ABSTRACT
Although several in vitro eye irritation models exist, none have demonstrated the ability to predict eye stinging. The NociOcular assay, a novel neuronal in vitro model with high expression of capsacin-responsive Transient Receptor Potential Vanilloid type 1 (TRPV1) channels, has been shown to distinguish stinging from non-stinging baby bath products. We sought to evaluate the eye stinging potential of additional surfactant-based products and sunscreen formulations. In the assay, SH-SY5Y neuroblastoma cells are cultured in 96-well plates and exposed to serially diluted test substance and TRPV1 channel activation is measured by acute increases in the intracellular free calcium. In separate wells, cell viability is determined using the MTT assay, and TRPV1 antagonist capsazepine is used to confirm TRPV1-mediated calcium influx. The positive control, an adult shampoo that contains cocamide DEA, a known stinging ingredient, was the most active surfactant-based test substance evaluated in the assay. The negative control, a baby shampoo, was negative in the NociOcular assay and clinical tests. Four shampoo products resulted from a range of responses between these controls and were classified as either stinging or non-stinging based on the percentage calcium influx as compared to capsazepin over the dose-response. During plot studies with sunscreen formulations, several technical challenges arose including insolubility in assay buffers and pipetting the subsequent dilutions onto the cells. In order to achieve greater solubility, alternate solvents composed of detergents along with assay buffers were reviewed with no success. These formulations were allowed for increased solubility and dilutions were successfully administered onto the cells. Ten sunscreen formulations were evaluated and ranked according to TRPV1 response and compared to available consumer experience reviews for eye stinging. Future research aims to assess the accuracy of the predictions for both the shampoos and sunscreen products through clinical data comparison.

EXPERIMENTAL PLAN

We planned to test sunscreens, with emphasis on those designed for babies and kids.

- Sunscreens designed for children are typically viscous and were therefore evaluated in the Step 1: Seeding image to conduct 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.
- We also evaluated vehicle (0%) as well as vehicle + TRPV1 antagonist capsazepine treated as a non-stinging control.

- 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 determine if the vehicle 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

In vitro assay capable of predicting eye stinging would be very beneficial as a preclinical 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 chosen as an in vitro model, using puroycin-containing selection medium. The transfection of TRPV1 expression was visualized by primary TRPV1 antibodies and Alexa fluor red 568-conjugated secondary antibodies (red) as shown 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 Ca2+ level was measured in a semi-HTS fluorescence reader (FlexStation, Molecular Devices) using Fura-2/AM. The ratio of fluorescence at 340 (Ca2+-bound Fura-2) vs. 380 (Fura-2) nm excitation wavelengths was registered without interruption before and during the 2 minute exposure with test compounds. The data was saved and analyzed using GraphPad Prism, Microsoft Excel and Prim 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.

<table>
<thead>
<tr>
<th>Test Parameter</th>
<th>Cut off Level</th>
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<tbody>
<tr>
<td>EmA (% of capsain response)</td>
<td>≥ 24</td>
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<tr>
<td>EC50 (concentration inducing 50% effect of EmA)</td>
<td>≤ 0.03</td>
</tr>
<tr>
<td>Effect at the concentration 0.032%</td>
<td>≥ 22</td>
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SUNSCREEN DILUTIONS

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

RESULTS & CONCLUSIONS

In a trial comparing surfactant- based sunscreens, the adult shampoo (red) demonstrates the strongest stinging response and Johnson Baby shampoo (blue) demonstrates a non-stinging response. The adult shampoo and Baby shampoo were similarly classified as stingers and the remaining three are classified as non-stingers. In the Table B the criteria for classification of eye stinging 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.

NOCIOcular IN VITRO ASSAY


Use of a Non-stinging Detergent

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 test substance and diluted test substance. 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 stinging response (Figure 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 curve in (B) displays the change in response associated with increased solubility of a baby sunscreen product.