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# Evaluation of EpiDerm full thickness-300 (EFT-300) as an in vitro model for skin irritation: Studies on aliphatic hydrocarbons

Ramya Mallampati [a,1], Ram R. Patlolla [a,1], Saurab Agarwal [a], R. Jayachandra Babu [b], Patrick Hayden [c], Mitchell Klausner [c], Mandip S. Singh [a,*]

[a] College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, FL 32307, USA
[b] Harrison School of Pharmacy, Auburn University, Auburn, AL 36849, USA
[c] MatTek Corporation, 200 Homer Avenue, Ashland, MA 01721, USA

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ABSTRACT

The aim of this study was to understand the skin irritation effects of saturated aliphatic hydrocarbons (HCs), C9–C16, found jet fuels using in vitro 3-dimensional EpiDerm full thickness-300 (EFT-300) skin cultures. The EFT-300 cultures were treated with 2.5 μl of HCs and the culture medium and skin samples were collected at 24 and 48 h to measure the release of various inflammatory biomarkers (IL-1α, IL-6 and IL-8). To validate the in vitro results, in vivo skin irritation studies were carried out in hairless rats by measuring trans epidermal water loss (TEWL) and erythema following un-occlusive dermal exposure of HCs for 72 h. The MTT tissue viability assay results with the EFT-300 tissue show that 2.5 μl/tissue (≈4.1 μl/cm$^2$) of the HCs did not induce any significant changes in the tissue viability for exposure times up to 48 h of exposure. Microscopic observation of the EFT-300 cross-sections indicated that there were no obvious changes in the tissue morphology of the samples at 24 h, but after 48 h of exposure, tridecane, tetradecane and hexadecane produced a slight thickening and disruption of stratum corneum. Dermal exposures of C12–C16 HCs for 24 h significantly increased the expression of IL-1α in the skin as well as in the culture medium. Similarly, dermal exposure of all HCs for 24 h significantly increased the expression of interleukin-6 (IL-6) and IL-8 in the skin as well as in the culture medium in proportion to the HC chain length. As the exposure time increased to 48 h, IL-6 concentrations increased 2-fold compared to the IL-6 values at 24 h. The in vivo skin irritation data also showed that both TEWL and erythema scores increased with increased HCs chain length (C9–C16). In conclusion, the EFT-300 showed that the skin irritation profile of HCs was in the order of C9 ⩽ C10 ⩽ C11 ⩽ C12 < C13 ≈ C14 ≈ C16 and that the tissue was an excellent in vitro model to predict in vivo irritation and to understand the structural activity relationship of HCs.

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## 1. Introduction

Skin irritation is a reversible inflammatory response observed following exposure to noxious stimuli, which causes the release of various inflammatory cytokines and chemokines by skin keratinocytes and fibroblast cells (Kinkead et al., 1992; Kanikkannan et al., 2000). Kerosene based fuels are widely used as jet propellants (JPs) and several studies have shown that dermal exposure of these fuels can cause skin irritation and/or sensitization. Occupational exposure to jet fuels may occur during fuel transport, aircraft fueling and de-fueling, and maintenance of equipment (Subcommittee on Jet-Propulsion 8 Fuel of Committee on Toxicology, 2003). Jet fuels used in commercial and military aviation are complex multi-component mixtures consisting of hundreds of aromatic and aliphatic hydrocarbons. The skin absorption and permeation of aromatics and aliphatic compounds depend on the chemical nature of the individual component. However, aliphatic hydrocarbons (HCs) have very high skin partitioning and retention compared to aromatics mostly due to their longer residence time in skin (McDougal et al., 2000). Our earlier in vivo studies with nonane, dodecane and tetradecane showed that these chemicals can cause skin irritation following a single occlusive dose and that nonane was the most irritating of these three chemicals (Babu et al., 2004a). Very few studies have been carried out to evaluate the structural activity relationship and skin irritation of HCs and it is very important to understand the skin toxicological behavior of individual aliphatic components of jet fuels.

Most of the reported jet fuel skin irritation studies have been carried out using laboratory animals such as rabbits, rats and mice.

* Corresponding author. Tel.: +1 850 561 2790; fax: +1 334 599 3347.
*E-mail addresses:* rjbabu68@gmail.com (R.J. Babu), mandip.sachdeva@gmail.com (M.S. Singh).
[1] Both authors contributed equally.

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The main problems with in vivo animal experiments are that: (a) these animal models produce higher skin permeation and irritation than human skin, (b) there are differences between human and animal skin, (c) a large number of animals are required to produce unbiased results, and (d) in vivo protocols are cumbersome to carry out in industrial setting due to restrictions placed on the use of animals, especially in European countries. Hence, there is a need to look for alternate in vitro toxicity screening models to minimize the animal use in the skin toxicological studies. We already have demonstrated that three dimensional skin models (EpiDerm EPI-200, MatTek Corporation, Ashland, MA) can be used to study the skin irritation of jet fuels (Chatterjee et al., 2006); however, because of absence of dermis in the EPI-200 skin cultures, we were unable to measure changes in cytokine and chemokine levels following exposure to jet fuels that completely reflect the in vivo situation. This study utilizes the EFT-300 tissue which is a full thickness skin model and contains well-differentiated stratum corneum, epidermis and dermis. There are many other skin cultures such as Alloderm®, Apligraft®, Biobrane®, Celaderm™, Dermagraft®, Epicel®, EZ Derm™, Laserskin®, OrCel®, TransCyte®, etc. are available from various commercial sources. These skin substitutes are widely used for skin burns, wound healing and diabetic ulcer applications, whereas EFT-300 model can be used for studying the skin permeation and irritation applications of drug products and chemicals.

The EFT-300 cultures are derived from human neonatal foreskin tissue and consist of normal human-derived epidermal keratinocytes and fibroblasts, which are cultured to form a multilayered, highly differentiated model of human dermis. The dermal compartment is composed of a collagen matrix containing viable normal human dermal fibroblasts and keratinocytes are cultured atop the dermal component to form the epidermis. Ultra structurally, the EFT-300 skin model closely resembles human skin, thus providing a useful in vitro means to assess dermal irritation and skin toxicity (Asbill et al., 2000; Hayden et al., 2009). EFT-300 model consists of organized basal, spinous, granular, and cornified epidermal layers analogous to those of human skin. Epidermal and dermal interactions will have significant effect on cytokine and chemokine secretion following exposure to an irritant signal and these interactions are important in understanding the skin irritation processes. Significant differences in pro-inflammatory mediator secretion exist, depending on the presence or absence of dermal fibroblasts. According to Welss et al. (2004) the epidermal skin equivalents such as EPI-200 skin are unable to release the secondary cytokines (such as IL-6), whereas due to presence of fibroblasts the EFT-300 full thickness skin model is known to release range of pro-inflammatory markers including IL-1α, IL-6, IL-8, IL-10 and GM-CSF (Bernhofer et al., 1999).

In vitro skin irritation testing methods could be useful in preclinical safety screening as well in ranking chemicals for their irritation potential even at the low end of irritation spectrum (Hayden et al., 2003). Human epidermal keratinocytes (NHEK) exposed to three jet fuels, jet A, JP-8 and JP-8+100 in a culture medium demonstrated that these chemicals induce the release of pro-inflammatory cytokines as TNF-α and IL-8 (Allen et al., 2000). Similar results were obtained with porcine keratinocytes (PKC) exposed to jet fuels; both TNF-α and IL-8 were up-regulated (Allen et al., 2001). Jet fuel aliphatic hydrocarbons (C6–C16) were dosed on NHEK to evaluate their effect on cytotoxicity and IL-8 expression in the tissue. Short chain hydrocarbons (C6–C11) were more cytotoxic, while C9–C13 hydrocarbons were more effective in inducing pro-inflammatory cytokine IL-8 in the cultures (Chou et al., 2002). The exposure of the aromatic hydrocarbon components of jet fuels (e.g. cyclohexylbenzene, trimethylbenzene, xylene, dimethylnaphthalene, ethylbenzene, toluene and benzene) to NHEK resulted in a dose-related differential response in IL-8 release (Chou et al., 2003). All the above studies utilized monolayer keratinocytes growing submerged in a culture medium. In contrast to cells in monolayer culture, engineered skin equivalents mimic human epidermis in terms of tissue architecture and barrier function (Andreadis et al., 2001).

In this study, we have used 3-dimensional EFT-300 skin culture to systematically study the structural activity relationship (SAR) of saturated HCs (from nonane (C9) to hexadecane (C16)) and their effect on tissue viability, skin morphology and cytokine release. In addition, this study assessed if the aliphatic HC structure–dermal irritancy relationship obtained by EFT-300 is comparable to that produced in a rodent (rat) model. Therefore, we have conducted in vivo skin irritation (TEWL and erythema) studies of these chemicals in hairless rats and compared the in vivo and in vitro results.

## 2. Materials and methods

### 2.1. Materials

The EpiDerm full thickness-300 (EFT-300) was obtained from MatTek Corporation (Ashland, MA). A Dulbecco's Modified Eagle (DME) based medium for maintaining cultures was supplied by manufacturer (EFT-300-MM). Aliphatic hydrocarbons (nonane, decane, dodecane, tridecane, tetradecane and hexadecane) were obtained from Wright Patterson AFB, OH. Human IL-1α, IL-6 and IL-8 enzyme immunoassay (EIA) kits were procured from Pierce Biotechnology Inc., Rockford, IL. All chemicals used in these studies were analytical grade.

### 2.2. Animals

CD® (SD) hrBi hairless rats (250–300 g; Charles River Laboratories) were utilized for the animal studies. The protocol for in vivo experiments was approved by the Animal Care and Use Committee, Florida A&M University. The animals were given standard animal chow and water *ad libitum* and were acclimated to laboratory conditions for one week prior to experiments. The temperature of the room was maintained at 22 ± 1 °C and the relative humidity varied between 35% and 50%. After completion of the study animals were sacrificed with an overdose of halothane anesthesia.

### 2.3. Chemicals exposure

EFT-300 culture inserts were placed in 6-well plates and equilibrated with 1 ml of EFT-300-MM medium at 37 °C. Following overnight pre-incubation, the culture medium was replaced with fresh 5 ml of medium and skin cultures were placed on top of two stainless steel washers in 6-well plates. Tissues were treated by topically applying 2.5 μl of HCs (C9–C16) for 24 and 48 h and at each time interval culture medium and tissues were collected for analysis. To spread the chemical evenly on the surface of the tissue, the chemical was mixed with equal amount of Johnsons® Baby Oil (Johnson and Johnson Co., Langhorne, PA). This mixture equal to 2.5 μl of the HC chemical was applied on the tissue. The control samples were treated with Baby Oil alone. Tissue samples were either used for the MTT tissue viability assay or harvested and stored in buffered formalin for histological and biomarkers analyses.

### 2.4. MTT tissue viability assay

The MTT assay (MTT-100, MatTek Corporation) was carried out as per manufacturer's instructions. In brief, at the end of 24 and 48 h of treatment, EFT-300 tissue samples were washed twice with