Brief Articles

Photoaffinity Labeling of the Anionic Sites in Caco-2 Cells Mediating Saturable Transport of Hydrophilic Cations Ranitidine and Famotidine

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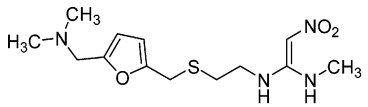
The H_2 antagonists, ranitidine and famotidine, exhibit saturable absorptive transport across Caco-2 cell monolayers and human intestine via a yet unidentified mechanism. A photoreactive derivative of famotidine has been synthesized and evaluated as a photoaffinity probe for the putative transporter protein(s). The probe irreversibly inhibited ranitidine transport across Caco-2 cell monolayers and irreversibly increased the transepithelial electrical resistance (TEER) after UV activation. Photoaffinity labeling was protected by a molar excess of famotidine.

Introduction

Hydrophilic cationic drugs such as ranitidine and famotidine (Figure 1) cross the intestinal epithelium via a predominantly paracellular route of transport as determined by studies in the Caco-2 cell culture model of intestinal epithelia.[1−3] Recent work in our laboratory has revealed both saturable and nonsaturable components to the absorptive transport of such compounds across Caco-2 cell monolayers.[2] A recent clinical study confirmed that ranitidine and famotidine are absorbed across the intestinal epithelium, at least in part, via a saturable transport mechanism as evidenced by the observation that orally administered ranitidine (300 mg) attenuated the oral bioavailability (absorption) of co-administered famotidine (40 mg).[4] Caco-2 studies also revealed that ranitidine and famotidine caused a saturable, concentration-dependent increase in the transepithelial electrical resistance (TEER) across the cell monolayers. This phenomenon was attributed to blocking of anionic sites in the paracellular space that facilitate transport of Na^+ ions (and other cations) across Caco-2 cell monolayers.[2] These results suggest an intriguing possibility that saturable transport may occur in the paracellular space, perhaps via interactions with anionic residues of the tight junction proteins, occludin and/or claudins. Thus, a mechanism has been proposed in which the cationic moieties of ranitidine and famotidine interact with anionic sites within the paracellular space and facilitate cation-selective transport across the intestinal epithelium.[2] Alternatively, one must consider the possibility that the saturable component of the overall transport may occur via a yet unidentified transporter in the apical membrane. In either scenario, the specific cellular components which facilitate this transport remain to be identified.



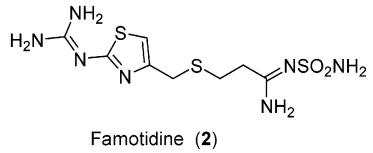


Figure 1. Structures of the H_2 antagonists, ranitidine (**1**) and famotidine (**2**).

One approach to identify the cellular component(s) mediating the saturable absorptive transport of ranitidine and famotidine is through photoaffinity labeling. This approach requires design of a probe that retains the structural motifs necessary for binding and incorporates a photoreactive moiety required for the covalent modification of cellular macromolecules.[5] Such a probe interacts selectively with the target protein and then reacts covalently via the photoreactive moiety upon irradiation with an appropriate light source. Utilizing a radioisotope-labeled probe, the radiolabeled macromolecule(s) can then be isolated, characterized, and identified.

In this first report of photoaffinity labeling of an intact cell monolayer system, we provide evidence for selective and irreversible modification of putative transporter protein(s) for ranitidine and famotidine in Caco-2 cells.

Chemistry. The photoaffinity probe, 4-azido-*N*-[2-[[[2-[(aminoiminomethyl)amino]-4-thiazolyl]methyl]thio]-ethyl]benzamide (**6**), was synthesized as outlined in Scheme 1. Commercially available aminothiourea (**3**) was reacted with 1,3-dichloroacetone in acetone to form [4-(chloromethyl)-2-thiazolyl]guanidine (**4**).[6,7] Compound **4** was then reacted with 2-aminoethanethiol hydrochloride in a solution containing sodium ethoxide in ethanol to give [4-[[(2-aminoethyl)thio]methyl]-2-thiazolyl]guani-

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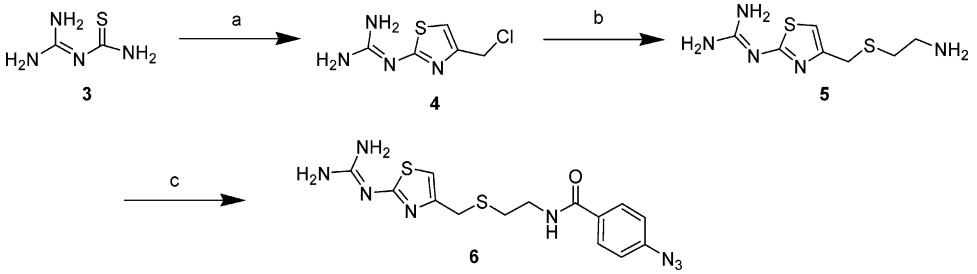
Scheme 1



(a) 1,3-Dichloroacetone/acetone. (b) 2-aminoethanethiol hydrochloride/NaOEt/EtOH. (c) 4-azidobenzoic acid, succinimidyl ester/THF/DMF/triethylamine.

dine (**5**).[6,8] Finally, compound **5** was reacted with 4-azidobenzoic acid, succinimidyl ester in a mixture of THF/DMF upon addition of triethylamine, yielding the photoaffinity probe (**6**). Photoreactivity of **6** was demonstrated by a progressive decrease in absorbance at 272 nm with time upon UV irradiation at 254 nm (Supporting Information Figure S1).

Effect of Compound 6 on Apical (AP) to Basolateral (BL) Transport of Ranitidine (1) and Famotidine (2) across Caco-2 Cell Monolayers. Previous studies indicated that ranitidine (**1**) and famotidine (**2**) flux across Caco-2 cell monolayers was saturable and was inhibited by a range of guanidine containing compounds, suggesting a common mechanism of transport across the cell monolayers.[2] In the presence of **6** (300 μM), ranitidine (**1**) (100 μM) flux decreased by 40% and famotidine (**2**) (300 μM) flux decreased by 37% suggesting that **6** shares a common transport mechanism with these compounds (Supporting Information Figure S2). Importantly, incorporation of the aryl azide moiety does not interfere with the probe's ability to interact with the transport mechanism of these H_2 antagonists.

Effect of Photoaffinity Labeling on Transepithelial Electrical Resistance (TEER) and AP to BL Transport of Ranitidine across Caco-2 Cell Monolayers. Both ranitidine (**1**) and famotidine (**2**) are known to increase TEER across Caco-2 cell monolayers in a concentration-dependent fashion, presumably through interaction with anionic sites in the paracellular space.[2,9] After incubation with **6** (200 μM), TEER across the monolayers increased to approximately 120% of control (Figure 2). Irradiation of the cells from the AP side caused an irreversible increase in TEER, as evidenced by the observation that the TEER value remained at approximately 110% of control ($p < 0.05$) upon washing the cells after irradiation. In contrast, TEER returned to the initial value upon washing in the absence of UV irradiation (Figure 2). These results suggest covalent binding of **6** with the cellular targets such that the anionic sites are irreversibly blocked.

Incubation of **6** (200 μM) with Caco-2 cells, followed by photoactivation at 254 nm, resulted in approximately 35% attenuation of ranitidine flux across the monolayers that was not reversible upon washing the cells (**6** + UV, Figure 3). Without UV irradiation, the inhibition of ranitidine transport by **6** was completely reversible by washing with the transport buffer (Figure 3), providing evidence that photoactivation of **6** causes an irreversible change in the binding sites involved in ranitidine transport. UV irradiation alone had no effect on ranitidine flux (Figure 3).

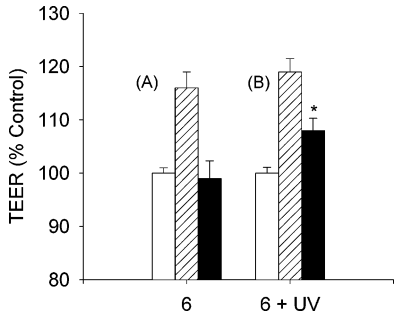


Figure 2. Irreversible increase in TEER after photoaffinity labeling. After measurement of initial TEER (open bars), the AP side of cell monolayers was treated for 10 min with **6** (200 μM), TEER was recorded (hatched bars), and cells were subjected to (A) ambient light or (B) UV irradiation. The AP solution was then aspirated, and cells were washed (3×) with 4 °C transport buffer. After replacement with 37 °C transport buffer, the AP side of cell monolayers was allowed to reequilibrate and a final TEER value recorded (solid bar). *, $p < 0.05$ compared to treatment with **6** without UV activation.

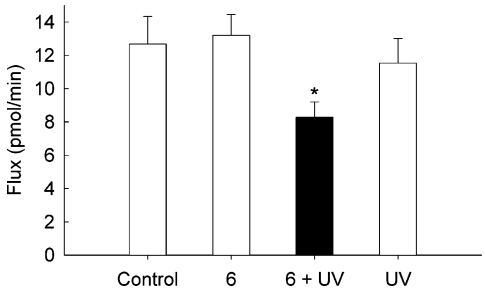


Figure 3. Irreversible inhibition of AP to BL transport of ranitidine (**1**) (0.1 mM) by photoaffinity labeling. Cell monolayers were incubated for 10 min either with transport buffer alone (control, UV) or transport buffer containing 200 μM **6** (6, 6+UV). After incubation, the monolayers were exposed to ambient light (control, **6**) or irradiated at 254 nm (**6** + UV, UV). Cells were washed (3×) with 4 °C transport buffer and reequilibrated at 37 °C prior to the measurement of ranitidine transport. *, $p < 0.05$ compared to control.

Selectivity of Photoaffinity Labeling. Incubation of **6** in the presence of a molar excess of famotidine (**2**) (5 mM) prevented both the irreversible increase in TEER and irreversible decrease in ranitidine flux observed after photolabeling with **6** alone (Figure 4). These results indicate that the photoaffinity probe causes irreversible changes in TEER and ranitidine/famotidine transport across Caco-2 cells by modifying the same sites that appear to interact with ranitidine and famotidine and mediate their transport across Caco-2 cell monolayers.

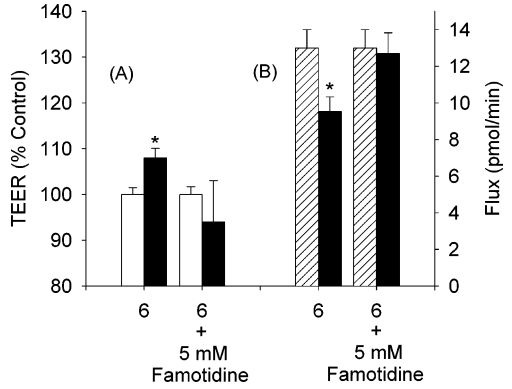


Figure 4. Selectivity of photoaffinity labeling. (A) After measurement of initial TEER (open bars), cells were incubated with **6** (200 μM) in the absence or presence of 5 mM famotidine (**2**). After irradiation at 254 nm, cells were washed (3×) with 4 °C transport buffer and reequilibrated at 37 °C prior to the measurement of final TEER (solid bars). (B) Cells were incubated with **6** (200 μM) in the absence or presence of 5 mM famotidine. After incubation, the monolayers were exposed to ambient light (hatched bars) or irradiated at 254 nm (solid bars). Cells were washed (3×) with 4 °C transport buffer and reequilibrated at 37 °C prior to the measurement of ranitidine transport. *, $p < 0.05$ compared to initial TEER or control ranitidine transport.

Discussion

We have demonstrated the ability of **6** to irreversibly increase TEER and inhibit ranitidine transport after photoactivation (Figures 2 and 3). Importantly, a molar excess of famotidine (**2**) can compete for and block this effect, indicating that the photoaffinity probe specifically and reversibly binds to sites mediating the transport of ranitidine (**1**). The molecular identity of these sites, however, is unknown. Claudins are a recently discovered family of membrane-bound tight junctional proteins whose extracellular loops protrude into the intercellular space forming the barrier properties of the tight junction.[10] Recent evidence points to negatively charged amino acid residues on the extracellular loops of claudin family members which facilitate the paracellular flux of positively charged ions through the paracellular space.[11] Interaction between these anionic residues and hydrophilic, cationic drugs such as ranitidine (**1**) and famotidine (**2**) may result in the facilitated and saturable transport of such molecules across the intestinal epithelium.

An alternative explanation consistent with the saturable transport behavior of H_2 antagonists across Caco-2 cells involves a carrier-mediated transport mechanism across the intestinal epithelium. Photoaffinity analogues of cimetidine, a related H_2 antagonist, have been used in attempts to identify organic cation transporter proteins involved in the active secretion of cimetidine in the rat renal brush-border membrane.[12] Similar transport mechanisms in the intestine, however, have not as of yet been identified. Nevertheless, we cannot rule out the possibility that the synthesized photoaffinity agent may interact with unknown organic cation uptake mechanisms at the apical membrane of Caco-2 cells.

To our knowledge, the photoaffinity labeling studies reported here represent one of the first successful attempts to label a polarized cell line grown as a cell monolayer (Caco-2). The majority of reported photoaffinity studies focus on the labeling of membrane fractions, membrane vesicles and/or whole cells in suspension.[13,14] Our monolayer system ensures the integrity of the intercellular junctions, thus allowing photolabeling of junctional components which are hypothesized to be involved in the translocation of ranitidine (**1**) and famotidine (**2**) across Caco-2 cells.

Conclusions

In conclusion, we have successfully designed and synthesized a photoaffinity derivative of famotidine (**2**) which covalently modifies the sites mediating saturable transport of ranitidine (**1**) and famotidine (**2**) across Caco-2 cell monolayers. The photoaffinity agent, **6**, may be a useful probe to isolate and identify the cellular components involved in the saturable transport of H_2 antagonists across Caco-2 cells.

Experimental Section

4-Azido-*N*-[2-[[[2-[(aminoiminomethyl)amino]-4-thiazolyl]methyl]thio]ethyl]benzamide (6). Triethylamine (0.11 mL, 2.5 mmol) was added to a solution of [4-[[(2-aminoethyl)-thio]methyl]-2-thiazolyl]guanidine (0.100 g, 0.328 mmol) and 4-azidobenzoic acid, succinimidyl ester (0.085 g, 0.328 mmol) in a mixture of THF (2 mL) and DMF (2 mL) and stirred overnight at room temperature. THF was removed using rotary evaporation and 10 mL water added to the solution. The resulting mixture was extracted with ethyl acetate (20 mL × 3). The combined organic layer was dried over sodium sulfate and concentrated using rotary evaporation. The product was purified using silica gel column chromatography [methylene chloride:methanol (93:7)] and evaporated to dryness and the residual solid dried, yielding 0.042 g of 4-azido-*N*-[2-[[[2-[(aminoiminomethyl)amino]-4-thiazolyl]methyl]thio]ethyl]benzamide (yield: 34%). Purity was greater than 99% as determined by two separate HPLC procedures: (1) t_R: 17.8 min; column: Waters μ Bondapak 3.9 × 150 mm C18 5 μm; mobile phase: (A) MeOH, (B) 50 mM KH_2PO_4, pH 6.0. Gradient: 10% (A) to 40% (A) from 0 to 10 min followed by 40% (A) from 10 to 30 min; flow rate: 1 mL/min; detection: 275 nm. (2) t_R: 8.9 min; column: PhaseSep Spherisorb 4.6 × 250 mm S5 SCX 5 μm; mobile phase: 35% ACN/65% 50 mM KH_2PO_4, pH 3.0; flow rate: 1 mL/min; Detection 275 nm. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.07 (1H, t, J = 7.0 Hz), 2.66 (2H, t, J = 6.7 Hz), 3.4−3.5 (2H, m), 3.64 (2H, s), 6.51 (1H, s), 6.85 (3H, br s), 7.20 (2H, dt, J = 8.5, 2.7 Hz),7.90 (2H, dt, J = 8.4, 2.6 Hz), 8.63 (1H, t, J = 5.5 Hz). MS (FAB): m/z 377 $(M + H)^+$. HRMS (CI) calcd for $C_{14}H_{16}N_8OS_2$ (M + 1), 377.0967; found, 377.0978.

Photoaffinity Labeling. Caco-2 cell monolayers were preincubated for 1 h at 37 °C in HBSS containing 25mM D-glucose and 10 mM HEPES, pH 7.4 (TBS). The AP buffer was replaced with 0.5 mL of TBS containing the photoaffinity probe (0.2 mM compound **6**). TEER was measured after 10 min, and the monolayers were subsequently cooled on ice for 15 min. The AP side was then irradiated at 254 nm for 4 min using a hand-held UV lamp (Model ENF-240C, Spectronics Inc., Wesbury, NY). Cell monolayers were washed 3× with 4 °C TBS and allowed to reequilibrate at 37 °C for 30 min before measurement of TEER and/or evaluation of ranitidine transport.

Data Analysis. Data are expressed as mean ± SD from three measurements. Statistical significance was evaluated using unpaired Student's t tests, with a significance level of $p < 0.05$.

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Supporting Information Available: Synthesis of key intermediates and their NMR. Degradation kinetics of photoaffinity agent. Detailed experimental procedures, measure-

ment of TEER and transport across Caco-2 cell monolayers. Purity of target compound **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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