Annex II to the CLH Report for Valifenalate (IR5885)

Valifenalate: Mode of Action Analysis using the WHO/IPCS MoA Framework

1. Problem formulation

Carcinogenicity studies in rats (See annex conf. 51) and mice (See annex conf. 52) have been completed for valifenalate. In Han Wistar rats there was no evidence of valifenalate-related carcinogenicity, whilst in CD-1 mice valifenalate induced hepatocellular adenomas and carcinomas in males. In female mice, valifenalate appeared to be less potent with a smaller, but statistically significant, increase in adenomas only being reported. With respect to human health, some mode of action (MOA) pathways that lead to the formation of hepatocellular carcinomas in rodents are considered to have little or no relevance, whilst other MOA pathways are considered to be potentially relevant to humans.

A series of investigative toxicology studies were undertaken in male mice with the aim of, firstly, shedding light on the likely mechanism of formation of hepatocellular carcinomas induced by valifenalate in male CD-1 mice, and secondly, to address the assessment of the relevance of these findings to human health.

2. Hypothesised Mode of action for Valifenalate-induced hepatocellular adenomas & carcinomas in CD-1 mice

The mode of action for the formation of liver tumours in mice, induced by valifenalate, has been considered following a series of investigative studies. In short, the initiating event is the co-activation of the nuclear receptors CAR/PXR/PPARα by valifenalate which in turn, mediates the induction of replicative DNA synthesis in the liver of CD-1 mice. This increased cell replication is critical to the subsequent induction of proliferative lesions of the liver including hepatocellular foci, adenomas, and eventually carcinomas (Holsapple *et al* 2006; Klaunig *et al* 2003, Cohen 2010, Elcombe *et al* 2014).

The following abbreviations for nuclear receptors are used in this document:

CAR = Constitutive androstane receptor
PXR = Pregnane X receptor
PPARα = Peroxisome proliferator-activated receptor alpha
AhR = Aryl hydrocarbon receptor

3. Summary of data for use in this Mode of Action Analysis

Species (strain)	Route/Dose	Incidence/Critical Endpoints	Comments	References
Mouse (CD-1)	Oral, diet, 0, 150, 850, 5000 ppm (time point = 78 weeks)	Mice examined after 78 weeks of treatment with valifenalate <u>Males (n=50)</u> Adenoma: 7, 2, 14, 16 Carcinoma: 2, 4, 4, 10 (Combined: 9, 6, 18, 26) <u>Females (n=50)</u> Adenoma: 0, 0, 2, 5 Carcinoma: 0, 1, 0, 0 (Combined: 0, 1, 2, 5)	Carcinogenicity study to GLP Historical Range (%) below Males (n=307) Adenoma: 7.8 - 21.2% Carcinoma: 1.9 – 8% Females (n=307) Adenoma: 0 – 1.9% Carcinoma: 0.0%	See annex conf 52 Valifenalate CLH report Annex I, Table 3.9.1.2-05. Note: Substance code IR5885 is valifenalate
Mouse (CD-1) Male animals	Oral, diet 0, 150, 1750, 7000 ppm valifenalate and 850 ppm phenobarbital as a positive control (time points: 3 and 14 days)	<u>Critical Endpoints:</u> Liver weight Clinical chemistry Histopathology Biochemistry: Enzyme activity of Cytochromes P450 (Cyp's) and Oxidative stress Gene expression Cyp mRNA Cell proliferation using bromodeoxyuridine (BrdU)	Initial investigations in CD-1 mice (non-guideline, GLP-compliant study)	Broich 2015 Valifenalate CLH report Annex I, Section 3.9.4.1 Note: Substance code IR5885 is valifenalate
Mouse (CD-1 & C57BL/6)	Oral, diet 0, 7000 ppm (time point 7 days)	<u>Critical Endpoints</u> Liver weight Clinical chemistry Biochemistry: Enzyme activity of Cyp2b and	Investigative Study Strain comparison - CD-1 and C57BL/6 (non-guideline, not fully GLP-	Vardy 2015a Valifenalate CLH report Annex I, Section 3.9.4.2

Species (strain)	Route/Dose	Incidence/Critical Endpoints	Comments	References
		Cyp4a, peroxisomal β-oxidation	compliant study)	
Mouse (C57BL/6 & C57BL/6 PPARα KO)	Oral, diet 0, 7000 ppm (time points 7 & 14 days)	<u>Critical Endpoints</u> Liver weight Clinical chemistry Histopathology Biochemistry: Cyp 2b10, Cyp3a11, Cyp 4a Gene expression: Cyp 2b10, Cyp3a11, Cyp4a Cell proliferation: BrdU	Investigative Study PPARα knockout - C57BL/6 wild type and PPARα knockout (non-guideline not fully GLP-compliant study)	Vardy 2015b Valifenalate CLH report Annex I, Section 3.9.4.3
Mouse (CD-1)	In vitro, hepatocytes 0, 10, 30, 100 & 300 µM valifenalate with phenobarbital (as Na salt at 100 and 1000 µM) and WY-14,643 (50 and 100 µM) as positive controls	<u>Critical Endpoints</u> Cell proliferation Gene expression: Cyp2b10, Cyp4a	Investigative Study CD-1 *in vitro* hepatocyte cultures (non-guideline, not fully GLP-compliant)	Vardy 2015c Valifenalate CLH report Annex I, Section 3.9.4.4.

Note: C57BL/6 PPARα KO = C57BL/6 (PPARα Knockout) strain,

4. Key events of the mode of action for the formation of Valifenalate-induced hepatocellular adenomas & carcinomas in CD-1 mice

Key Event 1 ***(Initiating event)***	**Nuclear receptor activation**, in particular, co-activation of the nuclear receptors **CAR, PXR and PPARα** by valifenalate.
Key Event 2	**Increased replicative DNA synthesis**
Key Event 3	**Formation of hepatocellular carcinoma** (via the development of altered, hyperplastic, hepatic foci, and the subsequent development of benign and, ultimately, malignant hepatocellular neoplasms)

Key Event 1: Activation of nuclear receptors, specifically CAR, PXR and PPARα

Hepatic xenobiotic metabolising enzymes have been considered as robust, surrogate, markers of nuclear receptor activation (Elcombe *et al* 2014, Lake 2009). In studies on valifenalate these diagnostic markers were used, alongside other information, as a 'fingerprint' to characterise activation of nuclear receptors. Complimentary to these diagnostic markers, analysis of gene expression was used to confirm the xenobiotic metabolising enzymes induced, or not induced, and hence confirm which nuclear receptors were activated, or not, on exposure of mice to high dietary concentrations of valifenalate. It became clear that nuclear receptors CAR, PXR and PPARα were all activated in male mice (CD-1 strain) exposed to higher concentrations of valifenalate administered in diet. In contrast, the aryl hydrocarbon (AhR) nuclear receptor was unaffected by all concentrations of valifenalate tested (up to 7000ppm) as measured by gene expression and Cyp 1a activity, hence Ahr was not investigated further. Table A1 details the enzyme and gene expression induction profile following dietary exposure of mice to high concentrations of valifenalate in the CD-1 mouse strain (Broich 2015) and Table A2 shows similar biochemical data for another mouse strain, C57BL/6, which was used in complimentary investigative studies (Vardy 2015b).

Considering that CD-1 or C57BL/6 strains of mice were fed valifenalate in the diet, in different laboratories, some years apart, and with exposure to different concentrations of valifenalate, the results of these studies (Broich, 2015; Vardy 2015a, Vardy 2015b) are remarkably consistent. In each strain of mouse, high dietary concentrations of valifenalate led to activation of CAR, PXR and PPARα.

Information from a study where a C57BL/6 (PPARα KO), strain of mouse was compared with the wild-type C57BL/6 confirmed involvement of the activation of PPARα, and at the same time confirmed that CAR/PXR receptors are also activated. Firstly, in both strains there was an increase in the liver:body-weight ratio but the relative liver weight increase in the wild type was considerably greater than in the knockout strain, consistent with activation of the PPARα receptor in the wild type (Table A3). However the knockout strain, treated with valifenalate, did show a small increase in the liver:body-weight ratio over the knockout –ve group, given control diet, consistent with the co-activation of other nuclear receptors, subsequently confirmed as CAR/PXR. Secondly, the hepatic hypertrophic response to valifenalate was very much greater in the wild type that in the knockout strain. This is consistent with most of the hypertrophic response being related to the PPARα activation, with only a relatively minor hypertrophic response expected through activation of CAR and PXR (Vardy 2015b).

Further information, to strengthen the hypothesis of co-activation of PPARα/CAR/PXR derived from the knockout study, was analysis of gene expression which confirmed induction of mRNA of Acox1 (PPARα), Cyp2b10 (CAR) and Cyp3a11 (PXR) in the wild-type strain. The consequential enzyme induction profile was also characterised.

1. Acox 1 mRNA (a marker of PPARα activation) was found to be reduced slightly in the valifenalate-treated knockout strain (1.3-fold relative to knockout –ve control) relative to the wild type strain (1.8-fold relative to wild type –ve control) confirming the presence of a relatively weak PPARα agonist. This relatively weak PPARα agonist activity was confirmed with higher 12-hydroxylauric acid (LAH) induction in the wild type (7.75 fold relative to –ve control) compared to the knockout (4-fold relative to knockout –ve control) (Table A4, Vardy 2015b).
2. For Cyp2b10 mRNA (a marker of CAR activation), there was a rather weak, if any, increase in the valifenalate-treated wild type (56-fold increase relative to the wild type –ve control) compared to the knockout strain (48-fold increase relative to the knockout –ve control) indicative of activation of CAR of similar magnitude, in both the wild type and knockout strains. This was confirmed with pentoxyresorifin-*O*-depentylation (PROD) induction (a mixed function oxidase enzyme specific for Cyp2b10 activity) being similar in the wild type (6-fold increase relative to the wild type –ve control) compared to the knockout (7-fold increase relative to the knockout –ve control) and was indicative of a relatively weak induction of Cyp2b10 (Table A4, Vardy 2015b).
3. The induction of Cyp3a11 mRNA (a marker of PXR activation) in valifenalate-treated C57BL/6 mice was similar in the valifenalate treated wild type (6.3-fold increase relative to the wild type –ve control) compared to the valifenalate-treated knockout strain (8.5-fold increase relative to the knockout –ve control) and indicated activation of PXR of similar magnitude, in both wild type and knockout strains. Confirming this interpretation, there was also induction of Cyp3a11 enzyme activity, as monitored by benzyloxyquinoline-*O*-debenzylation (BQ) to the same extent in the wild type strain (2.4-fold increase relative to the wild type –ve control) compared to the knockout strain (also 2.4-fold increase relative to the appropriate –ve control value) (Table A4, Table 39, Vardy 2015b).

In conclusion, for the initiating event, there is sufficient evidence from gene expression analysis, and enzyme activity of diagnostic markers, to support the co-activation of the nuclear receptors CAR/PXR/PPARα in both CD-1 (Broich 2015) and C57BL/6 (Vardy 2015b), and of CAR/PXR in the C57BL/6 (PPARα KO) strains of mice (Vardy 2015b).

Key Event 2: Induction of replicative DNA synthesis

Evidence for the second key event, induction of replicative DNA synthesis, was obtained from BrdU incorporation studies. In one study, where mice were fed valifenalate for 3 or 14 days, it was concluded that S-phase DNA synthesis was dose, and time-dependent, and was only present when mice (CD-1 strain) were exposed to valifenalate at concentrations above carcinogenic dose-levels (Table A5, Broich 2015). To stress, no increased cell proliferation was observed when valifenalate was administered to CD1 mice at the non-carcinogenic dose level.

From the study in which C57BL/6 wild type and PPARα knockout strains were exposed to high levels of dietary valifenalate, it was clear that replicative DNA synthesis was increased 8-fold on day 7 for the wild type (that is, PPARα/CAR/CXR co-activated) but remained 5-fold elevated in the knockout strain (that is, only CAR/PXR co-activated), and was indeed confirmatory evidence that PPARα was

not the only nuclear receptor involved in valifenalate-mediated hepatocarcinogenesis. As may be anticipated, by day 14 the initial wave of synthesis induced by valifenalate, had subsided to about 3-fold and 2-fold for the wild type and knockout strains, respectively (Table A6, Vardy 2015b).

All the above information was consistent with the hypothesis of valifenalate activating multiple nuclear receptors (PPARα/CAR/CXR) and being a relatively weak agonist of PPARα. In addition, when PPARα was activated, it appeared that the degree of the hepatic hypertrophic response was greater than the hyperplastic response whereas in contrast, with CAR/PXR activation, the hypertrophic response generally appeared less than the hyperplastic response.

Key event 3: Formation of hepatocellular carcinomas

Detailed studies on the development of rodent hepatic neoplasia have shown a direct correlation between the induction of hepatocyte DNA synthesis, the development of altered, hyperplastic, hepatic foci, and the subsequent development of benign and, ultimately, malignant hepatocellular neoplasms. For valifenalate, hepatocellular adenomas and carcinomas were reported with dietary incorporation levels of 850 and 5000ppm in a guideline 78-week carcinogenicity study in male CD-1 mice. In contrast in the same study, incorporation of valifenalate to diet of male CD-1 mice at the level of 150ppm did not produce hepatocellular adenomas or carcinomas. The incidences of hepatocellular eosinophilic foci, adenomas and carcinomas are detailed in Tables A7 & A8, derived from See annex conf. 52

5. Bradford Hill Considerations for Weight of Evidence Analysis of available data/information for Mode of Action Analysis in experimental species

5a. Dose Response Relationships and Temporal Association

Dose response relationship*: Are the key events observed at doses below or similar to those associated with the (adverse) effect?*

Key event 3 is defined as the formation of carcinomas. However, with increased utilisation of a potentially adverse pathway from altered, hyperplastic, hepatic, foci, to the subsequent development of benign and, ultimately, malignant hepatocellular neoplasms, it seems reasonable when considering dose-response information for this, final, key event to also consider the increased formation of other late-stage (pre-carcinoma) information. Therefore with respect to dose-response, and in the interests of clarity, the combined incidence of adenomas and carcinomas has been included under key event 3. Analysis of dose-response, using the combined incidence of adenomas and carcinomas brings some clarity, in particular, to the interpretation of the information at the intermediate dose-level, 850ppm, where the incidence of carcinomas, alone, is within the historical control range. For reference, the incidence of adenomas, carcinomas, and combined adenomas and carcinomas, are shown in the main CLH Report, Section 10.9 Carcinogenicity, Table 37.

An increased incidence of hepatocellular adenomas and carcinomas was reported for male CD-1 mice exposed to 5000ppm and 850ppm valifenalate for 78 weeks (See annex conf. 52 & CLH Report section 10.9). There was no increased incidence of tumours at the lower exposure level of 150ppm.

In a short-term investigative study male CD-1 mice were exposed to either 0, 150ppm, 1750ppm or 7000ppm valifenalate, or phenobarbital (850ppm) as a positive control. Both gene expression and enzyme activity for Cyp1a were unaffected by all concentrations of valifenalate exposure. In contrast, and reflecting co-activation of CAR/PXR/PPARα, gene expression of Cyp2b10 and Cyp3a11 (mRNA

of Cyp4a was not measured) and enzymatic activity of Cyp2b, Cyp3a and Cyp4a were induced in valifenalate-treated animals exposed to concentrations of 1750ppm and 7000ppm (Broich 2015).

Consistent with the lack of neoplastic effects at 150ppm in the carcinogenicity study, this short term investigative study also defined no, or marginal, induction of gene expression, or enzyme activity, at the lower dose level of 150ppm. In other words, the lower dose level of 150ppm valifenalate is insufficient to mediate key event 1, co-activation of CAR/PXR/PPARα, in CD-1 mice. Clearly the second key event, induction of replicative DNA synthesis, may occur after the initiating event is mediated by valifenalate. However, when valifenalate is present in insufficient concentrations to activate the appropriate nuclear receptors, there should be no induction of hepatocellular proliferation. The results of this short-term study in CD-1 mice are entirely consistent with these considerations (Table A5, Broich 2015).

It also follows that when activation of CAR/PXR/PPARα is maintained, and drives continual induction of replicative DNA synthesis, the longer term consequence may be the formation of altered hyperplastic foci, and eventually formation of hepatocellular adenomas and carcinomas. This is consistent with the results of the longer-term carcinogenicity study in CD-1 mice, where valifenalate-induced hepatocellular adenomas and carcinomas were reported at dose levels of 850 and 5000ppm, but not at 150ppm (See annex conf. 52).

Additional short-term information, concerning the first 2 key events is available in the C57BL/6 and C57BL/6 KO strains of mice. Once differential food intake and hence delivered dose, between the C57BL/6 strains and the CD-1 strain has been taken into account, the biological response with respect to the first and second key events is consistent at the high dose level tested, 7000ppm, and showed that there were no strain differences between the CD1 and C57Bl/6 mice in their hepatic response to valifenalate (Vardy 2015a & Vardy 2015b).

In conclusion, when all three key events were investigated across a range of exposure levels, the weight of evidence indicates that the observed effect (and no-effect) concentrations of valifenalate in short and in long-term studies in the CD-1 mouse, is entirely consistent with critical events of the hypothesised mode of action.

***Temporal association**:*

In valifenalate-treated CD-1 mice, the first 2 key events although consecutive, occur in quick succession. As the initiating event has been measured indirectly, through gene and enzyme induction analysis, absolute differentiation in time between the initiating event and key event 2 is less feasible in practice.

Hepatic xenobiotic metabolising enzymes have been considered as robust, surrogate, markers of nuclear receptor activation (Elcombe *et al* 2014, Lake 2009). Therefore to indirectly assess the activation of nuclear receptors (key event 1), experimental protocols are optimised to quantify the pattern of induction of hepatic xenobiotic metabolising enzymes which are a direct, downstream, consequence of activation. The time points used in these experimental protocols for valifenalate are 7, or 14 days (Tables A1 & A2, Broich 2015 & Vardy 2015b).

The second key event, an increase in replicative DNA synthesis, if it occurs following receptor activation, is initiated relatively quickly after receptor activation and hence was measured over a similar time scale with data available for 3, 7 and 14 days (Tables A5 & A6, Vardy 2015b & Broich 2015). When S-phase synthesis was measured after 14-days of treatment with valifenalate there was a

general decrease, relative to that recorded after exposure for the previous 3 (Broich 2015) or 7 days (Vardy 2015b), in the extent of the proliferative response in all valifenalate-treated mice, as the homeostatic control of internal conditions tended towards a resetting of the baseline. This phenomenon is common to all xenobiotics that induce hepatic hypertrophy including potent inducers of PPARα such as nafenopin and Wy14,643 and CAR/PXR such as phenobarbital.

The temporal association between this second key event, increased hepatocellular proliferation, and the third key event is more clearly defined from the long-term studies on valifenalate. Detailed studies on the development of rodent hepatic neoplasia have shown a direct correlation between the induction of hepatocyte DNA synthesis, the development of altered, hyperplastic, hepatic foci, and the subsequent development of benign and, ultimately, malignant hepatocellular neoplasms. This sequence of events is consistent with the database available from the 78 week carcinogenicity study with valifenalate in CD-1 mice. Male CD-1 mice are more severely affected than female mice, with a greater number of foci developing towards adenoma and carcinoma in the former gender. In the female mice, less sensitive to the effects of valifenalate, carcinomas did not develop, and consistent with this, the incidence of foci at the highest dose level was greater than in the males and a relatively small, but significantly increased, incidence of adenomas was reported in females (See annex conf. 52).

In summary the evidence generated for valifenalate in CD-1 mice is consistent with the temporal association of a mode of action with three, consecutive, key events.

The initiating event is the co-activation of multiple nuclear receptors, CAR/PXR/PPARα, and as a direct consequence, the associated induction of gene expression and enzyme activity of Cyp2b10, Cyp3a11 and Cyp4a.

The second key event, increased hepatocellular proliferation, is also initiated in CD-1 mice exposed to valifenalate, on a time scale not dissimilar to the appearance of induction of the hepatic metabolising enzymes.

The final key event is the longer-term formation of carcinomas via the development of altered, hyperplastic, hepatic, foci and the subsequent development of benign and, ultimately, malignant hepatocellular neoplasms. This is consistent with information from the 78 week carcinogenicity study in male and female CD-1 mice.

In another strain of mouse there is short-term, experimental, information consistent with key events 1 and 2, that is activation of CAR/CXR/PPARα and increased replicative DNA synthesis, in C57BL/6 and C57BL/6 (PPARα KO) mice. This information is also consistent with that described above for the CD-1 strain of mouse.

Summary of Dose Response (vertically) & Temporality (horizontally) of the Hypothesised Mode of Action

Dose		Time				
	Dose ppm (mg/kg bw/day)	**Initiating Event Activation of CAR/PXR/PPARα**	**Key Event 2 Increased replicative DNA synthesis**	**Associated event: Increased hepatocellular hypertrophy**	**Key Event 3 Formation of Carcinoma *** (refer to footnote)	**Study Reference**
		Measured indirectly from Day 7	*Measured from Day 3*	*Measured from Day 3 to 90*	*Key event: Measured at 78 weeks*	
	110 (15.3)			Negative in CD-1 strain of mouse 90 days		See annex conf. 50
	150 (20.7)	Negative on day 14 in male CD-1 strain of mouse	Negative on day 3 and day 14 in CD-1 strain of mouse	Negative in CD-1 strain of mouse at 3 & 14 days		Broich (2015)
	150 (16.8)			Negative in CD-1 strain of mouse at 78 weeks	**Negative at week 78 in CD-1 strain of mouse**	**See annex conf.52**
	850 (97.2)			Positive in CD-1 strain of mouse at 78 weeks	**Negative at week 78 in CD-1 strain of mouse**	**See annex conf. 52**
	900 (133.7)			Positive in CD-1 strain of mouse at 90 day		See annex conf. 50
	1750 (249)	Present on day 14 in male CD-1 strain of mouse	Positive (++) day 3 in CD-1 strain of mouse + day 14 in CD-1 strain of mouse	Positive in CD-1 strain of mouse at 3 and 14 days		Broich (2015)

	5000 (657)			Positive in CD-1 strain of mouse at 78 weeks	**Positive (++) week 78 in CD-1 strain of mouse**	**See annex conf. 52**
	7000 (1049.5)		Positive (++) day 3 in CD-1 strain of mouse Positive day 14 in CD-1 strain of mouse	Positive in CD-1 strain of mouse at 3 & 14 days		Broich (2015)
	7000 (995)			Positive in CD-1 strain of mouse at 90 days		See annex conf. 50
	7000 (1050)	Positive (++) day 14 in male CD-1 strain of mouse	Positive day 14 in CD-1 strain of mouse			Vardy A (2015a)
	7000 (1324-1636)	Positive (++) day 7 in male C57BL/6 and C57BL/6 (PPARα KO) strains of mouse	Positive (++) day 7 in C57BL/6 strain of mouse Positive day 7 in C57BL/6 (PPARα KO) strain of mouse	Positive in CD-1 strain of mouse at 7 & 14 days		Vardy A (2015b)

***** *Key event 3 is defined as the formation of carcinomas. However, with increased utilisation of a potentially adverse pathway from altered, hyperplastic, hepatic, foci, to the subsequent development of benign and, ultimately, malignant hepatocellular neoplasms, it seems reasonable when considering dose-response information for this, final, key event to also consider the increased formation of other late-stage (pre-carcinoma) information. Therefore with respect to dose-response, and in the interests of clarity, the combined incidence of adenomas and carcinomas has been included under key event 3. For reference, the incidence of adenomas, carcinomas, and combined adenomas and carcinomas, are shown above under section 3 .*

Within the main body of the table information in bold relates to the carcinogenicity study with valifenalate in CD-1 mice

Within the above table - represents no response, + represents a positive response and ++ represents a stronger positive response.

5b. Consistency & Specificity – Biological Plausibility

Consistency & Specificity

The database for valifenalate consists of a standard package of regulatory studies, to GLP, and four additional investigative studies aimed at addressing the mode of action and human relevance of the hepatocellular adenomas and carcinomas reported in the 78-week carcinogenicity study for valifenalate in male mice of the CD-1 strain. In female mice a low incidence of hepatocellular adenomas, outside of the historical control range, were also reported (See annex conf. 52). In a parallel carcinogenicity study for valifenalate, in male and female Han Wistar rats, there was no evidence of carcinogenicity (See annex conf. 51).

No adverse effects, reported in any repeat-dose mouse studies, were inconsistent with the proposed mode of action for valifenalate-induced carcinogenicity in mice – that is, there were no adverse effects in these mouse studies that cannot be attributed to the proposed mode of action. Another nuclear receptor, AhR, is not activated by valifenalate in CD-1 male mice (Broich 2015) and this information is consistent with the known structure-activity relationships associated with this receptor, where agonists tend to be relatively large, co-planar aromatic structures (McKinney & Singh, 1981).

Four investigative studies were designed to add information in support of the hypothesis that the tumours induced by valifenalate occur via a mechanism mediated by activation of three nuclear receptors, namely CAR/PXR/PPARα. In terms of dose-response relationship for valifenalate-induced carcinogenicity in mice, evidence from the short-term studies indicates that there is essentially no activation of CAR, PXR or PPARα at the lower dose level of 150ppm (Broich2015). As activation of these receptors is considered to be the initiating event for valifenalate-induced carcinogenicity, it follows that subsequent key events, dependent upon activation of key event 1, should not occur *in vivo*. This is supported by the outcome of the long-term carcinogenicity study where no tumours were reported at this dose level (150ppm). In short-term studies, as the exposure to valifenalate is increased and CAR/PXR/PPARα become co-activated, replicative DNA synthesis, the second key event, becomes measurable from as early as day 3 (Vardy 2015b) . This is the normal time period after initiation of dosing shown by the group of chemicals known to function through activation of these receptors. The long-term consequences of this increase in hepatocellular proliferation is the development of altered, hyperplastic, hepatic, foci, and the subsequent development of benign and, ultimately, malignant hepatocellular neoplasms. The incidence of these effects in male mice, although increased at the intermediate dose-level of 850ppm (considering combined adenomas & carcinomas), is markedly increased as exposure is increased to 5000ppm (considering combined adenomas & carcinomas), the highest dose-level tested in the carcinogenicity study. This is consistent with the analysis of gene expression and enzyme induction where these parameters were higher at the top dose than at the intermediate dose level. The database in female mice suggest that the same mechanism may operate but to a much lesser degree than in males.

In addition to this *in vivo* database in the CD-1 mouse (Broich 2015, Vardy 2015a), the strain used for the carcinogenicity study on valifenalate, some short-term information is available in another wild-type mouse strain (available knockout mouse strain), namely the C57Bl/6 strain (Vardy 2015a, Vardy 2015b). Considering that different strains of mice were fed valifenalate in diet, in different laboratories, some years apart, and with exposure to different concentrations of valifenalate, the results of the two investigative studies are remarkably consistent. In both CD-1 and C57BL/6 strains of mouse, high dietary concentrations clearly lead to co-activation of CAR, PXR and PPARα, (Key event 1) followed by increased replicative DNA synthesis (key event 2).

In conclusion the information generated, following exposure to valifenalate of the CD-1 strain of mouse, for which there are both short and long-term information, coupled with additional short-term mechanistic information in the C57BL/6 and C57BL/6 (PPARα KO) strains, are all consistent with the three key events proposed for the mode of action of tumour formation in valifenalate-exposed mice. Information from the knockout study, in conjunction with other considerations of potential alternative mechanisms (Section 6 of this Annex), provides a high degree of confidence relating to the correlation of the tumourigenic response to the hypothesised mode of action.

Biological Plausibility

The hypothesised mode of action is not unique but has been, and continues to be, a well-studied area of investigative toxicology. Other chemical entities, of which sulfoxaflor is a recent example, have been described as causing an increased incidence of hepatocellular adenomas and carcinomas in rats and/or mice through activation of one or more of the nuclear receptors involved in the biological action of valifenalate. In some instances, where the nuclear receptor activation is predominantly restricted to just the PPARα receptor, it has been shown using PPARα knockout mice that the second key event, increased hepatocellular proliferation, cannot proceed in the absence of activation of the receptor. Similarly with other chemistries, where nuclear receptor activation is predominantly restricted to the CAR/PXR receptors, it has been shown using CAR/PXR knockout mice that the second key event, increased hepatocellular proliferation, cannot proceed in the absence of activation of these receptors. Together, these studies have provided strong evidence that the initiating event for the formation of hepatocellular adenomas and carcinomas in rodents is activation of one or more of these nuclear receptors.

Other detailed studies on later aspects of the development of rodent hepatic neoplasia have shown a direct correlation between the induction of hepatocyte DNA synthesis with the development of altered, hyperplastic, hepatic foci, followed by the development of benign and, ultimately, malignant hepatocellular neoplasms (Holsapple *et al* 2006; Klaunig *et al* 2003, Cohen 2010, Elcombe *et al* 2014). For valifenalate, the second key event has therefore been shown to be increased replicative DNA synthesis which will ultimately lead to the final key step which is formation of malignant hepatocellular neoplasms. Studies with a range of different molecules, generally in hepatocytes from different species, have shown that although this increased replicative DNA synthesis may occur in rodents, it does not occur, or at least occurs to a much lesser degree, in humans (Gonzalez 1997; Marsman et al 1988; Maronpot 2010; Elcombe et al 2014).

The hypothesised mode of action, initiated by the activation of nuclear receptors CAR/PXR/PPARα is not unique to valifenalate and has been well studied by many researchers (Luisier et al 2014; Lake et al 2015; Hirose et al 2009).

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Summary of Consistency, Specificity and Biological Plausibility of the Hypothesised Mode of Action

	Key Event 1 ***Co—activation of CAR/PXR/PPARα***	***Key Event 2*** ***Increased hepatocellular proliferation***	***Key Event 3*** ***Formation of Carcinoma***
Consistency & Specificity	Significant evidence in short-term studies for activation of receptors from the 'fingerprint' of the induction of both gene expression and enzyme activities indicating activation of CAR/PXR/PPARα in the species and strain in which hepatocellular adenomas/carcinomas were reported. At dose-levels at which activation is absent in short-term studies, no tumours were reported in long-term studies. These short-term effects were present in another mouse strain (C57BL/6) used in investigative studies.	Increased replicative DNA synthesis was dependent on dose and time. Present in short-term studies at dose levels where tumours were reported in long-term studies, and absent in short-term studies at non-carcinogenic dose levels in male CD-1 mice. In PPARα KO mice the extent of replicative DNA synthesis was reduced relative to the wild type strain. Residual proliferative activity was expected, due to the activation of CAR/PXR, in the absence of PPARα.	There have been two guideline carcinogenicity studies with valifenalate: rats and mice. There was no evidence of carcinogenicity in rats.
Biological Plausibility	The role of nuclear receptor activation in the formation, in rodents, of hepatocellular adenomas and carcinomas has been much studied and is now a well-accepted mode of action. The relevance of activation of CAR/PXR/PPARα in these rodent findings to potential adverse human health continues to be investigated but is currently considered to be non-relevant for the carcinogenic outcome.	Detailed studies, reported in the scientific literature, on the development of rodent hepatic neoplasia have shown that induction of hepatocyte DNA synthesis is a critical precursor event in the development of hepatocellular adenomas and carcinomas. Such events may not be purely chemical-specific but occur after differing initiating events including cytotoxicity and nuclear receptor activation. Induction of hepatic DNA synthesis is a mode of action of relevance to the production of human hepatic cancer.	Following the induction of hepatic replicative DNA synthesis (2nd key event), the development of altered, hyperplastic, hepatic foci, and the subsequent development of benign and, ultimately, malignant hepatocellular neoplasms is a well-accepted mode of action for the formation of hepatocellular adenomas and carcinomas in rodents with potential relevance to humans.

5c. Qualitative and Quantitative human concordance

Key Event (name)	*(Evidence in Experimental Species)*	*(Evidence in Humans)*	*Quantitative Species Concordance (experimental species and humans)*	*Quantitative Dose Response*	*Confidence/ Uncertainty*
Key Event 1 ***Nuclear receptor activation***	Receptor activation (CAR/PXR/PPARα) confirmed in CD-1 mice through quantification of associated events. The 'fingerprint' of the induction profile of both Cyp mRNA and enzyme activity is consistent with the above receptors being activated in CD-1 mice treated with valifenalate. This key event was also confirmed in two other strains (C57BL/6 and C57BL/6 KO strains) used in the investigative studies. From the breadth of data generated in mice, this is confirmed as the initiating event of the hypothesised mode of action.	No direct evidence in humans for valifenalate. However, from the study of other chemistries, it is generally accepted that these nuclear receptors have the potential to be activated in humans. Experimental evidence would suggest that activation of these receptors can induce the hypertrophic but not the replicative hyperplastic response critical to the subsequent development of liver cancer.	There is no valifenalate-specific data in humans. For other compounds that activate the CAR/PXR/ PPARα receptors there are clear dose response relationships to receptor activation in animal and human hepatocyte studies in vitro whereby threshold doses exist below which nuclear receptor activation will not occur.	The dose-response for receptor activation in mice has been ascertained in short-term studies. This activation, correlates well with effect and no-effect information from the carcinogenicity study in the same mouse strain. This evidenced-based information for valifenalate is entirely consistent with the initiating event of the hypothesised mode of action.	The correlation between markers for this initiating event in short term studies and the final key event, derived from the carcinogenicity study in CD-1 mice, is well-founded. The data generated in short-term studies to confirm receptor activation derives from well-accepted associated events. There is no direct information on valifenalate relating to this key event in humans but it is assumed to be plausible by comparison with other chemicals that activate the three nuclear receptors. Overall, there is a high degree of confidence in this information in mice and an assumption of plausibility in humans.

Key Event 2 ***Increased hepatocellular proliferation***	Increased replicative DNA synthesis has been measured, directly, in short term studies in the CD-1 strain of mice. This key event was also confirmed in two other strains (C57BL/6 and C57BL/6 (PPARα KO) strains) used in the investigative studies.	No direct evidence in humans for valifenalate. For other CAR/PXR and PPARα agonists *in vitro* and *in vivo* evidence has shown the hypertrophic but not the hyperplastic consequences of nuclear receptor interaction	From experimental animal studies on Wy-14,643 and phenobarbital (and including human use of phenobarbital) and other chemistries (some in hepatocyte cultures), it is generally accepted that this key event is either not activated in humans, or at least activated to a significantly less extent in human cells in vitro.	The dose-response for the induction of replicative DNA synthesis in mice has been ascertained in short-term studies. This activation, (as for key event 1) correlates well with effect and no-effect information from the carcinogenicity study in the same mouse strain (i.e. the third key event). In contrast, it is generally accepted that this key event is either not activated in humans, or activated to a significantly less extent in humans	The correlation between this key event in short and long term studies in CD-1 mice is well-founded based on experimental evidence. There is no direct information for valifenalate relating to equivalent events in humans, *in vitro or in vivo*. Indirect evidence, relating to experimental investigations with other chemistries, with a similar mode of action in humans, has been used. Overall there is a high degree of confidence from evidence-based information in mice. In the absence of direct experimental evidence for valifenalate in humans there is high degree of confidence, based on evidence from other chemistries with a similar mode of action
Key Event 3 ***Formation of hepatocellular carcinomas***	Carcinogenicity studies in rats and mice reported valifenalate to be carcinogenic in	No evidence in humans for valifenalate.	There is no valifenalate-specific data in humans. Experimental data with other chemicals that	The incidence of hepatocellular adenoma and carcinoma in males was dose-related with	The guideline carcinogenicity study was well executed and reported.

	mice, particularly males. There was no evidence of carcinogenicity in rats.		work through activation of these nuclear receptors where the human orthologue of the respective nuclear receptor had been inserted into the mouse genome has shown that while the hypertrophic response continues to be seen, the hyperplastic, DNA replicative response, seen with the intact rodent orthologue of the nuclear receptors, is missing from those mice given the human receptors. This data strongly suggests that such a nuclear receptor mode of action (CAR/PXR/PPARα) is unlikely to be relevant to humans	increased tumour incidences at the top and middle dose-levels. For males exposed to valifenalate at the lower dose-level, there was no increased incidence over control. Female animals had a small but statistically significant increased incidence of adenomas. In contrast, it is generally accepted that such a nuclear receptor mode of action (CAR/PXR/ PPARα) is unlikely to be relevant to humans	The absence of tumours at the exposure level correlates well with the absence of key events 1 and 2 from short-term studies. Overall, there is a high degree of confidence in this evidence-based information in experimental animals. In the absence of direct experimental evidence for valifenalate in humans there is medium degree of confidence, based on evidence from other chemistries with a similar mode of action

The relevance to humans of valifenalate-mediated hepatocarcinogenesis in CD-1 mice

Overall, a mixed phenobarbital/peroxisome proliferation-type mode of action has been identified. Therefore relevance to humans, of the valifenalate-induced hepatic tumours in mice, has been considered within the context of valifenalate co-activating CAR/PXR and PPARα. For clarity, however, we have considered the relevance to humans of CAR/PXR activation independently from the relevance to humans of activation of the PPARα receptor. Alternative modes of action, such as activation of alternative nuclear receptors, the direct reactivity of valifenalate towards DNA, valifenalate-induced cytotoxicity and/or hormonal perturbation, have also been considered and discounted (see section 6 of this Annex).

The relevance to humans of valifenalate-mediated activation of CAR/PXR

Phenobarbital is typical of rodent hepatocarcinogens that induce tumours by a non-genotoxic mechanism, critically involving liver hyperplasia (Williams and Whysner, 1996). Phenobarbital, like valifenalate, is neither cytotoxic or genotoxic (Whysner *et al*., 1996; IARC, 2001). Induction of some Cyp enzymes, particularly of the Cyp2B family, are diagnostic for phenobarbital and are a consequence of activation of nuclear receptors, particularly CAR but also PXR and to a lesser extent PPARα. Phenobarbital also induces Cyp enzymes in human liver, although there are reports that it may act more through PXR than through CAR (Moore *et al*., 2003). It is this activation of CAR that is generally viewed as the first key event of phenobarbital-mediated hepatic tumours in rodents, and this event has been reported to occur in both rodents and humans (Yamada et al 2014; Lynch et al 2014; Elcombe et al 2014; Lake et al 2015).

There are some human studies that may shed light on the second key step, the increased replicative DNA synthesis. It has been reported using human hepatocytes that unlike rodent hepatocytes, these human cells are refractory to increased replicative DNA synthesis when exposed to phenobarbital (Parzefall *et al*., 1991; Hasmall & Roberts, 1999, Elcombe *et al*, 2014). Perhaps more importantly, there are clinical data on phenobarbital, where the substance has been used in patients for many years, exposed to plasma concentrations similar to those following a carcinogenic dose in rodents, and yet there is no evidence of a hepatocarcinogenic effect (IARC, 2001; Lamminpaa *et al*., 2002). Taken together this evidence indicates that, at least on a quantitative basis, taking into account kinetic and dynamic factors, that the phenobarbital-mediated (CAR/PXR-mediated) hepatocarcinogenicity, expressed in rodents, is not likely to be expressed in humans.

A study was designed to directly investigate the potential of valifenalate to activate CAR/PXR and/or PPARα nuclear receptors and stimulate cell proliferation, in isolated hepatocytes prepared from male CD-1 mice. Phenobarbital and Wy-14,643 were included as positive controls (Vardy 2015c).

An initial cytotoxicity assay (assessing ATP levels) was performed with valifenalate. Depletion of ATP levels, and therefore cytotoxicity, was measured at 300 μM making this a suitable top concentration to be assessed in the main enzyme induction and proliferation study.

Neither phenobarbital nor valifenalate had any impact on replicative DNA synthesis in the male CD-1 mouse hepatocytes *in vitro*. Phenobarbital has been reported to show a weak effect on mouse hepatocyte proliferation in vitro. However, Wy-14,643 increased replicative DNA synthesis by a maximum of 1.7-fold, and epidermal growth factor (EGF) also produced a robust response, demonstrating that the test system could respond to a proliferative stimulus.

In male CD-1 mouse hepatocytes phenobarbital induced Cyp2b10, as measured by Taqman® mRNA analysis and PROD activity, but as expected had little impact on the Cyp4a or peroxisome proliferation markers. Conversely, Wy-14,643 induced Cyp4a and peroxisome proliferation as measured by Taqman® mRNA analysis (Cyp4a10, Cyp4a14 and Acox1), 12-OH LA formation and PCoA oxidation. Valifenalate had essentially no impact on any of the biochemical markers assessed. It may be reasonable to consider that metabolism of valifenalate, and the production of a proximate metabolite is responsible for activation of CAR/PXR and PPARα, and that the hepatocyte culture system is incapable of producing the quantity(s) of the metabolite(s) necessary to co-activate CAR/PXR and PPARα. Evidence for this scenario comes from a comparison of the valifenalate-induced induction of the associated mRNAs and Cyp isozymes induced *in vivo*, but the absence, in this mouse (CD-1 strain) hepatocyte culture system, *in vitro*. Unfortunately these factors preclude a study to define a valifenalate-specific lack of induction of replicative DNA synthesis in human hepatocytes.

Despite the clear technical difficulties in assessing replicative DNA synthesis with valifenalate in cultured hepatocytes (Vardy 2015c), there is much information within the open literature that clearly identifies the relevance to humans of a phenobarbital-type mode of action in rodents, in the absence of other alternative mechanisms known to have potential relevance to humans.

The relevance to humans of valifenalate-mediated activation of PPARα

With valifenalate, as with many other substances, the finding of hepatic peroxisome proliferation is highly specific, and indicates that PPARα activation played a role in the initial stages of this mode of action. However, as discussed earlier, well-established indirect measures of PPARα activation were reported not only in the wild type CD-1 and C57BL/6 strains, but also in the C57BL/6 (PPARα knockout) strain.

The PPARα mode of action is a well- accepted, mode of action for the induction of liver tumours in rodents, and their carcinogenic non-relevance to humans is also scientifically well established (Klaunig *et al*., 2003).

The data available for valifenalate provide a high level of confidence that this mode of action is operative in male CD-1 and male C57BL/6 strains of mouse, in addition to activation of CAR/PXR.

The human relevance of the PPARα mode of action *in toto* may not as yet be reasonably excluded simply on the basis of fundamental qualitative differences in key events between animals and humans. Indeed the very basis for the use of fibrates as hypolipidemic agents in man is the activation of PPARα to produce some of the desired features of the pleiotropic responses shown by these chemicals, namely their hypolipidaemic and hypocholesterolaemic effects. Therefore, in the absence of further species-specific mechanistic data, it is relevant to consider a quantitative difference in kinetic or dynamic factors between animals and humans, rather than qualitative species difference in the response for this mode of action.

For PPARα agonists, the question of human relevance (amongst others) was the subject of a thorough review (Klaunig *et al*., 2003). The authors concluded that, after taking kinetic and dynamic factors into consideration, the overall weight of evidence suggested that the rodent mode of action for PPARα activator-induced liver tumours was not likely to occur in humans. Since this critical review by Klaunig *et al* (2003), many studies have been published on the subject of the PPARα mode of action, for example Lai, (2004), Ren *et al* (2010), Guyton *et al* (2009). A group of experts, aware of the increased understanding of the relationships between PPARα activation and liver cancer formation,

critically re-evaluated the state of the science for the induction of liver tumours in rodents (Corton *et al* 2014). The key events of the mode of action for PPARα, and the ultimate conclusions arrived at by this workshop remained consistent with the earlier publication of Klaunig *et al* in 2003.

The weight of evidence indicates that valifenalate acts as a PPARα agonist and that after taking kinetic and dynamic factors into consideration, any hepatocellular carcinomas developed through activation of this nuclear receptor by valifenalate in mice, is not likely to occur in humans.

The relevance to humans of valifenalate-mediated activation of CAR/PXR/PPARα

The evidence indicates that valifenalate acts as a co-activator of CXR/PXR and PPARα and, after taking kinetic and dynamic factors, such as the differential expression of hyperplastic and hypertrophic responses into account, any hepatocellular carcinomas developed through activation of these nuclear receptors by valifenalate in mice, is not likely to occur in humans.

6. Other potential Modes of Action

Several other modes of action have been associated with rodent hepatic carcinogenesis (Cohen 2010 & Klaunig *et al*., 2012). Such mechanisms can be subdivided into those that involve a direct interaction with DNA (so called genotoxic interactions), and those that do not. Examples of non-genotoxic mechanisms, that work in the absence of such DNA-reactivity include hepatic cytotoxicity, oestrogen-mediated, and receptor mediated mechanisms, such as those involving AhR, CAR, PXR and PPARα.

6.1 AhR-mediated carcinogenesis:

For valifenalate, an Ahr-mediated mode of action for the formation of adenomas and carcinomas in CD-1 mice can be ruled out since neither gene expression nor enzyme activity of hepatic Cyp1a was induced in CD-1 mice exposed to valifenalate at any dose level. Therefore AhR is not activated at dose levels where valifenalate induces hepatocellular carcinomas, and does not induce these effects, in male CD-1 mouse (Broich 2015).

6.2 Direct reactivity with DNA:

It has been clearly established from a panel of guideline *in vitro* and *in vivo* studies that valifenalate is not genotoxic (CLH Report Section 10.8) and hence such a mode of action is not relevant to valifenalate.

6.3 Oestrogen-mediated:

Although oestrogens have a receptor-mediated mode of action that includes hepatocellular proliferation, it has been proposed that this may be due to the formation of DNA adducts and the subsequent increased cell proliferation that occurs during regenerative hyperplasia (Cohen 2010). Clearly there is no structural similarity between valifenalate and oestrogen that might suggest a similar mode of action of hepatocarcinogenesis and there was no evidence of oestrogenic activity in the guideline two-generation toxicity study in the rat (CLH Report Section 10.10).

6.4 Cytotoxicity-mediated:

Many short and medium term oral mouse studies have been conducted using dietary incorporation of valifenalate. This includes some short-term studies conducted at very high levels of valifenalate exposure, up to about 1500mg/kg bw in some studies/strains of mouse. In most studies, ALP, AST

and ALT were measured as markers of hepatic necrosis in addition to standard histopathology. Throughout all these studies on valifenalate in the target species, mouse, there was no evidence of any significant perturbation of the measured plasma enzymes and no histopathological change that might indicate hepatic necrosis or cytotoxicity at any of the dose levels to which mice were exposed. Overall, the evidence in the mouse does not support a mechanism involving sustained cytotoxicity / repair / proliferation eventually leading to the formation of hepatocellular carcinomas.

Feeding studies were conducted in the rat of 28 or 90 day duration at estimated dose levels up to 1500 and 1000 mg/kg/day respectively (See annex conf. 48,49). In the 28 day study high serum aspartate aminotransferase levels were measured in the females only given the top dose level (15,000 ppm) but since there was no effect on liver weight or liver histopathology, the exact source of the enzyme was not determined. The 90 day study showed slight increases in liver weight at the top dose level of 1000 mg/kg/day only, but there was no evidence of hepatic cytotoxicity either by histopathology, or by changes in clinical chemistry parameters. All findings that were attributable to treatment on this study were completely reversed during the recovery period of four weeks after cessation of treatment with valifenalate. A 104 week carcinogenicity study, with a 12 month chronic toxicity arm, was conducted in the Han Wistar rat at dose levels up to 1000 mg/kg/day of valifenalate (See annex conf 51). There were no neoplastic effects of valifenalate in this study and no clinical chemistry or histopathology changes indicative of hepatic toxicity. Liver weights were increased at week 52 and 104 in rats of both sexes receiving 1000 mg/kg/day but there was no hepatic histopathology accompanying the liver weight increases.

Studies were conducted in the dog where valifenalate was dosed in capsules daily for 28 days at dose levels up to 1000 mg/kg/day (See annex conf. 7). Increased serum alanine aminotransferase, alkaline phosphatase and γ-glutamyltransferase and decreased serum total protein, cholesterol and albumin levels achieved statistical significance at the top dose level of 1000 mg/kg/day only in both sexes. At necropsy, liver weights were increased at all dose groups in both sexes and histologically hepatocellular hypertrophy, together with eosinophilic cytoplasmic inclusions were present in dogs at all dose levels. In addition single cell hepatocyte necrosis was present at the 1000 mg/kg/day dose level. The report concluded a NOAEL of 500 mg/kg/day.

In a 90 day study valifenalate was given in capsules to dogs at dose levels up to 750 mg/kg/day (See annex conf. 12). Higher group mean serum alkaline phosphatase levels were observed in both sexes at both weeks 6 and 13 of the study at all dose levels of valifenalate, higher alanine aminotransferase was present at weeks 6 and 13 in both sexes receiving 750 mg/kg/day and in females only receiving 250 mg/kg/day at week 13. Higher serum γ-glutamyltransferase was present in both sexes at the 6 and 13 week time point in animals receiving 750 mg/kg/day and at week 13 only for dogs receiving 250 mg/kg/day. These changes were accompanied by decreases in mean plasma cholesterol, total protein and albumin indicating hepatic effects of valifenalate at the dose levels applied in the study without a NOEL. Higher mean liver to body weight ratio was shown for both sexes at the 250 and 750 mg/kg/day dose level and for females only given the bottom dose level of 50 mg/kg/day. Histopathology showed hepatocyte hypertrophy in all dogs given 750 mg/kg/day valifenalate and eosinophilic cytoplasmic hepatocellular inclusions in dogs of both sexes given 250 and 750 mg/kg/day with a NOEL for the change at 50 mg/kg/day. The study concluded that the 250 mg/kg/day dose level was a NOAEL.

A one year dog study was conducted using oral capsules at dose levels up to 250 mg/kg/day (Saunders 2005). Higher group mean serum alkaline phosphatase levels were present from week 6 for both sexes receiving 250 mg/kg/day and from week 13 for dogs receiving 50 mg/kg/day. These levels

were significantly reduced following an 8 week recovery period following cessation of dosing with valifenalate. Alkaline phosphatase levels were also increased from weeks 39 and at week 52 for both sexes at the bottom dose level of 1 mg/kg/day. Higher group mean serum γ-glutamyltransferase levels were also observed from week 13 for both sexes receiving 250 mg/kg/day but these levels returned to normal by week 4 of the recovery period. These changes were accompanied by decreased total protein and serum albumin from week 6 onwards in both sexes given 250 mg/kg/day which also showed complete recovery by week 4 following cessation of valifenalate dosing. Higher group mean absolute liver weights were present at all dose levels and higher liver weights relative to body weights were present at the top dose of 250 mg/kg/day dose level only. Hepatic hypertrophy was present in all treated groups and was fully reversible in dogs following an 8 week recovery period. The study concluded a NOAEL of 50 mg/kg/day.

These data suggest that valifenalate can induce hepatocellular cytotoxicity in the dog at high dose levels of administration.

In conclusion, cytotoxicity has been associated with exposure to high levels of valifenalate in (oral, capsule) dog studies, however these effects were not evident after a recovery period. There is no indication of carcinogenic potential in the dog. With respect to cytotoxicity as an alternative mechanism for carcinogenicity in the mouse, all relevant information from rat, mouse and dog studies is entirely consistent with the hypothesised mode of action of carcinogenicity in mice and clearly indicates that cytotoxicity is unlikely to play a role in the carcinogenicity of valifenalate in CD-1 mice.

6.5 Iprovalicarb – a chemically related structure:

At first sight, the chemical structures of iprovalicarb and valifenalate may appear to share some similarity, however the differences in their chemical structures is sufficient to produce a very different mammalian toxicological profile for each molecule, ruling out any consideration of read-across of the toxicological profile, and hence a mechanism of toxicity in mammals, from one molecule to the other.

7. Uncertainties/Inconsistencies and Identification of Data Gaps

There are other chemical entities that have been described as causing an increased incidence of hepatocellular adenomas and carcinomas in rats or mice through activation of these receptors. In some instances, where the nuclear receptor activation is more prominently restricted to just the PPARα receptor, it has been shown using PPARα knockout mice that the second key event, increased hepatocellular proliferation, cannot proceed (Corton *et al* 2014). Similarly with other chemistries, where nuclear receptor activation is predominately restricted to the CAR/PXR receptor, it has been shown using CAR/PXR knockout mice that the second key event, increased hepatocellular proliferation, cannot proceed (LeBaron *et al 2014*). There is no doubt that there are other substances where all three receptors CAR/PXR/PPARα are co-activated, as for valifenalate, but as yet there is no reliable 'triple' knockout mouse strain available to clearly show that the second key event cannot proceed. Although this may appear to introduce some degree of uncertainty, there is good evidence, with respect to the mode of action, from dose-response relationships for valifenalate in male CD-1 mice that in the absence of the initiating event (activation of CAR/PXR/PPARα), the second key event, induction of replicative DNA synthesis, does not occur. Investigative studies with valifenalate in C57BL/6 and C57BL/6 (PPARα KO) strains have indicated that replicative DNA synthesis is significantly lower in the KO strain than in the wild-type, as might be expected when CAR/PXR may still be activated. It is considered that these data in conjunction with the lack of evidence, from the detailed analysis of other potential mechanisms, for an alternative mode of action for the formation of the hepatocellular carcinomas serve to increase the degree of certainty for this mode of action for valifenalate-induced liver tumours in CD-1 mice.

A comparison of *in vivo* and *in vitro* studies in CD-1 mice suggests that, in hepatocyte cultures, metabolism of valifenalate does not occur in sufficient quantities for activation of CAR/PXR/PPARα, therefore precluding induction of gene expression and enzyme induction and the subsequent key event, increased S-phase. In such a situation there is little value in using human hepatocyte studies to identify if the same receptors are activated and, in addition if the second key step, induced replicative DNA synthesis, occurs.

Human use of phenobarbital offers a valuable opportunity, on account of the extensive data available from its clinical use, relevant to this assessment. The mode of action for phenobarbital-like P450 inducers was determined to be unlikely in humans after kinetic and dynamic factors were considered (Holsapple *et al* 2006, Elcombe *et al* 2014). The weight of evidence from this assessment indicates that valifenalate is such a phenobarbital-like P450 inducer, albeit perhaps one with a somewhat increased induction of PPARα. However, PPARα induced rodent hepatocarcinogenesis has also been considered as not relevant to humans, on a quantitative basis.

8. Conclusions in relation to the human relevance of valifenalate-induced hepatocellular carcinomas and adenomas in CD-1 mice.

The key events of the mode of action for the formation of hepatocellular carcinomas induced by valifenalate in male CD-1 mice are summarised below.

1. The initiating event is **nuclear receptor activation**, in particular, co-activation of the nuclear receptors **CAR, PXR and PPARα**.
2. The second key event is **increased replicative DNA synthesis**.
3. The third key event is the formation of **hepatocellular carcinomas** (via the development of altered, hyperplastic, hepatic foci, and the subsequent development of benign and, ultimately, malignant hepatocellular neoplasms)

On the basis of quantitative differences in kinetic and/or dynamic factors between experimental animals and humans, the weight of evidence indicates that this mode of action is unlikely in humans.

TABLES

Table A1 (see also Annex I Tables 3.9.4.2-05 & 3.9.4.1-06): Gene expression and enzyme induction profiles following dietary exposure of mice to high concentrations of valifenalate in CD-1 mice for 14 days (Broich, 2015)

Group/sex	**1/M**	**2/M**	**3/M**	**4/M**	**5/M**
Dose (ppm)	**0**	**150**	**1750**	**7000**	**850**
Substance	**-**	**Val~**	**Val~**	**Val~**	**PB~~**
Number	**6**	**6**	**6**	**6**	**6**
Gene Expression					
Cyp1a1 mRNA	1.00	0.81	1.22	1.19	3.58
Cyp1a2 mRNA	1.00	0.68	0.83	0.31	2.96
Cyp2b10 mRNA	1.00	1.57	6.20	20.13	223.0
Cyp3a11 mRNA	1.00	1.09	6.08	9.54	12.12
Enzyme Activity					
MROD (Cyp 1a2)	100	108	126	118	312**
PROD (Cyp2b1)	100	79.5	165**	266**	1916**
6β-TOH (Cyp 3a)	100	93.1	150**	267**	420**
LA11OH (Cyp2e1)	100	101	206	384**	370**
LA12OH (Cyp4a1)	100	117	408**	1106**	214**
pCoA (Peroxisomal β-oxidation)	100	137	208**	308**	30**

** p <0.01
Val~=Valifenalate
PB~~=phenobarbital
MROD= 7-methoxyresorufin O-dealkylation
PROD=7-pentoxy-resorufin O-dealkylation
6β-TOH=testosterone 6β-hydroxylation
LA11OH=lauric acid 11-hydroxylation
LA12OH=lauric acid 12-hydroxylation
pCoA=palmitoyl CoA oxidation

Table A2 (See also Annex I Table 3.9.4.3-09): Gene expression and enzyme induction profiles following dietary exposure of mice to high concentrations of valifenalate in C57BL/6 mice (Vardy, 2015b)

Treatment	Control (0 ppm)	Valifenalate (7000 ppm)
Gene Expression		
Cyp2b10	1.0	55.59**
Cyp3a11	1.0	6.26**
Cyp4a10	1.0	5.89**
Cyp4a14	1.0	151.59**
Acox1	1.0	1.79**
Enzyme Activity		
PROD (Cyp2b1)	2.23 ± 0.58 (100.0 ± 25.8)	13.45 ± 3.34*** (603.5 ± 149.9)
BQ (Cyp 3a)	2.06 ± 0.55 (100.0 ± 26.9)	5.02 ± 0.43*** (244.0 ± 21.0)
LAH (Cyp4a1)	2.74 ± 0.95 (100.0 ± 34.7)	21.22 ± 3.12*** (774.6 ± 113.8)
PCoA (Peroxisomal β-oxidation)	17.03 ± 3.36[a] (100.00 ± 19.75)	34.49 ± 2.71*** (202.49 ± 15.91)

[a] Values are Mean ± SD (n=10 per group). Results are expressed as fold change relative to control, where control values were normalised to 1.00. Murine β-actin was employed as the internal control. A Student's t-test was performed on the results; *** statistically different from control $p<0.001$

Table A3 (See also Annex I Table 3.9.4.3-05): Bodyweights, bodyweight gains, liver weights and liver/bodyweight ratios from male wild-type and PPARα KO mice fed control diet and diet containing 7,000 ppm valifenalate for 14 days (Vardy, 2015b)

Parameter	WT mice Control (0 ppm)	WT mice valifenalate (7000 ppm)	PPARα KO mice Control (0 ppm)	PPARα KO mice valifenalate (7000 ppm)
Day 1 Body weight (g)	23.18 ± 0.65[a] (100.00 ± 2.81)	22.28 ± 0.59** (96.12 ± 2.53)	21.71 ± 1.47 (100.00 ± 6.77)	23.37 ± 1.74* (107.65 ± 8.01)
Terminal Body weight (g)	24.83 ± 1.11 (100.00 ± 4.46)	24.42 ± 1.20 (98.32 ± 4.84)	24.38 ± 1.55 (100.00 ± 6.35)	24.80 ± 1.60 (101.73 ± 6.57)
Body weight gain (g)	1.65 ± 1.01 (100.00 ± 60.95)	2.14 ± 0.89 (129.32 ± 54.03)	2.67 ± 1.37 (100.00 ± 51.36)	1.43 ± 0.81* (53.61 ± 30.37)
Liver Weight (g)	1.32 ± 0.06 (100.00 ± 4.54)	1.55 ± 0.15*** (116.95 ± 11.22)	1.29 ± 0.08 (100.00 ± 5.90)	1.45 ± 0.11** (112.42 ± 8.67)
Liver/Body weight ratio	5.33 ± 0.20 (100.00 ± 3.75)	6.33 ± 0.36*** (118.70 ± 6.73)	5.28 ± 0.15 (100.00 ± 2.90)	5.83 ± 0.21*** (110.42 ± 3.95)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain or control (n=10 per group). A Student's t-test was performed on the results; * statistically different from control p<0.05; ** p<0.01; *** p<0.001

Table A4 (See also Annex I Table 3.9.4.3-09):: Gene expression and enzyme induction profiles for male WT and PPARα KO mice administered valifenalate *via* the diet for 14 days (Vardy, 2015b)

Strain	WT		PPARα KO	
Treatment	**Control (0 ppm)**	**Valifenalate (7000 ppm)**	**Control (0 ppm)**	**Valifenalate (7000 ppm)**
Gene Expression				
Cyp2b10	1.0	55.59**	1.0	48.09
Cyp3a11	1.0	6.26**	1.0	8.49
Cyp4a10	1.0	5.89**	1.0	68.80
Cyp4a14	1.0	15.49**	1.0	151.59
Acox1	1.0	1.79**	1.0	1.34
Enzyme Activity				
PCoA (Peroxisomal β-oxidation)	17.03 ± 3.36[a] (100.00 ± 19.75)	34.49 ± 2.71*** (202.49 ± 15.91)	11.71 ± 0.86 (100.00 ± 7.38)	15.70 ± 1.76*** (134.12 ± 15.06)
PROD (Cyp2b1)	2.23 ± 0.58 (100.0 ± 25.8)	13.45 ± 3.34*** (603.5 ± 149.9)	2.70 ± 0.37 (100.0 ± 13.8)	19.10 ± 2.59*** (706.7 ± 95.9)
BQ (Cyp 3a)	2.06 ± 0.55 (100.0 ± 26.9)	5.02 ± 0.43*** (244.0 ± 21.0)	2.43 ± 0.33 (100.0 ± 13.4)	5.85 ± 0.52*** (240.6 ± 21.2)
LAH (Cyp4a1)	2.74 ± 0.95 (100.0 ± 34.7)	21.22 ± 3.12*** (774.6 ± 113.8)	1.02 ± 0.14 (100.0 ± 13.6)	4.09 ± 1.24*** (399.9 ± 121.4)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain (n=10 per group). A Student's t-test was performed on the results; *** statistically different from control $p<0.001$, ** $p<0.01$

Table A5 (See also Annex I Table 3.9.4.1-10): Replicative DNA Synthesis in CD-1 mice fed valifenalate in diet for 3 or 14 days (Broich, 2015)

Group/sex		**1/M**	**2/M**	**3/M**	**4/M**	**5/M**
Dose (ppm)		**0**	**150**	**1750**	**7000**	**850**
Substance		**-**	**Val~**	**Val~**	**Val~**	**PB~~**
Proliferating Hepatocytes (3 days)	**Number**	**6**	**6**	**6**	**6**	**6**
	Mean	18	34	65	69	164
	SD	14	16	43	12	57
	Min	5	15	19	49	112
	Max	39	62	143	86	256
Proliferating Hepatocytes (14 days)	**Number**	**6**	**6**	**6**	**6**	**6**
	Mean	15	37	51	60	80
	SD	9	17	31	30	28
	Min	5	10	8	13	42
	Max	25	55	101	97	124

Table A6 (See also Annex I Table 3.9.4.3-07): Replicative DNA synthesis in male WT and PPARα KO mice administered valifenalate *via* the diet for 14 days (Vardy, 2015b)

Strain	Day	Control 0 ppm	Valifenalate 7000 ppm
WT	7	1.90 ± 0.30[a] (100.00 ± 15.59)	15.46 ± 2.85***[#] (815.4 ± 150.3)
PPARα KO		0.79 ± 0.15 (100.00 ± 18.66)	4.24 ± 2.18*** (536.83 ± 276.28)
WT	14	2.66 ± 1.08 (100.00 ± 40.58)	9.42 ± 3.57*** (354.72 ± 134.53)
PPARα KO		1.68 ± 0.67 (100.00 ± 39.88)	3.14 ± 0.93*** (186.89 ± 55.35)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain (n=10 per group; [#] n=9 as mouse #29 was considered an outlier). A Student's t-test was performed on the results; *** statistically different from control p<0.001

Table A7 (See also Annex I Table 3.9.1.2-05): Neoplastic findings and historical control data in CD-1 mice treated with valifenalate for 78 weeks (See annex conf. 52).

Group/sex		**1/M**	**2/M**	**3/M**	**4/M**	**Historical Range (%)**	**1/F**	**2/F**	**3/F**	**4/F**	**Historical Range (%)**
Dose (ppm)		**0**	**150**	**850**	**5000**		**0**	**150**	**850**	**5000**	
Liver	**Hepatocellular adenoma**	7	2	14	16*	7.8 – 21.2	0	0	2	5*	0.0 – 1.9
	Hepatocellular carcinoma	2	4	4	10*	1.9 – 8.0	0	1	0	0	0.0
	Combined adenomas & carcinomas	9	6	18	26		0	1	2	5	
Number of animals examined		50	50	50	50	307	50	50	50	50	307

[*] $p < 0.05$ when compared to Group 1

Hepatocellular tumours are relatively common in male CD-1 mice, however the incidence of these tumours in males and females given 850 and 5000 ppm exceeded the background range seen in similarly performed studies at the performing laboratories.

Table A8 (See also Annex I Table) Non-neoplastic findings in the liver of valifenalate-treated CD-1 mice (78-week study):

Group distribution and severity of treatment related liver findings (See annex conf. 52).

Group/sex		**1/M**	**2/M**	**3/M**	**4/M**	**1/F**	**2/F**	**3/F**	**4/F**
Dose (ppm)		**0**	**150**	**850**	**5000**	**0**	**150**	**850**	**5000**
Centrilobular hepatocyte hypertrophy	**Total**	**21**	**34***	**26**	**8****	**8**	**9**	**12**	**25****
	Slight	19	21	18	5	8	8	12	22
	Moderate	2	13	8	2	0	1	0	3
	Marked	0	0	0	1	0	0	0	0
Generalised hepatocyte hypertrophy	**Total**	**3**	**6**	**13***	**29****	**2**	**2**	**7**	**5**
	Slight	3	5	10	18	2	2	6	5
	Moderate	0	1	3	11	0	0	1	0
Centrilobular hepatocyte vacuolation	**Total**	**11**	**14**	**33****	**32****	**2**	**8**	**8**	**2**
	Minimal	3	4	2	0	2	1	4	1
	Slight	7	7	20	11	0	7	4	0
	Moderate	1	3	11	20	0	0	0	1
	Marked	0	0	0	1	0	0	0	0
Increased hepatocytic cytoplasmic eosinophilia		**0**	**1**	**1**	**29****	**1**	**0**	**0**	**6**
Pigment in macrophages		**1**	**2**	**4**	**12****	**12**	**20**	**13**	**31****
Pigment in hepatocytes		**1**	**0**	**0**	**18****	**0**	**0**	**3**	**13****
Eosinophilic foci		**1**	**0**	**1**	**1**	**0**	**1**	**1**	**4**
Number of animal examined		**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**

* $p<0.05$; ** $p<0.01$ when compared to Group 1(Fisher's Exact Test)

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