Predicting Eye Stinging Using the Novel NociOcular Assay

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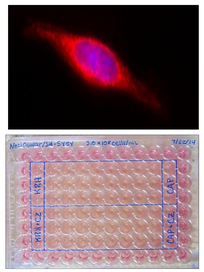
ABSTRACT

Several *in vitro* eye irritation models exist; however, no eye irritation models have demonstrated the ability to accurately predict eye stinging. The NociOcular assay, a novel neuronal model based on activation of the Transient Receptor Potential Vanilloid type 1 (TRPV1) channels, has been shown to distinguish stinging from non-stinging products. In the NociOcular assay, the TRPV1 channel expressing SH-SY5Y neuroblastoma cells are exposed to a serially-diluted test substance and TRPV1 channel activation is measured by acute increases in the intracellular free Ca^{2+}. Although the NociOcular assay was originally designed to predict the eye sting potential of surfactant ingredients and surfactant-based products, there are many other product types which may come in contact with the eyes, such as sunscreens. In this study, we sought to evaluate sunscreens and other products that are used near the eyes. We developed alternate solvents and a modified dilution method to overcome solubility and viscosity limitations and to more accurately model in-use exposures. Furthermore, we investigated the possibility that the alternate solvents and modified dilution method could affect the results of the assay. During proof-of-concept studies, the assay modifications allowed for greater solubility, and controls performed as expected. Additionally, using these modifications of the assay, we successfully measured TRPV1 channel activation caused by products which are hydrophobic, viscous, and may come into contact with the eyes at a high concentration. Future research will focus on evaluation of target ingredients in insoluble products and further modifications of the assay to assess final product formulations.

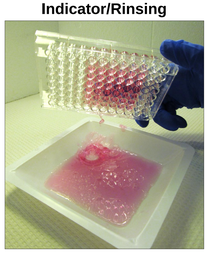
NOCIOCULAR *IN VITRO* ASSAY

Seeding



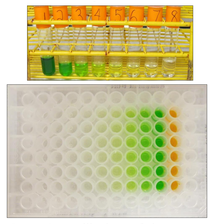
Step 1: TRPV1 transfected SH-SY5Y cells are seeded in 96-well plates and incubated until an appropriate confluency is achieved.

Addition of Calcium Indicator/Rinsing



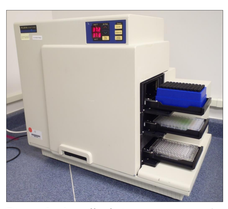
Step 2: The cells are treated with a calcium dye indicator and rinsed twice prior to the addition of assay buffer. Half of the wells receive buffer with a TRPV1 antagonist (capsazapein)

Test Article Preparation



Step 3: A dilution of the test article is prepared and added to a 96-well compound plate that is later used for dosing. The compound plate also contains the solvent control (assay buffer) and the positive control (TRPV1 agonist) for comparison.

Dose and Read Plate



Step 4: A cell plate, compound plate, and tips are loaded into the FlexStation Fluorometer. The cell plate is systematically dosed and the fluorescence intensity is recorded using SoftMax Pro software. The data is saved and analyzed using SoftMax Pro, Microsoft Excel, and Prism software.

DATA ANALYSIS

Table 1. Criteria for classification of a product to be stinging to the eye by using the NociOcular Assay. All three criteria must be met in order for a substance to be considered a stinger.

Test Parameter	Cut off Level
Emax (% of capsaicin response)	≥ 24
EC50 (concentration inducing 50% effect of Emax)	≤ 0.03
Effect at the concentration 0.032%	≥ 22

EXPERIMENTAL PLAN

- We planned to test sunscreens, with emphasis on those designed for babies and kids.
- Sunscreens designed for children are typically viscous and hydrophobic and present many challenges when conducting the assay including insolubility of the test substance in diluents typically used in the assay, and challenges for pipetting the diluted concentrations onto the cells using the robotic pipetting of the FlexStation.
- Our goals were to establish alternate solvents for use in the assay when handling these types of formulations which were also amenable to use in the FlexStation.
- Since these products are designed to be applied to the body without dilution, we sought to establish a dilution scheme which was more relevant to the exposure.
- Then, we planned to assess if these modified dilution schemes were compatible with the assay system and assess the products for eye stinging potential using the prediction models established for surfactant based products.

INTRODUCTION

An *in vitro* assay capable of predicting eye stinging would be very beneficial as a pre-clinical screening tool; the NociOcular assay is making advances to fill that gap. The TRPV1 channel is a well characterized pain–inducing receptor activated by chemical stimuli that is expressed in sensory nociceptors. A TRPV1 expressing clone of the human SH-SY5Y neuroblastoma cell line was obtained by stable transfection, using puromycin-containing selection medium. The transfection of TRPV1 expression was visualized by primary TRPV1 antibodies and Alexa fluor red 568-conjugated secondary antibodies (red) as seen in the Step 1: Seeding image to the left; the nucleus is stained with Hoechst (blue) (Forsby et al., Toxicol Sci. 2012, 129 (2):325-31). During the NociOcular assay, acute increase in the intracellular free Ca^{2+} level was measured in a semi-HTS fluorescence reader (FlexStation, Molecular Devices) using Fura-2/AM. The ratio of fluorescence at 340 (Ca^{2+}-bound Fura-2)/380 (Fura-2) nm excitatory wavelengths was registered without interruption before and during the 2 minute exposure to the test compounds. The mean value (% increase of basal Ca^{2+} level) from triplicate wells in the 96-well plate was monitored for each concentration from each experiment. The TRPV1 antagonist capsazepine was added simultaneously with each concentration of the chemicals in triplicate wells to confirm TRPV1-mediated Ca^{2+} influx. The intracellular Ca^{2+} increase induced by the specific TRPV1-agonist capsaicin was set to 100% response for each experiment and the effect of the test products was calculated as percent of the capsaicin induced response. All test compounds were diluted in KRH-buffer or a KRH-buffer solution containing a non-stinging detergent and the addition of the test compound to the cells was performed robotically during measurements by the FlexStation reader.

SUNSCREEN DILUTIONS

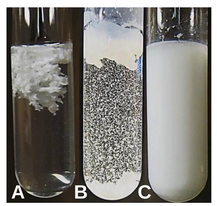
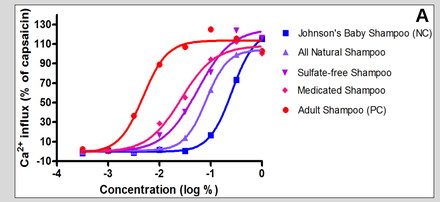


Figure 1. Sunscreen Products in KRH assay buffer. Many sunscreens, especially baby products, are viscous and insoluble in the NociOcular assay buffer (**A** and **B**). However, when the dilution is prepared using a known, non-stinging detergent, a homogenous mixture was obtained (**C**). This mixture was then used in the assay to create a serial dilution and to successfully dose the otherwise challenging test compounds.

RESULTS & CONCLUSIONS

Surfactant-Based Products

In a trial comparing surfactant-based products, the adult shampoo (red) demonstrates the strongest stinging response and Johnson's Baby shampoo (blue) demonstrates a non-stinging response. The adult shampoo and the medicated shampoo are classified as stingers and the remaining three are classified as non-stingers. In the table **(B)** the criteria for classification of eye stingers for surfactant-based products are listed along with the values obtained for each test substance. Only test substances that meet all three criteria are considered stingers in the assay model.



B

Test Substance	Emax ≥ 24	EC50 ≤ 0.03	Effect at conc. 0.032% ≥ 22
Adult Shampoo	113.5	0.004794	110.02
Medicated Shampoo	106.9	0.011	79.461
Sulfate-free Shampoo	125.8	0.05518	40.547
All-Natural Shampoo	104.6	0.1217	6.124
Johnson's Baby Shampoo	123.5	0.2593	1.633

Figure 2: Eye-Stinging Comparison of Surfactant-Based Products (Shampoos). The graph shows the concentration effect curves of five shampoo products **(A)**. The range of responses are graded by color ranging from red to blue. The table **(B)** displays the relevant values observed for each test substance. Highlighted in yellow are the values which meet the required criteria for stinging classification.

Use of a Non-stinging Detergent

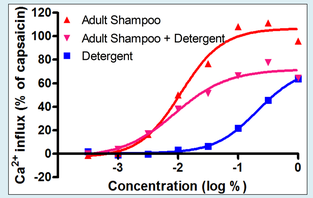


Figure 3: Standard curves displaying the effects of a non-stinging detergent. The adult shampoo, a non-stinging detergent, and a solution containing both were tested.

To determine if the use of a non-stinging detergent during sample preparation had any impact on stinging response, a dilution was prepared using a known stinger (adult shampoo). Results indicated that adult shampoo continued to meet all three criteria for classification as a stinger and therefore, the use of the detergent is a viable option for test substances which are insoluble in KRH assay buffer **(Figure 3)**.

Insoluble Sunscreen Products

Insolubility of test materials resulted in inadequate dosing and the inability of the cells to access the full range of ingredients in the sunscreen formulations. This resulted in inconclusive data concerning the stinging nature of the material **(A)**. Subsequently, dilutions containing varying concentrations of a detergent and an insoluble sunscreen were prepared and tested in the assay. Increasing the solubility of the product through the use of the detergent resulted in a sting response **(B)** that correlates to consumer reviews.

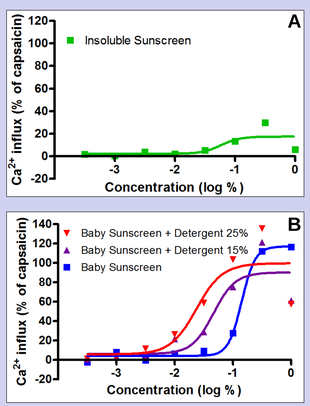


Figure 4: Insoluble Sunscreen Products. The concentration effect curve **(A)** provides an example of the result when products are insoluble in KRH buffer and incompatible with pipetting in the FlexStation. The curves in **(B)** display the change in response associated with increased solubility of a baby sunscreen product.

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