cooled to room temperature, and concentrated with silica gel *in*
27 *vacuo*. Purification of the residue by flash chromatography (CH₂Cl₂/MeOH = 10:1) afforded a
28 crude product. The crude product was recrystallized in methanol/Et₂O to give **B-12** as a pale yellow
29 solid (368 mg, 71%). The purity of the title compound was determined to be > 95% by qNMR. ¹H
30 NMR (500 MHz, DMSO-*d*₆) δ 9.92 (s, 1H), 8.96 (s, 1H), 8.21 (d, *J* = 8.8 Hz, 1H), 8.11 (t, *J* = 8.1
31 Hz, 1H), 7.78 (d, *J* = 8.3 Hz, 1H), 7.38 (d, *J* = 7.9 Hz, 1H), 7.12 (dd, *J* = 8.7, 2.6 Hz, 1H), 7.08 (d,
32 *J* = 2.5 Hz, 1H), 4.94 (t, *J* = 6.3 Hz, 2H), 4.13 (s, 3H), 3.88 (s, 3H), 3.31 – 3.26 (m, 2H). ¹³C NMR
33 (125 MHz, DMSO-*d*₆) δ 171.5, 166.7, 155.0, 149.8, 148.8, 147.9, 147.1, 137.7, 128.9, 128.8,
34 128.2, 127.2, 123.9, 122.7, 118.2, 66.2, 65.2, 64.3, 36.1. HRMS (ESI, *m/z*) calcd for C₁₉H₁₈NO₂,
35 [M]⁺: 292.1332, found: 292.1329.
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3 *5,6-Dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium chloride (B-1)*. This
4
5 compound was prepared from 2-bromobenzaldehyde and 2-(benzo[d][1,3]dioxol-5-yl)acetic acid
6
7 using the above method. The purity of the title compound was determined to be > 95% by qNMR.
8
9 ¹H NMR (500 MHz, CDCl₃) δ 8.44 – 8.39 (m, 1H), 7.62 (ddd, *J* = 8.3, 7.1, 1.3 Hz, 1H), 7.55 –
10
11 7.51 (m, 1H), 7.43 (ddd, *J* = 8.2, 7.1, 1.2 Hz, 1H), 7.26 (d, *J* = 1.6 Hz, 2H), 6.84 (s, 1H), 6.72 (s,
12
13 1H), 6.01 (s, 2H), 4.36 – 4.32 (m, 2H), 2.93 – 2.89 (m, 2H). ¹³C NMR (125 MHz, CDCl₃) δ 162.1,
14
15 148.7, 147.4, 137.4, 136.7, 132.2, 130.3, 127.9, 126.2, 126.0, 124.6, 123.7, 107.9, 105.0, 101.8,
16
17 101.5, 39.7, 28.5. ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.09 (d, *J* = 14.6 Hz, 1H), 9.04 (s, 1H), 8.43
18
19 (d, *J* = 8.3 Hz, 1H), 8.24 (d, *J* = 8.3 Hz, 1H), 8.19 (t, *J* = 7.6 Hz, 1H), 7.97 (t, *J* = 7.5 Hz, 1H), 7.87
20
21 (s, 1H), 7.11 (s, 1H), 6.18 (s, 2H), 4.88 (t, *J* = 6.0 Hz, 2H), 3.24 (t, *J* = 6.3 Hz, 2H). ¹³C NMR (125
22
23 MHz, DMSO-*d*₆) δ 150.4, 148.3, 147.8, 141.7, 138.4, 137.5, 136.7, 134.4, 131.1, 130.5, 129.9,
24
25 129.2, 127.2, 125.5, 120.5, 120.4, 114.3, 108.5, 106.4, 105.8, 105.1, 102.2, 55.2, 26.3. HRMS
26
27 (ESI, *m/z*) calcd for C₁₈H₁₄NO₂, [M]⁺: 276.1019, found: 276.1016. Spectroscopic data were
28
29 consistent with those previously reported.⁴²
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35 *5,6-Dihydro-[1,3]dioxolo[4',5':6,7]isoquinolino[3,2-a][1,3]dioxolo[4,5-g]isoquinolin-7-ium*
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37 *chloride (B-2, pseudocoptisine)*. This compound was prepared from 6-
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39 bromobenzo[d][1,3]dioxole-5-carbaldehyde and 2-(benzo[d][1,3]dioxol-5-yl)acetic acid using the
40
41 above method. The purity of the title compound was determined to be > 95% by qNMR. ¹H NMR
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43 (400 MHz, DMSO-*d*₆) δ 9.57 (s, 1H), 8.77 (s, 1H), 7.74 (s, 1H), 7.71 (s, 1H), 7.54 (s, 1H), 7.09 (s,
44
45 1H), 6.42 (s, 2H), 6.17 (s, 2H), 4.76 (t, *J* = 6.4 Hz, 2H), 3.18 (t, *J* = 6.4 Hz, 2H). ¹³C NMR (100
46
47 MHz, DMSO-*d*₆) δ 156.4, 151.3, 150.4, 148.1, 146.3, 139.2, 139.1, 131.3, 124.0, 120.7, 119.4,
48
49 108.9, 105.9, 104.3, 104.2, 103.1, 102.5, 54.9, 26.8. HRMS (ESI, *m/z*) calcd for C₁₉H₁₄NO₄, [M]⁺:
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51 320.0917, found: 320.0915. Spectroscopic data were consistent with those previously reported.³⁷
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3 *3,10,11-Trimethoxy-5,6-dihydroisoquinolino[3,2-a]isoquinolin-7-ium chloride (B-3)*. This
4 compound was prepared from 2-bromo-4,5-dimethoxybenzaldehyde and 2-(3-
5 methoxyphenyl)acetic acid using the above method. The purity of the title compound was
6 determined to be > 95% by qNMR. ¹H NMR (500 MHz, CD₃OD) δ 9.35 (s, 1H), 8.59 (s, 1H), 8.09
7 (d, *J* = 8.8 Hz, 1H), 7.62 (s, 1H), 7.60 (s, 1H), 7.09 (dd, *J* = 8.8, 2.5 Hz, 1H), 7.03 (d, *J* = 2.5 Hz,
8 1H), 4.82 (t, *J* = 6.6 Hz, 2H), 4.12 (s, 3H), 4.06 (s, 3H), 3.91 (s, 3H), 3.32 (t, *J* = 6.6 Hz, 2H). ¹³C
9 NMR (125 MHz, CD₃OD) δ 162.6, 158.5, 153.1, 144.6, 139.2, 137.6, 136.9, 127.4, 122.8, 119.4,
10 117.7, 114.4, 113.0, 105.9, 104.9, 56.1, 55.7, 54.8, 54.7, 27.2. HRMS (ESI, *m/z*) calcd for
11 C₂₀H₂₀NO₃, [M]⁺: 322.1438, found: 322.1435.

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24 *10,11-Dimethoxy-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium*
25 *chloride (B-4, pseudoberberine)*. This compound was prepared from 2-bromo-4,5-
26 dimethoxybenzaldehyde and 2-(benzo[d][1,3]dioxol-5-yl)acetic acid using the above method. The
27 purity of the title compound was determined to be > 95% by qNMR. ¹H NMR (500 MHz, DMSO-
28 *d*₆) δ 9.57 (s, 1H), 8.77 (s, 1H), 7.73 (s, 1H), 7.72 (s, 1H), 7.59 (s, 1H), 7.10 (s, 1H), 6.17 (s, 2H),
29 4.78 (t, *J* = 6.3 Hz, 2H), 4.07 (s, 3H), 4.00 (s, 3H), 3.20 (t, *J* = 6.3 Hz, 2H). ¹³C NMR (125 MHz,
30 DMSO-*d*₆) δ 157.5, 152.3, 149.8, 147.7, 145.5, 138.2, 136.6, 130.7, 122.1, 118.2, 108.5, 106.6,
31 105.3, 102.1, 56.7, 56.4, 54.5, 26.4. HRMS (ESI, *m/z*) calcd for C₂₀H₁₈NO₄⁺, [M]⁺: 336.1236,
32 found: 336.1234. Spectroscopic data were consistent with those previously reported.⁵¹

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45 *2,3,10,11-Tetramethoxy-5,6-dihydroisoquinolino[3,2-a]isoquinolin-7-ium chloride (B-5,*
46 *pseudopalmatine)*. This compound was prepared from 2-bromo-4,5-dimethoxybenzaldehyde and
47 2-(3,4-dimethoxyphenyl)acetic acid using the above method. The purity of the title compound was
48 determined to be > 95% by qNMR. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.58 (s, 1H), 8.88 (s, 1H),
49 7.72 (s, 1H), 7.67 (s, 1H), 7.62 (s, 1H), 7.10 (s, 1H), 4.80 (t, *J* = 6.3 Hz, 2H), 4.08 (s, 3H), 4.00 (s,
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3 3H), 3.94 (s, 3H), 3.87 (s, 3H), 3.22 (t, $J = 6.3$ Hz, 2H). ^{13}C NMR (125 MHz, DMSO- d_6) δ 157.4,
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5 152.2, 151.5, 148.7, 145.6, 138.4, 136.6, 128.6, 122.0, 118.9, 118.0, 111.3, 108.5, 106.5, 105.3,
6
7 56.6, 56.3, 56.0, 55.9, 54.6, 26.1. HRMS (ESI, m/z) calcd for $\text{C}_{21}\text{H}_{22}\text{NO}_4$, $[\text{M}]^+$: 352.1543, found:
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9 352.1540. Spectroscopic data were consistent with those previously reported.⁵¹

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12 *2,3-Dimethoxy-5,6-dihydroisoquinolino[3,2-*a*]isoquinolin-7-ium chloride (B-6)*. This
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14 compound was prepared from 2-bromobenzaldehyde and 2-(3,4-dimethoxyphenyl)acetic acid
15
16 using the above method. The purity of the title compound was determined to be > 95% by qNMR.
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18 ^1H NMR (500 MHz, CD_3OD) δ 9.81 (s, 1H), 8.93 (s, 1H), 8.38 (d, $J = 8.3$ Hz, 1H), 8.30 (d, $J =$
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20 8.4 Hz, 1H), 8.17 – 8.13 (m, 1H), 7.96 – 7.92 (m, 1H), 7.72 (s, 1H), 7.07 (s, 1H), 4.95 (t, $J = 6.4$
21
22 Hz, 2H), 4.01 (s, 3H), 3.95 (s, 3H), 3.34 (t, $J = 6.4$ Hz, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 154.2,
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24 151.1, 151.0, 142.0, 140.7, 138.0, 131.7, 131.0, 130.5, 128.6, 127.4, 121.7, 120.3, 112.3, 110.3,
25
26 57.3, 57.1, 56.7, 27.7. HRMS (ESI, m/z) calcd for $\text{C}_{19}\text{H}_{18}\text{NO}_2$, $[\text{M}]^+$: 292.1332, found: 292.1330.
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31 *3-Methoxy-5,6-dihydroisoquinolino[3,2-*a*]isoquinolin-7-ium chloride (B-7)*. This compound
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33 was prepared from 2-bromobenzaldehyde and 2-(3-methoxyphenyl)acetic acid using the above
34
35 method. The purity of the title compound was determined to be > 95% by qNMR. ^1H NMR (500
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37 MHz, CD_3OD) δ 9.83 (s, 1H), 8.81 (s, 1H), 8.38 (d, $J = 8.3$ Hz, 1H), 8.26 (d, $J = 8.4$ Hz, 1H), 8.16
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39 – 8.09 (m, 2H), 7.94 – 7.87 (m, 1H), 7.07 (dd, $J = 8.8, 2.6$ Hz, 1H), 7.02 (d, $J = 2.5$ Hz, 1H), 4.95
40
41 (t, $J = 6.3$ Hz, 2H), 3.89 (s, 3H), 3.36 (t, $J = 6.3$ Hz, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 164.2,
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43 151.0, 142.0, 140.6, 138.5, 138.0, 131.6, 131.0, 129.4, 128.5, 127.3, 121.4, 120.6, 116.0, 114.3,
44
45 57.0, 56.3, 28.4. HRMS (ESI, m/z) calcd for $\text{C}_{18}\text{H}_{16}\text{NO}$, $[\text{M}]^+$: 262.1226, found: 262.1224.
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50 *3-Methoxy-5,6-dihydro-[1,3]dioxolo[4,5-*g*]isoquinolino[2,1-*b*]isoquinolin-7-ium chloride (B-*
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52 *8)*. This compound was prepared from 6-bromobenzo[*d*][1,3]dioxole-5-carbaldehyde and 2-(3-
53
54 methoxyphenyl)acetic acid using the above method. The purity of the title compound was
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determined to be > 95% by qNMR. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.57 (s, 1H), 8.79 (s, 1H), 8.11 (d, *J* = 8.7 Hz, 1H), 7.71 (s, 1H), 7.60 (s, 1H), 7.12 (dd, *J* = 8.7, 2.3 Hz, 1H), 7.08 (d, *J* = 2.2 Hz, 1H), 6.41 (s, 1H), 4.79 (t, *J* = 6.0 Hz, 2H), 3.88 (s, 3H), 3.27 (t, *J* = 6.0 Hz, 2H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 164.1, 158.3, 153.0, 146.5, 141.2, 138.4, 129.0, 125.6, 120.6, 119.9, 119.8, 115.8, 114.4, 105.4, 104.7, 103.9, 64.3, 56.2, 28.4. HRMS (ESI, *m/z*) calcd for C₁₉H₁₆NO₃, [M]⁺: 306.1125, found: 306.1121.

10-Methoxy-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium chloride (B-9). This compound was prepared from 2-bromo-5-methoxybenzaldehyde and 2-(benzo[d][1,3]dioxol-5-yl)acetic acid using the above method. The purity of the title compound was determined to be > 95% by qNMR. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.84 (s, 1H), 8.99 (s, 1H), 8.17 (d, *J* = 9.1 Hz, 1H), 7.85 (dd, *J* = 9.1, 2.4 Hz, 1H), 7.81 (s, 1H), 7.77 (d, *J* = 2.4 Hz, 1H), 7.10 (s, 1H), 6.18 (s, 2H), 4.87 (t, *J* = 6.3 Hz, 2H), 4.00 (s, 3H), 3.22 (t, *J* = 6.3 Hz, 2H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 160.0, 149.9, 147.8, 147.7, 138.0, 134.4, 130.6, 129.8, 129.1, 127.3, 120.4, 108.4, 106.5, 105.5, 102.1, 56.2, 55.6, 55.2, 26.3. HRMS (ESI, *m/z*) C₁₉H₁₆NO₃, [M]⁺: 306.1125, found: 306.1122. Spectroscopic data were consistent with those previously reported.⁴²

3,9,10-Trimethoxy-5,6-dihydroisoquinolino[3,2-a]isoquinolin-7-ium chloride (B-10). This compound was prepared from 6-bromo-2,3-dimethoxybenzaldehyde and 2-(3-methoxyphenyl)acetic acid using the above method. The purity of the title compound was determined to be > 95% by qNMR. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.96 (s, 1H), 8.32 (s, 1H), 8.18 (d, *J* = 9.2 Hz, 1H), 8.14 (d, *J* = 8.7 Hz, 1H), 8.04 (d, *J* = 9.1 Hz, 1H), 7.10 (dd, *J* = 8.7, 2.6 Hz, 1H), 7.07 (d, *J* = 2.3 Hz, 1H), 4.96 (t, *J* = 6.2 Hz, 2H), 4.09 (s, 3H), 4.06 (s, 3H), 3.87 (s, 3H), 3.28 (t, *J* = 6.2 Hz, 2H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 162.0, 150.7, 145.8, 138.3, 137.6,

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3 133.6, 128.2, 127.2, 124.0, 121.9, 120.2, 120.0, 114.9, 113.7, 79.7, 62.4, 60.2, 57.5, 56.1, 27.2.

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5 HRMS (ESI, m/z) calcd for C₂₀H₂₀NO₃, [M]⁺: 322.1438, found: 322.1439.

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8 *2,3,9,10-Tetramethoxy-5,6-dihydroisoquinolino[3,2-a]isoquinolin-7-ium chloride (B-11,*
9
10 *palmatine)*. This compound was prepared from 6-bromo-2,3-dimethoxybenzaldehyde and 2-(3,4-
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12 dimethoxyphenyl)acetic acid using the above method. The purity of the title compound was
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14 determined to be > 95% by qNMR. ¹H NMR (500 MHz, CD₃OD) δ 9.73 (s, 1H), 8.76 (s, 1H), 8.06
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16 (d, *J* = 8.8 Hz, 1H), 7.99 (d, *J* = 8.8 Hz, 1H), 7.59 (s, 1H), 7.01 (s, 1H), 4.93 (s, 2H), 4.19 (s, 3H),
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18 4.07 (s, 3H), 3.97 (s, 3H), 3.92 (s, 3H), 3.27 (s, 2H). ¹³C NMR (125 MHz, CD₃OD) δ 153.7, 151.8,
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20 150.8, 146.3, 145.6, 139.7, 135.2, 130.0, 127.9, 124.5, 123.2, 121.2, 120.4, 112.2, 109.9, 62.6,
21
22 57.6, 57.3, 57.1, 56.7, 27.8. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.90 (s, 1H), 9.08 (s, 1H), 8.21 (d,
23
24 *J* = 9.1 Hz, 1H), 8.04 (d, *J* = 9.1 Hz, 1H), 7.73 (s, 1H), 7.09 (s, 1H), 4.96 (t, *J* = 6.3 Hz, 2H), 4.10
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26 (s, 3H), 4.07 (s, 3H), 3.94 (s, 3H), 3.87 (s, 3H), 3.23 (t, *J* = 6.3 Hz, 2H). ¹³C NMR (125 MHz,
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28 DMSO-*d*₆) δ 151.5, 150.2, 148.7, 145.4, 143.6, 137.7, 133.1, 128.6, 126.8, 123.4, 121.3, 119.9,
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30 118.9, 111.3, 108.8, 61.9, 57.0, 56.2, 55.9, 55.3, 26.0. HRMS (ESI, m/z) calcd for C₂₁H₂₂NO₄,
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32 [M]⁺: 352.1543, found: 352.1539. Spectroscopic data were consistent with those previously
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34 reported.³⁷

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40 *2,3,9-Trimethoxy-5,6-dihydroisoquinolino[3,2-a]isoquinolin-7-ium chloride (B-13)*. This
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42 compound was prepared from 2-bromo-6-methoxybenzaldehyde and 2-(3,4-
43
44 dimethoxyphenyl)acetic acid using the above method. The purity of the title compound was
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46 determined to be > 95% by qNMR. ¹H NMR (500 MHz, CD₃OD) δ 9.80 (s, 1H), 8.83 (s, 1H),
47
48 7.92 (d, *J* = 8.3 Hz, 1H), 7.86 (t, *J* = 8.0 Hz, 1H), 7.63 (s, 1H), 7.56 (d, *J* = 7.7 Hz, 1H), 7.07 (s,
49
50 1H), 4.94 (t, *J* = 6.3 Hz, 2H), 4.17 (s, 3H), 4.00 (s, 3H), 3.94 (s, 3H), 3.35 (t, *J* = 6.3 Hz, 2H). ¹³C
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52 NMR (125 MHz, CD₃OD) δ 154.5, 152.8, 149.5, 149.2, 140.1, 131.2, 129.2, 126.7, 120.7, 119.0,
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3 114.8, 113.7, 111.0, 108.9, 55.9, 55.80, 55.4, 29.3, 26.3. HRMS (ESI, m/z) calcd for C₂₀H₂₀NO₃,
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5 [M]⁺: 322.1438, found: 322.1440.
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7 *2,3,12-Trimethoxy-5,6-dihydroisoquinolino[3,2-a]isoquinolin-7-ium chloride (B-14)*. This
8 compound was prepared from 2-bromo-3-methoxybenzaldehyde and 2-(3,4-
9 dimethoxyphenyl)acetic acid using the above method. The purity of the title compound was
10 determined to be > 95% by qNMR. ¹H NMR (500 MHz, CD₃OD) δ 9.82 (s, 1H), 8.81 (s, 1H), 8.07
11 (t, *J* = 8.1 Hz, 1H), 7.79 (d, *J* = 8.3 Hz, 1H), 7.67 (s, 1H), 7.32 (d, *J* = 8.0 Hz, 1H), 7.05 (s, 1H),
12 4.96 – 4.91 (m, 2H), 4.18 (s, 3H), 4.00 (s, 3H), 3.94 (s, 3H), 3.32 – 3.28 (m, 2H). ¹³C NMR (125
13 MHz, CD₃OD) δ 157.8, 152.7, 149.4, 145.2, 140.7, 140.0, 138.5, 129.2, 119.5, 118.8, 118.6, 118.4,
14 110.9, 108.9, 108.1, 62.8, 55.9, 55.7, 55.3. HRMS (ESI, m/z) calcd for C₂₀H₂₀NO₃, [M]⁺:
15 322.1438, found: 322.1439.
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28 *12-Methoxy-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium chloride (B-*
29 *15)*. This compound was prepared from 2-bromo-3-methoxybenzaldehyde and 2-
30 (benzo[d][1,3]dioxol-5-yl)acetic acid using the above method. The purity of the title compound
31 was determined to be > 95% by qNMR. ¹H NMR (500 MHz, CD₃OD) δ 9.80 (s, 1H), 8.71 (s, 1H),
32 7.90 (d, *J* = 8.2 Hz, 1H), 7.84 (t, *J* = 8.0 Hz, 1H), 7.58 (s, 1H), 7.54 (d, *J* = 7.7 Hz, 1H), 6.93 (s,
33 1H), 6.08 (s, 2H), 4.97 – 4.90 (m, 3H), 4.15 (s, 3H), 3.29 – 3.24 (m, 2H). ¹³C NMR (125 MHz,
34 CD₃OD) δ 154.5, 151.0, 149.2, 148.5, 139.9, 131.4, 131.2, 130.9, 126.8, 120.7, 120.3, 114.9,
35 113.7, 108.1, 105.3, 102.4, 55.8, 55.7, 26.7. HRMS (ESI, m/z) calcd for C₁₉H₁₆NO₃, [M]⁺:
36 306.1125, found: 306.1121.
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51 ■ BIOLOGICAL ASSAY PROTOCOLS

52 *Chemicals and reagents*

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3 Berberine (cat. #141433-60-5) and 9-*cis*-RA (cat. #5300-03-8) were purchased from Sigma-
4 Aldrich, St. Louis, MO, USA. Rabbit anti-p21^{WAF1/CIP1} (cat. #2947), anti-Ki67 (cat. #9449) and
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6 mouse anti- β -catenin (cat. #6582), anti-Cdc2 (cat. #9116) antibodies were purchased from Cell
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8 Signaling Technology, Boston, MA, USA. Rabbit anti-c-Myc (cat. #40764) antibodies were
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10 purchased from Signalway Antibody, Baltimore, USA. Rabbit anti-PCNA (cat. #ab2426) antibody
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12 was purchased from Abcam, Boston, MA, USA. Rabbit anti-RXR α was purchased from Santa
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14 Cruz Biotechnology, Santa Cruz, CA, USA.
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21 *Molecular docking study*

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23 To further understand the relationship between berberine (and its analogues) and protein
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25 RXR α /LBD, molecular docking simulations were performed. The 3D structure of RXR α /LBD
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27 protein was obtained from the Protein Data Bank using PDB ID: 3FUG. Then the 3D structure
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29 was prepared by removing the water molecules from the structure and defining binding sites using
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31 APOPDB2RECEPTOR utility implemented in Openeye software suits. Small molecule structures
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33 were prepared by SYBYL2.0 and conformational enumeration was done by OMEGA utility
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35 implemented in Openeye software suits, with all parameters set default. After that FRED docking
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37 tool was used for molecular docking simulations and its Chemgauss4 score was used for ranking
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39 the pose of each docked compound. Conformation with the highest score was used for further
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41 evaluation. Interaction between small molecule and protein was shown by VIDA.
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49 *Cell culture*

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51 Human colon cell lines HIEC-6 were obtained from ATCC (Virginia, USA). The colon cancer
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53 cell lines HCT116 and SW620 were obtained from Cell Bank of Type Culture Collection of
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3 Chinese Academy of Sciences (Shanghai, China). Human KM12C colon cancer cell line,
4 established from a Duke's B2 primary colon cancer, was kindly provided by Professor I. J. Fidler
5 (M.D. Anderson Cancer Center, Houston, TX, USA). Mouse embryonic fibroblast MEF cell lines
6 were kindly provided by Professor Bo-an Li (Xiamen University, China). Mycoplasma testing was
7 regularly conducted to assure that all these cell lines were mycoplasma-free at all times. KM12C
8 were maintained in MEM culture medium (Gibco, Grand Island, NY, USA) supplemented with 10
9 % fetal bovine serum (FBS, Invitrogen; Thermo Fisher Scientific), 100 IU/mL penicillin (Life
10 Technologies, Carlsbad, CA, USA), 100 µg/mL streptomycin (Life Technologies), 1% non-
11 essential amino acids (Gibco) and 1% vitamin solution (Gibco). HIEC-6 were maintained in MEM
12 culture medium (Gibco, Grand Island, NY, USA) supplemented with 4 % fetal bovine serum (FBS,
13 Invitrogen; Thermo Fisher Scientific), 100 IU/mL penicillin (Life Technologies, Carlsbad, CA,
14 USA), 100 µg/mL streptomycin (Life Technologies), 20 mM HEPES (Gibco), 10 mM GlutaMAX
15 (Gibco Catalog No. 35050) and 10 ng/mL Epidermal Growth Factor (EGF) (Gibco). MEF cells
16 were cultured in DMEM high-glucose medium (Gibco) prepared with 10% of fetal bovine serum,
17 100 IU/mL penicillin and 100 µg/mL streptomycin. All cell lines were maintained at 37 °C in an
18 atmosphere of 95% air and 5% CO₂.

41 42 *Transfection and dual-luciferase reporter assays*

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44 The cells were inoculated into 24 well culture plates. After 24 h, different luciferase reporter
45 genes and other different expression vectors as required were transfected with liposome
46 transfection reagent Lipfectamine 2000 (Invitrogen, Gaithersburg, MD, USA) according to the
47 manufacturer's recommendations. Luciferase assays were carried out according to the instructions
48 of the Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA). Briefly, after 20 h of
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3 transfection, berberine or berberine analogues were added for 15 h and then the medium was
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5 carefully removed. 100 μ L $1 \times$ Passive Lysis Buffer was added into each hole at 4 $^{\circ}$ C for 1 h, the
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7 Firefly and renilla luciferase activity was detected by GloMax 20/20 Luminometer (Promega).
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10 11 12 *Cell proliferation assay*

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14 Cell proliferation rates were determined by MTT assay or EdU assay. In MTT assay, briefly, the
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16 cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After incubated at 37 $^{\circ}$ C
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18 overnight, the cells were treated with berberine or **B-12** at different concentrations for 15 h, then
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20 20 μ L MTT (5 mg/mL) solution was added to each well and incubated for 4 h at 37 $^{\circ}$ C. Four hours
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22 later, the medium was removed carefully. 150 μ L DMSO was added to each well to dissolve
23
24 formazan crystals. The absorbance at 570 nm was measured using a Model 680 microplate reader
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26 (Bio-Rad, Hercules, CA, USA). The EdU assay was performed in a 96-well plate by EdU assay
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28 kit (Ribobio, China) according to the instruction, and the cells were examined under a fluorescence
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30 microscope.
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38 *Purification of His-RXR α -LBD fusion proteins*

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40 The constructed pET15b-RXR-LBD expression plasmid was transformed into Ecoli strain BL21
41
42 (DE3) to express the target protein. The strain was cultured in fresh LB medium containing
43
44 ampicillin at 37 $^{\circ}$ C and 250 rpm until OD600 reached 0.6. Isopropyl-1-thio-b-D-galactopyranoside
45
46 (IPTG) with final concentration of 0.1 mM was added. After 16 h of induction at 16 $^{\circ}$ C, the strain
47
48 was centrifuged at 5000 rpm for 20 min to collect. The protein was purified according to the
49
50 standard His-tag fusion protein method. Briefly, the bacteria were resuspended with lysis buffer
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52 (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.5% (v/v) Triton X-100)
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3 supplemented with PMSF (1 mM), sonicated, and centrifuged at 10000 rpm, at 4 °C for 30 min.
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5 The supernatant was added to NiNTA resin chromatography affinity column, then incubated at 4
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7 °C for 1 h, and washed twice with 800 μ L wash buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl,
8
9 50 mM imidazole). Finally, the target protein was eluted with 100 μ L elution buffer (50 mM
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11 NaH₂PO₄ pH 8.0, 300 mM NaCl, 250 mM imidazole). After the purity of the collected samples
12
13 was detected by discontinuous SDS-PAGE, the components with a purity of more than 90% were
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15 dialyzed into the required buffer for the following experiments.
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21 *Saturation Transfer Difference (STD) NMR experiment*

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23 The NMR samples used for STD experiment contain 600 μ L 0.01 mM PBS buffer solution (pH
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25 7.4) consisting of 10 μ M RXR α /LBD, 200 μ M berberine or **B-12**, 5% DMSO and 5% D₂O. All
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27 NMR experiments were performed on Bruker 800 MHz AVANCE III HD equipped with a
28
29 cryoprobe at 25 °C. The Bruker pulse program stddiffgp19.3 was used to collect STD NMR data
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31 and the saturation time was set to 2 sec. The STD spectra were processed and analyzed by Topspin
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40 *Fluorescence titration measurements*

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42 The fluorescence titration measurements were carried out on a VARIAN spectrofluorometer
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44 model Varian Eclipse. The sample quartz cuvette path length was 1 cm, and the excitation and
45
46 emission slit widths were set as 5 nm. Then the fluorescence spectra of RXR α and the RXR α -
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48 berberine complex were recorded in the wavelength range 290–600 nm after excitation at 280 nm.
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50 The change in fluorescence intensity was observed by continuous stirring varying concentrations
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52 of berberine (2-30 μ M) to the fixed concentration of RXR α (1 μ M) in PBS buffer solution (pH
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3 7.4). The DMSO titrated with an equal amount of berberine was also measured and subtracted
4 from their respective samples to correct its effect. Each spectrum was measured three times.
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10 *Circular dichroism (CD) spectroscopy*
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12 CD spectra were measured with a Jasco J-810 spectropolarimeter (Jasco Corporation, Tokyo,
13 Japan) at 25 °C. Instrument path length was set to 0.1 cm, and wavelength range at 180-260 nm
14 (protein secondary structure). The band width was set to 1 nm with the response time of 1 s and a
15 scanning speed of 20 nm/min. All measurements were detected in the 0.01 M phosphate buffer.
16
17 Purification of His-RXR α -LBD was dilute to 1 μ M in phosphate buffer, and the concentration of
18 berberine or B-12 was 5 μ M (DMSO concentration was 0.01%). For the displacement studies, the
19 spectra were recorded 5 min after addition of the berberine to the His-RXR α /LBD solution.
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21 Average of 3 scans were collected for each spectrum.
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33 *Real-time PCR*
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35 Total RNA was extracted with Trizol (Invitrogen) reagent according to the manufacturer's
36 instructions. Then 1 μ g mRNA was reversely transcribed to cDNA by Reverse Transcription
37 System (Promega). The mRNA expression levels of target genes were detected by LightCycler 96
38 Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany) with SYBR Premix
39 EX Taq (TaKaRa, Japan). GAPDH gene level was set as an internal control.
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47 The primers were listed below:
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49 *GAPDH:*
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51 Forward: 5' - TGCACCACCAACTGCTTAGC-3'
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54 Reverse: 5' GGCATGGACTGTGGTCATGAG-3' ;
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c-Myc:

Forward: 5' GGGGCTTTATCTAACTCGC 3'

Reverse: 5' CTATGGGCAAAGTTTCGTG 3'

Cdc2:

Forward: 5' AA ACTACAGGTCAAGTGGTAGCC3'

Reverse: 5' TCCTGCATAAGCACATCCTGA3'

p21^{WAF1/CIP1}:

Forward: 5'-CCACTGGAGGGTGA CTTCG-3'

Reverse: 5'-TGCAGCAGAGCAGGTGAGG-3'

RXR α shRNA

The lentiviral vector-based shRNA technique was used to knockdown endogenous RXR α in KM12C cells. The targeted sequences of human RXR α shRNA was 5'-GTGTTGTCACCCTCCTTATTT-3'; 5'-CCTGTCTGGAGTGACATCT-3'. The negative control shRNA sequence was 5'-GGCTACGTCCAGGAGCGCACC-3'.

Mouse xenograft models

Male six-week-old nude mice (BALB/c, SPF grade, 19-23 g) were maintained in Xiamen University Experimental Animal Center. The mice were allowed to get accustomed to the new environment for one week before the experiment. All animal studies were handled in accordance with the Institutional Animal Use and Care Committee of Xiamen University (China). KM12C cells (1×10^6 per mouse) resuspended in 100 μ L Hank's Balanced Salt Solution (HBSS) were subcutaneously injected into both flanks of nude mice. The tumor-bearing mice were randomly

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3 divided into three groups after seven days and were then treated daily with Vehicle
4 (Carboxymethyl Cellulose Sodium-Na, CMC-Na), berberine (100 mg/kg body weight), and **B-12**
5 (100 mg/kg body weight) by intragastric administration daily for 15 days. Then the mice were
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8 euthanized and the tumors were removed for follow-up study.
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14 *Determination of Serum biochemical indexes*

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18 Serum samples were collected from mice and the following biochemical indexes were
19 measured: liver function indexes (Bilirubin total (TBIL), indirect Bilirubin (IBIL), alanine
20 aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP);
21 kidney function indexes (urea formaldehyde (UREA), and creatinine (CREA)); glycolipid function
22 indexes (total cholesterol (TC), and triglyceride (TG)); and Calcium (Ca) concentration. All the
23 indexes were measured by using Automatic Chemistry Analyzer (BS-240vet, Mindray Bio-
24 Medical Electronics Co., Ltd. Shenzhen, China).
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38 *Immunohistochemical assay*

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40 Tumor tissue specimens were fixed with neutral formalin and embedded in paraffin. Tissue
41 sections (4 μm thick) were dewaxed and deparaffinized in xylene, followed by treatment with a
42 graded series of alcohol (100, 95, 85, and 70%) and rehydration in PBS (pH 7.5). After that, tissue
43 sections were immersed in 3% hydrogen peroxide for 10 min to suppress endogenous peroxidase
44 activity, and then successively incubated with 0.01 M natrium citricum (pH 6.0) for antigen
45 retrieval. The slides were rinsed in PBS (pH 7.5) and incubated with diluted antibody incubating
46 at 4 $^{\circ}\text{C}$ overnight and performed with the immunostaining kit according to the manufacturer's
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3 instructions. DAB Detection Kit (cat. #Kit-0014) was purchased from Maixin Biotech (Fuzhou,
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5 China).

6 7 8 9 10 *Pharmacokinetics Study*

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12 SD rats weighed 200 ± 20 g were housed at 28 °C in a laminar flow under sterilized conditions.
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14 Rats were randomly divided into three groups and then treated with berberine (100 mg/kg), **B-12**
15 (100 mg/kg) or vehicle (0.5% CMC-Na) by intragastric administration. Blood samples (0.5 mL)
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17 were collected via the tail vein at 0.083、0.25、0.5、1、2、4、8、12、24、36 h after
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19 intragastric administration. After centrifugation at 2000 rpm for 10 min, the resulting plasma was
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21 frozen at -80 °C until LC-MS/MS quantification. Pharmacokinetic data analyses were conducted
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23 using a noncompartmental analysis model (DAS 2.0, T.C.M., Shanghai, China). All animal
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25 procedures were approved by the Animal Care and Use Committee of Xiamen University.
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32 33 *Solubility measurements*

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35 The gravimetric method was used to detect the solubility of berberine and its analogues. In brief,
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37 excess amount of powder was added into 10 mL deionized water in three parallel groups for each
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39 compound. Samples were shaken at 37 °C for 24 h, and centrifuged at 10,000 rpm for 5 min. Then
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41 5 mL supernatant was taken out and put into an evaporating dish (dried and weighed in advance,
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43 whose net weight was labeled as W1 (with the unit of grams), which was subsequently placed in
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45 the oven at 60 °C until the weight became constant. The total mass of the compound and the
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47 evaporating dish was then weighed (in the accuracy of 0.0001 g) and labeled as W2 (with the unit
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49 of grams). The solubility of berberine and berberine analogues in water (mg/mL) is calculated
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3 according to the following formula: $X = (W_2 - W_1) / 5 \times 10^3$, in which 5 is the volume of the
4 supernatant (mL).
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10 *Statistical analyses*

11 All results are shown as mean \pm SEM. Statistical significance is analyzed by one-way or two-
12 way ANOVA by GraphPad Prism software (version 5.0, GraphPad Software Inc., La Jolla, CA,
13 USA). The statistical significance was expressed as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.
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22 ■ ABBREVIATIONS

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24
25 Retinoid X receptor α (RXR α); structure-activity analysis (SAR); adenomatous polyposis coli
26 (APC); ligand-binding pocket (LBP); ligand-binding domain (LBD); 9-*cis*-retinoid acid (9-*cis*-
27 RA); berberine (Ber); hydrogen bonds (H-bonds); 5-Ethynyl-2'-deoxyuridine (EdU); proliferating
28 cell nuclear antigen (PCNA); saturation transfer difference (STD); nuclear magnetic resonance
29 (NMR); far-ultraviolet circular dichroism spectrum (far-UV CD spectrum); area under the curve
30 (AUC)
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55 ■ ANCILLARY INFORMATION

Supporting Information.

The following files are available free of charge.

^1H and ^{13}C NMR spectra for all analogues and supplementary figures (PDF)

Molecular Formula Strings for **B1-B15** (CSV)

X-ray data for compound **B-12** (CIF)

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T.H., Y.Z. and Y.-Y.Z. designed the experiments and wrote the manuscript. B.X., X.J, J.X., J.L., Y.T., L.Z., X.W., N.X., H.C., W.Z., H.Z., and X.H. carried out the experiments. The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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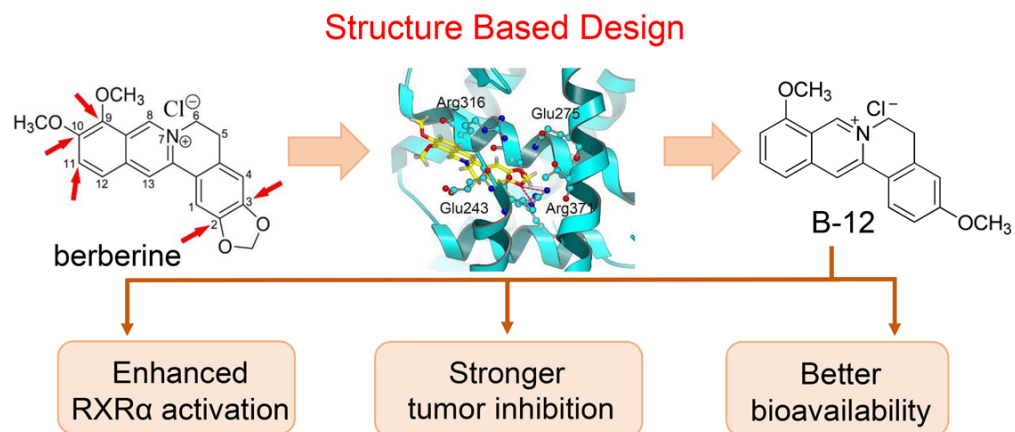


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