Structural Requirements for Optimized Delivery, Inhibition of Oxidative Stress, and Antiapoptotic Activity of Targeted Nitroxides

Jianfei Jiang, Igor Kurnikov, Natalia A. Belikova, Jingbo Xiao, Qing Zhao, Andrew A. Amoscato, Rebecca Braslau, Armido Studer, Mitchell P. Fink, Joel S. Greenberger, Peter Wipf, and Valerian E. Kagan

Center for Free Radical and Antioxidant Health, Departments of Environmental and Occupational Health (J.J., I.K., N.A.B., Q.Z., V.E.K.), Chemistry (J.X., P.W.), Pathology (A.A.A.), Critical Care Medicine (M.P.F.), and Radiation Oncology (J.S.G.), University of Pittsburgh, Pittsburgh, Pennsylvania; Department of Chemistry and Biochemistry, University of California, Santa Cruz, California (R.B.); and Institute of Organic Chemistry, University of Münster, Münster, Germany (A.S.)

Received September 28, 2006; accepted December 18, 2006

ABSTRACT

Suppression of mitochondrial production of reactive oxygen species is a promising strategy against intrinsic apoptosis typical of degenerative diseases. Stable nitroxide radicals such as 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl (TEMPOL) and its analogs combine several important features, including recyclability, electron acceptance from respiratory complexes, superoxide dismutase mimicry, and radical scavenging. Although successful in antioxidant protection, their effective concentrations are too high for successful in vivo applications. Recently (J Am Chem Soc 127:12460, 2005), we reported that 4-amino 2,2,6,6-tetramethyl-1-piperidinoloxyl, covalently conjugated to a five-residue segment of gramicidin S (GS), was integrated into mitochondria and blocked actinomycin D (ActD)-induced superoxide generation and apoptosis. Using a model of ActD-induced apoptosis in mouse embryonic cells, we screened a library of nitroxides to explore structure-activity relationships between their antioxidant/antiapoptotic properties and chemical composition and three-dimensional (3D) structure. High hydrophobicity and effective mitochondrial integration are necessary but not sufficient for high antiapoptotic/antioxidant activity of a nitroxide conjugate. By designing conformationally preorganized peptidyl nitroxide conjugates and characterizing their 3D structure experimentally (circular dichroism and NMR) and theoretically (molecular dynamics), we established that the presence of the β-turn/β-sheet secondary structure is essential for the desired activity. Monte Carlo simulations in model lipid membranes confirmed that the conservation of the o-Phe-Pro reverse turn in hemi-GS analogs ensures the specific positioning of the nitroxide moiety at the mitochondrial membrane interface and maximizes their protective effects. These new insights into the structure-activity relationships of nitroxide-peptide and -peptide isostere conjugates are instrumental for development of new mechanism-based therapeutically effective agents.

Supplemental material to this article can be found at: http://jpet.aspetjournals.org/content/suppl/2006/12/20/jpet.106.114769.DC1

Received September 28, 2006; accepted December 18, 2006

Supplemental material to this article can be found at: http://jpet.aspetjournals.org/content/suppl/2006/12/20/jpet.106.114769.DC1

P poorly controlled and excessive generation of reactive oxygen species (ROS), coupled with their ability to cause oxidative damage to phospholipids, proteins, and DNA, has been associated with the pathogenesis of a number of major human cardiovascular (Ambrosio and Tritto, 1999) and neurodegenerative diseases (Andersen, 2004) as well as cancer (Szatrowski and Nathan, 1991). Not surprisingly, significant efforts have been directed toward the use of radical scavengers and antioxidants in preventive and therapeutic strategies, albeit with limited success. The search for new protective remedies has been focused on molecules combining antioxidant utilities with recycling capacities (Mitchell et al.,

ABBREVIATIONS: ROS, reactive oxygen species; TEMPOL, 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl; SOD, superoxide dismutase; CL, cardiolipin; cyt, cytochrome; TEMPO, 2,2,6,6-tetramethyl-1-piperidinoloxyl; 4-AT, 4-amino TEMPO; GS, gramicidin S; hemi-GS-TEMPO, hemi-GS conjugates of 4-AT; ActD, actinomycin D; Amplex Red, N-acetyl-3,7-dihydroxyphenoxazine; MP, microperoxidase; DHE, dihydroethidium; HPTLC, high-performance thin layer chromatography; PS, phosphatidylserine; HPLC, high-performance liquid chromatography; ESR, electron spin resonance; ESI, electrospray ionization; CD, circular dichroism; EPR, electron paramagnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; MD, molecular dynamics; MC, Monte Carlo; 3D, three-dimensional; MS, mass spectrometry.
Among others, stable nitroxide radicals such as 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl (TEMPOL) have been extensively investigated as cytoprotectors in a number of experimental models of oxidative stress (Hahn et al., 1992; Rachmilewitz et al., 1994; Beit-Yannai et al., 1996; Howard et al., 1996; McDonald et al., 1999). By accepting one electron, nitroxide radicals are converted to their respective hydroxylamines. These hydroxylamines act as effective ROS scavengers to produce nitroxides, i.e., they undergo recycling. These hydroxylamines act as effective ROS scavengers to produce nitroxides, i.e., they undergo recycling.

Furthermore, nitroxide radicals exert antioxidative effects on mammalian cells and mitochondria are necessary before their therapeutic potential can be fully exploited.

Electron carriers, particularly those present in mitochondria, can generate massive amounts of superoxide radicals, \( \mathcal{O}_2^- \), during a dysregulation of the electron transport chain. Mitochondrial proteins, oxidatively modified by ROS, perpetuate the production of increasing concentrations of \( \mathcal{O}_2^- \), thus causing cell damage and death (Choksi et al., 2004; Keeney et al., 2006). In particular, mitochondrial ROS production is characteristic of early stages of programmed cell death (apoptosis). The molecular mechanisms by which ROS trigger and participate in the mitochondrial stages of apoptosis are still under debate. Mounting evidence indicates that dysregulated electron transport and generation of ROS are linked to the oxidation of cardiolipin (CL), a mitochondria-specific phospholipid (Shidoji et al., 1999; Kagan et al., 2005). Oxidation of CL has been suggested to be essential for release of proapoptotic factors, including cytochrome (cyt) \( c \) from mitochondria that ultimately lead to cellular apoptosis (Shidoji et al., 1999; Kagan et al., 2005). Therefore, antioxidants that function to prevent the formation of or scavenging ROS in apoptotic mitochondria are of great interest for drug development. Nitroxide radicals targeted to mitochondria may be effective in preventing apoptotic cell death. Indeed, the latest developments in the field indicate that targeting of nitroxides to mitochondria enhances their antiapoptotic activity (Dessolin et al., 2002; Dhaneanarakaran et al., 2005; Wipf et al., 2005). Dessolin et al. (2002) have reported that accumulation of TEMPOL in mitochondria was substantially increased by conjugating it with a Salen-Mn(III) complex of o-vanillin (EUK-134), and the targeted nitroxide delayed apoptosis after an exogenous oxidative insult. Dhaneanarakaran et al. (2005) have also demonstrated that mitochondria-targeted mitocarboxy peroxy (coupled with the triphenylphosphine adduct of 11-bromo-undecanol) inhibited peroxy-induced oxidative damage and apoptosis. We have previously reported a significant increase of cytoprotective effects of 4-amino TEMPO (4-AT) by coupling it to a segment of the membrane-active compound gomericidin S (GS) (Wipf et al., 2005).

However, the structural requirements for optimized targeting, proper orientation in membranes, as well as the molecular mechanisms of protective and antiapoptotic properties of nitroxides, including those of hemi-GS conjugates of TEMPOs (hemi-GS-TEMPOs) and the corresponding peptide bond isosteres, remain to be elucidated. In the present study, we performed the screening of a library of nitroxides with different structures, and hydrophobicity using a model of actinomycin D (ActD)-induced apoptosis in mouse embryonic cells. We established that the targeting of nitroxides to mitochondria is associated with the maximized antiapoptotic potential. We further investigated structural requirements that determine maximal inhibition of ROS generation, suppression of oxidative damage to mitochondrial lipids, and antiapoptotic protection due to proper positioning of selected TEMPO conjugates in the membrane.

**Materials and Methods**

**Materials.** Hemi-GS or control peptide-conjugated 4-ATs were synthesized as described previously (Wipf et al., 2005; Xiao et al., 2005). ActD, microperoxidase (MP)-11, and ATP bioluminescent somatic cell assay kit were purchased from Sigma-Aldrich (St. Louis, MO). Annexin V kit was from Biovision (Mountain View, CA). N-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red), dihydroethidium (DHE), and TEMPO choline were purchased from Invitrogen (Carlsbad, CA). Antibodies against cyt \( c \) and Bax were from BD Biosciences PharMingen (San Diego, CA). HPTLC plates (5 × 5 cm) were purchased from Whatman (Florham Park, NJ). All other reagents were purchased from Sigma-Aldrich unless indicated.

**Cell Culture and Treatment.** Mouse embryonic cells (courtesy of X. Wang, University of Texas, Dallas, TX) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum, 25 mM HEPES, 50 mg/ml uridine, 110 mg/ml pyruvate, 2 mM glutamine, 1× nonessential amino acids, 0.05 mM 2-mercaptoethanol, 0.5 × 10^6 U/ml mouse leukemia inhibitory factor, 100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin. For apoptosis induction, cells were treated with 100 ng/ml ActD alone or in combination with nitroxides under the indicated conditions. At the end of incubation, attached cells were harvested by trypsinization and pooled with detached cells from supernatant.

**Phosphatidylserine Externalization.** Externalization of phosphatidylserine (PS) was analyzed by flow cytometry using Annexin V kit. In brief, harvested cells were stained with Annexin V-fluorescein isothiocyanate and propidium iodide 5 min in the dark before flow cytometry analysis. Ten thousand events were collected on a FACScan flow cytometer (BD Biosciences, Rutherford, NJ) supplied with CellQuest software.

**Translocation of Cyt \( c \) and Bax.** Translocations of cyt \( c \) and Bax were examined by Western blot assay. At the end of incubation, harvested cells were resuspended in lysis buffer containing 250 mM sucrose, 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 \mu g/ml aprotinin, 1 \mu g/ml leupeptin, and 0.05% digitonin for 3 min on ice, and then centrifuged at 8500g for 5 min. The resulting supernatants were collected as cytosolic fractions. The pellets were then washed with lysis buffer containing 1% of Triton X-100. The supernatants, after 10-min centrifugation at 8500g, were collected as mitochondrial fractions. All the samples were subjected to 12% SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane, which was probed with anti-cyt \( c \) (clone J7H8.2C12) or anti-Bax (clone 6A7) antibody followed by horseradish peroxidase-coupled detection.

**Superoxide Generation.** Superoxide-mediated oxidation-sensitive fluorescent dye DHE was used to evaluate intracellular production of superoxide radicals. DHE is cell-permeable and, in the presence of \( \mathcal{O}_2^- \), is oxidized to fluorescent ethidium, which intercalates...
into DNA. In brief, cells were washed once with serum/phenol red-free medium at the indicated time point and then incubated with 5 μM DHE for 30 min in serum/phenol red-free medium. At the end of incubation, cells were collected by trypsinization and resuspended in phosphate-buffered saline. The fluorescence of ethidium was measured using a FACScan flow cytometer supplied with CellQuest software. Mean fluorescence intensity from 10,000 cells were acquired using a 585/42-nm bandpass filter (FL-2 channel).

Cl. Oxidation. Cl hydroperoxides were determined by fluorescence HPLC of products formed in MP-11-catalyzed reaction with a fluorogenic substrate, Amplex Red. In brief, lipids were extracted from cells and resolved by two-dimensional HPTLC as described previously (Tyurina et al., 2004). Spots of phospholipids were scraped from the HPTLC plates, and phospholipids were extracted from silica. Lipid phosphorus was determined by a micro-method (Tyurina et al., 2004). Oxidized phospholipids were hydrolyzed by pancreatic phospholipase A2 (2 U/ml) in 25 mM phosphate buffer containing 1.0 mM CaCl2, 0.5 mM EDTA, and 0.5 mM SDS (pH 8.0 at room temperature for 30 min). After that, Amplex Red and MP-11 were added, and samples were incubated for 40 min at 4°C. A Shimadzu LC-10AT vp HPLC system equipped with fluorescence detector (RF-10Ax, excitation/emission = 560/590 nm) and autosampler (SIL-10AD vp) was used for the analysis of products separated by HPLC (Eclipse XDB-C18 column, 5 μm, 150 × 4.6 mm). The mobile phase was composed of 25 mM NaH2PO4, pH 7.0/methanol (60:40, v/v).

Electron Paramagnetic Resonance-Based Analysis of Integration and Distribution of Nitroxides. To compare the integration efficiency, cells (1 × 10⁷/ml) were incubated with 10 μM hemi-GS-TEMPOs for 15 min. ESR spectra of nitroxide radicals in the incubation medium, cell suspension, or mitochondrial suspension were recorded after mixing with anestetinelle (1:1, v/v) after 5-min incubation (Jeol, with 2 mM K3Fe(CN)6 by using JEOLEI XPR spectrometer (Jeol, Tokyo, Japan) under the following conditions: 3350-G center field, 25-G scan range, 0.79-G field modulation, 20-mW microwave power, 0.1-s time constant, and 4-min scan time. Integration efficiency was calculated as (Einit - Emedium/ Einit × 100%. Mitochondria were isolated using a mitochondria isolation kit (Pierce Chemical, Rockford, IL) according to the manufacturer’s instruction. Amounts of nitroxide radicals integrated into mitochondria were normalized to the content of cyclooxygenase IV.

Electrospray Ionization Mass Spectrometry of Hemi-GS-TEMPOs in Mitochondrial Fractions. Mitochondrial fractions were extracted with chloroform/methanol (2:1, v/v). The chloroform phase was collected and evaporated under nitrogen and then resuspended in chloroform/methanol and directly used for acquisition of positive ion ESI mass spectra using a syringe pump RCK100 (KD Scientific, Holliston, MA) at flow rate of 2 μl/min. ESI-mass spectra of GS-TEMPOs were acquired by direct infusion into a triple-quadrupole mass spectrometer (Micromass, Inc., Manchester, England) equipped with an electrospray interface. Sheath flow was adjusted to 5 μl/min. The electrospray probe was operated at a voltage differential of −3.5 kV in the positive ion mode. Source temperature was maintained at 70°C. Mass spectra of GS-TEMPOs were obtained by scanning in the range of 400 to 1700 every 3.5 s and summing individual spectra.

NMR and Circular Dichroism Measurement. Circular dichroism (CD) spectra were obtained on a Jasco-J715 spectrometer (Jasco, Tokyo, Japan) at a 0.1 mM concentration in anhydrous ethanol solution. The correlation spectroscopy, NOESY, heteronuclear multiple quantum coherence spectroscopy, and heteronuclear multiple-bond correlation spectroscopy NMR spectra were obtained on a 600-MHz instrument (Bruker, Newark, DE) at 298 or 338 K.

Molecular Dynamics Simulations. Molecular dynamics (MD) simulations of hemi-GS-TEMPOs have been performed using the SANDER module of the AMBER8 molecular modeling package. Amber-94 force-field parameters were used for standard amino acids in TEMPO-peptide conjugates. Parameters for atoms of nonstandard groups (Boc, Cbz, modified alkene derivatives, and TEMPO) were set from general AMBER force field. Atomic charges for these groups were set from results of Hartree-Fock calculations using 6-31G* basis set. Calculations modeling peptides in solution for comparison with results of CD experiments were done by placing TEMPO-peptide conjugates in a cubic box of water (37.5 × 37.5 × 37.5 Å). Calculations were performed at constant pressure (1 atm) and constant temperature (300 K) conditions, and 2-ns MD trajectories were generated for each compound studied.

Monte Carlo Simulations. Monte Carlo (MC) simulations of TEMPO-peptide conjugates at the polar/nonpolar interface of the model membranes have been performed using the HARLE program (http://www.harlemprog.org). Two types of MC moves have been used. In one type of the MC moves, the 3D structure of a peptide was changed by selecting randomly among a set of peptide conformations generated in a long MD trajectory. In a second type of moves, the peptide was translated and rotated as a solid body. The energy of the system was computed as a sum of the conformational energy of the compound computed using Amber-94 force field, and the energy of solvation was computed using finite-difference solution of Poisson-Boltzmann equation (Honig et al., 1993).

Determination of Intracellular ATP Levels. Cells were incubated with 10 μM 5-125 and 7-75 for indicated periods (2, 4, 6, 12, and 14 h). At the end of incubation, cells were collected, and the content of intracellular ATP was determined using a biosiluminescent somatic cell assay kit. As a positive control, cells were incubated with 2 mM 2-deoxy-d-glucose, a glucose analog that competitively inhibits cellular uptake and utilization of glucose, for 12 and 14 h.

Results

Screening of Nitroxides for Antiapoptotic Activity. Using a prototypical model of ActD-induced intrinsic apoptosis in mouse embryonic cells, we screened the antiapoptotic properties of a library of nitroxide radicals (Fig. 1). Cellular apoptosis was evaluated by measuring the exposure of PS on the surface by flow cytometry. As shown in Fig. 2, ~32.4% of cells displayed externalized PS after a 14-h incubation in the presence of 100 ng/ml ActD. Our library of nitroxide radicals included derivatives with various degrees of steric hindrance, polarity, molecular weight, electrophilicity, acidity, and hydrophobicity (Fig. 1). Although some of the candidate compounds demonstrated their superoxide scavenging capacities in a xanthine/xanthine oxidase system (Supplemental Table 1), none of these nitroxide radicals afforded protection against ActD-induced apoptosis in a broad range of concentrations (0.01, 0.1, 1, and 10 mM; data not shown) in our assay system. Notably, a positively charged nitroxide-choline conjugate (TEMPO choline) was also ineffective in spite of the known affinity of mitochondria to organic cations. Moreover, some of the nitroxides, such as 4-(2-bromoacetamido)-TEMPO, exerted a significant toxicity at low micromolar range.

Because the screened nitroxides spanning a significant range of hydrophobicity and redox properties were inactive as protectors against apoptosis, and considering our previous findings on the high activity and specificity of mitochondria-targeted hemi-GS-nitroxide conjugates, we were anxious to explore further their possible protective effects and a establish structural basis for their action. We used two different segments (Leu-d-Phe-Pro-Val-Orn and d-Phe-Pro-Val-Orn-Leu) of GS in conjugation with 4-AT (5-125 and 7-75, Fig. 3). Incubation of the cells with low concentrations of GS-TEMPOs 5-125 and 7-75 attenuated ActD-induced PS external-
ization in a dose-dependent manner (2.5–20 μM) (Fig. 2). In contrast, the hemi-GS moiety alone (5-194; Fig. 3) afforded no protection (Fig. 2). It is noteworthy that untargeted 4-AT protected against ActD-induced apoptosis only at concentrations ~1000 times higher (10 mM) than those of hemi-GS-TEMPOs (Fig. 2). The high antiapoptotic effectiveness of GS-nitroxide 5-125 was confirmed by its ability to inhibit the release of cyt c from mitochondria as evidenced by Western blots of cyt c in the cytosolic fractions from ActD-challenged cells in the presence and absence of hemi-GS-nitroxide (Fig. 4A). 4-AT did not protect against ActD-induced cyt c release. ActD-induced translocation of a proapoptotic member of the Bcl-2 family of proteins, Bax, from the cytosol into mitochondria. The conjugate 5-125 did not affect Bax translocation (Fig. 4A), indicating that its protective action was not directly associated with Bax.

To assess the extent to which the hydrophobicity of hemi-GS-TEMPOs determines their cytoprotective properties, we studied the antiapoptotic properties of 5-125 derivatives (5-131, 5-197, 7-53, and 7-55; Fig. 3) as well as nitroxides conjugated with peptides or peptide isosteres having shortened or modified sequences (5-208, 7-32, 7-36, and 7-77; Fig. 5). The octanol-water partition coefficients (logP) (Hansch and Fujita, 1964) that characterize the lipophilic properties of these compounds were estimated using the online calculator of molecular properties and drug likeness on the Molinspirations Web site (www.molinspiration.com/cgi-bin/properties). We synthesized several derivatives of 5-125 covering a broad range of logP values from 6.4 to 9.8 (Fig. 3). Evaluations of their antiapoptotic properties clearly demonstrated that hydrophobicity did not correlate with the antiapoptotic properties of these compounds (Fig. 4A and Supplemental Fig. S1). Although the hydrophobicity of 5-208 was comparable with that of 5-125 (computed logP values 5.5 versus 4.5), no antiapoptotic effect was detected for 5-208 (Fig. 2). Likewise, negative results were obtained in the antiapoptotic
tests of conjugates 7-32 and the hemi-GS-nitroxide derivatives of 7-75 (7-36 and 7-77), in which prolines were substituted with alanine or an (E)-alkene moiety (Supplemental Fig. S2). Apparently, the unique structural features of hemi-GS derivatives rather than their increased hydrophobicity alone contributed to the antiapoptotic propensities of these targeted TEMPOs (see below).

**Hemi-GS-TEMPOs Inhibit ActD-Induced Superoxide Generation in Mouse Embryonic Cells.** Execution of the apoptotic program is closely linked to the disruption of mitochondrial electron transport coupled with the production of superoxide radicals. Antia apoptotic effects of GS-nitroxides may be associated with their electron-scavenging capacities, i.e., their ability to prevent superoxide generation. Therefore, we next tested whether antia apoptotic activity of hemi-GS-TEMPOs was also effective in inhibiting superoxide production in ActD-treated mouse embryonic cells. A significant increase of superoxide generation was detected following a 10-h treatment with 100 ng/ml ActD, as revealed by an increase (~2.1-fold versus nontreated cells) of ethidium fluorescence (Fig. 6). Concurrent treatment of the cells with 5-125 and 7-75 almost completely inhibited ActD-induced superoxide generation (Fig. 6), whereas no effect was observed with untargeted 4-AT, hemi-GS peptide 5-194, or the negative control peptidyl conjugates 5-208 and 7-77 under the same condition.

**Hemi-GS-TEMPOs Inhibit ActD-Induced CL Oxidation.** We have previously demonstrated that ROS generated by disrupted electron transport in apoptotic cells are used in the cyt c-catalyzed oxidation of a mitochondria-specific phospholipid, CL (Kagan et al., 2005). Oxidized CL is essential for the release of proapoptotic factors from mitochondria into the cytosol during apoptosis (Kagan et al., 2005). Therefore, we assessed CL oxidation in mouse embryonic cells after treatment with ActD. Predictably, ActD-challenged cells contained approximately 4-fold higher levels of CL hydroperoxides (68.5 ± 13.8 and 17.2 ± 4.4 pmol/nmol CL in treated versus control cells; Fig. 4B). Hemi-GS-TEMPO 5-125 and its derivative 5-131 were able to markedly suppress the ActD-induced CL peroxidation (Fig. 4B). In contrast, neither 4-AT nor the negative control peptide 5-194 (which is devoid of the nitroxide moiety) exerted any protective effect under the same condition.

**Accumulation of Hemi-GS-TEMPOs in Cells and Mitochondria.** We further determined the levels of internalization of the synthesized hemi-GS-TEMPOs (5-125 and 7-75) into cells and mitochondria after their incubation with mouse embryonic cells (10^5/ml; 15 min at 37°C). For this, we used ESR spectroscopy and ESI-MS. Figure 7A shows typical ESR spectra of nitroxide radicals of 4-AT, 5-125, and 5-208 in the medium and cells. A characteristic triplet signal with superfine splitting constants of 16.6 gauss was observed. After incubation with cells, the signals were detectable in cell pellets for 5-125 and 5-208 but not for 4-AT, indicating that 4-AT was not appreciably integrated into cells. Furthermore, both 5-125 and 5-208 were readily integrated into mitochondria (Fig. 7B). Importantly, treatment of the samples with a one-electron oxidant, ferricyanide, significantly increased the magnitude of the signals in both cells and mitochondria (Fig. 7A and B). This suggests that both 5-125 and 5-208 underwent reduction to the respective hydroxylamines, likely by the electron transport chain, resulting in the loss of their radical nature and hence of the ESR signal. The radicals were recovered by one electron oxidation with ferricyanide. In line with this interpretation, MS analysis (Fig. 7C) revealed the presence of two signals corresponding to hemi-GS-nitroxides in each of the spectra for 5-125 and 5-208, respectively. Based on the m/z values for these two conjugates (998.64, 999.55 and 537.40, 538.32, respectively), these signals are identifiable as hemi-GS-nitroxides and their respective hydroxylamines. Based on a quantitative analysis of ESR data, we found all the peptidyl nitroxides tested effectively integrated into cells and mitochondria, although to various degrees (Table 1). The integration efficiency of 5-125 and 5-208 into cells averaged 62.3 ± 16.1 and 52.9 ± 12.7%, respectively. Importantly, a significant amount of integrated 5-125 (~17.8 ± 9.1%) was recovered in the mitochondrial fraction. In contrast, the integration efficiency of 4-AT into cells was estimated to be 1.8 ± 1.1% (Table 1).

**Preservation of the β-Turn Secondary Structure in Hemi-GS Peptides Is Required for the Antiapoptotic Properties of GS-TEMPOs.** Clearly, the simple accumulation of nitroxides in mitochondria is not sufficient for protection against ActD-induced apoptosis. We next analyzed the
secondary structure of hemi-GS peptides and their derivatives using $^1$H NMR and CD spectroscopy. The chemical shifts of all amide protons in bis-Cbz-protected hemi-GS (2-73; Fig. 8A) and the (E)-alkene isostere at the β-Phe-Pro position (2-121; Fig. 8A) were assigned using a combination of correlation spectroscopy, NOESY, heteronuclear multiple quantum coherence spectroscopy, and heteronuclear multiple-bond correlation spectroscopy data sets collected in dimethyl sulfoxide-d$_6$ at 338 K, because $^1$H NMR signals of some amide functions were obscured at 298 K. The NOESY data for both 2-73 and 2-121 showed strong NOE interstrand couplings between N-H(Leu) and N-H(Val) as well as between N-H(Val) and H$_\alpha$ (β-Phe) (Fig. 8A). These data suggested the existence of a hydrogen bonding interaction between the N-H(Val) and C=O(Leu) groups, indicating that the type II β-turn conformation of native GS is preserved in the 2-121 analog. The presence of a hydrogen bond between the N-H(Val) and C=O(Leu) groups in 2-73 and 2-121 is also supported by the lack of a strong temperature dependence of the NH chemical shifts (Xiao et al., 2006).

Analysis of CD spectra (Fig. 8B) revealed that both hemi-GS peptides 2-70 and 2-300 (corresponding to vehicle-peptides for hemi-GS-nitroxides 5-125 and 7-75; Fig. 3), have very similar native-GS-like CD spectra with a large trough in the 200- to 215-nm region and a shoulder in the 215- to 225-nm region. This indicates that the hemi-GS peptides with the intact β-Phe-Pro motif largely preserve the native reverse turn secondary structure. The CD spectrum of the cyclic pseudo-peptide 2-121 indicates that it also maintains a secondary structure similar to native GS. However, a comparison of the CD spectra of 2-121 with the corresponding segment 2-119 (Fig. 8B) revealed a dramatic spectral change, indicating a different backbone fold of the hemi-GS derivative compared with the cyclic compound. The random-coil-like CD spectrum of 2-119 indicates a loss of the preference for a native GS β-turn/β-sheet secondary structure. A similar conclusion was derived from the consideration of the CD spectrum of a hemi-GS derivative in which the proline was replaced by an alanine residue (2-301; Fig. 8B).

A relative destabilization of the β-turn/β-sheet secondary structure in the hemi-GS derivatives with a modified β-Phe-Pro motif was also found in an MD simulation in water. Snapshots of the MD trajectory were taken in regular 1-ps intervals, and the geometrical criteria for the presence of hydrogen bonds characteristic of the β-turn of GS were probed and displayed as a dichotomic process (1 if the hydrogen bond was present in the conformation and 0 if the conditions for the presence of the hydrogen bond were not fulfilled). Figure 9 shows significant statistical populations of N-H(Val) to C=O(Leu or Boc) hydrogen bonds in peptide 2-70, whereas this hydrogen bond forms only intermittently in 2-119.

Combined with the results presented above, we can conclude that both the nitroxide functionality, as well as the preservation of the reverse turn structure of the targeting sequence, are essential features for the antiapoptotic properties of hemi-GS-TEMPOs.
Stability of β-Turn/β-Sheet Structure in Hemi-GS Peptides Determines Their Positioning at the Interface between Nonpolar and Polar Groups of the Lipid Membrane. To further understand the observed relationship between the secondary structure of hemi-GS peptides and their antiapoptotic activity, we theoretically analyzed the membrane positioning and the peptide conformational changes associated with immersion of the compounds into the nonpolar compartment of the membrane. We performed MC simulations of the hemi-GS-TEMPOs in contact with a lipid membrane model as a continuum with low dielectric constant ($\varepsilon = 4$). The interactions between peptidyl 4-ATs and the polar and nonpolar parts of lipid membranes have been estimated using a continuum electrostatics approach (the finite-difference solution of Poisson-Boltzmann equation). The energy associated with conformational perturbation of peptidyl 4-ATs has been estimated using a molecular mechanics approach. Figure 10 shows the positions of the center of mass of 5-125 and 7-77 relative to the polar/nonpolar interface of the lipid membrane computed for points of MC trajectories ($10^5$ steps long). Figure 11A demonstrates that hemi-GS-TEMPO 5-125 were positioned at the interface between nonpolar and polar regions of the lipid membrane, whereas compound 7-77 with the Pro residue substituted by Ala stayed preferentially inside the nonpolar part of the membrane (Fig. 11B). This compound readily changes its conformation upon the immersion into a nonpolar part of the membrane that greatly increases its nonpolar solvation energy.

Antiapoptotic Doses of Hemi-GS-TEMPOs Do Not Deplete ATP and Are Not Cytotoxic. Because the pronounced antiapoptotic properties of 5-125 or 7-75 make them potentially interesting candidates for further therapeutic applications, we examined whether their action is associated with impairments of bioenergetic functions—production and maintenance of sufficient ATP levels in cells. We found that 5-125 and 7-75 (Fig. 12A) did not significantly affect the intracellular ATP content under the experimental conditions used. Neither 5-125 nor 7-75 demonstrated any significant toxicity (assessed by PS externalization) in mouse embryonic cells, even at a higher concentration of 20 μM (Fig. 12B).

Discussion

The well known contribution of ROS, reactive nitrogen species, and other species with unpaired electrons to pathogenetic mechanisms of tissue and organ damage in acute and chronic disease conditions suggests that radical scavengers can act as clinically effective remedies based on their ability to prevent oxidative stress. Indeed, in vitro studies revealed a variety of molecules with useful and remarkably effective antioxidant properties. Unfortunately, in vivo applications of these findings as well as results of clinical trials have been much less rewarding (Flaherty et al., 1994; Rosenstock et al., 2004; Miller et al., 2005). These shortcomings serve as motivation to continue the search for conceptually new protective molecules, combining several antioxidant features with a delivery vehicle directing them to specific intracellular targets—the sources of oxidative stress.

Mitochondrial Targeting of Hemi-GS-Nitroxide Conjugates Protects against Oxidative Stress and Cellular Apoptosis. An important factor limiting the effectiveness of currently existing antioxidants in ameliorating oxidative stress is the poor accumulation of these agents in mitochondria—the major site of ROS generation. This may be particularly important in apoptosis where mitochondria and associated ROS production play a central role (Shideji et al., 1999; Madesc and Hajnoczy, 2001; Clayton et al., 2005; Kagan et al., 2005). Recently, mitochondria-targeted antioxidants have emerged as a new drug development strategy (Adlam et al., 2005; Garber, 2005). A number of delivery strategies, including targeting based on biophysical properties of mitochondria, the unique mitochondrial localization of enzymes that catalyze the release of drugs from prodrugs, and transporter-dependent delivery of prodrugs have been developed (Sheu et al., 2006).

We chose nitroxide radicals as an antioxidant cargo due to their well documented electron-scavenging, SOD-mimicking, and radical-scavenging capacities as well as their ability to be recycled (Samuni et al., 1988; Krishna et al., 1992; Zhang et al., 1999). In the present study, we used two hemi-GS peptides (Leu-\(\beta\)-Phe-Pro-Val-Orn and \(\beta\)-Phe-Pro-Val-Orn-Leu) as delivery vehicles and targeting sequences to achieve a high level of accumulation of nitroxide radicals in mitochondria. Based on our EPR and MS data, we report that both conjugates caused efficient accumulation of nitroxides in mouse embryonic cells and their mitochondria where they acted as effective 1) electron scavengers yielding hydroxylamines, 2) inhibitors of superoxide production, and 3) anti-
These antioxidant features of hemi-GS-TEMPOs were accompanied by an effective suppression of different stages of ActD-induced apoptosis—cyt c release, PS externalization, and nuclear condensation and fragmentation (Wipf et al., 2005). Release of cyt c from mitochondria is regulated by Bcl-2 family proteins with the essential requirement of Bax/Bak (Wei et al., 2001; Kuwana et al., 2002). The permeabilization function of Bax was shown to be dependent on the Bax-associated production of ROS (superoxide radicals) (Kirkland et al., 2002; Kirkland and Franklin, 2003) and the presence of CL (Kuwana et al., 2002) or oxidized CL (Kagan et al., 2005). In line with this, our data showed that both 5-131 and 5-125 effectively inhibited ActD-induced superoxide production and CL oxidation and prevented release of cyt c from mitochondria into the cytosol without substantially affecting the translocation of Bax from the cytosol into mitochondria.

It is possible that the mechanism(s) by which hemi-GS-TEMPOs block superoxide generation and protect CL oxidation are closely associated with their ability to scavenge electrons from a disrupted electron transport chain in mitochondria, hence preventing univalent reduction of molecular oxygen into superoxide. Consequently, the dismutation of superoxide radicals resulting in H$_2$O$_2$ is inhibited. Because H$_2$O$_2$ feeds the catalytic cycle of the cyt c/CL complex causing CL oxidation, successful electron scavenging also explains the inhibition of CL oxidation.

Structural Requirements for Antiapoptotic Properties of Hemi-GS-Nitroxide Conjugates. We showed that various chemically modified 5-125 derivatives with increased hydrophobicity provided no additional cytoprotection against ActD-induced apoptosis. Furthermore, we found that control compounds that contain shorter or modified GS segments exerted no effect on ActD-induced ROS generation and cellular apoptosis, although for some of them such as 5-208 the level of their integration into cells and mitochondria was comparable with that of 5-125. These data rule out the possibility that the cytoprotective effects of hemi-GS-TEMPOs are mainly due to the hydrophobicity of the active compounds and suggest that the effective partitioning of nitroxides is necessary but not sufficient for their protection against oxidative stress in mitochondria.
We further focused on the structural analysis of hemi-GS-nitroxide derivatives with expressed antiapoptotic activity versus those that failed to protect cells against apoptosis. GS has a rigid 3D structure consisting of an antiparallel β-sheet with two type II β-turns. It has an amphipathic spatial organization of its amino acids. As a result, GS is positioned at the interface between polar and nonpolar regions of lipid membranes. The nonpolar side chains of Leu, Val, D-Phe, and Pro are immersed into a nonpolar part of the lipid membrane, whereas the positively charged Orn side chains and the polar N-H and C-O groups of exposed amide groups are in contact with polar lipid head groups and intercalating water mole-

Fig. 7. Examination of the nitroxides integration in mouse embryonic cells. Cells (10^7/ml) were incubated with 10 μM nitroxides for 15 min at 37°C. Nitroxide radicals in medium, cells, or mitochondrial fraction were estimated in 50% of acetonitrile with or without addition of 2 mM K₃Fe(CN)₆. A, representative EPR spectrum of 4-AT, 5-125, and 5-208 in cells and medium after incubation. B, representative EPR spectra of 5-125 and 5-208 in mitochondrial fraction. C, typical positive-ion ESI-MS profiles of 5-125 (left) and 5-208 (right) in mitochondrial fraction. Samples were extracted with chloroform/methanol (2:1, v/v). The chloroform phase was collected and evaporated under nitrogen and then resuspended in chloroform/methanol (2:1 v/v) for MS analysis.

Fig. 8. Spectroscopic characterization of secondary structure of hemi-GS peptides and their derivatives. A, observed NOE contacts involving N-H of Leu in GSCbz 2-73 and GSCbz Pro-[Me-alkene] 2-121, supporting the preservation of native GS secondary structure upon Pro modification. B, comparison of CD spectra of GS and hemi-GS peptides. Compounds 2-70, 2-300, 2-73, and 2-121 generally preserve the characteristic CD spectrum of native GS, whereas the spectra of 2-119 and 2-301 close to CD spectrum of the random coil.
cules. Solid-state NMR studies (Salgado et al., 2001) and molecular dynamics simulations (Mihailescu and Smith, 2000) confirmed that GS molecules in lipid membranes are positioned at the interface between polar lipid head groups and hydrophobic lipid tails, with the plane of the GS β-sheet parallel to the plane of the lipid membrane. Hemi-GS peptides with an intact ß-Phe-Pro motif largely preserve the characteristic ß-turn structure as demonstrated by our comparison of CD spectra of cyclic GS analogs and the corresponding hemi-GS compounds as well as by molecular dynamics simulations. The substitution of the proline residue by alanine or a Me-alkene peptide bond isostere modifies the ß-turn/β-sheet secondary structure and/or eliminates the polar character of the central amide bond in the reverse turn motif. Consequently, conjugates of 4-AT with these modifications provided no protection against ActD-induced cellular apoptosis.

The location of hemi-GS derivatives in the lipid membrane is determined by a combination of several physicochemical features that include the difference in solvation energies of the compounds in the polar solution, in the nonpolar part of the lipid membrane and at the interface between polar and nonpolar regions of the membrane as well as conformational flexibility. Native cyclic GS has a very rigid 3D structure that is insensitive to solvent polarity. Hemi-GS compounds are not as rigid as their parent GS molecules because they do not have a cyclic scaffold. Therefore, their average 3D structure will depend on the surrounding environment. For sufficiently flexible hemi-GS compounds, an amphipathic spatial organization of polar and nonpolar chemical groups will be perturbed upon immersion into a nonpolar part of the membrane. If the free energy gains of the increased nonpolar solvation inside the membrane exceed the free energy cost of the conformational change and a loss of favorable contacts of polar groups of the hemi-GS derivative with polar lipid head groups, the compound will be positioned inside the nonpolar part of the membrane.

Monte Carlo simulations showed that nitroxide adducts of hemi-GS peptides with intact ß-turn structure were positioned at the interface between polar and nonpolar regions of the lipid membrane, whereas peptides with greatly destabi-
lized β-turn secondary structure were immersed in the hydrophobic portion of the membrane. Apparently, positioning of the nitroxide group at the polar/nonpolar interface of the membrane is essential for its antiapoptotic activity. Mitochondrial one-electron reduction of oxygen to superoxide by electron transport protein complexes and (ubiquinone) radicals is unlikely to readily proceed inside the nonpolar portion of the lipid membrane because formation of the charged superoxide anion would be strongly unfavorable in the nonpolar environment, whereas formation of protonated neutral superoxide (HO₂⁺) is energetically much less favorable than the formation of O₂⁻ in a polar solvent. It is therefore probable that optimized quenching of chemical groups responsible for oxygen reduction requires that nitroxides should be positioned inside the polar region of the mitochondrial membranes, a task that is accomplished by hemi-GS carrier groups with intact β-turn structure.

Overall, our results demonstrate that hemi-GS peptides can be used as vehicles for effective delivery and “parking” of nitroxides in mitochondrial membranes, thereby controlling ROS generation and preventing oxidative damage of mitochondria and apoptosis. Specific positioning of nitroxide groups within the mitochondrial membranes is important for optimizing the antiapoptotic activity of GS-nitroxides and can be accomplished by sequence and conformational control—information essential for the creation of new effective antiapoptotic drugs based on nitroxide conjugates.

References


Address correspondence to: Dr. Valerian E. Kagan, Department of Environmental and Occupational Health, University of Pittsburgh, Bridgeside Point, 100 Technology Drive, Suite 350, Pittsburgh, PA 15219. E-mail: vkagan@eooh.pitt.edu