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# Luminescent iridium(III) complexes as COX-2-specific imaging agents in cancer cells†

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**Two luminescent iridium(III) complexes, 1 and 2, were synthesized and evaluated for their ability to probe COX-2 in human cancer cells. This is the first application of iridium(III) complexes as imaging agents for COX-2. We demonstrate that complex 1 differentiates cancer cells from normal cells with high stability and low cytotoxicity.**

Cancer has become one of the greatest causes of death in humans worldwide.[1] Early diagnosis of cancer is particularly important to minimize cancer mortality. Consequently, efforts have intensified over the last decade to develop imaging agents and inhibitors to diagnose and treat cancers.[2] Molecular fluorescence imaging has attracted particular attention for cancer cell imaging by its high sensitivity, high cell permeability, and noninvasive nature.[3]

Cancer cells often overexpress enzymes, which therefore act as biomarkers for cancer cell detection.[4] For example, γ-glutamyltranspeptidase (GGT),[5] membrane type 1-matrix metalloproteinase (MT1-MMP),[3a] quinone oxidoreductase isozyme 1 (NQO1),[6] and cyclooxygenase-2 (COX-2)[7] are all overexpressed in cancerous cells. In particular, COX-2 is highly expressed in stomach, colon, pancreas, and other cancers.[8] Importantly, although COX-2 is barely expressed in normal cells, clinical data show that the level of COX-2 increases as cancer progresses.[9]

Chemical probes can be used to monitor cancer biomarkers, such as COX-2. Recently, Uddin et al. reported a $CF_3$-fluorocoxib A-based fluorescence molecule for COX-2-specific imaging.[10] Marnett and co-workers studied the structure-activity relationship (SAR) of COX-2-specific imaging dyes.[11] Peng and co-workers developed several fluorescence probes targeting COX-2 in cancer cells,[12] while Zhang and co-workers prepared a naphthalene-based two-photon optical probe for real-time bioimaging of COX-2 in living biosystems.[13] However, to the best of our knowledge, no metal-based imaging agent of COX-2 has been previously reported.

In recent years, luminescent transition metal complexes have found significant use as biological and chemical imaging agents and probes by virtue of their desirable photophysical and structural properties, including, for example, (i) adjustable emission and excitation spectra, (ii) long phosphorescent lifetimes, (iii) relatively high luminescent quantum yields, and (iv) well-defined three-dimensional structures that interact specifically with biomolecules.[14] As a result, transition metal complexes have emerged as promising alternative choices to organic dyes as diagnostic imaging agents for diseases.[15]

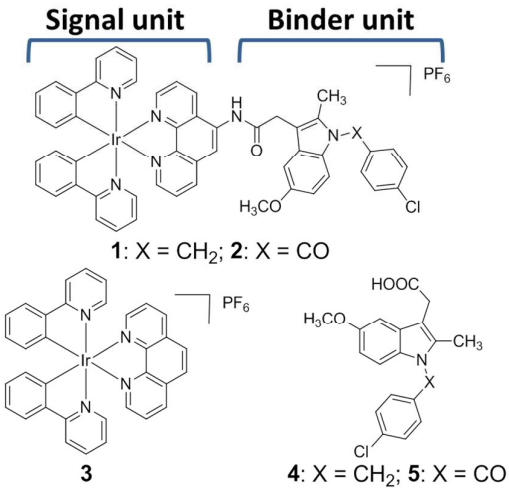


**Fig. 1.** Chemical structure of Ir(III) complexes **1**, **2** and **3** and compounds **4** and **5**.

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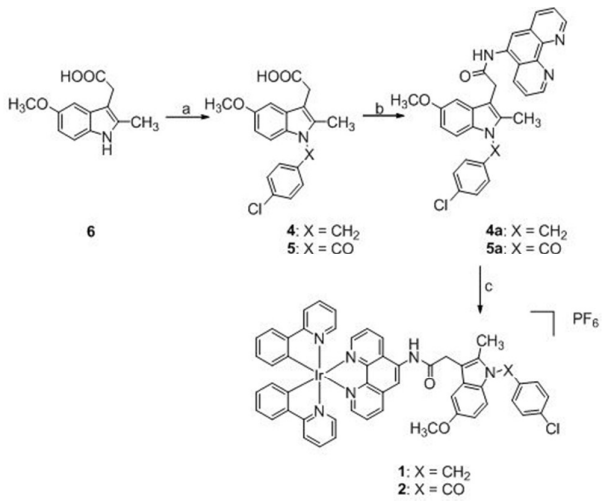


**Scheme 1.** Syntheses of Ir(III) complexes **1** and **2**. Reagents and conditions: (a) 4-chlorobenzyl chloride, NaH, DMF, 0 to 25 °C, 2 h, **4**: 52% yield; Compound **5** is commercially available. (b) 1,10-phenanthrolin-5-amine, oxalyl chloride, DMF, DCM, room temperature, overnight, **4a**: 55% yield, **5a**: 42% yield; (c) $[Ir(ppy)_2Cl]_2$, DCM/MeOH (1:1, *v*/*v*), 25 °C, overnight, then $NH_4PF_6$, 2 h, **1**: 53% yield, **2**: 65% yield. DMF = *N*,*N*-dimethylformamide, DCM = dichloromethane.

In this study, we designed two novel Ir(III) complexes, **1** and **2**, through conjugating an initial luminescent Ir(III) complex **3** with compound **4** or **5**, respectively (Fig. 1). Compound **4** is 2-(1-(4-chlorobenzyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)acetic acid, a structural analogue of the well-known COX-2 inhibitor indomethacin (compound **5**).[16] Therefore, complexes **1** and **2** can be considered to consist of a "signal unit" **3** conjugated to a "binder unit" **4** or **5** via an amide bond linkage. We anticipated that such design could generate suitable probe molecules to sense COX-2 and still retain specific binding to the target enzyme.

The structures of complexes **1** and **2**, as well as all intermediates, were characterized by $^1$H NMR, $^{13}$C NMR and HRMS spectrometry. The photophysical properties of **1** and **2** were then investigated. Complex **1** shows a maximum emission wavelength at 575 nm with excitation at 295 nm, while complex **2** exhibits a maximum emission at 580 nm at an excitation wavelength of 290 nm. Both metal complexes possess large Stokes shifts of around 280 nm based their metal-to-ligand charge-transfer (MLCT) states, which are significantly higher than those of organic dyes. Complexes **1** and **2** were stable at 298 K in a DMSO-$d_6$/$D_2O$ (9:1, *v*/*v*) solution for at least seven days, according to $^1$H NMR spectroscopy (Fig. S1). They are also stable in acetonitrile/$H_2O$ (9:1, *v*/*v*) solution at 298 K for at least seven days, as shown by UV/Vis spectroscopy (Fig. S2). Moreover, complexes **1** and **2** displayed long lifetimes of *ca.* 4.56 μs and 4.28 μs, respectively (Table S1), virtually on the same order as that exhibited by other Ir(III) complexes,[17] whereas organic chemosensors usually show nanosecond lifetimes. Employing time-resolved emission spectroscopy (TRES), it was shown that the long-lived phosphorescence of transition metal complexes, such as that of complexes **1** and **2**, allowed emission to be differentiated

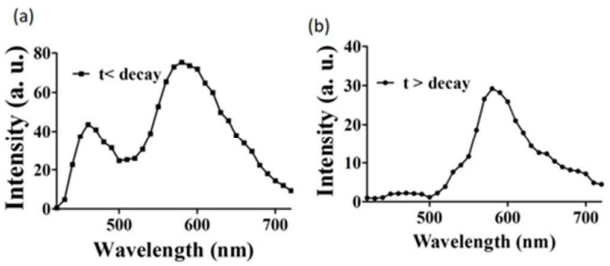


**Fig. 2.** Time-resolved emission spectra of complex **1** in the presence of coumarin 460 fluorescent media. (a) t < decay (b) t > decay. Excitation wavelength = 355 nm.

from strong autofluorescence. To validate this hypothesis, we used the fluorescent organic dye coumarin 460 (Cm-460) as a model matrix interference. For TRES measurement, the time gate was set after the complete fluorescence decay of Cm-460. When the time gate was shorter than the decay time, results showed that Cm-460 exhibited a strong emission peak at 455 nm and that the peak of **1** was partially obscured by the trailing edge of the Cm-460 peak (Fig. 2a). However, when the time gate was set to be longer than the decay time, fluorescence of Cm-460 was eliminated, and the emission of complex **1** became more obvious (Fig. 2b).

In consideration of the promising luminescent behavior shown by complexes **1** and **2**, we employed **1** and **2** as imaging agents to detect COX-2 in living cells. A Western blotting assay was first employed to determine the expression of COX-2 in two normal human cell lines, human embryonic kidney 293 (HEK293) and human liver cell (LO2) cells, and two human cancer cell lines, human breast cancer (MCF-7) and human cervical cancer (HeLa) cells. The results showed that COX-2 was strongly expressed in both cancer cell lines, but not in the two normal cell lines (Fig. S3).[12, 16a] Subsequently, HeLa cells and LO2 cells were incubated with complex **1**, **2** or DMSO for 4 h. Fluorescence imaging of the cells showed that the HeLa cells exhibited a strong and stable florescence upon excitation at 405 nm, while LO2 cells showed negligible fluorescence towards complexes **1** and **2** (Figs. 3a and 3b). These results indicate that complexes **1** and **2** could potentially differentiate between cancer and normal cells. However, complex **2** showed lower solubility in the cell imaging assays. As seen in Figure S4, insoluble particles, presumably from complex **2**, were found when HeLa cells were stained with complex **2**. Thus, in further experiments, complex **1** was used a as a model to demonstrate the imaging ability of Ir(III) complexes for COX-2. We presume that the mechanism of the differential luminescence enhancement in the imaging experiments is due to the binding of complex **1** to COX-2 in the cells. In normal cells, COX-2 expression is low. Hence, complex **1** would not accumulate within normal cells and no luminescence enhancement would be observed. On the other hand, cancer cells exhibit strong expression of COX-2. Complex **1** is therefore able to bind to COX-2 and is accumulated within the cells, thus generating a high level of luminescence in cancer cells. We also tested the luminescence of complex **1** in the presence of $H^+$, $K^+$, $Na^+$, $Mg^{2+}$, amino acids, bovine serum albumin (BSA) and human serum

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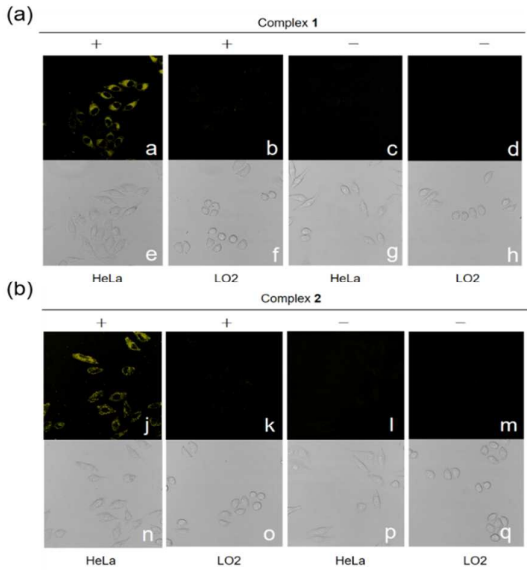


**Fig. 3.** Living cells stained by (a) complex **1** and (b) complex **2** (1.0 μM). (a, c, e, g, j, l, n, p) HeLa cells and (b, d, f, h, k, m, o, q) LO2 cells. The upper row is luminescence imaging, and the lower row is bright filed imaging. Excitation wavelength = 405 nm.

albumin (HSA) in the absence of cells. The experiment demonstrated that these ions, amino acids, BSA and HSA did not dramatically affect the luminescence of complex **1** (Figs. S5a and b).

In order to confirm that complex **1** could specifically target COX-2, the expression of COX-2 was blocked by treatment with curcumin, which specifically inhibits the expression of COX-2.[16a, 18] After treatment of HeLa cells with curcumin (0–40 μM) for 24 h, Western blotting showed that the expression of COX-2 in the cells was markedly reduced (Fig. 4a). After 24 h of

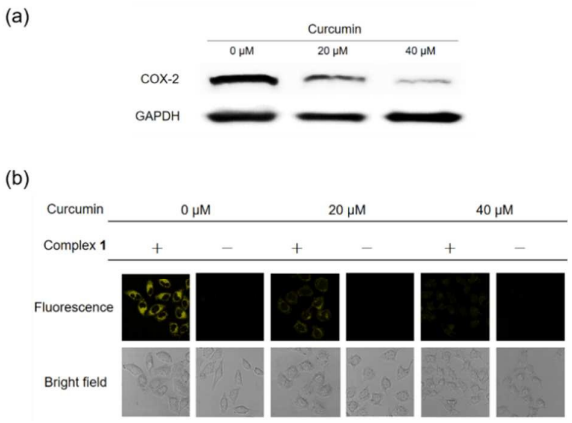


**Fig. 4.** (a) Immunoblotting analysis of the effect of curcumin treatment on HeLa cells after 24 h. Densitometry analysis revealed that curcumin inhibited COX-2 expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control enzyme. (b) HeLa cells stained by complex **1** (1.0 μM) in the presence of different concentration of curcumin. The upper row is luminescence imaging, and the lower row is bright filed imaging. Excitation wavelength = 405 nm.

curcumin treatment, HeLa cells were incubated for another 4 h with complex **1** (1 μM). Cell imaging showed that the fluorescence intensity of the HeLa cells decreased with increasing concentration of curcumin (Fig. 4b). Taken together, this evidence shows that the luminescence of complex **1** is linked to COX-2 expression, suggesting, in turn, that complex **1** could specifically recognize COX-2 in human cancer cells.

We then performed a dose-response experiment to study the staining ability of complex **1**. The results of the fluorescence imaging experiment showed that the luminescence intensity of the cancer cell line HeLa, after incubation with complex **1** for 2 h, increased with the concentration of complex **1** over the range of 0.3–30 μM (Fig. 5a). On the contrary, normal cell line LO2 showed only negligible luminescence, even with the highest concentration of complex **1** (Fig. S6). Next, the effect of incubation time on cell imaging was evaluated. The luminescence intensities of complex **1**-treated HeLa cells increased with incubation time (Fig. 5b). On the other hand, no luminescence was observed in

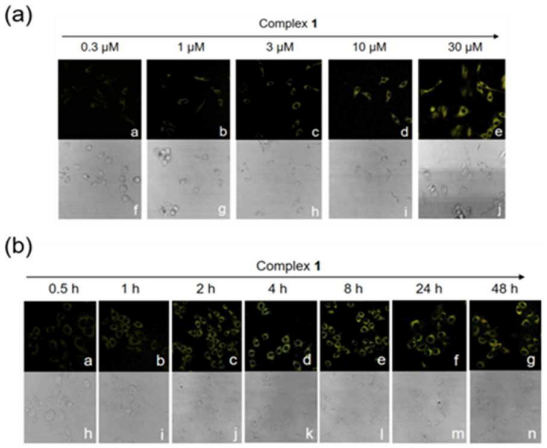


**Fig. 5.** (a) Cancer cell line (HeLa) stained by different concentrations of complex **1** (0.3, 1, 3, 10 and 30 μM). (b) Cancer cell line (HeLa) stained by complex **1** (1.0 μM) at different incubation time (0.5, 1, 2, 4, 8, 24 and 48 h).

LO2 cells, even up to the maximum incubation time of 48 h (Fig. S7).

It is noteworthy that Ir(III) complex **1** showed high stability and photostability. HeLa cells stained by complex **1** preserved their luminescence intensity for at least 24 h (Fig. S8). This indicates that complex **1** could potentially detect and image COX-2 in living cells over relatively longer time scales, which contrasts favorably with reported organic fluorescence probes.[13]

The cytotoxicity of complexes **1** and **2** was examined by using the (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) (XTT) assay (Fig. S9). HEK293 cells, LO2 cells, MCF-7 cells and HeLa cells were incubated with different concentrations of Ir(III) complexes **1** and **2** for 72 h, and cell viability was examined. The $IC_{50}$ value of complex **1** was estimated to be over 100 μM after exposure for 72 h. The estimated $IC_{50}$ values against all four cell lines were higher

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compared to the concentration required for staining these cells. This suggests that the presence of an imaging agent for COX-2 in living cells will not significantly damage cancer or normal cells. On the other hand, complex **2** inhibited the growth of the LO2 cells with an $IC_{50}$ value of 55.6 μM, indicating that it would be less suitable for use as an imaging agent (Fig. S10).

In conclusion, two novel luminescent Ir(III) complexes, **1** and **2**, containing indomethacin analogue (**4** or **5**)-functionalized N^N ligands, were explored as imaging probes for COX-2 based on the unique molecular structures comprising a signal unit and a binder unit. Ir(III) complexes **1** and **2** both possess superior photophysical characteristics and high stability in living cells. Complex **1**, however, showed higher solubility and lower cytotoxicity compared to complex **2**. Therefore, we anticipate that complex **1** could be developed as a tool to monitor COX-2 in living cells and/or a cancer diagnostic agent.

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## Notes and references

1 (a) R. L. Siegel, K. D. Miller and A. Jemal, *CA Cancer J. Clin.*, 2015, **65**, 5; (b) J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D. M. Parkin, D. Forman and F. Bray, *Int. J. Cancer*, 2015, **136**, E359; (c) C. E. DeSantis, R. L. Siegel, A. G. Sauer, K. D. Miller, S. A. Fedewa, K. I. Alcaraz and A. Jemal, *CA Cancer J. Clin.*, 2016, **66**, 290.

2 (a) A. B. Chinen, C. M. Guan, J. R. Ferrer, S. N. Barnaby, T. J. Merkel and C. A. Mirkin, *Chem. Rev.*, 2015, **115**, 10530; (b) H. Ma, J. Liu, M. M. Ali, M. A. I. Mahmood, L. Labanieh, M. Lu, S. M. Iqbal, Q. Zhang, W. Zhao and Y. Wan, *Chem. Soc. Rev.*, 2015, **44**, 1240.

3 (a) J. Park, J. Yang, E. K. Lim, E. Kim, J. Choi, J. K. Ryu, N. H. Kim, J. S. Suh, J. I. Yook and Y. M. Huh, *Angew. Chem. Int. Ed.*, 2012, **51**, 945; (b) R. Siegel, D. Naishadham and A. Jemal, *CA Cancer J. Clin.*, 2013, **63**, 11; (c) K. W. Plaxco and H. T. Soh, *Trends Biotechnol.*, 2011, **29**, 1; (d) Y. Zhang, W. Chen, D. Feng, W. Shi, X. Li and H. Ma, *Analyst*, 2012, **137**, 716.

4 (a) L. Wu and X. Qu, *Chem. Soc. Rev.*, 2015, **44**, 2963; (b) W. S. Shin, J. Han, P. Verwilst, R. Kumar, J.-H. Kim and J. S. Kim, *Bioconjugate Chem.*, 2016, **27**, 1419.

5 (a) T. Mizushima, S. Ohnishi, Y. Shimizu, Y. Hatanaka, K. C. Hatanaka, H. Hosono, Y. Kubota, M. Natsuizaka, M. Kamiya and S. Ono, *BMC cancer*, 2016, **16**, 1; (b) C. Sato, S. Abe, Y. Saito, E. So Tsuruki, H. Takamaru, M. Makazu, Y. Sato, H. Sasaki, H. Tanaka and N. Ikezawa, *Digestion*, 2015, **91**, 70.

6 (a) S. U. Hettiarachchi, B. Prasai and R. L. McCarley, *J. Am. Chem. Soc.*, 2014, **136**, 7575; (b) Y.-M. Sung, S. R. Gayam, P.-Y. Hsieh, H.-Y. Hsu, E. W.-G. Diau and S.-P. Wu, *ACS Appl. Mater. Interfaces*, 2015, **7**, 25961.

7 (a) O. Tietz, A. Marshall, M. Wuest, M. Wang and F. Wuest, *Curr. Med. Chem.*, 2013, **20**, 4350; (b) M. J. Uddin, T. A. Werfel, B. C. Crews, M. K. Gupta, T. E. Kavanaugh, P. J. Kingsley, K. Boyd, L. J. Marnett and C. L. Duvall, *Biomaterials*, 2016, **92**, 71.

8 (a) R. G. Kurumbail, A. M. Stevens, J. K. Gierse, J. J. McDonald, R. A. Stegeman, J. Y. Pak, D. Gildehaus, J. M. Miyashiro, T. D. Penning and K. Seibert, *Nature*, 1996, **384**, 644; (b) M. Tsujii, S. Kawano and R. N. DuBois, *Proc. Natl. Acad. Sci. U.S.A.*, 1997, **94**, 3336.

9 (a) X. P. He, Y. Shao, X. L. Li, W. Xu, G. S. Chen, H. H. Sun, H. C. Xu, X. Xu, D. Tang and X. F. Zheng, *FEBS J.*, 2012, **279**, 4201; (b) J. Cheng and X.-M. Fan, *World J. Gastroenterol.*, 2013, **19**, 7361.

10 M. J. Uddin, B. C. Crews, I. Huda, K. Ghebreselasie, C. K. Daniel and L. J. Marnett, *ACS Med. Chem. Lett.*, 2014, **5**, 446.

11 (a) M. J. Uddin, B. C. Crews, K. Ghebreselasie and L. J. Marnett, *Bioconjugate Chem.*, 2013, **24**, 712; (b) A. L. Blobaum, M. J. Uddin, A. S. Felts, B. C. Crews, C. A. Rouzer and L. J. Marnett, *ACS Med. Chem. Lett.*, 2013, **4**, 486.

12 (a) H. Zhang, J. Fan, J. Wang, B. Dou, F. Zhou, J. Cao, J. Qu, Z. Cao, W. Zhao and X. Peng, *J. Am. Chem. Soc.*, 2013, **135**, 17469; (b) H. Zhang, J. Fan, J. Wang, S. Zhang, B. Dou and X. Peng, *J. Am. Chem. Soc.*, 2013, **135**, 11663.

13 H. Zhang, J. Fan, K. Wang, J. Li, C. Wang, Y. Nie, T. Jiang, H. Mu, X. Peng and K. Jiang, *Anal. Chem.*, 2014, **86**, 9131.

14 (a) D.-L. Ma, H.-Z. He, K.-H. Leung, D. S.-H. Chan and C.-H. Leung, *Angew. Chem. Int. Ed.*, 2013, **52**, 7666; (b) E. Baggaley, J. A. Weinstein and J. G. Williams, *Coord. Chem. Rev.*, 2012, **256**, 1762; (c) V. W.-W. Yam, V. K.-M. Au and S. Y.-L. Leung, *Chem. Rev.*, 2015, **115**, 7589; (d) M. Chen, Y. Wu, Y. Liu, H. Yang, Q. Zhao and F. Li, *Biomaterials*, 2014, **35**, 8748; (e) L. Yao, J. Zhou, J. Liu, W. Feng and F. Li, *Adv. Funct. Mater.*, 2012, **22**, 2667; (f) J. C. Hidalgo-Acosta, M. A. Méndez, M. D. Scanlon, H. Vrubel, V. Amstutz, W. Adamiak, M. Opallo and H. H. Girault, *Chem. Sci.*, 2015, **6**, 1761.

15 (a) E. Meggers, *Curr. Opin. Chem. Biol.*, 2007, **11**, 287; (b) E. Meggers, *Chem. Commun.*, 2009, 1001; (c) E. Meggers, *Angew. Chem. Int. Ed.*, 2011, **50**, 2442; (d) N. P. E. Barry and P. J. Sadler, *Chem. Soc. Rev.*, 2012, **41**, 3264; (e) P. C. A. Bruijnincx and P. J. Sadler, *Curr. Opin. Chem. Biol.*, 2008, **12**, 197; (f) G. Gasser, I. Ott and N. Metzler-Nolte, *J. Med. Chem.*, 2010, **54**, 3; (g) I. Ott and R. Gust, *Arch. Pharm.*, 2007, **340**, 117; (h) Z. Chen, H. Ji, C. Liu, W. Bing, Z. Wang and X. Qu, *Angew. Chem.*, 2016; (i) N. Gao, H. Sun, K. Dong, J. Ren, T. Duan, C. Xu and X. Qu, *Nat. Commun.*, 2014, **5**.

16 (a) A. Goel, C. R. Boland and D. P. Chauhan, *Cancer Lett.*, 2001, **172**, 111; (b) J. Tibble, G. Sigthorsson, R. Foster and I. Bjarnason, *Scand. J. Gastroenterol.*, 2000, **35**, 802.

17 (a) H.-J. Zhong, L. Lu, K.-H. Leung, C. C. Wong, C. Peng, S.-C. Yan, D.-L. Ma, Z. Cai, H.-M. D. Wang and C.-H. Leung, *Chem. Sci.*, 2015, **6**, 5400; (b) L. Lu, M. Wang, L.-J. Liu, C.-Y. Wong, C.-H. Leung and D.-L. Ma, *Chem. Commun.*, 2015, **51**, 9953.

18 M. Niu, S. Wu, L. Mao and Y. Yang, *Traffic*, 2013, **14**, 1042.

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