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Structuring expert review using AOPs: Enabling robust weight-of-evidence assessments for carcinogenicity under ICH S1B(R1)

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ABSTRACT

There is widespread acceptance that non-animal studies can be used to assess chemical safety in humans. These New Approach Methodologies (NAMs) typically integrate data from multiple sources including *in silico* and *in vitro* models. Regulatory guidelines are being updated to recognise that these scientific advances are allowing animal studies to be replaced without compromising human safety. One such regulation, ICH S1B(R1), was updated in 2022 to include the provision for a weight-of-evidence assessment for carcinogenicity, using six factors to determine if there was sufficient evidence to waive the need to run a rat carcinogenicity assay. The volume of data and evidence, however, can be hard to organise and interpret into a cohesive evaluation. To aid such assessments, software has been developed that combines adverse outcome pathways (AOPs) and reasoning, to organise and contextualise knowledge, and provide an outcome based on the data available. Using this framework, a workflow has been developed to assess the initial outcome and structure expert review to investigate the factors, and potential biological mechanisms which could contribute to a compound's carcinogenic potential (or lack thereof). The framework was used to structure expert review of three examples of differing activity and levels of supporting evidence. This highlighted where AOPs supported expert review by showing 1) the value in using AOPs to analyse data, 2) the importance of expert review to strengthen confidence in outcomes, and 3) how this approach can accurately predict experimental results. Therefore, using this approach to assess evidence for ICH S1B(R1) will give transparent, scientifically robust, and reproducible calls, and thus reduce the need for rat carcinogenicity studies.

1. Introduction

Animal testing has been the foundation for assessing carcinogenic risk for decades to ensure the safety of substances to which humans are exposed. A key study in many of these regulatory guidelines has been the rodent lifetime bioassay, which has served to determine the carcinogenic potential of pharmaceuticals, agrochemicals, and industrial chemicals for use in or around a human population [1]. However, the value of these studies has been widely questioned, given the sometimes limited or questionable relevance to human health of the results they produce, and costs both in terms of time and animals [2,3]. Thus, there is a drive to move away from these assays towards new approach methodologies (NAMs) to reduce, and eventually replace the requirements for animal testing in favour of more human relevant assessments. It is, however, unlikely that animal models will be replaced by a single study and, therefore, a suite of different assays will be required to ensure suitable protection of human health requiring methods that allow data from different assays to be combined in order to reach a conclusion. Approaches that have been used include defined approaches, weight-of-evidence (WoE) [4–6], or integrated approaches to testing and assessment (IATA) [7–11]. These initiatives look to not only replicate the outcomes from the current animal models but improve upon them by providing more human-relevant conclusions.

The results of one such initiative are now embedded as an addendum

Abbreviations: DA, defined approaches; IATA, integrated approached to testing and assessment; AOP, adverse outcome pathway; NAM, new approach methodology; WoE, weight-of-evidence; MoA, mode-of-action; MIE, molecular initiating event; AO, adverse outcome; KE, key event; NDA, new drug application; CYP, cytochrome P450; AhR, aryl hydrocarbon receptor; PXR, pregnane X receptor; CA, chromosomal aberration; MN, micronucleus; MLA, mouse lymphoma assay; S1P, sphingosine-1-phosphate receptor; TSH, thyroid stimulating hormone; T4, thyroxine; CAR, constitutive androgen receptor.

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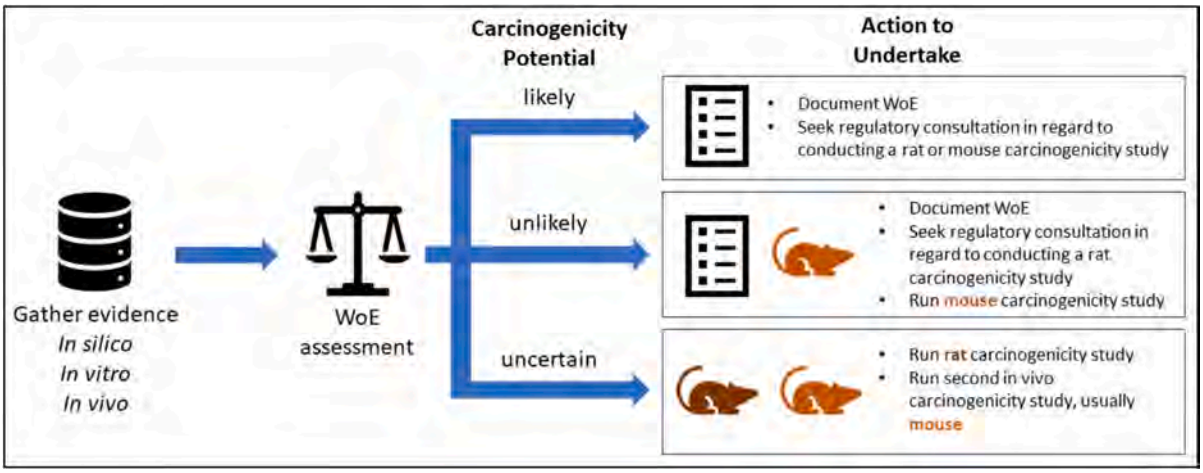


Fig. 1. Illustration of ICH S1B(R1) addendum approach highlighting the important steps and potential outcomes of a carcinogenicity assessment strategy under ICH S1B(R1) [5].

within the ICH S1B(R1) guideline for assessing the carcinogenic potential of active pharmaceutical ingredients [5]. This addendum states that, when a comprehensive assessment of all the data from public sources and those generated during the drug development process are considered, a WoE analysis can support the conclusion that a 2-year rat carcinogenicity study is unlikely to add value to the wider drug safety assessment and is not required. The evidence examined to make this decision is framed in the context of six factors:

1) Drug target biology and pharmacologic mechanism
2) Secondary pharmacology
3) Histopathology information from repeated dose toxicity studies
4) Hormonal perturbation
5) Genetic toxicity
6) Immunomodulation

After considering the available data relating to these criteria, there are three scenarios where a lifetime rat carcinogenicity study would not add value, beyond what can be predicted from the available data: 1) the compound is likely to be carcinogenic in rats and humans, 2) the compound is likely to be carcinogenic in rats but only via a human irrelevant mechanism, or 3) the compound is unlikely to be carcinogenic in rats and humans. Where one these conclusions can be reached with sufficient confidence, a regulatory consultation can be undertaken to confirm waiving the rat study. However, if the totality of data cannot give a clear indication of carcinogenic potential, the ICH S1B(R1) guidance for conducting the rat carcinogenicity study should be adhered to (Fig. 1).

Collating multiple pieces of evidence from diverse sources and reaching a reliable conclusion which can be clearly explained and replicated can be a challenge. A framework upon which evidence can be contextualised and reasoned between to reach a scientifically robust, consistent, and transparent outcome is therefore required. While the six factors outlined in the addendum go some way to grouping evidence strands into appropriate categories for comparison and interpretation of results, their level of granularity means that the same evidence may contribute to more than one factor and mode-of-action (MoA) hypotheses, which can aid in the interpretation and conclusions drawn from results. To enable contextualisation of data, adverse outcome pathways (AOPs) have been advocated as a framework [12]. AOPs are a way of capturing and visualising knowledge of biological pathways leading from the external perturbation of the biological system in the molecular initiating event (MIE) through to an adverse outcome (AO), indicating measurable key events (KEs) which occur as nodes along the pathway [13]. Models and assays can be associated to the corresponding KEs, allowing data and evidence to be organised on the network to give a mechanistic interpretation of the observations for a given chemical [12,14,15]. Contextualising the knowledge to enable hypothesis-based testing can direct (Fig. 2, scenario A), and rationalise (Fig. 2, scenario B) the following:

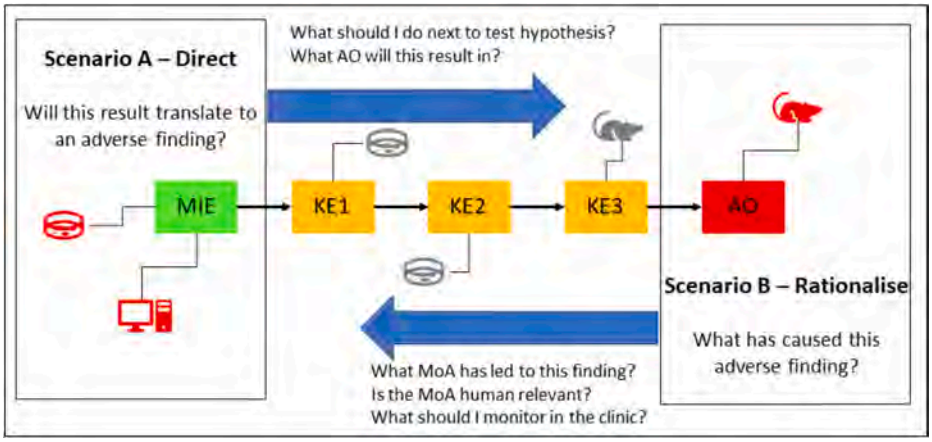


Fig. 2. Illustration of how AOPs can be used to direct testing of mechanistic hypotheses (Direct) or help give context to adverse findings coming from assays measuring AOs (Rationalise).

1) new hypotheses based on an existing knowledgebase of mechanistic information and data, which can be investigated and used to design subsequent testing approaches.
2) meaningful integration of new evidence types, including NAMs.
3) adverse findings to establish a MoA.
4) MoA information to argue human relevance.
5) what to monitor in the clinic.

Furthermore, the way AOPs are structured to organise and contextualise information supports more consistent, scalable, and reproducible results, that in turn supports defensible decisions [14].

Expert review should also be a critical step in this process, as this can increase confidence in initial outcomes, give indicators as to where more evidence is required to decrease uncertainty, or overturn calls if individual results seem unreliable. Indeed, the need for expert review is already embedded in some regulatory guidance, such as ICH M7, where such a review may be warranted to provide a rationale to support a conclusion for submission [16–19].

In previous work, it has been shown how an AOP network for carcinogenicity was derived [15] and how evidence can be reasoned between using AOPs [19]. Herein we bring these two concepts together with three case studies showing that the historic conclusions can be reached using available preclinical evidence for chemicals representing marketed pharmaceuticals. The case studies demonstrate how weight-of-evidence assessments can be built and hypotheses investigated. While the human carcinogenic potential of these chemicals has already been established, the analyses only use evidence available prior to running the rat lifetime bioassay. This shows the benefit of using AOPs as a framework to anchor the assessment, how to make confident testing decisions, how conclusions are reached, and the importance of expert review throughout this process.

2. Materials and methods

Three examples were selected for retrospective analysis in accordance with the ICH S1(R1) addendum WoE guidance to illustrate different scenarios with varying levels of available evidence and to explore how AOPs can help draw conclusions from the available evidence to aid expert review, direct testing and rationalise outcomes:

- Lansoprazole is a well-established pharmaceutical which is data-rich and has well-known, human-relevant carcinogenic properties.
- Siponimod is a recently developed pharmaceutical with preclinical data available that has some species-specific carcinogenic properties.
- Case Study 2 from the ICH S1B(R1) Addendum, an antagonist of a neuronal G-protein coupled receptor.

2.1. Data

Data for each compound and their major human metabolites (where available) was gathered from available databases, *in silico* systems and literature, including the following sources:

- New Drug Applications (NDAs) from the Drugs@FDA database [20]. This database contains information about FDA-approved products from 1938 to present day, including drug labels, pharmacology, and non-clinical safety toxicology reviews (*in vitro* and *in vivo* evidence) which were used in these analyses.
- Relevant *in vitro* and *in vivo* assay data from Vitic [21]. Vitic is a structure-searchable toxicity database provided by Lhasa Limited, which contains data for thousands of compounds, including data from *in vitro* and *in vivo* genotoxicity, repeated dose toxicity, and carcinogenicity studies.
- *In silico* predictions from Derek Nexus [22]. Derek Nexus is an expert-knowledge based software provided by Lhasa Limited, which can give predictions for multiple toxicity endpoints based on chemical structure.
- Information on the target from Open Targets Platform [23]. This platform brings together information on drug targets from multiple sources which can then be explored further. These sources include Expression Atlas [24], MGI [25], and Ensembl [26].
- ToxCast bioactivity data available within the CompTox Chemicals Dashboard provided by the U.S. EPA [27]. The data consists of results for multiple compounds tested against a variety of *in vitro* assays which were performed as part of the ToxCast program [28].
- Bioactivity data available within ChEMBL, provided by EMBL-EBI [29]. This is a database of bioactive molecules with drug-like properties, and includes bioactivity data, such as binding assays.

For the third example, the identity of the compound studied was masked in the ICH S1B(R1) addendum [5]. The listed evidence was extracted and structured to enable assessment within Kaptis.

2.2. Analysis

A workflow was established to conduct the assessment of each factor using Kaptis [30] and expert review (Fig. 3). The structure of this aims to follow the order in which safety pharmacology and toxicology assessments are conducted within the pharmaceutical drug development process [31]. At each stage of the workflow, the appropriate evidence was gathered to support the assessment of each factor for the ICH S1B (R1) addendum [5] (Table 1).

For all examples, the appropriate data was entered into Kaptis

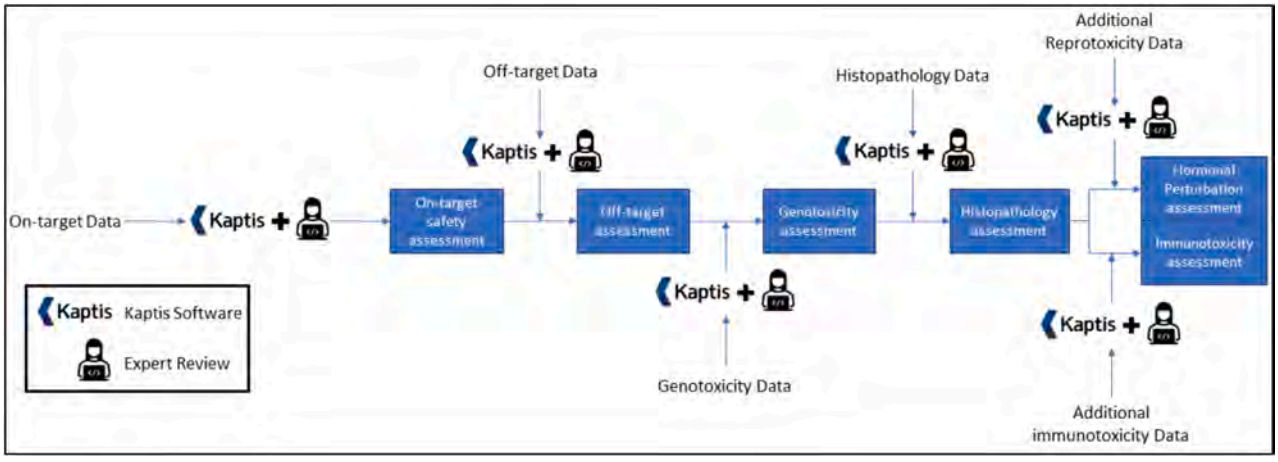


Fig. 3. Workflow for factor assessment using gathered data, Kaptis and expert review.

Table 1
ICH S1B(R1) factors considered for the WoE assessment for carcinogenicity, the suggested evidence required for analysis, and the sources used for each factor to make the evaluation.

Factor	Evidence needed (where available)	Source of data used in these studies:
Target Biology	Drug target biology Pharmacologic mechanism of parent compound and major human metabolites Drug target distribution in rats and humans Genetically engineered model findings Human genetic association studies Carcinogenicity information on class effects	NDAs Open Targets Platform (and sources therein)
Secondary Pharmacology	Secondary pharmacology screens (especially those linked to cancer e.g. nuclear receptors) for parent compound and major metabolites	NDAs ChEMBL ToxCast
Histopathology Chronic Studies	Repeated dose toxicity studies in rodent and non-rodent species	NDAs Vitic
Hormonal Effects	Repeated dose toxicity studies (focussing on endocrine and reproductive organs) Reproductive toxicology studies	NDAs Vitic Derek Nexus
Genotoxicity	Genetic toxicology data from studies associated with ICH S2(R1) [32]	NDAs Vitic Derek Nexus
Immune Modulation	Standard toxicity studies in accordance with ICH S8 [33]	NDAs Vitic

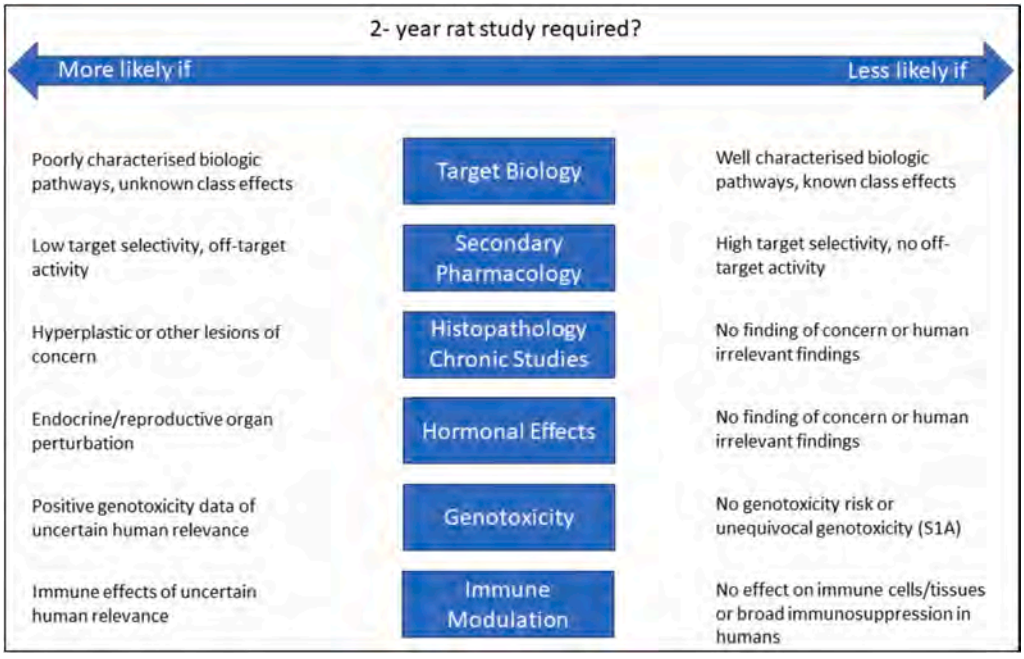


Fig. 4. WoE assessment adapted from ICH S1B(R1).

(illustrations of outcomes in results section), reviewed, and expert analysis conducted alongside the software outputs at each stage to give an outcome for each factor. Once an outcome for each factor was established, the result for each were reviewed against the integrated analysis figure presented in the ICH S1B(R1) addendum [12] (Fig. 4). Using this information, an overall assessment of whether a 2-year rat carcinogenicity study is necessary was made, which was then compared to the known experimental outcomes.

3. Results

3.1. Lansoprazole (CAS no. 103577-45-3)

Lansoprazole is a drug used to treat several gastric issues, including stomach ulcers. This compound acts as a proton pump inhibitor by covalently binding to cysteine residues on H^+/K^+-ATPase, thus inhibiting proton pump action. Depending on the condition, the drug can be taken orally, or via injection, over the short-term, or longer-term [34,35].

3.1.1. WoE expert review

3.1.1.1. On-target. The intended target of Lansoprazole is the ATP4A protein, a catalytic subunit of the gastric H^+/K^+-ATPase pump, which transports hydrogen and potassium ions across the apical membranes of parietal cells in the stomach, thus acidifying the stomach [23]. Lansoprazole is a pro-drug, requiring protonation before it can bind to ATP4A [36]. This requires an acidic environment; explaining why active forms of Lansoprazole only form in the stomach. Once bound, proton pump action is inhibited, the gastric acid secretion reduced [37]. Metabolites of Lansoprazole are the hydroxylated sulfinyl and sulfone analogues, neither of which are pharmacologically active nor are formed extensively in the liver via specific Cytochrome P450 (CYP) enzymes [5].

The intended target (ATP4A) has been found to be orthologous

Table 2
Carcinogenicity data for compounds pharmacologically similar to Lansoprazole.

Similar Compound	Carcinogenicity	FDA Labels/Reviews
Omeprazole	Rat – stomach (gastric mucosa) [21,41,42]	Gastric malignancy precaution [43]
Pantoprazole	Rat, Mouse – Positive [21]	Mouse – liver (female only) Rat – stomach, liver, thyroid Gastric malignancy precaution [44]
Esomeprazole	Rat – Positive [21]	Gastric malignancy precaution [45]
Rabeprazole	Rat – Positive [21]	Rat – stomach (female only) Gastric malignancy precaution [46]

across species [23,26], including rats, humans and dogs and is predominantly expressed in the stomach of humans [24,38], mice [24,39] and rats [40]. The gene is expressed in other organs [24,38], at levels several orders of magnitude lower than in the stomach, thus adverse on-target effects are less likely in other organs. Supporting this supposition, mutation of the ATP4A gene causes abnormalities in the parietal cells, achlorhydria, hypergastrinemia, and hyperplasia and metaplasia when the gene is knocked out in one mouse knockout model [25].

Lansoprazole is one of several drugs that act as proton pump inhibitors by targeting ATP4A, thus these were assessed for their carcinogenic potential (Table 2).

Carcinogenicity is observed for all the similar compounds which are proton pump inhibitors in rats, and the FDA labels for these drugs all carry a precaution for gastric malignancy [43–46]. Three out of four have evidence that stomach tumours are observed in rats, whereas esomeprazole is unspecified.

Using Kaptis [30], no automated links were found to exist between the targets and known KEs in the AOP network, based upon public ontologies; in fact, no AOPs involve this target. However, data from mouse knockout studies suggests that mutation of the ATP4A gene causes abnormalities in the parietal cells, achlorhydria, hypergastrinemia, and hyperplasia and metaplasia [25]. Hypergastrinemia and hyperplasia are known KEs within the AOP network, and both relate to a specific AOP which results in the induction of stomach-specific tumours. Therefore, the target can be indirectly linked to these KEs (Fig. 5). By integrating the target into the framework, it can be clearly shown how downstream events could lead to carcinogenicity in the stomach. Indeed, hypergastrinemia has been shown to be causally linked to these downstream effects, with prolonged gastrin exposure to enterochromaffin cells triggering progression of hyperplasia, and ultimately malignancy [47]. Thus, if this was a prospective analysis, the investigator could devise a directed testing strategy, specifically looking for serum gastrin increases in the blood and/or hyperplasia in the stomach in a rodent *in vivo* study to confirm or refute that Lansoprazole binding to ATP4A protein could be a concern for carcinogenic hazard.

From the evidence provided, it is possible to conclude that Lansoprazole will produce an on-target carcinogenic effect in the stomach. This is based on 1) the expression of the gene ATP4A being predominantly in the stomach, 2) the stomach carcinogenicity findings in rats for similar compounds acting via the same pharmacological target, and 3) the linking of the target to known KEs in the AOP network. The pharmacologically active protonated form of Lansoprazole is the only active species and it is only formed in the stomach. Given that expression of ATP4A is orthologous across species and is similarly distributed between tissues, it is likely that any effects in rats would also be observed in humans, and other animal species.

3.1.1.2. Off-target. For binding assays, Lansoprazole is negative for relevant nuclear receptors, except for the aryl hydrocarbon receptor (AhR) and pregane X receptor (PXR) (Fig. 6). No data could be found corresponding to binding assays and the major metabolites of Lansoprazole, however, for the purposes of this assessment, it can be assumed that the off-target profile of major metabolites has been assessed and no activity in the screening panels were observed. Functional assays for AhR and PXR were conducted, indicating modulation of the AhR (positive for agonism), but not the PXR (negative for agonism) may be responsible for any downstream activity. Framing this onto Kaptis can enable a testing strategy to be developed, using the relevant AOPs and downstream KEs to determine if the effect of Lansoprazole on AhR drives carcinogenicity. For example, testing specific CYP induction in the liver identifying AhR effects in repeated-dose rat studies could help in the understanding of whether the activation of AhR is an adaptive response to the exposure to the drug, or if it can be linked to an adverse effect.

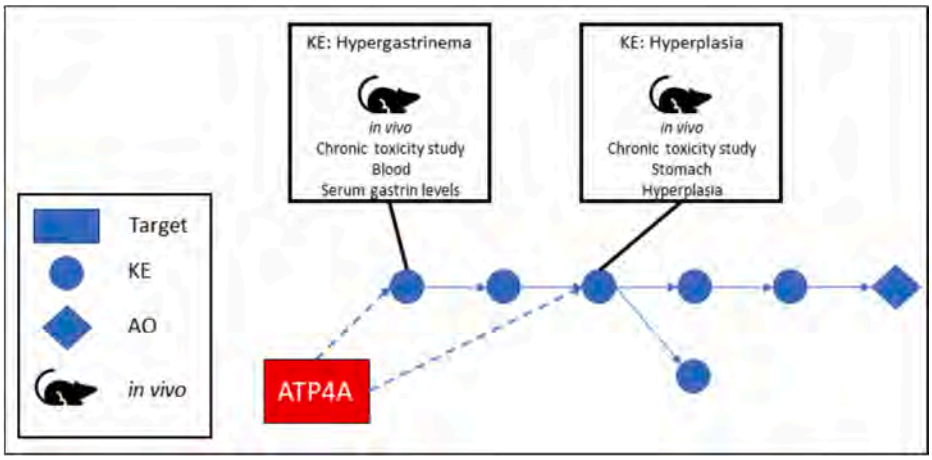
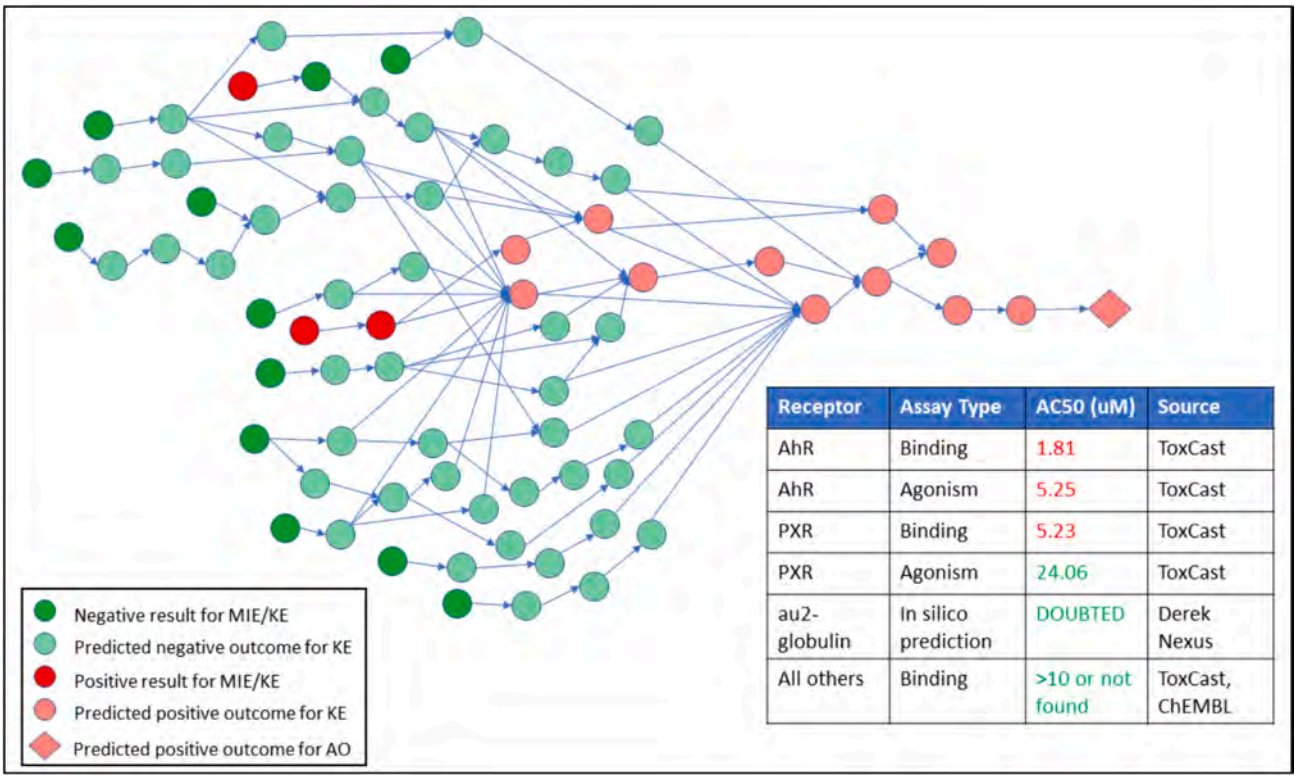


Fig. 5. Linking of ATP4A (target of Lansoprazole) to KEs within the Kaptis AOP network, and potential assays which could be used to test downstream KEs which could strengthen the hypothesis.



Receptor	Assay Type	AC50 (uM)	Source
AhR	Binding	1.81	ToxCast
AhR	Agonism	5.25	ToxCast
PXR	Binding	5.23	ToxCast
PXR	Agonism	24.06	ToxCast
au2-globulin	In silico prediction	DOUBTED	Derek Nexus
All others	Binding	>10 or not found	ToxCast, ChEMBL

Fig. 6. Off-target evidence for Lansoprazole [29,48] mapped onto the relevant subset of carcinogenicity AOPs, and the predicted AO. For binding and agonism assays findings which were less than 10 μM were classed as positive, and above this, negative.

3.1.1.3. Genotoxicity. The data collected for the genotoxicity of Lansoprazole fulfils the ICH S2(R1) guidance for option 1 (Table 3) [32]. Different combinations of the evidence can give a complete option 1 assessment for genotoxicity; however, the overall outcome of the assessment could be different depending on the data selected (here *in vitro* CA has a positive outcome, but MLA can be used instead, which would give a negative outcome) and follow-up strategies required dependent on the *in vitro* assays conducted. For example, taking the Ames test, *in vitro* chromosomal aberration (CA) assay and *in vivo* micronucleus (MN) assay would end up with questions around the positive *in vitro* CA result, but if the mouse lymphoma assay (MLA) was conducted instead of the *in vitro* CA assay, then the S2 assessment would result in a clear negative.

Even with the totality of evidence available, the positive *in vitro* MN and *in vitro* CA results could be questioned and would require further investigation. However, taking all this evidence and framing it on AOPs related to genotoxicity using Kaptis [30] can provide context, and provide rationalisation of evidence which describes activity of the same KE (Fig. 7).

Table 3
ICH S2(R1) option 1 data available for Lansoprazole.

Option 1		
test for gene mutation in bacteria	Ames Test	Negative
cytogenetic test for chromosomal damage (the *in vitro* metaphase chromosome aberration test or *in vitro* micronucleus test), or an *in vitro* mouse lymphoma Tk gene mutation assay.	CA assay MN assay MLA	Positive Positive Negative
An *in vivo* test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells	CA assay MN assay	Negative Negative

Despite positive results for *in vitro* CA and MN assays, these are overturned by *in vivo* studies (which are considered more biologically relevant models for human genotoxicity [19]) thereby giving an overall negative conclusion for genotoxic carcinogenicity. This is consistent with the approach taken in the ICH S2(R1) guidance [32]. This shows the value of framing the data on AOPs, as this can use all the data available and resolve to a robust and transparent outcome.

Further expert review of the Lansoprazole result was conducted, using genotoxicity data for biologically and structurally similar compounds (by target and 50 % structural similarity) [21], and following a literature review for mechanistic information. Based on the available genotoxicity, the profile of Lansoprazole largely matches those of similar compounds (Table 4). This helps to increase confidence in the overall call for genotoxicity given as it shows that other compounds in this pharmacological class present similar genotoxicity profiles, and thus are mechanistically similar, increasing confidence in the findings for Lansoprazole. Discordance between results in various genotoxicity studies, including differences between *in vitro* and *in vivo* CA, can happen for many reasons. It has been shown that *in vitro* assays are often positive for chemicals considered not to present a significant genotoxic or carcinogenic risk *in vivo* [49]. It has been theorised that differences for Lansoprazole in the *in vitro* and *in vivo* MN assays are due to inherent metabolism *in vivo* that cannot be simulated by *in vitro* assays [50], which may also be the case with the difference in results observed for the *in vitro* and *in vivo* CA assays. However, the mechanistic rationale for

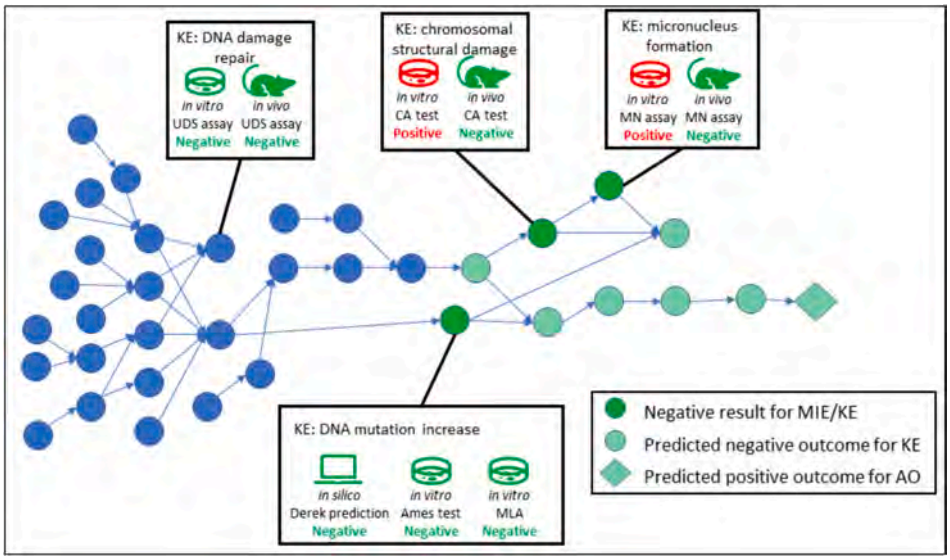


Fig. 7. Association of genotoxicity data for Lansoprazole with the relevant subset of the AOP network and the predicted AO.

Table 4
Genotoxicity data for compounds structurally and pharmacologically similar to Lansoprazole. Red = positive, Green = negative, yellow = Equivocal.

	Ames Test	CA *in vitro*	MN *in vitro*	MLA	UDS *in vitro*	CA *in vivo*	MN *in vivo*	UDS *in vivo*
Lansoprazole								
Omeprazole								
Pantoprazole								
Esomeprazole								
Rabeprazole								

why Lansoprazole causes *in vitro* CA and MN responses remains unresolved.

Therefore, it can be concluded that Lansoprazole is non-genotoxic. AOPs allow for the integration and resolution of conflicting experimental data to reach a confident decision that can inform an assessment under ICH S2(R1).

3.1.1.4. Histopathology. Repeated-dose toxicity study findings for Lansoprazole show evidence of preneoplastic lesions which have the potential to progress to malignant neoplasms in multiple species [21,37,51]. The location, time of finding, and types of finding might help establish whether these are likely to be relevant, what might have caused them, and if they are transient in nature, or likely to progress to cancer.

3.1.1.4.1. Rat studies

3.1.1.4.1.1. Stomach

From 3-, 6-, and 12-month repeated dose toxicity studies in rats, there is a clear effect on the stomach caused by Lansoprazole, with hyperplasia, hypertrophy and relative organ weight increase observed at nearly all time points (Fig. 8). Organising this on the Kaptis AOP framework shows that these findings are temporally consistent with study duration, do not appear to be transient and could progress to cause stomach cancer.

3.1.1.4.1.2. Other Organs

Organ weight increases were also observed in multiple other organs, but only in the liver and testes were other effects observed. Liver hypertrophy and organ weight increases were observed, however as liver hypertrophy was only observed in the shorter term 3-month study and not in the longer-term studies, this finding may not be deemed relevant. Without clear supportive findings of hypertrophy or hyperplasia, the organ weight increase alone may not be indicative of a cancerous effect for the liver. Testicular organ weight increases and hyperplasia may be indicative of hormonal perturbation effects in rats so should be considered within the hormonal perturbation factor.

Overall, using Kaptis and expert review facilitated by the tool, there are findings that indicate that Lansoprazole may pose a carcinogenic hazard to rats, with stomach being the main organ of concern. Testicular findings are less clear as to their relevance to a tumorigenic effect because the response over time is less consistent. The hyperplasia seen at 12 months needs to be associated with other findings to further

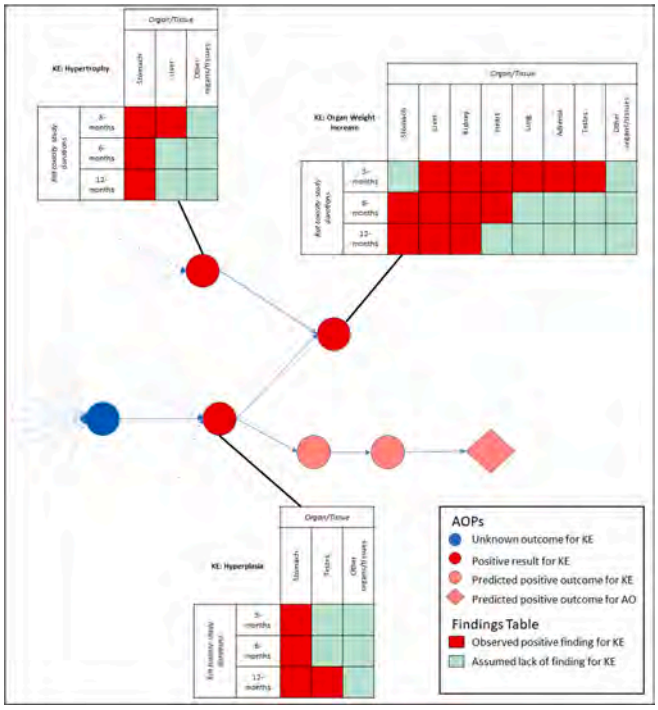


Fig. 8. Rat histopathology data for Lansoprazole mapped on to AOPs with details of tissues affected in assays of different durations.

understand its relevance.

3.1.1.4.2. Dog Studies

3.1.1.4.2.1. Stomach

As with the rat studies, there is an effect on the stomach caused by Lansoprazole, with hyperplasia and hypertrophy observed in 3- and 12-month studies (Fig. 9). These effects were observed in rats suggesting cross-species relevance, thereby increasing the likelihood of similar effects being observed in humans.

3.1.1.4.2.2. Other Organs

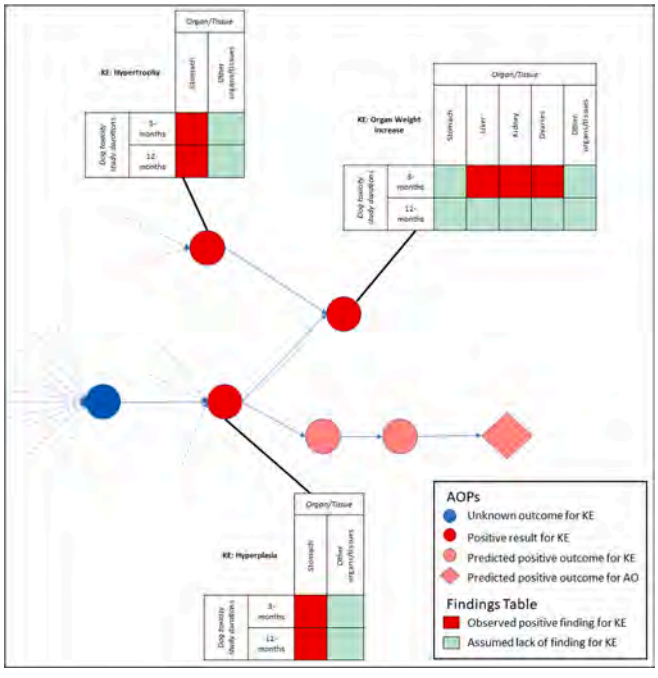


Fig. 9. Dog histopathology data for Lansoprazole mapped on to AOPs with details of tissues affected in assays of different durations.

While organ weight increases are observed in multiple organs at sub-chronic doses, these are not observed in chronic studies. As with the rat, these findings are not reflected in the hypertrophy, or hyperplasia findings, reducing a concern for their progression to carcinogenicity. The findings in rats for hypertrophy and hyperplasia in other organs are not mirrored in the dog, suggesting they are species specific.

Overall, using Kaptis and expert review, these findings indicate that Lansoprazole may pose a carcinogenic hazard to dogs, with the stomach being the key target organ.

3.1.1.4.3. Mechanistic Rationale. Combining the findings from Kaptis and expert review for the on-target factor and the histopathology evidence in the stomach, the findings observed *in vivo* in terms of location are consistent with the binding of the protonated form of Lansoprazole to ATP4A, causing a biological cascade, that could potentially result in malignant neoplasms of the stomach. As this is seen in both rat and dog studies, and with the knowledge that ATP4A receptor is ubiquitous across species, this increases confidence in this supposition. Additionally, evidence from epidemiological studies links long-term treatment with proton pump inhibitors to increased risk of gastric cancer [52].

Combining findings from the off-target assessment with the histopathological evidence in the liver, suggests that AhR binding, and activation could be responsible for the changes seen. The AOP for this receptor in Kaptis specifies that binding and activation of the AhR receptor, which is orthologous across species, could result in changes to the liver (including hypertrophy and organ weight increase). Additional expert review found that Lansoprazole binding to AhR can induce CYP1A [53], which is known to cause changes in the liver, including organ weight increase [54].

Histopathological findings indicate that Lansoprazole will cause changes to stomach tissues which could result in malignant neoplasms. This is supported by findings from the on-target factor and epidemiological observations for similar compounds. Liver findings are unlikely to progress to malignancy given 1) the transient nature of the liver hypertrophy findings, and 2) organ weight increase does not point to cancer given the lack of other evidence (hypertrophy, hyperplasia).

3.1.1.5. Hormonal perturbation. Expert review of off-target binding studies from secondary pharmacology screening, luteinising hormone and thyroid stimulating hormone levels data from a human 8-week study [37] and the *in vivo* observations from the sub-chronic and chronic toxicity studies for both rat and dog (Fig. 10), would suggest no thyroidal changes are observed. This confirms that the biochemical effects that may happen at the molecular level for Lansoprazole-PXR binding do not propagate down the pathway to thyroid hormone changes (as observed in toxicity studies in Kaptis), hence it may not represent a risk of thyroid-mediated tumour development. Along with the binding data and lack of human hormonal changes, it could be surmised that the few observations in endocrine organs in rats and dogs may not be relevant for humans. Testicular findings in rats may indicate a reproductive hormonal effect. This mechanism may be species specific based on the difference between findings in dog and rat hormonal tissues, questioning the potential of a hormone-mediated mechanism that is relevant to tumour development in humans.

3.1.1.6. Immunotoxicity. The data from chronic toxicity studies in rats for immunotoxicity-related tissues [37] in Kaptis indicates that Lansoprazole is unlikely to cause an immunotoxicological effect (Fig. 11). Although there is a lack of upstream data, the downstream evidence gives confidence that a carcinogenic MoA has not been missed, as the KEs which indicate proliferation and progression of cancer are not activated.

3.1.2. Weight-of-evidence call

Based on the information for each factor, it is unlikely that a 2-year

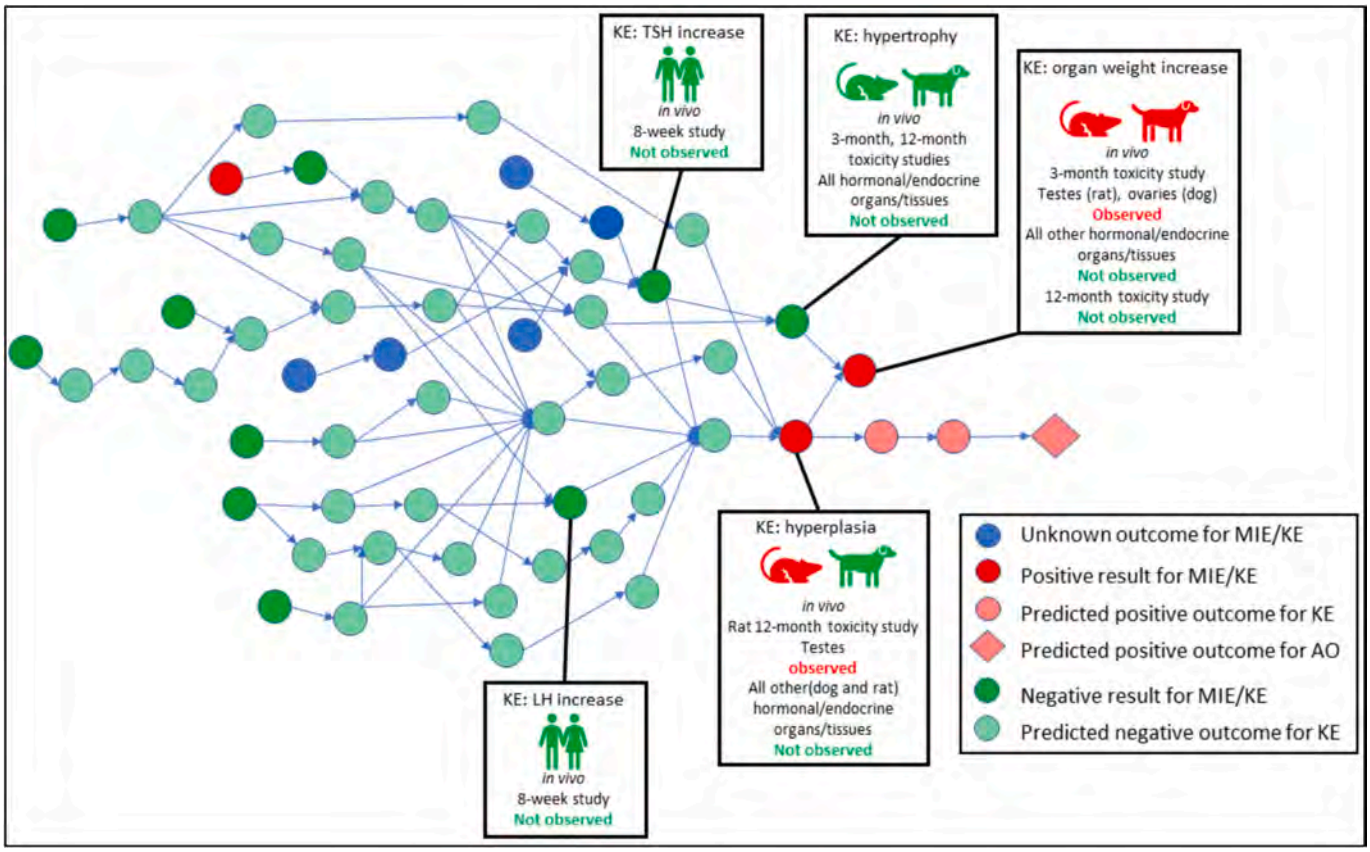


Fig. 10. Data relating to hormonal perturbation for Lansoprazole mapped onto a subset of Kaptis AOPs, and the predicted AO (without considering species differences).

rat carcinogenicity study will need to be conducted as the compound is likely to be carcinogenic in humans and rats (Fig. 12). This is likely related to the pharmacological activity of the compound. Key conclusions are:

- The primary pharmacology is well known, and likely to be responsible for effects observed in repeated dose studies; compounds in the same pharmacological class are carcinogenic in the stomach of rats.

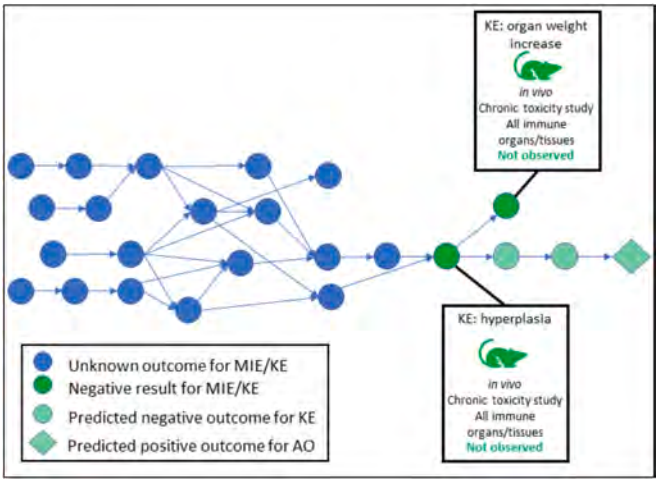


Fig. 11. Data relating to immunotoxicity from rat chronic toxicity studies for Lansoprazole mapped onto the relevant subset of AOPs and the predicted AO.

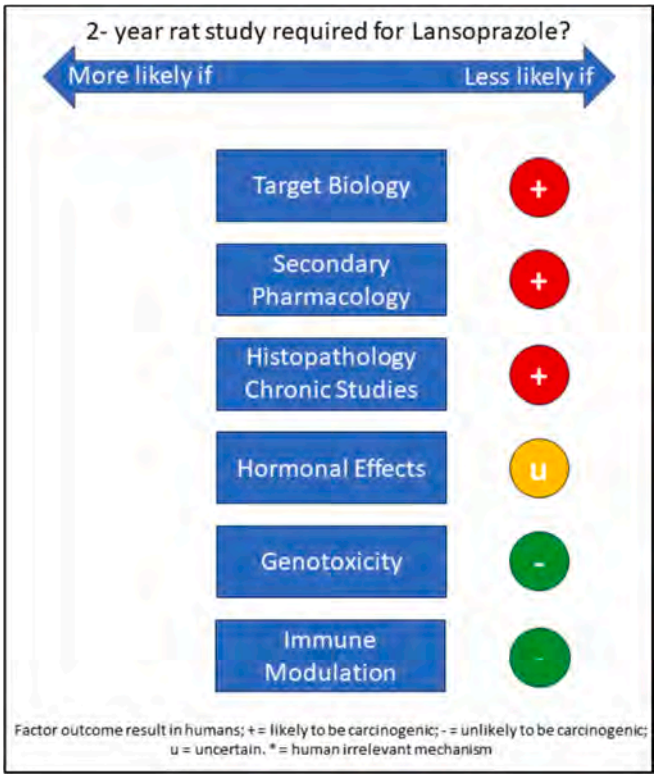


Fig. 12. Summary of WoE assessment for Lansoprazole for each factor mapped as to whether it is less likely or more likely a rat carcinogenicity study would add value.

- There is high target selectivity; two off-target findings found, but evidence from histopathology shows that these are unlikely to cause carcinogenicity.
- Histopathological lesions of concern can be mechanistically explained and rationalised for human relevance.
- Some potentially human-irrelevant hormonal effects were observed.
- Genotoxicity was only seen *in vitro*; *in vivo* data indicates no genotoxic concern.
- No immune-related lesions of concern.

While the relevance or impact of observed hormonal effects are uncertain, this does not affect the overall conclusion since there is already strong evidence to suggest stomach tumours are likely with high confidence in the assessment.

3.1.3. Carcinogenicity experimental call

2-year rat carcinogenicity study – tumours in stomach, testes (benign) [37].

3.2. Siponimod (CAS no. 1230487-00-9)

Siponimod is a drug used in the treatment of secondary progressive multiple sclerosis, supressing central nervous system inflammation. This is achieved by selectively targeting the sphingosine-1-phosphate receptor (S1P) and modulating the movement of lymphocytes. The drug is given orally, dosing in a stepped fashion, then maintaining until treatment ends [55,56].

3.2.1. WoE expert review

3.2.1.1. On-target. The intended targets of Siponimod are S1P1 (gene code S1PR1) and S1P5 (gene code S1PR5), which are G-protein coupled receptors for the lysosphingolipid S1P. S1P plays an important role in many biological functions, including the transport of lymphocytes into the blood stream [23]. Siponimod acts by promoting internalisation and degradation of S1P1, which inhibits the signal needed for egress of lymphocytes to the blood stream, thereby preventing their circulation [57] and suppressing central nervous system inflammation.

The major human metabolites of Siponimod are a glucuronide of the hydroxylated form of Siponimod, and a cholesterol ester [58]. Neither have an affinity for S1P1 or S1P5 [57]. These metabolites are not active for the target [4], therefore, the metabolites are unlikely to play a role in the action of Siponimod.

S1PR1 and S1PR5 are orthologous across species [23,26], including rats, humans, monkeys, and mice. These are expressed across multiple tissues in each species [24], the most being seen in immune organs [23]. Mouse knockout studies indicate multiple changes occur when S1PR1 or S1PR5 is knocked out, including abnormalities associated with the immune, haematopoietic, and nervous systems [25].

Siponimod is one of a few compounds that has been developed as an S1P receptor agonist. Thus, other pharmacologically similar compounds were assessed to determine if there is a common carcinogenic class effect for S1P receptor agonists (Table 5).

Table 5
Carcinogenicity data for compounds pharmacologically similar to Siponimod, with data from the relevant FDA drug labels and NDAs.

Similar Compound	Carcinogenicity	FDA Labels
Fingolimod hydrochloride	Mouse – Malignant lymphoma, hemangiosarcoma and hemangioma Rat – Negative [59]	Malignancies [59]
Ozanimod hydrochloride	Mouse – Hemangiosarcoma and hemangioma Rat – Negative [60]	No warning [60]
Ponesimod	Mouse – Hemangiosarcoma and hemangioma Rat – Negative [61]	Cutaneous malignancies [61]

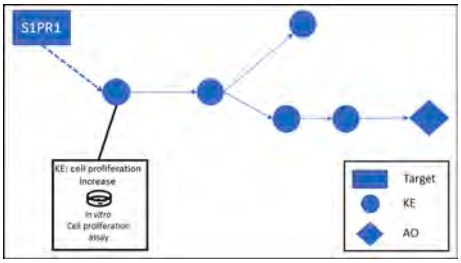


Fig. 13. Linking of S1PR1 (target of Siponimod) to KEs within the Kaptis AOP network.

For S1P receptor agonists, literature data indicates that tumours are commonly seen in mice, with all the similar compounds inducing hemangiosarcomas (endothelial cell tumours which occur in blood vessels) across multiple tissues [59–61].

Using Kaptis [30], an automated link was made between S1PR1 and the KE cell proliferation increase, based on the association of S1PR1 with the Gene Ontology Resource term 'cell population proliferation in mice and rats' (GO:0008283) (Fig. 13) [62]. This link supports the hypothesis that affecting the target can potentially induce carcinogenicity if the signal is propagated down the pathway. Using the same method, no associations was found between S1PR5 and the AOP network.

Using data gathered about the target function, its expression across species, distribution in tissues, knockout study information, carcinogenicity of similar compounds, and linkage of the target to known KEs in the Kaptis AOP carcinogenicity network, it is possible that Siponimod will produce an on-target carcinogenic effect. However, based on the carcinogenicity data from similar compounds, the class effect indicates that any potential carcinogenic effect caused by Siponimod would only be observed in mice, and not rats. As S1PR1 and S1PR5 are conserved across species and biological systems, the variation in carcinogenicity of compounds that target this receptor could be because the AOP linking these receptors to a carcinogenic outcome is species specific or because carcinogenicity is not driven by these receptors. Literature review suggests that these findings are related to vascular endothelial cell activation, and persistent proliferation of these cells is only observed in mice and not rats. The persistent proliferation is likely to lead to angiogenesis, which can result in hemangiosarcomas given lifelong treatment [63]. The lack of persistent cell proliferation in human cells also suggests this mechanism is not relevant to humans.

The linking of the target to a KE in the AOP network provides additional support to a user by being able to indicate what can be done next to increase confidence in calls made in the WoE assessment. In this case, to strengthen the hypothesis made above, the cell proliferation increase KE which S1PR1 links to can be tested by conducting *in vitro* cell proliferation assays. Given the species difference observed for carcinogenicity in pharmacologically similar compounds, and the types of neoplasms observed, testing this across vascular epithelial cells from multiple species would be ideal. These studies have indeed been conducted and show that cell proliferation increases are observed in mouse cells, but not rat or human cells. Thus, this increases confidence in the supposition that there could be an on-target effect observed based on the interaction of Siponimod with S1P receptors, which could result in malignancies like those seen for pharmacologically similar compounds.

3.2.1.2. Off-target. Details of secondary pharmacology screening were not published although it may be assumed that the relevant targets (including nuclear receptors) were analysed [64]. Where details have been reported, Siponimod has demonstrated an affinity for with adrenergic receptors, histamine H2 and a serotonin receptor [57] (Table 6). From the targets identified, the histamine H2 receptor is of interest given that it is linked to carcinogenic hazard (and is part of the

Table 6
Known affinity of Siponimod for secondary pharmacology targets. For assays, findings which were less than 10uM were classed as positive.

Receptor	Assay Type	IC50 (μM)
Histamine H2	Binding	0.12
Adrenergic alpha2A	Binding	0.56
Adrenergic alpha2B	Binding	0.21
Serotonin transporter	Binding	0.60

carcinogenicity AOP network). This receptor is expressed in the multiple organs and is orthologous across species [23]. In addition, the major human metabolites were also screened against targets and had no affinity; thus, it may be assumed that no off-target activity was observed for the targets relevant for this assessment.

Based on the qualitative data outcomes and assumptions made, Kaptis indicates that there may be a possibility that interaction of Siponimod with the histamine H2 receptor introduces some off-target carcinogenic potential. To strengthen, or refute this hypothesis, the use of the downstream KEs in the identified AOP can direct the testing strategy. In this case, information from chronic toxicity studies will inform the outcome for off-target hazards. As the AOP indicates that the antagonism of the histamine H2 receptor can cause stomach tumours, any positive observations in the stomach are of particular importance.

3.2.1.3. Genotoxicity. Based on the data (which fulfils the ICH S2(R1) guidance for option 1 [32] (Table 7)), it can be concluded that genotoxicity is unlikely to be the cause for any carcinogenicity findings. The use of AOPs in Kaptis facilitates the integration of additional evidence to support and increase confidence in the call (Fig. 14).

Table 7
ICH S2(R1) option 1 data available for Siponimod [57].

Option 1		
test for gene mutation in bacteria	Ames Test	Negative
cytogenetic test for chromosomal damage (the *in vitro* metaphase chromosome aberration test or *in vitro* micronucleus test), or an *in vitro* mouse lymphoma Tk gene mutation assay.	CA assay	Negative
An *in vivo* test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells	MN assay	Negative

In this case, an *in silico* Derek Nexus prediction can be incorporated into the assessment which further supports the case for Siponimod being non-genotoxic. Therefore, it can be concluded that genotoxicity would not be a concern in the overall assessment of the carcinogenicity of Siponimod.

3.2.1.4. Histopathology. Repeated dose toxicity findings in rats, mice and monkeys were reported for Siponimod [57]. In each species, relevant findings suggest some potential for carcinogenicity, but this is not replicated across species and tissues looked at. Again, as this is a recently marketed pharmaceutical, an assumption has been made that if findings were not reported, then they were not observed.

3.2.1.4.1. Rat Studies

3.2.1.4.1.1. Liver

In the 26-week study in rats, liver hypertrophy and organ weight increase were observed (Fig. 15). This evidence is supported by shorter-term studies, where similar observations were seen in the liver for hypertrophy and organ weight increase, even at 4-weeks. Organising this data on the AOPs in Kaptis allows us to contextualise this data and demonstrate that hypertrophy, and not hyperplasia, is the likely cause of the organ weight increases observed. There is no direct causal link between hypertrophy and cancer – in fact it has been reported that hypertrophy is not a good indicator on its own for carcinogenicity [57]. A lack of hyperplasia, supported by negative cell proliferation data in rat cells, suggests that it is unlikely for tumours to be observed in the liver in a two-year carcinogenicity rat study.

3.2.1.4.1.2. Other organs

In the 26-week study, hypertrophy is observed in thymus tissue, and hyperplasia observed in the spleen. These are not observed in shorter term studies. The finding of hyperplasia should coincide with the upstream observation of cell proliferation, however in rat vascular epithelial cells, cell proliferation increase is not seen which decreases confidence in the hyperplasia finding being relevant in this case.

In shorter term studies, there were no hyperplasia findings. Hypertrophy in thyroid tissue was observed in 4-week and 91-day exploratory studies, and organ weight increase in the thyroid in the 91-day study as well. However, none of these findings were reported in the 26-week study. These are considered as part of the hormonal effects factor.

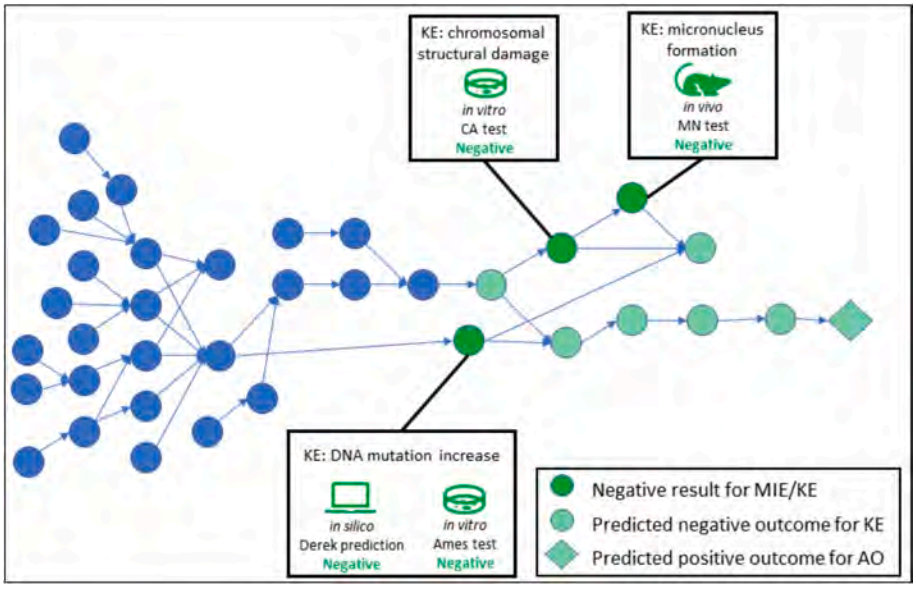


Fig. 14. Association of genotoxicity data for Siponimod with the relevant subset of the AOP network and the predicted AO.

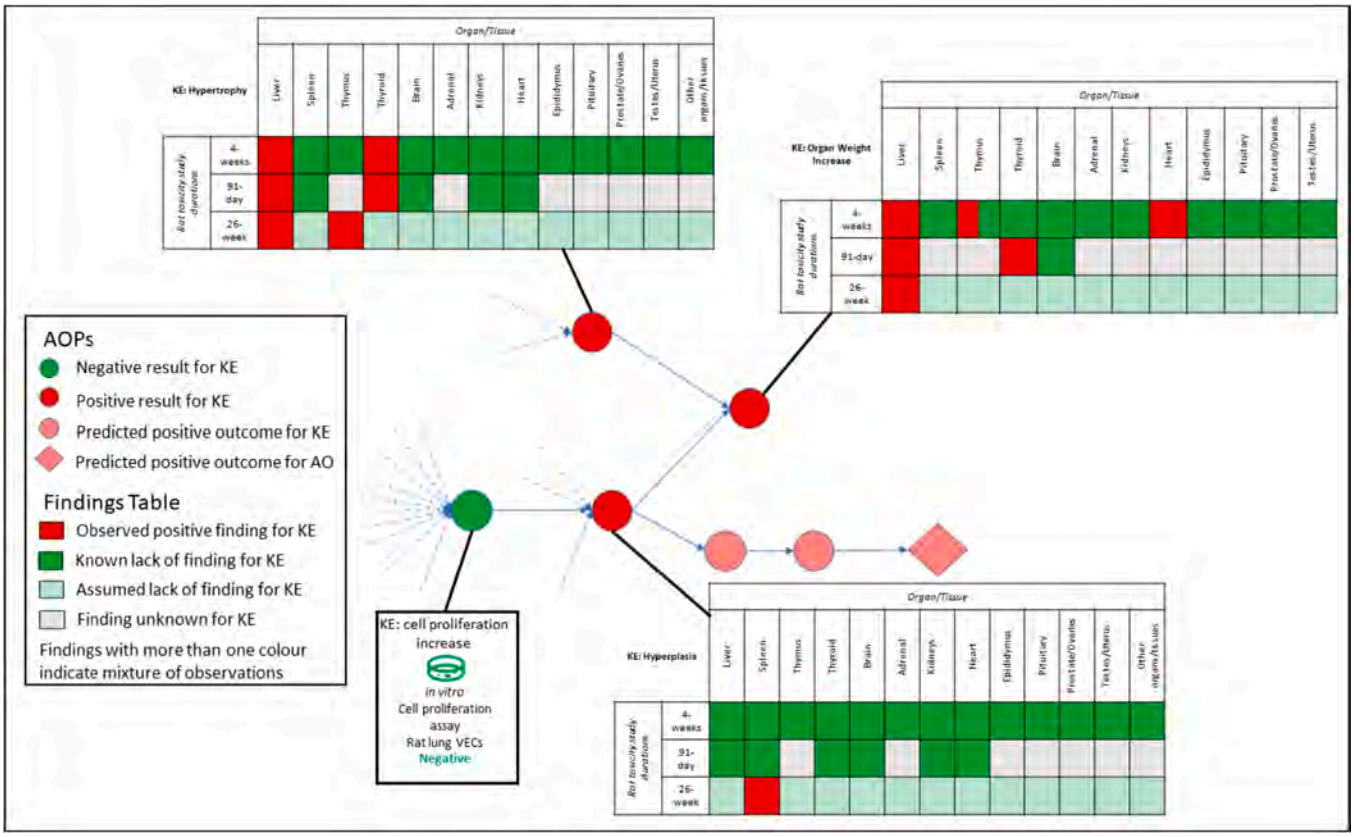


Fig. 15. Rat histopathology data for Siponimod mapped on to AOPs in Kaptis with details of tissues affected in assays of different durations.

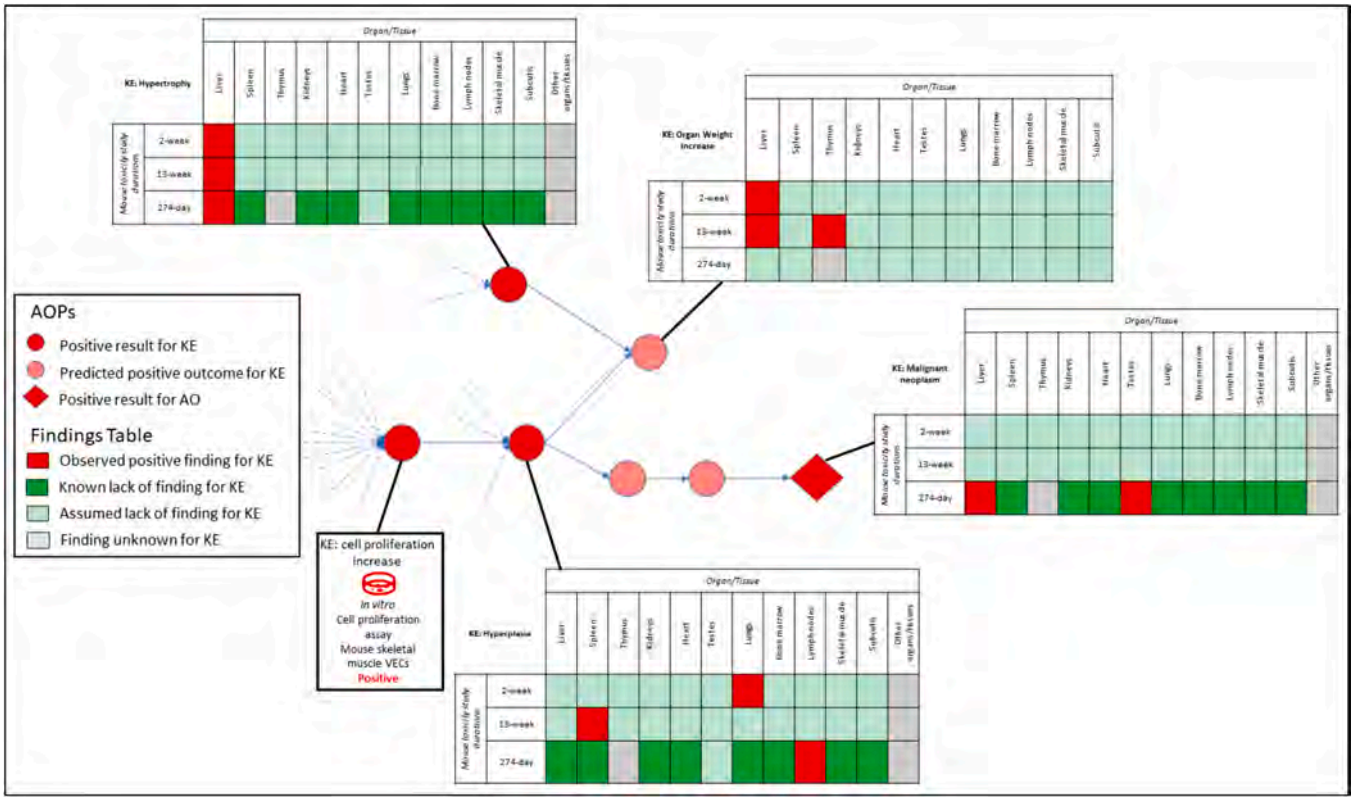


Fig. 16. Mouse histopathology data for Siponimod mapped on to AOPs in Kaptis with details of tissues affected in assays of different durations.

Therefore, the transient nature of this finding and lack of hyperplasia in this tissue indicates that the evidence might not impact on the WoE assessment for carcinogenicity. Organ weight increases were observed in the heart and thymus (in one study but not another), but not observed in the 26-week study, and not checked for in the 91-day study.

Using Kaptis, the lack of any findings in the stomach indicates that the binding of Siponimod to the histamine H2 receptor is unlikely to propagate to downstream effects and is thus unlikely to cause stomach tumours in rats.

Overall, the findings indicate that Siponimod does not pose a carcinogenic hazard to rats. Liver findings of hypertrophy and organ weight increase are not causally linked to cancer but should be noted.

3.2.1.4.2. Mouse Studies

3.2.1.4.2.1. Liver

In concordance with the observations in rats, hypertrophy and organ weight increases were observed in the liver, although organ weight increases were seemingly not reported in the 274-day exploratory study (Fig. 16). Hyperplasia was not observed, but contrary to what was observed in rats, cell proliferation increase was (in vascular epithelial cells from mouse skeletal muscle). In addition, one mouse showed a liver hemangiosarcoma in the exploratory study.

3.2.1.4.2.2. Other Organs

In the 274-day exploratory study, hyperplasia was only reported in lymph nodes, whilst for all other organs examined (excluding the liver) there were no reports for hyperplasia, organ weight increase, or hypertrophy. Additionally, hemangiosarcomas in the testes were observed in one mouse in the 274-day exploratory study. However, the list of tissues examined for histopathological changes was not exhaustive, potentially resulting in exclusion of relevant evidence. In shorter term studies, hyperplasia in the lung and spleen, and hypertrophy in the thymus were reported, but were not found or not examined in the longer study. The absence of any observations in the stomach limits our ability to link histamine H2 receptor to carcinogenicity in mice.

Overall, the findings indicate that Siponimod may pose a carcinogenic hazard to mice. Hemangiosarcomas (the same tumours as observed in the pharmacologically similar compounds) were observed in the liver and testes. Cell proliferation increases were also observed in mouse vascular epithelial cells, thus strengthening the evidence for carcinogenic hazard. Hyperplasia was observed in different organs but as a transient effect. Liver findings of hypertrophy and organ weight increase are not causally linked to cancer but should be noted.

3.2.1.4.3. Non-rodent studies. In monkeys, only liver organ weight was observed, without reports of hypertrophy, nor hyperplasia (Fig. 17). Given the comprehensive list of tissues examined in the 52-week study, we can be confident that Siponimod does not pose a carcinogenic hazard in monkeys based on the lack of adverse histopathology findings in the chronic repeated dose studies (Fig. 17). Findings taken at a shorter time suggest lung hyperplasia may be of concern, but as this is not reflected in longer-term studies, thus is not considered a risk.

The lack of any findings in the stomach indicates that the binding of Siponimod to the histamine H2 receptor is unlikely to cause any carcinogenicity in monkeys.

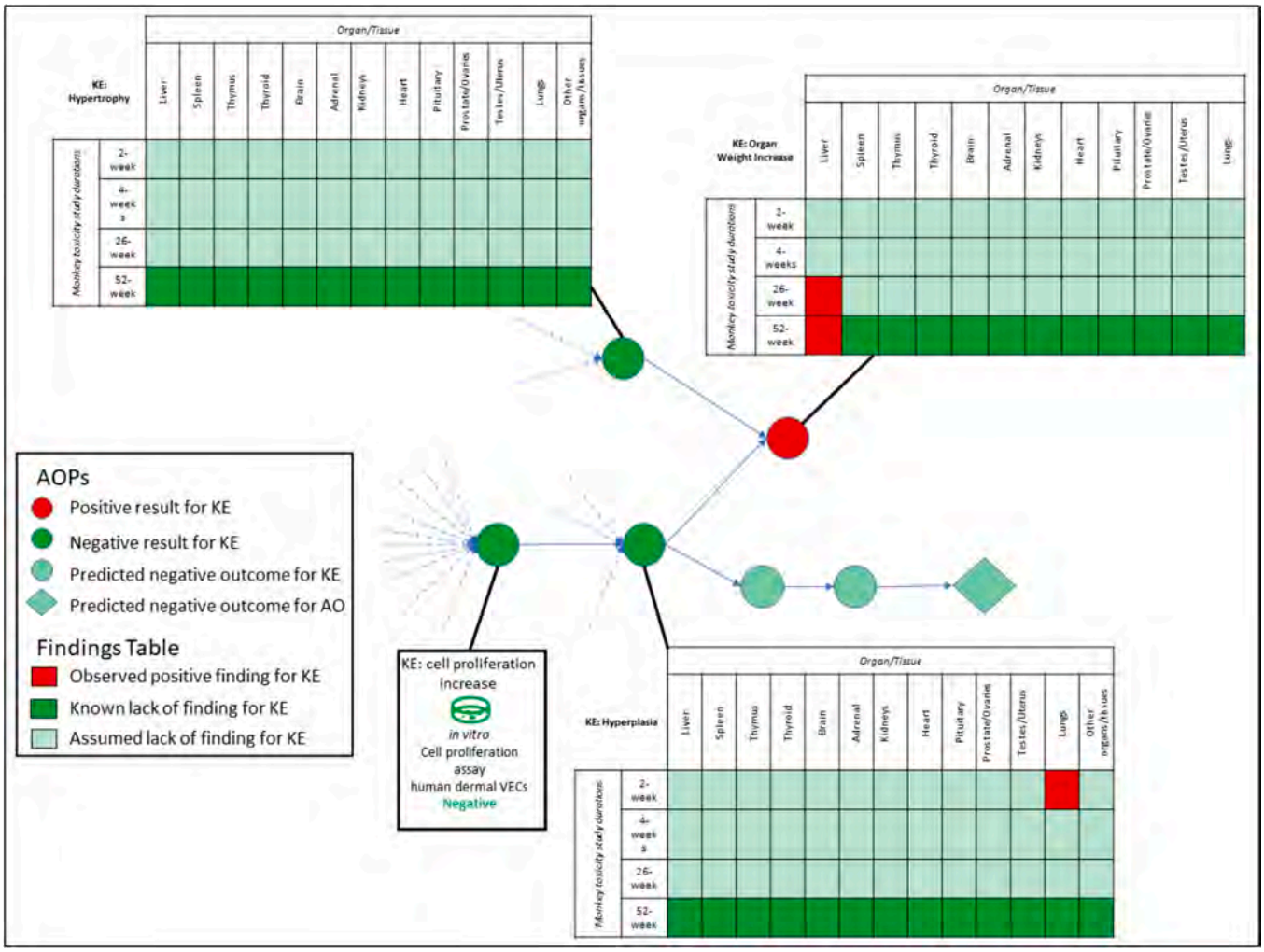


Fig. 17. Monkey histopathology data for Siponimod mapped on to AOPs in Kaptis with details of tissues affected in assays of different durations.

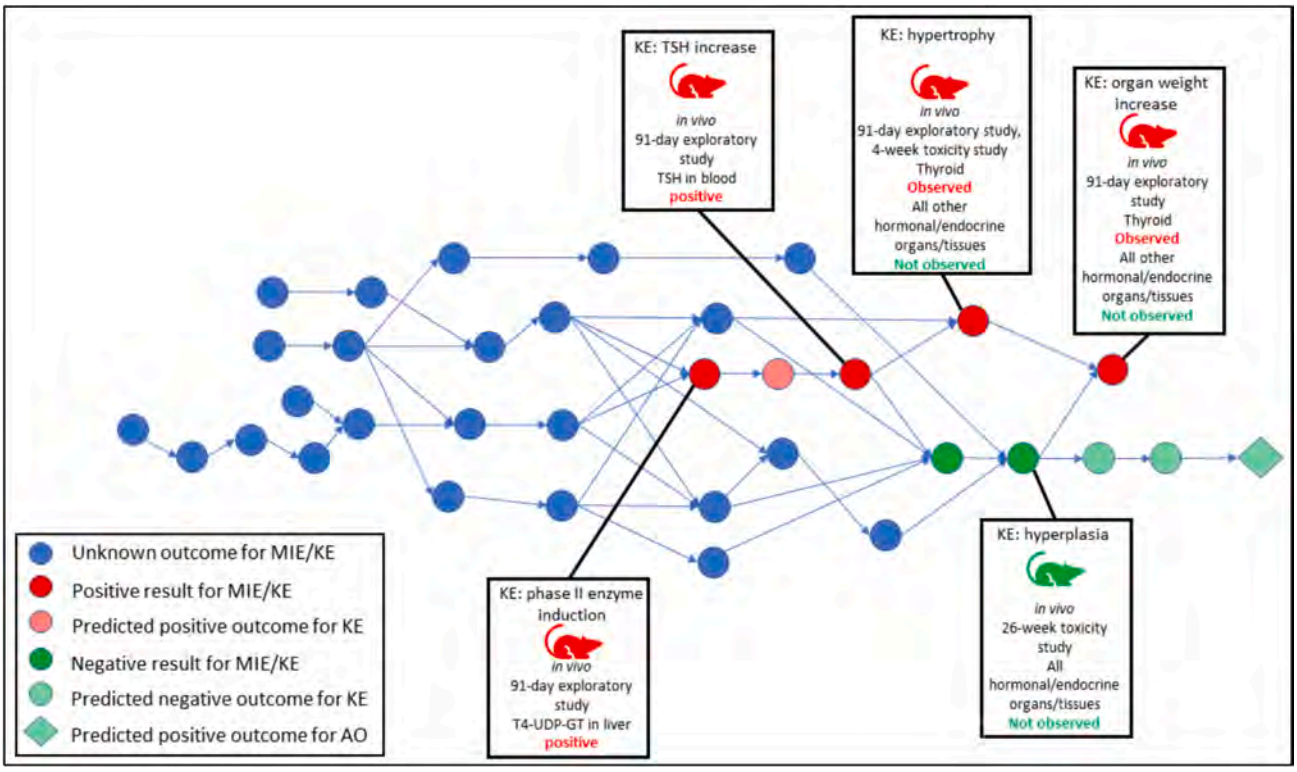


Fig. 18. Data relating to hormonal perturbation for Siponimod mapped onto a subset of Kaptis AOPs, and the predicted AO.

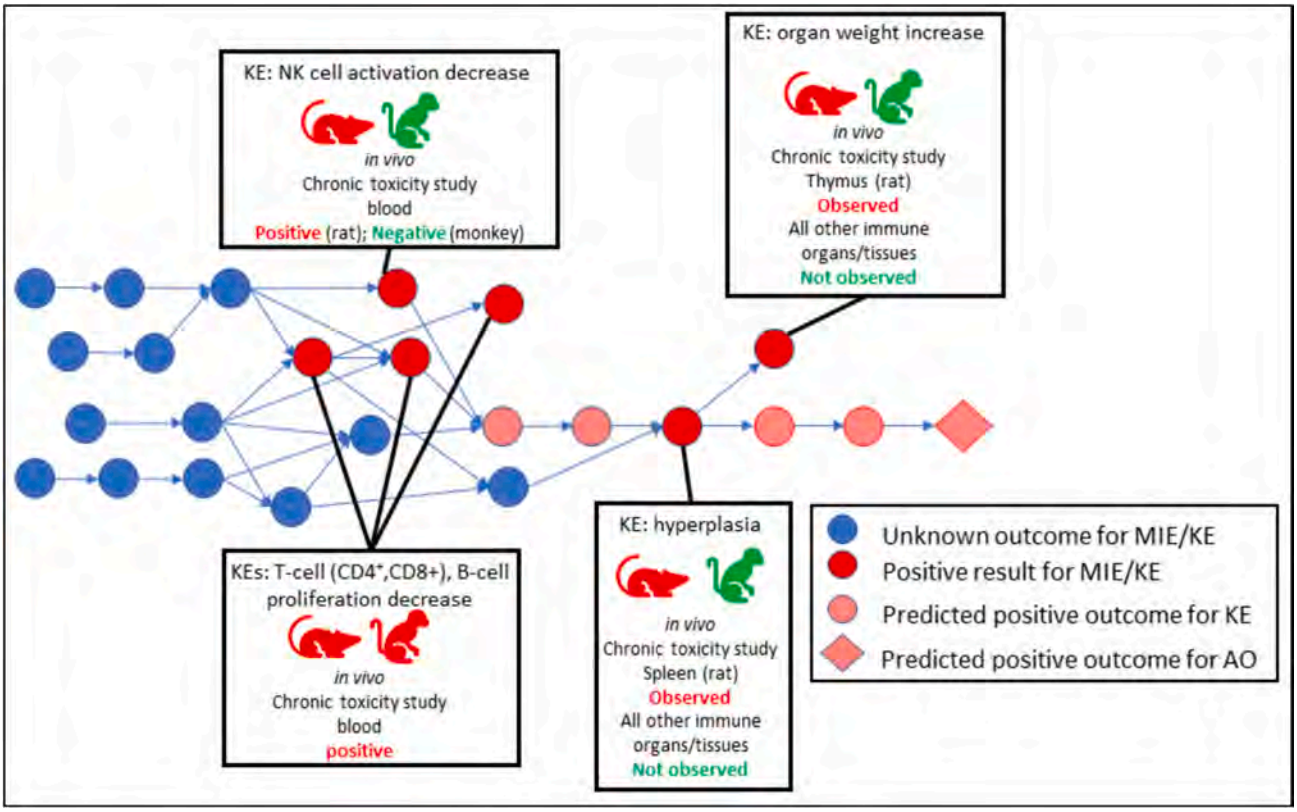


Fig. 19. Data relating to immunotoxicity from chronic toxicity studies mapped onto a subset of Kaptis AOPs, and the predicted AO (without considering species differences).

Expert review analysis of all chronic animal studies taken together (rat 26-week, mouse 274-day and monkey 52-week) indicates that Siponimod may pose a carcinogenic hazard based on histopathological observations which are cancer relevant. However, when the data is reviewed by species, there are some species differences, suggesting that the mechanisms may be species specific, or that some species are more sensitive to the mechanistic effects than others. In this instance, mice are more susceptible to adverse effects from Siponimod, which can potentially cause cancer, than rats, and in turn monkeys. This call is strengthened based upon carcinogenicity findings of pharmacologically similar compounds. Given that rat and monkeys are more reflective for modelling human scenarios, it can be hypothesised that any carcinogenic potential in mice is not human relevant. The downstream effects for the binding of Siponimod to the histamine H2 receptor are not observed in both rats and monkeys, thus this information together resolves that off-target findings are unlikely to be of carcinogenic concern.

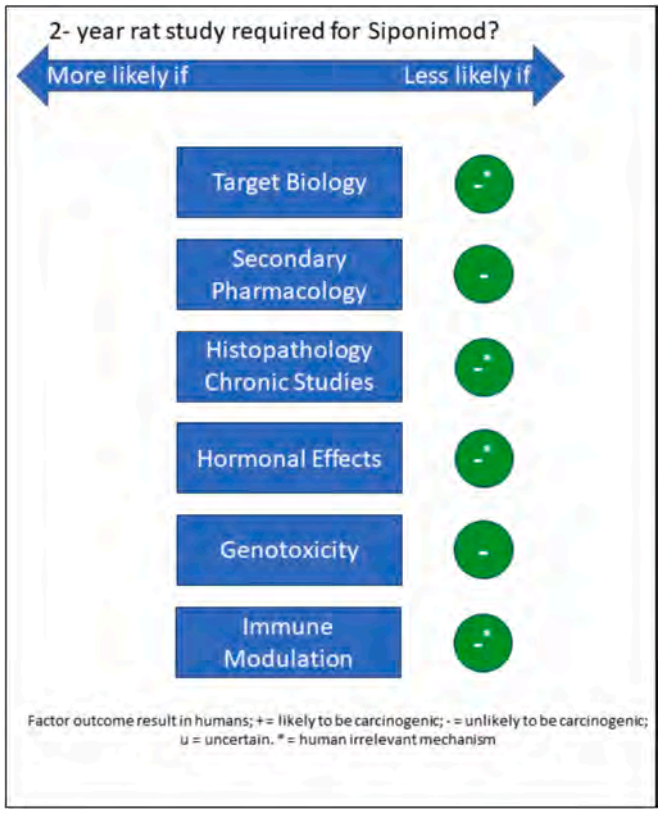


Fig. 20. Summary of WoE assessment for Siponimod for each factor as to whether it is less likely or more likely a rat carcinogenicity study would add value.

3.2.1.5. Hormonal perturbation. The hormonal effect data for Siponimod from chronic toxicity studies indicates a potential thyroid hormone effect [57] (Fig. 18). Thyroid stimulating hormone (TSH) and phase II enzyme induction (which glucuronidate thyroxine (T4)) are both increased in rats, and subsequent thyroidal hypertrophy and organ weight increase are observed (although not consistently across time). However, hyperplasia is not observed at any time point, so when framed on AOPs, the effects do not translate to neoplastic findings. Further to this, the additional information on species-specificity provided in Kaptis for the relevant AOPS indicates that the mechanism that may be occurring is considered not to be relevant in humans [57]. Additionally, the lack of histopathology findings in the mouse and monkey indicates these effects are specific to rats. While tumours of the testes are observed in the 274-day investigative study, the nature of the tumour (hemangiosarcoma) is not related to hormonal effects. Therefore, it is possible that, given the evidence available, hormonal effects are unlikely to raise carcinogenic concern.

3.2.1.6. Immunotoxicity. As seen in the histopathology assessment, there are some findings from the repeat-dose studies in immune tissues [57] (Fig. 19). In the long-term rat study, splenic hyperplasia and an organ weight increase in the thymus are observed, but in monkeys no such changes are seen [4]. In the same chronic toxicity studies, T-cell (CD4 and CD8) and B-cell levels are shown to decrease in blood in both monkeys and rats, but natural killer cell activation decreases are observed in rats. Linking these data through AOPs indicates that this

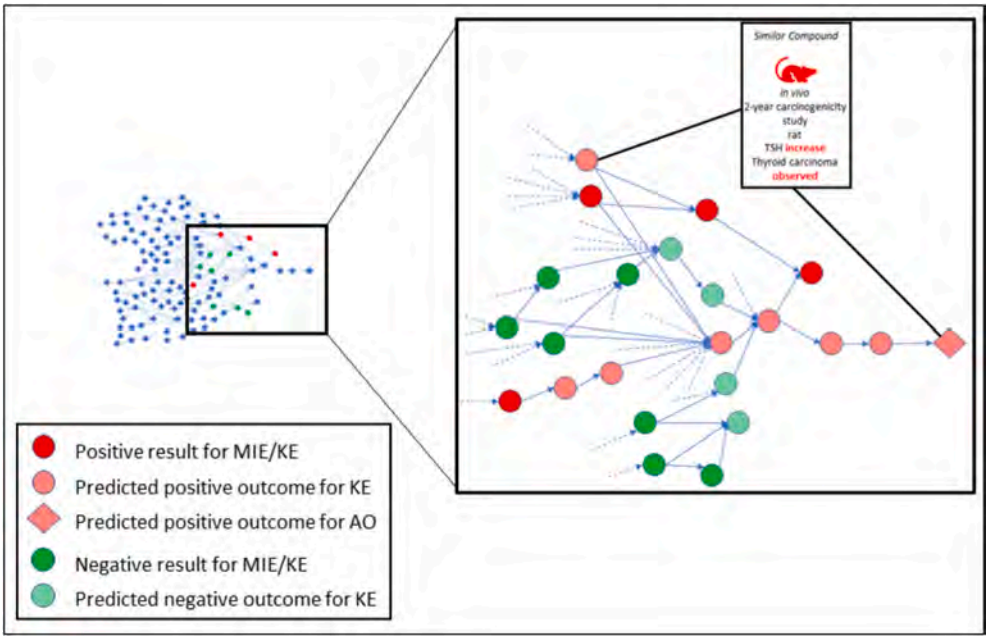


Fig. 21. AOP Network view and section showing the associated data for the example in ICH S1B(R1). Data for a similar compound (the identity of which was not specified in the case study) also added.

may be as a consequence of the intended MoA of Siponimod. No mouse blood chemistry data was available, so the same assessment could not be made for this species.

For Siponimod, the evidence indicates that at least some of the relevant observed histopathology are driven by a MoA involving perturbation of the immune system. However, given the transient nature of histopathology findings in rats and mice, these effects may not be sufficient to translate into neoplastic lesions. The lack of any findings in monkeys related to immunotoxicity and histopathology indicate that this mechanism could also be species specific.

3.2.2. *WoE call*

Based on the information for each factor, it is unlikely that a 2-year rat carcinogenicity study will need to be conducted as the compound is unlikely to be carcinogenic in humans, despite carcinogenicity in rats (and mice) based on species-specific mechanisms (Fig. 20):

- Pharmacologic action, similar compounds in the class, and potential mechanism for hemangiosarcoma formation in mice suggests human irrelevant carcinogenic potential
- Siponimod binds to four secondary targets; only one is potential carcinogenic concern. This concern was mitigated by lack of associated histopathology.
- While there are lesions of concern, most are species-specific (liver lesions in rats only; vascular epithelial cell changes in mice only; no changes in monkeys).
- Human-irrelevant hormonal effects in the thyroid were only seen in rats.
- No genotoxicity was observed.
- Some immune-related lesions of concern are potentially caused by some on-target effects in rats, but are only observed in sub-chronic, and not chronic, studies. Thus, these effects may not translate to neoplastic findings.

It is worth noting that the nature of the tumours formed for pharmacologically similar compounds may not be easily predicted for in the models suggested in the ICH S1B(R1) addendum. Hemangiosarcomas formation typically follows angiogenesis and cell proliferation in vascular epithelial cells, but does not follow from hyperplastic lesions, or other typical precancerous histopathology [65]. Alternative assays and monitoring outcomes in the clinic could help affirm the carcinogenic potential of Siponimod.

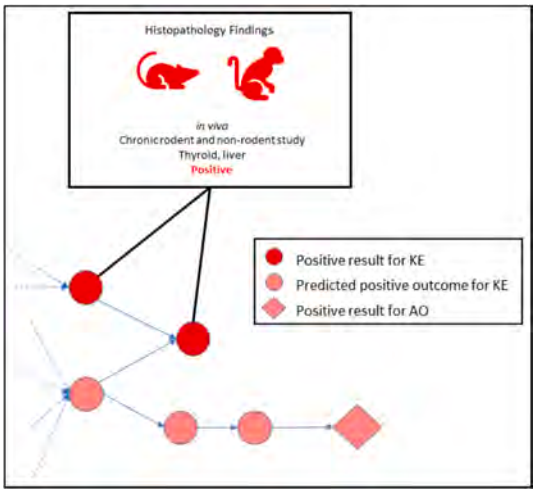


Fig. 23. Histopathology data for the ICH S1B(R1) example mapped onto the relevant KEs in the AOP network, and the predicted AO.

3.2.3. *Carcinogenicity experimental calls*

2-year rat carcinogenicity study – thyroid follicular cell adenoma and carcinoma (males only) [57].

18-month mouse carcinogenicity study – hemangiosarcoma, hemangioma and malignant lymphoma [57].

3.3. *Case study 2 from the ICH S1B(R1) Addendum: An antagonist of a neuronal G-protein coupled receptor*

Evidence from case study 2 presented in the ICH S1B(R1) addendum supplementary information was presented as a set of conclusions for each factor [5] was digitised and contextualised in Kaptis [30]. Projecting the evidence from case study 2 of the ICH S1B(R1) addendum [5] on the whole network shows how the relevant information relates to each other in the context of carcinogenicity (Fig. 21). Highlighting the adverse effects from the similar compound increases confidence in the projected carcinogenic potential of the antagonist being assessed.

3.3.1. *WoE expert review*

3.3.1.1. On-target and off-target. According to the evidence provided, there are no on-target or off-target effects which can be associated to the AOPs in the network.

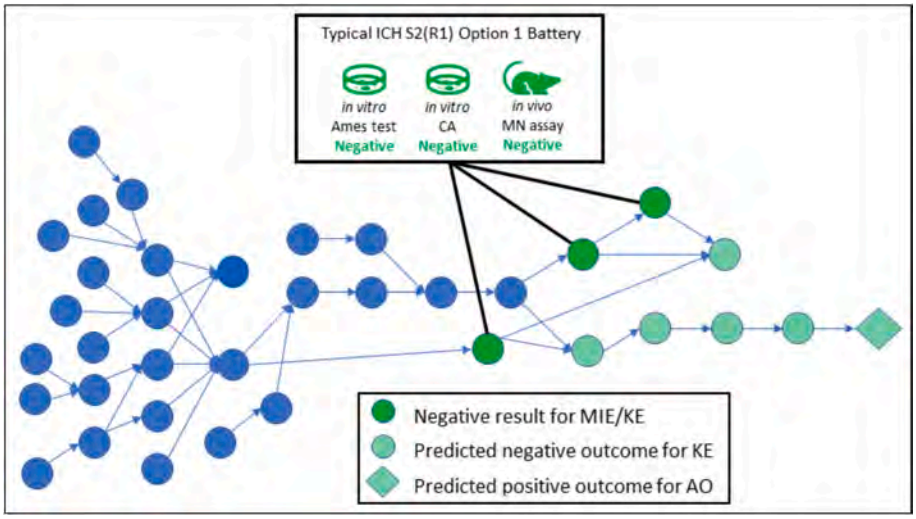


Fig. 22. Association of assumed genotoxicity data for the example in ICH S1B(R1) mapped on to the relevant subset of the AOP network and the predicted AO.

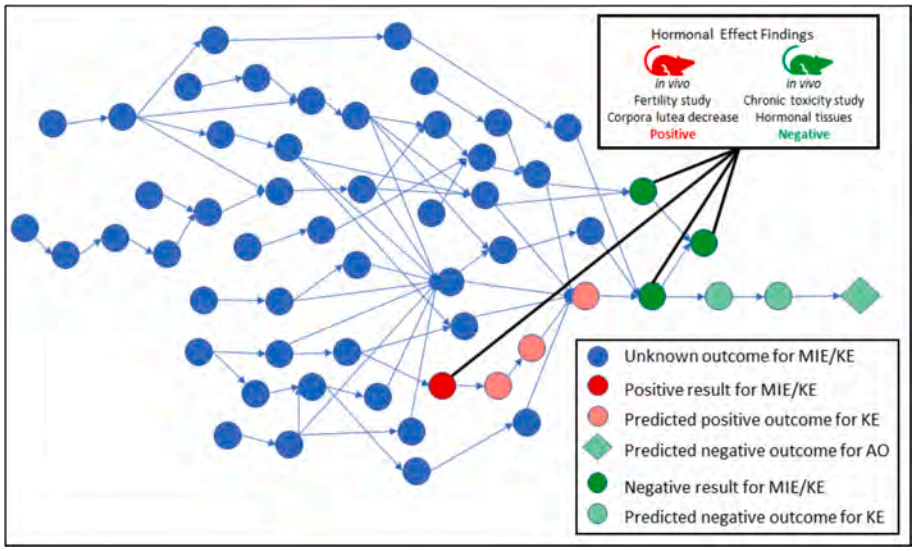


Fig. 24. Data for reproductive hormonal effects mapped on the relevant subset of AOPs related to hormonal perturbation, and the predicted AO.

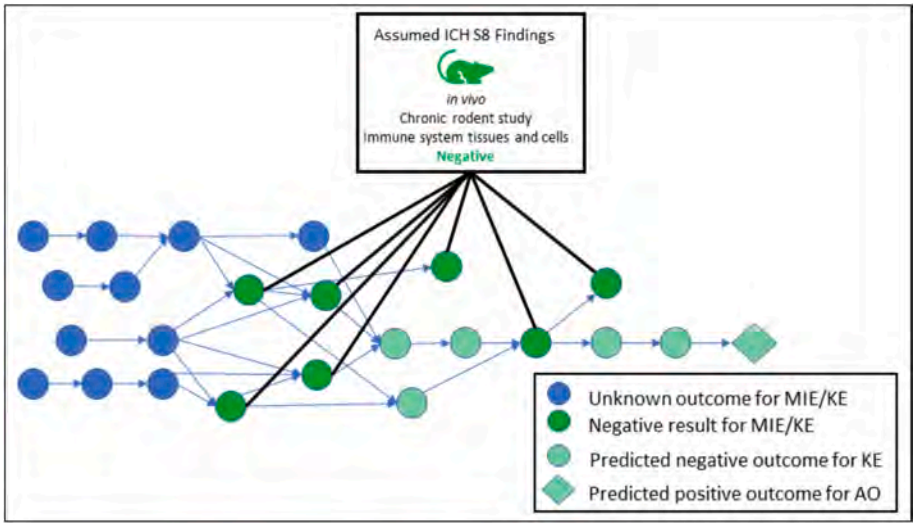


Fig. 25. Association of assumed immunotoxicity data for the example in ICH S1B(R1) mapped on to the relevant subset of AOPs in the network, and the predicted AO.

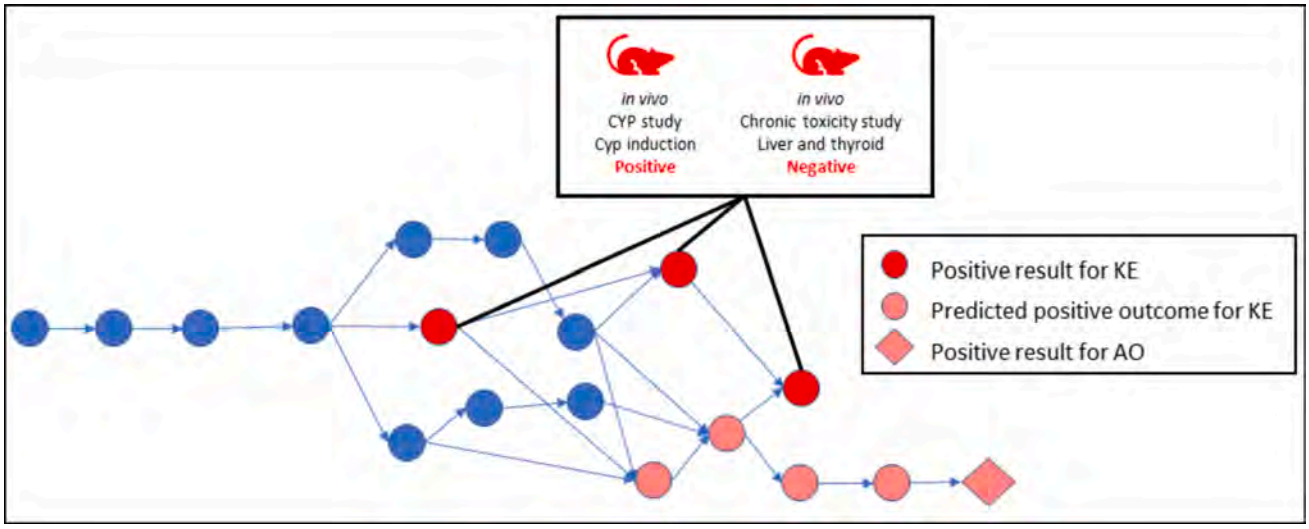


Fig. 26. CAR AOP and the relevant evidence associated, giving a predicted AO.

3.3.1.2. Genotoxicity. While there are no details as to what genotoxicity assays were conducted, it is specified that the ICH S2(R1) guidance was adhered to. By assuming an option 1 battery was run (Ames test, *in vitro* CA assay, *in vivo* MN assay) we can add this evidence to the AOPs (Fig. 22). As no genotoxicity was observed, we can assume the evidence was all negative and can be associated to the AOPs. This confirms that genotoxicity is unlikely to contribute to any potential for carcinogenicity induced by the antagonist.

3.3.1.3. Histopathology. The evidence indicates that liver and thyroid hypertrophy are observed in rats, and liver hypertrophy in the non-rodent study (Fig. 23). Although hypertrophy and organ weight increase alone are not directly causally linked to malignancy, hyperplasia is not mentioned in the text, and as the source of the data is unknown, it cannot be assumed that the report absence means there were no findings. Without this data, the potential for carcinogenic hazard based on histopathology findings cannot be eliminated. The thyroidal findings are only observed in rats, so there may also be a species-specific mechanism operating.

3.3.1.4. Hormonal perturbation. According to the case evidence, no histopathological findings were observed in tissues associated with the reproductive system. The only other finding of note was degradation in corpora lutea, which could indicate a reproductive hormonal mechanism operating, but its effects do not translate downstream (Fig. 24). The findings in the thyroid from histopathology however indicates some endocrinal disruption (Fig. 23).

3.3.1.5. Immunotoxicity. The case study states that there were no treatment-related changes in clinical pathology, lymphocyte subsets, or histopathology of immune tissues. Assuming the studies conducted adhered to ICH S8 guidance (findings from standard toxicology studies, which usually come from chronic rodent studies), the evidence was converted and added to the AOP network, thus confirming that immune modulation is unlikely to contribute to any potential for carcinogenicity induced by the antagonist (Fig. 25).

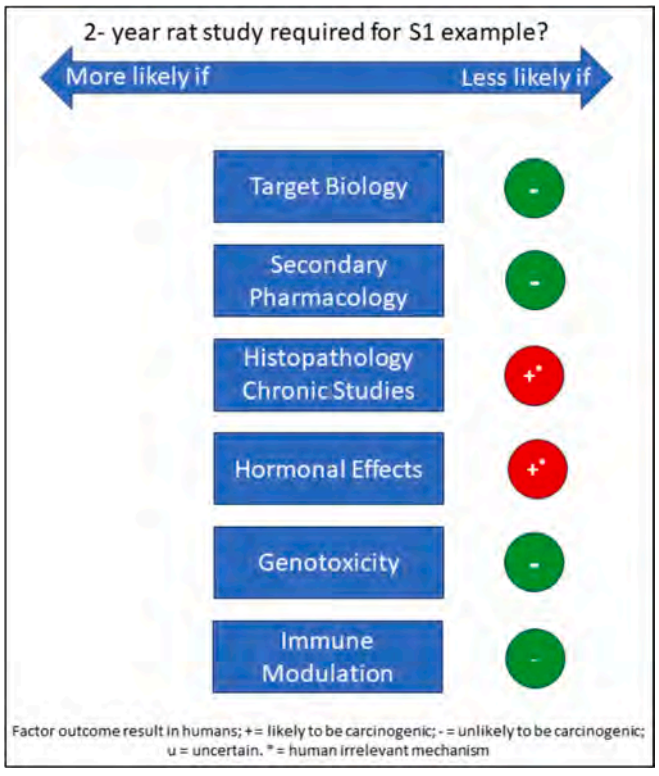


Fig. 27. Summary of WoE assessment for the example in the ICH S1B(R1) addendum for each factor mapped as to whether it is less likely or more likely a rat carcinogenicity study would add value.

Positive findings from the investigative study of CYP induction were also integrated into the AOP network based on the additional investigative studies conducted. The results could indicate any potential active mechanisms, so to probe this further, individual AOPs were reviewed.

One such AOP is for the constitutive androgen receptor (CAR), which within Kaptis, is shown to be applicable to liver and thyroidal findings. The data for the compound (liver and thyroidal hypertrophy), along with that for a similar compound (TSH increase, thyroidal adenoma/carcinoma) also supports this as a potential mechanism for effects observed (Fig. 26). More importantly, this MoA is only thought to be relevant in rats, with the probability of this mechanism operating in humans being low. Therefore, if this is the MoA responsible for observations, the lack of human relevance could be used to mitigate the need for carcinogenic studies in rats.

The evidence suggests that there is a potential concern for carcinogenicity based on the reasoning between data and propagation to the AO. It is possible that the tumours could be thyroidal, based on the thyroidal hypertrophy observed in rats, supported by the evidence from the similar compound, and/or liver tumours induced by the antagonist, based on the gross pathology and histopathology changes, and supported by liver CYP induction.

3.3.2. WoE call

Based on the information for each factor, it is unlikely that a 2-year rat carcinogenicity study will need to be conducted as the compound is unlikely to be carcinogenic in humans, but carcinogenic in rats based on species-specific mechanisms (Fig. 27):

- No evidence of on-target activity related to carcinogenicity.
- No evidence of off-target adverse activity related to carcinogenicity.
- While there are lesions of concern, most are likely species-specific based on potential mechanism.
- Human-irrelevant hormonal effects in the thyroid seen in rats based on potential mechanism.
- No genotoxicity.
- No immuntoxicity.

The unmasked data for this antagonist has not been published, so not all the findings can be compared, however, this example illustrates the value of using AOPs for such an assessment, based on the additional mechanistic hypothesis which was deduced from the published case evidence.

4. Discussion

The case studies presented highlight how organising and analysing available data, conducting expert review of the resulting WoE and drawing conclusions on the carcinogenic potential of a pharmaceutical can all be carried out in a systematic and robust way using an AOP network when assessing ICH S1B(R1) factors. Employing this approach for these case studies reflected many of the key advantages associated with using AOPs whilst performing chemical safety assessments (Fig. 2).

1) **Establishing a MoA hypothesis based on a drugs perturbation of the biological system early in drug development, testing this and monitoring for findings of concern in subsequent studies.**

Findings made early in the drug development process can be associated with KEs and AOPs, so that MoA hypotheses can be generated, contextualised and tested appropriately within an AOP construct. Associating the (observed or predicted) activity of a compound to a specific KE provides mechanistic insight that can inform a testing

strategy, or the ability to contextualise and resolve contradictory or inconsistent results. Understanding and acting on risks associated with a compound can lead to efficient compound prioritisation ('which analogue doesn't have that risk?') or to identify the assay that will confirm or refute it ('fail-fast'). *In silico* systems like Kaptis provide access to expert-curated knowledge of the biological mechanisms and the most appropriate test systems and offers the potential to reduce the knowledge-barrier to decision-making.

This is highlighted in the Lansoprazole and Siponimod case studies, where associations between the drug and experimental results allowed the potential carcinogenic risks to be identified and addressed. In both cases, adverse histopathology findings could be flagged for confirmation in later *in vivo* studies. While the current exercise did not allow the for the generation and integration of new data into the risk assessment, a more iterative approach ('hypothesize-test-review') would allow gaps in knowledge to be addressed strengthening the evidence for a mechanistic link between experimental observation and adverse outcome. Projecting data onto an AOPs both increases the depth of understanding of the assessment and supports efficient communication decisions made.

2) **Rationalising findings of concern observed in animal studies later in drug discovery through probing of potential MoAs**

Unexpected toxicity findings of concern discovered later in development (e.g. through repeat-dose studies) can be contextualised and evidence relating to the biological context of the findings assembled to suggest potential AOPs that with directed testing could ultimately determine the mechanistic basis of the observed toxicity. This knowledge can support a decision on compound progression or inform a screening strategy for subsequent analogues. This is highlighted in the example from the ICH S1B(R1) addendum, where a repeat-dose study resulted in findings of concern in the liver and thyroid. Analysis of the tissue context of these findings led to the identification of potential MoAs that could be evaluated within an AOP context. Working backwards from a toxic result from *in vivo* studies can help provide support for a proposed mechanism and identify earlier and generally cheaper / faster screens. If this approach were used in an ICH S1B(R1) assessment it would be possible to generate further data, to provide a mechanistic rationale for the toxicity including the MIE that led to the adverse findings observed.

3) **Using knowledge of MoAs to make better informed regulatory decisions**

Access to a systematic knowledge base containing the MoAs and their associated adverse outcomes (either predicted or observed) would support an informed approach to regulatory decision-making.

Firstly, knowledge of the MoA allows for potentially human relevance of mechanisms leading to findings of concern to be assessed, and the relevance of observations in different species to be considered. If there is likely to be limited human relevance, it may be possible to set aside species-specific findings. For example, employing the rationale that the drug substance is likely to be carcinogenic in rats, but unlikely to translate to humans, as described in the ICH S1B(R1) addendum [5]. Successful application of this argument may allow for continued progression of the drug substance without the requirement for further long-term rodent studies. This scenario is exemplified by the Siponimod and the ICH S1B(R1) examples, where the MoA implicated in rodent repeat-dose studies may have a limited human relevance due to compensatory mechanisms, or activation pathways that only operate in a given species.

Secondly, knowledge of the MoA can highlight limitations in existing test data and testing regimes relating to a specific mechanism of toxicity. For Siponimod, the AOP linking of the target to the AO through perturbation of the immune system connects together the on-target and immunotoxicity factors. This MoA is not well predicted in traditional assays [65] and therefore results from histopathology-related assays may not hold much weight during the assessment, hence other assays described along the AOP may better inform the risks caused by this MoA [66].

4) **Establishing consistency in how to contextualise results and assess their relevance**

Displaying available evidence as findings relating to specific KEs on an AOP allows for the direct comparison of different pieces of data that measure the same perturbation. This identifies contradictions in evidence, such as in Lansoprazole genotoxicity data, where *in vitro* and *in vivo* results measuring the same KE could be quickly and clearly shown to be in direct contradiction. It can also allow for more sophisticated relationships between different data points to be explored such as the temporal relationships between findings measuring the same KE. This allows for transience and reversibility of findings to be easily communicated and used as an argument for why a concerning finding may not translate into a cancer outcome. Indeed, advice in the ICH S1B(R1) addendum guidance recommends the consideration of this temporal relationship between findings and assessment of their transient nature [5]. This was demonstrated with Lansoprazole and Siponimod, where a number of concerning histopathology findings in specific tissues were found to be transient in nature, being observed only in shorter term repeat dose studies but not in longer term studies.

5) **Presenting evidence, arguments and interpretation in a consistent and logical manner**

Realising the aims of the ICH S1B(R1) addendum requires expert review to assess all available evidence to come to a safe, defensible and reproducible conclusion. To achieve this in more complex cases where there is contradictory or inconsistent data without reverting to animal studies requires a level of formalisation in both the approach and the principles behind these decisions. This can be achieved with software that encodes best practice within a systematic and logical framework developed in a collaborative pre-competitive manner. Alignment between submitter and reviewer of submissions under ICH S1B(R1) becomes more likely if this is in place and is reinforced when the evidence, arguments and interpretation is displayed in a consistent and logical manner. Decision-support software will play an increasingly important role in providing this capability.

5. Conclusions

This works describes how an AOP framework can aid in the organising, contextualising, and expert review of a WoE assessment which is required for the ICH S1B(R1) guidance. This was achieved by establishing a workflow for the assessment based on the normal drug development process, gathering required data, and using Kaptis to organise and contextualise this on an AOP network. The organisation of data on AOPs gives the ability to find relationships quickly, address data gaps, and using expert review, establish transparent and robust conclusions. Three examples are presented, which show the value of AOPs in this process (Fig. 2) to 1) establish MoA hypotheses which can be tested, 2) rationalise findings of concern, 3) use the appropriate knowledge of MoAs to inform decisions, 4) establish consistency of results, and 5) present evidence in a robust and consistent manner. Additionally, these studies highlight the importance of expert review (by injecting evidence, interpreting data, using observations from similar compounds, and applying defensible and explainable reasoning to derive conclusions. In the case of ICH S1B(R1), this can strengthen calls on the value a rat carcinogenicity study would add to a preclinical safety toxicology package. Thus, as seen in the examples, the calls made and neoplastic lesions predicted are more likely to reflect human relevance, and the findings in experimental rat studies. Providing access to knowledge and establishing best practice approaches on decision-making will support

safe human protective decisions whilst minimising the use of animals for testing carcinogenicity.

CRediT authorship contribution statement

Susanne A. Stalford: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alex N. Cayley:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Conceptualization. **Adrian Fowkes:** Writing – review & editing. **Antonio Anax F. de Oliveira:** Writing – review & editing, Supervision, Conceptualization. **Ioannis Xanthis:** Writing – review & editing. **Christopher G. Barber:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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