Bioconjugate Chemistry

Communication

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Development of a Folate Receptor (FR)-Targeted Indenoisoquinoline Using a pH-Sensitive *N*-Ethoxybenzylimidazole (NEBI) Bifunctional Cross-Linker

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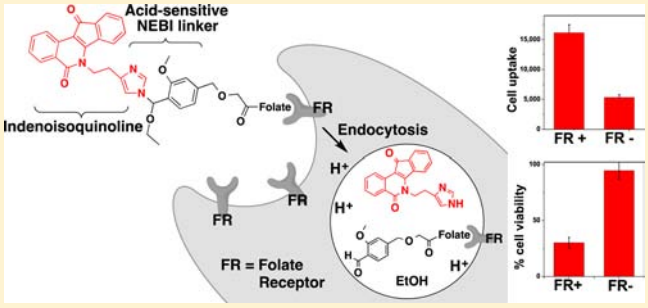
Supporting Information

ABSTRACT: This Communication describes the synthesis and evaluation of a folate-conjugated drug delivery system (DDS) that incorporates an acid-sensitive *N*-ethoxybenzylimidazole (NEBI) bifunctional linker and a novel imidazole-containing indenoisoquinoline. Indenoisoquinolines are a class of TOP1 inhibitors that exhibit broad anticancer activity. Here, we examined whether a DDS that comprised an indenoisoquinoline attached to a folate moiety could help target activity to cancer cells that naturally overexpress the folate receptor (FR), thereby increasing the specificity of these compounds. Evaluation of the DDS revealed an 11-fold increased toxicity in folate receptor (FR)-overexpressing cells compared to in FR-knockdown cancer cells. Microscopy studies demonstrate enhanced internalization and localization of the DDS in acidic lysosomal compartments of FR-overexpressing cells, supporting a receptor-mediated mechanism for uptake and activation. Together with control experiments, the results support that the cytotoxic activity of this DDS is dependent on both the presence of the folate group as well as the presence of the acid-sensitive hydrolyzable group. This work represents the first example of a cell receptor-targeted indenoisoquinoline, which could help pave the way for the use of this class of compounds in anticancer therapy.



INTRODUCTION

We previously reported the development of *N*-ethoxybenzylimidazole (NEBIs) as pH-sensitive linkers for potential use in drug delivery applications.[1] Since a common mechanism for internalization of drugs into cells involves subcellular localization to acidic endosomes (pH 5.5−6.0) and lysosomes (pH 4.5−5.0),[2,3] we designed these NEBI linkers to undergo accelerated hydrolysis in mild aqueous acidic solutions (e.g., pH 5.5) compared to in solutions at neutral pH (e.g., pH 7.4). We hypothesized that covalent attachment of drugs to drug carriers via NEBI linkers would lead to accelerated release of active drug upon uptake of the drug delivery system (DDS) in cells. We previously demonstrated that an analogue of doxorubicin (i.e., a model drug) conjugated to a model protein-based drug carrier, human serum albumin (HSA), via a NEBI linker showed improved uptake and cytotoxic activity to cancer cells compared to the unconjugated drug alone.[4] While these initial proof-of-concept studies are encouraging, doxorubicin (an FDA-approved amine-containing chemotherapeutic drug) was not released in free form. Additionally, HSA does not specifically target cancer cells and has been reported to internalize in cells through a nonreceptor, mediated pathway.[5]

Here, we report the first example of a cell-receptor targeted drug delivery system that comprises a NEBI linker. To achieve this goal, we also developed a novel imidazole-containing indenoisoquinoline to expand the scope of drug candidates that could be targeted for delivery using NEBI groups. The indenoisoquinolines are a class of non-camptothecin topoisomerase 1 (TOP1) inhibitors that have shown promising antitumor activity in animal models for cancer.[6] Indenoisoquinolines have several potential advantages over camptothecin for providing prolonged drug action in that they (1) are chemically more stable, (2) can form TOP1 cleavage complexes at different genomic locations, and (3) can form more stable TOP1 complexes.[6] To date, over 400 indenoisoquinolines have been reported, two of which are currently in clinical trials (indotecan and indimitecan, Figure 1).[7,8] To our knowledge, however, attempts to enhance the targeting of indenoisoquinolines to cell receptors that are naturally overexpressed in cancer cells has not been reported.

We chose to incorporate a folate targeting moiety into a DDS because of its capability to specifically interact with cells expressing folate receptors (FRs).[9−11] FRs are cell surface glycoproteins that bind folate with high affinity and mediate the cellular uptake of both folate and folate conjugates.[12] Once bound to the FR, folate conjugates are transported into the cell via receptor-mediated endocytosis.[13] FRs are attractive targets for anticancer therapy because they are expressed in high levels

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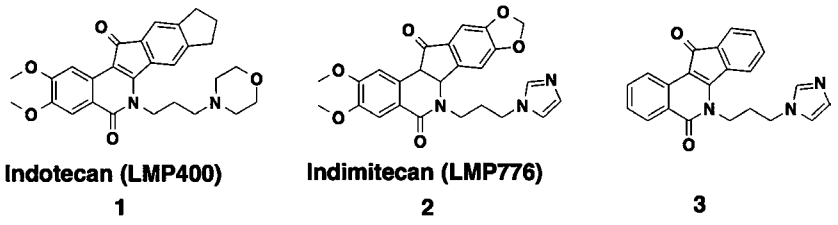

Figure 1. Representative examples of indenoisoquinoline TOP1 inhibitors.

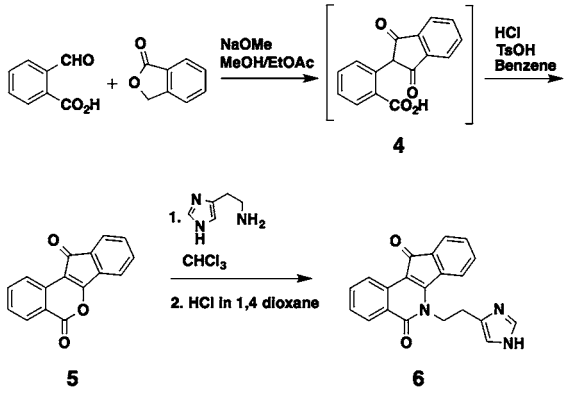

Figure 2. Scheme for the synthesis of indenoisoquinoline compound **6**.

in many types of cancers.[14] Folate conjugation shows great promise for selective delivery of nonspecific drugs into cancer cells while reducing toxic effects to healthy tissue. For instance, EC145 (vintafolide), a folate-conjugated vinca alkaloid desacetylvinblastine hydrazide (DAVLBH), is currently undergoing phase II clinical studies in patients with advanced epithelial ovarian cancer and non-small cell lung cancer.[15]

In this work, we designed and synthesized a novel indenoisoquinoline that is covalently attached to a folate group via a NEBI linker. We examined the hydrolysis of the NEBI linker in mild acidic solutions compared to at neutral pH. We evaluated the uptake of this folate-targeted indenoisoquinoline in FR-overexpressing cells. Finally, we evaluated the cytotoxic activity of this DDS in FR-overexpressing and FR-knockdown cells.

RESULTS AND DISCUSSION

Cushman and co-workers previously reported potent and broad anticancer activity of an indenoisoquinoline compound containing an *N*-linked imidazole group (compound **3**, Figure 1).[16] In order to design an indenoisoquinoline that could be reversibly conjugated to a DDS via a NEBI linker, we hypothesized that a

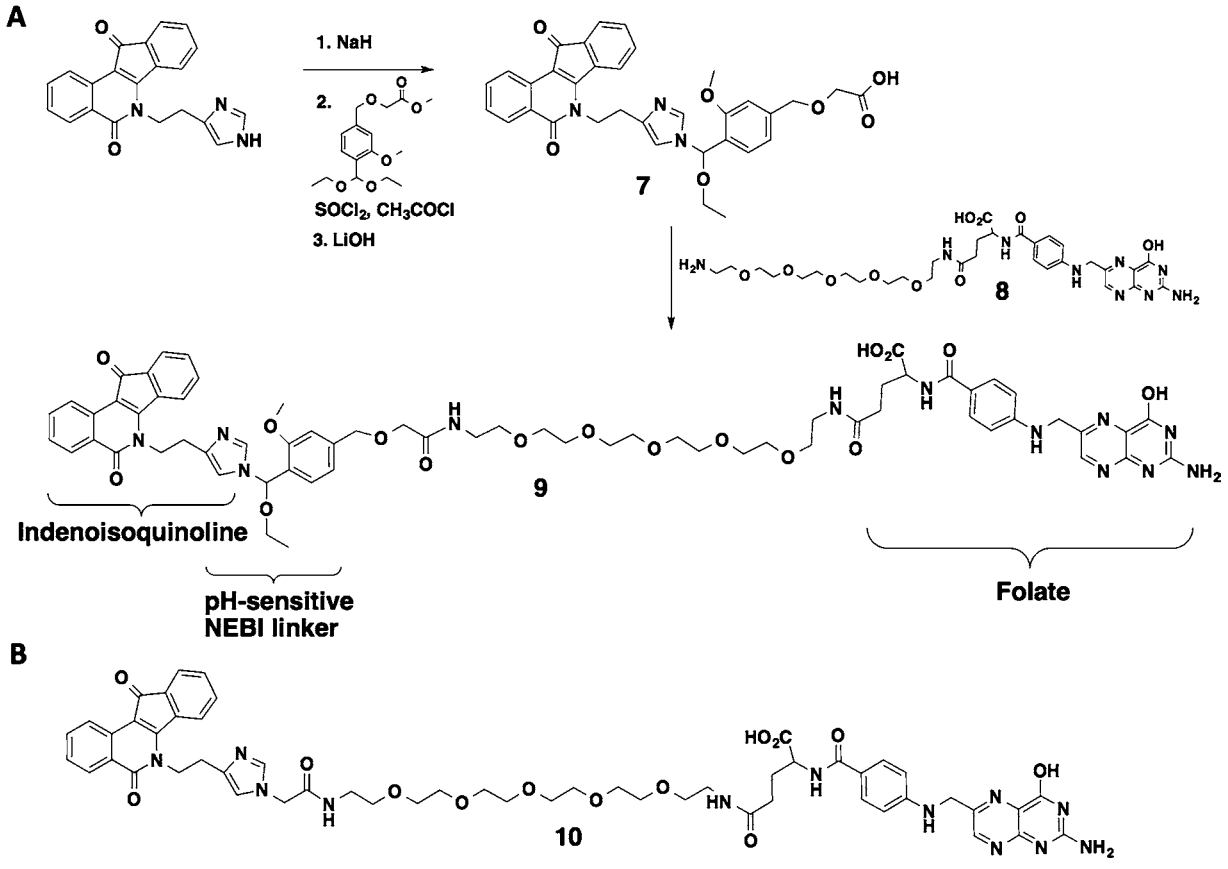

Figure 3. Folate-conjugated pH-sensitive bifunctional cross-linkers. (A) Synthetic scheme of the folate-conjugated NEBI drug delivery system (**9**) incorporating the indenoisoquinoline **6**. (B) Structure of a control molecule (**10**) comprising folate directly attached to the indenoisoquinoline **6** via an acid-stable amide bond.

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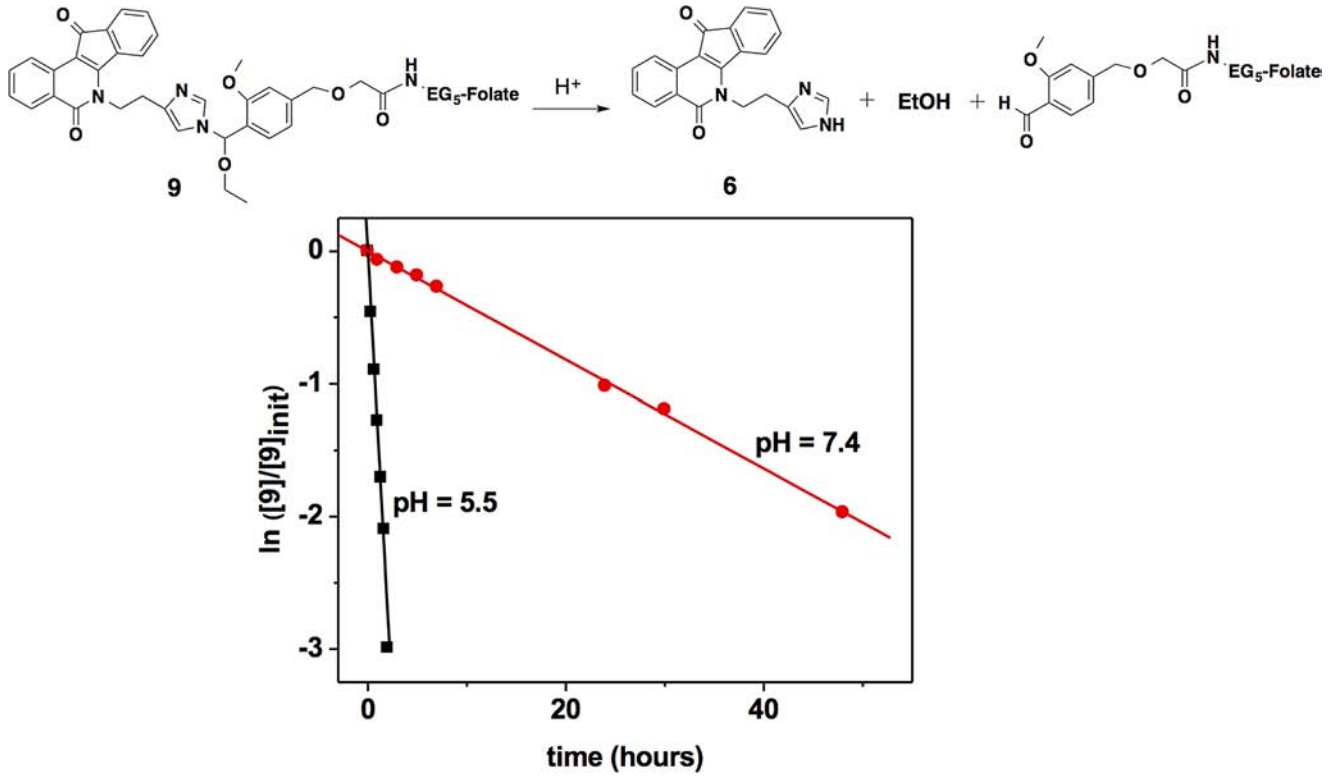

Figure 4. Kinetic studies showing the acid-catalyzed hydrolysis of folate-conjugated NEBI DDS **9** under mildly acidic conditions (MES buffer, pH 5.5, $t_{1/2}$ = 32 min) and physiological pH (HEPES buffer, pH 7.4, $t_{1/2}$ = 17 h). The pH-dependent rate of hydrolysis of the DDS was determined by RP-HPLC. See Supporting Information for details.

derivative of indenoisoquinoline (**3**) containing an imidazole linked at C-4/5 to the indenoisoquinoline group would retain anticancer activity while providing a chemical handle for ready incorporation into a NEBI group. To test this hypothesis, we synthesized molecule **6** using the procedure outlined in Figure 2. In the first step, the condensation of phthalide with phthaldehydic acid (2-carboxybenzaldehyde) afforded the intermediate **4**, which was converted to indenopyran **5** upon acidification.[17] We then formed indenoisoquinoline **6** via a condensation reaction between the indenopyran and histamine.[16] This simple procedure made it possible to synthesize >10 g of **6** with 71% overall isolated yield from inexpensive and readily available starting materials.

Indenoisoquinoline **6** was incorporated into a NEBI group and attached to a folate-containing conjugate to produce DDS **9** (Figure 3A). We designed the DDS with the consideration that the NEBI group should be hydrolyzed within the duration of FR recycling, estimated to be 12−24 h.[18] Previous work demonstrated that the hydrolysis rates of the NEBI can be easily tuned by the incorporation of electron donating or electron withdrawing groups on the phenyl ring of the NEBI.[1] We, therefore, designed DDS **9** to contain an ortho-methoxy group on the phenyl ring of the NEBI because preliminary studies revealed that the presence of this electron-donating group accelerates the hydrolysis rate by more than 2-fold.[1] Additionally, earlier unpublished work suggested that the solubility of the DDS has a direct negative effect on the hydrolysis rate of the NEBI group. Due to the poor water solubility of both the indenoisoquinoline (**6**) and folate by themselves, we designed **9** to contain a 5-unit ethylene glycol (EG_5) spacer in order to improve the overall solubility of the DDS and to maintain desired rates of hydrolysis. This EG_5 spacer was attached via the γ-carboxylate of the folate moiety, since previous reports demonstrate that binding of folate to the FR receptor is not affected upon derivatization of folate at the gamma position.[19] As a control, we synthesized folate-indenoisoquinoline conjugate **10** (Figure 3B), which does not contain an acid-labile linker, in order to evaluate the role of the acid-sensitive NEBI group on the activity of DDS **9**.

We performed hydrolysis studies to determine the rate of cleavage of the NEBI group in DDS **9** under mild acidic conditions compared to at physiological pH. The hydrolysis half-life of DDS **9** at 37 °C was determined to be 32 min at pH 5.5 and 17 h at pH 7.4 (Figure 4). This result suggests that if DDS **9** can be internalized in cells and localized to the acidic endosomes or lysosomes through a FR-mediated endocytotic mechanism, indenoisoquinoline **6** should be released in free, active form within the expected FR recycling time of the cell (12−24 h).[18] Additionally, previous studies have shown that 4-substituted benzaldehydes[4] (i.e., the product after hydrolytic release of **6** from the NEBI) and poly(ethylene glycol) conjugates of folate[20] are not toxic to cells. We, therefore, expect that any toxicity observed by DDS **9** would arise only from the indenoisoquinoline **6** that is released after uptake in cells.

To determine whether the folate-conjugated DDS **9** targets cells via a FR-mediated pathway, we next evaluated its cytotoxic activity and uptake in KB cells (a subline of HeLa cells), which naturally overexpress FR. As a control, we created a FR-knockdown KB cell line by transfecting KB cells with FOLR1 siRNA (see Supporting Information for more details). A modified pulse−chase sulforhodamine B (SRB) assay[21] was used to evaluate the cytotoxicity of **9** versus control compounds. In this assay, cells were incubated with the compounds for 5 h

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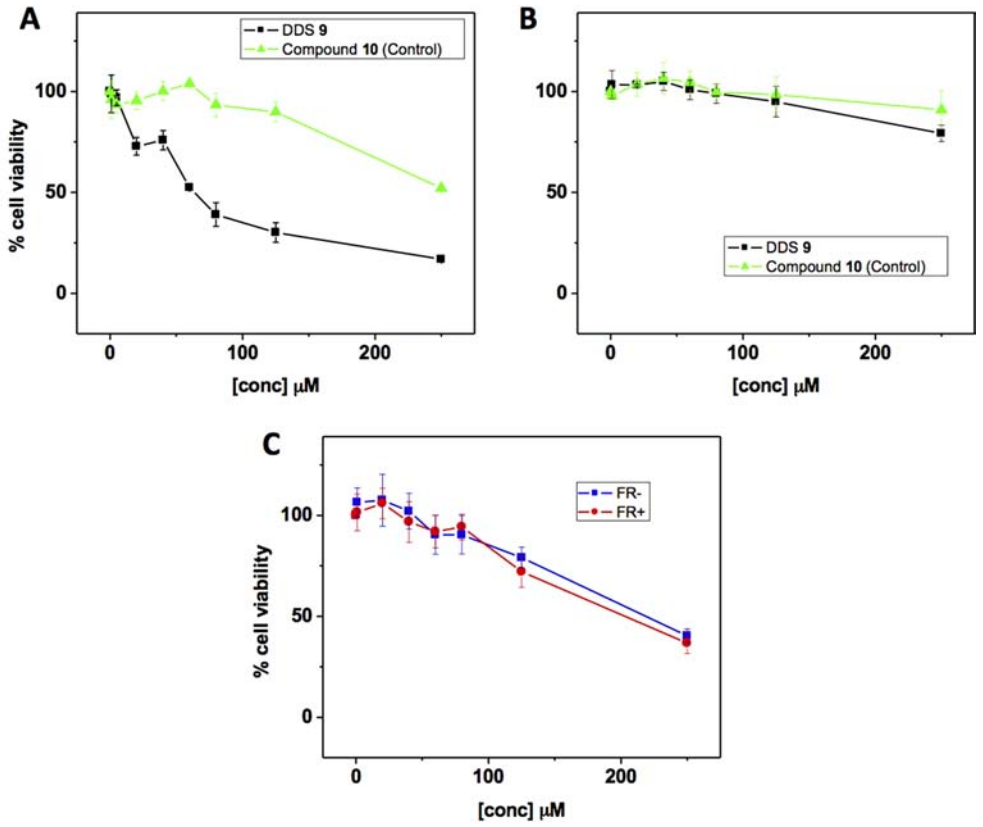

Figure 5. *In vitro* cytotoxicity studies of the DDS **9** and indenoisoquinoline−folate conjugate (control) **10** in folate-deficient media (A) KB (FR+) cells and (B) FR− knockdown KB cells (i.e., treated with FOLR1 siRNA). (C) Cell viability in the presence of compound **7** on FR+ and FR− cells. In all experiments, a pulse−chase SRB assay was performed in which the compounds were dosed for 5 h (pulse), washed with media, and allowed to incubate for an additional 67 h (chase) with fresh media.

(pulse), washed to remove unbound compound, and then incubated for an additional 67 h (chase) in fresh media.[22] The pulse−chase assay is considered to be more appropriate for the evaluation of folate-targeted compounds than a long exposure (e.g., 72 h) assay, since folate conjugates are commonly cleared from both the vasculature and the interstitial spaces within a short amount of time. This assay also provides additional insight into the basis for cellular uptake of the DDS.

When exposed to increasing concentrations of DDS **9** in folate-deficient media, an 11-fold enhancement of cell toxicity was observed in KB cells (IC_{50} = 60 μM, Figure 5A) compared to in FR-knockdown KB cells (extrapolated IC_{50} = 655 μM, Figure 5B). We attribute this selective toxic activity to increased cellular uptake of the folate-conjugated DDS **9** within the KB cells compared to the FR-knockdown KB cells during the 5 h pulse period, presumably due to FR-mediated endocytosis.

As a control, the indenoisoquinoline−folate conjugate **10** (without a pH-sensitive NEBI group) showed much lower or no cytotoxic activity in KB cells (IC_{50} = 250 μM, Figure 5A) or in FR-knockdown KB cells (no significant toxicity observed up to 250 μM, Figure 5B). *This result demonstrates that the NEBI linker plays an essential role in the cytotoxic activity of DDS* **9**. As a second control, the cytotoxic activity of the indenoisoquinoline−NEBI conjugate **7** (without a folate group) to KB and FR-knockdown KB cells was indistinguishable ($IC_{50} \approx 200$ μM in both cell types, Figure 5C).[23] This result further supports that the selectivity of DDS **9** for cytotoxic activity in KB cells is due, at least to a large extent, to FR-mediated endocytosis.

Since folate is a natural vitamin that is found throughout the body, we further examined the toxicity of DDS **9** in FR-positive KB cells in the presence of externally added folate. Although the concentration of folate in blood plasma has been reported to be 2−20 ng·mL^{-1} (4.5−45 nM),[24] we evaluated the efficacy of DDS **9** in folate-rich media that contained ∼1 μg·mL^{-1} folate (corresponding to ∼2.2 μM) in order to demonstrate that DDS **9** could perform effectively in the presence of folate at a concentration that was 50−500 times greater than the concentration of folate present *in vivo*. We found that the toxicity of DDS **9** was similar in folate-rich and folate-deficient media (see Figure S1A in the Supporting Information). Additionally, we performed a competition assay for the toxicity of 80 μM DDS **9** in KB cells in the presence of increasing concentrations (2.2−1000 μM) of folate. As expected, we observed that the toxicity of DDS **9** in KB cells decreased as a function of increasing concentrations of folate (Figure S1B), with complete loss of activity of DDS **9** at folate concentrations >100 μM. This observed reduction in toxicity in folate-rich solutions further supports the hypothesis that the cytotoxic activity of **9** depends on the binding of the DDS to folate receptors present on the cell surface.

Finally, we also examined the cellular uptake of DDS **9** in KB cells using fluorescence deconvolution microscopy (Figure 6). Incubation of the KB cells with DDS **9** for 2 h resulted in significant cellular uptake. Co-localization analysis with Lysotracker blue[4] showed that DDS **9** was able to internalize and localize substantially in the acidic lysosomal compartments (Pearson coefficient of correlation[25,26] = 0.606). Incubation of DDS **9** in FR-knockdown KB cells showed a significantly reduced (>3-fold) cellular uptake compared to exposure of DDS **9** to folate-overexpressing KB cells under exactly the same conditions

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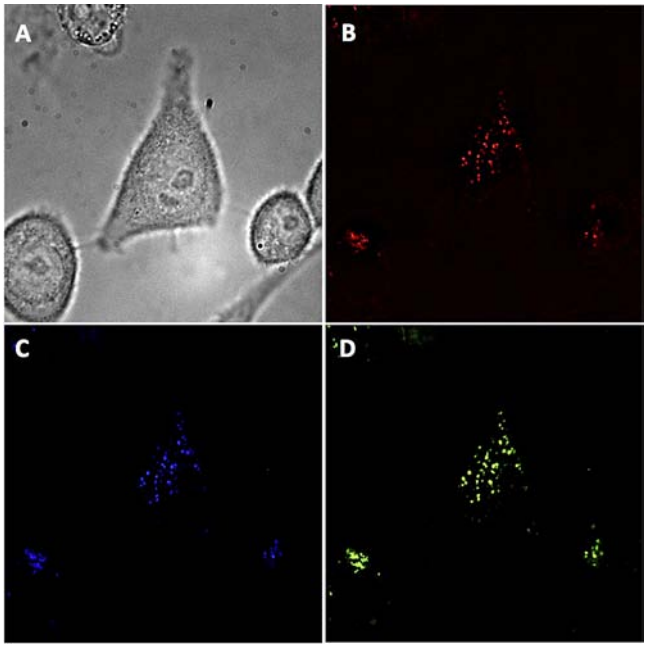

Figure 6. Brightfield and fluorescence micrographs showing the internalization and localization of DDS **9** in KB cells. (A) Differential interference contrast image of KB cells treated with DDS **9**. (B) Fluorescence micrograph of a *z*-slice through the cells showing the location of DDS **9** (red) inside the cells. (C) Fluorescence micrograph of the same *z*-slice through the KB cells shown in (B), except showing the location of the lysosomes (blue) stained with Lysotracker blue. (D) A merged fluorescence image of (B) and (C) indicating the areas of co-localization (green) of DDS **9** and Lysotracker blue.

(Figure 7). Taken together, these results again support a folate receptor-mediated mechanism of uptake of DDS **9** in cells.

We, thus, demonstrate the design, synthesis, and *in vitro* evaluation of a novel imidazole-containing indenoisoquinoline conjugated to a folate via a pH-sensitive NEBI linker. The folate-NEBI-indenoisoquinoline DDS **9** exhibited higher levels of cellular uptake and toxicity in FR-overexpressing KB cells compared to in FR-knockdown KB cells, supporting the important role of the folate group in the cell specific activity of DDS **9**. A folate−indenoisoquinoline conjugate **10**, which lacked an acid-sensitive hydrolytic group, did not exhibit significant cytotoxic effects on KB cells or FR-knockdown KB cells, highlighting the importance of the pH-sensitive NEBI linker in DDS **9**.

This work represents the first example for the incorporation of NEBI linkers in a receptor-targeted DDS. Some potential advantages of these NEBI linkers for drug delivery applications are (1) they are easy to synthesize, (2) they have tunable rates of hydrolysis, and (3) they are amenable to attaching drugs containing a variety of functionalities (e.g., amines, alcohols, or imidazoles) to drug carriers. Here, we also demonstrate the first example of a receptor-targeted indenoisoquinoline, which may further enable the use of these novel TOP1 inhibitors for the treatment of cancer. Since several imidazole-containing drugs[27−29] have already been developed for the treatment of a number of diseases including cancer (e.g., dacarbazine),[30] this work represents a promising step toward improving their efficacy through incorporation into targeted DDSs.

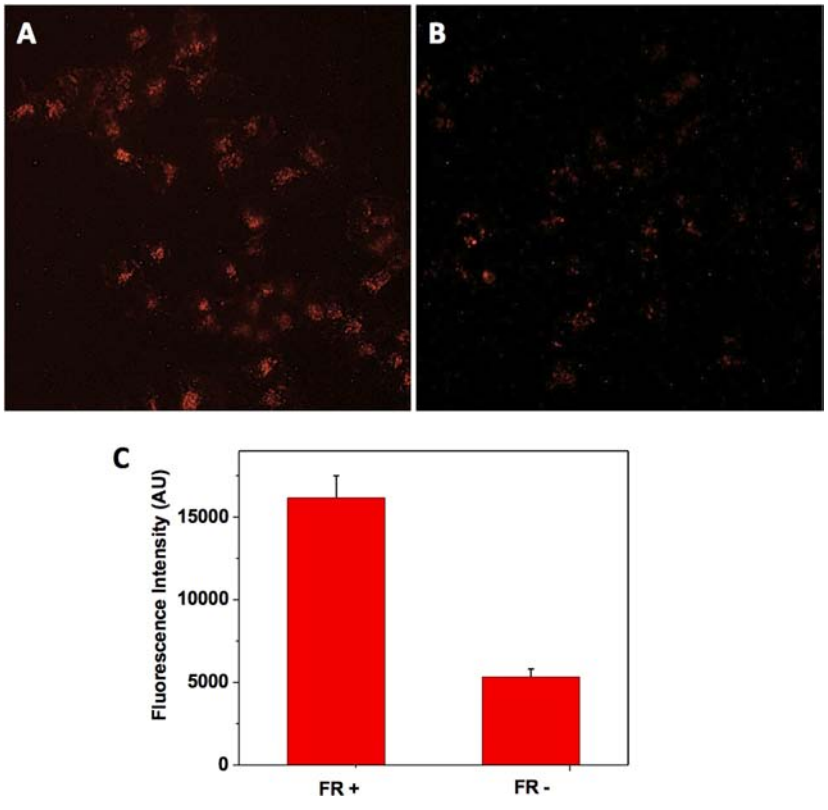

Figure 7. Analysis of the cellular uptake of DDS **9** by (A) KB cells and (B) FR-knockdown KB cells. The intrinsic fluorescence of indenoisoquinoline **6** is shown in red. (C) Quantification of the average total fluorescence per cell, determined from analysis of 10 cells in each sample image.

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■ ASSOCIATED CONTENT

Supporting Information

Additional experimental details and characterization of molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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