Cytotoxicity Effects and Apoptosis Induction by Bisbenzylisoquinoline Alkaloids from *Triclisia subcordata*

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ABSTRACT

Triclisia subcordata Oliv (Menispermeaceae) is a medicinal plant traditionally used for the treatment of various diseases in West Africa. The ethanol extract of *T. subcordata* and its fractions were screened for *in vitro* anti-ovarian cancer activities using the Sulforhodamine B assay. The crude alkaloids showed the strongest activity in cell growth assays on A2780 and Ovcar-8 cell lines (IC_{50} < 2.4 µg/mL). A bisbenzylisoquinoline alkaloid-cycleanine was isolated using HPLC and identified by MS and NMR analyses. The IC_{50} values of cycleanine and tetrandrine (an alkaloid previously reported from this plant) ranged from 7 to 14 µM on A2780, Ovcar-8, Ovcar-4 and Igrov-1 ovarian cancer cell lines. The IC_{50} of cycleanine on human normal ovarian surface epithelial cells was 35 ± 1 µM hinting at modest selectivity towards cancer cells. Both cycleanine and tetrandrine caused apoptosis as shown by activation of caspases 3/7 and cleavage of poly (ADP) ribose polymerase (PARP) to form PARP-1. Flow cytometry analyses showed that the percentages of apoptotic cells and cells in $subG_1$ phase increased after exposure of cycleanine and tetrandrine to Ovcar-8 cells for 48h compared to control. Cycleanine, like its isomer – tetrandrine isolated from *Triclisia subcordata,* could be a potential new anti-ovarian cancer agent acting through the apoptosis pathway.

Keywords: *Triclisia subcordata*, cycleanine, tetrandrine, anti-proliferation, apoptosis, ovarian cancer

INTRODUCTION

Ovarian cancer continues to be one of the main causes of death in all gynecologic malignancies (Siegel *et al.*, 2013). Natural products remain an important source of clinically approved anti-cancer drugs (e.g. paclitaxel from yew tree) (Cragg *et al.*, 2009; Li *et al*., 2015) suggesting they may provide a source of novel treatments for ovarian cancer. The bisbenzylisoquinoline (BBIQ) alkaloids are a large group of natural products occurring in many plant species in Families like Menispermeaceae, Ranunculaceae, Annonaceae, and Monimiaceae (Schiff, 1997). These alkaloids are made up of two isoquinonines ("head part") linked to two benzyl moieties ("tail part") through two types of linkage such as head-to-tail (e.g. cycleanine) and head-to-head (e.g. tetrandrine) (Fig. S1). The BBIQ alkaloids possess a range of biological activities including anticancer (Sun and Wink, 2014; Wang et al., 2010), inhibition of histamine release (Nakamura *et al.*, 1992), antiplasmodial (Angerhofer *et al.*, 1999), and antileishmanial and antitrypanosomal effects (del Rayo Camacho *et al*., 2002).

Triclisia subcordata Oliv (Menispermeaceae) has been reported to have anti-ulcer (Asuzu and Anaga, 1996), antihistamine and antimicrobial activities (Abo *et al.*, 2011). Early phytochemical studies in the 1970s revealed the presence of only three BBIQ alkaloids: tricordatine (Tackie *et al.*, 1973), fangchinoline (Tackie *et al*., 1974), and tetrandrine (Dwuma-Badu *et al.*, 1975) from this plant species. However, no anti-cancer activity of extracts of *T. subcordata* constituent alkaloids or further phytochemical study has been reported. Therefore, in continuation of our search for anticancer compounds from medicinal plants (Johnson-Ajinwo *et al*., 2015; Li *et al.*, 2013; Li *et al*., 2015), this study aimed to evaluate the *in vitro* anti-ovarian cancer activities of the crude extracts and pure compounds of *T. subcordata* and bioassay-guided fractionation

was used to isolate the bioactive compound. Furthermore, the cytotoxic effects and possible signalling pathways of cycleanine and tetrandrine involved in their cytotoxity on A2780, Ovcar-8, Ovcar-4 and Igrov-1 ovarian cancer cell lines were investigated.

EXPERIMENTAL

Plant Materials

The plant materials of *Triclisia subcordata* were collected in 2012 from Imo state, Nigeria and identified by a taxonomist, H.D Onyeachusim. The voucher specimen (Voucher number UUH 1817) was deposited at the herbarium of University of Uyo, Akwa Ibom state, Nigeria. The root bark of the plant part was cut into pieces, air-dried and pulverized.

Extraction and fractionation

The powdered root bark of *T. subcordata* (250 g) was extracted with 1 L of 50 % ethanol by Soxhlet apparatus. The extract was concentrated to dryness using rotary evaporator. The total *T. subcordata* extract (TSS) was subjected to further fractionation according to a published procedure (Kikueta *et al.*, 2013) to yield the chloroform fraction containing enriched alkaloid (TSS1), the aqueous extract (TSS2) and the aqueous methanol fraction (TSS3) (Fig. S2).

Isolation of cycleanine

The TSS1 (273 mg), which showed most cytotoxic activity, was subjected to silica gel chromatography eluting with $CHCl_3$ with increasing amount of MeOH to give 40 fractions. The eluted fractions were tested, identified and combined as guided by Dragendorff's reagent test, thin layer chromatography and UV detector at 254 nm and 365 nm wavelength. The combined fractions were concentrated in vacuum with rotary evaporator. Cycleanine (145 mg) was recrystallized from 1-4th fraction (TSS1.1). Fractions 5-8were combined as TSS1.2 and further purified by high performance liquid chromatography (HPLC). Purification of TSS1.2 (27.8 mg) was carried out using HPLC on an Agilent 1220 LC system (USA). UV detector was set at 254 nm wavelength. The mobile phase was a mixture of 0.1 % trifluoroacetic acid (TFA) (A) and 80% Acetonitrile containing 0.1 % TFA (B). The composition of the mobile phase was rising from 10 % to 70% B over a period of 25 min and kept at 100 % B for 6 min on a semi-preparative HPLC column (Phenomenex, UK; Jupiter C18 reverse phase, 300 A, 5 μm particle size, 9.4 × 250 mm) at a flow rate of 4 mL/min. Eluted fractions at different retention time were collected and freeze-dried on a lyophylizer. For analysis of the purity of the isolated alkaloids, a gradient from 10 % B to 70 % B over a period of 25 min and 100 % B for 6 min was applied on an analytical HPLC column (Phenomenex, UK; Jupiter C18 reverse phase, 300 A, 5 μm particle size, 4.6 × 250 mm) at a flow rate of 1 mL/min.

Characterization of cycleanine and TFA salt of cycleanine

Cycleanine, white powder (purity > 98%), ^{1}H and ^{13}C NMR ($CDCl_3$), see Table S1 and S2; LC-ESI-MS, *m/z*: 623.3118 $[M + H]^{+}$; 312.1610 $[M + 2H]^{2+}$. TFA salt of

cycleanine, white powder. ^{1}H and ^{13}C NMR ($CDCl_3$), see Table S1 and S2; LC-ESI-MS, *m/z*: 623.3074 $[M + H]^+$; 312.1579 $[M + 2H]^{2+}$.

Cell culture

The human ovarian cancer cell lines (Ovcar-8, Ovcar-4, A2780 and Igrov-1) and normal human ovarian surface epithelial (OSE) cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin-streptomycin (50 U/ml) and glutamine (2 mM).

Cell proliferation assay

The Sulforhodamine B (SRB) assay (Johnson-Ajinwo *et al*., 2015) was used to evaluate the effects of the plant extracts and pure compounds on the growth of ovarian cancer cell lines (A2780, Ovcar-8, Ovcar-4, Igrov-1) and human normal OSE cells. 2000 cells (A2780, Ovcar-8), and 5000 (Ovcar-4, Igrov-1, or OSE) cells were seeded in 80 µl of growth medium in each well of a 96-well plate. After 24 hours 20 µl of plant extracts dissolved in DMSO (final concentration 0.2%) or pure compounds were added. After 72 hours the medium was decanted and the cells were fixed with 10% trichloroacetic acid on ice for 30 min before drying. The cells were stained with 0.4% SRB in 1% acetic acid for 30 min, washed with 1% acetic acid and dried. Then, 200 µL of Tris-base (10 mM) were added to the dried plates and shaken for 5 min to solubilise the SRB dye. The absorbance at 540 nm was measured using a spectroscopic plate reader. The data was analysed by non-linear regression using the GraphPad PRISM 6 software to fit a 4 parameter sigmoidal concentration-response curve.

Cell counting

The viability of A2780 cancer cells was measured using trypan blue exclusion method (Invitrogen) before seeding cells and after drug treatment of the cells. The cells (A2780, Ovcar-8, Ovcar-4, or Igrov-1) (200, 000 cells/well) were seeded into 6 well plates, and medium (control), carboplatin, cycleanine or tetrandrine (20 μM) were administered to the cells after 24hr incubation. Then cell viability was determined using haemocytometer and trypan blue exclusion after 48h treatment. Briefly, 20 μL cells culture were collected after trypsinization and mixed with and equal volume of 0.4% trypan blue thoroughly. Then 20 μL of the suspension mixture was analysed with a Countess™ Automated Cell Counter (Life Technologies) to determine cell viability.

Caspase – Glo 3/7 activity assay

The effect of cycleanine and tetrandrine on the caspase-3/7 activities was carried out using assay kits Caspase-Glo 3/7 (Promega Corp., Madison, WI, USA) on a 96-well microplate as described previously with little modification (Robinson *et al.*, 2013). Briefly, A2780 and Ovcar-8 (2000 cells/well), and Igrov-1 and Ovcar-4 (5000 cells/well) cells were incubated in 80 μL growth medium in a 96-well plate for 24 hours. Cells were then supplemented with growth medium (control), carboplatin, cycleanine or tetrandrine (final concentration 20 μM) for 48 hours. Before adding caspase agents, the morphological changes were observed and the images were captured under an inverted light microscope. Then, 100 μL of the culture was treated with 25 μL caspase-Glo 3/7 reagent and incubated at room temperature on a Rocker (Gyro shaker) for 30 minutes, protected from light. The reading was measured at 570 nm by Multi-Mode Microplate Reader BioTEK Synergy 2 (USA).

Western blot assay for PARP cleavage

To evaluate apoptosis, 200,000 Ovcar-8 cells /ml were seeded in a 6-well plate and were treated with cycleanine, tetrandrine, carboplatin (20 μM) or medium for 48 h. Cells were collected by trypsinization, lysed, protein concentration determined by the bicinchoninic acid assay. Equal amounts of proteins (10 μg) from total cellular extracts were resolved on 4-20% Tris-Glycine polyacrylamide gradient gel (Nusep) with hepes running buffer (100 mM hepes, 100 mM Tris and 1% sodium dodecyl sulphate). After electrophoresis, the proteins on the gel were transferred to PVDF membrane. The membrane was then incubated with Tris buffered saline with tween buffer (50 mM Tris hydrochloride (Tris HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk powder for 1.0 hour with gentle rocking on a Stuart Scientific Platform Shaker STR6 at room temperature. The membrane was then probed with antibodies against poly (ADP) ribose polymerase (PARP) (Cell Signaling Technology Inc., USA) (1:1000) for 16 hours at 4°C overnight or glyceraldehde-3-phosphate dehydrogenase (GAPDH) from mouse (Millipore, USA) (1:5000) for 1 hour at room temperature. Protein were visualised using UptiLight HRP chemiluminescent substrate (Uptima) on a FluorChem M Imager, quantified by using AlphaView SA software (Protein Simple) and then normalised to the loading control, GAPDH.

Apoptosis assay with flow cytometry analysis

0.5 ml of Ovcar-8 cells (300,000 cells/ml) were seeded in 12 well plates for 24h, and exposed to medium as control, carboplatin, cycleanine and tetrandrine (20 μM). After incubation for 48 h, the cells were washed with PBS, trypinsized and stained with fluorescein isothycanate (FITC)-conjugated annexin V using Annexin V-FITC kit (Miltenyi Biotec, Germany) and popidium iodide (PI) following the manufacturer's protocol. Apoptotic and live cells were measured using

a Beckman Coulter Cytomics 500 flow cytometer with CXP software (High Wycombe UK). Flow cytometry data was analysed using Flowing Software (Perttu Terho, Turku Centre for Biotechnology, University of Turku, Finland). The percentage of early and later apoptotic, and live cells in three independent experiments were statistically analyzed using GraphPad Prism 6 software.

Cell cycle analysis by flow cytometry

Ovcar-8 1×10^6 cells/well seeded in a 6-well plates were treated with cycleanine, tetrandrine, carboplatin (20 µM), and medium (control) for 48 hr. The adherent cells were trypinized and washed with PBS. The cells were then fixed with 70% ethanol in cold PBS, and incubated at 4^oC for 24 hr. The fixed cells were centrifuged at 200 g for 10 mins and the cell pellets were washed twice with PBS. Cells were suspended in PBS containing propidium iodide (PI) (50 µg/mL), Triton X-100 (0.1%, v/v), and DNase-free RNase (1 µg/mL). DNA contents were determined by flow cytometry and analysed by Flowing software.

RESULTS

The extraction and fractionation of *T. subcordata* (Fig. S2) yielded the total crude extract (TSS), the total alkaloid (TSS1), the aqueous extract (TSS2) and the aqueous methanol fraction (TSS3). Both TSS1 and TSS3 showed positive Dragendorff's tests, which indicated the presence of alkaloids in these fractions. All of these fractions inhibited the growth of Ovcar-8 and A2780 ovarian cancer cells (Table 1 and Fig. 1A), but the most potent activity was exhibited by the alkaloid enriched TSS1. TSS1 also showed some selectivity for the cancer cells because the growth of normal OSE was inhibited with almost 10-fold lower potency (Table 1).

Bioassay-guided fractionation of TSS1 resulted in isolation of a single, highly pure alkaloid using silica gel chromatography and/or HPLC (Fig. S3), which was identified on the basis of spectroscopic data including LC-MS, ^{1}H NMR, and ^{13}C NMR (Table S1 and S2) as the bisbenzylisoquinoline alkaloid cycleanine (Fig. S1). Cycleanine was also isolated as a TFA salt form when using HPLC where the solvent system contained 0.1 % TFA. ^{1}H NMR and ^{13}C NMR of the TFA salt of cycleanine showed slight difference from those of cycleanine (Table S1 and S2).

Cell growth assays

The potency of cycleanine and tetrandrine in cell growth assay was investigated. They both inhibited the growth of 4 ovarian cancer cell lines with microMolar potencies (Fig. 1B and Table 2). The selective index (SI) of cycleanine ranged between 3 and 5. Consistent with the preliminary evidence for moderate selectivity of TSS1 for cancer cells, cycleanine showed modestly less potency against human OSE cells than the cancer cells.

Cytotoxicity

The trypan blue exclusion method was employed to distinguish cytotoxic and cytostatic effects. The viability of A2780, Ovcar-8, Igrov-1 and Ovcar-4 cells was reduced substantially by cycleanine and tetrandrine, as well as by the positive control carboplatin, when the four cancer cell lines were exposed to the two alkaloids for 48 h (Fig. 1C).

Apoptosis

To evaluate whether the decrease in viability caused by cycleanine was due to apoptosis, the activation of caspase 3/7 activity by cycleanine and tetrandrine was evaluated. Increased level of caspase activity in Ovcar-8, A2780 and Igrov-1 cells were observed (Fig. 2). Cycleanine increased caspase activity comparably to carboplatin, whereas tetrandrine augmented caspase activity noticeably more, up to 8 fold (Fig. 2). Morphological alterations of Ovcar-8 cells treated with carboplatin, cycleanine and tetrandrine were also consistent with apoptosis including membrane blebbing and cell detachment (Fig. S4).

To confirm the cells underwent apoptosis, PARP cleavage, a marker of apoptosis, was assessed by immunoblotting. Exposure of Ovcar-8 cells to cycleanine, tetrandrine, or carboplatin resulted in significant PARP cleavage (Fig. 3).

To further quantify the percentage of apoptotic cells after treatment of these alkaloids, the cells were stained by Annexin V and PI and analysed by flow cytometry (Fig. 4). Carboplatin, cycleanine and tetrandrine treatment after 48 h caused significant increase of the population of both early and late apoptotic cells compared to the control cells.

Cell cycle analysis

The effects of cycleanine, tetrandrine, and carboplatin on cell cycle distribution of Ovcar-8 cells are shown in Fig. 5. The percentage of Ovcar-8 cells in subG_1 increased

after exposure to cycleanine, tetrandrine, and carboplatin to for 48h compared to control, consistent with apoptosis (Fig. 5).

DISCUSSION

Cycleanine has been isolated from many other plant species of the Menispermaceae family (Schiff, 1997). Cycleanine was shown to increase the intracelluar doxorubicin accumulation in the resistant MCF-7/Adr cell lines (Tian and Pan, 1997).

Tetrandrine is also a common BBIQ alkaloid found in different plants as well as in *T. subcordata* (Dwuma-Badu *et al.*, 1975). It showed various biological activities including anticancer (Chen, 2002), anti-allergic, anti-inflammatory, anti-malarial, and cardiovascular effects (Schiff, 1997). Tetrandrine inhibited the growth of various types of cancer cells (Chen, 2002; He *et al.*, 2011; Xu *et al.*, 2011) and could reverse multidrug resistance by inhibiting P-glycoprotein activity (Sun and Wink, 2014).

Here, cycleanine has been isolated and characterized from *T. subcordata*, as part of novel contribution to knowledge. The analytical data (ESI-MS and NMR) were consistent with literature data for cycleanine (Kanyinda *et al.*, 1997). The change of ^{1}H NMR signals of cycleanine by TFA was observed and such features were often found for the other alkaloids (Verpoorte, 1986). Furthermore, we showed that both a head-to-tail BBIQ cycleanine (including TFA salt of cylcleanine) and a head-to-head BBIQ tetrandrine which is an isomer of cycleanine and was previously isolated from *T. subcordata* (Fig. S1) showed potent anti-ovarian cancer activities. While such a structural difference in BBIQ alkaloids was key to the inhibition of histamine release as

only the head-to-tail BBIQ such as tetrandrine showed the inhibition (Nakamura *et al.*, 1992).

The induction of apoptosis by tetrandrine was observed previously in human lung carcinoma (Lee *et al.*, 2002), bladder cancer (Li *et al.*, 2011), colorectal cancer (He *et al.*, 2011), gallbladder carcinoma (Zhu *et al.*, 2014), and prostate cancer cells (Liu et al., 2015). The combination of tetrandrine and cisplatin caused enhanced cytotoxicity through apoptosis as well as redistribution of the cell cycle in ovarian cancer cells in vitro and vivo (Zhang *et al.*, 2011). To confirm that these two types of BBIQ alkaloids (cycleanine and tetrandrine) could induce apoptosis in ovarian cancer cell lines, two markers of apoptosis including caspases 3/7 and cleavage of PARP to its 89 kDa fragment were measured. Cycleanine and tetrandrine significantly increased caspase 3/7 activity in all cell lines tested, indicating the induction of apoptotic cell death (Fig. 2). The apoptotic activity was further confirmed by the detection of the PARP-1 fragment in cycleanine and tetrandrine-treated Ovcar-8 cells using immunoblotting assay (Fig. 3). The percentage of the apoptotic cells caused by tetrandrine is greater than those by cycleanine and carboplatin (Fig. 4). Tetrandrine increased population of both apoptotic sub-G_1 and G_1 phase in the lung cancer cells (Lee *et al.*, 2002). The increase of cell fraction of sub-G_1 by tetrandrine was also observed in ovarian cancer cells in this study indicating the induction of apoptosis because such sub-G_1 peak is often regarded as a characteristic indicator of apoptosis (Kajstura et al., 2007). Interestingly, cycleanine was shown in this studay to cause apoptosis in cancer cells similar as tetrandrine but with less potency (Fig. 2, 4, 5).

In this study, the extracts and BBIQ alkaloids of *T. subcordata* were demonstrated to exert significant *in vitro* anti-ovarian cancer activities, which provided a scientific basis

for future potential use of this plant for the treatment of ovarian cancer. Importantly, cycleanine showed a modest selectivity towards ovarian cancer cells over normal cells via apoptosis, which may be used as a hit to modify its structure by semi-synthesis in order to improve its anti-cancer activity. To be approved as anticancer drugs, in vivo activities of these alkaloids and analogues must be evaluated. This work is ongoing in our laboratory and will be reported in the future.

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Conflict of Interest

All authors have no conflict of interest to disclose.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web site.

Table legends

Table 1. The IC_{50} and SI values of the total extracts and fractions of *T. subcordata* on Ovcar-8, A2780, and normal human OSE cells (the data are expressed as mean ± SEM (n=3).

Sample and SI	IC_{50} (µg/ml)		
	Ovcar-8	A2780	OSE
TSS	4.2 ± 1.1	2.6 ± 1.2	ND
TSS1	2.4 ± 0.5	2.1 ± 0.9	20 ± 1.3
SI for TSS1	8.2	9.5	-
TSS2	53 ± 3.0	39 ± 2.4	ND
TSS3	6.0 ± 2.1	3.5 ± 1.1	8.2 ± 1.1
SI for SS3	1.4	2.4	-

Selective index (SI) is defined as the ratio of IC_{50} in OSE cells to that in the cancer cells. ND represents not determined.

Table 2. The IC_{50} and SI values of the BBIQ alkaloids of *T. subcordata* on Ovcar-8, A2780, Igrov-1 and Ovcar-4 cells (the data are expressed as mean ± SEM (n=3).

Compound and SI	IC_{50} (μM)			
	Ovcar-8	A2780	Igrov-1	Ovcar-4
Cycleanine	10 ± 0.6	7.6 ± 0.7	14 ± 1.0	7.2 ± 0.4
SI for cycleanine	3.4	4.6	2.6	4.8
Tetrandrine	12 ± 1.4	7.7 ± 0.2	6.9 ± 0.6	9.3 ± 0.4
Paclitaxel	5.2 nM	4.6 nM	ND	8.7 nM

IC_{50} of cycleanine on normal human OSE cells was determined as 35 ± 1.0 μM. Paclitaxel served as positive control. ND represents not determined.

Fig. Legends

Fig. 1. Cytotoxicity of extracts and alkaloids from *T. subcordata*. Concentration vs response curves of total alkaloid fraction (TSS1) from *T. subcordata* (A), and isolated cycleanine (B) on Ovcar-8 ovarian cancer cell line. The mean values of their IC_{50} are expressed as mean ± SEM (n = 3). Cell viability tests for A2780, Ovcar-8, Igrov-1 and Ovcar-4 cell lines post-treatment with carboplatin, cycleanine and tetrandrine (20 µM) using countess automated cell counter (C).

Fig. 2. Enzymatic activities of caspase 3/7 after 48 h treatment of Ovcar-8 (A), A2780 (B) and Igrov-1 (C) cells with medium, carboplatin, cycleanine, and tetrandrine (20 µM). The caspase activity is expressed as fold increase relative to control cells (mean ± S.D., n = 3). The caspase activity was significantly increased following carboplatin, cycleanine and tetrandrine exposure compared to cells treated with medium (control) where indicated (one way ANOVA for group analysis, $P<0.0001$ for Ovcar-8 and Igrov-1 cells, and $P=0.0044$ for A2780 cell line; paired *t*-test relative to control, *, $P<0.05$; **, $P<0.005$; ***, $P<0.0001$).

Fig. 3. Western blotting analysis of the PARP cleavage marker in Ovcar-8 cells after treatment with carboplatin, cycleanine and tetrandrine. GAPDH was used as a loading control (n = 3). Quantification showed that the treatment of carboplatin, cycleanine and tetrandrine led to 1.1-, 1.5- and 1.4-fold increase in PARP compared to control cells, respectively. Cycleanine and tetrandrine induced 1.1- and 1.3-fold increase in PARP-1 cleavage compared to carboplatin, respectively.

Fig. 4. Flow cytometry analysis of apoptotic effects of carboplatin, cycleanine, and terandrine 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),

cycleanine (C), and tetrandrine (D) (20 μM) for 48 hours by using Annexin V & PI assay. The percentages of the total apoptotic cells caused by carboplatin, cycleanine, and tetrandrine were compared (E) (one way ANOVA for group analysis, $P<0.0001$; paired *t*-test relative to control, *, $P<0.05$; **, $P<0.005$).

Fig. 5. The effects of carboplatin, cycleanine, and tetrandrine on the cell cycle. Flow cytometry analysis of the DNA content in Ovcar-8 cells exposed to medium (control) (A), carboplatin (B), cycleanine (C), and tetrandrine (D) (20 μM) for 48 hours by using only PI staining. The percentage of carboplatin, cycleanine, and tetrandrine-treated cells in subG_1 phase of the cell cycle was significantly increased compared to cells exposed to medium alone.