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Title: Relative potency ranking of azoles altering cranio-facial morphogenesis in rat: an *in vitro* data modelling approach.

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Highlights:

- Whole embryo culture as an alternative method for pesticide hazard evaluation.
- Ranking of azole pesticide activity on embryo development
- In vitro evaluation of craniofacial primordia defects induced by azoles
- Comparison between azole and retinoic acid dysmorphogenic activity

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 cranio-facial 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 cranio-facial structures (fused branchial arches), similar to those induced by RA. Collected data were modelled by using PROAST 65.5 software in order to characterize 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 signaling perturbation to be further investigated. Moreover, the present results indicate the approach used in this work as an interesting tool applicable to the hazard evaluation of novel compounds or assessment of combined exposure to azoles or other dismorphogens.

Keywords:

- Azole, retinoic acid, malformation, potency, PROAST.

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 about 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 cranio-facial morphogenesis and of their disturbance by different chemicals is fundamental in order to evaluate the contribution of multiple exposures to the incidence of cranio-facial 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 cranio-facial morphogenesis. Both deficiency and excess of embryonic RA are related to malformations at multiple districts, including cranio-facial 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 has been observed in laboratory animals, such as effects on liver, on reproduction, and developmental defects. *In vivo* experimental studies, case reports and a recent birth defect prevention study relate i*n utero* exposure to some azole fungicides to cranio-facial 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 cranio-facial defects of the teratogenic antifungal azoles is the inhibition of embryonic CYP26, that metabolizes 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 relative potency factor (RPF) approach has been suggested (Hardy et al., 2017). 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 RA-relative potency of four antifungal azoles by fitting WEC experimental data by using PROAST software analysis (www.proast.nl) (modelling procedure according to Slob, 2002; Moretto et al., 2015). The chemicals known to induce cranio-facial defects selected in the present work are RA and azole antifungals (cyproconazole, CYPRO; flusilazole, FLUSI; triadimefon, FON; prochloraz, PCZ).

Three of these azoles (CYPRO, FON, FLUSI) are known to induce cranio-facial 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). Prochloraz (PCZ), an azole with broad spectrum fungistatic activity, induced maternotoxicity and embryotoxicity but not malformations after exposure *in utero* during the entire 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 in order to evaluate its intrinsic activity on embryo development in absence of maternal compartment. RA has been chosen in the present work as index compound in light of 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 by Sigma, Italy. Azoles were dissolved in ethanol (Sigma), RA was dissolved in DMSO (Sigma). The medium used for the extraction of embryos from the uteri was sterilized Tyrode solution (Sigma); the medium used for the postimplantation whole embryo culture was undiluted heat inactivated rat serum added with antibiotics (penicillin 100 IU/mL culture medium and streptomycin 100µg/mL culture medium, Sigma).

2.2. Selection of compound concentrations.

The concentrations of test molecules were selected from previous published experiments on rat WEC in order to gradually achieve the maximum degree of severity for branchial malformations: RA 0.025-1µM (Menegola et al., 2004), CYPRO 7.8-250µM (Di Renzo et al. 2011), FON 6.25-125µM (Menegola et al. 2000, Di Renzo et al. 2011b), FLUSI 1.56-125µM (Menegola et al. 2001). PCZ concentrations were selected on the basis of an unpublished range-finding test (6.25-50µM). For each dose-response experiment a group exposed to the relative solvent (dose 0) was performed.

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 cycle (light from 6.00 a.m. to 6.00 p.m.), 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. Animals were treated humanely and with regard for 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's method (1978) in 20 ml glass

bottles (5 embryos/bottle), containing 5 mL culture medium. At least a triplicate was performed 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 culture, embryos were morphologically examined under a dissecting microscope in order to evaluate any branchial or extra-branchial abnormality.

2.4. PROAST modelling.

The statistical analysis is done by dose-response modeling using PROAST. PROAST is a software package that has been 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 in order to obtain dose-response curves for single compounds setting the benchmark dose (BMD) at 50% response (BMR). Exponential model family equations have been selected in order to describe the dose-response curves and obtain RPFs versus the index compound (in our case, RA). Log-likelihood ratio test has been applied in order to assess the equal steepness assumption.

3. RESULTS

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

After 48 h in culture, normal embryos are dorsally convex and reached the phylotypic stage: tripartite encephalon (forebrain, midbrain, hindbrain) with enlarged ventricles, open posterior

neuropore, 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 concentration level≥ 0.125μM. The affected districts were encephalon (swollen and short romboencephalon, microcephalia), branchial apparatus (fused branchial arches with different severity degrees, as listed in Tab. 1), somites (fused) and tail (hook-shaped tail) at any effective concentration. By contrast, the exposure to FON at concentrations ≥12.5μM and FLUSI at concentrations ≥3.13μM affected only the branchial apparatus (fused branchial arches). Branchial arches resulted malformed after exposure to CYPRO at concentrations ≥ 7.8μM and PCZ at concentrations ≥ 6.25μM, while 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). Branchial defects are listed in Tab. 1 and consisted in different degrees of fusion among branchial arches: 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 an unique mass (Fig. 1 B-E).

3.2. PROAST analysis

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

Data were modelled to obtain the single dose-response curves (Figs 2-6); from these curves, the benchmark dose (BMD) for benchmark response (BMR) at 50% have been calculated (Tab. 2).

Data from all compounds were analyzed together by using the exponential model to calculate the RPFs (Tab. 3; Fig. 7). Log-likelihood ratio test showed that equal steepness assumption is not rejected (p=0. 498 with log-likelihood of separate fits=132.31, log-likelihood of the overall

fit=129.13, degrees of freedom=7) (Tab. 2). All the tested azoles were similar among each other and less potent than RA (Tab.3).

4. DISCUSSION

The WEC is a validated toxicological *in vitro* method (Corvi et al., 2006; ECVAM, 2006; Flick and Klug, 2006) that can be useful also 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 (EFSA, 2008). In agreement to 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, affected also by RA exposure. The branchial arches are typical embryonic structures involved in cranio-facial 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 by using the PROAST software, 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 index compound (IC) have been then calculated.

While RA was definitively the most potent, the potencies of the tested azoles are very similar. This result is of particular interest because suggests that the tested azoles have similar potency in inhibiting CYP26. As previously hypothesized, the specific inhibition of CYP26 could be the molecular initiating event, producing specific effects mainly at the branchial apparatus level because in rodents, at the evaluated stages, CYP26 isoforms are predominantly expressed in cranial region (Lee et al., 2017). As a consequence, a local increase of RA in that target region after azole exposure might be postulated. This interesting hypothesis needs to be more thoroughly investigated in *ad hoc in vitro* and *in silico* investigations. Finally, it is worth to keep in mind 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 was able to induce malformations in the present work 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). These may also include comparison of animal vs 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 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 cranio-facial 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 (authorization number 14/2015).

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FIGURE LEGEND

Fig 1: Evaluation of general and branchial morphology (in the rectangles) 48 h in culture. On 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 20x .

Fig 2: single dose-response curves of RA.

Fig 3: single dose-response curves of CYPRO.

Fig 4: single dose-response curves of FON.

Fig 5: single dose-response curves of FLUSI.

Fig 6: single dose-response curves of PCZ.

Fig 7: 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.

Table

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 µM	RA 0.025 µM	RA 0.05 µM	RA 0.125 µM	RA 0.25 µM	RA 0.5 µM	RA 1 µM		
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 µM	CYPRO 7.8 µM	CYPRO 15 µM	CYPRO 31 µM	CYPRO 46.8 µM	CYPRO 62.5 µM	CYPRO 125 µM	CYPRO 250 µM	
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 µM	FLUSI 1.56 µM	FLUSI 3.125 µM	FLUSI 4.8 µM	FLUSI 6.25 µM	FLUSI 7.7 µM	FLUSI 9.375 µM	FLUSI 10.1 µM	FLUSI 12.5 µM
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 µM	FON 6.25 µM	FON 12.5 µM	FON 25 µM	FON 26.7 µM	FON 42.85 µM	FON 50 µM	FON 56 µM	FON 125 µM
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 µM	PCZ 6.25 µM	PCZ 12.5 µM	PCZ 25 µM	PCZ 50 µM				
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				

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

Table 3. Relative potency factors obtained by PROAST analysis, fitting combined dataset.
RPF= relative potency factor.

	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)

Figure

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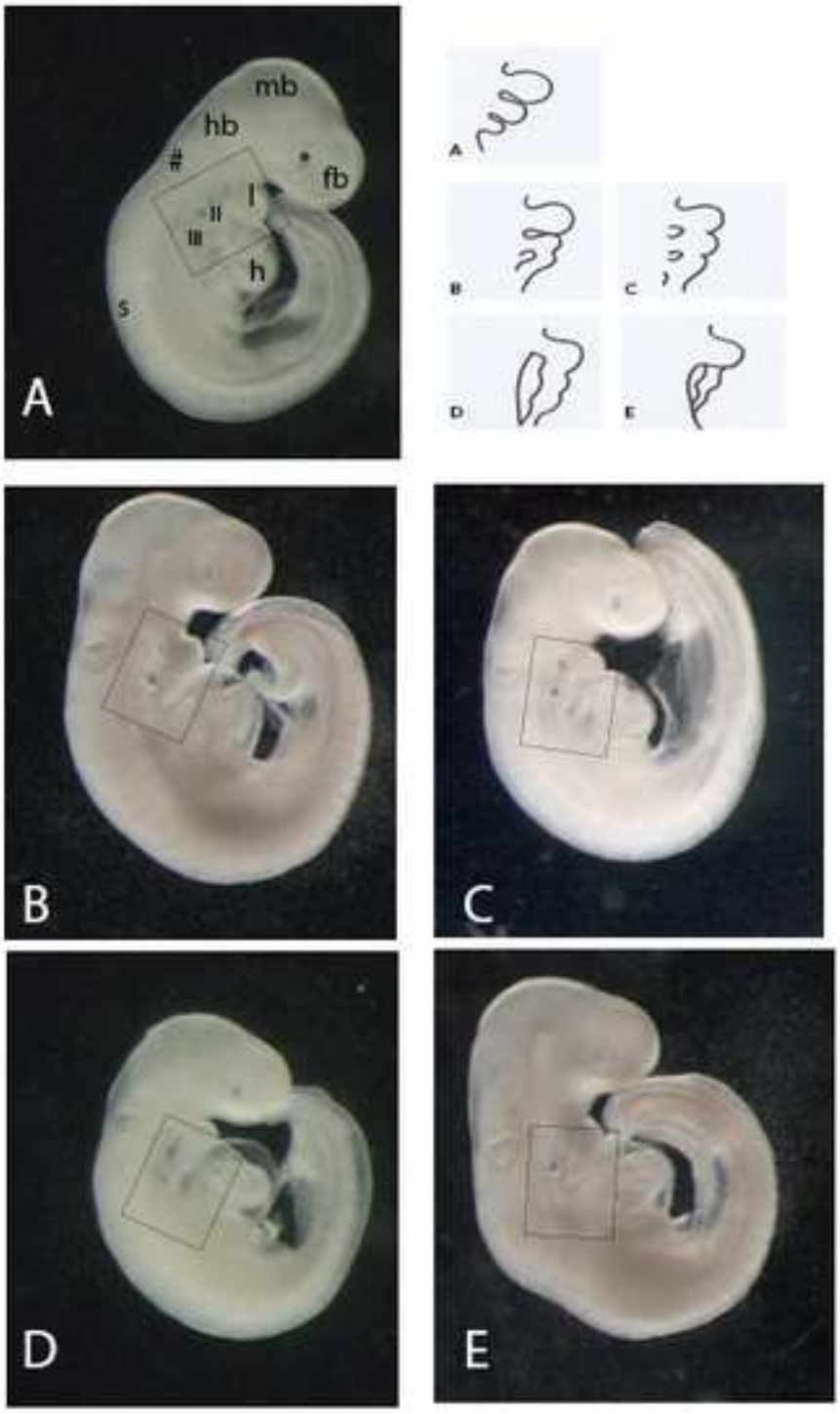

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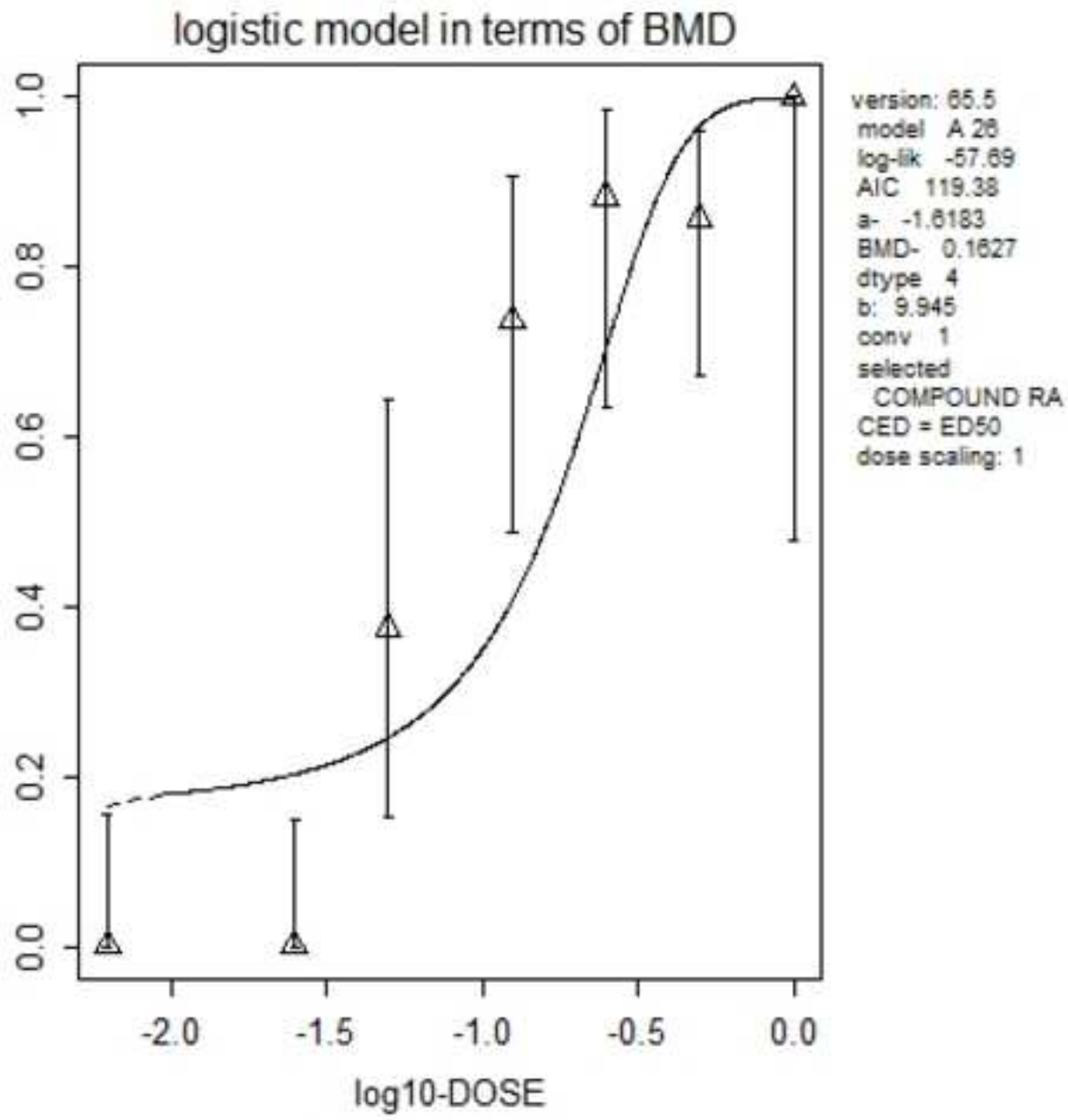

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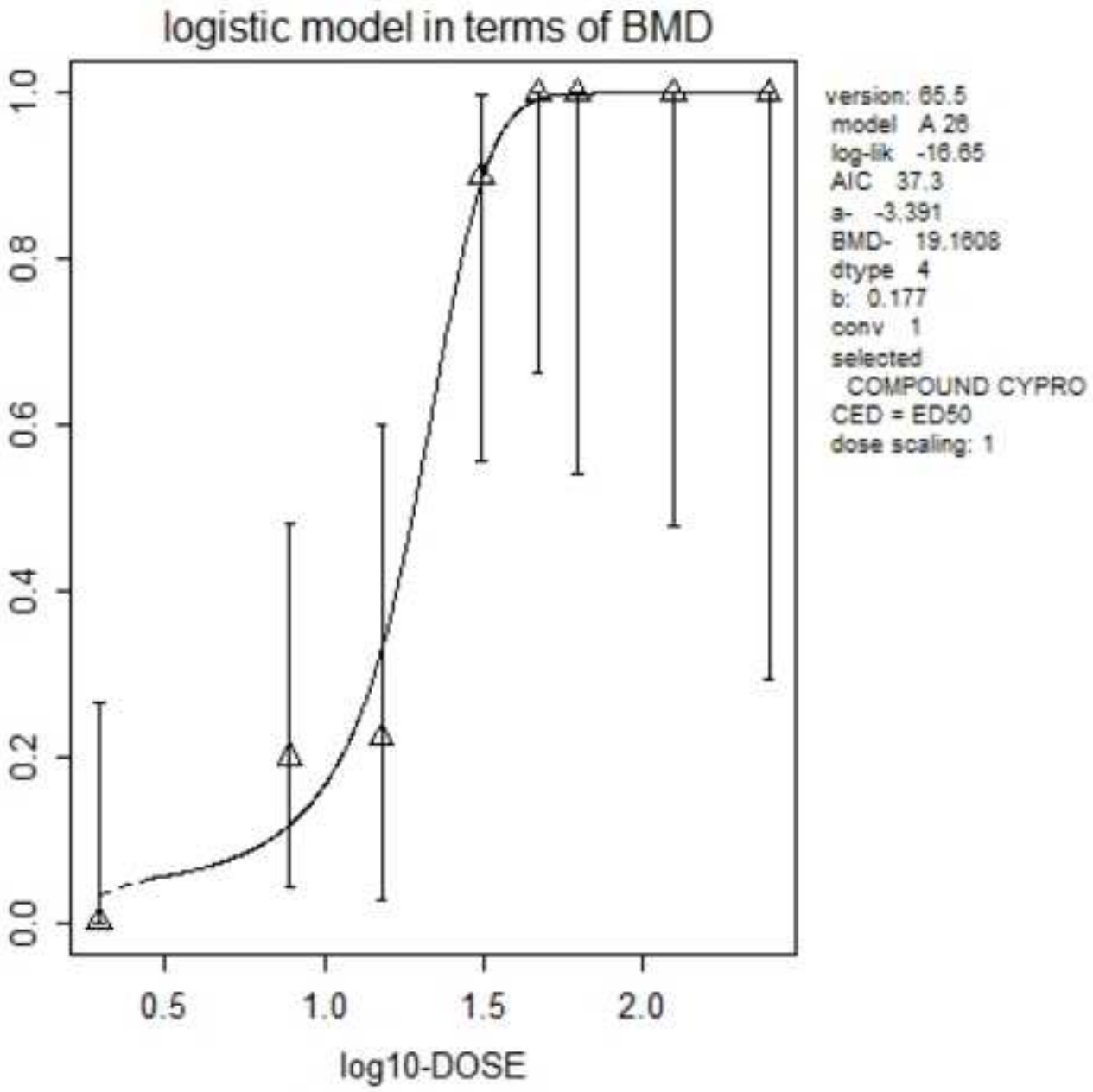

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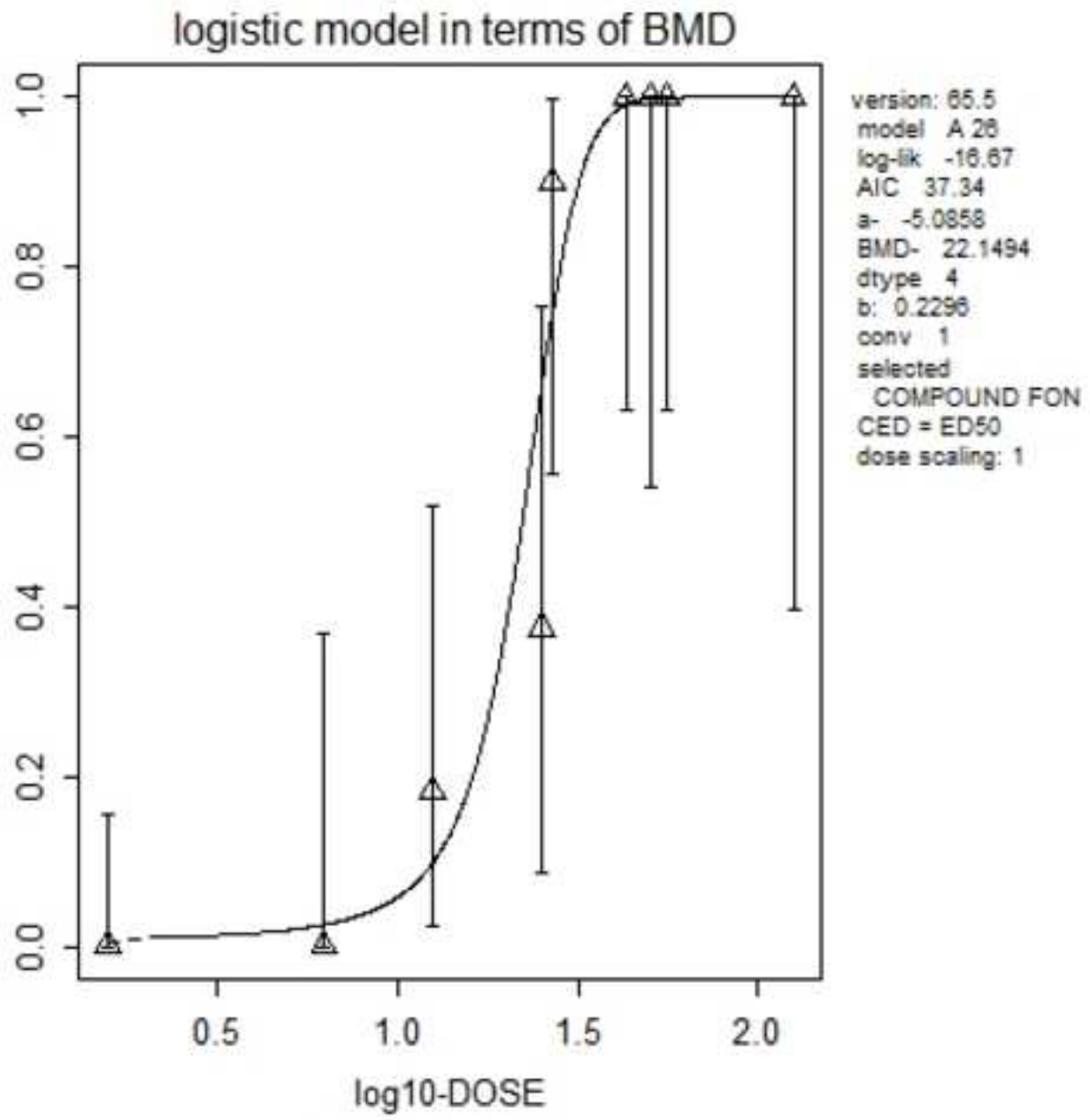

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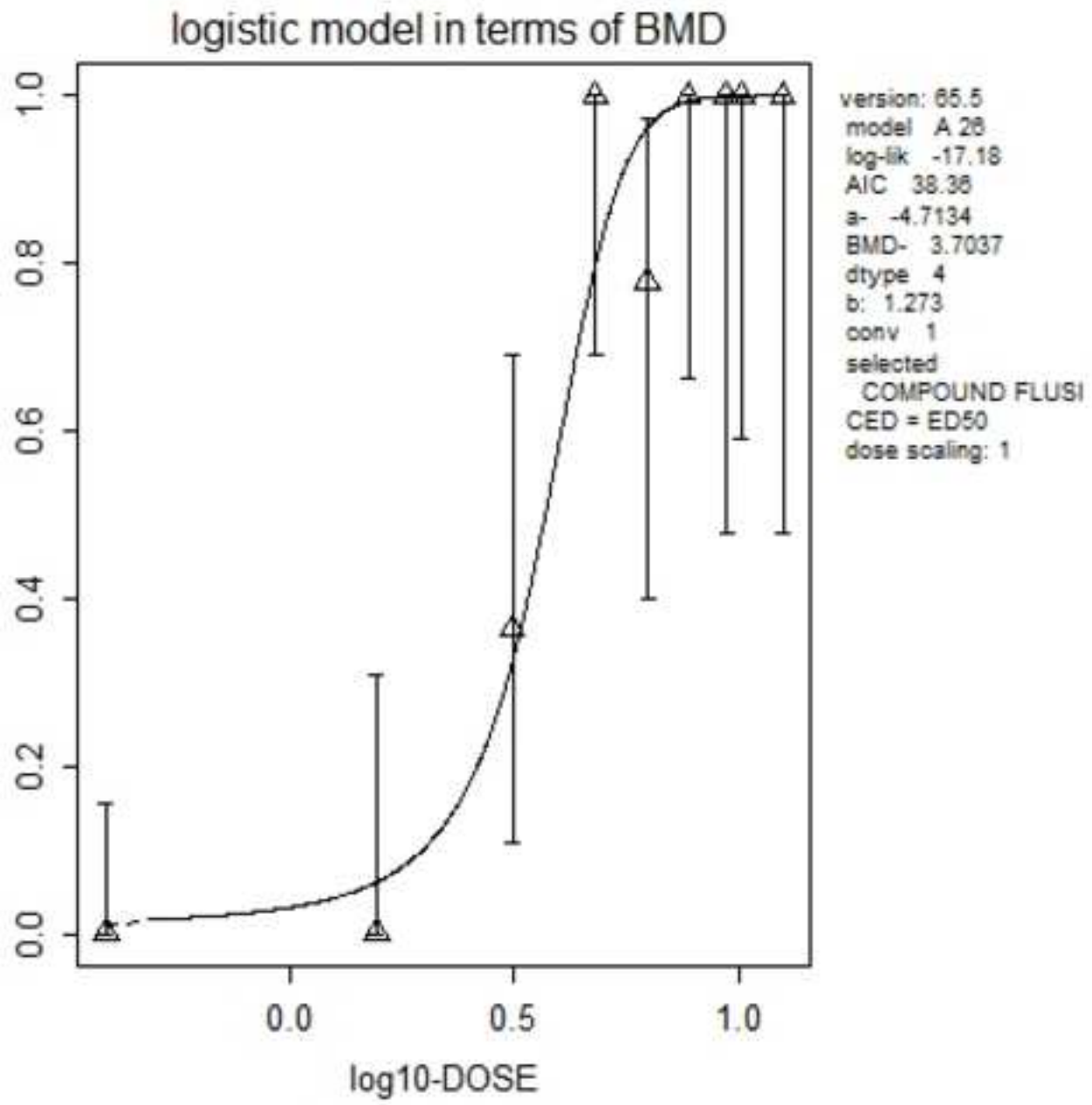

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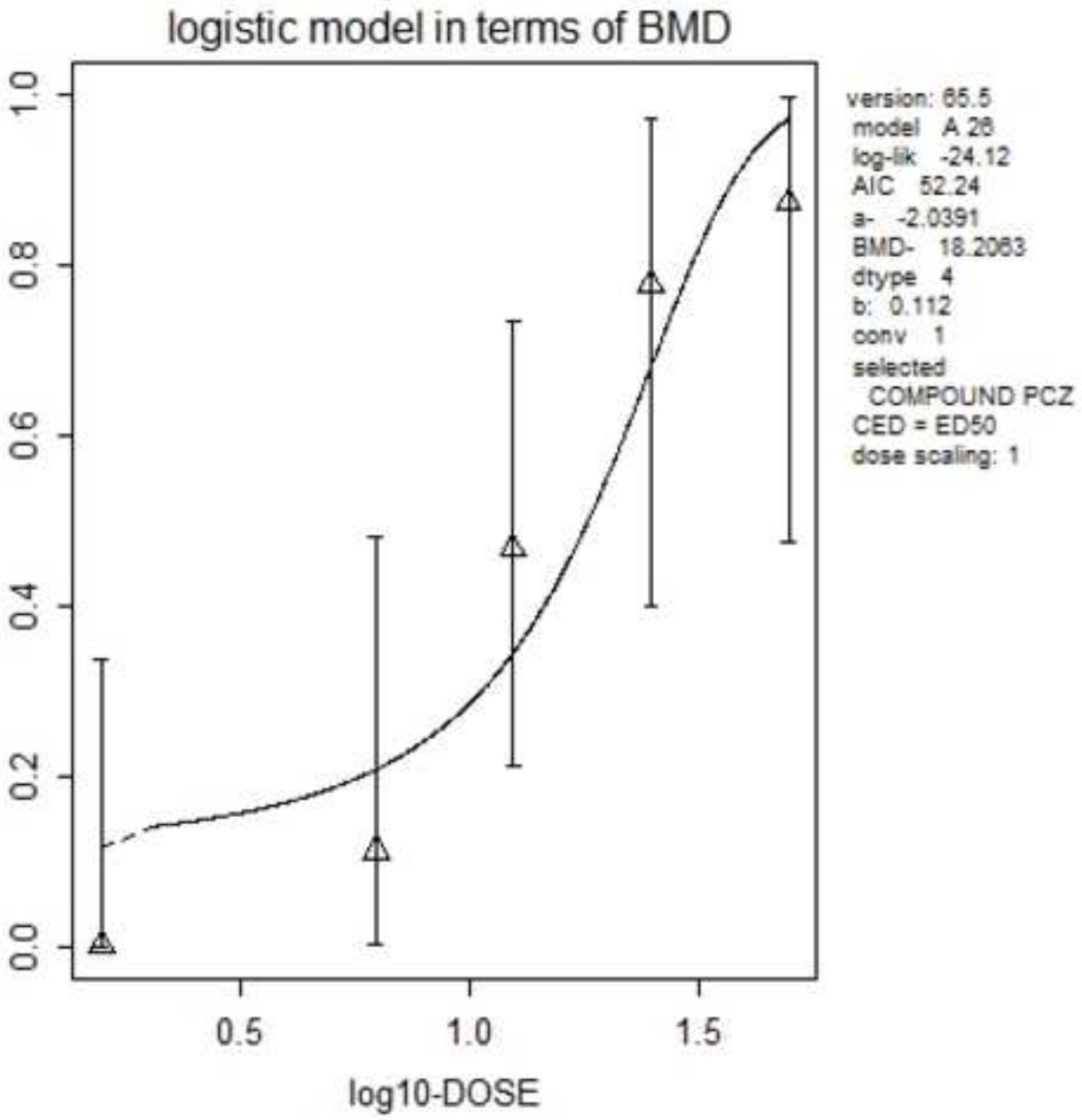

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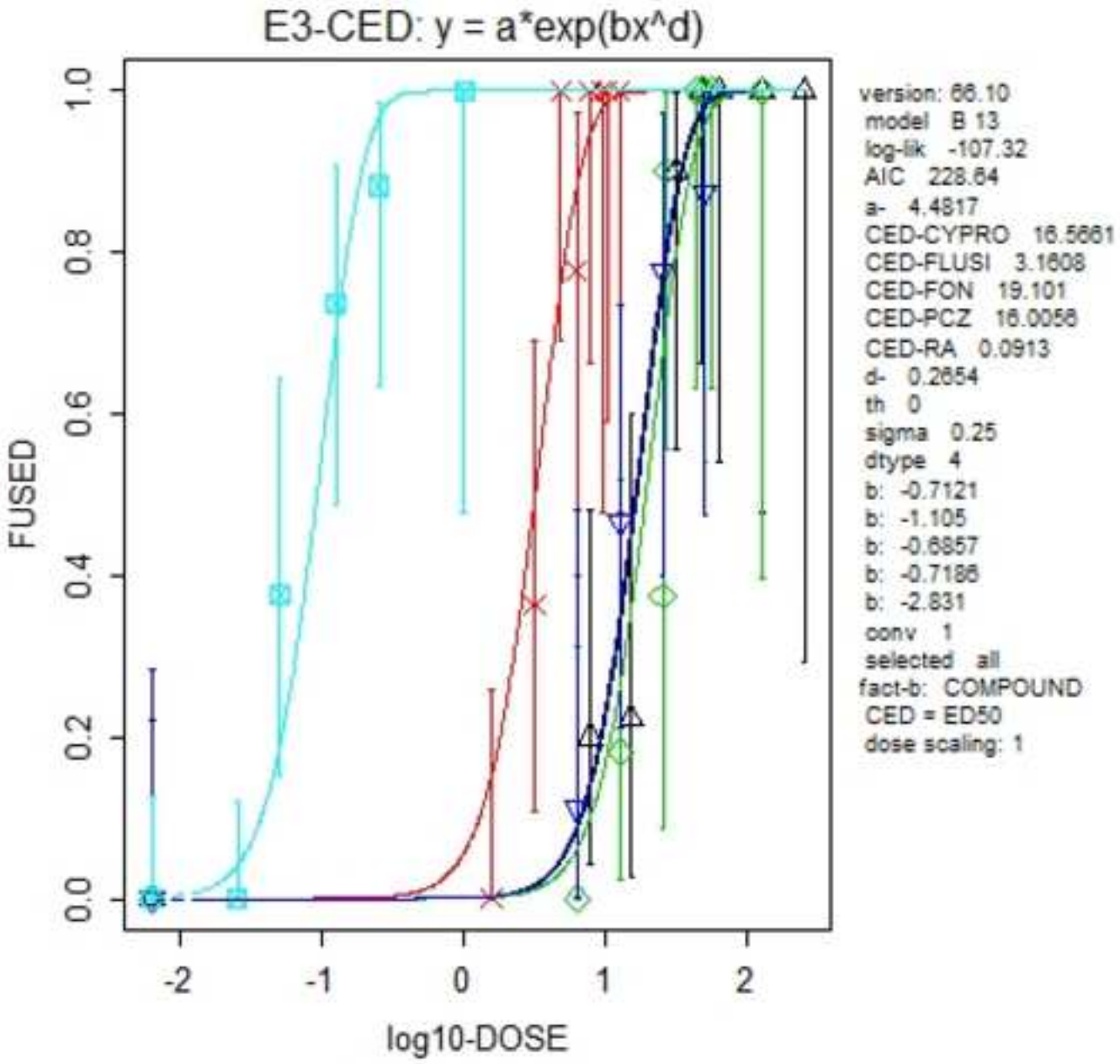

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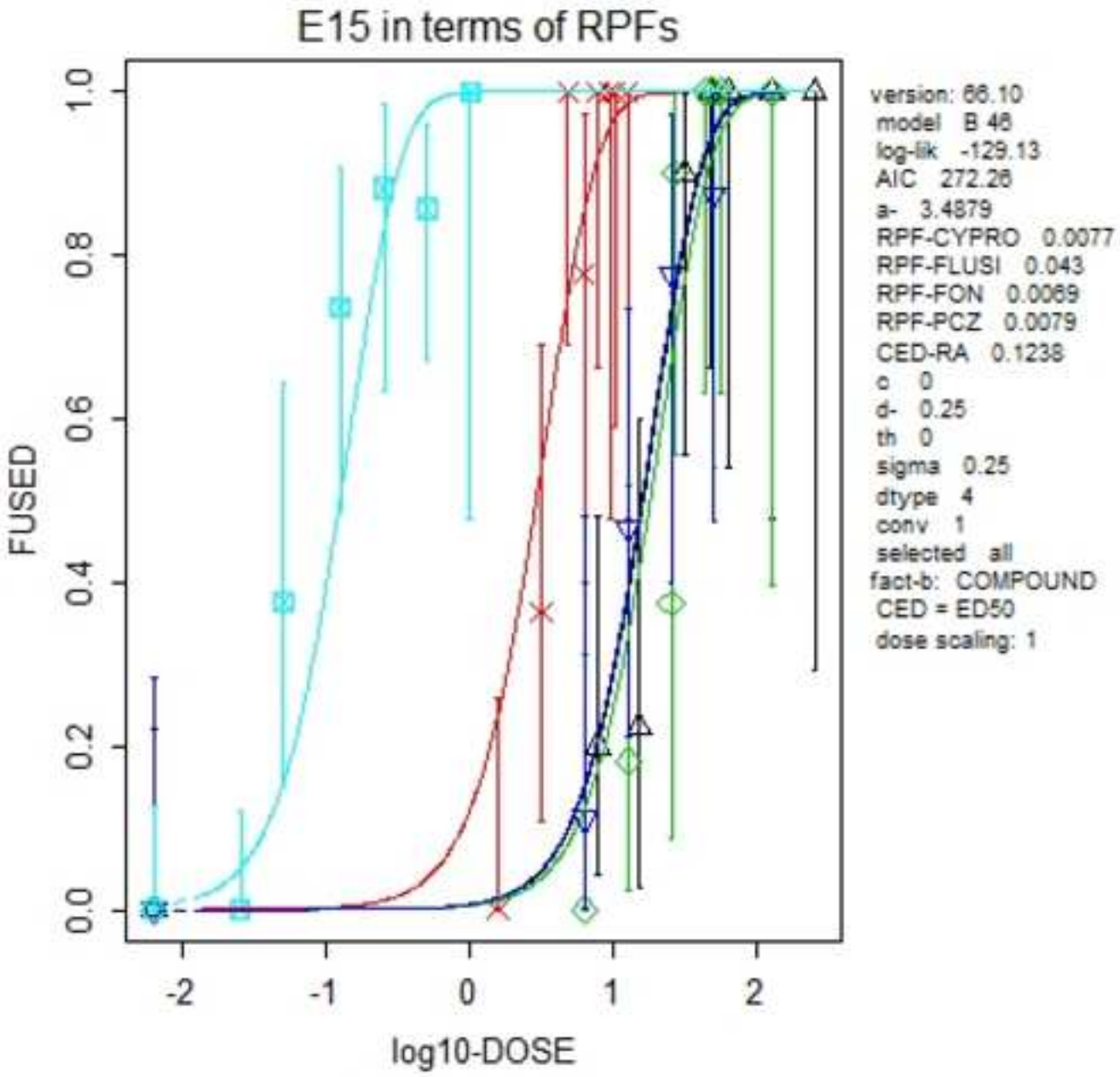

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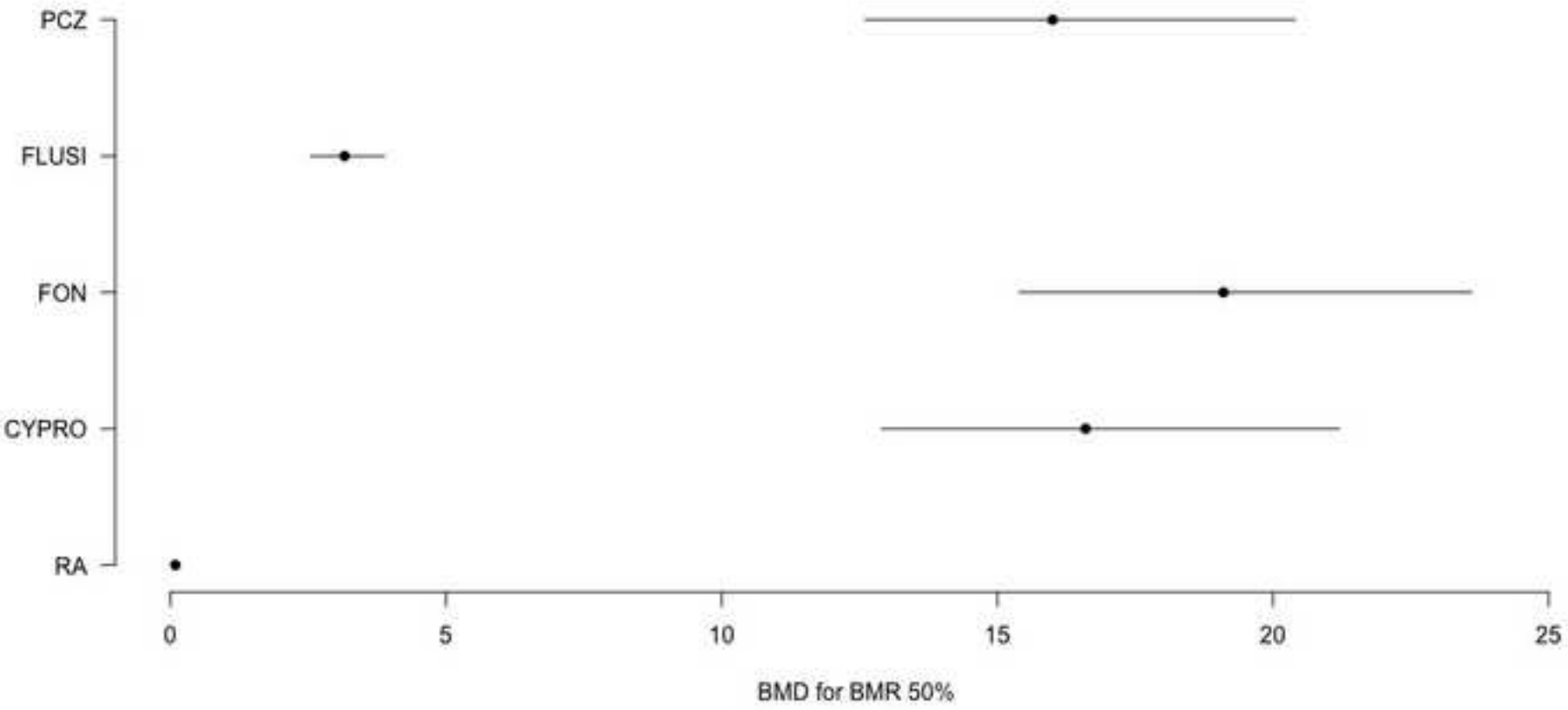

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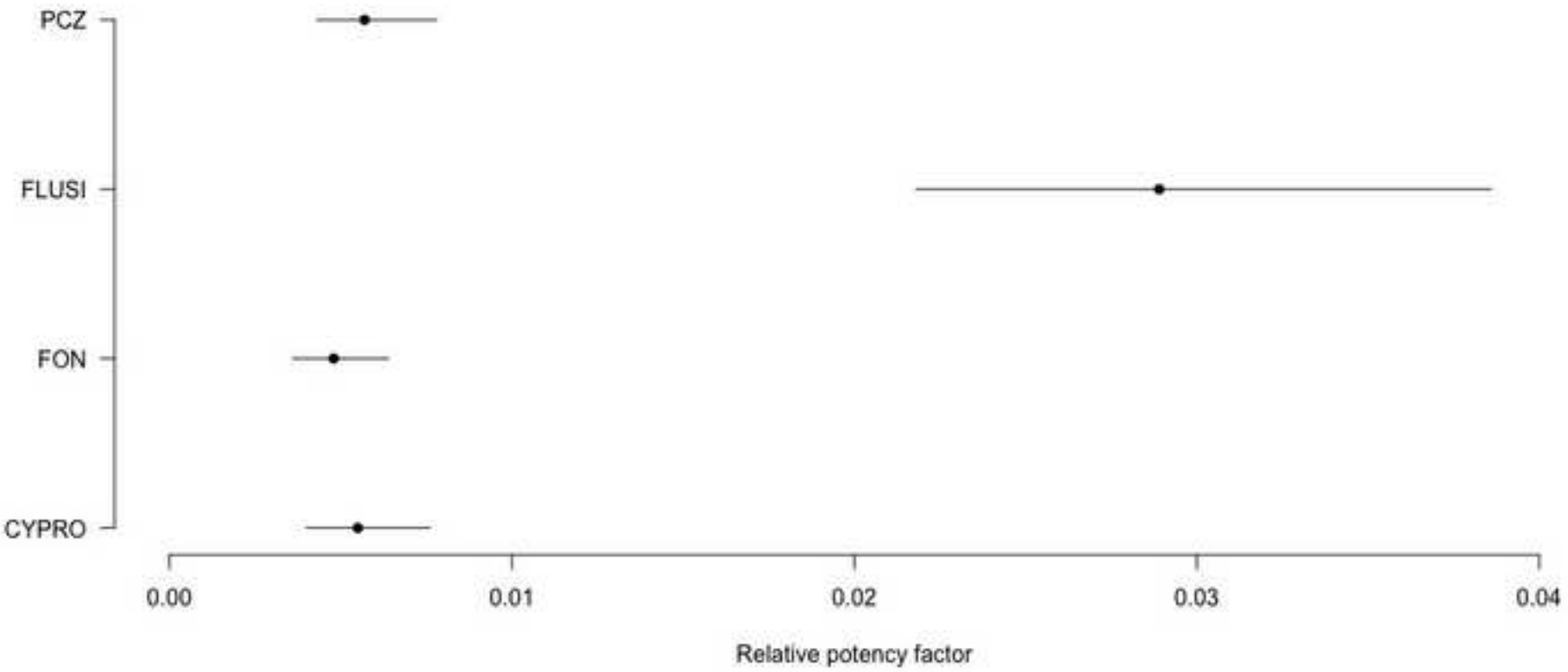

***Conflict of Interest Statement**

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