Interim Science Policy: Use of Alternative Approaches for Skin Sensitization as a Replacement for Laboratory Animal Testing

DRAFT FOR PUBLIC COMMENT
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EPA's Office of Chemical Safety and Pollution Prevention:

Office of Pesticide Programs
Office of Pollution Prevention and Toxics

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Background

EPA's Office of Chemical Safety and Pollution Prevention (OCSPP)'s mission is to protect public health and the environment from potential risks due to pesticides (through the Office of Pesticide Programs, or OPP) and commercial chemicals (through the Office of Pollution Prevention and Toxics, or OPPT). Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and listed in 40 CFR part 158, OPP requires significant laboratory animal toxicity tests to support registration of pesticides in the U.S. In response to the 2007 National Research Council (NRC) report on Toxicity Testing in the 21st Century[1], OPP developed a strategic vision for developing and implementing computational and predictive modeling approaches, *in vitro* techniques, and moving towards more limited, targeted *in vivo* testing, to supplement or replace the existing toxicity tests required in support of pesticide registration. In 2016, OPP's Office Director wrote an open letter to stakeholders committing OPP to significantly reducing the number of animals used in acute oral, dermal, and inhalation lethality toxicity testing along with skin irritation, eye irritation, and skin sensitization testing (often collectively known as the "6-pack"). Over the last several years, OPP has worked intensively and collaboratively with numerous domestic and international stakeholders to develop and refine new approach methodologies (NAMs) for use in modernizing the 6-pack.

On June 22, 2016, the Frank R. Lautenberg Chemical Safety for the 21st Century Act amended the Toxic Substances Control Act (TSCA). OPPT is responsible for implementing TSCA. A new section was added to the statute in 2016, Section 4(h) (Reduction of Testing in Vertebrates). Section 4(h)(1) states that EPA "…shall reduce and replace, to the extent practicable (and) scientifically justified…the use of vertebrate animals in the testing of chemicals substances or mixtures…". The law also requires OPPT to "…develop a strategic plan to promote the development and implementation of alternative test methods and strategies to reduce, refine, or replace vertebrate animal testing and provide information of equivalent or better scientific quality and relevance for assessing risks of injury to health or the environment…" (Section 4(h)(2)).

Consistent with OCSPP's commitment to advancing the implementation of NAMs in human health risk assessment, this document describes the science supporting an interim science policy on the acceptance of alternative *(in vitro, in silico, in chemico*) approaches for identifying skin sensitization hazard. These approaches will be accepted in lieu of laboratory animal studies, most often the local lymph node assay (LLNA) in the mouse (OECD TG 429; OECD 2010a), and Buehler or maximization tests in the guinea pig (OECD TG 406, OECD 2010a). Although this document is a draft for public comment, given the substantial scientific evidence and international activities supporting NAMs for skin sensitization, OPP and OPPT will begin accepting these approaches immediately under the conditions described below.

Introduction

Data needs and requirements within the United States and internationally for skin sensitization testing have been recently reviewed (Strickland et al. 2018; Daniel et al. 2018). In the US, as described in 40 CFR

[1] https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/strategic-vision-adopting-21st-century-science

part 158, skin sensitization testing is generally required for both pesticide active ingredients and pesticide formulations although waivers and exceptions are possible (EPA, 2012). OPPT does not have any specific data requirements *per se* but does receive skin sensitization information, mostly for some chemicals in the new chemicals program. In addition, if a new chemical is suspected of being a skin sensitizer, studies or information may be requested.

This draft science policy was developed in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and builds on international activities with member countries of the International Cooperation on Alternative Test Methods (ICATM), notably the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and Health Canada. This draft science policy also builds upon the collaborative efforts of EPA's Office of Pesticide Programs and Health Canada's Pest Management Regulatory Agency[2]. This draft policy also takes advantage of substantial work done by the OECD to establish the adverse outcome pathway (AOP) for skin sensitization, to develop guidelines for several assays (OECD 2012a; OECD 2012b), and establish a collaboration between NICEATM and Cosmetics Europe. Moreover, ICCVAM recently published "A Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States", which emphasizes the need for active engagement by both regulators and industry. EPA's interim policy described here provides an example of how active engagement of regulators and cross-sector collaboration can lead to successful implementation of NAMs to support public health decision making.

In October 2016, EURL ECVAM hosted an ICATM workshop entitled "International Regulatory Applicability and Acceptance of Alternative Non-Animal Approaches to Skin Sensitization Assessment of Chemicals", the first such event of its kind. The workshop was attended by international regulatory authorities from 14 countries, test method validation authorities, and supporting organizations, such as the OECD and the Scientific Committee on Consumer Safety. Workshop participants reviewed the performance of multiple non-animal integrated strategies for skin sensitization hazard assessment, discussed regulatory requirements for skin sensitization testing among various global regions by chemical sector, discussed obstacles to implementing non-animal approaches, and planned the path forward for evaluating and accepting integrated approaches in lieu of animals for skin sensitization (Casati et al. 2017). Follow-up activities from the ICATM workshop have led to publications (Casati et al. 2017, Daniel et al. 2018) and an OECD proposal to develop a new performance-based test guideline (PBTG) for defined approaches[3] (DAs) for assessing the skin sensitization potential of chemicals[4]. This proposed PBTG is being sponsored by the US, European Union, and Canada. In addition, NICEATM has been collaborating with Cosmetics Europe to review the scientific quality of 12 DAs available for skin sensitization, resulting in a comprehensive database of *in vivo* laboratory animal*, in chemico, in silico,*

[2] Related to the Regulatory Cooperation Council work plan on Integrated Approach to Testing and Assessment (IATA) (https://www.trade.gov/rcc/documents/2016_Pesticides_Work_Plan.pdf).

[3] Per the OECD, a defined approach (DA) to testing and assessment consists of a fixed data interpretation procedure (DIP) applied to data generated with a defined set of information sources to derive a result that can either be used on its own, or together with other information sources within an IATA [integrated approach to testing and assessment], to satisfy a specific regulatory need. (OECD GD 255).

[4] http://www.oecd.org/chemicalsafety/testing/TGP%20work%20plan_August%202017.pdf

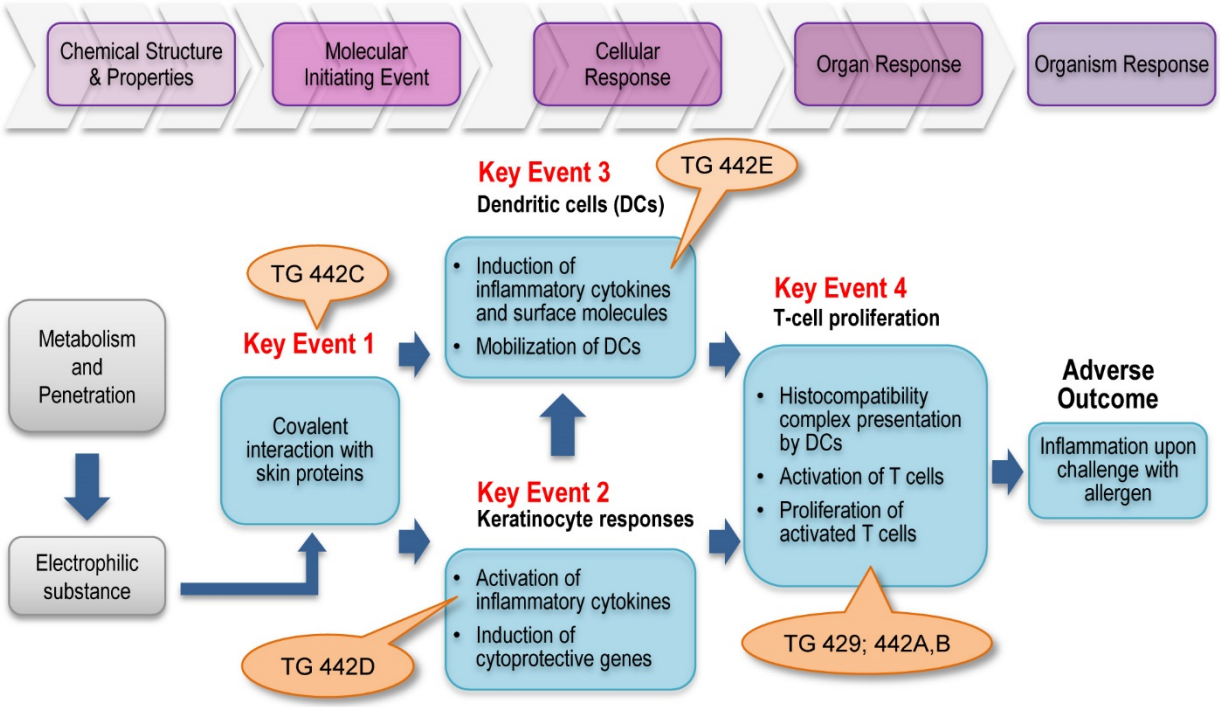
and *in vitro* data for 128 cosmetic ingredients (Supplemental Material, Kleinstreuer et al., 2018; Hoffman et al., 2018).

Scientific Evaluation & Support

1. Adverse Outcome Pathway & OECD Guidelines

The OECD AOP for skin sensitization is described in "The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins" (OECD 2012a; OECD 2012b). The AOP for skin sensitization (Figure 1) is initiated by key event 1 (KE1), which is followed sequentially by three KEs with well-accepted biological significance: (KE2) keratinocyte activation, (KE3) dendritic cell activation, and (KE4) proliferation of antigen-specific T cells.

Figure 1. The Adverse Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins (Adapted from Strickland et al. 2018)



Several non-animal methods with internationally recognized test guidelines adopted by OECD member countries assess the ability of chemicals to activate the first three KEs (OECD 2015a; OECD 2015b; OECD 2017). Examples of these methods are shown in Figure 1, and are detailed below. There are currently no validated non-animal methods that assess the ability of substances to activate KE4, the proliferation of activated T cells.

- OECD TG 442C covers assays that assess the ability of a substance to form a hapten–protein complex, i.e. the molecular initiating event, KE1 (Figure 1) (OECD 2015a). The direct peptide reactivity assay (DPRA) is an example of an *in chemico* test that maps to KE1.

- OECD TG 442D covers assays that assess the ability of substances to activate cytokines and induce cytoprotective genes in keratinocytes, KE2 (OECD 2015b). The KeratinoSens™ and the LuSens are ARE-Nrf2 luciferase test methods that map to KE2.
- OECD TG 442E covers assays addressing activation of dendritic cells, KE3. The human Cell Line Activation Test (h-CLAT), Interleukin-8 Reporter Gene Assay (IL8-Luc) and Myeloid U937 Skin Sensitization Test (U-SENS™) all assess the ability of substances to activate and mobilize dendritic cells in the skin, KE3. (OECD 2017).

Each of the OECD TGs associated with the skin sensitization AOP have been, or are in the process of being, updated to cover multiple assays that measure a particular KE. For example, the original OECD test guideline for h-CLAT was recently revised to include the IL8-Luc and U-SENS™ assays under OECD TG 442E, "In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation" (OECD TG 442E; OECD 2017), which is now a test guideline for *in vitro* skin sensitization assays addressing KE3. However, none of these methods are currently accepted as stand-alone replacements for the animal methods (OECD 2015a; OECD 2015b; OECD 2017a). Therefore, it is generally assumed that only a combination of methods will obviate the need for animal testing, although methods that can serve as stand-alone replacements may be developed in the future (Kleinstreuer et al. 2018, See Table 4). As such, approaches are needed that integrate data from *in vitro, in silico,* and/or *in chemico* sources.

2. Characteristics of Defined Approaches

Per OECD Guidance Document 256 (OECD GD 256), "a defined approach consists of a fixed data interpretation procedure (DIP) (e.g. statistical, mathematical models) applied to data (e.g., *in silico* predictions, *in chemico*, *in vitro* data) generated with a defined set of information sources to derive a prediction. In contrast to the assessment process within Integrated Approaches to Testing and Assessment (IATA), that necessarily involves some degree of expert judgment, predictions generated with defined approaches are rule-based and can either be used on their own if they are deemed fit-for-purpose or considered together with other sources of information in the context of IATA." (OECD 2016a).

A DA should contain the following: defined endpoint, defined purpose, description of the underlying rationale, description of the individual information sources used, description of how data from the individual information sources are processed, and consideration of the known uncertainties. As described in Casati et al. (2017), at the 2016 ICATM workshop, the following evaluation framework was proposed for skin sensitization defined approaches and their inclusion within IATA (extracted from Casati et al, 2017):

- The reproducibility of a DA should provide a level of confidence no less than that provided by the reproducibility of the reference animal test.
- If judging the predictive capacity of a DA directly against reference animal data is necessary, the DA should not be expected to show better predictivity than the animal test itself is able to predict. The DA should provide an equivalent level of information as the reference animal test method, depending on the decision context of the sector/regulatory framework. For example,

the DA should, at minimum, provide hazard information and should ideally provide sufficient information for classification and labeling.

- The DA should be mechanistically and biologically relevant, preferably with respect to an existing AOP framework. The DA should cover at least one molecular initiating event or key event of the AOP.
- The DA should be transparently described using the template provided in OECD GD 255 (OECD 2016b) (e.g., the chemical space/applicability domain for which the DA works and its known limitations must be clearly described, including applicability to multi-constituent substances, mixtures, substances of unknown and variable composition, etc.). OECD GD 255 also recommends that if non-OECD TG methods are used, they should be reported according to OECD GD 211 (OECD 2014a) and *in silico* models should be characterized according to the five OECD principles for QSAR model validation (OECD 2014b) and reported using the QSAR Model Reporting Format, accessible at: https://eurl-ecvam.jrc.ec.europa.eu/laboratories-research/predictive_toxicology/qsar_tools/qrf.
- Independent evaluation and implementation by third parties must be possible (i.e., all the DA components must be readily accessible and all the relevant protocols must be available).
- Ideally, the DA should include one or more OECD TG methods to facilitate acceptance.
- Conflicting results between reference *in vivo* laboratory animal data and DA information sources should be properly discussed and, if possible, explained.
- Uncertainty (both at the level of the DA and of the reference data against which the DA is assessed) should be described to the fullest extent possible. The DA and its individual information sources should undergo a quality assured, independent scientific review to raise confidence in the approach.
- Criteria for selecting reference chemicals should be defined for the particular regulatory area, to cover the relevant applicability domain, rather than developing one definitive list.
- DA predictions should be considered in the context of IATA together with all available and relevant information, when available.

3. Variability of Laboratory Animal Studies: LLNA & Skin Sensitization

All toxicity tests, animal and non-animal, have strengths and limitations. It is therefore important to keep in mind the limitations of the reference animal data when using them for assessing the performance of NAMs and defined approaches. Specifically, NAMs and defined approaches should not be expected to provide more accurate predictions than the reference method (i.e., based on the variability of the reference data in the method of interest) (Dumont et al. 2016). In the case of skin sensitization, LLNA data are most often used as the *in vivo* reference method. ICCVAM reviewed LLNA variability in a 1999 report (ICCVAM 1999), and several more recent reviews provide an evaluation of LLNA variability, specifically for use in assessing predictivity of NAMs and defined approaches (Dumont et al. 2016; Hoffmann 2015; Roberts et al. 2016; Kleinstreuer et al. 2018). The results for comparisons to hazard or potency classifications are summarized:

- Based on a dataset of LLNA studies collected by the European Commission's Joint Research Center, Dumont et al. (2016) proposed a level of accuracy for identifying negative chemicals of ~70% as comparable to the performance of the LLNA.

- Hoffmann (2015) evaluated the variability of the LLNA assay using data reported in the NICEATM LLNA database. In determining hazard potential (i.e., sensitizer vs. non-sensitizers), the false positive rate ranged from 14-20%, while the false negative rate was 4-5%.
- Most recently, using the Cosmetics Europe database (Hoffman et al. 2018), the reproducibility of the LLNA for hazard classification was ~78%.

In summary, the LLNA is the most typical *in vivo* animal reference dataset for benchmarking predictive performance of NAMs and defined approaches, and the inherent reproducibility of the LLNA has been shown by multiple analyses to be in the range of 70-80% for hazard prediction and 60-70% for potency prediction, depending on the summary statistic used for comparison (e.g., median, mean, etc.). This reflects the variability in the animal data and provides an indication of the uncertainty that exists when comparing DA predictions to the LLNA endpoint, especially in the case of substances that only have one LLNA study. Further, these numbers provide a baseline for comparison when quantitatively assessing the predictive performance of the defined approaches in Figure 2 below. This quantitative performance must also be combined with fulfillment of the qualitative evaluation criteria outlined above.

4. Evaluation of Existing Defined Approaches for Skin Sensitization by NICEATM and Cosmetics Europe

Annex I of OECD GD 256 contains twelve case studies submitted by international stakeholders, covering various DAs and IATA for skin sensitization (OECD 2016c). NICEATM and Cosmetics Europe collaborated to evaluate various technical and practical aspects, along with predictive performance, of these proposed alternative approaches (Kleinstreuer et al. 2018, See Table 4). The evaluation was conducted in two phases and considered a variety of data interpretation procedures, ranging from simple (e.g. decision trees) to complex (e.g. machine learning algorithms). In the first phase, six qualitative evaluation categories were used: characteristics (e.g., purpose of the approach); input data (e.g., *in vitro, in chemico, in silico* and expert systems); prediction algorithm; mechanistic relevance with respect to the OECD AOP and the relevant key event(s); applicability domain; and practical aspects (e.g., relative cost and availability through contract research organizations, CROs). The investigators emphasized transparency in terms of input data availability and the feasibility of reproducing the algorithm using open-source software.

In the second phase, six of the twelve DAs were quantitatively assessed for their ability to predict skin sensitization, using the maximum subset of 128 substances from the Cosmetics Europe dataset that had a complete set of *in vitro, in chemico, in silico*, and *in vivo* laboratory animal data. **All six DAs evaluated for performance demonstrate comparable or superior performance to the LLNA.**

5. Scope & Defined Approaches for Submission Under this Interim Policy

Under this interim policy, OPP and OPPT will accept submissions for single chemicals (e.g., pesticide active ingredients or pesticide inert ingredients) that can be tested using the methods noted in Section 1 that make up the two DAs identified below. Formulations will not yet be accepted under this interim policy. However, EPA expects expansion of this interim policy in the near term to include some pesticide formulations or other mixtures evaluated by OPPT, upon completion of ongoing testing (which includes

>20 pesticide formulation products and mixtures identified by OPPT) at the National Institute of Environmental Health Sciences (NIEHS) National Toxicology Program (NTP).

This interim policy focuses on those that will be accepted for regulatory use in the near term due to their simplicity, ease of implementation, and their use will not compromise the protection of public health. Based on the evaluation criteria outlined in Section 2 and the work done in Kleinstreuer et al. (2018), the following DAs are deemed acceptable alternatives to the LLNA for regulatory submission.

- **AOP "2 out of 3"** (Figure 2a). This DA was initially submitted to OECD by BASF and was first described in Bauch et al. (2012) and subsequently applied to a larger dataset by Urbisch et al. (2015). The approach predicts skin sensitization hazard by sequential testing, in an undefined order, in up to three internationally accepted non-animal methods that map to KEs 1-3 of the AOP. First assays are run for two KEs. If these assays provide consistent results, then the chemical is categorized accordingly as positive or negative. If the first two assays provide discordant results, an assay for the third KE is run. The overall result is based on the two concordant findings. While the first version of the DA relied upon the DPRA, KeratinoSens, and h-CLAT, here, following the model of the updated KE-based OECD TGs, any assay that is included under the same OECD TG may be considered a drop-in replacement. For example, submitters could run the LuSens instead of the KeratinoSens, and the U-SENS instead of the h-CLAT.
- **KE 3/1 sequential testing strategy (STS)** (Figure 2b). This DA, originally developed by Kao (Nukada et al. 2013, Takenouchi et al. 2015) is a simple decision tree that requires KE 1 (e.g., DPRA) and KE3 (e.g., h-CLAT, IL8-Luc, U-SENS) data as inputs. First, the assay for KE3 is conducted; if the response is positive, the test substance is classified as a sensitizer. If a negative result is obtained from a KE3 assay, an assay for KE1 is conducted. A negative study for KE1 confirms that it is a non-sensitizer and a positive result for KE1 leads to a finding of sensitizer. As with the "2 out 3" DA, only assays included in the respective KE-based OECD TGs will be considered as part of this STS DA.

EPA acknowledges that the 3/1 STS is simpler, will often lead to fewer studies conducted, and is thus more efficient. However, some chemicals are likely to have existing evaluations using the 2 out of 3 DA; the agency will accept these. As noted above, a project proposal was submitted to OECD jointly by the US, EU, and Canada to develop a new PBTG for defined approaches for skin sensitization. As part of the work on the future OECD PBTG guideline, refinements and updates to some of the defined approaches are expected in the near-term. OPP and OPPT expect to update this interim policy to accept more defined approaches as the OECD PBTG guideline develops, which includes potential defined approaches for the purpose of risk assessment/addressing potency. Some pesticides or chemicals may need skin sensitization evaluations for use in quantitative risk assessment. A stakeholder interested in conducting quantitative risk assessment for skin sensitization using NAMs is recommended to first consult with the agency.

Figure 2a. Schematic of the AOP "2 out of 3" defined approach. OECD TG methods for Key Events (KE) 1-3 are run in an undefined order until at least two of the three methods show consensus.

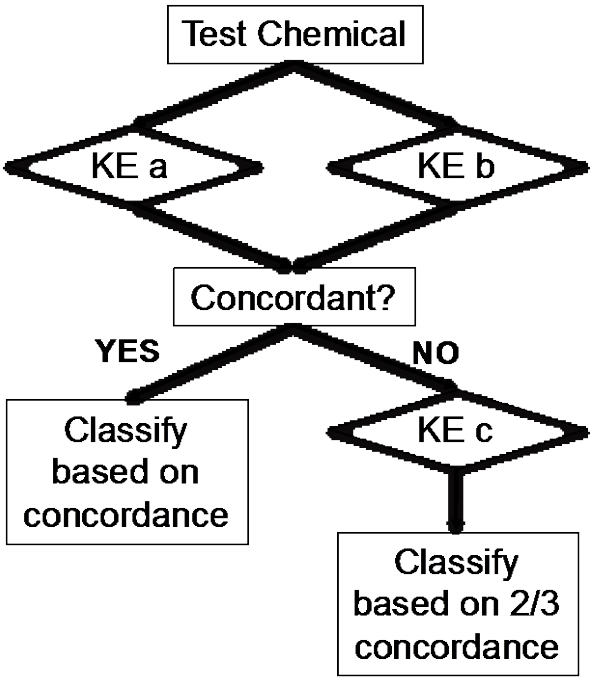
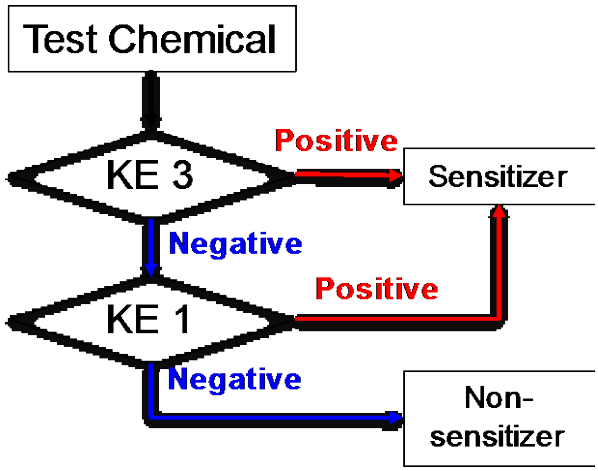


Figure 2b. Schematic of the Key Event (KE) 3/1 Sequential Testing Strategy (STS) defined approach



Conclusions & Next Steps

OPP and OPPT will immediately begin to accept submissions of NAMs and defined approaches as described in Section 5. There are multiple domestic and international activities ongoing that will allow for refinement and expansion of this interim policy to other DAs and additional NAMs and support global harmonization of defined approaches for skin sensitization. OPP and OPPT will continue to be active participants in these activities to ensure regulatory acceptance and will continue to support cross-

sector collaborations that enhance animal welfare, and accelerate the implementation of NAMs. OPP and OPPT invite public comment on this draft, interim science policy for a 60-day public comment period.

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