ORGANIC LETTERS

2010
Vol. 12, No. 7
1420−1423

Subcellular Localization of a Fluorescent Artemisinin Derivative to Endoplasmic Reticulum

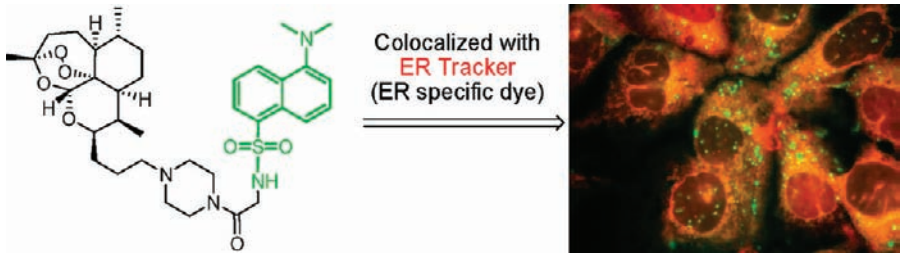
Yungen Liu,[†] Chun-Nam Lok,[†] Ben Chi-Bun Ko,[†] Tina Yuen-Ting Shum,[†] Man-Kin Wong,*[,‡] and Chi-Ming Che*[,†]

Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong, China, and Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Hong Kong, China

cmche@hku.hk; bcmkwong@inet.polyu.edu.hk

Received December 15, 2009

ABSTRACT



A cytotoxic artemisinin derivative conjugated with a fluorescent dansyl moiety was synthesized and its subcellular localization in Hep3B cells was examined. Comparison of the localization signals of the fluorescent artemisinin derivative with organelle specific dyes revealed that endoplasmic reticulum (ER) is the main site of its accumulation.

Artemisinin (Qinghaosu, **1**) is a sesquiterpene lactone endoperoxide isolated from an ancient Chinese herb *Artemisia annua* (Sweet wormwood).[1] Artemisinin and its derivatives are highly effective against multidrug resistant malaria caused by *Plasmodium falciparum*, and are currently used for clinical treatment of malaria.[2] Given the remarkable success of artemisinin in the treatment of malaria, there is a growing interest in exploring the anticancer activities of this class of compounds.[3] Despite hundreds of cytotoxic artemisinin compounds known in the literature, studies on their mechanism of action are in the rudimentary stage. It is generally accepted that the endoperoxide moiety is crucial to the biological activities. Decomposition of the endoperoxide in a reducing environment (Fe^{2+}, GSH, etc.) leads to reactive oxygen species (ROS) which could be cytotoxic.[2b,3b,c,4] A

[†] University of Hong Kong.
[‡] Hong Kong Polytechnic University.

(1) (a) Klayman, D. L. *Science* **1985**, *228*, 1049–1055. (b) Renslo, A. R.; McKerrow, J. H. *Nat. Chem. Biol.* **2006**, *2*, 701–710.

(2) (a) Robert, A.; Dechy-Cabaret, O.; Cazelles, J.; Meunier, B. *Acc. Chem. Res.* **2002**, *35*, 167–174. (b) Li, Y.; Wu, Y. L. *Curr. Med. Chem.* **2003**, *10*, 2197–2230. (c) O'Neill, P. M.; Posner, G. H. *J. Med. Chem.* **2004**, *47*, 2945–2964. (d) Posner, G. H.; O'Neill, P. M. *Acc. Chem. Res.* **2004**, *37*, 397–404. (e) Haynes, R. K.; Ho, W. Y.; Chan, H. W.; Fugmann, B.; Stetter, J.; Croft, S. L.; Vivas, L.; Peters, W.; Robinson, B. L. *Angew. Chem., Int. Ed.* **2004**, *43*, 1381–1385. (f) Haynes, R. K. *Angew. Chem., Int. Ed.* **2005**, *44*, 2064–2065. (g) Haynes, R. K.; Fugmann, B.; Stetter, J.; Rieckmann, K.; Heilmann, H. D.; Chan, H. W.; Cheung, M. K.; Lam, W. L.; Wong, H. N.; Croft, S. L.; Vivas, L.; Rattray, L.; Stewart, L.; Peters, W.; Robinson, B. L.; Edstein, M. D.; Kotecka, B.; Kyle, D. E.; Beckermann, B.; Gerisch, M.; Radtke, M.; Schmuck, G.; Steinke, W.; Wollborn, U.; Schmeer, K.; Romer, A. *Angew. Chem., Int. Ed.* **2006**, *45*, 2082–2088. (h) Haynes, R. K. *Curr. Top. Med. Chem.* **2006**, *6*, 509–537. (i) Haynes, R. K.; Wong, H. N.; Lee, K. W.; Lung, C. M.; Shek, L. Y.; Williams, I. D.; Croft, S. L.; Vivas, L.; Rattray, L.; Stewart, L.; Wong, V. K. W.; Ko, B. C. B. *ChemMedChem* **2007**, *2*, 1464–1479.

(3) (a) Haynes, R. K.; Chan, H. W.; Lam, W. L.; Tsang, H. W.; Hsiao, W. L. PCT Int. Appl. WO 2000004026 A1, 2000. (b) Lai, H.; Sasaki, T.; Singh, N. P. *Expert Opin. Ther. Targets* **2005**, *9*, 995–1007. (c) Disbrow, G. L.; Baege, A. C.; Kierpiec, K. A.; Yuan, H.; Centeno, J. A.; Thibodeaux, C. A.; Hartmann, D.; Schlegel, R. *Cancer Res.* **2005**, *65*, 10854–10861. (d) Posner, G. H.; D'Angelo, J.; O'Neill, P. M.; Mercer, A. *Expert Opin. Ther. Pat.* **2006**, *16*, 1665–1672. (e) Lee, S. *Mini-Rev. Med. Chem.* **2007**, *7*, 411–422.

10.1021/ol902890j
Published on Web 03/01/2010

recent study revealed that carbon-centered radicals generated from activation of the endoperoxide triggered apoptosis in cancer cell lines.[5]

We have previously reported a series of artemisinin compounds that are cytotoxic to cancer cells.[6] Artemisinin derivative **2** bearing a lipophilic carbon chain ($C_{16}H_{33}$) exhibits potent cytotoxicity against the HepG2 cell line (IC_{50} = 0.46 μM, Figure 1) whereas the natural artemisinin (**1**) is

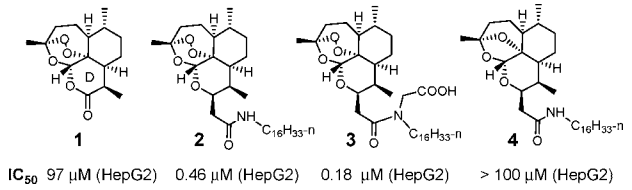


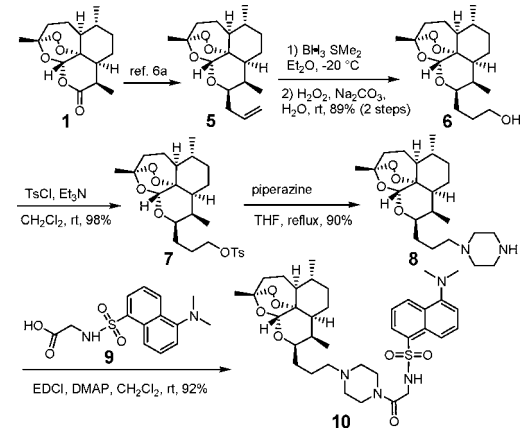
Figure 1. Artemisinin (**1**) and its derivatives (**2**−**4**).

only weakly cytotoxic against the same cell line (IC_{50} = 97 μM). By incorporation of a carboxylic acid group at the amide linker, water-soluble derivative **3** (IC_{50} = 0.18 μM, see the Supporting Information) was prepared and found to display cytotoxicity comparable to that of **2**. The deoxyartemisinin derivative **4** was found to be nontoxic toward the HepG2 cell line (Figure 1).

Comparing with the natural artemisinin (**1**), the strong cytotoxicity displayed by artemisinin derivatives **2** and **3** reveals that the latter two compounds may exert cytotoxic activities on specific cellular targets. In the literature, fluorescent derivatives of natural products have been employed as probes to identify their subcellular localizations. There are only several fluorescent artemisinin compounds reported and these have been used in the antimalarial mechanistic studies. For examples, O'Neill and Bray[7] prepared artemisinin-*O*-acridine, artemisinin-*N*-acridine, and artemisinin-*N*-NBD and employed these compounds to study the antimalarial mechanism of action (see the Supporting Information, Figure S1). Cooper and co-workers[8] synthesized artemisinin-dimer-*O*-coumaryl and artemisinin-dimer-*O*-dansyl compounds. Very recently, Haynes and co-workers[2i] reported the synthesis of 11-*N*-artemisinin dansyl amide. The objective of this work was to prepare a fluorescent cytotoxic artemisinin derivative and employ it in cellular localization study. A modular approach[6a] of "artemisinin + linker + lipophilic alkyl carbon chain" was used for the synthesis of fluorescent and cytotoxic artemisinin compounds. We proposed to prepare a small sized fluorescence probe with moderate lipophilicity and chose the dansyl fluorophore which has previously been used by Cooper[8] and us[9] in the synthesis of fluorescent derivatives of natural products. The fluorescent artemisinin compound **10** was designed. Amide bond was selected as the linker and the C10 site of artemisinin was linked to dansyl glycine using a carbon chain rather than an ether group to avoid hydrolysis of the probe under physiological conditions. Comparison of the colocalization of **10** with organelle specific dyes revealed that endoplasmic reticulum (ER) is the main subcellular site of accumulation of the fluorescent artemisinin compound **10**.

As depicted in Scheme 1, alcohol **6** was obtained from 10β-allylartemisinin (**5**)[6a] by hydroboration. *p*-Toluene-

Scheme 1. Synthesis of Fluorescent Artemisinin **10**



sulfonate artemisinin **7** was synthesized by coupling of alcohol **6** with *p*-toluenesulfonyl chloride in 98% yield. Nucleophilic substitution of **7** with piperazine gave **8** in 90% yield. The fluorescent artemisinin derivative **10** was obtained in 92% yield by coupling of amine **8** with dansyl glycine **9**.

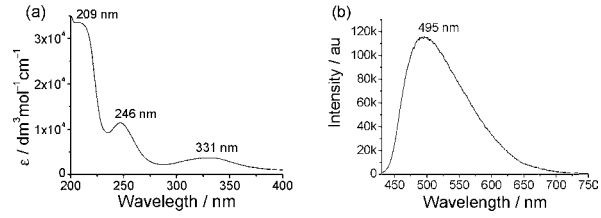


Figure 2. (a) Electronic absorption spectrum of **10** in H_2O. (b) Emission spectrum of **10** (5×10^{-5} M) in H_2O at 298 K (λ_{ex} = 331 nm).

(4) (a) Efferth, T.; Oesch, F. *Biochem. Pharmacol.* **2004**, *68*, 3–10. (b) Efferth, T. *Curr. Drug Targets* **2006**, *7*, 407–421.

(5) Mercer, A. E.; Maggs, J. L.; Sun, X. M.; Cohen, G. M.; Chadwick, J.; O'Neill, P. M.; Park, B. K. *J. Biol. Chem.* **2007**, *282*, 9372–9382.

(6) (a) Liu, Y.; Wong, V. K. W.; Ko, B. C. B.; Wong, M. K.; Che, C. M. *Org. Lett.* **2005**, *7*, 1561–1564. (b) Lo, V. K. Y.; Liu, Y.; Wong, M. K.; Che, C. M. *Org. Lett.* **2006**, *8*, 1529–1532. (c) Liu, Y.; Xiao, W.; Wong, M. K.; Che, C. M. *Org. Lett.* **2007**, *9*, 4107–4110. (d) Lo, V. K. Y.; Wong, M. K.; Che, C. M. *Org. Lett.* **2008**, *10*, 517–519.

(7) (a) Eckstein-Ludwig, U.; Webb, R. J.; van Goethem, I. D. A.; East, J. M.; Lee, A. G.; Kimura, M.; O'Neill, P. M.; Bray, P. G.; Ward, S. A.; Krishna, S. *Nature* **2003**, *424*, 957–961. (b) Stocks, P. A.; Bray, P. G.; Barton, V. E.; Al-Helal, M.; Jones, M.; Araujo, N. C.; Gibbons, P.; Ward, S. A.; Hughes, R. H.; Biagini, G. A.; Davies, J.; Amewu, R.; Mercer, A. E.; Ellis, G. *Angew. Chem., Int. Ed.* **2007**, *46*, 6278–6283. (c) Jones, M.; Mercer, A. E.; Stocks, P. A.; La Pensée, L. J. I.; Cosstick, R.; Park, B. K.; Kennedy, M. E.; Piantanida, I.; Ward, S. A.; Davies, J.; Bray, P. G.; Rawe, S. L.; Baird, J.; Charidza, T.; Janneh, O.; O'Neill, P. M. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2033–2037.

(8) Hartwig, C. L.; Rosenthal, A. S.; D'Angelo, J.; Griffin, C. E.; Posner, G. H.; Cooper, R. A. *Biochem. Pharmacol.* **2009**, *77*, 322–336.

The electronic absorption and emission spectra of **10** are depicted in Figure 2. Compound **10** in water shows absorption peak maxima at 209 ($\varepsilon = 33\,500$ dm^3 mol^{-1} cm^{-1}), 246 ($\varepsilon = 11\,500$ dm^3 mol^{-1} cm^{-1}), and 331 nm ($\varepsilon = 3\,700$ dm^3 mol^{-1} cm^{-1}). Upon excitation at 331 nm, an aqueous solution of **10** shows an emission at 495 nm (peak maximum) with a lifetime of 6.7 ns and a quantum yield of 0.053.

Compound **10** was found to be cytotoxic to a panel of cancer cell lines (Table 1) after 6 days treatment at the micromolar level. As compound **10** is both cytotoxic and fluorescent, it was employed as a molecular probe for subcellular localization study in this work.

Table 1. Cytotoxicities of **10** against a Panel of Cell Lines

cell line	HepG2	Hep3B	HeLa	SUNE1	MCF-7	CCD-19Lu
IC_{50} (μM)	8.7	7.8	10.0	1.4	3.4	22.0

To examine the subcellular localization of **10**, ER Tracker (ER specific dye, Figure 3), BODIPY TR ceramide (Golgi specific dye, Figure 4), and MitoTracker (mitochondria specific dye, Figure 5) were employed for colocalization studies. The subcellular localization of **10** in Hep3B cells was examined. The minimum concentration of **10** for the study was optimized at 5 μM. The cells were treated with

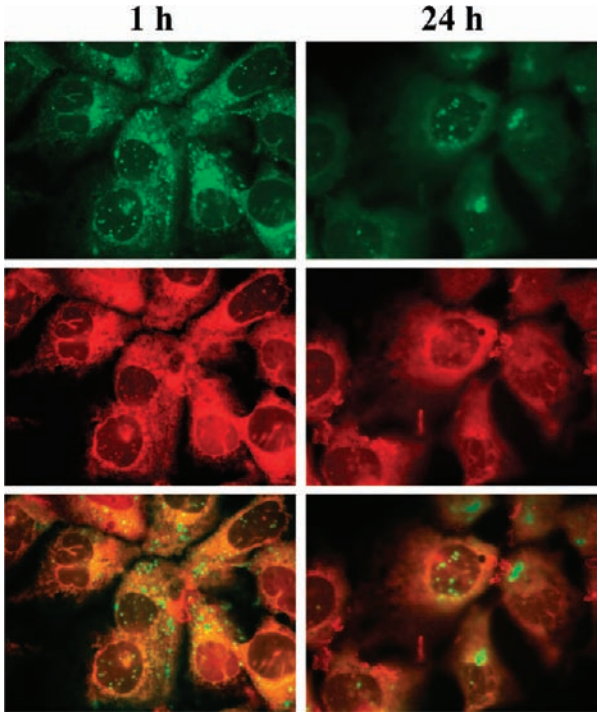


Figure 3. Colocalization of **10** with ER Tracker (ER specific dye) in Hep3B cells at different time points (1 and 24 h). Top panel: Cellular localization of **10**. Middle panel: Cellular localization of ER Tracker. Bottom panel: Overlay of the upper two images.

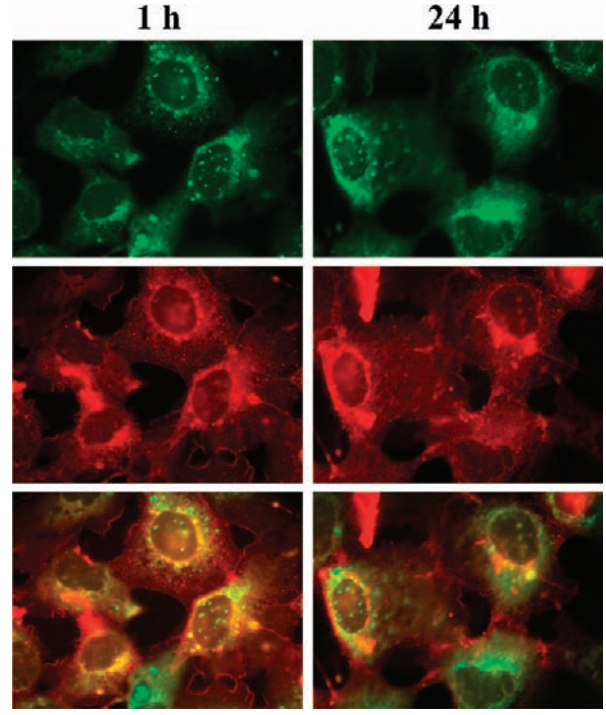


Figure 4. Colocalization of **10** with BODIPY TR ceramide (Golgi specific dye) in Hep3B cells at different time points (1 and 24 h). Top panel: Cellular localization of **10**. Middle panel: Cellular localization of BODIPY TR ceramide. Bottom panel: Overlay of the upper two images.

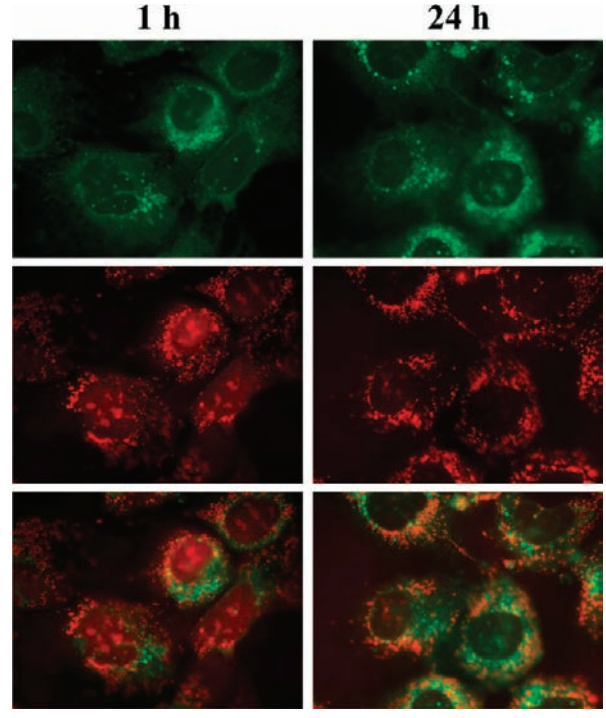


Figure 5. Colocalization of **10** with MitoTracker (mitochondria specific dye) in Hep3B cells at different time points (1 and 24 h). Top panel: Cellular localization of **10**. Middle panel: Cellular localization of MitoTracker. Bottom panel: Overlay of the upper two images.

10 (5 μM) and ER Tracker for 1 h and examined with fluorescence microscopy. The localization of **10** was viewed as green fluorescent spots upon excitation at 350 nm (Figure 3, top-left). The perinuclear and reticulate fluorescent image is suggestive of location at the endoplasmic reticulum (ER). Then, the same area was excited at 550 nm to specifically locate the ER using ER Tracker (red, middle-left). Overlapping the fluorescent images of **10** and ER Tracker showed an extensive orange image in the same area, revealing the colocalization of these two probes (bottom-left). It is likely that ER is a major site at which **10** was located. Similar results were obtained for cells subjected to incubation of **10** for 24 h (Figure 3, right column). ER has an extensive membrane network. When using a lipid stain Nile Red to stain the membranous structure,[8] extensive colocalization of **10** with cytoplasmic membranous network resembling endoplasmic reticulum has been observed (see the Supporting Information, Figure S2).

Overlapping of the fluorescent images arising from **10** and BODIPY TR ceramide (Golgi specific dye) also showed partial colocalization of these two fluorescent compounds (Figure 4, bottom-left), which may be related to vesicular connection between Golgi and ER. After 24 h, the colocalization became less obvious (Figure 4, bottom-right). The fluorescent images of **10** and MitoTracker (mitochondria specific dye) showed little colocalization, revealing that **10** is unlikely localized at the mitochondria (Figure 5).

The colocalization of **10** with organelle specific dyes (Figures 3−5) was quantified by Pearson's correlation analysis (see the Supporting Information, Table S1). The Pearson's coefficient values (*Rr*) for colocalization of **10** (1-h treatment) with ER tracker, BODIPY TR ceramide, and MitoTracker are 0.81, 0.68, and 0.39, respectively, suggesting that ER is the major site at which compound **10** is located.

The specialized functions of ER include translation, folding, and transport of proteins.[10] ER chaperones and enzymes are responsible for folding, disulfide bond formation, post-translational modification of the secretary, and integral membrane proteins. As depicted in Figure 3, the preferential accumulation of artemisinin compounds in ER might alter the efficiency of protein processing functions, and contribute to the cytotoxicities.[10] In particular, protein thiols could be the targets of the reactive endoperoxide of artemisinins.[11]

It has been reported that artemisinin and its derivatives specifically target the *Plasmodium falciparum* PfATP6, an ortholog of sarco/endoplasmic reticulum membrane calcium ATPase SERCA.[7a,12] Nonetheless, there are also reports revealing that artemisinin may have nonspecific molecular targets.[8,13] Although our fluorescent artemisinin derivative appears to target ER in cancer cells, it still remains to be determined to what extent the antimalarial and anticancer subcellular or molecular targets of diversified artemisinin derivatives are in common.[14]

We have examined the cellular localization of **10** in the absence or presence of a 10-fold excess of compound **1**, **2**, or **3**. No significant difference in both fluorescence intensity and localization of **10** was noted. We also observed that the cellular localization of **10** was not affected by the presence of iron chelator desferrioxamine.[7,8,14b,15] Apparently, the subcellular localization of **10** was determined by its lipophilicity but not affected by the trace free iron ions present in the cells.

In summary, a cytotoxic artemisinin compound conjugated with a fluorescent dansyl moiety was synthesized and its subcellular localization in Hep3B cells was investigated. Colocalization by organelle specific dyes revealed that ER is the main site of its accumulation.

Acknowledgment. We are thankful for the financial support of The University of Hong Kong and Hong Kong Polytechnic University.

Supporting Information Available: Experimental procedures and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

OL902890J

(9) Yang, Z.; Wong, E. L. M.; Shum, T. Y. T.; Che, C. M.; Hui, Y. *Org. Lett.* **2005**, *7*, 669–672.

(10) (a) Sanders, S. L.; Schekman, R. *J. Biol. Chem.* **1992**, *267*, 13791–13794. (b) Ellgaard, L.; Molinari, M.; Helenius, A. *Science* **1999**, *286*, 1882–1888. (c) Ellgaard, L.; Helenius, A. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 181–191. (d) Boelens, J.; Lust, S.; Offner, F.; Bracke, M. E.; Vanhoecke, B. W. *In Vivo* **2007**, *21*, 215–226.

(11) (a) Wu, Y.; Yue, Z. Y.; Wu, Y. L. *Angew. Chem., Int. Ed.* **1999**, *38*, 2580–2582. (b) Wang, D. Y.; Wu, Y. L. *Chem. Commun.* **2000**, 2193–2194. (c) Wang, D. Y.; Wu, Y. L.; Wu, Y.; Liang, J.; Li, Y. *J. Chem. Soc., Perkin Trans. 1* **2001**, 605–609. (d) Wu, W. M.; Chen, Y. L.; Zhai, Z.; Xiao, S. H.; Wu, Y. L. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1645–1647.

(12) (a) Uhlemann, A. C.; Cameron, A.; Eckstein-Ludwig, U.; Fischbarg, J.; Iserovich, P.; Zuniga, F. A.; East, M.; Lee, A.; Brady, L.; Haynes, R. K.; Krishna, S. *Nat. Struct. Mol. Biol.* **2005**, *12*, 628–629. (b) Jambou, R.; Legrand, E.; Niang, M.; Khim, N.; Lim, P.; Volney, B.; Ekala, M. T.; Bouchier, C.; Esterre, P.; Fandeur, T.; Mercereau-Puijalon, O. *Lancet* **2005**, *366*, 1960–1963. (c) Haynes, R. K.; Chan, W. C.; Lung, C. M.; Uhlemann, A. C.; Eckstein, U.; Taramelli, D.; Parapini, S.; Monti, D.; Krishna, S. *ChemMedChem* **2007**, *2*, 1480–1497.

(13) (a) O'Neill, P. M.; Rawe, S. L.; Borstnik, K.; Miller, A.; Ward, S. A.; Bray, P. G.; Davies, J.; Oh, C. H.; Posner, G. H. *ChemBioChem* **2005**, *6*, 2048–2054. (b) Garah, F. B. E.; Stigliani, J. L.; Coslédan, F.; Meunier, B.; Robert, A. *ChemMedChem* **2009**, *4*, 1469–1479.

(14) (a) Riganti, C.; Doublier, S.; Viarisio, D.; Miraglia, E.; Pescarmona, G.; Ghigo, D.; Bosia, A. *Br. J. Pharmacol.* **2009**, *156*, 1054–1066. (b) Stockwin, L. H.; Han, B.; Yu, S. X.; Hollingshead, M. G.; ElSohly, M. A.; Gul, W.; Slade, D.; Galal, A. M.; Newton, D. L. *Int. J. Cancer* **2009**, *125*, 1266–1275.

(15) Wu, Y. *Acc. Chem. Res.* **2002**, *35*, 255–259.