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Comparative embryotoxicity of different antimalarial peroxides: *In vitro* study using the rat whole embryo culture model (WEC)☆

Monica Longo[a,*], Sara Zanoncelli[a], Marco Brughera[a], Paolo Colombo[a], Sergio Wittlin[b], Jonathan L. Vennerstrom[c], Joerg Moehrle[d], J. Carl Craft[e]

[a] *Accelera S.r.l., Viale Pasteur 10, 20014 Nerviano, Milan, Italy*
[b] *Swiss Tropical Institute, Socinstrasse 57, CH-4002 Basel, Switzerland*
[c] *College of Pharmacy, University of Nebraska Medical Center, 986025 Nebraska Medical Center, Omaha, NE 68198-6025, USA*
[d] *Medicines for Malaria Venture, Route de Pre-Bois 20, CH-1215 Geneva, Switzerland*
[e] *MMV, 318 Camelot Lane, Libertyville, IL, USA*

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ABSTRACT

Three groups of compounds: (i) active peroxides (artemisinin and arterolene), (ii) inactive non-peroxidic derivatives (deoxyartemisinin and carbaOZ277) and (iii) inactive peroxide (OZ381) were tested by WEC system to provide insights into the relationship between chemical structure and embryotoxic potential, and to assess the relationship between embryotoxicity and antimalarial activity.

Deoxyartemisinin, OZ381 and carbaOZ277 did not affect rat embryonic development. Artemisinin and arterolane affected primarily nucleated red blood cells (RBCs), inducing anemia and subsequent tissue damage in rat embryos, with NOELs for RBC damage at 0.1 and 0.175 μg/mL, respectively. These data support the idea that only active antimalarial peroxides are able to interfere with normal embryonic development. In an attempt to establish whether and to what extent activity as antimalarials and embryotoxicity can be divorced, IC_{50}s for activity in *Plasmodium falciparum* strains and the NOELs for RBCs were compared. From this comparison, arterolane showed a better safety margin than artemisinin.

1. Introduction

Semi-synthetic artemisinins have become a cornerstone of contemporary malaria treatment. The current WHO recommended first line treatment of uncomplicated malaria is based on combinations of artemisinin compounds with other, longer lasting antimalarials (artemisinin-containing combination treatment, ACT) [1].

Artemisinin-based compounds are very potent inhibitors of parasite growth and are significantly faster in clearing the parasite, fever, and symptoms than any other known antimalarial [2]. In addition, elimination from the patients' blood is very rapid increasing the tolerability of these compounds [3]. As a monotherapy, they should be given for 7 consecutive days in order to cover at least three parasite replication cycles, thus making treatment impractical and difficult to adhere to. Combination with other antimalarials with longer residence time allows both shorter treatment courses (3 days) and mutual protection against resistance. A short half-life also minimizes the risk of parasites being exposed to drug levels that could select for drug-tolerant parasites.

However, currently available drugs of this class have some limitations and liabilities: (i) the source of active ingredients and (ii) the contraindication in the first trimester of pregnancy [4].

All in-use compounds are either extractive artemisinin or semi-synthetic derivatives, thus relying upon agricultural sources of the compound, a system that has shown limitations in the supply/demand response [5]. The active moiety of artemisinins is a peroxide bond embedded in a 1,2,4-trioxane heterocycle [6].

Arterolane (also called RBx11160 or OZ277), a fully synthetic peroxide, was selected from a series of 1,2,4-trioxolane molecules [7] by the Medicine for Malaria Venture (MMV) and Ranbaxy as a candidate for development in 2003 as a part of collaborative research program. This compound is still in development by Ranbaxy and is in Phase III clinical trials as an arterolane maleate-piperaquine phosphate combination since 2009.

The use of ACTs is currently contraindicated in the first trimester of pregnancy [1]. The reason for this contraindication is that artemisinin compounds cause embryo deaths and dysmorphogenic events in experimental animals when administered over a narrow window of sensitivity corresponding to the clonal production

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* Corresponding author. Tel.: +39 0331 1984269; fax: +39 0331 1984200.
E-mail address: monica.longo@accelera.org (M. Longo).

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of primitive red blood cells (RBCs) by the yolk sac [8–11]. This occurs on gestational days (GD) 9–14 in the rat and 24-h to 4 days post fertilization in *Xenopus Laevis*, corresponding to GD 18.5 to weeks 4–6 of pregnancy in humans [12–15]. While no developmental toxicity has yet been reported from use of artemisinin-type compounds in pregnant women including some cases in the first trimester [16–27], and while the relevance of animal data to human pregnancy is uncertain, this contraindication holds.

The liability appears to be a "class effect" related to the peroxide moiety, although the subcellular and molecular mechanisms of toxicity are not completely understood.

However, one element to be considered in selecting drug development candidates within this class of compounds would be to check if and to what extent potency against plasmodia can be divorced from embryotoxicity.

We have successfully applied the rat whole embryo culture (WEC) system in evaluating the embryotoxic potential of dihydroartemisinin (DHA) [8,9]. The WEC system allows the measuring of effects during the window of highest rat embryo sensitivity (GD 9.5–11.5) and thus helps define dose/response relationships and the identification of the no observed effect level (NOEL).

Here, we used the WEC system to assess and compare the potential for embryonic toxicity of the following compounds:

- Artemisinin and its inactive non-peroxidic isostere deoxyartemisinin [28].
- Arterolane (synthetic 1,2,4-trioxolane) and its inactive non-peroxidic isostere carbaOZ277 [29] and the inactive peroxidic hydroxylated metabolite OZ381 [30] (Fig. 1). We also include previously published data [8] for DHA for comparison.

These compounds were tested in order to provide insights into the relationship between chemical structure and embryotoxic potential, and to assess the relationship between embryotoxicity and antimalarial activity.

2. Materials and methods

2.1. Whole embryo culture

The method applied for whole embryo culture (WEC) was the same as previously described [8,9]. Briefly, embryos from pregnant Crl:CD(SD)BR rats were explanted in the afternoon of gestation day (GD) 9 (9.5-day-old embryos considering the day on which spermatozoa were found in the vaginal smear as Day 0 of pregnancy). Embryos from 1 to 3 somite stage were randomly distributed to experimental groups and cultured according to the New's method [31] in a 25-mL glass bottle (5 embryos/bottle) containing 5 mL of heat-inactivated sterile rat serum and antibiotics (penicillin 100 IU/mL, streptomycin 100 μg/mL) placed in a thermostatic (37.8 ± 0.1 °C) roller (~20 rpm) apparatus for 48 h.

The medium was periodically equilibrated with gas mixture containing increasing concentration of O_2.

All compounds were dissolved in DMSO (Sigma) at the appropriate concentrations immediately before use, and then diluted 1:1000 in the culture medium to reach the final appropriate concentrations. Test articles were added to the culture medium at the start of the culture on GD 9.5. The medium was not renewed during the 48-h culture.

The groups are as follows: Group 1: negative control, DMSO 0.1%; Group 2: artemisinin; Group 3: deoxyartemisinin; Group 4: arterolane; Group 5: OZ381; Group 6: CarbaOZ277.

The main characteristics of these test items and the different concentrations used are summarized in Table 1 (Fig. 1).

At the end of the culture period, the embryos (11.5 days old) were examined under the stereomicroscope. Heartbeat and yolk sac circulation were observed; embryos without heartbeat were considered dead. Yolk sac diameter, crown-rump length and head length were measured and the somites were counted. Live embryos were scored using the method of Brown and Fabro [32].

For each embryo, observed morphological abnormalities were divided into major abnormalities and minor abnormalities as previously described [8,9].

After measuring yolk sac diameters and scoring, the first two or three embryos of each bottle were used to collect embryonic blood cells. Embryos with intact yolk sac were immersed in phosphate buffered saline (PBS), pH 7.4. Yolk sac vessels were cut at the level of the vitelline artery and vein allowing blood cells to pool in the PBS, where they were collected with a pipette. Collected cells were concentrated by centrifugation at 700 rpm for 3 min onto slides using a Cytospin 3. The slides were immediately fixed in methanol and stained by the Wright–Giemsa method. Embryonic red blood cells (RBCs) were then analysed under a light-microscope to detect possible alterations. After blood collection, embryos were scored.

The remaining embryos of each bottle, after scoring, were fixed in 4% formaldehyde overnight and then embedded in paraffin, sectioned and stained with hematoxylin and eosin and histologically examined under a light-microscope to detect possible tissue alterations.

Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons or the chi-square test. The level of significance was set at $P \leq 0.05$.

2.2. In vitro antimalarial activity

In vitro antimalarial activity of the tested compounds was measured using the [^{3}H]-hypoxanthine incorporation assay [30,33] with various strains of *Plasmodium falciparum* obtained from F. Hoffmann-LaRoche AG (Basel). *P. falciparum* was cultivated in a variation of the medium previously described [34,35], consisting of RPMI 1640 supplemented with 0.5% ALBUMAX® II, 25 mM Hepes, 25 mM NaHCO3 (pH 7.3), 0.36 mM hypoxanthine, and 100 g/mL neomycin. Human erythrocytes served as host cells. Cultures were maintained at 37 °C in an atmosphere of 3% O_2, 4% CO_2, and 93% N_2 in humidified modular chambers. Compounds were dissolved by sonication in DMSO (10 mg/mL) and diluted in hypoxanthine-free culture medium. Infected erythrocytes (100 μL per well with 2.5% hematocrit and 0.3% parasitemia) were added to each drug titrated in 100 μL duplicates over a 64-fold range. After 48 h incubation, 0.5 μCi of [^{3}H]-hypoxanthine in 50 μL medium was added and plates were incubated for an additional 24 h. Parasites were harvested onto glass-fiber filters and radioactivity was counted using a Betaplate liquid scintillation counter (Wallac, Zurich). The results were recorded as counts per minute (cpm) per well at each drug concentration and expressed as a percentage of the untreated controls. Results were expressed as the concentration resulting in 50% inhibition (IC_{50}).

3. Results

3.1. Whole embryo culture

3.1.1. Control embryos (Tables 2–5)

All embryos in the control group cultured from gestation day (GD) 9.5 to GD 11.5 were alive at the end of the culture period. They showed normal growth and development and no abnormalities were detected either macroscopically or histologically.

In particular, the yolk sac was fully vascularized and red blood cells (RBCs) were easily visible.

Analysis of cytospin preparations of RBCs showed numerous well developed large nucleated spherical RBCs, some of which with mitotic figures.

The rare occurrence of control embryos with situs inversus viscerum as minor abnormality was ascribed to the early stage of embryos at the time of explantation (1 somite stage).

3.1.2. Deoxyartemisinin, OZ381 and carbaOZ277 (Table 3; Fig. 2)

All embryos exposed to deoxyartemisinin, OZ381 and carbaOZ277 from 1 to 10 μg/mL were alive at the end of the culture period. They showed normal heartbeat and yolk sac circulation and normal growth and development of all the principal embryonic and extra-embryonic parameters. No major abnormalities were seen. Histological examination did not show tissue alteration nor did cytospin RBC preparations reveal appreciable changes in the number and shape of RBCs.

In summary, deoxyartemisinin, OZ381 and carbaOZ277 did not affect rat embryonic development *in vitro* up to the highest concentration tested of 10 μg/mL.

3.1.3. Artemisinin (Tables 2–5; Fig. 3)

All embryos exposed to artemisinin at all concentrations were alive at the end of the culture period. At artemisinin concentrations ≥0.175 μg/mL, the visceral yolk sac was well vascularized but pale; yolk sac vessel formation and circulation appeared normal but blood was visibly paler than controls. This effect increased with increasing artemisinin concentrations and it was graded as slight at 0.175 μg/mL, moderate at 0.25 μg/mL and marked starting