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Effectiveness of Analogs of the GS-Nitroxide, JP4-039, as Total Body Irradiation Mitigators

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Abstract. *Background/Aim: Mitochondrial-targeted gramicidin S (GS)-nitroxide, JP4-039, has been demonstrated to be a potent radiation mitigator, and safe over a wide dose range. In addition, JP4-039 has organ-specific effectiveness when locally applied. Materials and Methods: We tested the effect of another GS-nitroxide, XJB-5-131, which has more effective mitochondrial localization, and compared these results to those for radiation mitigation against the hematopoietic syndrome, and two analogs of JP4-039, which have the same mitochondrial localization signal, but different chemical payloads: JRS527.084 contains a second nitroxide per molecule, and TK649.030 contains an ester group attached to the nitroxide. Results: The results demonstrate the superiority of JP4-039 as a systemic radiation mitigator. Conclusion: Structure–activity relationships and bioassays demonstrate that JP4-039 is an optimized small-molecule radiation mitigator.*

Ionizing irradiation damage to cells, tissues, organs, and organ systems has been demonstrated to have several phases, the initial phase being programmed cell death or apoptosis (1-6).

Using mouse models, we previously demonstrated that a mitochondrial-targeted antioxidant and free radical scavenger based on the core nitroxide, 4-amino-Tempo, and targeted to mitochondria by a segment of a cyclopeptide antibiotic, gramicidin S (1, 7-9), was broadly effective against total body irradiation-induced hematopoietic syndrome (10), when drugs are administered before or as late as 72 h after irradiation (6, 10-17).

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Recently, we demonstrated that the mechanism of radiation mitigation and protecion is due to mitochondrial localization, and abrogation of oxidative stress-mediated events in the mitochondrial membrane (2-4), which led to inhibition of cardiolipin binding to cytochrome *c* (16, 17), and leakage of cytochrome *c* into the cytoplasm followed by activation of the caspase system and apoptosis (6, 13, 14). Gramicidin S (GS)-nitroxide, JP4-039, has been shown to be effective when delivered systemically in a novel emulsion F14 (1, 10). This emulsion is also effective in ameliorating beta irradiation-induced skin damage (18).

In addition to the effectiveness of JP4-039/F14 delivered systemically or through the skin, a second formulation, F15, has been demonstrated to localize active drug in the oral cavity/oropharynx and significantly ameliorates single-fraction or fractionated irradiation-induced mucositis (19-24).

Previous studies have demonstrated a 30- to 33-fold concentration of 4-amino-Tempo nitroxide at the mitochondria when linked to JP4-039 (14, 15, 23). Another GS-nitroxide, XJB-5-131, has been shown to have a 600-fold mitochondrial concentration in the mitochondria (24), attributable to the longer mitochondrial localization sequence, which was also derived from the natural antibiotic GS.

The mitochondrial-targeted GS-nitroxide, JP4-039, delivered *i.v.* 24-72 h after $LD_{50/30}$ total body irradiation (TBI) mitigates irradiation damage (1). To optimize mitochondrial-targeted nitroxide irradiation mitigation, we designed two structurally related, but distinct mitochondrial-targeted nitroxides and compared each with JP4-039, and another GS-nitroxide, XJB-5-131 (24).

In the present studies, we tested whether XJB-5-131 compared to JP4-039 was a TBI mitigator. We also determined whether chemically-synthesized analogs of JP4-039, one using the same targeting sequence, but carrying two nitroxides per molecule (JRS527.084) or a prodrug of JP4-039 (TK649.030), were equivalent or superior to the lead compound (Figure 1).

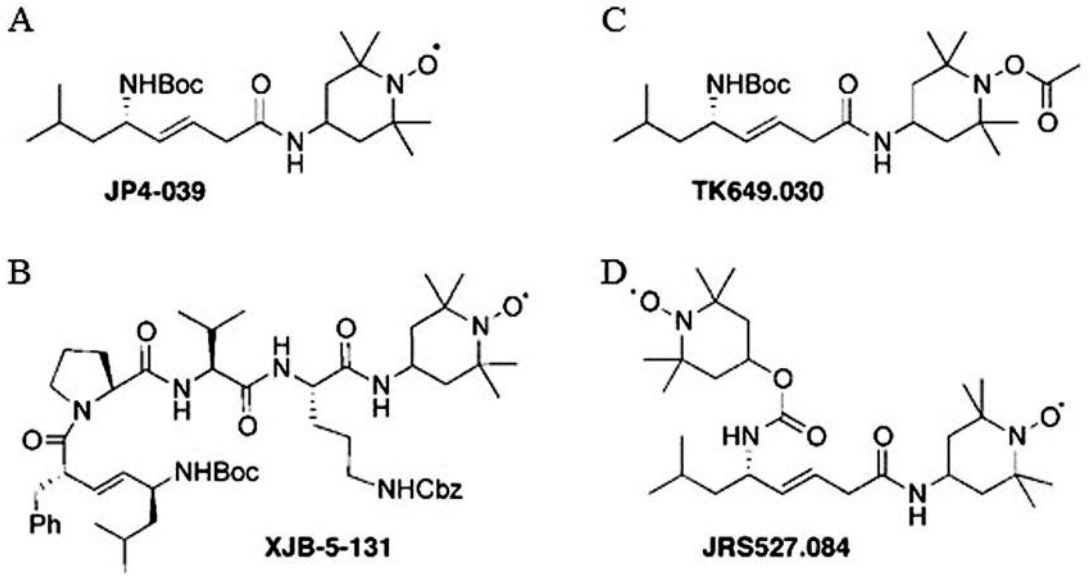

Figure 1. *Structure of gramicidin S (GS)-nitroxide radiation mitigators. Small molecules containing 4-amino-Tempo and each of two mitochondrial targeting linkers were constructed: A: JP4-039, B: XJB-5-131, C: ester attached to nitroxide generating an ester pro-drug (TK649-030) and D: new construct with two nitroxides per molecule (JRS527.084).*

Materials and Methods

Drug design and synthesis. JP4-039 and XJB-5-131 contain both a 4-amino-Tempo nitroxide linked to an alkene peptide isostere segment derived from the antibiotic GS (Figure 1). These compounds have proven significant and differential affinity for mitochondria *in vivo*. XJB-5-131 has a longer peptide-targeting sequence and results in a 600-fold enrichment in the mitochondria compared to cytoplasm, while JP4-039 contains a shortened alkene dipeptide isostere moiety attached to the nitroxide, which results in a 32-fold enrichment of nitroxide to the mitochondria compared to the cytoplasm (23).

We also tested JRS527.084, which has the identical shortened alkene peptide isostere backbone to that of JP4-039, but with a second nitroxide attached to the allylic amine function. TK649.030 has the same sequence as JP4-039 but is linked to an acetate at the nitroxide oxygen, forming a base-cleavable hydroxylamine/nitroxide prodrug moiety. The synthesis of JP4-039, XJB-5-131, JRS527.084, and TK649.030 has been previously reported (25). The methods for preparation of the formulation of F14 have been published previously (26, 27). Briefly, each GS-nitroxide was prepared in 100 μl volumes of F14 for intravenous injection according to published methods (26, 27). The molarity of each GS-nitroxide analog was calculated to standardize the molarity of drug delivery in a 100 μl. JP4-039 was formulated at 2 mg/ml, TK649.030 at 2.2 mg/ml, JRS527.084 at 2.5 mg/ml, and XJB-5-131 at 4.0 mg/ml.

Animals, their care and irradiation. C57BL/6NTac female mice (30-33 g, 6-8 weeks old, n=15 per group; Taconic Biosciences, Hudson, NY, USA) were housed four per cage according to Institutional IACUC regulations and fed standard laboratory chow and deionized water (PHS Assurance Number A3187-01).

Mice were irradiated to the radiation dose which killed 50% of animals at 30 days ($LD_{50/30}$) due to death from the hematopoietic syndrome (1) using a gamma cell cesium-137 irradiator (JL Shepherd and Associates, San Fernando, CA USA) at a dose rate of 343 cGy/per minute. The dose rate was increased to 343 cGy/per minute over the baseline 70 cGy/per minute (according to the manufacturer's specifications) by removing the filter in the irradiator. This resulted in a change in x-ray beam quality with respect to the number of cesium gamma rays in the spectrum.

Mice were irradiated to 9.5 Gy TBI and injected intravenously 24 h later with 10 mg/kg JP4-039/F14, or equimolar doses of another GS-nitroxide to give the same dose in 100 μl F14 to provide groups treated with XJB-3-131/F14, JRS527.084/F14, TK649.030/F14, or F14 alone.

Mice were monitored for signs of weakness and weight loss according to Institutional IACUC protocols and were euthanized at the time of loss of 20% body weight.

Statistical analysis. Calculation of differences in survival after TBI (n=15 per group) and the effect of each drug delivered at 24 h after irradiation were calculated according to previous publication (1).

Results

The molecular structure of JP4-039 and XJB-5-131 compared to the new test drugs JRS527.084 and TK649.030 are shown in Figure 1. JRS527.084 and TK649.030 were tested for the first time in a radiation model. JRS527.084 has the same GS-derived alkene peptide isostere backbone as JP4-039, but twice the nitroxide loading. TK649.030 has an identical backbone structure as JP4-039, but its nitroxide is masked by

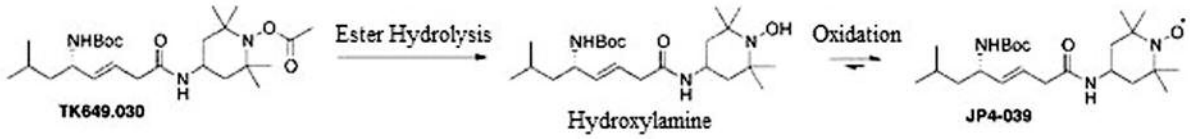

Figure 2. *Mechanism of prodrug activation in TK649.030 by ester hydrolysis followed by hydrogen atom abstraction/oxidation of the hydroxylamine to give the nitroxide.*

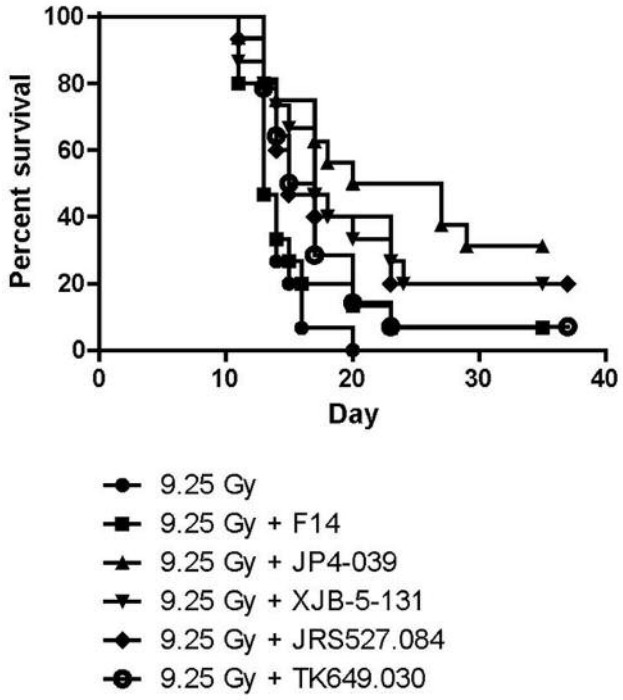

Figure 3. *Comparison of each nitroxide for mitigation of the effect of total body irradiation in vivo. JP4-039, XJB-5-131, TK649.030 and JRS527.084 were encapsulated in F14 liposomes (sesame seed oil, soy phosphatidyl choline and Dulbecco's PBS). C57BL/6NTac mice were irradiated to a dose of 9.5 Gy total body irradiation. Twenty-four hours later, the mice were injected i.v. with F14 only, JP4-039/F14, XJB-5-131/F14, TK649.030/F14, or JRS527.084/F14 and followed for the development of hematopoietic syndrome. All four small molecules led to increased survival compared to mice treated with 9.5 Gy alone (p<0.05) (see Table I).*

Table I. *Effect of gramicidin S (GS)-nitroxides on mitigation of the effect of total body irradiation. Time to median survival (50%) and the percentage of mice surviving at 35 days after 9.5 Gy were determined. Mice injected with JP4-039/F14 had the greatest probability of increased survival, a median survival of 20 days after irradiation, while all other groups had a median survival of 17 days or less. Mice treated with JP4-039/F14 also had more survivors at 35 days (33%) compared to the other treatment groups.*

Treatment group (n=15/group)	Median survival (days)	Survival at 35 days after 9.5 Gy (%)
9.5 Gy	13	0
9.5 Gy + F14	14	2.2 p=0.1544 *vs*. 9.5 Gy
9.5 Gy + JP4-039/F14	20	33 p=0.0003 *vs*. 9.5 Gy p=0.0027 *vs*. F14
9.5 Gy + XJB-5-131/F14	17	20 p=0.0017 *vs*. 9.5 Gy p=0.0343 *vs*. F14
9.5 Gy + TK649.030/F14	15	6.6 p=0.0058 *vs*. 9.5 Gy p=0.0547 *vs*. F14
9.5 Gy + JRS527.084	17	20 p=0.0051 *vs*. 9.5 Gy p=0.0800 *vs*. F14

a base-labile ester group that releases the Tempo-hydroxylamine upon spontaneous hydrolysis or by conversion with a lipase or peptidase. The hydroxylamine is further oxidized to the nitroxide in biological media containing molecular oxygen or other natural redox systems (Figure 2).

Comparison of JP4-039 to other GS-nitroxides as radiation mitigators *in vivo*. JP4-039 was a highly effective radiation mitigator when delivered at 24 h after TBI. As shown in Figure 3, comparison of the effect of equimolar concentrations of each of the four GS-nitroxides also showed JP4-039 to be the superior mitigator.

Compared to mice treated with 9.5-Gy irradiation only, each nitroxide demonstrated significant mitigation. There was no significant mitigation by F14 alone (p=0.1544 for F14 alone, 0.0003 for JP4-039/F14, 0.0017 for XJB-5-131/F14, 0.0058 for JRS527.084/F14, and 0.0051 for TK649.030/F14). There were no significant differences between the four nitroxides; however, JP4-039 led to the best median survival (Table I).

Discussion

Mechanism of JP4-039 mitigation. The mitochondrial-targeted nitroxides tested in this study all mitigate irradiation damage to different extents. In the absence of significant additional mitigation by the three new GS-nitroxides, the

development of the small molecule, JP4-039, as a radiation mitigator will be initiated. A possible explanation for the observed superior effects of JP4-039 *vs*. the more highly mitochondrially targeted XJB-5-131 is that radiation damage requires the presence of significant quantities of mitigator both in the cytosol as well as in mitochondria, a requirement that puts the almost exclusively mitochondrial-targeting XJB-5-131 at a disadvantage. Furthermore, the removal of the prodrug function from TK649.030 might not be sufficiently fast to rival the effective concentration of JP4-039. Finally, we hypothesize that due to the additional nitroxide moiety in JRS527.084 and the accompanying loss of the Boc-group on the allylic amine, this compound has a greater translocation into the mitochondria than JP4-039, and consequently a lower effective concentration in the cytosol, but this hypothesis remains to be experimentally verified. Alternatively, the observed effects could be due to differential *in vivo* absorption, distribution, metabolism, and excretion properties of these analogs.

The present data support moving forward with JP4-039 as our lead compound for drug development of a mitigator for the acute radiation syndrome (hematopoietic failure after TBI).

Conflicts of Interest

MWE, PW, and JSG have been awarded a United States Patent for the use of Mitochondrial Targeted Nitroxide Agents as Radiation Mitigators.

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