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Visualizing Dermal Permeation of Sodium Channel Modulators by Mass Spectrometric Imaging

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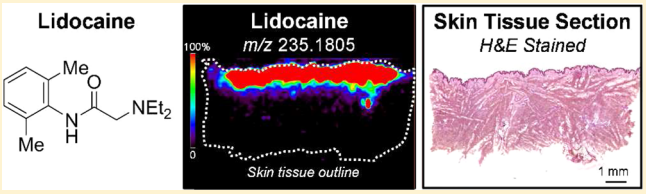
Supporting Information

ABSTRACT: Determining permeability of a given compound through human skin is a principal challenge owing to the highly complex nature of dermal tissue. We describe the application of an ambient mass spectrometry imaging method for visualizing skin penetration of sodium channel modulators, including novel synthetic analogs of natural neurotoxic alkaloids, topically applied ex vivo to human skin. Our simple and label-free approach enables successful mapping of the transverse and lateral diffusion of small molecules having different physicochemical properties without the need for extensive sample preparation.



INTRODUCTION

Transdermal delivery of therapeutic agents represents an attractive alternative to oral or parenteral delivery and has witnessed increased clinical application in the past two decades.[1] Transdermal delivery has many advantages in comparison to other methods for drug administration.[1,2] Although oral analgesics are commonly prescribed for the treatment of acute and chronic pain, adverse, potentially fatal effects such as respiratory depression, nausea, and addiction can ensue from systemic drug exposure.[3] Clinically effective drug concentrations can be introduced at a peripherally located site of injury or inflammation by topical administration without resulting in high systemic concentrations that may increase the likelihood of untoward side effects.[3,4] Understanding drug penetration and diffusivity through skin, however, poses significant challenges given the structural complexity of this organ and the many disparate mechanisms that transport small molecules across the dermal layers.[2] Methods for determining transdermal drug absorption in skin include skin extraction measurements,[5] quantitative autoradiography,[6] and spectroscopic methods (fluorescence[7] and FTIR[8]). In this work, we present a desorption ionization mass spectrometric imaging technique for identifying small molecule analytes in skin and for mapping compound distribution through the dermal layers. We suggest that this technique represents a superior approach to measuring the extent of skin permeation of topically applied compounds.

Many clinically employed analgesics and anesthetics for relieving pain modulate voltage-gated sodium channels (Na_Vs), which are a family of integral membrane proteins responsible for the rising phase of action potentials in electrically conducting cells.[9] One such example is lidocaine, a prescription-based injectable and topical pharmaceutical agent. In addition to clinically employed anesthetics, natural neurotoxic alkaloids, including saxitoxin, neosaxitoxin,[10] batrachotoxin,[11] and aconitine,[12,13] are sodium channel modulators, some of which are currently being investigated for potential usage as pain therapeutics owing to the high affinity and specificity that such compounds demonstrate for Na_Vs.[10,14−16] Little is known about the ability of these compounds to penetrate human skin to produce a topical analgesic effect. Mass spectrometry imaging is a label-free method with which the distribution of a multitude of compounds can be mapped in biological tissues with high specificity and offers an efficient, straightforward method for this type of analysis.[17−19]

We have used high mass resolution/mass accuracy desorption electrospray ionization mass spectrometric imaging (DESI-MSI) to investigate the permeation of natural sodium channel blockers and novel synthetic analogues following topical application of these compounds to ex vivo human skin. DESI-MSI enables two-dimensional mapping of a sample analyte in the ambient environment, without the need for extensive sample preparation. Samples are bombarded with microdroplets of acetonitrile that dissolve hundreds of

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endogenous lipids and metabolites as well as exogenous compounds present in tissue. The splash forms secondary microdroplets that enter a mass spectrometer, providing a detailed chemical map of the distribution of molecules within the sample surface.[20] After DESI-MSI, the same tissue section can be stained, optically imaged, and compared to selected 2D DESI-MS ion images.[21] The compounds investigated in this study include common analgesics such as lidocaine and prilocaine as well as aconitine, an herbal neurotoxin used in Chinese medicine, saxitoxin, a sodium channel inhibitor synonymous with paralytic shellfish poisoning, and novel synthetic analogues of both saxitoxin and batrachotoxin. The effect of sunburn injury on the penetration of these sodium channel blocking agents was also examined. Using DESI-MSI, we demonstrate that the compound of interest can be detected with unambiguous determination among the hundreds of other endogenous skin compounds concurrently being analyzed. The spatial distribution of these agents has been mapped and directly compared to the morphology of the skin sections to assess transdermal penetration.

RESULTS AND DISCUSSION

Dermal Permeation in ex Vivo Human Skin. Human skin samples were obtained from four patients undergoing surgery at Stanford Medical School. All patients gave written informed consent following an approved IRB protocol. The skin graft was placed in a shallow dish partially filled with synthetic interstitial fluid. Confined circular treatment areas were created by delineating the application site with a felt-tip pen and encircling this mark with petroleum jelly. The compounds were directly applied to the treatment area with a pipet, and after 1, 4, or 10 h of application, the skin was frozen and cross sectional tissue samples carefully prepared to avoid cross-contamination. Positive ion mode DESI-MSI was performed using an Orbitrap for mass analysis at a mass resolution of 60 000 (see the Supporting Information for experimental details). Rich and distinctive molecular profiles were observed from the different layers of skin (epidermis, dermis, hypodermis) by high-resolution DESI-MS imaging. Many of the ions were identified as sodium or potassium adducts of complex glycerophospholipids: glycerophosphocholines (PC), lyso-PC, sphingomyelins, glycerophosphoethanolamines, and glycerophosphoglycerols; and glycerolipids: triacylglycerols (TG) and diacylglycerols (Table S1). Ion images showing the distribution of these compounds within the different layers of the skin samples are shown in Figure S1, alongside the optical image of the hematoxylin and eosin (H&E) stained tissue sections. Pathological evaluation of the H&E stained human skin tissue sections reveals the presence of epidermis (30−60 μm thickness), superficial dermis (250−450 μm thickness), deep dermis (2200−3000 μm thickness) and hypodermis (600−1200 μm thickness) layers within all the human samples analyzed.

Topical application of local anesthetics and Na_V modulators was initially performed using ethanol as the vehicle, the alcohol most commonly used as a permeation enhancer.[22] Despite the complexity of the DESI mass spectra obtained from the different layers of the human skin, high mass resolution/high mass accuracy measurements allowed for clear mass spectral separation and identification of the compounds of interest. Saxitoxin was detected at m/z 282.1303 (mass error of 2.14 ppm), corresponding to the protonated molecule with loss of an equivalent of H_2O, whereas lidocaine, aconitine, and an analogue of batrachotoxin, BTX-A,[23] were observed as protonated molecules at m/z 235.1805 (mass error of 0.09 ppm), m/z 646.3220 (mass error of 0.35 ppm), and m/z 504.2373 (mass error of 1.44 ppm), respectively. Errors in these measurements were calculated from an average of a few mass spectra. Figure 1 shows the DESI-MSI results obtained for

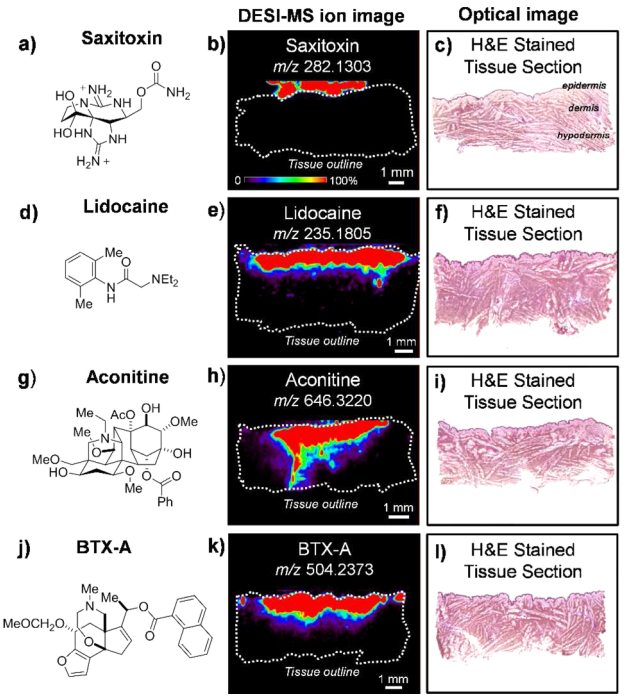

Figure 1. Penetration of the sodium channel blocker compounds tested in human skin of the same donor using ethanol as the vehicle. Chemical structures are shown for (a) saxitoxin, (d) lidocaine, (g) aconitine, and (j) BTX-A. Positive ion mode DESI-MS ion images of the compounds are shown for human skin in which (b) saxitoxin, (e) lidocaine, (h) aconitine, and (k) BTX-A were topically applied. Optical images of cross sections of the same tissue sections imaged by DESI after H&E stain are shown in (c) for saxitoxin, (f) for lidocaine, (i) for aconitine, and (l) for BTX-A.

human skin tissue sections from the same patient in which saxitoxin, lidocaine, aconitine, and BTX-A were topically applied following the protocol outlined above. The optical image of the H&E stained skin sample following DESI-MSI is also depicted to assist visualization of the outline of the tissue section. As observed in the DESI-MS ion image, saxitoxin did not exhibit any penetration in the human skin tissue (Figure 1b). The molecule is completely localized to the application site at the top of the tissue section. The low molecular weight of this compound notwithstanding,[1] the inability of saxitoxin to penetrate the epidermis is unsurprising given its charged, hydrophilic nature. By contrast, the DESI-MS ion image of lidocaine (Figure 1e) shows that this molecule readily diffuses throughout the tissue section, penetrating to the deep dermis layer (as determined by comparison with the H&E stained tissue section). Similar to lidocaine, DESI-MS images of aconitine and BTX-A also reflect extensive tissue penetration. Both compounds were detected deep within the tissue section, the former reaching the hypodermis of the skin. Ion images for samples treated with either aconitine or BTX-A reveal a higher relative signal intensity in the top layers of the skin than in the hypodermis (Figure 1h,k, respectively).[1] DESI-MS ion images

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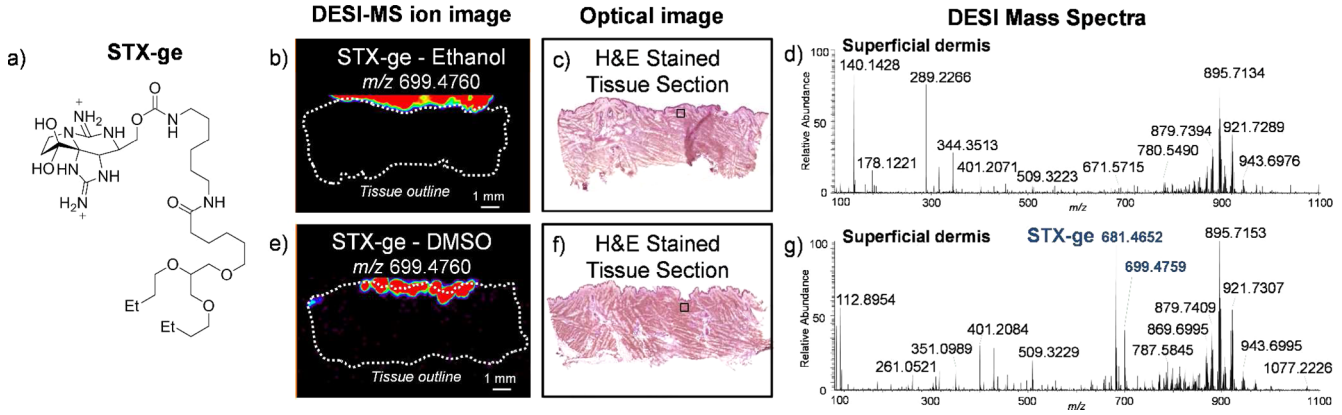

Figure 2. Penetration of novel synthetic analogue tested in human skin of the same donor. The chemical structure of STX-ge is shown in (a). Positive ion mode DESI-MS ion images of the compound are shown for human skin in which STX-ge was topically applied using (b) ethanol and (d) DMSO as vehicles. Optical images of the same tissue sections imaged by DESI after H&E stain are shown in (c) and (f), respectively. DESI mass spectra of selected skin regions outlined with a small black box in the optical images in (c) and (f) are shown for STX-ge (d) in ethanol and (g) in DMSO.

for saxitoxin, lidocaine, aconitine, and BTX-A using ethanol as vehicle were reproducible in skin sections obtained from the three different donors. Overall, the average penetration depth of lidocaine was 1.24 mm (5 samples), of aconitine was 3.05 mm (6 samples), of BTX-A was 2.00 mm (4 samples), and no penetration was observed for saxitoxin (7 samples).

A synthetic derivative of saxitoxin bearing a long chain, glycerol ether group (STX-ge) was also examined in human skin DESI-MS imaging experiments.[24,25] The attachment of a lipophilic chain to saxitoxin was expected to facilitate transdermal penetration of this compound. Detection of STX-ge was observed in the positive ion mode with protonation and loss of H_2O at m/z 681.4652 (mass error of 0.56 ppm) and in its protonated form m/z 699.4760 (mass error of 0.55 ppm). An endogenous lipid, ubiquitously distributed throughout the skin tissue, was detected at the same nominal m/z 699.4 of STX-ge. This lipid (measured at m/z 699.4082) could be completely resolved from toxin derivative using high mass resolution DESI-MSI, thereby enabling accurate mapping of the exogenous compound in tissue (Figure S2). The DESI-MS ion image for STX-ge is shown in Figure 2b. As is noted in Figure 2b, despite the hydrocarbon appendage, STX-ge does not penetrate human skin, similar to the results with saxitoxin (ethanol as vehicle, 6 samples). This compound was only detected outside of the tissue section, and only lipids were identified in the mass spectra of the epidermis and superficial dermis of the skin (Figure 2d).

In an attempt to improve the transdermal penetrability of STX-ge, ethanol was replaced with dimethyl sulfoxide (DMSO) as the vehicle. DMSO is a well-known skin penetration enhancer that interacts with lipids in the stratum cornea and epidermis and induces conformational changes in keratin.[26] Our DESI-MSI results show that some degree of skin penetration by STX-ge is achieved when DMSO is employed (Figure 2e). Figure 2g shows the mass spectra of the superficial layer of the skin dermis in which STX-ge can be clearly detected together with the complex phospholipids that characterize this layer of tissue. An average penetration depth of 0.62 mm was measured for STX-ge using DMSO (3 samples).

In the clinic, eutectic mixture of local anesthetics (EMLA) cream is prescribed as a dermal anesthetic. EMLA cream is an oil phase emulsion containing a eutectic mixture of lidocaine and prilocaine in a ratio of 1:1 by weight. We have investigated the skin penetration of these compounds in EMLA cream applications to human skin in addition to samples in which aconitine, BTX-A, saxitoxin, and STX-ge were each added. DESI-MS ion images of lidocaine and prilocaine (m/z 221.1651, mass error of 0.3 ppm) show complete transverse skin penetration to the hypodermis as well as diffuse lateral distribution within the skin section in all 14 samples analyzed (Figure 3). When aconitine is included to the EMLA cream

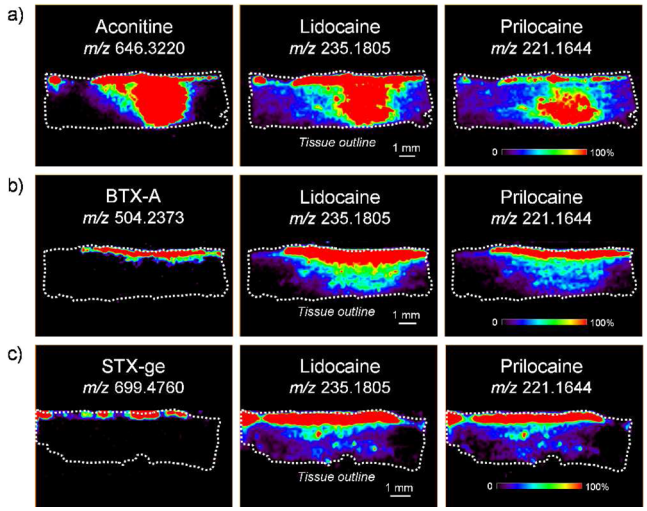

Figure 3. DESI-MS ion images showing the penetration of (a) aconitine, (b) BTX-A and (c) STX-ge tested in human skin of the same donor using EMLA cream. Ion images showing the penetration of lidocaine and prilocaine, compounds with are both present in the EMLA cream formulation, are also presented for the three sections analyzed.

formulation, similar penetration behavior was observed (average depth 2.49 mm, 4 samples); however, lateral spread of the compound was far less pronounced (Figure 3a). BTX-A also shows complete skin penetration (average depth 1.10 mm, 4 samples), albeit not to the same depth as lidocaine/prilocaine (Figure 3b); no penetration was observed for saxitoxin (4 samples). STX-ge showed partial skin penetration, with permeation observed through the skin epidermis reaching the

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superficial dermis, similar to results obtained using DMSO as the vehicle (Figure 3c). In a few skin sections in which less compound was detected, complete penetration of the STX-ge compound up to the superficial dermis was seen (average penetration depth of 0.45 mm for 4 samples) (Figure S3).

Evaluation of Dermal Permeation in a UV Burn Injury Model. Topical application of analgesics has been used for the treatment of dermal pain caused by skin injuries such as sunburn. To test if the penetrability and diffusivity of small molecules in human skin is altered due to UV burn injuries, we have performed DESI-MS experiments on a burn injury model by applying UV radiation (300−450 nm) to ex vivo human skin.[27] Following exposure to UV light, compounds of interest were applied as ethanol solutions. UV radiation of human skin sufficient to induce DNA damage, as measured by increase in growth arrest and DNA-damage-inducible Gadd45a protein, did not significantly alter skin penetration of the five compounds tested (Figure S4) when compared to our earlier findings (see Figures 1 and 2).

Dermal Permeation in ex Vivo Human Skin at Different Application Times. We have also evaluated the penetration behavior of aconitine, lidocaine, and BTX-A at different application times (1, 4, and 10 h) in ex vivo human skin. Interestingly, aconitine was the only compound that showed a pronounced change in penetration depth with application time, with decreased depth penetration occurring at 1 h of application in comparison to 4 and 10 h (Figure S5). For all the other compounds, no significant temporal difference was observed.

Dermal Permeation in in Vivo Rat Skin. Transdermal permeation experiments using live murine subjects were performed for comparative purposes against ex Vivo data. Solutions of aconitine and STX-ge in either ethanol or DMSO were applied to the shaved back of male Sprague−Dawley rats under general anesthesia (see Supporting Information for experimental details).[28] The animals were sacrificed, their skin removed, sectioned, and analyzed following the same experimental procedures used for ex vivo experiments. Pathologic evaluation of the tissue sections revealed that in all samples analyzed, besides skin epidermis, dermis, and hypodermis layers, muscle tissue was also present. Complete skin penetration of aconitine was noted using both ethanol and DMSO as vehicles, and in both cases the compound reached the muscle layer of the tissue section. Interestingly, many samples showed a different spatial distribution of analyte within the sectioned tissue, which varied depending on the choice of vehicle (Figure 4). Ion images of aconitine applied in ethanol to rat skin suggest that transverse diffusion of this compound is facilitated through hair follicles, an important pathway for the penetration of topically administered substances.[29] The same experiment performed with DMSO reveals a very different ion map that is much more reminiscent of the ex vivo data. Pathologic evaluation of the H&E stained tissue sections in comparison with the DESI-MS ion images confirms that aconitine is colocalized with the regions of hair follicles within the tissue section. In the case of STX-ge, complete penetration of this compound occurs using both ethanol and DMSO, reaching the deep dermis layer of the skin (Figure S6). The results from ethanol experiments stand in contrast to those obtained with human skin. Overall, deeper tissue penetration of both aconitine and STX-ge was achieved in vivo using DMSO as vehicle (1.90 mm in ethanol for 7 samples versus 2.60 mm for 3 samples in DMSO for aconitine; 0.90 mm in ethanol for 5 samples versus 1.12 mm for 7 samples in DMSO for STX-ge) (Table S2).

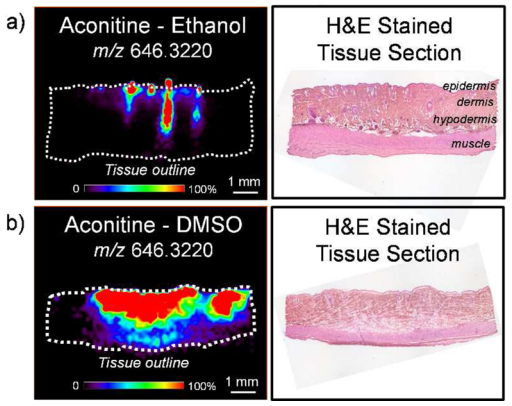

Figure 4. DESI-MS ion images showing the penetration of aconitine tested in in vivo rat skin using (a) ethanol and (b) DMSO as vehicles. Optical images of the same tissue sections imaged by DESI after H&E stain are also shown.

CONCLUSIONS

Our findings demonstrate that DESI-MSI is an operationally simple yet sensitive and powerful tool for investigating transdermal permeation of small molecules. We have successfully employed high mass resolution DESI-MSI to visualize the permeation of topically applied sodium channel modulators on ex vivo human skin samples and on live animal skin. These results lead the way for subsequent animal behavioral studies to assess the local antinociceptive effect of these agents when applied in different vehicles. We believe DESI-MSI methodology has the potential to play a guiding role in topical drug development and formulations research.

ASSOCIATED CONTENT

Supporting Information

Experimental details, supporting figures and tables are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): J.V.M., D.C.Y., and J.D.B. have an equity interest in SiteOne Therapeutics, Inc., a start-up company focused on pain research. The other authors declare no competing financial interests.

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