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Malarial PI4K inhibitor induced diaphragmatic hernias in rat: Potential link with mammalian kinase inhibition

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Abstract

Background: MMV390048 is an aminopyridine *plasmodial* PI4K inhibitor, selected as a *Plasmodium* blood-stage schizonticide for a next generation of malaria treatments to overcome resistance to current therapies. MMV390048 showed an acceptable preclinical safety profile and progressed up to Phase 2a clinical trials. However, embryofetal studies revealed adverse developmental toxicity signals, including diaphragmatic hernias and cardiovascular malformations in rats but not rabbits.

Methods: In vivo exposures of free plasma concentrations of compound in rats were assessed in relation to in vitro human kinase inhibition by MMV390048, using the ADP-Glo™ Kinase Assay.

Results: We demonstrate a potential link between the malformations seen in the embryofetal developmental (EFD) studies and inhibition of the mammalian PI4Kβ paralogue, as well as inhibition of the off-target kinases MAP4K4 and MINK1. PI3Kγ may also play a role in the embryofetal toxicity as its in vitro inhibition is covered by in vivo exposure. The exposures in the rabbit embryofetal development studies did not reach concentrations likely to cause PI4K inhibition. Overall, we hypothesize that the in vivo malformations observed could be due to inhibition of the PI4K target in combination with the off-targets, MAP4K4 and MINK1. However, these relationships are by association and not mechanistically proven.

Conclusions: Deciphering if the EFD effects are dependent on PI4K inhibition, and/or via inhibition of other off-target kinases will require the generation of novel, more potent, and more specific PI4K inhibitors.

KEYWORDS

diaphragmatic hernia, EFD toxicity, malaria, MV390048, PI4K

1 | INTRODUCTION

Malaria still inflicts extensive morbidity and mortality in low-middle income countries. Its eradication poses major intertwined challenges at the logistical, political, and scientific levels. The agent of the disease is a unicellular protozoon of the genus *Plasmodium (P.)* that causes the symptoms of malaria by infecting and destroying human erythrocytes. Nearly half of people in the world are at risk of malaria, most of them are in sub-Saharan Africa. Complications and mortality as a result of malaria are most common in children under 5 years of age, pregnant and lactating women, and women of childbearing potential (WoCBP). In 2019, according to World Health Organization, there were an estimated 33 million pregnancies, of which 35% (12 million) were exposed to malaria infection, resulting in 822,000 children with low birth weight (World Health Organization, 2020). Pregnant women are more likely to be bitten (Dobson, 2000) and are more likely to suffer severe consequences of malaria. Pregnancy-associated malaria, caused primarily by infection with *P. falciparum*, results from a massive accumulation of *P. falciparum*-parasitized erythrocytes and monocytes in the intervillous spaces of the placenta. This can cause severe clinical symptoms in the expectant mother (e.g., anemia, cerebral malaria, pulmonary edema, and kidney failure) and trigger the recruitment of inflammatory cells and production of cytokines, which are strongly associated with intrauterine growth retardation and low birth weight in infants (<2,500 g; Umbers et al., 2011), which in turn can lead to child growth retardation and poor cognitive outcomes, as well as being a major risk factor for perinatal, neonatal, and infant mortality (Guyatt & Snow, 2001, 2004; Samak, 2020). Since pregnant, lactating, and nonpregnant WoCBP are one of the most affected populations, a key step for the success of the malaria eradication program is the development of new antimalarial medicines with low probability of developmental toxicity (teratogens), which can be used throughout pregnancy and lactation to reduce maternal and fetal mortality rates.

Several antimalarial candidates have been developed or are currently in development (Burrows et al., 2017). One of these is MMV390048, a new class of antimalarial chemotherapy that selectively inhibits *Plasmodium* phosphatidylinositol-4-kinase (PI4K), required across all *Plasmodium* life cycle stages, except hypnozoites (Ghidelli-Disse et al., 2014; McNamara et al., 2013; Younis et al., 2012). PI4K is an essential enzyme responsible for the generation of specific phosphorylated variants of phosphatidylinositol lipids that are involved in membrane ingression during merozoite formation (Paquet et al., 2017) and are critical for second messenger signaling and cellular membrane remodeling, making *Plasmodium* PI4K an attractive target for antimalarial drug development. Furthermore, the chemoproteomic profiling of MMV390048 demonstrated no binding to the human PfPI4K orthologs PI4Kβ and PI4Kα. Binding affinity for MMV390048 was reported for human PIP4K2C (similar affinity to that for *P. falciparum* PI4K) and for two other kinases, ATM and TNIK, with lower binding affinity (Paquet et al., 2017).

Having successfully completed the early preclinical program, MMV390048 became the first PI4K inhibitor to enter the clinical development stage (McCarthy et al., 2020; Paquet et al., 2017; Sinxadi et al., 2020). In support of later-stage clinical trials, MMV390048 was progressed into embryofetal developmental (EFD) studies in pregnant rats and rabbits. There were no signals of concern in the rabbit EFD studies, but in the rat EFD studies in which higher systemic exposures were achieved, embryofetal toxicities, including congenital diaphragmatic hernia (CDH), were observed that contributed to the discontinuation of the progression of the compound. Here, we describe these embryofetal abnormalities and the investigative work undertaken to elucidate their origin.

It was suspected (based on initial kinase screening, data not shown) that the rat fetal malformations could be due to lack of selectivity of MMV390048 for plasmodial PI4K and that it could be inhibiting the mammalian homolog of the PI4K parasite target. A key consideration was to determine whether the embryofetal abnormalities, especially the CDH, could be linked to activity against the human homolog of the PI4K parasite target since this information could profoundly influence the specificity screening of backup programs. The IC_{50}'s against human PI4K (α and β paralogues) and a panel of off-target kinases were compared against in vivo exposures achieved in the EFD studies.

2 | MATERIALS AND METHODS

2.1 | Data sources

Three rat developmental toxicity studies were performed for MMV390048 in accordance with relevant institutional and national guidelines for the care and use of laboratory animals, ensuring the protection of the animals used in this work. These three studies met ICH S5(R2) guideline requirements and were done in compliance with Good Laboratory Practice (GLP) standards. Studies were performed at two different test facilities: (a) an initial dose range finding embryofetal developmental (DRF EFD; ref IG13166) study, (b) the main EFD study (ref G115064)

TABLE 1 Experimental design of the embryofetal developmental studies in rats

Study type	Study ref	CRO	Dosing regimen	Doses (mg/kg/day)	No. of animals per group	Major maternal endpoints	Major fetal endpoints
DRF EFD	IG13166	KIT	GD6-GD17	3, 10, 30, and 60	8	BW, BWG, FC, pregnancy status, postimplantation loss, corpora lutea, and live fetuses	BW, external, visceral, and skeletal examination
Main EFD	G115064	KIT	GD6-GD17	2, 6, and 20	24	BW, BWG, FC, pregnancy status, postimplantation loss, corpora lutea, and live fetuses	BW, external, visceral, and skeletal examination
Investigative EFD	20196008	CRL	GD6–GD17 GD8–GD11[a]	20, 40, and 60[a]	25	BW, BWG, FC, pregnancy status, postimplantation loss, corpora lutea, and live fetuses	BW, external and visceral examination

Abbreviations: BW, body weight; BWG, body weight gain; CRL, Charles River Laboratories; DRF, dose range finding; EFD, embryofetal development; FC, food consumption; GD, gestational day; KIT, Korea Institute of Toxicology.
[a]Group of animals dosed with 60 mg/kg/day daily during the specific period of diaphragm formation from GD8 to GD11.

were carried out at the Korea Institute of Toxicology (KIT) in South Korea, and (c) a follow-up investigative EFD study (ref 20196008) conducted at Charles River Laboratories (CRL) in France (Table 1). Animals were dosed daily via oral administration from gestational day 6 (GD6) to GD17 in all studies. Moreover, in the investigative EFD study, a designed group of animals were dosed daily during the specific period of diaphragm formation from GD8 to GD11. Blood samples were taken on GD6 and GD17 for toxicokinetic (TK) analysis in rat. The rat exposures were compared to the pregnant rabbit exposures in the EFD studies; samples in this case were taken in GD11.

2.2 | Test animals

Naïve time-mated females were used at both facilities; Sprague–Dawley rats (Crl:CD SD) at KIT and Sprague–Dawley rats (Crl: OFA SD) at CRL. The number of animals for each study was selected based on the ICH guidelines. The day on which a vaginal plug was detected was considered GD0. All surviving rats were euthanized at GD21 via carbon dioxide inhalation and then necropsied.

2.3 | Test item formulation

Oral administration was chosen as it was the route of administration of MMV048 to humans. Vehicle (20% w/w Kolliphor™ HS 15, 50% w/w PEG 400 in purified water) was prepared. The same batch of solvent control article components was used for the entire study. The test article was suspended in the vehicle, adjusted for purity, to reach the target concentration. The formulations were dispensed into amber glass containers for use and then stirred by a magnetic stirrer at room temperature until the completion of formulation. The test article was formulated at least once every week and was stored at room temperature until it was given to the animals once daily from GD6 or GD7 through GD17.

2.4 | Assessment of kinase inhibition

Unanticipated, off-target pharmacological activities of MMV390048 were investigated in several in vitro screens which included receptors, enzymes, ion channels, amine transporters, and some lipid and protein kinases (data not shown). In all assays (two CEREP screening panels of c.155 targets and an earlier Glaxo SmithKline in-house experimental screening panel of 49 targets), MMV390048 was initially tested at 10 μM and the criterion for significant activity was greater than 50% displacement of the labeled ligand or inhibition of activity. MMV390048 (10 μM) was profiled further for potential inhibitory activity against >333 protein and lipid kinases. The biochemical inhibitory activities (IC_{50}s) of human kinases were measured at ProQuinase/Reaction Biology with an ADP-Glo™ Kinase Assay platform (Promega) for PI4Kα, β and a panel of off-target kinases. A detailed description of the ADP-Glo™ assay is available from https://www.promega.co.uk/products/cell-signaling/kinase-assays-and-kinase-biology/adp-glo-kinase-assay/?catNum=V6930.

2.5 | Assessment of kinase inhibition potential at equivalent in vivo exposures in pregnant rats

Plasma TK data from all three EFD studies were converted into free compound μM amounts of MMV390048 (based on rat plasma protein binding levels of 85%). Exposures post dose were plotted for all dose groups tested. This in vivo TK data was overlaid with human in vitro IC_{50} μM data for individual kinases that are inhibited by MMV390048 in cell-free enzyme assays to compare them to the exposures achieved in the animal studies where abnormalities were observed. Kinases that had $IC_{50}s \geq 20$ μM were considered not to be relevant based on exposure data from EFD in vivo studies (maximum concentrations achieved in vivo were 8 μM free).

3 | RESULTS

3.1 | In vivo exposures

Maximum exposures of MMV390048 in the in vivo rat studies were as follows on GD17: DRF EFD KIT study (8 μM/L at 60 mg/kg 4 hr post dose), EFD main KIT study (2 μM/L at 20 mg/kg 4 hr post dose), and investigational EFD CRL study (6.5 μM/L at 40 mg/kg 7 hr post dose). The systemic exposures (C_{max} and AUC_{0-24}) achieved in the rat were at least fivefold higher than in the rabbit (Table 2).

In comparison, the clinical exposure (AUC_{0-Inf}) measured in a single ascending dose study was 2.6-fold lower than systemic exposures achieved in the rat (Tables 2 and 3; McCarthy et al., 2020) at an efficacious dose of 120 mg MMV390048.

3.2 | Study 1: DRF EFD (Ref IG13166)

Systemic exposure to MMV390048 in the pregnant rat increased with increasing dose on GD6 and GD17 (Table 2).

A statistically significant decrease in mean maternal body weight was observed on GD21 in the 60 mg/kg/day group (data not shown). However, this was influenced by low mean gravid uterine weight in that group and there was no effect after correction for gravid uterus weight.

Developmental toxicity at 60 mg/kg/day consisted of a 24% decrease in litter size (10.6 vs. 14 in the control group, data not shown), a 16% decrease in fetal weight (male and female average), delayed ossification and increased incidences of fetal alterations affecting primarily the visceral organs and axial skeleton, notably diaphragmatic hernia and ventricular septum defects (VSDs). Treatment-related visceral malformations in the 60 mg/kg/day group consisted of VSD which occurred in 3 fetuses from 3 litters and hernia of the diaphragm which occurred in 12 fetuses from 3 litters (Table 4).

Treatment-related skeletal malformations in the 60 mg/kg/day group consisted of fused rib, short rib, split sternebra, and fused cervical arch.

This first study demonstrated that at the highest dose (60 mg/kg/day), MMV390048 is a developmental toxicant in the rat. Since in the DRF study, at the highest dose (60 mg/kg/day), several malformations were observed for the next study (main EFD study), a lower dose for the highest dose was chosen (20 mg/kg/day).

3.3 | Study 2: Main EFD study (Ref G115064)

Systemic exposure to MMV390048 in the pregnant rats increased more than proportionally to the increase in the dose (Table 2).

Decreased body weight gains were observed between GD6 and GD9 which also correlated with decreased food consumption at 6 and 20 mg/kg/day. At this interval, weight gain was decreased to 20.2% and 41.1% of control at 6 and 20 mg/kg/day, respectively, and food consumption was decreased to 8.9% and 11.5% of control at 6 and 20 mg/kg/day, respectively.

At external, visceral, and skeletal examinations on live fetuses, diaphragmatic hernia was observed in one fetus of the 20 mg/kg/day group (1/154; Table 4). Although there was only this one single incidence, it was considered an effect of MMV390048 since diaphragmatic hernia was observed in 12 fetuses from 3 litters at the highest dose level (60 mg/kg/day) in the DRF EFD study (ref IG13166; Table 4).

In conclusion, the maternal no-observed-adverse-effect level (NOAEL) was 2 mg/kg/day since decreased weight gain and food consumption was seen at 6 and 20 mg/kg/day, and the NOAEL for EFD was 6 mg/kg/day based on the single fetus with a diaphragmatic hernia at 20 mg/kg/day.

3.4 | Study 3: Investigative EFD (Ref 20196008)

Based on these results, an investigational EFD study was performed to provide complementary information on the effect of MMV390048, on EFD toxicity when pregnant rats were administered the test item during the period of organogenesis GD6–GD17 or during the specific period

TABLE 2 Toxicokinetic exposure data from embryofetal developmental studies

Study type[a]	Dose (mg/kg/day)	Gestation day 6			Gestation day 17			Gestation day 11		
		T_{max} (hr)	C_{max} (ng/ml)	$AUC_{(0-t)}$ (hr*ng/ml)	T_{max} (hr)	C_{max} (ng/ml)	$AUC_{(0-t)}$ (hr*ng/ml)	T_{max} (hr)	C_{max} (ng/ml)	$AUC_{(0-t)}$ (hr*ng/ml)
Rat										
Main EFD ($n = 22$)	2	2	679.5	3,749.5	2	680.6	3,767.5	–	–	–
DRF EFD ($n = 8$)	3	4	626	3,749	8	876	13,191	–	–	–
Main EFD ($n = 23$)	6	2	1,382.6	19,615.6	2	1,602.4	21,374	–	–	–
DRF EFD ($n = 8$)	10	4	2,160	32,894	4	2,384	39,165	–	–	–
Main EFD ($n = 22$)	20	4	4,949.9	69,995.1	4	5,189.4	74,434	–	–	–
Investigative EFD ($n = 24$)	20	7	5,230	92,900	7	7,550	134,000	–	–	–
DRF EFD ($n = 7$)	30	8	9,116	126,134	8	8,613	140,565	–	–	–
Investigative EFD ($n = 22$)	40	7	11,300	213,000	7	17,100	310,000	–	–	–
DRF EFD ($n = 8$)	60	8	15,581	228,818	4	21,292	361,170	–	–	–
Investigative EFD ($n = 25$)	60	ND	ND	ND	ND	ND	ND	–	–	–
Rabbit										
DRF EFD ($n = 6$)	1	–	–	–	–	–	–	2	267	3,747
Main EFD ($n = 18$)	1	–	–	–	–	–	–	4	250	4,094
DRF EFD ($n = 6$)	3	–	–	–	–	–	–	4	857	14,876
Main EFD ($n = 18$)	3	–	–	–	–	–	–	2	894	13,575
DRF EFD ($n = 5$)	10	–	–	–	–	–	–	4	3,794	64,549
Main EFD ($n = 21$)	10	–	–	–	–	–	–	2	3,409	53,334

Abbreviations: EFD, embryofetal developmental; ND, not determined.
[a]The number of animals for each study was selected based on the ICH guidelines.

of diaphragm formation from GD8 through GD11. The dose levels to be tested in this study were selected to reproduce the malformations observed in previous studies and thus validate the results. The aim of this study was to clarify the potential teratogenic effect of MMV390048, especially on diaphragmatic hernia since the previous studies results were not clear as the CDH findings were not dose-related and the number of fetus affected was low in the EFD (only one in 154 fetuses).

Systemic exposure to MMV390048 in the pregnant rat increased with increasing dose on GD6 and GD17 (Table 2).

In both dosing intervals (GD6 through GD17 or GD GD8 through GD11), there was an observed reduction in

TABLE 3 Human pharmacokinetic parameters

Subject no.	Sex	Dose (mg)	T_{max} (hr)	C_{max} (ng/ml)	$AUC_{(0-inf)}$ (hr*ng/ml)
6	M	40	1.5	272.7	30,320
6	M	80	2	561	82,680
6	M	120	1	1,094	137,800

Source: McCarthy et al. (2020).

food consumption and a markedly lower mean body weight gain at 40 and 60 mg/kg/day, decreasing to 41% and 49% of controls, respectively, during the dosing and post dose periods (data not shown). However, the effect was entirely due to markedly high percentage of resorptions since there was no effect after correction for gravid uterus weight.

Fetotoxic potential of MMV390048 was evidenced by a high postimplantation loss resulting in a markedly reduced mean live litter size at 40 and 60 mg/kg/day and lower mean fetal weight in all treated groups. There was a markedly higher percentage of postimplantation loss, 66.45% and 79.22% of control in the 40 and 60 mg/kg/day groups, respectively, due to a high number of both early and late resorptions (Table 4). Only 16 and 14 females had viable fetuses in the 40 and 60 mg/kg/day groups, respectively (6 and 11 females with no viable fetuses).

In addition, there were higher incidences of external (absent/small anus, omphalocele, and/or edema) and visceral abnormalities (diaphragmatic hernia, lung, heart, major blood vessels, and urogenital defects) in all treated groups clearly indicative of a teratogenic potential of MMV390048 in the rat (Table 4).

In conclusion, in this investigational study, the low dose of 20 mg/kg/day was considered a NOAEL for maternal toxicity due to the observed reduction in food consumption and marked reduction in mean body weight gain at 40 mg/kg/day; a NOAEL for developmental toxicity was not established.

3.5 | Assessment of kinase inhibition potential at in vivo exposures in pregnant rats

To explore if off-targets, including kinases, may be potentially responsible for the observed fetal malformations in rat studies, human kinase inhibition data with MMV390048 were analyzed to assess if in vivo exposures achieved were greater than IC_{50} inhibition values and were therefore covered by exposure of free MMV390048 in pregnant rats.

Data were plotted from all the above EFD in vivo studies and compared to human in vitro IC_{50} data for individual kinases as described in Section 2. Off-targets, including kinases that had IC_{50}s $\geq$ 20 μM, were not considered to be relevant based on exposure data from in vivo studies (maximum concentrations achieved were 8 μM free). Several other human kinases showed IC_{50}s $\leq$ 20 μM: PI3Kα, PI3Kδ, PI3Kγ, mitogen-activated protein kinase 4 (MAP4K4), misshapen/nik-related kinase 1 (MINK1), Serine/Threonine Kinase 16 (STK16), and the ligand-activated transcription factor aryl hydrocarbon receptor (AhR). IC_{50} in vitro inhibition of MMV390048 by kinases ≤20 μM is shown in Figures 1–3 and includes the human target paralogues, PI4Kβ (1 μM) and PI4Kα (18.7 μM), and off-targets MAP4K4 (0.8 μM), MINK1 (0.7 μM), PI3Kγ (4.3 μM), TSF1 (7.3 μM), PI3Kα (7.8 μM), AhR (7.9 μM), and PI3Kd (8.1 μM). All other off-targets including kinases tested had IC_{50}s $\geq$ 20 μM. The homology between human kinases and rodents and/or rabbits is high (70–99% Percent identity).

In summary, MMV390048 exposure in rat studies where EFD toxicities were observed exceeded the in vitro inhibition assay IC_{50} values of the human target, PI4Kβ, and two off-target kinases, MAP4K4, and MINK1 (Figures 1–3), supporting the hypothesis that inhibition of these kinases may contribute to the EFD pathologies. MMV390048 exposure in the DRF EFD study (ref IG13166) and investigative EFD study (20196008) exceeded the in vitro inhibition IC_{50} value of PI3Kγ at the top doses of 60 and 40 mg/kg, respectively, indicating a possible, albeit less likely, role for PI3Kγ in the EFD pathologies. MMV390048 exposure in rat studies did not exceed the in vitro inhibition assay IC_{50} values of TSF1, PI3Kα, AhR, or PI4Kα indicating that these targets have a lower probability of being involved in the EFD toxicities seen.

4 | DISCUSSION

MMV390048 was developed as a blood-stage schizonticide designed to be part of a new treatment to replace artemisinin combination therapies, with a high potential for good post-treatment prophylaxis potential, long half-life, and high oral bioavailability. Despite reaching initial clinical studies in African patients with a

TABLE 4 Embryo developmental studies maternal and fetal findings

Study type	Dose (mg/kg day)	Maternal observations		Fetal BW	Fetal observations[a]							
							Heart		Blood vessels			
		Female with viable fetuses	Postimplantation loss (%)	% from CTL	Edema	Hernias	VSD	Thick VW	AARS	ADA	NPT	IAA
Main EFD	2	22/22	6.9	–	–	–	–	–	–	–	–	–
DRF EFD	3	8/8	5.1	–	–	–	–	–	–	–	–	–
Main EFD	6	23/23	3.5	–	1/336 (1/23)	–	–	–	–	–	–	–
DRF EFD	10	8/8	1.7	−2	–	–	–	–	–	–	–	–
Main EFD	20	22/22	2.9	–	–	1/154 (1/22)	–	–	–	–	–	–
Investigative EFD	20	24/24	9.6	−10	1/280 (1/24)	35/280 (16/24)	1/280 (1/24)	–	2/280 (2/24)	4/280 (3/24)	1/280 (1/24)	2/280 (2/24)
DRF EFD	30	7/7	5.9	−5	–	–	1/43 (1/7)	–	–	–	–	–
Investigative EFD	40	16/22	66.45	−24	1/97 (1/16)	63/97 (15/16)	21/97 (8/16)	1/97 (1/16)	8/97 (8/16)	9/97 (7/16)	9/97 (6/16)	4/97 (4/16)
DRF EFD	60	8/8	21.9	−16	–	12/40 (3/8)	7/40 (3/8)	–	–	–	–	1/40 (1/8)
Investigative EFD[b]	60	14/25	79.2	−26	2/65 (1/14)	31/65 (11/14)	–	3/65 (3/14)	12/65 (12/14)	10/65 (6/14)	10/65 (6/14)	1/65 (1/14)

Abbreviations: AARS, aortic arch right-sided; ADA, absent ductus arteriosus; BW, body weight; CTL, control group; EFD, embryofetal developmental; IAA, interrupted aortic arch; NPT, narrow pulmonary truck; VSD, ventricular septum defect; VW, ventricular wall.

[a]Number of fetuses with malformation/total number of fetuses examined in group (number of litters with malformation/total number of litters examined in group).

[b]Only dosed from GD8 to GD11 (i.e., critical window for diaphragm formation; Babiuk, Zhang, Clugston, Allan, & Greer, 2003).

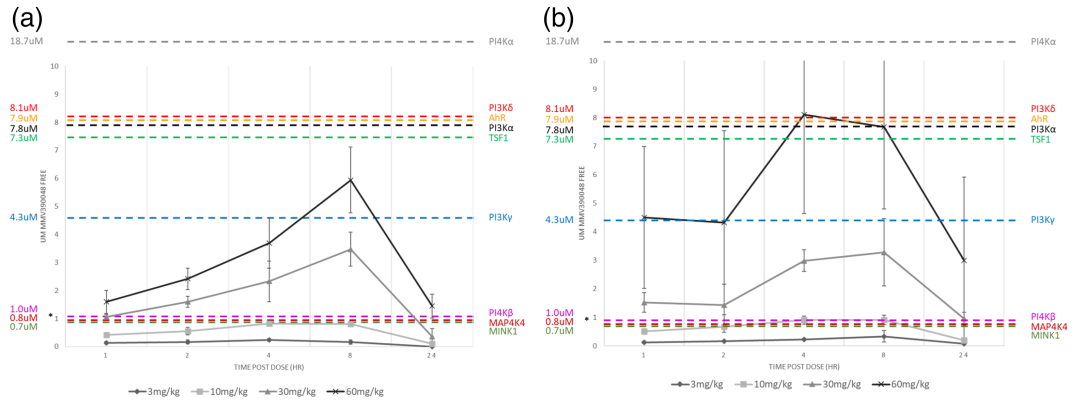

FIGURE 1 Rat DRF EFD study (KIT). Free μM exposure of MMV390048 achieved 1–24 hr post dose compared to MMV390048 IC_{50} inhibition of kinases. (a) Gestational day 6, (b) Gestational day 17. Diaphragmatic hernias occurred at 60 mg/kg in this study. EFD, embryofetal developmental

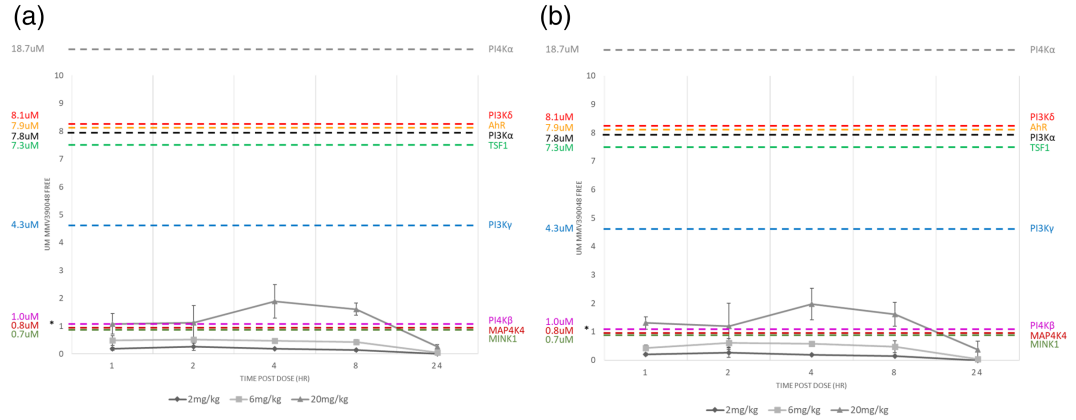

FIGURE 2 Rat main EFD study (KIT). Free μM exposure of MMV390048 achieved 1–24 hr post dose compared to MMV390048 IC_{50} inhibition of kinases. (a) Gestational day 6, (b) Gestational day 17. One diaphragmatic hernia occurred in a fetus at 20 mg/kg in this study. EFD, embryofetal developmental

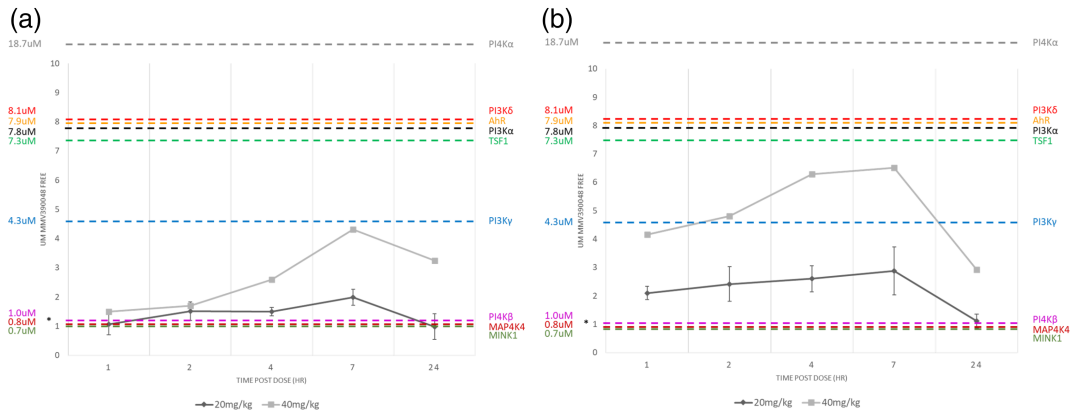

FIGURE 3 Rat investigative EFD study (CRL). Free μM exposure of MMV390048 achieved 1–24 hr post dose compared to MMV390048 IC_{50} inhibition of kinases. (a) Gestational day 6, (b) Gestational day 17. Diaphragmatic hernias occurred at 20 and 40 mg/kg in this study. EFD, embryofetal developmental

favorable safety profile, EFD studies in rats indicated developmental toxicity. This along with a testicular toxicity signal led to the decision to discontinue the program. There were no signals of concern in rabbit EFD studies; however, exposures achieved in the rabbit were below the lowest observed adverse effect level in rats. Developmental safety margins calculated based on the rat NOAEL of 6 mg/kg/day and the observed clinical PK data (McCarthy et al., 2020) were <1 in human volunteers and patients, respectively.

Studies in pregnant rats revealed external and visceral malformations such as diaphragmatic hernias and/or cardiovascular malformations. The first DRF EFD study revealed developmental toxicity consisting of decreased litter sizes, decreased fetal weight, delayed ossification, and increased incidences of fetal alterations affecting primarily the visceral organs and axial skeleton, notably diaphragmatic hernia, and VSDs. Similar findings were also seen in both main and investigational EFD studies. Although in the main EFD study, it was possible to define the NOAEL for embryofetal development (6 mg/kg/day); in the following investigational EFD study, a NOAEL was not established due to the various fetal malformations observed at higher exposures with lower doses than before.

It was suspected that the rat fetal malformations could be due to lack of selectivity of MMV390048 for plasmodial PI4K and that it could be inhibiting the mammalian homolog of the PI4K parasite target. Therefore, the IC50s against human PI4K (paralogues α and β) and a panel of kinases were measured (rat assays were not available) on the basis that the kinases tested are highly conserved across species. Results were compared against in vivo exposures achieved in the EFD studies. Human kinase assays were used, rat assays were not available, on the basis that the kinases tested are highly conserved across species. In order to further aid in the selection of a backup chemical series with minimal liability for the EFD pathologies observed with MMV390048, we undertook an investigation into potential kinases involved in the EFD toxicity by analyzing which kinases are inhibited by 50% at similar exposures achieved by MMV390048 in the in vivo rat studies.

MMV390048 exposure in all rat studies where EFD toxicities were observed exceeded the in vitro inhibition assay IC_{50} values of PI4Kβ, MAP4K4, and MINK1 (Figures 1–3) by 0.3–8.1-, 0.3–10.1-, and 0.4–11.6-fold, respectively. IC_{50} in vitro inhibition of PI3Kγ was covered by in vivo MMV390048 exposure, where EFD toxicities were observed in two out of three rat studies (Study #IG13166; Study #20196008) at the top dose only. In both studies, malformations (EFD toxicity) were also observed at lower dose levels where exposures achieved did not cover IC_{50} in vitro inhibition of PI3Kγ (Figures 1–3). In contrast, the IC_{50} in vitro values of PI3Kα, PI3Kδ, AhR, TSF1, and PI4Kα were higher than the MMV390048 exposures achieved in the EFD toxicity studies (Figures 1–3).

Therefore, a combination of human lipid kinase and human protein kinase could potentially contribute to MMV390048 malformations seen in the EFD studies in rats. Of the kinases, we studied the most likely kinase targets implicated in the EFD are PI4Kβ, the human orthologue of *Plasmodium* PI4K, in addition to the off-targets MAP4K4 and MINK1 as the in vitro IC_{50} data from these kinases was consistently covered by the level of exposure to MMV390048 seen when malformations were observed.

PI4Kβ plays a major role in producing phosphoinositide lipids (PPIns), which are critical in organizing trafficking pathways in all eukaryotic cells, including the Golgi. Phosphatidylinositol-4-phosphate (produced primarily by PI4Kβ and PI4K2α) is the major PPIn in the Golgi (Boura & Nencka, 2015). Our hypothesis that inhibition of mammalian PI4Kβ may contribute to the development of fetal malformations is based on the plausible inhibition of the enzyme at exposures achieved in the rat EFD study. However, at the time of writing, no evidence has been found to directly link embryofetal teratogenesis with PI4Kβ inhibitors. It would be valuable to validate this hypothesis with in vivo morphology data from PI4Kβ rodent or human mutant phenotypes but currently no such information is available.

As well as the target itself, our in vitro data implicate MAP4K4 and MINK1 as having possible roles in the malformations. Although there are no reports of loss or inhibition of MAP4K4 leading to embryofetal diaphragmatic hernias, MAP4K4 is known to play an essential role in mammalian development, providing further evidence that MAP4K4 inhibition by MMV390048 could be involved in the malformations seen in the EFD studies. Loss of MAP4K4 has been shown to decrease membrane dynamics, slow endothelial cell migration, and impair angiogenesis in vitro and in vivo in an endothelial cell conditional mouse knockout. MAP4K4 also has an essential role in skin wound healing and epidermal migration (Vitorino et al., 2015; J. Yue et al., 2014). MAP4K4$^{-/-}$ mice die postgastrulation between embryonic days 9.5 and 10.5, embryos fail to develop somites or a hindgut and are truncated posteriorly (Xue et al., 2001). Embryonic lethality during organogenesis and embryonic growth retardation have also been reported (Mouse Genome Informatics: 1933044). MINK1 is involved in the regulation of actin cytoskeleton reorganization, cell–matrix adhesion, cell–cell adhesion, and cell migration; however, homozygous knockout mice are viable (M. Yue et al., 2016), and there is no mention of a diaphragmatic hernia phenotype or cardiovascular malformations.

PI3Kγ is a less likely target as its in vitro IC_{50} is not consistently covered by exposures known to be associated with fetal malformations. However, a dual selective PI3Kγ/δ inhibitor used to treat leukemia (Duvelisib; Food and Drug Administration; Duvelisib Label, 2018) is described as a developmental toxicant causing fetal losses and malformations in both rats and rabbits leading to the labeling requirement for pregnancy testing prior to use in patients. Therefore, inhibition of PI3Kγ cannot be completely ruled out as a possible target kinase involved in the EFD malformations. Other targets that cannot be ruled out but are less likely contributors to the toxicity are: PI3Kα, PI3Kδ, AhR, TSF1, and PI4Kα. These targets are less likely to be involved in the EFD malformations caused by MMV390048 as their IC_{50} values are not covered by exposure in the rat in vivo studies. However, although IC_{50}s are a standard scientific measurement used to analyze kinase inhibition data, it is not known if 50% inhibition is needed to cause an effect, indeed low doses of PI3K inhibitors have shown EFD abnormalities in preclinical studies (Food and Drug Administration; Alpelisib Label, 2019; Food and Drug Administration; Copanlisib Label, 2017; Food and Drug Administration; Idelalisib Label, 2014).

It is known that CDH in humans is associated with other types of defects and some of them are the same malformations observed in our EFD studies, such as VSD. CDH is a congenital malformation that occurs with a frequency of 0.08–0.45 per 1,000 births (Kling & Schnitzer, 2007). Although the etiology of CDH is largely unknown, several advances have been made in understanding its molecular mechanisms using different models. Several studies have highlighted the critical role of vitamin A (retinol) homeostasis (Anderson, 1941; Major et al., 1998; Wilson, Roth, & Warkany, 1953) and retinoic acid (RA) signaling (Chen, MacGowan, Ward, Bavik, & Greer, 2003; Greer, Babiuk, & Thebaud, 2003; Major et al., 1998; Mendelsohn et al., 1994) for proper fetal development providing a rationale link with the etiology of CDH. Administration of teratogens such as nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether), 4-biphenyl carboxylic acid (BPCA), bisdiamine [N,N-octamethylenebis (dichloroacetamide)] and SB-210661 to pregnant rats exhibited congenital anomalies in the offspring, including CDH defects similar to the pathologic findings in humans (Mey, Babiuk, Clugston, Zhang, & Greer, 2003). Furthermore, all four CDH-inducing compounds inhibit retinal dehydrogenase-2 (RALDH2), a key enzyme necessary for the production of RA expressed in the developing diaphragm, in a dose-dependent manner providing evidence that the primordial diaphragm tissue, the pleuroperitoneal fold, is dependent on retinoid-mediated signaling for its proper formation (Mey et al., 2003).

Interestingly pathways relevant to nitrofen-induced lung hypoplasia include the PI3K pathway (Mahood, Johar, Iwasiow, Xu, & Keijzer, 2016). The serine/threonine-protein kinase B (AKT) plays a key role in lung morphogenesis through epithelial–mesenchymal interaction in a PI3K-dependent manner. Prenatal treatment with RA has been reported to stimulate alveologenesis in hypoplastic lungs in the nitrofen model of CDH and prenatal treatment with RA upregulates pulmonary gene expression of PI3K and AKT in nitrofen-induced pulmonary hypoplasia (Doi, Sugimoto, Ruttenstock, Dingemann, & Puri, 2010). Furthermore, nitrofen inhibits PI3K-AKT signaling during mid-to-late lung morphogenesis in the nitrofen-induced hypoplastic lung (Doi, Ruttenstock, Dingemann, & Puri, 2010). No such links to the RA signaling pathway and processes involved in abnormal diaphragm development were found at the time of writing for PI4K, MAP4K4, or MINK1.

In conclusion, our data suggest that inhibition of the mammalian PI4Kβ target itself, as well as some off-target kinases could contribute to the malformations seen in the EFD studies. Homology between the human and rodent kinases is highly conserved and therefore if this hypothesis is correct, if pregnant patients were to receive similar exposures to MMV390048 as the rat, there is a risk of developmental toxicity. However, these relationships are by association and not mechanistically proven. Overall, these data suggest that increased selectivity for *Plasmodium* PI4K over mammalian PI4K is likely to be important to protect the health of the unborn child. Deciphering if the embryofetal effects are dependent on the inhibition of PI4Kβ, or through other off-target kinase effects (or indeed a different hypothesis not linked to kinase inhibition) requires the generation of novel, more potent, and specific PI4K inhibitors.

AUTHOR CONTRIBUTIONS

Claudia Demarta-Gatsi and Belen Tornesi were responsible for the experimental design of this study. Jane A. Barber and Claire Sadler conducted the analysis of kinase activity relative to in vivo exposure. This manuscript was written and edited by Claudia Demarta-Gatsi, Belen Tornesi, Jane A. Barber, and Jane Stewart.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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