Mitochondrial targeting of radioprotectants using peptidyl conjugates

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Ionizing radiation activates a mitochondrial nitric oxide synthase, leading to inhibition of the respiratory chain, generation of excess superoxide, peroxynitrite production and nitrosative damage. We have measured the radioprotective effects of a nitric oxide synthase antagonist (AMT) *versus* a free radical scavenger (4-amino-TEMPO) using electrochemical detection of nitric oxide and peroxynitrite. To enhance their efficacy, we have conjugated these compounds to peptides and peptide isosteres—derived from the antibiotic gramicidin S—that target the mitochondria. The targeting ability of these peptidyl conjugates was measured using quantitative mass spectrometry.

Introduction

Each year a large number of patients undergo radiation therapy for various pelvic malignancies. It has been reported that as many as 75% of these patients develop radiation cystitis.[1]

Ionizing radiation activates a variety of cytoplasmic transduction pathways, some of which are believed to be mediated by reactive nitrogen and oxygen species (RNS and ROS).[2] We have evidence that ionizing radiation turns on a mitochondrial nitric oxide synthase (mtNOS) in the uroepithelial cells that line the urinary bladder.[3,4] As a consequence, nitric oxide (NO) is produced in large amounts and can inhibit respiration, resulting in superoxide (O_2^-) production. NO and O_2^- can react to form peroxynitrite (ONO_2^-) which damages complexes I and III of the respiratory chain.[5,6] We have demonstrated that the presence of a NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM), in the bladder during irradiation, is radioprotective.[4]

Protection against irradiation damage using systemic drug administration can result in unwanted side effects. One approach to limit or prevent these adverse side effects is to target drug delivery to the mitochondria. For example, a structural motif based on alternating aromatic and basic amino acid residues (*i.e.*, *Dmt*-D-*Arg*-*Phe*-*Lys*-NH_2 and D-*Arg*-*Dmt*-*Lys*-*Phe*-NH_2) can be employed.[7] These peptide conjugates are cell-permeable in a passive manner and concentrate up to 1000 fold in the inner mitochondrial membrane,[8] thereby allowing the use of lower drug concentrations. We have developed an alternative strategy by taking advantage of the naturally occurring membrane-active antibiotic, gramicidin S (GS). The bioavailability of a GS fragment was improved by replacing critical amide bonds with (*E*)-alkene peptide isosteres.[9,10] The length of the GS-derived segment can be varied and the ornithine side chain amines can be acylated to modulate cell membrane passage *vs.* mitochondrial targeting. By accumulating the prodrugs at the inner membrane, the active drugs are in close proximity to the sites of mitochondrial NO and O_2^- production when released into the matrix. The targeting sequences themselves are not toxic to cells even when present at 100 μM concentrations, which is consistent with their lack of effect on $\Delta\Psi$.[7] A targeted delivery strategy is essential since some NOS antagonists and most antioxidants, including nitroxide derivatives, are poorly cell-permeable and would require therapeutically effective concentrations >100 μM if used without a conjugate. A further advantage of targeted delivery is that a prodrug timed-release function can be incorporated that increases the duration of action of these radioprotectants.[11]

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Results and discussion

For this study, one of the more potent NOS inhibitors, the non-arginine analog 2-amino-6-methylthiazine (AMT), was selected because of its small size and the presence of the terminal amino group which could readily be acylated. Since we have previously demonstrated that irradiation results in increased production of both NO and O_2^- in the uroepithelial cells that line the bladder, we treated these cells with AMT (10 to 100 μM) or 4-amino-2,2,6,6-tetramethyl-piperidine-*N*-oxyl (4-amino-TEMPO; 10 to 100 μM) to determine if inhibition of NO or scavenging of free radicals is more protective. We found both compounds to be ineffective (not shown). Therefore, we took the unconjugated and conjugated AMT (100 μM; not shown) and unconjugated and conjugated 4-amino-TEMPO (100 μM; Fig. 1a and 1c) and incubated them for two hours at 37 °C with 32Dcl3 hemopoietic cells to determine their ability to get into mitochondria. Following incubation, the cells were lysed and the mitochondria isolated for mass spectrometry analysis. The compounds isolated from mitochondria typically were identified as Na^+ adducts (+23 Daltons). The spectra for mitochondria incubated with conjugated-TEMPO (which is partially converted into the reduced, hydroxylamine form) show a peak at 999.59 (975.59 + H + Na^+; Fig. 1d) while the unconjugated 4-amino-TEMPO (reduced) does not show a peak at 173.17 (M + H + H^+) (Fig. 1b). This discrepancy demonstrates that 4-amino-TEMPO only enters the mitochondria by means of the attached GS-derived targeting sequence. The spectra for mitochondria incubated with conjugated AMT show a peak at 957.49 (934.49 + Na^+) and there is also no peak in the case of unconjugated AMT at 131.06 (M + H^+), indicating that AMT by itself also does not enter the mitochondria. Thus, the targeting peptides successfully direct a NOS antagonist and a nitroxide free radical scavenger to the mitochondria.

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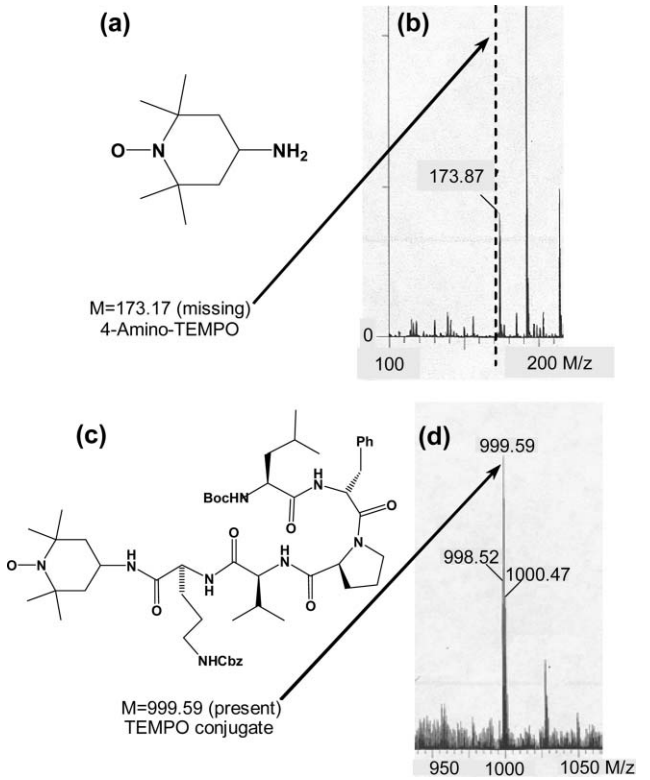

Fig. 1 Unconjugated (a) and conjugated (c) 4-amino-TEMPO (XJB-5-125), at 100 μM and spectra for mitochondria incubated with unconjugated (b) and conjugated (d) 4-amino-TEMPO.

Physiological studies were then conducted to determine the effects of peptide-targeted AMT and 4-amino-TEMPO on NO and ONO_2^- production in irradiated uroepithelial cells. The cells were cultured in 8-well slide chambers (250 μl/well) for 3 days and then microsensor measurements were taken 24 hours after irradiation. In untreated irradiated cells (not shown) and cells treated with unconjugated 4-amino-TEMPO (100 μM; Fig. 2b)

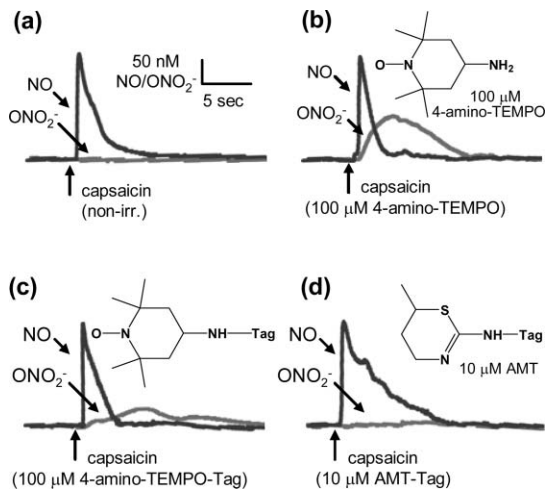

Fig. 2 Capsaicin evoked NO production and formation of ONO_2^- simultaneously measured by microsensors in (a) non-irradiated cells, and irradiated cells treated with (b) unconjugated 4-amino-TEMPO (100 μM), (c) high-dose conjugated 4-amino-TEMPO (XJB-5-125; 100 μM) and (d) conjugated-AMT (XJB-5-127; 10 μM).

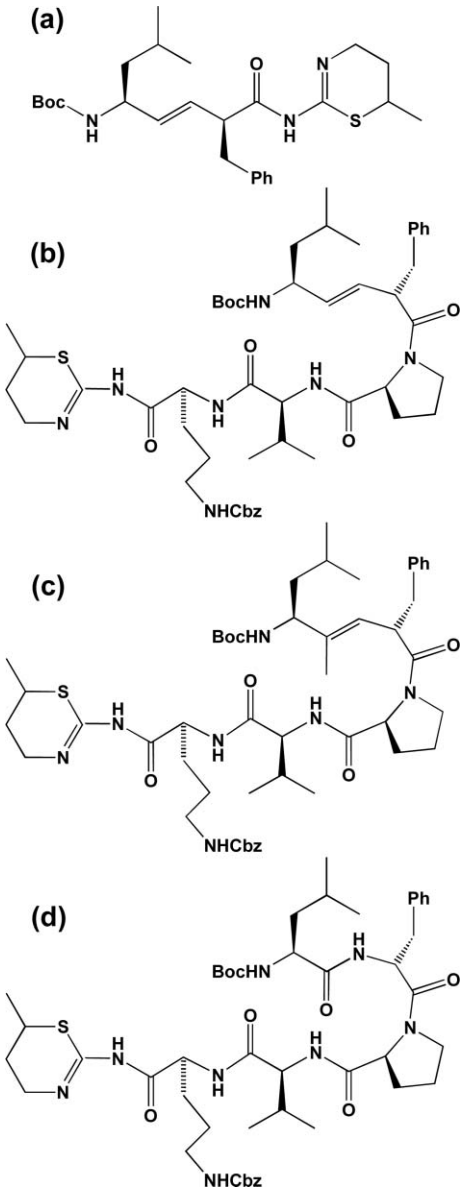

Fig. 3 Chemical structures of compounds (a) XJB-5-234, (b) XJB-5-133, (c) XJB-5-241 and (d) XJB-5-127.

or unconjugated AMT (100 μM; not shown), agonist (capsaicin, 1 μM) evoked NO production resulted in the formation of comparable amounts of ONO_2^-. In cells treated with high-dose conjugated 4-amino-TEMPO (100 μM; Fig. 2c), ONO_2^- production was significantly decreased. However, in non-irradiated cells (Fig. 2a), or cells treated with conjugated-AMT (10 μM; Fig. 2d), NO induced ONO_2^- formation was almost completely abolished or not observed ($n = 6$).

These findings suggest that peptide conjugates drag membrane impermeant 4-amino-TEMPO and AMT across the mitochondrial membrane and that they do not interfere with the free radical scavenging activity of 4-amino-TEMPO or the NOS inhibitory activity of AMT.

In addition, quantitative mass spectroscopy was used to compare the effectiveness of different AMT conjugates in penetrating the mitochondria. For these studies, compounds XJB-5-234, XJB-5-133, XJB-5-241 and XJB-5-127 (Fig. 3) were used.

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Table 1 Quantification of compound localized in the mitochondrial fraction (see Fig. 3 for structures)

Compound	Fmole/10 μg mitochondrial protein
XJB-5-234 (a)	1.45
XJB-5-133 (b)	89.8
XJB-5-241 (c)	103.3
XJB-5-127 (d)	50.8

As mentioned above, when unconjugated AMT was administered, it was not detected in the mitochondrial fraction (at or below noise level). The amounts of the other compounds are shown in Table 1.

As indicated in Table 1, the most efficacious conjugate was compound XJB-5-241. The trisubstituted (*E*)-alkene moiety embedded in XJB-5-241 has a much stronger conformational effect than the less biologically active disubstituted (*E*)-alkene (XJB-5-133) or the GS peptidyl fragment XJB-5-127.[12–14] Therefore, we hypothesize that a defined secondary structure and an appropriate conformational preorganization is important in accomplishing efficient mitochondrial delivery. The presence of non-hydrolyzable alkene isostere functions in place of labile peptide bonds is also significant for a prolonged mechanism of action. The least efficacious conjugate was XJB-5-234. This may be due to the lack of a complete targeting sequence.

In this study, the targeting of a NOS antagonist was more radioprotective than the targeting of a free radical scavenger. However, previous studies have demonstrated that when a NOS antagonist and a free radical scavenger were administered as a dual-function molecule, therapeutic effects were greater than when given together but unlinked.[15,16] We hypothesize that this may be a consequence of the fact that when mtNOS is undergoing inhibition, it can produce both NO and O_2^- resulting in ONO_2^- formation. The dual-action drug may locally inhibit both NO and O_2^- production, thereby preventing ONO_2^- formation which protects the mtNOS enzyme. Accordingly, future plans include the synthesis and analysis of the radioprotective effects of targeted dual-action compounds.

Conclusion

While it is easy to deliver NOS antagonists or free radical scavengers intravesically to the bladder, their systemic administration can cause adverse side-effects such as hypertension or altered stomach motility. Mitochondrial targeting of these compounds, using a peptide dragging strategy, enhances their radioprotective effects and avoids these adverse complications. While not evaluated in this study, these novel targeting peptides can be optimized for prodrug timed-release that can increase their duration of action when used to treat, rather than protect, against radiation damage. This feature is dependent upon the structure of the peptide chain which can change the time-release profile from minutes to hours to obtain prolonged therapeutic effects.

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References

1 P. F. Schellhammer, G. H. Jordan and A. M. el-Mahdi, *World J. Surg.*, 1986, **10**, 259–268.
2 M. W. Epperly, C. A. Sikora, S. J. DeFilippi, J. A. Gretton, Q. Zhan, D. W. Kufe and J. S. Greenberger, *Radiat. Res.*, 2002, **157**, 568–577.
3 A. J. Kanai, L. L. Pearce, P. R. Clemens, L. A. Birder, M. M. VanBibber, S. Y. Choi, W. C. de Groat and J. Peterson, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 14126–14131.
4 A. Kanai, M. Epperly, L. Pearce, L. Birder, M. Zeidel, S. Meyers, J. Greenberger, W. de Groat, G. Apodaca and J. Peterson, *Am. J. Physiol.*, 2004, **286**, H13–21.
5 L. L. Pearce, A. J. Kanai, M. W. Epperly and J. Peterson, *Nitric Oxide*, 2005, **13**, 254–263.
6 L. L. Pearce, M. W. Epperly, J. S. Greenberger, B. R. Pitt and J. Peterson, *Nitric Oxide*, 2001, **5**, 128–136.
7 K. Zhao, G. M. Zhao, D. Wu, Y. Soong, A. V. Birk, P. W. Schiller and H. H. Szeto, *J. Biol. Chem.*, 2004, **279**, 34682–34690.
8 J. Dessolin, M. Schuler, A. Quinart, F. De Giorgi, L. Ghosez and F. Ichas, *Eur. J. Pharmacol.*, 2002, **447**, 155–161.
9 J. Xiao, B. Weisblum and P. Wipf, *J. Am. Chem. Soc.*, 2005, **127**, 5742–5743.
10 P. Wipf, J. Xiao, J. Jiang, N. A. Belikova, V. A. Tyurin, M. P. Fink and V. E. Kagan, *J. Am. Chem. Soc.*, 2005, **127**, 12460–12461.
11 P. Wipf, W. Li, C. M. Adeyeye, J. M. Rusnak and J. S. Lazo, *Bioorg. Med. Chem.*, 1996, **4**, 1585–1596.
12 P. Wipf, T. C. Henninger and S. J. Geib, *J. Org. Chem.*, 1998, **63**, 6088–6089.
13 P. Wipf, J. Xiao and G. S. J., *Adv. Synth. Catal.*, 2005, **347**, 1605–1613.
14 P. Wipf and J. Xiao, *Org. Lett.*, 2005, **7**, 103–106.
15 P. E. Chabrier, M. Auguet, B. Spinnewyn, S. Auvin, S. Cornet, C. Demerle-Pallardy, C. Guilmard-Favre, J. G. Marin, B. Pignol, V. Gillard-Roubert, C. Roussillot-Charnet, J. Schulz, I. Viossat, D. Bigg and S. Moncada, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 10824–10829.
16 S. Auvin, M. Auguet, E. Navet, J. J. Harnett, I. Viossat, J. Schulz, D. Bigg and P. E. Chabrier, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 209–212.