Novel Melphalan and Chlorambucil Derivatives of 2,2,6,6-Tetramethyl-1-piperidinyloxy Radicals: Synthesis, Characterization, and Biological Evaluation *in Vitro*

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A series of spin-labeled melphalan and chlorambucil derivatives, coupling the alkylating agents with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) radicals, were synthesized, characterized, and their biological properties *in vitro* were evaluated. These compounds showed much higher cytotoxic activity against human leukemia cell line K562 *in vitro* than their parent compounds.

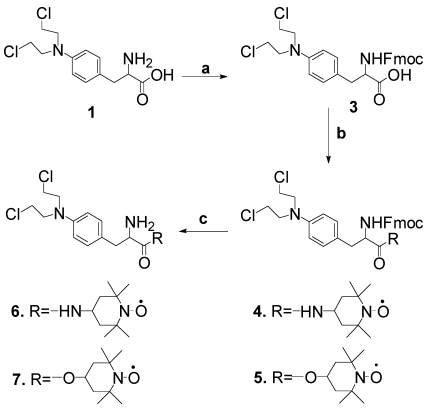
Key words melphalan; chlorambucil; 2,2,6,6-tetramethyl-1-piperidinyloxy radical; cytotoxic activity

In recent years nitroxide free radicals have attracted a great deal of attention in many research fields because, compared to most other free radicals, they exhibit a chemical inertness and are wildly used in biological and pathological areas.[1,2] At present, the stable nitroxide radicals such as 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and its derivatives are intensively studied in such fields as spin label/trapping in electron paramagnetic resonance (EPR) spectroscopy,[2] ionizing radiation prevention,[3,4] cancer prevention,[5—7] hypertension,[8,9] and chemical synthetic studies.[10,11]

Alkylating agents are one of the oldest anticancer agents in clinical use. Melphalan and chlorambucil, two members of this family, are employed in various cancer treatments.[12] However, these alkylating agents have numerous drawbacks because of their high reactivity and the ability to kill cells at whole using stage,[13—15] both of which greatly hinder their benefits. In an attempt to address their weaknesses, one of the most effective methods is to design and synthesize novel compounds by introducing functional groups into the molecular structures of alkylating agents.[16,17] TEMPO radical derivatives have demonstrated anticancer activities and can induce apoptosis of tumor cells in some ways.[18] Also, based on their chemical structures, the new nitroxide compounds can be designed easily. Furthermore, TEMPO radicals, as less toxic stable free radicals, can improve the properties of new compounds, such as the toxicity, antioxidant capability and anticancer activities. In our lab, a series of novel melphalan and chlorambucil derivatives conjugating TEMPO radicals were designed in order to improve the properties of those anticancer drugs.

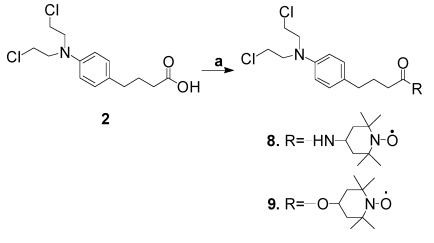
In this paper we wish to report our work on synthesis and characterization, and *in vitro* cytotoxic activity of the TEMPO conjugates of melphalan and chlorambucil. Although Prabhutendolkar *et al.*[19] reported the synthesis of one chlorambucil-tempol (2,2,6,6-tetramethylpiperidin-*N*-oxyl-4-yl-{4-[bis(2-chloroethyl)amino]phenyl}butanoate) adduct, its biological properties were not evaluated. In our investigation, more novel melphalan/chlorambucil-TEMPO spin-labeled compounds were synthesized and studied. Through the combination of the two different anticancer mechanisms, we hope to obtain novel drugs possessing more potency.

Chemistry Novel spin-labeled melphalan and chlorambucil compounds were obtained through 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl) coupling reaction, in which the carboxylic group of melphalan (**1**)/chlorambucil (**2**) was conjugated with the hydroxyl or amino group of 4-hydroxyl-2,2,6,6-tetramethyl-1-piperidinyloxy (4-OH-TEMPO)/4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy (4-NH_2-TEMPO). Before the coupling reaction occurred, the amino group of the melphalan was protected by 9-fluorenylmethyl chloroformate (Fmoc-Cl) and then the *N*-(9-fluorenylmethoxycarbonyl) (N-Fmoc) derivatives were formed, followed by the esterification or acylation



Reagents and conditions: a: Fmoc-Cl, dioxane, $NaHCO_3$ solution, 0 °C, 8 h (88.6%); b: (1) EDC·HCl, HOBT, 4-NH_2-TEMPO, CH_2Cl_2, 8 h (85.0%), (2) EDC·HCl, DMAP, 4-OH-TEMPO, CH_2Cl_2, 8 h (62.0%); c: piperidine, DMF, room temperature, 1 h (**6**: 83.0%, **7**: 86.0%).

Chart 1. Synthesis of Compounds **6** and **7**



Reagents and conditions: a: (1) EDC·HCl, HOBT, 4-NH_2-TEMPO, CH_2Cl_2, 8 h (87.0%), (2) EDC·HCl, DMAP, 4-OH-TEMPO, CH_2Cl_2, 8 h (72.0%).

Chart 2. Synthesis of Compounds **8** and **9**

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reaction. In dichloromethane solution, the N-protected melphalan and the chlorambucil reacted with 4-NH_2-TEMPO in the presence of EDC·HCl·1-hydroxybenzotriazole (HOBT) and 4-OH-TEMPO in EDC·HCl·4-dimethylaminopyridine (DMAP), respectively. Finally, the N-Fmoc derivatives of the melphalan were deprotected in the piperidine/*N*,*N*-dimethylformamide (DMF) with a very high yield. (Charts 1, 2).

Results and Discussion

Partition Coefficient In our assays, the partition coefficient (log *P*) values of the studied compounds were obtained from the *n*-octane/water system (Table 1).[20] The log *P* values of compounds **6** and **7** were much bigger than their parent melphalan, and similarly, compounds **8** and **9** exhibited increasing lipophilicity compared to chlorambucil. The results indicated that the introduction of the TEMPO radical led to an increase of the lipophilicity of the novel compounds. Because the carboxyl groups of the parent melphalan and chlorambucil were replaced during the reaction of esterification or amidation by the TEMPO group which has much weaker polarity, the lipophilicity of the novel compounds increased.

Kinetics of Hydrolysis In our experiment, a water/acetone hydrolysis system was selected, and the hydrolysis was conducted mainly under a neutral environment because the rate of the hydrolysis is temperature and pH dependent.[21] The major hydrolytic pathway of the alkylating agents was the formation of the aziridinium ion, followed by a rapid hydrolysis to yield the monohydroxy, and subsequently the dihydroxy products. All hydrolysis profiles were found to exhibit linear relationships ($R>0.96$) in our assays. The kinetics of the hydrolysis could be approximated by consecutive first-order kinetics, and the pseudo-first-order rate constants (K_H) of the hydrolysis reaction are given in Table 1. The attachment of TEMPO moiety to the melphalan and the chlorambucil introduced an ester or amide bond which is also easily hydrolyzed in the aqueous solution, and the hydrolysis reaction process could be demonstrated by HPLC. But according to the rate constants K_H shown in Table 1, the hydrolysis rate of the modified compounds did not greatly change, compared with the counterpart parent drugs. The results showed that the introduction of TEMPO moiety to melphalan or chlorambucil exerted very little effect on the hydrolysis rate of the parent drugs, and the pathway of hydrolysis that they underwent may be similar under a neutral environment.

Cytotoxicity Studies The cytotoxic activities of the compounds *in vitro* against human leukemia cell line K562 were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[14] Figure 1 gives the results of the assays. According to our measurements, compared to the parent drugs melphalan and chlorambucil, their derivatives modified by TEMPO radicals exhibited improved anti-leukemia activities. For human leukemia cell line K562, IC_{50} values could be obtained from the assay results (Table 1) according to the median effect equation described by Chou.[22] Consequently, the IC_{50} values of the TEMPO derivatives were almost twice as low as those of the parent drugs. The results, firstly, may be related to the introduction of the TEMPO moiety which led to an increase of the lipophilicity known by our measurements of log *P* of the compounds, enhancing the ability to cross the cell membrane. Secondly, we know that the alkylating agents form covalent adducts with DNA *via* the formation of a transient aziridinium species that attack the nucleophilic N^7 position of guanine nucleotides.[13] When the parent drugs were modified by TEMPO radicals, the active groups in the structures of melphalan and chlorambucil were protected before they reached the target DNA. This way greatly reduced the chances of the hydrolyzation and the reactions with other biomolecules in the body. Finally, as known, the piperidine nitroxides can exhibit anticancer ability to a certain extent.[7,23,24] Therefore, we concluded that the combination of the nitroxide radicals and melphalan/chlorambucil bearing two different anti-cancer mechanisms would improve the antiproliferative potency, compared to each single compound.

Table 1. Physicochemical Properties and Cytotoxic Activities of the Compounds

Compound	log *P*	$K_H/min^{-1}\times10^3$	Cytotoxic activities/ K562/IC_{50} (μM)
1	−0.97	9.08	65.66
2	2.78	16.28	134.4
6	0.22	11.60	36.26
7	1.34	9.45	20.03
8	3.13	11.53	47.53
9	2.94	9.24	38.61

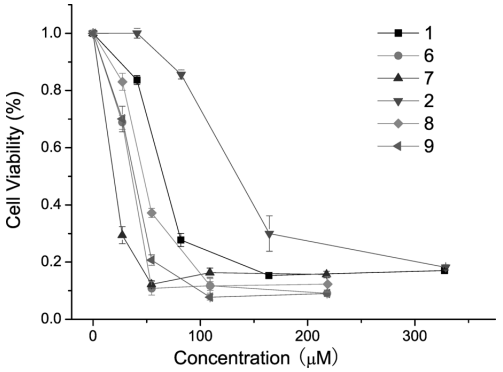

Fig. 1. Effects of the Studied Compounds on Viability of K562 Cell

Conclusion

A series of novel spin-labeled melphalan and chlorambucil derivatives were synthesized and characterized in our lab. The novel compounds obviously held the properties of both the alkylations and the TEMPO radicals through the physicochemical measurements and biological evaluation. Because the hydrolysis pathway of the alkylations and their TEMPO conjugations was similar, the attachment of the TEMPO radicals to melphalan and chlorambucil had very little effect on the hydrolytic stability of the parent compounds. Furthermore, the results of cytotoxic assays *in vitro* indicated that the novel compounds functionalized by TEMPO could exert a strong effect on the cytotoxic potency and improve this potency against the human leukemia K562 cell line.

Experimental

General Methods and Materials The ^{1}H-NMR spectra were recorded with a Bruker AM-500 NMR Spectrometer and the traditional ^{1}H-NMR spectra were of very limited help in detecting the final compounds because the spectra of the nitroxide-containing molecules are broad.[1] Melting points were determined by an X-5 micro-melting point apparatus and uncorrected.

EI-mass spectra were obtained on a Micromass GCT TOF GC/MS spectrometer. Elemental analyses were realized with an elementar vario EL III analyser. IR spectra were measured on a Nicolet AVATAR360 FT-IR spectrophotometer. The UV–visible spectra were recorded on a UNICO UV-2102PC spectrophotometer. Analytical thin-layer chromatography (TLC) was conducted on precoated silica gel plates (Merck silica gel F254, 0.25 mm thick) with detection both by ultraviolet light and visualization with iodine. EPR measurements were performed at X-band, 9.85 GHz, using a Bruker EMX EPR spectrometer and the measurement of g-factors of the compounds was carried out in contrast to a standard g-marker valued of 1.9800 provided by the Bruker Company. High performance liquid chromatography (HPLC) was conducted on an Agilent Technologies 1200 Series HPLC using a 0.4×30 cm p-Bondapak C-18 column (Agilent Eclipse XDB-C18, Agilent Ltd. Company, U.S.A.).

Melphalan and chlorambucil used as building block-drugs in this study were purchased from Beijing Lunarsun Pharmaceutical Co., Ltd. 4-Amino-2,2,6,6-tetramethyl-1-piperidinyloxy (4-NH_2-TEMPO, 97%) radical and 4-hydroxyl-2,2,6,6-tetramethyl-1-piperidinyloxy (4-OH-TEMPO, 99.9%) radical were commercially available and were bought from Sigma Corp. All solvents and reagents obtained from commercial sources were analytical reagent (AR) grade and used without further purification. New compounds synthesized were tracked by TLC. Human leukemia cell line K562 was purchased from the Institute of Biochemistry and Cell Biology, SIBS, CAS.

Chemistry 1) *N*-(9-Fluorenylmethoxycarbonyl)-4-[bis(2-chloroethyl) amino]phenylalanine (**3**): Ten milliliters of saturated $NaHCO_3$ solution was added to an ice-cooled solution of melphalan **1** (2 g, 6.55 mmol) and 9-fluorenylmethyl chloroformate (Fmoc-Cl, 1.69 g, 6.55 mmol) in dioxane (20 ml). After stirring for 8 h at room temperature (r.t.), the reaction mixture was diluted with brine (200 ml), and extracted with ethyl acetate (200 ml). The aqueous layer was acidified to a pH of 2 and extracted with ethyl acetate (200 ml) again. The combined organic extracts were dried and concentrated. The solid residue was purified by column chromatography (hexane : ethyl acetate=3 : 1, v/v) to give the compound **3** (3.06 g, 88.6%) as white solid, mp 116—118 °C. IR γ_{max}^{KBr} (cm^{-1}): 3330.23 (N–H, O–H), 2950.2 (C–H); 1708.7 (C=O); 1520.0, 1445.5 (C=C); 1350.6 (=N–O); 1047.9 (C–O–C) cm^{-1}. MS *m/e*: $[M]^+$=526.2. ^{1}H-NMR ($CDCl_3$) δ: 3.0 (m, 2H, CH_2Ph), 3.5—3.7 (m, 8H, N(CH_2CH_2)$_2$), 4.3 (1H, t, CH[Fmoc]), 4.4 (2H, m, CH_2[Fmoc]), 5.2 (1H, d, NH), 6.8 (d, 2H, arom *meta*), 7.0 (d, 2H, arom *ortho*), 7.2—7.7 (8H, m, Ar[Fmoc]), 9.7 (1H, COOH). *Anal.* Calcd for $C_{28}H_{28}Cl_2N_2O_4$: C, 63.76; H, 5.35; N, 5.31. Found: C, 63.81; H, 5.27; N, 4.96.

2) 1-Oxyl-4-{*N*-(9-fluorenylmethoxycarbonyl)-4-[bis(2-chloroethyl) amino]phenylalanyl}amino-2,2,6,6-tetramethylpiperidine (**4**): 4-NH_2-TEMPO (648.7 mg, 3.79 mmol), 1-hydroxy-benzotriazole (HOBT, 512 mg, 3.79 mmol) and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride(EDC·HCl, 726 mg, 3.79 mmol) was successively added to a solution of **3** (2 g, 3.79 mmol) in CH_2Cl_2 (20 ml) at 0 °C. After stirring for 8 h at r.t., the mixture was concentrated, and the residue was taken up in brine and extracted with ethyl acetate. The organic phase was dried and purified by column chromatography (hexane : ethyl acetate=1 : 1, v/v) to yield an oil and then crystallized with diethyl ether to give the golden solid **4** (2.2 g, 85%), mp 139—140 °C. IR γ_{max}^{KBr} (cm^{-1}): 3331.8 (N–H); 2970.4, 2928.7 (C–H); 1702.5 (C=O); 1674.6, 1520.1, 1450 (C=C); 1360 (=N–O); 1250.7, 1185.5, 1040.7 (C–O–C) cm^{-1}. MS *m/e*: $[M-Fmoc]^+$=457.3. EPR: α_N in CH_2Cl_2: 15.54G; g value=2.0073.

3) 1-Oxyl-4-{*N*-(9-fluorenylmethoxycarbonyl)-4-[bis(2-chloroethyl) amino]phenylalanyl}oxo-2,2,6,6-tetramethylpiperidine (**5**): Using the same procedure employed for the synthesis of compound **4** and starting from 4-OH-TEMPO (1.95 g, 11.37 mmol), 4-dimethyl-aminopyridine (DMAP, 92.6 mg, 0.758 mmol), EDC·HCl (1.453 g, 7.58 mmol) and compound **3** (2 g, 3.79 mmol) in CH_2Cl_2 (20 ml) at 0 °C, compound **5** was synthesized, and purified by column chromatography (dichloromethane : hexane : ethyl acetate=1 : 4 : 1, v/v/v) to yield a golden solid **5** (1.6 g, 62%), mp 62—63 °C. IR γ_{max}^{KBr} (cm^{-1}): 3330.2 (N–H); 2971.4, 2929.8 (C–H); 1723.0 (C=O); 1615.5, 1519.2, 1448.0 (C=C); 1350.6 (=N–O); 1249.3, 1178.6, 1047.9 (C–O–C) m^{-1}. MS *m/e*: $[M]^+$=681.3. EPR: α_N in CH_2Cl_2: 15.25G; g value=2.0073.

4) 1-Oxyl-4-{4-[bis(2-chloroethyl)amino]phenylalanyl}amino-2,2,6,6-tetramethylpiperidine (**6**): Piperidine (2 ml) was added dropwise to a stirred solution of compound **4** (2 g, 2.94 mmol) in DMF (10 ml) at r.t. After 1 h, the solvent were removed *in vacuo* by co-evaporating with toluene, and the residue was purified by column chromatography (hexane : methanol : dichloromethane=5 : 1 : 5, v/v/v) to give an oil and then crystallized with diethyl ether to give the orange solid **6** (1.1 g, 83%), mp 112—113 °C. IR γ_{max}^{KBr} (cm^{-1}): 3375.3 (N–H); 2973.4, 2945.6 (C–H); 1680.8 (C=O); 1620.4, 1521.8 (C=C); 1369.6 (=N–O) cm^{-1}. MS *m/e*: $[M]^+$=457.3. EPR: α_N in CH_2Cl_2: 15.54G; g value=2.0073. *Anal.* Calcd for $C_{22}H_{35}Cl_2N_4O_2^{\cdot}$: C, 57.64; H, 7.70; N, 12.22. Found: C, 57.67; H, 7.95; N, 12.33.

5) 1-Oxyl-4-{4-[bis(2-chloroethyl)amino]phenylalanyl}oxo-2,2,6,6-tetramethylpiperidine (**7**): Under similar conditions to the synthesization of **6**, compound **7** was prepared as an orange solid, yield 86% and mp 117—118 °C. IR γ_{max}^{KBr} (cm^{-1}): 2972.7, 2932.8 (C–H); 1731.4 (C=O); 1614.6, 1519.1, 1462.3 (C=C); 1359.4 (=N–O); 1179.4 (C–O–C) cm^{-1}. MS *m/e*: $[M]^+$=459.2. EPR: α_N in CH_2Cl_2: 15.54G; g value=2.0073. *Anal.* Calcd for $C_{22}H_{34}Cl_2N_3O_3^{\cdot}$: C, 57.51; H, 7.46; N, 9.15. Found: C, 57.46; H, 7.26; N, 9.03.

6) 1-Oxyl-4-{4-[bis(2-chloroethyl)amino] phenylbutanoyl}amino-2,2,6,6-tetramethyl-piperidine (**8**): 4-NH_2-TEMPO (562.12 mg, 3.28 mmol), HOBT (444.21 mg, 3.28 mmol) and EDC·HCl (630.17 mg, 3.28 mmol) were successively added to a solution of chlorambucil **2** (1 g, 3.28 mmol) in CH_2Cl_2 (20 ml) at 0 °C. After stirring for 8 h at r.t., the mixture was concentrated, and the residue was taken up in brine and extracted with ethyl acetate. The organic phase was dried and purified by column chromatography (hexane: ethyl acetate=4 : 1, v/v) to yield an oil and then crystallized with diethyl ether to give the orange solid **8** (1.3 g, 87%), mp 97—98 °C. IR γ_{max}^{KBr} (cm^{-1}): 3310.3 (N–H); 2937.5, 2875.5 (C–H); 1642.4 (C=O); 1547.5, 1525.4, 1460.6 (C=C); 1375.4 (=N–O) cm^{-1}. MS *m/e*: $[M]^+$=457.3. EPR: α_N in CH_2Cl_2: 14.96G; g value=2.0073. *Anal.* Calcd for $C_{23}H_{36}Cl_2N_3O_2^{\cdot}$: C, 60.39; H, 7.93; N, 9.19. Found: C, 60.48; H, 7.64; N, 9.06.

7) 1-Oxyl-4-{4-[bis(2-chloroethyl)amino] phenylbutanoyl}oxo-2,2,6,6-tetramethyl-piperidine (**9**): Using the procedure employed for the synthesis of compound **8** and starting from 4-OH-TEMPO (1.7 g , 9.84 mmol), DMAP (40 mg, 0.328 mmol), EDC·HCl (693.19 mg, 3.608 mmol) and chlorambucil **2** (1 g, 3.28 mmol) in CH_2Cl_2 (20 ml) at 0 °C, compound **9** was prepared, and purified by column chromatography (hexane : ethyl acetate=1 : 10, v/v) to yield an oil and then crystallized with diethyl ether to give the orange solid **9** (1.1 g, 72%), mp 59—61 °C. IR γ_{max}^{KBr} (cm^{-1}): 3437.5 (N–H); 2973.5, 2932.5 (C–H); 1731.5 (C=O); 1615.4, 1520.5, 1465.4 (C=C); 1350.6 (=N–O); 1184.5 (C–O–C) cm^{-1}. MS *m/e*: $[M]^+$=458.2. EPR: α_N in CH_2Cl_2: 15.25G; g value=2.0073. *Anal.* Calcd for $C_{23}H_{35}Cl_2N_3O_3^{\cdot}$: C, 60.26; H, 7.70; N, 6.11. Found: C, 60.23; H, 7.80; N, 5.98.

Determination of log *P* Oil/water partition coefficients (log *P*) of the drugs in the *n*-octane/water system were determined by the shake-flask method as described in the literature[25], using a UV–Vis spectrophotometer. The absorption values (corresponding to the maximum absorption wavelength) of the two phases were obtained. The distribution ratio of one compound between 1-octanol and water was calculated from the ratio of its absorption values in the oil phase to that in the water phase. Four separate measurements for the *P* values were performed. And the reported values of log *P* were the arithmetic averages of the four independent measurements. The standard deviation of these replicated measurements was taken to be the estimate of the uncertainty in the reported mean value.

Hydrolysis of the Compounds The studied compounds were dissolved in dimethyl sulfoxide (DMSO) to make 2 mM solutions, and 50 μl of DMSO solution was added to 950 μl of 50% acetone/water (w/w) in 1.5-ml capped microcentrifuge tubes at 66 °C[14]; 50 μl aliquots of the solution were then analyzed by HPLC. The system was eluted at 1 ml/min with a mobile phase of 70% MeOH, 10% CH_3CN, and 20% 1 M aqueous ammonium acetate, containing 1 mM triethylamine and 1 mM heptanesulfonic acid. The absorbance was monitored at 258 and 436 nm, and peaks corresponding to the parent alkylating agents, half alkylating agents, and the diol were integrated. The percentage of parent compound remaining at each time point was determined and used to calculate a pseudo-first-order rate constant for the hydrolysis reaction.

Cytotoxicity Assays K-562 cells were maintained in exponential growth phase by subculturing in RPMI 1640 containing 10% fetal calf serum and 2 mM L-glutamine at 37 °C, 5% CO_2, 95% air, 100% relative humidity. 1×100 cells/well were seeded onto 96-well plates, treated using a range of compound concentrations after 24 h incubation. Then MTT was added to each well to the final concentration of 0.5 mg/ml and cells were incubated for an additional 3 h at 37 °C. Formazan crystals were dissolved in DMSO and absorbance was read at 492 nm using a Universal Microplate Reader 550 (Bio-Rad Instruments).

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