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Synthesis of analogs of the radiation mitigator JP4-039 and visualization of BODIPY derivatives in mitochondria

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Abstract

JP4-039 is a lead structure in a series of nitroxide conjugates that are capable of accumulating in mitochondria and scavenging reactive oxygen species (ROS). To explore structure-activity relationships (SAR), new analogs with variable nitroxide moieties were prepared. Furthermore, fluorophore-tagged analogs were synthesized and provided the opportunity for visualization in mitochondria. All analogs were tested for radioprotective and radiomitigative effects in 32Dcl3 cells.

Introduction

The 4-amino-TEMPO (4-AT) derivative JP4-039 is a promising lead among an emerging family of mitochondria-targeted nitroxides whose structures were inspired by the antibiotic gramicidin S (GS) and its microbial membrane affinity (Figure 1).^{1,2} The first generation hybrid molecule XJB-5-131 is composed of an alkene peptide isostere that mimics the leucyl-D-phenylalanine dipeptide in GS, a side chain-protected ornithylvalylproline tripeptide taken directly from GS, and a nitroxide subunit. The truncated analog JP4-039 contains a shortened alkene dipeptide isostere moiety directly attached to the nitroxide. The nitroxide fulfills several critical functions. It is effective at catalyzing the dismutation of superoxide radical anions and other reactive oxygen species (ROS) generated in mitochondria into H₂O₂.³ Nitroxides also trap electrons and scavenge radicals escaping from the oxidative phosphorylation (OXPHOS) pathway. In solution, nitroxides are in a dynamic equilibrium with hydroxylamines and nitroxonium species, which also respond to the redox state of the environment. GS adopts a type II' β -turn structure that buries several polar amide groups inside the molecule and thus may facilitate membrane transport.^{4,5} Both XJB-5-131 and JP4-039 fold into a β -turn secondary structure in the solution and solid state, respectively, and exhibited enrichments of 30- to 600-fold in mitochondria.^{1,6} Localization

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of ROS trapping agents to mitochondria is important since ca. 90% of all ROS are generated in these ATP-producing cell organelles.⁷

Our previous studies demonstrated that XJB-5-131 is superior to 4-AT or TEMPO alone in preventing actinomycin D-induced DNA fragmentation and upregulation of caspase 3, thus reducing apoptosis and enhancing cell survival in mouse embryonic cells (MECs).^{1a} In addition to its antiapoptotic^{1a} and radioprotective² activities in cells and in mice, XJB-5-131 can also prolong survival in a rat model of lethal hemorrhagic shock.⁸ XJB-5-131 readily partitions into the brain and shows promising efficacy in improving neurocognitive outcome after traumatic brain injury (TBI) in rats by preventing cardiolipin oxidation and reducing neuronal death.^{9a,b} Furthermore, XJB-5-131 proved to be an effective therapeutic in a mouse model of Huntington's disease (HD) by enhancing neuronal survival and suppressing oxidative damage to mitochondrial DNA as well as motor decline.^{9c} JP4-039, in addition to having a lower molecular weight and greater solubility, retains many of the desired physiological effects of XJB-5-131, including radiation damage prevention and mitigation.^{2,10,11}

Aside from some early, closely related analogs of XJB-5-131 that established the function of the peptide isostere, the SAR of these promising compounds has not been thoroughly studied. The recent development of a large-scale JP4-039 synthesis allowed for the preparation of several cyclopropane and fluorinated analogs,¹² but we had yet to modify the radical/electron scavenging nitroxide moiety of these molecules. Alternatives to 4-AT, namely the more stable isoindoline-based TMIO (**1**),¹³ as well as the more reactive azabicyclic ABNO (**5**)¹⁴ and the azaadamantane 1-Me-AZADO (**7**)¹⁵ were selected for this purpose (Figure 2). ABNO and 1-Me-AZADO were first synthesized by Dupeyre and Rassat,^{14a,15a} and then further developed as oxidation catalysts by Iwabuchi *et al.*^{14d,15b,c} They oxidize alcohol substrates more quickly and more efficiently than TEMPO or TEMPOL (**3**) due to their decreased steric hindrance at the nitroxide/nitroxonium reaction center. The different ring sizes and electron-withdrawing effects of the R-substituents in nitroxides **1-8** may also contribute to their modified redox properties.^{13a,16}

For the detection of GS-nitroxides in cell tissue samples, we had previously utilized spectroscopic methods such as EPR and MS.^{1,2,6} In order to more thoroughly investigate the ability of these nitroxides to partition in tissue and cells and localize in mitochondria, we required a fluorescence-based visualization tool. Many fluorophores are charged, and since charge can affect mitochondrial uptake, an overall net charge of zero should allow for unaffected mitochondrial uptake studies. We selected BODIPY® fluorophores for this purpose.^{17,18}

Results and Discussion

Synthesis of New Nitroxide Analogs

Starting with commercially available alcohol **9**, homoallylic alcohol **10** was synthesized in 5 steps according to known procedures.¹² Nitroxide analogs of JP4-039 were synthesized in two steps by Jones oxidation of **10** to the acid and EDCI-mediated coupling with amine-

functionalized building blocks. In this fashion, TMIO analog **12** could be readily obtained from **10** and 5-amino-TMIO (**2**)^{13c-e} in 67% yield (Scheme 1).

Preparation of 3-amino-ABNO (**6**) was envisioned from the known ketone **13**, which was easily accessible in one step from acetonedicarboxylic acid and glutaraldehyde (Scheme 2).¹⁹ Conversion of **13** to oxime **14**, followed by reduction to the amine with nickel boride and *N*-Boc protection provided carbamate **15** in high yield. Removal of the benzyl group by catalytic hydrogenolysis and oxidation of the resulting secondary amine in the presence of urea-hydrogen peroxide complex (UHP) furnished the Boc-protected nitroxide **16**.²⁰ However, once the Boc group was removed, the resulting 3-amino-ABNO (**6**) was found to be unstable,^{21,22} thus preventing its use in the coupling step with **11**.

To avoid the problem of the instability of amino nitroxide **6**, the synthetic route to obtain this ABNO analog was modified so that the nitroxide could be formed in the last step after *N*-acylation. Reduction of oxime **14** and EDCI coupling of the resulting primary amine with acid **11** were readily accomplished and gave amide **18** in 57% yield over 3 steps (Scheme 3). Benzyl amine cleavage with ceric ammonium nitrate (CAN), followed by UHP-assisted oxidation to the nitroxide then provided the desired ABNO analog **17** in 69% yield.²⁰

The preparation of the azaadamantyl-based nitroxide **8** (6-amino-1-Me-AZADO) was achieved in 10 steps from adamantane carboxylic acid (Scheme 4).²³ Selective dibromination of the adamantane ring in neat bromine at reflux required the presence of 2 equiv of AlBr₃ and was further accelerated by addition of catalytic BBr₃.²⁴ Curtius rearrangement of dibromo acid **19** provided the amine function for the attachment of the alkene peptide isostere targeting sequence. Grob fragmentation²⁵ of **20** in an autoclave at 160 °C followed by *N*-Boc protection led to the keto-alkene **21** in 42% yield over 4 steps. According to Iwabuchi's procedure,^{15b,c} oxime formation led to **22**, and reduction with NaBH₄/MoO₃ followed by iodoamination of the alkene furnished the azaadamantane core **23**. After removal of the iodine atom by catalytic hydrogenation, oxidation of the crude secondary amine with sodium tungstate and hydrogen peroxide led to the Boc-protected nitroxide **24** in 58% yield over 2 steps. Deprotection of **24** with HCl in dioxane completed the synthesis of **8**.

Unfortunately, similar to the difficulties encountered with **6**, coupling of **8** with acid **11** failed to furnish the desired analog **25**, possibly due to an in situ reduction of the nitroxide to the hydroxylamine followed by double *N*- and *O*-acylations (Scheme 5). Accordingly, an alternative synthetic route was used to overcome the high reactivity of the AZADO nitroxide. Diamine **26** was generated from precursor **23** in 2 steps. Selective coupling of the primary amine with acid **11**, and subsequent oxidation of the secondary amine allowed the isolation of the desired AZADO analog **25** in acceptable yield. The structure of **25** was confirmed by X-ray analysis (Figure 3).

Synthesis of Fluorophore-Tagged Analogs

Our first fluorophore labeled derivative contained a BODIPY®-FL substituent in place of the *N*-Boc group (Scheme 6). The direct synthesis of the labeled derivative from JP4-039 failed, since, after the Boc group of JP4-039 was successfully removed with HCl, treatment

of the free amine with the *N*-hydroxysuccinimide activated ester of BODIPY®-FL provided an 17:83 mixture of the desired compound and the undesired doubly-coupled side product. According to an LC/MS/UV analysis of the crude reaction mixture, an additional acylation had occurred at the nitroxide oxygen. In an alternative strategy, we attached a BODIPY®-FL label to the alkene peptide isostere prior to coupling to the 4-AT group. Accordingly, carboxylic acid **11** was protected as the methyl ester to give **27**. Removal of the Boc-protecting group with TFA followed by coupling to BODIPY®-FL-NHS (**28**) provided **29** in 82% yield. Saponification of the methyl ester under standard basic conditions resulted in extensive decomposition; therefore, pig liver esterase (PLE)²⁶ in acetone/pH 7 phosphate buffer was used to effect a chemoselective transformation. Coupling of the resulting acid to 4-AT provided the desired BODIPY®-FL-labeled compound **30** in 56% yield over 2 steps.

We expected that the formation of the biscoupling side product could be avoided in the fluorophore conjugation by carefully monitoring the equivalents of the BODIPY® *N*-hydroxysuccinimides. Thus, to streamline the synthesis of BODIPY®-R6G-JP4-039, we first reduced the nitroxide to the hydroxylamine with ascorbic acid (Scheme 7). This step was introduced since nitroxides can undergo acid-catalyzed disproportionations²⁷ and also display broadened NMR spectra leading to challenging characterization problems.²⁸ Removal of the Boc group with TFA and subsequent coupling to the NHS-derivative of BODIPY®-R6G provided **32**. Oxidation to the nitroxide provided BODIPY®-R6G-JP4-039 (**33**).

The BODIPY®-labeled agents were used to visualize GS-nitroxide distribution in mitochondria as a complement to the previously used EPR and MS methods. Compound **30** was applied for imaging in FancD20F (human Fanconi anemia) adherent cells.^{10a} In comparison to Mitotracker and BODIPY® alone, significant colocalization of the labeled nitroxide in mitochondria was clearly visible (Figure 4). Compound **33**, which minimizes spectral overlap with GFP, has also been shown to colocalize in mitochondria of KM101 (human bone marrow stroma) cell lines (see ESI, page S31).

Radioprotective and Radiomitigative Effects

The study of small molecules that ameliorate radiation damage is pressing due to the lack of selective and potent development candidates as well as the multiple therapeutic applications that such small molecules could find. Radiation protectors, administered prior to irradiation to prevent normal tissue damage, can be applied in radiation oncology. Clinical applications of radiation protectors also include treatments that decrease tissue damage and prevent accidental death in workers and patients exposed to high energy beams, such as α -, α -, and γ -radiation, cosmic and particle radiation, as well as UV radiation. Alternatively, compounds that are effective at mitigating radiation injury even when administered 24 to 48 h after the exposure event could be applied as a countermeasure in a radiation disaster situation such as a nuclear accident. These agents have to meet multiple safety requirements, such as selectivity for protection of normal tissue versus tumor tissue, ease of administration, and minimal toxicity. Only the phosphorothioate prodrug amifostine is currently in use for this purpose.²⁹

Irradiation survival assays were used to test the ability of selected nitroxide-conjugates to protect against, or mitigate, irradiation-induced damage of 32Dcl3 murine hematopoietic cells. The results for TEMPO- (JP4-039), TMIO- (**12**), ABNO- (**17**) and 1-Me-AZADO- (**25**) type nitroxides demonstrate that all structural classes are able to protect cells from multiple killing events if given 1 h before irradiation, as shown by their increased D_0 (Table 1).³⁰ If the nitroxide is given before irradiation, it may react with the free radicals produced by the photon of X-rays or γ -rays reacting with water in the cell, resulting in more photons having to be delivered to result in the one inactivating event, and thus increasing the dose. Notably, the protective activity of the nitroxides used herein can be classified as follows: **12** (TMIO) < JP4-039 (TEMPO) **25** (1-Me-AZADO) < **17** (ABNO). This order closely reflects their expected reactivity based on steric hindrance of the nitroxide species.

When given shortly after irradiation, all agents were also able to mitigate cell damage, according to the increase observed in $\tilde{n}$.³⁰ The $\tilde{n}$ is the low dose range of the survival curve where cell death results from an accumulation of events, none of which are lethal by themselves. These may be events which occur subsequently to the irradiation, such as production of free radicals due to damage to the mitochondria or other organelles and damage due to production of cellular cytokines from the initial injury to the cell. By treating with nitroxide after irradiation, if an inactivating event has already occurred, the nitroxide will have no effect on the D_0 . If the nitroxide is given at the time that the sublethal damage is occurring, then more sublethal events may be necessary to kill the cell, thus increasing the $\tilde{n}$ or shoulder on the curve. However, a simple correlation between the mitigative effect of the test compounds and the chemical reactivity of the nitroxide subunits could not be established. Further studies will be necessary to determine the SAR of nitroxides in radiation mitigation.

The fluorophore labeled compounds **30** and **33** maintained similar activity, indicating that they should be suitable tools with which to study the GS-nitroxides in cells. It is important to note that TEMPOL (**3**) alone does not have a significant protective or mitigative effect at a concentration of 10 μ M, displaying similar protection and mitigation as the negative control. The conjugation of the GS-derived peptide isostere sequence to the nitroxide is crucial for the observed irradiation survival properties.

Conclusion

We have designed a new series of mitochondria-targeted nitroxide conjugates as therapeutic lead structures for diseases where oxidative stress overcomes the natural biological defense mechanisms against superoxide accumulation, electron leakage, and radical escape. Aging, ischemia, neurodegeneration, inflammatory- and radiation-induced ailments are among the results of cellular damage that could be countered by appropriate electronically tuned and subcellular-targeted nitroxides.

Although the relative instability of ABNO and 1-Me-AZADO derivatives required optimizations of the synthetic processes, these new types of nitroxides were successfully tethered to the mitochondria-targeting sequence used for JP4-039. The resulting stable conjugates demonstrate comparable or improved biological activities in a cellular assay of

radioprotection and radiation damage mitigation. The activity of these nitroxides depends mainly on the steric and electronic environment in the immediate vicinity of the nitroxide center.

Overall, the new nitroxides described in this work represent new tool compounds for testing hypotheses for mechanism of action and developing more effective countermeasures against redox stress and mitochondrial decay.³¹ Further synthetic and biological studies on these lead structures are ongoing in our laboratories and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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 20. The NMR spectra of the hydroxylamines show two isomers, presumably corresponding to conformational equilibria of the azabicyclononane ring. Coalescence of the ^1H signals was observed by VT-NMR.
 21. The relative instability and difficult isolation of ABNO and other sterically relatively unshielded nitroxides were observed previously.^{14b–d} Recovery of the hydroxylamine, alone or as a mixture with the nitroxide, has also been noted^{14b,d}.
 22. The isolated yellow oil decomposed into a microcrystalline, colorless solid upon standing. The same observation was made by Mendenhall and Ingold for solutions of 8-azabicyclo[3.2.1]octane *N*-oxyl (ABOO), and explained by an irreversible dimerization process^{14b}.
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Figure 1.
Structural correlations between gramicidin S, XJB-5-131, and JP4-039.

Figure 2.
Structures of stable cyclic nitroxides.

Figure 3.
X-ray structure of **25** (CCDC 928524).

Figure 4.

Visualization of BODIPY®-JP4-039 (**30**) alone, BODIPY® alone, MitoTracker alone, and combinations of **30** at 1 μ M, 2.5 μ M, and 5 μ M concentrations with MitoTracker in FancD20F adherent cells. Yellow-orange coloring demonstrates MitoTracker/**30** colocalization in mitochondria. At higher concentrations of the labeled nitroxide, some cytoplasmic BODIPY®-JP4-039 is also visible (green). White arrows point toward mitochondria and show colocalization.

Scheme 1.
Synthesis of TMIO analog **12**.

Scheme 2.
Synthesis of unstable nitroxide **6** (3-amino-ABNO).

Scheme 3.
Synthesis of ABNO analog **17**.

Scheme 4.
 Synthesis of **8** (6-amino-1-Me-AZADO).

Scheme 5.

Synthesis of 1-Me-AZADO analog **25**: **A)** failed coupling of **8** with **11**, and **B)** successful coupling of **26** with **11**.

Scheme 6.
Synthesis of BODIPY[®]-FL-JP4-039 (**30**).

Scheme 7.
Synthesis of BODIPY®-R6G-JP4-039.

Table 1Irradiation survival curve parameters in 32Dcl3 cells^a.

Compound	Before Irradiation ^b		After Irradiation ^c	
	D ₀ (Gy)	$\tilde{n}$	D ₀ (Gy)	$\tilde{n}$
Control ^d	1.3 ± 0.1	1.0 ± 0.1	1.6 ± 0.2	1.1 ± 0.1
TEMPOL (3)	1.3 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	1.6 ± 0.3
JP4-039	2.3 ± 0.3 (p = 0.043)	1.0 ± 0.1	1.2 ± 0.1	3.5 ± 0.2 (p = 0.0001)
30 (BODIPY®-FL-JP4-039)	2.6 ± 0.5 (p = 0.048)	1.0 ± 0.1	1.5 ± 0.2	4.5 ± 1.1 (p = 0.0026)
33 (BODIPY®-R6G-JP4-039)	N.D.	N.D.	1.3 ± 0.1	4.8 ± 1.0 (p = 0.020)
12 (TMIO)	1.8 ± 0.1 (p = 0.029)	1.0 ± 0.1	1.4 ± 0.1	2.5 ± 0.3 (p = 0.007)
17 (ABNO)	3.1 ± 0.6 (p = 0.048)	1.0 ± 0.1	1.3 ± 0.1	2.7 ± 0.7 (p = 0.088)
25 (1-Me-AZADO)	2.6 ± 0.4 (p = 0.041)	1.0 ± 0.1	1.3 ± 0.1	2.5 ± 0.3 (p = 0.0065)

^a 32Dcl3 cells were placed in methylcellulose and incubated for 7 d at 37 °C in a CO₂ incubator. Colonies of > 50 cells were counted and data were analyzed using linear quadratic or single-hit, multi-target models. For a detailed description, see ESI (p. S29-S31).

^b Cells were incubated in the presence of 10 μM nitroxides for 1 h and then irradiated to doses ranging from 0 to 8 Gy.

^c Cells were irradiated and then placed in media containing 10 μM nitroxides.

^d Control = untreated 32Dcl3 cells.

N.D. = not determined.