Anodal Iontophoresis of a Soluble Guanylate Cyclase Stimulator Induces a Sustained Increase in Skin Blood Flow in Rats

Sylvain Kotzki, Matthieu Roustit, Claire Arnaud, Jean Boutonnat, Sophie Blaise, Diane Godin-Ribuot, and Jean-Luc Cracowski

INSERM U1042, Grenoble, France (S.K., M.R., C.A., S.B., D.G.-R., J.-L.C.); Université Joseph Fourier, CNRS UMR 5525, Grenoble, France (J.B.); Université Joseph Fourier, Clinical Pharmacology Unit–INSERM CIC03, Grenoble, France (S.K., M.R., J.-L.C.); and Vascular Medicine Department, Grenoble University Hospital, Grenoble, France (S.B.)

Received April 26, 2013; accepted July 8, 2013

ABSTRACT

The treatment of systemic sclerosis–related digital ulcers is challenging. Although the only effective drugs are prostacyclin analogs, their use is limited by vasodilation-related adverse reactions. In this study, we assessed the local iontophoresis administration of three soluble guanylate cyclase [A-350619 [3-[2-[[4-chlorophenyl]sulfanyl]phenyl]-N-[4-(dimethylamino)butyl]-2-propenamide hydrochloride], SIN-1 [α-morpholino-1, 2,3-oxadiazolium chloride], and CMF 1571 [3-[3-[dimethylamino]propoxy]-N-[4-methoxyphenyl]-1-(phenylmethyl)-1H-pyrazole-5-carboxamide hydrochloride] and two nonprostanoid prostaglandin I2 (prostacyclin) receptor agonists (MRE-269 [4-[[6, 6-diphenylpyrazinyl][1-methylthyl]aminobutoxy]-acetic acid] and BMY 45778 [3-(4,5-diphenyl[2,4-bioxazol]-5-yl)phenoxycetic acid]) to induce vasodilation onto the hindquarters of anesthetized rats. Skin blood flow was quantified using laser Doppler imaging during the whole experience, and safety was assessed by continuous recording of blood pressure and histopathological examination. Anodal iontophoresis of A-350619 (7.54 mM) induced a sustained increase in cutaneous blood flow (P = 0.008 vs. control). All other drugs exhibited poor or no effect on skin blood flow. Vasodilation with A-350619 iontophoresis was concentration-dependent (7.5, 0.75, and 0.075 mM; P < 0.001, Jonckheere-Terpstra trend test), and repeated administrations do not suggest any risk of tolerance. This study also compared continuous versus intermittent iontophoresis protocols. Continuous anodal iontophoresis of A-350619 at 7.5 mM increases cutaneous blood flow with good local tolerance. Iontophoresis of soluble guanylate cyclase stimulators should be investigated as potential local therapy for digital ulceration in patients with scleroderma.

Introduction

Systemic sclerosis (SSc) is a rare disease affecting the skin microcirculation. Its pathophysiology involves an early microvascular dysfunction, associated with autoimmunity and cutaneous collagen deposition. Raynaud’s phenomenon is the early manifestation of the vasculopathy associated with the disease, and may develop into digital ulcerations and gangrene (Herrick, 2000). Digital ulcerations affect 43% of patients with limited cutaneous systemic sclerosis and 51% of those with diffuse cutaneous systemic sclerosis (Hachulla et al., 2007). The treatment of SSc-related ulcers remains challenging. Bosentan, a nonspecific endothelin receptor antagonist, has been indicated to prevent digital ulcers in patients at risk, but has no effect on existing ulcers (Korn et al., 2004). In addition, an elevated aminotransferase level is the main adverse effect, with an annual rate of 10.1%, leading to therapy discontinuation in 3.2% of bosentan-naive patients (Humbert et al., 2007). Prostacyclin analogs are used intravenously (Wigley et al., 1992), and iloprost is the only drug available for treating existing ulcerations (Wigley et al., 1998). However, the therapeutic effect is counterbalanced by serious dose-limiting side effects related to the potent induced vasodilatation (e.g., severe headaches, flushing, tachycardia, and hypotension). In a recent study, sildenafil, a phosphodiesterase type 5 inhibitor, seemed to show benefits in digital ulcer healing, but the lack of a placebo group in the design was a major limitation to a formal conclusion (Brueckner et al., 2010).

The topical administration of these drugs may be a way of getting around the toxicity of systemic treatments. Iontophoresis is a simple, noninvasive transdermal drug delivery method using a low-intensity electric current (Kalina et al., 2004). Some authors have highlighted the potential interest in iontophoresis of vasodilating drugs as a treatment of digital ulcers in SSc (Murray et al., 2005, 2008). Previous work from our laboratory showed that bosentan was not an appropriate candidate for iontophoresis (Roustit et al., 2012). In addition, although sodium nitroprusside, an indirect nitric oxide (NO)
donor, can be delivered through iontophoresis (Blaise et al., 2010), its short half-life does not make it a promising candidate. On the other hand, the iontophoresis of prostacyclin analogs (iloprost and treprostinil) showed promise at inducing a sustained increase in cutaneous flow in rats (Blaise et al., 2011). A first study with iloprost in healthy volunteers was performed to confirm these results in humans; unfortunately, the study was aborted because of significant side effects. Afterward, a second study with treprostinil was realized in healthy volunteers. In this study, we demonstrated the ability of treprostinil to increase cutaneous flow without any side effect (Blaise et al., 2012).

Although the iontophoresis of treprostinil is currently being tested in patients with SSc (ClinicalTrials.gov identifier: NCT01554540), screening other agonists with vasodilator potency could open new perspectives. The first target is the prostaglandin I2 receptor (IP). In addition to the classic prostacyclin analogs, nonprostanoid IP agonists are smaller molecules that have an effect of comparable intensity to that of treprostinil (Woodward et al., 2011). The second target is soluble guanylate cyclase (sGC), a key signal-transduction enzyme activated by NO. Compounds that activate sGC in an NO-independent manner lead to vascular smooth muscle cell relaxation, and may provide considerable therapeutic advantages (Evgenov et al., 2006). Moreover, recent findings have suggested an antifibrotic effect of sGC stimulators in different experimental models of SSc (Beyer et al., 2012). Finally, these nonprostanoid IP agonists and sGC stimulators could be interesting candidates for therapeutic iontophoresis in SSc.

The main objective of this study was to assess whether iontophoretically administered nonprostanoid IP agonists and sGC stimulators increase cutaneous blood flow in rats. As secondary objectives, we also tested their safety, tolerance after repeated administrations, as well as the effect of different drug concentrations and different protocols of administration to enhance drug transport through the skin.

**Materials and Methods**

**Animals**

Forty-four male Wistar rats (8 weeks old, 275–290 g; CERJ, Le Genest-St-Isle, France) were housed in controlled conditions conforming to the current French legislation, and were fed with standard rat chow. The protocol was approved by the Grenoble Animal Ethics Committee. Rats were kept in a day/night cycle of 12/12 hours with food and water at will. Rats were shaved as previously described (Blaise et al., 2011; Roustit et al., 2012).

**Drugs**

**Virtual Screening.** We selected potential candidate drugs with a three-step filter procedure using the following criteria: 1) good affinity for the target site (sGC or IP receptor); 2) suitable physical and chemical properties with molecular mass $<500$ Da, $\log D_0 < 5$ ($\log P$ for pH 6), and polar surface area $<140$ Å²; and 3) ionized molecules at cutaneous physiologic pH.

**Drug Supply and Preparation.** Five candidates were retained as follows: two nonprostanoid IP agonists [BMY 45778 (3-[2-(4-chlorophenylsulfonyl)phenyl]-N-(4-dimethylaminobutyl)acrylamide hydrochloride) (Miller et al., 2003); SIN-1 (amino-3-morpholinyl-1,2,3-oxidiazolium chloride) (Maurice and Haslam, 1990); and CFM 1571 (3-[3-(dimethylamino)proproxyl]-N-(4-methoxyphenyl)-1-phenylmethyl)-1H-pyrazole-5-carboxamide hydrochloride) (Evgenov et al., 2006) (Fig. 1)]. A-350619 hydrochloride ($M_r$ 425.41), SIN-1 chloride ($M_r$ 521.13), CFM 1571 hydrochloride ($M_r$ 444.95), and BMY 45778 ($M_r$ 438.44) were purchased from Tocris Bioscience (Bristol, UK). MRE-269 ($M_r$ 419.5) was purchased from Cayman Chemical (Ann Arbor, MI). Inorganic sodium chloride (0.9% NaCl; Aguet, Lyon, France) was used as vehicle for sGC stimulators. We prepared specific vehicles for MRE-269 (1:3 solution of ethanol:phosphate-buffered saline, pH 7.2) and for BMY 45778 (0.9% NaCl with 0.1% dimethylsulfoxide and 1% ethanol), as advised in the product information. These vehicles were used as controls for the corresponding drugs.

**Experimental Procedures**

The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and were maintained in the prone position for the duration of the experiment. Experiments were performed in a temperature-controlled room, and the rats were placed on a thermal pad, with the temperature
maintained at 37.5°C (Harvard Apparatus, Holliston, MA). Before iontophoresis, each rat was inspected to ensure that the hairless skin on the back and the hind legs was intact. Rats were then equipped with three 1.2-cm\(^2\) circular iontophoresis AgCl electrodes (LI 611, PeriIont System; Perimed, Järnäilla, Sweden) containing drugs or vehicles, as described previously (Blaise et al., 2011). Passive electrodes (PF 384, PeriIont System; Perimed) were placed on the back of the neck, and both electrodes were connected to a current generator (PF 382b, PeriIont System; Perimed).

**Experiment 1: Initial Screening.** Iontophoresis of 7.5 mM A-350619, 10 mM SIN-1, and 7.5 mM CFM 1571 was performed for 20 minutes using an anodal current (100 \(\mu A\)) because these agents are negatively charged at the skin and physiologic pH. They were compared with anodal iontophoresis of NaCl. To minimize the number of sacrifices, each animal received two electrodes filled with two different drugs and a third electrode filled with NaCl. With this distribution, only 12 animals were used to obtain \(n = 8\) for each drug and its simultaneous control (NaCl). Distribution of drugs was randomly generated. Iontophoresis of BMY 45778 and MRE-269 and their respective vehicles was performed for 20 minutes using a cathodal current (100 \(\mu A\)) because these agents are negatively charged at the skin and physiologic pH (\(n = 8\) for each series). Since vehicles were not the same for both drugs, the number of animals could not be reduced. We finally tested whether passive diffusion of the molecules had any significant effect on skin blood flow. The same iontophoretic protocol was used as follows: active electrodes were filled with drugs or vehicles but they were not connected to the current generator. The 20-minute application was followed by a 40-minute recording of skin blood flow.

**Experiment 2: Effect of Concentration and Safety.** We tested three different concentrations of the molecule that had shown significant effects on skin blood flow in experiment 1 (i.e., A-350619). Rats were equipped as described earlier. In addition, a catheter was inserted in the left carotid artery to continuously record blood pressure (Powerlab; ADInstrument, Colorado Springs, CO) from 5 minutes before to 60 minutes after iontophoresis. Photographs were taken before iontophoresis and immediately after iontophoresis to assess cutaneous tolerance. Negative reactions were coded grade 0; weak reactions were coded as grade 1 and are characterized by nonvesicular erythema. Strong positive reactions (grade 2) are characterized by erythema associated with vesicles. Extreme positive reactions (grade 3) are bullous reactions. Irritant reactions (coded grade 4) are characterized by necrosis.

Histopathologic examination of full-thickness skin biopsies from drug-treated and from one nontreated skin area was realized. Biopsies were fixed in acidified formal alcohol fluid (5% acetic acid, 75% absolute ethyl alcohol, and 18% water; Carlo Erba Reagents, Val de Reuil, France), paraffin embedded, and stained with hematoxylin, eosin, and safran. Specific features were sought to evaluate the effect of the treatment on the skin, including hyperkeratosis and epidermolytic aspects. In the stratum corneum, the degree of hyperkeratosis was evaluated, and the presence of any parakeratosis was noted. The granular layer was evaluated for perineurial vacuolar changes, cytolysis, and the appearance of keratohyalin granules. The spinous layer was investigated for the development of these features. We also assessed vasculitis, a histological diagnosis defined as inflammation targeting blood vessel walls and compromising their function, leading to hemorrhagic and/or ischemic events. Furthermore, any inflammation accompanied by infiltration of neutrophils, lymphocytes, or mast cells at the dermo-epidermal interface was evaluated.

**Experiment 3: Protocol Optimization and Cutaneous Resistance.** To determine whether an intermittent current could enhance the effect of iontophoresis (Nair and Panchagnula, 2004), we assessed the effect of an intermittent iontophoresis protocol for 7.5 mM A-350619 and NaCl (\(n = 8\)) on skin blood flow. We repeated 20 1-minute cycles of 10 seconds at 600 \(\mu A\) ("on") followed by 50 seconds without current ("off"). This series was compared with the continuous current condition (100 \(\mu A\), 20 minutes) in experiment 2.

In both protocols, the same amount of current (120 millicoulombs) was delivered.

Cutaneous resistance was determined during continuous-current iontophoresis with an amperometric biosensor unit. For these measurements, one electrode was connected to the passive probe on the neck of the rat and the other to a probe positioned on a hind leg. Measurements started 5 minutes after starting iontophoresis. This delay was chosen in pilot experiments and was aimed at monitoring the voltage plateau. Voltage (expressed in volts) was recorded, and skin resistance was calculated and expressed in ohms (Perrell et al., 2002).

**Experiment 4: Evaluation of Tolerance after Repeated Administrations.** As tolerance to sGC activation is a major issue with treatments involving the NO pathway, we tested whether repeated administrations of A-350619 decrease the vascular response over time. Eight animals were prepared as previously described. Two circular skin sites were marked and subsequently equipped with electrodes. Anodal iontophoresis of 7.5 mM A-350619 and NaCl was then simultaneously performed for 20 minutes at 100 \(\mu A\). The procedure was repeated 1 hour (H1) and 24 hours (H24) later, with the electrodes positioned exactly on the previously used skin sites.

**Skin Blood Flow Measurement and Data Analysis**

Skin blood flow was measured with laser Doppler imaging (PeriScan PIM3; Perimed). The scan resolution was 1.59 mm. Flow was averaged over 0.5-cm\(^2\) regions of interest, and scans were taken every minute for at least 5 minutes for the baseline (BL) recording and then for the next 60 minutes from the beginning of iontophoresis.

Data are expressed as arbitrary perfusion units. To take into account interindividual BL variations, data were subsequently expressed as a percentage change from BL (%BL), and then we calculated the area under the curve (AUC) from the beginning of iontophoresis to the end of the recording (AUC\(_{0-60}\)). Since %BLs, as primary outcome) and during the 20-minute iontophoresis (AUC\(_{0-20}\)) as secondary outcome). This choice reflects our objective to screen drugs exhibiting a sustained effect beyond the duration of iontophoresis itself.

**Statistical Analysis**

Continuous data are expressed as the mean ± S.D. Data were analyzed by repeated-measures analysis of variance (ANOVA) and paired \(t\) tests for \(2 \times 2\) comparisons. When the conditions of application of parametric tests were not fulfilled, nonparametric tests were used (Friedman test and Wilcoxon test for paired comparisons).

The Jonckheere-Terpstra trend test was used to assess the effect of concentration (experiment 2). A two-way ANOVA was used to assess tolerance (experiment 4), comparing AUCs over time between the two skin sites (A-350619 and control). Mauchly’s test of sphericity was used to assess equality of variance. As inequality of variance could not be excluded, Greenhouse-Geisser adjustment was used. We tested the effect of time, drug, and the interaction between time and drug. Two-sided significance tests were used throughout. We considered \(P\) values <0.05 as significant, corrected by Bonferroni’s method for multiple comparisons. Statistical analysis was performed with SPSS 13.0 for Windows (SPSS Inc., Chicago, IL).

**Results**

Effect of Iontophoresis of sGC Stimulators and Nonprostanoid IP Agonists: Initial Screening (Experiment 1). Anodal iontophoresis of A-350619 (7.5 mM) significantly increased AUC\(_{0-20}\) and AUC\(_{0-40}\) compared with NaCl (Fig. 2A; Table 1). On the other hand, iontophoresis of the other sGC stimulators (CFM 1571 at 7.5 mM and SIN-1 at 10 mM) as well as nonprostanoid IP agonists (MRE-269 at 0.6
mM and BMY 45778 at 0.052 mM) did not induce any significant change in skin blood flow compared with their respective vehicles (Fig. 2, B–E; Table 1). However, a transient vasodilation was observed for CFM 1571, as shown by the significant increase in AUC$_{0-20}$ versus NaCl (79,088 ± 27,383 and 15,812 ± 15,856 %BL, respectively; $P = 0.001$). Neither A-350619 nor CFM 1571 increased cutaneous flow when passively applied, i.e., in the same condition but without current (electrodes not connected to the generator).

**Concentration-Dependent Effect of Iontophoresis of A-350619 on Cutaneous Flow (Experiment 2).** There was a significant concentration-dependent effect between the three concentrations of A-350619 (7.5, 0.75, and 0.075 mM). AUC$_{0-60}$ were 26,580 ± 16,470, 72,701 ± 49,345, and 34,203 ± 77,796 %BLs, respectively ($P < 0.001$, Jonckheere-Terpstra trend test) (Fig. 3). Paired comparisons showed that A-350619 at 7.5 mM induced significantly increased skin blood flow as compared with 0.75 mM ($P = 0.025$) and 0.075 mM ($P = 0.017$) solutions.

**Comparison between Intermittent and Continuous Iontophoresis of A-350619 (Experiment 3).** Continuous and intermittent iontophoresis of A-350619 at 7.5 mM induced comparable vasodilation (AUC$_{0-60}$ were 265,807 ± 164,703 and 211,959 ± 136,821 %BLs, respectively; $P = 0.49$) (Fig. 4A). However, intermittent iontophoresis of 0.9% NaCl significantly increased skin blood flow as compared with continuous iontophoresis (111,696 ± 59,591 and 44,051 ± 55,963 %BLs, respectively; $P = 0.03$).

Cutaneous resistance between continuous iontophoresis of 7.5 mM A-350619 and 0.9% NaCl was not significantly different (28.5 ± 7 and 29.3 ± 3 kΩ, respectively; $P = 0.79$).

**Evaluation of the Tolerance after Repeated Administrations (Experiment 4).** Repeated administrations of 7.5 mM A-350619 and NaCl at T0, T1, and T24 did not significantly change skin blood flow over time. AUC$_{0-60}$ were 277,583 ± 145,094, 216,336 ± 157,945, and 303,332 ± 171,202 %BLs, respectively, for A-350619, and 19,888 ± 41,866, 21,283 ± 54,847, and 16,615 ± 58,921 %BLs, respectively, for NaCl. The influence of time was not significant ($P = 0.63$, two-way ANOVA), but the effect of the drug was significant ($P < 0.001$, two-way ANOVA). There was no significant interaction between time and drug ($P = 0.29$).

---

**Fig. 2.** Vascular effect of 20-minute 100-μA anodal iontophoresis of the soluble guanylate cyclase stimulators SIN-1 (10 mM) (A), CFM 1571 (7.5 mM) (B), and A-350619 (7.5 mM) (C) in comparison with 0.9% NaCl. Effect of 20-minute 100-μA anodal nonprostanoid prostacyclin receptor agonists BMY 45778 (0.052 mM) (D) and MRE-269 (0.6 mM) (E), in comparison with their respective vehicles, on cutaneous blood flow expressed as percentage of baseline.
two-way ANOVA), suggesting that the effect of the drug persists over time.

Skin and Systemic Safety of the Iontophoresis of A-350619. No side effects were observed on the skin. No significant drop in mean arterial pressure after iontophoresis with A-350619 was observed. During experiment 2, mean arterial pressure was 133.70 ± 16.79 mm Hg before and 126.63 ± 14.29 mm Hg during iontophoresis (n = 6; P = 0.2). Twenty-four skin biopsies were collected to evaluate skin tolerability (experiment 2). None of the histopathological features listed in Materials and Methods were found in any of the skin biopsies.

Discussion

For the first time, we show that anodal iontophoresis of an sGC stimulator (A-350619) induces a large, sustained, concentration-dependent increase in cutaneous blood flow in rats without skin or systemic toxicity.

TABLE 1
Effect of iontophoresis of nonprostanoid prostacyclin receptor agonists and stimulators of sGC on cutaneous blood flow
Data are expressed as the area under the curve of the percentage change from baseline (expressed as %BLs) during 60 minutes (20-minute iontophoresis and 40-minute rest).

<table>
<thead>
<tr>
<th></th>
<th>Active Drug</th>
<th>Vehicle</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sGC stimulators</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-350619 (7.5 mM)</td>
<td>319,896 ± 169,386</td>
<td>69,307 ± 50,999</td>
<td>0.008</td>
</tr>
<tr>
<td>CFM 1571 (7.5 mM)</td>
<td>141,533 ± 43,688</td>
<td>81,701 ± 74,991</td>
<td>0.06</td>
</tr>
<tr>
<td>SIN-1 (10 mM)</td>
<td>114,162 ± 46,056</td>
<td>76,488 ± 60,704</td>
<td>0.2</td>
</tr>
<tr>
<td>Nonprostanoid IP agonists</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMY 45778 (0.052 mM)</td>
<td>259,412 ± 73,032</td>
<td>236,622 ± 70,855</td>
<td>0.48</td>
</tr>
<tr>
<td>MRE-269 (0.6 mM)</td>
<td>214,540 ± 38,531</td>
<td>204,932 ± 67,030</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Fig. 3. Dose-dependent effect of anodal iontophoresis of the soluble guanylate cyclase stimulator A-350619 at 7.5, 0.75, and 0.075 mM on cutaneous blood flow expressed as percentage of baseline.
Fig. 4. (A) Comparison of continuous and intermittent anodal iontophoresis of the soluble guanylate cyclase stimulator A-350619 (7.5 mM) on cutaneous blood flow expressed as percentage of baseline. (B) Comparison of continuous and intermittent anodal iontophoresis of 0.9% NaCl on cutaneous blood flow expressed as percentage of baseline.
Iontophoresis refers to the facilitated movement of ionized molecules through application of an electrical field (Kalia et al., 2004). It has the advantage over passive diffusion of enabling the delivery of polarized and/or larger drugs across the dermal barrier. Iontophoresis enhances the transport of drugs by two major mechanisms in addition to passive diffusion, electronepulsion, and electro-osmosis. Electronepulsion refers to the ion-electric field interaction that provides a force, which drives ionized drugs through the skin. Electro-osmosis refers to the bulk motion of the solvent that carries ionic or neutral solutes with the solvent stream, and is mostly observed when iontophoresis is applied using an anodal current (Dixit et al., 2007). Many factors are critical for transdermal drug delivery using iontophoresis; among these, the most important are the drug concentration and molecular weight, the charge on the molecule, the pH of the solution (which directly influences ionization), and the hydrophobic factor (Dixit et al., 2007).

In this study, we selected molecules with theoretically high potential for iontophoretic transport. Nonetheless, we were only able to demonstrate an effect on skin blood flow for two of the drugs, A-350619 and CFM 1571. As all compounds were highly ionized (99% or higher) at cutaneous pH, this result may be due to the molecular physical and chemical differences between candidates. Concerning the sGC stimulators, SIN-1 is a small hydrophilic molecule (Mr 206.63 and logD6 = −1.5), whereas A-350619 and CFM 1571 are larger but are more lipophilic (Mr 425.41 and 444.95, logD6 = 2.1 and 0.9, respectively). In contrast, the low polar surface area of A-350619 (32.34 Å²), compared with that of SIN-1 and CFM 1571 (68.4 and 68.62 Å², respectively), is not a barrier to iontophoretic transfer. With regard to the nonprostanoid IP agonist, both candidates are lipophilic (logD6 > 2), with large polar surface areas (>75 Å²) and high molecular weight (>400), but they have poor solubility. For this reason, the highest concentration we could test was lower than that of the sGC stimulators, which could explain why we did not observe any effect. Taken together, these data suggest that the partition coefficient and the concentration are crucial parameters to enhance iontophoresis. This is consistent with the good iontophoretic transfer of treprostinil (Mr 390.51 and logD6 1.4) in the same rat model (Blaise et al., 2011).

Pharmacological differences between A-350619, CFM 1571, and SIN-1 may also explain the absence of vasodilation with the latter. Indeed, the molecular mechanism of sGC activation by SIN-1 involves the release of NO and superoxide in equimolar amounts. (Schrammel et al., 1998). Considering the extinction coefficients of these compounds, their interaction to generate peroxynitrite is likely more rapid than the reaction of NO with the heme moiety of sGC.

Among the two sGC stimulators with significant effect on skin blood flow, CFM 1571 showed a significant difference from its vehicle when blood flow was recorded over the 20-minute iontophoresis period (secondary outcome). However, this difference did not reach significance over 60 minutes (primary outcome) because of a rapid decrease in skin blood flow within a few minutes after the end of iontophoresis. Analysis of individual tracings confirmed this pattern. We did not pursue the study of this compound as we focused on molecules with a sustained effect.

We tested three concentrations of A-350619 and observed a concentration-dependent effect. However, paired comparisons revealed that only the highest concentration (7.5 mM) was significantly different from the others. We also tested different iontophoresis protocols (i.e., continuous vs. intermittent current delivery) with the same overall amount of current. The effect of A-350619 was similar with both protocols; however, intermittent current induced significant current-induced vasodilation with NaCl. Indeed, iontophoresis has been associated with a confounding nonspecific current-induced vasodilation suggested to be mediated through axon reflex (Durand et al., 2002a). This is consistent with previous observations that intermittent anodal current potentiates current-induced vasodilation (Durand et al., 2002b). Therefore, we conclude that continuous iontophoresis of A-350619 is more appropriate.

Repeated administrations of A-350619 do not suggest any risk of tolerance. There is a nonsignificant trend toward decreased vasodilation at H1, which could reflect a short-term desensitization of sGC, as observed on isolated rat aorta after a 30-minute exposure to nitroglycerin (Kakutani et al., 2005). However, skin vascular reactivity at H24 is fully restored; this is encouraging as daily administration (or less) is a more feasible dose regimen in a clinical setting.

Transdermal iontophoresis is considered to be a safe procedure, associated with only moderate erythema and tingling sensations (Kumar and Lin, 2008). In the present work, we did not observe any significant toxicity of A-350619 iontophoresis on histopathologic examination of the skin. Moreover, the effect on blood pressure was slight and not significant.

Nonetheless, there are several limitations. First, we did not quantify the concentration of drugs in the dermis. As the concentrations used in experiment 1 are known to induce a significant pharmacological effect, we speculate that iontophoresis does not allow sufficient dermal concentrations to be reached in the skin to be able to show any effect on the microvasculature (except for A-350619 and, to a lesser extent, CFM 1571). The quantification of drug concentration in the dermis would address this issue, either in vivo (using microdialysis) or in skin biopsies. In both cases, this implies setting up the drug assay (e.g., by liquid chromatography–tandem mass spectrometry). A simpler method could be to directly deliver into the dermis the same concentration of drug with intradermal injections or microdialysis, and to compare the response to that obtained with iontophoresis. However, the benefit of such experiments is minimized by significant bias such as nonspecific, inflammation-induced vasodilation.

We observed rapid decrease in skin blood flow after the end of iontophoresis. Indeed, despite a large and sustained vasodilator response at 7.5 mM, the response was not as prolonged as that previously described with treprostinil, for which a plateau was still present at least 60 minutes after the end of iontophoresis (Blaise et al., 2011). This could be due to an elevated subcutaneous clearance of A-350619, or to a transient pharmacodynamic effect. Such transient effect on skin blood flow might be potentiated by inhibiting the degradation of cGMP with phosphodiesterase type 5 inhibitors, as was shown with NO donors (Blaise et al., 2010).

Another issue that must be raised is that sGC stimulators depend on a reduced heme group in sGC to be active (Evgenov et al., 2006). Therefore, oxidative stress, which is a hallmark of the pathophysiology of SSC (Gabrielli et al., 2009), may decrease the effect of iontophoresis of sGC stimulators in the disease state. On the other hand, sGC activators are heme-independent and could be an interesting alternative in SSc.
This hypothesis should be tested in animal models of SSc exhibiting elevated production of reactive oxygen species (e.g., bleomycin or HOCl models) (Batteux et al., 2011).

In the same way, recent work demonstrated that sGC stimulation potently inhibits fibroblast activation and progressive fibrosis in a model of inflammation-driven fibrosis but also plays a role in preventing the progression of fibrosis and induces regression of established inflammation-dependent fibrosis (Beyer et al., 2012). Thus, as we show that iontophoresis allows drug delivery directly into the dermis, we may expect a benefit on both the vascular and the fibrotic components of the disease, making sGC stimulators/activators good candidates for therapeutic iontophoresis in patients with SSc.

In conclusion, the present study demonstrates that local drug delivery of A-350619, an sGC stimulator, using iontophoresis induces a sustained increase in skin blood flow. Systemic and cutaneous safety was good. Further work is needed to test whether iontophoresis of sGC stimulators may present a new therapeutic option in SSc.

Acknowledgments
The authors thank Claire Arnaud and Océane Combe for technical support and Alison Poote for editing the manuscript.

Authorship Contributions
Participated in research design: Kotzki, Roustit, Blaise, Godin-Ribout, Cracowski.
Conducted experiments: Kotzki, Roustit, Arnaud.
Performed data analysis: Kotzki, Roustit, Arnaud, Boutonnat.
Wrote or contributed to the writing of the manuscript: Kotzki, Roustit, Arnaud, Cracowski.

References

Iontophoresis of a Soluble Guanylate Cyclase Stimulator 431


Address correspondence to: Matthieu Roustit, Unité de Pharmacologie Clinique, Centre d’Investigation Clinique de Grenoble–INSEERM CIC003, CHU de Grenoble, 38043 Grenoble Cedex 09, France. E-mail: M.Roustit@chu-grenoble.fr