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Chemical Constituents from *Macleaya cordata* (Willd) R. Br. and Their Phenotypic Functions against a Parkinson's Disease Patient-derived Cell Line

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Abstract

Cytological profiling (CP) assay against a human olfactory neurosphere-derived (hONS) cell line using a library of traditional Chinese medicinal plant extracts gave indications that the ethanolic extract of *Macleaya cordata* elicited strong perturbations to various cellular components. Further chemical investigation of this extract resulted in the isolation of two new benzo[*c*]phenanthridine alkaloids, (6*R*)-10-methoxybocconoline (**1**) and 6-(1-hydroxyethyl)-10-methoxy-5,6-dihydrochelerythrine (**2**). Their planar structures were elucidated by extensive 1D and 2D NMR studies, together with MS data. The absolute configuration for position C-6 of **1** and relative configurations for position C-6 and C-1' of **2** were assigned by density functional theory (DFT) calculations of ECD and NMR data, respectively. Also isolated were fourteen known metabolites, including ten alkaloids (**3–12**) and four coumaroyl-containing compounds (**13–16**). Cytological profiling of the isolates against Parkinson's Disease (PD) patient-derived olfactory cells revealed bocconoline (**3**) and 6-(1-hydroxyethyl)-5,6-dihydrochelerythrine (**4**) significantly perturbed the features of cellular organelles including early endosomes, mitochondria and autophagosomes. Given that hONS cells from PD patients model some functional aspects of the disease, the results suggested that these phenotypic profiles may have a role in the mechanisms underlying PD and signified the efficacy of CP in finding potential chemical tools to study the biological pathways in PD.

Keywords: Parkinson's disease, phenotypic screening, *Macleaya cordata*, DFT, NMR.

1. Introduction

Recognized as the world's second most common neurodegenerative disorder after Alzheimer's Disease, Parkinson's Disease (PD) is pathologically characterized by the progressive degeneration of dopaminergic neurons and the aggregation of the α synuclein protein (Lewy bodies) in the brain.^{1, 2} Unfortunately, PD is currently incurable, mainly due to our limited understanding of its underlying molecular mechanisms. In other words, there are no confirmed or validated targets which have disease-modifying effects for PD drug discovery to date.^{3–5} Cytological profiling^{6–9} (CP) is a multiparametric, imaged-based phenotypic screening approach that is capable of effectively quantifying multiple biological responses of cells. This technique integrates automated fluorescence microscopy and image analysis to evaluate the effects of small molecules on multiple cellular components, thus generating invaluable phenotypic information.² Owing to its

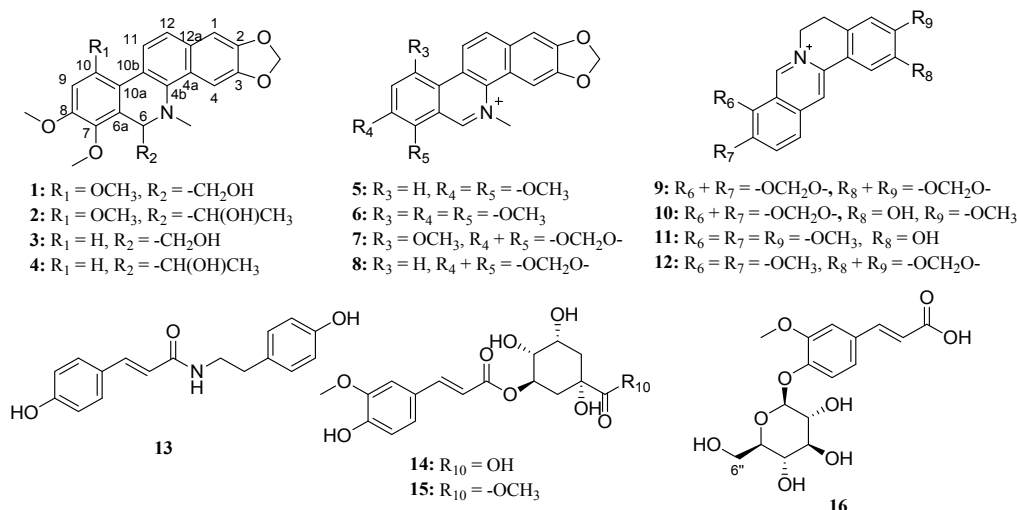
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ability to capture a global, unbiased view of cellular functions,¹⁰ CP has been widely incorporated into the studies of several complex diseases such as cancers,¹¹⁻¹³ bacterial infections^{14, 15} and neurological diseases.¹⁶ The non-transformed and non-immortalized human olfactory neurosphere-derived (hONS) cells, which are primary cells biopsied from Parkinson's disease patients, are thought to be able to recapitulate some functional aspects of PD, proposedly due to the disease-specific differences in term of metabolic features and phenotypes between PD patient-derived cells and those of healthy controls.¹⁷⁻²⁰ Constitutively, given the intricate molecular networks of PD and the distinctions of hONS cells, phenotypic research of these cells using CP could potentially uncover important biological pathways underlying PD.

As part of our aim to identify small molecules for PD phenotypic study, a library of plant extracts was screened against hONS cells using CP.²¹ One extract sourced from a Chinese herbal plant, *Macleaya cordata*, showed significant perturbations to cellular components and therefore was selected for further investigation. *Macleaya cordata* (Willd) R. Br. belongs to the genus *Macleaya* of the family Papaveraceae.^{22, 23} Like many other plants, *M. cordata* possesses various medicinal values and is a rich source of structurally diverse, biologically active natural products. To date, more than one hundred metabolites have been identified in this species, most of which are isoquinoline alkaloids including benzophenanthridines, protopines, protoberberines, and a few other alkaloid-type compounds with interesting bioactivities.²³

Chemical investigation of the crude ethanolic extract of *M. cordata* resulted in the identification of two new benzo[*c*]phenanthridine alkaloids, (6*R*)-10-methoxybocconoline (**1**) and 6-(1-hydroxyethyl)-10-methoxy-5,6-dihydrochelerythrine (**2**), together with fourteen known metabolites, namely bocconoline (**3**), 6-(1-hydroxyethyl)-5,6-dihydrochelerythrine (**4**), chelerythrine (**5**), chelilutine (**6**), chelirubine (**7**), sanguinarine (**8**), coptisine (**9**), tetrahydroscoulerine (**10**), columbamine (**11**), berberine (**12**), *p*-coumaroyltyramine (**13**), 3-*O*-feruloylquinic acid (**14**), methyl 3-*O*-feruloylquinic acid (**15**) and ferulic acid 4-*O*-glucoside (**16**).

All isolates were subject to an unbiased phenotypic assay against hONS cells, followed by statistical analysis to determine the compounds inducing strong responses. In this paper, the isolation and structure elucidation of the two new compounds (**1** and **2**), as well as the phenotypic effects of the isolated natural products from *M. cordata* are reported.



2. Results and discussion

The air-dried and ground *M. cordata* (750 g) was extracted with 95% ethanol in water for 24 h (5 L x 3) to obtain the crude extract (40 g). A portion of this extract (20 g) was subject to polyamide flash column chromatography with step wise gradients from H₂O to MeOH. Fractions 30% MeOH and 40% MeOH contained diagnostic ¹H NMR signals of benzo[*c*]phenanthridine alkaloids, while fractions 70% MeOH and 90% MeOH gave MS signals of potentially new compounds. These fractions were further purified by RP-HPLC (MeOH/H₂O/0.1%TFA) to obtain compounds **3–16** and by preparative TLC to isolate the new compounds **1** and **2**.

Compound **1** was obtained as white powder (0.1 mg, 0.00002% dry wt.). Its (+)-HRESIMS data gave a [M+H]⁺ ion at *m/z* 410.1600 consistent with a molecular formula of C₂₃H₂₃NO₆ (calcd. 410.1598) with thirteen degrees of unsaturation. The ¹H NMR and HSQC data showed the presence of three aromatic singlets (δ_{H} 7.66, 7.11, and 6.59), two aromatic doublets with *ortho*-coupling system (δ_{H} 8.32 and 7.47), one pair of geminal-coupled methylene doublets (δ_{H} 6.06 and 6.04), one methine doublet of doublets (δ_{H} 4.64), one set of two methylene multiplets (δ_{H} 3.48 and 3.02), three methoxy singlets (δ_{H} 3.95, 3.93 and 3.89), one *N*-methyl singlet (δ_{H} 2.71) and one exchangeable doublet (δ_{H} 2.75). The pair of methylene doublets at δ_{H} 6.06 and 6.04, along with their HSQC correlation to the carbon signal at δ_{C} 101.2 suggested the existence of an oxymethylene group which was further confirmed by its characteristic IR absorptions at 1041 and 947 cm⁻¹.²⁴ This information, together with the *N*-methyl and aromatic proton signals gave a partial indication of the dihydrochelerythrine skeleton.^{25, 26} Further analysis of the NMR spectral data revealed that there was a high degree of similarity between the ¹H NMR spectrum of **1** and that of bocconoline (**3**). The most notable differences were an addition of a methoxy group and a conversion of two *ortho*-coupled aromatic doublets in **3** to one aromatic singlet in **1**. These observations strongly suggested that **1** was a methoxy-substituted analogue of bocconoline, and the substitution would happen at one in four positions C-9, C-10, C-11, and C-12. The HMBC data showed correlations from the above aromatic singlet (δ_{H} 6.59) to the two oxygenated aromatic carbons C-7 (δ_{C} 140.5) and C-8 (δ_{C} 152.5), indicating that the singlet was at position C-9. This led to the assignment of the additional methoxy group to position C-10, which was further confirmed by the ROESY correlations from the protons of that methoxy group (δ_{H} 3.93) to the aromatic doublet (δ_{H} 8.32) of C-11. Constituently, the planar structure of **1** was determined as 10-methoxybocconoline. **Table 1** summarized the NMR spectral data of **1**, and **Figure 1** displayed key correlations for this compound.

Position	1		2	
	δ_{H} (ppm), J (Hz)	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)	δ_{C} (ppm)
1	7.11 (s, 1H)	104.5	7.11 (s, 1H)	104.6
2	-	148.3	-	148.4
3	-	147.7	-	147.7
4	7.66 (s, 1H)	99.8	7.65 (s, 1H)	99.5
4a	-	127.0	-	126.7
4b	-	138.2	-	138.4
6	4.64 (dd, 4.8, 10.4, 1H)	60.0	4.18 (d, 9.6, 1H)	64.9
6a	-	128.6	-	128.2
7	-	140.5	-	141.1
8	-	152.5	-	152.9
9	6.59 (s, 1H)	97.5	6.60 (s, 1H)	97.4
10	-	153.7	-	153.5
10a	-	113.4	-	113.0
10b	-	123.1	-	123.4
11	8.32 (d, 8.8, 1H)	124.6	8.31 (d, 8.8, 1H)	124.6
12	7.47 (d, 8.8, 1H)	123.5	7.46 (d, 8.8, 1H)	123.5
12a	-	130.7	-	130.7
-OCH ₂ O-	6.06 (d, 1.6, 1H, H _a)	101.2	6.06 (d, 1.6, 1H, H _a)	101.2
	6.04 (d, 1.6, 1H, H _b)		6.05 (d, 1.6, 1H, H _b)	
N-CH ₃	2.71 (s, 3H)	42.4	2.68 (s, 3H)	41.8
7-OCH ₃	3.89 (s, 3H)	61.5	3.87 (s, 3H)	61.0
8-OCH ₃	3.95 (s, 3H)	56.3	3.96 (s, 3H)	56.0
10-OCH ₃	3.93 (s, 3H)	56.0	3.93 (s, 3H)	56.2
1'	3.48 (m, 1H, H _{1'a})	61.6	3.14 (dq, 9.6, 5.6, 1H)	66.6
	3.02 (t, 10.4, 1H, H _{1'b})			
1'-OH	2.75 (d, 10.4, 1H)	-	-	-
2'	-	-	1.09 (d, 5.6, 3H)	18.8

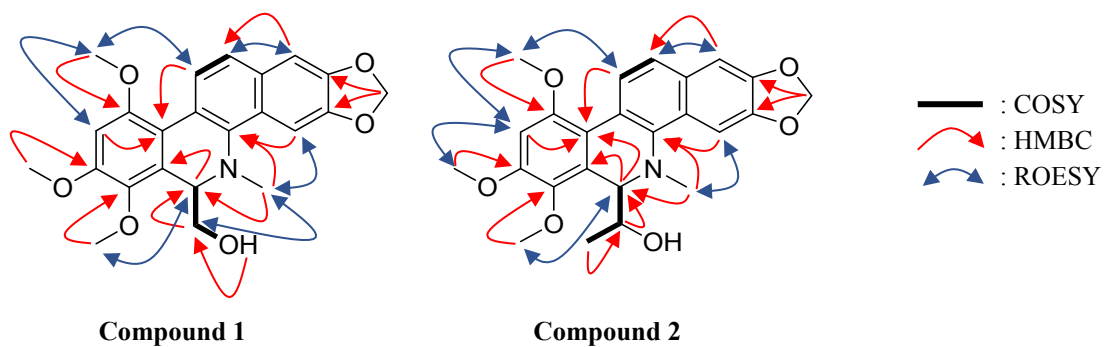


Figure 1 Key ^1H - ^1H COSY (—), HMBC (→) and ROESY (↔) correlations of 10-methoxybocconoline (**1**) and 6-(1-hydroxyethyl)-10-methoxy-5,6-dihydrochelerythrine (**2**).

The absolute configuration (AC) of **1** was established by DFT calculations of electronic

circular dichroism (ECD) data. DFT is a powerful technique in computational chemistry that contends with problems regarding electronic structure theory.²⁷ The ability of DFT in generating a theoretical electron density can lead to predictions of many physical properties of a molecule such as vibrational frequencies,²⁸ optical properties²⁹ or NMR chemical shifts.^{30, 31} DFT has been increasingly implicated in recent research owing to the improvement of computational technologies,³² and in several instances this has confirmed regiochemistry of functional groups or the stereochemistry of various small organic molecules.^{30, 33–36} In this study, time-dependent (TD)-DFT at the level B3LYP/6-31G(d) was employed to generate theoretical ECD spectra which were used to assist the AC assignment of position C-6 in compound **1**. Having been described in many previous literatures, the concept of this approach is based on the comparison of the experimental and DFT-calculated ECD data.^{34, 37, 38} Basically, the more closely they match, the more confidently or reliably the correct structure is assigned.³² As displayed in **Figure 2**, the experimental ECD spectrum of compound **1** (black graph) exhibited four positive Cotton

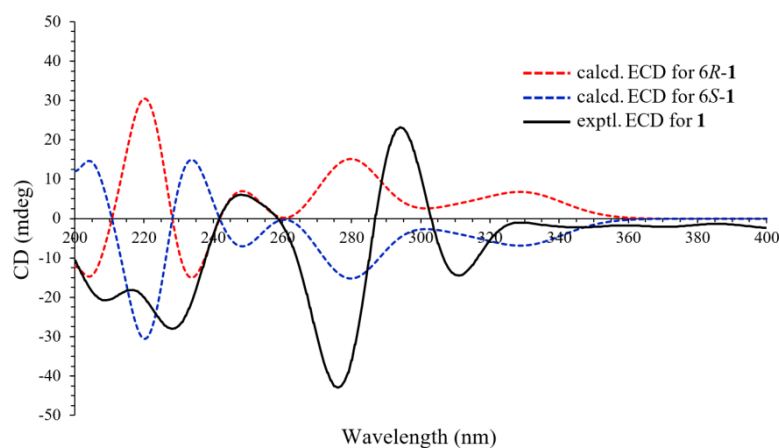


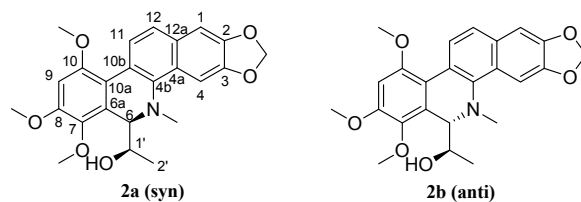
Figure 2 Calculated ECD spectra of 10-methoxybocconoline (red and blue dotted curve) and experimental ECD data obtained for compound **1** (black curve). The ECD spectrum of the 6*S* configuration was generated by reflecting that of the 6*R* configuration over the wavelength axis. The intensity of the experimental spectrum was scaled by a factor of 6, followed by a deconvolution transformation.

effects at 215–225 nm, 245–250 nm, 290–295 nm, and 325–335 nm. These effects were relatively corresponding to those seen in the calculated ECD spectrum of the (6*R*) conformer (red dotted graph). Nonetheless, it was noteworthy that the negative Cotton effect at 270–275 nm and the positive one at 290–295 nm in the experimental data were shifted toward higher wavelengths compared to those at 260 and 280 nm in the (6*R*)-calculated spectrum, respectively. This was probably due to variation of algorithms used in DFT. Although the hybrid functional B3LYP with the basis set 6-31G(d) are commonly used and believed to give satisfactory outcomes in ECD calculations,^{32, 37} it has been reported that in some cases, data generated from this combination did not match well with those observed in experiments and that computing at a different level provided better results.^{32, 39} Consequently, given that both experimental and (6*R*)-calculated ECD spectra of **1** exhibited the same number of ECD maxima at relatively similar wavelength regions, compound **1** was proposed to be (6*R*)-10-methoxybocconoline.

Compound **2** was isolated as white powder (0.1 mg, 0.00002% dry wt.). The (+)-HRESIMS analysis for this compound returned a protonated molecular ion ($[\text{M}+\text{H}]^+$) at m/z 424.1752 consistent with a molecular formula of $\text{C}_{24}\text{H}_{25}\text{NO}_6$ (calcd. 424.1755).

Diagnostic features of the dihydrochelerythrine scaffold were also present in the ^1H NMR spectral data of **2**, such as the dioxolane doublets at δ_{H} 6.05 and 6.06, the methoxy singlets at δ_{H} 3.87, 3.93 and 3.96, the aromatic resonances from δ_{H} 6.50 to δ_{H} 9.00, and the *N*-methyl singlet at δ_{H} 2.68. This was also supported by the ^{13}C NMR data (**Table 1**). ^1H NMR comparison between **1** and **2** revealed that the $-\text{CH}_2\text{OH}$ group at position C-6 in **1** was replaced by a $-\text{CH}(\text{OH})\text{CH}_3$ moiety in **2**. This was evident by an additional methyl doublet at δ_{H} 1.09 and the presence of a methine doublet of quartets at δ_{H} 3.14; both signals showed COSY correlations to each other with a coupling constant of 9.6 Hz. The HMBC data confirmed that the $-\text{CH}(\text{OH})\text{CH}_3$ moiety was connected to position C-6 of the dihydrochelerythrine skeleton by a correlation from the doublet of quartets to the carbon C-6 at δ_{C} 64.9. This assignment was also allowed by the ROESY data which exhibited compatible correlations. **Figure 1** shows main 2D NMR (COSY, HMBC and ROESY) correlations for **2**, and **Table 1** gives a summary of the spectroscopic data for this compound. The planar structure of **2** was therefore elucidated as 6-(1-hydroxyethyl)-10-methoxy-5,6-dihydrochelerythrine.

Efforts were made to assign the absolute configuration for **2** by using the same ECD calculation strategies as for **1**. Unfortunately, this was unsuccessful due to the inconsistencies between the experimental and calculated ECD data of **2**. The limited amount of this compound has also prevented other techniques such as chemical derivatization or crystallography. Our interest was then shifted to determining the relative configuration for compound **2** by comparing its experimental and theoretical NMR data.^{30, 40} DFT was again employed to calculate the NMR shielding constants for **2**. Specifically, a pair of corresponding diastereomeric structures of **2** (**2a** and **2b**) were subject to systematic conformational search using the OPLSe3 forcefield⁴¹ and simulated water solvent (GB/SA).⁴² Each conformer with minimum energy was further optimized by DFT calculations at the B3LYP/6-31G(d,p) level. The M062X/def2-TZVP level incorporating IEF-PCM solvation modelling^{43, 44} was used to calculate single-point energies of the optimized conformers which were subsequently utilized to scale the calculated NMR parameters relative to their Boltzmann populations. NMR shielding tensors were generated by MPW1PW91/6-311+G(2d,p) for proton and wB97XD/6-311+G(2d,p) for carbon using gauge including atomic orbitals (GIAO) method.⁴⁵ To obtain the scaled theoretical NMR chemical shifts for **2a** and **2b**, the calculated shielding constants were empirically corrected using a linear regression model of the relationship between the observed chemical shifts and their corresponding calculated NMR data of known compounds.^{31, 46} The reported theoretical chemical shifts (**Table S1, Supporting Information**) were the Boltzmann-weighted average of all values generated from the minimal energy conformers of each isomer.



It was our interest to see whether the stereocenters C-6/C-1' in the syn arrangement (**2a**) could be distinguished from the C-6/C-1' in the anti arrangement (**2b**). Analysis of the calculated NMR values revealed that there were little differences in the ^{13}C NMR

chemical shifts for the positions C-6 and C-1' between the two isomers. However, the ^1H NMR data for H-6 and H-1' in **2a** were significantly different to those seen in **2b**. More importantly, the computed NMR data for the syn isomer were much closer to the observed data compared to those for the anti isomer, especially at the two stereocenters, suggesting that the relative configuration for compound **2** would be the syn isomer. This was supported by the mean absolute errors (MAE) and maximum deviations (MD) calculated for both isomers (**Table 2**). As seen in **Table 2**, the MAEs for ^1H NMR (0.04 ppm) and ^{13}C NMR (1.07 ppm) of **2a** appeared much smaller than those of **2b** (0.12 and 1.48 ppm, respectively). Similar results were also seen for MDs, which helped confirm the syn arrangement of compound **2**.

To add more evidence to the preferred arrangement, the proton coupling constants $^3J_{6-1'}$ were also calculated for the above two diastereomers by DFT and subsequently compared with that derived from experiment. For each contributing conformer of **2a** and **2b**, the dihedral angle between H-6 and H-1' was calculated and used to obtain a $^3J_{\text{H-H}}$ coupling constant according to the method of Haasnoot et al.⁴⁷ The final coupling constants for **2a** and **2b** (**Table S1, Supporting Information**) were weighted based on the Boltzmann distributions using the corresponding energies described above. The **2a** and **2b** diastereomers had a $^3J_{6-1'}$ coupling constant of 9.8 and 6.0 Hz, respectively, whereas the experimental value was 9.6 Hz. This again was in favor of the syn configuration. Furthermore, the analysis also incorporated DP4 probability,^{48, 49} which is a formulation commonly integrated into DFT NMR calculations to estimate the probability that a chosen solution is correct.⁵⁰ There have been a number of research that successfully utilized DP4 in solving problems related to stereochemistry.^{30, 48-51} For example, the structure of a fungal metabolite acremine P was recently corrected by comparison of DFT-calculated and experimental ^{13}C NMR data coupled with DP4 probability.⁵¹

DP4 analysis in this experiment (**Table 2**) showed that the syn isomer was in good agreement with the experimental data, with a probability of 99.96% for ^1H NMR and 99.87% for ^{13}C NMR, further adding confirmation to the correct relative configuration of compound **2** being the syn conformer. In other words, the isolated compound would likely exist either in the syn arrangement or in the enantiomeric form of the syn configuration. Pursuant to this assignment, it was our anticipation that the absolute configuration for this compound might be (6*R*/1'*R*) as the position C-1' of its known analogue, 6-(1-hydroxyethyl)-5,6-dihydrochelerythrine, was previously determined to be the *R* configuration by Mosher's method.⁵²

Table 2 Mean absolute error (MAE), maximum deviation (MD) and DP4 probability of theoretical NMR data* for **2**

Formula	δ_{H} calcd 2a	δ_{H} calcd 2b	δ_{C} calcd 2a	δ_{C} calcd 2b
MAE (ppm)	0.04	0.12	1.07	1.48
MD (ppm)	0.12	0.45	2.70	4.20
DP4 Probability (%)	99.96	0.04	99.87	0.13

*Theoretical NMR data were calculated in chloroform.

The isolated compounds were submitted to cytological profiling to investigate their effects on cellular organelles. PD hONS cells were treated with 30 μM of each compound for 24 h and subsequently stained with fluorescence probes targeting several cellular components believed to be implicated in PD. DMSO was used as negative control.

Rotenone and chloroquine worked as positive controls. An automated microscopy imaging system was employed to capture images of cells, which in turn generated numerical data for several cellular parameters describing nuclear morphology, intensity, and texture of cellular components as well as overall cell morphology. After staining and imaging, there were not enough cells in the wells containing compounds **5**, **6**, **7** and **8** to conduct data analysis, suggesting that these compounds were cytotoxic to hONS cells at the above concentration and therefore were excluded from further analysis. Binary logarithms ($\log_2$) of the ratio of the compounds to DMSO were calculated for each parameter. In total, 22 biological descriptors associated with mitochondria, lysosome, early endosome, nuclei, and cytoskeleton were evaluated for their phenotypic changes. The results were summarized in **Figure 3** and visualized in **Figure 4**.

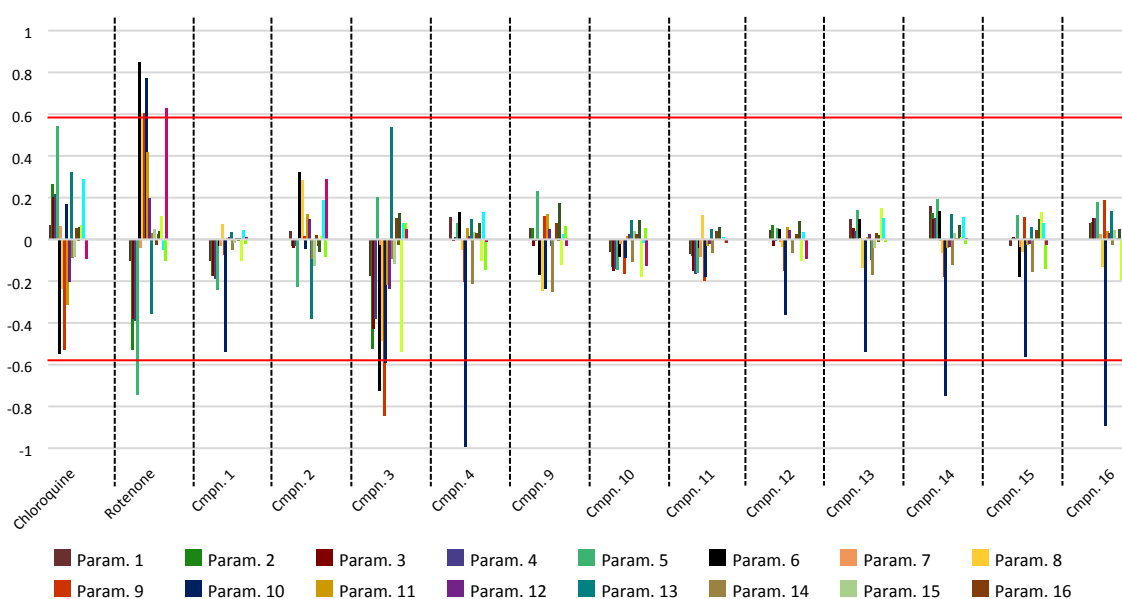


Figure 3 Bar chart depicting the cytological profiles against hONS cells of the positive controls chloroquine (10 μ M) and rotenone (20 μ M), and of compounds **1-16** (30 μ M), except for **5**, **6**, **7** and **8**. y-axis: $\log_2$ ratios of compound to DMSO. Red horizontal lines indicate $\log_2$ ratios of 0.58 and -0.58. x-axis: 12 compounds (**1 - 4** and **9 - 16**) subject to CP. 22 cytological parameters were: 1. EEA1 marker texture index. 2. Number of EEA1 marker spots per area of inner region of cytoplasm. 3. Number of EEA1 marker spots per area of outer region of cytoplasm. 4. Number of EEA1 marker spots per area of cytoplasm. 5. Number of EEA1 marker spots in cytoplasm. 6. EEA1 marker intensity in the cytoplasm. 7. Lysosome marker texture index. 8. Lysosomal marker intensity mean. 9. Mitochondria marker intensity in inner region of the cytoplasm. 10. LC3b marker intensity in inner region of cytoplasm. 11. Cell morphology ratio width to length. 12. Cell morphology roundness. 13. Cell morphology area (μm^2). 14. Nucleus marker texture index. 15. Nucleus morphology ratio width to length. 16. Nucleus morphology length (μm). 17. Nucleus morphology roundness. 18. Nucleus morphology area (μm^2). 19. Nucleus marker intensity. 20. LC3b marker texture index. 21. Mitochondrial marker texture index. 22. Tubulin marker texture index.

The $\log_2$ ratios represented the strength of the effects that the compounds induced to the cellular components compared to the DMSO control. The positive controls were used to ensure that the experiment behaved correctly. Chloroquine is a well-known antimalarial agent and has been found to disrupt the fusion of endosome and lysosome via inhibiting the acidification of these organelles.^{53, 54} In **Figure 3**, chloroquine indeed induced strong $\log_2$ ratio deviations at parameters 5 and 6 which were associated with early endosome antigen 1 (EEA1), a biological marker of early endosome. Similarly, rotenone, a broad-spectrum pesticide, inhibits complex I of the mitochondrial respiratory chain by

interfering with the electron transport in mitochondria.⁵⁵ As a result, there was a significant $\log_2$ ratio value of the mitochondria marker intensity in inner region of the cytoplasm (parameter 9) for rotenone in **Figure 3**. In term of imaging, hONS cells treated with chloroquine displayed altered EEA1 staining compared to DMSO-treated cells (**Figure 4A**), and those dosed with rotenone resulted in changes in MitoTracker, a fluorescent probe used to label mitochondria, in comparison with the cells on DMSO treatment (**Figure 4B**).

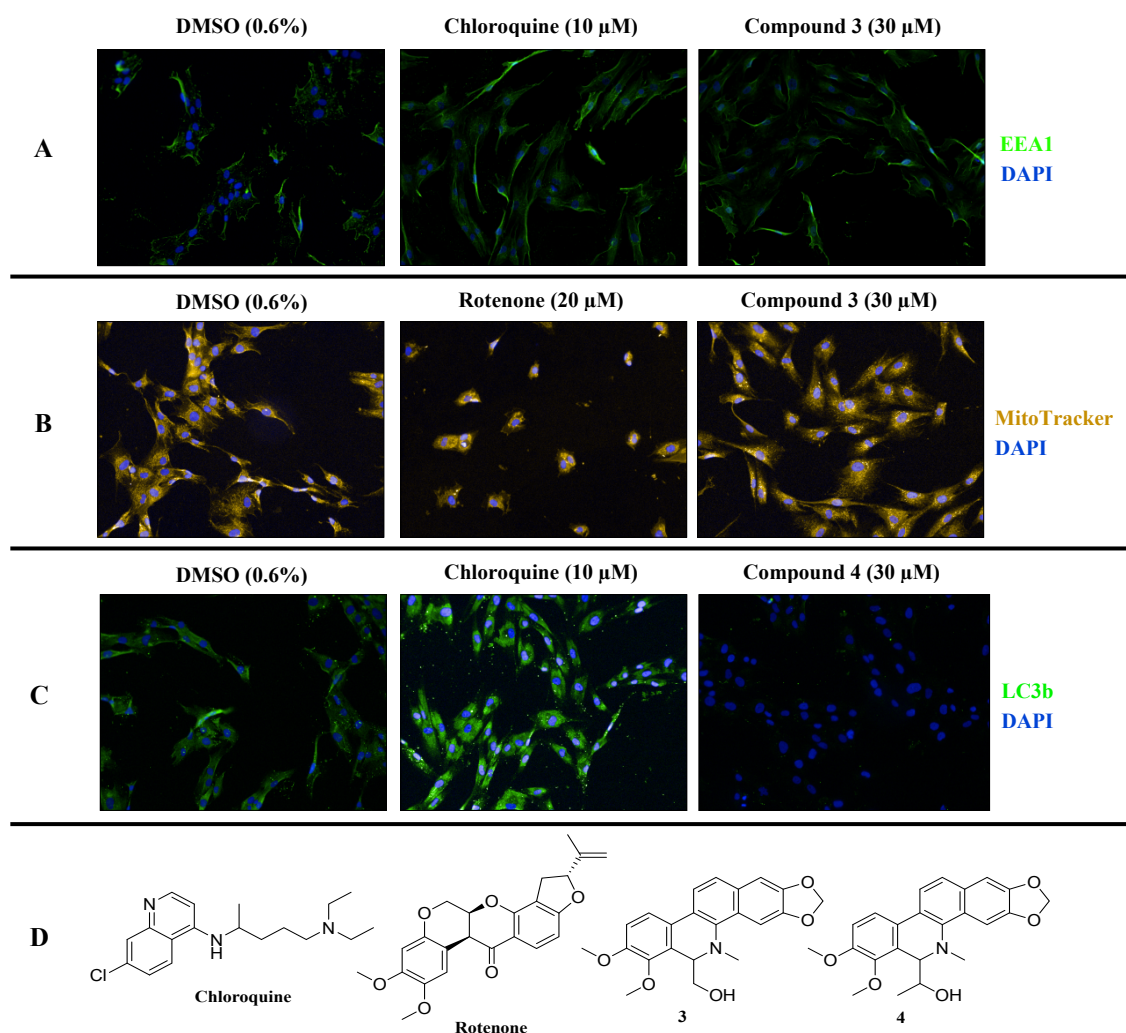


Figure 4 Phenotypic profiling of chloroquine, rotenone, bocconoline (**3**) and 6-(1-hydroxyethyl)-5,6-dihydrochelerythrine (**4**) on PD hONS cells. **A**. Cytological responses of hONS cells to chloroquine at 10 μ M and to compound **3** at 30 μ M compared to DMSO 0.6%; cells were stained with anti-EEA1 antibody (green) and DAPI (blue). **B**. Cytological responses of hONS cells to rotenone at 20 μ M and to compound **3** at 30 μ M compared to DMSO 0.6%; cells were stained with MitoTracker (yellow), anti- α -tubulin antibody (red) and DAPI (blue). **C**. Cytological responses of hONS cells to chloroquine at 10 μ M and to compound **4** at 30 μ M compared to DMSO 0.6%; cells were stained with anti-LC3b antibody (green), MitoTracker (yellow) and DAPI (blue). **D**. Chemical structures of chloroquine, rotenone, compound **3** and compound **4**. All images were acquired using 20X objective on the Operetta CLS system; scale bar 50 μ m.

In this experiment, compounds that influenced at least 1.5-fold change in the ratio with statistical significance were of interest. The ratio of 1.5 can be converted into $\log_2$ as $\log_2(1.5) \approx 0.58$. This meant that any compound whose $\log_2$ ratios at any parameters were greater than 0.58 or less than -0.58 were further considered. As seen in **Figure 3**, aside from the controls, there were four compounds which met the expected $\log_2$ ratio values,

including bocconoline (**3**), 6-(1-hydroxyethyl)-5,6-dihydrochelerythrine (**4**), 3-*O*-feruloylquinic acid (**14**) and ferulic acid 4-*O*-glucoside (**16**). More specifically, these compounds affected primarily on EEA1 marker intensity in the cytoplasm, mitochondria marker intensity in inner region of the cytoplasm and microtubule-associated protein light chain 3B (LC3b) marker intensity in inner region of cytoplasm (parameter 6, 9, and 10, respectively). Statistical analysis, particularly Wilcoxon tests, were conducted to measure the significances. As a result, compound **3** exhibited a significant effect (p -value < 0.05) on EEA1 and MitoTracker responses, while compound **4** displayed a significant difference on LC3b feature (**Table 3**). The other two compounds, **14** and **16**, did not show significances compared to DMSO (p -value > 0.05), proposedly because of large variation in the technical repeats. Thus, compound **3** and **4** satisfied the selection criteria and were further analyzed.

Table 3 Cellular response of compound **3** and **4** with $\log_2$ ratios and p -values

Compound	Response	$\log_2[\text{compd.}/\text{DMSO}]$	p -value
3	EEA1 marker intensity in the cytoplasm	-0.73	0.022
	Mitochondria marker intensity in inner region of the cytoplasm	-0.85	0.022
4	LC3b marker intensity in inner region of cytoplasm	-0.99	0.044

More detailed analysis showed that compound **3** greatly reduced MitoTracker and EEA1 staining intensities (**Figure 4A & 4B**), while compound **4** strongly depleted LC3b staining in hONS cells (**Figure 4C**). Interestingly, as seen in **Figure 4A**, there was little difference in the EEA1 staining between hONS cells with compound **3** and those with chloroquine treatment; this was in agreement with the $\log_2$ ratio analysis (**Figure 3**) in which compound **3** and chloroquine both showed similar deviations at EEA1-associated parameters (parameter 5 and 6), suggesting that compound **3** and chloroquine may share some similarities in the mechanisms of action (MOA) affecting the early endosomes of hONS cells. This could also be supported by the fact that chloroquine functions by increasing the pH of intracellular vesicles such as autophagosome, endosome and lysosome,⁵⁶ owing to the basicity caused by the nitrogen atoms⁵⁷ which is also found in compound **3** (**Figure 4D**). In contrast, the effect of **3** on MitoTracker staining was contrary to that caused by rotenone. This was observed in **Figure 3** which showed opposite $\log_2$ ratio values at parameter 9 representing mitochondrial features for the two compounds. The difference was also visualized in **Figure 4B** where the MitoTracker in cells with **3** became punctate or fragmented and had different texture compared to that in DMSO- and rotenone-treated cells, respectively, possibly due to changes in mitochondrial morphology and distribution. Given that rotenone is a neurotoxin with known MOA on mitochondria⁵⁵ and has been used to elicit PD-like features in many animal studies,⁵⁸ the ability of **3** to influence opposite cytological responses to rotenone could give an indication that it may work in different mechanisms leading to the observed phenotypes and may potentially be involved in neuroprotection. Nonetheless, further studies are necessary to confirm or validate its neuroprotective effects.

Figure 4C gave the comparison on the LC3b staining for DMSO, chloroquine and compound **4**. LC3b is a protein involved in autophagy, a process of elimination of damaged cellular organelles and protein aggregates.^{59, 60} Since chloroquine is also an autophagy inhibitor functioning through the same MOA described above,^{61, 62} cells

treated with this compound were reported to experience alternations in autophagosome markers.⁶¹ However, the effects on LC3b staining and LC3b-related parameter (parameter 10) seen for chloroquine were quite weak in both **Figure 4C** and **Figure 3**, respectively. This could be because the chloroquine concentration used in this experiment (10 μ M) was not sufficient to show apparent effects on the LC3b markers, or the PD hONS cell line employed in this assay possessed distinct phenotypes related to autophagy that affected the staining. Compared to both DMSO and chloroquine, compound **4** greatly reduced LC3b staining intensity in hONS cells, which was supported by a strong negative deviation at parameter 10 in **Figure 3**, suggesting that it may have a role in the autophagic pathways of the tested cell line.

Besides, accumulating evidence indicates that the above-mentioned cellular components, mitochondria, early endosomes and autophagosomes, are closely related to the pathogenesis of PD. For instance, impairment of mitochondrial dynamics and deficiency of mitochondrial complex I, resulting in increased free radical production and oxidative stress, as well as decreased ATP production, has been found in the substantia nigra (SN), skeletal muscles and platelets of patients with PD.^{63, 64} Similarly, abnormal autophagy function has been identified in the SN of PD patients;^{60, 65} and upregulating autophagy can reduce aggregation of α -synuclein *in vivo*.^{66, 67} Consequently, provided that hONS cells from PD patients are believed to model some functional features of the disease, the ability of **3** and **4** to induce phenotypic changes in biological compartments of hONS cells signified that they might be involved in some underlying disease-relevant mechanisms and can potentially serve as chemical probes to interrogate the biological pathways of PD.

3. Conclusion

Benzo[*c*]phenanthridine is an interesting class of alkaloids which possesses unique scaffold and bioactivities. In this research we reported for the first time the isolation of two benzo[*c*]phenanthridine alkaloids, (6*R*)-10-methoxybocconoline and 6-(1-hydroxyethyl)-10-methoxy-5,6-dihydrochelerythrine from the TCM herbal plant *Macleaya cordata*. Apart from standard spectroscopic methods, theoretical ECD and NMR calculations greatly added confirmation to the proposed structures.

Cytological profiling is indeed a sensitive and robust technique to unbiasedly profile small molecules against several organelles within the cells, which in turn generates much valuable biological information. In this project, CP platform was utilized to investigate the phenotypic responses of hONS cellular components following treatments of pure natural products derived from the studied plant. From the analysis of the primary screen, two out of the sixteen isolates, bocconoline and 6-(1-hydroxyethyl)-5,6-dihydrochelerythrine, induced strong responses in various cytological parameters. Both compounds have been previously studied for their anticancer activity;^{68, 69} however, their abilities to influence phenotypic responses in cells have not yet been reported. By analyzing the cytological profiles, we anticipated that bocconoline might possess similar MOA in term of affecting the early endosomes and different MOA in respect of impacting the mitochondria of the hONS cells used, compared to chloroquine and rotenone, respectively. It was also our prediction that 6-(1-hydroxyethyl)-5,6-dihydrochelerythrine might function via pathways related to autophagy of the cell line employed. Since these features are believed to be implicated in PD pathogenesis, further research on the

compounds' MOA and their effects on large numbers of hONS cell lines from different PD patients and healthy controls could reveal disease-specific phenotypes, potentially leading to the discovery of new biological targets for PD.

4. Experimental

4.1 General Experimental Procedures

NMR spectra were recorded in DMSO-*d*₆, methanol-*d*₄ or chloroform-*d* at 298 K on a Bruker Ascend 800 MHz spectrometer coupled to a TCI CryoProbe. Chemical shifts (δ) were referenced to solvent residual signals.⁷⁰ All UV spectra were conducted on a CAMSPEC M501 UV/vis spectrometer. IR spectra were recorded on a Bruker Tensor 27 spectrometer, and CD spectra on a JASCO J-715 spectrometer. LC-LRESIMS utilized an Accucore C18 LC column (2.6 μ m, 150 $\times$ 2.1 mm) and a Thermo Scientific MSQ Plus single quadrupole ESI mass spectrometer. HRESIMS data were acquired on a Bruker MaXis Q-TOF mass spectrometer. An Alltech Hyperprep PEP C18 (300 $\text{\AA}$, 8 μ m, 250 mm $\times$ 22 mm) and a Phenomenex Luna C18 (100 $\text{\AA}$, 5 μ m, 250 mm $\times$ 10 mm) column were used for semipreparative HPLC. The HPLC system used for purification was Thermal Scientific Dionex Ultimate 3000 RS UHPLC. Thin-layer chromatography (TLC) was performed on Merck Silica gel 60 F₂₅₄ Aluminium sheets and Merck Aluminium oxide 60 F₂₅₄ basic glass plates. All solvents used for chromatography, UV, IR, CD and MS were Honeywell or RCI Labscan HPLC grade; the water used was filtered through a Millipore Milli-Q PF system.

4.2 Plant Material

The whole plant of *Macleaya cordata* was collected in Wufeng County, Yichang City, Hubei Province, China in August 2013. Taxonomic identification of *M. cordata* was performed by Prof. Jingquan Yuan (Guangxi Botanical Garden of Medical Plants, Nanning, China).

4.3 Extraction and Purification of Compounds 1-16

The air-dried and ground *M. cordata* (750 g) was extracted with 95% ethanol in water for 24 h (5L $\times$ 3) to obtain the crude extract (40 g). A portion of this extract (20 g) was initially fractionated with a polyamide flash column, yielding six fractions by eluting with MeOH/H₂O gradients (0:100, 30:70, 40:60, 70:30, 90:10 and 100:0, respectively). The 30:70 and 40:60 fraction were combined and subsequently subject to an open Sephadex LH-20 column using gradient of 100% MeOH to obtain 85 subfractions which were later combined into eight subfractions (A1 – 8) based on TLC analysis. Subfraction A2 was chromatographed by semipreparative RP-HPLC using the C₁₈ Luna column with linear gradient of MeOH/H₂O/TFA from 25:75:1 to 65:35:1 within 50 minutes at the flow rate of 4 mL/min to afford chelerythrine (**5**) and chelilutine (**6**). Similar RP-HPLC procedures with varied gradients were repeatedly performed on subfraction A3 to obtain bocconoline (**3**), columbamine (**11**), berberine (**12**), 3-*O*-feruloylquinic acid (**14**), methyl 3-*O*-feruloylquinic acid (**15**) and ferulic acid 4-*O*-glucoside (**16**), and on subfraction A4 to yield chelirubine (**7**), sanguinarine (**8**), coptisine (**9**) and tetrahydroscoulerine (**10**). The Sephadex column was also used to fractionate the combined fraction of MeOH/H₂O 70:30 and 90:10, resulting in 80 subfractions which were subsequently combined by TLC into 6 subfractions (B1 – 6). *p*-Coumaroyltyramine (**13**) was obtained by repeated RP-HPLC on fraction B2. Fraction B3 was chromatographed with the Hyperprep PEP C18 column

at the flow rate of 9 mL/min with MeOH/H₂O/TFA linear gradient from 50:50:1 to 65:35:1 within 50 minutes, followed by 10 minute wash from 65:35:1 to 100:0:1. HPLC-subfractions 53 to 56 contained 6-(1-hydroxyethyl)-5,6-dihydrochelerythrine (**4**). LC-MS detected that HPLC-subfractions 35 to 41 contained 10-methoxybocconoline (**1**) and 6-(1-hydroxyethyl)-10-methoxy-5,6-dihydrochelerythrine (**2**). These fractions were purified by repeated RP-HPLC and finally by preparative TLC on Merck Aluminium oxide 60 F254 basic glass plates with CH₂Cl₂/MeOH 99:1 as the mobile phase to afford **1** and **2**.

10-Methoxybocconoline (1): White powder; UV (CH₂Cl₂) λ_{max} (log ϵ) 233 (4.76), 280 (4.69), 329 (4.41); IR ν_{max} 2929.7, 1466, 1335.7, 1239.5, 1041 cm⁻¹; ¹H and ¹³C NMR data (CDCl₃) see Table 1; (+)-LRESIMS m/z 410 [M+H]⁺, 392 [M+H-H₂O]⁺; (+)-HRESIMS m/z 410.1600 [M+H]⁺ (calcd. for C₂₃H₂₄NO₆, 410.1598).

6-(1-Hydroxyethyl)-10-methoxy-5,6-dihydrochelerythrine (2): White powder; UV (MeOH) λ_{max} (log ϵ) 201 (4.16), 225 (4.11), 279 (4.10), 327 (3.77); IR ν_{max} 3395, 2985.6, 1593.2, 1466, 1382.2, 1248.8, 1081.3 cm⁻¹; ¹H and ¹³C NMR data (CDCl₃) see Table 1; (+)-LRESIMS m/z 424 [M+H]⁺, 406 [M+H-H₂O]⁺; (+)-HRESIMS m/z 424.1752 [M+H]⁺ (calcd. for C₂₄H₂₆NO₆, 424.1755).

4.4 Computational Chemistry

4.4.1 ECD Calculations

The preliminary conformational analysis was carried out in HyperChem 7.5. TD-DFT calculations were performed in Gaussian 03.⁷¹ The corresponding geometries with minimum energies were further optimized at the B3LYP/6-31G(d) level. Conformers within a 2 kcal/mol energy threshold from the global minimum were selected to calculate the electronic excitation energies and rotational strengths at the B3LYP/6-31G(d) level. SCRF/PCM solvation model^{72, 73} was used to evaluate solvent effects of methanol at the same DFT level. The overall theoretical ECD spectra were averaged based on Boltzmann weighting of each conformer.

4.4.2 NMR Calculations

A Monte Carlo conformational search was undertaken using the OPLSe3 forcefield with H₂O solvation in MacroModel.⁷⁴ Conformers with less than 3 kcal/mol of the global minimum were selected for further geometry optimization by DFT using the B3LYP hybrid functional and 6-31G(d,p) basis set in Gaussian 16.⁷⁵ A single-point energy of each optimized conformer was calculated using M062X/def2-TZVP with IEF-PCM implicit solvent model for chloroform; duplicate conformers and conformers with greater than 3 kcal/mol of the global minimum were removed. The computed energies were used to scale the calculated NMR parameters relative to the Boltzmann populations of the conformers. NMR shielding constants were calculated using GIAO at the MPW1PW91/6-311+G(2d,p) level for proton and at the wB97XD/6-311+G(2d,p) level for carbon with chloroform solvent using the integral equation formalism version of the polarizable continuum model (IEF-PCM). The unscaled chemical shifts were computed referenced to the shielding tensors of TMS which were calculated at the same level of theory as for the conformers. The final chemical shifts were empirically scaled using the slope and intercept resulting from the multireference linear regression model.^{31, 46} The theoretical shifts reported for each diastereomer were the Boltzmann-weighted average

of all values calculated for the corresponding conformers with respect to their Boltzmann populations.

4.5 Cell Culture

Human olfactory neurosphere-derived (hONS) cell line C1200080013 obtained from an idiopathic PD patient was used for cytological profiling assays throughout this study. Initially, a frozen aliquot of cells at passage five was thawed and grown in DMEM/F12 (Invitrogen) with 10% fetal bovine serum FBS (Invitrogen) in 75 mm² cell culture flasks (ThermoFisher) at 37 °C and 5% CO₂. The medium was refreshed every other day until the cells reached 70 – 90% confluence. At reaching the desired confluence, the cells were then expanded from passage five to passage seven. Cells at passage eight were used in this experiment.

4.6 Compound Transferring

Initially, 300 nL of stock solutions of compounds (5 mM in DMSO) were robotically transferred into two optically clear bottom CellCarrier 384-well plates (PerkinElmer) by Compounds Australia⁷⁶ in triplicate layout. hONS cells from the culture were counted, and 1350 cells were added to the wells already containing the compounds, leading to a final concentration of 0.6% for DMSO and 30 µM for compounds in 50 µL of growth medium (DMEM/F12, 10% FBS) for each well. DMSO 0.6% was utilized as negative control; Rotenone (20 µM in 0.6% DMSO) and Chloroquine (10 µM in 0.6% DMSO) worked as positive controls. The cells were then incubated for 24 hours at 37 °C under 5% CO₂.

4.7 Cell Staining

Following 24 hours of incubation, one 384-well plate was stained with MitoTracker Orange CMTMRos (Life Technologies) (400 nM) for 30 minutes away from light at 37 °C under 5% CO₂. The other 384-well plate was stained with LysoTracker Red DND-99 (Life Technologies) (100 nM) for 1 hour protected from light at 37 °C under 5% CO₂. Cells in both plates were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, Sigma-Aldrich) (pH 7.4) for 10 minutes at room temperature (rt), and subsequently washed twice with PBS, followed by treatment with 3% goat serum (Sigma-Aldrich) and 0.2% Triton X-100 (Sigma Aldrich) in PBS for 45 minutes at rt. Antibodies, including primary and secondary, were then used to stain cellular components. For primary antibodies, mouse anti-EEA1 1/200 (Sigma-Aldrich) was added to the plate previously treated with LysoTracker. Mouse anti- α -tubulin 1/4000 (Sigma-Aldrich) and rabbit anti-LC3b 1/335 (Sigma-Aldrich) were added to the plate treated with MitoTracker. Plates were incubated in the dark at room temperature for 1 h, then washed twice with PBS. Secondary antibodies goat anti-mouse Alexa-647 1/500 (Life Technologies) and goat anti-rabbit Alexa-488 1/500 (Life Technologies) were added to the MitoTracker-treated plate, and goat anti-mouse Alexa-488 1/500 (Life Technologies) was added to the plate previously treated with Lysotracker for 30 minutes at rt. Cells in both plates were washed twice with PBS and stained with 4',6'-diamidino-2-phenylindole 1/5000 (Dapi, Life Technologies) and with CellMask Deep Red 1/5000 (Life Technologies) for the LysoTracker-treated plate and incubated for 10 minutes at rt. Cells were finally washed twice with PBS, and plates were covered with aluminium foil and stored in the dark at 4 °C with 25 µL of PBS per well until imaging.

4.8 Cell Imaging and Image Analysis

Cell images were captured automatically using a 20X objective on a PerkinElmer Operetta High-Content Imaging system, which were later analyzed to generate numerical data using PerkinElmer Harmony High-Content Imaging and Analysis, and Columbus Image Data Storage and Analysis software through a set of analysis sequences that effectively quantified biological features and cellular components based on the sizes and shapes of nuclei and cells, textures and intensities of mitochondria, nuclei, lysosome, tubulin, autophagosomes and early endosomes.

RStudio was utilized throughout the analysis stage to statistically process the data. The preliminary analysis involved the removal of invalid and zero values. Normalization of intensity features (e.g. LC3b marker intensity, nucleus marker intensity) was performed against cellular or nucleus areas. All the intensity features were normalized to the cellular area except DAPI intensity which was normalized to the nuclear area. Binary logarithmic transformation was subsequently executed on the dataset. Log₂ ratios of compounds to control were calculated by subtracting the log₂ of data for compounds to those for DMSO. Wilcoxon rank statistic was subsequently employed to measure significances and identify strong biologically active compounds in the experiment.

Declaration of Competing 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.

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