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Relative potency ranking of azoles altering craniofacial morphogenesis in rats: An *in vitro* data modelling approach

Francesca Di Renzo[a,1], Francesca Metruccio[b,1], Maria Battistoni[c], Angelo Moretto[b,c,*], Elena Menegola[a]

[a] Department of Environmental Sciences and Policy, Università degli Studi di Milano, Milan, Italy
[b] International Centre for Pesticides and Health Risks Prevention (ICPS), ASST Fatebenefratelli Sacco, Milan, Italy
[c] Department of Biomedical and Clinical Sciences, Università degli Studi di Milano, Milan, Italy

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ABSTRACT

Facial malformations represent one of the most frequent abnormality in humans. The adverse outcome pathway involved in facial defects seems to be related to retinoic acid (RA) pathway imbalance. Environmental agents inducing craniofacial malformations in experimental models include pesticides (especially azole fungicides). By using the *in vitro* alternative method postimplantation rat whole embryo culture (WEC), we evaluated the intrinsic embryotoxic activity of some azole antifungals (cyproconazole, CYPRO; triadimefon, FON; flusilazole, FLUSI; and prochloraz, PCZ), in comparison to RA. All the tested molecules induced in a dose-related manner specific defects of the craniofacial structures (fused branchial arches), similar to those induced by RA. Collected data were modelled using PROAST 65.5 software to characterise the relative potency factors (RPFs) versus RA. In comparison to RA, all the evaluated azoles were less potent, showing among them a similar potency. Our data suggest a possible azole-related RA signalling perturbation to be further investigated. Moreover, the present results indicate the approach used in this work to be an interesting tool applicable to the hazard evaluation of novel compounds or the assessment of combined exposure to azoles or other dismorphogens.

1. Introduction

Among congenital anomalies in humans, oral clefts in any form (cleft lip and/or palate alone or associated with other head skeletal defects) occur in approximately 1:700 live births, both as isolated anomalies and in syndromic conditions (Mossey et al., 2009). Evidence suggests that oral clefts are multifactorial in origin, involving both genetic and environmental risk factors (Mossey et al., 2009).

The elucidation of the different pathways contributing to craniofacial morphogenesis and of their disturbance by different chemicals is fundamental to evaluate the contribution of multiple exposures to the incidence of craniofacial defects.

Retinoic acid (RA), the active metabolite of vitamin A, is the main regulator of embryo development (Metzler and Sandell, 2016) controlling also the normal craniofacial morphogenesis. Both deficiency and excess of embryonic RA are related to malformations at multiple districts, including craniofacial defects in humans and animals (Warkany and Schraffenberger, 1944; Lammer et al., 1985; Hathcock et al., 1990; Morriss-Kay, 1992; Browne et al., 2014; Piersma et al., 2017). In the presence of excess of RA, postimplantation rodent whole embryo cultures (WEC) show specific defects, including branchial arch (the embryonic facial primordial) abnormalities (Klug et al., 1989; Menegola et al., 2004).

Antifungal azoles are a group of fungistatic agents used both in clinical and veterinary practice as well as in agriculture. A number of adverse effects via non-genotoxic mechanisms have been observed in laboratory animals, such as effects on the liver, on reproduction, and developmental defects. *In vivo* experimental studies, case reports, and a recent birth defect prevention study relate *in utero* exposure to some azole fungicides to craniofacial defects (Tachibana et al., 1987; Lee et al., 1992; Pursley et al., 1996; Aleck and Bartley, 1997; Sanchez and Moya, 1998; Lopez-Rangel and Van Allen, 2005; Menegola et al., 2005; Di Renzo et al., 2011a; Howley et al., 2016). The teratogenic effect of certain azole fungicides in WEC has been documented in the past, describing abnormalities at the branchial arch apparatus level (Tiboni, 1993; Menegola et al., 2000, 2001; 2003, 2004; 2005; Di Renzo et al., 2011b). The hypothetical mode of action for craniofacial defects of the teratogenic antifungal azoles is the inhibition of embryonic CYP26,

* Corresponding author. Department of Biomedical and Clinical Sciences, University of Milano, Via GB Grassi 74, 20157, Milano. Italy.
E-mail address: angelo.moretto@unimi.it (A. Moretto).
[1] These authors contributed equally to this work.

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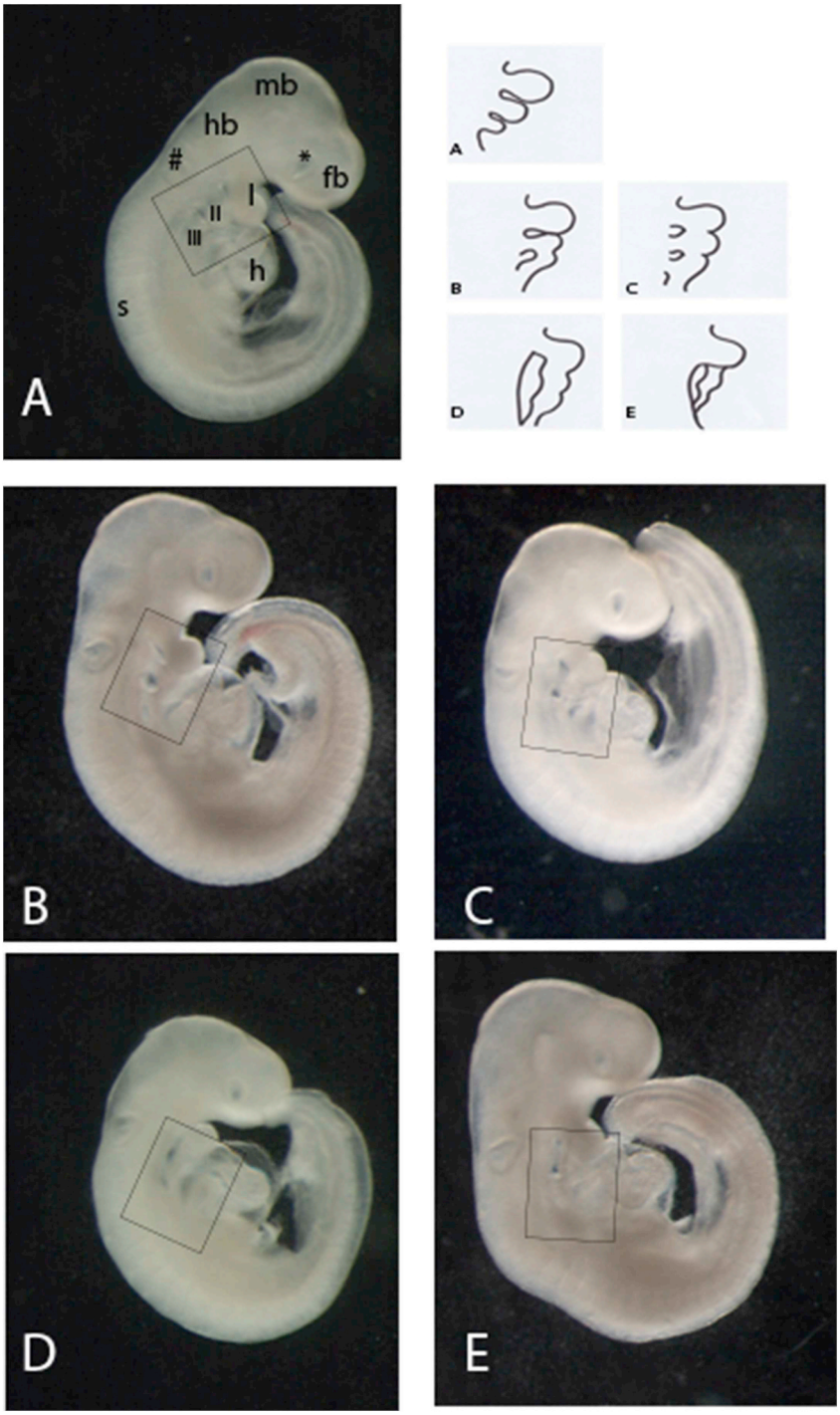

Fig. 1. Evaluation of general and branchial morphology (in the rectangles) 48 h in culture. In the right corner: scheme illustrating branchial arch morphology.
A) normal embryo dorsally convex with a tripartite encephalon with enlarged ventricles (fb, forebrain; mb, midbrain; hb, hindbrain); otic (#) and optic (*) vesicles; regular somite disposition (s); looped heart (h) three well separated branchial arches (I– II– III); B-E) abnormal branchial arch morphology: B) II-III unseparated branchial arches; C) I-II-III branchial arches ventrally fused (dorsal radicles visible)
D) I-II-III branchial arches totally fused (dorsal radicles undistinguishable); E) I-II-III branchial arches forming an unique mass.Magnification 20.

which metabolises RA, with consequent increase of endogenous RA content (Tiboni et al., 2009; Giavini and Menegola, 2010; Robinson et al., 2012; Piersma et al., 2017). An azole-related RA pathway imbalance has been recently described by using a transcriptomic approach (Dimopoulou et al., 2017a, 2017b).

Increasingly, the focus of risk assessment is on combined exposures, and for those molecules sharing the same mode of action, dose additivity with the use of the relative potency factor (RPF) approach has been suggested (Hardy et al., 2017). The RPF method entails scaling the toxicity of each individual component in the combined exposures to the toxicity of an index compound.

The aim of the present work is to define the RA-relative potency of four antifungal azoles by fitting WEC experimental data using PROAST software analysis (www.proast.nl) (modelling procedure according to Slob, 2002; Moretto et al., 2015). The chemicals known to induce craniofacial defects selected in the present work are RA and azole antifungals (cyproconazole, CYPRO; flusilazole, FLUSI; triadimefon, FON; and prochloraz, PCZ).

Three of these azoles (CYPRO, FON, FLUSI) are known to induce craniofacial defects after *in utero* exposure (FAO/WHO, 2005; 2008, 2011; Menegola et al., 2005) and branchial defects *in vitro* (Menegola et al., 2000, 2001; Di Renzo et al., 2011b). PCZ, an azole with broad-spectrum fungistatic activity, induced maternotoxicity and embryotoxicity but not malformations after exposure *in utero* during the entire

Table 1
Percentage of embryos with malformations at the branchial arches (BA).
Grey columns indicate concentration levels at which extra-branchial defects were also observed.

RETINOIC ACID	RA 0 □M	RA 0.025 □M	RA 0.05 □M	RA 0.125 □M	RA 0.25 □M	RA 0.5 □M	RA 1 □M		
BA abnormalities	0.0	0.0	37.5	73.7	88.2	85.7	100.0		
II-III BA unseparated	0.0	0.0	25.0	5.3	0.0	0.0	0.0		
I-II-III BA vetrally fused	0.0	0.0	12.5	57.9	41.2	7.1	0.0		
I-II-III BA fused without dorsal part visible	0.0	0.0	0.0	5.3	11.8	39.3	0.0		
I-II-III BA fused in a unique mass	0.0	0.0	0.0	5.3	35.3	39.3	100.0		
CYPROCONAZOLE	**CYPRO 0 □M**	**CYPRO 7.8 □M**	**CYPRO 15 □M**	**CYPRO 31 □M**	**CYPRO 46.8 □M**	**CYPRO 62.5 □M**	**CYPRO 125 □M**	**CYPRO 250 □M**	
BA abnormalities	0.0	20.0	22.2	90.0	100.0	100.0	100.0	100.0	
II-III BA unseparated	0.0	13.3	22.2	70.0	0.0	0.0	0.0	0.0	
I-II-III BA vetrally fused	0.0	6.7	0.0	20.0	22.2	66.7	20.0	0.0	
I-II-III BA fused without dorsal part visible	0.0	0.0	0.0	0.0	66.7	33.3	0.0	0.0	
I-II-III BA fused in a unique mass	0.0	0.0	0.0	0.0	11.1	0.0	80.0	100.0	
FLUSILAZOLE	**FLUSI 0 □M**	**FLUSI 1.56 □M**	**FLUSI 3.125 □M**	**FLUSI 4.8 □M**	**FLUSI 6.25 □M**	**FLUSI 7.7 □M**	**FLUSI 9.375 □M**	**FLUSI 10.1 □M**	**FLUSI 12.5 □M**
BA abnormalities	0.0	0.0	36.4	100.0	77.8	100.0	100.0	100.0	100.0
II-III BA unseparated	0.0	0.0	27.3	40.0	33.3	44.4	40.0	0.0	0.0
I-II-III BA vetrally fused	0.0	0.0	9.1	20.0	33.3	22.2	40.0	42.9	20.0
I-II-III BA fused without dorsal part visible	0.0	0.0	0.0	40.0	11.1	33.3	20.0	42.9	80.0
I-II-III BA fused in a unique mass	0.0	0.0	0.0	0.0	0.0	0.0	0.0	14.3	0.0
TRIADIMEFON	**FON 0 □M**	**FON 6.25 □M**	**FON 12.5 □M**	**FON 25 □M**	**FON 26.7 □M**	**FON 42.85 □M**	**FON 50 □M**	**FON 56 □M**	**FON 125 □M**
BA abnormalities	0.0	0.0	18.2	37.5	90.0	100.0	100.0	100.0	100.0
II-III BA unseparated	0.0	0.0	9.1	37.5	70.0	50.0	50.0	0.0	0.0
I-II-III BA vetrally fused	0.0	0.0	9.1	0.0	20.0	50.0	16.7	37.5	0.0
I-II-III BA fused without dorsal part visible	0.0	0.0	0.0	0.0	0.0	0.0	33.3	50.0	0.0
I-II-III BA fused in a unique mass	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.5	100.0
PROCHLORAZ	**PCZ 0 □M**	**PCZ 6.25 □M**	**PCZ 12.5 □M**	**PCZ 25 □M**	**PCZ 50 □M**				
BA abnormalities	0.0	11.1	46.7	77.8	77.8				
II-III BA unseparated	0.0	11.1	46.7	44.4	33.3				
I-II-III BA vetrally fused	0.0	0.0	0.0	33.3	33.3				
I-II-III BA fused without dorsal part visible	0.0	0.0	0.0	0.0	11.1				
I-II-III BA fused in a unique mass	0.0	0.0	0.0	0.0	0.0				

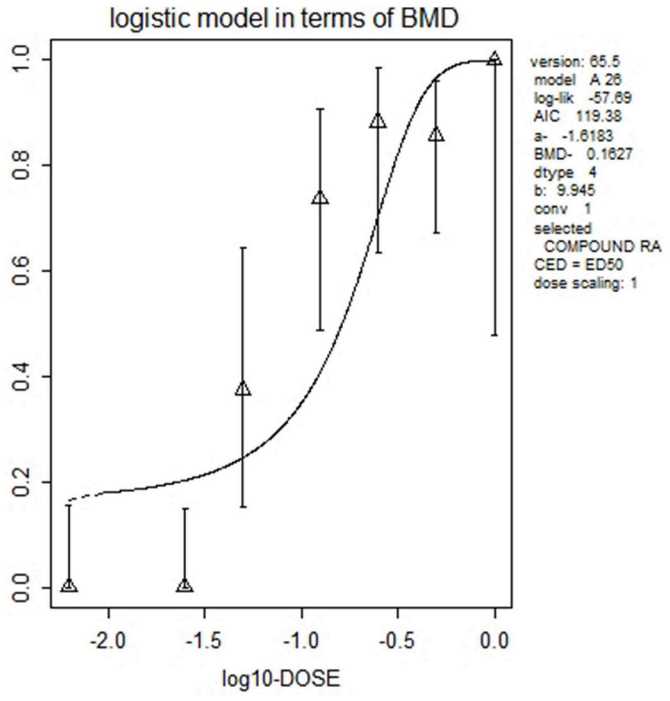

Fig 2. Single dose-response curves of RA.

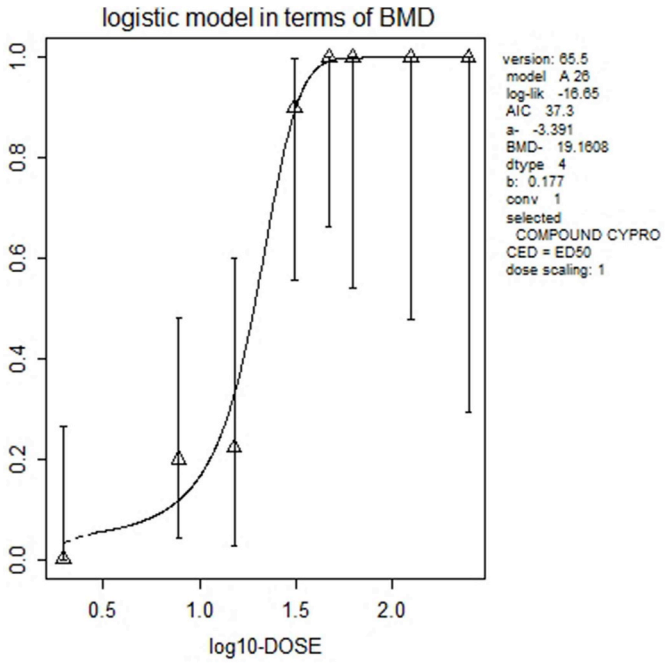

Fig 3. Single dose-response curves of CYPRO.

gestation (FAO/WHO, 2002). No data are available on PCZ embryotoxicity when exposure duration was limited to the organogenetic period or after embryo exposure *in vitro*. PCZ has been selected to evaluate its intrinsic activity on embryo development in the absence of a maternal compartment. RA has been chosen in the present work as an index compound due to the fact that azoles are suspected to interfere with RA catabolism, thereby causing an increase in RA levels in specific embryo segments.

2. Materials and methods

2.1. Materials and compound preparation

All the tested compounds were purchased from Sigma, Italy. The azoles were dissolved in ethanol (Sigma), RA was dissolved in dimethyl sulfoxide (DMSO; Sigma). The medium used for the extraction of embryos from the uteri was sterilised Tyrode solution (Sigma); the medium used for the post-implantation whole embryo culture was undiluted heat inactivated rat serum added with antibiotics (penicillin 100 IU/mL

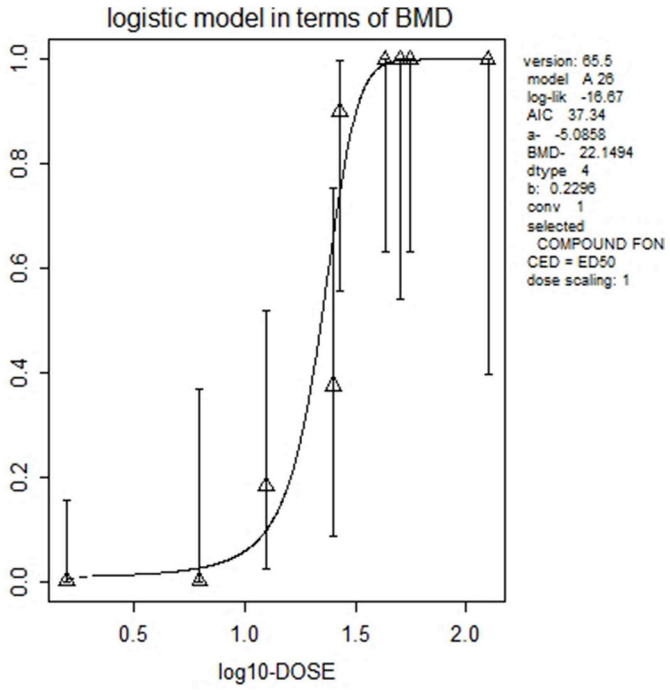

Fig 4. Single dose-response curves of FON.

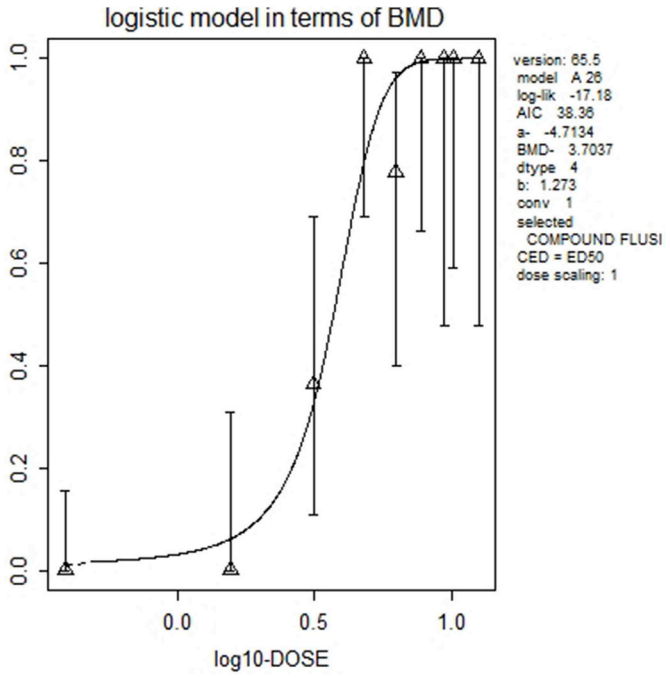

Fig 5. Single dose-response curves of FLUSI.

culture medium and streptomycin 100 μg/mL culture medium, Sigma).

2.2. Selection of compound concentrations

The concentrations of test molecules were selected from previous published experiments on rat WEC to gradually achieve the maximum degree of severity for branchial malformations: RA 0.025–1 μM (Menegola et al., 2004), CYPRO 7.8–250 μM (Di Renzo et al. 2011a,b), FON 6.25–125 μM (Menegola et al., 2000; Di Renzo et al., 2011b), and FLUSI 1.56–125 μM (Menegola et al., 2001). PCZ concentrations were selected on the basis of an unpublished range-finding test (6.25–50 μM).

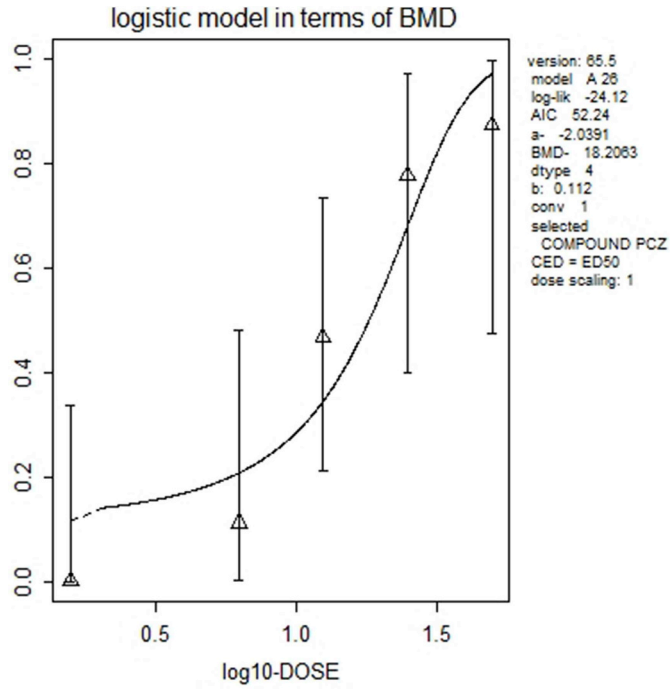

Fig 6. Single dose-response curves of PCZ.

For each dose-response experiment, a group was exposed to the relative solvent (dose 0).

2.3. Embryo culture

Virgin female CD:Crl rats (Charles River, Calco, Italy), housed in a thermostatically maintained room (T = 22 ± 2 °C; relative humidity 55 ± 5%) with a 12-h light-dark cycle (light from 6:00 a.m. to 6:00 p.m.), and free access to food (Italiana Mangimi, Settimo Milanese, Italy) and tap water, were caged overnight with males of proven fertility. All animal use protocols were approved by the Ministry of Health, Department for Veterinary Public Health, Nutrition and Food Safety committee. The animals were treated humanely and with regard for the alleviation of suffering.

Embryos were explanted from untreated pregnant rats at E9.5 (early neurula stage, 1–3 somites; day of positive vaginal smear = 0) and cultured according to the New method (1978) in 20-mL glass bottles (five embryos/bottle), containing 5 mL of culture medium. The culturing was performed at least in triplicate for each group.

The bottles, inserted in a thermostatic (37.8 °C) roller (30 rpm) apparatus, were periodically gas equilibrated according to Giavini et al. (1992). After 48 h of culturing, the embryos were morphologically examined under a dissecting microscope to evaluate any branchial or extra-branchial abnormality.

2.4. PROAST modelling

The statistical analysis was done by dose-response modelling using PROAST, a software package developed by the Dutch National Institute for Public Health and the Environment (RIVM) (www.proast.nl) for the statistical analysis of dose-response data (65.5 version). Results on branchial arch abnormalities have been modelled according to the dose-response analysis for quantal data.

The first step was the analysis of individual datasets to obtain dose-response curves for single compounds, setting the benchmark dose (BMD) at 50% response (BMR). The exponential model family equations were selected to describe the dose-response curves and obtain RPFs versus the index compound (in our case, RA). The log-likelihood

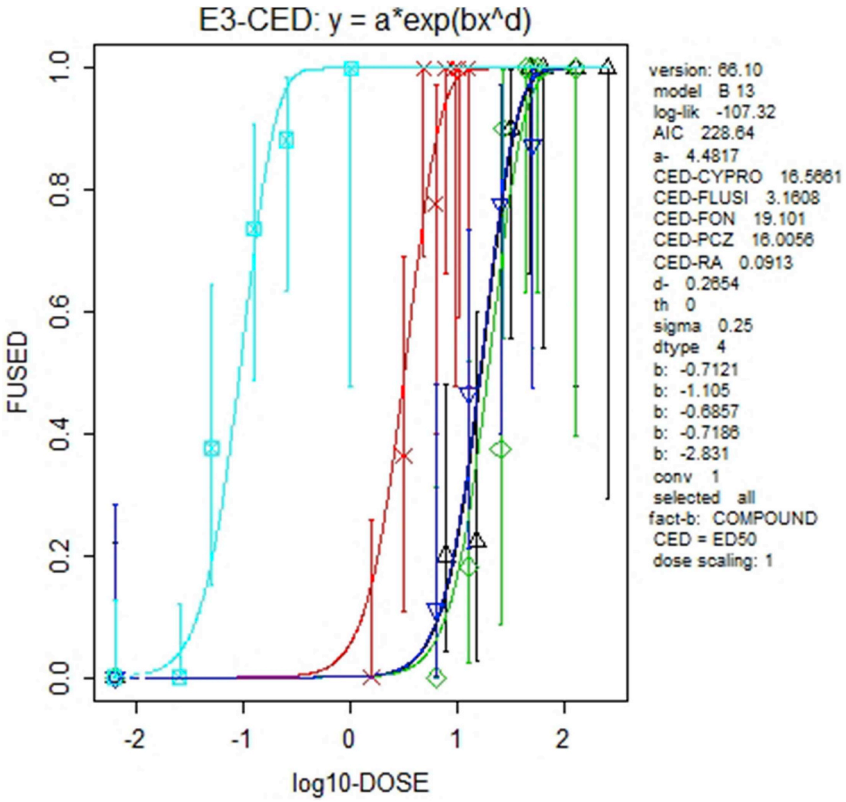

Fig 7. Evaluation of the benchmark doses (BMDs) for benchmark response at 50% of CYPRO, FON, FLUSI, PCZ in respect to RA. From left to right: RA-FLUSI-PCZ-CYPRO-FON.

Table 2
Parameters obtained by PROAST analysis, fitting separate dataset for each compound and combined dataset for all. BMD = benchmark dose; BMR = benchmark response.

	BMD for BMR 50% (μM)	log-likelihood
RA	0.16	−57.69
CYPRO	19.16	−16.65
FON	22.15	−16.67
FLUSI	3.7	−17.18
PCZ	18.21	−24.12
COMBINED (RA as index)	0.1238	−129.13

ratio test was applied to assess the equal steepness assumption.

3. Results

3.1. Effects of exposure to RA, CYPRO, FON, FLUSI, and PCZ on rat embryo development

After 48 h in culture, normal embryos were dorsally convex and reached the phylotypic stage: tripartite encephalon (forebrain, midbrain, hindbrain) with enlarged ventricles, an open posterior neuropore, and three well-separated branchial arches (the embryonic precursors of facial structures) (Fig. 1 A).

Dose-related teratogenic effects were detected in embryos exposed to the different chemicals. A syndromic picture was observed after RA exposure at a concentration level of 0.125 μM or greater. The affected districts were the encephalon (swollen and short romboencephalon, microcephalia), the branchial apparatus (fused branchial arches with different degrees of severity, as listed in Table 1), somites (fused), and tail (hook-shaped tail) at any effective concentration. By contrast, the exposure to FON at concentrations of 12.5 μM or greater and FLUSI at concentrations of 3.13 μM or greater affected only the branchial apparatus (fused branchial arches). Malformed branchial arches resulted after exposure to CYPRO at concentrations of 7.8 μM or greater and PCZ at concentrations of 6.25 μM or greater, whereas other districts were affected only at the highest concentrations (CYPRO 250 μM, tail malformations; PCZ 25–50 μM, abnormalities at the encephalon, somites, and tail). The branchial defects are listed in Table 1 and consisted of fusion among branchial arches of different degrees, as follows: II-III unseparated branchial arches; I-II-III branchial arches ventrally fused (dorsal radicles visible); I-II-III branchial arches totally fused (dorsal radicles undistinguishable); or I-II-III branchial arches forming a unique mass (Fig. 1B–E).

3.2. PROAST analysis

PROAST analysis was exclusively limited to branchial outcomes, because this apparatus was the common target for all the tested substances.

Table 3
Relative potency factors obtained by PROAST analysis, fitting combined dataset.

	RA	CYPRO	FON	FLUSI	PCZ
RPF (CI)	1	0.0077 (0.00543–0.011)	0.0069 (0.005–0.0095)	0.043 (0.0315–0.0591)	0.0079 (0.0055–0.0113)

RPF = relative potency factor.

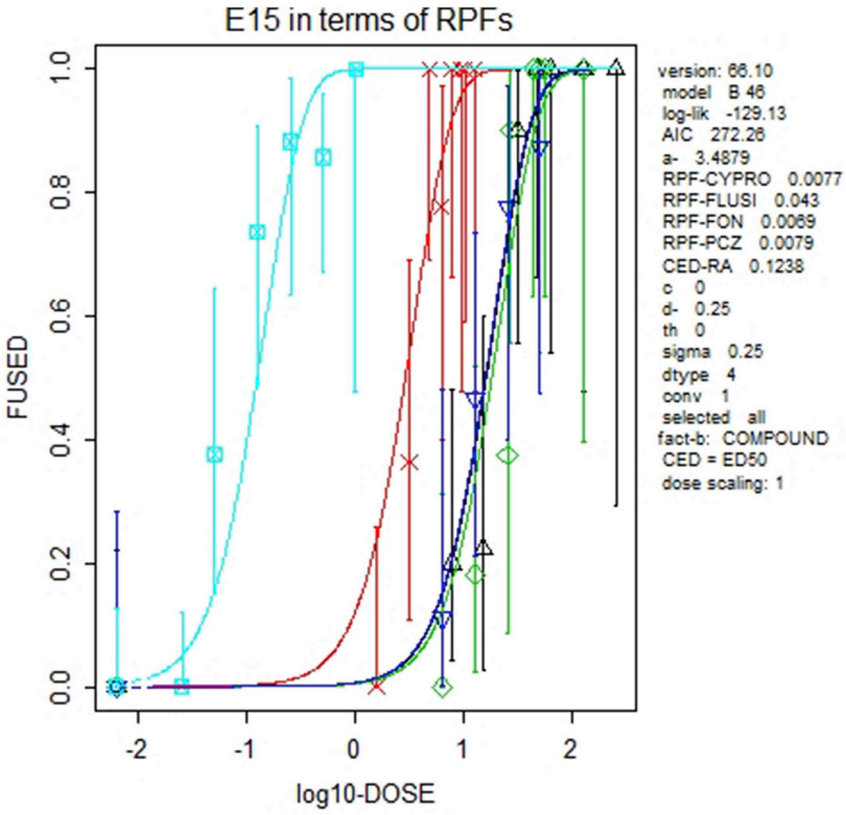

Fig 8. Evaluation of the relative potency factors (RPFs) of the effects of CYPRO, FON, FLUSI, PCZ in respect to RA. From left to right: RA-FLUSI-PCZ-CYPRO-FON.

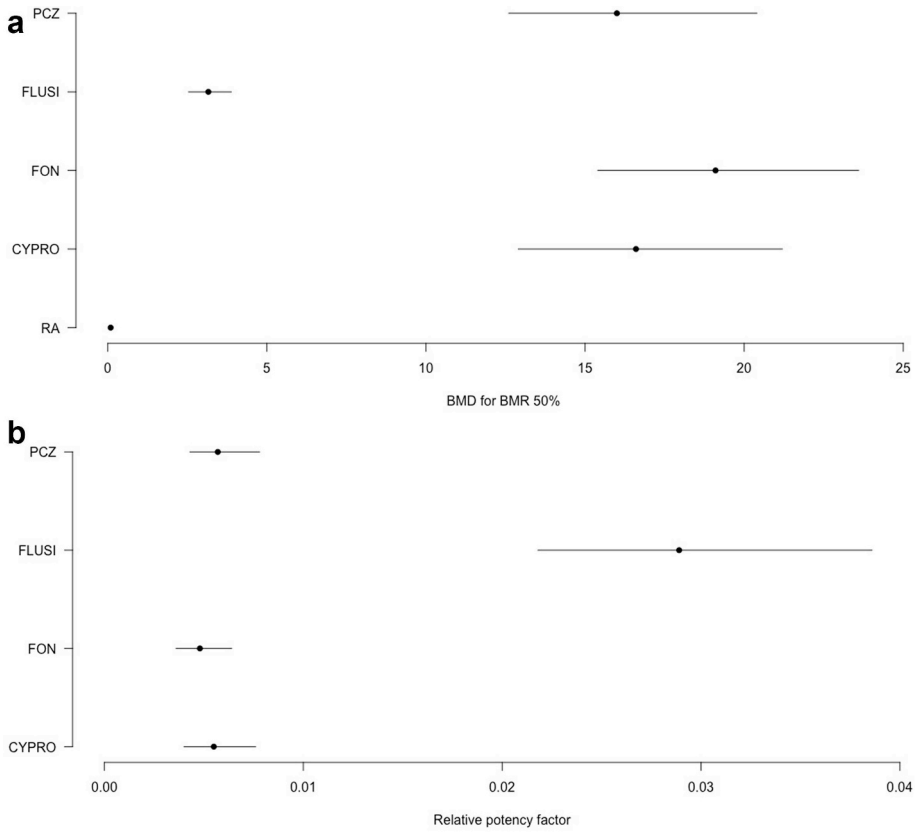

Fig. 9. Plot of benchmark doses (BMDs) for benchmark response at 50% with CIs (a) and plot of relative potency factors (RPFs) with CIs (b).

The first step consisted of comparing the fits to the single datasets (Figs. 2–6) with the fit to the combined dataset (Fig. 7), using in both cases the exponential model. The log-likelihood ratio test showed that the equal steepness assumption was not rejected ($p = 0.14$ with log-likelihood of separate fits = −101.78, log-likelihood of the overall fit = −107.32, degrees of freedom = 7) (Table 2).

As the steepness result was homogenous, the benchmark doses (BMDs) for benchmark response (BMR) at 50% and relative potency factors (RPFs) were estimated by the combined model fit (Table 3; Figs. 7 and 8). The evaluation of BMD CIs and RPF CIs suggests that, with the exception of FLUSI, all the tested azoles were similar among each other and at least two magnitude orders less potent than RA (Table 3, Fig. 9). FLUSI was more potent than the other azoles, but at least one magnitude order less potent than RA (Table 3, Fig. 9).

4. Discussion

The WEC is a validated toxicological *in vitro* method (Corvi et al., 2006; ECVAM, 2006; Flick and Klug, 2006) that also can be useful for mechanistic and modelling approaches (Robinson et al., 2012; Menegola et al., 2013; Zhang et al., 2016; Dimopoulou et al., 2017a, 2017b). *In vitro* methods are in general encouraged in toxicity assessment to apply the RPF approach (European Food Safety Authority (EFSA), 2008).

In agreement with previous WEC studies (Tiboni, 1993; Menegola et al., 2000, 2001; 2003, 2004; 2005; Di Renzo et al., 2011b), we observed a clear dose-response in rat embryos exposed *in vitro* to RA and to the tested azoles, including the newly studied PCZ. The specific target for all tested azoles was the branchial apparatus, which was also affected by RA exposure. The branchial arches are typical embryonic structures involved in craniofacial morphogenesis. Local perturbation of endogenous RA levels, mediated by the azole-dependent inhibition of RA catabolism (inhibition of CYP26 enzyme), is the proposed mode of action for azoles (Tiboni et al., 2009; Giavini and Menegola, 2010; Robinson et al., 2012; Piersma et al., 2017). As previously suggested, the inhibition of embryonic CYP26 isoenzymes could indirectly increase the local endogenous concentration of RA at the branchial apparatus level (Menegola et al., 2004). Using a transcriptomic approach, Dimopoulou et al. (2017a, 2017b) indicate a correlation between CYP26-dependent RA-pathway perturbation and azole embryotoxicity in rat embryos cultured *in vitro*.

In the present work, experimental data have been modelled using PROAST software, which is considered a useful tool for an in-depth analysis of concentration-response data from *in vitro* studies (Piersma et al., 2008). First, we tested all dose-response curves for parallelism.

RPFs versus RA chosen as the index compound (IC) was then calculated.

While RA was definitively the most potent, the potencies of the tested azoles are very similar, resulting in similar RPFs. This result is of particular interest because it suggests that the tested azoles have similar potency in inhibiting CYP26. As previously hypothesised, the specific inhibition of CYP26 could be the molecular initiating event, producing specific effects mainly at the branchial apparatus level because in rodents, CYP26 isoforms are predominantly expressed in the cranial region during the stages evaluated in this study (Lee et al., 2017). As a consequence, a local increase in RA in those target regions after azole exposure i postulated. This hypothesis needs to be more thoroughly investigated by *ad hoc in vitro* and *in silico* investigations. Finally, it is worth reminding that RPFs obtained *in vitro* reflect only the potency of compounds at the target (internal dose), since they do not take into account differences in toxicokinetics. This could also justify the fact that PCZ could induce malformations in WEC (this paper) but not after *in utero* exposure, where only maternotoxicity and embryolethality were reported (FAO/WHO, 2002). Therefore, comparison with the external dose can be performed only if toxicokinetic information is available (Tan et al., 2018). This may also include comparison of animal versus human *in vitro* metabolic information (Punt et al., 2017). Hence, the selected *in vitro* method (WEC) could be an alternative method applicable to hazard evaluation of novel chemicals, as an initial screening not influenced by the species-specific maternal toxicity. As previously suggested by the EFSA scientific committee (Hardy et al., 2017), simple but predictive alternative methods should be applicable to derive relative RPFs, essential also to evaluate the exposure to mixtures. On these bases, the WEC can be used to test both single azoles and mixtures and identify their effects on craniofacial morphogenesis.

Conflicts of interest

There are no conflicts of interest to declare.

Animal use

All animal use protocols were in accordance to EU Directive 2010/63, approved by the Ministry of Health - Department for Veterinary Public Health, Nutrition and Food Safety committee (authorisation number 14/2015).

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.004.

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