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# Synthesis and anticancer activity of novel 9,13-disubstituted berberine derivatives

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**ABSTRACT**

Novel berberine derivatives with disubstituents on positions C9 and C13 were synthesized and evaluated for antiproliferative activities against human prostate cancer cell lines (PC3 and DU145), breast cancer cell line (MDA-MB-231) and human colon cancer cell lines (HT29 and HCT116). All compounds showed significantly enhanced antiproliferative activities compared with berberine. Notably, compound 18e exhibited the strongest cytotoxicity against PC3 cells with an $IC_{50}$ value of 0.19 μM, and the highest selectivity index ($SI^{PC3}$>20). Further studies showed that 18e could arrest the cell cycle at G1 phase, and significantly inhibit tumor cell colony forming and migration even at low concentrations. Interestingly, 18e could significantly induce cytoplasmic vacuolation, suggesting a different mode of action from berberine.

**Keywords:** berberine derivatives, lipophilic group, anticancer activity, colony forming, cell migration

Cancer is one of the most serious clinical problems and global health burden with increasing incidence every year[1]. In order to find new anticancer agents, natural products have been widely used in anticancer drug screening studies. Berberine (Chart 1), an isoquinoline alkaloid extracted from *Coptis chinensis*, has a variety of pharmacological effects, such as anti-inflammatory[2,3], antibacterial[4-6], antiviral[7-9], neuroprotective[10,11], and hypoglycemic[12,13] activity. After its anti-tumor activity was reported in 1959 for the first time, berberine has been proved to be potent against multiple tumor cells[14-18]. It has been confirmed that berberine can induce apoptosis[19-21], autophagy[20,22], cell cycle arrest[15,23] and suppress migration[23] to affect the development of cancer cells, but the mechanism is complicated, involving a large number of signaling pathways.

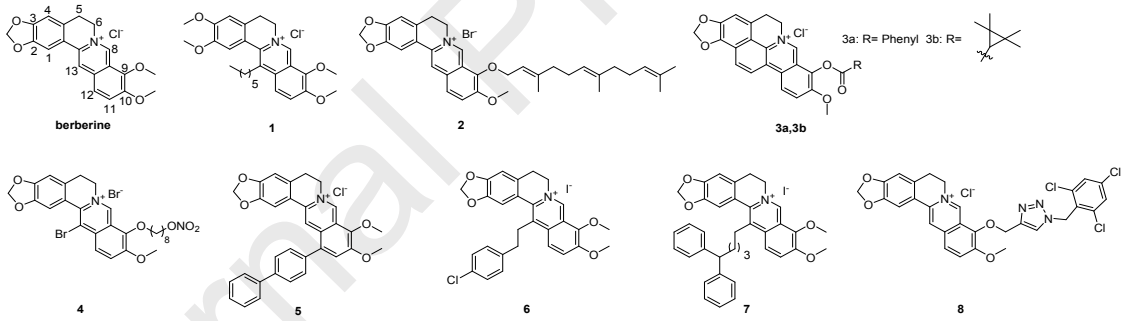



**Chart 1.** Berberine and reported berberine derivatives with anti-tumor activity

In the past decade, different types of berberine derivatives have been reported, with antiproliferative activity on various tumor cell lines, such as lung cancer cells[24], leukemic cells[25], colon cancer cells[26,27], breast cancer cells [26-30], glioma cells[31], and liver cancer cells [32-34]. As shown in Chart 1, most of the structural modifications focused on monomodification at C9 or C13

position of berberine. And in the modification at C9, most of the reported works preferred to remove the methyl group first, followed by nucleophilic substitution with halo alkane or acid. Hence, almost all the reported anti-tumor derivatives maintained the O atom at C9 position of berberine.

In our previous work, a series of 9,13-disubstituted berberine derivatives were designed and synthesized in order to search for new chemical classes of potential antibacterial agents[35]. Unlike the reported anti-tumor berberine derivatives, these compounds have disubstituted modifications at both C9 and C13 positions. More attractively, the oxygen atom at C9 is replaced by a nitrogen atom, which may result in a different hydrogen bonding with the potential target. In this case, some compounds were selected and tested for anti-tumor activity.

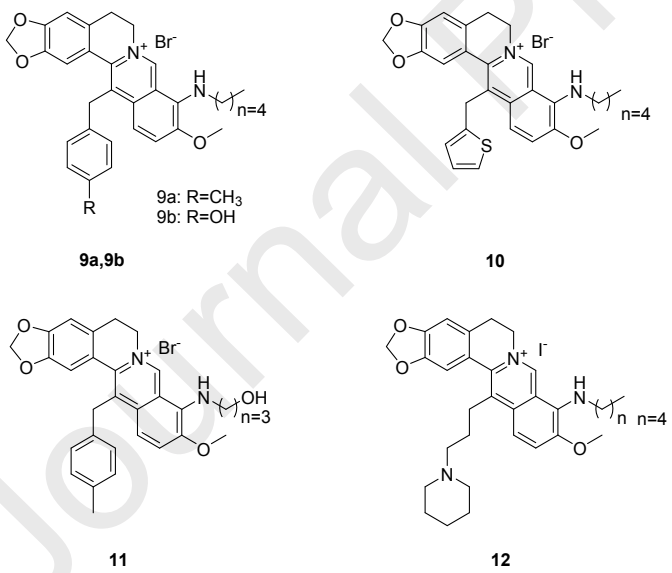



**Chart 2.** 9,13-disubstitute berberine derivatives in our previous report

With berberine as the positive control, their antiproliferative activities on human prostate cancer cell lines (PC3 and DU145), breast cancer cell line (MDA-MB-231) and human colon

cancer cell lines (HT29 and HCT116) were shown in Table 1. These disubstituted derivatives showed enhanced antiproliferative activity compared with berberine, and the most potent compound 9a has $IC_{50}$ values lower than 1μM on PC3 and HT29 cells. Compared with 9a, the increase in substituent's hydrophilicity of 9b, 10, 11 and 12 is accompanied by a decrease in the antiproliferative activity. Therefore, we suspected that increased lipophilicity may favor the anti-tumor activity of these derivatives. Thus, a series of 9,13-disubstituted berberine derivatives with different lipophilic groups were designed and synthesized, in order to reveal structure-activity relationship and search for new anticancer agents.

**Table 1**

Antiproliferative activity of compounds 9-12 against five tumor cell lines.

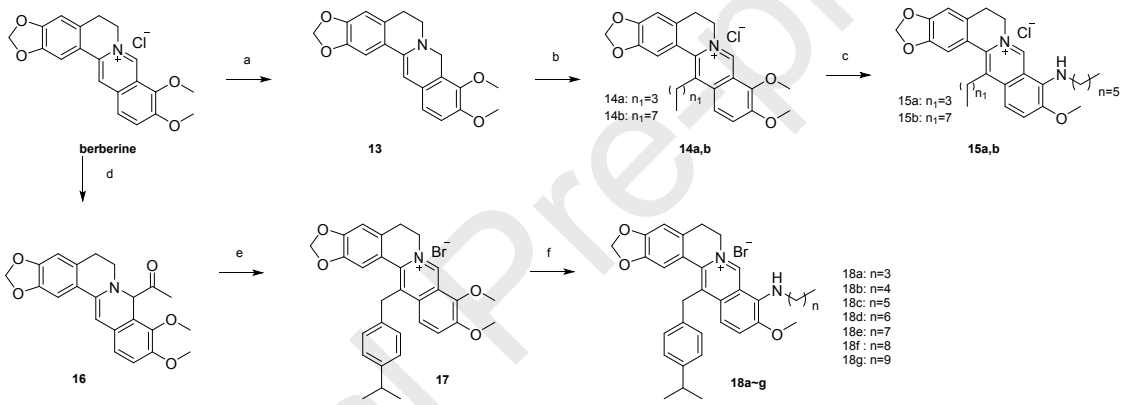
| Comp | $IC_{50}$[a] (μM) | | | | |
|---|---|---|---|---|---|
| | PC3 | DU145 | MDA-MB-231 | HCT-116 | HT-29 |
| 9a | 0.71±0.08 | 1.82±0.21 | 1.27±0.17 | 1.92±0.07 | 0.97±0.10 |
| 9b | 4.36±0.16 | 6.03±0.20 | 6.23±0.32 | 4.01±0.16 | 5.21±0.27 |
| 10 | 2.51±0.07 | 3.08±0.15 | 3.98±0.78 | 1.19±0.05 | 1.34±0.06 |
| 11 | 4.20±0.28 | 5.91±0.38 | 5.75±0.79 | 3.84±0.10 | 4.97±0.07 |
| 12 | 18.32±0.82 | > 10 | > 10 | > 10 | > 10 |
| BBR[b] | 14.39±3.14 | >20 | 20.6±3.093 | > 10 | > 10 |

[a] $IC_{50}$: concentration of the test compound that inhibits 50% of cell growth. Results are expressed as the mean ± SD (n=3).

[b] BBR is the abbreviation of berberine.

All the compounds were synthesized according to Scheme 1. The commercially available

berberine was reduced by $NaBH_4$ in 5% NaOH to afford 13 in 71% yield. 13 was dissolved in EtOH and heated with acetic acid and *n*-butyl aldehyde or *n*-octanal at 85°C for 5 h to get 14a and 14b in 58% and 68% yields, respectively. 15a and 15b were synthesized in 60% and 82% yields by heating 14a and 14b with *n*-hexylamine at 100℃ for 8h. Treating berberine with 5N NaOH and acetone for 1h afforded 16, then 16 reacted with 4-isopropyl benzyl bromide in acetonitrile in the presence of NaI for 4h to give 17 in 63% yield over two steps. 17 reacted with various amine without solvent at 100℃ about 8-15h to afford 18a~18g in 60%-85%.




**Scheme 1.** Synthesis of 9,13-disubstituted berberine derivatives. Reagents and conditions: (a) $NaBH_4$, 5% NaOH, rt, 1h, 71%; (b) *n*-butyl aldehyde or *n*-octanal, acetic acid, EtOH, 85°C, 5 h; HCl, $H_2O$, 1h, 58%-68% ; (c) *n*-hexylamine,100°C, 8h, 60%-82%; (d) 5N NaOH, acetone, rt, 1 h; (e) 4-isopropyl benzyl bromide, NaI, $CH_3CN$, 80°C, 4h, 63% (over two steps); (f) various amine, 100℃, 8-15 h, 60%-85%.

The chemical structure of representative compound **18e** was further confirmed by an X-ray crystallography assay[36] (Figure 1).

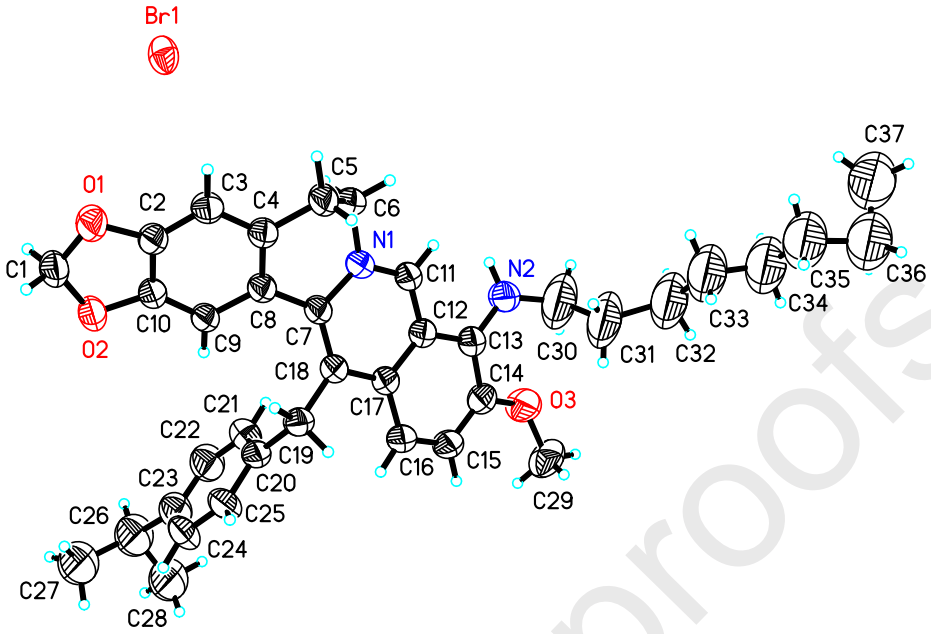



**Figure 1.** X-ray single crystal structure of **18e**

The antiproliferative activities of compounds 15a-b and 18a-g were analyzed using SRB assay on human prostate cancer cell lines (PC3 and DU145), breast cancer cell line (MDA-MB-231) and human colon cancer cell lines (HT29 and HCT116) as well as human aortic fibroblasts (HAF) with berberine as a positive control. The results were expressed as $IC_{50}$ values that were calculated using GraphPad software (Table 2). Compared with that of berberine, the antiproliferative activities of these derivatives were greatly enhanced due to the introduction of the lipophilic groups at C9 and C13 positions. All tested compounds showed strong cytotoxicity against PC3, DU145, HCT-116 and HT-29 cell lines with $IC_{50}$ values lower than 1μM. Moreover, these compounds appeared to be more cytotoxic to PC3 cells while MDA-MB-231 cells less sensitive to them.

**Table 2**

Inhibitory effect ($IC_{50}$ values) of 9,13-disubstituted berberine derivatives on five cancer cell lines and human aortic fibroblasts.

| Comp | $IC_{50}$[a] (μM) | | | | | | [b]$SI^{PC3}$ |
|---|---|---|---|---|---|---|---|
| | PC3 | DU145 | MDA-MB-231 | HCT-116 | HT-29 | HAF | |
| 15a | 0.87±0.23 | 0.92±0.33 | 1.33±0.42 | 0.86±0.27 | 0.70±0.06 | n.t.[c] | - |
| 15b | 0.30±0.08 | 0.81±0.15 | 1.16±0.29 | 0.53±0.03 | 0.59±0.98 | 1.59±0.98 | 5.3 |
| 18a | 0.64±0.03 | 0.97±0.01 | 3.64±0.16 | 0.90±0.09 | 0.69±0.04 | n.t. [c] | - |
| 18b | 0.45±0.01 | 0.91±0.01 | 1.27±0.08 | 0.92±0.11 | 0.57±0.02 | n.t. [c] | - |
| 18c | 0.41±0.01 | 0.88±0.06 | 1.28±0.06 | 0.62±0.01 | 0.58±0.06 | n.t. [c] | - |
| 18d | 0.38±0.03 | 0.72±0.11 | 1.08±0.11 | 0.48±0.03 | 0.54±0.01 | 3.79±0.64 | 10.0 |
| 18e | 0.19±0.01 | 0.74±0.07 | 1.31±0.17 | 0.55±0.03 | 0.56±0.02 | 3.95±0.29 | 20.8 |
| 18f | 0.20±0.05 | 0.51±0.03 | 1.55±0.62 | 0.46±0.03 | 0.51±0.01 | 3.52±0.41 | 17.6 |
| 18g | 0.20±0.02 | 0.68±0.06 | 0.94±0.19 | 0.46±0.02 | 0.52±0.03 | 2.69±0.12 | 13.4 |
| BBR | 14.39±3.14 | >20 | 20.6±3.10 | > 10 | > 10 | 86.12±2.75 | 6.0 |

[a]: $IC_{50}$: Results are expressed as the mean ± SD (n=3).

[b]: Selectivity $Index^{PC3}$ ($SI^{PC3}$) = $IC_{50}$(HAF)/$IC_{50}$(PC3)

[c]: n.t = not tested

In order to improve the lipophilicity, the *p*-methylphenyl group at C13 position of compound 9a was replaced by a *p*-isopropylphenyl substituent. The obtained 18b showed stronger anti-tumor activity than 9a with approximately halved $IC_{50}$ values on PC3, DU145, HCT-116 and HT-29 cell lines. Similarly, 15b, with a longer carbon chain at C13, is more potent than 15a, which suggested

the introduction of a more lipophilic group at C13 enhanced the anti-tumor activity as we expected. Unfortunately, 15b also exhibited strong cytotoxicity on HAF cells with selectivity index (SI) as low as 5.3.

The relationship between the carbon chain's length at C9 position and their anti-tumor activity has also been explored. Compared the antiproliferative activity against PC3 cells, we found that a longer carbon chain at C9 position means a better activity. For example, 18a, 18c and 18e, with 2, 4, and 6 carbon chains, give $IC_{50}$ values of 0.64μM, 0.41μM and 0.19μM respectively. This indicates that an increase of the lipophilicity at C9 position results in the promotion of their antiproliferation activity on PC3 cells. The same rule was also found on other cells. Consequently, 18f and 18g, with further lengthened carbon chain, were synthesized and tested. However, no obvious ameliorative activity was observed with the cytotoxicity on HAF lightly enhanced. Among all these compounds, 18e achieved a balance of anti-tumor activity and cytotoxicity on HAF, with the strongest antiproliferative activity against tumor cells and the highest selectivity index ($SI^{PC3}$>20).

Given the strong cytotoxicity of 18e against PC3 cell line, cell cycle arrest caused by 18e was examined. After treating PC3 cells with 18e in different concentrations for 24h, the results were analyzed using a flow cytometer. Compared with the control (54.67% of cells in G1 phase), the percentage of cells in G1 phase has increased to 57.16% (0.5μM), 59.08% (1μM), and 66.21% (2μM), along with the reduction of cells in S phase (Figure 2). This indicates that 18e could arrest the cell cycle in G1 phase in a dose-dependent manner.

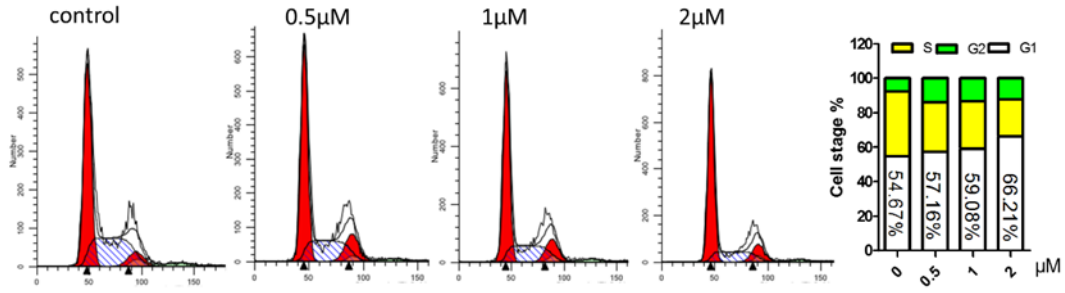



**Figure 2.** Cell cycle analysis of PC3 cells treated with compound 18e at concentrations of 0.5, 1 and 2 μM for 24 h, showing enhanced dose-dependent G1 arrest with treatment of 18e.

PC3 cells were treated with 18e at different concentrations（0.1, 0.5, 1, 2μM）for a week, and the colony forming was examined. As shown in Figure 3, 18e inhibited PC3 cell colony forming in a dose-dependent manner. Treatment of 18e caused ~50% inhibition on the colony forming at 0.1μM and ~90% inhibition at 0.5μM. We couldn't find any colonies even at a medium concentration (1μM), demonstrating the remarkable inhibition to colony forming.

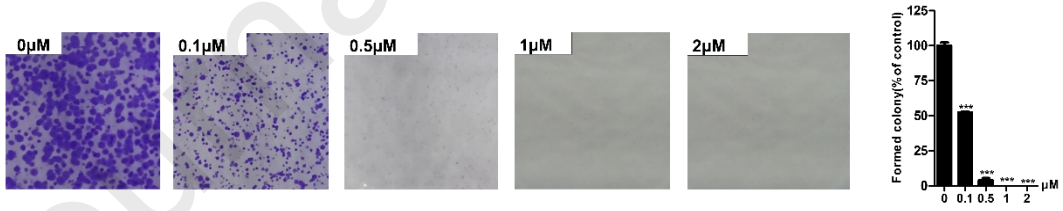



**Figure 3.** Colony forming assay of PC3 cells with treatment of 18e at concentrations of 0.1, 0.5, 1, 2μM. Number of colonies counted in experiments repeated three times. Results were represented the average of three replications, **P<0.01; ***P<0.001.

Cell migration is a fundamental and necessary step in the process of cancer metastasis, and inhibition of migration is significant in anticancer chemotherapy. Anti-migration ability of 18e

was assessed by a transwell cell migration experiment. PC3 cells were treated with different concentrations of 18e and their migration was examined after 24 h. As shown in Figure 4, compared to the blank contrast group, 18e inhibited cell migration to ~60% at 0.5μM, ~40% at 1μM and ~10% at 2μM, indicating 18e inhibited migration in a dose-dependent manner.

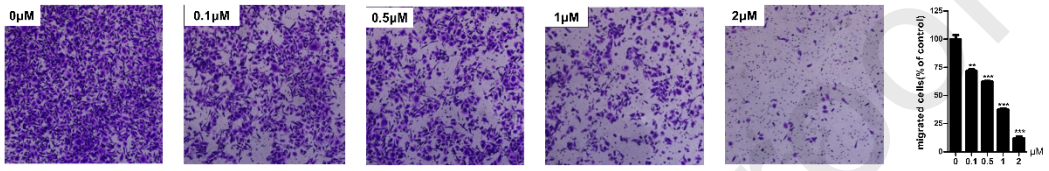



**Figure 4.** Anti-migration effect of compound 18e on PC3 cells at concentrations of 0.1, 0.5, 1 and 2μM via transwell migration assay. Results were represented the average of three replications, **P<0.01; ***P<0.001.

Observing cell morphological changes is a simple way to distinguish cell death patterns. To explore whether 18e induces cell death in the same way with berberine, morphological changes were observed using a white light microscope. PC3 cells were treated with different concentrations of 18e and berberine for 24 hours respectively, and their morphological features were photographed as shown in Figure 5. Compared with the control group, 18e could significantly cause cytoplasmic vacuolization at low concentrations (0.5μM, 1μM), while berberine couldn't cause vacuolization even at a high concentration (50μM), suggesting that they may not share the same mode of action. In order to learn more about the mechanism, some biological experiments are underway.

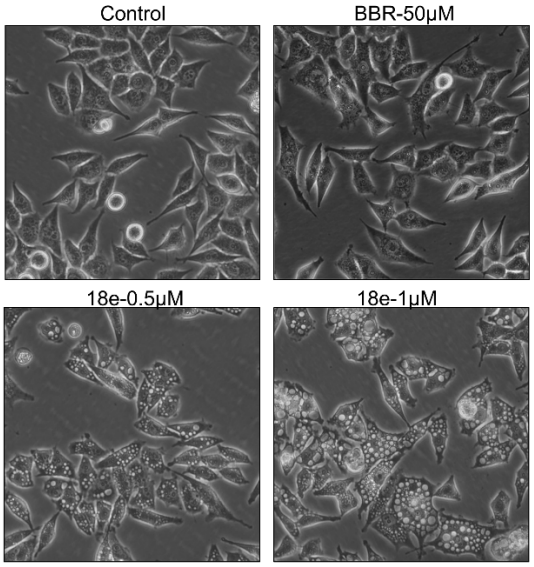



**Figure 5.** Cell morphological change of PC3 cell lines after 24 h exposure with 50μM berberine and 0.5 or 1.0μM compound 18e. Photographs were obtained with microscope (Olympus).

As a result, all of the newly synthesized 9,13-disubstituted berberine derivatives exhibited enhanced antiproliferative activity compared to the initial compound 9a. The structure-activity relationship showed that the introduction of lipophilic substituents at both C9 and C13 positions favored their anti-tumor activity as we expected. The most potent compound, 18e, not only showed strong antiproliferative activity and acceptable selectivity, but also inhibited cell migration and colony forming at low concentrations. Due to its remarkable drug efficacy in vitro, a study on the anti-tumor effect in vivo is underway. In addition, 18e could arrest the cell cycle in the G1 phase. And interestingly, it could induce cytoplasmic vacuolation, implying a different mode of action from berberine. A further research on the mechanism is also in progress.

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36. CCDC number of **18e**: CCDC 1939417.

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

| |
|---|
| Declarations of interest: none' in the template. |

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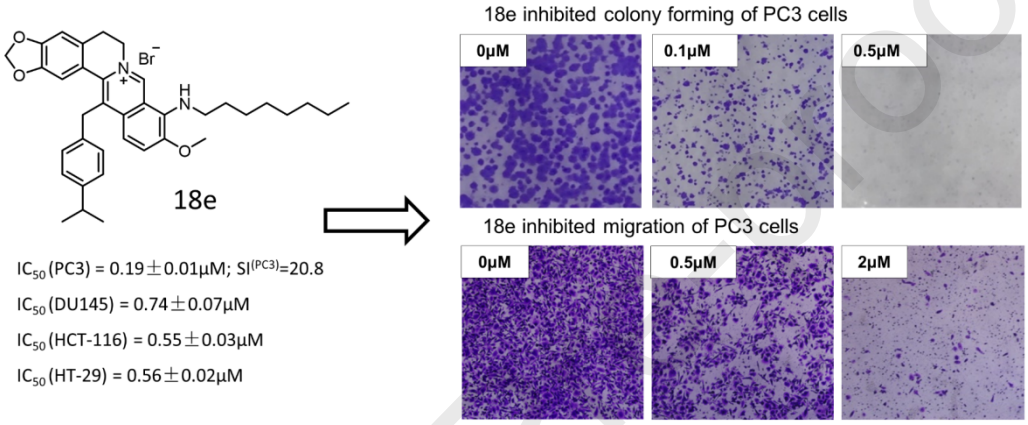


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