JPET Fast Forward. Published on July 9, 2013 as DOI: 10.1124/jpet.113.205484

JPET #205484

Anodal iontophoresis of a soluble guanylate cyclase stimulator induces a sustained increase in skin blood flow in rats.

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JPET #205484

Running title:

Iontophoresis of a soluble guanylate cyclase stimulator.

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Document statistics:

Text pages: 21

Words in text: 4042

Words in abstract: 190

Words in introduction: 631

Words in discussion: 1333

Tables: 1

Figures: 4

References: 30

Abbreviations:

ANOVA, analysis of variance; IP, prostaglandin I2 receptor; sGC, soluble guanylate cyclase; LDI, laser Doppler imaging; SSc, systemic sclerosis.

Abstract

The treatment of systemic sclerosis-related digital ulcers is challenging. While the only effective drugs are prostacyclin analogues, 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, SIN-1 and CFM 1571) and two non-prostanoid prostaglandin I_2 (prostacyclin) receptor (IP) agonists (MRE-269 and BMY 45778) to induce vasodilation onto the hindquarters of anaesthetized rats. Skin blood flow was quantified using laser Doppler imaging during the whole experience, safety was assessed by continuously recording blood pressure and histopathologic 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 mM, 0.75 mM 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 vs intermittent iontophoresis protocols. Continuous, anodal iontophoresis of A-350619 at 7.5 mM, increases cutaneous blood flow with good local tolerance. Iontophoresis of sGC 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 therapy of SSc-related ulcers remains challenging. Bosentan, a non-specific endothelin receptor antagonist, has been indicated to prevent digital ulcers in patients at risk, but has no efficacy 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 analogues 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 ulcers healing but the lack of a placebo group in the design was a major limitation to conclude formally (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, non-invasive transdermal drug delivery method using a low-intensity electric current (Kalia et al., 2004). Some authors have highlighted the potential interest of iontophoresis of vasodilating drugs as a treatment for 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, while sodium nitroprusside, an indirect 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 analogues (iloprost and treprostinil) showed promise to induce a sustained increase in cutaneous flow in rats (Blaise et al., 2011). A first study with iloprost on healthy volunteers was engaged to confirm these result on human, unfortunately study was aborted because of important side effects. Afterward, a second study with treprostinil was realized on healthy volunteers. In this study we demonstrated treprostinil ability to increase cutaneous flow without any side effect (Blaise et al., 2012).

While the iontophoresis of treprostinil is currently being tested in patients with SSc (ClinicalTrials.gov Identifier: NCT01554540), screening other agonists with vasodilatator potency could open new perspectives. The first target is the prostacyclin receptor (IP). In addition to the classical prostacyclin analogues, non-prostanoid 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 nitric oxide (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 anti-fibrotic effect of sGC stimulators in different experimental models of SSc (Beyer et al., 2012). Finally, these compounds are small, hydrophilic polarized molecules with a high potential for transdermal delivery through iontophoresis. All considered, these non-prostanoid 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 non-prostanoid 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.

Methods

1. Animals

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

2. 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 weight <500 Da, log D_6< 5 (logP for pH 6), polar surface area <140 $Å^2$; 3) ionized molecules at cutaneous physiological pH.

Drug supply and preparation

Five candidates were retained, two non-prostanoid IP agonists: BMY 45778 ([3-(4,5-Diphenyl[2,4'-bioxazol]-5'-yl-)phenoxy]acetic acid) (Seiler et al., 1997) and MRE-269 ([4-[(5,

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6-diphenylpyrazinyl)(1-methylethyl)amino]butoxy]-acetic acid) (Morrison et al., 2010); and three sGC stimulators: A-350619 (3-[2-[(4-Chlorophenyl)thiophenyl]-N-[4-(dimethylamino)butyl]-2-propenamide hydrochloride) (Miller et al., 2003), SIN-1 (Amino-3-morpholinyl-1,2,3-oxadiazol-ium chloride) (Maurice and Haslam, 1990) and CFM 1571 (3-[3-(Dimethylamino)propoxy]-N-(4-m-ethoxyphenyl)-1-(phenylmethyl)-1H-pyrazole-5-carbo-xamide hydrochloride) (Evgenov et al., 2006) (Figure 1). A-350619 hydrochloride (MW 425.41), SIN-1 chloride (MW s211.13), CFM 1571 hydrochloride (MW 444.95) and BMY 45778 (MW 438.44) were purchased from Tocris Bioscience, Bristol, United Kingdom. MRE-269 (MW 419.5) was purchased from Cayman Chemical, Ann Arbor, MI, USA. Isotonic sodium chloride (NaCl 0.9%) (Aguettant, Lyon, France) was used as vehicle for sGC stimulators. We prepared specific vehicles for MRE-269 (1:3 solution of ethanol:PBS, pH 7.2) and for BMY 45778 (NaCl 0.9% with DMSO 0.1% and ethanol 1%), as advised in the product information. These vehicles were used as controls for the corresponding drugs

Solutions of sGC stimulators were prepared extemporaneously by diluting 10 mg of A-350619 in 3.13 ml of NaCl 0.9%, 10 mg of CFM 1571 in 3 ml of NaCl 0.9% and 10 mg of SIN-1 in 4.7 ml of NaCl 0.9%, to obtain 7.5 mM, 7.5 mM and 10 mM solutions, respectively. We also diluted A-350619 7.5 mM to obtain 0.75 mM and 0.075 mM solutions (experiment 2). MRE-269 solution was prepared extemporaneously by diluting 1 mg in 1 ml of 100%, ethanol, heating the solution for 10 minutes at 45 °C and diluting it in 3 ml of PBS 1X at pH 7.2 to obtain a 0.6 mM solution. BMY 45778 solution was prepared by diluting 1 mg of powder in 88 µl of 1:1 solution of DMSO:ethanol then further diluting it in 44.3 ml of NaCl 0.9% to obtain an 0.052 mM solution.

3. Experimental procedures

The rats were anesthetized with sodium pentobarbital (50 $mg.kg^{-1}$ i.p.) and were maintained in the prone position for the whole duration of the experiment, with the back uppermost. 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). Before iontophoresis each rat was inspected to ensure that the hairless skin in 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, Järfälla, Sweden) containing drugs or vehicles, as previously described (Blaise et al., 2011). Passive electrodes (PF 384, PeriIont System, Perimed, Järfälla, Sweden) were placed on the back of the neck, and both electrodes were connected to a current generator (PF 382b, PeriIont System, Perimed, Järfälla, Sweden).

Experiment 1: Initial screening

Iontophoresis of A-350619 7.5 mM, SIN-1 10 mM, and CFM 1571 7.5 mM were performed during 20 min using an anodal current (100 µA) because of their positive charge at skin and physiological pH. They were compared to 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 were randomly generated. Iontophoresis of BMY 45778 and MRE-269 and their respective vehicles was performed during 20 min using a cathodal current (100 µA) because of their negative charge at skin and physiological 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: active electrodes were filled with drugs or

vehicles but they were not connected to the current generator. The 20-min application was followed by a 40-min 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 above. In addition, a catheter was inserted in the left carotid artery to continuously record blood pressure (Powerlab, ADInstrument) from 5 min before, to 60 min after iontophoresis. Photographs were taken before iontophoresis, and immediately after iontophoresis to assess cutaneous tolerance. Negative reactions were coded grade 0; weak reactions as grade 1 and are characterized by non-vesicular 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 non-treated skin area was realized. Biopsies were fixed in AFA fluid (5% acetic acid, 75% absolute ethyl alcohol, and 18% water; Carlo Erba), paraffin-embedded and stained with hematoxylin, eosin, and safran. Specific features were sought in order 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 perinuclear vacuolar changes, cytolysis and the appearance of keratohyaline 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

JPET Fast Forward. Published on July 9, 2013 as DOI: 10.1124/jpet.113.205484
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infiltration of neutrophils, lymphocytes or mast cells at the dermo-epiderma interface was evaluated.

Experiment 3: Protocol optimization and cutaneous resistance

To determine if an intermittent current could enhance the effect of iontophoresis (Nair and Panchagnula, 2004), we assessed the effect of an intermittent iontophoresis protocol for A-350619 7.5 mM and NaCl (n=8) on skin blood flow. We repeated twenty 1-min cycles of 10 seconds at 600µA ("on") followed by 50 seconds without current ("off"). This series was compared to the continuous current condition (100µA, 20 min) in experiment 2. In both protocols the same amount of current (120mC) 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 min 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 calculated and expressed in Ω (Ferrell 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 A-350619 7.5 mM and NaCl was then simultaneously performed during 20 min at 100 µA. The procedure was repeated 1 hour (H1) and 24 hours (H24) later, positioning the electrodes exactly on the previously used skin sites.

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4. Skin blood flow measurement and data analysis

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

Data are expressed as arbitrary perfusion units (PU). In order to take into account inter-individual BL variations, data were subsequently expressed as a percentage change from BL (%BL). Then, we calculated area under the curve from the beginning of iontophoresis to the end of the recording ($AUC_{0\text{-}60}$, in %BL.s; as primary outcome) and during the 20 min iontophoresis ($AUC_{0\text{-}20}$, as secondary outcome). This choice reflects our objective to screen drugs exhibiting a sustained effect beyond the duration of iontophoresis itself.

5. Statistical analysis

Continuous data are expressed as mean ±standard deviation. Data were analyzed by repeated measures ANOVA, and paired t tests for 2x2 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, of drug, as well as 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, USA).

Results

1. Effect of iontophoresis of sGC stimulators and non-prostanoid IP agonists: initial screening (experiment 1)

Anodal iontophoresis of A-350619 (7.5 mM) significantly increasedAUC_{0-20} and AUC_{0-60} compared to NaCl (Table 1; Figure 2 (A)). On the other hand, iontophoresis of the other sGC stimulators (CFM 1571 at 7.5 mM and SIN-1at 10 mM) as well as non-prostanoid IP agonists (MRE-269 at 0.6mM and BMY 45778 at 0.052mM) did not induce any significant change in skin blood flow compared to their respective vehicles (Table 1; Figure 2 (B-E)). However, a transient vasodilation was observed for CFM 1571, as shown by the significant increase in AUC_{0-20} versus NaCl (79088 ±27383 %BL and 15812±15859 %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).

2. 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 (7.5mM, 0.75 mM and 0.075mM). AUC_{0-60} were 265807 ±164703%BL.s, 72701 ± 49345%BL.s and 34203± 77796%BL.s, respectively (P<0.001, Jonckheere-Terpstra trend test) (Figure 3). Paired comparisons showed that A-350619 at 7.5 mM induced significantly increased skin blood flow as compared to 0.75 mM (P=0.025) and 0.075 mM (P=0.017) solutions.

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3. Comparison between intermittent and continuous iontophoresis of A-350619 (experiment 3)

Continuous and intermittent iontophoresis of A-350619 at 7.5mM induced comparable vasodilation (AUC_{0-60} were 265807±164703%BL.s and 211959 ± 136821%BL.s, respectively; P=0.49) (Figure 4 (A)). However, intermittent iontophoresis of NaCl 0.9 % significantly increased skin blood flow as compared to continuous iontophoresis (111696 ± 59591 %BL.s and, 44051 ±55963 %BL.s respectively; P=0.03).

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

4. Evaluation of the tolerance after repeated administrations (experiment 4)

Repeated administrations of A-350619 7.5 mM and NaCl at T0, H1 and H24 did not significantly change skin blood flow over time. AUC_{0-60} were 277583±145094%BL.s, 216336±157045%BL.s and 303332±171202%BL.s, respectively, for A-350619; and 19888±41866%BL.s, 21283± 54847%BL.s and 16615± 58921%BL.s, 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, two-way ANOVA) suggesting that the effect of the drug persists over time.

5. 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 II, 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 the Methods section were found in any of the skin biopsies.

Discussion

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

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, electrorepulsion and electro-osmosis. Electrorepulsion 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

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differences between candidates. Concerning the sGC stimulators, SIN-1 is a small hydrophilic molecule (MW 206.63 and $logD_6$ = -1.5), while A-350619 and CFM 1571 are larger but are more lipophilic (MW 425.41 and 444.95, $logD_6$= 2.1 and 0.9 respectively). In contrast, the low polar surface area of A-350619 (32.34 Å²), as compared to that of SIN-1 and CFM 1571 (68.4 and 68.62 Å² respectively), is not a barrier to iontophoretic transfer. Concerning the non-prostanoid IP agonist; both candidates are lipophilic ($logD_6$> 2) with large polar surface areas (PSA > 75Å²) and high molecular weight (MW > 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 (MW 390.51 and $logD_6$ 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 involve 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 min iontophoresis period (secondary outcome). However, this difference did not reach significance over 60 min (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 3 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 non-specific current-induced vasodilatation 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). We therefore 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 non significant trend to decreased vasodilation at H1, which could reflect a short term desensitization of sGC, as observed on isolated rat aorta after a 30 min 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.

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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, for 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 non-specific, inflammation-induced vasodilation.

We observed rapid decrease of 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 min 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 PDE-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 contributes 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 the vascular but also 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, a 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.

Acknowledgements

We thank Claire Arnaud and Océane Combe for technical support, and Alison Foote for editing the manuscript.

Authorship Contributions

Participated in research design: Kotzki, Roustit, Blaise, Godin-Ribuot and Cracowski.

Conducted experiments: Kotzki, Roustit and Arnaud.

Performed data analysis: Kotzki, Roustit, Arnaud, Boutonnat.

Wrote or contributed to the writing of the manuscript: Kotzki, Roustit, Arnaud and Cracowski.

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Footnotes

This research was supported by a grant from the patients' association 'Association des Sclérodermiques de France'.

Legends for Figures

Figure 1. Candidates for the initial screening among non-prostanoid prostacyclin receptor agonists and stimulators of soluble guanylatecyclase.

Figure 2. Vascular effect of 20 min 100 µA anodal iontophoresis of the soluble guanylate cyclase stimulators A-350619 7.5 mM (A), CFM 1571 7.5mM (B), SIN-1 10mM (C) in comparison with NaCl 0.9%. Effect of 20 min 100 µA anodal non-prostanoid prostacyclin receptor agonists BMY 45778 0.052mM (D) and MRE-269 0.6mM (E) in comparison with their respective vehicle, on cutaneous blood flow expressed as % of baseline.

Figure 3. Dose-dependent effect of anodal iontophoresis of the soluble guanylate cyclase stimulator A-350619 at 7.5, mM, 0.75 mM and 0.075 mM on cutaneous blood flow expressed as % of baseline.

Figure 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 % of baseline. (B) Comparison of continuous and intermittent anodal iontophoresis of NaCl 0.9% on cutaneous blood flow expressed as % of baseline.

JPET Fast Forward. Published on July 9, 2013 as DOI: 10.1124/jpet.113.205484
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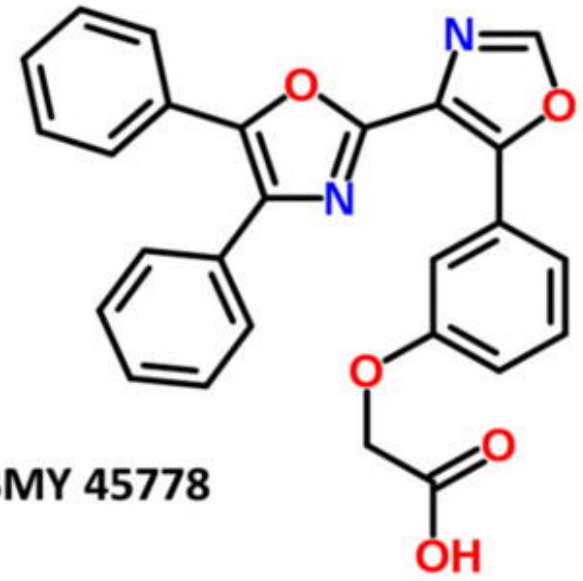
Tables

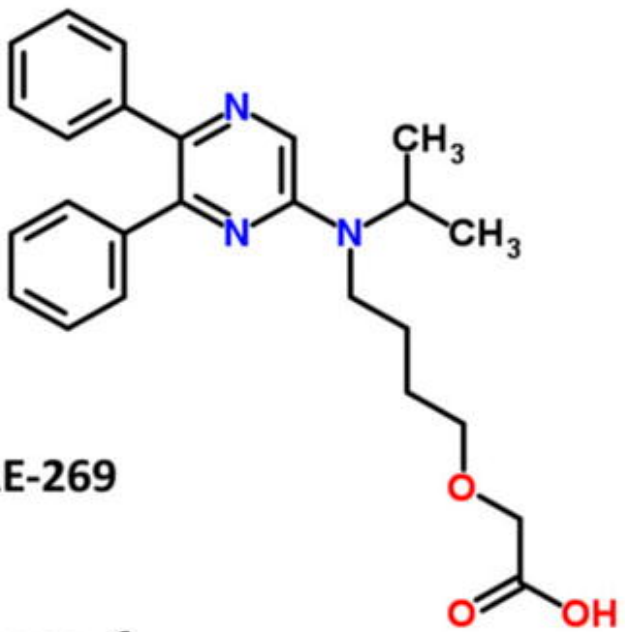
Table 1. Effect of iontophoresis of non-prostanoid prostacyclin receptor (IP) agonists and stimulators of soluble guanylate cyclase (sGC) on cutaneous blood flow. Data are expressed as area under the curve of the percentage change from baseline (expressed as %BL.s) during 60 min (20-min iontophoresis and 40-min rest)

	Active drug	Vehicle	P-value
sGC stimulators			
A-350619 (7.5 mM)	319896 ±169386	69307 ± 50999	0.008
CFM 1571 (7.5 mM)	141533 ± 43688	81701 ± 74991	0.06
SIN-1 (10 mM)	114162 ± 46036	76488 ± 60704	0.2
Non-prostanoid IP agonists			
BMY 45778 (0.052 mM)	259412 ± 73032	236622 ± 70855	0.48
MRE-269 (0.6 mM)	214540 ± 38531	204932 ± 67030	0.77

Non-prostanoid IP Agonists



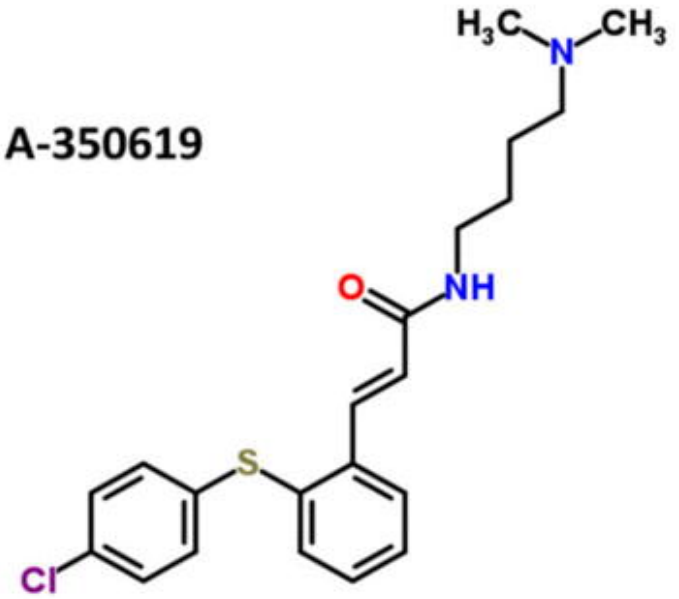
BMY 45778



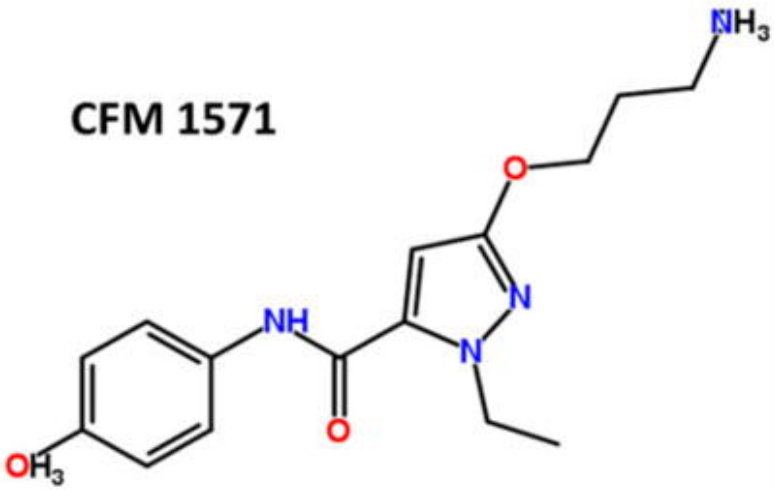
MRE-269

sGC Stimulators

A-350619



CFM 1571



SIN-1

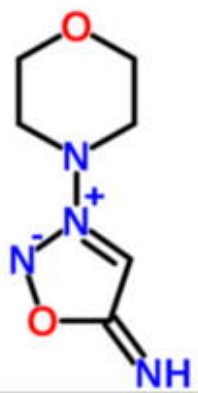


Figure 1.

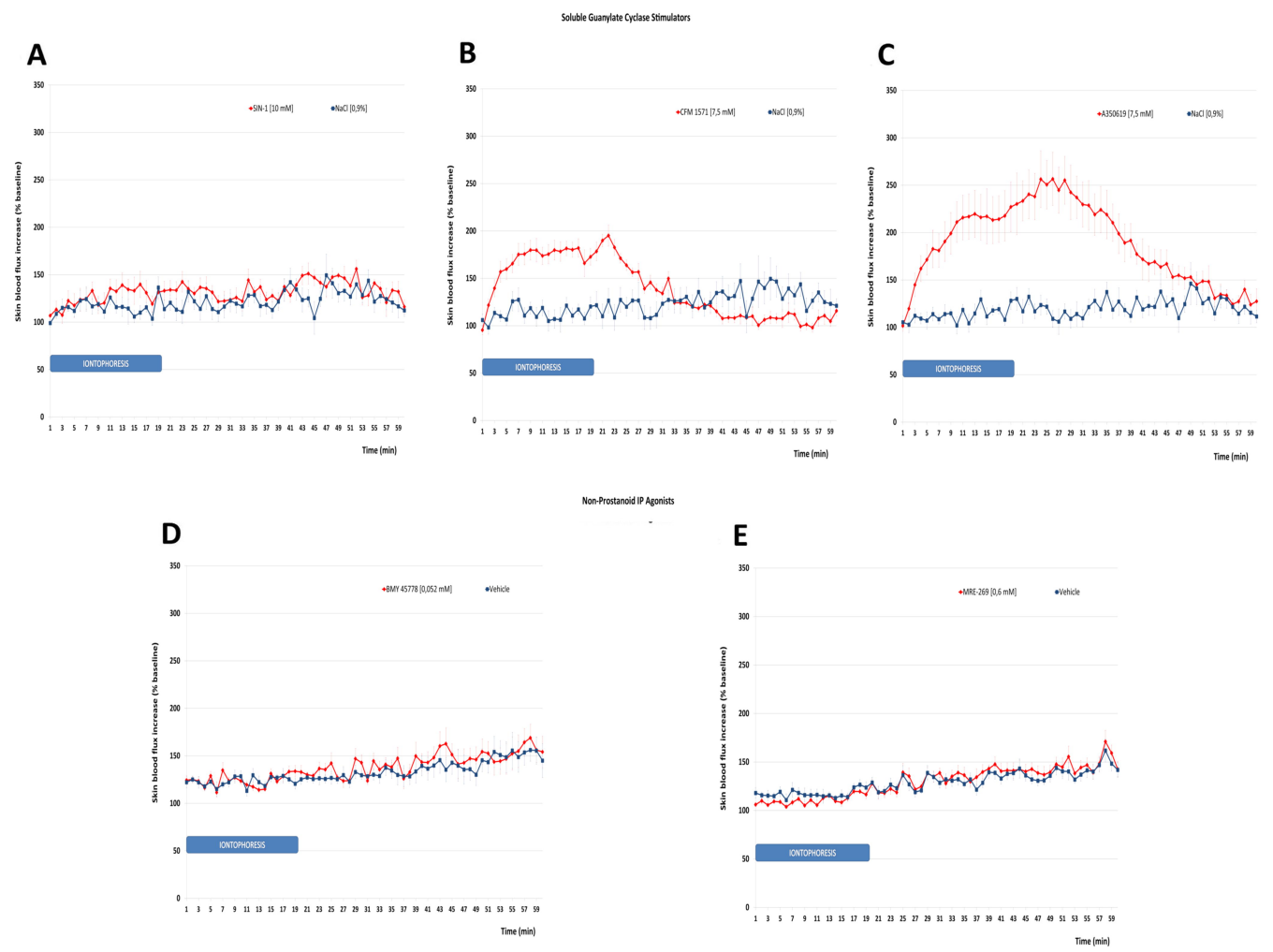

Soluble Guanylate Cyclase Stimulators
A
SIN-1 [10 mM]
NaCl [0,9%]
Skin blood flux increase (% baseline)
IONTOPHORESIS
Time (min)
B
CFM 1571 [7,5 mM]
NaCl [0,9%]
Skin blood flux increase (% baseline)
IONTOPHORESIS
Time (min)
C
A350619 [7,5 mM]
NaCl [0,9%]
Skin blood flux increase (% baseline)
IONTOPHORESIS
Time (min)
Non-Prostanoid IP Agonists
D
BMY 45778 [0,052 mM]
Vehicle
Skin blood flux increase (% baseline)
IONTOPHORESIS
Time (min)
E
MRE-269 [0,6 mM]
Vehicle
Skin blood flux increase (% baseline)
IONTOPHORESIS
Time (min)

Figure 2.

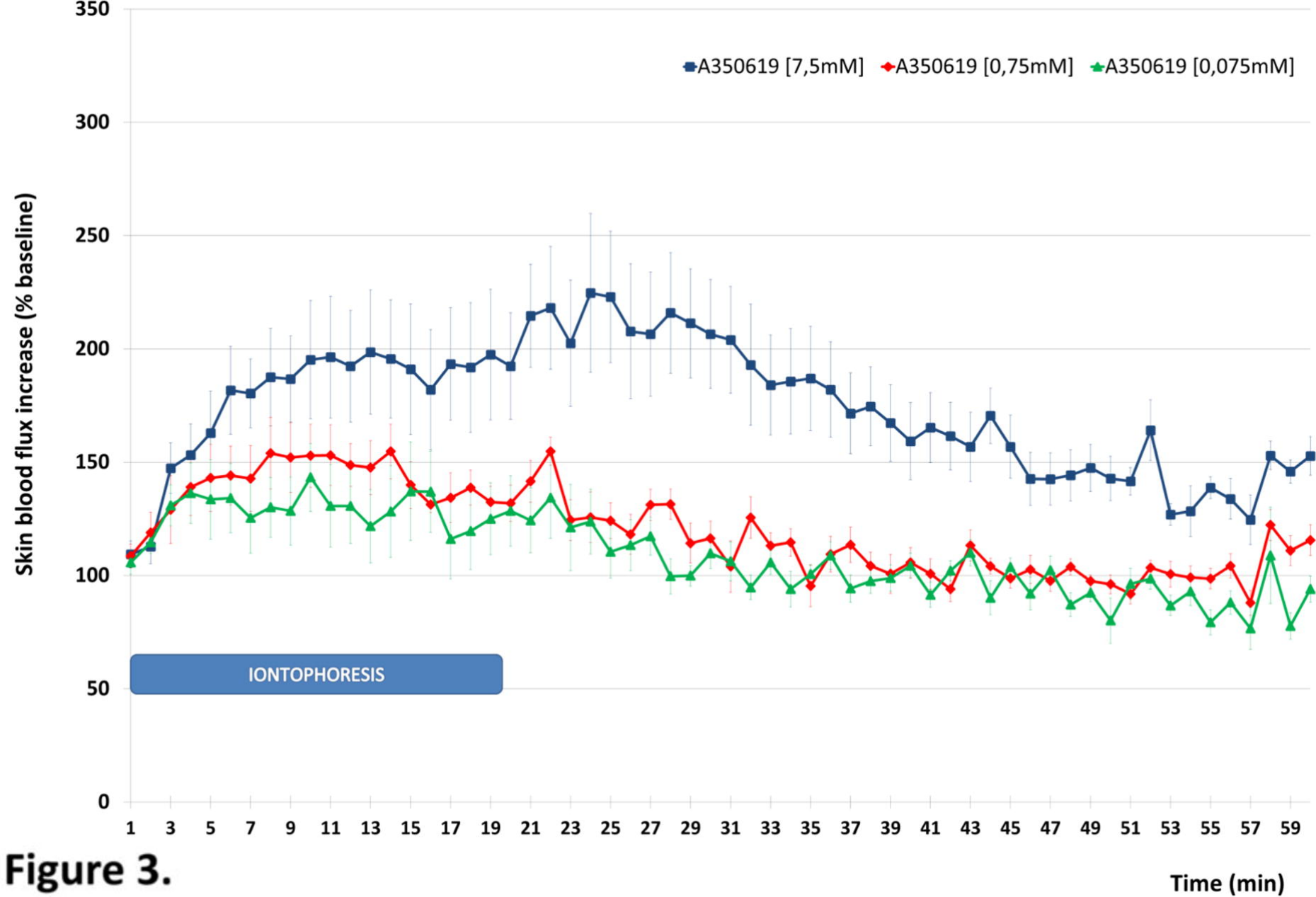

A350619 [7,5mM]
A350619 [0,75mM]
A350619 [0,075mM]
Skin blood flux increase (% baseline)
350
300
250
200
150
100
50
0
IONTOPHORESIS
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
Time (min)

Figure 3.

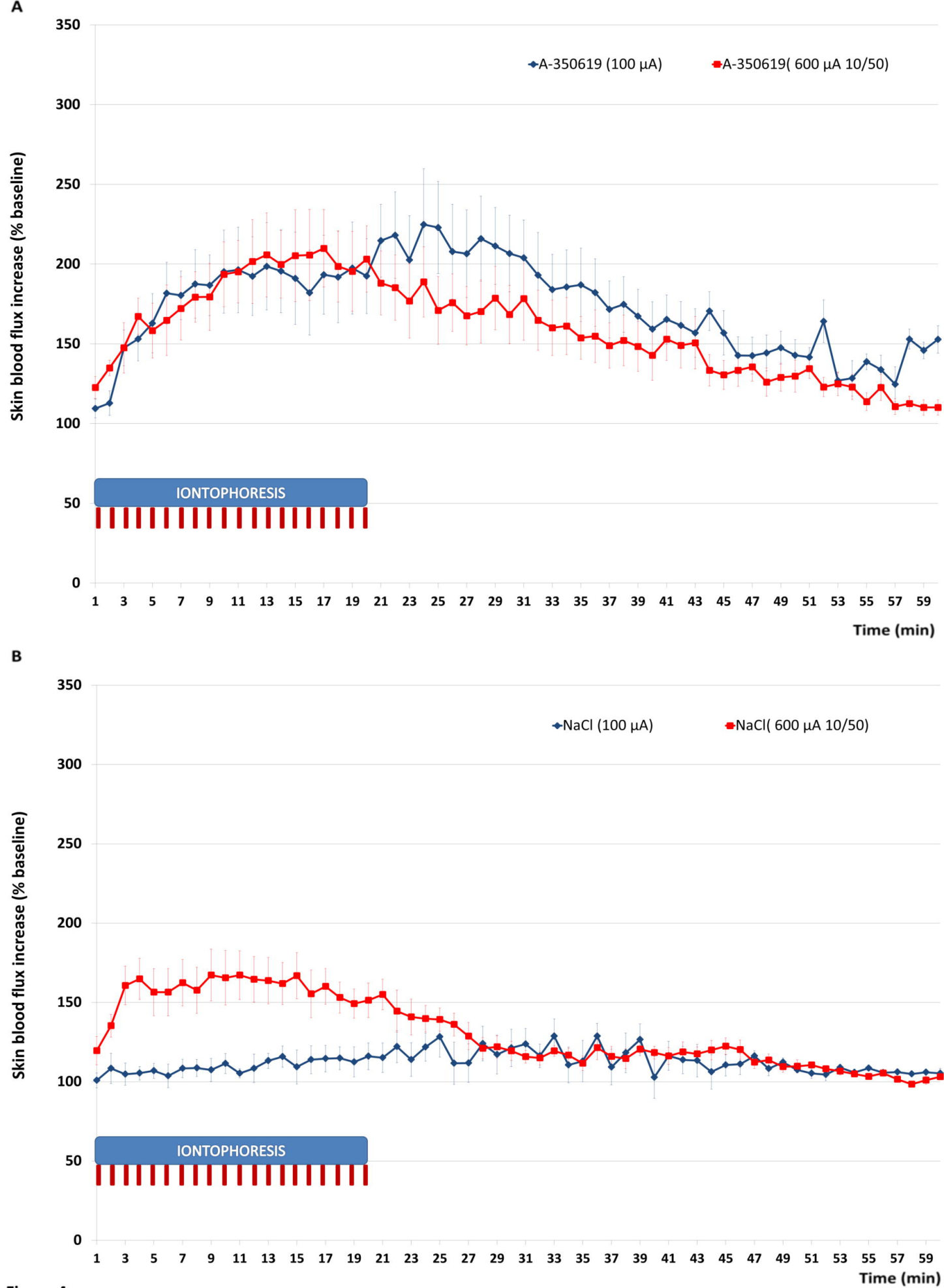

A
A-350619 (100 µA)
A-350619(600 µA 10/50)
Skin blood flux increase (% baseline)
350
300
250
200
150
100
50
0
IONTOPHORESIS
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
Time (min)
B
NaCl (100 µA)
NaCl(600 µA 10/50)
Skin blood flux increase (% baseline)
350
300
250
200
150
100
50
0
IONTOPHORESIS
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
Time (min)

Figure 4.