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Design, synthesis, and biological evaluation of novel Tempol derivatives as effective antitumor agents

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Abstract Two series of novel Tempol derivatives **T1–T6** based on the piperidine nitroxide Tempol and phenolic acids were designed and synthesized, and their biological evaluation is also described. The chemical structure was verified by HRMS, IR, and EPR analysis. The antitumor activity was tested against two tumor cell lines (A549 and Hela cells). Simultaneously, HK-2 cells were selected to investigate cytotoxicity and selectivity of synthetic compounds to the normal cells. The antioxidant property was also studied by DPPH radical scavenging assay and hydrogen peroxide-induced cell injury assay. The results demonstrated that most of the Tempol derivatives exhibited more active antioxidant activity than Tempol, and all synthesized Tempol derivatives exhibited more potent antitumor activity than Tempol. Among them, compound **T6** displayed the highest antitumor activity (IC_{50} = 29.4 μg/mL for A549 cells; IC_{50} = 16.2 μg/mL for Hela cells). The results indicated that **T6** exhibited efficient antitumor performance, having the potential of being excellent antitumor agents for cancer treatment.

Keywords Piperidine nitroxide · Phenolic acids · Antitumor activity · Growth inhibition · Antioxidant activity

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Introduction

In recent years, cancer incidence and morbidity are steadily increasing even though great progress has been made in the treatment of tumors. In 2015, an estimated 1,658,370 new cancer cases diagnosed and 589,430 cancer deaths occurred in the US [1]. In addition to surgical intervention, current cancer treatments mainly rely on radiation and chemotherapeutic agents. The design of antitumor agents is very important for cancer treatment. Much evidence suggests that multiple metabolites and metabolic by-products such as ATP, acetyl-CoA, α-ketoglutarate (α-KG), and reactive oxygen species (ROS) can have a crucial role in tumor development [2]. Carefully considering these points will allow for the development of efficient agents for cancer therapy.

4-Hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempol, Fig. 1) is a piperidine nitroxide analogue, which is widely used as biological agent [3–7] and has various biological activities, such as radio-protection [8–12] and anti-proliferation properties [13]. It has been extensively studied for its antitumor activity by inducing cell apoptosis in breast cancer and human leukemia [14]. Antitumor activity is potentiated by pretreatment with Tempol in the colon carcinoma cell lines [15]. The paradoxical pro-oxidant action of Tempol has accounted for provoking cancer cell apoptosis [16]. More detailed studies indicate that Tempol can inhibit the tumor growth, which seems to be related to the reactivity of the nitroxyl group [3, 4, 17, 18]. It suggested that we can prepared Tempol derivatives as antitumor drugs by introduction of a piperidine nitroxide radical into pharmaceutical molecules.

In recent years, there has been a revival of interest in the medical properties of phenolic acids because they exhibit a broad spectrum of activity including antioxidant, antibacterial, antifungal, antitumor, and antiinflammatory properties. It has been reported that caffeic acid [19, 20] and ferulic acid [20] can induce apoptosis in tumor cells and exerts anticarcinogenic effects. Oxidative stress and free radical damage are recognized to induce malignant transformation and the occurrence of cancer. It has been reported that the supplementation with antioxidants can inhibit the carcinogenesis [21]. Tempol and phenolic acids have a great effect on the antioxidant activities and anti-free radical effect. However, so

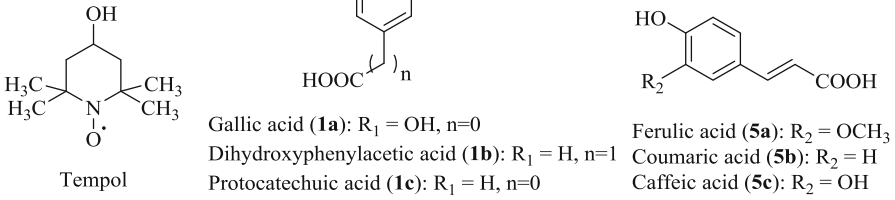

Fig. 1 Structure of 4-hydroxy-2,2,6,6,tetramethylpiperidine-*N*-oxyl (Tempol) and phenolic acids used to design and synthesize Tempol derivatives

far, Tempol and phenolic acid used as starting materials to prepare new conjugated derivatives as antitumor agents has not been paid much attention.

To explore the potential of this idea in the antitumor agents design and improve antitumor potency of Tempol, in this paper, we firstly designed and synthesized two series of novel Tempol derivatives as antitumor agents based on piperidine nitroxide Tempol and phenolic acids (Fig. 1). The antitumor activity of Tempol derivatives against A549 and Hela tumor cells was investigated, and the antioxidant capacity was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay and hydrogen peroxide-induced cell injury assay.

Experimental

General

4-Hydroxy-2,2,6,6-tetramethyl-piperidinooxy (Tempol) was purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical was purchased from TCI (Shanghai, China). All of the different phenolic acids, as well as other reagents and solvents were purchased from Energy Chemical (Shanghai, China). A549 (human adenocarcinoma lung cells), Hela (human cervical carcinoma cells), and HK-2 (human proximal tubular epithelial cells) cell lines were procured from the culture collection of the Chinese Academy of Science (Shanghai, China). Cell culture media, Opti-MEM, and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). The progress of the reactions was monitored by thin-layer chromatography on silica gel plates from Kehao (Xi'an, China). Column chromatography was performed with silica gel (200–300 or 300–400 mesh, Kehao). ^{1}H NMR spectroscopy were recorded in $CDCl_3$ or CD_3OD solution on a Bruker Spectrospin 400 (400 MHz) instrument. EPR spectra were recorded on a JES-FA200 EPR spectrometer (JEOL, Japan, Measurement conditions: frequency 9.0514 GHz, normal pressure and temperature, CH_2Cl_2 as solvent). Fourier transform infrared (FT-IR) spectroscopy was performed on a Paragon 1000 (Perkin-Elmer) instrument using KBr plates. High-resolution mass spectra (HRMS) were measured with ESI.

General procedure for preparation of Tempol derivatives T1–T3

*Preparation of **2a–2c***

Gallic acid (**1a**, 7.53 g, 40 mmol) and acetic anhydride (22.7 mL, 240 mmol) were combined in a 100 mL round-bottom flask. The slurry was stirred with a magnetic stirrer and catalytic amount of sulfuric acid (500 μL) was added. The reaction mixture was stirred at 75 °C for 1.5 h, and the slurry became a clear yellow solution. The mixture was further stirred for 20 min at room temperature, and 60 mL of water was added. After stirring for 2.5 h, a white crystalline product was isolated by filtration and washed with water (20 mL × 3). The product was dried in

a vacuum drying oven overnight to obtain the acetyl-protected gallic acid **2a** (8.7 g). **2b**, **2c** were synthesized using similar method as **2a**.

2a (73 %) was obtained as a white crystalline solid. mp 166–167 °C (lit [22]. 166–168 °C); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.31 (s, 9H, –OCH_3), 7.87 (s, 2H, Ar–H); IR (KBr, cm^{-1}) ν_{max}: 2983, 2955, 2930, 1778, 1693, 1433, 1372, 1322, 1183, 1056, 899.

2b (46 %) was obtained as a white solid. mp 105–106 °C (lit [23]. 104–106 °C); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.24 (s, 3H, –OCH_3), 2.25 (s, 3H, –OCH_3), 3.63 (s, 2H, –CH_2–), 7.14–7.20 (m, 3H, Ar–H); IR (KBr, cm^{-1}) ν_{max}: 2939, 2922, 2854, 1770, 1708, 1508, 1373, 1261, 1211, 1184, 1114, 1014, 906.

2c (76 %) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.33 (s, 6H, –OCH_3), 7.32 (d, J = 8.4 Hz, 1H, Ar–$H_{5'}$), 7.94 (d, J = 2.0 Hz, 1H, Ar–$H_{2'}$), 8.02 (dd, J_1 = 8.4 Hz, J_2 = 2.0 Hz, 1H, Ar–$H_{6'}$); IR (KBr, cm^{-1}) ν_{max}: 2985, 2945, 2854, 1766, 1685, 1373, 1242, 1164, 1049, 914, 779.

*Preparation of **3a–3c***

To the solution of **2a** (2.96 g, 10 mmol) in dry dichloromethane (25 mL), $SOCl_2$ (0.88 mL, 12 mmol) and DMF (0.2 mL, 2.5 mmol) were added dropwise. The mixture was refluxed for 3 h. After the solvent was evaporated under reduced pressure, **3a** (3.1 g, 98 %) was obtained as a white solid. mp 105–107 °C (lit [24]. 106–107 °C). **3b**, **3c** were synthesized using similar method as **3a**. **3b** (98 %), **3c** (99 %) were obtained as a yellow oil, which used without purification for the next reaction.

*Preparation of **4a–4c***

4-Hydroxy-2,2,6,6-tetramethyl-piperidinooxy (Tempol, 1.85 g, 10.73 mmol) and pyridine (1.2 mL, 15 mmol) were dissolved in dry dichloromethane (15 mL) and the mixture was stirred for 1 h at room temperature. **3a** (3.1 g, 9.5 mmol) dissolved in dry dichloromethane (15 mL) was added to the above mixture at 0 °C. Then, the reaction mixture was stirred for 3 h at room temperature and monitored by TLC. The reaction mixture was repeatedly washed with saturated copper sulfate solution and the organic phase was dried under magnesium sulfate. After filtering, the solvent was removed in a vacuum. The residue was purified by flash chromatography on silica gel with petroleum ether/ethyl acetate (100/1 to 20/1) to give **4a** (2.32 g). **4b**, **4c** were synthesized using a similar method as **4a**.

4a (53 %) was obtained as a red solid. mp 101–102 °C; IR (KBr, cm^{-1}) ν_{max}: 2975, 2937, 2869, 1739, 1463, 1365, 1236, 1180, 1031, 889; HRMS (ESI$^+$) Calcd for $[M + Na]^+$: 473.1662 *m/z*. Found: 473.1659 *m/z*.

4b (56 %) was obtained as a red solid. mp 86–87 °C; IR (KBr, cm^{-1}) ν_{max}: 2977, 2935, 2854, 1770, 1731, 1504, 1369, 1261, 1203, 1180, 1110, 1010, 902; HRMS (ESI$^+$) Calcd for $[M + Na]^+$: 429.1763 *m/z*. Found: 429.1750 *m/z*.

4c (67 %) was obtained as a red solid. mp 118–119 °C; IR (KBr, cm^{-1}) ν_{max}: 2977, 2939, 2854, 1770, 1708, 1612, 1504, 1373, 1288, 1195, 1164, 1114, 1091, 1010, 910, 763, 736.

*Typical procedure of preparation of **T1–T3***

Guanidine hydrochloride (764 mg, 8 mmol) was added to a solution of **4a** (450 mg, 1 mmol) in 20 mL mixed solvent (dichloromethane/methanol = 5/1). The mixture was refluxed for 48 h and monitored by TLC. The solvent was evaporated and the residue was purified by flash chromatography on silica gel with dichloromethane/ethyl acetate (20/1 to 2/1) to give **T1** (150 mg, 43 %) as a brownish red solid. mp 78–79 °C; IR (KBr, cm^{-1}) ν_{max}: 3371, 2977, 2935, 2873, 1701, 1612, 1465, 1319, 1226, 1180, 1033, 771; ESR: $g_{iso} = 2.0044$, $A_N = 15.39$ Gs (CH_2Cl_2); HRMS (ESI^-) Calcd for $[M–H]^-$: 323.1369 *m/z*. Found: 323.1386 *m/z*. **T2**, **T3** were synthesized by the method as **T1**.

T2 (38 %) was obtained as a pale yellow solid. mp 139–140 °C; IR (KBr, cm^{-1}) ν_{max}: 3394, 2977, 2939, 2854, 1731, 1604, 1519, 1365, 1284, 1242, 1145, 1045, 798; ESR: $g_{iso} = 2.0044$, $A_N = 15.53$ Gs (CH_2Cl_2); HRMS (ESI^-) Calcd for $[M–H]^-$: 321.1576 *m/z*. Found: 321.1576 *m/z*.

T3 (90 %) was obtained as a yellow solid. mp 191–192 °C; IR (KBr, cm^{-1}) ν_{max}: 3309, 2977, 2939, 2854, 1708, 1604, 1442, 1292, 1218, 1176, 1091, 767; ESR: $g_{iso} = 2.0044$, $A_N = 15.61$ Gs (CH_2Cl_2); HRMS (ESI^-) Calcd for $[M–H]^-$: 307.1420 *m/z*. Found: 307.1420 *m/z*.

General procedure for preparation of Tempol derivatives T4–T6

*Preparation of **6a–6c***

Acetic anhydride (0.59 mL, 6.25 mmol) was added slowly to a solution of ferulic acid (**5a**, 970 mg, 5 mmol) and DMAP (62 mg, 0.5 mmol) in pyridine (5 mL) at 0 °C. After stirring for 1.5 h, the mixture was poured into ice water. The aqueous phase was acidified with hydrochloric acid (2 M) to pH 2 and then extracted with ethyl acetate (3 × 150 mL). The combined organic layers were dried over Mg_2SO_4. After filtering and concentrating in vacuo, the residue was treated with petroleum ether/ethyl acetate (95/5; v/v; 10 mL) to give **6a** (1.04 g, 88 %) as a white powder. mp 198–199 °C (lit [25, 26]. 197–200 °C); ^{1}H NMR (400 MHz, CD_3OD): δ 2.26 (s, 3H, –OCH_3), 3.84 (s, 3H, –OCH_3), 6.84 (d, $J = 16.0$ Hz, 1H, –CH=CH–), 7.05 (d, $J = 8.4$ Hz, 1H, Ar–$H_{6'}$), 7.18 (dd, $J_1 = 8.4$ Hz, $J_2 = 2.0$ Hz, 1H, Ar–$H_{5'}$), 7.30 (d, $J = 1.6$ Hz, 1H, Ar–$H_{2'}$), 7.65 (d, $J = 16.0$ Hz, 1H, –CH=CH–); IR (KBr, cm^{-1}) ν_{max}: 2977, 2944, 2873, 1761, 1684, 1632, 1507, 1261, 1223, 1199, 1034, 855. **6b** and **6c** were synthesized using a similar method as **6a**.

6b (97 %) was obtained as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 2.28 (s, 3H, –OCH_3), 6.46 (d, $J = 16.0$ Hz, 1H, –CH=CH–), 7.15 (d, $J = 8.4$ Hz, 2H, Ar–$H_{3,5'}$), 7.64 (d, $J = 8.8$ Hz, 2H, Ar–$H_{2,6'}$), 7.68 (d, $J = 15.6$ Hz, 1H, –CH=CH–); IR (KBr, cm^{-1}) ν_{max}: 2972, 2935, 2854, 1743, 1677, 1508, 1209, 1170, 916, 838.

6c (89 %) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.31 (s, 3H, –OCH_3), 2.32 (s, 3H, –OCH_3), 6.39 (d, $J = 16.0$ Hz, 1H, –CH=CH–), 7.26 (d, $J = 2.8$ Hz, 1H, Ar–$H_{6'}$), 7.39 (d, $J = 2.0$ Hz, 1H, Ar–$H_{5'}$), 7.43 (dd, $J_1 = 8.4$ Hz, $J_2 = 2.0$ Hz, 1H, Ar–$H_{2'}$), 7.72 (d, $J = 16.0$ Hz, 1H, –CH=CH–); IR (KBr, cm^{-1}) ν_{max}: 2952, 2925, 2854, 1755, 1674, 1631, 1434, 1261, 1190, 1118, 827.

*Preparation of **7a–7c***

Thionyl chloride (0.88 mL, 12 mmol) was added to a suspension of **6a** (2.36 g, 10 mmol) in dry chloroform (25 mL) and DMF (0.2 mL). The mixture was refluxed for 4 h. After the solvent and excess thionyl chloride were removed under reduced pressure, **7a** (2.45 g, 92 %) was obtained as a pale yellow solid. This material was used without further purification. mp 129–130 °C (lit [25]. 129–131 °C). **7b**, **7c** were synthesized using similar method as **7a**. **7b** (99 %) and **7c** (98 %) were obtained as a white powder and used without further purification.

*Preparation of **8a–8c***

8a, **8b,** and **8c** were synthesized using similar methods as **4a**. **8a** (85 %) was obtained as a pale red solid. mp 142–143 °C; IR (KBr, cm^{-1}) ν_{max}: 2974, 2933, 2854, 1766, 1704, 1635, 1506, 1259, 1197, 1155, 1124, 1031, 831; HRMS (ESI^+) Calcd for $[M + Na]^+$: 413.1814 *m/z*. Found: 413.1800 *m/z*. **8b** (69 %) was obtained as a red solid. mp 104–105 °C; IR (KBr, cm^{-1}) ν_{max}: 2952, 2925, 2854, 1774, 1708, 1635, 1506, 1199, 1012, 910, 835; HRMS (ESI^+) Calcd for $[M + Na]^+$: 383.1709 *m/z*. Found: 383.1694 *m/z*. **8c** (69 %) was obtained as a pale red solid. mp 115–116 °C; IR (KBr, cm^{-1}) ν_{max}: 2975, 2937, 2854, 1774, 1712, 1635, 1506, 1371, 1205, 1178, 1112, 1012, 900; HRMS (ESI^+) Calcd for $[M + Na]^+$: 441.1763 *m/z*. Found: 441.1754 *m/z*.

*Typical procedure of preparation of **T4–T6***

8a (2.34 g, 6 mmol) was dissolved in a 20 mL mixed solvent (dichloromethane/methanol = 6/1) at room temperature. Potassium tert-butoxide (741 mg, 6.6 mmol) was added and the mixture was stirred for 15 min at 0 °C. Then, ice water was added into the reaction mixture. The product was extracted using dichloromethane (3 × 100 mL), dried over sodium sulfate, and concentrated under reduced pressure. The crude product was purified by flash chromatography using hexane and ethyl acetate as eluent (100/1 to 10/1) to give **T4** (1.08 g). **T5** was synthesized by the method as **T4**. **T6** was synthesized by the method as **T1**.

T4 (52 %) was obtained as a golden solid. mp 183–184 °C; IR (KBr, cm^{-1}) ν_{max}: 3413, 2974, 2925, 2854, 1704, 1635, 1515, 1157, 1027, 985, 848; ESR: $g_{iso} = 2.0049$, $A_N = 15.67$ Gs (CH_2Cl_2); HRMS (ESI^+) Calcd for $[M + Na]^+$: 371.1709 *m/z*. Found: 371.1718 *m/z*.

T5 (34 %) was obtained as a golden solid. mp 178–179 °C; IR (KBr, cm^{-1}) ν_{max}: 3394, 2977, 2937, 2854, 1704, 1631, 1604, 1585, 1515, 1240, 1166, 1045, 833; ESR: $g_{iso} = 2.0049$, $A_N = 15.47$ Gs (CH_2Cl_2); HRMS (ESI^-) Calcd for $[M–H]^-$: 317.1627 *m/z*. Found: 317.1615 *m/z*.

T6 (56 %) was obtained as a brownish red solid. mp 90–91 °C; IR (KBr, cm^{-1}) ν_{max}: 3371, 2974, 2931, 2869, 1704, 1685, 1635, 1604, 1515, 1446, 1272, 1157, 1114, 979, 813, 736; ESR: $g_{iso} = 2.0044$, $A_N = 15.64$ Gs (CH_2Cl_2); HRMS (ESI^-) Calcd for $[M–H]^-$: 333.1576 *m/z*. Found: 333.1577 *m/z*.

Biological studies on cell growth inhibition

A549 cells and Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10 % fetal bovine serum (FBS) in T25 culture flasks (Nunc, Denmark) and were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. HK-2 cell was cultured in Dulbecco's modified eagle essential medium and F-12 nutrient mixture (DMEM/F-12) (Hyclone, Thermo Scientific, Barrington, IL) supplemented with 10 % fetal bovine serum (FBS) in T75 culture flasks (Nunc, Denmark) and were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. Cells were regularly passaged by 0.1 % trypsin (EDTA 0.02 % and trypsin 0.1 %) in PBS (pH 7.4).

The antitumor activity and selectivity of Tempol derivatives was evaluated by MTT assays through determining the inhibition effect on the proliferation of A549 cells, Hela cells or HK-2 cells. Tempol and Dox were used as the positive control. The in vitro antitumor activities of each Tempol derivatives was determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay following the literature procedures [27, 28]. Nearly 2×10^4 cells/well were plated in 96-well plate. The concentration of compounds was set as 5–100 μg/mL. After 24 h of incubation, test samples were added to the wells containing the appropriate tumor cells and incubated for an additional 24 h. Twenty microliters of MTT (5 mg/mL) solution was added, and the cells were incubated for another 3 h. The old media were removed and 150 μL of DMSO was added per well. The reduced crystal in violet was completely dissolved in DMSO, and then the absorbance of the solution was determined at 490 nm using a microtiter plate reader (Bio-Rad Laboratories, Richmond, CA, USA). The samples were assayed in independent experiments to calculate either percent inhibition or IC_{50} values. All of the tested compounds were dissolved in 0.1 % DMSO, and the drug solution was diluted from the stock solution for the MTT assay.

Evaluation of antioxidant activity by DPPH radical scavenging assay

The antioxidant activity of the Tempol derivatives, based on the scavenging activity of the stable 2,2′-diphenyl-1-picrylhydrazyl (DPPH) free radical, was determined by the method described by Yen et al. [29] and Yokozawa et al. [30]. The Tempol derivatives, Tempol solutions (60 μL) in methanol at different concentrations were added to a 240 μL 1.6×10^{-4} mol/L solution of DPPH in methanol, total volume of the reactive mixture is 1.5 mL, previously prepared daily and protected from light. The reaction mixture was incubated at room temperature in the dark for 30 min, and then the absorbance of the reactive mixture was recorded using spectrophotometer at 517 nm. Inhibition of the DPPH free radical in percent was calculated in following way:

$$\text{Scavenging effect } (\%) = [1 - (A_s - A_r)/(A_m - A_0)] \times 100;$$

where A_m the absorbance of the control reaction (containing all reagents except the test compound), A_s the absorbance of the test compound, A_0 the absorbance of the

blank control, and A_r the absorbance of the drug control group (containing all reagents except the DPPH). Compound concentration providing 50 % inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against solution concentration. Tests were carried out in triplicate. Five different concentration (0.05, 0.09, 0.19, 0.38, 0.75 mmol/L) of each Tempol derivatives and control studied have been assayed in order to check the linearity of response and to establish the antioxidant activity values in the adequate linear range. Methanol was tested against DPPH radical and this resulted in null effect on the absorbance at 517 nm.

Effect of Temple derivatives on protecting HK-2 cells against hydrogen peroxide-induced cell injury

Considering the complexity of antioxidant activity in biological systems, hydrogen peroxide (H_2O_2)-induced cell injury model was further used to evaluate the antioxidant activity of these compounds. The protective effects of the compounds on HK-2 cells against hydrogen peroxide-induced cell injury were assessed by MTT assay as reported literature procedure [31, 32]. Tempol was used as the positive control. HK-2 cells were cultured at 37 °C in a humidified atmosphere containing 5 % of CO_2. Cells were seeded into 96 well cell culture plates at a density of 8×10^3 cells/well in DMEM/F12 medium. Following protocols were carried out 12 h after cells were seeded. H_2O_2 was newly prepared in phosphate buffered saline (PBS) on the day of application to cultures at a final concentration of 400 μM. Tempol and Tempol derivatives were dissolved in DMSO and diluted with DMEM/F12. The HK-2 cells were preincubated with samples for 2 h before the H_2O_2 was added. A fresh solution of MTT (5 mg/mL) prepared in PBS (pH 7.4) was added to the 96-well cell culture plates 3 h after H_2O_2 was added. The plates were incubated in the CO_2 incubator for 3 h, and the formazan was lysed with DMSO. The plates were analyzed in a multi-well-plate reader (Bio-Rad Laboratories, Richmond, CA, USA) at 490 nm. The samples were assayed in sextuplicate.

Statistical analysis

Results are expressed as mean ± SD. Differences between experimental groups were evaluated by using analysis of variance (ANOVA). The statistical significance of the difference between groups was evaluated by one-way ANOVA and *T* test. Values of $P < 0.05$ were accepted as significant.

Results and discussion

Chemistry

The synthesis of Tempol derivatives **T1**–**T6** was outlined in Figs. 2 and 3. Acetic anhydride was used as a hydroxyl protection reagent to protect the hydroxyl group of the phenolic acids, and acetyl-protected phenolic acid (**2a**–**2c**, **6a**–**6c**) was successfully prepared. After the reaction of **2a**–**2c** or **6a**–**6c** with 1.1 equiv of thionyl

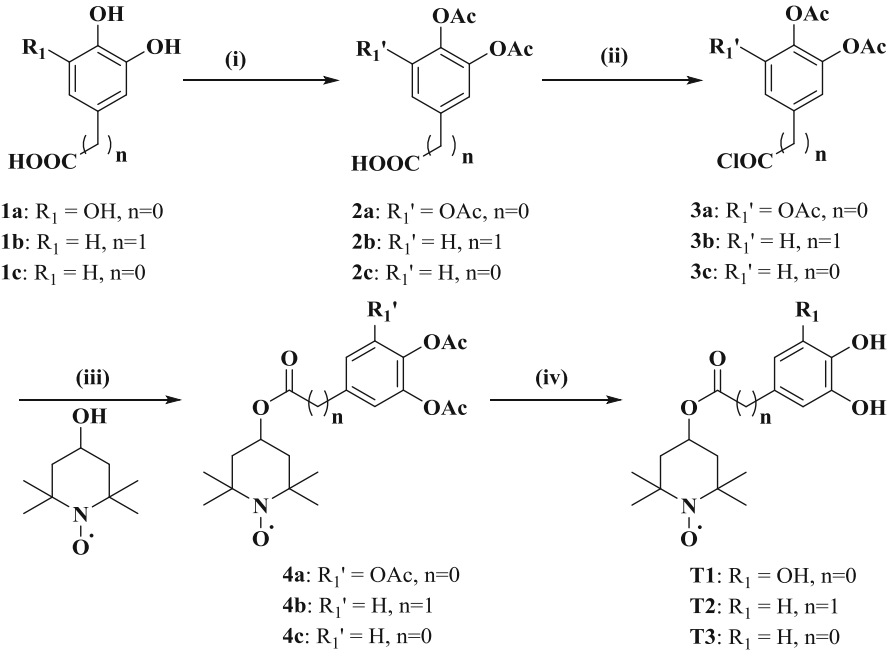

Fig. 2 Synthesis of Tempol derivatives **T1**–**T3**. Reagents and conditions: (*i*) Ac_2O, H_2SO_4, 85 °C, 1.5 h; (*ii*) $SOCl_2$, DMF/CH_2Cl_2, reflux 3 h; (*iii*) pyridine/CH_2Cl_2, rt, 5 h; (*iv*) guanidine hydrochloride, CH_2Cl_2, 40 °C, 48 h

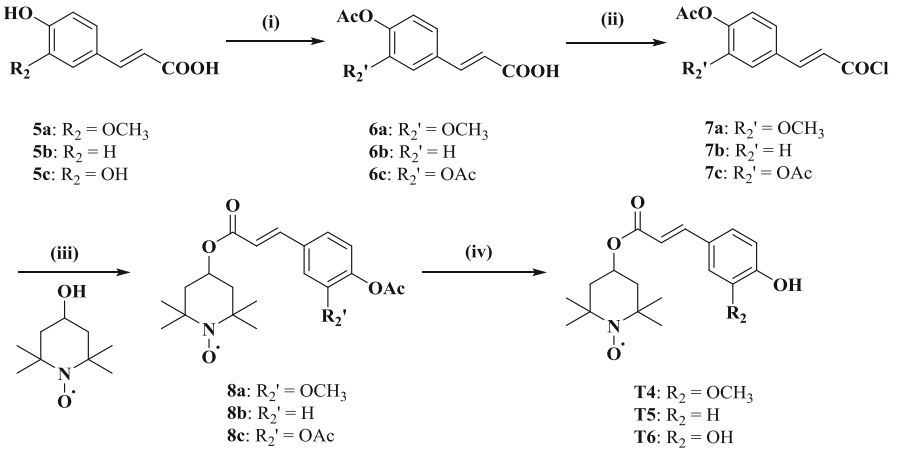

Fig. 3 Synthesis of Tempol derivatives **T4**–**T6**. Reagents and conditions: (*i*) Ac_2O, DMAP, pyridine, rt, 1.5 h; (*ii*) $SOCl_2$, DMF/$CHCl_3$, reflux 4 h; (*iii*) Pyridine/CH_2Cl_2, rt, 5 h; (*iv*) potassium tert-butylate, CH_2Cl_2/CH_3OH, 0 °C, 15 min for **T4**, **T5**; guanidine hydrochloride, CH_2Cl_2, 40 °C, 48 h for **T6**

chloride in the presence of a catalytic amount DMF in $CHCl_3$ or CH_2Cl_2 at refluxing temperature, **3a**–**3c** and **7a**–**7c** was obtained in a good yield. Then chloride of acetyl-protected phenolic acid (**3a**–**3c**, **7a**–**7c**) and Tempol was carried out in the

presence of a stoichiometry amount pyridine in CH_2Cl_2 at room temperature to gave **4a**–**4c** and **8a**–**8c**. Finally, target compounds **T1**–**T6** were successfully prepared in a moderate yield using potassium tert-butylate or guanidine hydrochloride as the deacetylase reagent. All synthesized target Tempol derivatives **T1**–**T6** were purified by column chromatography, and confirmed by the melting point, IR, ESR, and HRMS analyses.

Antitumor activity of Tempol derivatives

The anti-proliferative activities of Tempol derivatives **T1**–**T6** were tested against two tumor cells A549 and Hela cells using Tempol and Doxorubicin (Dox) as the positive control. As shown in Figs. 4 and 5, all of the Tempol derivatives **T1**–**T6** with a concentration range of 5–100 μg/mL exhibited a dose dependent manner against the tumor cells growth. Remarkably, all of the Tempol derivatives were more potent than Tempol. The same results are also confirmed in Tables 1 and 2. Among them, **T1** and **T6** showed stronger growth inhibition for A549 cells with IC_{50} values of 42.3, 29.4 μg/mL, respectively. Other Tempol derivatives showed growth inhibition with IC_{50} values of 99.8 (**T2**), 225.3 (**T3**), 142.0 (**T4**), and 72.7 (**T5**) μg/mL, respectively. Among them, **T4** and **T6** showed stronger growth inhibition for Hela cells with IC_{50} values of 30.1, 16.2 μg/mL, respectively. Other Tempol derivatives showed growth inhibition with IC_{50} values of 363.3 (**T1**), 62.4 (**T2**), 388.7 (**T3**), and 64.8 (**T5**) μg/mL, respectively. These results indicated that introduction of phenolic acids to Tempol derivatives can improve the antitumor activities significantly. Furthermore, the antitumor activity of these Tempol derivatives (**T1**–**T6**) was correlated with their structure. Tempol derivatives **T2**, with a longer linker moiety, showed markedly increased antitumor activity

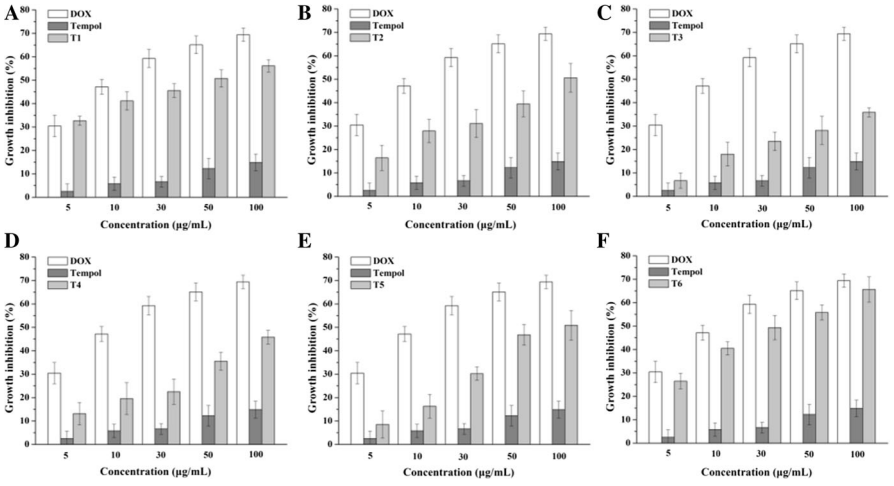

Fig. 4 The antitumor effect of Tempol derivatives **T1**–**T6** against A549 cells determined by MTT assay. Each *bar value* represents the mean ± SD ($n = 6$)

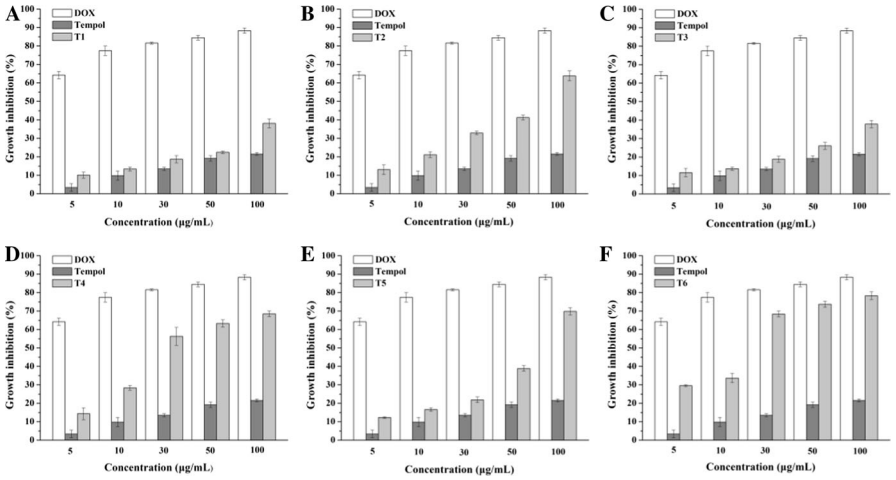

Fig. 5 The antitumor effect of Tempol derivatives **T1**–**T6** on the growth of Hela cells determined by MTT assay. Each *bar value* represents the mean ± SD ($n = 6$)

Table 1 The value of IC_{50} against A549 cells

Entry	Tempol derivatives	IC_{50} (μg/mL)
1	**T1**	42.3 ± 5.8**,##
2	**T2**	99.8 ± 8.7**,##
3	**T3**	225.3 ± 18.0**,##
4	**T4**	142.0 ± 7.8**,##
5	**T5**	72.7 ± 9.3**,##
6	**T6**	29.4 ± 4.8**,#
7	Tempol	1637.9 ± 246.8##
8	Dox	16.2 ± 2.8

Each value is presented as mean ± SD ($n = 6$). ** $P < 0.01$, compared with Tempol. # $P < 0.05$, ## $P < 0.01$, compared with Dox

Table 2 The value of IC_{50} against Hela cells

Entry	Tempol derivatives	IC_{50} (μg/mL)
1	**T1**	363.3 ± 25.1**,##
2	**T2**	62.4 ± 5.0**,##
3	**T3**	388.7 ± 24.5**,##
4	**T4**	30.1 ± 0.6**,##
5	**T5**	64.8 ± 3.0**,##
6	**T6**	16.2 ± 0.7**,##
7	Tempol	559.7 ± 15.4##
8	Dox	1.0 ± 0.1

Each value is presented as mean ± SD ($n = 6$). ** $P < 0.01$, compared with Tempol. ## $P < 0.01$, compared with Dox

compared with **T3**. Tempol derivatives **T6**, with an hydroxyl group at the C-3 position, showed markedly increased antitumor activity compared with **T4** and **T5**. In addition, we also used HK-2 cells, the human proximal tubular epithelial cells, as

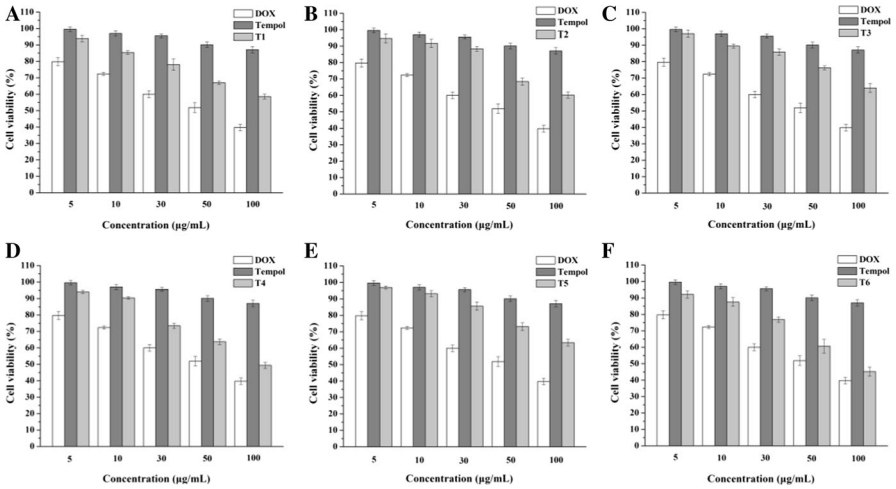

Fig. 6 The cytotoxicity of Tempol derivatives **T1**–**T6** for HK-2 cells determined by MTT assay. Each *bar value* represents the mean ± SD ($n = 6$)

the model to evaluate the cytotoxicity and selectivity of the compounds for normal cells. As shown in Fig. 6, all of the Tempol derivatives **T1**–**T6** with the concentration range of 5–100 μg/mL, exhibited lower cytotoxicity than Doxorubicin (Dox) for normal cells.

Antioxidant activity of Tempol derivatives

Antioxidant activity of Tempol derivatives are closely related to their bioactivities, such as the inhibition of mutagenesis and tumor cell growth, which is often associated with the termination of free radical transmission in biological systems [33, 34]. Thus, the antioxidant capacity of Tempol derivatives was investigated by DPPH radical scavenging assay and hydrogen peroxide-induced cell injury assay. The method is based on the reduction of DPPH in the presence of a hydrogen donating antioxidants, and the non-radical form DPPH-H was eventually obtained by the reaction. The reduction of DPPH radical occurs with a decrease in its absorbance at 517 nm induced by Tempol derivatives. The antioxidant activity of Tempol derivatives was tested by DPPH radical scavenging and is shown in Fig. 7 and Table 3. All of the Tempol derivatives **T1**–**T6** with concentration range of 0.01–0.15 μmol/mL showed dose–response relationship (Fig. 7), and the antioxidant activity of Tempol derivatives were more potent than Tempol ($IC_{50} = 21.5$ - μM) except for **T5** ($IC_{50} = 123.9$ μM). The IC_{50} values of Tempol derivatives **T1**, **T2**, **T3**, **T4**, **T6** were 7.8, 7.4, 9.6, 19.7, and 10.2 μM, respectively.

Since biological systems are complex, we made further evaluation of the antioxidant properties of Tempol derivatives by hydrogen peroxide-induced cell injury assay in HK-2 cells. As shown in Fig. 8, compared with negative control, the cell viability of H_2O_2-injury group was significantly reduced. Compared with H_2O_2-

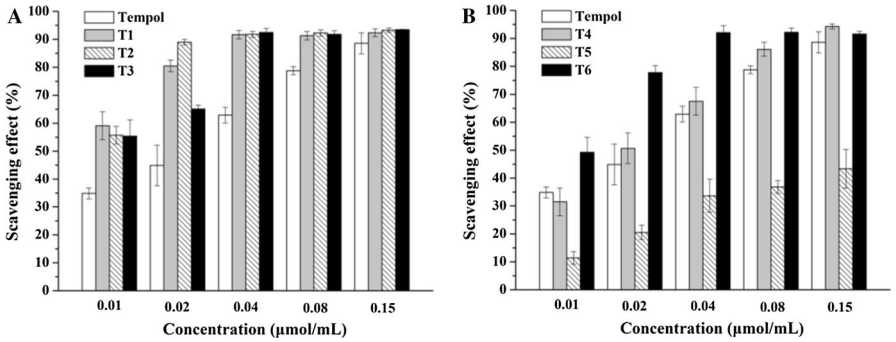

Fig. 7 Antioxidant activity of Tempol derivatives measured by DPPH radical scavenging assay. Each *bar value* is presented as mean ± SD ($n = 3$)

Table 3 The IC_{50} of antioxidant activity measured by DPPH radical scavenging assay

Entry	Tempol derivatives	IC_{50} (μM)
1	**T1**	7.8 ± 1.0**
2	**T2**	7.4 ± 0.5**
3	**T3**	9.6 ± 0.8**
4	**T4**	19.7 ± 3.4
5	**T5**	123.9 ± 24.3**
6	**T6**	10.2 ± 0.9**
7	Tempol	21.5 ± 2.4

Each value is presented as mean ± SD ($n = 3$).
** $P < 0.01$, compared with Tempol

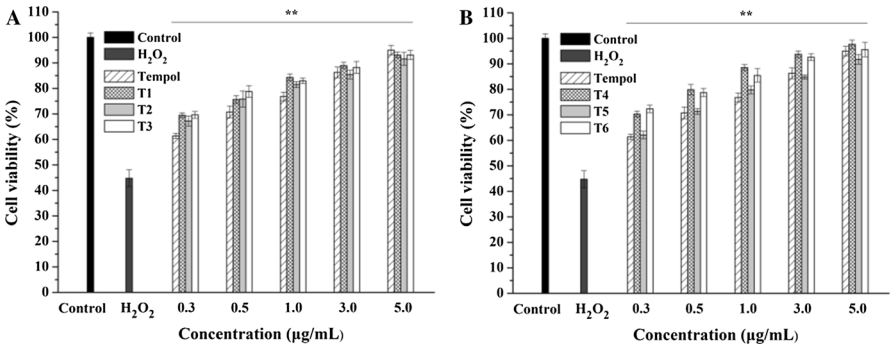

Fig. 8 The protective effect of Tempol derivatives **T1**–**T6** for the injury of HK-2 cells induced by H_2O_2. Each *bar value* represents the mean ± SD ($n = 6$). **$P < 0.01$, compared with H_2O_2-injury group

injury group, Tempol and **T1**–**T6** could significantly improve the cell viability, and exhibited a dose dependent manner against the injury protection of HK-2 cells and improved the cell viability to 90 % at 5 μg/mL. Like the antitumor activity, the substituent on the benzene ring influences the antioxidant activity of Tempol derivatives. Tempol derivatives **T1** and **T6** with a hydroxyl group at the C-3

position of the benzene ring showed markedly increased antioxidant activity compared with other compounds. This might be related to their quick reduction to hydroxylamines in the presence of OH groups.

Conclusions

In summary, two series of novel Tempol derivatives were synthesized successfully, their antitumor activity evaluated by the MTT assay, and their antioxidant activity investigated using the DPPH radical scavenging assay and hydrogen peroxide-induced cell injury assay, respectively. The antitumor results showed that all of the Tempol derivatives exhibited more potent antitumor activity than Tempol and most of the Tempol derivatives exhibited better antioxidant activity than Tempol. The results showed that Tempol derivatives designed by introduction of phenolic acids are promising lead compounds in antitumor chemotherapy. Among them, Tempol derivatives **T6** is the promising derivative and can be selected as effective antitumor agent for further investigation.

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