

Reviewer information

Name of country/agency of reviewer (including regulatory sector): Crop Life Europe Developmental Neurotoxicity Subgroup

Document Title: Case Studies for the Integrated Approaches for Testing and Assessment for AOP3 based Risk assessment of Tebufenpyrad mitochondrial Complex I inhibition mediated neurotoxicity

Please find below a comprehensive review including (4) comments pertaining to use in the regulatory context, (9) major comments pertaining to content of the document, and (41) minor comments pertaining to editorial elements of the document.

Please find below key elements to consider for regulatory use:

	Comment
1	Biological relevance of the selected in vitro endpoint: The use of neurite area reduction in LUHMES cells as a proxy for dopaminergic neurodegeneration ○ represents an early and indirect endpoint, ○ does not capture chronic neurodegeneration, ○ may reflect developmental or acute neuronal perturbation rather than degenerative processes
2	Use of BMC derived from galactose (not glucose) conditions: ○ Galactose conditions are used to enhance sensitivity to mitochondrial toxicants which creates a non-physiological metabolic state ○ High likelihood for a more conservative PoD/Potential overestimation of in vivo potency ○ BMCL25 selection: No established quantitative relationship between in vitro effect size and in vivo hazard ○ introduces additional uncertainty in IVIVE
3	Impact of exposure duration on endpoints ○ How to (quantitatively) relate vitro exposures between minutes up to 24 hours to in vivo chronic exposure pattern? ○ The 24-hour time-point for the neurite outgrowth (most plausible PoD) is not really scientifically justified (more a pragmatic/technical choice) à impact of exposure time on PoD unclear (assumption: higher PoD with shorter exposures, lower PoD with longer exposures) ○ Clear (scientifically justified) guidance is required on in vitro exposure conditions for regulatory implementation

4	Validation of PBK-derived internal exposure: ○ Model not validated against in vivo data ○ Reliance on NAM-based parameterization, assumptions and correction factors ○ exposure values reported are indicative, not predictive

Please find below (9) major and (41) minor comments for consideration:

#	Section - Page	Comments	Proposed changes for improvement
Major comments			
1	Section 10.3.1, Page 68	The document states “For each scenario, results were derived for BMR equal to 0.05, 0.15, 0. 20, 0. 25, 0.30, 0.35, 0.50.” A statement is made in the introduction (Page 14) that a 50% reduction in SNpc cell bodies and their terminal projections in the putamen is observed at [parkinsonian] symptom onset. Was that observation used to determine the upper limit of benchmark responses (BMRs) investigated in the AOP3-IVB KE890 in vitro POD sensitivity analysis in section 10.3.1? The authors should explicitly state their reasoning for using an upper limit BMR value of 0.50 in the data modeling exercise.	Include justification for the use of an upper limit BMR value of 0.50 in the in vitro POD sensitivity analysis.
2	Executive Summary Page 8 & Conclusion Page 89	In the Executive Summary (Page 8) the phrase “most probable in vitro POD” is used. In the Conclusion (Page 89) the phrase “most plausible in vitro POD” is used. Neither of these phrases are formally defined, but they refer to the same thing, namely decreases in neurite area in the AOP3-IVB KE890 assay.	Please consider which phrase is correct and revise the document to use consistent terminology. Consider including a definition for whichever phrase is chosen by the IATA team.

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3	Executive Summary Page 8	The document states “Based on the distribution of the highest concentration of internal brain exposure relative to the distribution of the lowest concentration required to trigger KE890, there is a 99.5% probability that the MoIE is below 1 for unprotected operators”. Two comments regarding this sentence. First, there is room for refinement to make this sentence more clear to the audience. Second, the value of 99.5% is inaccurate. In Table 22, the probability that the MoIE < 1 is stated as 0.997 which would equate to 99.7%, not 99.5 %.	Consider revision of the sentence for clarity. Here is a suggested revision “Based on the highest concentration of brain exposure from the distribution of PBK-modeled estimates relative to the lowest concentration from the distribution of AOP-IVB KE890 assay in vitro PODs, there is a 99.7% probability that the MoIE is below 1 for unprotected operators.” The suggested revision corrects the inaccuracy in the reported probability.
4	Section 3.1, Page 22	The document states “To fill data gaps, in vitro experimental data were generated for Tebufenpyrad: 1. fu (fraction unbound), fu,mic (fraction unbound in microsomes) and fu,p (fraction unbound in plasma); 2. In vitro intrinsic clearance (CL,int).” State whether in vitro intrinsic clearance data was generated using microsomes, primary hepatocytes (human or rat) or a combination of these. This would improve the clarity regarding methodology for the audience. It is unclear in the document if the hepatic clearance information generated for the rat is used in any capacity. If it is not used in any way to inform the IATA, then why mention it?	Clarify methodological details.

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5	Section 4, Page 28	The document states “Upon differentiation, LUHMES cells develop key biochemical, morphological, and functional features of post mitotic dopaminergic neurons, including dopamine metabolism, complex neuronal architecture, high energetic demand, calcium dependent pacemaking activity, and marked sensitivity to mitochondrial complex I inhibition.”. Many claims are made in this sentence without supporting references. Adding references will allow the audience to assess the veracity of these statements.	Suggest adding supporting references for the key biochemical, morphological and functional properties of LUHMES cells listed in this sentence.
6	Section 6.1.4, Page 35 & Table 3 & Section 10.1.1, Page 61 & Table 4	The document states “Six exposure scenarios were modelled, accounting for factors such as outdoor or indoor exposure, crop type, application method, application equipment, population exposed (e.g., operator, worker, resident (children and adults)), and presence or absence of personal protective equipment (PPE).” This statement is somewhat confusing when compared to results in Table 3 on Page 61. It is accurate to say that six exposure scenarios were modeled based on exposure setting (i.e. indoor / outdoor), crop type, application method and application equipment. However, each of these six combinations were modeled or considered for six different subpopulations (e.g. operator w/ PPE, operator w/out PPE, worker w/ PPE, worker w/o PPE, resident adult and resident child). This sentence would be clearer if the subpopulations were not included as a factor that defined the exposure scenario. Rather the six exposure scenarios were applied to each subpopulation (i.e. receptor). Did the IATA author’s consider using a conceptual site model (CSM) to illustrate the	Review instances in the document where the number of population sub-groups evaluated is described and edit where necessary for consistency and accuracy. Also capitalize the acronym “PLETHEM” in the heading of the fourth column of Table 4 to be consistent with the rest of the document.

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		receptors and exposure route combinations that were considered? Also, in Section 10.1.1, the document states “The aim of this module was to estimate the aggregate external exposure to Tebufenpyrad for five population subgroups (operators with and without protections, workers and residents, the latter split in adults and children)”. Is the stated number (5) of population subgroups correct. A worker with and without PPE is presented in Table 3, yet the list in this sentence only says workers. Should this be revised to say “workers with and without protections”? Table 4 also lists a concentration at steady state (Css) for a worker with and without PPE. Please review instances in the document where the number of population sub-groups evaluated is described and edit where necessary for consistency and accuracy.	
7	Section 10.1.4, Page 76	The document states “The performance of the theoretical distributions was assessed in terms of goodness-of-fit (i.e. AIC and the Bayesian Information criterion, BIC, the smaller the value the better - seeTable 19 and 20) to identify the distribution that best represents each of the two uncertainty frequency distributions”. The text indicates that AIC and BIC values were generated for all five theoretical parametric distributions for both the POD for neurite area” and “highest internal exposure operators”.	Add BIC values to Table 20.

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		Table 19 corresponds to “POD for neurite area” and contains both AIC and BIC values. Table 20 corresponds to “highest internal exposure operators” and only contains AIC values. Please add the appropriate BIC values to Table 20 or revise the text on page 75 for accuracy. The BIC values for “highest internal exposure operators” is important to include because it is part of the decision logic that define the best-fit theoretical parametric distribution to the expert elicitation determined frequency distribution.	
8	Section 10.1.4, Page 77	The authors state “The first 5000 samples were discarded to achieve a better convergence to the expected distributions, leading to a final sample of 95,000 values for each of the two parameters.” Why was this step necessary? Is this a standard practice for this type of simulation. The IATA team should provide context and/or justification for why a “burn in” period of 5,000 iterations is needed for this data simulation.	Add supporting information and/or justification for this step in the process
9	Section 14, Page 89	The document states “Experts concluded that the steady-state brain concentration is highly likely to fall within the range of 0.02–5.556 μM. there is a 1.2% probability that the MoIE is below 1 for unprotected operators and a 52% probability that the MoIE is below 10” The conclusion that the probability of the MoIE being below 10 is 52% was not stated in section 10.2 where the actual analysis was conducted. This statement also appears to be in	Review the conclusion for accuracy and revise if necessary.

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		conflict with Table 22 which states that the probability of the MoIE being below 10 for the most extreme exposure scenario (unprotected operators) is 1. Where in the results of the uncertainty analysis shows the 52% probability value? If this is an important finding, it should be stated in the section where the analysis was conducted in addition to the conclusion.	
Minor comments			
1	Executive Summary Page 8	At the end of the second paragraph, the word “Absorption” in the phrase “Absorption, distribution, metabolism and excretion (ADME)” is capitalized.	This word does not need to be capitalized.
2	Executive Summary, Page 8.	In the third paragraph, the document states “This may indicate a <u>potential concern of degeneration of dopaminergic neurons that can potentially result in degeneration of DA neurons</u> and Parkinsonian syndrome neurodegeneration for unprotected operators.” The underlined verbiage in this sentence is repetitive. Suggest revision for brevity.	Revise the sentence to remove repetitive verbiage.
3	Executive Summary, Page 8.	In third paragraph, the document states “These conclusions <u>useare</u> based on the most conservative estimates for an in vitro PoD”. The underlined word “useare” is a typo.	Correct the typo.
4	Executive Summary Figure & Figure 1 in section 3.2.	Module 2, Step 1 states “PBK NAM-based model to translate UB of the external exposure estimate <u>in</u> an UB for the internal exposure (Henri et al., 2023)”.	Change “in” to “into”.
5	Abbreviations and Acronyms, Page 10 & Section 9.4 Module 4 Table, Page 49-59	In section 9.4 in the Module 4 table, the acronym “cl” is used multiple times. This acronym is not defined in the Abbreviations list on Page _____. This acronym refers to mitochondrial complex I.	Given that there are several other abbreviations similar to “cl” (e.g. confidence interval (CI) and clearance (CL)”, suggest either:

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			1) not using the “cl” acronym in place of mitochondrial complex I or 2) using a different acronym for mitochondrial complex I that is not so similar to the others. If an acronym for mitochondrial complex I is to be used, add it to the abbreviation list.
6	Section 1, Page 18.	The document states “Since then, new information on the relationship between complex I inhibition and PD <u>had become available</u> with AOP3.”	Suggest changing “had become available” to “has become available”.
7	Section 1, Page 18.	The document states “The methods, results and raw data from these two projects are respectively published in Henri et al., (2023) and Alimohammadi et al. (2023), and available in Annex III and II of this IATA.” Annex II and III are associated with the PBK modeling performed in Henri et al. (2023). Annex IV and V are associated with the AOP3-IVB developed and implemented by Alimohammadi et al. (2023).	Revise the statement for accuracy. The statement should refer to Annex II and III corresponding to Henri et al. (2023) and Annex IV and V corresponding to Alimohammadi et al. (2023).
8	Section 1, Page 18.	There is a formatting error at the bottom of the page. The phrase “operators without protection equipment” has an unintentional indentation in the middle of the phrase.	Correct the formatting error.
9	Section 1, Page 19.	The document states “To address the problem formulation, EFSA and its Working Group on Neurotoxicity have conducted an independent uncertainty analysis on the methods and experimental data produced in the context of the two outsourced <u>project</u> and integrated the available lines of	Correct the grammatical error.

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		evidence to derive a MoIE.” The word “project” should be plural.	
10	Section 2, Page 20	In the first sentence of the second paragraph, the word “mitochondrial” is misspelled and the word “Complex” is improperly capitalized.	Correct the spelling and capitalization issues.
11	Section 3, Page 22	The document states “This IATA case study has been developed to test the hypothesis that the inclusion of mechanistic information in the <u>postulated AOP3-IVB</u> would enhance the certainty for the neurotoxicity hazard identification and characterization of Tebufenpyrad...”. This sentence is unclear. The AOP3-IVB is not postulated. It actually exists and is used herein. Is this sentence supposed to say “AOP3”, not “AOP3-IVB”?	Review the sentence for content and clarity and revise if necessary. Suggested revision “...mechanistic information determined from the AOP3-IVB would enhance...” or “...mechanistic information from the postulated AOP3 would enhance...”
12	Section 3.1, Page 22	The document states “The methods, results and raw data of these projects are published in Henri et al. (2023) and Alimohammadi et al. (2023) and reported in full in Annexes III, IV and V”. Annex II is also associated with the Henri et al. 2023 study. Should Annex II be cited in this sentence as well?	Review the sentence and revise if necessary by adding Annex II to the list of Annexes cited.
13	Section 3.1, Page 22	The document states “The PBK model developed for Tebufenpyrad was then applied to estimate the internal brain concentration exposure”. Recommend revision for clarity. Internal brain concentrations were estimated from an estimated (or modeled) exposure.	Revise sentence for clarity. Suggested revision “The PBK model developed for Tebufenpyrad was then applied to estimate the internal brain concentration from a modeled external exposure”

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14	Section 3.1, Page 22	The document states: “Generation of experimental data in the AOP3-IVB with Tebufenpyrad. In particular, in vitro assays were developed to describe and quantify effects in KE887, KE177 and KE890 (AOP3)”. It may be difficult for the audience to remember what the actual key events are that are associated with the KE numbers. Suggest adding succinct parenthetical descriptions of KE887, KE177 and KE890 to aid comprehension by the reader.	Review the sentence and consider revising to increase readability and comprehension by the audience.
15	Section 3.2, Page 24	The document states: “Pillar 2: based on the outcome of tests within the <u>AOP3-In Vitro Battery (IVB)</u> which measure the chemical perturbations of either MiE/KE/AO along AOP3”. There is no need to define the IVB acronym at this point. It has already been defined at first use. Also, in other parts of the document the authors explicitly state what MIE, KEs and AO are being assessed using alphanumeric codes (i.e. KE887, KE177, KE890, etc.). For clarity, do the same here. They are highlighted in the graphic in for Pillar 2 in Figure 1.	Revise sentence by removing the acronym description and by being more explicit about the key events being assessed.
16	Section 3.2, Page 24	In two places on Page 24, the document has a parenthetical reference of “(EFSA OECD IATA Case Study, 2026)”. Is this statement self-referential? i.e., is it referring to the same document that the audience is reading or a different document? Is self-referential, can the phrase be restated to let the audience know that? Otherwise this annotation is confusing.	Review the parenthetical statement and revise for clarity.
17	Section 3.2, Page 24	The document states “Pillar 2 deals with the estimate of the lowest concentration able to affect neurite area in <u>Lund</u>	Review the sentence and revise appropriately. Define the

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		<u>human mesencephalic (LUHMES)</u> cells using the in vitro assays of the AOP3-IVB (module 3)...”. The acronym LUHMES had already been used several times up to this point in the document. Define the acronym at first use. Not here.	LUHMES acronym at first usage in the document.
18	Section 3.2, Page 25	The document states “Starting from in silico predictions, in vitro experimental <u>workdata</u> and complementary PBK modelling,...”. The underlined text is a typo.	Correct the typo.
19	Section 4, Page 27	The document states “Regarding Impaired proteostasis (KE889), although the EFSA 2017 Scientific Opinion provides a comprehensive description of this KE and available methods to assess <u>UPS</u> and autophagic disruption, standardised and easily applicable methodologies remain limited.” This is the first usage of the acronym UPS referring to the ubiquitin proteasome system. Define the acronym at first use.	Define the UPS acronym at first use.
20	Section 4, Page 27	The document states “The AOP3-IVB tested in Alimohammadi et al., (2023) relies on Lund human mesencephalic (LUHMES) cells as a model of human derived dopaminergic cells.” The acronym LUHMES had already been used several times up to this point in the document. Define the acronym at first use. Not here.	Review the sentence and revise appropriately. Define the LUHMES acronym at first usage in the document.
21	Section 4, Page 28	The document states “ <u>They</u> have been characterized extensively with regards to their sensitivity to the parkinsonian toxicant MPP + /MPTP as well as their metabolism and transcriptome changes triggered by MPP +.” Does the “they” that begins this sentence refer to the LUHMES cells. If so, suggest being explicit.	Review sentence and consider revision. Example “The LUHMES cells have been characterized...”

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22	Section 4, Page 28	The document states “In the second <u>one</u> , the 120h exposure scenario, LUHMES cells were differentiated for another 3 days after reseeding before they were treated on day 5 (d5) and d7 by a half medium exchange.” A minor revision changing “one” to “protocol” would make this sentence more explicit and easier to understand. It would also make the terminology consistent with the preceding sentence.	Review sentence and consider revision. Example “In the second protocol...”
23	Section 4, Page 28	The third paragraph on this page begins “Limitations related to the use of a single neuronal test system...”. I seems that this paragraph was added in response to a criticism regarding the use of the LUHMES cells that were not fully differentiated in the AOP3-IVB KE890 assay. The information in this paragraph is somewhat distracting from the overall narrative of the case study. Would Section 9 on uncertainties be a better place for this information to be included?	Consider moving paragraph to a section of the document that discusses uncertainties.
24	Section 4.1, Page 29	The document states “The pesticide rotenone has the same binding site on complex I as MPP+. It has also been shown to cause nigrostriatal dopaminergic neuronal loss associated with Parkinsonian motor deficits in rats.” No reference is provided for this statement.	Suggest adding a reference that supports this statement.
25	Section 4.1, Page 29	The last paragraph on Page 29 discusses “Uncertainties in <i>in vivo</i> methodologies for hazard identification (experimental toxicological studies).” The paragraph describes the difficulty in using traditional animal models to identify hazards associated with parkinsonian sequelae, specifically loss of dopaminergic neurons in a specific region of the brain. The paragraph does not have a conclusion sentence indicating	Consider adding a conclusion sentence to this paragraph such as “Because <i>in vivo</i> methodologies may not be suitable for detecting degeneration of dopaminergic neurons, alternative approaches

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		the needs for alternative approaches for detecting this type of hazard.	such as the AOP3-IVB are needed for putative hazard characterization?"
26	Section 4.1, Page 30	The document states "They are <u>provided with</u> very long and dense arborisations projecting into the striatum where DA is released." The grammar in this sentence could be improved. Change "...are provided with very long..." to "...possess very long..." In the same paragraph the document also states "Dopaminergic SNpc neurons are <u>provided with</u> specific calcium channels, the L-type Cav 1.3 which are intended to regulate the autonomous firing as "pacemaker". The grammar in this sentence could also be improved. Change "...are provided..." to "...have..." or "...express..."	Consider revising the sentences for clarity.
27	Section 5, Page 31	The document states "There are also known limitations in terms of the metabolic competence in the AOP3-IVB." This sentence is somewhat ambiguous as written. Suggest more precisely describing what is meant by "metabolic competence" (i.e. the ability to metabolize xenobiotics in a way that is reflective of in vivo processes).	Review sentence and consider revising for clarity.
28	Section 6.1.3, Page 34	The document states "As reported in Henri et al., (2023), for instance, PBK simulations for oral route of exposure <u>require to provide</u> the oral absorption rate (ka, in hour-1) as input parameter, which are representative of the parent compound." The grammar in this sentence could be improved for clarity.	Consider revising the sentence for clarity. Here is an example revision "As reported in Henri et al., (2023), for instance, PBK simulations for the oral route of exposure require an oral absorption rate (ka, in hour-1)

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			that is representative of the parent compound as an input parameter.”
29	Section 6.1.3, Page 34	The document states “Performing these calculations would result in a ka representative of Tebufenpyrad <u>associated to all</u> its metabolites instead of Tebufenpyrad specifically...”. Suggest changing the underlined phrase “...associated with all...”.	Revise the sentence for clarity.
30	Section 6.1.4, Page 35	The first paragraph “b) PBK model parameterisation” uses the acronym “fu,hep”. This is the first use of this acronym in the document. Define the acronym at first use. Also, in the abbreviations table, a space is included between “fu,” and “hep”. Also, in other places in the document (ex. Page 100), the “hep” is subscripted. Suggest a global search of the document for this acronym and revision for constancy. Use the same acronym formatting in each instance in the document.	Review document for consistency in acronym usage.
31	Section 6.1.4, Page 35	The document states “For further details, please <u>check</u> results in Tables 9a and 9b published in Henri et al. (2023).” Suggest changing the word “check” to “refer to”.	Review sentence and consider revision.
32	Section 10.1.2, Page 62	In the first two paragraphs of this section, there is one sentence where all the words in the phrase “expert knowledge elicitation” are lower-case and one sentence where all the words in this phrase are capitalized. Also, the acronym EKE is defined in multiple places in the document. Only need to define the acronym at first usage.	Review capitalization of the phrase “expert knowledge elicitation” throughout the document and use consistent formatting.

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33	Figure 4 Caption, Page 66	An acronym “a.s.” is used. This acronym is not defined in the Acronyms and Abbreviations section. The assumption is that a.s. stands for “active substance”.	Define the acronym in the Acronyms and Abbreviations section at the beginning of the document or consider not using it if it is not used very often.
34	Section 10.1.3, Page 70	The document states “ <u>Table 8</u> shows the estimates of the BMC 90%CI bounds for scenario2.” The table citation is incorrect. This sentence should reference Table 9.	Correct the table citation.
35	Section 10.1.3, Page 71	The document states “. The goodness-of-fit statistics indicates that the best fit is achieved when parameter estimates of are derived from individual biological replicates (in <u>Table 9</u> , models with letters abv are reflecting this situation). As displayed in <u>Table 9</u> , differently from the previous scenarios, now only three models contribute to the model average, one of which with a very limited weight (exponential with three parameters, Hill with three parameters and log-normal with three parameters)” The table citations are incorrect. The sentences should reference Table 10.	Correct the table citations.
36	Section 10.1.3, Page 71	The document states “The AIC in Table 9 shows that the inclusion of the biological replicate as covariate in the model (i.e. BMC estimates stratified by biological replicate) improves the fit of the model, compared to treating the biological replicate as random variable or ignoring it as, respectively, in scenario 1 and 2 where only the residual variance is affected.” The table citation is incorrect. The sentence should refer to table 10.	Correct the table citation.

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38	Section 10.1.4, Page 77 & Figure 9	Figure 9 is not mentioned anywhere in the text. Is the figure actually needed? If so, it needs to be mentioned (briefly) somewhere in the narrative of the document.	Mention Figure 9 somewhere in the document or delete it.
39	Section 10.1.4, Page 77 & Figure 11 & Figure 12.	The last panel of Figure 11 showing the MoIE uncertainty probability distribution is redundant with the information presented in Figure 12. Can Figure 12 be deleted since there is no new information displayed?	Consider whether Figure 12 is necessary or whether the last panel in Figure 11 could be deleted and information combined with Figure 12.
40	Figure 8, Figure 9, Figure 10 and elsewhere in the document.	All figure titles, legends and captions should use terminology that is consistent with what is stated in the text. In the text, it is stated that the phrases “highest internal exposure operators” and “POD for neurite area” will be used to describe the two halves of the margin of internal exposure (MoIE) analysis. Yet, the title of Figure 8 uses the phrase “<i>Highest internal exposure in the brain of operators</i>”, the title of Figure 9 uses the phrase “<i>Internal exposure in the brain of the operators not using PPE</i>” and the title of Figure 10 uses the phrase “<i>internal exposure for operators not using PPE</i>”. In the same vein, the title of Figure 9 uses the phrase “PoD for KE4” while the title of Figure 10 and Figure 11 uses the phrase “PoD for KE890”. KE4 and KE890 refer to the same endpoint measurement. Also, the legends in Figure 9 and Figure 10 as well as the panel titles rendered by ggplot (i.e. the black text directly above each graph), also use inconsistent terminology. Use of inconsistent terminology can confuse an audience and make interpretation of results more difficult.	Review the entire document and use consistent terminology defined in the text as to not confuse the audience.

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41	Section 14, Page 89	The document states “Interpretation of this endpoint requires consideration of assay-specific limitations, including metabolic restrictions, cell <u>permeabilization</u>, and the differentiation status of the test system.” Permeabilization refers to the process of puncturing or dissolving the plasma membrane of a cell. The concept that the IATA team is describing is more accurately referred to as “partitioning into cells” or “accumulating in cells”. Suggest revising the phrase “cell permeabilization” to one of these other options throughout the document.	Change the phrase “cell permeabilization” to a more accurate description of test chemicals partitioning to the components of an in vitro test system.