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Antioxidative and antitumor activity of derivatives of 4-β-amino-4′-demethylepipodophyllotoxin and their structure-activity relationship

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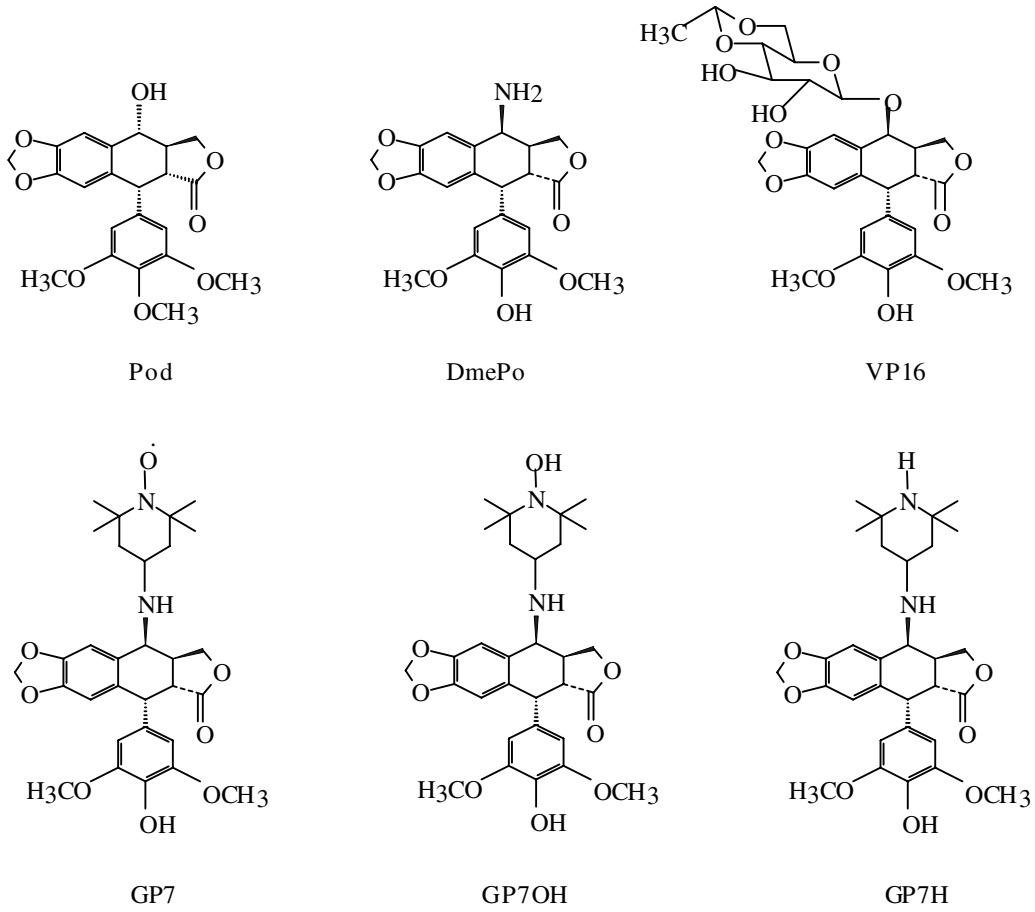
The purpose of this study was to investigate antioxidative and antitumor activity of derivatives of 4-β-amino-4′-demethylepipodophyllotoxin (DmePod) and to analyze their structure-activity relationship. Homogenates of liver, heart and kidney of rats were used to measure malondialdehyde (MDA) generation spontaneously formed or induced by a hydroxyl free radical generation system (Fe^{2+}-ascorbic acid) using thiobarbituric acid (TBA) assay. H_2O_2-induced red blood cells (RBC) hemolysis was determined spectrophotometrically. Superoxide anion ($O_2^{\cdot-}$) from zymosan-stimulated neutrophils of rats was evaluated by nitroblue tetrazolium (NBT) reduction assay. Microculture tetrazolium (MTT) assay was used to determine the antitumor effects on K562 and K562/DOX cells. The results showed that all the tested compounds strongly inhibited MDA formation from tissue homogenates in a concentration-dependent manner following the rank GP7OH > GP7 > VP16 and GP7H > DmePod > Pod. The potency of antihemolysis for DmePod, GP7, GP7OH, GP7H and VP16 was similar among them according to their IC_{50} values by 13.6, 8.6, 11.7, 10.3, and 9.47 $\mu mol \cdot L^{-1}$, respectively, whereas the potency for Pod was the weakest ($IC_{50} > 320\ \mu mol \cdot L^{-1}$). GP7, GP7OH and VP16 ($160 \sim 320\ \mu mol \cdot L^{-1}$) significantly inhibited $O_2^{\cdot-}$ formation following the potency rank VP16 > GP7 > GP7OH. However, $320\ \mu mol \cdot L^{-1}$ of DmePod, Pod or GP7H had no effect on $O_2^{\cdot-}$ formation. Meanwhile, all the tested compounds strongly inhibited K562 and K562/DOX cell proliferation for 96 h in a concentration-dependent manner. The resistance magnitude of GP7, GP7OH, VP16, and DmePod was 2.05, 2.21, 14.29, and 3.26, respectively, while antitumor activity of Pod and GP7H on K562/DOX cells was the weakest in all compounds. Taken together, the introduction of nitroxyl radical moieties into DmePod greatly enhances antioxidative and antitumor activity, and reverses drug resistance. Both NO$^{\cdot}$ and NOH groups are essential active moieties.

1. Introduction

Podophyllotoxin (Pod) is a traditional chemotherapeutic agent. Its semisynthetic derivatives such as etoposide, teniposide and etopophos, are widely used as antineoplastic agents and useful in the clinical treatment of several types of neoplasms including testicular cancer, small-cell lung cancer, Hodgkin and non-Hodgkin or other lymphomas, leukaemia, Wilms tumour, Kaposi's sarcoma and so on (Gordaliza et al. 2004; Damayanthi and Lown 1998). Nonetheless, their clinical effectiveness is restricted due to several limitations including myelosuppression, drug resistance, cytotoxicity towards normal cells and inactivation caused by metabolism. According to the presumption that introduction of nitroxyl radicals into antineoplastic agents may enhance their antitumor activity and reduce their toxicity, in order to overcome the limitations of these compounds and to develop new compounds with stronger antitumor activity and lower toxicity, a number of spin labeled derivatives of Pod was obtained through structural modifications. Furthermore, the spin labeled derivatives of Pod had significant antitumor activities and could diminish marked toxicity as compared with their parent. For example, GP-1 ($LD_{50} = 392.0$ mg/kg), a spin labeled derivative of Pod, had less toxicity than the parent compound Pod ($LD_{50} = 50$ mg/kg) and a weaker effect on mice WBC than its amino derivative GP-1-H. Meanwhile, GP-1 possessed higher antitumor activity than its parent compound Pod and corresponding hydroxylamine and amine compounds including GP-1-OH, GP-1-H. They all showed the same antitumor mechanisms (Chen et al. 1987, 1989; Wang et al. 1993; Tian et al. 1997; Jia et al. 1999). These results could account for a possible correlation between tissue distribution and the selective anti-tumor activity of the spin labeled derivatives.

Recently, it was shown that nitroxides had a beneficial effect on the toxicity and anticancer activity of antineoplastic agents due partly to their antioxidative activity (Konovalova



et al. 1996; Li et al. 2002; Tian et al. 2002; Gadjeva et al. 2005; Gadjeva 2002; Wu et al. 1997; Jia et al. 1999). However, to date the precise mechanisms are still unclear. Therefore, the experiment was designed to investigate the antitumor and antioxidative actions of derivatives of 4-β-amino-4′-demethylepipodophyllotoxin (DmePod) with the chemical structures displayed, and to analyze the relationship between their structure and activity.

2. Investigations and results

2.1. Inhibitory effects on malondialdehyde (MDA) formation

MDA was spontaneously formed from rat liver homogenate after 2 h incubation. Pod, DmePod, GP7, GP7OH, GP7H, and VP16 inhibited MDA generation in a concentration-dependent manner. Among all the tested compounds, Pod was the weakest with the threshold concentration of inhibiting MDA formation for 40 $\mu mol \cdot L^{-1}$ whereas GP7 was the strongest with the threshold concentration for 1.25 $\mu mol \cdot L^{-1}$ (Table 1).

The compounds as mentioned above strongly inhibited MDA formation from tissue homogenates stimulated by Fe^{2+}-AA in a concentration-dependent manner. The threshold concentrations of Pod, DmePod, GP7, GP7OH, GP7H and VP16 in inhibiting MDA formation from heart homogenate were 20, 20, 1.25, 1.25, 2.5, and 5 $\mu mol \cdot L^{-1}$, those from liver homogenate were 20, 20, 5, 2.5, 10, and 2.5 $\mu mol \cdot L^{-1}$, and those from kidney homogenate were 20, 20, 2.5, 1.25, 2.5, and 2.5 $\mu mol \cdot L^{-1}$, respectively (Table 1).

2.2. Inhibitory effects on H_2O_2-induced RBC hemolysis

DmePod, GP7, GP7OH, GP7H and VP16 (5 $\sim$ 20 $\mu mol \cdot L^{-1}$) significantly inhibited H_2O_2-induced RBC hemolysis from rats in a concentration-dependent manner. However, Pod 320 $\mu mol \cdot L^{-1}$ had no effect (Fig. 1).

2.3. Inhibitory effects on superoxide anion ($O_2^{\cdot-}$) formation from activated neutrophils

The reduced NBT product (formazan) from rat activated neutrophils in the control group was markedly increased after stimulation by zymosan compared to basal group (without zymosan). GP7, GP7OH and VP16 (160 –

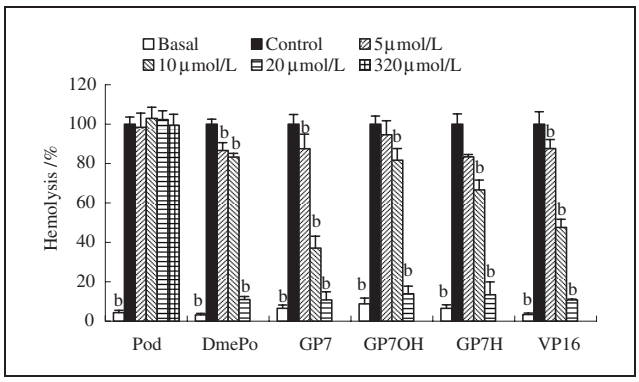


Fig. 1: Effects of spin labeled derivatives of DmePod on rat RBC hemolysis stimulated by H_2O_2. Results are expressed as mean ± SD (n = 5). [a] $P < 0.05$, [b] $P < 0.01$ compared with control group (ANOVA, LSD-t test)

Table 1: Inhibitory effects of spin labeled derivatives of DmePod on malondialdehyde (MDA) generation in tissues of rats

Concentration ($\mu mol \cdot L^{-1}$)	MDA content ($nmol \cdot L^{-1}$/g tissues)			
	Induced by Fe^{2+}-AA in			Spontaneously formed in liver
	Heart	Liver	Kidney	
Pod				
Basal	53.4 ± 6.7^{b}	55.6 ± 7.4^{b}	97.5 ± 6.4^{b}	134.1 ± 13.2
Control	169.1 ± 6.8	144.7 ± 8.5	310.8 ± 10.4	
20	151.9 ± 12.4^{a}	125.1 ± 7.2^{b}	260.6 ± 16.9^{b}	121.0 ± 3.9
40	140.5 ± 9.7^{b}	122.3 ± 3.2^{b}	263.2 ± 3.8^{b}	114.7 ± 4.4^{d}
80	129.9 ± 7.9^{b}	113.8 ± 2.9^{b}	245.2 ± 10.2^{b}	105.8 ± 6.5^{d}
160	103.1 ± 10.2^{b}	91.7 ± 2.5^{b}	203.1 ± 6.4^{b}	70.8 ± 5.4^{d}
320	76.6 ± 4.3^{b}	69.7 ± 11.7^{b}	162.2 ± 5.3^{b}	41.0 ± 4.1^{d}
DmePod				
Basal	57.0 ± 5.9^{b}	56.4 ± 6.2^{b}	100.4 ± 4.7^{b}	132.2 ± 7.2
Control	175.7 ± 6.7	145.3 ± 5.1	314.8 ± 12.4	
20	140.2 ± 7.4^{b}	114.7 ± 15.5^{b}	236.1 ± 6.7^{b}	92.1 ± 8.3^{d}
40	128.7 ± 9.8^{b}	93.5 ± 2.1^{b}	219.2 ± 5.6^{b}	71.4 ± 2.8^{d}
80	91.4 ± 7.3^{b}	60.4 ± 6.3^{b}	170.7 ± 3.8^{b}	50.9 ± 7.3^{d}
160	55.3 ± 6.8^{b}	21.4 ± 4.9^{b}	94.8 ± 1.5^{b}	24.0 ± 2.4^{d}
320	55.2 ± 6.7^{b}	15.1 ± 1.2^{b}	104.2 ± 9.0^{b}	20.9 ± 5.9^{d}
GP7				
Basal	61.4 ± 6.0^{b}	43.9 ± 9.0^{b}	136.6 ± 6.1^{b}	170.7 ± 11.9
Control	196.9 ± 11.9	154.1 ± 19.7	348.8 ± 18.1	
1.25	166.9 ± 17.2^{a}	150.2 ± 12.0	329.3 ± 11.4	125.8 ± 9.3^{d}
2.5	128.1 ± 5.4^{b}	136.8 ± 22.2	312.7 ± 20.2^{a}	118.7 ± 13.3^{d}
5	91.7 ± 12.8^{b}	118.3 ± 3.1^{b}	288.6 ± 16.0^{b}	106.1 ± 6.7^{d}
10	45.8 ± 16.1^{b}	83.6 ± 9.8^{b}	165.8 ± 15.8^{b}	58.8 ± 11.0^{d}
20	30.0 ± 9.0^{b}	38.9 ± 7.9^{b}	121.2 ± 11.6^{b}	34.4 ± 9.2^{d}
GP7OH				
Basal	51.9 ± 7.6^{b}	46.9 ± 4.3^{b}	124.8 ± 6.2^{b}	166.5 ± 6.5
Control	178.3 ± 13.2	145.8 ± 9.6	302.4 ± 16.4	
1.25	101.3 ± 12.2^{b}	134.1 ± 12.4	239.4 ± 22.4^{b}	166.2 ± 5.4
2.5	64.5 ± 8.9^{b}	113.2 ± 5.3^{b}	121.4 ± 10.8^{b}	144.9 ± 6.6^{d}
5	30.8 ± 4.7^{b}	71.8 ± 7.1^{b}	86.2 ± 6.6^{b}	69.6 ± 12.2^{d}
10	36.3 ± 4.8^{b}	36.8 ± 5.1^{b}	79.6 ± 5.4^{b}	53.3 ± 6.1^{d}
20	27.8 ± 6.0^{b}	32.6 ± 5.4^{b}	66.1 ± 2.6^{b}	48.8 ± 3.8^{d}
GP7H				
Basal	62.3 ± 6.0^{b}	40.6 ± 2.0^{b}	133.5 ± 6.3^{b}	144.6 ± 18.7
Control	180.5 ± 6.5	165.9 ± 4.0	301.8 ± 10.2	
2.5	138.1 ± 2.8^{b}	166.1 ± 4.2	271.9 ± 13.5^{b}	145.9 ± 4.8
5	121.3 ± 11.8^{b}	168.3 ± 15.1	258.1 ± 3.3^{b}	123.8 ± 12.7
10	83.6 ± 11.2^{b}	133.8 ± 6.3^{b}	228.9 ± 8.1^{b}	113.8 ± 15.3^{c}
20	29.4 ± 2.8^{b}	90.4 ± 15.8^{b}	135.1 ± 10.4^{b}	62.8 ± 6.3^{d}
40	25.6 ± 3.9^{b}	21.6 ± 3.4^{b}	98.9 ± 4.8^{b}	31.8 ± 2.3^{d}
VP16				
Basal	62.1 ± 1.9^{b}	42.2 ± 4.5^{b}	116.5 ± 8.3^{b}	231.1 ± 8.1
Control	142.3 ± 4.5	166.2 ± 8.2	294.4 ± 9.6	
2.5	134.8 ± 7.8	153.4 ± 3.4^{a}	276.5 ± 8.2^{a}	206.0 ± 5.7^{d}
5	122.7 ± 10.6^{a}	106.5 ± 5.6^{b}	236.8 ± 5.8^{b}	139.1 ± 7.3^{d}
10	111.6 ± 6.9^{b}	97.4 ± 7.2^{b}	176.6 ± 7.4^{b}	111.8 ± 3.7^{d}
20	100.9 ± 5.3^{b}	50.8 ± 4.1^{b}	131.5 ± 5.5^{b}	90.1 ± 7.6^{d}
40	46.2 ± 4.2^{b}	34.5 ± 5.2^{b}	108.2 ± 5.3^{b}	61.5 ± 5.2^{d}

Results are expressed as mean $\pm$ SD (n = 4). [a] $P < 0.05$, [b] $P < 0.01$ compared with control group; [c] $P < 0.05$, [d] $P < 0.01$ vs basal group (ANOVA, LSD-t test). AA: ascorbic acid

320 $\mu mol \cdot L^{-1}$) significantly inhibited $O_2^{\bullet -}$ formation, whereas 320 $\mu mol \cdot L^{-1}$ of DmePod, Pod and GP7H had no effect on $O_2^{\bullet -}$ formation (Table 2).

2.4. Inhibitory effects on K562 and K562/DOX cell proliferation

As shown in Fig. 2, all the tested compounds significantly inhibited K562 cell proliferation in a concentration-dependent manner for 96 h. The potency rank was as follows: VP16 > GP7 > GP7OH > GP7H > DmePod > Pod with their IC_{50} values by 0.7 (0.1 – 5.1), 2.2 (1.8 – 2.6), 2.4 (0.5 – 10.9), 3.3 (1.6 – 6.9), 5.8 (2.7 – 12.2), and 13.1 (4.9 – 34.7) $g \cdot L^{-1}$, respectively. They also inhibited K562/DOX cell proliferation in a concentration-dependent manner following the rank GP7 > GP7OH > VP16 > DmePod > Pod > GP7H with their IC_{50} values by 4.5 (1.4 – 14.9), 5.3 (0.6 – 45.4), 10.0 (4.4 – 22.9), 18.9 (4.5 – 79.7), >27, and >27 $g \cdot L^{-1}$, respectively. The resistance fold of GP7, GP7OH, VP16, and DmePod was 2.05, 2.21, 14.29, and 3.26, respectively, while that of Pod and GP7H on K562/DOX was very high.

2.5. Structure-activity relationship

Our results showed that all the tested compounds strongly inhibited MDA formation spontaneously or stimulated by Fe^{2+}-AA in a concentration-dependent manner. Both GP7

Table 2: Effects of spin labeled derivatives of DmePod on $O_2^{\cdot-}$ production from zymosan-stimulated neutrophils of rats

Drugs	Concentration/ $\mu mol \cdot L^{-1}$	Reduced NBT (OD_{515nm})	Inhibitory rate/%
Basal	–	0.138 ± 0.042^b	
Control	–	0.354 ± 0.020	
Pod	320	0.352 ± 0.001	–
DmePod	320	0.355 ± 0.020	–
GP7H	320	0.350 ± 0.011	–
GP7	80	0.340 ± 0.003	6.5
	160	0.298 ± 0.015^b	25.7
	320	0.231 ± 0.006^b	56.7
GP7OH	80	0.347 ± 0.010	3.2
	160	0.280 ± 0.015^b	34.1
	320	0.257 ± 0.020^b	44.7
VP16	80	0.302 ± 0.016^b	16.3
	160	0.210 ± 0.017^b	66.3
	320	0.178 ± 0.010^b	81.3

Results are expressed as mean ± SD (n = 5). [a] $P < 0.05$, [b] $P < 0.01$ compared with control group (ANOVA, LSD-t test). NBT: nitroblue tetrazolium

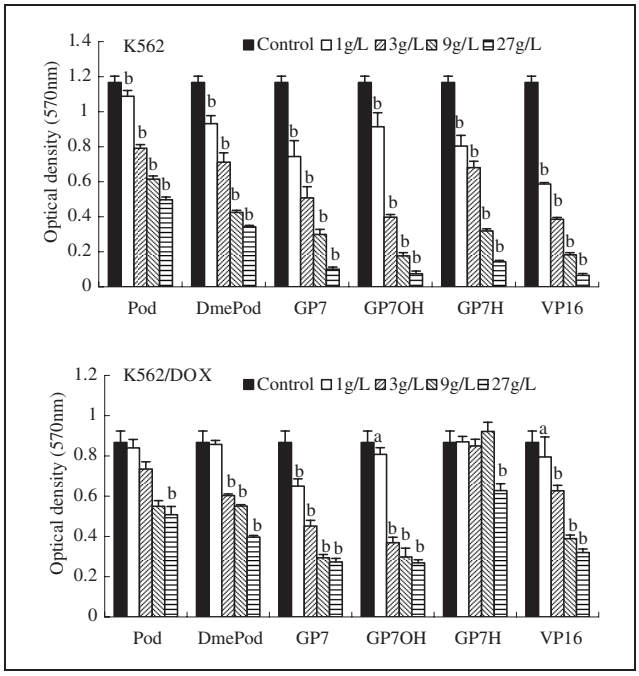


Fig. 2: Inhibitory effects of derivatives of DmePod on K562 and K562/DOX cell proliferation for 96 h. Results are expressed as mean ± SD (n = 4). [a] $P < 0.05$, [b] $P < 0.01$ compared with control group (ANOVA, LSD-t test)

and GP7OH were the strongest, whereas Pod was the weakest in inhibiting MDA formation. In general, their potency was in the order GP7OH > GP7 > P16 > GP7H > DmePod > Pod. The potency of antihemolysis for DmePod, GP7, GP7OH, GP7H and VP16 was similar among them with IC_{50} values by 13.6, 8.6, 11.7, 10.3, and 9.47 $\mu mol \cdot L^{-1}$, respectively, while Pod was the weakest ($IC_{50} > 320\ \mu mol \cdot L^{-1}$, Table 2). GP7, GP7OH and VP16 ($160 - 320\ \mu mol \cdot L^{-1}$) significantly inhibited $O_2^{\cdot-}$ formation with the potency rank VP16 > GP7 > GP7OH, whereas DmePod, Pod and GP7H ($320\ \mu mol \cdot L^{-1}$) had no effect (Table 3). The potency of GP7, GP7OH, GP7H, and VP16 was stronger than their parental DmePod, while DmePod was stronger than Pod. In general, GP7 and GP7OH had similar antioxidative effect which was stronger than that of the other compounds, especially GP7H. These results indicate that DmePod with a hydroxyl group at 4′-position in E-ring has stronger antioxidative activity than Pod. Introduction of nitroxide into DmePod markedly enhances the antioxidative activity, and a nitroxide moiety and a hydroxyl group are essential groups in DmePod derivatives.

Based on Fenton reaction, Fe^{2+} and AA produce hydroxyl radical ($^{\cdot}OH$), induce lipid peroxidation and form MDA in tissue homogenates. MDA generation indirectly reflects the amount of $^{\cdot}OH$ formation. The formazan, NBT-reduced product by $O_2^{\cdot-}$, represents the amount of $O_2^{\cdot-}$ generation from activated neutrophils. H_2O_2-induced RBC hemolysis mainly reflects the extent of lipoperoxidation of RBC membrane. In the present experiment, all the tested compounds had inhibitory effects on MDA formation and hemolysis. GP7, GP7OH and VP16 ($160 - 320\ \mu mol \cdot L^{-1}$) significantly inhibited $O_2^{\cdot-}$ formation, while DmePod, Pod and GP7H ($320\ \mu mol \cdot L^{-1}$) had no effect (Table 2). Therefore, introduction of nitroxide into DmePod can influence the activity on scavenging reactive oxygen species (ROS).

Moreover, all compounds strongly inhibited K562 and K562/DOX cell proliferation in a concentration-dependent manner. The resistance fold of GP7, GP7OH, DmePod and VP16 was 2.05, 2.21, 14.29, and 3.26, respectively. This indicates that the introduction of nitroxide into DmePod not only enhances the antitumor activity, but also reverses drug resistance.

In general, all the tested compounds had the similar rank in both the antioxidative and antitumor activities. This indicates that there is a relationship between the antioxidative and antitumor activities of derivatives of DmePod, and the same active groups are responsible for antioxidative and antitumor activities.

Table 3: Comparison of IC_{50} values ($\mu mol \cdot L^{-1}$) of spin labled derivatives of DmePod

Drugs	Inhibition of MDA formation				Anti-hemolysis
	Induced by Fe^{2+}-AA in			Spontaneously formed in liver	
	Heart	Liver	Kidney		
Pod	112.2 (74.0 – 170.2)	99.1 (51.5 – 166.2)	150.6 (99.6 – 227.7)	178.6 (115.9 – 274.1)	>320.0
DmePod	42.54 (24.00 – 75.41)	28.92 (17.85 – 46.85)	38.07 (22.62 – 64.07)	47.59 (32.16 – 70.42)	13.6 (3.3 – 42.1)
GP7	2.45 (1.84 – 3.26)	5.90 (3.79 – 9.18)	5.05 (2.78 – 9.16)	5.51 (3.20 – 9.50)	8.6 (5.5 – 13.6)
GP7OH	0.78 (0.49 – 1.23)	3.15 (2.04 – 4.87)	1.02 (0.39 – 2.7)	6.63 (3.35 – 13.11)	11.7 (4.3 – 31.4)
GP7H	4.1 (2.2 – 7.2)	14.07 (9.32 – 21.25)	7.5 (4.4 – 12.9)	18.3 (10.7 – 28.5)	10.3 (5.0 – 21.1)
VP16	10.53 (4.91 – 22.59)	6.74 (4.60 – 9.90)	9.5 (5.7 – 7.4)	11.35 (7.23 – 17.83)	9.47 (7.97 – 11.26)

The IC_{50} (50% inhibition concentration) values and its 95% confidence limits (in parentheses) were calculated by linear regression analysis. MDA: malondialdehyde; AA: ascorbic acid

3. Discussion

Free radicals are produced exogenously by ionizing radiation or specific chemical agents and endogenously as a result of oxygen metabolism. Oxygen is an abundant electron sink that sustains life, yet oxygen metabolites such as $O_2^{\bullet-}$, $^{\bullet}OH$ and hydrogen peroxide (H_2O_2) are potentially toxic reactive oxygen species (ROS). ROS are major promoters of lipid peroxidation (LPO). LPO is a major damaging process in membranes and liposomal dispersions containing polyunsaturated acyl chains. Prevention of LPO may become an important therapy in many related diseases that involve LPO, and is an important aspect in the preparation and preservation of liposomes and similar lipid assemblies. Fe^{2+}-AA system produces $^{\bullet}OH$ according to the Fenton reaction (Krishna et al. 1998):

$$\text{ascorbic acid} + H^+ + 2\,O_2^{\bullet-} \rightarrow H_2O_2 + \text{dehydroascorbic acid}$$

$$Fe^{2+}\text{-chelate} + H_2O_2 \rightarrow Fe^{3+}\text{-chelate} + OH^- + {}^{\bullet}OH$$

$$^{\bullet}OH + LH \rightarrow L^{\bullet} + H_2O \text{ (initiation)}$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \text{ (propagation)}$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \text{ (propagation)}$$

$$LOOH \rightarrow LO^{\bullet} + {}^{\bullet}OH \text{ (propagation)}$$

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow \text{products (termination)}$$

From these equations, it follows that excess Fe^{2+}-AA can generate most toxic $^{\bullet}OH$, cause LPO and tissue injury accompanying MDA formation. Therefore, MDA is a convenient index for indirectly detecting hydroxyl radicals. In our experiment, all the tested derivatives of DmePod inhibited spontaneous MDA generation from rat liver homogenate in a concentration-dependent manner. After being stimulated by Fe^{2+}-AA for 30 min, the MDA generation was 2-4-fold higher than in unstimulated liver, heart, and kidney homogenates. All the tested derivatives of DmePod also concentration-dependently inhibited Fe^{2+}-AA-induced MDA formation in diverse tissue homogenates. This indicates that the tested compounds are scavengers of hydroxyl radicals. In general, the order of inhibitory effect on MDA formation was as follows: GP7OH > GP7 > VP16 and GP7H > DmePod > Pod. It is believed that Pod and DmePod with hydroxyl moiety in their structures possess antioxidative activities. Our experiment showed that DmePod and its derivatives with a hydroxyl group in the E-ring were stronger than Pod with a hydroxyl group in the C-ring, indicating that the phenolic hydroxyl group at 4′-position in E-ring is the active group against oxidation. GP7, the spin-labeled derivative of DmePod, and its corresponding hydroxylamine compound GP7OH had stronger antioxidative activities than its corresponding amine compound GP7H. Our previous experiment has demonstrated that $NO^{\bullet}$ and NOH groups of nitroxides are essential active groups (Wu et al. 1997; Jia et al. 1999; Li et al. 2006). Moreover, the spin labeled derivatives of Pod, podophyllic acid-[4-(2,2,6,6-tetramethyl-1-piperidyloxy)] hydrazone (GP1) and its congeners podophyllic acid-[4-(2,2,6,6-tetramely-1-piperidyloxyamine)] hydrazone (GP1OH), podophyllic acid-[4-(2,2,6,6-tetramethyl-1-piperidyl)] hydrazone (GP1H) had the similar results (Li et al. 2002). These results also suggested that the amino moiety in the GP1H and the hydroxylamine moiety in the GP1OH can be oxidized to the corresponding nitroxide free radical GP1 (Tian et al. 2002). Nitroxide stable radicals function as antioxidants by operating through a cyclic mechanism of electron transfer among three oxidation states – the oxoammonium cation, the nitroxide and the hydroxylamine as the following equations (Krishna et al. 1998):

$$RRNO^{\bullet} + Fe^{2+}\text{-chelate} + H^+ \rightarrow RRNOH + Fe^{3+}\text{-chelate}$$

$$RRNOH + {}^{\bullet}OH \rightarrow RRNO^{\bullet} + H_2O$$

$$RRNO^{\bullet} + H^+ + HO_2^{\bullet} \rightarrow RRNO^+ + H_2O_2$$

$$RRNO^+ + O_2^{\bullet-} \rightarrow RRNO^{\bullet} + O_2$$

It is confirmed that the nitroxides are more rapidly reduced to the corresponding intracellular hydroxylamine than amine (Wu et al. 1997; Hahn et al. 2000; Krishna et al. 1998). This indicates that introduction of nitroxide greatly increases the activity of DmePod derivatives.

Although we observed that the potency of GP7 and GP7OH was relatively similar to that of VP16, DmePod and GP7H (no both $NO^{\bullet}$ group and NOH group but with phenolic hydroxyl group in E-ring) in antihemolysis test, they all were stronger than Pod (without phenolic hydroxyl group in E-ring), indicating that antihemolysis activity of these derivatives of DmePod contributes mainly to the phenolic hydroxyl group in E-ring of their parent.

Our experiment also showed that 160 – 320 $\mu mol \cdot L^{-1}$ of GP7, GP7OH and VP16, which was lower than that of their cytotoxicity concentration significantly inhibited $O_2^{\bullet-}$ formation through scavenging superoxide but not exerting an influence on neutrophil function with the potency rank VP16 > GP7 > GP7OH, whereas 320 $\mu mol \cdot L^{-1}$ of DmePod, Pod and GP7H had no effect, indicating that inhibitory effects on $O_2^{\bullet-}$ formation of DmePod derivatives maybe contribute mainly not to the phenolic hydroxyl group in the E-ring of their parent but the $NO^{\bullet}$ group, NOH group or the piperidine group. It is concluded that there are different mechanisms and selectivity among scavenging $O_2^{\bullet-}$, $^{\bullet}OH$ and H_2O_2.

It was reported that the concentration of MDA, the final product of LPO in carcinomatous tissues, was higher than that of normal organs (Yagi 1991). Antioxidants such as nitroxides and some plant derived extracts containing antioxidants showed cytotoxicity towards tumor cells and antitumor activity in experimental animals (Ruby et al. 1995; Mitchell et al. 2003; Gariboldi et al. 2003; Gupta et al. 2004). Consistent with this notion, our results demonstrated that all the tested compounds significantly inhibited K562 and K562/DOX cell proliferation in a concentration-dependent manner. The potency rank was as follows VP16 > GP7 > GP7OH > GP7H > DmePod > Pod in K562 cells, and GP7 > GP7OH > VP16 > DmePod > Pod > GP7H in K562/DOX cells. The resistance fold of GP7, GP7OH, DmePod, and VP16 in K562/DOX cells was 2.05, 2.21, 3.26, and 14.29, respectively, and was lower than that of DOX (Zhang et al. 2005). However, the antitumor activity of Pod and GP7H on K562/DOX cells was the weakest in all compounds. In summary, introduction of a $NO^{\bullet}$ or NOH moiety into DmePod can enhance the antitumor activity and reverse the resistance. Because all tested derivatives had the similar rank in both the antioxidative and antitumor activities, there is a relationship between antioxidative and antitumor activities of derivatives of DmePod. The antitumor mechanism may be due partially to the antioxidative activity.

Our results indicate that all derivatives of DmePod strongly inhibited MDA generation from homogenates, and antagonized H_2O_2-induced RBC hemolysis from rats, GP7, GP7OH and VP16 (160 – 320 $\mu mol \cdot L^{-1}$) significantly inhibited $O_2^{\bullet-}$ release from neutrophils stimulated by zymosan. Meanwhile, all the tested derivatives strongly

inhibited K562 and K562/DOX cell proliferation. The resistance magnitude of GP7, GP7OH, VP16, and DmePod was 2.05, 2.21, 14.29, and 3.26, respectively, while that of Pod and GP7H was too high for K562/DOX cells. In general, all the tested compounds had the similar rank in both the antioxidative and antitumor activities. It is concluded that introduction of nitroxide into DmePod may enhance its antioxidative and antitumor activities, and both NO$^{\bullet}$ and NOH groups are essential active moieties. There is a relationship between antioxidative and antitumor activities of DmePod derivatives.

4. Experimental

4.1. Drugs and chemicals

Podophyllotoxin (Pod), 4-β-amino-4′-demethylepipodophyllotoxin (DmePod), etoposide (VP16), 4′-(4′′-(2′′,2′′,6′′,6′′-tetramethyl-1′′-piperidinooxyl) amino-4′-demethylepipodophyllotoxin (GP7), 4′-(4′′-(2′′,2′′,6′′,6′′-tetramethyl-1′′-hydroxypiperidine) amino-4′-demethylepipodophyllotoxin (GP7OH), and 4-(4′′-(2′′,2′′,6′′,6′′-tetramethylpiperidine) amino-4′-demethylepipodophyllotoxin (GP7H) were synthesized by the State Key Laboratory of Applied Organic Chemistry, Lanzhou University in according to the method of Chen et al. 1989 (Lanzhou, China). The purities of these compounds were approximately 98%. They were dissolved in 5% dimethyl sulfoxide (DMSO). Doxorubicin (DOX) was obtained from Meiji Pharmaceutical Co Ltd (Tokyo, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Zymosan A (Sigma) was opsonized with rat serum and suspended in 0.15 mmol $\cdot$ L^{-1} phosphate buffer (pH 7.4). RPMI-1640 medium was obtained from GIBCO BRL (Grand Island, NY, USA). Bovine serum was purchased from Hangzhou Si-Ji-Qing Biotechnology Co. (Hangzhou, China). Other chemicals were of analytical purity.

4.2. Animals

Female and male Wistar rats (8 weeks old) weighing 175 ± 13 g were provided by the Animal Breeding Center of Lanzhou University. The animals were housed two per cage and maintained in climate controlled and circadian rhythm-adjusted rooms, and provided with standard laboratory chow and water *ad libitum*. All animal experiments were approved by the University Committee on Use and Care of Animals.

4.3. Determination of malondialdehyde (MDA)

The homogenates of liver, heart and kidney of rats were prepared with Tris-KCl buffer (0.1 mol $\cdot$ L^{-1} Tris, 1 mmol $\cdot$ L^{-1} KCl, pH 7.4). Fe^{2+}-ascorbic acid (Fe^{2+}-AA, $FeSO_4$/AA = 50/50 μmol/L) was used as a hydroxyl free radical generation system. The experiment was divided into basal tubes (DMSO), control tubes (DMSO and Fe^{2+}-AA) and drug tubes (different concentration of tested compounds and Fe^{2+}-AA). After incubation of tissue homogenates with compounds at 37 °C for 10 min, Fe^{2+}-AA was added. Following 30 min incubation, MDA was assayed by the thiobarbituric acid (TBA) method (Tanizaw et al. 1981; Ohkawa et al. 1979). The spontaneous MDA generation from rat liver homogenate was measured after 2 h incubation with the tested compounds and without Fe^{2+}-AA.

4.4. Hemolysis test

Rat red blood cells (RBC) were washed 3 times with normal saline and made into 0.5% suspension. H_2O_2 (100 mmol $\cdot$ L^{-1})-induced hemolysis was tested after 1 h incubation of RBC suspension with tested compounds at 37 °C as described previously (Li et al. 2002; Wu et al. 1997). Optical density (OD) at 415 nm of control tubes was defined as 100%, then hemolysis percentage of other groups was calculated with the following equation: hemolysis (%) = $OD_{415\ Treated}/OD_{415\ Control} \times 100$ (Wu et al. 1997; Li et al. 2002)

4.5. Superoxide anion ($O_2^{\cdot-}$) formation assay

Neutrophils from rat abdominal cavity were prepared as described previously (Wu et al. 1997; Li et al. 2002) and suspended in Hanks' solution ($CaCl_2$ 1.3, $MgSO_4$ 0.8 and Glucose 10 mmol/L, pH 7.4) to a density of 1×10^{10} cells/L. The neutrophils viability, assessed by trypan blue exclusion, was more than 98%. All the procedures were carried out below 4 °C. The reaction system includes 0.1 ml of 10 mmol $\cdot$ L^{-1} potassium cyanide (KCN), 0.4 ml of 0.1% nitroblue tetrazolium (NBT)-saline, 0.4 ml of neutrophil suspension, 0.1 ml of 2.5 g $\cdot$ L^{-1} zymosan A and 50 μl of tested compound or solvent. After incubated at 37 °C for 35 min, OD of reduced NBT product (formazan) from neutrophils was measured at 515 nm with a spectrophotometer.

4.6. Cell cultures and microculture tetrazolium (MTT) assay in vitro

DOX-resistant human chronic myelogenous leukemia K562/DOX and its parental K562 cells, were purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were grown in complete RPMI-1640 medium containing 10% heat-inactivated bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2, and routinely passaged every other day. DOX 3 μg/L was regularly added in K562/DOX culture medium to maintain drug resistance. K562/DOX cells were incubated in DOX-free medium for two weeks and used for experiment.In order to determine anti-tumor activity of tested compounds *in vitro*, cytotoxicity was measured by MTT assay (Mosmann 1983). In brief, exponentially-growing cells were washed and resuspended in complete RPMI-1640 medium to a concentration of 1×10^8 cells/L. 100 μl aliquots of cells containing tested compounds were seeded in quadruplicate into a 96-well flat bottom microculture plate (Costar, Corning, USA) for 96 h. At the end of the incubation period, MTT (5 g/L) 10 μl was added to each well and further cultured for another 4 h, then SDS 100 μl (10%, w/v, in 0.01 M HCl) was added and mixed thoroughly to dissolve formazan crystals at 37 °C. OD was measured by Microplate Reader (EL × 800 Instruments, Bio-TEK, USA) at 570 nm after shaking plates for 5 min. The percentage of cell growth inhibition and resistance fold was calculated with the following equation:
Inhibitory rate (IR %) = $(1 - OD_{570\ Treated}/OD_{570\ Control}) \times 100$. Resistance fold = IC_{50} (K562/DOX)/IC_{50} (K562).

4.7. Statistical analysis

Results were expressed as mean ± SD. Data analysis was performed using analysis of variance (ANOVA) followed by LSD-t post-hoc test for multiple comparisons with the computer statistical package SPSS 12.0 (for Window). Differences were considered statistically significant at $P < 0.05$. 50% inhibition concentration (IC_{50}) and its 95% confidence limits were calculated by linear regression analysis (Tallarida and Murray 1981).

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