ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2018, **54**, 256

Received 6th November 2017,
Accepted 1st December 2017

DOI: 10.1039/c7cc08482j

rsc.li/chemcomm

Oxidative activation of leinamycin E1 triggers alkylation of guanine residues in double-stranded DNA†

Maryam Imani Nejad,[a] Dong Yang,[bc] Ben Shen*[bcd] and Kent S. Gates*[ae]

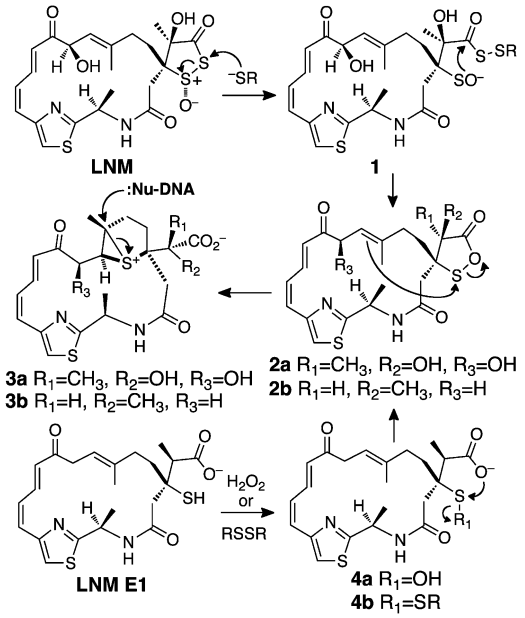
It may be useful to develop prodrugs that are selectively activated by oxidative stress in cancer cells to release cell-killing reactive intermediates. However, relatively few chemical strategies exist for the activation of prodrugs under conditions of oxidative stress. Here we provide evidence for a novel process in which oxidation of a thiol residue in the natural product leinamycin E1 by H_2O_2 and other byproducts of cellular oxidative stress initiates generation of an episulfonium ion that selectively alkylates guanine residues in duplex DNA.

In cancer chemotherapy, it would be advantageous to develop prodrugs that are selectively activated in tumor tissue. This requires identification of features that are unique to cancer cells or the tumor microenvironment.[1] For example, there have been longstanding efforts to identify agents that are transformed to cytotoxic DNA-damaging intermediates in the hypoxic tissue found in some solid tumors.[2–4] Alternatively, evidence indicates that some cancer cells reside in a state of oxidative stress characterized by higher than normal levels of reactive oxygen species (ROS) such as H_2O_2 due to increased growth factor signaling, metabolic rate, and mitochondrial dysfunction.[5–8] This has led to the idea that oxidative stress may represent a new "therapeutic target" in cancer and has inspired efforts to develop oxidatively-activated prodrugs that release cytotoxic DNA-damaging agents following reaction with H_2O_2.[6,9–14] To date, however, there are few chemical strategies for the activation of prodrugs by H_2O_2 or other byproducts of oxidative stress in cells.[9–14] In the work described here, we characterize a novel process involving oxidatively-activated generation of a cytotoxic DNA-alklyating agent by an analog of the natural product leinamycin (LNM).

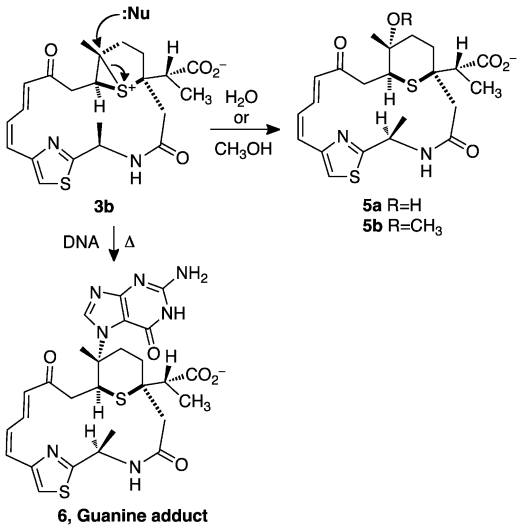
LNM is a *Streptomyces*-derived natural product with an unprecedented molecular architecture that includes an 18-membered, thiazole-containing macrolactam spiro-fused to a 1,2-dithiolan-3-one 1-oxide heterocycle.[15–17] LNM displays activity in the low nanomolar concentration range against various human cancer cell lines[15,16,18,19] and activity *in vivo* against tumors that are resistant to important anticancer drugs such as cisplatin, doxorubicin, mitomycin, and cyclophosphamide.[18,20] LNM is a prodrug that is rapidly converted to a potent DNA-damaging agent when it enters the thiol-rich intracellular environment.[18,21–25] Attack of thiolate on the central sulfur atom of the 1,2-dithiolan-3-one 1-oxide heterocycle in LNM generates the ring-opened compound **1**, followed by cyclization involving attack of the sulfenate residue on the adjacent carbonyl group to give the 1,2-oxathiolan-5-one derivative **2** (Scheme 1).[26–28] The electrophilicity of the sulfur center in **2** drives an intramolecular rearrangement involving the C6–C7 alkene of the 18-membered macrolactam to produce the DNA-alkylating episulfonium ion (**3**, Scheme 1). Alkylation of guanine residues in duplex DNA by **3** is enabled by noncovalent association of LNM with the double helix involving intercalation of the extended thiazolyl-2,4-pentadienone pi-system on the 3′-face of target nucleobase residues.[29–31] The 7-alkylguanine residues generated by LNM undergo rapid depurination to leave abasic sites on the DNA backbone.[32,33]

We recently isolated and characterized the first biosynthetically engineered LNM analog, LNM E1, from SB3033, a Δ*lnmE* mutant strain of *S. atroolivaceus* S-140.[34] LNM E1 lacks the 1,2-dithiolan-3-one 1-oxide heterocycle that is critical for thiol-initiated generation of the DNA-alkylating episulfonium ion in the parent natural product. Interestingly, under oxidative conditions, LNM E1 undergoes rearrangement to give the hydrolysis and methanolysis products **5a** and **b** (Scheme 2) that are diagnostic for episulfonium ion formation.[34] Indeed, LNM E1 is selectively toxic to cancer cell lines with high levels of oxidative stress.[34] It was proposed that oxidation of LNM E1 to the sulfenic acid derivative **4a** affords an

[a] *Department of Chemistry, University of Missouri, 125 Chemistry Bldg, Columbia, MO 65211, USA. E-mail: gatesk@missouri.edu*
[b] *Department of Chemistry, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA. E-mail: shenb@scripps.edu*
[c] *Natural Products Library Initiative at The Scripps Research Institute, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA*
[d] *Department of Molecular Medicine, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA*
[e] *Department of Biochemistry, University of Missouri, Columbia, MO 65211, USA*
† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7cc08482j



Scheme 1 Activation of LNM and LNM E1.



Scheme 2 Fates of the LNM E1-derived episulfonium ion.

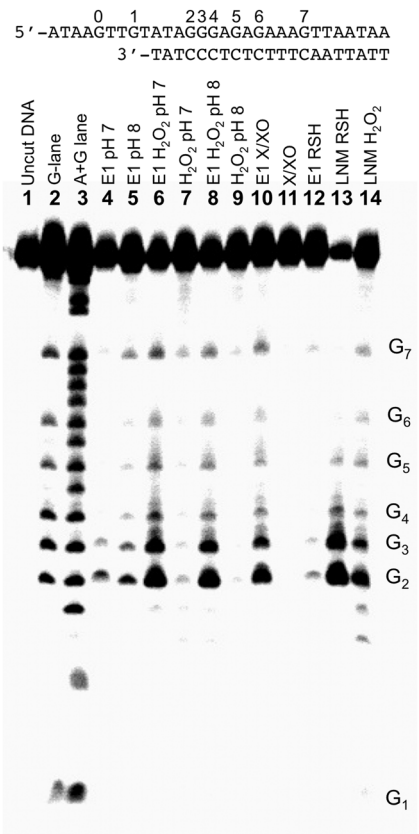


Fig. 1 Oxidatively-activated DNA damage by LNM E1. The DNA duplex shown was 5′-^{32}P-labeled on the top strand. Reactions were incubated for 24 h, followed by piperidine workup and 20% denaturing polyacrylamide gel electrophoretic analysis. Lane 1: untreated duplex; lane 2: Maxam–Gilbert G reaction; lane 3: A + G reaction; lane 4: LNM E1 (200 μM), pH 7; lane 5: LNM E1 (200 μM), pH 8; lane 6: LNM E1 (200 μM) + H_2O_2 (500 μM) pH 7; lane 7: H_2O_2 (500 μM) pH 7; lane 8: LNM E1 (200 μM) + H_2O_2 (500 μM) pH 8; lane 9: H_2O_2 (500 μM) pH 8; lane 10: LNM E1 (200 μM) + xanthine (250 μM) + xanthine oxidase (1 unit); lane 11: xanthine (250 μM) + xanthine oxidase; lane 12: LNM E1 (200 μM) + 2-mercaptoethanol (500 μM); lane 13: LNM (100 μM) + 2-mercaptoethanol (500 μM); lane 14: LNM (100 μM) + H_2O_2 (500 μM).

alternative entry into the LNM rearrangement reaction manifold (Scheme 1); however, the ability of H_2O_2 and various byproducts of oxidative stress to activate DNA alkylation by LNM E1 under physiologically-relevant conditions has not been examined until now.

We incubated LNM or LNM E1 with 5′-^{32}P-labeled DNA duplexes in the presence of various activating agents, followed by piperidine workup (0.1 M, 90 °C, 25 min) and gel electrophoretic analysis of the labeled DNA fragments resulting from cleavage at alkylation sites. As expected,[29,31] a control experiment in which the parent natural product LNM was activated by 2-mercaptoethanol generated high yields of thiol-triggered DNA strand cleavage products that were consistent with alkylation of guanine residues in the target duplex (Fig. 1, lane 13). In contrast, LNM generated relatively weak strand cleavage under oxidative conditions, in the presence of H_2O_2 (lane 14). LNM E1 generated low levels of strand cleavage at guanine residues alone or in the presence of 2-mercaptoethanol (lanes 4 and 12). Importantly, LNM E1 in the presence of H_2O_2 produced substantial yields of strand cleavage that were well above the background levels generated by H_2O_2 alone (lanes 6 and 8). The products were consistent with strand cleavage arising from alkylation of guanine residues in the DNA duplex. Comparative time course and concentration profiles revealed that the yield of DNA alkylation by LNM E1 + H_2O_2 was similar to that generated by the parent natural product LNM + thiol (Fig. S1–S6, ESI†).

Previous studies showed that the LNM-derived episulfonium ion displays sequence preferences that are distinct from other DNA-alkylating agents.[31] Thus, it is significant that the sequence specificity of the DNA damage generated by LNM E1 in duplex DNA closely mirrors that caused by the parent compound LNM (Fig. 2B and Fig. S7, ESI†).[31] Damage is primarily located

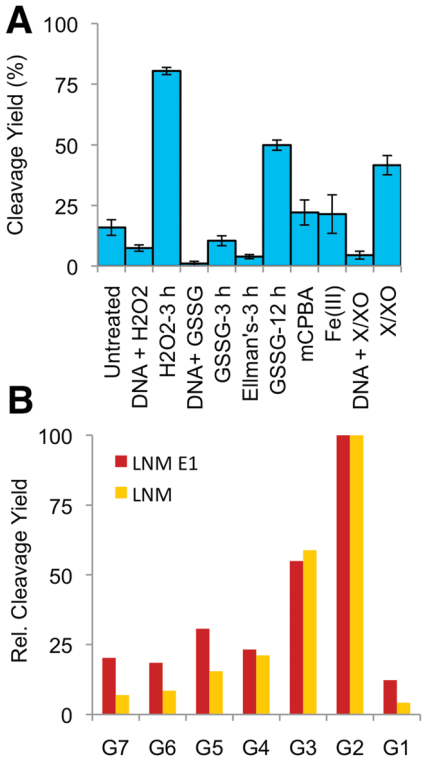


Fig. 2 DNA alkylation by LNM E1 and LNM. The DNA duplex shown in Fig. 1 was 5′-^{32}P-labeled on the top strand. Following incubation, reactions were subjected to piperidine workup and 20% denaturing polyacrylamide gel electrophoretic analysis. Panel A: strand cleavage yields under various activation conditions. The bars depict fraction of material found in cleavage products under each condition. Panel B: sequence-specificity of DNA alkylation by LNM E1 compared to LNM. The bars depict the relative amount of cleavage generated by LNM E1 + H_2O_2 and LNM + 2-mercaptoethanol at each guanine residue in the duplex shown in Fig. 1. To allow comparison, the amount of cleavage at G_2 was normalized to 100 in each case.

at guanine residues in regions of duplex DNA, with relatively little damage observed in the single-stranded overhangs. Furthermore, the two agents share common alkylation "hotspots" in the duplexes examined here. These results suggest that LNM and LNM E1 share similar noncovalent DNA-binding modes and alkylate guanine residues *via* the closely related episulfonium ion intermediates **3a** and **3b** (Scheme 1).

We examined the ability of several biologically-relevant oxidizing systems other than H_2O_2 to activate LNM E1. First, we showed that the xanthine/xanthine oxidase enzyme system, a physiologically-relevant generator of the reactive oxygen species (ROS) superoxide radical and hydrogen peroxide,[35] activates DNA alkylation by LNM E1 (Fig. 1, lanes 10 and 11, Fig. 2). Second, we found that LNM E1 can be activated by $FeNH_4(SO_4)_2$ (Fig. 2). Iron salts are known to catalyze the oxidation of thiols.[36,37] Finally, we examined the ability of disulfides to activate LNM E1 (Fig. 2 and Fig. S8, ESI†). Oxidative stress can cause elevated levels of intracellular disulfides, such as glutathione disulfide.[38] We found that glutathione disulfide,[39] 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent),[40] and (bis)2,4-dinitrophenyl disulfide were able to trigger DNA alkylation by E1. Rapid disulfide exchange reactions[39,40] presumably generated a LNM E1 disulfide **4b** that afforded access to the episulfonium ion alkylating agent **3b** *via* the oxathiolanone intermediate **2b** (Scheme 1). The activation of LNM E1 by the disulfide pathway is slower than the H_2O_2 activation pathway (Fig. S8, ESI†).

To more clearly elucidate the chemical events underlying DNA strand cleavage induced by LNM E1, we used LC-MS to characterize the reactions of LNM E1 with H_2O_2 both in the presence and absence of duplex DNA. We incubated LNM E1 with the DNA duplex shown in Fig. 1 and H_2O_2 in pH 7.4 buffer, followed by thermal workup (90 degrees, 40 min) to induce depurination of *N*7-alkylguanine residues. The mixture was extracted with *n*-butanol, and the extract analyzed using LC-MS to reveal a prominent product whose mass-to-charge (*m*/*z*) ratio was consistent with the guanine adduct **6** (Fig. 3).

LC-MS analysis of the reaction between LNM E1 and H_2O_2 in the absence of DNA in either methanol or aqueous buffer showed that the natural product was consumed rapidly to give a mixture that includes the symmetrical LNM E1 disulfide (**4b**, R = LNM E1) and the hydrolysis or methanolysis products **5** (Fig. S9 and S10, ESI†). Upon further incubation, we observed disappearance of the symmetrical E1 disulfide accompanied by a corresponding increase in the rearranged product **5b** (Fig. S9, ESI†). These results were consistent with the idea that disulfides of LNM E1 (**4b**) can undergo conversion to the DNA-alkylating episulfonium ion **3b** as illustrated in Scheme 1.

Our studies characterized new bioactivation processes in which oxidation of a thiol residue in LNM E1 by H_2O_2 and other byproducts of cellular oxidative stress initiates generation of a DNA-alkylating episulfonium ion. Thus, the ability of LNM E1

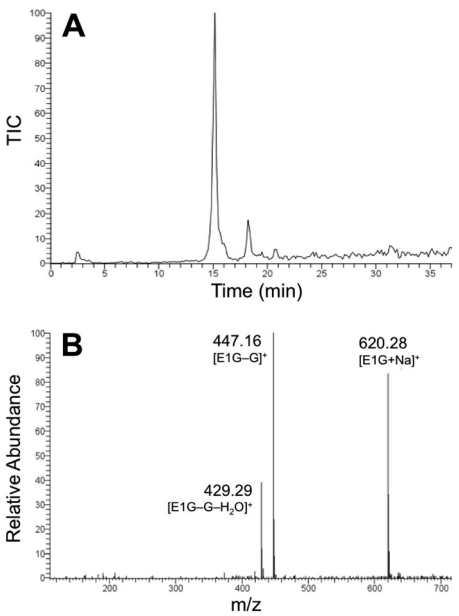


Fig. 3 LC-MS analysis of the reaction between LNM E1 + H_2O_2 and DNA. The modified DNA was subjected to thermal workup, the products extracted into *n*-butanol and the extract analyzed by LC-MS in the positive ion mode. Panel A shows the total ion chromatogram (TIC) and panel B shows the mass spectrum of the product eluting at 15 min that displays an *m*/*z* value and fragmentation pattern consistent with a LNM E1-guanine adduct (M + Na calcd 620.6).

to kill cancer cell lines that exist in a state of elevated oxidative stress may stem from oxidatively-activated DNA alkylation. The oxidative activation of LNM E1 provides indirect support for a mechanism proposed previously to explain thiol-independent, hydrolytic activation of the parent natural product LNM.[41] The LNM scaffold may be unique in its ability to support the generation of a common reactive intermediate *via* reductive (RSH), oxidative (H_2O_2, RSSR), and hydrolytic routes (Scheme 1).[28,34,41,45] While the detailed mechanisms of these activation processes require further study, this multifaceted behavior likely is made possible by the ambiphilic nature[42–44] of the sulfenic acid group (RSOH) that can function as either a nucleophile (LNM) or an electrophile (LNM E1) in different routes to the episulfonium ions **3** (Scheme 1). These fascinating features of LNM and LNM E1 will surely inspire the continued search for new members of the LNM family of natural products for the purpose of anticancer drug discovery.[45]

Conflicts of interest

There are no conflicts to declare.

Notes and references

1 X. Zhang, X. Li, Q. You and X. Zhang, *Eur. J. Med. Chem.*, 2017, **139**, 542–563.
2 F. W. Hunter, B. G. Wouters and W. R. Wilson, *Br. J. Cancer*, 2016, **114**, 1071–1077.
3 Y. Chen and L. Hu, *Med. Res. Rev.*, 2009, **29**, 29–64.
4 W. R. Wilson and M. P. Hay, *Nat. Rev. Cancer*, 2011, **11**, 393–410.
5 B. Kumar, S. Koul, L. Khandrika, R. B. Meacham and H. K. Koul, *Cancer Res.*, 2008, **68**, 1777–1785.
6 D. Trachootham, J. Alexandre and P. Huang, *Nat. Rev. Drug Discovery*, 2009, **8**, 579–591.
7 V. Nogueira and N. Hay, *Clin. Cancer Res.*, 2013, **19**, 4309–4314.
8 P. A. Cerutti, *Science*, 1985, **227**, 375–381.
9 Y. Kuang, K. Balakrishnan, V. Gandhi and X. Peng, *J. Am. Chem. Soc.*, 2011, **133**, 19278–19281.
10 J. L. Major Jourden and S. M. Cohen, *Angew. Chem., Int. Ed. Engl.*, 2010, **122**, 6947–6949.
11 H. Hagen, P. Marzenell, E. Jentzsch, F. Wenz, M. R. Veldwijk and A. Mokhir, *J. Med. Chem.*, 2012, **55**, 924–934.
12 W. Chen, K. Balakrishnan, Y. Kuang, Y. Han, M. Fu, V. Gandhi and X. Peng, *J. Med. Chem.*, 2014, **57**, 4498–4510.
13 L. Wang, S. Xie, L. Ma, Y. Chen and W. Lu, *Eur. J. Med. Chem.*, 2016, **116**, 84–89.
14 C. Perez, J.-P. Monserrat, Y. Chen and S. M. Cohen, *Chem. Commun.*, 2015, **51**, 7116–7119.
15 M. Hara, K. Asano, I. Kawamoto, T. Takiguchi, S. Katsumata, K. Takahashi and H. Nakano, *J. Antibiot.*, 1989, **42**, 1768–1774.
16 M. Hara, I. Takahashi, M. Yoshida, I. Kawamoto, M. Morimoto and H. Nakano, *J. Antibiot.*, 1989, **42**, 333–335.
17 Y.-Q. Cheng, G.-L. Tang and B. Shen, *J. Bacteriol.*, 2002, **184**, 7013–7024.
18 M. Hara, Y. Saitoh and H. Nakano, *Biochemistry*, 1990, **29**, 5676–5681.
19 T. Liu, M. Ma, H.-M. Ge, C. Yang, J. Cleveland and B. Shen, *Bioorg. Med. Chem. Lett.*, 2015, **25**, 4899–4902.
20 T. Ashizawa, K. Kawashima, Y. Kanda, K. Gomi, M. Okabe, K. Ueda and T. Tamaoki, *Anticancer Drugs*, 1999, **10**, 829–836.
21 K. S. Gates, *Chem. Res. Toxicol.*, 2000, **13**, 953–956.
22 P. Sinha, Y. Shin, A. M. Hays, K. S. Gates and D. Sun, *J. Cancer Sci. Ther.*, 2012, DOI: 10.4172/1948-5956.S8-003.
23 V. Viswesh, K. S. Gates and D. Sun, *Chem. Res. Toxicol.*, 2010, **23**, 99–107.
24 S. Bassett, R. Urrabaz and D. Sun, *Anti-Cancer Drugs*, 2004, **15**, 689–696.
25 V. Viswesh, A. Hayes, K. S. Gates and D. Sun, *Bioorg. Med. Chem.*, 2012, **20**, 4413–4421.
26 L. Breydo and K. S. Gates, *J. Org. Chem.*, 2002, **67**, 9054–9060.
27 S. J. Behroozi, W. Kim and K. S. Gates, *J. Org. Chem.*, 1995, **60**, 3964–3966.
28 A. Asai, M. Hara, S. Kakita, Y. Kanda, M. Yoshida, H. Saito and Y. Saitoh, *J. Am. Chem. Soc.*, 1996, **118**, 6802–6803.
29 M. Fekry, J. Szekely, S. Dutta, L. Breydo, H. Zang and K. S. Gates, *J. Am. Chem. Soc.*, 2011, **133**, 17641–17651.
30 L. Breydo, H. Zang and K. S. Gates, *Tetrahedron Lett.*, 2004, **45**, 5711–5716.
31 H. Zang and K. S. Gates, *Chem. Res. Toxicol.*, 2003, **16**, 1539–1546.
32 T. Nooner, S. Dutta and K. S. Gates, *Chem. Res. Toxicol.*, 2004, **17**, 942–949.
33 M. Imani-Nejad, K. M. Johnson, N. E. Price and K. S. Gates, *Biochemistry*, 2016, **55**, 7033–7041.
34 S.-X. Huang, B.-S. Yun, M. Ma, H. S. Basu, D. R. Church, G. Ingenhorst, Y. Huang, D. Yang, J. R. Lohman, G.-L. Tang, J. Ju, T. Liu, G. Wilding and B. Shen, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 8278–8283.
35 E. E. Kelley, N. K. H. Khoo, N. J. Hundley, U. Z. Malik, B. A. Freeman and M. M. Tarpey, *Free Radical Biol. Med.*, 2010, **48**, 493–498.
36 H. P. Misra, *J. Biol. Chem.*, 1974, **249**, 2151–2155.
37 P. W. Albro, J. T. Corbett and J. L. Schroeder, *J. Inorg. Biochem.*, 1986, **27**, 191–203.
38 C. Hwang, A. J. Sinskey and H. F. Lodish, *Science*, 1992, **257**, 1496–1502.
39 R. P. Szajewski and G. M. Whitesides, *J. Am. Chem. Soc.*, 1980, **102**, 2011–2026.
40 G. M. Whitesides, J. E. Lilburn and R. P. Szajewski, *J. Org. Chem.*, 1977, **42**, 332–338.
41 L. Breydo, H. Zang, K. Mitra and K. S. Gates, *J. Am. Chem. Soc.*, 2001, **123**, 2060–2061.
42 D. R. Hogg and A. Robertson, *Tetrahedron Lett.*, 1974, **43**, 3783–3784.
43 W. S. Allison, *Acc. Chem. Res.*, 1976, **9**, 293–299.
44 V. Gupta, H. Paritala and K. S. Carroll, *Bioconjugate Chem.*, 2016, **27**, 1411–1418.
45 G. Pan, Z. Xu, Z. Guo, Hindra, M. Ma, D. Yang, H. Zhou, Y. Gansemans, X. Zhu, Y. Huang, L.-X. Zhao, Y. Jiang, J. Cheng, F. Van Nieuwerburgh, J.-W. Suh, Y. Duan and B. Shen, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**, DOI: 10.1073/pnas.1716245115.