Synthesis of (aminoalkyl)cycleanine analogues: cytotoxicity, cellular uptake, and apoptosis induction in ovarian cancer cells

Fidelia Ijeoma Uche,[1,2] James McCullagh,[3] Timothy W.D. Claridge,[3] Alan Richardson,[1] Wen-Wu Li[1*]

Affiliation

[1]Institute for Science and Technology in Medicine, Keele University, Stoke-on-Trent ST4 7QB, United Kingdom

[2]Faculty of Pharmaceutical Sciences, University of Port Harcourt, Nigeria

[3]Chemical Research Laboratory, University of Oxford, Oxford OX1 3TA, United Kingdom

*__Corresponding author:__

Dr. Wen-Wu Li

E-mail: w.li@keele.ac.uk

Phone: +44 (0)1782 674382

Fax: +44 (0)1782 747319

Abstract

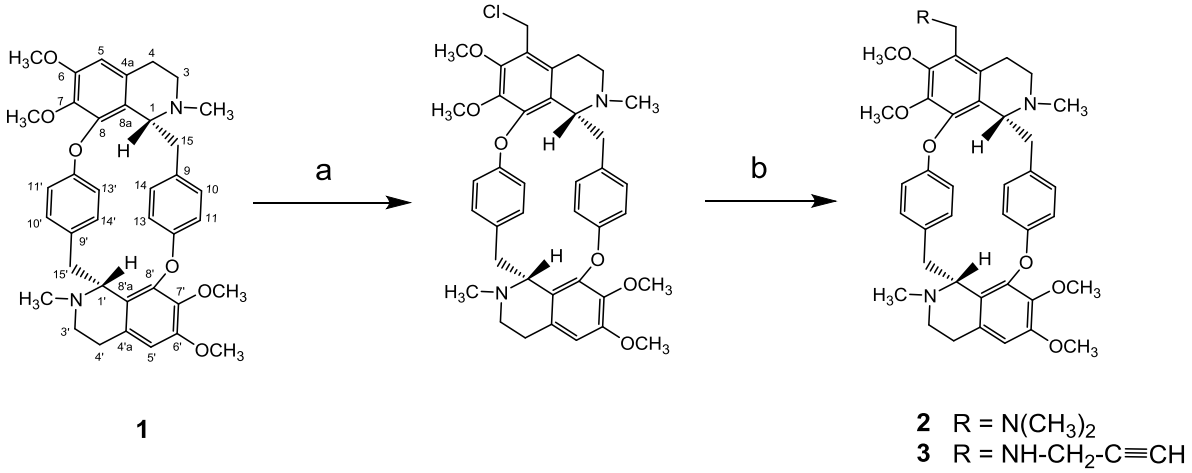
Our previous studies demonstrated that cycleanine, a macrocyclic bisbenzylisoquinoline (BBIQ) alkaloid, showed potent anti-ovarian cancer activity via apoptosis induction. Here, we synthesized two novel (aminoalkyl)cycleanine analogues (**2** and **3**) through a simple and efficient two-step reaction starting from cycleanine isolated from *Triclisia subcordata* Oliv.. These analogues showed greater potency than the unmodified cycleanine in three human ovarian cancer cell lines. Both **2** and **3** induced apoptosis in ovarian cancer cells by activations of caspases 3/7, cleavage of PARP, increase in subG_1 cell cycle phase and in the percentage of apoptotic cells. Further confocal fluorescence microscopy analysis confirmed the cellular uptake of alkaloids in ovarian cancer cells by using the unique alknylcycleanine (**3**) via click chemistry reaction. Our results suggest that cycleanine could be a hit compound for the future development in attacking ovarian cancer.

Keywords: Cycleanine; bisbenzylisoquinoline alkaloid; ovarian cancer; semi-synthesis; cellular uptake; apoptosis

The macrocyclic bisbenzylisoquinoline (BBIQ) alkaloids are among different large classes of alkaloids common in plant species. BBIQ alkaloids are prevalent in the families like Menispermeaceae, Annonaceae, Berberidaceae, Monimaiaceae and Ranunuclaceae.[1-3] The BBIQ alkaloids were reported to exhibit anti-ovarian,[4-7] –lung,[8] –bladder,[9] –colorectal,[10] -gallbladder carcinoma and –prostate[11] cancer activities.[12, 13] The use of isolated natural products as scaffolds to generate their analogues via chemical transformation is a promising and successful approach in drug discovery.[14, 15] So far, only a small number of semi-synthetic BBIQ alkaloids (e.g. tetrandrine and fanginoline analogues,[16-19] C14-urea-tetrandrine,[20] cycleanine mono-N-oxides,[21] and berbamine derivatives.[22]) have been made, which show potent cytotoxicity or can reverse P-glycoprotein-mediated multidrug resistance in cancer cells[17, 23]. The making of more potent cytotoxic BBIQ alkaloids with improved activities and investigation of their mechanisms of action (e.g. cellular uptake, target identification) by using the synthetic probes are desired. [24, 25] Previously, several BBIQ alkaloids were isolated from *Triclisia subcordata* Oliv. by us and they showed potent *in vitro* anti-ovarian cancer activity.[4-6] Among them, cycleanine (**1**) is bioactive, with the highest therapeutic index and the most abundant component (1.1%).[4] Therefore cycleanine was preferred as a hit compound for chemical modification.[14] In this study, we semi-synthesized analogues of cycleanine and evaluated their cytotoxicity, induction of apoptosis, and cellular uptake.

The semi-synthesis of cycleanine analogues was achieved via a simple two-step reaction (Scheme 1). Reaction of cycleanine (**1**) under paraformaldehyde and concentrated HCl produced the intermediate 5-chloromethylcycleanine through a Blanc chloromethylation reaction.[26] Without purification 5-chloromethylcycleanine reacted with

dimethylamine and propargylamine via SN2 nucleophilic substitution reaction to yield 5-[(dimethylamino)methyl]cycleanine (**2**), and 5-[(propargylamino)methyl]cycleanine (**3**), respectively (Supporting information).



Scheme 1. Semi-synthesis of (aminoalkyl)cycleanine analogues via modification of cycleanine.

Reagents and conditions: (a) paraformaldehyde, conc. HCl, 0 °C, 3h; (b) CH_3CN, NaOH, dimethylamine or propargylamine, rt, 3h.

Compounds **2** and **3** were purified by silica gel chromatography to a high purity (>98%) as determined by analytical high performance liquid chromatography. The structure of 5-[(dimethylamino)methyl]cycleanine (**2**) and 5-[(propargylamino)methyl]cycleanine (**3**) was confirmed on the basis of ^{1}H NMR (Table S1, Figure S1 and S2), ^{13}C NMR (Table S2), 2D NMR and high resolution liquid chromatography mass spectrometric (LC-MS) analysis. The symmetry of cycleanine was lost after the chemical modification, as evidenced by that the single peak for H-5 or 5' in the ^{1}H NMR of cycleanine[4] was shifted and other peaks for aromatic protons were split into two pairs of peaks (Table S1,

Figure S1 and S2). The resulting single peak around 6.80 ppm in the ^{1}H NMR of **2** or **3** corresponds to only one proton (H-5') which indicates the substitution at C-5 of cycleanine (**1**) by the alkylamino groups. The addition of aminoalkyl group to cycleanine could improve its water solubility as found in the approved anticancer drug such as (aminoalkyl)camptothecin (Toptecan).[27, 28]

To assay the cytotoxicity or anti-proliferative activities of **2** and **3**, the IC_{50} of these new compounds were evaluated on different ovarian cancer cell lines (OVCAR-8, A2780 and IGROV-1) and a normal human ovarian epithelial (HOE) cell line after treatment for 72h using Sulforhodamine B (SRB) colorimetric assay.[29, 30] The results reveal the IC_{50} of **2** and **3** ranging from 3.6 ± 0.5 to 5.2 ± 0.6 µM and from 5.6 ± 0.2 to 6.3 ± 0.6 µM, respectively (Table 1; Figures S3 and S4). The results suggest that **2** and **3**, exert about 2-3 times improved potency against three ovarian cancer cells than cycleanine (**1**) with IC_{50} ranging from 7 to 14 µM and the approved anti-ovarian cancer drug-carboplatin with IC_{50} ranging from 8 to 16 µM. Cycleanine (**1**) and **3** seem to be slightly more tolerant to HOE cells than compound **2**. Overall, compound **3** showed milder selectivity index (SI) than **1** and **2** (Table 1).

Table 1 The IC_{50} of cycleanine (**1**), 5-[(dimethylamino)methyl]cycleanine (**2**) and 5-[(propargylamino) methyl]cycleanine (**3**) in ovarian cancer cells. Data represented as mean ± SEM (n= 5). SI means selective index.

Compound	Ovarian cancer cell lines (μM)			HOE (μM)
	OVCAR-8	A2780	IGROV-1	
1	10 ± 0.6	7.6 ± 0.7	14 ± 1.0	35 ± 1.0
SI (**1**)	3	5	3	
2	5.2 ± 0.6	3.6 ± 0.5	4.4 ± 0.1	10 ± 0.2
SI (**2**)	2	3	2	
3	5.6 ± 0.2	6.3 ± 0.6	6.1 ± 0.5	32 ± 1.6
SI (**3**)	6	5	5	
Carboplatin	16 ± 1.0	8.0 ± 0.7	12 ± 0.9	-

The effects of **2** and **3** on morphology of OVCAR-8 cancer cells were also found to be time- and concentration-dependent (Figure S5 and S6). Both of them caused the shrinkage of the cell, cell detachment, and the nucleus condensation, which are characteristic features of apoptosis of cells. The induction of apoptosis in cancer cells by **2** and **3** was further investigated by evaluation of caspase 3/7 activation, cleavage of PARP, flow cytometry, and cell cycle assays.

2 and **3** after exposure to OVCAR-8, A2780 and IGROV-1 for 24h caused activation of caspase Glo 3/7 activities, which was manifested as elevation of caspase levels (Figure 1).

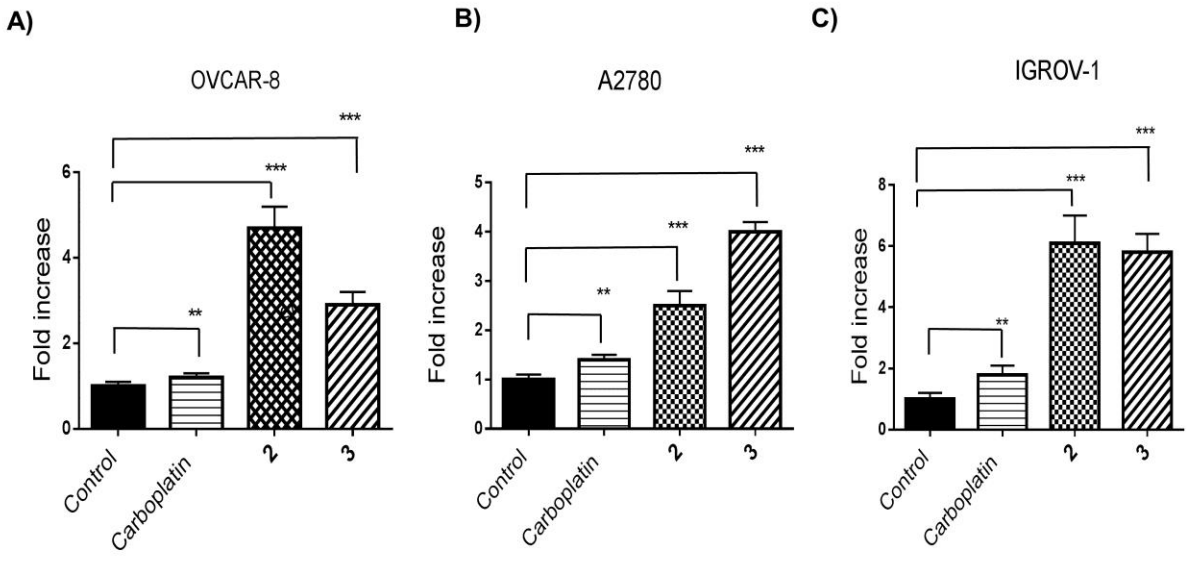


Figure 1 The apoptotic effects (increase of caspase 3/7 activities) of carboplatin, 5-[(dimethylamino)] cycleanine (**2)** and 5-[(propargylamino) methyl] cycleanine (**3**) on three ovarian cancer cell lines. Data represented as mean ± SEM, n=6 observations, negative control was the DMSO (0.2%), carboplatin used as positive control, **2** and **3** (20 μM). Results were significant compared to control (*** P<0.005, one-way ANOVA) *post hoc* analysis multiple t-test compared with control (**P<0.01, *** P<0.001).

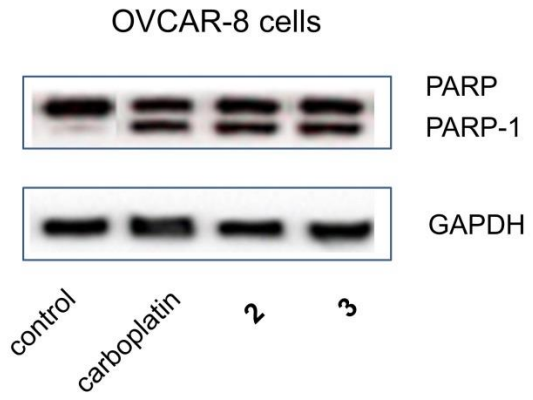


Figure 2 Western blotting analysis of the PARP-1 cleavage (apoptosis marker) in OVCAR-8 cells after treatment with carboplatin, 5-[(dimethylamino)methyl]cycleanine (**2**) and 5-[(propargylamino)methyl]cycleanine (**3**). GAPDH was used as a loading control. A representative of three independent experiments is shown.

Western blotting results showed that OVCAR-8 cancer cells by treatment of **2** and **3** caused a significant cleavage of PARP to produce PARP-1 fragment (Figure 2) in a similar way as cycleanine[4]. There was no significant PARP-cleavage observed in negative control.

For quantification of the percentage of apoptotic cells post-treatment with **2** and **3**, OVCAR-8 cells were treated and stained with both Annexin V-FITC and propidium iodide (PI) and analysed by flow cytometry. Treatment of OVCAR-8 with carboplatin, **2** and **3** over 48h caused significant increase in the population of both early and late apoptotic cells compared to the control cells (Figure 3).

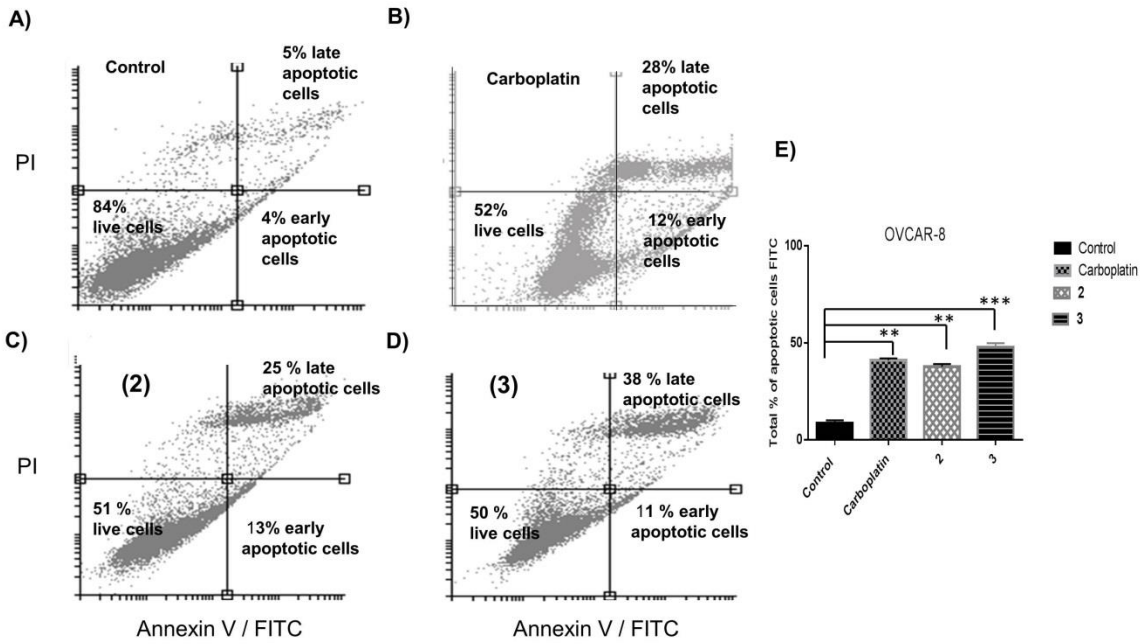


Figure 3 Flow cytometry analysis of apoptotic effects of 5-[(dimethylamino)methyl]cycleanine (**2**) and 5-[(propargylamino)methyl]cycleanine (**3**) on OVCAR-8 cells. The scattering dot plots indicating the percentage of live, early and late apoptotic cells under treatment of medium (control) (**A**), carboplatin (**B**), compound **2** (**C**), and compound **3** (**D**) (20 μM) for 48 hours by using Annexin V-FITC & PI assay. The percentages of the total apoptotic cells (E) caused by **2**, **3** and carboplatin (positive control) were significant compared to negative control (one way ANOVA for group analysis, *P*<0.001; post hoc paired *t*-test relative to control was done (* P<0.05)).

The effect on cell cycle by **2** and **3** in OVCAR-8 cells was analysed using PI and flow cytometry. The percentage of OVCAR-8 cells in subG_1 increased after exposure to carboplatin, **2** and **3** for 48h compared to negative control. The percentage of cells at the G_0/G_1 phase (live cells) decreased significantly compared to control (Figure 4). This was similar to result of cycleanine as reported previously[4].

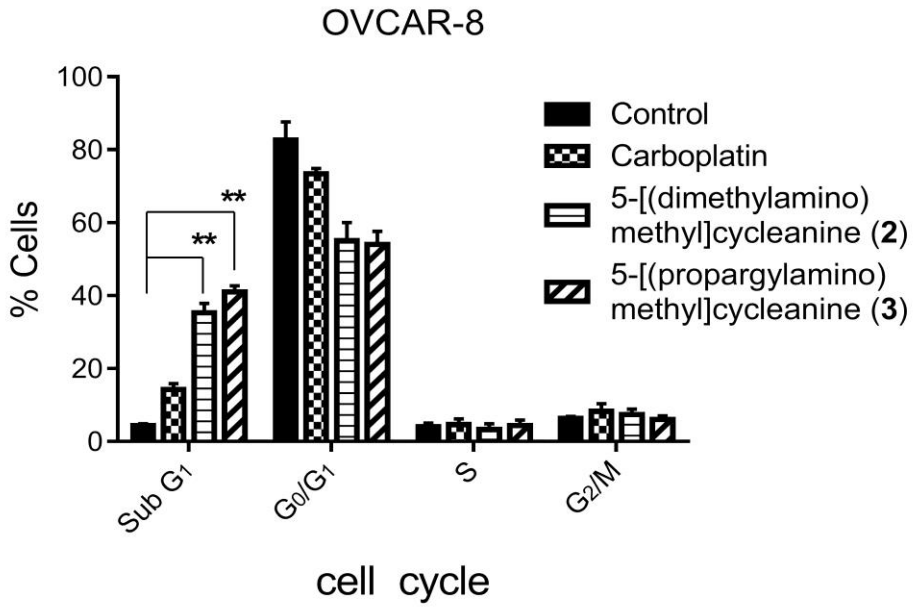


Figure 4 Cell cycle distribution analysis of apoptotic effects of 5-[(dimethylamino)methyl]cycleanine (**2**) and 5- [(propargylamino)methyl]cycleanine (**3**) on OVCAR-8 cell lines. Data represented as mean ± SEM, n = 4 observations. The percentage of subG_1 phase (apoptotic cells) of the OVCAR-8 cell cycle was significantly increased compared to cells exposed to control, while the percentage of G_0/G_1 phase cells decreased significantly compared to control ($P< 0.001$, one-way ANOVA).

Because compound **3** with modification of a propargyl group showed similar potency and induction of apoptosis as cycleanine (**1**), it was used as a model compound to investigate the cellular uptake of BBIQ alkaloids. Therefore medium (control), cycleanine (**1**) and **3** were administered in OVCAR-8 cells for 48h. Alexa 488 azide (a fluorescent dye) was used to monitor the intracellular uptake of 5-[(propargylamino)methyl]cycleanine (**3**) via specific click chemistry reaction[25]. Cycleanine is not fluorescent compound. The cells of control and after treatment of cycleanine showed little fluorescence (Figure 5A and B), while the cells treated by **3**

showed very strong fluorescence (Figure 5C). The fluorescence intensity was used as a measure of the distribution and uptake of **3** in OVCAR-8 cells. Treatment with **3** caused dramatic increase in mean fluorescence intensities (Figure 5D) compared to control cells. There was no significant change in fluorescence intensity observed in cycleanine-treated cells compared to control (Figure 5D), because they both lack an alkynyl group which is necessary for the click chemistry reaction with Alexa 488 azide dye. Only **3** reacted with Alexa 488 azide dye, thus causing the intense fluorescence. Increase of concentration of **3** indicated the increase of fluorescence (Figure S7). These results confirmed the intracellular uptake of cycleanine derivative (**3**).

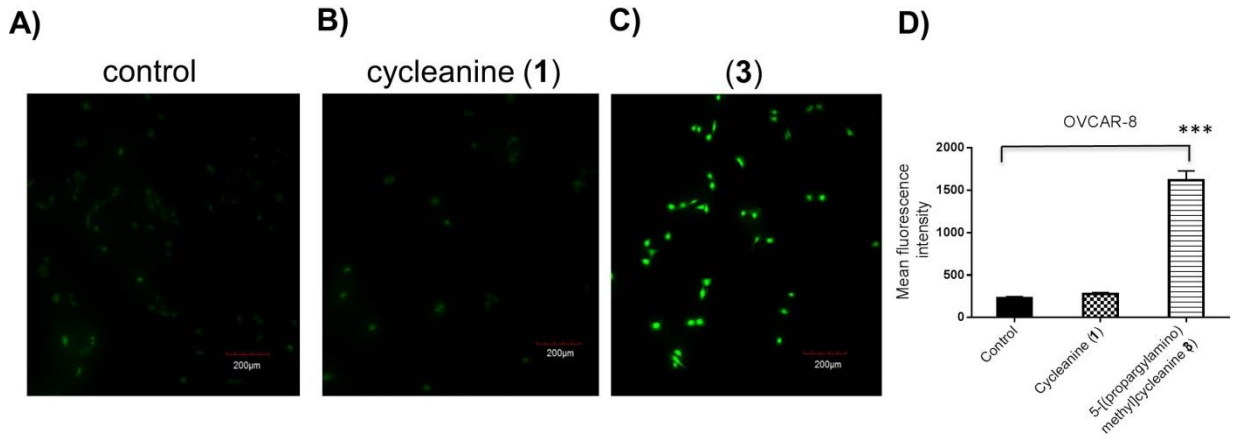


Figure 5 Analysis intracellular uptake of cycleanine (**1**) and 5-[(propargylamino)methyl]cycleanine (**3**) in OVCAR-8 cells using confocal fluorescence microscopy. Graph **A, B** and **C** represents cell images after treatment with medium only (control), cycleanine, and **3** (each 20 μM), respectively. **D** represents for the mean fluorescence intensity of OVCAR-8 cells after 48h treatment. The fluorescence data were analyzed by the determination of the average fluorescence intensities of the cells in an area by use of Image J software and evaluation of the difference from the

background fluorescence values. Values were evaluated from 30-90 cells determined in five independent experiments. Data represented as mean ± SEM, n = 5 independent experiments. Results were significant compared to control (*** P<0.0001, one-way ANOVA)).

In summary, we have successfully semi-synthesized two novel aminoalkyl BBIQ alkaloids (**2** and **3**) starting from an isolated natural product-cycleanine through a simple and efficient method of site-specific chemical modification at C-5 position of cycleanine. The new modification approach has advantages over some reported transformation methods for BBIQ alkaloids.[16, 17, 21] Because it can be applied to cycleanine as well as other BBIQ alkaloids to make a large number of analogues using a range of other nucleophilic compounds (e.g. primary and secondary amines, thiols). Furthermore, the available alkynylcycleanine (**3**) could react with any azide-containing compounds/anticancer drugs such as platinum derivatives to generate dual BBIQ alkaloid and platinum conjugates via click chemistry,[31] which may produce potentially stronger synergic effects between them[32]. The observed anti-proliferative activities and selectivity (Table 1) of the semi-synthetic BBIQ alkaloids were found to be higher (about 2-3-fold increase) than the observed effect with cycleanine (a hit compound). This suggests that substitution at 5-position of cycleanine with a dimethylaminoalkyl or an alkynylaminoalkyl group could lead to improved cytotoxicity of cycleanine. The mechanism of cancer cell death induced by the new BBIQ alkaloids has been determined as apoptosis induction by caspase 3/7 activation, PARP-cleavage, increase in percentage of both early and late apoptotic cells; and increase in $SubG_1$ with decrease in G_0/G_1 cell cycle phase of the OVCAR-8 cells. These were consistent with

unmodified cycleanine and other BBIQ alkaloids.[4, 5] Importantly, using analogue **3** appended with a propargyl group its cellular uptake in cancer cells was clearly demonstrated via click chemistry and fluorescence microscopy. The semi-synthesis of additional BBIQ alkaloid analogues using the established approach to gain structure-activity relationship, and identification of protein targets in the cancer cells using a chemical proteomics approach[33, 34] are ongoing in our laboratory. *In vivo* studies also need to be performed to evaluate the preclinical and clinical effects of these novel compounds.

Acknowledgments

Fidelia I. Uche was supported by Nigerian Education trust fund (ETF) and Niger Delta Development Commission (NDDC) with a PhD fellowship. We thank Dr. Joshua Price for training in confocal fluorescence microscopy.

Appendix A. Supplementary data

Supplementary data (the semi-synthesis of the compounds **2** and **3** and their characterizations, and biological assays of **2** and **3**) associated with this article can be found in the online version.

References

1. Schiff PL, Jr. Bisbenzylisoquinoline alkaloids. *J Nat Prod.* 1987;50(4): 529-599.
2. Pachaly P. [Recent advances in bisbenzylisoquinoline alkaloids]. *Planta Med.* 1990;56(2): 135-151.
3. Schiff PL, Jr. Bisbenzylisoquinoline Alkaloids. *J Nat Prod.* 1997;60: 934-953.

4. Uche FI, Drijfhout FP, McCullagh J, Richardson A, Li WW. Cytotoxicity effects and apoptosis induction by bisbenzylisoquinoline alkaloids from Triclisia subcordata. *Phytother Res.* 2016;30(9): 1533-1539.

5. Uche FI, Abed M, Abdullah MI, et al. Isochondodendrine and 2'-norcocsuline: additional alkaloids from Triclisia subcordata induce cytotoxicity and apoptosis in ovarian cancer cell lines. *RSC Advances.* 2017;7(70): 44154-44161.

6. Uche FI, Abed M, Abdullah MI, et al. Isolation, identification and anti-cancer activity of minor alkaloids from Triclisia subcordata Oliv. *Biochem Pharmacol.* 2017;139: 112-112.

7. Choi SU, Park SH, Kim KH, et al. The bisbenzylisoquinoline alkaloids, tetrandine and fangchinoline, enhance the cytotoxicity of multidrug resistance-related drugs via modulation of P-glycoprotein. *Anticancer Drugs.* 1998;9(3): 255-261.

8. Lee JH, Kang GH, Kim KC, et al. Tetrandrine-induced cell cycle arrest and apoptosis in A549 human lung carcinoma cells. *Int J Oncol.* 2002;21(6): 1239-1244.

9. Li X, Su B, Liu R, Wu D, He D. Tetrandrine induces apoptosis and triggers caspase cascade in human bladder cancer cells. *J Surg Res.* 2011;166(1): e45-51.

10. He BC, Gao JL, Zhang BQ, et al. Tetrandrine inhibits Wnt/beta-catenin signaling and suppresses tumor growth of human colorectal cancer. *Mol Pharmacol.* 2011;79(2): 211-219.

11. Liu W, Kou B, Ma ZK, et al. Tetrandrine suppresses proliferation, induces apoptosis, and inhibits migration and invasion in human prostate cancer cells. *Asian J Androl.* 2015;17(5): 850-853.

12. Liu T, Liu X, Li W. Tetrandrine, a Chinese plant-derived alkaloid, is a potential candidate for cancer chemotherapy. *Oncotarget.* 2016;7(26): 40800-40815.

13. Bhagya N, Chandrashekar K, R. . Tetrandrine and cancer - An overview on the molecular approach. *Biomed Pharmacother.* 2018;97: 624-632.

14. Barnes EC, Kumar R, Davis RA. The use of isolated natural products as scaffolds for the generation of chemically diverse screening libraries for drug discovery. *Nat Prod Rep.* 2016;33(3): 372-381.

15. DeCorte BL. Underexplored Opportunities for Natural Products in Drug Discovery. *J Med Chem.* 2016;59(20): 9295-9304.

16. Wang FP, Wang L, Yang JS, Nomura M, Miyamoto K. Reversal of P-glycoprotein-dependent resistance to vinblastine by newly synthesized bisbenzylisoquinoline alkaloids in mouse leukemia P388 cells. *Biol Pharm Bull.* 2005;28(10): 1979-1982.

17. He P, Sun H, Jian XX, et al. Partial synthesis and biological evaluation of bisbenzylisoquinoline alkaloids derivatives: potential modulators of multidrug resistance in cancer. *J Asian Nat Prod Res.* 2012;14(6): 564-576.

18. Li DC, Liu HZ, Liu YF, et al. Design, synthesis and biological activities of tetrandrine and fangchinoline derivatives as antitumer agents. *Bioorg Med Chem Lett.* 2017;27(3): 533-536.

19. Lan JJ, Wang N, Huang L, et al. Design and synthesis of novel tetrandrine derivatives as potential antitumor agents against human hepatocellular carcinoma. *Eur J Med Chem.* 2017;127: 554-566.

20. Lan J, Huang L, Lou H, et al. Design and synthesis of novel C14-urea-tetrandrine derivatives with potent anti-cancer activity. *Eur J Med Chem.* 2018;143: 1968-1980.

21. Kashiwaba N, Ono M, Toda J, Suzuki H, Sano T. Synthesis of cycleanine mono-N-oxides. *Journal of Natural Products.* 1998;61(2): 253-255.

22. Xie JW, Ma T, Gu Y, et al. Berbamine derivatives: A novel class of compounds for anti-leukemia activity. *Eur J Med Chem.* 2009;44(8): 3293-3298.

23. Tian H, Pan OC. Modulation of multidrug resistance by three bisbenzyl-isoquinolines in comparison with verapamil. *Zhongguo Yao Li Xue Bao.* 1997;18(5): 455-458.

24. Thirumurugan P, Matosiuk D, Jozwiak K. Click chemistry for drug development and diverse chemical-biology applications. *Chem Rev.* 2013;113(7): 4905-4979.

25. Luo Z, Tikekar RV, Nitin N. Click chemistry approach for imaging intracellular and intratissue distribution of curcumin and its nanoscale carrier. *Bioconjug Chem.* 2014;25(1): 32-42.

26. Belen'kii LI, Vol'kenshtein YB, Karmanova IB. New Data on the Chloromethylation of Aromatic and Heteroaromatic Compounds. *Russian Chemical Reviews.* 1977;46: 891-903.

27. Kingsbury WD, Boehm JC, Jakas DR, et al. Synthesis of water-soluble (aminoalkyl)camptothecin analogues: inhibition of topoisomerase I and antitumor activity. *Journal of medicinal chemistry.* 1991;34(1): 98-107.

28. Li WW, Johnson-Ajinwo OR, Uche F. *Potential of Phytochemicals and their Derivatives in the Treatment of Ovarian Cancer. In Handbook on Ovarian Cancer: Risk*

Factors, Therapies and Prognosis. (ed. Collier, B.R.) USA: Nova Science publishers; 2015.

29. Johnson-Ajinwo OR, Richardson A, Li WW. Cytotoxic effects of stem bark extracts and pure compounds from Margaritaria discoidea on human ovarian cancer cell lines. *Phytomedicine.* 2015;22(1): 1-4.

30. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc.* 2006;1(3): 1112-1116.

31. Pathak RK, McNitt CD, Popik VV, Dhar S. Copper-free click-chemistry platform to functionalize cisplatin prodrugs. *Chemistry.* 2014;20(23): 6861-6865.

32. Zhang Y, Wang C, Wang H, Wang K, Du Y, Zhang J. Combination of Tetrandrine with cisplatin enhances cytotoxicity through growth suppression and apoptosis in ovarian cancer in vitro and in vivo. *Cancer Lett.* 2011;304(1): 21-32.

33. Simon GM, Niphakis MJ, Cravatt BF. Determining target engagement in living systems. *Nat Chem Biol.* 2013;9(4): 200-205.

34. Wright MH, Sieber SA. Chemical proteomics approaches for identifying the cellular targets of natural products. *Nat Prod Rep.* 2016;33(5): 681-708.