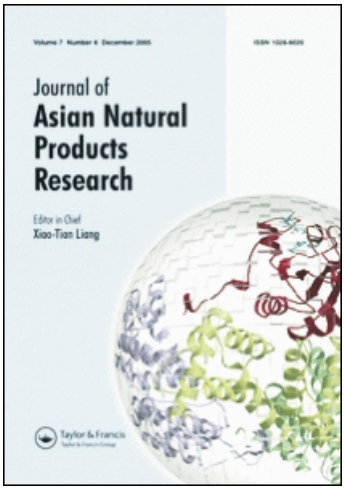

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Growth inhibition and induction of early apoptosis by arenicolsterol A, a novel cytotoxic enolic sulphated sterol from the marine annelid, *Arenicola cristata*

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Growth inhibition and induction of early apoptosis by arenicolsterol A, a novel cytotoxic enolic sulphated sterol from the marine annelid, *Arenicola cristata*

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Arenicolsterol A (ASA), a novel cytotoxic enolic sulphated sterol, was isolated from the marine annelid, *Arenicola cristata* (AC). Growth inhibition of this compound on cancer cell lines was determined by MTT assay and suppression of tumour stem cells colony formation. The results showed that ASA was selectively cytotoxic on HeLa cell line ($IC_{50} = 6.00 \pm 1.16\,\mu mol\,L^{-1}$ on HeLa cell line, $IC_{50} = 10.85 \pm 0.97\,\mu mol\,L^{-1}$ on 929 cell line and $14.72 \pm 1.55\,\mu mol\,L^{-1}$ on NCI-h6 cell line). In addition, the apoptosis induced by ASA was verified from monitoring the stainability with Annexin V and propidium iodine by a fluorescence-activated cell sorter. The experimental data confirmed that ASA could induce apoptosis in HeLa cells by arresting early stage in apoptosis. Meanwhile, the apoptosis was found to be correlative with the inhibition of the protein tyrosine phosphatases (cdc25A, cdc25B, JSP1, etc). Therefore, ASA might be a novel promising precursor of anticancer medicines.

Keywords: Natural product; *Arenicola cristata*; Arenicolsterol A; Anticancer; Apoptosis

1. Introduction

Marine organisms are an important source of bioactive compounds [1]. Some have shown very strong cytotoxic activity on certain cancer cell lines and have been applied as important anticancer medicines, such as the well-known bryostatin 1, isolated from bryozoan *Bugula neritina* [2], which can work on carcinoma cells through inducing apoptosis. For example, the flavonoids from *Vitex trifolia* L. inhibit cell cycle progression and induce apoptosis in mammalian cancer cells [3], and CFP-2, a novel lichenin from *Cladonia furcata*, performs as a telomerase inhibitor and induces apoptosis in human leukaemia cells (HL-60) [4]. Although many compounds inducing apoptosis of carcinoma cell lines have been found, it is still a big challenge to find new anticancer compounds in order to realize the effective chemotherapy of various sorts of cancers.

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Arenicola cristata (AC) is a widely distributed polychaete annelid in the eastern coastland of China. The most famous Chinese traditional medical book *Pen-ts'ao Kan-mu* illustrated that it was used as an effective raw medicine material with the function of de-tumescence and promotion of wound healing [5]. Some research has focused on AC. Lloyd Waxman from Harvard University separated the haemoglobin from AC and characterized the structure [6]. George Raymond Parker and Ying Ming Lin isolated four proteases that activate cyclic AMP phosphodiesterase from lugworm (AC) and reported the characterisation and the peptidase specificity of protease C [7]. However, the anti-proliferative activity of AC on tumours has never been demonstrated.

In our previous work, a novel enolic sulphated sterol, arenicolsterol A (ASA), has been isolated and identified from the marine annelid, AC [8]. Here we present the tumour-specific cytotoxic activity of ASA, by using both the normal cells (mouse skin fibroblast 929) and human tumour cells (human cervix cancer cell line HeLa and human non-small cell lung cancer cell line NSCLC). In addition, the early apoptosis induced by ASA was investigated in detail by using Annexin V as a probe of aminophospholipid exposure.

2. Results and discussion

2.1 Preparation and identification of ASA

Extraction, isolation and purification were performed under the bioassay-guide. Strong polar components, which showed highly biological activity, were obtained from the extraction of 85% aqueous ethanol. Combination *via* silica gel columns with filtration by Sephadex LH-20 made the isolation more effective. The structure of the novel sulphated sterol was elucidated by interpretation of spectral data and comparison with those of stellasterol (figure 1). Details about the elucidation of the novel enolic sulphated sterol have been published elsewhere [8].

2.2 Cytotoxic activity of ASA

MTT assay showed that ASA in all different concentrations inhibited the growth of human cervix cancer cell line (HeLa) and human non-small cell lung cancer cell line (NCI-h6). The control cell line is mouse skin fibroblast (929). At the highest concentration of 15.50 μmol L^{-1}, the inhibition rate (IR%) was 98.44% on HeLa cell line, 51.8% on NCI-h6

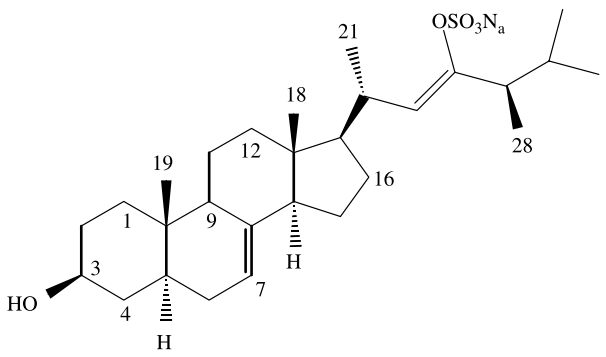

Figure 1. The structure of arenicolsterol A (ASA).

Table 1. Suppression of ASA on HeLa stem cells colony formation (929 cell line is employed as the control).

Group	*Concentration ($\mu mol\,L^{-1}$)*	*Clone number (/dish)*	*Inhibition rate (%)*
Control (HeLa)	–	224.50 ± 9.52	–
a (HeLa)	11.62	0	100
b (HeLa)	5.81	0	100
c (HeLa)	1.16	4.20 ± 1.32	98.13
Control (929)	–	112.60 ± 11.46	–
a (929)	11.62	0	100
b (929)	5.81	35.35 ± 5.23	68.61
c (929)	1.16	92.30 ± 8.32	18.03

cell line and 33.6% on 929 cell line. The half-maximal inhibitory concentration values (IC_{50}) were listed as follows: HeLa, 6.00 ± 1.16 $\mu mol\,L^{-1}$; NCI-h6, 1.47 ± 0.15 $\mu mol\,L^{-1}$, and 929, 1.08 ± 0.01 $\mu mol\,L^{-1}$. ASA showed selective cytotoxicity on HeLa cell as compared with the control cell, 929 cell line.

2.3 Suppression of ASA on the colony formation of HeLa stem cells

After incubation with ASA (11.62, 5.81 and 1.16 $\mu mol\,L^{-1}$) or vehicle for 9 days, the clones that HeLa stem cells formed were strongly inhibited (see table 1 and figure 2). At the lowest concentration of 1.16 $\mu mol\,L^{-1}$, the inhibition rate of the colony formation on HeLa stem cells was 98.13%; While the inhibition rate of the colony formation on 929 stem cells was 18.03%. The result showed selective inhibition of ASA on the cancer cells.

2.4 Assay for early apoptosis induction

Apoptosis of HeLa cells treated for 12, 16, 20 h with ASA (34.86, 17.43, 8.72, 4.36, 2.18 $\mu mol\,L^{-1}$) or vehicle control was analysed using flow cytometry. Results showed that ASA mediated a time-dependent and dose-dependent change in apoptosis (shown in tables 2 and 3 and figures 3–5). As shown in figure 3, after the cells were treated for 12 h with the samples, the cell viability changed from 95.44 ± 5.45% to 96.37 ± 4.57% (mean ± SE (%)) and thus apoptosis could hardly be observed. But after the cells were treated for 16 h, the total number of early and late apoptotic cells changed significantly from 5.53 ± 0.67% to 22.90 ± 2.54% ($p < 0.05$). In addition, after cells were treated for 20 h (figure 5), there were

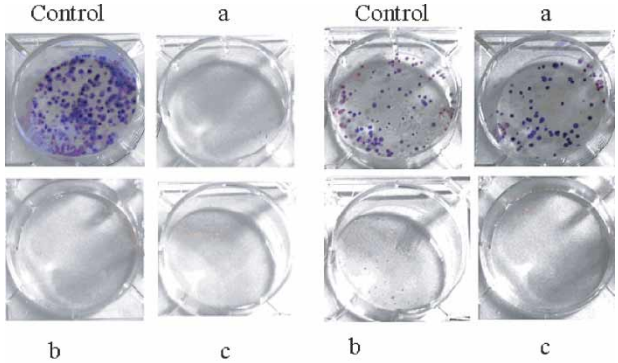

Figure 2. Suppression of ASA on HeLa stem cells colony formation. A. Human cervix cancer cell line (HeLa), B. Mouse skin fibroblast (929). a. 1.16 $\mu mol\,L^{-1}$, b. 5.81 $\mu mol\,L^{-1}$, c. 11.62 $\mu mol\,L^{-1}$.

Table 2. Inhibition of ASA on protein tyrosine phosphatases.

PTPs	*IC_{50} ($\mu mol\,L^{-1}$)*	*PTPs*	*IC_{50} ($\mu mol\,L^{-1}$)*
cdc25A	0.69 ± 0.42	cdc25B	0.27 ± 0.05
JSP1	0.21 ± 0.01	SHP1	0.34 ± 0.03
PTP1B	0.22 ± 0.04	mPTPσ	2.90 ± 0.06
TCPTP	1.48 ± 0.11	PTP-PEST	1.07 ± 0.05
PTP-LAR	38.62 ± 3.56	PTPα	>40

Table 3. ASA mediates a dose-dependent change in apoptosis by cell-flow cytometry.

	Annexin-V positive cells, %Total	
Concentration ($\mu mol\,L^{-1}$)	*Late apoptotic and necrotic (PI +)*	*Early apoptotic (PI −)*
0	5.36 ± 0.58	0.07 ± 0.01
2.18	6.17 ± 1.02	9.72 ± 2.20
4.36	6.35 ± 1.81	14.92 ± 0.88
8.72	12.19 ± 0.81	10.41 ± 2.78
17.43	15.80 ± 1.54	10.33 ± 1.92
34.86	26.94 ± 3.58	2.76 ± 0.37

hardly any early apoptotic cells observed and the total number of early and late apoptotic cells changed significantly from 17.30 ± 3.42% to 49.52 ± 6.33% ($p < 0.05$). As shown in figures 3–5, it was observed that ASA mediated a time-dependent change in early apoptosis. A dose-dependent change in early apoptosis mediated by ASA is shown in table 3. HeLa cells treated for 16 h with ASA (2.18, 4.36, 8.72, 17.43 and 34.86 $\mu mol\,L^{-1}$) or vehicle

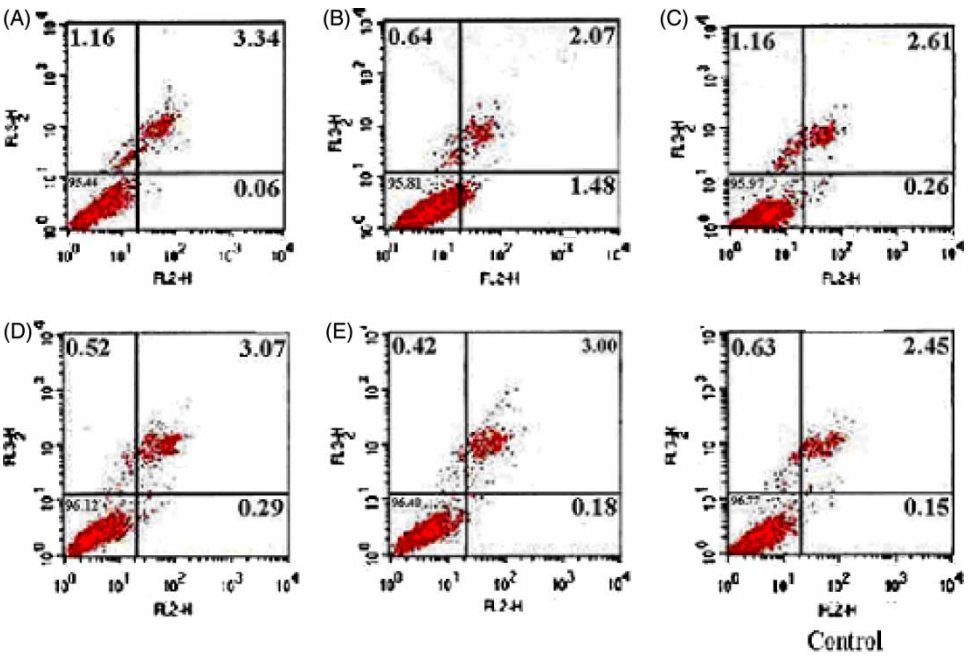

Figure 3. FACS analysis of the appearance of apoptotic and necrotic cell populations after 12 h treatment with ASA in HeLa cell line. The number of each quadrant represents the percentages of cells (A. 34.86 $\mu mol\,L^{-1}$, B. 1.743 $\mu mol\,L^{-1}$, C. 8.72 $\mu mol\,L^{-1}$, D. 4.36 $\mu mol\,L^{-1}$, E. 2.18 $\mu mol\,L^{-1}$). Two independent experiments were done in triplicate.

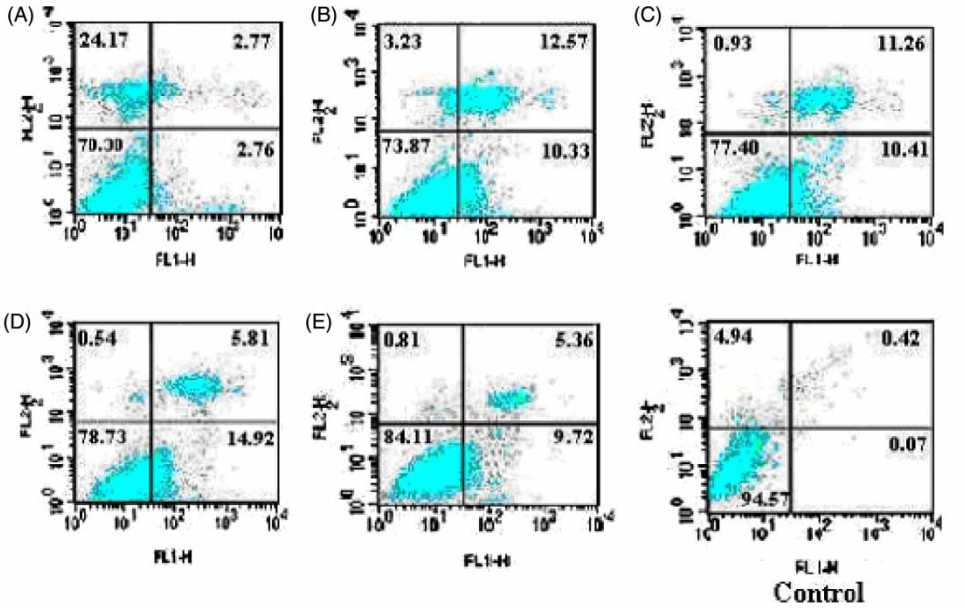

Figure 4. FACS analysis of the appearance of apoptotic and necrotic cell populations after 16 h treatment with ASA in HeLa cell line. The number of each quadrant represents the percentages of cells (A. 34.86 μmol L^{-1}, B. 1.743 μmol L^{-1}, C. 8.72 μmol L^{-1}, D. 4.36 μmol L^{-1}, E. 2.18 μmol L^{-1}). Two independent experiments were done in triplicate.

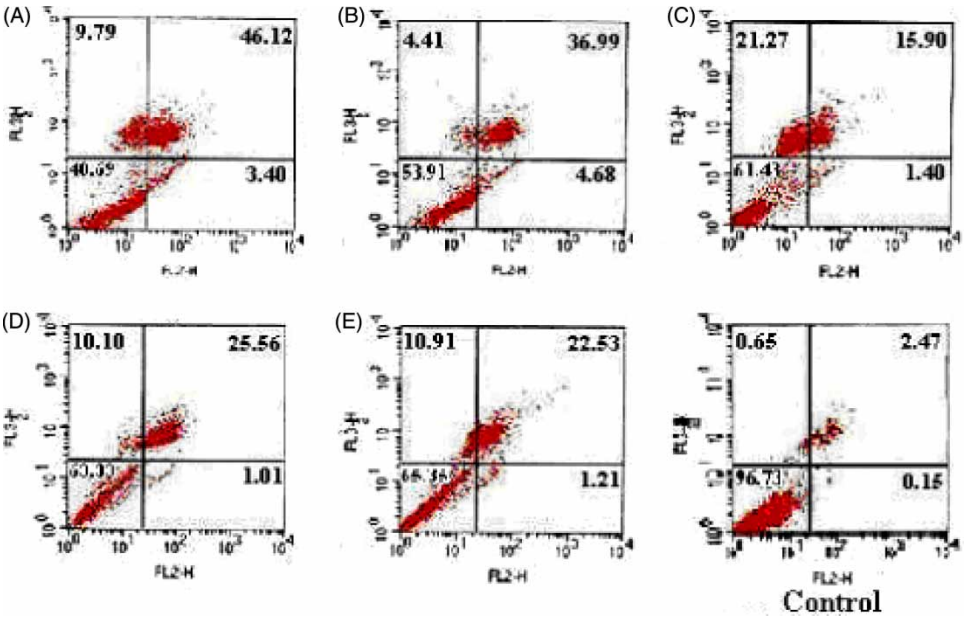

Figure 5. FACS analysis of the appearance of apoptotic and necrotic cell populations after 20 h treatment with ASA in HeLa cell line. The number of each quadrant represents the percentages of cells (A. 34.86 μmol L^{-1}, B. 1.743 μmol L^{-1}, C. 8.72 μmol L^{-1}, D. 4.36 μmol L^{-1}, E. 2.18 μmol L^{-1}). Two independent experiments were done in triplicate.

control were simultaneously stained *in situ* with annexin V-FITC antibodies and PI. Annexin membrane-stained cells and PI − or PI + nuclear stained cells were counted using a fluorescent microscope and expressed as a percentage of the total number of cells in the same field determined by phase microscope (mean ± SE). Results showed a dose-dependent change ($p < 0.05$, ANOVA) in early apoptotic cells (annexin +/PI −) and necrotic cells. With sample concentration increasing, the total number of the late apoptotic cells and necrotic cells increased significantly from 5.36 ± 0.58% to 26.94 ± 3.58%. The early apoptosis can be observed obviously at 8.72 μmol L^{-1} (the number of apoptotic cells is 10.41 ± 2.78%) and 4.36 μmol L^{-1} (the number of apoptotic cells is 14.92 ± 0.88%). With sample concentration increased, the number of the early apoptotic cells is firstly increased and then decreased.

2.5 Inhibition of ASA on protein tyrosine phosphatases

To elucidate the possible cellular molecular target of ASA, the inhibitory effect of compound ASA on cdc25A and cdc25B was determined, and the results are listed in table 2. ASA inhibits the cdc25 enzymes with an IC_{50} of submicromolar (cdc25A, 0.69 ± 0.42; cdc25B, 0.27 ± 0.05). On further study of its selectivity on other members of protein tyrosine phosphatase family, we found ASA brought inhibition on intracellular JSP1, SHP1, and PTP1B with a similar potency to cdc25s, weaker inhibition on mPTPσ, TCPTP, and PTP-PEST, and almost no inhibition on receptor-like PTP-LAR and PTPα.

3. Discussion

In this work, growth inhibition of ASA, a novel enolic sulphate sterol isolated from the marine annelid AC, was determined on cancer cell lines by MTT assay and suppression of tumour stem cell colony formation. ASA was higher cytotoxic on HeLa ($IC_{50} = 6.00 \pm 1.16$ μmol L^{-1}) than the other two cell lines ($IC_{50} = 10.85 \pm 0.97$ μmol L^{-1} on 929 cell line, 14.72 ± 1.55 μmol L^{-1} on NCI-h6 cell line), which showed that ASA was a selective cytotoxicity on HeLa. At the same time, the present study also demonstrated that ASA produced Annexin V-positive cells in which phosphatidylserine was exposed on the other cell membrane at an early stage of apoptosis. Apoptosis induced by ASA could be correlative with the inhibition of the protein tyrosine phosphatases (cdc25A, cdc25B, JSP1, etc).

As addressed by other research groups, many cytotoxic sterols can kill the cancer cells through inducing apoptosis. For example, dietary alpha-linolenic acid reduces COX-2 expression and induces apoptosis of hepatoma cells [9]. Another sterol from plant, called beta-sitosterol, induces apoptosis by activating key caspases in MDA-MB-231 human breast cancer cells [10]. However, no sulphated sterols that can induce apoptosis in cancer cells has been reported yet. ASA, a novel enolic sulphated sterol, was demonstrated to induce apoptosis in HeLa cancer cell lines.

It is very important to study the mechanism of apoptosis by arresting the early stage in apoptosis. High level and over-expression of stimulative-apoptotic genes (Bax, Bak, Bad, Fas, etc) or relative low level and expression of anti-apoptotic genes (Bcl-2, Bcl-xL, Al/Bfl-1, Mcl-1, etc) are the characteristics of the early apoptotic cancer cells, which independently and subsequently induce the emergence of the characteristics of the late apoptosis, such as the activation of caspases. The above-mentioned signal transmitting process forms the

apoptotic pathway [11,12]. The mechanism of HeLa apoptosis induced by ASA was elucidated by the inhibition assay on protein tyrosine phosphatases.

Over-expression of cdc25A and cdc25B was observed in a variety of cancers with a striking association with tumour aggressiveness and poor prognosis, which made cdc25s an attractive drug target for cancer therapy. Over the past few years, some irreversible small molecule cdc25 inhibitors have been described in the literature [13], and have been reported to arrest the cell cycle in G1 or S/G2 phase. JSP1 and PTP1B are also potentially involved in the development of cancer [14,15]. In this study, the novel enolic sulphate sterol, ASA, potently inhibited cdc25A, cdc25B, JSP1 and PTP1B, suggesting that these protein tyrosine phosphatases could play important roles on apoptosis induction and the anticancer effect.

In conclusion, ASA was confirmed to be an ideal growth inhibitor of HeLa cancer cells in our present study. ASA has potential to be employed as a valuable starting compound in order to develop novel anti-cancer medicines.

4. Experimental

4.1 Preparation of ASA

The marine annelid AC was collected from the Beach of Zhou Shan Island of the Mainland of China and immediately frozen. After the frozen samples were dried (5 kg), they were extracted with 85% aqueous ethanol. The extract was dried and sequentially partitioned between water and ethyl acetate and *n*-butanol. The *n*-butanol soluble part was firstly chromatographed over silica gel, and followed by a gel filtration step with a Sephadex LH_{20} column. Final purification was achieved by reverse-phase HPLC and yielded the pure ASA ($C_{28}H_{45}O_5SNa$, 16 mg) [8]. The yield rate is 3.2×10^{-4}%.

The vacuum-dried ASA was kept at -20°C for not more than 1 week before further testing. In *in vivo* and *in vitro* experiments, the vehicle for the tested materials was 0.5% DMSO in PBS.

4.2 Cell culture

The lung cancer cell line (NCI-h6), the cervix carcinoma cell line (HeLa), and the normal mouse skin fibroblast cell line (929) were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All cell lines were maintained in Dulbecco's modified Eagle medium, containing 1% non-essential amino acids and 10% foetal calf serum. They were cultured in the humidified 5% carbon dioxide in air at 37°C.

4.3 Assay for cytotoxic activity

About 2000 NCI-h6, HeLa cells or mouse skin fibroblasts were seeded separately in wells of a 96-well flat-bottomed plate. On the second day, different concentrations of ASA (each concentration in triplicate) were added and cells were incubated for another 48 h. Afterwards, the medium was discarded and 100 μl of the MTT solution (Sigma, 500 μg/ml in 1-fold Dulbecco's modified Eagle medium) was added and incubated for 4 h at 37°C. The medium was carefully removed and 150 μl of DMSO was added. After gently shaking at room

temperature for 10 min, optical absorbance at 570 nm was recorded using a microplate reader (Bio-Rad). Each experiment was done in triplicate and repeated 3 times.

4.4 Suppression on the colony formation of the tumour stem cell

HeLa cancer cells were seeded at 800 cells per 75-mm dish and the next day ASA in different concentrations or the vehicle was added. After 9 days, cells were removed with trypsin–EDTA, rinsed with ice-cold PBS (NaCl 171 mmol L^{-1}, KCl 3.35 mmol L^{-1}, Na_2HPO_4 10 mmol L^{-1}, KH_2PO_4 1.84 mmol L^{-1}, pH 7.2) twice, and fixed for at least 20 min after drop-wise addition of methanol. Then the cells were stained with 10% Giemsa for 20 min, rinsed with 3 × -distilled water, and dried in air. Finally the clones containing more than 20 cells per dish were counted with dissection lens. Two independent experiments were done in triplicate. The inhibition rate (IR%) of colony formation was calculated using the following equation [16].

$$\text{IR}\,(\%) = (\text{Mean treated clone number})/(\text{Mean control clone number}) \times 100\,\%$$

4.5 Assay for early apoptosis induction

PI/Annexin V double fluorescence staining method was adopted [17]. After treatment of ASA in different concentrations or control for different times, 1×10^6 cells were trypsinised and washed twice with ice-cold PBS. Cells were resuspended with 1 × binding buffer (10 mmol L^{-1} Hepes/NaOH (pH 7.4), 140 mmol L^{-1} NaCl, 2.5 mmol L^{-1} $CaCl_2$, Becton Dickinson, Franklin Lakes, NJ, USA). Cells suspended in 100 μL solution were stained with 5 μL Annexin V-FITC (Cat. No. 51-65874X, Becton Dickinson) and 5 μL propidium iodide (Cat. No. 51-66211E, Becton Dickinson Co.). The stained cells were held at room temperature for 15 min in the dark. Samples were analysed using a fluorescence-activated cell sorter (FACS) (Becton Dickson) at a laser setting of 36 mW and an excitation wavelength of 488 nm. Two independent experiments were done in triplicate.

The cell populations were separated into four groups (figures 3–5). The cells that were stained with Annexin V but not with PI were classified as early apoptotic cells (lower right quadrant); the cells that were positive for Annexin V and PI were classified as late apoptotic cells (upper right quadrant); the cells that were negative for Annexin V but positive for PI were classified as necrotic cells (upper left quadrant); the cells that were negative for both Annexin V and PI were classified as normal viable cells (lower left quadrant).

4.6 Inhibition assay of ASA on protein tyrosine phosphatases

In our efforts to elucidate the possible cellular mechanism of anti-tumour activity of ASA, we tested the compound on the identified molecular targets in tumour occurrence. The dual specificity phosphatases (cdc25s) play a pivotal role in the regulation of the cell cycle by activating cyclin dependent kinases (CDK) and participating in Raf-1-mediated cell signalling [18]. Cdc25A and cdc25B were prepared as GST fusion protein as in a previous report [19], and the typical inhibition assay was carried out in a 100 μl system containing 50 mmol L^{-1} Tris–HCl pH 8.0, 50 mmol L^{-1} NaCl, 2 mmol L^{-1} DTT, 2 mmol L^{-1} EDTA, 1% glycerol, 10 mmol L^{-1} OMEP, 500 nmol L^{-1} GST-cdc25A or 100 nmol L^{-1} GST-cdc25B, and the inhibitor diluted around the estimated IC_{50} values in 2% DMSO. The reaction was monitored by Victor^2 (Perkin–Elmer; excitation filter 485 nm, emission filter

530 nm) at room temperature. IC_{50} was calculated from the non-linear curve fitting of percent inhibition (% inhibition) *vs* inhibitor concentration [I] by using the following equation:

$$\text{Inhibition}\,\% = 100/\{1 + (IC_{50}/[I])k\},$$

where k is the Hill coefficient.

To study the inhibition selectivity of ASA on other PTPase family members, mouse PTP and human PTP1B, TCPTP, PTP-PEST, PTP-LAR, SHP1, JSP1 and PTP were prepared. Assays were performed for PTP-LAR, SHP1, PTP, PTP1B, TCPTP using 2 mmol L^{-1} pNPP as substrate, JSP1, mouse PTP and PTP-PEST using 10–20 μmol L^{-1} OMFP as substrate in 50 mmol L^{-1} Tri–HCl at their optimal pH in the presence or absence of the compound.

Acknowledgements

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