

# Isothiocyanate Inhibits Restitution and Wound Repair after Injury in the Stomach: Ex Vivo and in Vitro Studies

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## ABSTRACT

The role of isothiocyanate (ITC) in blocking epithelial restitution after injury and in the recovery of round wounds was examined in the ex vivo guinea pig stomach and in rat gastric mucosal-1 (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 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) or 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid (H<sub>2</sub>DIDS) (two ITC groups), 4-acetamido-4-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) (one ITC group), or 4,4'-diinitrostilbene-2,2'-disulfonic acid (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  $\mu$ M, DIDS or H<sub>2</sub>DIDS blocked restitution and wound repair by 100%, SITS blocked wound repair by 50%, and DNDS blocked wound repair by 2%. These results demonstrate the dependence of restitution and wound repair on ITC. ITC-binding

purino (ATP) receptors and K<sub>ATP</sub> channels were investigated as potential sites of inhibition, but they 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 cotransporter (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. Reverse transcription-polymerase chain reaction 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.

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 they 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*, 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, such as Caco-2 cells, skin, endothelial cells, and epithelial cells of the gastrointestinal tract, including the stomach (Lacy and Ito, 1984; Critchlow et al., 1985; Ito and Lacy, 1985), small intestine (Donowitz and Madara, 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 lamellipodia

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**ABBREVIATIONS:** pH<sub>i</sub>, intracellular pH; SITS, 4-acetamido-4-isothiocyanatostilbene-2,2'-disulfonic acid; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; ITC, isothiocyanate; RGM1, rat gastric mucosal-1; NBC, sodium bicarbonate cotransporter; MCT, monocarboxylate transporter; TER, transmucosal electrical resistance; H<sub>2</sub>DIDS, 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid; DNDS, 4,4'-diinitrostilbene-2,2'-disulfonic acid; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; STD, standard buffer;  $\alpha$ , $\beta$ -meATP,  $\alpha$ , $\beta$ -methyl-ATP; 2-MeSATP, 2-methylthio-ATP; SUR, sulfonyleurea receptor; bp, base pairs; GLUT, glucose transporter; PKC, protein kinase C.

and filopodia 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),  $\text{Ca}^{2+}$  (Critchlow et al., 1985), actin (Critchlow et al., 1985), glycolysis (Cheng et al., 2001), and ion transport (Joutsu et al., 1996; Yanaka et al., 2002; Hagen et al., 2004). Ion transport is required to regulate intracellular pH ( $\text{pH}_i$ ) in migrating cells after injury, a process that is particularly important in the stomach due to the acidic luminal environment.

It was recently reported that both amiloride, an  $\text{Na}^+/\text{H}^+$  exchange blocker, and 4-acetamido-4-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), a bicarbonate transport antagonist, inhibit restitution of the guinea pig gastric mucosa (Joutsu 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 inhibits 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.

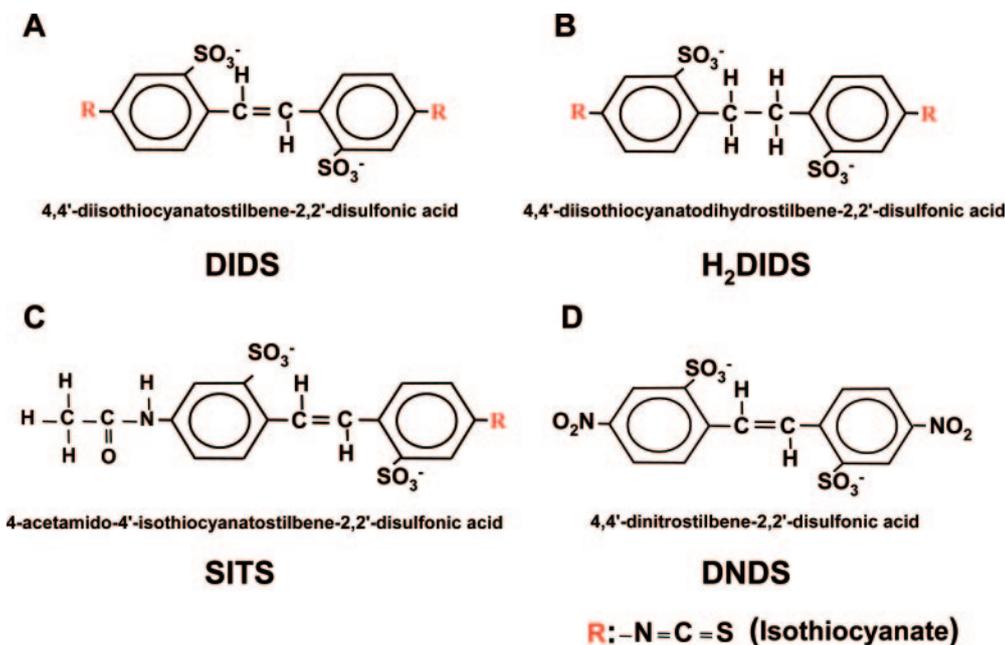
In the present study, we examined the role of SITS and DIDS in restitution of the mammalian gastric mucosa, ex vivo, and in an in vitro cell wounding model using rat gastric mucosal-1 (RGM1) cells. Because many cellular functions, in addition to bicarbonate transport by the sodium bicarbonate cotransporter (NBC), are blocked by the ITC group on stilbenes such as SITS and DIDS, we explored the role of three

important DIDS-inhibitable pathways, including purino (ATP) receptors, barium-sensitive  $\text{K}_{\text{ATP}}$  channels such as Kir6.1/SUR6A, and the monocarboxylate transporter MCT-1. Our data suggest that MCT-1, along with NBC, may be the relevant binding sites for DIDS/ITC in gastric surface epithelial cells after injury, and they support the notion that MCT-1 plays an important role in restitution and the maintenance of barrier integrity after injury.

## Materials and Methods

**Animals.** Male guinea pigs (300–350 g, Hartley strain; Elm Hill, Chelmsford, MA) were used in this work. Studies were done with six animals per treatment and without fasting. All animals in this study were maintained in accordance with the guidelines of the Committee on Animals at Beth Israel Deaconess Medical Center and with those prepared by the Committee on Care and Use of Laboratory Animals of the National Research Council (Institute of Laboratory Animal Resources, 1996).

**Ussing Chamber Studies.** After anesthesia, the stomach was removed, and it was 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 a dissecting microscope. Stripped mucosae were mounted in Lucite Ussing chambers (exposed area of  $0.636 \text{ cm}^2$ ) connected to water-jacketed gas-lift reservoirs maintained at  $37^\circ\text{C}$ . The serosal side was bathed with buffer containing  $147 \text{ mM Na}^+$ ,  $5.0 \text{ mM K}^+$ ,  $131 \text{ mM Cl}^-$ ,  $1.3 \text{ mM Mg}^{2+}$ ,  $1.3 \text{ mM SO}_4^{2-}$ ,  $2 \text{ mM Ca}^{2+}$ ,  $25 \text{ mM HCO}_3^-$ ,  $15 \text{ mM HEPES}$ , and  $20 \text{ mM D-glucose}$ , and it was continuously gassed with  $95\% \text{ O}_2$ ,  $5\% \text{ CO}_2$ . The luminal side was bathed with  $150 \text{ mM NaCl}$ , gassed with  $100\% \text{ O}_2$ , and kept at  $\text{pH } 4.0$  with  $\text{pH-stat}$  device (Radiometer Copenhagen GmbH, Copenhagen, Denmark). Transmucosal electrical resistance (TER) and potential difference were monitored throughout the experiment. After potential difference reached a stable value, injury was induced by exposing the luminal side of tissues to  $150 \text{ mM NaCl}$  containing  $0.5\% \text{ Triton X-100}$  for 5 min. Subsequently, the mucosa was washed three 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 h in the absence or presence of DIDS, 4,4'-diisothiocyanatodihydrostilbene-2,2'-disul-



**Fig. 1.** Structure of stilbene compounds with or without isothiocyanate "R" group(s), which have a chemical structure of  $-\text{N}=\text{C}=\text{S}$ . DIDS (A) and  $\text{H}_2\text{DIDS}$  (B) have two and SITS (C) has one isothiocyanate group(s). DIDS and  $\text{H}_2\text{DIDS}$  have a different backbone structure, where the linking carbons in  $\text{H}_2\text{DIDS}$  are oxidized. DNDS (D) has the same molecular backbone as DIDS and SITS, but it has no isothiocyanate groups.

fonic 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 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 hematoxylin 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 to 50% of the apical surface was covered with flattened surface epithelial cells. A score of 3 indicated that 50 to 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. Finally, 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.** RGM1 cells, established by Dr. H. Matsui [Institute of Physical and Chemical Science (RIKEN) Cell Bank and Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan] (Kobayashi et al., 1996), are nontransformed gastric surface epithelial cells. RGM1 cells were cultured in DMEM/F-12 (1:1) supplemented with heat-inactivated 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B. Confluent monolayers of RGM1 cells were starved for 24 h in culture medium without fetal bovine serum (DMEM/F-12 containing 15 mM HEPES, pH 7.4) at 37°C under 5%  $CO_2$  in air, and then they were used as a confluent monolayer for experiments. All experiments were performed at pH 7.4 in the presence 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, Osaka, Japan), resulting in a cell-free area of uniform size after wounding. The monolayer was then washed with standard (STD) buffer, containing 147 mM  $Na^+$ , 5.0 mM  $K^+$ , 131 mM  $Cl^-$ , 1.3 mM  $Mg^{2+}$ , 1.3 mM  $SO_4^{2-}$ , 2 mM  $Ca^{2+}$ , 25 mM  $HCO_3^-$ , 15 mM HEPES, and 20 mM 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 to 300  $\mu$ M DIDS or  $H_2DIDS$  or 3 to 500  $\mu$ M SITS or DNDS was 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 of the nonhydrolyzable analogs of ATP  $\alpha,\beta$ -methyl-ATP ( $\alpha,\beta$ -meATP) or 2-methylthio-ATP (2-meSATP) to determine whether ATP accelerates wound repair. Furthermore, 10 to 300  $\mu$ M suramin was used to determine whether blockade of purinoreceptors inhibits wound repair. To determine whether barium-sensitive  $K_{ATP}$  channels such as Kir6.1/SUR6A are involved in wound repair, injured monolayers were incubated with 0.1 to 5 mM barium chloride ( $BaCl_2$ ) in sulfate-free buffer that contained 160 mM  $Na^+$ , 5.0 mM  $K^+$ , 143 mM  $Cl^-$ , 1.3 mM  $Mg^{2+}$ , 2 mM  $Ca^{2+}$ , 25 mM  $HCO_3^-$ , 15 mM HEPES, and 20 mM D-glucose. Barium chloride precipitated in STD buffer, so sulfate-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  $\mu$ M. 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 and 8 h with a Nikon TE300 microscope (MicroVideo Instruments, Avon, MA) outfitted with an Orca charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan). Change in the cell-free area was quantified using IPLab software (Scanalytics, 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 phosphate-buffered saline, 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 a kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

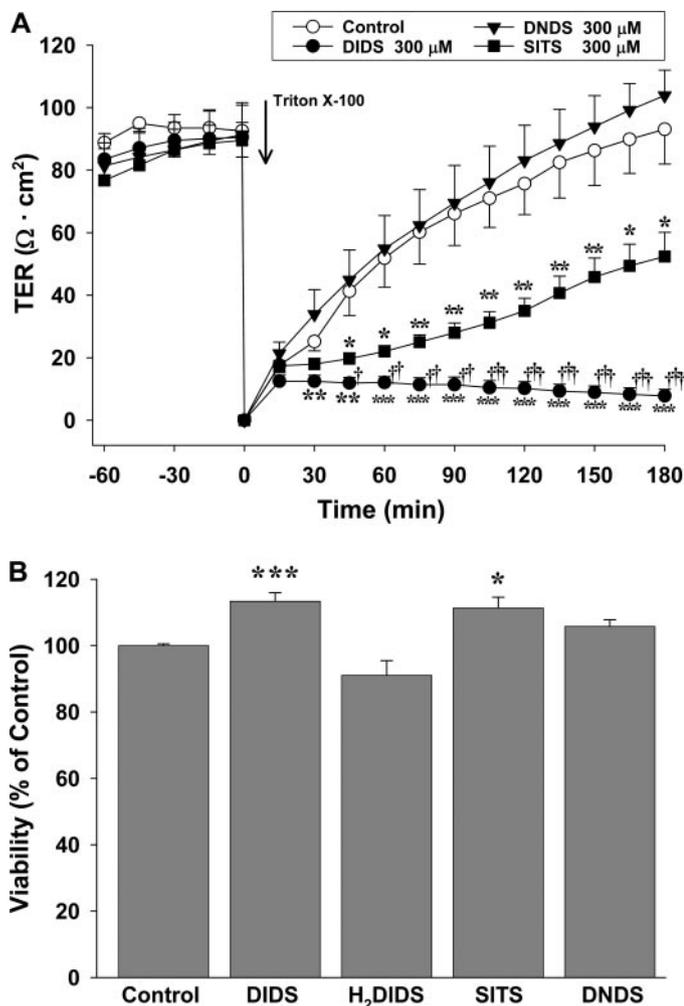
**Analysis of MCT Expression by Reverse Transcription-Polymerase Chain Reaction.** RGM1 cells were grown to confluence, and then they were isolated from the culture plate by using 0.1% trypsin in 0.5 mM EDTA at 37°C for 10 min. Isolated cells were pelleted and frozen at  $-80^\circ C$  before use. Total RNA from the thawed cells was extracted with the single-step acid phenol-chloroform extraction procedure using TRIzol (Invitrogen). 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 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'-TCTGGCCTCGTGCCAT-3' to obtain a 427-bp product; and 4) rat MCT4 (U87627) were 5'-GGCAGTCCCGTTCCTT-3' and 5'-GCACCTCTTGAGCCCTGTTAT-3' to obtain a 369-bp product.

**Reagents.**  $H_2DIDS$  was purchased from Invitrogen. All other chemicals, including DIDS, SITS, and DNDS, were purchased from Sigma-Aldrich (St. Louis, MO), unless noted otherwise.

**Statistical Analysis.** In RGM1 cell experiments, the data represent means  $\pm$  S.E. for two wells from three different experiments. In Ussing chamber experiments, the data represent means  $\pm$  S.E. obtained from at least six experiments. Statistical differences were evaluated by analysis of variance, Dunnett's multiple comparison test, and Student's *t* test using SigmaPlot (SPSS Inc., Chicago, IL). Significance of the data is denoted as \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , and \*\*\*,  $P < 0.001$  compared with time-matched control samples or as †,  $P < 0.05$ , ††,  $P < 0.01$ , and †††,  $P < 0.001$  compared with time-matched samples from another treatment.

## Results

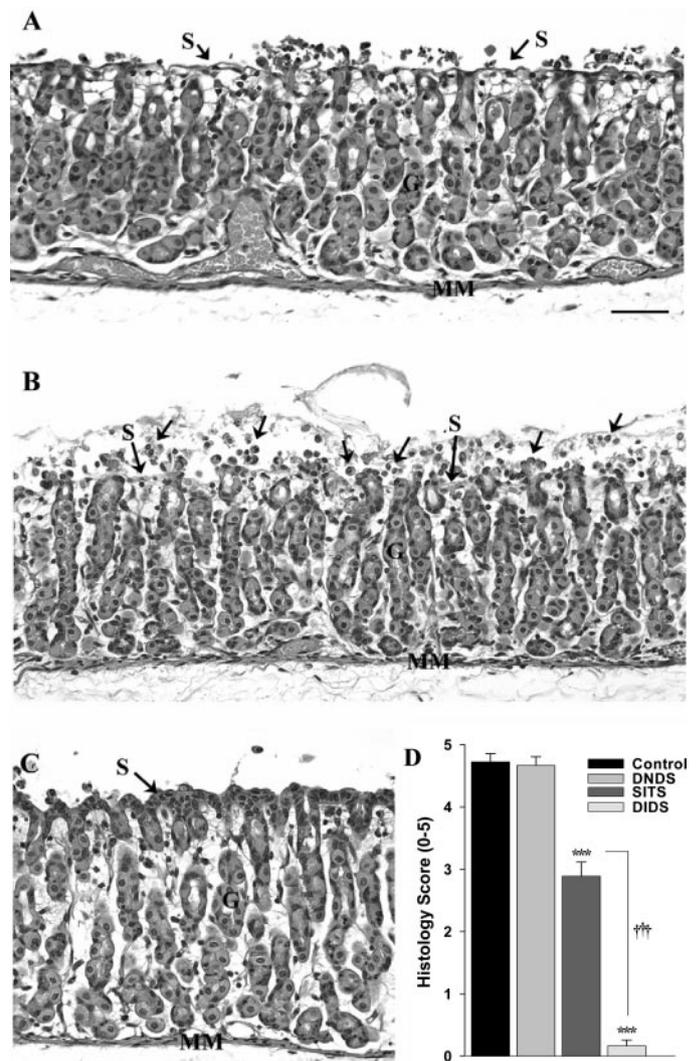
**Effects of Various Stilbene Compounds on Restitution after Injury in the ex Vivo Guinea Pig Gastric Mucosa.** When injured with Triton X-100, TER dropped to 0  $\Omega cm^2$  in 5 min, and it returned to pretreatment levels within 180 min in the presence of control buffer or with DNDS, a stilbene compound that contains no ITC groups (Fig. 2A). In contrast, tissues treated with Triton X-100 followed by DIDS, a stilbene with two 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



**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 h (180 min) by measuring recovery of 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 h. Values represent means  $\pm$  S.E. of six experiments (A) or two wells from three different experiments (B). \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; or \*,  $P < 0.05$  compared with standard buffer containing 0.1% DMSO (control) or ††† comparing DIDS with SITS.

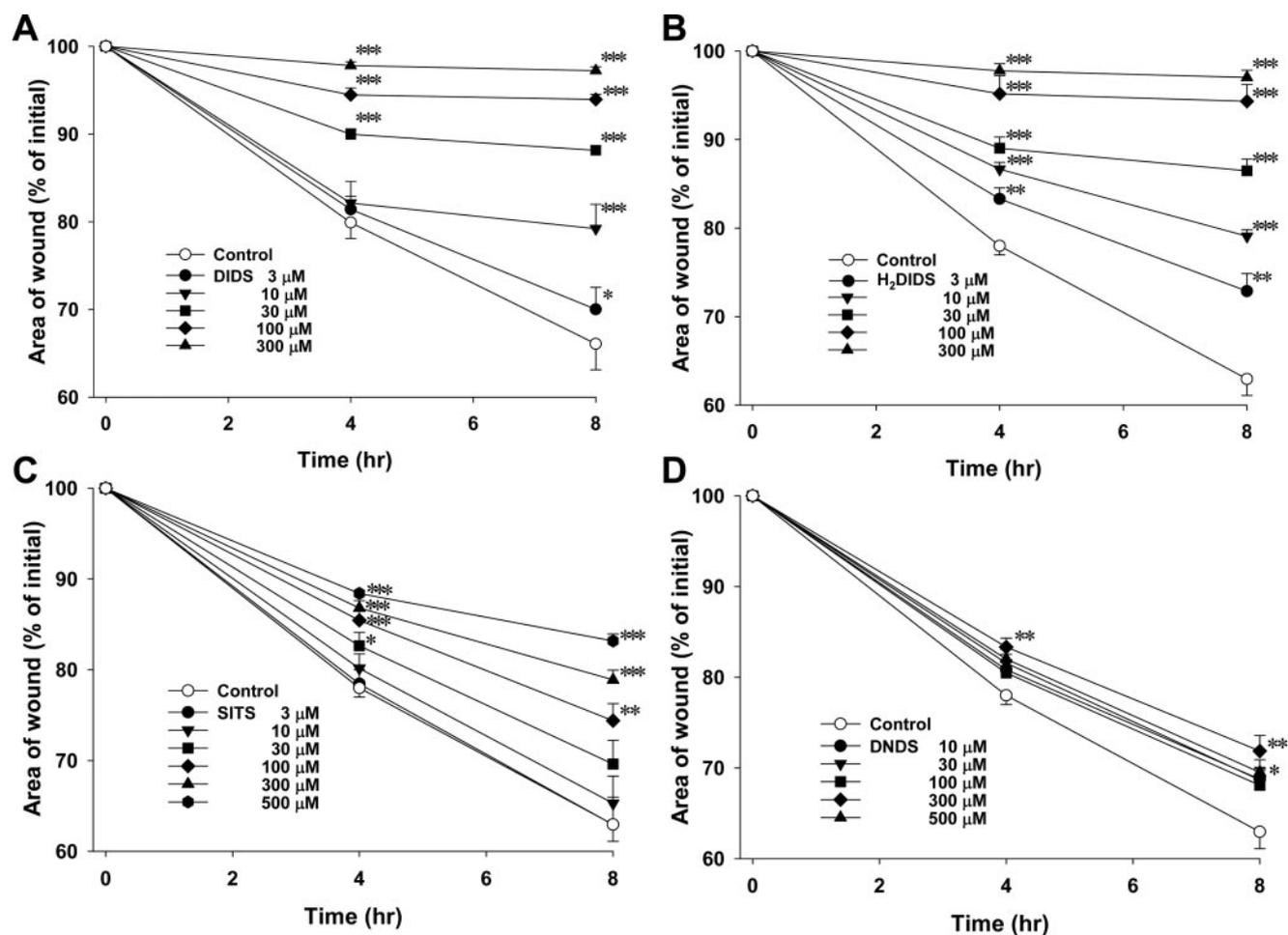
followed by migration and repolarization during repair, the morphology of tissues treated with each stilbene compound was examined at 180 min after injury (Fig. 3). With SITS (Fig. 3A), some recovery occurred, and the histology score was  $2.89 \pm 0.23$  (Fig. 3D). With DIDS (Fig. 3B), no recovery occurred, and the histology score was  $0.167 \pm 0.09$  (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 (Fig. 3, C 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<sub>2</sub>DIDS, and SITS inhibit wound repair in a reductionist model of injury and repair, RGM1 cells were used (Fig. 4). Consistent with re-



**Fig. 3.** Effect of DIDS (A), SITS (B), and DNDS (C) on morphological recovery of guinea pig gastric mucosa at 3 h after injury. Tissues were fixed in formalin, and they were processed for paraffin sectioning and analysis after the experiment described in Fig. 2. **D**, histological scores for this experiment were derived after analysis of all tissues by four independent graders without foreknowledge of source. Values represent means  $\pm$  S.E. from six tissues per stilbene compound. Criteria for grading tissues were described in detail under *Materials and Methods*. \*\*\*,  $P < 0.001$  compared with standard buffer containing 0.1% DMSO (control) or ††† comparing DIDS with SITS.

sults from the guinea pig ex vivo model in Fig. 2, DIDS (Fig. 4A) and H<sub>2</sub>DIDS (Fig. 4B), stilbene compounds with two 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 repair was dose-dependently inhibited by approximately 50% with SITS (Fig. 4C), a stilbene with one 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 manner. Instead, there was some inhibition of wound repair at all doses tested, which reached significance at 300 to 500 μM compared with controls (Fig. 4D). As shown in Fig. 2B, the stilbene compounds tested did not reduce the viability of RGM1 cells when examined 8 h after wounding, so the results cannot be attributed to toxicity of drugs at the concentrations used for this study. In the



**Fig. 4.** Dose-dependent inhibition of wound recovery in RGM1 monolayers by the stilbene compounds DIDS (A), H<sub>2</sub>DIDS (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<sub>2</sub>DIDS, SITS, or DNDS in 0.1% DMSO for 8 h. These compounds did not affect the viability of RGM1 cells (Fig. 2). Values represent means  $\pm$  S.E. of two wells from three different experiments. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; or \*,  $P < 0.05$  compared with cells incubated with standard buffer containing 0.1% DMSO (control).

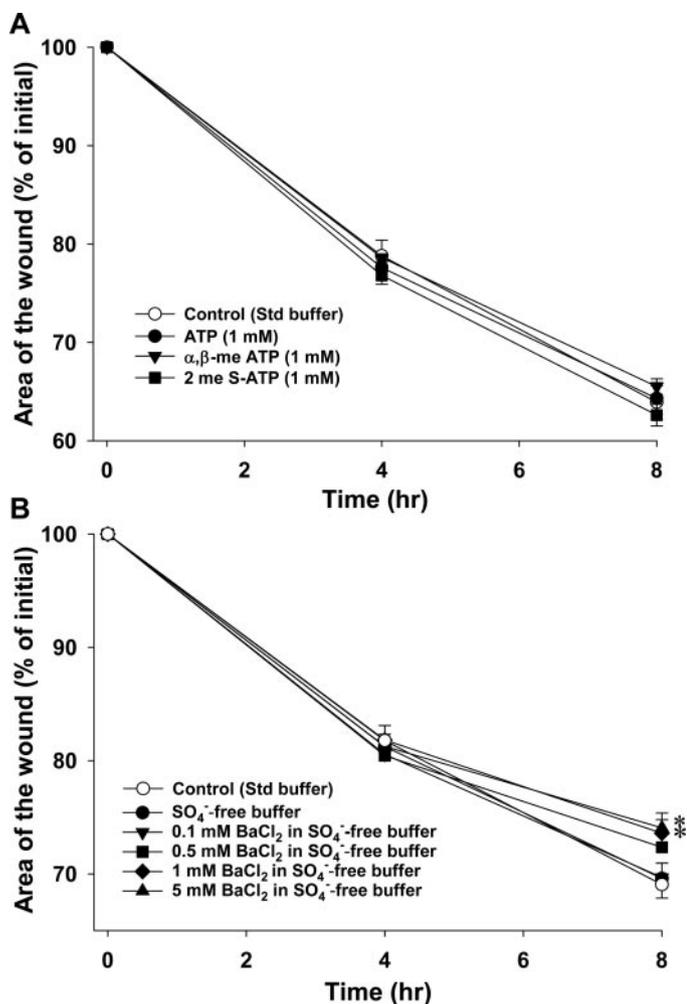
RGM1 cell model, bicarbonate-free conditions resulted in a significant but modest inhibition of wound repair (See Fig. 6).

**Role of Purino (ATP) Receptors and K<sub>ATP</sub> Channels on Wound Repair in the in Vitro RGM1 Cell Model.** ATP binding to purinoreceptors and the activity of K<sub>ATP</sub> channels such as Kir6.1/SUR6A are both known to be inhibited by DIDS (Furukawa et al., 1993; Bültmann and Starke, 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 analogs of ATP, or with barium, a nonspecific antagonist of Kir6.1/SUR6A channels (Fig. 5). ATP and nonhydrolyzable analogs of ATP, such as  $\alpha,\beta$ -methyl-ATP and 2-methylthio-ATP would be expected to bind P2-type purinoreceptors on the cell surface (Bültmann and Starke, 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<sup>+</sup> 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 below (data not shown). Sulfate-free conditions alone did not inhibit wound repair nor did sulfate-free conditions with 0.1 to 0.5 mM barium (Fig. 5B).

Higher doses of barium, including 1 to 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<sub>2</sub>DIDS, and SITS (Poole and Halestrap, 1993; Halestrap and Price, 1999; Halestrap and Meredith, 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  $\mu$ M) 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 also 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



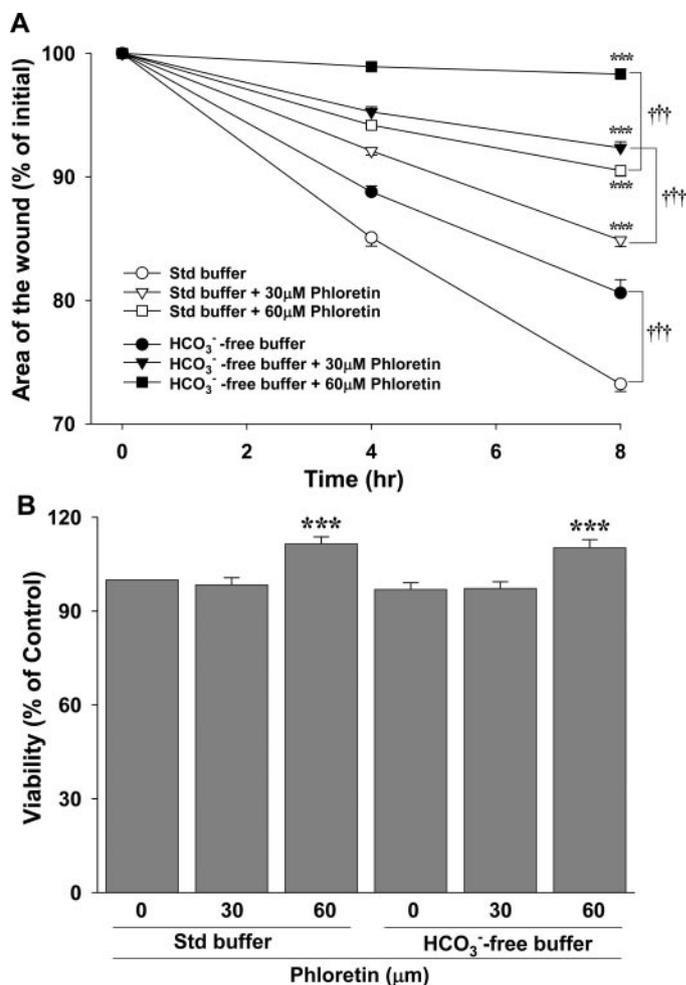
**Fig. 5.** Effects of ATP receptors (A) or barium-sensitive SUR6/Kir6.1  $\text{K}^+$  channels (B) on recovery of wounds induced in RGM1 cell monolayers. After the induction of a round wound, the recovery of RGM1 cells was monitored in the presence of ATP or ATP analogs  $\alpha, \beta$ -meATP or 2-me-SATP (A) or  $\text{BaCl}_2$  (B) for 8 h. Barium precipitated in standard buffer, so the experiment required buffer that was sulfate ( $\text{SO}_4^{2-}$ )-free. Values represent means  $\pm$  S.E. of two wells from three different experiments, and they are expressed as percentage of the initial wounded area. \*\*,  $P < 0.01$  compared with the STD buffer control containing  $\text{SO}_4^{2-}$ .

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).

To determine whether RGM1 cells have MCT transporters, reverse transcription-polymerase chain reaction was performed (Fig. 8). Using primers specific for rat MCTs that are published in GenBank, our results show that the message for MCT-1 was expressed in RGM1 cells (Fig. 8).

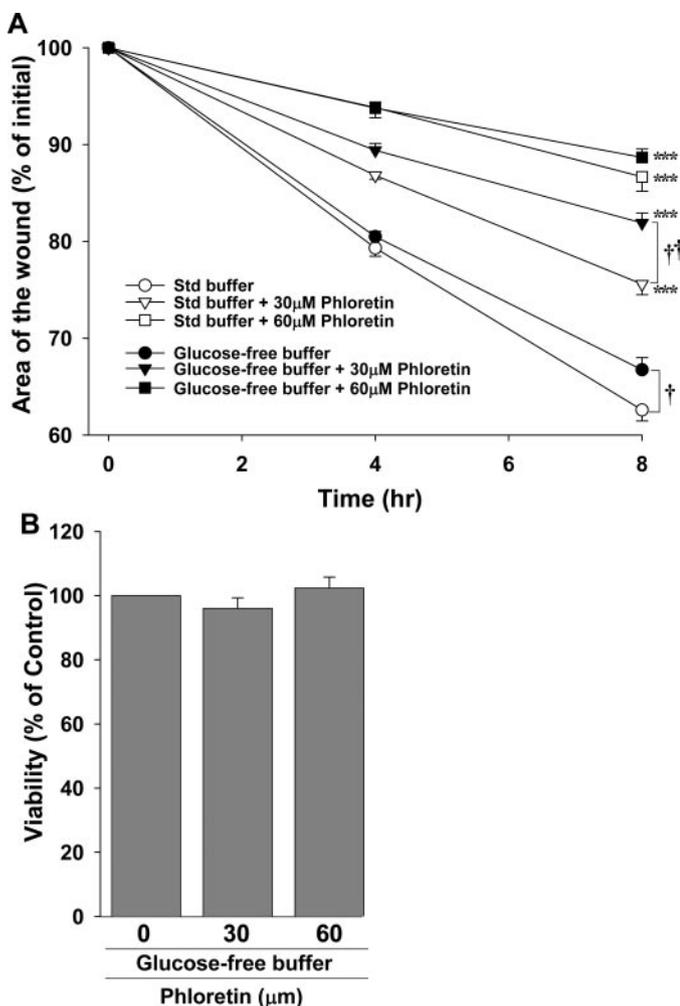
## Discussion

Although previous work showed that stilbene compounds such as DIDS and SITS block restitution of the ex vivo frog and guinea pig gastric mucosa (Joutsu 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

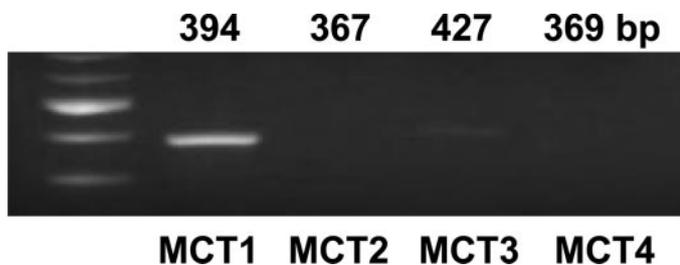


**Fig. 6.** Effect of the monocarboxylate transporter antagonist phloretin on recovery of wounds induced in RGM1 cell monolayers. A, after the induction of a round wound, the recovery of RGM1 cell monolayers was monitored in the presence of phloretin in either STD buffer or in bicarbonate-free buffer. 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 h after the conclusion of experiments described in A. Values represent means  $\pm$  S.E. of two wells from three different experiments, and they are expressed as percentage of the initial wounded area (A) or as the percentage of viability compared with STD buffer (B). \*\*\*,  $P < 0.001$  compared with treatment control; †††,  $P < 0.001$  comparing treatments.

an efficacy of DIDS =  $\text{H}_2\text{DIDS}$  (two ITC) > SITS (one ITC). 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, such as sulforaphane from broccoli and broccoli sprouts, have been proposed to treat *H. 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, 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 compared with those who



**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 h after the conclusion of experiments described in A. Values represent means  $\pm$  S.E. of two wells from three different experiments, and they are expressed as percentage of the initial wounded area (A) or as the percentage of viability compared with standard buffer. \*\*\*,  $P < 0.001$  compared with treatment control; ††,  $P < 0.01$  comparing treatments; or †,  $P < 0.05$  comparing treatments.



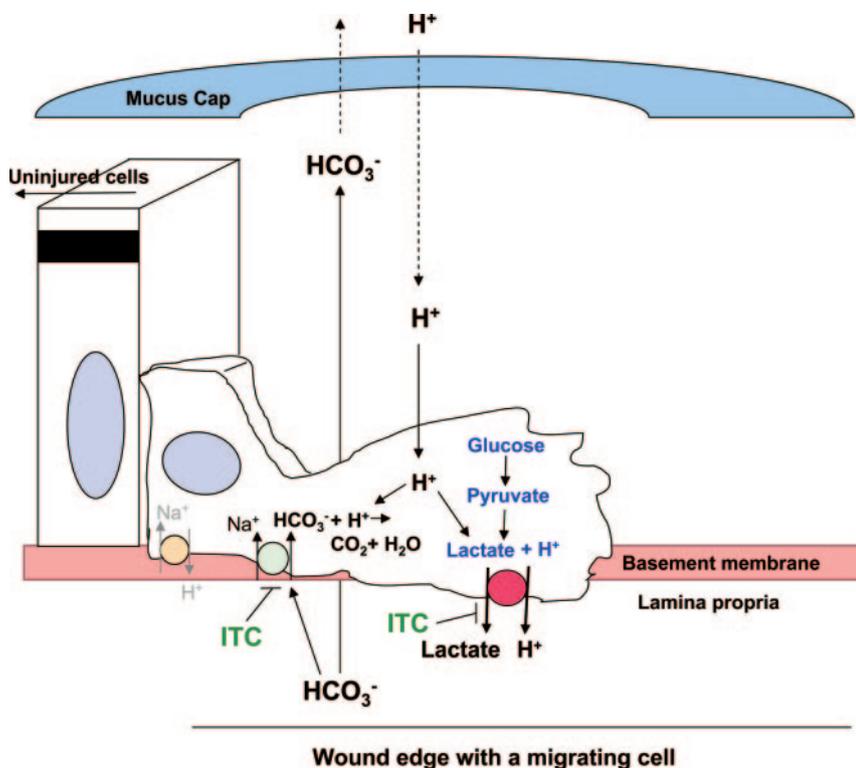
**Fig. 8.** Expression of MCT1 to 4 in RGM1 cells. RGM1 cells were grown to confluence, isolated from the culture plate, and then they were frozen at  $-80^{\circ}\text{C}$  before use. Total RNA from thawed cells was extracted with the single-step acid phenol-chloroform extraction procedure using TRIzol,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

consumed a negligible amount of this 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  $\text{pH}_i$  and be potently inhibited by DIDS,  $\text{H}_2\text{DIDS}$ , 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,  $\text{H}_2\text{DIDS}$ , or SITS during restitution and wound repair.

Three other potential ITC-binding receptors/transporters were investigated in this work to determine why DIDS,  $\text{H}_2\text{DIDS}$ , or SITS blocks restitution. First, it is well known that the P-type purinoreceptors  $\text{P}_{2X}$  and  $\text{P}_{2Y}$  are blocked by DIDS and that  $\text{P}_2$  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 they 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 nonhydrolyzable analogs of ATP, each of which binds to purinoreceptors, and we found that exogenous ATP does not accelerate wound repair in RGM1 cells. To verify this negative finding, we also blocked purinoreceptors with suramin, and we saw no effect on the rate of wound repair, or on viability, in RGM1 cells (data not shown). Because ATP did not accelerate nor did blockade of purinoreceptors inhibit wound repair in our study, we conclude that  $\text{P}_2$ -type purinoreceptors are probably not the target of DIDS,  $\text{H}_2\text{DIDS}$ , or SITS. Likewise,  $\text{K}_{\text{ATP}}$  channels such as Kir6.1/SUR2A have been shown to regulate cell migration, and they 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  $\text{K}^+$  channels are also not the major target of DIDS,  $\text{H}_2\text{DIDS}$ , or SITS in this study. However, our results suggest that one or more barium-sensitive  $\text{K}^+$  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 it was cloned, sequenced, and renamed MCT-1 (Garcia et al., 1994; Wang 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  $\text{H}^+$  and monocarboxylate cotransporter that transports lactate and  $\text{H}^+$  against a concentration gradient, especially during glycolysis when lactate efflux is required (Poole et al., 1993; Halestrap and Meredith, 2004). MCT is blocked not only by 4,4'-substituted stilbene-2,2'-disulfonates such as DIDS but also by bulky or aromatic monocarboxylates such as  $\alpha$ -cyano-4-hydroxycinnamate and by amphiphilic compounds such as



**Fig. 9.** 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.  $Na^+/H^+$  exchange, a "housekeeping" 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.

quercetin and phloretin (Poole et al., 1993; Halestrap and Meredith, 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  $60 \mu M$  phloretin inhibited wound repair in RGM1 cells by approximately 60%. In liver cells,  $60 \mu M$  phloretin inhibited lactate/ $H^+$  transport by approximately 85% (Jackson and Halestrap, 1996), which may explain why wound repair was not blocked completely by phloretin in our experiments. Although ITC groups on DIDS,  $H_2$ DIDS, and SITS can inhibit MCT-1 activity, they can also bind to NBC, suggesting that two pathways could be blocked simultaneously to affect wound repair and restitution in live cell experiments (Fig. 9). 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 it 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  $pH_i$ . 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 nonspecific drug, and it 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 it has been used to study PKC-dependent pathways. However, when used to block PKC activation, it was determined that 200 to  $250 \mu M$  phloretin is required (Emel-

janova et al., 2002), which is far above the highest concentration used 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 GLUT1 and GLUT2 activity (Hersey et al., 1982; Kellett and Helliwell, 2000; Walker et al., 2005). Therefore, we tested the role of glucose transport in wound repair, and we 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 use internal glucose stores to drive glycolysis in the absence of extracellular glucose transport for at least 24 h (Hagen et al., 2004).

In conclusion, the results presented here demonstrate that the ITC group(s) on stilbenes such as DIDS,  $H_2$ DIDS, and SITS block restitution and wound repair in a dose-dependent manner, and they 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 it 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 that 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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