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Targeting the Cubilin Receptor through the Vitamin B_{12} Uptake Pathway: Cytotoxicity and Mechanistic Insight through Fluorescent Re(I) Delivery

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The intrinsic factor (IF) vitamin B_{12} ileum anchored receptor, cubilin, mediates endocytotic uptake of the IF complex of vitamin B_{12} to the blood serum. This receptor was targeted for the selective delivery and accumulation of a new bioprobe, a B_{12} conjugate of rhenium **2**, in the cubilin expressing placental choriocarcinoma BeWo cell line. Competitive uptake and cytotoxicity assays of **2** were investigated and interactions with nuclear DNA explored. In addition, the mechanism of internalization of **2** was confirmed to proceed in an IF-cubilin mediated fashion via siRNA transfection experiments. These studies show the great potential of cubilin as a new target for the delivery of B_{12} based conjugates for cancer diagnostics and/or treatment.

Introduction

Delivery of chemotherapeutics by selective tropism offers specificity and selectivity toward malignant cells and factors that drive their tumorigenicity.[1] Release of a drug payload by such a selective biomarker directly in the tumor provides enhanced response to treatment while minimizing side effects normally experienced with chemotherapy (i.e., improved therapeutic index).[2] Because of the benefits of targeted delivery, pharmaceutical paradigms have shifted toward combining cytotoxic drugs with molecular vehicles with subsequent in-depth investigations of the concomitant pharmacokinetic and pharmacodynamic properties of such systems.[3] This need to deliver treatments and/or diagnostics to a specific site of interest has resulted in measures exploring such targeting moieties as antibodies, peptides, hormones, and natural cofactors such as vitamins.[4]

Site-specific delivery of pharmaceutics via the vitamin B_{12} [a] uptake pathway has generated considerable interest in recent years.[5,6] B_{12} (see Figure 1) is a highly water-soluble, nontoxic vitamin that plays a key role in reactions ranging from the conversion of homocysteine to methionine, to DNA methylation.[7] Since it is an important requirement for rapidly growing cancer cells, B_{12} makes for a plausible carrier for a drug payload delivery to these tissues, assuming receptors involved in its uptake can be targeted.

B_{12} is absorbed during digestion and transported to the plasma via three binding proteins listed here: haptocorrin (HC, $K_d \approx 0.01$ pM), transcobalamin II (TCII, $K_d \approx 0.005$ pM), and intrinsic factor (IF, $K_d \approx 1.0$ pM).[8,9] TCII and HC are transport proteins of B_{12} in the plasma.[10] The receptor for TCII (TCII-R) is reported to be overexpressed in a number of malignant cells such as human (K562 and HL-60)[11] leukemic cell lines, breast (MCF-7),[12,13] ovarian (NIH-OVCAR3),[13] and methionine-independent glioma (P60H)[14] cells. The structure of TCII was recently published in a seminal paper by Randaccio et al.[15] Several reports have taken advantage of TCII-R overexpression as a means of delivery of B_{12} conjugates.[5,13,16,17] TCII-R targets have a disadvantage, however, as this receptor is present in high concentrations in the kidneys, placenta, intestine, and liver.[18] TCII mediated uptake of B_{12} involves not just transport but also subsequent retention,[19] so targeting this uptake pathway has a disadvantage especially for radiopharmaceutical drugs, which require rapid clearance from the body.

IF is a 44 kDa glycosylated protein produced in gastric cells and has the highest specificity for B_{12} ($K_D \approx 10^{-9}$)[9,20] of the three binding proteins.[21] IF is responsible for transport of B_{12} across the intestinal enterocyte via the receptor, cubilin.[8] The structure of IF was solved recently by Alpers et al.[22] Cubilin is a ~460 kDa protein composed of eight epidermal growth factor domains and 27 CUB domain proteins (see Figure 2). CUB domains are typically ~110−115 amino acid residues in size and are derived from Clr/Cls, Uegf, and bone morphogenic protein-1 complements.[23] Binding of IF-B_{12} to cubilin occurs primarily at CUB domains 5−8[24] and appears to be calcium dependent.[25] This receptor is expressed in select tissues including the placental membranes,[26] the renal proximal tubular cells[27] and brush border,[28] gall bladder,[29] and the gastrointestinal tract,[30,31] making it an intriguing target for selective delivery of cytotoxic or imaging agents.

The use of an IF-B_{12} system to specifically target cubilin is, however, relatively unexplored. Some seminal studies, conducted by C. B. Grissom and G. J. Russell-Jones, have demonstrated the dissociation kinetics of IF and B_{12} [32] and uptake, among other studies, of nanoparticles in Caco-2 cells, respectively.[33]

Inspired by the limited tissue expression of cubilin and its putative importance for cancer cell growth, we decided to explore IF as a means to mediate cubilin targeting and delivery of B_{12}. We focused on this transport mechanism as a means of

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[a] Abbreviations: IF, intrinsic factor; TCII, transcobalamin II; siRNA, small interfering RNA; BeWO, placental choriocarcinoma line; CHO, Chinese hamster ovary cancer line; BQBA, *tert*-butyl 4-(bis((quinolin-2-yl)methyl)amino)butylcarbamate; CDT, 1,1′-carbonyl-di-(1,2,4-triazole); B_{12}, vitamin B_{12}.

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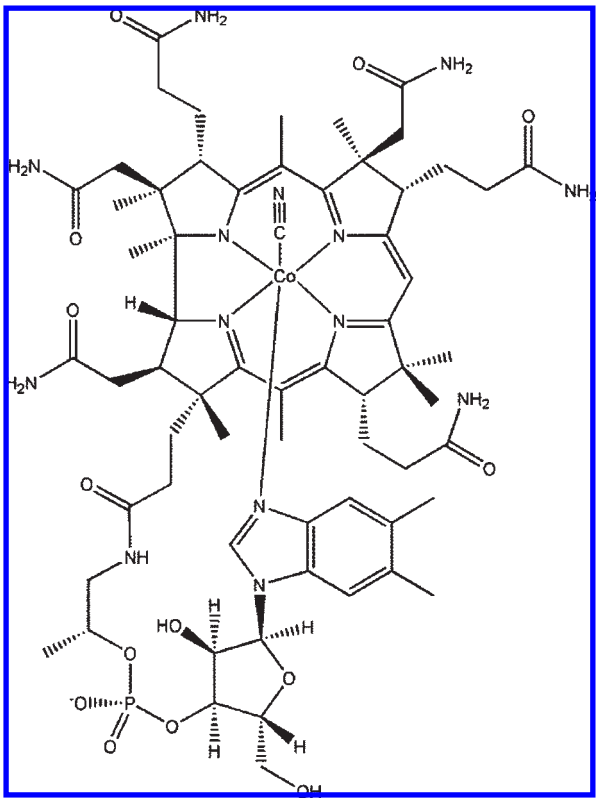


Figure 1. The structure of cyano-B_{12} includes a Co(III) bound to a cyano group and to N-donors of a tetradentate corrin ring and 5,6-dimethylbenzimidazole with the latter containing a pendent phosphoribose unit.

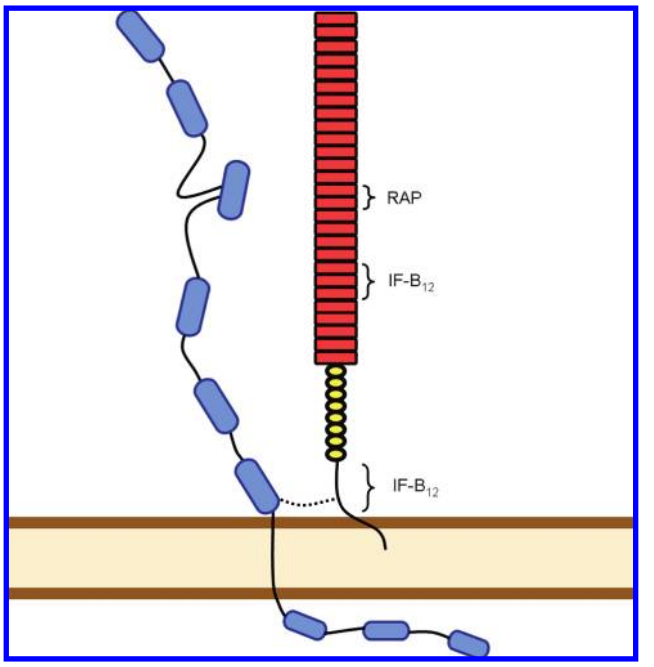


Figure 2. The cubilin receptor has 27 CUB domains (red) and 8 epidermal growth domains (yellow). CUB domains 5−8 are involved primarily in IF-B_{12} binding: blue = megalin; RAP = receptor associated protein.

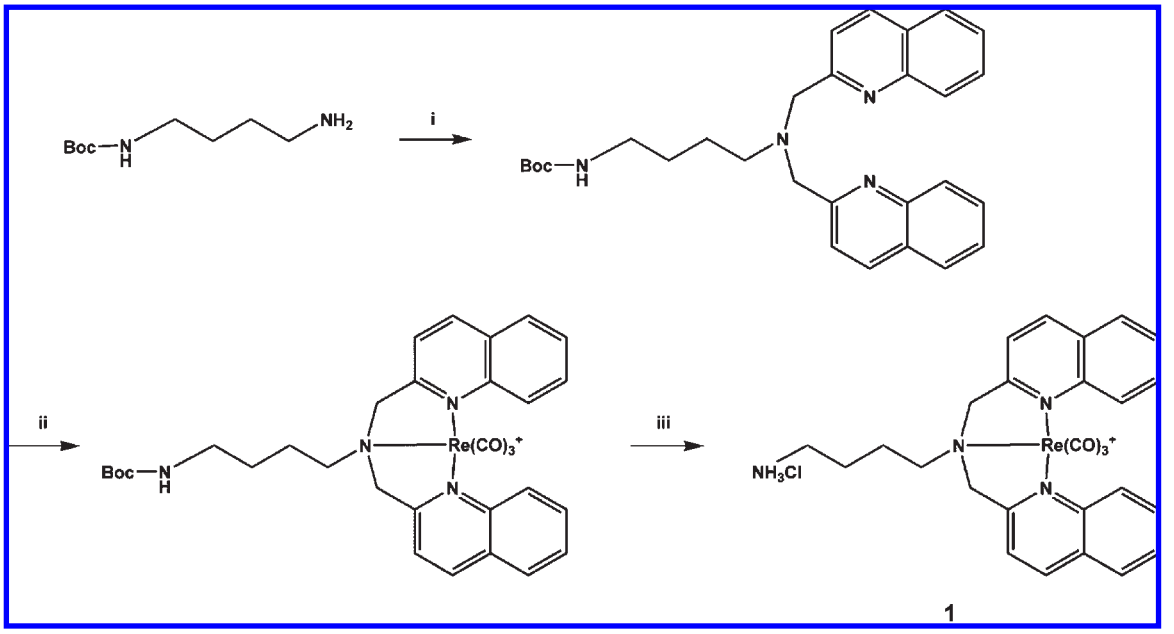
facilitating cellular entry of a rhenium fluorescent probe tethered to B_{12}. We present herein the synthesis and characterization of a rhenium(I) tricarbonyl core complexed to *N*,*N*′-bis(6,7-dihydroquinolin-2-ylmethyl)butane-1,4-diamine, **1**, and its B_{12} conjugate, **2**. In vitro antiproliferative assays and uptake studies via fluorescent confocal microscopy were conducted on two different cell lines: human placental choriocarcinoma (BeWo) expressing cubilin and (cubilin (−)) Chinese hamster ovary (CHO) cells. siRNA mediated inhibition of cubilin was conducted to demonstrate cubilin mediated uptake pathway of **2**. Following IF mediated B_{12} bioconjugate delivery through cubilin has also not been extensively investigated. So, using the rhenium conjugate, we also ventured to follow the process in depth.

Results and Discussion

Synthesis and Characterization of 1. It was previously shown that the rhenium(I) tricarbonyl core coordinated to a *N*,*N*-bis(quinolinoyl) moiety exhibits fluorescent properties.[34] The introduction of the rhenium fluorophore to B_{12} was accomplished by the preparation of a bifunctional chelate having a *N*,*N*-bis(quinolinoyl) (BQBA) moiety for the coordination of the metal core and an amino terminus for the coupling to B_{12}. The tridentate chelate functionality to *N*-Boc-1,4-diaminobutane was introduced by a standard reductive amination technique. The protected bifunctional chelate was reacted with the rhenium metal precursor $[Re(H_2O)_3(CO)_3]Br$ at 60 °C in methanol for 3 h. In the ^1H nuclear magnetic resonance (NMR) spectrum, a downfield shift for the protons adjacent to the metal core compared to the spectrum of the free ligand was observed upon metal coordination. This has been reported for several rhenium tricarbonyl complexes.[35] The downfield shift is most distinctive for the methylene protons in α-position to the tertiary nitrogen atom of the chelate moiety that are shifted from 3.92 ppm to 5.18 and 5.00 ppm, respectively. Moreover, the proton NMR data show additional evidence of site specific binding of the rhenium tricarbonyl core to the ligand. As expected, the methylene protons in the α-position to the tertiary nitrogen atom of the *N*,*N*-bis(quinolinoyl) residue of the uncoordinated ligand show a singlet. After coordination of the rhenium tricarbonyl precursor, these resonances split into two sets of doublets with coupling constants consistent for geminal coupling ($J = 17.74$ and 17.81 Hz), reflecting an AB spin pattern. The removal of the Boc protecting group was accomplished in the final synthetic step by reacting the intermediate in 3 M hydrochloric acid overnight (Scheme 1). The infrared (IR) spectrum of **1** exhibits a sharp band at 2017 cm^{-1} and a broad, intense band around 1880 cm^{-1}, which can be assigned to the *fac*-$Re(CO)_3$ moiety. While the stretching frequency at 2017 cm^{-1} remains unchanged compared to the IR spectrum of the $[Re(CO)_3(H_2O)_3]Br$ precursor (2017 and 1936 cm^{-1}), an extensive red shift was observed for the stretching vibration at 1880 cm^{-1}. In the mass spectra, the highest *m*/*z* ratios were consistent with calculated masses of the corresponding complex (M^+) and the isotope patterns were in excellent agreement with the calculated distributions.

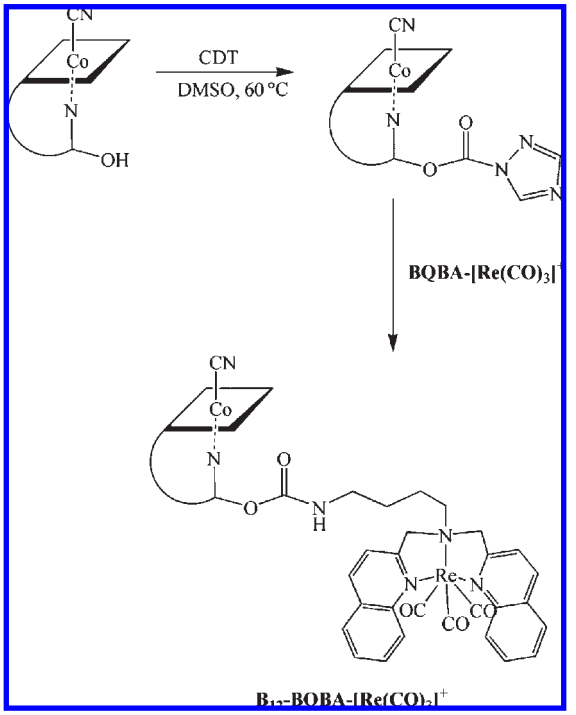
Synthesis and Characterization of 2. Coupling of **1** to B_{12} focused on the ribose 5′-hydroxy group because different derivatizations on the corrin functional groups have been reported to provide a decrease in affinity for both TCII and IF.[36] Conjugation through the 5′-hydroxyl group of the ribose moiety of B_{12}, however, was reported to least affect affinity. B_{12} was conjugated to **1** via a two-step procedure. Briefly, B_{12} was activated with 1.2 equiv of 1,1′-carbonyl-di-(1,2,4-triazole) (CDT) in dimethyl sulfoxide (DMSO) at

Scheme 1. Synthesis of the Bifunctional Chelate **1**[a]



[a] Reagents and conditions: (i) 2-quinolinecarboxaldehyde, $NaBH(OAc)_3$, DCE, room temp, overnight; (ii) $[Re(H_2O)_3(CO)_3]Br$, MeOH, 60 °C, 3 h; (iii) 3 M hydrochloric acid, room temp, overnight.

Scheme 2. 1,1′-Carbonyl-di-(1,2,4-triazole) Coupled Reaction of B_{12} to **1**



60 °C over 30 min in an oil bath under inert N_2. To this solution, **1** previously dissolved in DMSO was added dropwise. This mixture was left to stand while being stirred under N_2 for 6 h (see Scheme 2). Removal of DMSO was achieved by precipitating the crude product in a 1:15 acetone/ether solution. A crimson solid was collected via centrifugation at ~4000 rpm over 10 min. If the supernatant remained pink, addition of 1−2 mL of acetone aids in the precipitation. The red solid was then redissolved in 1:4 acetonitrile/water and subsequently purified via C_{18} reverse phase high pressure liquid chromatography. **2** eluted at retention time $t_R \approx 13.7$ min. The percentage yield of **2** ranged from 12% to 17%. Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (see Supporting Information), ^1H NMR spectroscopy, and inductively coupled plasma (ICP) were employed to establish the identity of **2**.

The MALDI-TOF mass spectrum of **2** (see Supporting Information) displayed three sets of peaks with the parent peak centered at ~2022.3 *m*/*z*. The parent peak at ~2022.3 *m*/*z* is consistent with the calculated theoretical molecular mass value of $[M^+] \approx 2022.09$ for $C_{91}H_{112}CoN_{18}O_{18}PRe$. The base peak centered at ~1994.9 *m*/*z* was determined to be the parent peak less a carbonyl moiety ($MW_{CO} \approx 28$ *m*/*z*) with a calculated $[M^+ - CO] \approx 1994$ *m*/*z*. The third set of peaks centering at ~1966.9 *m*/*z* was in agreement with $[M^+ - 2CO]$ with a theoretical value of ~1966 *m*/*z*. Obtaining the mass spectrum of the *fac*-tricarbonylrhenium(I) core proved to be a challenge, as the carbonyl groups are readily discharged from the complex upon ionization in situ. Closer inspection of the isotopic patterns of **2** shows peaks in close agreement with the theoretical isotope patterns. ^1H NMR data were consistent with **2**, and ICP results indicated the presence of rhenium. The carbamate linker formed is stable over at least 24 h between pH 5.5 and pH 7.4 as followed by HPLC.

Intrinsic Factor Binding to 2. Binding of the protein to **2** was monitored by electron absorption spectroscopy (see Figure 3). Upon each addition of IF, an increase in absorption due to binding was observed consistent with previous literature.[37,38] Saturation of the IF binding site was reached when the absorbance intensity remained constant. The total overall volume was noted, and the final concentration of **2** bound to IF (**2-IF**) was calculated.

Uptake Experiments via Fluorescent Confocal Microscopy of 2. To the BeWo cell line, a total volume of ~1.5 mL of 10 μM **2-IF** was added. The cells were then incubated at 37 °C over two separate periods of ~45 min or 6 h to allow drug

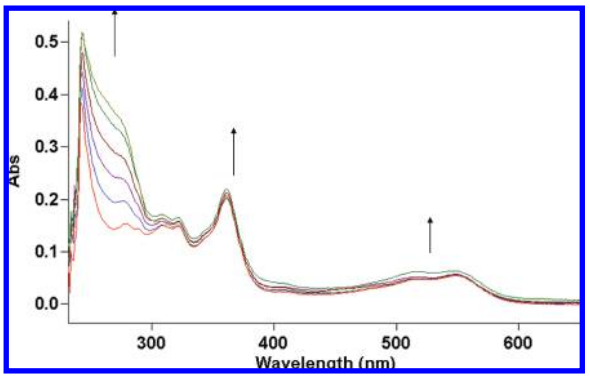


Figure 3. Intrinsic factor binding of **2** is indicated by an increase in absorption intensity due to further lowering of symmetry of B_{12}.

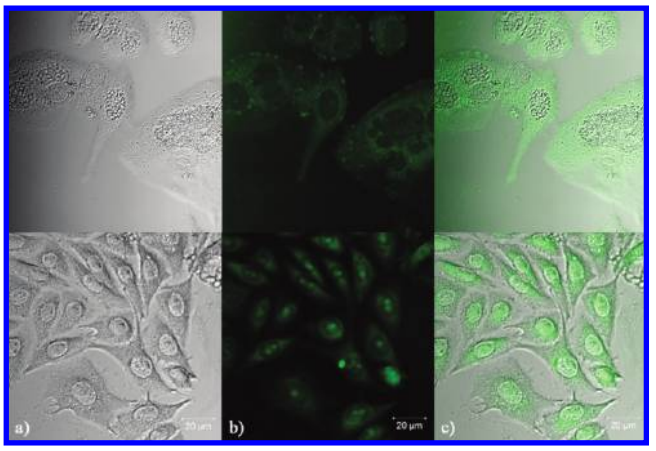


Figure 4. Cell binding and internalization of **2-IF** after ~45 min of incubation taken at 63× showing images of BeWo (a) collected by a monochromatic transmitted light photomultiplier tube (TMPT-1) and (b) after excitation at 488 nm with fluorescent green emission at ~560 nm, consistent with rhenium(I). (c) The merged images after simultaneous scans showed illumination in the nucleus and in the cytosol.

influx. The solution of **2-IF** was then removed via pipet. Subsequent washing with RPMI 1640 and 1× PBS buffer (pH ≈ 7.4) in triplicate was performed with and without ethanol fixation. Intracellular fluorescence was observed after 45 min of drug exposure as shown in Figure 4. This indicates rapid entry of the drug into the cells and is consistent with receptor mediated endocytosis.

To further prove that internalization indeed occurred, optical slicing at ~1 μm per slice was conducted as shown in Figure 5 as a gallery view (see Supporting Information for movie). Fluorescence in the cellular milieu was only observed in the middle sections, confirming the internalization of **2-IF**.

Competitive binding with B_{12} against **2-IF** was investigated by adding excess of vitamin. Excess B_{12} at concentrations of ~100 μM and ~10 mM were added first to the cells and incubated at room temperature for ~5 min. Then, **2-IF** was added to the cells. The final concentration of **2-IF** was ~10 μM. The cells were then incubated for ~45 min, after which the medium containing **2-IF** and B_{12} was discarded. The cells were then washed with medium 1× PBS (pH ≈ 7.4) in triplicate to remove residual **2-IF**. On the basis of observations, addition of a 100-fold excess of 100 μM B_{12} did little to block binding of **2-IF** complex to the cubilin receptor. At higher concentrations of B_{12} (10 mM) however, cellular access and accumulation of **2-IF** were completely inhibited. In addition, there was no observed fluorescence even after 6 h of incubation under these conditions, indicating that the excess B_{12} was clearly inhibiting uptake of **2-IF**. To further support evidence that IF is critical for the B_{12}-conjugate transport and that entry is gained through the cubilin receptor, the BeWo cells were exposed to **2** unbound to IF (i.e., **2** only) and with prior addition of 10 mM excess B_{12} to determine uptake. No fluorescence was observed at 45 min and even after 6 h for cells with prior exposure to excess B_{12}. By saturating the cells with a 1000-fold excess of B_{12} however, we noted that transport of **2** was shut down, presumably since now no free IF is available and the fact that the B_{12}-transport proteins have a greater affinity for the unmodified B_{12}.[32] This lower affinity of derivatized B_{12} was previously established in the binding kinetics studies of Fedosov et al., wherein a B_{12} analogue modified with rhodamine at the 5′-OH ribosyl group of B_{12} binds to IF at a lower affinity by 2 orders of magnitude ($K_D \approx 10^{-13}$) than cyano-B_{12} ($K_D \approx 10^{-15}$).[32]

Chinese hamster ovary cells were investigated for any uptake of **2-IF** as a control. There was no observed fluorescence in this cell line for **2-IF**, indicating a lack of uptake of the drug via the IF receptor. Hence, this points to an absence of the cubilin receptor, as supported by the literature.[39]

Finally, to further support the hypothesis that **2-IF** gained cellular entry via receptor mediated endocytosis, a pH-sensitive cyanine dye CypHer5E was conjugated to IF. This cyanine dye has a pK_a of ~7.3 and is fluorescent (red) only in acidic conditions. Internalization of this dye through receptor mediated endocytosis provides an acidic environment of ~5.5 in the endosome. Conjugation of the *N*-hydroxysuccinimide ester of this dye to IF was conducted according to manufacturer's (GE Sciences) instructions. A volume of 1 mL was then added to a plate containing ~200000 BeWo cells and incubated for 45 min. As shown in Figure 6, fluorescence was observed. The red flouresence of the dye counterstained with the green of **2** appears mostly yellow, although some red can still be seen. This provides further evidence that uptake of **2** occurs by an IF receptor (cubilin) mediated process.

In Vitro Antiproliferative Cell Assay. To determine whether cytotoxicity was significant for the transported conjugates, BQBA, **1**, **2**, and **2** bound to IF preincubation (**2-IF**) were screened at a range of concentrations against BeWo and CHO cells. The cells were cultured to ~90% confluence. Approximately, 8000 cells/well were inoculated into a 96-well plate. The plate was then incubated for 24 h to assist in adherence. Stock solutions of the drugs were prepared with RPMI 1640 media. A range of concentrations for each drug was prepared with a volume of ~100 μL of each added to the cells. The cells were then exposed to the drugs over a 6 h time period. The drug solutions were then removed from the plates, and the cells were washed with media to remove any drug residues. A 10% solution of CCK-8 dye in RPMI 1640 supplemented with fetal bovine serum and penicillin streptomycin solution was prepared. A volume of 100 μL of this dye was then added to the plates and incubated for 24 h. The calculated IC_{50} values are shown in Table 1.

No IC_{50} values were obtained for BQBA up to 5 mM. The rhenium complex **1**, however, displayed an IC_{50} of 376 μM, 10-fold more toxic than the B_{12} conjugate **2**. **1** is rationalized to have greater, more rapid access to the intracellular pool due to passive diffusion.[40] Passive diffusion of nutrients in this cell line was reported to be indirectly proportional to size, with the permeability coefficient, P_e, displaying a linear

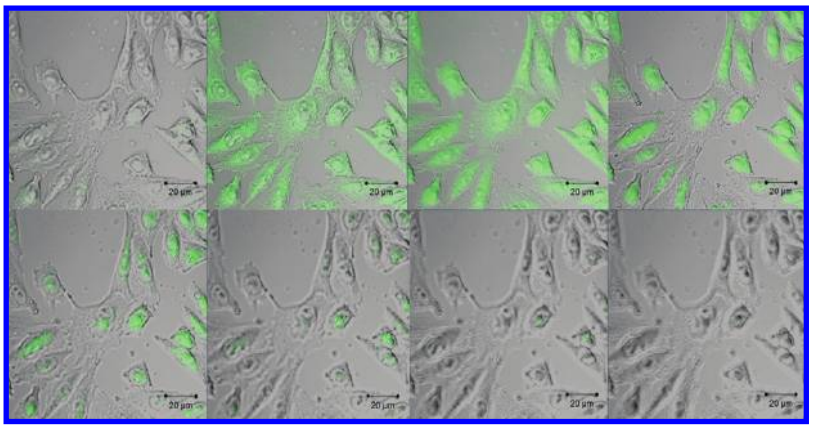


Figure 5. Random depth laser optical slices at ~1 μm per slice of BeWo cells show fluorescence in the middle slices of the cell, confirming internalization of **2-IF** after 45 min of incubation at 37 °C.

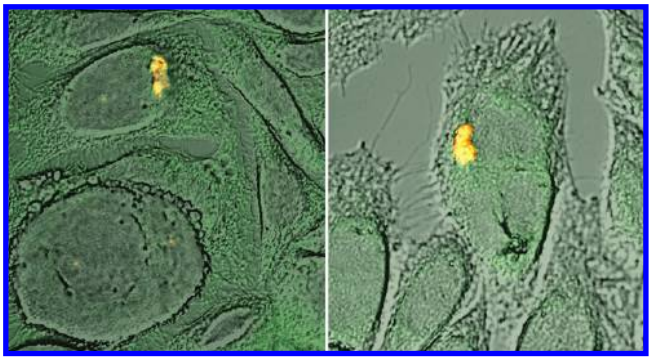


Figure 6. Red fluorescence was displayed upon exposure of an IF-CypHer5E conjugate to endosomal pH of ~5.5. **2** was bound to this conjugate via IF (confirmed by electronic absorption assay as in Figure 3 (data not shown)).

decrease with increasing molecular weights (MW: = 400−70000).[41] In addition, the positive charge of rhenium(I) may be providing electrostatic interactions with membrane proteins, providing an enhanced uptake of **1** in synergy with the passive permeation of the drug. Cell membrane studies conducted by Patillo et al. showed the BeWo cell lines as possessing a negative transmembrane potential of about −35 mV.[42] This faster diffusion of **1** in BeWo cells may also explain the difference in IC_{50} values compared to CHO (2.627 mM) for the same compound, although rapid efflux may also play a role. **2** displayed an IC_{50} of 3.180 mM in BeWo cells, while no toxicity was observed for **2** in the CHO line. This is supported by confocal microscopy experiments of **2** with prior exposure to lower B_{12} concentrations in BeWo cells showing minimal fluorescence, indicating lower uptake of **2**. In CHO cells, there was no observed fluorescence, consistent with **2** not permeating the plasma membrane. The IC_{50} values for **2-IF** in BeWo cells are calculated to be ~1.844 mM however, significantly lower than that for **2** not previously bound to IF. This increased toxicity of **2-IF** is most likely due to the greater uptake through the cubilin receptor. The higher IC_{50} concentration displayed in CHO cells is most likely a consequence of this cell's inability to internalize **2-IF**, in agreement with the lack of fluorescence observed in CHO cells upon exposure to **2-IF**.

siRNA Gene Knockdown of Cubilin Receptor. To fully establish the route of uptake as cubilin based, small interfering RNA (siRNA) specific for cubilin mRNA was transfected into the BeWo cells to knockdown expression of the cubilin receptor. siRNA mediated inhibition of cubilin with corresponding loss of uptake of **2-IF** would provide conclusive evidence that internalization of **2-IF** in BeWo cells proceeds via the cubilin receptor uptake pathway and establish **2** as a specific bioprobe for this receptor. A fluoroscein conjugate of random sequence siRNA, known not to inhibit any mRNA, was employed as a transfection uptake marker. After transfection of the cubilin siRNA, the cells were incubated at 37 °C. Uptake experiments were conducted after allowing the cells to grow over 24 h. The growth medium was aspirated and 10 μM **2-IF** was added to the transfected cells and control cells (no siRNA of any type added) and incubated over 45 min. After incubation, the drug-containing medium was discarded and the cells were then washed with 1× PBS in triplicate to remove residual **2-IF**. At both time points, confocal microscopy experiments showed illumination for both control and transfected cells. Fluorescence intensity values differed markedly however. The control plate displayed a mean intensity of ~4.0, whereas the transfected cells had an average recorded intensity value of ~0.4. This significant decrease (~10-fold) in fluorescence intensity in the transfected cells is indicative of a significant reduction of drug uptake and correlates with knockdown of the cubilin gene. We also conducted uptake experiments after 48 h of transfection. Optical slices (~1 μm) of the transfected BeWo cells revealed internalization with varying fluorescence intensities ranging from 0.81 to as much as 3.7, consistent with the cubilin receptor switching back to expression upon multiple passages (the BeWo line has a doubling time of ~20 h).

Table 1. IC_{50} Values (mM) of BeWo and CHO Cells after 6 h of Drug Exposure

	IC_{50} (mM)	
drug	BeWo	CHO
BQBA	*a*	2.979 ± 0.210
1	0.376 ± 0.028	2.627 ± 0.280
2	3.180 ± 0.258	*a*
2-IF	1.844 ± 0.478	4.860 ± 0.283

[a] Not cytotoxic at concentrations of up to 5 mM.

Propidium Iodide Counterstaining. To look at a possible mechanism of toxicity, propidium iodide (PI) counterstain was utilized to determine whether nuclear localization of **2**, once transported, occurred. PI is a known intercalator for DNA[43] and RNA.[44] In addition, this dye is an excellent nuclear stain with cells that have been previously stained

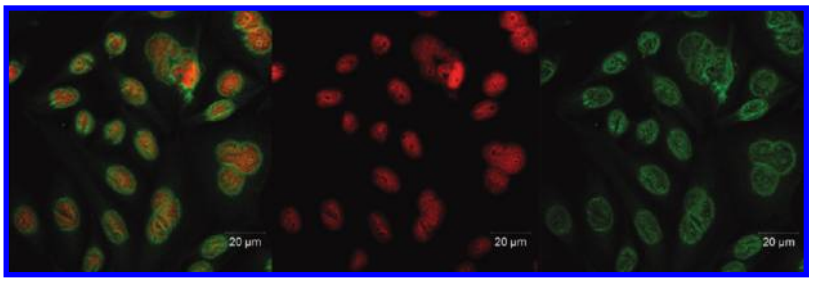


Figure 7. Counterstaining with propidium iodide along with **2-IF** shows nuclear and cytosolic accumulation of **2** as shown in the merged (left) and separate fluorescent images of cells marked with propidium iodide (center) and **2** (right).

with a fluorescent dye such as **2**. RNase A digestion for ~5 min at room temperature was conducted along with staining with PI to prevent staining of RNA and the cytoplasm.[45] Since this fluorophore is not able to permeate through the cell membrane, PI is dissolved in a solubilizing detergent such as Triton X-100 and added to the cell pool. Confocal images, obtained subsequently, of the BeWo cells show aggregation of **2** in the cytosol but also in the nucleus (see Figure 7). It is probable that the positively charged rhenium chelate component can interact with the negatively charged DNA backbone, and this may play a role in the observed toxicity.

Conclusion

We have successfully demonstrated targeted delivery of a rhenium based bioprobe to the cubilin receptor. The new B_{12} conjugate of rhenium, **2**, requires intrinsic factor and uses the IF-cubilin import mechanism of B_{12}. Confocal microscopy results show internalization and accumulation of **2** bound to IF in the nuclear and cytosolic region of the placental BeWo cell line. Saturation of this uptake mechanism with excess B_{12} shuts down the route of entry of **2**. This is indicative and consistent with the endocytotic mechanism of cubilin. In addition, siRNA inhibition of the cubilin gene significantly knocked down uptake of **2-IF**. This conclusively points to the key role that the cubilin receptor is playing in the internalization of **2-IF**. IF mediated delivery of B_{12} conjugates to cubilin overexpressing malignancies is achievable and shows immense potential. This receptor in particular makes for a suitable biomarker for targeting cancers in the gastrointestinal[26] and renal tissues,[27] and the bioprobe itself can be readily switched to a ^{99m}Tc derivative inspired by the B_{12}-^{99m}Tc conjugate of Alberto et al.[46]

Experimental Section

Materials. All reactions were performed under an inert atmosphere of argon or nitrogen using standard Schlenk techniques. Dimethyl sulfoxide (DMSO, 99%, Sigma) was dried by charging through a column of 4 Å molecular sieves (Mallinckrodt) previously dried overnight at 120 °C. Reagents listed below were purchased and used without further manipulations: vitamin B_{12} (B_{12}, 99%, Sigma), rhenium(I) pentacarbonyl bromide (98%, Sigma) 1,1′-carbonyl-di-(1,2,4-triazole) (≥90%, CDT, Fluka), quinoline-2-carboxaldehyde (98%, Alfa Aesar), sodium triacetoxyborohydride (95%, Sigma), 1,2-dichloroethane (DCE, ≥99%, Sigma), 1,2-dichloromethane (DCM, ≥ 99.5%, Sigma), methanol (MeOH, ≥99.8%, Sigma), acetonitrile (MeCN, ≥99.8%, Sigma), sodium sulfate (≥99.9%, anhydrous, Sigma), intrinsic factor (IF, MP Biomedicals LLC). siRNA transfection reagents were purchased from Santa Cruz Biotechnology. Calf-thymus (CT-DNA) and propidum iodide (PI) were purchased from Calbiochem. Compounds were confirmed to be >95% pure by HPLC, ^{1}H NMR, and/or ICP.

All in vitro cell experiments were performed in an air-filtered and UV-irradiated Labconco Purifier I laminar flow hood. Chinese hamster ovarian cells (CHO) cells were obtained from the American Type Culture Collection (ATCC) culture code CHO-K1. The A2780/AD cell line used for testing was provided by the Fox Chase Cancer Centre, Philadelphia, PA. The BeWo choriocarcinoma human cell line (ATCC number CCL-98) was purchased from ATCC. Fetal bovine serum (FBS) and Cellgro Cellstripper were purchased from Mediatech from Manassas, VA. Penicillin−streptomycin solution with 10 000 units penicillin and 10 mg/mL streptomycin in 0.9% NaCl was obtained from Sigma. Invitrogen supplied RPMI 1640 1× growth media containing L-glutamine and phenol red with and without folic acid. 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt solution (WSK-8), was purchased from Dojindo.

Purification using reverse phase high-pressure liquid chromatography (RP-HPLC) was conducted using an Agilent 1100 with manual injection and automated fraction collector. A Zorbax C_{18} analytical column (42 mm × 10 mm) was utilized for analytical runs with a flow rate of 0.7 mL/min. Purification of **2** was made with a C_{18} semipreparative column (9.4 mm × 250 mm) at a flow rate of 2 mL/min. Detection was by ultraviolet monitoring at 254 or 280 nm. Elution was performed using water (A) and MeCN (B) as solvents and with the following gradient: (1) 30% B and 70% A to 40% B and 60% A over 8 min, (2) 40% B and 60% A to 50% A and B over 8 min,[42] 50% A and B to 100% B over 5 min (t_R = 13.7 min). Proton nuclear magnetic resonance (^{1}H NMR) was performed using Bruker Avance DPX 500 MHz and Bruker Avance DPX 300 machines. Electrospray ionization (ESI) and matrix assosted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analyses were carried out on a Shimadzu LCMS-2010 A mass spectrometer and Bruker Autoflex MALDI-TOF, respectively. A Perkin-Elmer ELAN 6100 was used to conduct inductively coupled plasma analysis (ICP). A Varian Cary 50 Bio UV−vis spectrophotometer recorded the electronic absorption spectra of the compounds and was also used for intrinsic factor binding studies. Centrifugation was performed for 10 min at 4000 rpm at 4 °C with a Sorvall Legend RT centrifuge. Circular dichroism (CD) experiments were conducted with an Aviv model 202 spectrometer at 25 °C in the wavelength range of 200−350 nm. The CD experiments were performed using a quartz cuvette with a path length of 2 mm. Confocal microscopy experiments were conducted with a Zeiss LSM 700 Pascal confocal microscope with Zen 2008 image analysis software equipped with argon ion and HeNe lasers. IR spectra were obtained on a Perkin-Elmer series 1600 FT-IR spectrometer in the region 400−4000 cm^{-1} with polystyrene as a reference.

Synthesis of $[BQBA(Re(CO)_3]^+$ (1). Amounts of 0.1968 g (1.045 mmol) of *N*-Boc-1,4-diaminobutane and 0.3450 g (2.195 mmol) 2-quinolinecarboxaldehyde were dissolved in 30 mL of dichloroethane under argon and allowed to stir for 30 min. Then, 0.6646 g (3.136 mmol) of sodium triacetoxyborohydride was added to the purple reaction mixture, and stirring

was continued overnight. After completion of the reaction, the solvent was removed followed by the addition of 30 mL of methanol to destroy excess reducing agent. After the gas evolution ceased the solvent was removed once more and the crude reaction mixture purified by basic alumina chromatography using ethyl acetate as eluent. The first fraction eluted contained the purple product. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.04−7.96 (m, 4H), 7.70−7.56 (m, 6H), 7.40 (m, 2H), 4.67 (s, 1H), 3.92 (s, 4H), 2.96 (m, 2H), 2.54 (t, J = 7.07 Hz, 2H), 1.53 (m, 2H), 1.32 (m, 11H) ppm. Yield: 74% (0.3640 g).

Amounts of 0.3640 g (0.774 mmol) of *tert*-butyl 4-(bis-((quinolin-2-yl)methyl)amino)butylcarbamate (BQBA) and 0.4042 g (0.774 mmol) of $[Re(H_2O)_3(CO)_3]Br$ were heated under argon at 60 °C in 30 mL of methanol for 3 h. The solvent was removed and the crude mixture purified by basic alumina chromatography with a gradient of ethyl acetate/methanol. Unreacted ligand was eluted with ethyl acetate followed by the elution of the metal complex with ethyl acetate/methanol, 10:1. ^{1}H NMR (300 MHz, MeOH-d_4): δ = 8.46 (t, J = 8.92 Hz, 4H), 7.94 (dd, J = 8.09, 1.37 Hz, 2H), 7.80 (ddd, J = 8.70, 7.06, 1.57 Hz, 2H), 7.64 (m, 2H), 5.18 (d, J = 17.74 Hz, 2H), 5.00 (d, J = 17.81 Hz, 2H), 3.09 (t, J = 6.72 Hz, 2H), 1.95 (m, 2H), 1.57 (m, 2H), 1.33 (s, 9H) ppm. ESI MS: 740.65 m/z [M$^+$], calculated 741.21 m/z. Yield: 38% (0.241 g).

An amount of 0.241 g (0.294 mmol) of rhenium tricarbonyl *tert*-butyl 4-(bis((quinolin-2-yl)methyl)amino)butylcarbamate bromide was dissolved in 50 mL of 3 M hydrochloric acid, and the mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure to give **1** as a brownish powder. ^{1}H NMR (300 MHz, MeOH-d_4): δ = 8.45 (dd, J = 13.56, 8.62 Hz, 4H), 7.95 (d, J = 7.34 Hz, 2H), 7.80 (m, 2H), 7.66−7.59 (m, 4H), 5.23 (d, J = 17.80 Hz, 2H), 5.09 (d, J = 17.96 Hz, 2H), 3.88 (m, 2H), 3.01 (m, 2H), 2.07 (m, 2H), 1.80 (m, 2H) ppm. ESI MS: 640.90 m/z [M$^+$], calculated 641.16 m/z. IR (KBr pellet): ν = 3447, 2017, 1880 cm^{-1}. Yield was quantitative.

Synthesis of B_{12}-[BQBA(Re(CO)$_3$)]$^+$ (2). B_{12} (0.0250 g, 0.0369 mmol) was activated with 1.2 mol equiv of CDT (0.0440 g, 0.0244 mmol) in 3 mlL of DMSO. The solution was heated while being stirred at 60 °C under $N_{2(g)}$ for 30 min. **7** (0.0142 g, 0.0221 mmol) in 2 mL of DMSO was added dropwise to this solution, and the mixture was stirred at room temperature for 6 h. A crimson solid was collected after the solution was precipitated in a 1:15 acetone/ether mix. The powder was then purified using C_{18} RP-HPLC with a retention time t_R = 13.7 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.83 (d, 2H), 8.50 (d, 2H), 8.32 (d, 2H), 8.22 (d, 2H), 8.08 (m, 3H), 7.88 (t, 2H), 7.83 (s, 1H), 6.93 (s, 1H), 6.68 (s, 1H), 6.60 (s, 1H), 6.06 (s, 1H) ppm. MALDI-TOF MS: 2022.37 m/z [M$^+$], calculated 2022.09 m/z. Yield: 15.3% (3.8 mg) based on B_{12}.

Cell Culture and Growth. Cells were grown in Millipore 250 mL culture bottles with vented lids. Chinese hamster ovary (CHO, ATCC code CCL-61) cell line and human BeWo choriocarcinoma cell line (ATCC code CCL-98) were cultured as adherent monolayers in RPMI 1640 growth media (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and 10 000 units of penicillin and 10 mg/mL streptomycin (Sigma). Cells were harvested using Cellstripper (Mediatech, Manassas, VA). The cells were then collected via centrifugation, and the solution containing Cellstripper and media was disposed. Resuspension of the cells proceeded by addition of 5 mL of complete growth medium and aspirated gently by pipetting. All cell cultures were grown and incubated in mammalian cell incubator maintained at 37 °C with 5% CO_2 and 95% humidity. All cell-based assays were performed using cells in exponential growth.

Intrinsic Factor Binding to 2. IF weighing 10 mg was dissolved in 2 mL of 1× phosphate buffered saline (PBS) (pH ≈ 7.4) and rotated overnight in 4 °C. The undissolved solids were removed via centrifugation. The electronic absorption spectra of **2** (20−50 μM) were then monitored upon addition of 5−10 μL increments of the solution containing IF. An increase in absorbance indicates formation of a binary B_{12}-IF complex.[34,43] The resulting final solution with IF-B_{12} binary complex formed was subsequently used for confocal microscopy studies and cytotoxicity assays.

Confocal Microscopy Uptake Experiments. The human choriocarcinoma placental cell line (BeWo) and Chinese hamster ovary cells (CHO) were grown and cultured as adherent monolayers in a vented flask with a 175 cm^2 culture area, filter vented close cap, and angled neck. These cells were grown to >85% confluence.

BeWo placental cells and Chinese hamster ovary cells (∼200000 cells per dish) were plated on 35 mm × 100 mm glass bottom vented dishes (Martek). The cultures were allowed to adhere onto the dish surface over 24 h in RPMI-1640 media at 37 °C. To these cells, **2-IF** with a final concentration of ∼10 μM with and without prior addition of excess B_{12} (100 μM and 10 mM) was added. Cells were also incubated with 10 μM **2** (i.e., unbound to IF) and excess B_{12} (10 mM). The dishes were then incubated at 37 °C over 45 min and 6 h. The solutions were then removed, and the cells were washed with 1 mL 1× PBS (pH ≈ 7.4) in triplicate. Fluorescence was then observed with a fluorescent confocal microscope with intensities recorded at 63× magnification.

Propidium Iodide Staining. BeWo cells were previously plated (∼200000 cells per dish) in 35 mm × 100 mm glass bottom vented dishes (Martek) and incubated overnight. A volume of 1 mL of 10 μM **2-IF** was added to the cells and incubated at 37 °C for 45 min. The medium containing the drug was discarded and replaced with ∼1 mL of ethanol (chilled at ∼20 °C). After 3 min, the ethanol was removed. A solution containing 20 μL of 1 mg/mL PI, 200 μL of 10 μg/mL RNase A, 5 μL of Triton X-100, and 4.78 mL of 1× PBS buffer was prepared. A volume of 1 mL of this solution was added to the BeWo cells, and the mixture was incubated in the dark for 5 min. Images were collected at 63× magnification using a fluorescent confocal microscope.

Drug Cytotoxicity. The proliferation of the exponential phase cultures of BeWo and CHO cells was assessed by WSK-8 colorimetric assay. This assay follows ATP production and indicates mitochondrial health. WSK-8 was performed according to manufacturer's instructions (Dojindo). Adherent cell cultures were harvested by stripping of culture flasks using a nonenzymatic cell stripper and after a 30 min incubation period. To each well, aliquots of 100 μL were inoculated, resulting in 8000 cells per well. The plates were then incubated over a 24 h period to facilitate adherence. Serial dilutions of drugs **1**, **2**, **2-IF**, and BQBA were prepared. A volume of 100 μL of the different drug concentrations was then added to the wells and incubated for 6 h. The drugs were removed from the wells and rinsed with growth medium. A solution containing 10% WSK-8 dye in growth medium with a volume of 100 μL was then added to each of the microcultures and incubated over 24 h. Optical densities were measured using a plate reader (Thermo Multiskan EX plate reader equipped with Ascent software, version 2.6). The percentage of cell viability was determined relative to untreated control microcultures. The IC_{50} concentrations were calculated on the basis of an exponential fit using OriginLabs 8 software with $R^2 \geq 0.80$ in all cases. This assay was conducted in triplicate with each point in triplicate on separate "batches" of compound on cells.

siRNA Gene "Knockdown" of the Cubilin Receptor. The siRNA transfection experiment was conducted according to manufacturer's instructions. The cubilin siRNA was dissolved in 330 μL of RNase-free buffer. For each transfection, the following solutions were prepared. Solution A was prepared by mixing 6 μL of the cubilin siRNA with 100 μL of siRNA transfection medium. Solution B contained 6 μL of siRNA transfection reagent and 100 μL of transfection medium. The two solutions were mixed and incubated at room temperature for 30 min. A volume of 2 mL of transfection medium was used to wash a plate of cells (∼200000) previously prepared and

allowed to grow in penicillin−streptomycin free growth medium overnight. To the mixture containing solutions A and B, 0.8 mL of transfection medium was added. This solution was then added to the cells and incubated for 5−7 h at 37 °C. A volume of 1 mL of growth medium containing 2× FBS and penicillin−streptomycin was added to the cells without removing the transfection solution. The cells were then incubated for an addition 18 h, after which the medium was discarded and replaced with fresh 1× growth medium. After 24 h, the transfected cells were then exposed to 10 μM **2-IF** for 45 min. The medium containing **2-IF** was removed, and the cells were washed with 1× PBS in triplicate. Uptake of **2-IF** on cubilin knocked down versus cubilin expressing BeWo cells (i.e., no transfection performed) was compared via fluorescence confocal microscopy.

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Supporting Information Available: ESI mass spectrum of **1**, IR spectrum of **1**, MALDI-TOF mass spectrum for **2**, and a movie file of the depth optical scanning of BeWo cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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