Executive Summary:

Artemisinin combination therapies are currently the first-line treatment for P. falciparum malaria as they have high efficacy against blood-borne multidrug-resistant P. falciparum. Experiments on laboratory animals have shown potential embryotoxicity associated with artemisinins during defined windows of sensitivity in the first trimester of pregnancy. The key cellular target is thought to be embryonic red blood cells which are depleted and the consequent anaemia and oxygen deprivation is responsible for the embryotoxicity observed. The risk to humans is currently uncertain and therefore these drugs are contra-indicated for use in the first trimester of pregnancy.

The aim of the ARTEMIP project was to gain an understanding of the safety pharmacology of artemisinin antimalarials in pregnancy by elucidating the mechanisms of toxicity and to inform recommendations on the risk: benefit balance in the first trimester of pregnancy in humans.

This was achieved through the following project objectives:

1. Synthesis of semi synthetic and synthetic peroxide probe molecules
2. Establishing reproducible in vitro and in vivo models of embryotoxicity
3. Defining the metabolism of peroxide antimalarials including characterisation of reactive metabolites
4. Establishing a role for reactive oxygen species (ROS) in embryotoxicity
5. Establishing if apoptosis is a key event in artemisinin embryotoxicity
6. Establishing the targets and molecular consequences of drug action
7. Defining drug distribution in the pregnant rat
8. Collating all available evidence to establish human risk

The results of the project were integrated with all current knowledge in the field at an informal consultation meeting on the safety of artemisinins in pregnancy, hosted by WHO/TDR on 2011-08-23. Experts from within and outside the ARTEMIP consortium reviewed the data generated within ARTEMIP together with data from other sources.

It was concluded from this review that the critical three-day period in rat and mouse that encompasses all the processes of early erythropoesis, covers a significantly longer period of real time in humans, perhaps 10-14 days. This longer period may, in itself, be sufficient to reduce sensitivity to artemisinin-induced developmental effects when the drugs are used clinically for a period of only three days. However, if artemisinin derivatives with slower elimination profiles are generated the risk: benefit ratio could change. No human data was presented; the results of the MiP Consortium on the safety of the artemisinins in pregnancy will be available in 2013.

In view of the fact that the MiP consortium data was not available to the consortium (this was outside the control of ARTEMIP) and the WHO studies had just concluded, but were not yet analysed, the WHO will hold a separate clinical meeting to review the clinical data and relate the information to the experimental data in 2012. This will then inform recommendations on the risk: benefit balance for the use of these drugs in the first trimester of pregnancy.

Due to the involvement of the WHO as a beneficiary in the project, the information generated during ARTEMIP has the potential to impact the recommendation for the use of these drugs in pregnant women or women of child bearing age in malaria endemic settings. The overriding conclusion derived from the project was a confirmation that this drug class does represent a potential risk to the foetal development within the first trimester and perhaps at periods beyond this. Mechanistic data generated as part of the project indicate a clear role for oxidative stress and foetal red cell damage as central to this toxicity. The observation that peroxide bridge activation is a core feature of both antimalarial action and toxicity suggests that it will be difficult to disassociate these two pharmacological responses in second and third generation molecules.

Based on the data available it was not possible at this time to make a recommendation to change the label use of artemisinins and the recommendation that these drugs should not be used in the first trimester of pregnancy remains in place.

Project Context and Objectives:

Concept and project objectives

The objective of this Collaborative Project was to provide a mechanistic explanation for the embryotoxicity and teratogenticity associated with artemisinin based antimalarials as seen in rat, rabbit and non-human primate, to establish if these toxicities are shared with the new generation of synthetic peroxide/trioxolane antimalarials and provide an indication of the risk these drugs pose to the developing human foetus (there is one report of a single case of an early first trimester abortion in a subject in a phase II clinical trial with an artesunate based combination which was attributed as drug related - Ward personal communication). The outputs of this research will shape recommendations on global malaria in pregnancy treatment and prevention policy.

Artemisinin based drug combinations are currently being considered as alternatives to failing drugs in the treatment and prevention of malaria in pregnancy. The studies completed in this collaborative project are central to assessing the potential hazard posed by these drugs to the developing human foetus. Thereby making evidence based recommendations on the risk: benefit of these drugs, this is a critical knowledge gap in pregnancy malaria.

Artemisinin based antimalarial drug combinations are recommended for the treatment of P. falciparum malaria infections throughout all malarial endemic areas of the world and in all populations, including women of child bearing age. Although clinical experience to date indicates the artemisinins to be safe, the area of reproductive toxicology demands special consideration. Data from the Chinese literature and our own studies confirm that the artemisinins are embryotoxic (rat, rabbit and non-human primate) and potentially teratogenic (rat and rabbit) in animal species at drug doses within the human therapeutic range. Our hypothesis which could explain the embryotoxic effects is based on the generation of reactive oxygen species (ROS) from cleavage of the artemisinin peroxide bridge and consequent embryo foetal damage to key biological macromolecules. This hypothesis draws on parallels with the metabolic activation and teratogenic effects of the established teratogens such as phenytoin.

Hypothesis

The hypothesis was proposed based on our previous studies and those of others in non-embryonic model systems, that the embryotoxicity and teratogenicity of the artemisinins is mediated via production of free radicals within the developing embryo with the consequent formation of reactive oxygen species and/or irreversible modification of vital cellular bio-molecules. We further propose that these modifications result in apoptosis of critical cells. Comparison of the chemical, biochemical and molecular effects of the semi-synthetic artemisinins such as artesunate with fully synthetic molecules such as the trioxolanes and established teratogens such as phenytoin will establish if these novel peroxides represent a new pharmacophore for embryotoxicity and teratogenicity. The purpose of the collaborative project is to establish the mechanism behind this toxicity, establish if it is applicable to all peroxide based antimalarials and relate the effects to human drug exposure levels. This

information will facilitate hazard and risk assessment in humans of this important drug class. This data will contribute to the design and revision of recommended treatment and prevention policies for malaria in pregnancy.

The working hypothesis

In this Collaborative Project we integrated much of the available molecular and biochemical data on the artemisinin class into a hypothesis to explain embryotoxicity and teratogenicity. Many mechanisms have been proposed to explain drug-induced teratogenicity. With almost all of the mechanisms proposed to date there is mounting evidence implicating bioactivation and the involvement of reactive oxygen species (ROS) in the teratogenic effects of the established teratogens such as thalidomide and phenytoin. It is the generation of free radical intermediates which initiate the formation of ROS that in turn have the potential to damage and/or alter function of a range of biological macromolecules including critical transcription factors and DNA. ROS are short-lived unstable molecules incapable of significant escape from their site of generation. Thus it must be assumed that their generation occurs within the embryo system. Central to our working hypothesis are the demonstrations made by members of this Collaborative Project (and others) for a role of iron in the bioactivation of the artemisinins. We have already investigated the role of drug metabolism and iron dependent reductive peroxide bridge cleavage in the disposition, antimalarial activity and toxicological potential of various endoperoxide drugs (Ward and Park Groups 32-36). Of relevance to this proposal are the observations that these peroxides undergo Fe(II) dependent cleavage to reactive species including carbon centred radical species as demonstrated by a combination of biomimetic Fe(II) degradation chemistry and EPR spin-trapping techniques. We have fully characterised the secondary carbon centred radical derived from the peroxide analogue arteflene and the primary and secondary carbon radicals derived from artemisinin analogues. Furthermore we have confirmed the role of free radical initiated reactive oxygen species (ROS) in the antiparasitic and the neurotoxic actions of these drugs. Indirect evidence for the generation of reactive species has been derived from experiments using fluorescent tagged synthetic peroxide produced in-house. The selective loss of nucleated embryonic red cells from the visceral yolk sac of artemisinin treated whole embryo cultures (Oliarro) is in keeping with a mechanism based on iron activation to reactive species which induce apoptosis of these replicating cells. In agreement with this suggestion are the observations of artemisinin induced apoptosis in certain tumour cells.

The ferrous mediated homolytic cleavage of the peroxide bridge leads to the formation of carbon radical species that can be efficiently characterised by spin-trapping techniques. Apart from the generation of carbon centred radicals, other workers have suggested that the 1,2,4-trioxane unit acts as a masked source of 'free hydroperoxide'. Heterolysis of the C-O bond followed by a homolytic cleavage (Fenton reaction) on this species would lead to the generation of the hydroxyl radical (with the potential to generate the superoxide anion via interaction of .OH with another molecule of hydroperoxide). An alternative route to ROS is also possible in which the ring opened hydroperoxide moiety interacts directly with peroxidises via the mechanism proposed for phenytoin derived hydroperoxides. The ROS species produced (HO*, O2* - , H2O2) could mediate oxidation of embryonic protein thiols or DNA as a mechanism of endoperoxide induced teratogenesis.

We propose the hypothesis, based on our previous studies and those of others in non-embryonic model systems, that the teratogenicity of the artemisinins is mediated via production of free radicals within the developing embryo with the consequent formation of reactive oxygen species and/or irreversible modification of vital cellular bio-molecules. We further propose that these modifications result in apoptosis of critical cells. Comparison of the chemical, biochemical and molecular effects of the semi-synthetic artemisinins such as artesunate with fully synthetic molecules such as the trioxolanes and established teratogens such as phenytoin will establish if these novel peroxides represent a new pharmacophore for teratogenicity. The collaborative Project will establish the mechanism behind this toxicity, establish if it is applicable to all peroxide antimalarials and relate the effects to human drug exposure levels. This information will facilitate hazard and risk assessment in humans of this important drug class.

Overall strategy and general description

The overall objective of this project is to understand the basis for the teratogenic and embryotoxic potential of artemisinin based and related peroxide antimalarials. Furthermore this data will be used to inform on the potential risk associated with the use of these drugs in pregnancy. To achieve this objective this project has addressed seven specific S&T objectives. Each has been tackled by an individual workpackage (WP). Together with the demonstration activity in WP 8 these form a cohesive intellectual and technical framework, which together with effective project management and integration have been designed to deliver the overall project objective. Each of these objectives is described as follows:

S&T Objective 1

To Synthesis of Artemisinin and Ozonide (synthetic peroxide) probe molecules: The semi-synthetic artemisinins and more recently the fully synthetic peroxides such as the ozonides are predicted to be deployed in millions of doses per year for the treatment of malaria. However, the base molecules are less than ideal for studies into drug metabolism, drug activation; cellular distribution and drug adduct formation. To overcome these limitations we will synthesise probe molecules based on these structures which can be shown to retain antiparasitic activity (we have already generated similar molecules with appropriate pharmacological profiles). The probes will be based on chemistry expertise within the assembled group and will include radio labelled molecules, fluorescent tagged molecules and biotin tagged molecules.

S&T Objective 2

To establish reproducible in vitro and in vivo models Embryotoxicity and teratogenicity studies rely on specialised model systems of pregnancy. Expertise within the group will provide validated model systems for further biochemical and molecular mechanistic studies. The micromass rat limb bud culture will be used as a representative foetal cell line and the Whole embryo culture (WEC) model will allow evaluation of the visceral yolk sac, its circulating nucleated red cells and the embryo per se as targets for the toxic effects of these drugs. In addition we will use pluripotent mouse embryonic stem cells as a more simplified model, which allows genetic manipulation for more mechanistic investigations.

S&T Objective 3

To define the metabolism of peroxide antimalarials including characterisation of reactive metabolites: The basis for our working hypothesis is based on metabolism of these drugs to reactive metabolites within relevant cellular systems. Consequently the first experiments will be designed to confirm this. These studies will focus on the quantitative identification of stable drug metabolites and activation rearrangement products as well as indirect measures of activation such as GSH depletion.

Using the base standard molecules (artesunate and our trioxolane peroxide derivative) we will establish the metabolic profile within our experimental model systems, evaluate oxidative stress from GSH depletion and identify rearrangement products by LCMS. The Park and Ward groups have already performed similar experiments with peroxides in other models systems

S&T Objective 4

To establish a role for Role of Reactive Oxygen Species (ROS) and artemisinin induced embryotoxicity Based on our data and data in the literature there is clear evidence that the peroxide based antimalarials can result in elevated ROS in many model systems. It is considered by many that this ROS generation has important implications in the antimalarial and anticancer effects of these drugs. We have experience in a range of biochemical and imaging techniques that can demonstrate, characterise and locate ROS within cellular systems. These strategies will be used to look at these peroxides in our model systems of foetal development.

WP4 complements and is a logical extension of WP3. Again the Project team will use methodologies that they have already used to establish the importance of reactive oxygen species in drug toxicity. The distribution of activated peroxide drug in these studies will rely on the successful synthesis and evaluation of probe molecules from WP1

S&T Objective 5

To establish if apoptosis is a key event in artemisinin embryotoxicity: Studies in tumour cells have demonstrated the potential of the artemisinins to induce apoptosis. Using the LBC and nucleated red cells from the VYS as well as mouse embryonic stem cells the dose dependent and iron dependent potential for the semi-synthetic and synthetic peroxides to induced apoptosis will be established. In addition, by using over expression or down regulation of anti-oxidant genes or DNA repair genes using lentiviral (RNAi) approaches, the role of oxidative stress and DNA damage in the progression of apoptosis will be evaluated

WP5 focuses in on a specific mechanism of toxicity through the apoptotic pathway. We have brought together two groups with extensive experience in this area of toxicology (van de Water and Park). The experimental approach will be as used by these groups already. Information delivered from WPs 3 and 4 about metabolic routes and rates will inform some aspects of study design in WP5.

S&T Objective 6

To establish the targets and molecular consequences of drug action Understanding the molecular targets of drug adduct formation and the cellular response to the toxic effects of

drug is critical to establishing the mechanism/s by which drugs exert toxicity (or activity). We propose a series of studies to address this question. DNA adduct formation will be assessed by the formation of 8-hydroxy-2'-deoxyguanosine. Protein targets will be characterised using biotin tagged probe drug. Biotin tagged proteins will be purified on streptavidin beads, digested with trypsin and formally characterised by either MALDI or ESI mass spectrometry. Studies in tumour cells have demonstrated the value of transcriptional analysis using gene chip technology to highlight the cellular response to chemical insult with artemisinins, we will adopt a similar strategy to characterise the response to an embryotoxic insult.

These studies represent the most challenging aspects of the Project relying on state of the art proteomic and transcriptomic technologies. Again we have three independent research teams within the Project with the necessary competencies to ensure that these studies are a success.

S&T Objective 7

To define drug distribution in the pregnant rat: We have already demonstrated the dose and time dependency of the embryotoxic effects of the artemisinins in vivo. However it is not clear what the relationship is between administered does, circulating plasma and blood drug levels and drug penetration into the embryo system. Using a combination of radiolabelled drug and highly sensitive LCMS methods we will establish this relationship after oral administration of radiolabelled drug to pregnant rats.

In WP7 we will apply standard drug disposition and pharmacokinetic approaches to establish drug embryo exposure in our models systems. Again the two groups working on this have significant experience in this specific area of drug analysis and drug disposition. The outputs of this WP complement those from WPs 3, 4, 5 and 6 in relating observations on cellular exposure and cellular response to the drug concentration exposure profile achieved after oral drug administration to our target animal species (the pregnant rat).

S&T Objective 8

Establishing human risk Using the data generated by the Project on the relative embryotoxic potential of semi-synthetic and synthetic peroxides, the potentials targets of drug action (VYS, embryonic red cell of foetal tissue), mechanisms of drug action and the drug dose exposure relationship, together with emerging data in the literature the consortium will provide an integrated view on the relative risk of artemisinin/peroxide use to the pregnant woman.

The animal model systems selected for use in this project are those considered by international regulatory authorities to be the best at screening for potential human risk and the cellular models are those which have most recently been applied to investigating teratogenic and embryolethal mechanisms for a range of established human teratogens. The hallmark effect of artesunate exposure was a dramatic induction of embryo loss, apparent as abortions in rabbits and resorptions in both rats and rabbits. In addition, low incidences of cardiovascular malformations and a syndrome of skeletal defects were induced at or close to embryolethal doses of artesunate in both rats and rabbits. The cardiovascular malformations consisted of ventricular septal and vessel defects. The skeletal syndrome consisted of shortened and/or bent long bones and scapulae, misshapen ribs, cleft sternebrae and incompletely ossified pelvic bones. Where these defects have been seen in these model systems before, they have always translated into human risk.

In the current project the morphological, biochemical, transcriptional and proteomic changes seen in our experimental models will be extrapolated to their human equivalents. This will determine if the process perturbed in the model systems have parallels in man and will direct future investigative studies in humans. This understanding will enable relative risk to be quantified and disseminated to policy makers and will identify possible solutions/interventions which can be considered as a means to reduce or remove the risk.

WPs 1-6 provide the mechanistic basis for the embryotoxicity and teratogenicity seen with the peroxides with WP7 linking these cellular effects with exposure. In WP8 we have identified WHO/TDR with responsibility for monitoring and advising on the use of artemisinins in pregnancy. Experimental data will be reviewed alongside all the available data on artemisinin/peroxide drug usage in man, including pregnant women, in order to provide policy makers with a risk: benefit analysis on the unsupervised community deployment of these important drugs.

Project Results:

The consortium was set up on the 1st February 2009 with a total of 6 beneficiaries.

1. Liverpool School of Tropical Medicine, Professor Steve Ward
2. University of Liverpool, Professor Kevin Park
3. Universidade do Algarve, Dr Maria Lurdes Sanots Cristiano
4. Leiden University, Professor Bob van de Water
5. Goteborg University, Professor Michael Ashton
6. WHO/TDR, Professor Pierro Olliaro

Summary of achievements

WP 1 Synthesis of probe molecules

This was one of two work-packages that were established to support the consortium as a whole. The principle requirement from this work-package was to develop synthetic organic chemistry strategies that would generate probe molecules that could be used as tools to investigate the underlying chemical and biological basis of artemisinin (and related peroxidic antimalarial) embryotoxicity and antimalarial activity.

A series of artemisinin and ozonide conjugates bearing fluorescent, biotinylated and radiolabelled moieties were prepared, with the aim of producing adequate probes for the investigation of potential teratogenicity and embryotoxicity of endoperoxide-based antimalarial drugs and drug candidates. In order to introduce chemical diversity and assess structural effects on toxicity, a small library of conjugates was produced. In these conjugates, the pharmacophore (artemisinin or ozonide) is connected to the probe (NBD, DNS, biotin or tritium-labelled benzoate) through a linker. The structure of this linker was altered. Flexible alkyl and more rigid aromatic benzyl linkers were used. For the ozonide conjugates two sets were considered, one with the probe connected to the cyclohexyl ring and the other with the probe connected to the more rigid adamantly ring. In total, 7 fluorescent conjugates (4 derived from artemisinin and 3 from ozonide), 2 biotin-labelled conjugates (one derived from artemisinin and the other from ozonide) and 3 radiolabelled conjugates (one derived from artemisinin and 2 from ozonide) were produced and delivered to the consortium.

It is established that for artemisinin and trioxolane-based antimalarials the anti-plasmodial activity is dependent on the presence of the peroxide pharmacophore. However, it was important to establish if the toxicity of the probe compounds was also peroxide-dependent. It was decided by the consortium to expand the initial objectives of WP1 and include the preparation of deoxygenated conjugates that could be used as negative controls. Six deoxygenated conjugates derived from artemisinin were produced and delivered to the consortium.

All compounds were tested at the University of Liverpool for stability in biological media, and then subjected to cytoxicity assays using the HL60 leukaemia cell line model. In these assays, the toxicity of the artemisinin-derived conjugates was compared to that of artesunate and the toxicity of the ozonide-based conjugates was compared to that of an active trioxolane.

Selected intermediate compounds were also tested to assess the independent toxicity of the fluorophore/biotin label and of the linker. All conjugates were tested for anti-plasmodial activity against PF parasites, at LSTM.

From the results obtained, it was possible to extract the following general conclusions:

1. Artemisinin/ozonide derivatives synthesised by UOA demonstrated concentration-dependent toxicity towards HL60 cells and appear to be relatively stable in biological media.
2. The peroxide bridge represents the toxicophore for the artemisinins; molecules lacking the peroxide did not show toxicity and could serve as useful negative controls, with good results.
3. All peroxide-derived probes exhibited similar antimalarial activity to the unlabelled peroxides.
4. The conjugates where the pharmacophore is connected to the probe through a flexible alkyl linker have demonstrated altered toxicity and/or pharmacology. These conjugates show lower toxicity than unlabelled molecules.
5. The conjugates where the pharmacophore is connected to the probe through a rigid aromatic benzyl linker appear to show greater similarity to unlabelled molecules in terms of toxicity/ pharmacology profile and are therefore suitable as probes for peroxide-dependent toxicity
6. The benzyl-linked DNS-tagged probes are more toxic than their unlabelled counterparts. The DNS-fluorophore alone and b-linker alone, exhibit little toxicity but when DNS is attached to the b-linker, toxicity is increased.
7. Radioactive probes have been made and have been used to undertake mass balance and drug adduct studies.
8. The observation that a-linked probes are much less toxic towards HL60 cells than b-linked probes, but both classes of compounds exhibit similar antimalarial activity against FP parasite is relevant in terms of anti-malarial drug optimization.
9. For ozonide's, substitution by fluorophore at the adamantly system may be more adequate than at the cyclohexyl ring. This preliminary indication will require further investigation.

The work-package more than achieved its role within the consortium. Through this work-package we were able to establish new synthetic routes to endoperoxide-labelled hybrid molecules. Moreover, we were able to generate these molecules using strategies that did not have any significant impact on in vitro antimalarial activity. In contrast we demonstrated that the inherent cytotoxicity of the probes did have a profound impact on mammalian cell cytotoxicity. This is an area that is still being investigated by UOA as a follow on to ARTEMIP. It suggests that subtle chemical modification does influence the therapeutic index of these molecules. The need to generate non-peroxidic analogues of all our probe molecules became obvious very early in the project. These are essential to defining the peroxide bridge as the driver of toxicity and activity. All the probe pairs generated in the project fully support the view that the pharmacophore and unfortunately the toxicophore is the peroxide moiety. This conclusion remained the same even when we looked at fully synthetic molecules of the trioxolane and tetraoxane class. This has profound importance in terms of strategic considerations for second and third generation peroxidic antimalarials and their risk/benefit ratios. These conclusions indicate the requirement to continue to look at embryotoxic potential, both pre-clinically and clinically, with any new molecule under development.

WP 2 In vitro and in vivo models

The second workpackage was established to provide biological support to the consortium. It was designed to establish Whole Embryo Cultures (WEC), cell based models and in vivo models of embryotoxicity and teratogenicity using the rat as the target species. The models established within the consortium have previously been used to investigate embryotoxicity and teratogenicity by the biomedical and pharmaceutical community. Furthermore other groups have used these models to demonstrate the effects of artemisinin based antimalarials. The models were used by the consortium to confirm and validate observations in the literature, and to further investigate the observed toxicity in terms of oxidative stress and glutathione depletion.

The first model to be validated was the in vivo pregnant rat model. In this model time-mated (pregnant) rats are treated with drug at a specific time in the gestational period. Foetal material can then be harvested for further analysis. We were able to establish the model and confirm the embryotoxic profile of the artemisinins in line with data from the literature. The in vivo rat model was also able to demonstrate effects on haematopoietic blood cell development and vascularisation in embryos at gestation day 13. Histology of artesunate-treated embryos and foetal material from in vivo experiments following explantation at gestation day 11, 12 & 13 demonstrated toxic effects in line with the accepted toxicity of these drugs.

We adopted a modified strategy to look at this. Initial in vivo experiments were conducted to evaluate effects on haematopoietic blood cell development and vascularisation in embryos at gestation day 13. Then clinical haematological protocols were used to analyse blood from the embryos explanted at gestation day 13.5 from dams dosed with either carboxymethyl cellulose vehicle or artesunate at 17mg/mL. Similarly histological analyses (haematoxylin and eosin (H&E) staining for morphological evaluation and alcian blue staining to investigate the somites and limb bud cells for chondrogenesis were developed to ascertain drug effects on overall foetal development and also specifically the skeletal development. These studies, although taking a different approach, replicated the data reported in the literature with respect to embryotoxicity following drug administration to dams

During funding period 3 an in vivo experiment was conducted to measure glutathione levels in embryos from dams treated with DHA. The rationale behind the study was if, as has been speculated, toxicity is due to generation of oxidative stress then this should be reflected in reduced cellular glutathione levels. Ten groups of five dams were set up over three days alongside an untreated control group. The following groups were assessed. 1 mg/kg DHA, 2 mg/kg DHA, 5 mg/kg DHA, 10 mg/kg DHA, 20 mg/kg, DMSO (vehicle group) and DEM (depletion group). In this experiment, dams were dosed only on gestation day 9.5 (previously, dams were dosed on gestation day 9.5 and 10). Surprisingly we did not see any glutathione depletion. This suggests that on a global level there is no gross effect on glutathione signifying that the biological system as a whole may not be under oxidative stress. However these studies do not negate the idea of local effects within specific cell populations that might result in altered glutathione levels. This question will require more detailed investigation in the future but the models we have established will be an excellent starting point for such studies.

Alternative in vitro models were also established based on whole embryo cultures (WEC) and limb bud cultures (LBC) to allow interrogation of the visceral yolk sac, foetal red cells and other foetal cell populations. In general the WEC was found to be a robust model for investigation but the LBC posed technical and biological challenges that we concluded limited its usefulness in the context of our principle set of questions.

The WEC screening assay has undergone many stages of optimisation ranging from improvements in serum collection methodologies, negotiation with the animal's suppliers to ensure dams were mated at 10 weeks rather than 9 weeks old, adoption of totally aseptic embryo explantation conditions following caesarean section as used in industry and changing strains from Wistar to Sprague Dawley. In addition, WEC was successfully extended to 70+ hours where the embryos were exposed to DHA at the beginning of embryogenesis at gestation day 9.5 and cultured until gestation day 12. Collection of embryos at this stage enabled better comparison of vasculogenesis and haematopoiesis as sufficient red blood cell and progenitors were generated to facilitate comparison between drugs exposed and control embryo cultures. Collection of 12 day old embryo cultures also enabled better evaluation of heart, brain and limb development as older embryos are robust enough to withstand histological (H&E) procedures.

Experiments have been performed using the WEC model to determine if oxidative stress occurs in response to treatment with artemisinin derivatives. Hydrogen peroxide and phenytoin were used as positive controls for oxidative stress. A variety of approaches was attempted including Cryo-sectioning, and whole mounts microscopy with two different oxidative stress stains (DCFA and DHE). DHE appeared to give the most reliable results when examined using confocal microscopy. Artesunate and DHA linked to fluorescent molecules (DNS and NBD), produced by UOA were also used in the WEC model to identify cell localisation. Whole mount microscopy was used to view fluorescence with a confocal microscope.

To assist the analysis of bioactivation a series of WEC cultures were set up and embryos were analysed after 24 and 48 hours. Unfortunately morphological scoring was difficult at 24 hours resulting in this time point being abandoned after the third bioactivation experiment. Embryos treated with 100 M pfDHA failed to grow/died during culture, therefore scoring 0 for morphological scoring.

The WEC was also used to analyse two new peroxide based antimalarial compounds, RKA182 and FBEG100. This was to address the question of embryotoxicity in the next generation of molecules. Cultures were set up at gestation day 9.5 and analysed at gestation day 11.5. Total morphological scoring was performed, indicating that these new compounds are also embryotoxic above 1.

The use of the WEC model successfully confirmed the embryotoxic potential of the artemisinins in the first trimester of pregnancy. We have demonstrated that at embryotoxic concentrations of peroxidic drug there was no GSH depletion, although there was bioactivation of probe molecules and the generation of oxidative stress. Furthermore these observations of embryotoxicity extended to novel fully synthetic peroxides. This data is critical as it confirms the concern that this is a class effect that will continue to be a risk even with newer drugs of this class. Depending on their pharmacokinetic properties (such as a longer elimination profile) toxicity could be even more pronounced.

The other in vitro model studied was the limb bud culture procedure. The selection of the model was based on the fact that it was a foetal cell line independent of red cell progenitors and was associated with limb development (a potential target of artemisinin embryotoxicity). Limb buds were collected from surplus embryos explanted following oral dosing and cells were dissociated and plated without further treatment in tissue culture well plates. Cultured cells were successfully stained with alcian blue staining for proteoglycans associated with chondrogenesis in embryonic progenitor cells and there were clear effects seen in cell populations derived from embryos explanted from artesunate-treated.

Limb bud cultures were established in tissue culture plates. However successful culture was only possible with a concomitant culture with fibroblasts. This unavoidable contamination significantly compromised the utility of this model. Although it was possible to image cells by confocal microscopy it was not possible to establish signals that were purely emanating from the limb bud cells. In addition probe sequestration within the fibroblast complicated interpretation of probe staining experiments to the point that the model was considered inappropriate. We successfully adapted the WEC model in order to perform some of the experiments in WP4 and WP6 instead of the limb bud model. Further analysis at UOL showed the presence of fibroblast derived cell populations surrounding and possibly supporting the growth of the pre-chondrogenic nodules which interfered with the interpretation of results and structural changes observed under drug pressure using high magnification fluorescence and LSCM generated ambiguous results.

WP3: Metabolic Activation Studies

The principle aim of this workpackage was to determine the metabolism and activation of the artemisinins in whole embryo culture from the rat. The role of metabolic bioactivation in the induction of cytotoxicity by a model artemisinin has been characterised previously in an in vitro model, namely HL60 human promyelocytic leukaemia cells (Mercer et al., 2007, J Biol Chem, 282, 9372-9382). From this work, we have established that bioactivation is required for toxicity, and that cells that are unable to bioactivate as the artemisinins are insensitive. To quantify levels of bioactivation, we used liquid chromatography tandem mass spectrometry (multiple reaction monitoring) to track formation of a stable end-product, the tetrahydrofuran (THF) acetate isomer that is indicative of carbon-centred radical formation. This approach was used due to the inherent instability and resulting difficulty of measurement of the radicals in biological systems. In embryos exposed to the model artemisinin PfDHA for 24 hours, evidence of parent compound degradation and THF-acetate isomer formation was obtained, indicating that bioactivation does occur under embryotoxic conditions. These observations were not recorded in samples consisting of culture media and PfDHA but lacking embryos, indicating that the bioactivation is dependent on the presence and biological activity of the embryo. We have also quantified the formation of the THF-acetate isomer, which is a stable rearrangement product of carbon-centred radicals formed following artemisinin bioactivation. By identifying this rearrangement product in the whole embryo culture model, we have provided evidence that bioactivation of the artemisinins to a primary carbon-centred radical is associated with embryotoxicity. This represents the first indication that bioactivation takes place under toxic conditions in an ex vivo model.

Work undertaken in our in vitro cell models indicates that the artemisinins do not significantly affect global cellular GSH levels. Indeed, GSH depletion is only observed following the onset of overt cytotoxicity. As a result, it appears that GSH depletion is a consequence, rather than direct cause, of cytotoxicity in mammalian cells. We have also provided evidence that the depletion of GSH is not important for the onset of artemisinin-induced embryotoxicity in the whole embryo culture model. The above observations provide important insights into the chemical and molecular mechanisms of artemisinin embryotoxicity and will require further investigation beyond the ARTEMIP project.

WP4: The role of oxygen species (ROS) in artemisinin induced embryotoxicity

The aim of this workpackage was to test the hypothesis that the artemisinins are capable of generating oxidative stress within the developing embryo and mouse embryonic stem cells. Using the fluorescent probe dichlorofluorescin diacetate together with flow cytometry analysis, we have demonstrated that artemisinins are capable of inducing a concentration and time-dependent generation of reactive oxygen species in cellular systems. We were able to generate data indicating that the generation of reactive oxygen species is an important underpinning mechanism in artemisinin-induced cell death. Attempts to further investigate this have confirmed that the embryonic limb bud cell model was not robust enough to ensure experimental reliability and reproducibility. As a result of this and due to technical difficulties in adapting existing methods for detection of oxidative stress probes within simple cell systems, we were forced to the more complex whole embryo culture tissue model. Although we could confirm the oxidative stress hypothesis in the model it has not been possible to reliably assess the intracellular localisation of oxidative stress within the timeframe of the project. This will require further investigation beyond ARTEMIP.

Further evidence for the role of oxidative stress in the embryotoxicity of the artemisinins was confirmed by the protective effect of the superoxide scavenger tiron demonstrated in a cell-based model. Tiron diminishes artemisinin-induced reactive oxygen species generation and protects against artemisinin-induced cell death, demonstrating the important role of oxidative stress in the propagation of artemisinin toxicity. The most compelling data for this mechanism relies on the evidence for the formation of the THF-acetate isomer of the model artemisinin PfDHA in whole embryo culture samples. This, in addition to our previous demonstration of an association between the formation of the THF-acetate isomer and sensitivity to artemisinin toxicity in other mammalian cells (Mercer et al., 2007, J Biol Chem, 282, 9372-9382), is taken as evidence indicating that a primary carbon-centred radical is formed upon bioactivation of PfDHA in complex embryonic and simple cellular systems. This is a major conclusion of our studies to date.

WP5 Artemisinin induced apoptosis

The aim of this workpackage was to unravel the type of cell death caused by artemisinins and the involvement of oxidative stress in this process using a range of model cellular approaches. The onset of apoptosis in embryonic stem cells following exposure to artemisinins (ART and DHA) and appropriate controls was investigated using flow cytometry. Apoptosis onset in embryonic stem cells was between 24 and 48 hours, except at high DHA concentrations

where at 24 hours there was already considerable apoptosis. The negative control deoxy-DHA induces similar apoptosis levels to the vehicle (DMSO) at 24 hours. Apoptosis induced by ART and DHA is caspase dependent, since blocking caspases with zVAD-fmk reduces apoptosis levels. Although limited apoptosis is observed at lower concentrations of ART and DHA in the embryonic stem cell model, the sulphorhodamine B assay indicated that cells do undergo cell cycle arrest and cease proliferating at these concentrations. Using our previously generated cell lines containing either a GFP-tagged oxidative stress marker, DNA damage marker, or a marker that responds to both oxidative stress and DNA damage, we have shown that the artemisinins induce an oxidative stress response with no evidence of DNA damage. Additional flow cytometry experiments as well as live cell imaging results clearly show that the stress caused by artemisinins has an oxidative stress signature. These findings are consistent with previous reports of artemisinin toxicity in non-embryonic cell systems.

As described earlier a key feature of the mechanism of artemisinin embryotoxicity we have observed is elevated oxidative stress. The Keap1-Nrf2 pathway represents the primary cellular mechanism for sensing and responding to oxidative stress. In CSB-/- embryonic stem cells, depletion of Keap1 by RNA interference protects against the induction of apoptosis by artemisinins, likely via the stabilization of Nrf2 and up-regulation of its downstream protective processes. Although it has not been possible to establish the whole embryo culture model in Nrf2-/- mice, we are further exploring the role of the Keap1-Nrf2 pathway in determining sensitivity to artemisinin toxicity through RNA interference screens in genotoxic and oxidative stress reporter cell lines. Using the fluorescent probe dichlorofluorescin diacetate, we have demonstrated that artemisinins provoke an increase in cellular reactive oxygen species in mammalian cells, and that blocking the generation or propagation of these species with the iron chelator desferrioxamine or the superoxide scavenger tiron, respectively, protects cells against artemisinin-induced cell death. A regular feature of apoptosis is DNA damage and we did look for such an effect with the artemisinins. Using a high-throughput screening approach, we have identified several novel phosphatases that control the onset of artemisinin toxicity. Functional genomics analysis revealed networks associated with apoptosis and the cell cycle, including signalling pathways associated with NF-kB, PI3K/AKT, ATM, p53 and apoptosis, as well as G1/S and G2/M checkpoint regulation and BRCA1 DNA damage responses. Additional functional genomics analysis showed differentiation related pathways such as Nanog pluripotency networks, Wnt/beta-catenin, BMP, HGF and TGF-beta signalling. We plan to study these in more detail for their effect in artemisinin-induced apoptosis at high concentrations and differentiation or proliferation at low concentrations of artemisinins.

WP6 Target molecule identification

The aim of this workpackage was to identify the molecular targets for peroxide/artemisinin binding and pathways that are activated in response to artemisinins using a range of approaches. We had initially proposed to look at DNA oxidation as a target for drug toxicity. However, exposure of mouse embryonic stem cell lines stably expressing a DNA damage reporter construct has revealed that DNA damage is not a primary mechanism of toxicity of the artemisinins and so this hypothesis was not pursued further within the project. A second target identification strategy was based on biotin tagged pull down probe molecules. A biotin-tagged artemisinin was synthesised at UOA and tested for toxicity at UOL and

antimalarial activity at LSTM. However, these tests revealed that the introduction of the biotin tag and/or linker adversely affected the toxicity of the probe, despite having little impact on pharmacological potency. A similar phenotype was observed when the toxicity and pharmacological activity of the fluorescent-tagged probes from UOA were tested under similar conditions. A common feature of these molecules is the incorporation of an alkyl linker between the test compound and the fluorescent/biotin tag. It was therefore determined that the alkyl linker was not suitable for use in these hybrids, as its incorporation clearly disrupts the toxic action of the probes. Although unfortunate, in terms of the unsuitability of these probes for their intended applications, we are currently preparing a manuscript in which we use the data generated from these pharmacology/toxicology screens to better understand the importance of the endoperoxide bridge in driving the pharmacological and toxicological actions of the artemisinins, as well as demonstrating the potential for incorporation of secondary chemical moieties that can dissociate pharmacological and toxicological potencies. A back-up biotin-tagged probe, containing a benzyl linker that we have shown to have no effect on pharmacological/toxicological action of other probes prepared at UOA, is in the latter stages of being synthesised. We will use this back-up probe to define the molecular targets of the artemisinins in relevant cellular and embryonic models. Furthermore we have designeda set of 'click' probes that selectively label parasite proteins and allow pull-down for mass spectrometric confirmation of target proteins. These probes are currently being used in the WEC model for target identification.

As an alternative approach to understanding the toxicological actions of the artemisinins we have investigated gene expression profiles after drug exposure. Following exposure of mouse embryonic stem cells to a range of concentrations of DHA, ART and deoxy-DHA, we have detected down regulation of the self-renewal markers Nanog, Dppa3, Sox1 and BMP4, whereas the differentiation markers vimentin, Wnt4, MCAM and Tpb9 are up-regulated. This response pattern, together with an observed up-regulation of the anti-proliferative gene Btg2, is consistent with the proliferation block observed following artemisinin exposure. Additional gene ontology mapping and pathway analysis indicates that artemisinins activate several pathways associated with endoplasmic reticular stress, unfolded protein response and oxidative stress. Principal component analysis reveals that the gene expression profiles associated with artemisinins are dissimilar to those of genotoxins and pro-oxidants, indicating differences in mechanisms of toxicity amongst these compounds. As pluripotent mouse embryonic stem cells represent a more simplified model of the whole embryo culture, we sought to validate our gene expression profiling data using mRNA extracts from artemisinin-exposed embryonic tissue. Whilst yields of good quality RNA from embryo samples were sufficient for a large number of qPCR experiments, there were persistent reproducibility issues with this work, possibly as a result of genetic variability among embryos. However, from an early pilot study, results indicate that VEGFA is substantially up-regulated in embryonic tissue in response to treatment with DHA. Further work will be required to fully validate the gene expression profiles determined in mouse embryonic stem cells.

WP7 Artesunate/dihydroartemisinin toxicokinetics

The final experimental workpackage was designed to put the cellular experiments in the earlier WPs into the context of therapeutic drug exposures and plasma drug concentration in the dam and tissues of the foetus under conditions that generate clear embryotoxicity.

In order to achieve this University of Gothenburg has developed a sensitive and selective analytical method for the quantification of artesunate and dihydroartemisnin in rat plasma, amniotic fluid and foetal material that are suitable for pharmacokinetic and toxicokinetic studies in rodents. Because of the rapid and complete turnover of artesunate to DHA in biological systems all model analysis is based on DHA exposures, as has been the practice employed by other research groups. Bio analysis was performed using a sensitive and selective LCMSMS methodology that was capable of quantifying DHA at concentrations as low as 5ng/ml from 150a of plasma. These limits of sensitivity were subsequently shown to be adequate for the studies performed. An extensive in vivo study involving 120 animals was undertaken. Artesunate or DHA was administered at a clinical relevant dose (20mg/kg) or a high dose (100mg/kg) at gestation day (GD) 10 or 20 either orally or IV. Non pregnant rats were used as a control. Plasma samples, foetal tissue and amniotic fluid were collected and stored at -80oC.

Analysis of samples from control (non-pregnant) animals receiving a low IV dose of DHA showed a large variation in DHA levels. The level of DHA from one animal was approximately 50x that of the other two rats. This variation was also seen in the gestation day 10 group dosed orally with a low concentration of DHA. The animals were dosed immediately after each other using the same DHA solution and this observation is seen both in pregnant and in non-pregnant animals and in oral in IV administration. (see Tables below). This huge inter-subject variability has been previously reported in a number of animal species and in man. Peak drug concentrations in control IV treated rats ranged from approximately 100 to 10,0000 ng/ml. Similarly after oral administration to pregnant rats circulating plasma concentrations ranged from approximately 50 to 5000ng/ml. It was not possible to establish the relevance to this large inter-animal variability in drug exposure to embryotoxicity in this study (the study was not designed or powered to look at this). What is clear from the literature is that foetal resorption and effects on the developing foetus are related to the administered dose and time of gestation. Extrapolating to the current study this would suggest that even very moderate exposures (approximating 50 -100ng/ml peak concentrations and measurable exposures over a few hours) is sufficient to cause toxicity. The analysis of foetal and amniotic fluid samples is currently underway and so are studies to define the underlying basis for the large inter-subject variability.

To further assist in the interpretation of the data a physiologically based pharmacokinetic, PBPK, model has been developed. The data that is being generated to form the large rat study will be incorporated into a model that will allow identification of all co -variants. In a PBPK model the drug disposition in the body is described by a set of equations that together characterize the absorption, tissue distribution and elimination of the drug. The concentration-time profile of the drug can be simulated for any tissue in the model, e.g. as a measure of toxic exposure. Data from the rat plasma samples is being used to validate the PBPK model. The model will have value in predicting/understanding changes in pharmacokinetic and the pharmacodynamics behaviour of these drugs and it will allow for extrapolation across species.

WP8 Integration of data and assessment of human risk

The purpose of the work of WHO in relation to the ARTEMIP Consortium was to integrate the data created by the project together with all pre-clinical and clinical data on artemisinin

reproductive toxicology to provide an assessment of the risk: benefit ratio for the use of these drugs in humans at the end of the project. In order to achieve this a meeting was convened with the ARTEMIP consortium members and key opinion leaders in the area to discuss the findings of ARTEMIP alongside other pre-clinical data on the subject. The conclusions of the meeting (see impact below) was that although we now knew more about the underlying mechanisms of artemisinin based embryotoxicity it was not possible to mitigate the risk to pregnant women at this time.

Conclusion:

We have carried out a series of mechanistic studies in order find an explanation for the observed embryotoxicity and teratogenicity of the artemisinin class of antimalarial in pre-clinical animal models. The importance of this work is to establish a risk: benefit framework that will be useful in assessing the risk to the pregnant woman and her unborn child should they be exposed to this drug class during pregnancy. We were able to confirm the importance of the embryonic red cell as a target of toxicity and we were able to demonstrate activation of the peroxide through a possible C centred radical and also concomitant increases in oxidative stress as central to these toxic actions. We have generated genomic level data that provide some insight into the global cellular response to mammalian cells under drug exposure. This will require further investigation before we can come up with a definitive set of biochemical events that lead to toxicity. Very important outcomes include confirmation that the peroxide bridge is essential to both toxicological and antimalarial actions. However we have generated molecules where there are subtle alterations in the pattern and potency of the cytotoxic response without any change in antimalarial potency. If we can reach a point where we fully understand the relationship between these effects and the chemistry it may be useful in the design of future peroxidic antimalarials. It had been hoped that the new fully synthetic based peroxide-like antimalarials might have a cleaner embryo toxicological profile in comparison to the semi-synthetic drugs. The data generated in this project indicate that the liability is still present in these drugs although there may be some subtle but helpful differences in potency

Potential Impact:

At the outset of the project the view of the community was that malaria during pregnancy causes serious clinical effects on the mother and the foetus and constitutes a significant public health problem. The consequences of malaria include maternal anaemia, low birth weight increased mortality, and cause as many as 300,000 foetal and infant deaths, in addition to 2,500 maternal deaths each year.

The World Health Organization (WHO) currently recommends that first line treatment for uncomplicated malaria should be an artemisinin-based combination (ACT). However it is accepted that the safety of these drugs in pregnancy has not been definitively established. It is well recognised that there are data from treatment in the 2nd and 3rd trimesters with the artemisinins which encouragingly have shown no adverse outcomes or adverse event signals to date. The main anxiety relates to preclinical data that suggests that there is potential risk for loss of the foetus or for birth defects in the first trimester. In response to this concern these drugs are not recommended by WHO in the first trimester of pregnancy except when the mother's life is at stake. Thus, an important population at high risk for malaria continues to be excluded from treatment with artemisinin-based combination therapy unless these treatments are shown to be effective and safe in pregnancy.

In 2002 long before the start of ARTEMIP the mechanism of the developmental toxicity in animals was not known. It was not clear whether the initial site of action was in the mother, on the foetus or on the placenta. Detailed knowledge of the mechanism/s involved in embryotoxicity in animals was recognized to be of value in extrapolating the risks for humans. In an Informal Consultation convened in 2002 by the WHO it was concluded that 'artemisinin compounds cannot be recommended for treatment of malaria in the 1st trimester. However, they should not be withheld if treatment is considered lifesaving for the mother and other antimalarials are considered unsuitable.' In a follow up Informal Consultation in 2006, a re-review of the experimental and human data was undertaken which is now being updated to incorporate the additional data generated by ARTEMIP. At this point in time the overall conclusions stand and conclude from studies in vitro with dihydroartemisinin (DHA) and artesunate that DHA was a direct acting embryo toxicant in rat whole embryo cultures (WEC), and artesunate was more toxic to isolated primitive erythrocytes than to foetal or adult erythrocytes in vitro. Studies identified a target cell population, the primitive erythroblasts, and a chain of pathogenesis which, on its own could account for the embryo-toxicity and lethality observed in animal studies. As pointed out earlier and reassuringly clinical data from more than 1500 second and third trimester exposures did not find adverse effects.

The time window of sensitivity observed in the animal studies would correspond in the human to part of the first trimester during organogenesis. The available information in 2006 and now is inadequate to define precisely the likely period of maximum sensitivity in the human. Yolk sac haematopoiesis extends from day 14 to week 6 in humans, but it was not known whether the clonal production of primitive erythroblasts occurs in a similar way to that observed in the rat.

The background, to the working hypothesis of the experimental work of the ARTEMIP Consortium on the developmental toxicity of artemisinins in animals was that primitive embryonic red blood cells, erythroblasts, are especially sensitive to the cytotoxic effects of the artemisinins. The experimental work carried out by the ARTEMIP Consortium (and others) has focussed on establishing the mechanism of action of this toxicity with an aim of devising strategies to avoid or circumvent the risk, possibly through rational medicinal chemistry re-design.

The WHO initiated an expert review of the experimental data produced by the ARTEMIP Consortium in the context of other scientific findings. The specific focus was discussion around in-vitro studies using whole embryo culture as well as isolated cells that might elucidate the mechanism of toxic action of the artemisinins on organ development and cell viability. During a one-day discussion, experts in reproductive and developmental toxicity discussed recently published and unpublished studies on the toxicity of artemisinins. In addition, related studies in vitro using whole embryo culture as well as isolated cells was also reviewed to help elucidate the mechanism of toxic action of the artemisinins in pregnancy. Experts discussed:

- Recently published and unpublished studies on the toxicity of artemisinins and their mechanism of action
- Genetic studies using the artemisinin derivatives
- Metabolism and bioactivation studies of artemisinins
- Oxidative stress studies in developing embryo and embryonic stem cells
- The role of the mitochondrion and heme in the induction of mammalian cell death by the artemisinins
- Toxicokinetics of the artemisinins and plasma protein binding and metabolism.

The main conclusions from the workshop and the principle ARTEMIP contribution outputs are as follows:

It was concluded that ad hoc in vivo studies in the rat helped identify a narrow window of susceptibility of the embryo to artemisinins (GD9-14). In vivo treatment during gestation day (GD) 9 and GD10 induced embryo-lethality and malformations: cardiovascular (great vessels abnormalities) and limb defects (shortened and/or bent long bones). Treatments after GD15 or 16 through parturition did not affect embryo-foetal development except at higher dose levels.

A sustained depletion of primitive red blood cells (RBCs) was identified as the primary mechanism of artemisinin induced embryotoxicity in the rat. It is hypothesised that anaemia can lead to tissue hypoxia and consequent cell death. Depending on its severity and location, anaemia can cause the embryo to die or to survive with or without sequelae. It was demonstrated that DHA was not cytotoxic per se on embryonic tissues. Embryotoxicity was not species-specific. DHA targets primitive metabolically active RBCs clonally released over a short period of time from primitive sites of hematopoiesis in rodents and also in frog embryo. Mitochondria appear to be the sub-cellular site of action in embryonic primitive RBCs (as well as in Plasmodium).

The objective of establishing if a relationship existed between potency against plasmodia and embryotoxicity was met. It has now been shown that only active antimalarial peroxides are

able to interfere with embryonic RBCs survival. Inactive molecules, even with an intact peroxide moiety do not interfere. Newer synthetic substances related to the artemisinins however have shown an increased margin between the concentrations inhibitory to the plasmodium and those inhibitory to the erythroblasts, which may increase the margin of safety. However the toxicological signal remains even with the new synthetic peroxides and the risk cannot be completely mitigated at this time. It is suggested that while a 48-hour exposure at a critical phase of an approximately 5-day period in rats and Xenopus may seriously affect embryonic RBCs, a few hours' exposure out of a development period in humans of at least 28 days may not result in equivalent damage. However attempts to generate newer fully synthetic peroxides with slow elimination profiles may push safety profiles in the wrong direction based on this working hypothesis.

Post-meeting, the WHO has asked the invited experts to undertake an updated literature search for recent information on the earliest stages of red blood cell formation in the human embryo. This review was completed and the WHO is working with ARTEMIP consortium members and other experts to finalise it for publication.

The overall conclusion and current 'state of the art' at the end of ARTEMIP is that the critical three-day period in rat and mouse that encompasses all the processes of early erythropoesis covers a significantly longer period of real time in humans, perhaps 10-14 days. This longer period may, in itself be sufficient to reduce sensitivity to artemisinin-induced developmental effects when the drugs are used clinically for a period of only 3 days. Human data is not currently available to support this hypothesis but results will be made available from the MiP Consortium on the safety of the artemisinins in pregnancy - due to report in 2013. The WHO proposes to hold a separate clinical meeting to review the clinical data and relate the information to the experimental data generated by ARTEMIP and the malaria community in general sometime in 2012.

List of Websites:

http://www.liv.ac.uk/lstm/lstm.html