JPET Fast Forward. Published on July 3, 2007 as DOI: 10.1124/jpet.107.121640
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JPET Fast Forward. Published on July 3, 2007 as DOI:10.1124/jpet.107.121640

Isothiocyanate Inhibits Restitution and Wound Repair after Injury in the Stomach: *ex vivo* and *in vitro* Studies

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Running title: Isothiocyanate Inhibits Restitution

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Number of text pages: 31 double spaced pages

Number of Tables: None

Number of Figures: 10

Number of References: 40

Number of words in the Abstract: 250

Number of words in the Introduction: 619

Number of words in the Discussion: 1,341

Non-standard abbreviations: DIDS, 4, 4'-diisothiocyanatostilbene-2, 2'-disulfonic acid; DNDS, 4,4-diinitrostilbene-2,2'-disulfonic acid; H_2DIDS, 4,4-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid; HP, *Helicobacter pylori*; ITC, isothiocyanate compound; MCT, monocarboxylate transporter; NBC, sodium bicarbonate co-transporter; NHE, sodium hydrogen exchanger; PD, potential difference; pHi, intracellular pH; RGM1, rat gastric mucosal-1 cells; SITS, 4-acetamido-4-isothiocyanatostilbene-2, 2'-disulfonic acid; STD, standard buffer; TER, transmucosal electrical resistance.

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Abstract

The role of isothiocyanate (ITC) in blocking epithelial restitution after injury and the recovery of round wounds was examined in the *ex vivo* guinea pig stomach and in rat gastric mucosal (RGM1) cells, respectively. For this, recovery of transepithelial electrical resistance and morphology after injury or the closure of round wounds was evaluated in the presence of DIDS or H_2DIDS (2 ITC groups), SITS (1 ITC group), or DNDS (no ITC groups). Wounded RGM1 cells were also incubated with bicarbonate-free buffer, ATP, barium, or phloretin to determine the mechanism of ITC inhibition. At 300 μM, DIDS or H_2DIDS blocked restitution and wound repair by 100%, SITS blocked by 50%, and DNDS blocked by 2%. These results demonstrate the dependence of restitution and wound repair on ITC. ITC-binding purino (ATP) receptors and K_{ATP}-channels were investigated as potential sites of inhibition but were found not to be the target of ITC in wound repair. Phloretin, blocking the monocarboxylate transporter (MCT), dose-dependently inhibited wound repair and this result was exacerbated when the sodium bicarbonate co-transporter (NBC) was also blocked by bicarbonate-free conditions, resulting in 100% inhibition of wound repair with no reduction in viability when both transporters were blocked simultaneously. ITC potently inhibits both MCT and NBC, which may account for the inhibitory action of DIDS during restitution and wound repair. RT-PCR data verified that MCT-1 is expressed in RGM1 cells. In conclusion, our results suggest that bicarbonate and monocarboxylate transport may work cooperatively to facilitate restitution of the gastric mucosa after injury.

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Introduction

Inflammation of the stomach and intestine is thought to occur because defects in the mucosal barrier allow permeation of luminal contents into the underlying connective tissue. In the stomach, defects in the mucosal barrier can occur at tight junctions, by impaired secretion of bicarbonate or mucus, or because the healing of superficial wounds is inhibited. Superficial wounds occur in the stomach from mild physical trauma or localized superficial injury, and heal rapidly by the migration of epithelial cells to cover gaps in continuity by a process termed restitution. During infection, such as with *Helicobacter pylori* (HP), the healing of superficial wounds may be particularly important to impede translocation of substances that trigger an immune response. Little is known about the mechanisms that regulate restitution, despite the importance of this process in maintaining mucosal integrity after injury.

In a variety of diverse cell systems including Caco-2 cells, skin, endothelial cells, and epithelial cells of the gastrointestinal tract including the stomach (Lacy et al., 1984; Critchlow et al., 1985; Ito et al., 1985), small intestine (Donowitz et al., 1982; Feil et al., 1987; Moore et al., 1989; Riegler et al., 1991), and colon (Feil et al., 1989; Prasad et al., 1997), repair of superficial wounds occurs rapidly and with similar characteristics whereby viable cells on either side of an injury flatten, spread thin actin-containing lamellapodia and filapodia along the basement membrane, migrate, and ultimately cover the denuded surface with polarized epithelial cells. At least five factors are known to influence restitution after injury including growth factors (Paimela et al., 1993; Yanaka et al., 1996), Ca^{2+} (Critchlow et al., 1985), actin (Critchlow et al., 1985), glycolysis (Cheng et al., 2001) and ion transport (Joutsi et al., 1996; Yanaka et al., 2002; Hagen et al., 2004). Ion transport is required to regulate intracellular pH (pH_i) in migrating cells after

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injury, a process that is particularly important in the stomach due to the acidic luminal environment.

It was recently reported that both amiloride, a Na^+/H^+ exchange (NHE) blocker, and 4-acetamido-4-isothiocyanatostilbene-2, 2'-disulfonic acid (SITS), a bicarbonate transport antagonist, inhibit restitution of the guinea pig gastric mucosa (Joutsi et al., 1996). In the frog, restitution is inhibited by a similar bicarbonate transport antagonist, 4, 4'-diisothiocyanatostilbene-2, 2'-disulfonic acid (DIDS), but ion substitution experiments showed that bicarbonate transport from the nutrient solution, *per se*, is not required for restitution to occur (Hagen et al., 2004). These results suggest that SITS and DIDS, in addition to blocking bicarbonate transport, inhibit other pathways important to restitution. To date, the pathway blocked by SITS and DIDS during restitution in the *ex vivo* gastric mucosa is unknown as is whether it is the isothiocyanate (ITC) group or another aspect of these compounds that inhibit restitution. In addition, it is not known whether blockade of wound repair occurs by SITS and DIDS in cultured gastric cells, results that would implicate the action of SITS and DIDS on epithelial cells rather than on other cells in the tissue.

In the present study, we examined the role of SITS and DIDS in restitution of the mammalian gastric mucosa, *ex vivo*, and in an *in vitro* cell wounding model using rat gastric RGM1 cells. Because many cellular functions, in addition to bicarbonate transport by the sodium bicarbonate co-transporter (NBC), are blocked by the ITC group on stilbenes like SITS and DIDS, we explored the role of three important DIDS-inhibitable pathways including purino (ATP) receptors, barium-sensitive K_{ATP}-channels like Kir6.1/SUR6A, and the monocarboxylate transporter, MCT-1. Our data suggest that MCT-1, along with NBC, may be the relevant binding

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sites for DIDS/ITC in gastric surface epithelial cells after injury and support the notion that MCT-1 plays an important role in restitution and the maintenance of barrier integrity after injury.

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Materials and Methods

Animals. Male guinea pigs (300-350g, Hartley strain) were used in this work. Studies were done with 6 animals per treatment and without fasting. All animals in this study were maintained in accordance with the guideline of the Committee on Animals at Beth Israel Deaconess Medical Center and those prepared by the Committee on Care and Use of Laboratory Animals of the National Research Council.

Ussing Chamber Studies. After anethesia, the stomach was removed and divided into paired halves by incision of the greater and lesser curvature. The muscularis propria was stripped from corpus mucosa with small scissors and fine forceps under dissecting microscope. Stripped mucosae were mounted in Lucite Ussing chambers (exposed area of 0.636 cm^2) connected to water-jacketed gas-lift reservoirs maintained at 37 °C. The serosal side was bathed with buffer containing (in mM) 147 Na^+, 5.0 K^+, 131 Cl^-, 1.3 Mg^{2+}, 1.3 SO_4^{2-}, 2 Ca^{2+}, 25 HCO_3^-, 15 HEPES, and 20 D-glucose and was continuously gassed with 95% O_2-5% CO_2. The luminal side was bathed with 150 mM NaCl, gassed with 100% O_2, and kept at pH 4.0 with pH-stat device. Transmucosal electrical resistance (TER) and potential difference (PD) were monitored throughout the experiment. After PD reached a stable value, injury was induced by exposing the luminal side of tissues to 150 mM NaCl containing 0.5% Triton X-100 for 5 min. Subsequently, the mucosa was washed 3 times in buffer and the serosal and luminal solutions were replaced. To determine whether it is the ITC group or other aspects of stilbenes (Fig. 1) that block restitution after injury, tissues were incubated for 3 hr in the absence or presence of DIDS, 4,4-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid (H_2DIDS), SITS, or 4,4-diinitrostilbene-2,2'-disulfonic acid (DNDS), which were added from a concentrated stock in DMSO. Control tissues were incubated with DMSO alone and

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the concentration of DMSO was 0.1% in all experiments. Luminal pH was maintained at 4.0 throughout the experiment by titration with 10 mM HCl or 10 mM NaOH. Restitution was evaluated quantitatively by the recovery of TER after injury. At the end of the experiment, tissues were fixed for light microscopy for 10 min at room temperature in the chamber and then overnight at 4° C in 4.0% phosphate-buffered formalin. Fixed tissues were embedded in paraffin, sectioned, and then stained with hematoxilin and eosin for light microscopy to evaluate restitution in each experiment. All samples were coded, and morphological evaluation was conducted without foreknowledge of their source by four investigators. Each tissue was evaluated from 0 to 5, as follows. A score of 0 indicated that the apical surface of the mucosa was denuded without cell migration from the gastric pits (no restitution). A score of 1 indicated that less than 25% of the apical surface was covered with flattened surface epithelial cells. A score of 2 indicated that 25-50% of the apical surface was covered with flattened surface epithelial cells. A score of 3 indicated that 50-75% of the apical surface was covered with flattened epithelial cells. A score of 4 indicated that 100% of the apical surface was covered with flattened epithelial cells. Last, a score of 5 indicated that 100% of the apical surface was covered by cuboidal or columnar, rather than flattened, epithelial cells. After analysis by each investigator, the code was broken, and the results were analyzed as described below.

RGM1 Cell Culture. Rat gastric mucosal (RGM1) cells, established by Dr. H. Matsui at the Institute of Physical and Chemical Science (RIKEN) Cell Bank and Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan (Kobayashi et al., 1996), are non-transformed gastric surface epithelial cells. RGM1 cells were cultured in DMEM/F12 (1:1) supplemented with heat-inactivated 10% FBS (Gibco/BRL, Gaithersburg, MA), 100 units/ml

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penicillin, 100 units/ml streptomycin and 0.25 μg/ml amphotericin B. Confluent monolayers of RGM1 cells were starved for 24 hr in culture medium without FBS (DMEM/F12 containing 15 mmol/L HEPES at pH 7.4) at 37 °C under 5% CO_2 in air and then used as a confluent monolayer for experiments. All experiments were performed at pH 7.4 in the presence and/or absence of reagent(s).

Recovery of a Round Wound Induced in RGM1 Cell Monolayers. A round artificial wound was induced in the center of confluent RGM1 cell monolayers by using a rotating silicon tip driven by a pencil-type mixer (Iuchi, Japan), resulting in a cell-free area of uniform size after wounding. The monolayer was then washed with standard (STD) buffer, containing (in mM) 147 Na^+, 5.0 K^+, 131 Cl^-, 1.3 Mg^{2+}, 1.3 SO_4^{2-}, 2 Ca^{2+}, 25 HCO_3^-, 15 HEPES, and 20 D-glucose and reagents were then added in STD buffer or in ion-substituted buffer to the wells. To determine whether the ITC group or other aspects of stilbenes block the recovery of wounds, 3-300 μM DIDS or H_2DIDS, or 3-500 μM SITS or DNDS were solubilized in DMSO and added so that the final concentration of DMSO did not exceed 0.1%. To determine the role of bicarbonate in the recovery of wounds, mannitol was substituted for bicarbonate in the STD buffer. To evaluate the role of purinoreceptors in wound repair, wounded RGM1 cell monolayers were incubated with 1 mM ATP or 1 mM the non-hydrolyzable analogues of ATP, α,β-me ATP or 2 me S-ATP, to determine whether ATP accelerates wound repair. Additionally, 10-300 μM suramin was used to determine whether blockade of purinoreceptors inhibits wound repair. To determine whether barium-sensitive K_{ATP} channels like Kir6.1/Sur6A are involved in wound repair, injured monolayers were incubated with 0.1-5 mM barium chloride in sulphate-free buffer that contained (in mM) 160 Na^+, 5.0 K^+, 143 Cl^-, 1.3 Mg^{2+}, 2 Ca^{2+}, 25 HCO_3^-, 15 HEPES, and 20 D-glucose. Barium

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chloride precipitated in STD buffer, so sulphate-free buffer was used to ensure that the barium remained in solution. Control STD buffer, to balance osmolarity, contained an additional 23.7 mM mannitol in experiments with barium. To evaluate the role of MCT in wound recovery, phloretin was used at 30 and 60 μM. Phloretin also blocks glucose transport, so to determine the role of glucose transport in wound repair, phloretin was added in both STD buffer and in glucose-free buffer, where mannitol was substituted for glucose. Closure of the wound was monitored at 4 hr and 8 hr with a Nikon TE300 microscope (MicroVideo Instruments, Avon, MA) outfitted with an Orca CCD camera (Hamamatsu Photonics, Japan). Change in the cell-free area was quantified using IP lab software (Scanalytics, Inc., Fairfax VA).

Determination of Cell Viability. The viability of RGM1 cells was evaluated by a colorimetric assay using crystal violet, a cytochemical stain that binds to chromatin. For this assay, RGM1 cells were washed with PBS, fixed with methanol, and then air-dried. The dried cells were stained with 1% crystal violet, washed in tap water, and then air-dried. Stained cells were solubilized with 0.5 % SDS and the absorbance was measured at 590 nm using micro plate reader.

Analysis of MCT expression by Reverse Transcription-Polymerase Chain Reaction. RGM1 cells were grown to confluence and then isolated from the culture plate by using 0.1% trypsin in 0.5 mM EDTA at 37°C for 10 min. Isolated cells were pelleted and frozen at -80°C before use. Total RNA from the thawed cells was extracted with the single-step acid phenol-chloroform extraction procedure using TRIzol (Invitrogen, Gaighersburg, MD). Total RNA was reverse transcribed with SuperScript preamplification (Invitrogen). The sequences for sense and antisense primers, obtained from GenBank, for 1) rat MCT1 (D63834) were 5'-GTCTACGACCTATGTTGGG-3' and 5'-CCTCCGCTTTCTGTTCT-3' to obtain a 394-bp

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product; 2) rat MCT2 (X97445) were 5'-GGGGCTGGGTTGTAGT-3' and 5'-GACGGTGAGGTAAGTTCTA-3' to obtain a 367-bp product; 3) rat MCT3 (AF059258) were 5'-CGCTGCTCTAAGAACATCTCATC-3' and 5'-TCTGGCCTCGTGCCTCAT-3' to obtain a 427-bp product; and 4) rat MCT4 (U87627) were 5'-GGCAGTCCCGTGTTCCTTT-3' and 5'-GCACCTTCTTGAGCCCTGTTAT-3' to obtain a 369-bp product.

Reagents. H_2DIDS was purchased from Molecular Probes, Inc. (Eugene, OR). All other chemicals, including DIDS, SITS, and DNDS, were purchased from Sigma Chemical Company (St. Louis, MO) unless noted otherwise.

Statistical Analysis. In RGM1 cell experiments, the data represent means ± SE for 2 wells from 3 different experiments. In Ussing chamber experiment, the data represent means ± SE obtained from at least 6 experiments. Statistical differences were evaluated by analysis of variance, Dunnett's multiple comparison test, and the Student's t test using SigmaPlot (SPSS Science). Significance of the data is denoted as * $P < 0.05$, ** $P< 0.01$, and ***$P < 0.001$ when compared to time-matched control samples or † $P < 0.05$, †† $P< 0.01$, and ††† $P <0.001$ when compared to time-matched samples from another treatment.

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Results

Effects of Various Stilbene Compounds on Restitution after Injury in the *ex vivo* Guinea Pig Gastric Mucosa

When injured with triton X-100, TER dropped to 0 Ohm cm^2 in 5 min and returned to pre-treatment levels within 180 min in the presence of control buffer or with DNDS, a stilbene compound that contains no ITC groups (Fig. 2A). In contrast, tissues treated with triton X-100 followed by DIDS, a stilbene with 2 ITC groups, showed no repair after injury and by SITS, a stilbene with 1 ITC group, showed repair intermediate to DIDS and control (Fig. 2A). No impairment of viability was found with any of the stilbene compounds tested (Fig. 2B). To verify that the electrical measurements were related to surface defects after injury followed by migration and repolarization during repair, the morphology of tissues treated with each stilbene compound was examined at 180 min after injury (Fig. 3). With SITS (Fig. 3A), some recovery occurred and the histology score was 2.89 ± 0.23 (Fig. 3D). With DIDS (Fig. 3B), no recovery occurred and the histology score was 0.167 ± 0.09 (Fig. 3D). Both scores were significantly different ($P < 0.001$) from control. Full recovery of epithelial integrity occurred with DNDS (Fig. 3D), which was not different from control (Figs. 3C and D).

Effects of Various Stilbene Compounds and Bicarbonate Transport Inhibitors on Wound Repair in the *in vitro* Rat Gastric RGM1 Cell Model

To determine whether the stilbene compounds DIDS, H_2DIDS, and SITS inhibit wound repair in a reductionist model of injury and repair, RGM1 cells were used (Fig. 4). Consistent with results from the guinea pig *ex vivo* model in Fig. 2, DIDS (Fig. 4A) and H_2DIDS (Fig. 4B), stilbene compounds with 2 ITC groups, dose-dependently inhibited wound repair with a maximal effect at 300 µM. Using the same concentration curve, from 3 to 300 µM, wound

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repair was dose-dependently inhibited by about 50% with SITS (Fig. 4C), a stilbene with 1 ITC group. Even at 500 μM SITS, there was not complete inhibition of wound repair (Fig. 4C). DNDS, a stilbene with no ITC groups (Fig. 4D), did not inhibit wound repair in a dose-dependent fashion. Instead, there was some inhibition of wound repair at all doses tested, which reached significance at 300-500 μM when compared to controls (Fig. 4D). As shown in Fig. 2B, the stilbene compounds tested did not reduce the viability of RGM1 cells when examined 8 hrs after wounding, so the results cannot be attributed to toxicity of drugs at the concentrations used for this study. In the RGM1 cell model, bicarbonate-free conditions resulted in a significant but modest inhibition of wound repair (Fig. 6).

Role of Purino(ATP) Receptors and K_{ATP} channels on Wound Repair in the *in vitro* RGM1 Cell Model

ATP binding to purinoreceptors and the activity of K_{ATP} channels like Kir6.1/SUR6A are both known to be inhibited by DIDS (Furukawa et al., 1993; Bultmann et al., 1994; Proks et al., 2001). To determine whether these pathways are involved in wound repair in the RGM1 cell model, we incubated RGM1 cells with ATP and analogues of ATP, or with barium, a non-specific antagonist of Kir6.1/SUR6A channels (Fig. 5). ATP and non-hydrolyzable analogues of ATP, such as α, β-methyl ATP and 2 methyl S-ATP would be expected to bind P2-type purinoreceptors on the cell surface (Bultmann et al., 1994; Suzuki et al., 2000), and to accelerate the rate of wound repair (Dignass et al., 1998; Chen et al., 2006). Figure 5A shows that wound repair in RGM1 cells is unaffected by ATP. Likewise, barium should inhibit wound repair if Kir6.1/Sur6A channels are required, even if many other K^+-channels are also blocked by barium. Sulfate-free conditions were used to maintain the solubility of barium and this condition did not reduce cell viability as long as the barium concentration was 5 mM or

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below (now shown). Sulphate-free conditions alone did not inhibit wound repair nor did sulphate-free conditions with 0.1-0.5 mM barium (Fig. 5B). Higher doses of barium, including 1-5 mM, resulted in a modest but significant inhibition of wound repair (Fig. 5B).

Role of Monocarboxylate Transporters, Especially MCT-1, on Wound Repair in the *in vitro* RGM1 Cell Model

Monocarboxylate transporters are potently inhibited by the ITC group(s) on DIDS, H_2DIDS, and SITS (Poole et al., 1993; Halestrap et al., 1999; Halestrap et al., 2004). To determine whether monocarboxylate transport pathways are involved in wound repair in the RGM1 cell model, we incubated RGM1 cells with phloretin, which potently and dose-dependently inhibited wound repair (Fig. 6A) without affecting the viability of RGM1 cells (Fig. 6B). Because DIDS would block both NBC and MCT in live cells, we also determined whether inhibition of wound repair would be greater if both transporters were blocked simultaneously by using phloretin and bicarbonate-free conditions (Fig. 6A). Phloretin (60 μM) in combination with bicarbonate-free buffer resulted in complete inhibition of wound repair as occurs with DIDS (compare Fig. 6A with Fig. 4A). Like with phloretin alone, there was no reduction in viability with phloretin in bicarbonate-free buffer (Fig. 6B).

Because phloretin blocks not only MCT but glucose transporters such as GLUT1, we tested whether inhibition of glucose transport in the RGM1 cell model would inhibit wound repair (Fig. 7). Although there was a significant but modest slowing of wound repair in glucose-free buffer, there was no difference in the rate of wound repair with or without phloretin that could account for the results with phloretin alone (Fig.7A). Like before, phloretin in glucose-free buffer did not affect the viability of RGM1 cells (Fig. 7B).

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To determine whether RGM1 cells have MCT transporters, RT-PCR was performed (Fig. 8). Using primers specific for rat MCT's that are published in GenBank, our results show that the message for MCT-1 is expressed in RGM1 cells (Fig. 8).

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Discussion

Although previous work showed that stilbene compounds like DIDS and SITS block restitution of the *ex vivo* frog and guinea pig gastric mucosa (Joutsi et al., 1996; Hagen et al., 2004), it was unclear whether blockade was by the ITC "R" group or due to other characteristics of the molecule. Here we provide evidence that ITC groups dose-dependently block restitution and wound repair of the gastric mucosa with an efficacy of DIDS=H_2DIDS (2 ITC)>SITS (1ITC). DNDS, with no ITC groups but an otherwise similar structure, has little effect on wound repair or on restitution, suggesting that the backbone of stilbene compounds contributes little to their overall inhibitory properties. These results are important to consider when designing new therapies to treat diseases of the stomach and perhaps of the intestine and colon, where mechanisms that regulate restitution are similar. In particular, ITC-containing compounds from cruciferous vegetables like sulforaphane from broccoli and broccoli sprouts have been proposed to treat *Helicobacter pylori* infection of the stomach (Fahey et al., 2002; Haristoy et al., 2003; Galan et al., 2004), or sinigrin from Brussels sprouts to inhibit carcinogenesis in the colon (Bonnesen et al., 2001; Smith et al., 2003; Smith et al., 2004). Although efficacious *in vitro*, ITC-containing compounds may inhibit important repair processes *in vivo* to affect mucosal integrity. To support this idea, chronic atrophic gastritis was shown to be twice as high in subjects who ate broccoli once or more weekly when compared to those who consumed a negligible amount of the vegetable (Sato et al., 2004).

Elucidating the mechanism by which ITC-containing compounds inhibit wound repair is complicated by the fact that many transporters and receptors avidly bind ITC groups. In restitution, the first obvious ITC-binding transporter that may be affected is NBC (Fig. 9), which is known to regulate intracellular pH (pHi) and to be potently inhibited by DIDS,

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H_2DIDS, and SITS. However, previous studies in the frog gastric mucosa demonstrated that bicarbonate-free conditions, which would inhibit bicarbonate transport by NBC, did not affect restitution (Hagen et al., 2004). Using cultured RGM1 cells in the present study, this result was confirmed, suggesting that transporters other than or in addition to NBC are targets of the ITC group on DIDS, H_2DIDS, or SITS during restitution and wound repair.

Three other potential ITC-binding receptors/transporters were investigated in this work to determine why DIDS, H_2DIDS, or SITS blocks restitution. First, it is well-known that the P-type purinoreceptors, P_{2X} and P_{2Y} are blocked by DIDS and that P_2 purinoreceptors are present on and affect the function of gastric surface cells (Ota et al., 1994). Purinoreceptors facilitate cell signaling after binding extracellular ATP and are known to accelerate wound repair in intestinal IEC-6 cells (Dignass et al., 1998) and chemotaxis in neutrophils (Chen et al., 2006). As such, we incubated RGM1 cells with either ATP or non-hydrolyzable analogues of ATP, each of which bind to purinoreceptors, and found that exogeneous ATP does not accelerate wound repair in RGM1 cells. To verify this negative finding, we also blocked purinoreceptors with suramin and saw no effect on the rate of wound repair, or on viability, in RGM1 cells (not shown). Because ATP did not accelerate nor did blockade of purinoreceptors inhibit wound repair in our study, we conclude that P_2-type purinoreceptors are probably not the target of DIDS, H_2DIDS, or SITS. Likewise, K_{ATP} channels like Kir6.1/SUR2A have been shown to regulate cell migration and are blocked by DIDS and by barium. Because our data showed that barium has only a minimal effect on wound repair, we conclude that Kir6.1/SUR2A K^+-channels are also not the major target of DIDS, H_2DIDS, or SITS in this study. Our results suggest, however, that one or more barium-sensitive K^+-

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channels are involved in wound repair in RGM1 cells and further studies would be required to investigate this interesting finding.

The remaining transport mechanism to test was MCT, which has been shown to be potently and irreversibly inhibited by the ITC group on DIDS. MCT is a protein catalyzed proton-linked pyruvate and lactate transporter that was initially called MEV because of its mevalonate transport properties, but was cloned, sequenced, and renamed MCT-1 (Wang et al., 1994; Garcia et al., 1994). In the stomach, MCT-1 is localized exclusively at the basolateral surface of gastric surface epithelial cells (Garcia et al., 1994), but no function for it has been elucidated. MCT-1 is a potent H^+ and monocarboxylate co-transporter that transports lactate and H^+ against a concentration gradient, especially during glycolysis when lactate efflux is required (Poole et al., 1993; Halestrap et al., 2004). MCT is blocked not only by 4,4'-substituted stilbene-2,2'-disulphonates like DIDS, but also by bulky or aromatic monocarboxylates like α-cyano-4-hydroxycinnimate and by amphiphilic compounds like quercitin and phloretin (Poole et al., 1993; Halestrap et al., 2004). We determined that phloretin is a good MCT antagonist for RGM1 cell studies because it did not kill cells in STD buffer or in bicarbonate-free conditions. We also demonstrated that, like in native surface cells, MCT-1 is present in RGM1 cells.

In the present work, we showed that 60 μM phloretin inhibited wound repair in RGM1 cells by about 60%. In liver cells, 60 μM phloretin inhibited lactate/H^+-transport by about 85% (Jackson et al., 1996), which may explain why wound repair was not blocked completely by phloretin in our experiments. Although ITC groups on DIDS, H_2DIDS, and SITS can inhibit MCT-1 activity, they can also bind to NBC, suggesting that 2 pathways could be blocked simultaneously to affect wound repair and restitution in live cell experiments (Fig. 9).

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Our data suggest that this is the case, because blocking MCT-1 with phloretin and NBC with bicarbonate-free buffer completely inhibits wound repair in RGM1 cells and these results are exactly the same as with DIDS. The mechanism by which this inhibition occurs is largely unknown but may be related to the role MCT-1 plays in H^+/lactate transport (export) during glycolysis, which is suggested to be the main energy source for cell migration during restitution (Cheng et al., 2001). Alternatively, both NBC and MCT-1 transporters may be involved in the maintenance of pHi. Further experiments will be required to test these two interesting possibilities in the RGM1 cell wounding model.

Although phloretin is commonly used to inhibit MCT-1 activity, it is a non-specific drug and can block a number of other pathways that may be important for wound repair. First, phloretin is a potent inhibitor of protein kinase C (PKC) and has been used to study PKC-dependent pathways. When used to block PKC activation, however, it was determined that 200-250 μM phloretin is required (Emelijanova et al., 2002), which is far above the highest concentration employed in the current study. Although unlikely, we cannot exclude that phloretin inhibits PKC activation to some extent in our study and further experiments would be required to test this condition in the RGM1 wounding model. Another target of phloretin is glucose transport, where small concentrations of phloretin inhibit GLUT-1 and GLUT-2 activity (Hersey et al., 1982; Kellett et al., 2000; Walker et al., 2005). We therefore tested the role of glucose transport in wound repair and showed that although glucose regulates the rate of wound repair in RGM1 cells, blockade of this process is not significant enough to explain the results obtained with phloretin. These results are consistent with studies in the *ex vivo* frog gastric mucosa where it was suggested that gastric cells can utilize internal glucose stores

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to drive glycolysis in the absence of extracellular glucose transport for at least 24 hours (Hagen et al., 2004).

In conclusion, the results presented here demonstrate that the ITC group(s) on stilbenes like DIDS, H_2DIDS, and SITS block restitution and wound repair in a dose-dependent fashion and suggest they do so by binding to both NBC and MCT-1 on gastric surface cells (Fig. 9). The precise mechanism of inhibition is yet to be defined, but is worthy of further investigation in light of the importance of restitution for the maintenance of mucosal barrier integrity. The results presented here also suggest that new drug therapies which include ITC derivatives must take into account the important pathways they block in live cell systems, including mucosal repair after injury.

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Footnotes

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This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases Grant R01 DK-15681, the Harvard Digestive Diseases Center Grant DK-34854 (S.J. Hagen), and the Kyoto Pharmaceutical University's 21st century COE program (K.Takeuchi).

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Legends for Figures

Fig. 1. The structure of stilbene compounds with or without isothiocyanate "R" group(s), which have a chemical structure of $^{-}N{=}C{=}S$. DIDS (A) and H_2DIDS (B) have two and SITS (C) has one isothocyanate group(s). DIDS and H_2DIDS have a different backbone structure, where the linking carbons in H_2DIDS are oxidized. DNDS (D) has the same molecular backbone as DIDS and SITS but no isothiocyanate groups.

Fig. 2. Effect of stilbene compounds DIDS, SITS, and DNDS on restitution of injured guinea pig gastric mucosa. (A) After induction of injury by 0.5% Triton X-100 for 5 min, tissues mounted in Lucite Ussing chambers were incubated in standard buffer alone (control) or in standard buffer containing the indicated stilbene compound and the restitution was monitored for 3 hr (180 min) by measuring recovery of transmucosal electrical resistance (TER). (B) Viability for each compound was tested using the crystal violet assay and RGM1 cell monolayers that were incubated in standard buffer alone (control) or in standard buffer containing the indicated stilbene compound for 3 hr. Values represent means ± SE of 6 experiments (A) or 2 wells from 3 different experiments (B). ***, $P<0.001$, **, $P<0.01$ or *, $P<0.05$ * when compared to standard buffer containing 0.1% DMSO (Control) or ††† when comparing DIDS to SITS.

Fig. 3. Effect of DIDS (A), SITS (B), and DNDS (C) on morphological recovery of guinea pig gastric mucosa at 3 hr after injury. Tissues were fixed in formalin and processed for paraffin sectioning and analysis after the experiment in Fig. 2. (D) Histological scores for this experiment were derived after analysis of all tissues by 4 independent graders without foreknowledge of source. Values represent means ± SE from 6 tissues per stilbene compound. Criteria for grading tissues were described in detail in Materials and Methods.

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***, *P*<0.001 when compared to standard buffer containing 0.1% DMSO (Control) or ††† when comparing DIDS to SITS.

Fig. 4. Dose-dependent inhibition of wound recovery in RGM1 monolayers by the stilbene compounds DIDS (A), H_2DIDS (B), SITS (C), or DNDS (D). After the induction of a round wound, the recovery of RGM1 cell monolayers was monitored in the presence of DIDS, H_2DIDS, SITS, or DNDS in 0.1% DMSO for 8 hr. These compounds did not affect the viability of RGM1 cells (Fig. 2). Values represent means ± SE of 2 wells from 3 different experiments. ***, *P*<0.001, **, *P*<0.01 or *, *P*<0.05 * when compared to cells incubated with standard buffer containing 0.1% DMSO (Control).

Fig. 5. Effects of ATP receptors (A) or barium-sensitive SUR6/Kir6.1 K^+-channels (B) on recovery of wounds induced in RGM1 cell monolayers. After the induction of a round wound, the recovery of RGM1 cells was monitored in the presence of ATP or ATP-analogues α,β-me ATP or 2 me-S-ATP (A) or barium chloride ($BaCl_2$, B) for 8 hr. Barium precipitated in standard buffer so the experiment required buffer that was sulfate (SO_4^-)-free. Values represent means ± SE of 2 wells from 3 different experiments and are expressed as % of the initial wounded area. **, *P*<0.01 when compared to the standard buffer (Std) control containing SO_4^-.

Fig. 6. Effect of the monocarboxylate transporter antagonist, phloretin, on recovery of wounds induced in RGM1 cell monolayers. (A) After the induction of a round wound, the recovery of RGM1 cell monolayers was monitored in the presence of phloretin in either STD buffer or in bicarbonate- free buffer. (B) Viability for phloretin in STD buffer or in bicarbonate- free buffer was tested using the crystal violet assay in RGM1 cell monolayers for 8 hr after the conclusion of experiments in (A). Values represent means ± SE of 2 wells from

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3 different experiments and are expressed as % of the initial wounded area (A) or as the % viability when compared to Std buffer. ***, $P<0.001$ when compared to treatment control; †††, $P<0.001$ comparing treatments.

Fig. 7. Effect of blockade of glucose transport with glucose-free buffer and with phloretin on recovery of wounds induced in RGM1 cell monolayers. (A) After the induction of a round wound, recovery of RGM1 cell monolayers was monitored in either STD buffer or in glucose-free (GF) buffer with or without phloretin. (B) Viability for phloretin in GF buffer was tested using the crystal violet assay in RGM1 cell monolayers for 8 hr after the conclusion of experiments in (A). Values represent means ± SE of 2 wells from 3 different experiments and are expressed as % of the initial wounded area (A) or as the % viability when compared to standard buffer. ***, $P<0.001$ when compared to treatment control; ††, $P<0.01$ comparing treatments; † $P<0.05$ comparing treatments.

Fig. 8. Expression of MCT1-4 in RGM1 cells. RGM1 cells were grown to confluence, isolated from the culture plate, and then frozen at -80°C before use. Total RNA from thawed cells was extracted with the single-step acid phenol-chloroform extraction procedure using TRIzol and then was reverse transcribed with a SuperScript preamplification kit. The sequences for sense and antisense primers, obtained from GenBank, were described in Materials and Methods. Note that MCT-1 is the main MCT expressed in RGM1 cells.

Fig. 9. A schematic diagram of the proposed action of ITC on restitution. ITC groups avidly bind to lactate/H^{+}-transporters (MCT-1) and to NBC. Chloride bicarbonate exchangers, also blocked by ITC, are not present on gastric surface cells. Although the mechanism is not fully understood, blocking both MCT-1 and NBC may affect intracellular pH regulation by inhibiting lactic acid efflux and bicarbonate influx during restitution. NHE, a "housekeeping"

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pH regulatory transporter, may not be able to effectively regulate pH in the absence of the other transporters. Glycolysis and energy production may also be impaired, by feedback inhibition, by the high lactic acid concentration in migrating cells after blockade of MCT-1.

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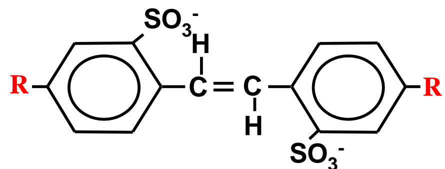
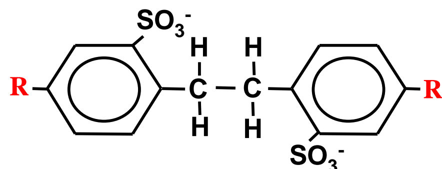
A



4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid

DIDS

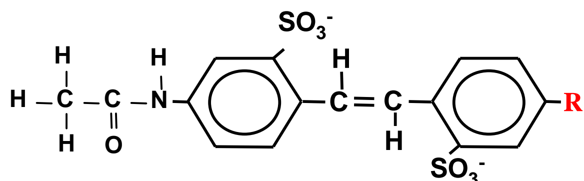
B



4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid

H_2DIDS

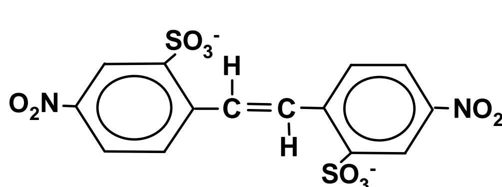
C



4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid

SITS

D



4,4'-dinitrostilbene-2,2'-disulfonic acid

DNDS

R: –N = C = S (Isothiocyanate)

Figure 1

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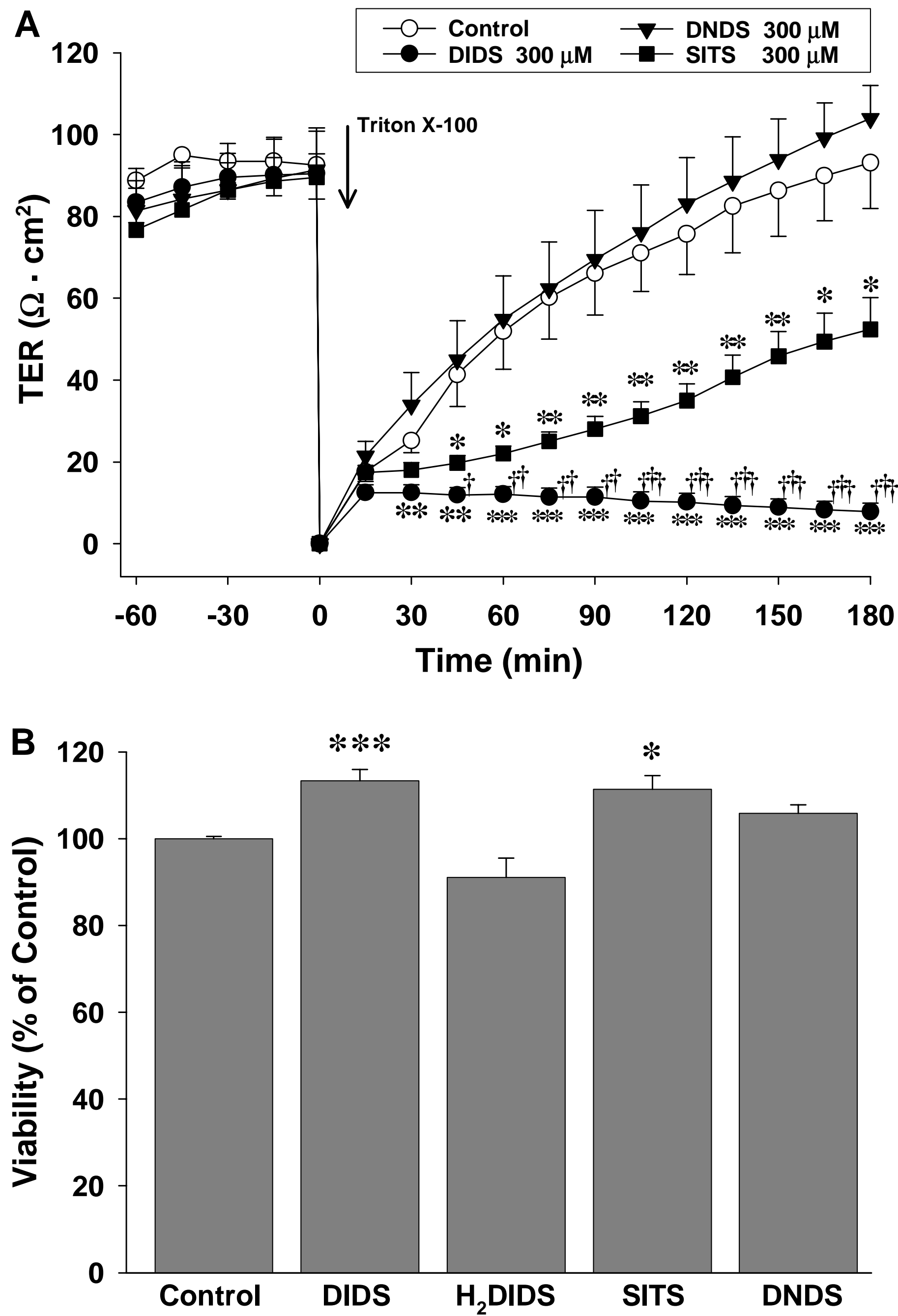

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Figure 2

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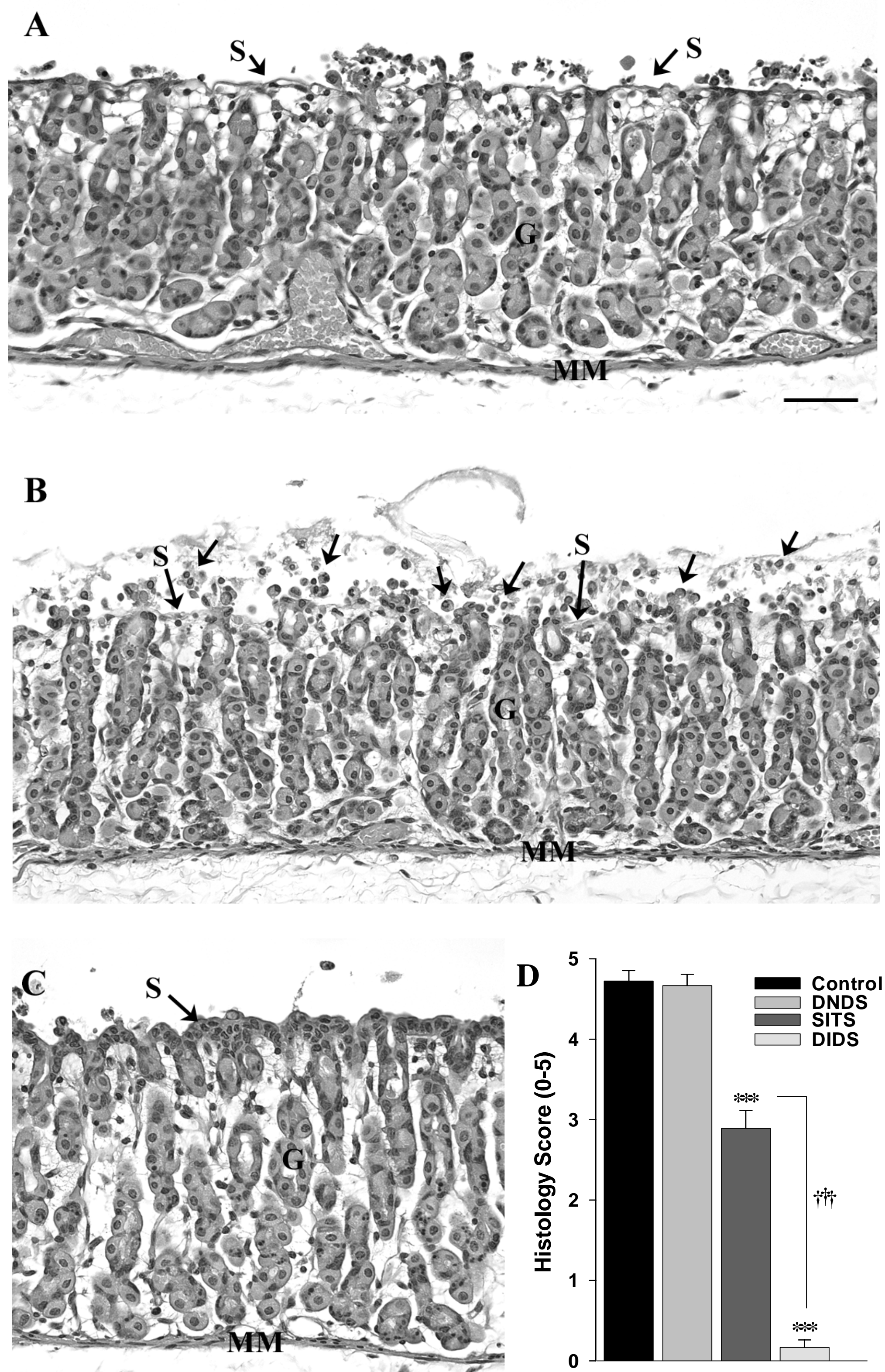

Figure 3

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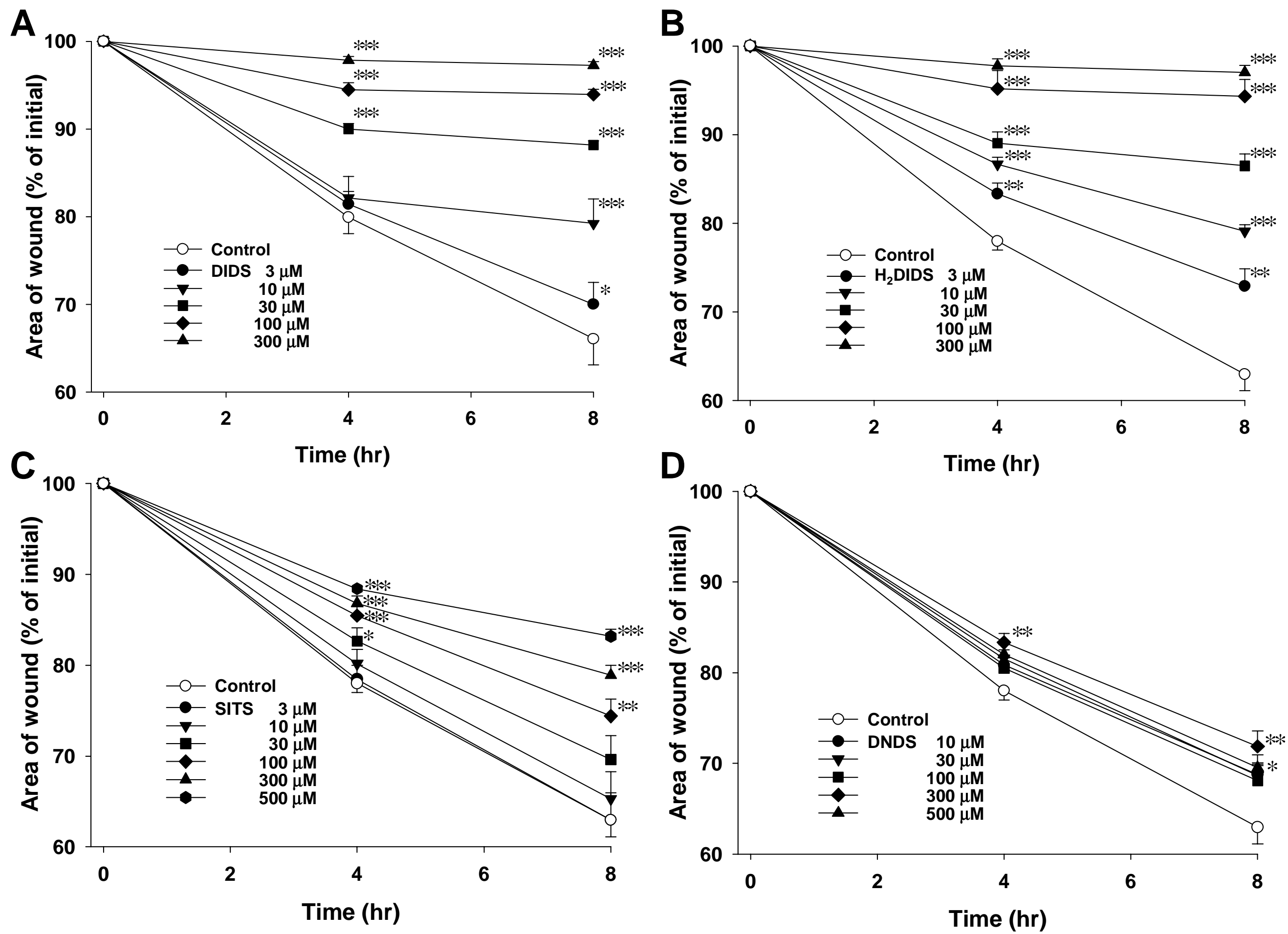

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Figure 4

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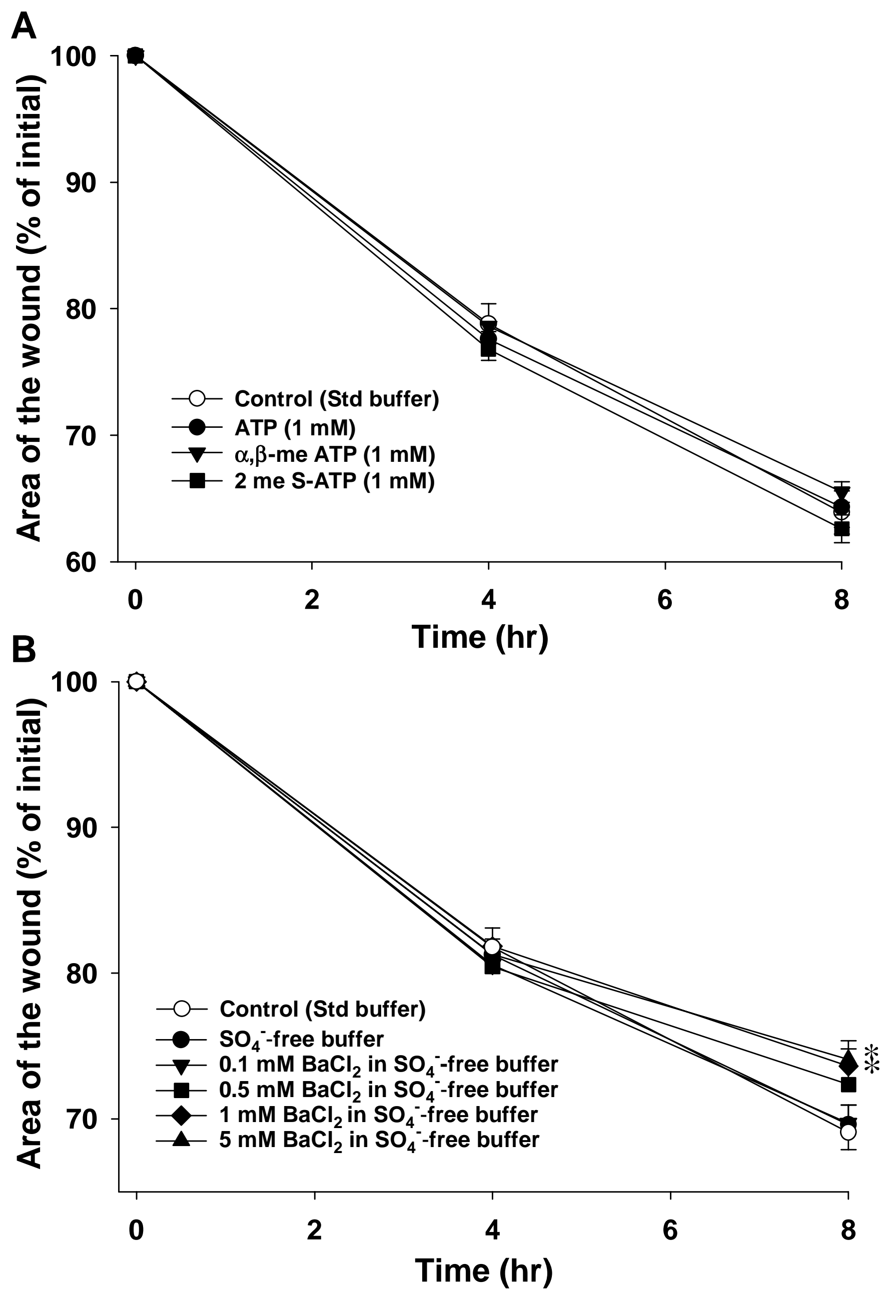

Figure 5

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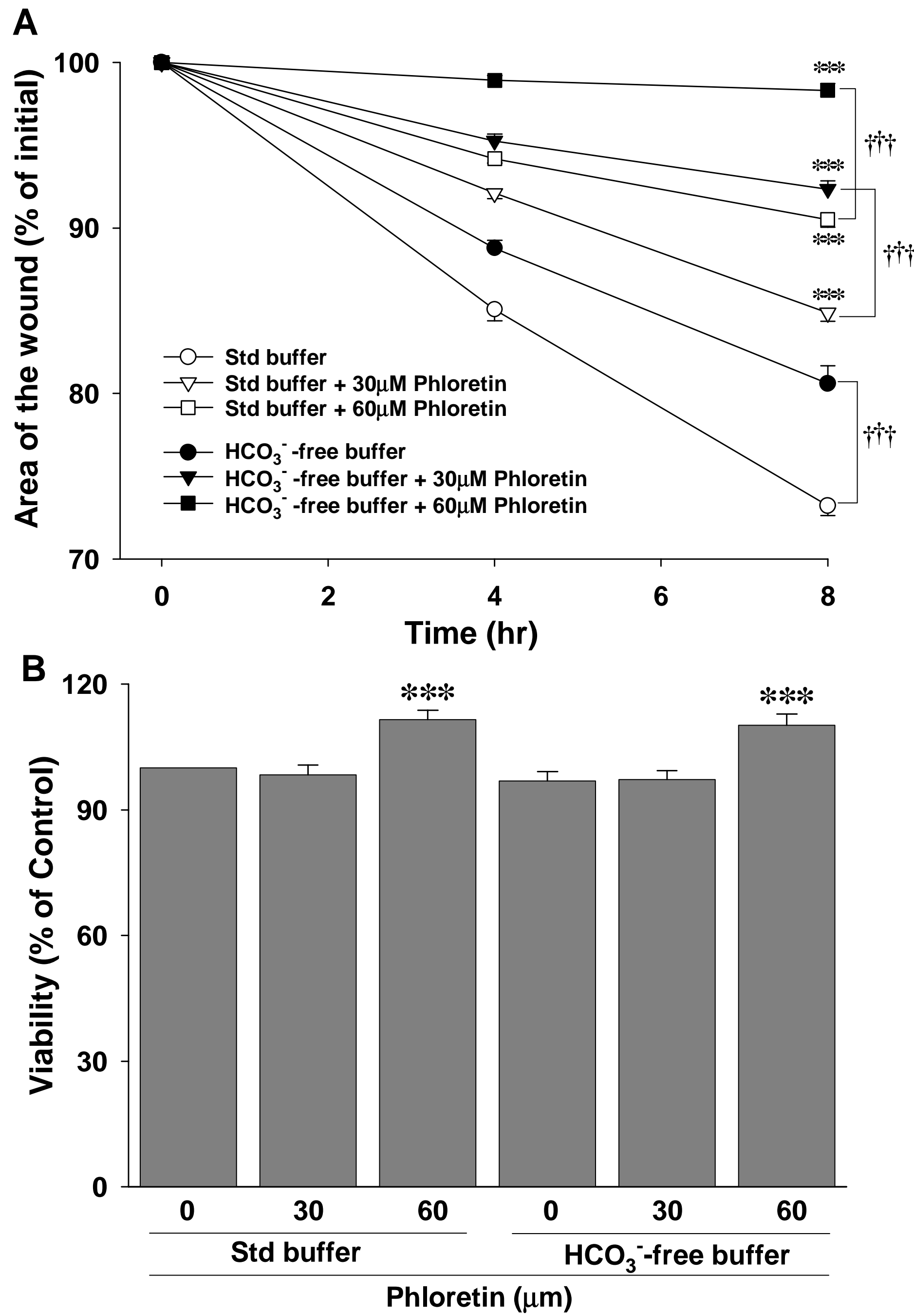

Figure 6

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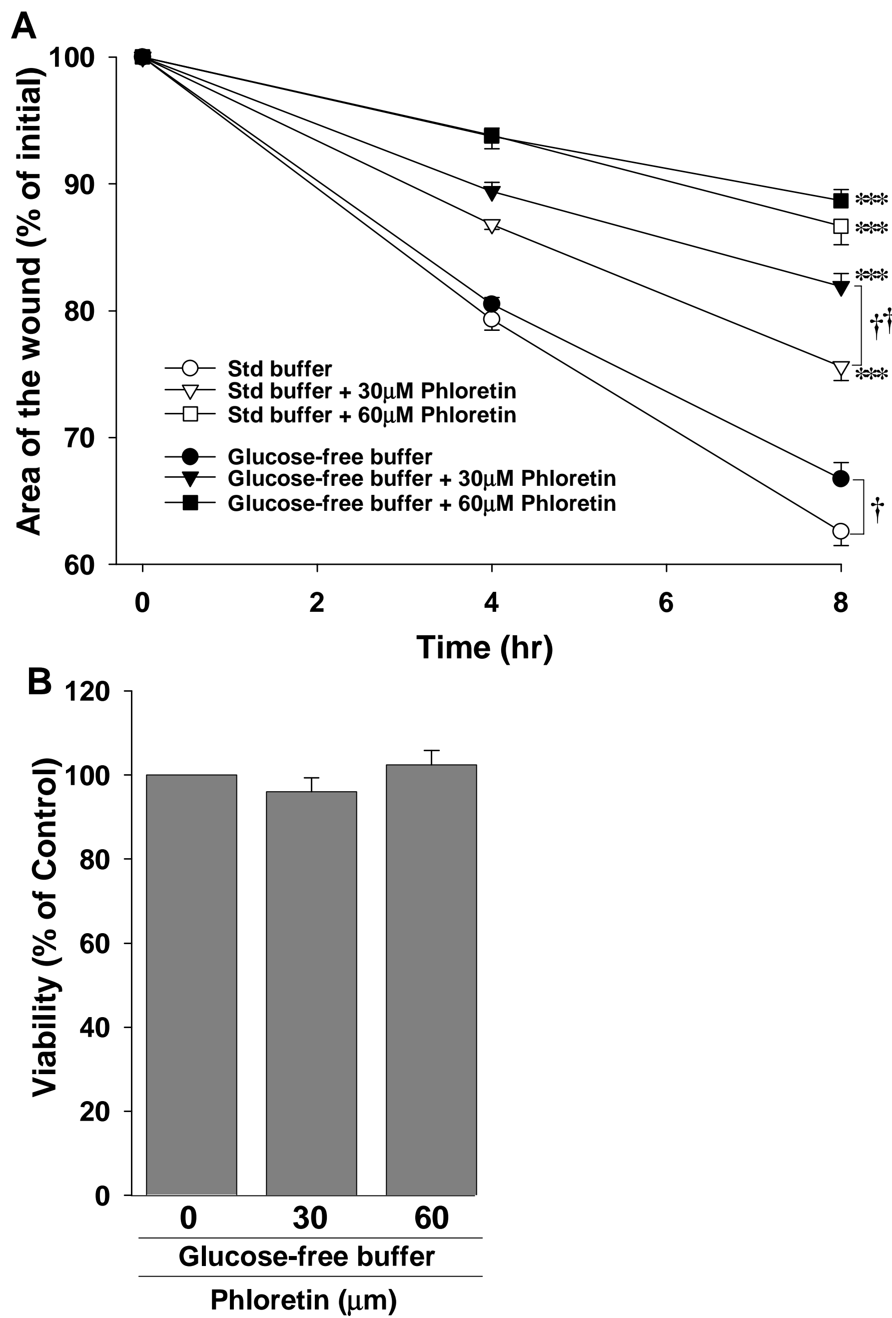

Figure 7

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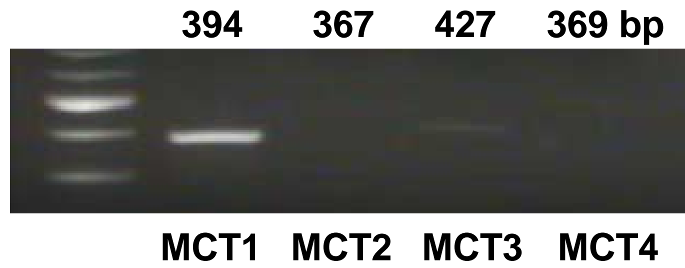

Figure 8

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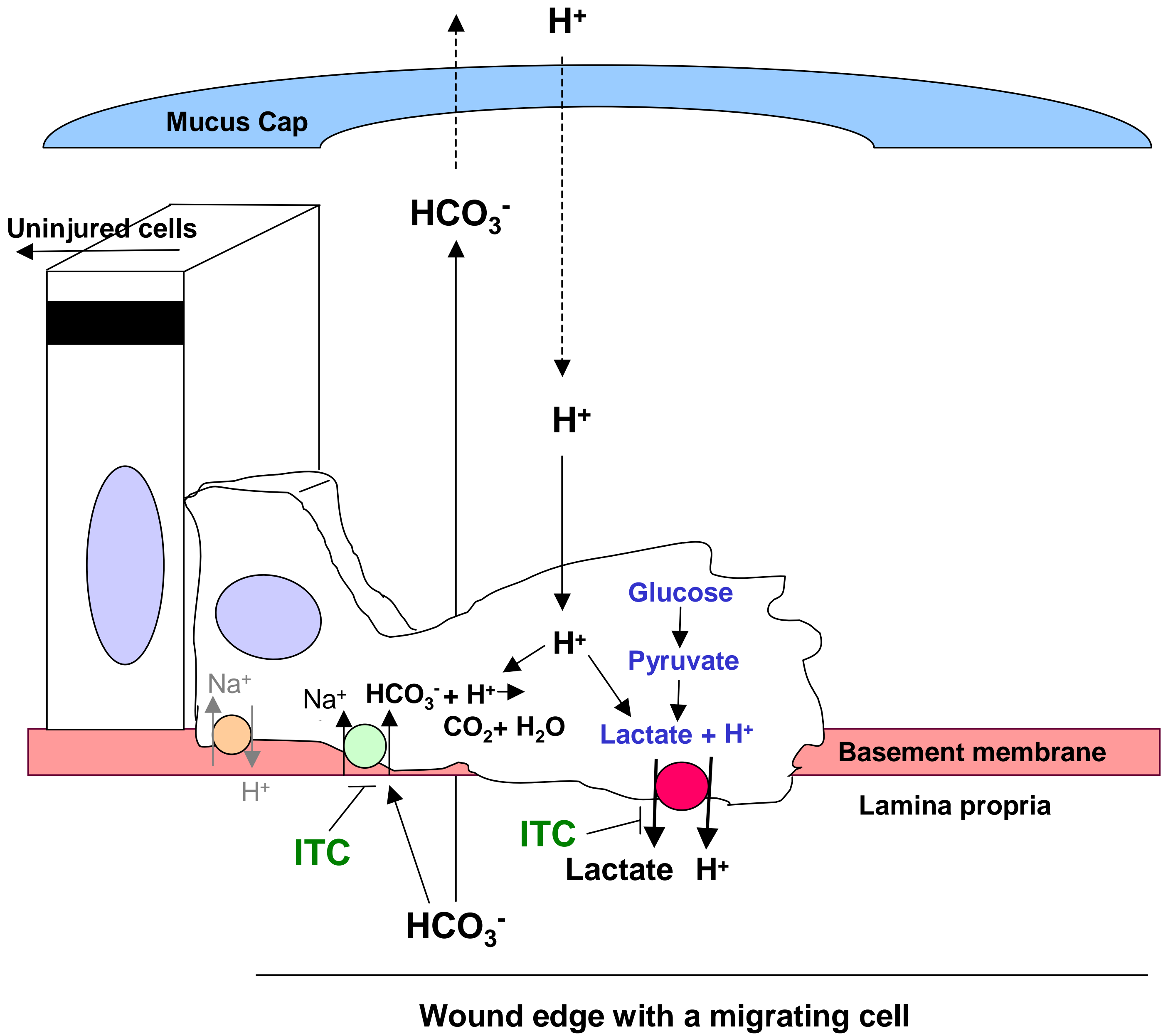

Figure 9

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