Polysaccharide from *Sepia esculenta* ink and cisplatin inhibit synergistically proliferation and metastasis of triple-negative breast cancer MDA-MB-231 cells

Hua-Zhong Liu [1], Wei Xiao [2, 3], Yi-Peng Gu [4], Ye-Xing Tao [5], Da-Yan Zhang [3], Hui Du [6], Jiang-Hua Shang [2]*

[1] College of Chemistry and Environment, Guangdong Ocean University, Zhanjiang 524088, China
[2] Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences, Nanning 530001, China
[3] College of Food Science & Technology, Guangdong Ocean University, Zhanjiang 524088, China
[4] Institute of Food Science & Engineering Technology, Hezhou University, Hezhou 542899, China
[5] Experimental Center for Sciences, Guilin Medical University, Guilin 541004, China
[6] Biochemisty Center, Guangdong Ocean University, Zhanjiang 524088, China

A R T I C L E I N F O	A B S T R A C T
	Objective(s): This paper aims to investigate synergistic inhibition of polysaccharide from *Sepia esculenta* ink (SIP), a newly isolated marine polysaccharide in our laboratory, on breast cancer MDA-MB-231 cells exposed to cisplatin. ***Materials and Methods:*** Cell viability of MDA-MB-231 cells was determined by CCK 8 assay. Median-effect concentration was analyzed using Chou-Talalay method that was also subjected to determine cell inhibition ratio and combined index, as well as interaction between SIP and cisplatin. Proliferation and migration abilities were detected with plate colony formation assay and cell wound scratch assay, respectively. Expression of MMP-2 and MMP-9 proteins was measured with Western blot assay. ***Results:*** Data showed that SIP not only suppressed proliferation and migration of MDA-MB-231 cells, and expression of MMP-2 and MMP-9 proteins, also promoted inhibition of cisplatin on proliferation, migration and MMPs expression of MDA-MB-231 cells, which indicates synergy inhibition of drug combination of SIP and cisplatin on breast cancer cells. The median-effect concentrations of cisplatin and SIP were 4.9 and 1659.6 μg/ml, respectively. Whereas the concentration of combination drug was 158.5 μg/ml. The data indicated that drug combination can decrease dosages of the two single agents, especially the usual dosage of cisplatin. ***Conclusion:*** This research demonstrated that SIP repressed proliferation and metastasis of MDA-MB-231 cells and promoted anticancer effect of cisplatin on the breast cancer cells. The data suggested that SIP is a potential natural drug that can be used as an auxiliary medicine alongside chemotherapy in treating breast cancer.

Introduction

Breast cancer, most common cancer in women, is treated with initial surgical resection and adjuvant chemotherapy, although the undesirable event is generally hard to avoid (1). As a common chemotherapeutic drug, cisplatin has been proved to be effective in treating metastatic breast cancer, used alone or combined with other anticancer drugs (2, 3). Despite the positive roles of cisplatin in destroying breast cancer cells, cytotoxicity also occurs on normal cells, mediating nuclear DNA to inter- and intra-strand cross-linkage and eventually resulting in apoptosis (4). Reduction of cisplatin dosage is adopted to impair toxicity on normal tissues/organs, which should decrease the killing effects on breast cancer cells as a result.

Squid ink polysaccharide (SIP) has been proved to be a multifunctional marine active substance. Besides antioxidation (5-7), UV resistance (5), antimutagenesis (8), antitumor (9-11), and immune-potentiation (11), chemopreventative activity was also found in previous reports. Our findings showed that SIP could effectively attenuate the toxicity of cyclophosphamide on testes, protecting reproductive ability of mice via activation of the Nrf2/ARE pathway (6, 7). Also protection of SIP towards intestine of mice exposed to cyclophosphamide was reported elsewhere (12-14).

Furthermore Zong *et al* (11) found that SIP could enhance the antitumor activity of cyclophosphamide, which indicates a synergistic enhancing effect through the combination of anticancer agent and SIP.

Corresponding author: Jianghua Shang. Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences, China, 530001. email: lgxdzh@163.com

Drug combination, commonly called cocktail therapy, is widely used to treat cancer. The therapeutic method can not only achieve synergistic therapeutic effect, but also reduce dosage and toxicity of antitumor agents, as well as reduce or delay the induction of drug resistance (15). The objectives should be based on optimal dose proportions of the drugs used, an important and necessary problem to be solved before therapy. The Chou-Talalay method for drug combination based on the median-effect equation is an analytical method, used *in vitro*, that can be used to quantify antagonism, synergism, and additive effect among the drugs (16). To take full advantage of the antitumor activities of cisplatin and SIP, as well as the chemopreventive activity of SIP, this study investigated the inhibitory effect of a combination drug of cisplatin and SIP on the proliferation and metastasis of breast cancer MDA-MB-231 cells. SIP used in this study is a newly isolated polysaccharide, with a different primary structure from the reported SIPs, from *Sepia esculenta* ink in our laboratory (17).

Materials and Methods

Cell culture

Breast cancer MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium (DMEM) and Ham F-12 (DMEM/F12 = 1:1) supplemented with 10% foetal bovine serum (FBS) at 37 ℃, 5 % CO_2 in a humidified atmosphere. Cells were harvested and seeded at a density of 1×10^6 cells/ml.

Proliferation and viability analysis of MDA-MB-231 cells

Cells (1×10^4 cells/ml) were seeded in 96-well cell culture plate, at 100 μl in each well, and subjected to determine cell viability when treated with cisplatin and/or SIP using cell counting kit 8 (CCK 8), 10 μl of CCK 8 solution were used in each well. At the end of this treatment, optical density values were read at a wavelength of 450 nm. Cell growth inhibiting rate (fa) was calculated as follows:

fa= (1-OD450nm in drug treatment cells/ OD450 nm in vehicle treatment cells)×100%

Results determination using median-effect principle

Results were determined on the basis of median-effect equation fa/fu = (D/Dm)m that was reduced to the best-fit equation y = bx + a. Thereinto, a = -m lg Dm, b = m, x = lg D, y = lg (fa/fu), fa expresses the drug inhibitory rate, fu is 1 – fa, D represents the applied drug dosage, m is the slope of the line, and Dm is used to indicate median-effect concentration. Based on the equation, calculating the median-effect concentration and the required concentration each effective time-point of the two drugs was done to find the combination index (CI) of the two drugs. CI <

1, CI = 1, or CI > 1 denoted synergistic, additive, or antagonistic effect, respectively (16).

Plate colony formation assay

Logarithmic growth phase MDA-MB-231 cells were harvested and reseeded in 96-well cell culture plates, at 500 cells per well. After incubation for 24 hr, cells were treated with vehicle, SIP (800 μg/ml), cisplatin (3 μg/ml) and SIP (800 μg/ml) plus cisplatin (3 μg/ml), respectively. After 7 days, medium-free cells were fixed with 10% paraformaldehyde for 10 min and stained for 2 to 3 min with Giemsa stain, and then washed three times with phosphate buffered saline. The formed cell clones were counted and used to calculate colony forming efficiency as follows:

Colony forming efficiency = (number of cell clone / seeded cell count) × 100 %.

Cell wound scratch assay

MDA-MB-231 cells in their logarithmic growth phase were harvested and transferred into 6-well cell culture plates at density of 1×10^5 cells/ml. While cells grew to 90% confluence, the well-bottom of the cell plate was scratched with a sterile pipette tip and then we measured the scratch width and recorded it as T_0. Cells were incubated for 15 min in drug-free medium followed by stimulated culture for 24 hr with vehicle, SIP and/or cisplatin. Finally scratch widths T_{24} were measured and used to calculate migration rate as follows:

$$\text{Migration rate} = (T_0 - T_{24}) / T_0 \times 100 \%$$

Western blotting assay

Protein lysate from cells lysed at 4 °C in non-denaturing cell lysis buffer was denatured in protein sample buffer for 5 min in boiling water, and subjected to SDS-polyacrylamide gel electrophoresis. Following transferring to nitrocellulose membranes, the target proteins were probed with antibodies. A chemiluminescence detection system was used to visualize the membranes. The target protein content was normalised with β-actin protein content.

Statistical analysis

Data were analysed using SPSS 19.0 software. One-way analysis of variance and the *post hoc* Tukey HSD testing were used to evaluate differences between groups, $P<0.05$ and $P<0.01$ were considered significant.

Results

Cisplatin and/or SIP inhibited viability of MDA-MB-231 cells

Data (Table 1) from CCK 8 assay system showed that cisplatin (1, 2, 4, 8, 16 μg/ml) and/or SIP (100, 200, 400, 800, 1600 μg/ml) suppressed proliferation of the breast cancer cells to a significant extent, and the inhibition was observed to behave in a dose-

Table 1. Inhibition of the two drugs used on proliferation of breast cancer cells in a dose-dependent manner

Cisplatin		SIP		Cisplatin + SIP	
C (µg/ml)	f_a (D_{x1})	C (µg/ml)	f_a (D_{x2})	C (µg/ml)	f_a ($D_{x1.2}$)
1	0.1698 ± 0.015 [Aa]	100	0.2191 ± 0.028 [Aa]	1 + 100	0.3753 ± 0.021 [Aa]
2	0.2885 ± 0.012 [Bb]	200	0.3555 ± 0.036 [Bb]	2 + 200	0.6276 ± 0.035 [Bb]
4	0.4636 ± 0.018 [Cc]	400	0.3922 ± 0.029 [BBbc]	4 + 400	0.7053 ± 0.048 [Bc]
8	0.6261 ± 0.211 [Dd]	800	0.4529 ± 0.041 [BCcd]	8 + 800	0.8418 ± 0.057 [Cd]
16	0.7568 ± 0.256 [Ee]	1600	0.4964 ± 0.039 [Cd]	16 + 1600	0.8770 ± 0.061 [Cd]
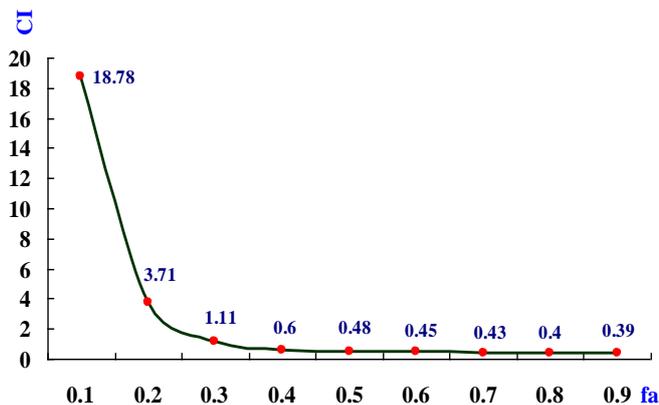
Figure 1. Combination indices of cisplatin and SIP with different effects. Under the combination of cisplatin and SIP with the scheduled concentrations, (cisplatin + SIP, 1 + 100, 2 + 200, 4 + 400, 8 + 800, and 16 + 1600, µg/ml), different effects (f_a) on viability of MDA-MB-231 cells produced the corresponding combination indices (CI) of the two drugs

dependent manner. Besides stronger inhibitory effect was visualized in combination drug treated cells, compared with the cells exposed to SIP or cisplatin.

Effect evaluation of the two drugs based on median-effect principle

According to the inhibitory effects of the two drugs on the cancer cells, linear equations describing the behaviour of the two drugs were fitted using the median-effect principle, respectively, and used to calculate m, Dm and r. The data are presented in Table 2. According to the Chou-Talalay method, the median-effect concentrations of cisplatin and SIP were 4.9 and 1659.6 µg/ml, respectively. Whereas the concentration of combination drug was 158.5 µg/ml, far lower than the sum of the two drugs. The data indicated that drug combination can reduce concentrations of the two single agents, especially the usual dosage of cisplatin.

Combination index of cisplatin and SIP with different effect

Based on the data in Table 2, a combination index (CI) was deduced from different effects (f_a) of combination of cisplatin and SIP, which was used to evaluate the interaction between the two combined drugs with their different effects (Figure 1). At *fa*<
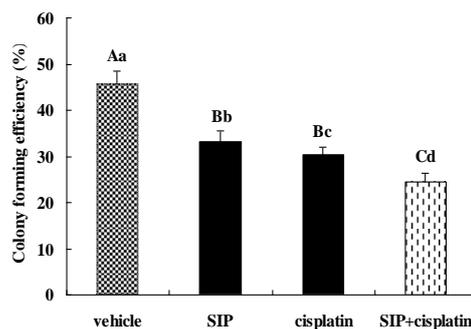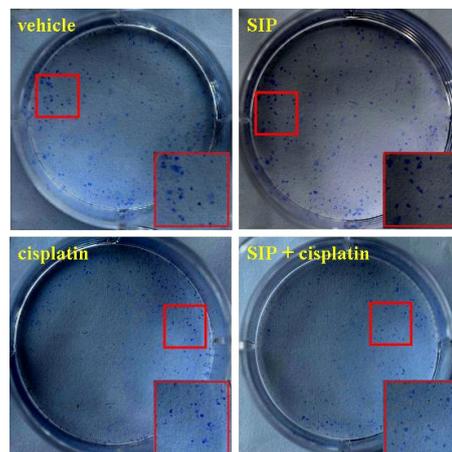
Figure 2. Inhibition of SIP and/or cisplatin on colony forming efficiency of MDA-MB-231 cells. Triple-negative breast cancer MDA-MB-231 cells were exposed to SIP (800 µg/ml), cisplatin (3 µg/ml), or a combination thereof (800 µg/ml of SIP + 3 µg/ml of cisplatin). While the colony number of combination-treated cells was more than 50, incubation of all groups of cells was stopped and cells were fixed, stained, and counted. [abcd]P<0.05, [ABC]P<0.01

0.3, CI>1 indicated antagonism between cisplatin and SIP. Whereas *fa*>0.4 showed CI<1, representing a synergetic effect of the two drugs.

For ensuring to achieve better results in the following work, concentration of cisplatin and SIP were selected to be 3 µg/ml and 800 µg/ml, respectively.

Inhibition of cisplatin and/or SIP on the clonogenic capacity of MDA-MB-231 cells

In Figure 2, the three colony forming efficiencies were all markedly lower than the vehicle-treated cells. Moreover obvious difference was observed between co-treated and single-treated cells. In

Table 2. Evaluation of the effects of two drugs based on median-effect principle

Drugs	Linear equations	Median effect concentration (D_m, μg/ml)	R^2
Cisplatin	$y = 0.9901x - 0.6816$	4.9	0.9988
SIP	$y = 0.2865x - 0.9227$	1659.6	0.9963
Cisplatin+SIP	$y = 0.8552x - 1.8801$	158.5	0.9871

Note: $y = \lg (f_a/f_u)$, $x = \lg D$

addition colony forming efficiency of SIP-exposed cells was significantly higher than that of cisplatin-stimulated cells.

Inhibition of cisplatin and/or SIP on migration of MDA-MB-231 cells

From Figure 3, it can be seen that both SIP and cisplatin clearly inhibited migration of cultured MDA-MB-231 cells, and the inhibition of the combination of the two drugs was more significant than their single use. Migration rates of SIP, cisplatin or combinations thereof were 28.6%, 19.6%, or 11.7%, respectively. Although cisplatin's suppression on migration of the breast cancer cells was more
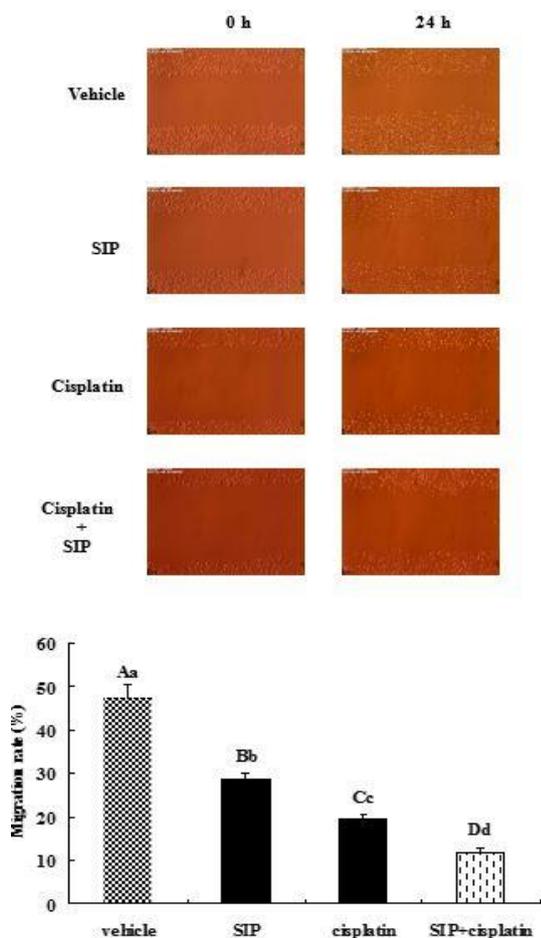
Figure 3. Inhibition of SIP and/or cisplatin on migration of MDA-MB-231 cells. MDA-MB-231 cells were treated with SIP (800 μg/ml), cisplatin (3 μg/ml), or a thereof (800 μg/ml of SIP + 3 μg/ml of cisplatin), respectively, for 24 hr. Scratch width at 0 or 24 hr was measured to calculate the migration rate of differently treated cells. abcd$P<0.05$, ABCD$P<0.01$
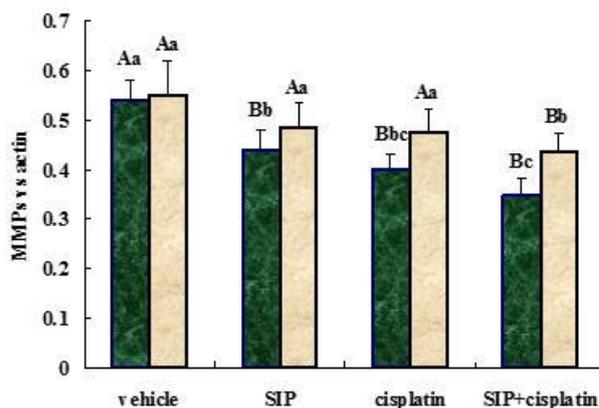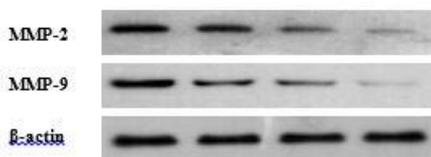
Figure 4. Inhibition of SIP and/or cisplatin on expression of MMPs proteins in MDA-MB-231 cells. MDA-MB-231 cells were treated with SIP (800 μg/ml), cisplatin (3 μg/ml), or a combination thereof (800 μg/ml of SIP + 3 μg/ml of cisplatin), respectively. Proteins in nitrocellulose membrane were probed using specific antibodies against MMP-2, MMP-9 and β-actin. The secondary antibodies were peroxidase-conjugated polyclonal antibodies. Darker and light-coloured bars represent MMP-2 or MMP-9 proteins, respectively. abc$P<0.05$, AB$P<0.01$

marked than that of SIP, the natural polysaccharide resulted in a significant reduction in cell mobility. Also, a more obvious inhibition was observed in the wells co-treated with SIP and cisplatin, the decreased degree of mobility rate was extremely significant and was larger than that with both cisplatin and SIP.

Inhibition of cisplatin and/or SIP on expression of MMP proteins in MDA-MB-231 cells

Figure 4 shows that cisplatin, SIP, and combinations thereof, all downregulated matrix metalloproteinase 2 (MMP-2) protein contents in the cells, and combinations of the drugs induced a more obvious reduction than the two single agents. From the data presented in Figure 4, the MMP-9 protein content was decreased in co-treated cells compared with vehicle-, SIP- or cisplatin-treated cells. However both SIP and cisplatin failed to inhibit expression of the MMP-9 protein in MDA-MB-231 cells.

Discussion

Breast cancer has become one of the most dangerous cancers and accounts for 30% of all the

female cancers (1). It is divided into three types according to different receptors, including human epidermal growth factor receptor-2 (HER-2) positive, hormone receptor (HR) positive and triple negative breast cancer (TNBC). TNBC cells cannot express HER-2, PR (progesterone receptor) and ER (oestrogen receptor). Clinical features of TNBC include earlier onset age, poor prognosis and high metastasis. Metastasis is diagnosed in 70% of patients, which is a main death cause of breast cancer (18, 19). The MDA-MB-231 cell derived from TNBC was used to undergo treatment by combination drug therapy using cisplatin and SIP.

Apart from surgical resection, chemotherapy is an important treatment among the therapies used towards the cancer. As a widely used chemotherapeutic agent, cisplatin can achieve a good therapeutic effect that should be the main basis for its clinical application for many years. Being a non-specific and cell cycle specific antitumor agent, the toxicity of cisplatin towards kidney, ear, and nerve increases gradually along with elevation of cisplatin content in serum (20, 21). The inevitable toxicity of the antitumor agent for its mechanisms is responsible for decreasing clinical dosages and resultantly declined treatment effects. In addition, resistance decreases the therapeutic effect of cisplatin on cancer (22, 23). So screening a low-toxicity agent to strengthen the antitumor activity of low-dosage cisplatin would be an effective method of curing cancer; SIP could be a potential anticancer agent that can be used as an adjunctive drug to strengthen the effects of cisplatin.

SIP was a naturally bioactive polysaccharide with a unique primary structure. Presently derived from squid ink, a discarded material in the course of squid processing. Presently, three laboratories have reported two types of SIP with different primary structures (8, 24, 25), but similar biofunctions, such as chemoprevention and antitumor activity (6, 7, 9-14). Based on our investigations over nearly ten years, a novel SIP with a different primary structure consisted of two major monosaccharides, galactosamine and arabinose, from those reports (8, 24, 25), was isolated from *Sepia esculenta* ink (17) and was proved to protect ovary of mouse from cyclophosphamide mediated cytotoxicity through regulating apoptosis and autophagy, via activation of PI3K/Akt and p38 MAPK pathways (17).

In this work we used the Chou-Talalay method to investigate the inhibitory effects of combination drug therapy using cisplatin and SIP on the proliferation and metastasis of breast cancer MDA-MB-231 cells. Combined, or single use of cisplatin and SIP inhibited proliferation of the breast cancer cells, furthermore, inhibition of combination drug therapy was superior to that arising from use of the two single agents and was so in a dose-dependent manner. Both cisplatin

and SIP suppressed MDA-MB-231 cells with the median-effect concentrations of them were 4.9 µg/ml and 1659.6 µg/ml, respectively, However, the two agents were combined, the median-effect concentration of the drug combination was far lower than the sum of those as single agents, which indicated that a lower dosage of the combination of drugs could achieve higher inhibitory effects and that resistance and toxicity of cisplatin could be impaired through dose reduction of single usage. Based on cell growth inhibiting rate (f_a) and combination index (CI) of the two drugs used here, it could be found that CI<1 corresponded to f_a>0.4 implying a synergistic effect between cisplatin and SIP, and CI>1 (f_a<0.3) indicated an antagonistic relationship between the drugs.

Inhibition of SIP on tumors was found in the few published papers (9-11), as was demonstrated once again by this study. Chen *et al* (9) reported that SIP did not affect HepG2 cell proliferation, but inhibited invasion and migration. Zong *et al* (10, 11) found that SIP inhibited tumor growth and metastasis in S180-bearing mice via inducing tumor cell apoptosis, inhibited metastasis of B16F10 cells, and caused angiogenesis. Although SIPs used in these trials had different primary structures, their inhibitory effects on tumour were similar. Our newly isolated SIP exhibited its inhibition on TNBC MDA-MB-231 cells, including proliferation and metastasis, which was similar to these reports (9-11). More than that, this study revealed that a combination of cisplatin and SIP showed a better inhibitory effect on colony formation ability and migration rate of MDA-MB-231 cells compared with cisplatin or SIP. Meanwhile, two key proteins promoting tumor metastasis, MMP-2 and MMP-9 (26), were both down-regulated more significantly in combination drug therapy on exposed cells than in two single-drug-treated cells. These data suggested that SIP strengthened antitumor activity of cisplatin on TNBC cells in a synergistic manner.

Conclusion

This work demonstrated that SIP can effectively promote the antitumor activity of cisplatin, a commonly used chemotherapeutic drug, on triple-negative breast cancer MDA-MB-231 cells. Some reports, including our previous research, have discovered that SIP can weaken cyclophosphamide-induced cytotoxicity in normal tissues/organs of model animals through regulating apoptosis and autophagy via activation Nrf2/ARE, PI3K/Akt, and p38 MAPK pathways. Therefore, a valuable hypothesis may be postulated as to whether or not, SIP impairs the killing effects of chemotherapeutic agents on cancer cells. Published research, and results in this paper, revealed that the speculation is false. SIP has been proved to be a double-faced functional natural bioactive polysaccharide towards

chemotherapeutic drug-induced cytotoxicity, giving chemoprevention effects on normal cells and synergy enhancement on cancer cells. The other is that, based on the median-effect principle, SIP can reduce the clinical dosage of chemotherapeutic drugs to harvest two inspiring outcomes, strengthening therapeutic effects and reducing cytotoxicity and resistance of anticancer agents. Consequently SIP is a potential natural, marine drug for cancer treatment.

Acknowledgment

Conflict of interest

The authors declare there are no conflicts of interest regarding the publication of this paper.

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