Selective A2A adenosine receptor activation reduces skin pressure ulcer formation and inflammation

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Peirce, Shayn M., Thomas C. Skalak, Jayson M. Rieger, Timothy L. MacDonald, and Joel Linden. Selective A2A adenosine receptor activation reduces skin pressure ulcer formation and inflammation. Am J Physiol Heart Circ Physiol 281: H67–H74, 2001.—Activation of A2A adenosine receptors (A2A-AR) by ATL-146e (formerly DWH-146e) prevents inflammatory cell activation and adhesion. Recurrent ischemia-reperfusion (I/R) of the skin results in pressure ulcer formation, a major clinical problem. ATL-146e was evaluated in a novel reproducible rat model of pressure ulcer. A 9-cm2 region of dorsal rat skin was cyclically compressed at 50 mmHg using a surgically implanted metal plate and an overlying magnet to generate reproducible tissue necrosis. Osmotic minipumps were implanted into 24 rats divided into four equal groups to infuse vehicle (control), ATL-146e (0.004 µg·kg−1·min−1), ATL-146e plus an equimolar concentration of A2A antagonist, ZM-241385, or ZM-241385 alone. Each group received 10 I/R cycles. In non-I/R-treated skin, ATL-146e has no effect on blood flow. I/R-treated skin of the ATL-146e group compared with the vehicle group had 65% less necrotic area, 31% less inhibition of average skin blood flow, and fewer extravasated leukocytes (23 ± 3 vs. 49 ± 6 per 500 µm2). These data suggest that ATL-146e, acting via an A2A-AR, reduces leukocyte infiltration and is a potent prophylactic for I/R injury in skin.

ischemia-reperfusion; leukocyte extravasation; magnetic force; skin necrosis

CHRONIC PRESSURE ULCERS occur in 16% of all hospitalized patients. A major causative factor is cyclic ischemia-reperfusion (I/R) injury or the cyclic attenuation of blood flow to the skin. In the clinic, the force of a patient’s bony prominence, such as a trochanter or sacrum, on a hospital bed compresses the interposed skin and muscle, and the amount of force applied over the compressed tissue creates pressure that is large enough to significantly reduce perfusion (8). When the pressure is applied and relieved in a frequent cyclic manner, as occurs in the bedridden patient, I/R injury to the skin and muscle can result in a chronic pressure ulcer (11).

The complete etiology of pressure ulceration is not yet fully understood; however, there are a number of proposed mechanisms for tissue damage resulting from I/R insult. When an ischemic tissue has been depleted of its blood supply for a significant amount of time, the tissue may reduce its metabolism to preserve function (12). Subsequent reperfusion of blood to the nutrient- and oxygen-deprived tissue can result in a cascade of harmful events including the overproduction of cytotoxic oxygen-derived free radicals that exceeds the capacity of normally present free-radical scavenging mechanisms and endothelial cell swelling that limits perfusion (19). Free radical oxidative injury can cause endothelial cells to release cytokines and express adhesion molecules that recruit circulating leukocytes to the injured vasculature (7). Adherent leukocytes release toxic reactive oxygen intermediates and can alter the permeability of postcapillary venules, thus propagating tissue damage.

Selective A2A adenosine receptor (A2A-AR) activation by 4-[3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxytetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl]-cyclohexanecarboxylic acid methyl ester (ATL-146e, formerly DWH-146e) (32) has been shown to significantly reduce I/R injury in rat kidney (20). This occurs at doses that are subthreshold for hemodynamic changes and probably results, at least in part, from a direct effect on neutrophils and/or endothelial cells that reduces inflammation. It has been shown that the binding of A2A agonists to receptors expressed on activated neutrophils reduces the release of reactive oxygen species and leukocyte adhesion (30). Furthermore, it has been shown that 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo [2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol (ZM-241385) is a potent and selective antagonist of A2A-ARs that blocks the renal protective effects of ATL-146e when administered simultaneously at A2A selective doses (20, 22). The purpose of this study was to use a novel animal model of the chronic pressure ulcer to test the hypothesis that the potent A2A-AR agonist ATL-146e will prevent I/R injury in the skin, and thus act as a prophylactic for pressure ulcer formation. The results show that this synthetic adenosine analog acts in ischemic skin through an A2A receptor to

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decrease the amount of tissue necrosis, limit loss of blood flow, and limit the number of extravasating leukocytes, thereby reducing overall skin injury in the pressure ulcer model.

MATERIALS AND METHODS

Surgery and experimental protocol. Ischemia is defined in this study as a reduction of tissue oxygen supply resulting from a compression-induced 50–100% decrease in blood flow to a region of skin. Relief of surface pressure and return of blood flow to the ischemic tissue region is termed the reperfusion event. I/R injury was induced by applying and removing a rectangular permanent magnet (4.0 cm × 2.25 cm × 1.0 cm) to a dorsal region of rat skin under which a ferromagnetic steel plate was implanted, as shown in Fig. 1. The application of the magnet compressed the skin and reduced blood flow, thus causing ischemia, whereas the removal of the magnet allowed reperfusion of blood to the ischemic skin region. A point-probe laser Doppler flowmeter was inserted through a small hole in the magnet to confirm that, when applied to the skin, the magnet reduced skin blood flow to <20% of normal flow for the duration of its application (21). When the 1,250-Gauss magnet was applied to the skin to cause the ischemic phase of the I/R cycle, it was held to the skin purely by magnetic attraction and produced 50 mmHg of pressure that approximates clinically relevant pressure (36).

Each of 24 adult male Sprague-Dawley rats (250–300 g; Hilltop Lab Animals; Scottsdale, PA) underwent the same surgical procedure for implantation of the steel plate. After the rat was anesthetized with 50 mg/kg of pentobarbital ip, the stabilized steel plate was inserted under the dorsal region of skin on the right side as previously described (21). No bandages were used because there was negligible bleeding. The contralateral side did not receive an implanted plate and served as a control for each animal.

Osmotic minipumps (model 1003D, Alzet; Palo Alto, CA) were filled with one of four solutions prepared in PBS-0.01% DMSO: 1) ATL-146e (0.004 μg·kg⁻¹·min⁻¹), 2) ATL-146e + ZM-241385, a selective A₂A antagonist (molar equivalent to the amount of ATL-146e), 3) ZM-241385, (molar equivalent to the amount of ATL-146e), and 4) vehicle (PBS-DMSO). Each rat received an osmotic minipump implanted subcutaneously under the region of skin located between the shoulder blades. The rats were returned to their cages and allowed to recover from anesthesia for 5 h after which the prescribed I/R cycles were initiated.

All 24 rats (divided into four groups of six rats each for the four treatments) in the 72-h study were subjected to the same I/R protocol (Fig. 2). This protocol was selected because the 2-h period of ischemia, followed by 0.5-h reperfusion, is clinically relevant. Patients at risk for developing pressure ulcers should be turned at least every 2 h (23). This I/R protocol in the rat was previously shown to produce significant skin necrosis with ulcer formation (21). All of the cycles were administered while the animals were unanesthetized. The animals were allowed free access to water and regular chow and were allowed to roam freely in their cages in a controlled vivarium throughout the compression cycles. Animals were individually housed and cared for under approved guidelines, with a protocol approved by the University of Virginia Animal Research Committee, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International Public Health Service Animal Assurance. After the second and final 12-h period of I/R treatment, 31 h of reperfusion preceded euthanization of the animals and analysis of their dorsal skin.

Skin blood flow. Average skin blood flow of the treatment site exposed to cyclic I/R injury and the contralateral untreated skin were quantified using a quantitative laser Doppler flowmeter (Lica Perfusion Image Monitor, Lica; North Brunswick, NJ). The laser Doppler flowmeter measures to a depth of ~300 μm into the skin (9). Blood flow scans were obtained before plate insertion for each rat. The average blood flow was obtained over the area targeted for subsequent ischemia and over the contralateral side. These images were used for baseline readings. Blood flow scans were also taken before the first compression (t = 5 h), immediately after the first compression (t = 7 h), after the first reperfusion period (t = 7.5 h), and at final analysis (t = 72 h after minipump implantation) in the ischemic nonnecrotic region.

Necrotic tissue. At analysis, each treatment site was photographed with a digital camera (model DC120, Kodak; Rochester, NY). The wound was digitized using computer software and the border of the necrotic area (black eschar) was manually traced. Computer software was used to calculate the necrotic area within the outlined border (WoundTrak98, Clinically Effective Outcomes; Charleston, SC). The area of the necrotic tissue was calculated as a percentage of total flap area (4.0 cm × 2.25 cm).

Histology. Full-thickness biopsies (including the panniculus carnosus muscle layers) for histological analysis were obtained from each treated site and the corresponding nonischemic contralateral site. For each treated site, three contiguous samples were obtained from nonischemic skin adjacent to ischemic zone, ischemic skin that was not necrotic, and ischemic skin that was necrotic. The samples were contiguous from the outside margin to the center of the treated area. All samples were fixed in neutral buffered formalin (10%), embedded in paraffin, sectioned perpendicular to the skin surface at 4 μm thickness, and stained with hematoxylin and eosin. The number of extravasated leukocytes was counted in sections taken from the ischemic nonnecrotic zone. Extravasated leukocytes were counted and subsequently averaged in fifty-four 500-μm² fields of view in 18 sections per experimental group under ×400 magnification. The total number of intravascular leukocytes was also counted in each venule with diameter 30 μm or larger in fifty-four 500-μm² fields of view in 18 sections per experimental group under ×400 magnification. Total intravascular leukocytes were counted because it was impossible to conclusively distinguish adherent leukocytes versus nonadherent leukocytes proximal to the vessel wall.

Cell culture. Transfected HEK cells were grown under 5% CO₂-95% O₂ humidified atmosphere at a temperature of 37°C. Transfected cells were maintained in DMEM supple-
A3 receptors, rat cortex (a rich source of A1 receptors), or rat
G418.

Using 500 A3 cDNAs were subcloned into pDoubleTrouble (25). The
described (6). Stable clones of A3 and A2B lines were selected
produced into HEK-293 cells by means of lipofectin as previously
reported here, ATL-146e, is a C2-modified derivative of aden-
osine-5'-ethyluronamide (NECA). Both C2 and 5'-hydroxyl
modifications to the adenosine core have been shown through
structure activity relationships (SAR) to be important in
imparting potency and selectivity for the adenosine A2A
receptor. The synthesis of ATL-146e was accomplished through
the palladium-catalyzed cross-coupling reaction of 2-ido-
NECA (3) and methyl-4-prop-2-ynylcyclohexanecarboxylate.
This alkyne was realized in five steps from commercially
available trans-[4-(hydroxymethyl)cyclohexyl]methan-1-ol (24).

Radioligand binding studies. Rat A2B receptor cDNA was
prepared by reverse transcription polymerase chain reaction
from rat bladder and sequenced on both strands in the
University of Virginia biomolecular core laboratory. A2B and
A3 cDNAs were subcloned into pDoubleTrouble (25). The
plasmids were amplified in competent JM109 cells, and plas-
mid DNA was isolated using Wizard Megaprep columns
(Promega; Madison, WI). Rat adenosine receptors were intro-
duced into HEK-293 cells by means of lipofectin as previously
described (6). Stable clones of A3 and A2B lines were selected
for A2A (10, 14), [125I]2-[2-(4-amino-3-iodobenzyl)-8-oxyacetate-1-propyl-xanthine for A2B
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imparting potency and selectivity for the adenosine A2A
receptor. The synthesis of ATL-146e was accomplished through
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NECA (3) and methyl-4-prop-2-ynylcyclohexanecarboxylate.
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available trans-[4-(hydroxymethyl)cyclohexyl]methan-1-ol (24).

Confluent monolayers of HEK cells expressing rat A2B and
A3 receptors, rat cortex (a rich source of A1 receptors), or rat striatum (a rich source of A2A receptors) were homogenized in
HE buffer (10 mM HEPES, 1 mM EDTA, pH 7.4) with
protease inhibitors (10 μg/ml benzamidine, 100 μM phenyl-
methanesulfonyl fluoride, and 2 μg/ml each of aprotonin,
pepsatin, and leupeptin). The membranes were homoge-
nized in a polytron (Brinkmann) for 20 s and centrifuged at
30,000 g, and the pellets were washed twice in HE buffer
with protease inhibitors. The final pellet was resuspended in
buffer HE, supplemented with 10% sucrose, and frozen in
aliquots at −80°C. For binding assays, membranes were
thawed and diluted 5- to 10-fold with HE to a final protein
concentration of ~1 mg/ml. To determine protein concentra-
tions, membranes and bovine serum albumin standards were
dissolved in 0.2% NaOH-0.01% SDS, and protein was deter-
mied using fluorescamine fluorescence (29).

Saturation binding assays for rat adenosine receptors were
performed, respectively, with 125I-labeled N6-aminobenzyl-
adenosine for A1 and A3 (10, 14), [125I]2-2-[2-(4-amino-3-iodo-
phenyl)ethylamino]adenosine for A2A (16), and [125I]3-(4-
amino-3-iodobenzyl)-8-oxoacetate-1-propyl-xanthine for A2B
(15). Nonspecific binding for all receptors was defined using
100 μM of NECA, except A2A (1 μM of ZM-241385). Radioli-
gand binding experiments were performed in triplicate with
20–25 μg of membrane protein in a total volume of 0.1 ml of
HE buffer supplemented with 1 U/ml adenosine deaminase
and 5 mM MgCl2. The incubation time was 3 h at 21°C. Competition experiments were carried out using 0.5–1 nM
radioligands and seven concentrations of competing ligands.
Membranes were filtered on Whatman GF/C filters using a
Brandel cell harvester (Gaithersburg, MD) and washed three
times for 15–20 s with ice-cold buffer (10 mM Tris, 1 mM
MgCl2, pH 7.4). Maximum binding and dissociation constant
values were calculated by Marquardt’s nonlinear least
squares interpolation for a single site-binding model (17).
Inhibitory constant values for different compounds were de-
ferred from IC50 values as described (13). Data from triplicate
experiments are tabulated as means ± SE.

Statistical analysis. A one-way analysis of variance test
was performed for each of the three experiments using Corel
Quattro Pro software, and each group had a P value <0.05. A
two-sample Student’s t-test assuming unequal variance was
performed on each subsequent group in the experiment to
determine significant differences within a 95% confidence
interval.

RESULTS

Repeated cycles of I/R sufficient to reduce blood flow by 80% (21) induced significant necrosis in the dorsal
rat skin. The progression of the tissue damage mimick-
ed that seen in patients confined to bed for extended
periods. The center of the I/R-treated skin presented
with erythema that gradually spread radially outward
from the center. The inflamed tissue became non-
blanchable over time, the wound edge became edema-
tous, and black eschar eventually formed in the wound.

The amount of necrotic tissue in the skin treated
with the I/R cycles was significantly reduced (P < 0.05)
in the group infused subcutaneously with ATL-146e,
to about one-third of that in the vehicle-treated, the ATL-146e +
ZM-241385, and the ZM-241385 alone groups (Fig. 3
and Table 1). The number of extravasated leukocytes
in the ischemic area following the number of intrava-
sated leukocytes follows the same
number of I/R cycles was significantly reduced (P < 0.05)
in the group infused subcutaneously with ATL-146e,
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in the ischemic area following the number of intrava-
sated leukocytes follows the same
number of I/R cycles was significantly reduced (P < 0.05)
in the group infused subcutaneously with ATL-146e,
The amount of blood flow in the ischemic zone was reduced after the cycles of I/R and the reduction was significantly less in animals receiving ATL-146e than in animals receiving vehicle, ATL-146e
2
ZM-241385, or ZM-241385 (Table 1). However, after a single I/R cycle there was no significant difference in blood flow reduction among the four treatment groups, as illustrated in Fig. 5, which shows the amount of blood flow reduction as a percentage of contralateral side blood flow after the first ischemic period (7 h) and after the first reperfusion period (5 h).

For comparison, Table 2 shows the corresponding leukocyte and blood flow data for the contralateral sides not exposed to any I/R cycles. In contrast to the I/R-treated side (Table 1), there was no significant difference in any of the measured parameters for the contralateral sides across all treatment groups. Additionally, there was no necrosis in the contralateral side.

Previous radioligand binding studies indicate that ATL-146e is a selective agonist and ZM-241385 is a selective antagonist of the human A2A-AR over the other three AR subtypes (32, 20). We determined for the first time the binding affinities of these compounds for the four rat AR subtypes. The results are shown in Fig. 6 and Table 3. ATL-146e is highly selective for rat A2A over A2B receptors but displays only moderate selectivity for A2A over A1 and A3 receptors. Table 3 shows that compared with the A2A receptor, ATL-146e has a sixfold lower affinity for the A1 receptor subtype and a 1.6-fold lower affinity for the A3 receptor subtype. However, agonist binding affinity to A2A receptors may be underestimated because these receptors are poorly coupled to G proteins (16). Furthermore, the affinity of ZM-241385 for the A1 and A3 receptor subtypes is 1,120- and 62,300-times lower than its affinity for the A2A subtype. Blockade of the effects of ATL-146e on skin ulceration by equimolar concentrations of ZM-241385 provides strong evidence that these effects are mediated by A2A receptors.

**DISCUSSION**

In this study, a model of reproducible I/R skin injury was used to evaluate the prophylactic capabilities of ATL-146e, an A2A-AR agonist, to prevent skin pressure ulcer formation. This model used unanesthetized rats subjected to applied pressure magnitudes, pressure durations, and I/R cycle times that are clinically relevant to pressure ulcer formation. ATL-146e, administered throughout the ischemia and reperfusion periods at doses below the threshold for hemodynamic responses, substantially reduced the amount of necrosis due to I/R injury, reduced the degree of leukocyte extravasation in the ischemic zone, and lessened the reduction in blood flow that occurred in the ischemic zone after I/R. When ATL-146e was administered with the A2A-AR antagonist ZM-241385, all of the protective effects were blocked: 1) blood flow in the ischemic skin during reperfusion was significantly reduced, 2) leukocyte extravasation was increased, and 3) the amount of necrotic area was significantly increased to control levels where no protective measures were taken.

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**Table 1. Summary of the effects of ATL-146e, ATL-146e + ZM-241385, and ZM-241385 on pressure-induced skin necrosis, leukocyte extravasation, and blood flow in the nonnecrotic ischemic zone**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Necrotic Area, % of Ischemic Zone</th>
<th>Number of Extravasated Leukocytes, per 500 μm²</th>
<th>Number of Intravascular Leukocytes, per 500 μm²</th>
<th>Blood Flow, % Reduction vs. Contralateral Side</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, no I/R</td>
<td>0</td>
<td>12 ± 4</td>
<td>2 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>Vehicle</td>
<td>23 ± 6</td>
<td>49 ± 6</td>
<td>8 ± 3</td>
<td>64 ± 5</td>
</tr>
<tr>
<td>ATL-146e</td>
<td>8 ± 4*</td>
<td>23 ± 3*</td>
<td>5 ± 2*</td>
<td>33 ± 4*</td>
</tr>
<tr>
<td>ATL-146e + ZM-241385</td>
<td>20 ± 4</td>
<td>42 ± 4</td>
<td>9 ± 3</td>
<td>67 ± 7</td>
</tr>
<tr>
<td>ZM-241385</td>
<td>21 ± 4</td>
<td>42 ± 3</td>
<td>10 ± 4</td>
<td>57 ± 9</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 rats in treated groups. Data were collected 72 h after the first ischemia-reperfusion (I/R) cycle was initiated. Leukocyte counts were measured in the nonnecrotic ischemic zone. *Significant difference from vehicle-treated group (P < 0.05).
Radioligand binding studies suggest that the protective effects conferred by ATL-146e are a result of specific $\text{A}_{2A}$ interactions and not of $\text{A}_1$, $\text{A}_{2B}$, or $\text{A}_3$ interactions as indicated by the relatively high affinity of ATL-146e for the $\text{A}_{2A}$ receptor subtype. Furthermore, the data indicate a more than 1,000-fold selectivity of the antagonist ZM-241385 for the rat $\text{A}_{2A}$-AR over the $\text{A}_1$ and $\text{A}_3$ subtypes. When ZM-241385 is administered with ATL-146e, the protection against pressure ulcer formation is completely reversed. Therefore, we conclude that the protective effects are related to the selective activation of the $\text{A}_{2A}$ receptor subtype by ATL-146e.

It is also important to note that in the absence of an agonist, ZM-241385 has no effect on purified neutrophils. This has been demonstrated in previous studies that measured cAMP accumulation, superoxide production, and adherence (31). Furthermore, our study with the $\text{A}_{2A}$ receptor antagonist alone confirms that it has no effect on ulcer formation as indicated by the

![Fig. 4. Photographs showing typical pressure-induced leukocyte extravasation in skin after I/R. A: control, B: vehicle, C: ATL-146e, D: ATL-146e + ZM-241385, (ZM-241385 group not pictured.) (See Table 1). The location where the section was obtained is designated on Fig. 3 by an asterisk for each panel. Dark globular dots are extravasated leukocytes. Venules are indicated by arrows (stained with hematoxylin and eosin, ×200 magnification). E: close-up view of a venule containing intravascular leukocytes (arrows) from ATL-146e group (stained with hematoxylin and eosin, ×400 magnification).](http://ajpheart.physiology.org/)

![Fig. 5. Reduction in blood flow as a percentage of contralateral side blood flow. Differences in blood flow reduction among the four treatment groups are insignificant after the first I/R cycle.](http://ajpheart.physiology.org/)

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Number of Extravasated Leukocytes, per 500 $\mu$m²</th>
<th>Number of Intravascular Leukocytes, per 500 $\mu$m²</th>
<th>Blood Flow, % Reduction vs. Flow at Time 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>$10 \pm 3$</td>
<td>$3 \pm 3$</td>
<td>$1 \pm 3$</td>
</tr>
<tr>
<td>ATL-146e</td>
<td>$13 \pm 3$</td>
<td>$3 \pm 2$</td>
<td>$4 \pm 4$</td>
</tr>
<tr>
<td>ATL-146e + ZM-241385</td>
<td>$10 \pm 4$</td>
<td>$2 \pm 3$</td>
<td>$4 \pm 3$</td>
</tr>
<tr>
<td>ZM-241385</td>
<td>$11 \pm 3$</td>
<td>$2 \pm 2$</td>
<td>$8 \pm 3$</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SD. Data were collected 72 h after the first I/R cycle was initiated.
insignificant differences in necrotic area, skin blood flow, and leukocyte extravasation from vehicle control values. Because the degree of ulceration was not augmented by administering the antagonist, we conclude that adenosine accumulation in the ischemic tissue during I/R injury does not offer significant protection against I/R injury by activating A2A receptors. Because adenosine is known to accumulate in ischemic tissues, this suggests that ATL-146e acts outside the ischemic zone, e.g., on circulating leukocytes. Failure by ZM-241385 to augment ischemic injury does not rule out the possibility that adenosine that does accumulate in the ischemic tissue may produce some degree of protection through other adenosine receptor subtypes.

The protection offered by ATL-146e against necrosis and skin inflammation seen in this study is similar to the type of response noted previously in a rat kidney model of I/R (20). The mechanism of this protection is uncertain, but it may be due in part to inhibition by A2A agonists of neutrophil adhesion to endothelial cells and inhibition of neutrophil activation (31). It is possible that suppression by A2A agonists of several different inflammatory cell types via various mechanisms contributes to this response (30).

Previous studies conducted on neutrophils in vitro suggest mechanisms for this protection. The A2A receptor subtype is responsible for inhibiting leukocyte adhesion to endothelial cells (5) possibly via an intracellular signaling mechanism involving cAMP (35). A2A-ARs have also been shown to inhibit the production of tumor necrosis factor-α, an inflammatory cytokine, by stimulated monocytes (34). In contrast to the
A2A receptor activation that inhibits neutrophils, activation of A1 or A3 receptor subtypes stimulates leukocyte chemotaxis (4), adhesion to endothelial cells (5), and phagocytosis of immunoglobulin particles (26). Hence, activation of A1 or A3 receptors is proinflammatory rather than anti-inflammatory.

Endothelial cells also express adenosine receptors, and the A2A-ARs have been reported to be involved with endothelial cell migration and proliferation (28). A2A-ARs diminish endothelial expression of E-selectin and secretion of the inflammatory cytokines interleukin-6 and interleukin-8 (2). Thus adenosine acting at A2 receptors inhibits leukocyte recruitment to inflamed tissue (27). This mechanistic evidence strengthens the conclusion that the ATL-146e interaction with the A2A adenosine receptor subtype, and not with A1 or A3 adenosine receptor subtypes, on both neutrophils and endothelial cells is responsible for the protection against skin ulcer formation in this study.

In speculating about a mechanism for the protective effect of ATL-146e, it should also be noted that the protection is not a consequence of a direct hemodynamic response. Previous studies using the ATL-146e in models of I/R in other tissues indicate that the compound has no effect on heart rate, cardiovascular output, and vascular resistance at the dose used in this study (20). Moreover, this study showed that immediately after the first ischemic event, the reduction in skin blood flow was not significantly different between any of the four experimental groups. Similarly, the blood flow reduction after the first reperfusion event was not significantly different between groups (Fig. 5). This suggests that because the A2A receptor agonist did not rapidly alter hemodynamics, the ATL-146e did not function as a direct vasodilator in this system. However, because the blood flow reduction was decreased significantly in ischemic skin of the ATL-146e-treated group compared with the other three treatment groups at the end of the 72-h study (Table 1), this late change in blood flow is likely secondary to the anti-inflammatory action of the compound. Therefore, we conclude that the improved perfusion in the ATL-146e-treated group over the 72-h study is not due to a short-term hemodynamic effect but may be secondary to its effects on inflammation.

A2A-AR agonist ATL-146e did not alter vascular reactivity. The contralateral side did not exhibit significantly altered blood flow at the end of the 72-h study for any of the four treatment groups: vehicle, ATL-146e, ATL-146e + ZM-241385, or ZM-241385 (Table 2). Similarly, numbers of extravasated and intravascular leukocytes at the contralateral site were not significantly different between any of the treatment groups (Table 2). Furthermore, histological full-thickness sections were obtained at analysis and stained with hematoxylin and eosin. No significant morphological differences were seen in the dermis, epidermis, or blood vessels of the contralateral side among the four experimental groups (data not shown). This evidence allows us to suggest that there is no effect of ATL-146e on normal skin. Adenosine can precondition against ischemic damage in heart and other tissues, but these effects are mediated by A1 or A3 receptors, not A2A receptors (1). Thus the results reported in this study suggest a direct effect of the adenosine receptor agonist during the I/R cycles on neutrophils and/or endothelial cells to reduce inflammation.

We therefore conclude 1) endogenous adenosine is not sufficient for optimally protecting against ulcer formation in this experimental model, and 2) addition of a highly selective A2A receptor agonist offers significant protection against ulcer formation by enhancing adenosine/A2A receptor interactions above that occurring in normal conditions.

Additional experimentation will be necessary to evaluate the effects of administering ATL-146e at different time points in the I/R cycle. The extent and duration of the prophylactic effect should also be examined. The potential therapeutic benefit of treating bedridden patients with ATL-146e or a similar compound are great. For example, the compound might be used as a preventative treatment that could be administered to any patient who is identified as “at risk” before entering the hospital. If treatment requires them to be bedridden or immobile for an extended period of time.

Over two million hospital and nursing home patients in the United States are annually afflicted with new chronic pressure ulcers (33). Furthermore, the estimated average total cost per year to treat pressure ulcers ranges from 1.5 billion dollars to 10 billion dollars across all treatment settings (18, 36). The widespread occurrence of pressure ulcers and high cost to treat them in the clinical setting underscores the need to develop new treatments. This clinically relevant animal model for a reproducible pressure ulcer has provided evidence that the A2A-AR agonist ATL-146e significantly reduces the amount of I/R injury in skin and has the potential to alleviate the formation of chronic pressure ulcers.

We especially thank Dr. George Rodeheaver (Dept. of Plastic Surgery, University of Virginia) for collaboration in the pressure ulcer model development and Li Ping (Dr. Mark Okusa Laboratory, University of Virginia) for help with the subcutaneous implant surgery. Dr. Dianne Rosin (Dept. of Pharmacology, University of Virginia) provided rat striatal membranes, and Mellissa Marshall

### Table 3. Dissociation binding constants of ATL-146e and ZM-241385 for the four subtypes of rat adenosine receptors

<table>
<thead>
<tr>
<th>Rat AR Subtype</th>
<th>$K_i, \text{ nM}$</th>
<th>Selectivity</th>
<th>$K_i, \text{ nM}$</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_1$</td>
<td>27.1 ± 4.1</td>
<td>6.0</td>
<td>541 ± 83</td>
<td>1,120</td>
</tr>
<tr>
<td>$A_{2A}$</td>
<td>4.5 ± 1.8</td>
<td>1</td>
<td>0.48 ± 0.08</td>
<td>1</td>
</tr>
<tr>
<td>$A_{2B}$</td>
<td>51,300 ± 10,300</td>
<td>11,400</td>
<td>707 ± 105</td>
<td>1,460</td>
</tr>
<tr>
<td>$A_3$</td>
<td>7.0 ± 1.7</td>
<td>1.6</td>
<td>30,200 ± 2,700</td>
<td>62,300</td>
</tr>
</tbody>
</table>

Values are means ± SD. Selectivity values represent a times less affinity relative to the $A_{2A}$ receptor. Data are derived from triplicate experiments, each consisting of 6–7 concentrations of competing compound assayed in triplicate.
Rat A2B cDNA was prepared by Yuan-Ji (Linden Laboratory). This work was supported by the Whitaker Foundation and National Heart, Lung, and Blood Institute Grant RO1-HL-37942.

REFERENCES


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