Neurotoxicity and Structure-Activity Relationships of Resveratrol and its two Natural Analogs, 4,4′-Dihydroxystilbene and Pinosylvin

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Abstract
Resveratrol (RES) and its two natural analogues, 4,4′-dihydroxystilbene (DHS) and pinosylvin (PIN), are very important polyphenols and have attracted considerable pharmaceutical interest because of their diverse biological activities. However, their adverse effects on motor nerves and glioma cells have not been properly assessed. Herein, we surveyed the toxicity and analyzed the structure-activity relationship of these three polyphenols using transgenic zebrafish (Danio rerio) and U87. Results indicated that, in zebrafish embryos, both DHS (1 and 10 μg/mL) with hydroxyl groups at the 4 and 4′ positions, and PIN (1 and 10 μg/mL) with hydroxyl groups at the 3 and 5 positions inhibited motor neuron growth more effectively than RES (1 and 10 μg/mL) with hydroxyl groups at the 3, 4′, and 5 positions, although their appearance is normal. Both the DHS- (10 μg/mL) and PIN (10 μg/mL) -treated groups significantly reduced the swimming distance of zebrafish compared with the RES (10 μg/mL) -treated group. In addition, DHS with the hydroxyl groups at the 4 and 4′ positions (0.002, 0.02, 0.2, 2, and 20 μM) inhibited U87 cell aggregation in a concentration-dependent manner; PIN with the hydroxyl groups at the 3 and 5 positions (0.002, 0.02, 0.2, 2, and 20 μM) promoted U87 cell aggregation in a concentration-dependent manner, while RES with three hydroxyl groups promoted U87 cell aggregation at concentrations from 0.2 to 2 μM. Taken together, DHS and PIN are more neurotoxic than RES. The position and number of hydroxyl groups significantly affected the ability of the polyphenols to aggregate into tumors in the U87 cell.

Keywords
resveratrol, 4, 4′-dihydroxystilbene, pinosylvin, neurotoxicity, u87 cell, structure-activity relationship

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Introduction
Natural polyphenols often have a series of good biological functions such as antioxidant,3–5 anti-radiation,4–6 anti-inflammatory,7–9 antibacterial,10–12 and anti-tumor,13–15 and are used for the treatment of various diseases, including obesity,16 diabetes,17,18 tumor and cardiovascular.19,20 Several studies suggest that dietary polyphenols can be a strategy for obesity management, helping to recover or preserve gut health through anti-inflammatory and antioxidant actions.7–9 Plants containing phenolic compounds have been shown to have therapeutic effects on type 2 diabetes by ameliorating insulin resistance and insulin secretion.21 Studies suggest that fruit peel polyphenols have substantial anti-tumor effects in the model of breast cancer.24 Studies have demonstrated that the intake of polyphenol-rich foods improves vascular health, thereby significantly reducing the risk of cardiovascular disease.25 Therefore, studies on the biological functions of natural polyphenols have attracted lots of global attention.

Resveratrol (RES, (E)-3,4′,5-trihydroxystilbene, Figure 1A), a naturally occurring stilbene polyphenolic derivative, is found in a limited number of plant species such as grape, pine, and peanut. It has been considered as a phytoalexin in plants, and many studies have also shown its health benefits such as blood thinning,26 antioxidant activity, cancer prevention, and life span extension.27 (E)-4,4′-Dihydroxystilbene (DHS, Figure 1B), a RES analog, is extracted from the bark of various Yucca species28 and has potential...
antioxidant, anti-inflammatory and anti-cancer activities.\textsuperscript{29–31} Pinosylvin (PIN, (\(E\))-3,5-dihydroxystilbene, Figure 1C), a RES analog, is extracted from the leaves and wood of \textit{Pinus densiflora}.\textsuperscript{32} It exhibits multiple biological functions and has been suggested as a promising agent for the treatment of inflammatory and tumor diseases.\textsuperscript{33,34} However, the side effects and structure-activity relationships of RES, DHS, and PIN have not been evaluated.

The zebrafish model, one of the important vertebrate models, has been used to evaluate drug effects in recent years.\textsuperscript{35,36} This model is applicable to multiple stages of drug evaluation, including absorption, metabolism, distribution, and toxicity studies.\textsuperscript{37} Here, the zebrafish model was used to investigate further the effect of RES, DHS, and PIN on neural development. So far, the adverse effects and structure-activity relationships of RES, DHS, and PIN have not been reported. Our study provides new insights into the toxicities, functions and structure-activity relationships of RES, DHS, and PIN.

Materials and Methods

\textbf{Materials}

RES and PIN (purity \(>98\%\)) were both purchased from Shanghai Macklin Biochemical Technology Co., Ltd, China, DHS (purity \(>97\%\)) from Shanghai Bide Pharmaceutical Technology Co., Ltd, China, and the U87 cell line (Catalogue Number: HTB-14) from ATCC, Virginia, USA.

\textbf{Zebrafish}

The transgenic zebrafish lines of Tg (hb9: EGFP), with motor neuron gene hb9 marked with EGFP, was offered by the Zebrafish Center Key Laboratory of Neuroregeneration of Nantong University. Zebrafish adults and embryos were maintained and raised in standard conditions, as previously described.\textsuperscript{38,39} We carried out all animal experimentation in accordance with ethically approved procedures by the Administration Committee of Experimental Animals, Jiangsu Province, China [Approval ID: SYXK (SU) 2007–0021].

\textbf{Zebrafish Drug Treatments}

The drug administration experiments were carried out as described in the literature.\textsuperscript{37} In brief, the (hb9: EGFP) zebrafish embryos were obtained by natural mating and maintained in E3 solution at 28.5 \(^\circ\)C. We screened and removed the morphologically abnormal embryos using an anatomical microscope, 6 h post fertilization (hpf). Each well of a 96-well plate was loaded with 10 healthy embryos, and then the E3 solutions were replaced with RES, DHS, and PIN treatment solutions at different concentrations (0.1, 1, 10 \(\mu\)g/mL). At 50 hpf, the zebrafish embryos were collected, anesthetized, and embedded for Leica TCS-SP8 LSM confocal imaging. Imaris software (version 7.2.3) was used for analysis.

\textbf{Locomotion Analysis of Zebrafish}

The swimming distance assays of zebrafish under different drug administrations were performed as previously reported.\textsuperscript{40} In brief, E3 solutions were replaced with 10 \(\mu\)g/mL concentrations of RES, DHS, and PIN treatment solutions at 96 hpf. One larval zebrafish was maintained in each well of a 24-well plate filled with 1 mL of E3 medium. The Zebralab Video-Track system was used to detect swimming distance, and ESOvision behavioral analysis software was used for analysis.

\textbf{U87 Cell Aggregation Assay}

U87 cells were cultured in DMEM/F12 supplemented with 1% penicillin/ streptomycin and 10\% FBS and maintained in 5%
CO₂ at 37°C. U87 cells were added to different concentrations (0.002, 0.02, 0.2, 2, 20 μM) of RES, DHS, and PIN culture medium, and then cultured for 48 h at 37°C. A Leica DM3000B microscope was used to observe the cell aggregation.

Statistical Analysis

GraphPad Prism (version 7.0, Graphpad Software, La Jolla, California) was used to statistically analyze the data. All data are expressed as mean ± S. D. A one-way analysis of variance (ANOVA) (P < .05) was used to perform statistical analysis.

Results

Effects of RES, DHS, and PIN Treatment on Zebrafish Neuronal Axon Development

Tg (hb9: EGFP) zebrafish neuronal axons, labeled with green fluorescence, were used to investigate the effect of RES, DHS, and PIN treatment on neural axon development. RES, DHS, and PIN treatments at 0.1, 1, and 10 μg/mL inhibited neuronal development in a concentration-dependent manner at 50 hpf (Figure 2, A-K, A'-K', L). There were no significant differences in the motor neuronal axon lengths caused by 0.1, 1, and 10 μg/mL RES treatment compared with the control group (P > .05) (Table 1, entries 1-3), whereas the axon length of motor neurons was significantly reduced by DHS and PIN treatment compared with the control group (P < .05) (Table 1, entries 4-9). Both the DHS and PIN treatments resulted in significantly reduced axon lengths of motor neurons compared with the RES treatment (P < .001) (Table 1, entries 11-15). Compared with the DHS treatment group, the motor neuronal axon length was significantly reduced in the PIN treatment group at concentrations of 0.1 and 1 μg/mL (Table 1, entries 16-18).

Effects of RES, DHS, and PIN Treatments on Zebrafish Embryos

To investigate further the effects of RES, DHS, and PIN on the appearance of zebrafish, the zebrafish embryos were treated with high concentrations of RES, PIN, and DHS solution (10 μg/mL) at 6 hpf. All the embryos appeared normal within 50 hpf (Figure 3A–D). Thus, the RES, DHS, and PIN treatment groups had very low deformity rates and mortality.

Effects of RES, DHS, and PIN Treatments on Zebrafish Swimming Distance

The zebrafish were treated with RES, DHS, and PIN solutions at a concentration of 10 μg/mL, respectively, and at 100 hpf the zebrafish swimming distance was measured. Results demonstrated that RES treatment reduced the zebrafish swimming distance, but there was no significant difference compared with the control group (P > .05) (Figure 4A and B). The swimming distance was obviously reduced by DHS and PIN treatments compared with the control group (P < .001, P < .001) (Figure 4A, C and D). Both DHS and PIN treatment significantly reduced the swimming distance compared with the RES treatment (P < .001, P < .001) (Figure 4B–E). Compared with the DHS treatment group, the PIN treatment appeared to reduce the swimming distance, but there were no significant differences (P > .05) (Figure 4C–E).

Effects of RES, DHS, and PIN Treatments on U87 Cell Aggregation

U87 cells were incubated with different concentrations (0.002, 0.02, 0.2, 2, 20 μM) of RES, DHS, and PIN culture medium for 48 h. The results demonstrated that RES promoted U87 cell aggregation at concentrations from 0.2 to 2 μM, DHS inhibited aggregation in a concentration-dependent manner, and PIN promoted U87 cell aggregation in a concentration-dependent manner (Figure 5).

Discussion

In recent decades, polyphenolic compounds have received widespread attention due to their various biological activities and functions. RES, DHS, and PIN are all natural trans-stilbene polyphenolic compounds. The structure of RES is characterized by three hydroxyl groups located at the 3, 5 and 4’ positions, respectively (Figure 1A). The structure of DHS is characterized by two hydroxyl groups, located at the 3 and 5 positions, respectively (Figure 1B), and the structure of PIN is characterized by two hydroxyl groups located at the 3 and 5 positions, respectively (Figure 1C). The Savio group has reported the structure-activity relationship between RES and DHS on the endothelin axis of human endothelial cells. The Marcello group has reported the structure-activity relationships of resveratrol and derivatives, including DHS, PIN, 4-hydroxystilbene, and 3,4’-dihydroxystilbene, in breast cancer cells. To our knowledge, this work is the first to investigate the structure-activity relationship of RES, DHS, and PIN on the nervous system using a zebrafish model and a U87 cell model. Numerous studies have reported that RES has neuroprotective effects. Our results demonstrated that RES was less toxic to motor neurons of zebrafish at concentrations ranging from 0.1 to 10 μg/mL (Figure 2), whereas DHS and PIN were more toxic to motor neurons and significantly inhibited neuronal development (Figure 2). The swimming distance experiments with zebrafish further verified this result (Figure 4). Interestingly, treatment with high concentrations of RES, DHS, and PIN resulted in early zebrafish embryos all looking normal (Figure 3). The structure-activity relationship indicated that the three-hydroxy structure (RES) makes it less neurotoxic than the two-hydroxy structure (PIN and DHS),...
and the hydroxyl at the 4′ position of RES results in less neurotoxicity compared to PIN. The hydroxyls at the 3 and 5 positions of RES result in less neurotoxicity compared with the hydroxyl at the 4 position of DHS. The hydroxyls at the 3 and 5 positions of PIN result in more neurotoxicity compared to the hydroxyls at the 4 and 4′ positions of DHS (Figure 6).

The results of U87 cell aggregation reveal that DHS inhibited U87 cell aggregation, while PIN promoted it. The hydroxyl at the 4′ position of RES inhibited the aggregation of U87 cells to a certain extent compared with PIN. The 4-hydroxyl group of DHS significantly inhibited the aggregation of U87 cells compared with the 3- and 5-hydroxyl groups of RES (Figures 5 and 6). The hydroxyls at the 3 and 5 positions of PIN promoted U87 cell aggregation, while the hydroxyls at the 4 and 4′ positions of DHS inhibited it (Figure 6). This result suggests that DHS and PIN may have different antitumor effects in different modes of action. Recently, several studies have shown that DHS inhibits tumor growth, cancer invasion and metastasis, including human neuroblastoma and lung cancer.29,46 Studies also have shown that PIN can inhibit the growth of human colorectal cancer cell migration and invasion of nasopharyngeal carcinoma cancer cells.47,48 These findings further support our view.

Table 1. Statistical Analyses of the Motor Neurons Axon Length in Control Group and RES-, PIN-, and DHS-Treated Groups at 50 hpf.

| Entry | Comparisons test* | P value | Significant |
|-------|-------------------|---------|-------------|
| 1     | Ctrl versus RES 0.1 μg/mL | >.999 | No |
| 2     | Ctrl versus RES 1 μg/mL | .641 | No |
| 3     | Ctrl versus RES 10 μg/mL | .368 | No |
| 4     | Ctrl versus DHS 0.1 μg/mL | .003 | Yes |
| 5     | Ctrl versus DHS 1 μg/mL | <.001 | Yes |
| 6     | Ctrl versus DHS 10 μg/mL | <.001 | Yes |
| 7     | Ctrl versus PIN 0.1 μg/mL | <.001 | Yes |
| 8     | Ctrl versus PIN 1 μg/mL | <.001 | Yes |
| 9     | Ctrl versus PIN 10 μg/mL | <.001 | Yes |
| 10    | RES 0.1 μg/mL versus DHS 0.1 μg/mL | .113 | No |
| 11    | RES 0.1 μg/mL versus PIN 0.1 μg/mL | <.001 | Yes |
| 12    | RES 1 μg/mL versus DHS 1 μg/mL | <.001 | Yes |
| 13    | RES 1 μg/mL versus PIN 1 μg/mL | <.001 | Yes |
| 14    | RES 10 μg/mL versus DHS 10 μg/mL | <.001 | Yes |
| 15    | RES 10 μg/mL versus PIN 10 μg/mL | <.001 | Yes |
| 16    | DHS 0.1 μg/mL versus PIN 0.1 μg/mL | .001 | Yes |
| 17    | DHS 1 μg/mL versus PIN 1 μg/mL | .015 | Yes |
| 18    | DHS 10 μg/mL versus PIN 10 μg/mL | .651 | No |

*One-way ANOVA with Holm-Sidak’s multiple comparisons test.
Figure 3. Effects of zebrafish embryo appearance phenotype by RES, DHS, and PIN (10 μg/mL) treatments. (A–D) The bright field images of zebrafish at 50 hpf.

Table

|       | Ctrl | RES, 10 μg/mL | DHS, 10 μg/mL | PIN, 10 μg/mL |
|-------|------|---------------|---------------|---------------|
| A     |      |               |               |               |
| B     |      |               |               |               |
| C     |      |               |               |               |
| D     |      |               |               |               |

Figure 4. Analysis of zebrafish swimming distance at 100 hpf (swimming behavior assay (5 min)). (A) Normal group, (B) RES, 10 μg/mL administration group, (C) DHS, 10 μg/mL administration group, (D) PIN, 10 μg/mL administration group, (n = 6).
In conclusion, our study has shown that DHS and PIN, two natural RES analogs, exhibit stronger neurotoxicity than RES in transgenic zebrafish. DHS and PIN can significantly reduce zebrafish motor nerve axon length, and the reduction is most obvious in the PIN-treated group. Both DHS and PIN treatments significantly shorten the swimming distance.
of zebrafish. DHS and PIN may be potential nerve growth inhibitors or anesthetics. DHS inhibited U87 cell aggregation, but PIN promoted it in a concentration-dependent manner. The position and number of hydroxyl moieties significantly affected neurotoxicity and the ability of aggregation. Additional work is needed to investigate the mechanisms underlying the inhibitory effects of DHS, and PIN on nerve growth. Additional work is also required to investigate the underlying mechanisms by which DHS inhibits U87 aggregation, whereas PIN promotes it.

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Declaration of Conflicting Interests

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