

Characterization of the pH of Folate Receptor-Containing Endosomes and the Rate of Hydrolysis of Internalized Acid-Labile Folate-Drug Conjugates^[S]

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ABSTRACT

Despite the widely accepted assumption that most endosomal compartments are acidic, evaluation of the efficiency of pH-dependent drug release from a ligand-targeted drug conjugate during receptor-mediated endocytosis is lacking. Therefore, we have characterized the kinetics of pH-dependent drug release from a model folate-drug conjugate during folate receptor (FR)-mediated endosomal trafficking. For this purpose, we synthesized an acid-labile folate-fluorescence resonance energy transfer reporter (ALFR) that emits green fluorescence (BODIPY FL, 6-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diazas-indacene-3-propionyl)amino)hexanoic acid) only after acid-catalyzed hydrolysis of the acyl hydrazone linker. In a cell-free system, cleavage of ALFR was found to be efficient only at acidic pH values ($t_{1/2} = 1.95,$

4.63, and 75 h at pH 4, 5, and 6, respectively) and essentially resistant to hydrolysis at pH 7. Curiously, when applied to folate receptor-expressing cancer cells, the acid-labile folate-linked probe exhibited little or no recovery of BODIPY FL fluorescence (green), even after 55 h of incubation, arguing very inefficient cleavage within the FR endocytic pathway. To understand this unanticipated observation, we measured the pH of FR-containing endosomes using ratiometric fluorescence microscopy and observed that most FR⁺ endosomes are only mildly acidic (average ~pH 6.5). Taken together, these data argue that the FR-trafficking pathway does not involve acidic compartments and that acyl hydrazone linkers may constitute a poor option for FR-mediated drug delivery.

Tumor-specific delivery of anti-cancer drugs can often be achieved by attaching the cytotoxic drug to a tumor-recognizing ligand (e.g., an antibody or a low molecular weight ligand). Such attachments, however, can potentially introduce steric hindrances and prevent association of the drug with its molecular target. Therefore, improved therapeutic efficacy is frequently only realized when the active agent is linked to its targeting ligand through a cleavable spacer that is stable in circulation but readily hydrolyzed upon entry into its target cell. In particular, linkers that are selectively hydrolyzed at acidic pH have received considerable attention, because the majority of receptor-directed drugs are delivered

to endosomal compartments or lysosomes where pH values are thought to be low (Kratz et al., 1999; Masson et al., 2004; Maxfield and McGraw, 2004). The slightly acidic microenvironment of some tumors (~pH 6.5) has also been proposed to assist in release of these drugs, especially when the conjugate is expected to be trapped within the tumor for prolonged periods (Ashby, 1966; Lavie et al., 1991; Gatenby et al., 2006).

Among the acid-labile linkers in common use today, acyl hydrazones have exhibited good activity in many preclinical studies. When exploited to link doxorubicin to tumor-recognizing antibodies, for example, an acyl hydrazone bridge was found to resist unwanted hydrolysis at neutral pH (>10% drug release at 5 h) but readily discharge free doxorubicin after only 2 h at pH 4.5 (>50% release) (Kaneko et al., 1991). Not surprisingly, when tested on tumor-bearing mice, the antibody-targeted, releasable doxorubicin exhibited a maximal tolerated dose approximately 3- to 4-fold higher than the unmodified drug (Trail et al., 1992). Similar improvement in

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ABBREVIATIONS: FR, folate receptor; FRET, fluorescence resonance energy transfer; BODIPY FL, 6-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diazas-indacene-3-propionyl)amino)hexanoic acid, fluorescein; GPI, glycosylphosphatidylinositol; FITC, fluorescein isothiocyanate; ALFR, acid-labile folate-FRET reporter; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry.

drug tolerance has also been observed with pH-sensitive antibody-conjugated vinca alkaloids (Laguzza et al., 1989; Johnson et al., 1990; Gutowski et al., 1991; Appleman and Frey, 1996).

The folate receptor (FR), a GPI-anchored membrane protein involved in folate endocytosis, is often overexpressed on tumor cells (Elnakat and Ratnam, 2004). Because folate and folate-drug conjugates bind FR with high affinity ($K_d \sim 1$ nM) and because FR rapidly recycles between the plasma membrane and the cell interior (Kamen and Smith, 2004; Sabharanjak and Mayor, 2004), strategies have been pursued to exploit folate for selective targeting of attached imaging and therapeutic agents to tumors (Jackman et al., 2004; Leamon and Reddy, 2004; Lu et al., 2004; Zhao and Lee, 2004; Hilgenbrink and Low, 2005; Reddy et al., 2005). Analyses of such drug uptake processes reveal that folate-drug conjugates are internalized into endosomes via receptor-mediated endocytosis (Chatterjee et al., 2001) and then released from FR before it resurfaces for the next round of delivery (Kamen and Smith, 2004; Paulos et al., 2004). It is noteworthy that a previous report by Lee et al. (1996) that the pH of FR-containing endosomes can drop below pH 5 offered encouragement that pH-labile linkers might be ideal for assuring efficient drug release only after uptake by targeted cancer cells. However, more recent studies of Fivaz et al. (2002) demonstrated that polyvalent ligands, such as those used by Lee et al. (1996) to measure endosomal pH, follow a different endocytic pathway than monovalent ligands for GPI-anchored receptors, such as FR, which raised questions regarding the applicability of the Lee et al. (1996) studies to delivery of monovalent folate-targeted drugs.

To evaluate the effectiveness of pH-sensitive acyl hydrazone linkers for intracellular drug release during FR-mediated endocytosis, we prepared a folate-dye conjugate [acid-labile folate-fluorescence resonance energy transfer (FRET) reporter (ALFR)] whose fluorescence is quenched via intramolecular FRET until the pH-sensitive hydrazone bond to the dye is cleaved at low pH. By measuring fluorescence, we first characterized the folate-FRET reporter's cleavage kinetics at different pH values in vitro and then monitored cleavage of the folate conjugate in real time in intact cancer cells

during folate receptor-mediated endocytosis. Because the rate of intracellular hydrazone hydrolysis was unexpectedly slow, we finally characterized the pH of monovalent folate conjugate-containing endosomal compartments in live cells.

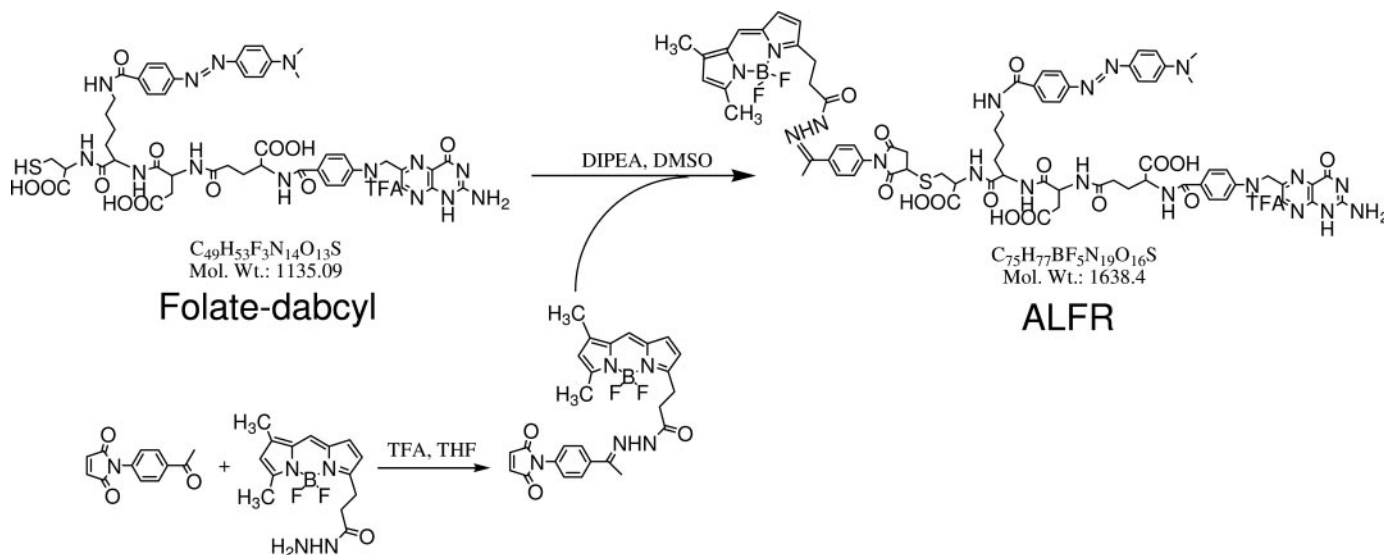
Materials and Methods

Materials. All reagents for the synthesis of the folate backbone were purchased from Novabiochem (San Diego, CA). 4-Maleimidophenylacetic acid was obtained from Sigma-Aldrich (St. Louis, MO). Pteronic acid and folate-FITC were generous gifts from Endocyte, Inc. (West Lafayette, IN). BODIPY FL hydrazide was purchased from Molecular Probes (Eugene, OR).

Cell Culture. KB cells, a human nasopharyngeal cancer cell line, was grown continuously as a monolayer using folate-free RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT) at 37°C in a 5% CO₂/95% air-humidified atmosphere in the presence of ampicillin and streptomycin. Cells were grown in 35-mm glass bottom Petri dishes (Mat Tek, Ashland, MA) for imaging studies. All cells were cultured to 50 to 60% confluence before each experiment.

Synthesis and Characterization of the ALFR. Synthesis of ALFR was carried out by following Scheme 1. In brief, folate-dabcyI was prepared by standard Fmoc chemistry on an acid-sensitive trityl resin loaded with Fmoc-L-Cys(Trt)-OH as described previously (Leamon et al., 2002). Crude folate-dabcyI was dissolved in water, pH 8.2, in the presence of argon to prevent disulfide bond formation. The resulting solution was loaded on a VYDAC "protein and peptide C18" HPLC column and separated using 10 mM ammonium acetate, pH 5.0, and acetonitrile as the mobile phase. HPLC-purified folate-dabcyI was characterized by LC-MS ($M_r = 1135.09$), using a Waters 2995 HPLC system coupled with a Waters Micromass ZQ Mass Spectrometer (Waters, Milford, MA).

4-Maleimidophenylacetic acid (2.1 mg) was reacted with BODIPY FL hydrazide (3 mg) in tetrahydrofuran using trifluoroacetic acid (1 Eq relative to 4-maleimidophenylacetic acid) as a catalyst. Reaction progress was followed by analytical HPLC (mobile phase: 10 mM ammonium acetate, pH 5.0, in acetonitrile) and found to reach 90% completion within 0.5 h. After removal of tetrahydrofuran in vacuo, folate-dabcyI (9 mg) and excess *N,N'*-diisopropylethylamine were added, and the reaction progress was again monitored by analytical HPLC (mobile phase: 10 mM ammonium acetate, pH 7.0, in acetonitrile). By 1 h, the reaction was found to reach completion, and the desired product was isolated by preparative HPLC using 1 mM



Scheme 1. Synthesis of the acid-labile folate-FRET reporter (i.e., ALFR).

sodium phosphate, pH 7.2, as the mobile phase. The final conjugate, ALFR, was characterized by LC-MS ($M_r = 1638.4$). Analytical and preparative HPLC runs were performed using a Waters 2996 system and a Waters 2487 system, respectively. All HPLC systems used in this study are coupled with a Waters Photodiode array.

The binding specificity and affinity of ALFR for the folate receptor was determined by a previously described competition assay with slight modification (Leamon et al., 2002). In brief, KB cells were incubated with folate rhodamine (25 nM; K_d for FR ~ 20 nM) in the presence of increasing concentrations of ALFR (0–200 nM) for 30 min on ice, followed by washes with fresh phosphate-buffered saline to remove unbound conjugate. Cells were then immediately lysed with 2% SDS and measured for rhodamine fluorescence using an Aminco Bowman Series 2 luminescence spectrometer (excitation: 545 nm, emission: 595 nm) (Thermo Electron, Waltham, MA). Analysis of the competitive binding curve yielded a K_d of ALFR for the folate receptor of ~ 17 nM (Supplemental Data).

Characterization of the pH-Dependent Cleavage of ALFR in Solution. To measure the pH dependence of ALFR hydrolysis in vitro, ALFR was dissolved in 10 mM sodium citrate, pH 4 and 5, or 10 mM sodium phosphate, pH 6 and 7. ALFR solutions (10 nM) were incubated at room temperature for the indicated lengths of time and then subjected to fluorescence spectrophotometry (excitation: 488 nm and emission: 520 nm) using an Aminco Bowman Series 2 luminescence spectrometer. The fluorescence intensities were plotted against incubation time, and SigmaPlot software (Systat Software, San Jose, CA) was used to calculate the release half-time assuming first-order kinetics at all pH values.

FRET Imaging. FRET imaging was performed using an Olympus IX-70 inverted confocal microscope system. KB cells were incubated with a mixture of ALFR (100 nM) and folate rhodamine (a known ligand for FR; 100 nM) for 30 min on ice to minimize diffusional uptake of the probe and to prevent any receptor-mediated endocytosis before imaging. Cells were then washed with fresh culture medium to remove unbound conjugate. After incubation at 37°C in culture medium for the desired lengths of time (0.5 to 55 h), cells were examined under an Olympus 60X/1.2 NA water objective (Olympus USA, Center Valley, PA) using an argon laser (488 nm)

and the proper emission filter (520/40 nm). As a positive control, the same cells were also imaged for folate-rhodamine fluorescence ($\lambda_{ex} = 543$ nm, $\lambda_{em} = 595$ nm) to assure that FR internalization occurred normally. Image processing was performed using FluoView software (Olympus USA).

pH Determination of FR-Containing Endosomes by Fluorescence Ratio Imaging. The pH of FR-containing endosomes was measured by following the previously described procedure with slight modifications (Lee et al., 1996). In brief, KB cells were incubated for 0.5 h at 0°C with an equimolar solution (100 nM each) of the pH-insensitive construct folate-rhodamine and the pH-sensitive construct folate-FITC. After washes with fresh medium to remove unbound folate conjugates, cells were then incubated at 37°C for an additional 3 h to allow endocytic trafficking to reach a steady state. Laser excitation bands at 488 and 543 nm were then used to independently excite fluorescein and rhodamine fluorescence, respectively, and images were acquired using an Olympus IX-70 inverted confocal microscope with appropriate filters. The intensity of each type of folate conjugate (i.e., folate-FITC and folate rhodamine) in individual endosomes was quantitated using Image J Software (<http://rsb.info.nih.gov/ij/>) (Abramoff, 2004). To construct the calibration curve (i.e., standard curve), equal molar folate-rhodamine and folate-FITC were mixed at different pH values and imaged using confocal microscopy under the same conditions used for the cell imaging. Endosome fluorescence was quantitated in several distinct areas in the microscope field and averaged. The ratio of green to red fluorescence was plotted against pH using SigmaPlot software.

Results

pH-Dependent Cleavage of the Acid-Labile Folate-FRET Reporter. As shown in Fig. 1, the ALFR comprises 1) the tumor-targeting ligand, folic acid; 2) a peptide spacer that improves water solubility of the conjugate; 3) the FRET donor, BODIPY FL (488/520 nm) that is linked to folate via the pH-sensitive acyl hydrazone linker; and 4) the quencher, dabcyf, that is attached to folate via a pH-insensitive amide

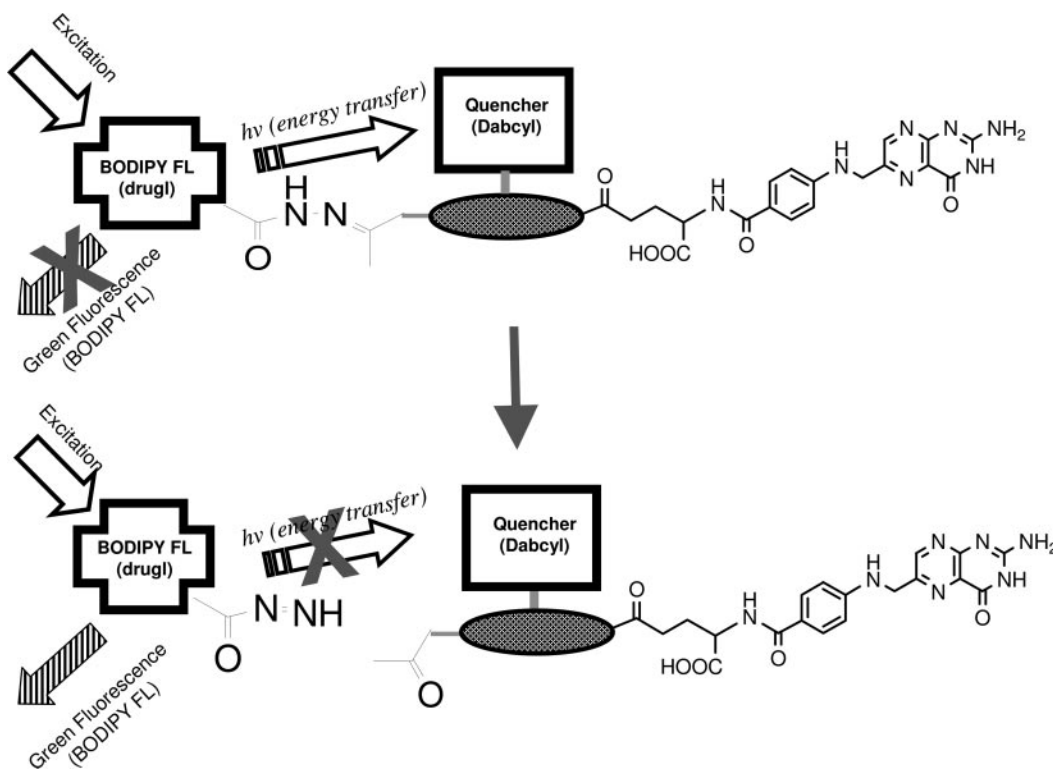


Fig. 1. Schematic representation of acid-triggered hydrolysis ALFR and its association with dequenching of BODIPY FL fluorescence. In the intact folate-FRET probe, emission of BODIPY FL fluorescence is depressed by the quencher, namely dabcyf, due to FRET from BODIPY FL to dabcyf. Upon hydrolysis of the acyl hydrazone, BODIPY FL is released from the folate backbone, resulting in the loss of FRET and concurrent recovery of BODIPY FL fluorescence.

bond. The probe is designed to assure quenching of BODIPY FL fluorescence as long as the fluorescence donor in the FRET pair is attached in close proximity to dabcyI, the quencher (fluorescence acceptor) in the FRET pair. Upon acid-catalyzed hydrolysis of the hydrazone linker, the green fluorescent dye will be released, whereas dabcyI remains attached to the folate-containing fragment via its amide bond. This separation of BODIPY FL from its quencher leads to a loss of FRET and the consequent recovery of BODIPY FL green fluorescence.

As an initial test of this methodology, the kinetics of the folate-FRET reporter dequenching was determined at various pH values (i.e., 4.0, 5.0, 6.0, and 7.0). As seen in Fig. 2, half-times for ALFR hydrolysis (assuming a first-order reaction in all cases) were found to be 1.95 h at pH 4, 4.63 h at pH 5, and 75 h at pH 6. No release was detected at pH 7. These data suggest that 1) recovery of BODIPY FL fluorescence constitutes a useful surrogate marker of the acyl hydrazone cleavage in ALFR, 2) the acyl hydrazone linker is stable at pH 7 but increasingly less stable as pH decreases, and 3) cleavage of the acyl hydrazone is reasonably rapid between pH 4 and 5.

Characterization of ALFR Cleavage in Intact Cancer Cells. We next examined acid-catalyzed hydrolysis of the folate-FRET reporter in live FR-expressing cancer cells by confocal fluorescence microscopy. In contrast to the rapid dequenching of ALFR seen in buffered solutions *in vitro*, hydrazone hydrolysis was not detectable until 5.5 h postadministration. Thus, at 5.5 h, a low level of green fluorescence appeared in a perinuclear region of the cell that was previously identified as the recycling endocytic compartment (Fig. 3B) (Chatterjee et al., 2001). Curiously, as incubation proceeded, fluorescence remained surprisingly weak. Careful analysis of serial micrographs acquired at different time points revealed that recovery of BODIPY FL fluorescence barely improved with time [i.e., 18.5 h (Fig. 3C) and 55 h (data not shown)]. As a positive control, the same cells were simultaneously incubated with folate-rhodamine (a known ligand for FR) and imaged for rhodamine fluorescence at various time points to assure that FR internalization occurred normally (Fig. 3D). The prominent rhodamine fluorescence both on the cell surface and within endocytic com-

partments demonstrates that both FR binding and conjugate internalization were proceeding unabated in these cells. Because a similar lack of ALFR dequenching was also observed in the absence of the added folate rhodamine, we concluded that acyl hydrazone cleavage is inefficient within the FR endocytic pathway.

Measurement of the pH of FR-Containing Endosomes. Lack of measurable cleavage of the ALFR conjugate in KB cells was unexpected in view of a previous article from our laboratory (Lee et al., 1996) reporting the pH of FR-containing endosomes ranging from 4.7 to 5.8 in the same KB cell line. However, because this previous pH analysis was obtained using a polymeric dye conjugate tethered to multiple folates and because polyvalent ligands have been subsequently shown to force GPI-anchored receptors to enter and traffic in cells by a distinct pathway (Fivaz et al., 2002), we recharacterized the intracellular pH of folate-containing endosomes using monovalent folate-dye conjugates. For this purpose, we incubated KB cells with a solution containing equimolar quantities of folate-FITC (a pH-sensitive dye conjugate) and folate-rhodamine (a pH-insensitive dye conjugate) and then measured endosomal pH by quantitating the ratio of green (fluorescein) to red (rhodamine) fluorescence in each endocytic vesicle. By comparing this ratio to the same ratio obtained for the same solution of dye conjugates in standard buffers (Fig. 3G), an estimate of intraendosomal pH could be obtained. As shown in Fig. 3, E and F, folate-FITC and folate rhodamine were both seen to internalize and cluster in the perinuclear region where previous monovalent folate-dye conjugates have been reported to collect (Chatterjee et al., 2001). However, due to quenching of FITC at low pH, some endosomes exhibited primarily rhodamine fluorescence, suggesting some decline in pH between FR on the cell surface and FR in the perinuclear endosomes. Ratiometric analysis revealed that the pH of FR-containing endosomes ranged from 5.6 to 7.2, with the most prominent pH values ranging from pH 6.6 to 6.8 (Fig. 4). This unexpectedly high compartmental pH range alone is probably sufficient to explain the very slow rate of ALFR hydrolysis in KB cells (compare Fig. 2). The results are also consistent with the observation that FR traffics through recycling endosomes that are known to have a pH of only 6.5 (Turek et al., 1993; Mayor et al., 1998; Chatterjee et al., 2001).

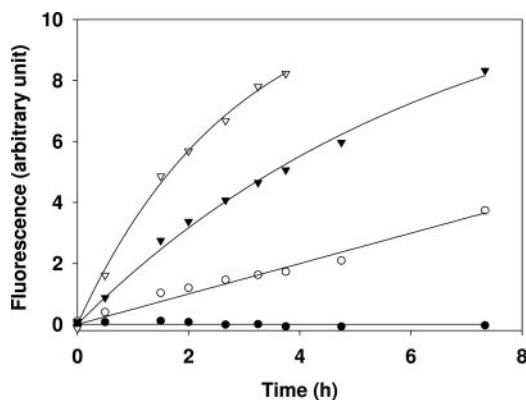


Fig. 2. pH-dependent hydrolysis of ALFR. ALFR was dissolved at various pH values (i.e., pH 4 (open triangles), pH 5 (solid triangles), pH 6 (open circles), and pH 7 (solid circles)), and incubated at room temperature for the indicated lengths of time. The solutions were then examined by fluorescence spectrophotometry (excitation: 488 nm and emission: 520 nm) to quantitate the rate of appearance of BODIPY FL fluorescence.

Discussion

In this study, we have characterized the pH dependence of model drug release from a folate-drug conjugate during FR endosomal trafficking. We have described the synthesis of an acyl hydrazone-linked folate-FRET conjugate, ALFR, that emits green fluorescence only after acid-catalyzed hydrolysis of the acid-labile linker. In a cell-free system, cleavage of ALFR was found to be efficient at acidic pH values but extremely slow at neutral pH. When little measurable ALFR conjugate was seen to hydrolyze during endocytosis by FR-expressing KB cells, the pH of FR-containing endosomes was reevaluated using monovalent folate-dye conjugates. The observation that FR-containing endosomes are only mildly acidic (average \sim pH 6.6–6.8) argued that acyl hydrazone hydrolysis during FR-mediated endocytosis was highly inefficient largely because FR⁺ endosomal pH values are too high to catalyze ALFR cleavage efficiently. The data also

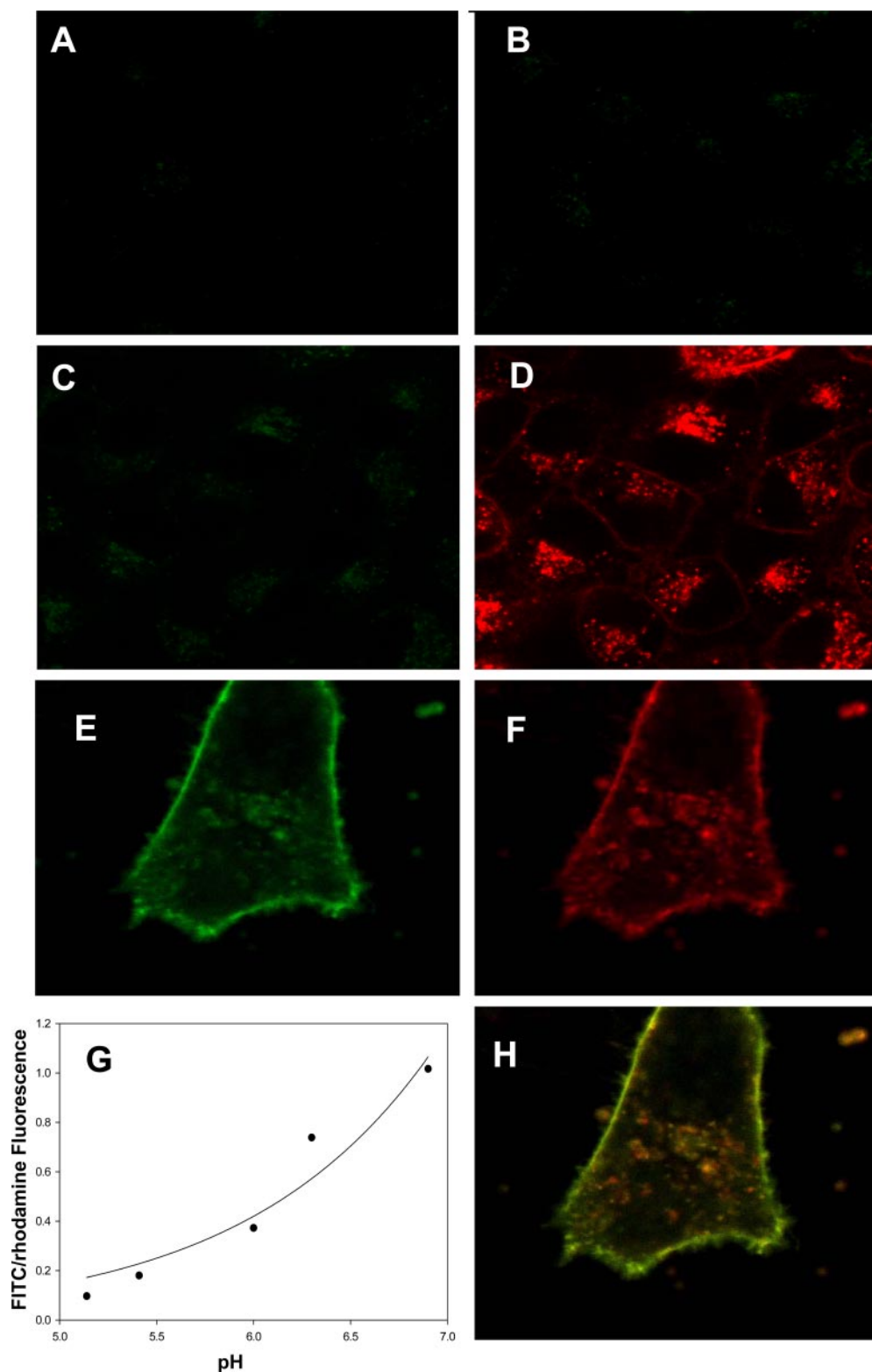


Fig. 3. Hydrolysis of ALFR during FR-mediated endocytosis and measurement of pH in FR-containing endosomes. A to D, hydrolysis of ALFR during FR-mediated endocytosis in KB cells. Cells were incubated for 0.5 h with a mixture of the ALFR (100 nM) and folate rhodamine (a known ligand for FR, 100 nM) and then washed to remove unbound conjugate. Cells were imaged by confocal microscopy for BODIPY FL fluorescence after 1.5 (A), 5.5 (B), or 18.5 h (C) of further incubation in culture medium ($\lambda_{\text{ex}} = 488 \text{ nm}$). As a positive control, the same cells were imaged for rhodamine fluorescence ($\lambda_{\text{ex}} = 543 \text{ nm}$) at the above time points (image at 18.5 h shown in D) to assure that FR internalization occurred normally. E to H, measurement of pH in FR-containing endosomes of KB cells by ratiometric imaging. Cells were incubated with an equal molar concentration of folate-FITC (a pH-sensitive conjugate) and folate-rhodamine (a pH-insensitive conjugate) (100 nM) for 0.5 h and washed to remove the unbound ligands. Cells were then incubated in fresh medium for another 3 h to allow receptor-mediated endocytosis to reach a steady state. Confocal images were obtained at excitation wavelengths for fluorescein (488 nm, E) and rhodamine (543 nm, F). H, an overlay of E and F. Calibration curve (G) was established by comparing fluorescein and rhodamine fluorescence from pH 5.2 to 6.8, as described under "Materials and Methods".

suggest that pH-sensitive linkers that release their therapeutic cargo only slowly between pH 6 and 7 may not enable maximal intracellular release of drugs from monovalent folate-drug conjugates. However, acid-labile spacers that hydrolyze rapidly within this pH range may yield effective folate conjugates if the targeted therapeutic agent can reach an FR on the tumor cell surface before it is hydrolyzed in the plasma (Leamon et al., 2006).

The finding that FR-containing endosomes do not experience low pH values distinguishes them from many other endocytic pathways. In general, endosome acidification is thought to occur within 2 to 3 min following receptor-mediated endocytosis due to an influx of protons via a vacuolar proton ATPase (Grabe and Oster, 2001; Beyenbach and Wiczorek, 2006; Hurtado-Lorenzo et al., 2006). As a result, the pH of early endosomes, sorting endosomes, and multivesicu-

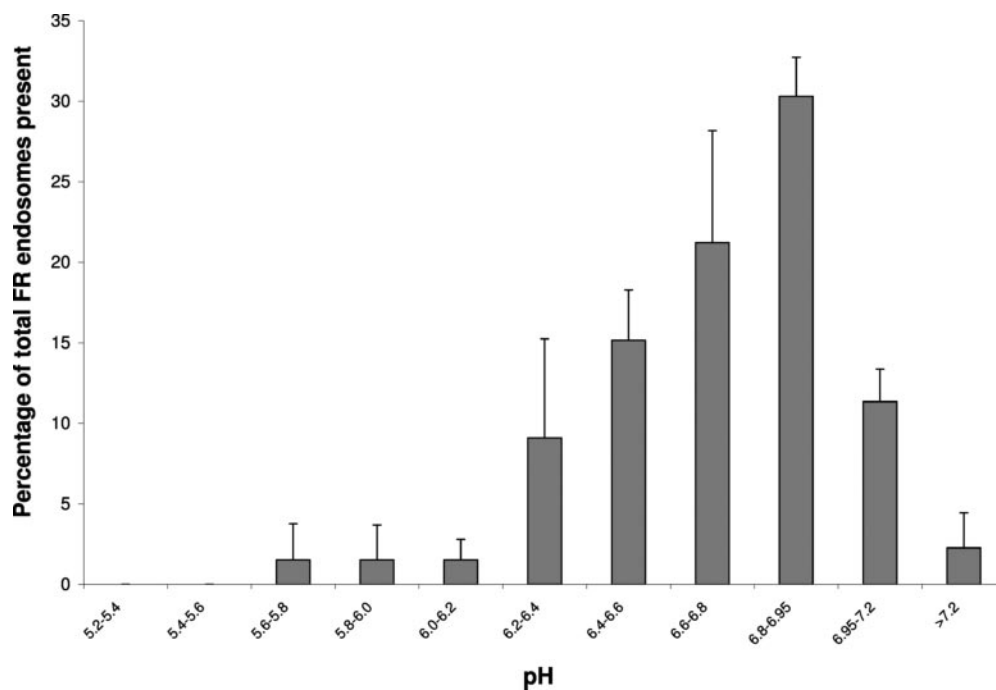


Fig. 4. Histogram of KB cell compartmental pH values. The histogram represents pH values calculated by the ratio of 132 endosomal areas (approximately 30–40 vesicles from each of four cells) in the folate-FITC and folate-rhodamine images, as described under “Materials and Methods”. The experiment was repeated three times with similar results.

lar bodies can drop rapidly to \sim pH 6.0 and below, allowing nonrecycling ligands (e.g., low-density lipoprotein, etc.) to dissociate from their receptors and sort into late endosomes (\sim pH 5.5) and lysosomes (\sim pH 5.0). Acidic conditions in the lysosomal system are then thought to facilitate protein denaturation and degradation (McCoy and Schwartz, 1988; Chapman, 2006). In contrast to such nonrecycling receptors, trafficking of recycling receptors is thought to proceed through a distinct sorting compartment, also known as the recycling center, before returning to the plasma membrane for reuse. Unlike lysosomes, these recycling compartments have been demonstrated to have pH values between 6 and 7 (Maxfield and McGraw, 2004). The fact that the FR endocytic pathway is characterized by compartmental pH values in this range can now be viewed as consistent with the fact that FR does not traffic to lysosomes but rather is recycled back to the cell surface for further use. It is noteworthy that this pH gradient along the FR endocytic pathway is conducive to folate acquisition by mammalian cells. Thus, unlike folic acid, which remains bound to FR throughout the endocytic pathway, 5-methyltetrahydrofolate, the common form of folate in the body, rapidly loses its affinity for FR upon endosomal acidification and is consequently released into the cytosol where it participates in various biological processes.

Finally, as noted above, when Lee et al. (1996) employed polymeric folate conjugates derivatized with multiple folates to measure the pH in FR⁺ endosomes, they observed pH values between pH 4.7 and 5.8, with the most frequent pH value around 5.0. It is noteworthy that subsequent research from other laboratories (Fivaz et al., 2002) has now shown that multivalent ligands traffic rapidly to late endosomes/lysosomes where the pH is normally in the range measured by Lee et al. (1996). This observation not only explains the apparent discrepancy between the data from Lee et al. (1996) and our own data but it also suggests that the choice of a releasable linker for folate-targeted drug delivery should be guided by the valence of the conjugate employed. Whenever a

multivalent folate conjugate, such as a dendrimer (Choi et al., 2005; Majoros et al., 2006), liposome (Reddy and Low, 2000; Shi et al., 2002; Sudimack et al., 2002), or lipoplex (Xu et al., 2001), is to be constructed, a pH-sensitive linker may prove optimal for efficient drug release. In contrast, where a monovalent folate conjugate is to be delivered, a disulfide-linked conjugate (Yang et al., 2006) should provide superior intracellular drug delivery.

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