CYANIDIN AND PEONIDIN INHIBIT SPCA-1 GROWTH *IN VITRO* VIA INDUCING CELL CYCLE ARREST AND APOPTOSIS

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Abstract: Non-small cell lung cancer (NSCLC) accounts for the majority (85%) of all lung cancers. Although many therapies are available, 35–50% of patients with stage I or II NSCLC develop recurrence and metastasis. This study was designed to investigate the anti-tumor activity of cyanidin (Cy) and peonidin (Pn) on NSCLC cells (SPCA-1). SPCA-1 cell proliferation, cell cycle and early apoptosis were investigated after treatment with Cy and Pn. The underlying signaling mechanism was also explored by detecting the levels of apoptosis-related proteins using enzyme-linked immunosorbent assay (ELISA). Cy and Pn inhibited the viability of SPCA-1 cells with an IC_{50} of 141.08 μ g/mL and 161.31 μ g/mL, respectively. Meanwhile, Cy and Pn induced cell cycle arrest at G2/M phase. Cy and Pn treatment significantly increased the levels of Bax, P53, and Caspase-3, while decreasing that of Bcl-2, thereby inhibiting the growth of SPCA-1 cells. In conclusion, Cy and Pn induced early apoptosis of NSCLC cells through regulation of the levels on Caspase-3, Bax, Bcl-2, and P53. These results suggest Cy and Pn as potential anticancer drugs for the treatment of lung cancer.

Keywords: SPCA-1, cyanidin, peonidin, lung cancer, apoptosis, cell cycle

Lung cancer is the most commonly diagnosed malignancy and the main cause of cancer-related deaths in Asian and Western populations, with a 5-year survival rate of only approximately 15% (1, 2). Non-small cell lung cancer (NSCLC) accounts for the majority (85%) of all lung cancers, and despite two decades of surgery, radiation, and chemotherapy interventions, 35–50% of patients with stage I or II NSCLC relapse and develop metastasis (3).

5-fluorouracil (5-FU), a pyrimidine analog, is one of the most widely used chemotherapy drugs in the treatment of a range of cancers, including breast cancer, gastrointestinal cancer, and NSCLC (4, 5). However, for 31% to 34% of patients, 5-FU (Figure 1a) presents dose-limiting toxicity and even leads to death due to complete Dihydropyrimidine dehydrogenase (DPD) deficiency or partial DPD deficiency (6, 7). Thus, alternative and bio-

compatible drugs are urgently needed to treat NSCLC.

Anthocyanidins are the most abundant water-soluble pigments present in fruit and vegetables (8). They are glycosides and acyl glycoside derivatives of six common anthocyanidins: pelargonidin, cyaniding (Cy), malvidin, delphinidin, peonidin (Pn), and petunidin, which are classified according to the number and position of hydroxyl groups on the flavan nucleus (9, 10). Some reports indicated that delphinidin and Cy (Figure 1b) were cytotoxic in a metastatic human colorectal cancer cell line (11, 12). Anthocyanidins have been increasingly explored for their anticancer effects. Evidence indicates that anthocyanidins have significant therapeutic activity against lung, breast, and prostate cancer (13, 14, 15). In recent years, various studies demonstrated that cyanidin 3-glucoside and peonidin 3-

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glucoside inhibit cell growth by blocking the cell cycle and inducing cell apoptosis of HS578T (breast cancer cell line), HT-29 (intestinal cancer cell line), and H1299 (lung cancer cell line) (16, 17, 18). Additionally, Cy suppresses ultraviolet B-induced COX-2 expression in epidermal cells by targeting MKK4, MEK1, and Raf-1 (19). Pn (Figure 1c) inhibits phorbol-ester-induced COX-2 expression and transformation in JB6 P(+) cells by blocking ERK-1 and -2 phosphorylation (20).

SPCA-1 cells have high metastatic ability and are the most representative NSCLC cells. Thus, this study was undertaken to evaluate the therapeutic potential of Cy and Pn for NSCLC treatment in comparison to that of 5-FU. We investigated whether Cy or Pn could inhibit SPCA-1 cell proliferation. We demonstrate for the first time that Cy and Pn inhibit cell growth and induce apoptosis of SPCA-1 *in vitro*.

EXPERIMENTAL

Materials

Cy and Pn were purchased from PhytoLab (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany). The compounds were stored at -20°C. The purity of Cy and Pn was above 95% and 99%, respectively, was measured by HPLC (The certificates were provided by the PhytoLab). SPCA-1 cell line was obtained from the Shanghai Academy of Agricultural Sciences, Shanghai, China. Culture medium (DMEM) and fetal calf serum were purchased from GIBCO (Carlsbad, CA, USA). Trypsin-EDTA solution 0.25% was purchased from Fermentas (Waltham, MA, USA). Alamar Blue was purchased from AbD Serotec (Oxford, UK). Apoptosis kit was purchased from KeyGEN BioTECH Co., Ltd. (Nanjing, China). P53, Caspase-3, Bcl-2, and Bax ELISA kits were purchased from Abcam (Cambridge, MA, USA). All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

Cell culture

A human NSCLC line, SPCA-1, was cultured in DMEM culture medium, supplemented with 10% FBS, 100 U/L penicillin, and 100 mg/mL streptomycin. The cells were cultured at 37°C in an incubator humidified with 5% CO₂ to reach 90% confluence and used for further experiments.

Alamar blue assay

SPCA-1 cells were diluted to a final concentration of 2×10^4 cells/mL and dispensed into 96-well plates (100 μ L per well) and incubated at 37°C in a 5% CO₂ atmosphere. Twenty-four hours later, the medium was replaced with fresh DMEM medium (100 μ L) containing 50, 100, 150, or 200 μ g/mL Cy and Pn. DMEM medium containing 5% DMSO and 50 μ g/mL 5-FU served as the negative and positive controls, respectively. The experiments were performed in triplicate. After incubation at 37°C in a 5% CO₂ atmosphere for 72 h, the medium was replaced with 180 μ L DMEM medium and 20 μ L Alamar blue for further incubation. When the color of the medium changed, absorbance was measured at 570 nm and 600 nm using a microplate reader (Bio-Tek Instruments, Inc, Winooski, VT, USA). The proliferation rate was calculated according to the following formula:

$$\text{Proliferation rate(\%)} = 1 - \frac{A_{570}(\text{sample}) - A_{600}(\text{sample})}{A_{570}(\text{control}) - A_{600}(\text{control})} \times 100\%$$

Cell-cycle analysis

SPCA-1 cells were diluted to a final concentration of 1×10^5 cells/mL and were dispensed into 12-well plates (1 mL per well). Medium (1 mL) containing 50, 100, and 200 μ g/mL Cy and Pn was added to the wells. Medium containing 5% DMSO and 50 μ g/mL 5-FU served as the negative and positive controls, respectively. Plates were incubated at 37°C in a 5% CO₂ atmosphere. Forty-eight hours later, the supernatant was discarded after centrifugation and the cells were washed with pre-cooled PBS once. Then, 70% ethanol (500 μ L) was added and

Figure 1. Chemical formulas of 5-FU (a), Cy (b) and Pn (c)

Figure 2. (A): Effect of Cy on the viability of SPCA-1 cells. (B) Effect of Pn on the viability of SPCA-1 cells. SPCA-1 cells were incubated for 72 h with different concentrations of cyanidin (Cy), peonidin (Pn), and 50 µg/mL 5-FU. Alamar Blue assay was conducted to determine the effect of Cy and Pn on the viability of SPCA-1 cells. Values are mean $\pm$ SD of at least three different experiments performed in duplicate. ** $p < 0.01$ compared with negative control, * $p < 0.05$ compared with negative control. Positive control: 5-FU (50 µg/mL)

the cells were incubated at 4°C overnight for fixation. Cells were collected by centrifugation, washed once with PBS, and 100 µL PBS containing 1 µg/mL propidium iodide (PI) and 500 µg/mL RNase A were added. After incubation at room temperature for 30 min, 300 µL PBS was added and Flow cytometry (counting 10000 cells) (BD Accuri C6) was performed. The results were analyzed with FlowJo software.

Annexin V-FITC/PI double staining analysis by flow cytometry

SPCA-1 cells were diluted to a final concentration of 1×10^5 cells/mL and dispensed into a 12-well plate (1 mL per well). Medium (1 mL) containing 50, 100, and 200 µg/mL Cy and Pn was added to the wells. Medium containing 5% DMSO and 50 µg/mL 5-Fu served as the negative and positive controls, respectively. After incubation at 37°C in a 5% CO₂ atmosphere for 48 h, 0.05% EDTA was added. The supernatant was removed by centrifugation and the cells were collected. Cells were washed once

with pre-cooled PBS. The supernatant was replaced with 300 µL Binding Buffer to suspend the cells. After addition of 2.5 µL Annexin V and 2 µL PI, the cells were incubated in the dark at room temperature for 15 min. Flow cytometry (counting 10000 cells) was performed and the results were analyzed with FlowJo software.

Enzyme-linked Immunosorbent Assay (ELISA)

The levels of Caspase-3, P53, Bax, and Bcl-2 were measured by ELISA following the manufacturer's protocols. The target protein concentration was calculated and then multiplied by the dilution factor.

Statistical analysis

The results are expressed as mean $\pm$ SD. One-way ANOVA in SPSS 19.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. The p -values less than 0.05 were considered to be statistically significant.

RESULTS

Cy and Pn inhibit the growth of SPCA-1 cells

Alamar Blue assay was conducted to determine the effect of Cy and Pn on SPCA-1 cell proliferation. As shown in Figure 2, Cy and Pn significantly inhibited SPCA-1 cell proliferation in a dose-dependent manner. When the concentration was 200 mg/mL, the inhibitory effect of Cy and Pn was similar to that of the positive control, 5-Fu (50 µg/mL). The IC₅₀ of Cy and Pn was 141.079 µg/mL and 161.312 µg/mL, respectively. The growth inhibitory ability of Cy on

SPCA-1 was better than that of Pn within a certain range. The results show that Cy and Pn suppress the growth of SPCA-1 cells, and the growth inhibitory ability of Cy on SPCA-1 was better than that of Pn within a certain range.

Influence of Cy and Pn on the cell cycle in SPCA-1 cells

Flow cytometry allows us to determine changes to the cell cycle, which is related to tumor cell proliferation. The results are shown in Figure 3, Cy and Pn induced a decrease in the percentage of cells in G0/G1 and S phases. Meanwhile, Cy and Pn

Figure 3. Effect of different concentrations of Cy and Pn on SPCA-1 cell cycle. SPCA-1 cells were incubated for 48 h with different concentrations of Cy, Pn, and 5-FU (50 µg/mL). Cell cycle was assessed by flow cytometry using PI staining

Figure 4. Effects of different concentrations of Cy and Pn on early apoptosis of SPCA-1 cells. SPCA-1 cells were incubated for 48 h with different concentrations of Cy, Pn, and 5-FU (50 µg/mL). Cell apoptosis was assessed by flow cytometry using Annexin V-FITC and PI double staining

induced an increase in the percentage of cells in G2/M phase. The results indicated that Cy and Pn induced cell cycle arrest at the G2/M phase.

Induction of apoptosis

Decrease in cell proliferation is closely related to early apoptosis. Thus, we assessed early apoptosis of SPCA-1 cells by Annexin V-FITC and PI double staining using a flow cytometer. The early apoptosis rates after treatment with 50, 100, 200 µg/mL Cy and Pn were 12.0%, 19.7%, 21.8% and 13.8%, 14.8%, 30.8%, respectively, which were higher than

that in the negative control (Figure 4). This result suggests that Cy and Pn cause early apoptosis in SPCA-1 cells in a dose-dependent manner.

Effect of Cy and Pn on the levels of apoptotic proteins (Caspase-3, P53, Bax, and Bcl-2) in SPCA-1 cells

Apoptosis is an active and programmed death process, which is precisely regulated by many genes under certain physiological or pathological conditions (21). Also, apoptosis is closely related to apoptosis-related proteins. Therefore, we investigated the

effect of Cy and Pn on the levels of the apoptotic proteins in SPCA-1 cells.

As shown in Figure 5A, SPCA-1 cells were treated with different concentrations of Cy, Pn, and 5-FU for 48 h. Compared with the negative group, the level of Caspase-3 increased in the treated groups. Cy and Pn at the concentration of 200 µg/mL significantly affected the level of Caspase-3 when compared with the negative group ($p < 0.01$). Furthermore, Pn at the concentration of 200 µg/mL increased the level of Caspase-3 to a level similar to that induced by 5-Fu (50 µg/mL).

As shown in Figure 5B, SPCA-1 cells were treated with different concentrations of Cy, Pn, and 5-FU for 48 h and the level of P53 increased compared to that in the negative control group. Compared with the negative control group, Cy (50 µg/mL) and Pn (50 µg/mL) showed no significant difference. However, 5-FU at 50 µg/mL as well as Cy and Pn at 100 and 200 µg/mL significantly affected the level of P53 ($p < 0.01$). Cy and Pn at 200 µg/mL had effects similar to that of 5-Fu at 50 µg/mL on the level of P53.

The level of Bax increased after Cy, Pn, and 5-FU treatment when compared to that in the negative

control (Figure 5C). Compared with the negative control group, 50 µg/mL 5-FU and 50, 100, and 200 µg/mL Cy significantly increased the level of Bax ($p < 0.01$), while Pn only had a significant effect at 200 µg/mL ($p < 0.01$). The effect of Cy on the level of Bax was more obvious than Pn. When the cells was treated with 200 µg/mL of Cy, the level of Bax was similar to that in cells treated with 5-FU (50 µg/mL).

However, when the Cy, Pn, and 5-FU groups were compared with the negative control group, the level of Bcl-2 was decreased (Figure 5D). A significant decrease in the level of Bcl-2 was observed after treatment with 50 µg/mL of 5-FU and 200 µg/mL of Cy and Pn ($p < 0.01$). Cy at concentrations of 50 and 100 µg/mL, but not Pn at the same concentrations affected the level of Bcl-2 ($p < 0.05$). The effect of Cy on the level of Bcl-2 was more obvious than Pn. The level of Bcl-2 was similar to that in the positive control (50 µg/mL 5-FU) when the cells were treated with 200 µg/mL of Cy.

DISCUSSION AND CONCLUSION

In recent years, chemotherapy has been widely used to treat and prolong the life of patients with

Figure 5. Effect of different concentrations of Cy and Pn on the level of Caspase-3 (Figure 5A), P53 (Figure 5B), Bax (Figure 5C) and Bcl-2 (Figure 5D) in SPCA-1 cells. The level of Caspase-3, P53, Bax, and Bcl-2 were measured with ELISA kits. ** $p < 0.01$ compared with negative control, * $p < 0.05$ compared with negative control

NSCLC. Numerous experiments confirmed that active ingredient from natural products such as triterpenoids, alkaloids, polysaccharides, saponins, and other active substances could be used to treat malignant tumors and reduce the side effects of chemotherapy (22, 23, 24, 25). Additionally, various studies recently reported that anthocyanins could inhibit cellular transformation and cell-cycle progression, and induced apoptosis in different human cell lines (13, 26, 27, 28).

Treatment with peonidin 3-glucoside and cyanidin 3-glucoside inhibits cell growth of human breast cancer and treatment with Cy inhibits the growth of human monocytic leukemia cells via G2/M arrest and induction of apoptosis (16, 29). In this study, Cy and Pn affected the cell cycle at the G2/M phase in SPCA-1 cells. Therefore, we considered that early apoptosis induced by Cy and Pn in SPCA-1 cells was associated with G2/M phase arrest. Meanwhile, our study suggests that Cy and Pn induce apoptosis of SPCA-1 cells in a dose-dependent manner. Furthermore, we explored the mechanism underlying Cy and Pn-induced apoptosis.

The Bcl-2 family includes Bax, Bad, and other pro-apoptotic proteins as well as anti-apoptosis proteins such as Bcl-2 and Bcl-xL. Most of them are composed of a C-terminal transmembrane domain and a Bcl-2 homology domain (30, 31). Moreover, the caspase family plays a key role in the execution of apoptosis (32). Caspases are the most important enzymes for apoptosis (33), inducing chromatin condensation, DNA fragmentation, and finally apoptosis. P53 is also an important regulator of apoptosis. It interacts with Bcl-2 in the process of apoptosis, thereby regulating cell apoptosis (34). The present study showed that Cy and Pn treatment dose-dependently induced the down-regulation of Bcl-2 level and the up-regulation of levels of Caspase-3, P53, and Bax in SPCA-1 cells. Furthermore, our results suggest differences between Cy and Pn in term of their anti-tumor function, which may be associated with their different structures.

In conclusion, this study demonstrated that Cy and Pn inhibit the growth of SPCA-1 cells by inducing cell cycle arrest and early apoptosis. Additionally, Cy and Pn may induce early apoptosis through up-regulation of Caspase-3, P53, Bax, and Bcl-2 down-regulation. Our findings suggest Cy and Pn as potential anticancer therapeutics for the treatment of lung cancer. Further studies are warranted to decipher the mechanism underlying the anti-cancer effect of anthocyanidins and for the development of

anthocyanidins as therapeutics for the treatment of NSCLC.

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Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

REFERENCES

1. Jemal A., Murray T., Ward E., Samuels A., Tiwari R.C. et al.: *CA Cancer J. Clin.* 55, 10 (2005).
2. Tang J.M., He Q.Y., Guo R.X., Chang X.J.: *Lung Cancer.* 51, 181 (2006).
3. Egermann U., Jaeggi K., Habicht J.M., Perruchoud A.P., Dalquen P. et al.: *Eur. Respir. J.* 19, 464 (2002).
4. Longley D.B., Harkin D.P., Johnston P.G.: *Nat. Rev. Cancer.* 3, 330 (2003).
5. Huang C.L., Yokomise H., Kobayashi S., Fukushima M., Hitomi S. et al.: *Int. J. Oncol.* 17, 47 (2000).
6. Bocci G., Barbara C., Vannozzi F., Di Paolo A., Melosi A. et al.: *Clin. Pharmacol Ther.* 80, 384 (2006).
7. Bocci G., Di Paolo A., Barbara C., Masi G., Fornaro L. et al.: *Br. J. Clin. Pharmacol.* 67, 132 (2009).
8. Abdel-Aal el S.M., Young J.C., Rabalski I.: *J. Agric. Food Chem.* 54, 4696 (2006).
9. Crozier A., Jaganath I.B., Clifford M.N.: *Nat. Prod. Rep.* 26, 1001 (2009).
10. Kong J.M., Chia L. S., Goh N.K., Chia T.F., Brouillard R.: *Phytochemistry.* 64, 923 (2003).
11. Tulio A.Z., Jr., Reese R.N., Wyzgoski F.J., Rinaldi P.L., Fu R. et al.: *J. Agric. Food Chem.* 56, 1880 (2008).
12. Cvorovic J., Tramer F., Granzotto M., Candussio L., Decorti G. et al.: *Arch. Biochem. Biophys.* 501, 151 (2010).
13. Hafeez B.B., Siddiqui I.A., Asim M., Malik A., Afaq F. et al.: *Cancer Res.* 68, 8564 (2008).
14. Chen P.N., Chu S.C., Chiou H.L., Kuo W.H., Chiang C.L. et al.: *Cancer Lett.* 235, 248 (2006).

15. Xu M., Bower K.A., Wang S., Frank J.A., Chen G. et al.: *Mol. Cancer.* 9, 285 (2010).
16. Chen P.N., Chu S.C., Chiou H.L., Chiang C.L., Yang S.F. et al.: *Nutr. Cancer.* 53, 232 (2005).
17. Serra D., Paixao J., Nunes C., Dinis T.C., Almeida L.M.: *PLoS One.* 8, e73001 (2013).
18. Ho M.L., Chen P.N., Chu S.C., Kuo D.Y., Kuo W.H. et al.: *Nutr. Cancer.* 62, 505 (2010).
19. Kim J.E., Kwon J.Y., Seo S.K., Son J.E., Jung S.K. et al.: *Biochem. Pharmacol.* 79, 1473 (2010).
20. Kwon J.Y., Lee K.W., Hur H.J., Lee H.J.: *Ann. N. Y. Acad. Sci.* 1095, 513 (2007).
21. Lowe S.W., Lin A.W.: *Carcinogenesis* 21, 485 (2000).
22. Mu L.H., Bai L., Dong X.Z., Yan F.Q., Guo D.H. et al.: *Biol. Pharm. Bull.* 37, 1035 (2014).
23. Wu G.S., Lu J.J., Guo J.J., Li Y.B., Tan W. et al.: *Fitoterapia* 83, 408 (2012).
24. Akl M.R., Ayoub N.M., Ebrahim H.Y., Mohyeldin M.M., Orabi K.Y. et al.: *Mar. Drugs.* 13, 288 (2015).
25. Yeh C.T., Wu C.H., Yen G.C.: *Mol. Nutr. Food Res.* 54, 1285 (2010).
26. Lazze M.C., Savio M., Pizzala R., Cazzalini O., Perucca P. et al.: *Carcinogenesis.* 25, 1427 (2004).
27. Han C., Ding H., Casto B., Stoner G.D., Ambrosio S.M.: *Nutr. Cancer.* 51, 207 (2005).
28. Huang C., Huang Y., Li J., Hu W., Aziz R. et al.: *Cancer Res.* 62, 6857 (2002).
29. Hyun J.W., Chung H.S.: *J. Agric. Food Chem.* 52, 2213 (2004).
30. Gross A., McDonnell J.M., Korsmeyer S.J.: *Genes Dev.* 13, 1899 (1999).
31. Farrow S.N., Brown R.: *Curr. Opin. Genet. Dev.* 6, 45 (1996).
32. Cryns V.L., Byun Y., Rana A., Mellor H., Lustig K.D. et al.: *J. Biol. Chem.* 272, 29449 (1997).
33. Thornberry N.A., Lazebnik Y.: *Science* 281, 1312 (1998).
34. Vazquez A., Bond E.E., Levine A.J., Bond G.L.: *Nat. Rev. Drug Discov.* 7, 979 (2008).

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