

The Hedgehog inhibitor cyclopamine antagonizes chemoresistance of breast cancer cells

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Abstract: Chemoresistance of cancer cells has been a severe problem in multiple types of cancers. One possibility is to combine different drugs with chemotherapy for improved efficacy. Cyclopamine blocks Hedgehog signaling by antagonizing Smo function, which induces tumor apoptosis. Here, we show that the combined use of cyclopamine and paclitaxel (chemotherapy drugs) was able to induce breast cancer cell apoptosis both in vivo and in vitro. The results suggest that Hedgehog signaling is a prospective drug target for chemoresistant cancer cells.

Keywords: chemoresistance, Hedgehog signaling, cyclopamine, paclitaxel, cancer stem cell

Introduction

Cyclopamine blocks Hedgehog signaling by antagonizing Smo function, which leads to apoptotic cell deaths.¹⁻⁵ The application of cyclopamine causes very few adverse effects in animals, and therefore demonstrates its usefulness in clinical applications. In recent years, cyclopamine has been used effectively in treatments for multiple types of cancers, both in vitro and in vivo, such as prostate cancer and pancreas cancer.⁶⁻¹⁰ Hedgehog signaling is also important for breast cancer cells,¹¹⁻¹³ contributing to tumor stem cell maintenance and recurrence.¹⁴⁻¹⁶ Here, we describe the use of cyclopamine to antagonize the growth and chemoresistance of breast cancer cells. The results suggest cyclopamine as a prospective conjugate in clinical therapies.

Materials and methods

Ethics statement

This study was approved by the Animal Research Committee of Zhejiang Xiaoshan Hospital (ZJXS2009-1073SJ).

Cell culture

MDA-MB-231 human breast cancer cells were purchased from Shengsheng Logistics (Shanghai, People's Republic of China) and maintained in Roswell Park Memorial Institute 1640 medium (Life Technologies, Carlsbad, CA, USA). Paclitaxel (Life Technologies) at 50 μ M was chosen as the chemotherapeutic drug, as previously described, to induce cell apoptosis.^{2,5} Cyclopamine at 20 μ M was included to examine the effects of conjugated treatments.

Cell viability and apoptosis

Cell viability was examined with an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The MTT assay was done with an MTT kit (EMD Millipore,

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Billerica, MA, USA), following the brochure carefully. Finally, each experiment was repeated at least three times.

The apoptotic cells were detected by a caspase-3 activity kit (Merck, Darmstadt, Germany) and a TUNEL (terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling) kit (Roche, Basel, Switzerland). The staining was performed as described in the kit. Then, the cells were counted for ten random sites at 40× magnification after staining. For confirmation of the results, DAPI (4',6-diamidino-2-phenylindole; 15 µg/mL, Sigma-Aldrich, St Louis, MO, USA) was occasionally employed for nuclei staining.

Xenograft

Sixty nude mice were injected with 2×10^6 cancer cells into the flank for tumor establishment for 3 weeks. Then, the mice were subdivided into three groups: control group with saline injection (every 3 days), paclitaxel (20 mg/kg/day)-treated (every 3 days), and paclitaxel (20 mg/kg/day) plus cyclopamine (25 mg/kg/day)-treated (every 3 days). The mice were killed 6 weeks and 9 weeks after the start of cancer cell transplantation for tumor harvesting. The size of the tumors was measured. Then, the tissue was fixed in 4% paraformaldehyde for 48 hours before being processed for paraffin embedding. Then, 5 µm sections were prepared for TUNEL staining, and the number of apoptotic cells within the tumor was determined by positive cells/hematoxylin and eosin-stained cells.

Statistics

The data are presented as means \pm standard deviation and were analyzed with SPSS 13.0 (IBM Corporation, Armonk, NY, USA) software. The group data were compared with analysis of variance and paired *t*-tests. $P < 0.05$ was determined as statistically significant.

Results

Cyclopamine-enhanced paclitaxel-induced cell death

We found at both the 24-hour and 48-hour time points that the addition of cyclopamine had further enhanced

paclitaxel-induced cell death, reflected by both decreased percentage of viable cells and increased percentage of apoptotic cells ($P < 0.05$; Table 1 and Figure 1).

Cyclopamine–paclitaxel combined treatment decreased tumor growth in xenograft

We further found that in xenograft-transplanted mice, the administration of paclitaxel reduced tumor growth and enhanced cell apoptosis significantly. Interestingly, the combined administration of cyclopamine promoted the observed antitumor effect ($P < 0.05$; Table 2 and Figure 2).

Discussion

Hedgehog signaling is important for breast cancer cells, contributing to tumor stem cell maintenance and recurrence in multiple models.^{11–13,15,17} It has been found that tumor stem cells are partly responsible for the chemoresistance of tumor cells in response to chemotherapy, which is maintained by Hedgehog-signaling pathways.^{4,18–22} Therefore, the antagonism of Hedgehog signaling might sensitize tumor cells to chemotherapy and reduce recurrence after surgical removal. This could be the same for breast cancer, given the fact that Hedgehog signaling has been well recognized in anti-breast cancer efforts.^{14,18,23,24} In addition, tumor stem cells have been suggested to promote breast cancer development and recurrence.^{11,18,19,25}

The present study examined the chemoresistance of a common chemotherapy drug – paclitaxel. Paclitaxel as a mitotic inhibitor targets tubulin, and has been employed in different types of cancers, including breast cancer.^{7,26} However, the chemoresistance represents a difficulty in clinical management of single-drug chemotherapy. Here, we found that the single administration of paclitaxel could reduce tumor cell survival and growth, both in vitro and in vivo, confirming previous reports. However, we showed that the combined use of cyclopamine, which blocks Hedgehog signaling, can further induce tumor cell apoptosis. This is possibly due to the loss of tumor stem cell maintenance. In future studies, it will be interesting

Table 1 Cyclopamine enhances paclitaxel-induced cell death

	24 hours			48 hours		
	Control	Paclitaxel	+Cyclopamine	Control	Paclitaxel	+Cyclopamine
Viable cells	99.4% \pm 0.1%	47.0% \pm 9.4%*	20.1% \pm 7.3%#	98.7% \pm 0.09%	35.6% \pm 2.8%*	16.4% \pm 10.2%#
Number of repeated experiments	3	6	6	3	6	6
Apoptotic cells	0.2% \pm 0.03%	42.1% \pm 7.7%*	71.9% \pm 6.4%#	0.6% \pm 0.07%	51.8% \pm 6.9%*	74.2% \pm 9.3%#
Number of repeated experiments	5	8	8	5	6	6

Notes: * $P < 0.05$ compared to control group; # $P < 0.05$ compared to paclitaxel-treated group.

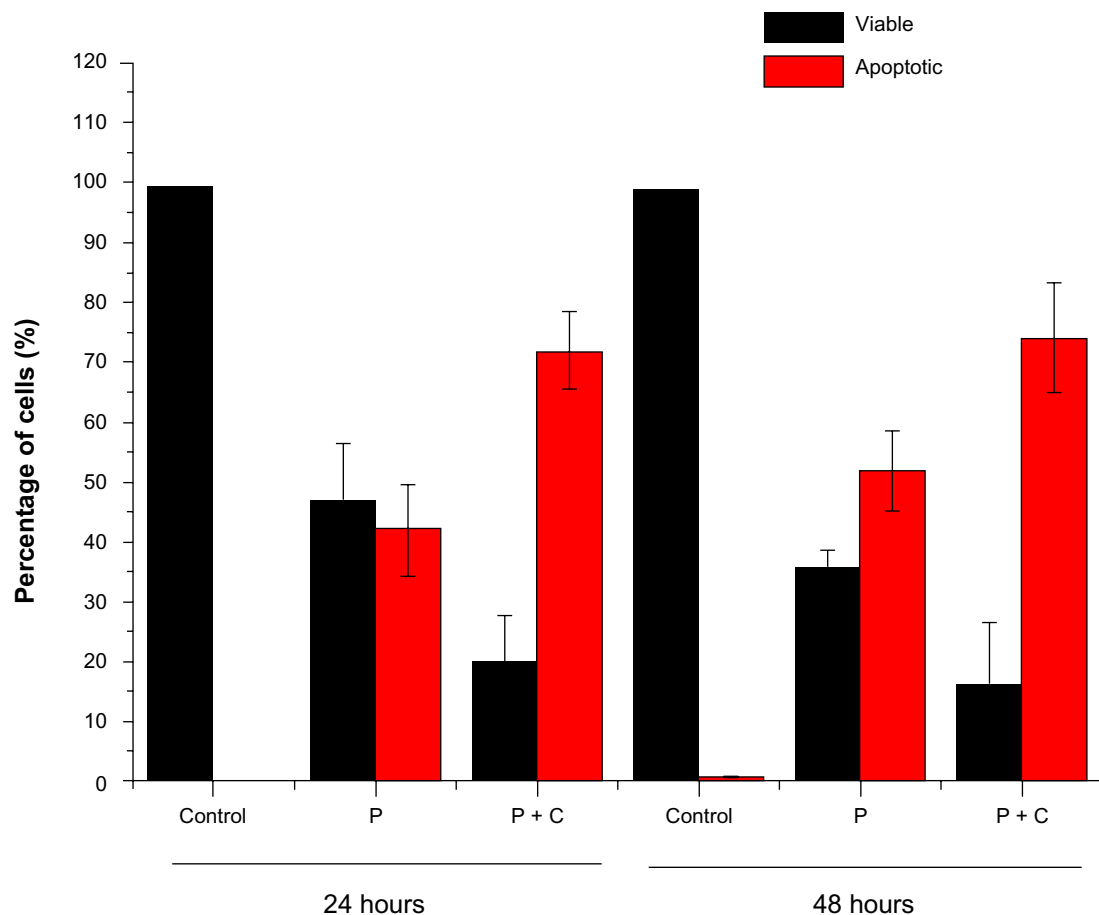


Figure 1 Cyclopamine-enhanced paclitaxel-induced cell death. Cyclopamine further enhances the cell apoptosis (red) and reduce the viable cells (black) at both the 24-hour and 48-hour time points.

Abbreviations: P, paclitaxel; C, cyclopamine.

to isolate these chemotherapy-resistant cells specifically for pharmacological and signaling pathway dissections. It should be noted that cyclopamine might also activate the Smo signaling pathway, and therefore partly contribute to the increased apoptosis of breast cancer cells. This requires further investigation to dissect downstream-signaling cascades.

Cyclopamine has been employed in different types of diseases, with proven use of safety.^{27,28} In the present study, we did not observe any adverse effects after 6 weeks of cyclopamine administration (nor in important organs by histological examination; data not shown). The combined

use of cyclopamine with other chemotherapy drugs, however, should still be evaluated for any potential harm.

In conclusion, this study firstly demonstrated that combined use of cyclopamine might act as the chemoresistance remover in paclitaxel administration for breast cancer. In pancreas cancer cells, the combination was found to be unique compared to the use of other combinations.²⁶ Whether this is the case for breast cancer is yet to be investigated. If so, this might further emphasize the importance of Hedgehog signaling and cancer stem cells in breast cancer chemoresistance to paclitaxel.

Table 2 Cyclopamine combined treatment decreases tumor growth in vivo

	6 weeks			9 weeks		
	Control	Paclitaxel	+Cyclopamine	Control	Paclitaxel	+Cyclopamine
Tumor size (mm ³)	1,792 ± 243	1,329 ± 289*	1,121 ± 163 [#]	2,260 ± 403	1,547 ± 274*	1,196 ± 209 [#]
Number of animals	10	10	10	10	10	10
Apoptotic cells	1.0% ± 0.2%	29.3% ± 4.7%*	56.7% ± 5.4% [#]	0.8% ± 0.2%	32.4% ± 3.3%*	67.9% ± 8.5% [#]
Number of animals	6	6	6	6	6	6

Notes: * $P < 0.05$ compared to control group; [#] $P < 0.05$ compared to paclitaxel-treated group.

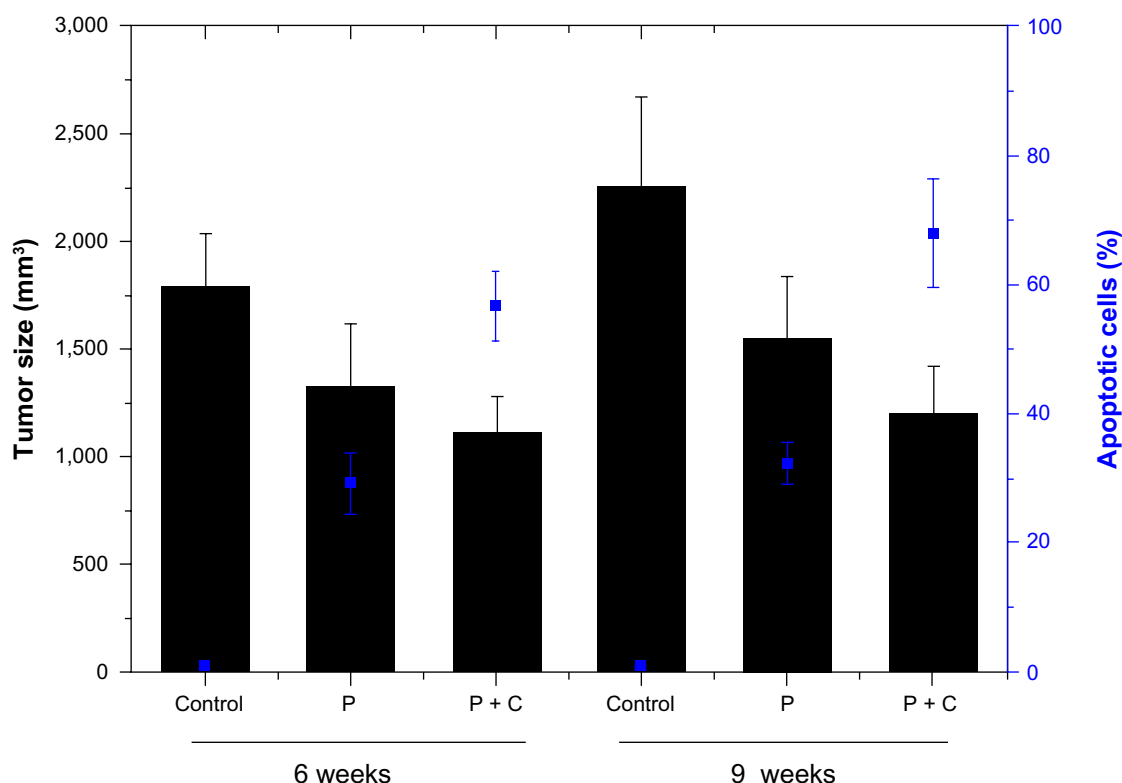


Figure 2 Cyclopamine-combined treatment decreased tumor growth in vivo. Cyclopamine further reduced the tumor size (black) and increased the cancer cell-apoptosis rate (blue), at both the 6-week and 9-week time points.

Abbreviations: P, paclitaxel; C, cyclopamine.

Author contributions

FC, JZ, CC, PS and JS designed the study; FC, JZ, CC, SX, XC and PS performed the study; FC, JZ, CC, SX, PS and JS analyzed the data; JS provided the funding for the study; FC, SX and JS wrote the draft of the paper; all authors have drafted, revised, and approved the final version of this manuscript.

Disclosure

The authors report no conflicts of interest in this work.

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