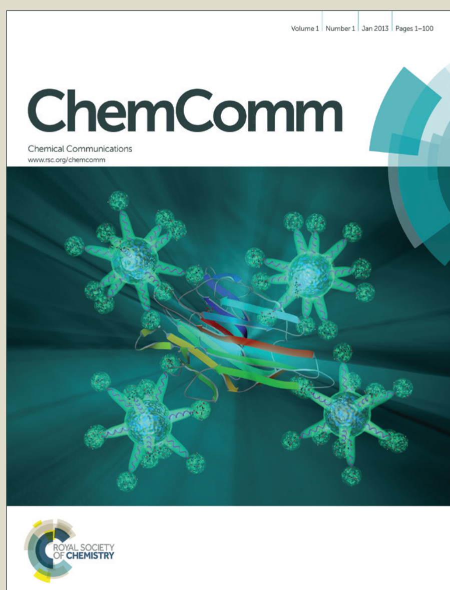
ChemComm

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

CREATED USING THE RSC COMMUNICATION TEMPLATE (VER. 3.1) - SEE WWW.RSC.ORG/ELECTRONICFILES FOR DETAILS
ARTICLE TYPE www.rsc.org/xxxxxx | XXXXXXXX

Organelle-selective fluorescent Cu^{2+} ion probes: revealing endoplasmic reticulum as reservoir for Cu-overloading

Yun Hak Lee,[a] Nayoung Park,[a] Young Bin Park,[b] Yu Jeong Hwang,[b] Chulhun Kang,*[,b] Jong Seung Kim*[,a]

Received (in XXX, XXX) Xth XXXXXXXXX 200X, Accepted Xth XXXXXXXXX 200X
First published on the web Xth XXXXXXXXX 200X
DOI: 10.1039/b000000xektl

Two novel Cu^{2+} sensors, 1 and 2, bearing naphthalimide and DPA moiety were synthesized to study copper accumulation in organelles by selective Cu^{2+} sensing. The ER-selective Cu^{2+} sensor 1 that we developed serves as valuable tool for understanding the subcellular compartmentalization and roles of copper ions in physiology and pathophysiology.

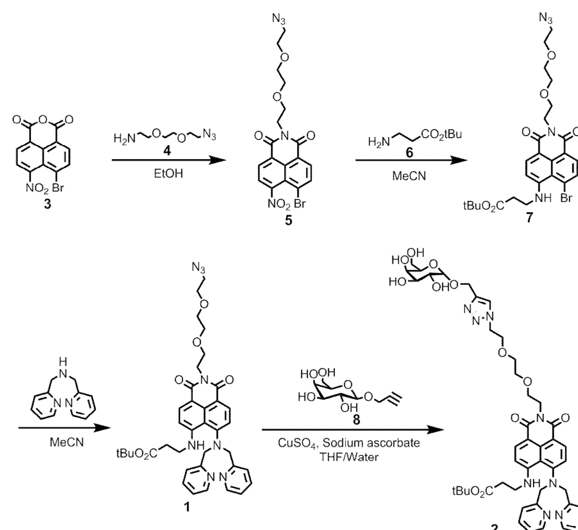
Copper ion is the third most abundant trace element present in the human diet and it plays essential roles in various biological processes, including the detoxification of reactive oxygen species (ROS), neurotransmitter biosynthesis and denaturation, and the functioning and maintenance of the structural stability of proteins.[1] Indeed, copper homeostasis is tightly controlled by factors, including copper transport proteins and chaperones.[2] However, its imbalance in the body caused by defects in copper export systems or long-term exposure leads to many diseases, including liver diseases.[3]

The cytotoxicity of copper in Wilson's disease has been well documented. Wilson's disease is a genetic disease caused by mutations in ATP7B gene present in the copper export system. In the disease, copper ion is accumulated in the liver (higher than 5-fold of the normal),[4] resulting in liver cirrhosis and hemolysis.[5] The toxicity of copper is thought to be mediated by ROS generated by copper ions *via* Haber-Weiss and Fenton reactions, which inhibit antioxidant enzymes, leading to oxidative cell death, denaturation of haemoglobin, and protein aggregation.[6] However, its effect on subcellular organelle functions or copper uptake pathways is poorly understood.[7] Although the lysosome is the main storage site for the excess copper ions in the hepatocytes of copper-overloaded rats,[8] its cytotoxic effects involve the malfunction of various organelles such as the mitochondria, endoplasmic reticulum (ER), as well as lysosome.[9] In fact, several reports concerning copper ion-induced ER stress have been explored where the ER stress leads to an accumulation of unfolded proteins and subsequent activation of the unfolded protein response, causing the disturbance of ER function as seen in hypoxia, glucose deprivation, oxidative stress, and cell death.[10] The apparent discrepancy that hepatic storage sites for copper is not consistent with its localization during the copper-induced damage demands investigations on the spatiotemporal dynamics of copper ion by using organelle-selective copper probes.

To date, a variety of Cu^{2+}-selective fluorescent probes, including small-molecule fluorophores, chemodosimeters, protein-based devices, nanocomposites, enzyme-based assays, and magnetic resonance imaging contrast agents have been developed for biological applications.[11] However, only few reports have described the development of sensitive fluorescent probes for the organelle-selective detection of Cu^{2+} ions.[12] Thus, the development of new techniques that will allow the visualization of the subcellular distribution of copper ions is critical for a better understanding of copper cytotoxicity. To this end, we attempted to develop novel sensors for the organelle-specific detection of copper ions.

Here, we report the development of two new fluorescence-based sensors for probing Cu^{2+} ions in the ER or lysosome of the living cells. We took advantage of the facts that lipophilic or glycosylated compounds are often delivered to ER or lysosome.[13] Using the newly developed probes, we demonstrate that the primary site for Cu^{2+} accumulation, when in excess, is not the lysosome, but the ER. To the best of our knowledge, this is the first report describing the ER-selective detection of copper ions in living cells.

The probes **1** and **2** were prepared according to the synthetic route outlined in Scheme 1. The compounds **3**[14], **4**[15], **6**[16], and **8**[17] were prepared according to the previously reported methods with minor modifications. Compound **5** was obtained by the condensation of **3** with **4,** and it was subsequently converted into compound **7** in the presence of **6.** The reaction of **7** with DPA afforded compound **1**. Compound **2** was then prepared by click reaction of **1** with propargyl β-D-galactopyranoside, **8**, in the presence of sodium ascorbate and copper(II) sulfate. The identities of all newly synthesized compounds were confirmed by ^1H NMR, ^{13}C NMR, ESI-MS and HRMS (ESI†).



Scheme 1. Synthesis of compounds **1** and **2**.

The spectroscopic properties of **1** were characterized in HEPES buffer solutions (20 mM, pH 7.4, DMSO 0.5%). As shown in Fig. 1A and 1B, the UV/vis and fluorescence spectra of probe **1** showed bands centered at 462 nm (pale yellow, $\varepsilon = 1.3 \times 10^4\ M^{-1}\ cm^{-1}$) and 547 nm, respectively. In the presence of Cu^{2+}, its

ChemComm Accepted Manuscript

absorption maximum red-shifted to 504 nm (pink, $\varepsilon = 0.9 \times 10^4$ M^{-1} cm^{-1}) and its fluorescence intensity at 547 nm significantly declined because of the heavy metal ion effect.[18] The probe **2**, an analogue of **1** bearing an additional galactose group, showed similar spectroscopic properties to those of compound **1,** regardless of Cu^{2+} ion (Fig. S1). As the concentration of Cu^{2+} increased to 20 µM, the fluorescence intensity of probe **1** at 547 nm following excitation at 485 nm, an isosbestic point in its absorption spectra, was quenched largely (Fig. 1C). The Job's plot shown in Fig. 1D indicated a 1:1 binding stoichiometry between **1** and Cu^{2+}. The dissociation constants of **1**-Cu^{2+} and **2**-Cu^{2+} complexes at pH 7.4 and 6.5 estimated from the titration experiments of its emission intensity against various concentrations of Cu^{2+} ion are summarized in Fig. S2 and Table S1. The dissociation constant for **1**-Cu^{2+} complex was 3.2×10^{-7} M, with a small increase at pH 6.5.

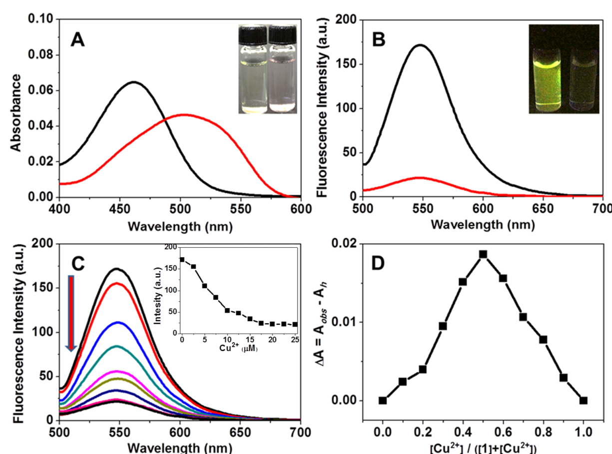


Fig. 1 (A) UV-Vis and (B) fluorescence spectra of **1** (5 µM) in the absence (black) and presence (red) of Cu^{2+} (20 µM). (C) Emission spectra of **1** (5 µM) in the presence of various concentrations of Cu^{2+} (0–25 µM). Inset: fluorescence intensity at 547 nm as a function of Cu^{2+} concentration. (D) Normalized Job's plot of **1** and Cu^{2+} in HEPES buffer (20 mM, pH 7.4, 0.5% DMSO). All spectra were acquired in HEPES buffer (20 mM, pH 7.4, 0.5% DMSO) following excitation at 485 nm, and the emission intensity was measured at 547 nm.

The possibility of quenching of probe **1** by other biologically relevant metal ions under physiological conditions (20 mM PBS at pH 7.4) was examined, and the result is shown in Fig. 2A. The results indicate that the quenching is Cu^{2+} ion-selective even in the presence of the other metal ions. Further, the fluorescence of probe **1** was quenched by 20 µM of Cu^{2+} ion in the pH range of 6 to 8, whereas it remained fluorescent in the absence of Cu^{2+} ion within the same pH range, indicating that probe **1** could serve as a Cu^{2+} ion-selective sensor for examining most biological processes (Fig. 2B). The probe **2** showed similar spectroscopic properties as that of the probe **1** (Fig S3, and S4).

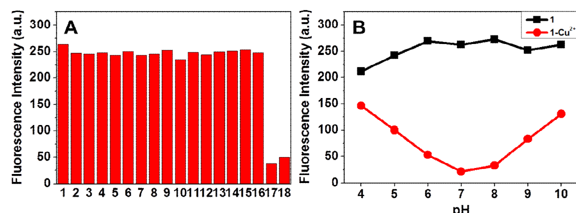


Fig. 2 (A) Metal ion selectivity of **1** (5 µM) toward 50 µM of various chloride salts (1, only probe; 2, Li^+; 3, Na^+; 4, K^+; 5, Ca^{2+}; 6, Sr^{2+}; 7, Ba^{2+}; 8, Co^{2+}; 9, Mg^{2+}; 10, Hg^{2+}; 11, Cd^{2+}; 12, Fe^{2+}; 13, Fe^{3+}; 14, Zn^{2+}; 15, Mn^{2+}; 16, Ni^{2+}; 17, Cu^{2+} (20 µM); 18, Cu^{2+} (20 µM) + other metals). (B) Fluorescence response of **1** (5 µM) at various pH (4−10) in the absence (black) or presence (red) of Cu^{2+} (20 µM). All spectra were acquired in HEPES buffer (20 mM, pH 7.4, 0.5% DMSO) following excitation at 485 nm, and emission intensity was measured at 547 nm.

As the copper ion accumulation occurs first in the liver, so we selected HepG2 as the model cell line to examine the subcellular localization of copper ion under copper overloading conditions.[19] Using confocal microscopy, the co-localization of the newly developed probes with the subcellular organelle markers, namely, LysoTracker Red DND-99, ER-Tracker Red, and MitoTracker, were analyzed. Fig. 3A and 3B show the co-localization of various organelle markers with probes **1** and **2**, respectively. We observed that the image of probe **1** overlapped with that of the ER-tracker (Fig 3A), whereas the image of probe **2** mostly overlapped with that of the Lyso-tracker with a minor overlap also observed with that of the ER-tracker (Fig 3B). Therefore, we conclude that probes **1** and **2** are predominantly localized to the ER and lysosome, respectively, and that these probes can be used to examine the accumulation of Cu^{2+} ions in the ER or lysosome of living cells.

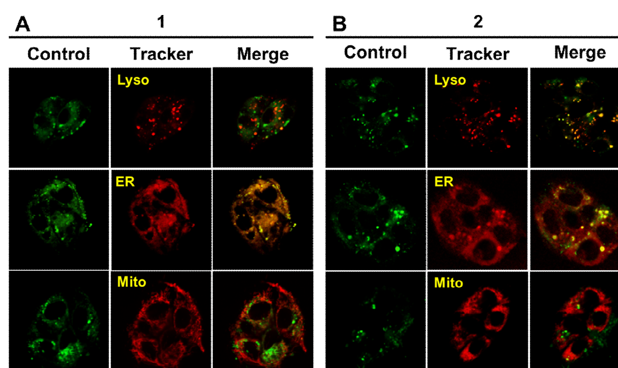


Fig. 3 Subcellular localization of (A) **1** and (B) **2** in HepG2 cells as revealed by confocal microscopy. HepG2 cells were stained with **1** (1 µM) or **2** (10 µM) for 10 min and stained for organelle markers (0.5 µM LysoTracker Red, 0.1 µM ER Red, and 0.1 µM MitoTracker Deep Red, respectively).

Because probes **1** and **2** selectively targeted the ER and lysosome, respectively, of HepG2 cells, we analyzed their responsiveness to copper overload. In the presence of each probe, the HepG2 cells were treated with three different copper overloading procedures which have been reported in the literatures, 200 µM of $CuCl_2$ for 15 min,[20] 1.0 mM of $CuCl_2$ for 7 h,[21] and 100 µM of $CuCl_2$ for 7 h with pretreated 100 µM of $NaVO_3$ for 24 h,[22] an inhibitor of the export system of cellular copper.[23] The results are shown in Fig 4. At all three copper overloading conditions, the fluorescence intensity of probe **1,** located in the ER, was significantly lower than that observed in the untreated control cells. In contrast, the fluorescence emission from probe **2** was not altered significantly by copper overload. These results clearly suggest that, under conditions of copper-ion overload, the primary site of copper accumulation is the ER. This finding was supported by the result that the fluorescence of probe **1,** but not that of **2,** was quenched in the presence of $NaVO_3$ (inhibitor of copper ion export), which implied that the copper accumulation following the blockade of copper export occurred in the ER and not in the lysosome. One possibility is that the acidic lysosomal environment may prevent fluorescence quenching by Cu^{2+} ion. However, this seemed unlikely as the metal ion was able to bind to the probe even in the acidic environments (Fig 2, S2, and Table S1). We found that, at a

ChemComm Accepted Manuscript

longer incubation time of 12 h, the fluorescence of both probes **1** and **2** in HepG2 cells were hardly quenched by Cu^{2+} ions (Fig. S5). Nonetheless, these results demonstrated that the primary intracellular copper accumulation site was ER. Our results provide important information in understanding the copper-induced ER stress or the related cytotoxicity.

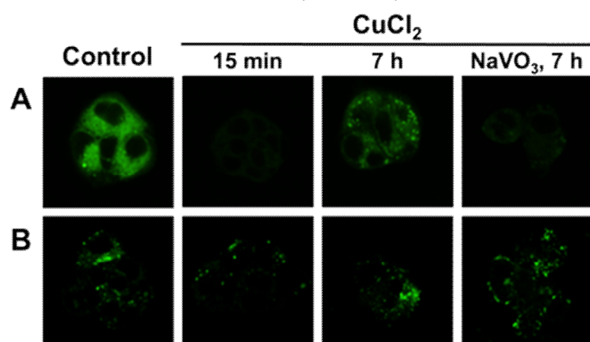


Fig. 4 Confocal microscopy images of (A) probe **1** and (B) probe **2** (1 µM and 10 µM, respectively) in HepG2 cells. The HepG2 cells were exerted with **1** or **2** under three different copper overloading conditions, 200 µM of $CuCl_2$ for 15 min, 1 mM of $CuCl_2$ for 7 h, and 100 µM of $CuCl_2$ for 7 h with pretreated 100 µM of $NaVO_3$ for 24 h.

In conclusion, we have developed two new fluorescent sensors, **1** and **2**, bearing naphthalimide as a fluorophore and a DPA moiety that selectively binds copper ion. The fluorescence of the probes was selectively quenched by copper ions. Following delivery into HepG2 cells, probes **1** and **2** were selectively localized to the ER and the lysosome, respectively. In HepG2 cells, copper overload quenched the fluorescence of probe **1**, whereas that of probe **2** was unaffected. Together with the fluorescence quenching of probe **1** observed in HepG2 cells following the blockade of copper export system by $NaVO_3$, our results demonstrate that the primary site of copper accumulation in hepatic cells under copper overload is the ER. Therefore, the ER-selective Cu^{2+} sensor that we developed serves not only as a reference, but also as a valuable tool for understanding the subcellular compartmentalization and diverse roles of copper ions.

This work was supported by CRI project (No. 2009-0081566, J.S.K.) and Basic Science Research Program (2012R1A1A2006259, C.K.) from the National Research Foundation of The Ministry of Science, ICT & Future Planning in Korea.

Notes and references

[a] *Department of Chemistry, Korea University, Seoul 136-701, Korea. E-mail: jongskim@korea.ac.kr*
[b] *The East-West Medical Science, Kyung Hee University, Yongin 446-701, Korea. E-mail: kangch@khu.ac.kr*
† *Electronic Supplementary Information (ESI) available: Experimental procedure, supporting figures*

1 (a) A. Mokhir, A. Kiel, D.-P. Herten and R. Kraemer, *Inorg. Chem.*, 2005, **44**, 5661; (b) J. Liu and Y. Lu, *J. Am. Chem. Soc.*, 2007, **129**, 9838; (c) A. Torrado, G. K. Walkup and B. Imperiali, *J. Am. Chem Soc.*, 1998, **120**, 609; (d) P. Grandini, F. Mancin, P. Tecilla, P. Scrimin and U. Tonellato, *Angew. Chem., Int. Ed.*, 1999, **38**, 3061; (e) M. M. Pena, J. Lee and D. J. Thiele, *J. Nutr.*, 1999, **129**, 1251; (f) E. Madsen and J. D. Gitlin, *Ann. Rev. Neurosci.*, 2007, **30**, 317.
2 W. I. Vonk, C. Wijmenga and B. van de Sluis, *Am. J. Clin. Nutr.*, 2008, **88**, 840S.
3 (a) D. G. Barceloux, *Clin. Toxicol.*, 1999, **37**, 217; (b) X.-B. Zhang, J. Peng, C.-L. He, G.-L. Shen and R.-Q. Yu *Anal. Chim. Acta*, 2006, **567**, 189; (c) E. L. Que, D. W. Domaille and C. J. Chang, *Chem. Rev.*, 2008, **108**, 1517.
4 (a) I. Sternlieb and I. H. Scheinberg, *N. Engl. J. Med.*, 1968, **278**, 352; (b) B. Sarkar, *Chem. Rev.*, 1999, **99**, 2535; (c) G. J. Brewer, *Proc. Soc. Exp. Biol. Med.*, 2000, **223**, 39; (d) E. A. Roberts and M. L. Schilsky, *Hepatology*, 2008, **47**, 2089.
5 S. M. Riordan and R. Williams, *J. Hepatol.*, 2001, **34**, 165.
6 (a) S. J. Stohs and D. Bagchi, *Free Radic. Biol. Med.*, 1995, **18**, 321; (b) A. Deiss, , G. R. Lee and G. E. Cartwright, *Ann. Intern. Med.*, 1970, **73**, 413; (c) C. S. Atwood, R. D. Moir, X. Huang, R. C. Scarpa, N. M. E. Bacarra and D. M. Romano, *J. Biol. Chem.*, 1998, **273**, 12817.
7 C. D. Vulpe and S. Packman, *Annu. Rev. Nulr.*, 1995, **15**, 293.
8 H. Hayashi, A. Hattori, Y. Tatsumi, K. Hayashi, Y. Katano, J. Ueyama, S. Wakusawa, M. Yano and H. Goto, *Med. Mol. Morphol.*, 2013, **46**, 133.
9 K. S. Squibb and B. A. Fowler, *Environ. Health Perspect.*, 1981, **40**, 181.
10 (a) S. Tardito, I. Bassanetti, C. Bignardi, L. Elviri, M. Tegoni, C. Mucchino, O. Bussolati, R. Franchi-Gazzola and L. Marchiò, *J. Am. Chem. Soc.*, 2011, **133**, 6235; (b) S. Tardito, C. Isella, E. Medico, L. Marchio, E. Bevilacqua, M. Hatzoglou, O. Bussolati and R. Franchi-Gazzola, *J. Biol. Chem.*, 2009, **284**, 24306; (c) C. N. Hancock, L. H. Stockwin, B. Han, R. D. Divelbiss, J. H. Jun, S. V. Malhotra, M. G. Hollingshead and D. L. Newton, *Free Radic. Biol. Med.*, 2011, **50**, 110; (d) C. Xu, B. Bailly-Maitre, J. C. Reed, *J. Clin. Invest.*, 2005, **115**, 2656.
11 (a) H. S. Jung, P. S. Kwon, J. W. Lee, J. I. Kim, C. S. Hong, J. W. Kim, S. Yan, J. Y. Lee, J. H. Lee, T. Joo and J. S. Kim, *J. Am. Chem. Soc.*, 2009, **131**, 2008; (b) Y.-R. Kim, H. J. Kim, J. S. Kim and H. Kim, *Adv. Mater.*, 2008, **20**, 4428; (c) T. Mizuno, K. Murao, Y. Tanabe, M. Oda and T. Tanaka, *J. Am. Chem. Soc.*, 2007, **129**, 11378; (d) R. Meallet-Renault, R. Pansu, S. Amigoni-Gerbier and C. Larpent, *Chem. Commun.*, 2004, 2344; (e) C. Ge, J. Chen, W. Wu, Z. Fang, L. Chen, Q. Liu, L. Wang, X. Xing and L. Zeng, *Analyst*, 2013, **138**, 4737; (f) G. J. Stasiuk and N. J. Long, *Chem. Commun.*, 2013, **49**, 2732.
12 K. A. Price, J. L. Hickey, Z. Xiao, A. G. Wedd, S. A. James, J. R. Liddell, P. J. Crouch, A. R. White and P. S. Donnelly, *Chem. Sci.*, 2012, **3**, 2748.
13 (a) A. L. Schwartz, *CRC Crit. Rev. Biochem.*, 1984, **16**, 207; (b) J. Moan, K. Berg, E. Kvam, A. Western, Z. Malik, A. Rück and H. Schneckenburger, *Ciba Found. Symp.*, 1989, **146**, 95.
14 Z. Xu, X. Qian, J. Cui and R. Zhang, *Tetrahedron*, 2006, **62**, 10117.
15 H. Zheng, F. Wang, Q. Wang and J. Gao, *J. Am. Chem. Soc.*, 2011, **133**, 15280.
16 R. Ruijtenbeek, J. A. Kruijtzer, W. van de Wiel, M. J. Fischer, M. Flück, F. A. Redegeld, R. M. Liskamp and F. P. Nijkamp, *ChemBioChem.*, 2001, **2**, 171.
17 J. Zhao, Y. Liu, H.-J. Park, J. M. Boggs and A. Basu, *Bioconjugate Chem.*, 2012, **23**, 1166.
18 (a) N. Malcik, P. Caglar and R. Narayanaswamy, *Quím. Anal.*, 2000, **19**, 94. (b) S. H. Lee, J. Y. Kim, J. Ko, J. Y. Lee and J. S. Kim, *J. Org. Chem.*, 2004, **69**, 2902; (c) Y. H. Lee, M. H. Lee, J. F. Zhang and J. S. Kim, *J. Org. Chem.*, 2010, **75**, 7159; (d) H. J. Kim and J. S. Kim, *Tetrahedron Lett.*, 2006, **47**, 7051; (e) K. C. Ko, J. -S. Wu, H. J. Kim, P. S. Kwon, J. W. Kim, R. A. Bartsch, J. Y. Lee and J. S. Kim, *Chem. Commun.*, 2011, **47**, 3165.
19 V. Mersch-Sundermann, S. Knasmüller, X. J. Wu, F. Darroudi and F. Kassie, *Toxicology*, 2004, **198**, 329.
20 Y. Chen, C. Zhu, J. Cen, J. Li, W. He, Y. Jiao and Z. Guo, *Chem. Commun.*, 2013, **49**, 7632.
21 C. S. Lim, J. H. Han, C. W. Kim, M. Y. Kang, D. W. Kang and B. R. Cho, *Chem. Commun.*, 2011, **47**, 7146.
22 M. Moriya, Y. H. Ho, A. Grana, L. Nguyen, A. Alvarez, R. Jamil, M. L. Ackland, A. Michalczyk, P. Hamer, D. Ramos, S. Kim, J. F. Mercer and M. C. Linder, *Am. J. Physiol. Cell Physiol.*, 2008, **295**, C708.
23 C. T. Cui, J. Y. Uriu-Adams, E. H. Tchaparian, C. L. Keen and R. B. Rucker, *Toxicol. Appl. Pharmacol.*, 2004, **199**, 35.

ChemComm Accepted Manuscript