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Antioxidative activity of spin labeled derivatives of podophyllic acid hydrazide

LI Wen-Guang[1], ZHANG Xiao-Yu[2], WU Yong-Jie, TIAN Xuan[3]

Department of Pharmacology, [2]Department of Physiology, Lanzhou Medical College;
[3]National Laboratory of Organic Chemistry, Lanzhou University, Lanzhou 730000, China

KEY WORDS podophyllic acid hydrazide; spin labels; doxorubicin; free radicals; antioxidants; malondialdehyde

ABSTRACT

AIM: To study the relationship between structure and antioxidation activity of spin labeled derivatives of podophyllic acid hydrazide (GP) in tissues and red blood cells (RBC) from rats. **METHODS:** The homogenate of liver, heart, and kidneys of rats was used to measure malondialdehyde (MDA) spontaneous generated and induced by hydroxyl free radical generation system (Fe^{2+}-ascorbic acid, FRGS) or doxorubicin (DOX) by TBA colorimetric method. H_2O_2-caused hemolysis was determined spectrophotometrically. Superoxide anion from zymosan-stimulated neutrophils of rats was evaluated by NBT-reduction assay. **RESULTS:** GP1 and GP1OH obviously inhibited MDA formation either spontaneously or induced by FRGS and DOX and antagonized hemolysis induced by H_2O_2, but GP and GP1H showed less potent activity. GP1 also inhibited the formation of superoxide anion from activated neutrophils of rats. **CONCLUSION:** Introduction of nitroxyl radical moieties into GP generated potent derivatives with antioxidative activity. The essential antioxidation active groups of spin labeled derivative of GP are NO or NOH group in nitroxyl radical moieties.

INTRODUCTION

The nitroxides, with low molecular weight and less toxic stable free radicals, had been widely used as spin labels[1,2], which also had been demonstrated to possess potent antioxidative action either in biological or nonbiological test systems[3,4], and antitumor action. Introduction of nitroxyl radicals into antitumor agent was demonstrated to enhance its antitumor activity and reduce its toxicity[5,6], and could also be used for the study of pharmacokinetics of bioactive spin labels by electron paramagnetic resonance (EPR) technique. Based on this fact, a series of spin labeled derivatives of podophyllic acid hydrazine (GP) had been synthesized and showed that introduction of nitroxyl radicals into GP could enhance the antitumor activity and lower the toxicity. However, it is unknown how the antitumor activity was enhanced and the toxicity was lowered by introduction of nitroxyl radicals into antitumor agent. The present study were designed to evaluate the

[1] Correspondence to Prof LI Wen-Guang.
Phn 86-931-862-3573. E-mail wyj@lz.gs.cninfo.net
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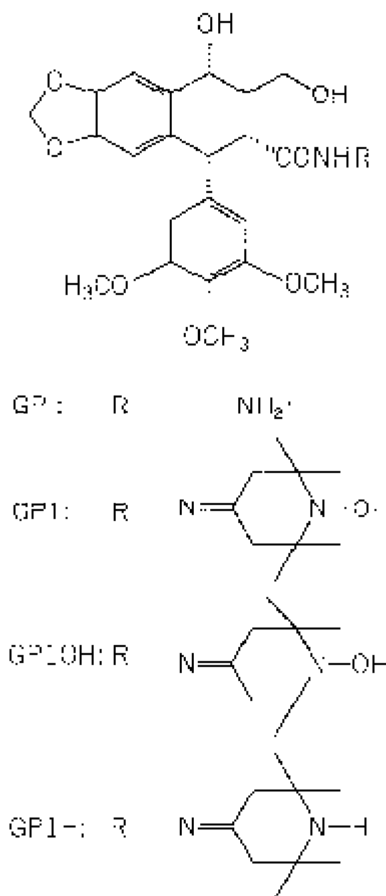
antioxidative effect of spin labeled derivatives of GP with following chemical structure and analyze the relationship between their structure and activity.



Spin labeled derivatives of podophyllic acid hydrazide (GP)

MATERIALS AND METHODS

Podophyllic acid hydrazide (GP), podophyllic acid [4-(2,2,6,6-tetramethyl -piperidinooxyl)]hydrazone (GP1), podophyllic acid [4 -(2,2,6,6 -tetramethyl-1-hydroxy piperidine)]hydrazone (GP1OH), and podophyllic acid [4-(2,2,6,6-tetramethyl-1-piperidine)] hydrazone (GP1H) were semi-synthesized[1,2] by National Laboratory of Organic Chemistry, Lanzhou University, China. The purity of them was approximately 98 %. They were dissolved into 5 % Me_2SO. Doxorubicin (DOX) was the product of Shenzhen Main Luck Pharmaceutical Inc. Zymosan A (Sigma) was opsonized with rat serum[7] and suspended in phosphate buffer 0.15 mmol/L (pH 7.4). All the other reagents were of analytical grade.

Wistar rats (♀ ♂, 8 weeks-old) weighing 182 $\pm s$ 16 g were provided by Animal Center of Gansu Academy of Medical Sciences (Grade II, Certificate № 14-004).

Determination of malondialdehyde The homogenate of liver, heart, and kidneys of rats was prepared[4] and MDA was assayed by thiobarbituric acid (TBA) method[8].

Hemolysis test Rat RBC was washed 3 times with normal saline and made into 0.5 % suspension. H_2O_2 (100 mmol/L) induced hemolysis was tested after 1 h incubation of RBC suspension at 37 ℃ with tested drugs as previously[4]. The absorbance (*A*) at 415 nm of control tubes was defined as 100 %. The hemolysis extent was calculated by referring to control tube.

Superoxide anion formation analysis Rat neutrophils from abdominal cavity were prepared[4] and the reduced NBT product formazan by O^{-}_2 from neutrophils was assayed by spectrophotometry at 515 nm.

Statistics Data were presented as mean±SD. Statistical analysis was performed using unpaired *t*-test. The IC_{50} and its 95 % confidence limits were calculated by liner regression analysis[9].

RESULTS

Effect on MDA formation MDA was spontaneously formed in liver homogenate after 2 h incubation. GP and GP1H 160 μmol/L inhibited spontaneous MDA formation by 26.5 % and 34.8 % respectively. In contrast, GP1 1.25, 2.5, 5, 10, and 20 μmol/L inhibited MDA formation by 7.6 %, 27.7 %, 59.8 %, 71.5 %, and 74.8 %; and GP1OH 2.5, 5, 10, and 20 μmol/L inhibited MDA formation by 21.9 %, 42.1 %, 75.2 %, and 84.2 % respectively (Tab 1).

After stimulated by FRGS (hydroxyl free radical generation system, Fe^{2+}-ascorbic acid 50/50 mmol· L^{-1}) for 30 min, homogenate of heart, liver, and kidney produced enormous amount of MDA (Tab 1,2). GP 160 μmol/L inhibited FRGS-induced MDA formation from heart and liver by 28.3 % and 16.6 % respectively, but failed to affect MDA formation from kidneys. GP1H 160 μmol/L inhibited MDA formation from heart, liver, and kidneys by 5.9 %, 8.5 %, and 25.4 %, respectively.

Tab 1. The effect of spin labeled derivatives of GP on malondialdehyde (MDA) formation induced by Fe^{2+}-ascorbic acid. n=4. Mean±SD. [a]P>0.05, [b]P<0.05, [c]P<0.01 *vs* control. [d]P>0.05, [f]P<0.01 *vs* basic tubes.

Drugs/ μmol·L^{-1}	MDA/nmol·g^{-1} tissues Induced by Fe^{2+}-ascorbic acid: Heart	Liver	Kidneys	Spontaneously in liver
GP				
Basic	73±6[c]	51±3[c]	90±4[c]	117±10
Control	352±10	290±16	279.5±2.8	
320	246±11[c]	196±9[c]	274±14[a]	68±5[f]
160	273±4[c]	242±9[c]	277±4[a]	86.2±2.9[f]
80	306±6[c]	294±8[a]	281±8[a]	102±5[f]
40	341±6[b]	298±8[a]	279±3[a]	118±6[d]
GP1				
Basic	82±4[c]	58±7[c]	108±5[c]	128±8
Control	369±12	272±8	233±6	
20	26±4[c]	43±5[c]	78±6[c]	32±9[f]
10	248±9[c]	78±8[c]	93±9[c]	37±9[f]
5	324.6±1.4[c]	133±12[c]	118.7±2.1[c]	52±5[f]
2.5	336±5[c]	223±15[b]	161±8[c]	93±4[f]
1.25	357±3[b]	240±18[c]	221±14[a]	118±13[d]
GP1H				
Basic	92±6[c]	94±7[c]	96.1±1.5[c]	126±7
Control	295±13	252±8	294±8	
160	278±10[b]	230±8[c]	219±8[c]	82±14[f]
80	282±8[a]	242±15[a]	234±9[c]	104±15[f]
40	289±9[a]	254±11[a]	279±8[c]	120±11[d]
GP1OH				
Basic	46±6[c]	52±4[c]	110.5±2.1[c]	127±15
Control	339±9	225±7	275±11	
20	90±8[c]	44±4[c]	109±7[c]	20±5[f]
10	212±7[c]	94±10[c]	246±8[c]	32±7[f]
5	331±7[a]	203±10[c]	273±8[a]	74±11[f]
2.5	335±4[a]	227±7[a]	274±8[a]	99±13[f]

Tab 2. The effect of spin labeled derivatives of GP on malondialdehyde (MDA) formation induced by DOX. n=4. Mean±SD. [a]P>0.05, [b]P<0.05, [c]P<0.01 *vs* control.

Drugs/ μmol·L^{-1}	Heart MDA/ nmol·g^{-1}	IR/%	Liver MDA/ nmol·g^{-1}	IR/%
GP				
Basic	112±14[c]		135±9[c]	
Control	160±11		198±4	
160	135±14[b]	52.5	165±15[c]	51.9
80	150±6[a]	20.8	176±6[c]	34.0
40	153±5[a]	14.8	184.7±2.4[c]	20.8
20	163±5[a]	0	188±4[c]	15.6
GP1				
Basic	174±4[c]		161±12[c]	
Control	236±11		249±6	
10	167±11[c]	110.7		
5	204±11[c]	52.2	84±6[c]	189.2
2.5	209±8[c]	43.8	142±22[c]	122.8
1.25	226±5[a]	16.8	204±11[c]	51.6
0.625			216±13[c]	38.0
GP1OH				
Basic	198±8[c]		117±9[c]	
Control	250±16		283±11	
20	196±13[c]	102.5	104±7[c]	107.9
10	196±5[c]	103.7	131±15[c]	91.8
5	206±5[c]	85.0	223±8[c]	36.4
2.5	213±13[c]	70.1	254±19[b]	17.4
1.25	236±13	26.0		
GP1H				
Basic	178±16[c]		104±26[c]	
Control	219±8		160±17	
250	146±6[c]	177.2	115.3±2.3[c]	80.2
125	168±6[c]	123.0	126±13[c]	60.1
62.5	178.1±2.1[c]	99.0	151.7±2.6[a]	14.2
31.25	190±10[c]	71.0	158±6[a]	2.0
15.625	204±7[c]	37.4		

GP1 1.25–20 μmol/L inhibited MDA formation from heart, liver, and kidneys concentration-dependently with MIC 1.25, 1.25, and 2.5 μmol/L, respectively. All the MDA formation induced by FRGS were inhibited by GP1 20 μmol/L in heart, liver, and kidneys. The MIC for GP1OH to inhibit MDA formation from heart, liver, and kidneys were 10, 5, and 10 μmol/L, respectively. GP1OH 20 μmol/L inhibited MDA formation from heart, liver, and kidneys by 85.2 %, 101.0 %, and 104.8 % respectively (Tab 1). MDA formation were elevated by DOX in rat heart and liver homogenate and were inhibited by all tested drugs with the potential rank order of GP1, GP1OH, GP1H, and GP (Tab 2).

Effect on hemolysis induced by H_2O_2 GP1 and

GP1OH 80 and 160 μmol/L inhibited hemolysis of rat RBC, but GP and GP1H showed no effect (Tab 3).

Tab 3. Effect of spin labeled derivatives of GP on the hemolysis of rat RBC stimulated by H_2O_2. n=5. Mean±SD. [a]P>0.05, [c]P<0.01 *vs* control.

Drugs/	Hemolysis		Extent/%	
μmol· L^{-1}	GP	GP1H	GP1OH	GP1
Basic	5.9±1.2[c]	11±4[c]	8±4[c]	8.2±1.7[c]
Control	100	100	100	100
160	101±6[a]	102±4[a]	24.5±1.2[c]	10±7[c]
80	99±6[a]	99±5[a]	79.3±2.0[c]	82±8[c]
40	101±6[a]	102±11[a]	102±4[a]	91±4[a]

Effect on superoxide anion formation from activated neutrophils The reduced NBT product (formazan) from neutrophils of rats was markedly increased after stimulated by zymosan. The specificity of assay for O^-_2 was demonstrated by the fact that SOD 150, 300 and 600 kU/L inhibited formazan formation by 28.6 %, 40.8 %, and 84.5 %, respectively. GP1 160 and 320 μmol/L also slightly inhibited superoxide anion formation from activated neutrophils by 18.3 % and 26.6 % with statistical significance (P<0.01), however, GP, GP1H, and GP1OH all did not show any action (Tab 4).

A comparison of IC_{50} values In both experiments of MDA test and anti-hemolysis, the rank order of IC_{50} values was GP1>GP1OH>GP1H>GP (Tab 5).

DISCUSSION

Fe^{2+}-ascorbic acid system produces hydroxyl radicals according to Fenton reaction, and the latter caused lipoperoxidation and damage of tissues accompanying MDA formation[10]. Therefore, MDA is a convenient index for indirectly detecting hydroxyl radicals. The spin labeled derivatives of GP were capable of inhibiting FRGS -induced MDA formation, and antagonizing H_2O_2-

Tab 4. Effect of spin labeled derivatives of GP on the release of superoxide anion from rat neutrophils stimulated by zymosan. n=4. Mean±SD. [a]P>0.05, [c]P<0.01 *vs* control.

Drugs/μmol· L^{-1}		A_{515}		
	GP1	GP	GP1OH	GP1H
Basic	0.154±0.023[c]	0.136±0.013[c]	0.154±0.023[c]	0.145±0.029[c]
Control	0.497±0.028	0.358±0.018	0.497±0.028	0.353±0.009
320	0.365±0.011[c]	0.355±0.020[a]	0.46±0.03[a]	0.357±0.010[a]
160	0.41±0.03[c]	0.354±0.010[a]	0.475±0.013[a]	0.359±0.011[a]

Tab 5. A comparison of IC_{50} values (95 % confidence limits, μmol/L) of spin labeled derivatives of GP.

	MDA formation						
Drugs	Spontaneously formed in liver	Induced by Fe^{2+}-AA liver	heart	kidneys	Induced by DOX liver	heart	Anti-hemolysis
GP	>160	>160	>160	NO	170.4 (65.2-445.5)	185.2 (107.2-319.8)	NO
GP1	5.4 (3.6-7.0)	3.8 (2.4-5.2)	7.5 (4.4-10.5)	2.4 (0.7-4.2)	0.9 (0.4-2.0)	3.1 (2.7-3.6)	88.9 (87.6-90.2)
GP1OH	5.9 (4.3-7.5)	7.3 (5.5-9.1)	10.5 (8.1-12.9)	10.9 (5.8-16.0)	5.3 (3.0-9.5)	2.0 (0.9-4.3)	103.0 (101.9-104.3)
GP1H	>160	>160	>160	>160	115.6 (67.3-198.7)	21.3 (14.3-31.7)	NO

AA: ascorbic acid. NO: no effect.

caused hemolysis, indicating that these drugs are scavengers against hydroxyl radicals.

DOX plays an important role in cancer chemotherapy. But its clinical use has been limited by its irreversible cardiotoxicity. It has been believed that the cardiotoxicity of DOX is caused by free radicals, which has little relation to its anticancer effects[11,12]. The semiquinone free radical formed from DOX in rat heart homogenate was demonstrated[13]. And it may further transfer electron to oxygen or H_2O_2 to produce O_2^- or •OH and induce MDA formation. GP, GP1, GP1OH, and GP1H all concentration-dependently inhibited the MDA escalation caused by DOX from heart and liver homogenate of rat, among which GP1 was shown to be the most potent.

Our previous experiment demonstrated that nitroxides 4-oxy-2,2,6,6 -tetramethylpiperidinooxyl (4-O-TEMPO) and 4-oxy-2,2,6,6-tetramethyl-1-hydroxy piperidine (4-O-TEMPOH) inhibited FRGS-induced formation of MDA in the same test system as present one[4]. However, they were relatively weaker than GP1OH and especially GP1 (Tab 4). Introduction of 4-O-TEMPO into GP produces GP1 and 4-O-TEMPOH into GP produces GP1OH [5,6]. In the experiment of FRGS-induced MDA formation in liver, heart, and kidneys of rats, the ratio of IC_{50} values (μmol/L) for GP1/4-O-TEMPO were 3.8/22.2, 7.5/38.5, and 2.4/18.8 respectively, and for GP1OH/4-O-TEMPOH were 7.3/8.3, 10.5/28.6, and 10.9/47.8, respectively, exhibiting that introduction of 4-O-TEMPO into GP1 or 4-O-TEMPOH into GP1OH greatly strengthened the antioxidative activity. However, GP1 was shown to be the most potent. Although the parent GP only had slightly inhibitory action against lipoperoxidation in FRGS system, its derivative GP1 was more potent than 4-O-TEMPO, also demonstrating that GP is capable of increasing the activity of nitroxides against oxidation. On the other hand, if the NO group of GP1 or NOH group of GP1OH was substituted by NH group, both GP1 and GP1OH were changed into GP1H almost without activity at concentration as high as 80 μmol/L, suggesting that NO group or NOH group should be essential active groups of antioxidation in nitroxyl radical moieties.

Nitroxides were observed not to affect superoxide anion radical formation from rat neutrophils-zymosan system[4]. The present study showed that GP1 inhibited superoxide anion radical formation in this test system. The results imply that GP1 is a scavenger for oxygen free radicals with more wide acting spectrum than nitroxides.

Podophyllotoxin and a number of its derivatives possess anticancer activity. It has been found that introduction of nitroxides into some antitumor drugs, such as podophyllotoxin, had significant antitumor activity with marked decrease in toxicity compared with the parent compounds[5,6]. It is not very clear why introduction of nitroxides group enhances the antitumor activity but reduces the toxicity. It is possibly related to its antioxidative activity. Administration of antineoplastic agents such as GP derivative VP16 results in oxidative stress[14], ie, the production of free radicals and other reactive oxygen species (ROS). ROS slow the rate of cell proliferation, and that occurring during chemotherapy may interfere with the cytotoxic effects of antineoplastic drugs, which depends on rapid proliferation of cancer cells for optimal activity[15]. Therefore, simultaneous antioxidative effect may sensitize the reaction of tumor cells to cytotoxic drugs. Introduction of nitroxides group into GP made their derivatives possess dual actions, ie, as a potent antioxidant to possibly abolish the suppression of cell proliferation by ROS, and simultaneously as an inhibitor of topoisomerase II to enhance the anticancer effects of GP derivatives. Beyond their antioxidative activity, nitroxides also possess antitumor activities. This kind of active groups may synergize with original active groups in GP derivatives each other. On the other hand, ROS cause or contribute to certain side effects that are common to many anticancer drugs. The reduction of toxicity of GP derivatives with nitroxide group may also result from their antioxidative activity.

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鬼柏酰肼自旋标记衍生物的抗氧化活性

李文广[1]，张小郁[2]，吴勇杰，田 暄[3]

(兰州医学院药理学教研室，[2]生理学教研室；[3]兰州大学国家重点有机化学实验室，兰州 730000，中国)

关键词 鬼柏酰肼；自旋标记物；多柔比星；自由基；抗氧化剂；丙二醛

目的：研究鬼柏酰肼自旋不达意标记衍生物的抗氧化活性及其结构活性关系．**方法：**用 TBA 比色法测自发性生成或 Fe^{2+}- 抗坏血酸和多柔比星刺激大鼠心、肝、肾组织匀浆生成的 MDA；用分光光度法测 H_2O_2 诱导的大鼠 RBC 溶血反应；用 NBT 还原法测大鼠活化中性粒细胞超氧阴离子生成．**结果：**GP1、GP1OH 明显抑制 MDA 生成，对抗 H_2O_2 诱导的溶血反应，而 GP 和 GP1H 作用微弱，GP1 尚抑制超氧阴离子生成．**结论：**氮氧自由基引入 GP 中，使其衍生物抗氧化作用大大增强，必需活性基团为 NO 基和 NOH 基．

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