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Combining the zebrafish embryo developmental toxicity assay (ZEDTA) with hemoglobin staining to accelerate the research of novel antimalarial drugs for pregnant women

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ABSTRACT

Background: Malaria during pregnancy implies a high risk for the mother and the developing child. However, the therapeutic options for pregnant women have historically been very limited, especially during the first trimester of pregnancy due to potential adverse effects on embryo-fetal development. Recently, there has been great controversy regarding these potential embryo-fetal adverse effects because the results of rodent studies were not in accordance with the clinical data available, and finally the WHO has changed the recommendations for pregnant women with uncomplicated *P. falciparum* malaria to treatment with artemether-lumefantrine during the first trimester. The discrepancy between pre-clinical and clinical studies has been attributed to species-differences in the duration of the window of susceptibility of circulating primitive erythroblasts.
Methods: Here we provide a tool based on an alternative method to animal experimentation that accelerates the research of novel drugs for pregnant women. We have adapted the zebrafish embryo developmental toxicity assay to include hemoglobin staining in the embryos and two time-points of lethality and dysmorphogenesis evaluation. These two time-points were selected to include one when the development is independent of and one when the development is dependent of erythrocytes function. The method was used to test four marketed antimalarial drugs and three new antimalarial drug candidates.
Results: Our combination of tests can correctly predict the teratogenic and non-teratogenic effects of several antimalarial marketed drugs (artemisinin, quinine, chloroquine, and dihydroartemisinin + desbutyl-lumefantrine). Furthermore, we have tested three new drug candidates (GS-GUAN, DONE3TCl, and YAT2150) with novel mechanisms of action, and different from those of the marketed antimalarial drugs.
Conclusions: We propose a decision tree combining the results of the two time-points of evaluation together with the information on significant erythrocyte depletion. The aim of this decision tree is to identify compounds with no or lower hazard on teratogenicity or erythrocyte depletion at an early phase of the drug development process.

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1. Background

Malaria in pregnancy is a major public health issue entailing very high risks for both the mother and the developing child [WHO, 2023]. Compared to other population groups, pregnant women are four times more likely to contract malaria, three times more likely to suffer a severe disease derived from it, and two times more likely to die from it [Schantz-Dunn et al., 2009]. Placental malaria is characterized by sequestration of *Plasmodium falciparum*-infected erythrocytes and infiltration of immune cells within the intervillous spaces of the placenta, resulting in an inflammatory environment and an altered materno-fetal exchange system [Sharma et al., 2017; Omer et al., 2021.]. This placental dysfunction leads to intrauterine growth restriction, preterm delivery, low birth weight, stillbirths, miscarriages and increased new-born mortality [Fairhurst and Wellems, 2015].

Each year, more than 30 million women become pregnant in malaria endemic areas [Reddy et al., 2023], but the therapeutic options for this population have historically been very limited, especially during the first trimester of pregnancy due to potential adverse effects on embryo-fetal development of several drugs available for the general population, as for example some artemisinin-based combination therapies (ACTs) [WHO, 2021]. However, in the last years there has been great controversy regarding these potential embryo-fetal adverse effects of ACTs, because animal studies demonstrating fetal death and congenital malformations were not in accordance with clinical data. Clinical administration of therapeutic doses of some ACTs during the first trimester of pregnancy was not associated with increased risk of miscarriage, stillbirth or congenital malformation, nor low birth weight by the available data in the given studies [D'Alessandro et al., 2020; Dellicour et al., 2017]. As a result of the revision of this clinical data, the World Health Organization (WHO) has very recently updated the guidelines for the treatment of malaria in pregnancy and has endorsed the use of one ACT in the first trimester, the combination of arthemeter + lumefantrine [WHO, 2022; Castro et al., 2024]. However, continued research is needed to increase the data available for the use of this combination of drugs during pregnancy [WHO, 2022], as well as new research on further drug candidates with a lower intrinsic hazard to cause reproductive toxicity to ensure safe use under all circumstances for this population.

Initiatives led by Medicines for Malaria Venture (MMV) and partners have already claimed for an early testing of embryo-fetal developmental toxicity in drug development strategies, to enable for prioritization or parallel selection of drug candidates that are safe during pregnancy [El Gaaloul et al., 2022; Demarta-Gatsi et al., 2023]. To increase the throughput, decrease the costs and the time invested, such an early screening step should be conducted in what are called New Approach Methodologies (NAMs), because the testing of hundreds/thousands of candidates would not be feasible/affordable with the classical reproductive toxicity studies in rats and rabbits. An alternative method to animal experimentation which allows to evaluate the teratogenic potential of drugs in a whole organism, but in short time and with low costs is the Zebrafish Embryo Developmental Toxicity Assay or ZEDTA [Hoyberghs et al., 2020; de Jong et al., 2011]. This method has also been recently endorsed as "qualified alternative assay" according to the Guideline ICH S5 (R3) on detection of reproductive and developmental toxicity for human pharmaceuticals [Europen Medicines Agency, 2020; Weiner et al., 2024; Song et al., 2021], meaning that its results can now also be used at regulatory level for the assessment of embryo-fetal developmental toxicity studies in selected cases. Some approaches have already applied the ZEDTA to investigate the effects of a single antimalarial candidate and compared the teratogenic effects with those observed in other species or other *in vitro* methods [Demarta-Gatsi et al., 2023]. Other works have used the Fish Embryo Acute Toxicity Test (FET) to assess 400 antimalarial drug candidates but for general toxicity after completion of organogenesis, with exposure starting at 3 days post-fertilization instead of covering the early developmental stages [Van Voorhis et al., 2016]. However, to the best of our knowledge, there has been no study to asses wether the method correctly predicts the developmental adverse or non-adverse effects of several marketed antimalarial drugs with an exposure that lasts the whole organogenesis period. Likewise, there are no reports on the use of the test to screen several new antimalarial candidates with developmental toxicity exclusion purposes, although an editorial reporting a single-case evaluation is available as example [Demarta-Gatsi et al., 2023].

So far, NAMs have a limited, most of the time not completely defined, biological or chemical applicability domain. In this context, we are developing further and testing a NAM for a specific applicability domain/mode of action related to erythrocyte depletion [Clark 2009; Longo et al., 2010], by including and hemoglobin staining (HbSt). With the aim to provide a tool that accelerates the research of novel antimalarial drugs for pregnant women and women of childbearing potential, in this study we have applied an adapted version of the ZEDTA, including dysmorphogenesis evaluation at two timepoints, behavioural testing and HbSt in the embryos, to investigate if it can correctly predict the teratogenic and non-teratogenic effects of several antimalarial marketed drugs, to test three new drug candidates, and to select the best one of them regarding their hazard and risk-benefit profile during pregnancy.

2. Methods

2.1. Ethics statement

All experiments described were conducted in accordance with the regulations of the Ethics Committee for Animal Experimentation of the University of Barcelona (CCEA-UB). The experimentation project, with number 9967 was approved on the May 31, 2018 by the CCEA-UB and the Department of Environment and Housing of the Generalitat de Catalunya with license order 334/18.

2.2. Zebrafish adult colony maintenance, egg harvest and selection

Adult zebrafish (*Danio rerio*; Tropical Center ICA S.A., Spain) were maintained in closed flow-through system aquariums with standardized water (as defined in ISO 7346-3 (1996)) at a temperature between 26.5 and 28 °C, and under a constant light:dark cycle (14:10 h). Fish were daily fed in the morning with *Artemia salina*, and with commercial flake food in the afternoon (SDS400, Special Diet Services, Dietex, France). Male and female fish in proportion 2:1 were placed overnight in a mating tank with artificial plants and marbles to stimulate spawning. Next morning, after lights turned on, eggs were collected by filtering the water with a sieve, and afterwards washed a minimum of three times with standardized ISO 7346–3 water diluted 1:5. Fertilized, non-coagulated, and synchronously divided eggs were selected using a stereomicroscope (Motic SMZ168, Motic China group, LTD., China) and transferred to 6-well plates (10 embryos/well).

2.3. ZEDTA

Zebrafish embryos were kept in standardized water diluted 1:5 (5 mL/well) until 5 h post-fertilization (hpf), when the exposure started.

2.3.1. Exposure

At 5 hpf water of all wells was replaced with 5 mL of freshly prepared solutions of the different concentrations (between 5 and 9) of the corresponding compounds in 0.3 × Danieau's solution.

In all cases control embryos were exposed to the corresponding solubilizing agent used, and this group was therefore named 'solvent control' (see Table 1). Embryos were incubated at 26 ± 1 °C with a light: dark cycle of 14:10 h until 77 hpf under semi-static conditions, as exposure solutions were renewed by freshly prepared solutions every 24 h (Fig. 1).

2.3.2. Evaluation of lethality, dysmorphogenesis and calculation of teratogenic index (TI)

At 29, 53 and 77 hpf embryos were observed under a stereomicroscope to evaluate lethality. Lethality criteria were applied according to OECD Test Guideline n.236 and were from 24 hpf on: coagulation of embryos, lack of somite formation, or non-detachment of the tail, and from 48 hpf on, the same three criteria or lack of heartbeat [OECD, 2013]. At the same time-points dysmorphogenesis was evaluated only in live embryos. The lethality and dysmorphogenesis results of 10 embryos per concentration was calculated in % and afterwards, the mean of at least three independent experiments was obtained. A sigmoidal (variable slope) curve-fit was applied to the means to obtain concentration-response curves and to calculate the LC_{50} (concentration causing lethality to 50% of larvae) and EC_{50Dysm} (concentration causing dysmorphogenesis in 50% of larvae). Teratogenic Index (TI) was calculated as follows when both, LC_{50} and EC_{50Dysm} could be obtained: $TI = LC_{50}/EC_{50Dysm}$. Compounds were defined as teratogenic if $TI > 2$.

2.3.3. Total morphological score (TMS)

At 77 hpf TMS was evaluated only in live embryos following the score proposed by Beekhuijzen et al. [2015], to have a measurement of morphological development in relation to the stage/hpf. The maximum score achievable was 15 points and significantly lower scores were interpreted as developmental delay. Although hatching was one of the parameters included in the TMS, it was also evaluated and represented in figures independently.

2.3.4. Touch-evoked response (TER) test

Since neurodevelopmental adverse effects might not be visible in a morphological evaluation, a simple behavioural test was added to the assessment. At 77 hpf the TER test was conducted following the protocol described in Guzman et al., [2020] and recorded with a videocamera (Casio Exilim EX-ZR200). Briefly, the distance swum by zebrafish embryos after three mechanical stimuli (each one every 10 s) in the tail with a forceps tip (FST, Dumont #5) was averaged and transformed from pixels to mm. This assay was only performed in larvae from concentration groups with lethality and dysmorphogenesis <20%. A total of 6 larvae were evaluated per each concentration group in at least 3 independent experiments.

Table 1
Test compounds with CAS RN, solvent used and origin.

Abbreviation	Name	CAS RN	Origin	Solvent
Artemisinin	Artemisinin	63968-64-9	Sigma-Aldrich	DMSO
Quinine	Quinine sulfate	207671-44-1	Sigma-Aldrich	DMSO
Chloroquine	Chloroquine diphosphate	50-63-5	Sigma-Aldrich	$_dH_2O$
DBL	(2)-Desbutyl-lumefantrine	355841-11-1	Santa Cruz Biotechnology	DMSO
DHA	Dihydroartemisinin	71939-50-9	Sigma-Aldrich	DMSO
DONE3TCl	6-Chloro-9-[(3-{4-[(5,6-dimethoxy-1-oxoindan-2-yl) methyl]piperidin-1-yl}propyl)amino]-1,2,3,4-tetrahydroacridine dihydrochloride	955993-18-7	Synthesized at affiliation "f" as described in [Camps et al., 2008]	DMSO
YAT2150	1,1'-(decane-1,10-diyl)bis{4-[(E)-4-(diethylamino) styryl]-3-methylpyridin-1-ium} dibromide	2924230-19-1	Synthesized at affiliation "f" as described in [Bouzón-Arnáiz et al., 2022]	DMSO
GS-GUAN	Methyl 6-deoxy-6-[(2-guanidinoethyl) thio]-α-D-glucopyranoside	2222816-02-4	Synthesized at affiliation "f" as described in [Alencar et al., 2018]	$_dH_2O$

2.3.5. Hemoglobin staining (HbSt)

After TER, hemoglobin staining was performed following the protocol described by [Paffet-Lugassy & Zon, 2005]. Dechorionated zebrafish larvae of 77 hpf were exposed to the o-dianisidine, NaOAc, $_dH_2O$ and H_2O_2 solution for 30 min (under darkness and room temperature (RT) conditions). Larvae were washed with $_dH_2O$ and fixed with paraformaldehyde (PFA) (4%) overnight and their natural pigmentation was bleached afterwards using a solution of KOH (0.8%), H_2O_2 (0.9%) and Tween-20 (0.1%) during 30 min at RT. After a second washing with phosphate-buffered saline (PBS) containing Tween-20 at 0.1% and a second fixation step (in 4% PFA overnight at 4 °C), larvae were stored in PBS at 4 °C until imaging. The staining was performed in 6 larvae per concentration in at least 3 independent experiments for each compound, and only at those concentrations with lethality <20%.

2.3.6. HbSt imaging and image analysis

Stained larvae were oriented dorsally and imaged using a camera (Imaging Source, DFK 72AUC02) connected to a stereomicroscope. Images were analysed using the ImageJ programme 1.53k (64-bit) [Schneider et al., 2012]. Images were converted to 8-bit, and contrast and brightness were adjusted with the aim to isolate the hemoglobin-stained area. At this point images were processed to black and white using the "make binary" function, the black area was selected and the black pixels measured (Fig. 1). Results, representing the area of hemoglobin stained, were expressed in percentage of control group.

2.4. Quality criteria

The following quality criteria were established to consider experiments as valid.

- The percentage of fertilized eggs in a spawn should be ≥ 70%.
- Survival of control group at 77 hpf should be ≥ 90%.
- Percentage of dysmorphogenesis in control group at 77 hpf should be ≤ 10%.
- In TER assay, the mean swum distance in control group should be ≥ 20 mm.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism v.8.2.1. Lethality, dysmorphogenesis, TMS, TER and HbSt were analysed using one-way ANOVA and the post-hoc multiple-comparison Dunnett test. Hatching was analysed using a two-way ANOVA (considering concentration and time as variables) and the Tuckey multiple comparison test. In all statistical analysis, significance threshold was established at $p < 0.05$.

3. Results

3.1. Evaluation of marketed antimalarial drugs

3.1.1. Artemisinin

Current first line antimalarial treatments for uncomplicated malaria caused by *P. falciparum* in general population are based on ACTs [WHO, 2023]. Several studies have described dysmorphogenic, teratogenic or lethal effects in animals *in vivo* and *in vitro* after developmental exposure to artemisinin [reviewed in Gomes et al., 2016; Clark 2009; Recht et al., 2023], and for this reason there has been for a long time the recommendation to exclude pregnant women during the first trimester from this treatment group. The teratogenic effects described in the literature are mainly cardiovascular and skeletal defects, which occur in

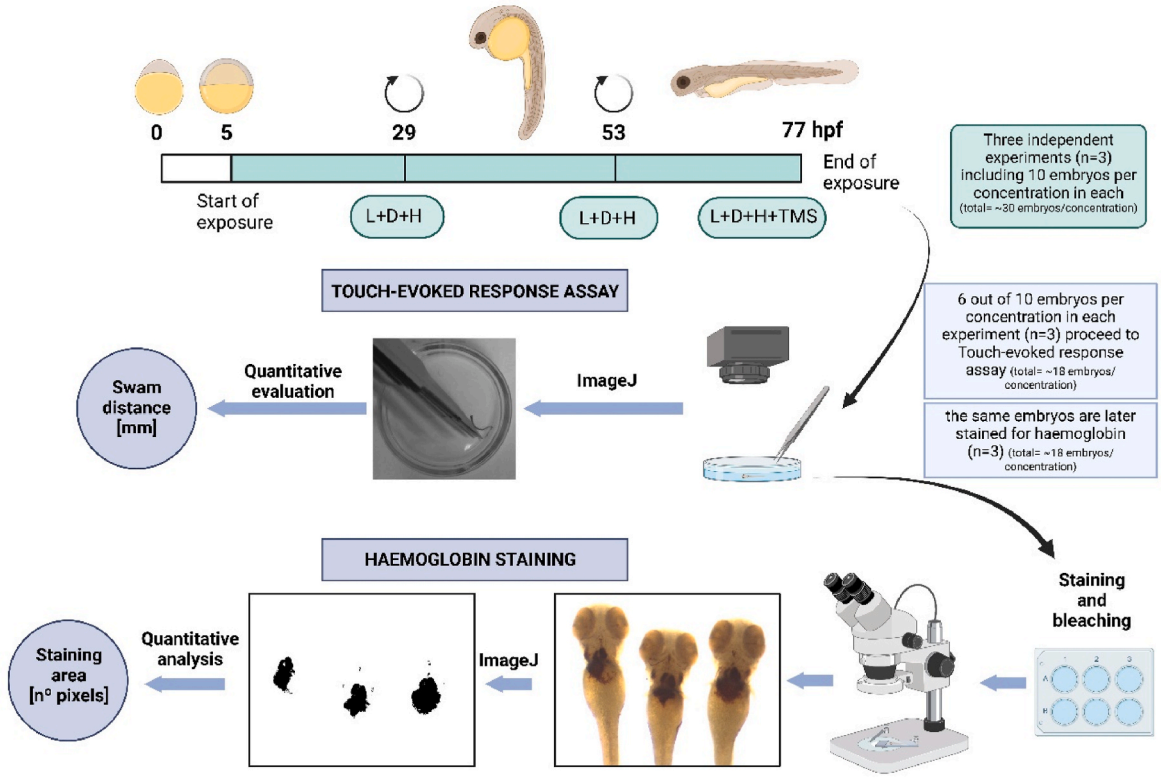

Fig. 1. Schematic representation of the experimental procedure including the endpoints evaluated at 29, 53 and 77 hpf. Circular arrows at 29 and 53 hpf indicate the renewal of water with the corresponding concentration of the tested drug, L: lethality, D: Dysmorphogenesis, H: hatching, hpf: hours postfertilization; TMS: total morphological score. Figure created with BioRender.com.

association with increased resorptions. Considering these results, artemisinin was selected as a positive control in our study. Because the mechanism of embryotoxicity of artemisinin is related to a depletion of circulating embryonic primitive erythroblasts [Gomes et al., 2016; Clark et al., 2008; White et al., 2006], artemisinin was also expected to be a positive control in the HbSt test added to the ZEDTA.

Indeed, a significant concentration-response relationship was observed after exposure to artemisinin for dysmorphogenic effects at 29 hpf, but not for lethality leading to a TI of at least 3.4. The lowest-observed adverse effective concentration (LOAEC) for dysmorphogenesis was 50 μM, which is far above relevant therapeutic concentrations in humans, but since the compound was soluble, high concentrations were tested. At 77 hpf a significant concentration-response relationship was found for both lethality (LOAEC = 200 μM; LC_{50} = 153 μM) and dysmorphogenic effects (LOAEC = 100 μM; EC_{50Dysm} = 75.15 μM) (Fig. 2). The different sensitivity of zebrafish larvae to both endpoints led to a TI = 2.04. Taking into consideration that the TI of both time-points was >2 the test was leading to the classification of artemisinin as potentially teratogenic. The observed dysmorphogenesis were mainly pericardial edema, cardiomegaly, and flexion alterations (Fig. 3). Besides these main effects, the TMS clearly indicated a subtler but significant developmental delay occurring already at 10 times lower concentrations than LC_{50} (LOAEC = 12.5 μM). However, a delay was not affecting hatching until 200 μM. No specific neurodevelopmental significant adverse effect was observed in the TER at any tested concentration (up to 25 μM according to the criteria defined in Materials and Methods section). A complete absence of HbSt was observed at all tested concentrations (LOAEC = 12.5 μM), indicating a specific adverse effect of artemisinin consisting on erythrocyte depletion. Therefore, with this combination of endpoints we could identify two hazards for artemisinin, teratogenicity and erythrocyte depletion.

3.1.2. *Quinine*

Quinine has been for many years and until 2022 the drug of first election recommended by the WHO for the treatment of *P. falciparum* malaria in pregnant women during the first trimester, coadministered with clyndamicin [WHO, 2021]. Standard doses of quinine during pregnancy do not increase the risk of abortion, preterm delivery or malformations [reviewed by Phillips-Howard and Wood, 1996]. For these reasons, quinine was selected as negative control in our study. That clyndamicin is a true negative control in the zebrafish embryo assay was already proved elsewhere [Song et al., 2021].

No significant adverse effects were observed in lethality, dysmorphogenesis, TMS, hatching or HbSt up to 400 μM quinine concentrations (Fig. 4). Therefore, it was not possible to calculate a TI and the compound was classified as non-teratogenic.

However, a concentration-dependent specific behavioural adverse effect was identified, since quinine significantly reduced the swum distance at all concentrations tested in the TER test. To better characterize this effect, and to be able to obtain a NOAEC (non-observed adverse effect concentration), the concentration range of this specific experiment was expanded to lower concentrations following a factor 2 dilution series until 1.5 μM, and the NOAEC = 12.5 μM was found (Fig. 4E). Quinine has not previously been described as a developmental neurotoxic in *in vivo* studies, but it is known to block acetylcholine (ACh)-evoked responses in human adult and fetal nAChR (nicotinic acetylcholine receptors) [Gisselmann et al., 2018], and in fact it has been clinically used as therapy and prophylaxys for nocturnal leg cramps [Diener et al., 2002]. To distinguish if the behavioural adverse effect in larvae was acute and transient (and just observed due to the performance of the TER directly after quinine exposure) or permanent (and thus with potential developmental neurotoxicity relevance), we performed two more types of TER tests with or without a 24 h wash-out period after 48 h of exposure to the drug (Fig. 5). The significant

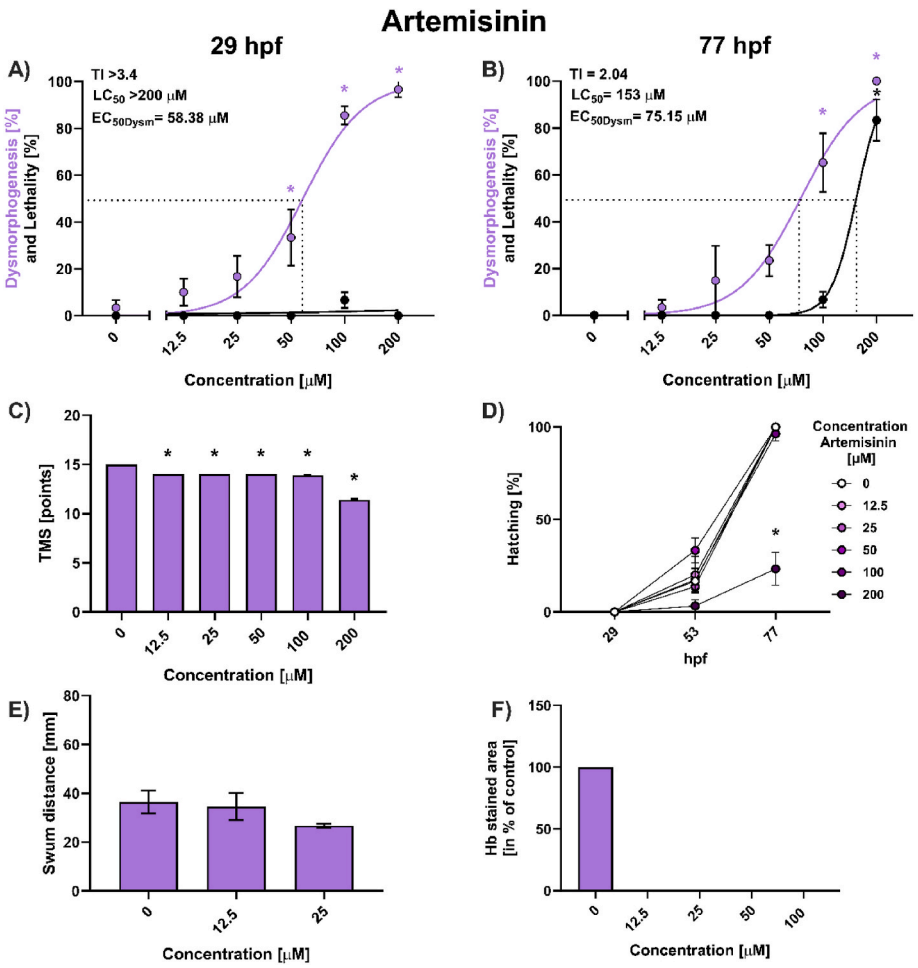

Fig. 2. Artemisinin is teratogenic in zebrafish embryos and causes depletion of erythrocytes. Results obtained in zebrafish larvae exposed to artemisinin for A) Lethality and dysmorphogenesis at 29 hpf, B) Lethality and dysmorphogenesis at 77 hpf, C) Total Morphological Score (TMS) at 77 hpf, D) Hatching over-time, E) Touch-evoked response at 77 hpf (TER) and F) Hemoglobin staining (HbSt) at 77 hpf. Results presented as mean ± SEM of at least three independent experiments including 10 (A to D) or 6 embryos (E and F) per concentration in each experiment (in total at least 30 or 18 embryos per concentration). Statistical comparisons performed by one-way ANOVA and Dunnett test (A to C and E) or by two-way ANOVA and Tukey test (D). No statistical analysis could be performed for HbSt (F) because all samples had a standard error of zero. LC_{50} = Lethal concentration 50, EC_{50Dysm} = Effective concentration 50 for Dysmorphogenesis, TI= Teratogenic Index. * indicates a p value < 0.05 in comparison to control.

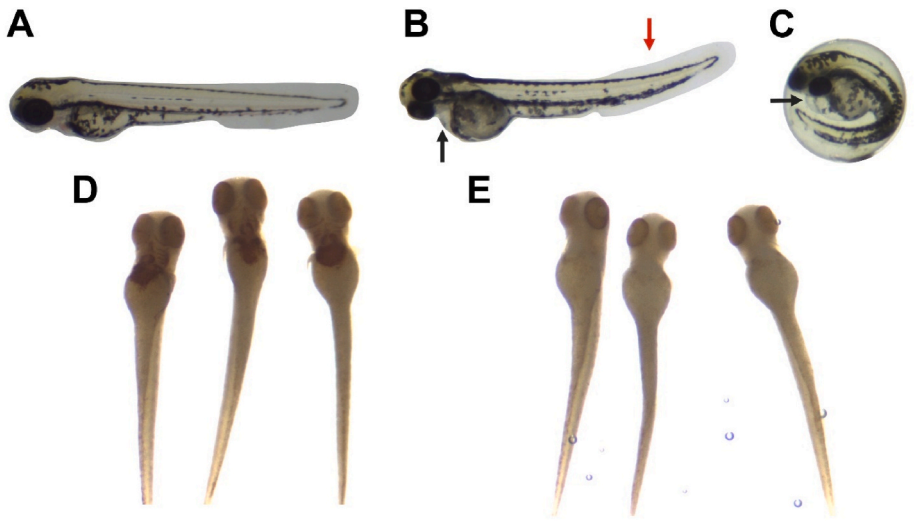

Fig. 3. Representative images of alterations induced by artemisinin at 77 hpf. A) Solvent control; B) 100 µM artemisinin; C) 200 µM artemisinin. D) HbSt solvent control, E) HbSt 12.5 µM artemisinin. Black arrow: pericardial edema; red arrow: abnormal flexion. Created with BioRender.com.

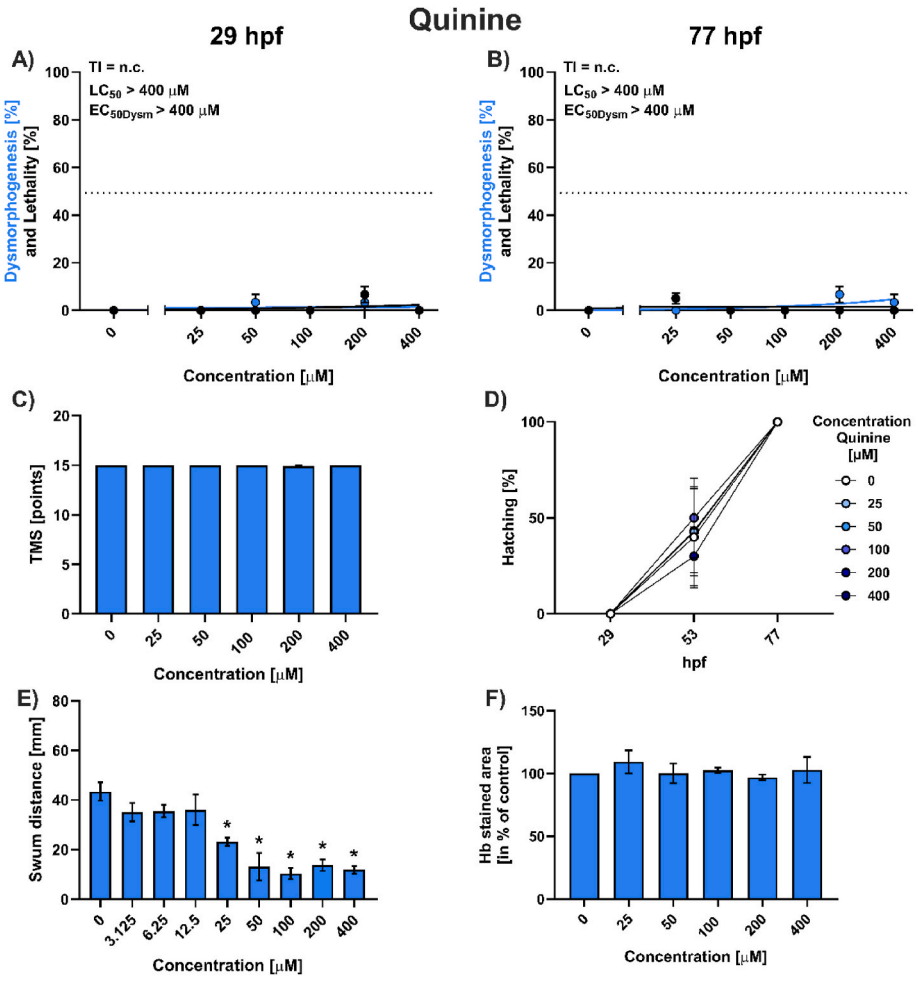

Fig. 4. Quinine is not teratogenic in zebrafish embryos. Results obtained in zebrafish larvae exposed to quinine. Details about results presented and statistical analysis performed are explained in the legend of Fig. 2, except for F), where a one-way ANOVA analysis followed by Dunnett test could be performed. *: $p < 0.05$. n. c.: not calculable.

reduction in swum distance detected in the TER assay after 48 h of quinine exposure without wash-out (from 24 to 72 hpf) (Fig. 5A) was reverted back to control levels when a 24 h wash-out period was added after the 48 h of exposure (from 4 to 56 hpf), proving that the effects were transitory (Fig. 5B).

3.1.3. *Chloroquine*

Chloroquine is another drug considered safe in the first trimester of pregnancy [WHO, 2021], and it is recommended to be used as weekly chemoprophylaxis during pregnancy until delivery and breastfeeding to prevent relapse in *P. vivax* or *P. ovale* malaria [WHO, 2021, 2023]. Therefore, this drug was also used as a negative control in our study.

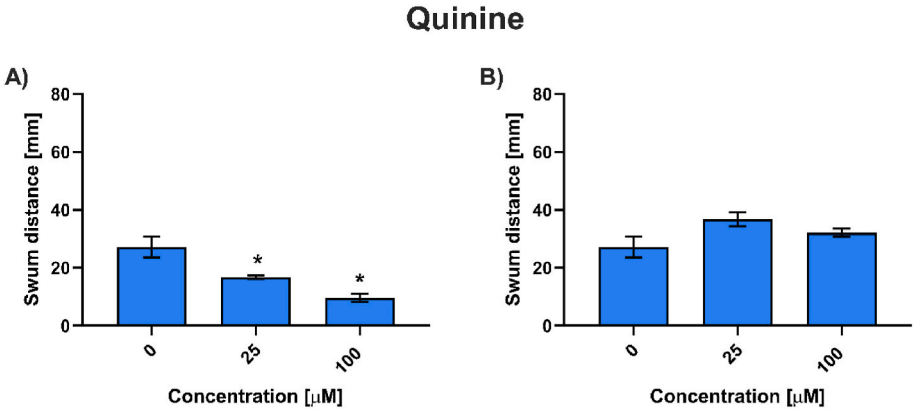

Fig. 5. Quinine behavioural effects in zebrafish larvae are reversible after a 24 h wash-out period. Results obtained at 77 hpf in the TER test in zebrafish larvae exposed to quinine for 48 h: A) exposure from 29 to 77 hpf, B) exposure from 5 to 53 hpf +24 h of wash-out. Results presented as mean ± SEM of at least three independent experiments including 6 embryos per concentration in each experiment (in total at least 18 embryos per concentration). Statistical comparisons performed by one-way ANOVA and Dunnett test. * indicates a p value < 0.05 in comparison to control.

Again, no significant adverse effects were detected in lethality, dysmorphogenesis, TMS, hatching or HbSt up to 400 μM chloroquine concentrations (Fig. 6); in consequence no TI could be calculated, and in view of these results the compound was classified as non-teratogenic.

However, again a concentration-dependent adverse effect in the behavioural study was found. This time only one concentration was significantly affected, and the NOAEC could be directly established at 50 μM, but we cannot exclude that this significant effect could be an artifact of the test (Fig. 6E). For chloroquine it was also already described in the literature that it blocks ACh-evoked responses, but less potently than quinine [Gisselmann et al., 2018].

3.1.4. *Desbutyl-lumefantrine + dihydroartemisinin*

The November 2022 version of the WHO guidelines for malaria treatment indicated for the first time that "pregnant women with uncomplicated *P. falciparum* malaria should be treated with artemether-lumefantrine during the first trimester of pregnancy" [WHO, 2022], as a consequence of the meta-analysis performed by Dellicour et al. (2017). This statement was indicated as a strong recommendation with low certainty evidence, while still mentioning that the current evidence is insufficient to make a recommendation for routine use of other ACTs in the first trimester of pregnancy. Previously, this combination was not recommended for use in early pregnancy because studies in animals demonstrated the potential adverse pregnancy outcomes associated with the use of artemisinins in general in the first trimester [Castro et al., 2024].

Dihydroartemisinin (DHA), the main metabolite of artemether, has already been tested in zebrafish embryos [Ba et al., 2013] and rat embryos *in vitro* [Longo et al., 2006] where it produces very severe dysmorphogenic effects at 48 but not at 24 hpf (mainly pericardial edema and curved trunk), and where the erythrocytic depletion mode of action was already confirmed. Therefore, the hazard of teratogenicity and erythrocyte depletion have already been identified for this compound. However, to help in the risk assessment procedure, it is important to take into account the exposure time and concentrations at which the effects occur, especially when the drug is used in combination with lumefantrine. To the best of our knowledge, the newly WHO-recommended combination has never been tested in zebrafish. Due to the low solubility and rapid metabolism of lumefantrine and artemether, their active metabolites desbutyl-lumefantrine (DBL), and DHA, respectively, were tested as a combination in the ZEDTA using the 6 to 1 proportion given as fixed dose combination for therapeutic dosage [WHO, 2022]. High concentrations of the drugs were tested in a preliminary range-finding study, by which 120 μM DBL +20 μM DHA and 60 μM DBL +10 μM DHA were directly discarded due to immediate precipitation. Four concentration groups from 30 μM DBL +5 μM DHA to 3.75 μM DBL +0.63 μM DHA (with dilution factor 2) were discarded due to precipitation, but 100% lethality was observed in the two highest concentration groups and 30% and 10% dysmorphogenesis was observed in the two following groups. Therefore, further studies were performed with 1.8 μM DBL +0.3 μM DHA as maximum concentration group with 4 decreasing concentrations with dilution factor 2, where no precipitation

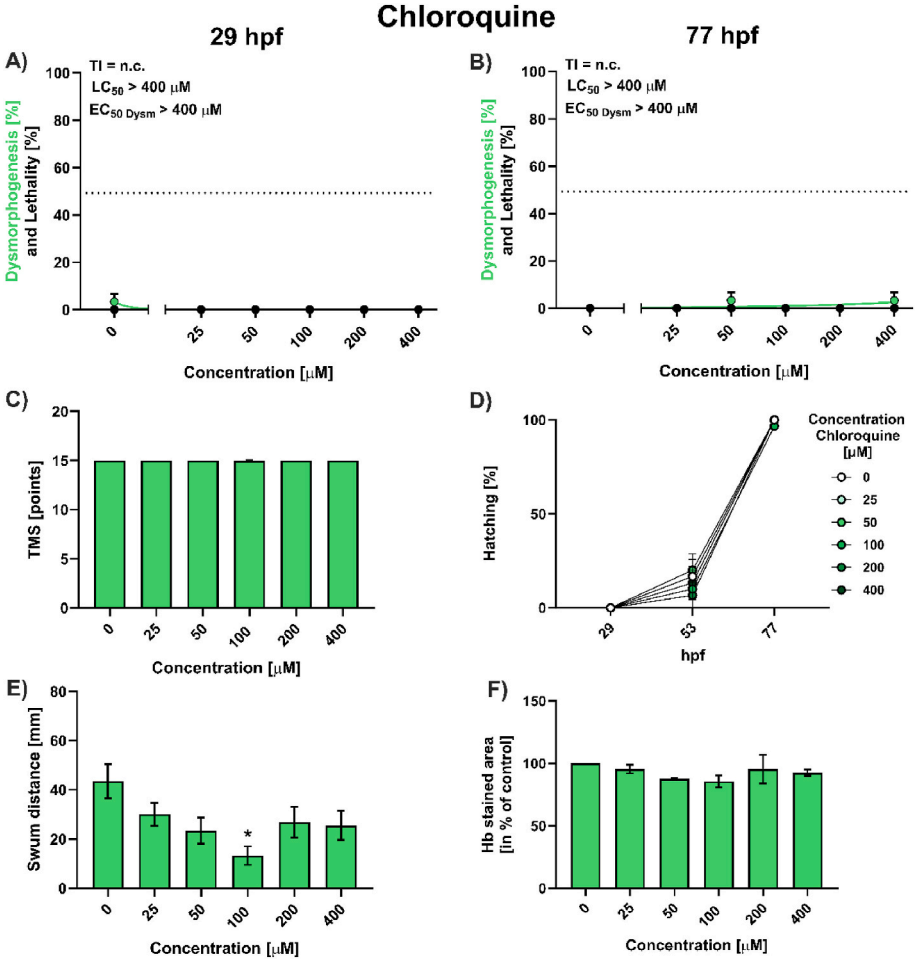

Fig. 6. Chloroquine is not teratogenic in zebrafish embryos. Results obtained in zebrafish larvae exposed to chloroquine. Details about results presented and statistical analysis performed are explained in the legend of Fig. 2, except for F) where in this case, a one-way ANOVA analysis followed by Dunnett test could be performed. *: $p < 0.05$. n.c.: not calculable.

occurred. At this concentration range (Fig. 7) no significant adverse effects in lethality or dysmorphogenesis were observed, and therefore no TI could be calculated. At 77 hpf LC_{50} and EC_{50Dysm} were occurring at the range of the precipitated concentrations, which means these results should be taken cautiously (they are presented in Fig. 7 only for orientation). A slight but significant decrease in TMS was observed at the highest soluble concentration, mainly related to the lack of visible circulation. No significant adverse effect was detected in the TER, although a general and not concentration-dependent decrease in swimming distance was noticed. The main effect "decrease in HbSt" related to the mechanism of embryotoxicity of artemisinin derivatives in animal studies "depletion of circulating embryonic primitive erythroblasts" [White et al., 2006] was clearly detected at the two highest concentrations tested. Therefore, this combination of tests was able to detect the hazard described in animal studies: erythrocyte depletion, but due to DBL solubility limitations, teratogenic effects were only observed at concentrations where DBL precipitated.

3.2. Evaluation of new antimalarial drug candidates

After testing several clinically used drugs against malaria which are recommended or not recommended for its use during the first trimester of pregnancy, and identifying the presence/absence of hazards in the ZEDTA and HbSt tests, we proceeded to test three investigational antimalarial drug candidates, which display novel mechanisms of action, different from those of the marketed antimalarial drugs and other drug candidates under development [Alencar et al., 2018; Sola et al., 2015; Bouzón-Arnáiz et al., 2022; Camps et al., 2008].

3.2.1. GS-GUAN

GS-GUAN is a member of a new family of antiplasmodial compounds based on the selective inhibition of the bifunctional enzyme glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase of *P. falciparum* (*Pf*G6PD-6PGL), which affords protection against reactive oxygen spieces and is essential for the survival of the parasite during the infection stage [Allen et al., 2015]. Alencar et al. (2018) described the first 3D structural homology model of the G6PD domain of the *Pf*G6PD-6PGL enzyme, which revealed a critical structural difference in the substrate binding site of the parasitic enzyme versus the human enzyme hG6PD. Indeed, an arginine in position 365 in hG6PD (R/Arg365), positively charged at physiological pH, is replaced by a negatively charged aspartate residue in the equivalent position of *Pf*G6PD (D/Asp750). This results in a drastic change of the charge and size of the side chains of these residues in *Pf*G6PD and hG6PD, which was leveraged for the

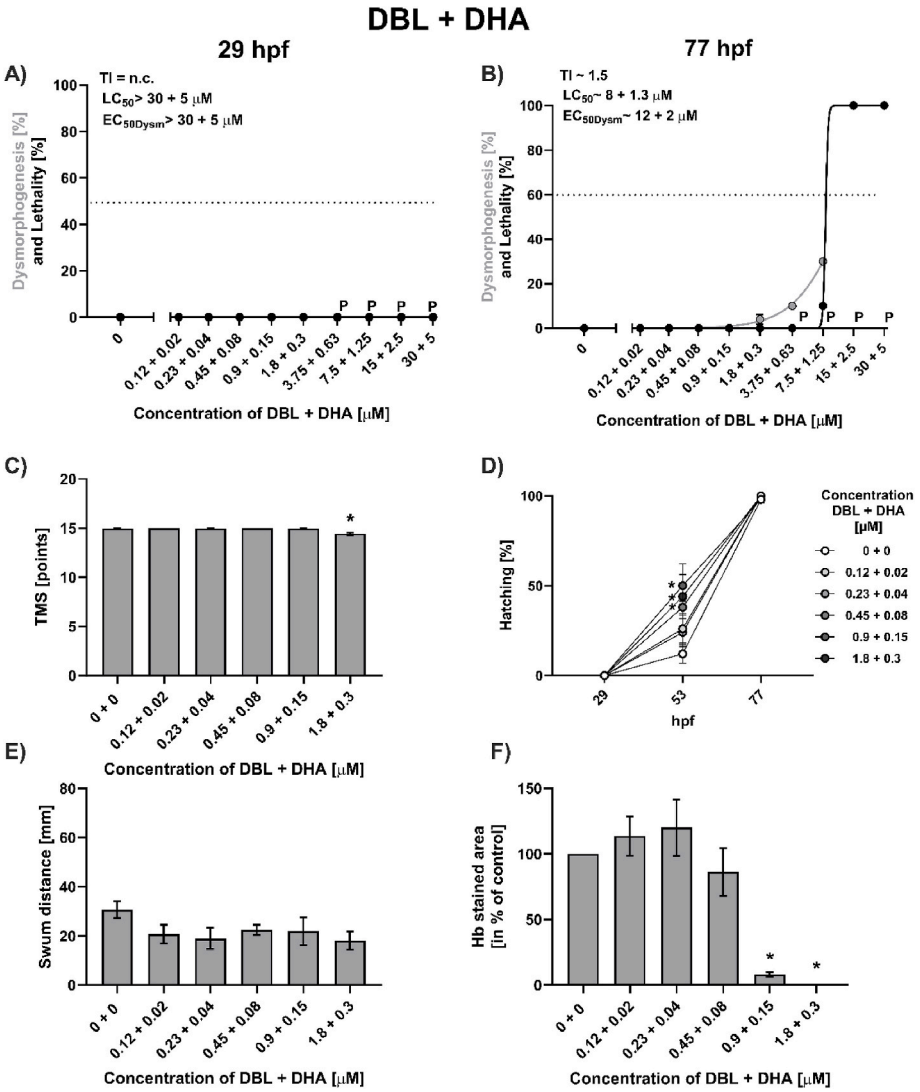

Fig. 7. Desbutyl-lumefantrine and dihydroartemisinin. Results obtained in zebrafish larvae exposed to desbutyl-lumefantrine and dihydroartemisinin. Details about results presented and statistical analysis performed are explained in the legend of Fig. 2, except for F) where in this case, a one-way ANOVA analysis followed by Dunnett test could be performed. *: $p < 0.05$. n.c.: not calculable. P: indicates precipitation at the corresponding concentration and therefore only $n = 1$.

rational design of this class of inhibitors with selectivity for the parasite over the human enzyme.

Here, we compared first the substrate binding site of hG6PD with that of zebrafish. Thus, the hG6PD sequence was compared with the sequence of the 4 isoforms of the enzyme that exist in zebrafish (X1 to X4) using NCBI BLAST software [BLAST NCBI software; Camacho et al., 2009]. As depicted in Supplementary Fig. 1, the sequence of the substrate binding site is identical in human and zebrafish proteins for all isoforms, thereby confirming that the zebrafish is a good model for testing this family of compounds. The next step was to test GS-GUAN, as the lead compound of this structural family, for developmental toxicity in our model assay. No lethality was observed at any tested concentration and all embryos underwent a normal development without any significant effect in dysmorphogenesis, TMS, hatching, or HbSt up to 1200 µM GS-GUAN (Fig. 8). However, again a significant decrease in the TER assay was observed, in this case starting at 600 µM.

3.2.2. *DONE3TCl*

DONE3TCl is a 4-aminoquinoline based compound that was initially developed as an anti-Alzheimer disease agent, in part by virtue of its ability to inhibit the aggregation of amyloidogenic proteins, such as the β-amyloid peptide [Camps et al., 2008]. Later, due to its structural relationship with known 4-aminoquinoline based antimalarial drugs, such as chloroquine, its antiplasmodial activity was tested and confirmed [Sola et al., 2015]. This compound seems to share the mechanism of action of chloroquine [Sola et al., 2015], even though another novel mechanism involving disruption of protein aggregation in *P. falciparum* (see below) could also play a role in the antiplasmodial activity of DONE3TCl. Thus, it was selected as potentially interesting for treatment during pregnancy. The range tested for DONE3TCl covered concentrations up to 100 µM, and that was sufficient to identify severe adverse effects at several endpoints. A significant concentration-response relationship for both lethality and dysmorphogenic effects was detected at both time-points 29 hpf (LOAEC = 100 µM; LC_{50} > 100 µM for lethality and LOAEC = 6.25 µM; EC_{50Dysm} = 5.1 µM for dysmorphogenesis) and 77 hpf (LOAEC = 50 µM; LC_{50} = 49.9 µM for lethality and LOAEC = 6.25 µM; EC_{50Dysm} = 6.1 µM for dysmorphogenesis) (Fig. 9). This large difference between both endpoints at both time-points led to the classification of DONE3TCl as potential teratogenic with a TI of 8.2 at 77 hpf and even higher at 29 hpf. The main dysmorphogenic effects detected (Fig. 10) were pericardial edema, abnormal flexion, craniofacial alterations (thickening of the jaw), and a bigger yolk sac, which normally is considered to be related to developmental delay. This developmental delay was in parallel clearly indicated by the TMS (LOAEC = 12.5 µM), and could be visually observed in the size of the larvae. For this reason, an extra endpoint was measured for this compound, the larvae length, as indicator of growth. Indeed, a significant reduction in length was already found at 6.25 µM DONE3TCl, although this delay was not affecting hatching. Moreover, a specific adverse effect in behaviour was observed in the TER at all tested concentrations, and a significant reduction of HbSt was detected at 6.25 µM and higher concentrations. With this information, two hazards were

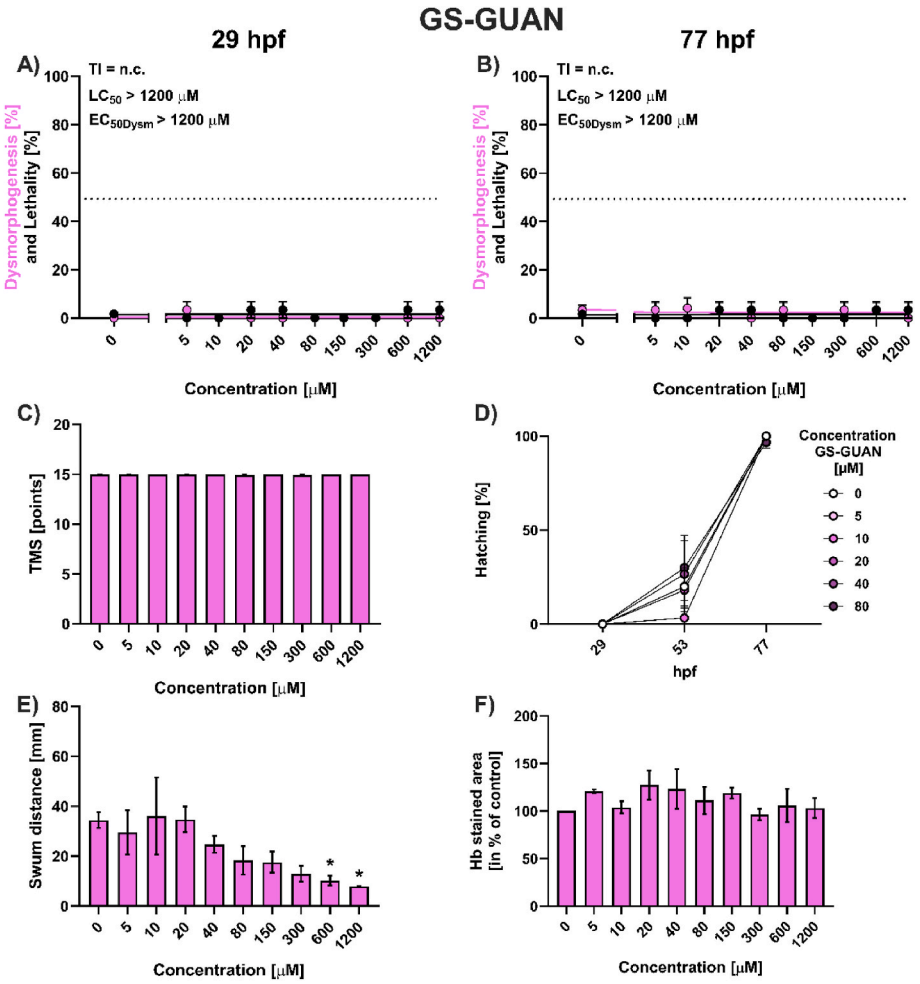

Fig. 8. GS-GUAN is not teratogenic in zebrafish embryos. Results obtained in zebrafish larvae exposed to GS-GUAN. Details about results presented and statistical analysis performed are explained in the legend of Fig. 2, except for F) where in this case, a one-way ANOVA analysis followed by Dunnett test could be performed. *: $p < 0.05$. n.c.: not calculable.

assigned to DONE3TCl: teratogenicity and erythrocyte depletion.

3.2.3. YAT2150

YAT2150 is a new antimalarial agent, whose mechanism of action seems to be the inhibition of protein aggregation, which is strikingly abundant in *P. falciparum*, where it seems to play a functional role [Bouzón-Arnáiz et al., 2022]. The maximum concentration tested for this compound was 8 μM due to its solubility limitations. A concentration-dependent effect in lethality and dysmorphogenesis induction was identified at 29 and 77 hpf, with a LC_{50} = 2.4 and 2.15 μM and EC_{50Dysm} = 2.8 and 1.87 μM, respectively (Fig. 11). This resulted in a TI = 0.86 at 29 hpf and a TI = 1.15 at 77 hpf, and the compound was therefore not considered as potentially teratogenic.

Regarding the TMS, a significant reduction was only observed in the few embryos that survived at 4 μM. A concentration-dependent effect in hatching was detected, but it was only significant at 0.0625 μM, producing an increase compared to the control group, while the decreases produced at higher concentrations did not reach a significant level. The swum distance was concentration-dependently decreased, with a LOAEC = 0.5 μM, and there was no alteration in HbSt at any of the tested concentrations. In view of these results, YAT2150 was considered to not have teratogenic, neither erythrocyte depletion hazard.

4. Discussion

In this study we have shown the applicability of the ZEDTA for the

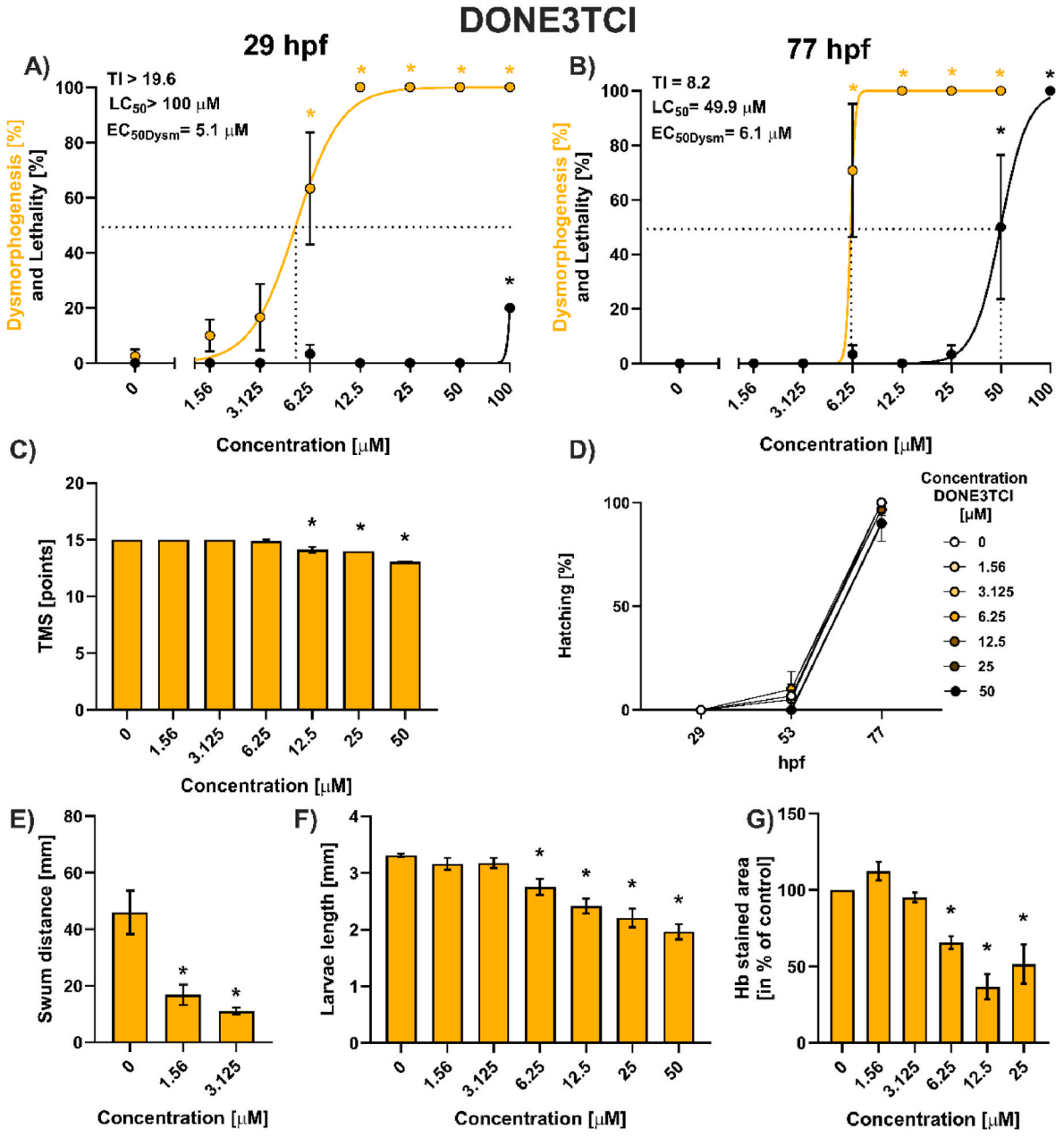

Fig. 9. DONE3TCl is teratogenic in zebrafish embryos. Results obtained at in zebrafish larvae exposed to DONE3TCl for A) Lethality and dysmorphogenesis at 29 hpf, B) Lethality and dysmorphogenesis at 77 hpf, C) Total Morphological Score (TMS) at 29 and 77 hpf, D) Hatching over-time, E) Touch-evoked response (TER) at 77 hpf, F) larvae length at 77 hpf, and G) Hemoglobin staining (HbSt) at 77 hpf. Results presented as mean ± SEM of at least three independent experiments including 10 (A to D and F) or 6 embryos (E and G) per concentration in each experiment (in total at least 30 or 18 embryos per concentration). Statistical comparisons performed by one-way ANOVA and Dunnett test (A to C, E, F and G) or by two-way ANOVA and Tukey test (D). LC_{50} = Lethal concentration 50, EC_{50Dysm} = Effective concentration 50 for Dysmorphogenesis, TI= Teratogenic Index. * indicates a p value < 0.05 in comparison with control.

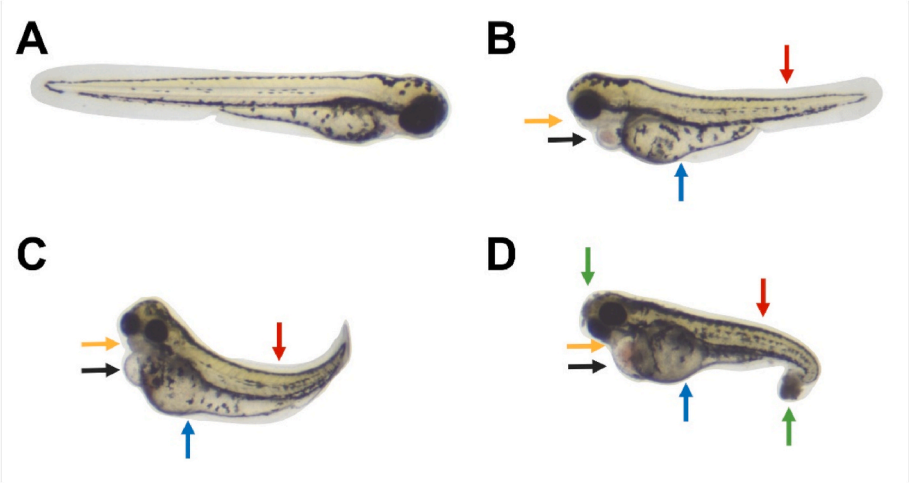

Fig. 10. Representative images of alterations induced by DONE3TCl at 77 hpf. Representative pictures of: A) Solvent control, B) 12.5 μM group, C) and D) 25 μM group. Alterations are indicated as follows: pericardial edema with black arrow, curved trunk with red arrow, thickening of the jaw with yellow arrow, enlargement of yolk sac with blue arrow, and coagulation of tissues at the cranial area and tail with green arrows. Created with BioRender.com.

drug discovery process of antimalarial compounds, and the advantages that it has for an early prioritization of drug candidates which are safe during pregnancy.

We have performed a comprehensive characterization of the developmental effects in zebrafish embryos of several commercialized antimalarial drugs, covering four drugs that have been recommended/accepted for their use during the first trimester of pregnancy (quinine, chloroquine, DBL + DHA) and one which is not recommended (artemisinin). We have followed a broad approach that covers not only the detection of lethality and dismorphogenesis, but also the evaluation of subtler endpoints like developmental delays (by means of TMS, hatching and in one case larvae length), neurodevelopmental alterations and erythrocyte depletion. With this strategy we have been able to identify hazards (summarized in Table 2) that correlate very well to those hazards identified in rat studies, mainly teratogenicity and erythrocytic depletion [Clark 2009; Longo et al., 2010].

However, to move from hazard identification to risk assessment it is important to know that in rat embryos, the target of artemisinin toxicity are the circulating primitive erythroblasts [D'Alessandro et al., 2020], and that there are differences in erythropoiesis during embryo-fetal development between rat and human species [Gomes et al., 2016]. While in rats, primitive erythroblasts are formed and predominant in the circulation for 48 h, in humans this period lasts for approximately 6 weeks [Gomes et al., 2016; D'Alessandro et al., 2020]. Therefore, antimalarial exposure in rats easily covers the whole susceptible period, and then erythrocyte depletion cannot be compensated and turns into severe deleterious effects for the embryos [Longo et al., 2006]. On the contrary, since the toxicity target cells are formed over a longer period of time in humans, which is much longer than the typical 3–7 days treatment period, damaged cells can be replaced by newly formed cells, and the toxic effects become marginal with minimal clinical consequences [D'Alessandro et al., 2020; Gomes et al., 2016]. For comparison, in zebrafish, primitive erythrocytes begin to enter circulation at approximately 24 hpf (with the onset of blood circulation) and primitive erythropoiesis accounts for all circulating erythrocytes for the first 4 days after fertilization [reviewed by Kulkeaw and Sugiyama 2012]. Since these differences between the proportion of timing of administration and susceptibility window seem to be key factors in the appearance of embryo damage [González et al., 2020] and they are proposed to be a main reason for the differences in effects observed between animal studies and clinical data [D'Alessandro et al., 2020], we have decided to include two time-points of assessment in our study. The first time-point is at 29 hpf, after a period when the morphological development is independent of primitive erythrocytes. It covers the first part of organogenesis and would compare to the organogenesis occurring during the first 22 pregnancy days in humans. The second time-point is at 77 hpf, when the morphological development depends on primitive erythrocytes function. It allows the performance of hemoglobin staining, covers almost all the organogenesis period and would better approximate to a morphological development in some aspects similar to the whole 1st trimester of pregnancy in humans.

By comparing the results of the clinically used drugs for these two time-points it can be observed that DBL + DHA is not inducing dysmorphogenesis at 29 hpf, but at 77 hpf, which is in agreement with previous studies exposing zebrafish embryos to DHA alone and observing dysmorphogenesis at 48 hpf at 3.5 μM but not at 24 hpf up to 35 μM [Ba et al., 2013]. This combination of time-points, together with the information on significant erythrocyte depletion at 77 hpf, helps to identify a compound whose mechanism of teratogenicity is linked to erythrocyte depletion and gives the opportunity to consider if the effects could be relevant for humans or not, for example in case longer exposures are expected due to relapse, reinfection or to preventive intermittent use. This is a major advantage that can be achieved in an economical, ethical, and fast way during the screening phase of drug candidates. Besides that, this combination of time- and endpoints allows to identify compounds with no or lower hazard on teratogenicity or erythrocyte depletion, which would be of utmost importance because, independently of the posterior risk assessment for clinical purposes, it would be more desirable to have drug candidates that do not entail any developmental hazard at all (summary of possibilities in Fig. 12), also no hazards in developmental delay (which could be related to the TMS endpoint) or to developmental neurotoxicity (related to the TER test). To perform a better risk assessment, it would be necessary to determine the internal concentrations of the test compounds in the zebrafish embryos or larvae, in order to better compare the real effective concentrations for all endpoints, and to better compare them with the expected concentrations *in vivo* (if the information is known) or with the IC_{50} *(P. falciparum)*.

By screening with the proposed tests three new investigational drug candidates with novel mechanisms of action, we have observed three very different profiles. On the one hand, GS-GUAN entails no hazard and induces no adverse effect up to 1200 μM, which is a very safe toxicological profile, but when the balance efficacy/toxicity is evaluated (data from literature included in Table 2), the compound has a low efficacy against *P. falciparum* and therefore other candidates with higher efficacy should be prioritized. On the other hand, DONE3TCl has teratogenic and erythrocytic depletion hazards and also induces teratogenicity at 29 hpf.

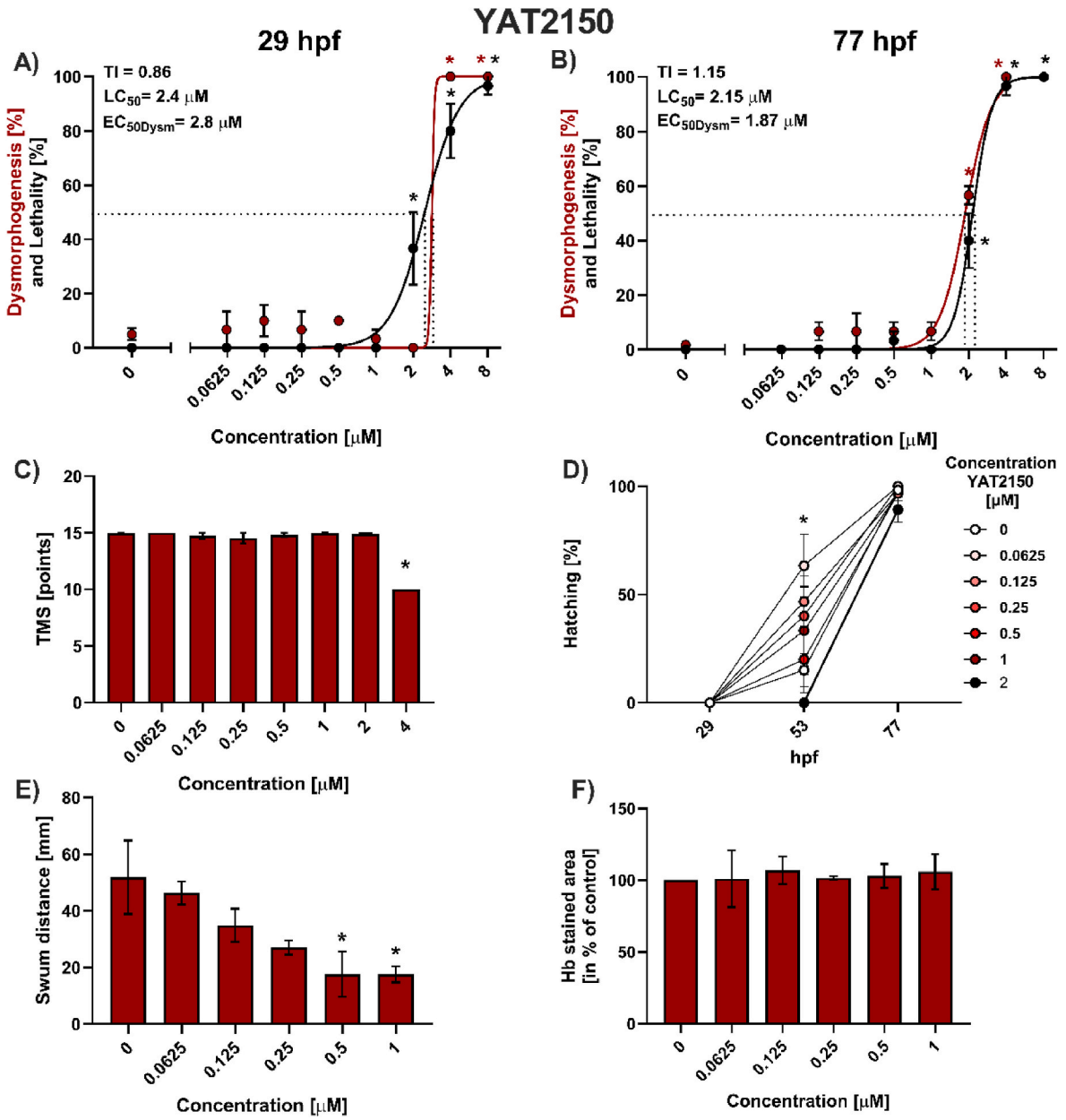

Fig. 11. YAT2150 is not teratogenic in zebrafish embryos. Results obtained in zebrafish larvae exposed to YAT2150. Details about results presented and statistical analysis performed are explained in the legend of Fig. 2, except for F) where in this case, a one-way ANOVA analysis followed by Dunnett test could be performed.

For these reasons it would directly be discarded as a candidate. Finally, YAT2150 has no teratogenic potential (TI < 2) at 29 or 77 hpf and does not display the erythrocytic depletion hazard. It induces lethality at 2 µM which in relationship with its efficacy against *P. falciparum*, in the low nM range, indicates a very promising efficacy/toxicity profile that would need to be further improved. That the drug presents adverse effects in behaviour already at 0.5 µM is a very relevant aspect that should also be improved in further development, or followed-up for studying if they are transitory.

Looking at the characteristics of the commercialized and potential new drugs, one can conclude that the best drug to treat malaria in pregnant women does not exist yet. Also, resistances associated to artemisinin and derivatives have already been described and therefore further research on safe candidates is needed. We believe that our test will help to accelerate the screening phase of these drug candidates and help to find drugs with no developmental hazard.

5. Conclusions

To conclude, we have established a combination of tests based on zebrafish embryos combining different time-points (29 hpf and 77 hpf) and endpoints (dysmorphogenesis, lethality, erythrocyte depletion, and behaviour among others) which allows to characterize relevant hazards related to antimalarial drugs during prenatal development and to distinguish if the adverse effects observed could be mainly related to erythrocyte depletion or not. We have also proposed an interpretation of the different possible combinations of results in the tests and the decision derived for drug screening purposes. With this testing strategy, we have been able to reproduce the main findings observed in the literature for five commercialized drugs, to screen three new drug candidates and to select the most promising of them. YAT2150 is a promising drug candidate to be further developed to improve its safety margin and to obtain a good alternative therapy for malaria in pregnant women and women of childbearing potential.

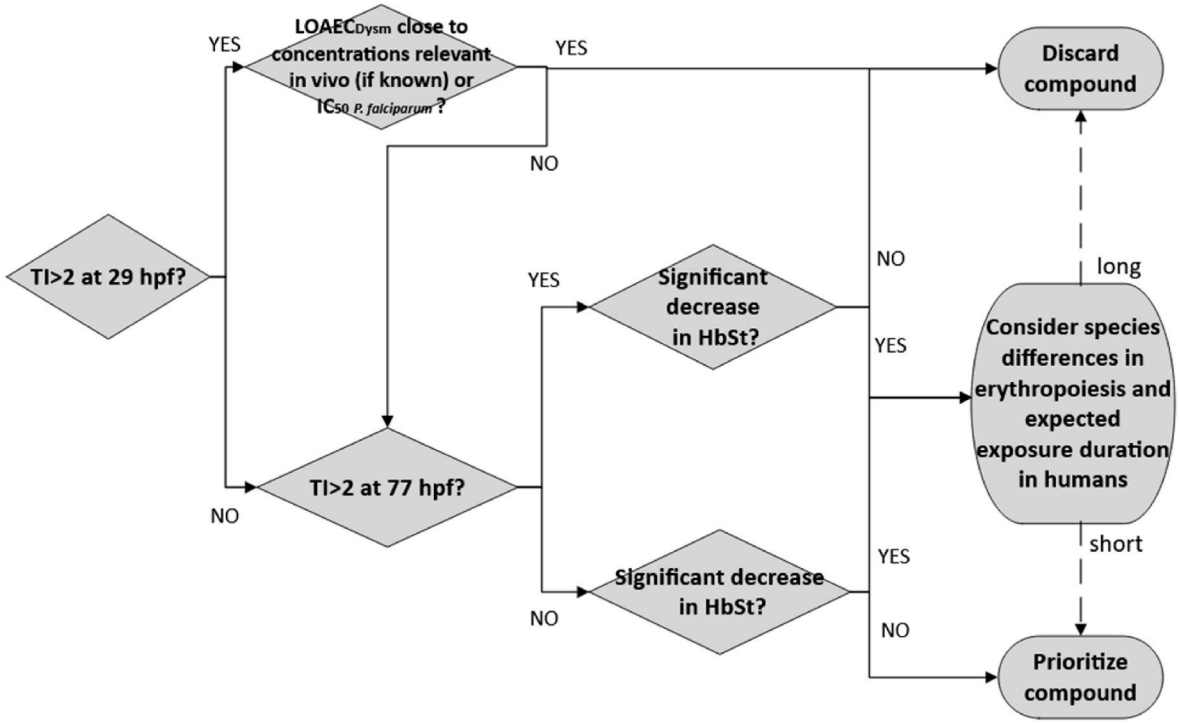

Fig. 12. Proposed decision chart for the screening of malaria drug candidates based on the results of the ZEDTA and HbS.

Table 2
Summary of toxicity results of this study and effectivity results from the literature.

Drug	Hazard identification		Risk assessment[§]		Effectivity (from literature)		
	Teratogenic (TI > 2) at any time-point	Erythrocyte depletion	Teratogenic (TI > 2) at 29 hpf	LOAEC$_{Dysm\ 29\ hpf}$	IC$_{50\ (P.\ falciparum)}$	Strain/ duration	Effectivity reference
Artemisinin	YES	YES	YES	50 μM	32 nM	3D7/48 h	Sanz et al. (2012)
Quinine	NO	NO	NO	>400 μM	75 nM	3D7/72 h	Zhao et al. (2022)
Chloroquine	NO	NO	NO	>400 μM	24 nM	3D7/48 h	Sanz et al. (2012)
DBL + DHA	(NO)^{L}	YES	NO	>30 + 5 μM	–	–	–
DHA	YES^{1}	YES^{2}	NO^{1}	>35^{1} μM	3.2 nM	3D7/48 h	Cui et al. (2012)
DBL	–	–	–	–	9 nM	3D7/48 h	Wong et al. (2011)
GS-GUAN	NO	NO	NO	>1200 μM	590 μM	3D7/48 h	Alencar et al. (2018)
DONE3TCl	YES	YES	YES	6.25 μM	80 nM	3D7/48 h	Bouzón-Arnáiz et al. (2022)
YAT2150	NO	NO	NO	4 μM	90 nM	3D7/48 h	Bouzón-Arnáiz et al. (2022)

[§] Risk assessment column includes the information necessary to distinguish if the hazard identified in the first column is related to the mechanism of action of artemisinins using Fig. 12 and comparing the LOAEC$_{Dysm}$ with the relevant concentrations (*in vivo* or IC$_{50\ (P.Faciparum)}$). L: limited concentration testing due to precipitation of DBL. 1: results described in [Ba et al., 2013]. 2: results described in [Longo et al., 2006].

CRediT authorship contribution statement

Lucia Borrallo-Lopez: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Laura Guzman:** Writing – review & editing, Methodology, Investigation, Writing – review & editing, Methodology, Investigation, Formal analysis. **Noelia G. Romero:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Anna Sampietro:** Resources, Methodology, Investigation. **Ana Mallo-Abreu:** Resources, Methodology, Investigation. **Elisabet Teixidó:** Writing – review & editing, Resources, Methodology, Investigation. **Burkhard Flick:** Writing – review & editing, Validation, Conceptualization. **Xavier Fernàndez-Busquets:** Writing – review & editing, Validation, Resources. **Diego Muñoz-Torrero:** Writing – review & editing, Validation, Resources. **Marta Barenys:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethical approval and consent to participate

The experimentation project, with number 9967 was approved on the May 31, 2018 by the Ethics Committee for Animal Experimentation of the University of Barcelona (CCEA-UB) and the Department of Environment and Housing of the Generalitat de Catalunya with license order 334/18.

Consent to participate is not applicable to this study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

A patent application (WO, 2023/067170 A1; filing date: October 21, 2022) has been filed to protect some of the results presented in the paper, which includes as inventors DM-T and XF-B. All other authors declare they have no competing interests.

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Declaration of competing interest

Marta Barenys is a Guest Editor for *Reproductive Toxicology* and was not involved in the editorial review or the decision to publish this article.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: A patent application (WO, 2023/067170 A1; filing date: October 21, 2022) has been filed to protect some of the results presented in the paper, which includes as inventors DM-T and XF-B.

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

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6. List of Abbreviations

ACT: artemisinin-based combination therapy; CAS RN: Chemical Abstracts Service Registry Number; D/Asp: aspartic acid; DMSO: dimethyl sulfoxide; Dysm: dysmorfogenesis; EC_{50Dysm}: effective concentration 50 for dysmorphogenic effect; FET: fish embryo acute toxicity test; G6PD: glucose-6-phosphate dehydrogenase; GS-GUAN: methyl 6-deoxy-6-[(2-guanidinoethyl)thio]-α-D-glucopyranoside; Hb: hemoglobin; HbSt: hemoglobin staining; hpf: hours post-fertilization; $IC_{50\ Pfalciparum}$: inhibitory concentration 50 for *Plasmodium falciparum*; L: lethality; LC_{50}: lethal concentration 50; LOAEC: lowest-observed adverse effective concentration; MMV: Medicines for Malaria Venture; NAMs: new approach methodologies; n.c.: not calculable; NOAEC: non-observed adverse effect concentration; OECD: Organisation for Economic Co-operation and Development; PBS: phosphate-buffered saline; PFA: paraformaldehyde; R/Arg: arginine; RT: room temperature; SEM: standard error of the mean; TER: touch-evoked response assay; TI: teratogenic index; TMS: total morphological score; WCS: worse case scenario; WHO: World Health Organization; ZEDTA: zebrafish embryo developmental toxicity assay.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2025.100582.

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