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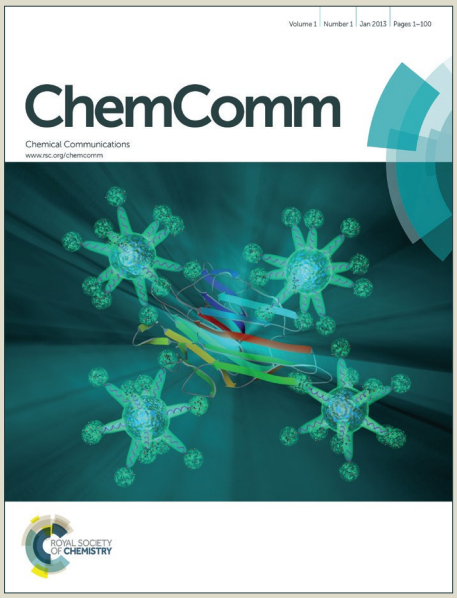

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View Article Online
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COMMUNICATION

Indomethacin-guided cancer selective prodrug conjugate activated by histone deacetylase and tumor-associated protease

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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An indomethacin guided drug delivery conjugate (IGDDC) has been designed by utilizing cancer associated elevated HDAC and CTSL activities as consective demasking ventures for drug activation. IGDDC exhibited preferential uptake by COX-2 positive cells both *in vitro* and *ex vivo* highlighting indomethacin role in designing new cancer-specific drug delivery frameworks.

The current thrust of the cancer treatment program is to improve the pharmacokinetics of the drug candidates.[1] Over a past few decades, tumor-targeted delivery strategies has been widely investigated in chemotherapy, so as to minimize adverse effect in perturbing normal cells function compared to the cancer cells, thereby augmenting therapeutic efficacy. The conventional approach in this context is to utilize the targeting ligands selective to the over-expressed receptors or transporters on the cancer cell surface,[2] further taking advantage of pathological tumor microenvironment such as acidic pH,[3] reactive oxygen species (ROS)[4] and overexpressed enzymes at or around cancer cell[5] to release anticancer drugs with regained cytotoxicity.

Cyclooxygenase (COX) is a prostaglandin-endoperoxide synthase (PTGS) enzyme, which plays an integral role in the formation of prostanoids.[6] As compared to the normal cells, overexpressed COX-2 levels have been associated with various cancers such as the pancreas, colon, stomach, breast, head/neck carcinoma, or inflammatory lesions, thus indicating their vital role in promoting tumor growth, invasiveness, angiogenesis and metastasis.[7] Indomethacin, a commercially available nonsteroid anti-inflammatory drug (NSAID) is a non-selective COX isoforms inhibitor (COX-1 and COX-2). Despite possessing such poor selectivity toward COX-2 cells, its fluorescent conjugate has been successfully utilized in tumor selective imaging.[8] Yet, to the best of our knowledge, no indomethacin-based tumor-targeted drug delivery conjugates has been attempted.[9]

The epigenetic enzyme, histone deacetylases (HDACs) has garnered much attention in oncology owing to its crucial roles in various biological processes.[10] HDACs modulate the chromatin structure and function by de-acetylation of the lysine residue on amino-terminal histone trails.[11] The involvement of altered HDACs expressions in cancer pathogenesis has been intensively scrutinized and validated.[12] Currently, several HDACs are in various development stages as monotherapy and combination therapy.[13] Likewise, upregulated cysteine cathepsins L (CTSL) has been perceived as a hallmark in cancer progression and metastasis at multiple stages.[14] On that account, CTSL with elevated activity and their localization exhibited the prognostic and diagnostic value clinically.[15] Collectively, combining HDAC and CTSL activities as a sequential demasking tool, Ueki *et al* has indeed developed a novel anticancer drug activation system with enhanced efficacy.[16]

Thus, in this report, we elucidate a novel design, synthesis and development of indomethacin-guided drug delivery conjugate (IGDDC) for tumor treatment. IGDDC comprises of three parts as shown in Scheme 1, incorporated intentionally to enhance the targeting effect. The first part adopts a NSAID (non-steroidal anti-inflammatory drug), indomethacin, as a cancer targeting unit. The second part consists of drug-releasing system *via* cancer abundant HDAC and CTSL activities where a lysine moiety can be exposed after deacetylation by HDAC and further its amide bond is selectively cleaved by CTSL to release the drug. The third part is doxorubicin (DOX), a well-known topoisomerase II inhibitor, for effective anti-cancer treatment. Thereby, the IGDDC reagent will be preferentially taken up by cancer cells, followed by stepwise activation by endogenous HDAC and CTSL activities resulting in DOX release with concomittent fluorescence enhancement. In this study, the *in vitro* and *ex vivo* cancer cell targeting ability of IGDDC is monitored by *in situ* fluorescence enhancement of DOX moiety.

The **1** (IGDDC) was synthesized as shown in Scheme 1. The EDC coupling of indomethacin with **5**[17] in DMF resulted in compound **6** (88% yield) followed by *tert*-butyl group deprotection in TFA/DCM yielding compound **2** (90 %). The selective acid protection of Lys(Ac)-OH using the *tert*-butylacetate/$HClO_4$ (26% yield) and EDC coupling with **2** produced compound **8** (60 % yield). The subsequent deprotection reaction followed by conjugation with DOX *via* HATU coupling resulted in the formation of the **1** in 24 % yield (detailed synthetic procedure in the ESI†). All compounds were well confirmed by ^1H, ^{13}C NMR spectroscopy and ESI-MS spectrometry (see the ESI†).

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Electronic Supplementary Information (ESI) available: Synthesis procedure, NMR, MS, fluorescence, and cell imaging data. See DOI: 10.1039/x0xx00000x
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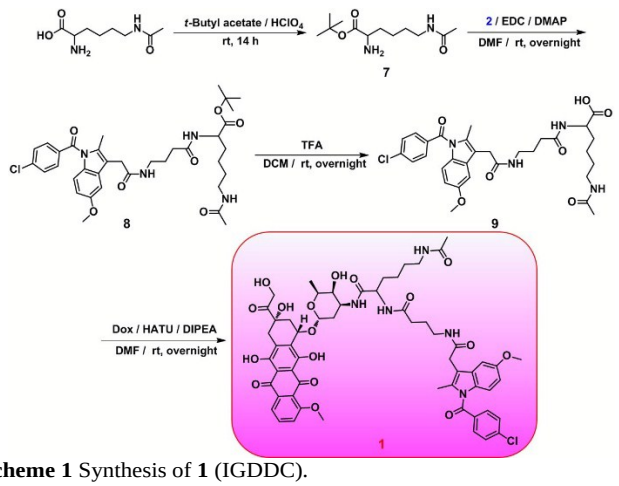
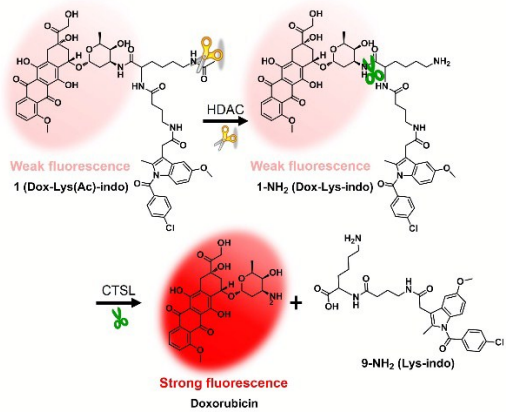


Scheme 1 Synthesis of **1** (IGDDC).

The drug release mechanism from **1** after stepwise HDAC and CTSL action was monitored through the anticipated spectral changes with ultra-visible and fluorescence microscopy. **1** exhibited a broad absorption band at 499 nm and weak emission band at 590 nm (Fig. S2 in the ESI†). After adding HDAC (1.25 mM) followed by trypsin (500 BAEE units/ml) (possessing a similar protease activity to CTSL[18]) to the solution, the fluorescence intensity was enhanced at 590 nm in time-dependent manner, mostly due to the DOX release from **1** (Fig. 1a). However, such fluorescence change was not observed either by HDAC and trypsin alone (Fig. 1b). These results are consistent with our anticipation in Scheme 2, the acetyl group is cleaved selectively by HDAC activity in the first step and the DOX is released by trypsin with the concomitant fluorescence enhancement. Curiosity drove us to prove the mechanism of stepwise action of the **1** towards two enzymes. Thus, we carried out a mass spectrometry analysis after addition of the HDAC and CTSL to **1** solution. The **1** possesses a single molecular ion peak at 1136.4 *m/z*. However, after addition of HDAC alone, the mass spectrum showed a peak at 1095.649 which corresponds to the *m/z* of **1-NH$_2$**, further upon the subsequent addition of trypsin, it shows the peaks at 543.201 and 571.140, corresponding to those of **Dox** and **9-NH$_2$**, respectively (Fig. S18-S21 in the ESI†). Further, by HPLC data, we have confirmed that **1** is cleaved by the cell extract to release DOX (Fig. S22 in the ESI†). Thus, from these results, we confirmed that **1** would release DOX only after the stepwise action of two enzymes, HDAC and trypsin, possibly CTSL in the cells.



Scheme 2 Sequential DOX releasing mechanism of **1**

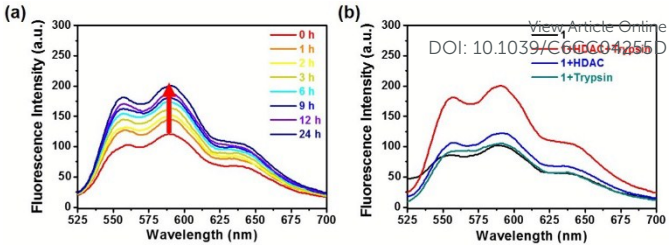


Fig. 1 Fluorescence spectra of **1** (5 μM) treated with HDAC and trypsin IX (a) b) Fluorescence spectra of **1** (5 μM) (Black line), **1** treated with HDAC and trypsin (Red line), **1** and HDAC (Blue line), **1** and trypsin (Green line), excitation at 499 nm in HDAC buffer with 1 % DMSO (Slit widths: ex 5 em 5)

To test the prominent role of indomethacin (a COX-selective anti-inflammatory drug), in **1** for its cancer-targeting ability, the fluorescence microscopic studies have been conducted towards selected cancer cell lines. After incubating the cells with **1** for 90 min, the fluorescence intensity of released DOX was recorded (Fig. 2). In the COX-2 positive cell lines (HeLa, HepG2), the fluorescence intensity is significantly enhanced whereas it is slightly or little increased in other cell lines (Fig. 2b). The fluorescence enhancement order observed as HeLa, HepG2 > HCT 116, MIA PaCa-2 > Caco-2, correspond to the increased COX-2 expression (Table S1 in the ESI†)[19] as well as elevated HDAC and CTSL activities in the cells. The time-dependent fluorescence change is shown in Fig. S3 in the ESI†. All the results together support that indomethacin in **1** exhibits a significant tumor targeting ability. The increased fluorescence intensity in HeLa and HepG2 cell lines further confirmed the efficient activation of **1**.

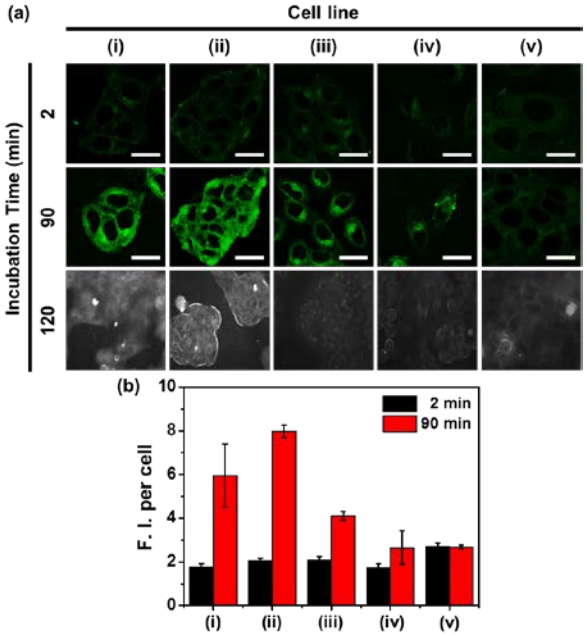


Fig. 2 (a) Confocal and fluorescence microscopy images of **1** depend on incubation time. Cell lines were treated with **1** (5 μM) and incubated for 2, 90 (confocal images) and 120 min (fluorescence microscopy images). Confocal microscopy images were obtained from which excitation wavelength was 488 nm (Laser power 10 %) and filter was long pass 505 nm and detector gain value was 850. Fluorescence microscopy image was obtained at 460-490 nm excitation and long pass 520 nm emission. (i) HeLa; (ii) HepG2; (iii) HCT 119; (iv) MIA PaCa-2; (v) Caco-2. The scale bars indicate 30 μm. (b) Comparison with the fluorescence absolute value per cell of respective cell line.

To further confirm the indomethacin's targeting ability, competition studies were carried out (Fig. 3a). Thus, when HepG2 was pre-treated with various concentrations of indomethacin up to 150 μM for 1 h, the fluorescence intensity for **1** was gradually diminished (Fig. 3a) whereas the intensity with HCT 116, a COX-2 negative cell line was steady under similar conditions. The evaluated results for both experiments are demonstrated in Fig. 3b and 3c. Together, these results confirmed that indomethacin moiety in **1** displayed a COX-2 positive cancer-targeting ability.

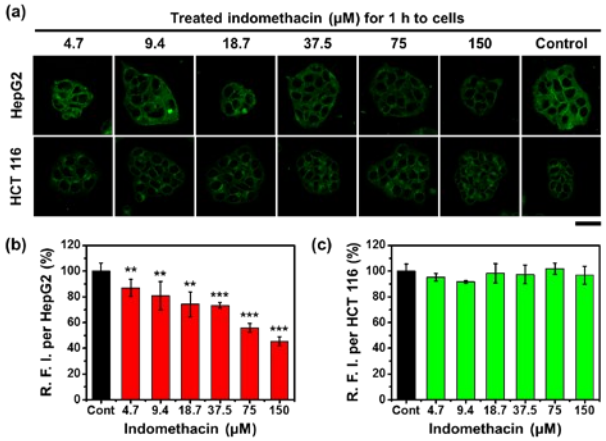


Fig. 3 (a) Confocal microscopy images of **1** depend on COX-2 inhibitor; indomethacin. Cell lines were treated with various concentration of indomethacin (μM) for 1 h at incubator. Collected media which treated before and dissolved **1** (5 μM) in it and incubated for 15 min. Control images were untreated indomethacin. The scale bar indicates 30 μm. Histogram of relative fluorescence intensity per cell of (b) HepG2 cells and (c) HCT 116 depend on various concentration of indomethacin was represented using image J program. Results represent the mean (±SEM) of five independent experiments (n=5). The statistical signification was marked as ** and *** for $p < 0.01$ and $p < 0.001$ respectively, compared with the control.

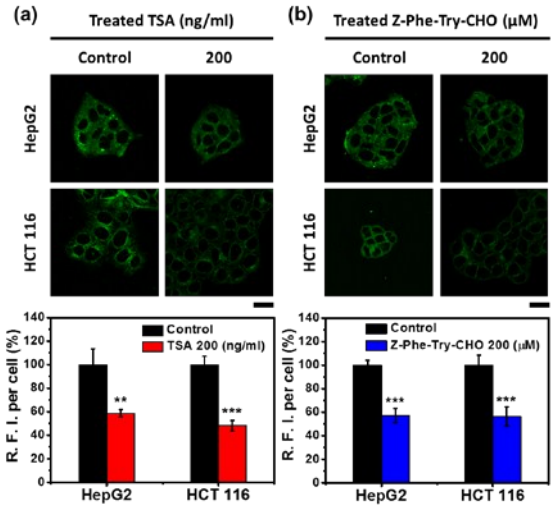


Fig. 4 Confocal microscopy images of **1** depend on (a) HDAC inhibitor; trichostatin A (TSA) (b) CTSL inhibitor; Z-Phe-Try-CHO. HepG2 and HCT 116 cell lines were treated with TSA (200 ng/ml) or Z-Phe-Try-CHO (200 μM) for 1 h at the incubator. Collected media which treated before and dissolved **1** (5 μM) in it and incubated for 15 min. Control images were untreated TSA or Z-Phe-Try-CHO. The scale bar indicates 20 μm. Histogram of relative fluorescence intensity per cell of HepG2 cells and HCT 116 was represented using image J program. Results represent the mean (±SEM) of five independent experiments (n=5). The statistical signification was marked as ** and *** for $p < 0.01$ and $p < 0.001$ respectively, compared with the control.

To investigate whether the release of DOX from **1** relies on HDAC and CTSL activities in the cells, we conducted inhibition studies using a well-known HDAC inhibitor (trichostatin A, TSA) and a CTSL inhibitor (Z-Phe-Try-CHO) and the confocal microscopic results are shown in Fig. 4. When the cells were pretreated with 200 ng/ml TSA[17] for 1 h, the intensity was decreased about 40 and 50 % for HepG2 and HCT 116 cell lines, respectively, compared to those of the untreated cells (Fig. 4a). Likewise, treatments of the cells with 200 μM Z-Phe-Try-CHO[17] resulted in diminished fluorescence intensities by 50 and 60 %, respectively (Fig. 4b). Taken together, these results demonstrate that the **1** possess a higher degree of selectivity towards cancer cells with elevated HDAC and CTSL activities over normal cells.

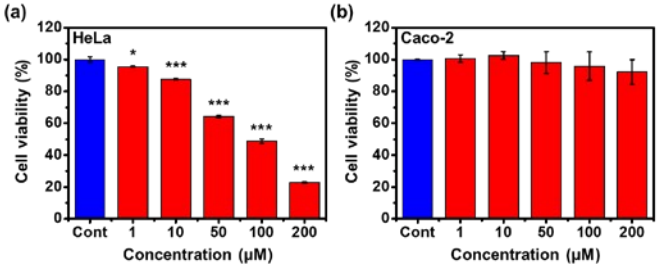


Fig. 5 Effect of **1** on cell viability in (a) HeLa (b) Caco-2 cell lines. The viability of cells, which treated with **1** was incubated for 72 h at 37 °C, 5 % CO_2 at different concentration. After treatment of the MTT during 30 min, the visible absorption of formazan crystals was measured at 570 nm. Blue bars represent the control (untreated probe) and cell viability was normalized by the control value. Results represent the mean (±SEM) of four independent experiments (n=4). The statistical signification was marked as * and *** for $p < 0.05$ and $p < 0.001$ respectively, compared with the control.

To confirm the anticancer drug effect of **1**, it was treated to HeLa and Caco-2 cells (Fig. 5). **1** exhibited significantly higher cytotoxicity for positive cell (HeLa) with concentration increase from 1 to 200 μM. In contrast, cytotoxicity of **1** is not observed for the Caco-2 cells in similar conditions. Taken together, these results demonstrated that **1** selectively went to enzyme positive cells.

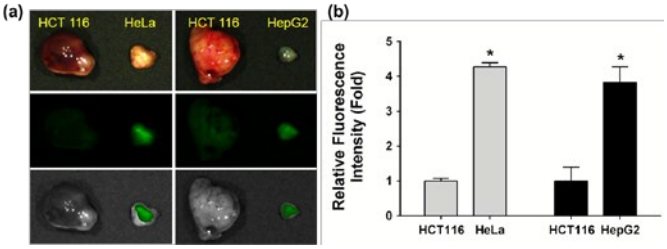


Fig. 6 *Ex-vivo* fluorescence of probe **1** from xenografted mice after tail intravenous injection. (a) Fluorescence images and (b) histogram of relative fluorescence intensity were obtained according to comparison with HCT 116 (cox-2 negative) and HeLa or HepG2 (cox-2 positive) cell line. From the top White, Blue Merge image. *$p < 0.05$ vs. COX-2 negative tumor HCT 116.

Last but not least, in order to examine the tumor targeting ability of **1** in live animals, we utilized a xenograft mice models using subcutaneously injected tumor cells. The cell lines used under this study were human colon cancer HCT 116 (COX-2 negative), human cervical cancer HeLa (COX-2 positive) and human hepatocellular carcinoma HepG2 (COX-2 positive). After tail-vein injection of **1**, the distribution of its fluorescence intensity was analyzed *ex vivo* (Fig. 6). As shown in Fig. 5,

compared with HCT 116 (COX-2 negative), strong fluorescence signals were observed from the dissected tumor tissues of the mice injected with COX-2 positive cells (HeLa and HepG2). The fluorescent signal was significantly enhanced up to 4.3 ± 0.1 (HeLa) and 3.8 ± 0.4 (HepG2) folds, compared to the COX-2 negative tumors (HCT 116). These data clearly demonstrate the ability of **1** in targeting COX-2 overexpressed tumor more adequately over the COX-2 negative tissues.

In conclusion, we present here a preliminary results of first indomethacin-guided drug delivery conjugate (IGDDC) for selective delivery of therapeutic agent to the malignant cancer cells exhibiting higher levels of COX-2, HDAC and CTSL activities. Our results demonstrated the possibility of NSAID as a guiding unit for cancer-targeting drug delivery system together with HDAC and CTSL-driven drug release mechanism. Further studies to explore the dose-dependent tumor growth inhibition, cellular uptake mechanism, the possible synergistic effect of NSAID with anticancer drug as a combinational therapeutic approach against cancer are in progress.

This work was supported by CRI (No. 2009-0081566, JSK), NRF (No. 2015R1A5A1037656, CK), the Basic Science Research Program (No. 20100020209, CK) project grants from the National Research Foundation of Korea.

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