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Discovery of mitochondria-targeting berberine derivatives as inhibitors ofproliferation, invasion and migration against rat C6 and human U87 glioma cells

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Abbreviations

GBM, Glioblastoma multiforme; MMP, matrix metalloprotease; Bcl-2, B-cell CLL/lymphoma 2; TLC,thin-layer chromatography; MTT, (3,4,5-dimethylthiazol-yl)-2,5-diphenyl tetrazolium; IC_{50}, half maximal inhibitory concentration.

Abstract

This research aims to synthesize lipophilic berberine derivatives and evaluate their antiglioma effects on C6 and U87 cells. Introduction of substituents with various carbon chain lengths on C-13- or C-9-*O*-position of the berberine scaffold led to the discovery of several potent inhibitors against glioblastoma cells. Derivatives substituted with carbon chains of moderate length (twelve carbons) displayed improved lipophilicity and the strongest inhibitory effects. Several compounds, presented dose-dependent repression against proliferation (IC_{50}, 1.12- 6.12 μM) and blocked migration and invasion by over 60% at lower dose levels. Further preliminary research about the underlying mechanism for the enhanced antiglioma ability indicated that these analogues preferentially localized into mitochondria, inducing up-regulation of ROS production. Overall, these compounds represent promising candidates to combat glioblastoma and highlight new sight into the antiglioma therapy through interaction with mitochondria.

Introduction

Glioblastoma multiforme (GBM), a grade Ⅳastrocytoma, is the

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most common and malignant primary brain tumor.[1] Despite multiple therapies, GBM claims its sufferers' lives typically less than 14 months after diagnosis.[2] One subtle characteristic of GBM is the highly invasive behavior, conferring glioma cells the propensity of rapidly infiltrating neighbor normal tissue and making the complete surgical resection impossible.[3] Actually, after surgical removal of a glioma, 96% of the recurrent tumors derived from the residual invasive glioma cells arise within 2 cm from the resection cavity[4]or immediately adjacent to the resection rim.[5] Further, radiotherapy, also seems to have reached its practical limit, due to at least partly inherent and/or acquired radioresistance of high-grade glioma.[6] Moreover, traditional non-selective chemotherapeutics such as temozolomide (an alkylating agent) and irinotecan (a topoisomerase 1 inhibitor), have been reported that the survival advantage brought by these chemicals is accompanied with certain observed severe side effects.[7] In addition, some low molecular inhibitors of matrix metalloproteases (MMPs) and antivascular pharmaceuticals were investigated to suppress cancer invasion, yet only achieving limited clinical results.[8]

Conclusively, the invasion and migration ability of glioma cells remains to be the main reason of current unsatisfactory therapies. Indeed, accumulated data substantiate that GBM invasion is regulated and influenced by extremely diversified and overlapping signal pathways instead of a single one.[9]Hence, it is extensively supported that future successful therapies in the treatment of GBM will target multiple signal transduction cascades, either by one drug aiming at the pivotal intersection of several pathways or by a group of agents aiming at multiple signaling pathways.[3]

Coincidentally, dozens of lethal signaling pathways converge on what seems to be a promising therapeutic target, the mitochondrion organelle.[10]Recent studies shed light on the crucial role that mitochondria, at least partially, play in tumor invasion. The high expression feature of Bcl-W (an mitochondrial outer membrane anti-apoptotic protein) has been revealed in invasive glioma cells *in vivo* [11] and depletion of Bcl-W or Bcl-X_L mediated by siRNA renders invasive glioma cells susceptible to cytotoxic-therapy-induced apoptosis.[12]Moreover, research showed that the active Akt controlling the balance between cell survival and apoptosis [13]predominantly localized at the leading edge of migrating cells,[14]suggesting the implication between tumor invasion and mitochondria. Hurd et al. have well validated the dual effects of intracellular reactive oxygen species (ROS) mainly produced by mitochondriaon triggering signal pathways for cell migration and invasion.[15] Additionally, inhibition of heat-shock protein 90 (HSP90), which preferentially localizes in malignant cell mitochondria but not in their counterparts of normal cells,[10] will reduce cell migration and invasion via decreasing MMP-9 expression in GBM.[9] Therefore, it may be possible to target mitochondria as the junction of multiple signal cascades to achieve improved therapeutic efficacy for GBM patients.

Berberine (**1**), an isoquinoline alkaloid, possesses many striking pharmacological effects elaborated in available literature.[16] Absorbingly, berberine can selectively accumulate in tumor cell mitochondria,[17]which could be attributed to its structural amphiphilicity and delocalized positive charge.[18] Besides, mitochondrial-based cancer cell apoptosis induced by berberine has been confirmed in several non-CNS malignant cell lines, with the underlying mechanisms interpreted as decreasing ATP levels and activating apoptotic cascades via lowering the mitochondrial membrane potential, inhibiting complex Ⅰ of the respiration chain, induction of mitochondrial permeability transition (MPT) and release pro-apoptotic proteins to cytosol.[19] Further, recent investigations start to uncover berberine's potential in suppressing migration and invasion in non-CNS cancer cell lines. For example, berberine attenuates human gastric tumor cell invasion and inhibits the metastatic potential of breast cancer cells through down-regulation of MMPs and Akt pathway modulation, respectively.[20] However, current literature about whether berberine derivatives could selectively interact with mitochondria and repress the proliferation, migration and invasion of malignant glioma cells is rare. In addition, a non-ignorable obstacle that limits berberine's clinical application is its innate poor solubility and lipophilicity.[16] A structure-activity relationship study on berberrubine derivatives indicated that presence of lipophilic alkyl substituents of moderate length presents the optimal antibacterial activity,[21] prompting us that the lipophilicity alteration of berberine may favor its performance in cytotoxicity and anti-migration activity.

Previously, we have evaluated the antiglioma ability of four berberine derivatives varying the alkyl chain length in the C-9-*O*-position of berberine (**3b-e)** and found that they showed inhibitory effects on C6 glioma cells in proliferation, migration and invasion.[22] In this study, five 13-O-substitued berberine analogs varying the alkyl chain length (**5f-g**) were synthesized and characterized. However, to comprehensively compare the antiglioma effects of ten berberine derivatives and achieve more reliable results, in other words to avoid time interference on experimental results, the MTT, migration and invasion assays previously used to evaluated four 9-*O*-substitued berberine derivatives (**3b-e**) on C6 cells were performed again. Simultaneously the same assays of another 9-*O*-substitued berberine derivative (**3a**) and five newly synthesized 13-*O*-substitued berberine derivatives (**5f-g**) on rat C6 glioma cells were added. Additionally, human U87 glioma cells were chosen to assess the total ten berberine derivatives in this study. The underlying mechanisms for the improved antiglioma activity were preliminarily explored by studying the subcellular localization of these compounds and measuring ROS production of the C6 and U87 glioma cells with or without berberine derivatives. The BBB penetration ability was also predicted since these compounds have to function inside the brain.

Results and Discussion

Chemistry

Synthesis and identification of 3a-e

The synthetic pathway for compounds **3a-e** and**5f-j** is illustrated in Scheme 1. To obtain **3a-e**, the first step involved pyrolysis of

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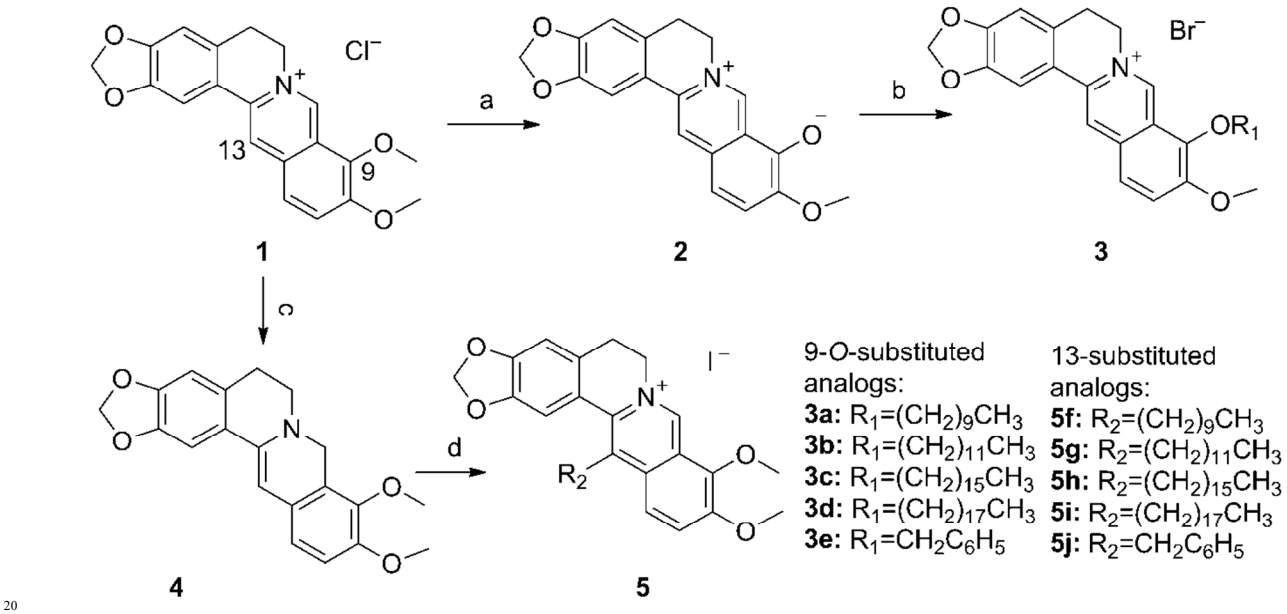
1to the intermediate berberrubine(**2**). Following alkylation of the resulting **2** by corresponding alkyl bromides or the benzyl bromide afforded five 9-*O*-substitued berberine analogs. We found the key determinant in synthesis of 9-*O*-substitued berberine derivatives was always keeping the reaction system completely absent of oxygen. It should be noted that in the alkylation step we simplified the reported condition of reflux at 120 ℃ [23]to stirring at 80 ℃ without compromising the yield.Besides, the mass spectra of **3a-e** show the molecular ions at *m/z* 462, 490, 546, 574, and 412 (M-Br, ESI-MS), which are 140, 168, 224, 252 and 90 mass units higher than that of **2** (M+H), respectively.

Synthesis and identification of5f-j

To synthesize **5f-j**, precursor**1** was first converted into intermediate dihydroberberine (**4**) bysodium borohydride-promoted reduction. Subsequent sodium iodide-promoted enamine alkylation of **4** by alkyl bromides of various chain lengths or the benzyl bromidefurnished five 13-substituted



Scheme 1. Synthesis of **3a-e**, **5f-j**. Reagents and conditions. (a) Decalin, N_2, 190 ℃. (b) R_1Br, DMF, N_2, stirring, 80 ℃. (c) $NaBH_4$, pyridine, stirring, rt. (d) R_2Br, anhydrous acetonitrile, NaI, N_2, stirring, 80 ℃.

berberine analogues. This enamine alkylation step is accompanied by restoring thearomaticity of one nitrogen-containing heterocycle, which is indispensible in maintaining the planar characteristic of the berberine scaffold as well as the pharmacological effects. The ^{1}H NMR spectra indicate that five compounds **5f-j** share the same parent nucleus with **1**.The singlet (~8.95 ppm) displayed by the C-13 position proton of **1** disappears in the ^{1}H NMR spectra of **5f-j**, suggesting that the substitution reaction occurs at C-13 position. Moreover, owing to the electron-withdrawing effect of C-7 position N^+, the deshielding and higher field ^{1}H chemical shifts of the protons in C-13 substituents are also observed in the ^{1}H NMR spectra of **5f-j**, similar with those of **3a-e**.

Apart from the above approach to synthesize **5f-j**, we attempted another method including reduction of **1** to an intermediate 8-acetonyldihydroberberine and then alkylation of this intermediate with bromides.[24] Nonetheless, the yield of 8-acetonyldihydroberberine was lower than that of the intermediate **4**, due to the former's easy rearrangement to **1** during the heating process. In addition, during preparation of **4** we compared two reaction solvents, pyridine and the methanol solution containing potassium carbonate, in respect of reaction temperatures, time and yields. Results manifested that the yield of **4** from the reaction with pyridine as the solvent at room temperature for 20 min exceeded that of the reaction with the latter as the solvent at 0 ℃ for 8 h.

The first obstacle we met in synthesis of **5f-i** was the considerably low yields that could be ascribed to the following reasons. Approximate 30%-40% of **4** converted back into **1**[25] in the enamine alkylation process which *per se* possesses the drawbacks of low yields and numerous byproducts. The second reason is that alkyl bromides with long carbon chains present poor reaction activity. To improve the yield, we tried to optimize the enamine alkylation in reaction solvent, pressure, temperature, catalyst, feeding sequence and feeding ratio. Firstly, using ESI-MS monitoring the reaction process, we finally chose acetonitrile to dissolve **4** instead of dichloromethane or DMF, which both exhibited excellent solubility to **4** though, but the molecular ion peaks of target compounds failed to appear using the latter two as solvents. Secondly, an elevated yield was achieved under high pressure using an airtight thick-walled flask as the reaction apparatus rather than the usual reflux method.[23]Thirdly, we selected 80 ℃ as the reaction temperature since no significant improvement of the yield was attained at 100 ℃ and 130 ℃. Fourthly, sodium iodide exhibited higher catalytic activity than potassium iodide and the possible explanation could be the higher

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solubility of sodium iodide in acetonitrile. Finally, distinctive feeding sequences and ratios were tried and the present sequence and ratio in the experimental section were the relatively advantageous combination in favor of the final yield.

Another obstruction came down to purification of **5f-i**. Highly pure intermediate **4** was difficult to obtain from either recrystallizationor chromatography since **4**,as mentioned earlier, mixture.[26] After extensive attempts, we developed a three-step approach to remove impurities. Firstly, most byproducts were separated byneutral alumina column chromatography(Petroleum ether/*n*-butanol). Secondly, column chromatography on silica gel (*n*-butanol/water/acetic acid) was harnessed to remove a byproduct whose polarity was next to those of target products. Finally, an unidentified impurity peak in ^{1}H NMR spectra was successfully eliminated after the purification by Sephadex™ LH-20 column chromatography (Methanol).

Log P values

In Table 1, the negative log *P* values of **1**, **3e** and **5j** demonstrate that introduction of benzyl to the berberine scaffold at the C-9-*O*position and C-13 position will moderately augment the lipophilicity of berberine, while alkyl substituents at both C-9-*O* and C-13 positions of berberine remarkably increase the lipophilicity of berberine. Further, berberine derivatives with moderate carbon chain length (**3b** and **5g**) show the most significant enhancement in lipophilicity, implying more preferable transmembrane permeability than berberine.

Table 1. Log *P* values, purity data[a], and effects on survival rate (IC_{50}, μM) of rat C6 and human U87 glioma cells by **1**, **3a-e** and **5f-j**[b]

compounds	Purity (%)	log *P*	IC_{50} (μM)	
			C6	U87
1	98.89	-1.68 ± 0.24	> 20	> 20
3a	99.12	2.26 ± 0.34	2.95 ± 0.22	5.73 ± 0.51
3b	99.08	3.43 ± 0.21	1.24 ± 0.13	4.55 ± 0.28
3c	96.23	3.41 ± 0.40	6.12 ± 0.64	15.2 ± 2.02
3d	99.16	3.01 ± 0.40	4.45 ± 0.57	22.49 ±3.38
3e	96.80	-0.86 ± 0.16	11.48 ± 2.05	> 20
5f	98.17	2.37 ± 0.27	1.12 ± 0.35	3.65 ± 0.61
5g	96.75	3.66 ± 0.30	1.65 ± 0.38	3.09 ± 0.54
5h	95.62	3.24 ± 0.22	1.67 ± 0.46	3.25 ± 0.27
5i	95.49	3.22 ± 0.31	2.37 ± 0.29	5.75 ± 0.68
5j	99.46	-0.73 ± 0.14	14.4 ± 1.48	> 20

[a]Log *P* means the logarithm of the *n*-octanol/water partition coefficients; Purity of these compounds was determined by High-performance LiquidChromatography.[b]IC_{50}: inhibitory concentration causing a 50% reduction in cell proliferation in μM. Data were expressed as mean ± SD.

was easily oxidized back to **1** under aerobic conditions. Consequently, adding impure **4** to the following enamine alkylation produced six byproducts per reaction, whose R_f values were very close to those of target product in silica gel-based TLC (Developing solvents: dichloromethane/methanol, 20:1, v/v). Neither recrystallization nor silica gel-based column or alumina column chromatography could separate the total resulting

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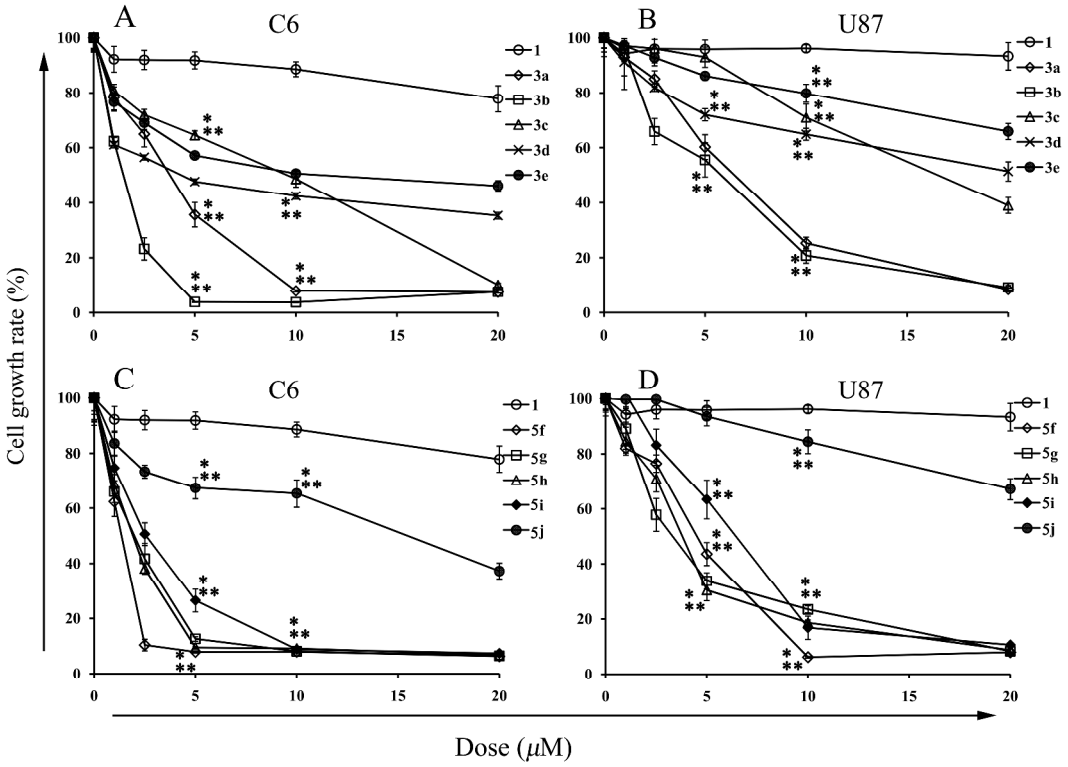

Fugure 1. Survival rate (%) of C6 (A, C) and U87 (B, D) cells after incubated with different compounds at various concentrations for 24 h (n = 10). Data are presented as mean ± standard deviation. $P < 0.05$; *versus control; **versus berberine.

Proliferation inhibition in MTT assays

The antiproliferative effects of compounds **1**, **3a-e** and **5f-j** was assayed in rat C6 and human U87 glioblastoma cells using the MTT ((3, 4, 5-dimethylthiazol-yl)-2, 5-diphenyl tetrazolium) assay that measures the number of metabolically active cells. Figure 1 illustrates the relative survival rates of both cell lines after incubation with various compounds at different dose levels for 24 h. Firstly, exposure of C6 cells to **1** with dose up to 20μM exhibits a minimal antiproliferative effect, while U87 cells are not sensitive to **1** at this dose level (Figure 1A and 1B). The rest derivatives **3a-e** and **5f-j** are active against both tumor cell lines in a dose-dependent manner. Specifically, in Figure 1A and 1B, **3a** and **3b** display the strongest anti-proliferation effects on both cell lines, while **3d** and **3e** have a moderate effect. In particular, **3c** achieves almost the same complete inhibition result against C6 cells as **3a** and **3b** do when the concentration of **3c** reaches at 20 μM whereas the survival rate of U87 cells exposed to **3c** is 38.95%. Further, for 13-substituted berberine analogs, **5f-i** also shows robust growth suppression on C6 and U87 tumor cells. For instance, the survival rates are decreased to lower than 20% by **5f-i** at 10 μM (Figure 1C and 1D). However, **5j** exerts the modest repression effect on C6 and U87 cells at 20 μM since the survival rates are as high as 37.24% and 67.20%, respectively. In general, the MTT results of these synthesized derivatives on C6 cells are in line with those of U87 cells and the C6 cells are more sensitive to all the tested compounds than U87 cells are.

Based on the MTT tests, IC_{50} values of the assayed compounds were calculated and shown in Table 1. Of note, **3a-c** and **5f-i** are superior to the clinically commonly used chemotherapeutics for glioblastoma in relation to cytotoxicity. Indeed, the IC_{50} values of temozolomide and irinotecan on U87 glioma cells are 19.38 μM and higher than 250 μM, respectively.[27]Recently,**3b** was synthesized and also proved to be a more robust inhibitor on human cancer HepG2 and HT29 cell lines.[28] Moreover, data trends in Figure 1 and Table 1demonstrate that the variation of cytotoxic activity of the synthetic analogues correlates with alternation of alkyl chain length. Namely, the presence of alkyl chains with moderate length (12 carbons) at C-9-*O*-position or C-13-position on the berberine scaffold guarantees the most

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appreciable antiglioma activity. Nonetheless, introduction of the benzyl group at aforementioned positions contributes to activity elevation inconspicuously. These highly similar varying patterns between the lipophilicity and cytotoxicity of compounds **1**, **3a-e** and **5f-j** led us to postulate that the elevated lipophilicity might promote the transmembrane permeability of these compounds, thus enhancing the intracellular concentrations and cytotoxicity.

Efficacy in transwell migration and invasion assays

As reported, berberine possesses potential repression on migration and invasion in non-CNS cancer cell lines. Hence, not only to identify, but to compare the possible suppression of berberine and its lipophiphilic derivatives on migration and invasion of glioma cells, we tested **1**, **3a-e** and **5f-j** using transwell migration and invasion assays on Milipore cell culture inserts with 8-μmpore size polycarbonate membranein C6 and U87 cell lines. Migration and

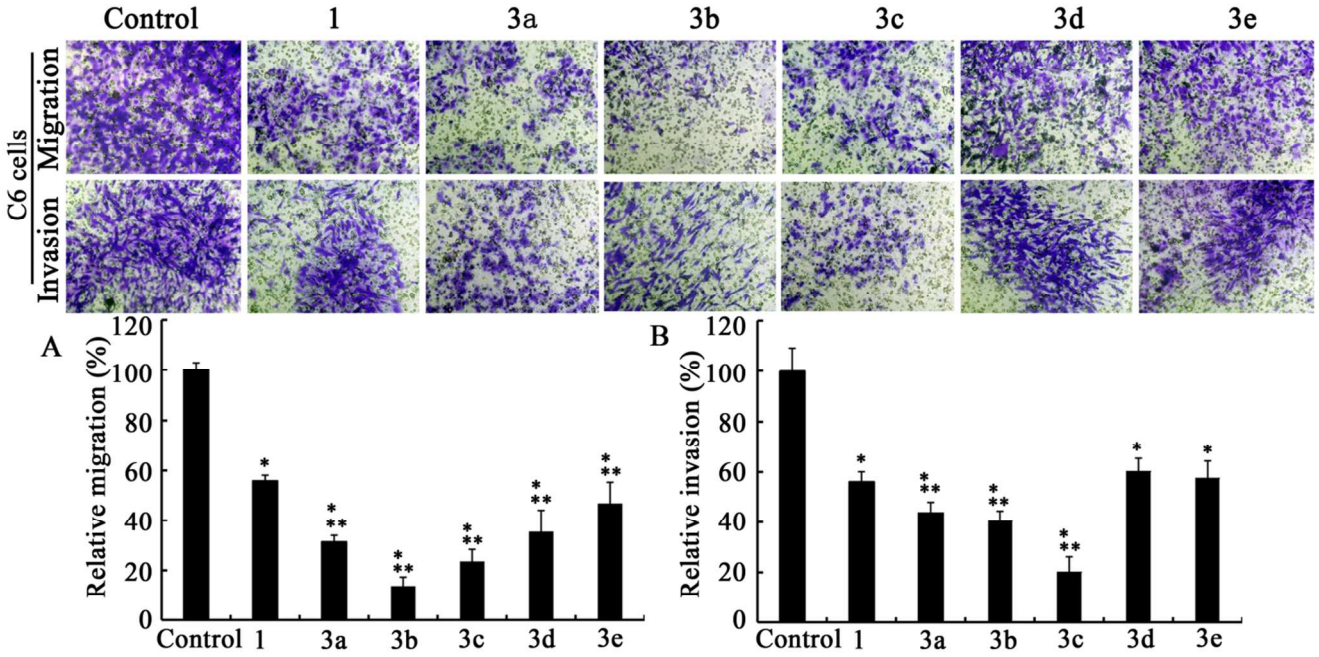

Figure 2. Transwell migration (A) and invasion (B) assays in C6 cell lines (n=3). Dose levels for **1**and**3a-e** are 5, 0.5, 0.5, 0.5, 0.5 and 0.5 μM, respectively. Data are presented as mean ± standard deviation. $P < 0.05$; *versus control; **versus berberine.

invasion experiments were performed in the absence or presence of matrigel on the polycarbonate membrane, respectively. To rule out cytotoxicity as the driver of decreasing tumor cell migration and invasion ability, first, we chose the minimum highest dose levels of all assayed compounds based on MTT assays so that all survival rates of all groups were higher than 85%. Second, the number of migrated and invaded tumor cells to the lower chambers was calibrated via being divided by the total cell numbers that were characterized through synchronous MTT assays at the same dose levels.

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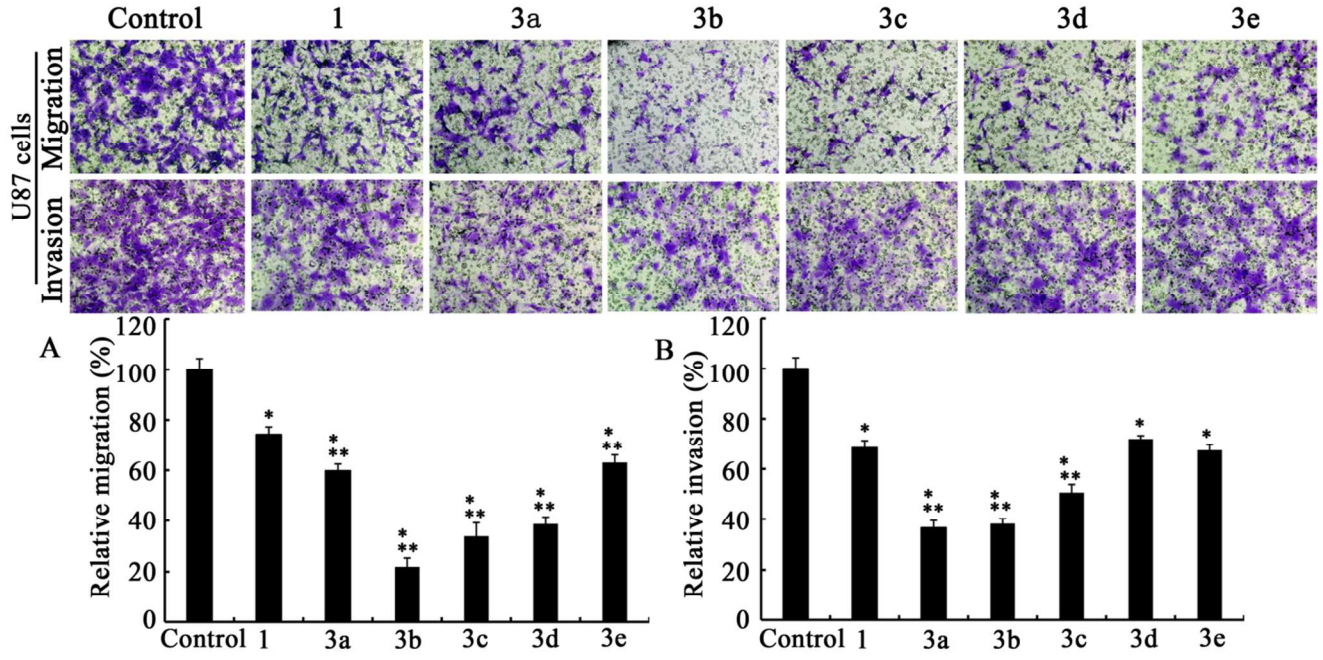

Figure 3. Transwell migration (A) and invasion (B) assays in U87 cell lines (n=3). Dose levels for **1**and**3a-e** are 5, 2.5, 2.0, 2.5, 2.5 and 2.5 μM, respectively. Data are presented as mean ± standard deviation. $P < 0.05$; *versus control; **versus berberine.

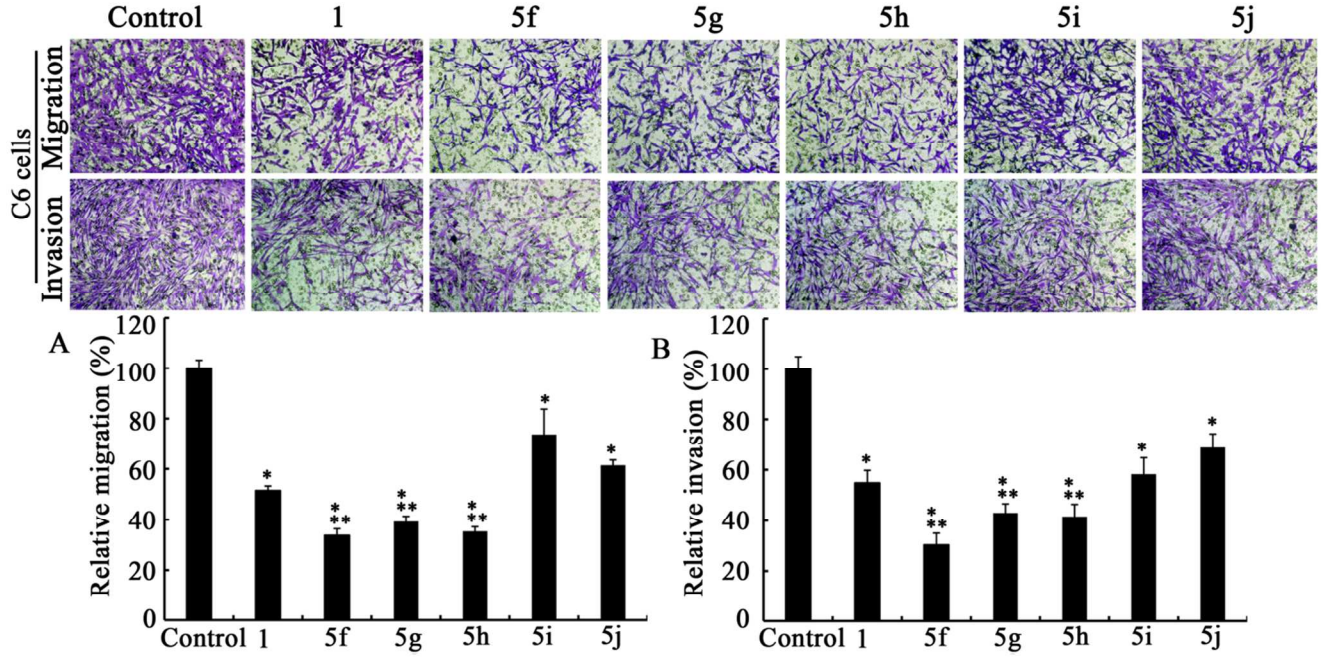

Figure 4. Transwell migration (A) and invasion (B) assays in C6 cell lines (n=3). Dose levels for **1**and**5f-j** are 5, 0.5, 0.5, 0.5, 0.5 and 1 μM, respectively. Data are presented as mean ± standard deviation. $P < 0.05$; *versus control; **versus berberine.

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As shown in Figure 2, 3, 4 and 5, all compounds exhibits robust or moderate inhibitory effects against invasion and

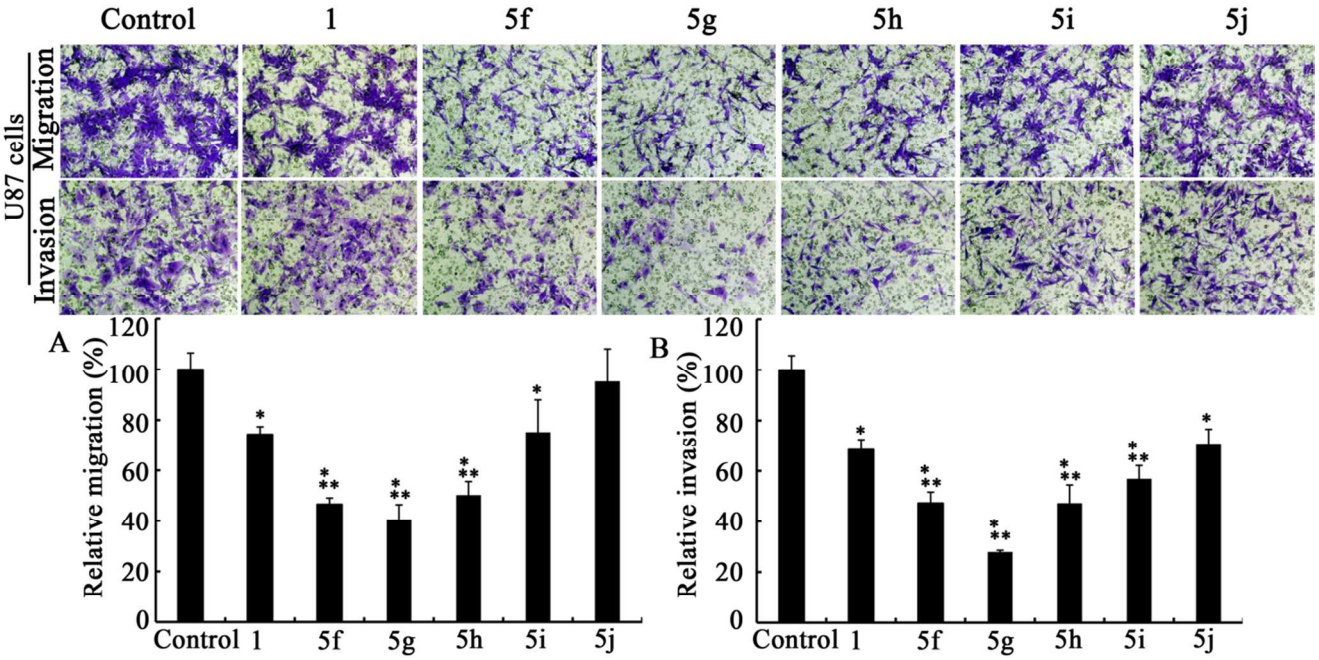

Figure 5. Transwell migration (A) and invasion (B) assays in U87 cell lines (n=3). Dose levels for **1**and**5f-j** are 5, 0.5, 0.5, 0.5, 2.5 and 2.5 μM, respectively. Data are presented as mean ± standard deviation. $P < 0.05$; *versus control; **versus berberine.

migration in both cancer cell lines except 13-benzyl-subtituted berberine analog, **5j**. A modest anti-migration and anti-invasion activity is observed in the presence of berberine, which agrees with previous proof that berberine could attenuate migration and invasion in other tumor cell lines.[20] Furthermore, **3d**, **3e**, **5i** and **5j** display greater than 30% suppression and three other potent derivatives (**3b**, **3c** and **5g**) block migration or invasion by over 60%. Overall, these experimental results substantiate that the tested compounds are effective migration and invasion inhibitors.

After comparing Figure 2A and Figure 3A, it could be concluded that only those analogs with moderate alkyl chain length (**3b**and**3c**) could significantly lower tumor migration tendency. Lengthening the carbon chain (**3d**) or shortening the substitute (**3a** and **3e**) will compromise the anti-migration potency. Likewise, the similar phenomenon is noted for **5f-j** in transwell migration assays (Figure 4A and Figure 5A). Meanwhile, there is no cancer type-specific mode of action revealed in migration inhibition evaluation. On the contrary, C6 and U87 cells are differently sensitive to the derivatives regarding invasiveness. For example, the most powerful anti-invasion agents for C6 cells are **3c** and **5f**, whereas for U87 cells **3a**, **3b** and **5g** are the most remarkable ones (Figure 2B, Figure 3B, Figure 4B and Figure 5B). Therefore, both substitutes with suitable carbon chain length and tumor cell types influence the activity of synthetic analogues in terms of subduing invasion ability. Intriguingly, agents **1**, **3d**, **3e** and **5j** could attenuate cancer cell migration and invasion without exert acute cytotoxicity. Using these effective but low cytotoxic inhibitors to block tumor cell metastasis will be more advantageous in long-term treatment of invasive tumor, since most chemotherapeutics targeting cancer cell proliferation provoke acquired chemoresistance and toxicity to healthy cells after long-time taking.[29]

Subcellular localization in mitochondria

To preliminarily examine the potential target of the synthetic derivatives and if they preferentially localize into mitochondria as berberine does, we selected four compounds (**3b**, **3c**, **5g** and **5h**) with high antigrowth, anti-migration and anti-invasion ability to incubate with C6 and U87 cells at 1 μM dose level followed by staining mitochondria with Mitotracker Green FM (500 nM) and photographing. Berberine was also included as a control group. Figure 6 depicts the laser confocal micrographs of double-stained C6 cells (the left panel) and U87 cells (the right panel).

A perfect superposition between Mitotracker Green FM dye images and those of berberine evidences the specific subcellular localization of berberine to cell mitochondria, which is in harmony with previous results.[17] The overlapping photographs for **3b**, **3c**, **5g** and **5h** unequivocally demonstrate that these berberine derivatives have a preference for mitochondrial organelle.Such a mitochondrion-targeting feature could be ascribed to two determinants. The primary reason is that the

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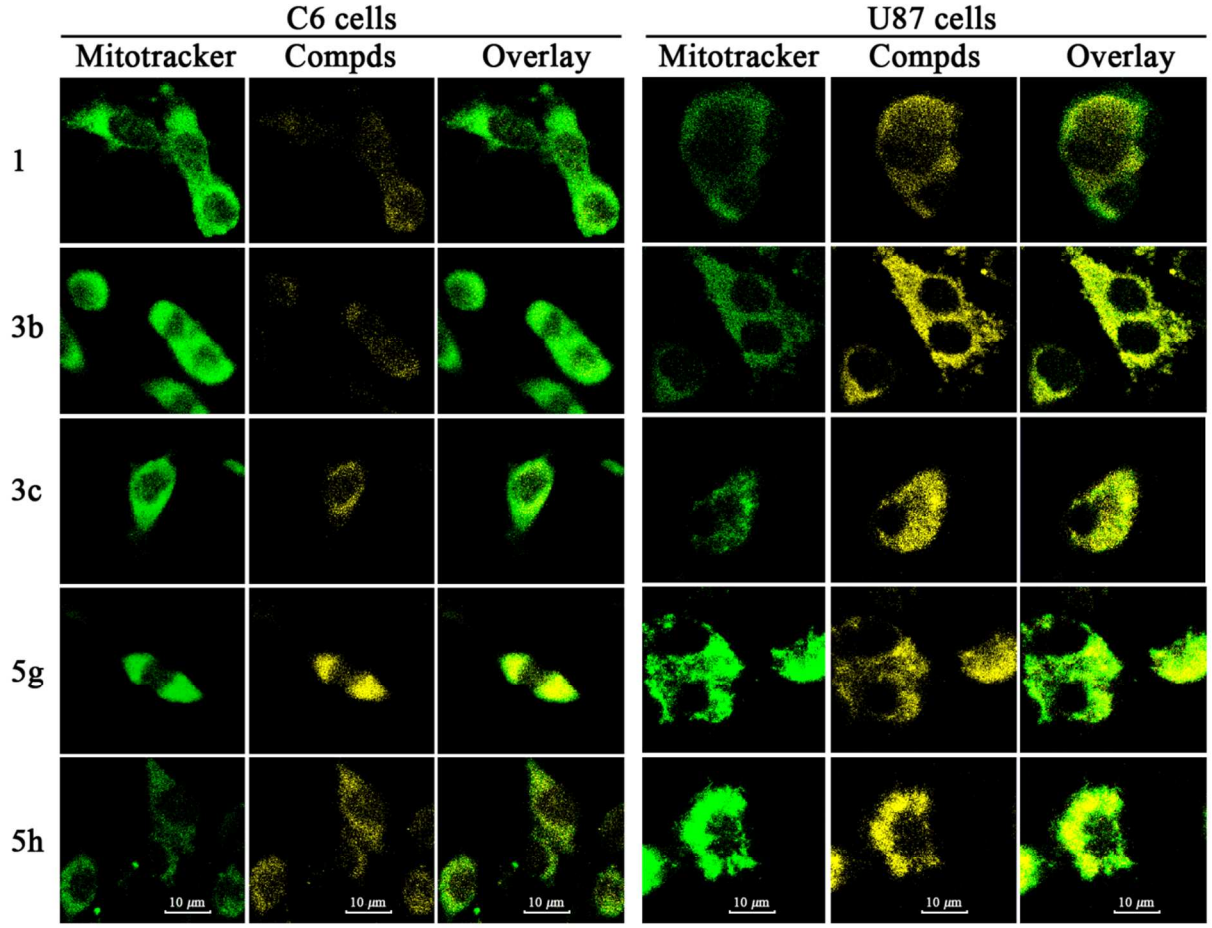

Figure 6. Laser confocal micrographs of double-stained C6 cells (Left panel) and U87 cells (Right panel). Notes: 1. Green channel: 500 nMMitotracker Geen FM stained mitochondria; 2. Yellow channel: 1 μM respective compounds; 3. Composite images of the former two channels, indicating the co-localization of synthesized derivatives into mitochondria in C6 or U87 cells.

amphiphilic berberine analogs retain the delocalized cationiccharge centers that are crucial to selective mitochondrial accumulation.[18] Secondly, it has been verified that the driving force for such selective accumulationis the mitochondrial transmembrane potential, which proves to be elevated in cancer cells, further favoring the mitochondrial localization propensity.[30] In addition, yellow fluorescence intensities of **3b**, **3c**, **5g** and **5h** are stronger than that of berberine in both tumor cell lines. The possible underlying mechanism for the enhanced fluorescence intensities may be that an increase in the lipophilicity from the alkyl substituents promotes thepenetration of the synthetic analogs through cytomembrane, thus improving mitochondrial accumulation.[26b]

High generation of reactive oxygen species

It is well established that reactive oxygen species are implicated in both cytotoxicity and cancer cell metastasis. Now that mitochondria are the main sources of intracellular ROS and the synthesized derivatives seem to preferentially locate to mitochondria, ROS production of C6 and U87 tumor cells after incubation with or without compounds **1**, **3a-e** and **5f-j** was measured by DHE assay. Cell-permeable DHE is oxidized by O_2^- to ethidium bromide, intercalating the cells' DNA and staining the nuclei a fluorescent red that reflects ROS levels. In agreement

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Table 2. Physical properties and prediction of BBB penetration of **1**, **3a**, **3b**, **5f**, and **5g**.

Calculation[a]	MW	Clog P	HBA	HBD	PSA	Log BB[b]
1	371.87	0.20	4	0	40.82	- 0.44
3a	542.51	4.665	4	0	40.82	0.23
3b	570.56	5.675	4	0	40.82	0.39
5f	603.54	5.019	4	0	40.82	0.28
5g	631.59	6.029	4	0	40.82	0.44
Lipinski's rules	≤ 500	≤ 5.0	≤ 10	≤ 5	≤ 90	>0.3 (readily cross the BBB);< - 1.0(only poorly distributed to the brain)

[a]MW: molecular weight; clogP: calculated logarithm of the octanol-water partition coefficient; HBA: hydrogen-bond acceptor atoms; HBD: hydrogen-bond donor atoms; PSA: polar surface area;[b]compounds with logBB > 0.3 are able to cross the BBB readily, with logBB < - 1.0 are only poorly distributed to the brain.

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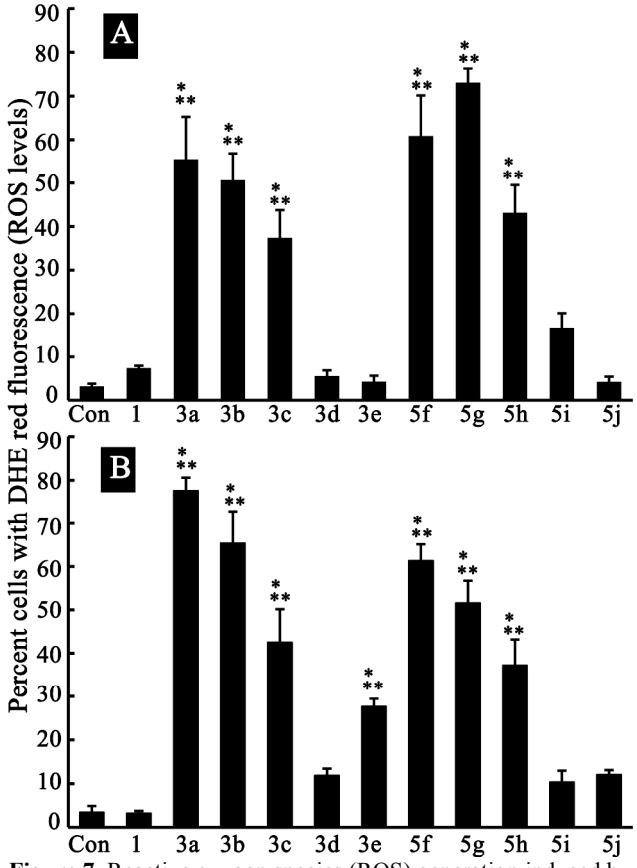

Figure 7. Reactive oxygen species (ROS) generation induced by 2 μM of compounds **1**, **3a-e** and **5f-j** (n=4). Data are presented as mean ± standard deviation. A: C6 cells; B: U87 cells. ROS levels were determined by detection of the dihydroethidium (DHE) red fluorescence in the nuclei resulting from the oxidation of DHE by intracellular ROS. $P < 0.05$; *versus control; **versus berberine. Con= Control group.

with the varying patterns of lipophilicity (Table 1) and cytotoxicity (Figure 1), ROS production of both cell lines after incubation with**1**, **3a-e** and **5f-j** presented similar changing trends (Figure 7). For instance, those derivatives (**3a, 3b, 5f** and **5g**) with alkyl chains of moderate length caused significantly higher ROS generation than that of compounds **1**, **3d**, **3e**, **5i** and **5j**, whose substituents were either longer or shorter alkyl chains or the benzyl at the C-9-*O*- or C-13-position on the berberine scaffold. Combined with the mitochondriotropic attribute, these data indicated that the cytotoxicity displayed by the synthesized analogues was derived from the induction of high ROS generation from the mitochondrial organelle.

Because mitochondria are the junction of multiple signal pathways responsible for cancer cell proliferation, migration and invasion, the mitochondrion-specific ability and high ROS production tend to be a tentative underlying mechanism for that certain derivatives such as **3b**, **3c**, **5g** and **5h** possess antiproliferation, anti-migration and anti-invasion activity at different dose levels spontaneously. To further understand the underlying mechanism by which ROS production induced by berberine derivatives regulates C6 and U87 glioma cells, studies about the mitochondrial membrane potential($\Delta\Psi_m$), release of cytochrome C and apoptosis-inducing factor (AIF) and permeabilization of lysosomes are underway.

Improved BBB penetration of berberine derivatives

To target gliomas, BBB penetration is necessary for these berberine derivatives. As shown in Table 2, berberine only displayed limited BBB penetration potential, while compounds **3a** and **5g** could readily cross the BBB. Therefore, compounds **3a** and **5g** fulfilthe necessary drug-like and brain penetration criteria. There results demonstrate that introducing the dodecyl to the C-9-*O*-position or C-13-position of the berberine scaffold will notably enhance the BBB penetration of synthetic derivatives, favouring their anti-glioma effects.

Conclusions

Glioblastoma still poses an urgent challenge to both patients and neuro-oncologists. Unfortunately, management of glioblastoma is mainly palliative all over the world. In this study, an effort was made to develop therapeutics against malignant gliomas through enhancing the lipophilicity of berberine and retaining the mitochondriotropic activity, potentially influencing the intersection of multiple signaling cascades in cancer pathology. Inspired by our previously evaluated 9-*O*-substitued berberine derivatives on C6 glioma cells,[22] 9-*O*-decyl-berberine (**3a**)and five cationic C-13-position berberine derivatives (**5f-j**) varying in the length of substituted alkyl chains were synthesized. The data of biological experiments on C6 and U87 cells suggested that introduction of the lipophilic substituents with moderate alkyl chain length to the C-9-*O*-position and C-13-position of the berberine scaffold led to the identification of several potent proliferation, migration and invasion inhibitors in two gliomablastoma cell lines. Indeed, the IC_{50} values for antiproliferation of several synthesized compounds reached low micromolar range (**3b**, 1.24 μM; **5f**, 1.12 μM; C6 cells). Over 60% of invasion and migration was blocked by the derivatives with moderate alkyl chain length (**3b** and **5g**). Further, the simultaneous inhibitory effects against glioma cell survival, migration and invasion should be an integrated result of the improved lipophilicity, the preferential localization into

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neoplastic mitochondria and elevated intracellular ROS production. Overall, these compounds provide novel chemotherapeutic candidates and also offer new sight into the antiglioma therapy through the mitochondrial pathway.

Notes and references

† Electronic Supplementary Information (ESI) available: experiments and characterisation of compounds are in the ESI.

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Conflict of interest

There is no conflict of interests in this work.

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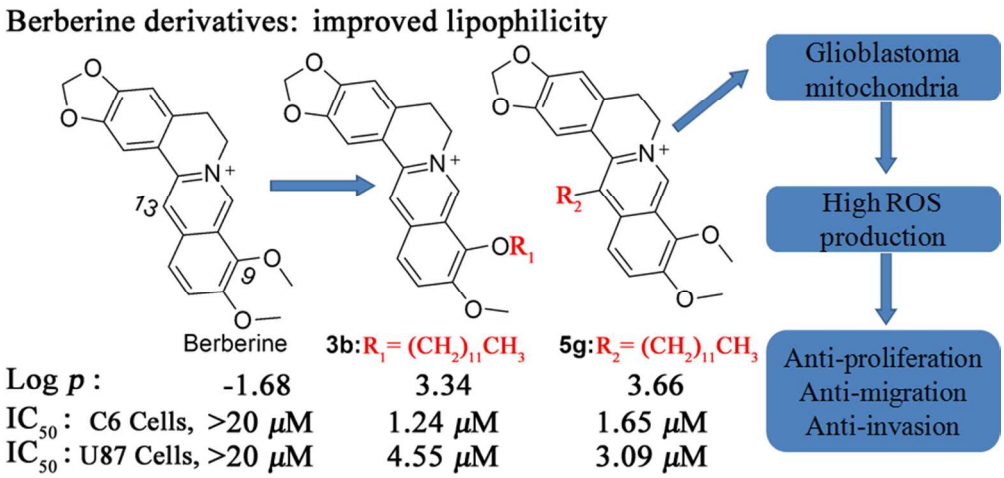

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