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

Authors: Francesca Di Renzo *[a], Francesca Metruccio*[b], Maria Battistoni[c], Angelo Moretto §[c], Elena Menegola[a].

Affiliations: [a]Department of Environmental Sciences and Policy, Università degli Studi di Milano-Milan Italy; [b]ASST Fatebenefratelli Sacco- Milan, Italy, [c]Department of Biomedical and Clinical Sciences, Università degli Studi di Milano- Milan Italy.

*These authors contributed equally to this work

§Corresponding author:

Angelo Moretto

Department of Biomedical and Clinical Sciences

University of Milano

Via GB Grassi 74

20157 Milano Italy

angelo.moretto@unimi.it

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).

FUNDS: our research project received funding from the European Union Horizon 2020 Framework Program under Grant Agreement 633172 (European Test and Risk Assessment Strategies for Mixtures, EuroMix). This publication reflects only the authors' views, and the European community is not liable for any use made of the information contained therein.

REFERENCES

Aleck, K.A., Bartley, D.L., 1997. Multiple malformation syndrome following fluconazole use in pregnancy: Report of an additional patient. Am. J. Med. Genet. 72, 253–256. https://doi.org/10.1002/(SICI)1096-8628(19971031)72:3<253::AID-AJMG1>3.0.CO;2-S

Belz, R.G., Hurle, K., Duke, S.O., 2005. Dose-Response—A Challenge for Allelopathy? Nonlinearity Biol. Toxicol. Med. 3, nonlin.003.02.0. https://doi.org/10.2201/nonlin.003.02.002

Browne, H., Mason, G., Tang, T., 2014. Retinoids and pregnancy: an update. Obstet. Gynaecol. 16, 7–11. https://doi.org/10.1111/tog.12075

Corvi, R. et al., 2006. Meeting report: Validation of toxicogenomics-based test systems: ECVAM-ICCVAM/NICEATM considerations for regulatory use. Environ. Health Perspect. 114, 420–429.

Di Renzo, F. et al, 2011a. Stage-dependent abnormalities induced by the fungicide triadimefon in the mouse☆. Reprod. Toxicol. 31, 194–199. https://doi.org/10.1016/j.reprotox.2010.10.011

Di Renzo, F. et al., 2011b. Early genetic control of craniofacial development is affected by the in vitro exposure of rat embryos to the fungicide triadimefon. Birth Defects Res. B. Dev. Reprod. Toxicol. 92, 77–81. https://doi.org/10.1002/bdrb.20284

Dimopoulou, M. et al., I.M.C.M., Piersma, A.H., 2017a. A transcriptomic approach for evaluating the relative potency and mechanism of action of azoles in the rat Whole Embryo Culture. Toxicology 392, 96–105. https://doi.org/10.1016/j.tox.2017.09.014

Dimopoulou, M. et al., 2017b. Embryotoxic and pharmacologic potency ranking of six azoles in the rat whole embryo culture by morphological and transcriptomic analysis. Toxicol. Appl. Pharmacol. 322, 15–26. https://doi.org/10.1016/j.taap.2017.03.001

ECVAM, 2006. ECVAM DB-ALM: invittox protocol. Embryotoxicity testing in postimplantation embryo culture- Method of Piersma INVITTOX n° 123.

EFSA, 2008. Opinion of the Scientific Panel on Plant Protection products and their Residues to evaluate the suitability of existing methodologies and, if appropriate, the identification of new approaches to assess cumulative and synergistic risks from pesticides to h: Opinion of the Scientific Panel on Plant Protection products and their Residues to evaluate the suitability of existing methodologies and, if appropri. EFSA J. 6, 705. https://doi.org/10.2903/j.efsa.2008.705

FAO/WHO JMPR, 2002. Report of the 2001 JMPR FA/WHO Meeting of Experts- Prochloraz, 144-149. http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Reports_1991-2006/REPORT2001.pdf

FAO/WHO JMPR, 2005. Pesticide Residues in Food - 2004 - Toxicological evaluations - Triadimefon, 325-386. http://apps.who.int/pesticide-residues-jmpr-database/pesticide?name=TRIADIMEFON

FAO/WHO JMPR, 2008. Pesticide Residues in Food – 2007- Toxicological Evaluations – Flusilazole, 317-347. http://apps.who.int/pesticide-residues-jmpr-database/pesticide?name=FLUSILAZOLE

FAO/WHO JMPR, 2011. Pesticide Residues in Food – 2010- Toxicological Evaluations – Cyproconazole, 117-202. http://apps.who.int/pesticide-residues-jmpr-database/pesticide?name=CYPROCONAZOLE

Flick, B., Klug, S., 2006. Whole Embryo Culture: An Important Tool in Developmental Toxicology Today. Curr. Pharm. Des. 12, 1467–1488. https://doi.org/10.2174/138161206776389822

Giavini, E. et al., 1992. Effects of ethanol and acetaldehyde on rat embryos developing in vitro. Vitro Cell. Dev. Biol. - Anim. 28, 205–210. https://doi.org/10.1007/BF02631093

Giavini, E., Menegola, E., 2010. Are azole fungicides a teratogenic risk for human conceptus? Toxicol. Lett. 198, 106–111. https://doi.org/10.1016/j.toxlet.2010.07.005

Hardy, A. et al., 2017. Update: use of the benchmark dose approach in risk assessment. EFSA J. 15. https://doi.org/10.2903/j.efsa.2017.4658

Hathcock, J.N.et al., 1990. Evaluation of vitamin A toxicity. Am. J. Clin. Nutr. 52, 183–202. https://doi.org/10.1093/ajcn/52.2.183

Howley, M.M. et al., 2016. Fluconazole use and birth defects in the National Birth Defects Prevention Study. Am. J. Obstet. Gynecol. 214, 657.e1-657.e9. https://doi.org/10.1016/j.ajog.2015.11.022

Klug, S. et al., 1989. All-trans retinoic acid and 13-cis-retinoic acid in the rat whole-embryo culture: abnormal development due to the all-trans isomer. Arch. Toxicol. 63, 440–444. https://doi.org/10.1007/BF00316445

Lammer, E.J. et al., 1985. Retinoic Acid Embryopathy. N. Engl. J. Med. 313, 837–841. https://doi.org/10.1056/NEJM198510033131401

Lee, B.E. et al., 1992. Congenital malformations in an infant born to a woman treated with fluconazole. Pediatr. Infect. Dis. J. 11, 1062–1064.

Lee et al., 2017. Perturbation of retinoid homeostasis increases malformation risk in embryos exposed to pregestational diabetes. Diabetes 66, 1041-1051.

Lopez-Rangel, E., Van Allen, M.I., 2005. Prenatal exposure to fluconazole: An identifiable dysmorphic phenotype. Birt. Defects Res. A. Clin. Mol. Teratol. 73, 919–923. https://doi.org/10.1002/bdra.20189

Menegola, E. et al., 2000. in vitro teratogenic potential of two antifungal triazoles: triadimefon and triadimenol. Vitro Cell. Dev. Biol. - Anim. 36, 88. https://doi.org/10.1290/1071-2690(2000)036<0088:IVTPOT>2.0.CO;2

Menegola, E et al., 2001. Antifungal triazoles induce malformations in vitro. Reprod. Toxicol. 15, 421–427. https://doi.org/10.1016/S0890-6238(01)00143-5

Menegola, E et al., 2003. Pathogenic pathways in fluconazole-induced branchial arch malformations. Birt. Defects Res. A. Clin. Mol. Teratol. 67, 116–124. https://doi.org/10.1002/bdra.10022

Menegola, E. et al., 2004. Relationship between hindbrain segmentation, neural crest cell migration and branchial arch abnormalities in rat embryos exposed to fluconazole and retinoic acid in vitro. Reprod. Toxicol. 18, 121–130. https://doi.org/10.1016/j.reprotox.2003.09.004

Menegola, E. et al., 2005. Study on the common teratogenic pathway elicited by the fungicides triazole-derivatives. Toxicol. In Vitro 19, 737–748. https://doi.org/10.1016/j.tiv.2005.04.005

Menegola, E. et al., 2013. Effects of mixtures of azole fungicides in postimplantation rat whole-embryo cultures. Arch. Toxicol. 87, 1989–1997. https://doi.org/10.1007/s00204-013-1048-y

Metzler, M., Sandell, L., 2016. Enzymatic Metabolism of Vitamin A in Developing Vertebrate Embryos. Nutrients 8, 812. https://doi.org/10.3390/nu8120812

Moretto, A. et al., 2015. The use of in vitro testing to refine cumulative assessment groups of pesticides: The example of teratogenic conazoles. Food Chem. Toxicol. 79, 65–69. https://doi.org/10.1016/j.fct.2014.07.006

Morriss-Kay, G., 1992. Retinoic Acid and Development. Pathobiology 60, 264–270. https://doi.org/10.1159/000163733

Mossey, P.A. et al.., 2009. Cleft lip and palate. The Lancet 374, 1773–1785. https://doi.org/10.1016/S0140-6736(09)60695-4

New, D.A.T., 1978. Whole-embryo culture and the study of mammalian embryos during organogenesis. Biol. Rev. 53, 81–122. https://doi.org/10.1111/j.1469-185X.1978.tb00993.x

Piersma et al., 2008 tox sci

Piersma, A.H., Hessel, E.V., Staal, Y.C., 2017. Retinoic acid in developmental toxicology: Teratogen, morphogen and biomarker. Reprod. Toxicol. 72, 53–61. https://doi.org/10.1016/j.reprotox.2017.05.014

Punt, A., Peijnenburg. A.A.C.M., Hoogenboom, R.L.A.P., Bouwmeester, H., 2017. Non-animal approaches for toxicokinetics in risk evaluations of food chemicals. ALTEX 34, 501-514. https://doi.org/10.1473/altex.1702211.

Pursley, T.J. et al., 1996. Fluconazole-Induced Congenital Anomalies in Three Infants. Clin. Infect. Dis. 22, 336–340. https://doi.org/10.1093/clinids/22.2.336

Robinson, J.F. et al., 2012. Triazole induced concentration-related gene signatures in rat whole embryo culture. Reprod. Toxicol. 34, 275–283. https://doi.org/10.1016/j.reprotox.2012.05.088

Sanchez, J.M., Moya, G., 1998. Fluconazole teratogenicity. Prenat. Diagn. 18, 862–863. https://doi.org/10.1002/(SICI)1097-0223(199808)18:8<862::AID-PD347>3.0.CO;2-F

Slob, W., 2002. Dose-Response Modeling of Continuous Endpoints. Toxicol. Sci. 66, 298–312. https://doi.org/10.1093/toxsci/66.2.298

Slob, W., Setzer, R.W., 2014. Shape and steepness of toxicological dose–response relationships of continuous endpoints. Crit. Rev. Toxicol. 44, 270–297. https://doi.org/10.3109/10408444.2013.853726

Tachibana, M., Noguchi, N., Monro, A., 1987. Toxicology of fluconazole in experimental animals., in: Fromtling RA, Editor. Recent Trends in the Discovery, Development, and Evaluation of Antifungal Agents. J Proust Science Publishers, Barcelona, Spain, pp. 93–102.

Tan, Y-M., Worley, R.R., Leonard, J.A., Fisher, J.W., 2018. Challenges associated with applying physiologically based pharmacokinetic modeling for public health decision-making. Tox. Sci. 162, 341-348. https://doi.org/10.1093/toxsci/kfy010.

Tiboni, G.M., 1993. Second branchial arch anomalies induced by fluconazole, a bis-triazole antifungal agent, in cultured mouse embryos. Res. Commun. Chem. Pathol. Pharmacol. 79, 381–384.

Tiboni, G.M., Marotta, F., Carletti, E., 2009. Fluconazole alters CYP26 gene expression in mouse embryos. Reprod. Toxicol. 27, 199–202. https://doi.org/10.1016/j.reprotox.2009.01.001

Warkany, J., Schraffenberger, E., 1944. Congenital Malformations of the Eyes Induced in Rats by Maternal Vitamin A Deficiency. Exp. Biol. Med. 57, 49–52. https://doi.org/10.3181/00379727-57-14695P

Zhang, C., Ball, J., Panzica-Kelly, J., Augustine-Rauch, K., 2016. In Vitro Developmental Toxicology Screens: A Report on the Progress of the Methodology and Future Applications. Chem. Res. Toxicol. 29, 534–544. https://doi.org/10.1021/acs.chemrestox.5b00458

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;

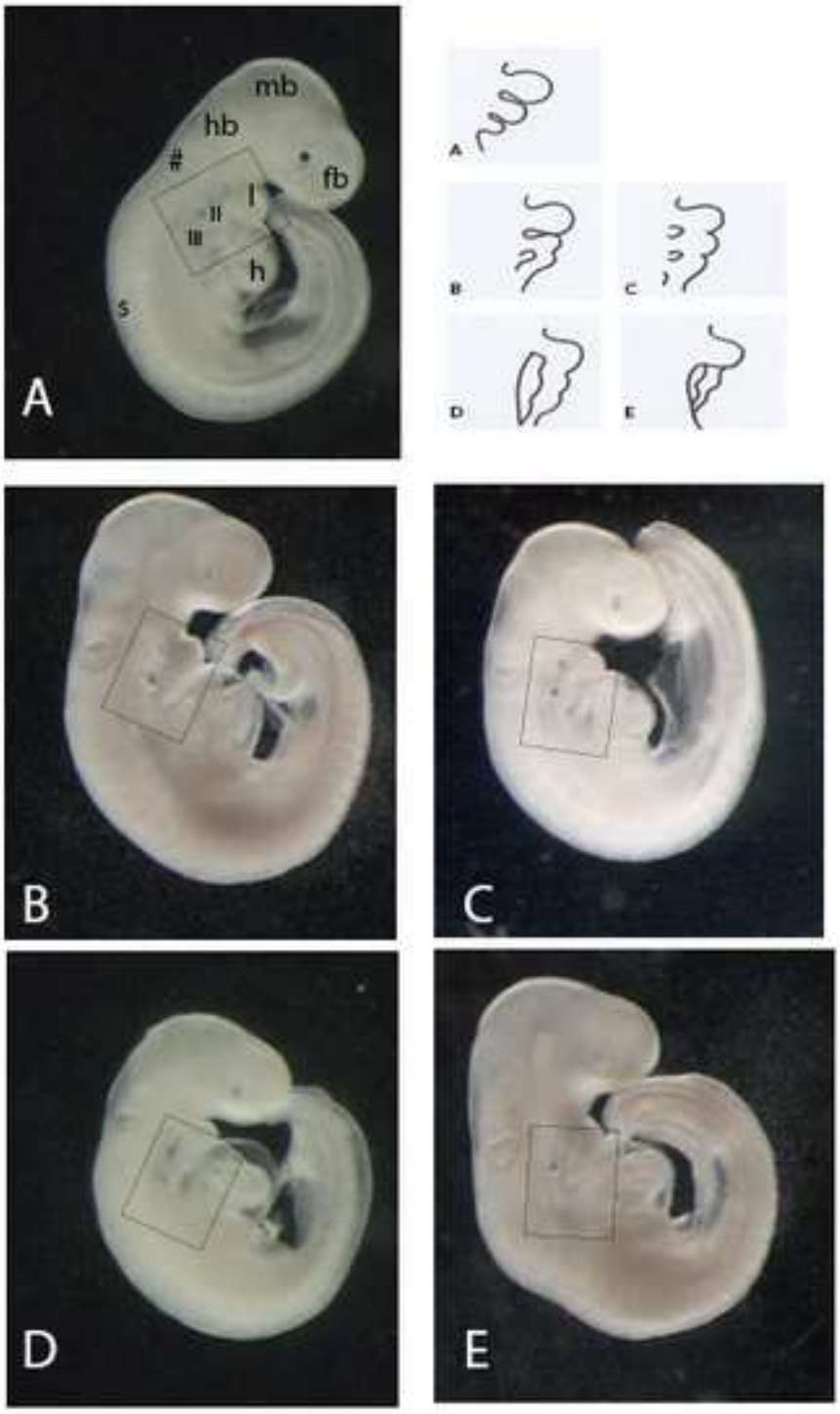
	BMD for BMR 50% (µM)	log-likelihood
RA	0.16	-57.69
CYPRO	19.16	-16.65
FON	22.15	-16.67
FLUSI	3.7	-17.18
PCZ	18.21	-24.12
COMBINED (RA as index)	0.1238	-129.13

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)

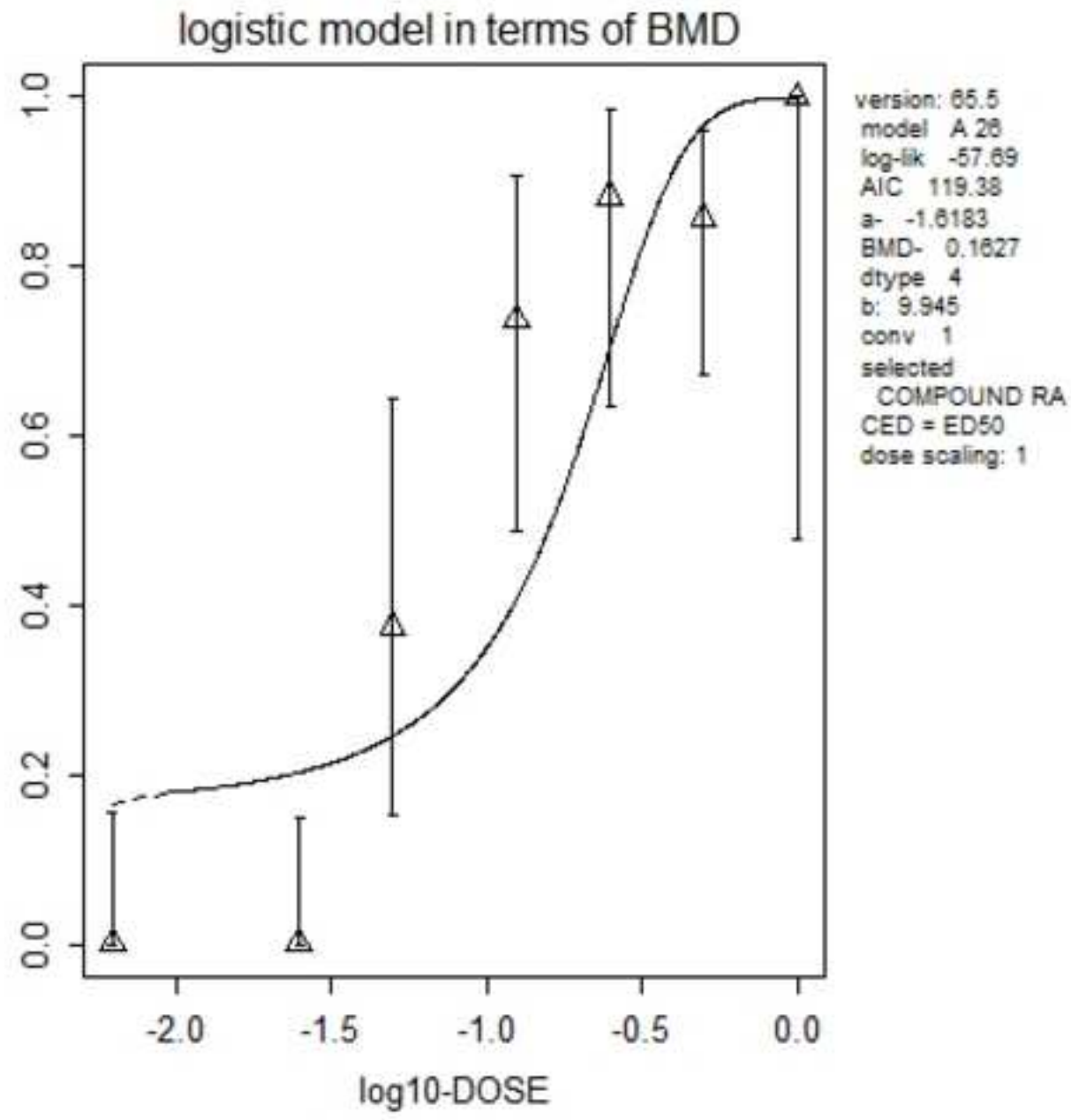
Figure

Click here to download high resolution image



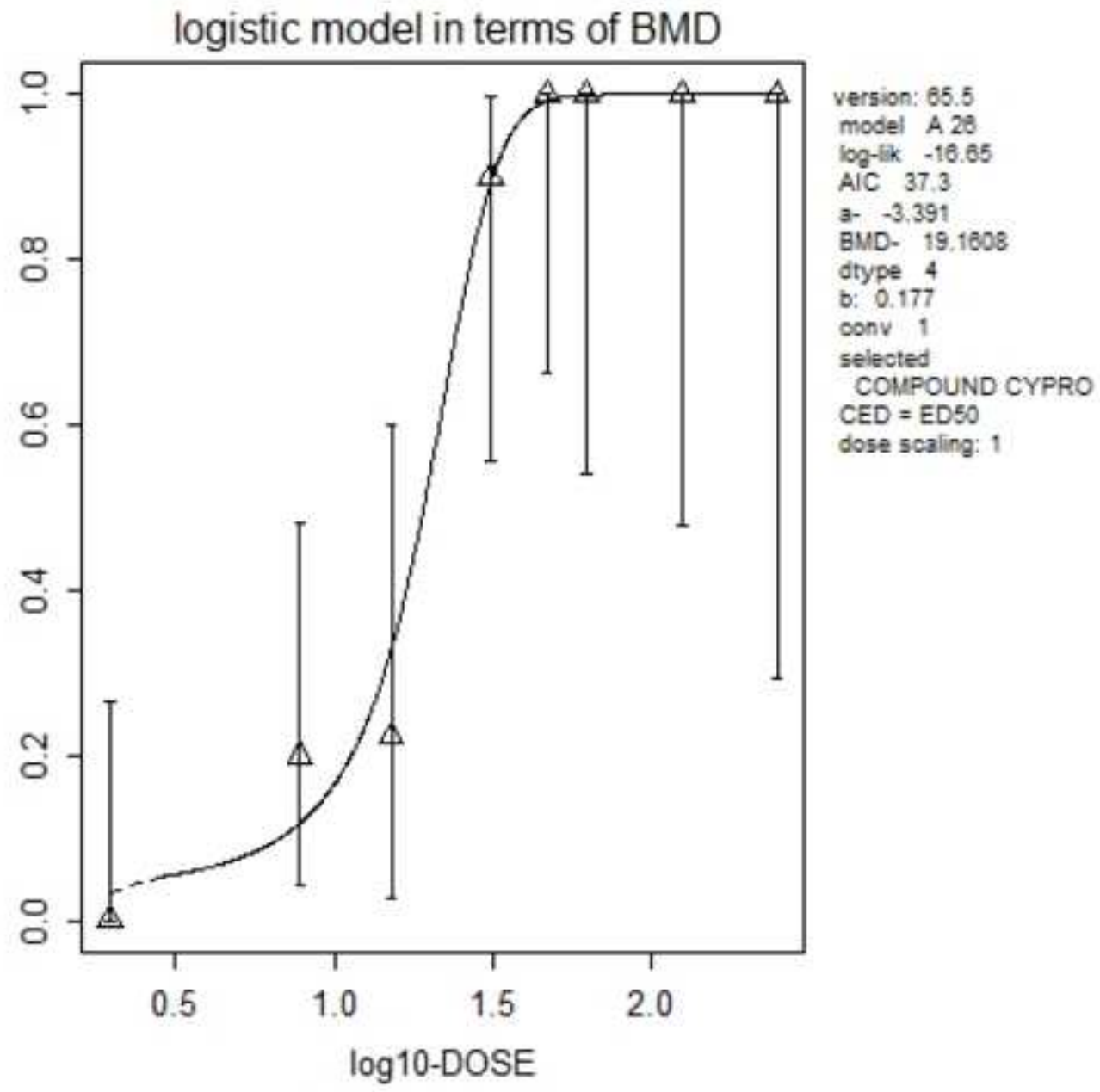
Figure

Click here to download high resolution image



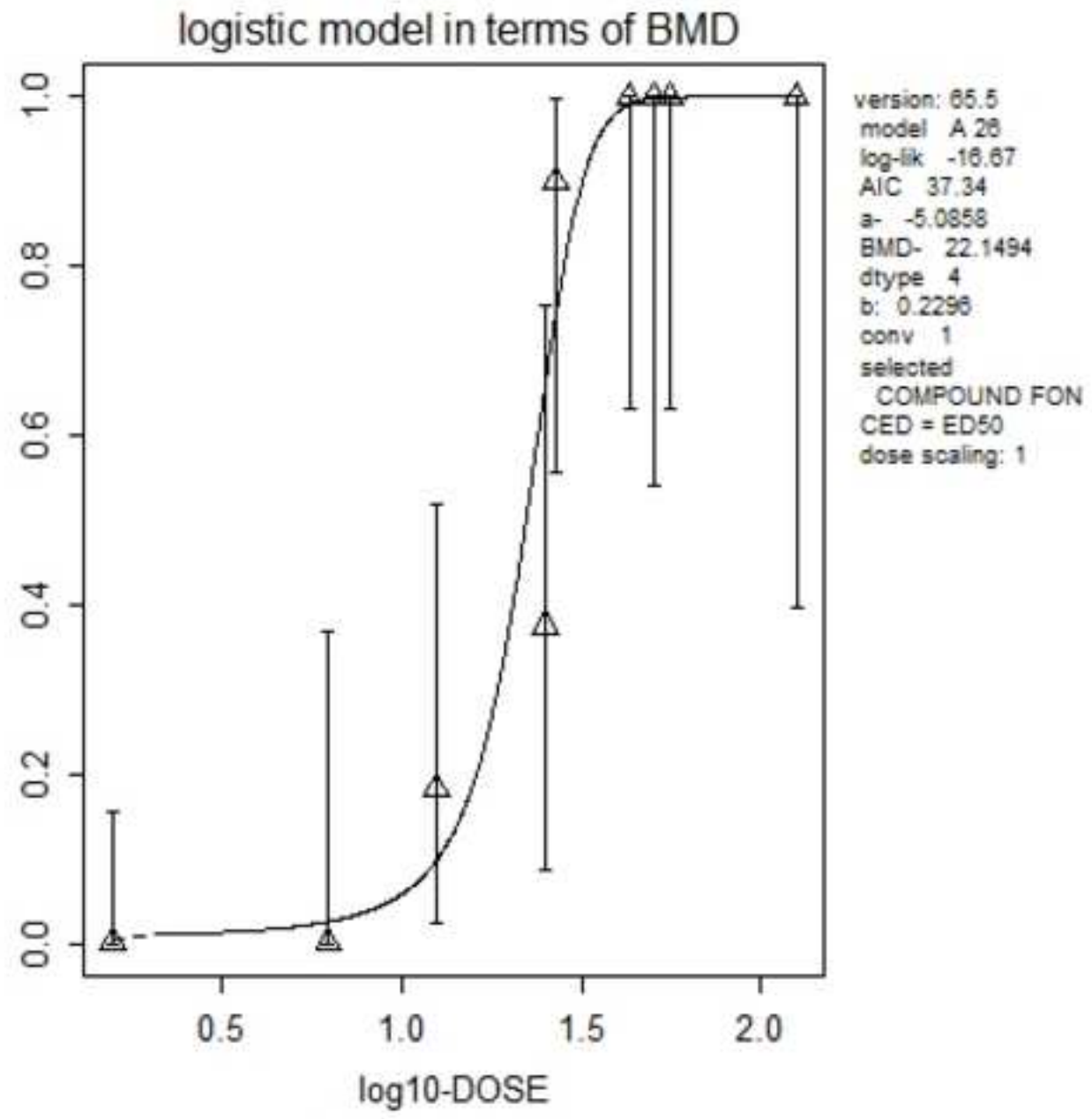
Figure

Click here to download high resolution image



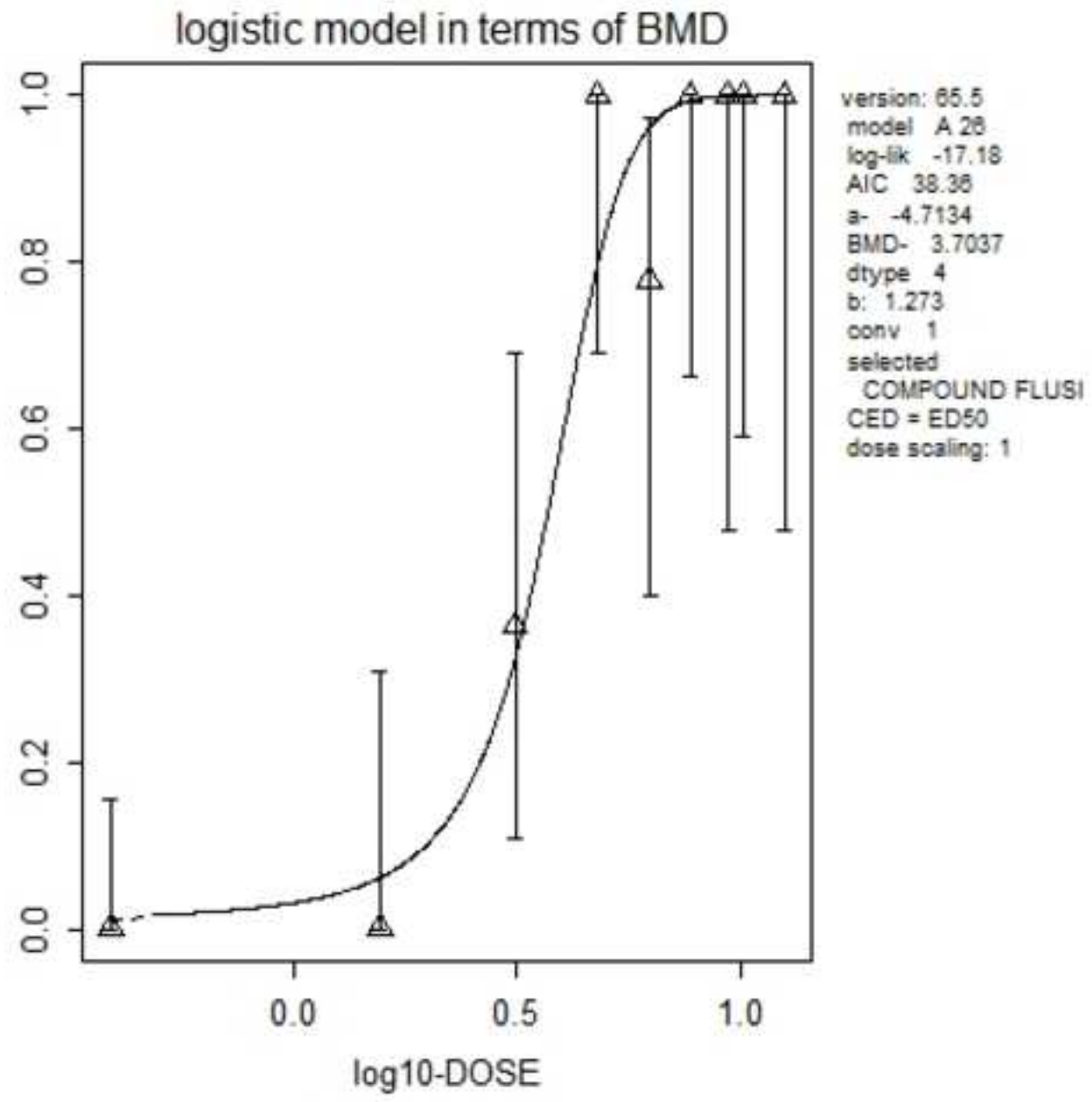
Figure

Click here to download high resolution image



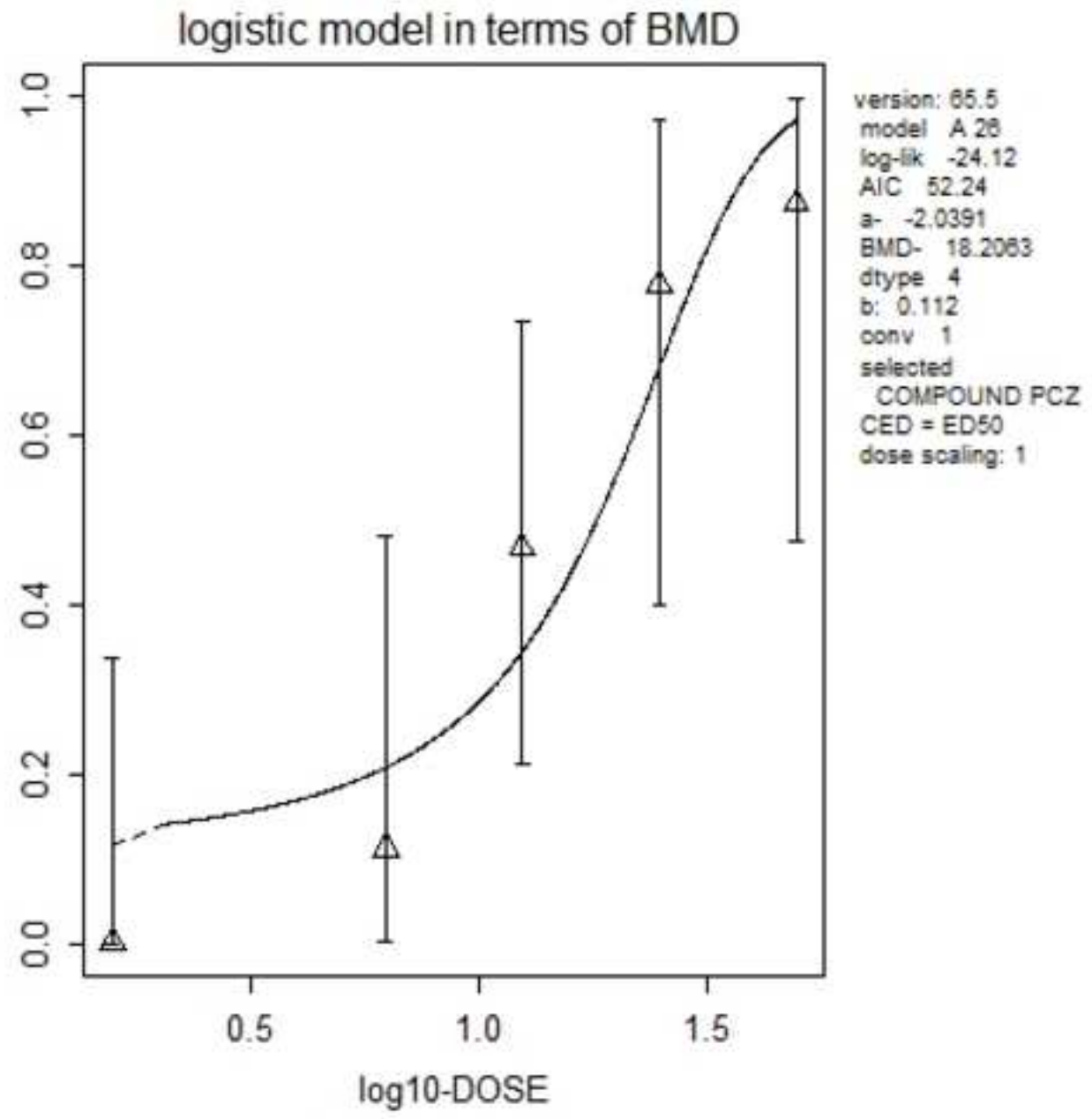
Figure

Click here to download high resolution image



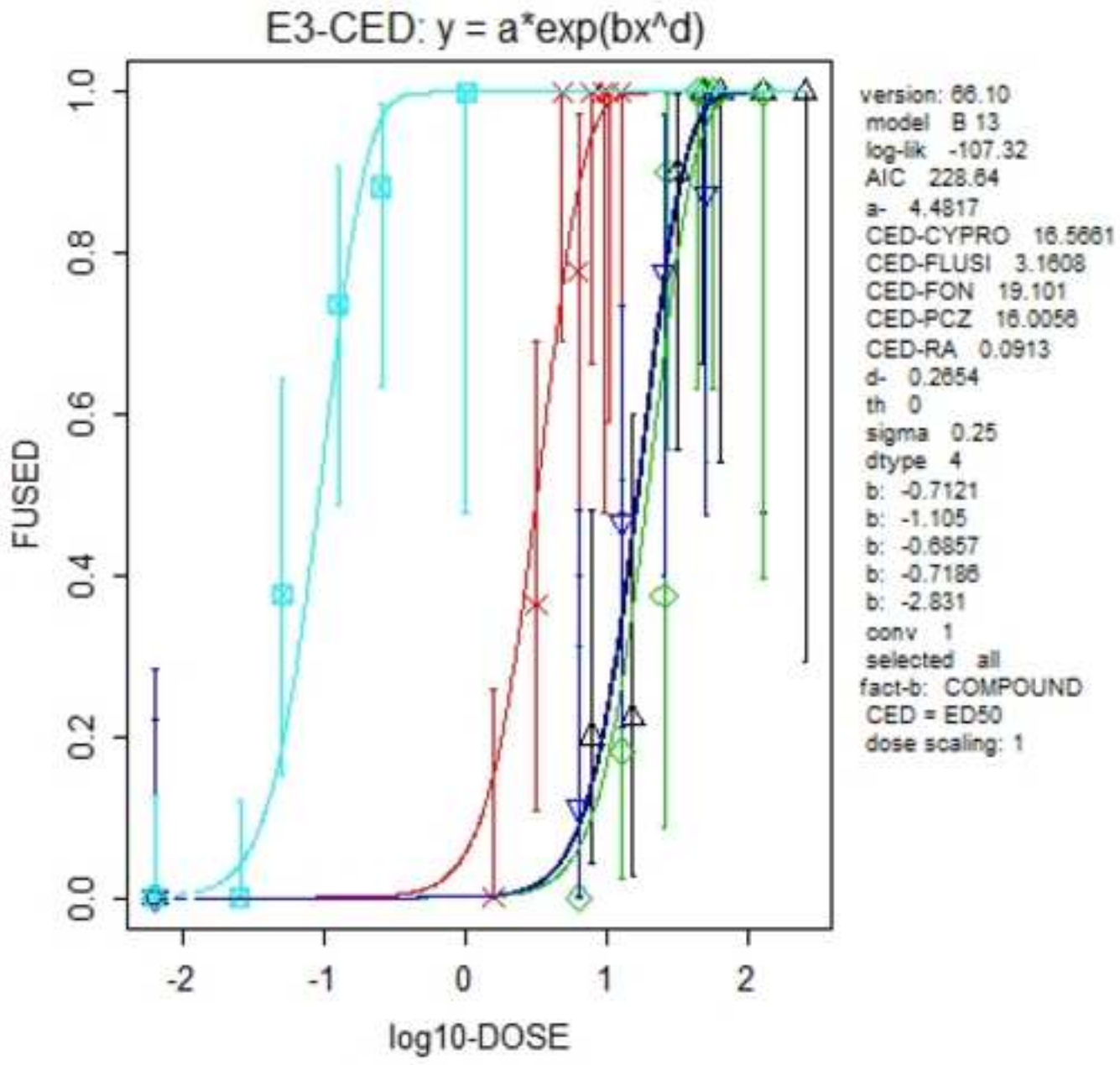
Figure

Click here to download high resolution image



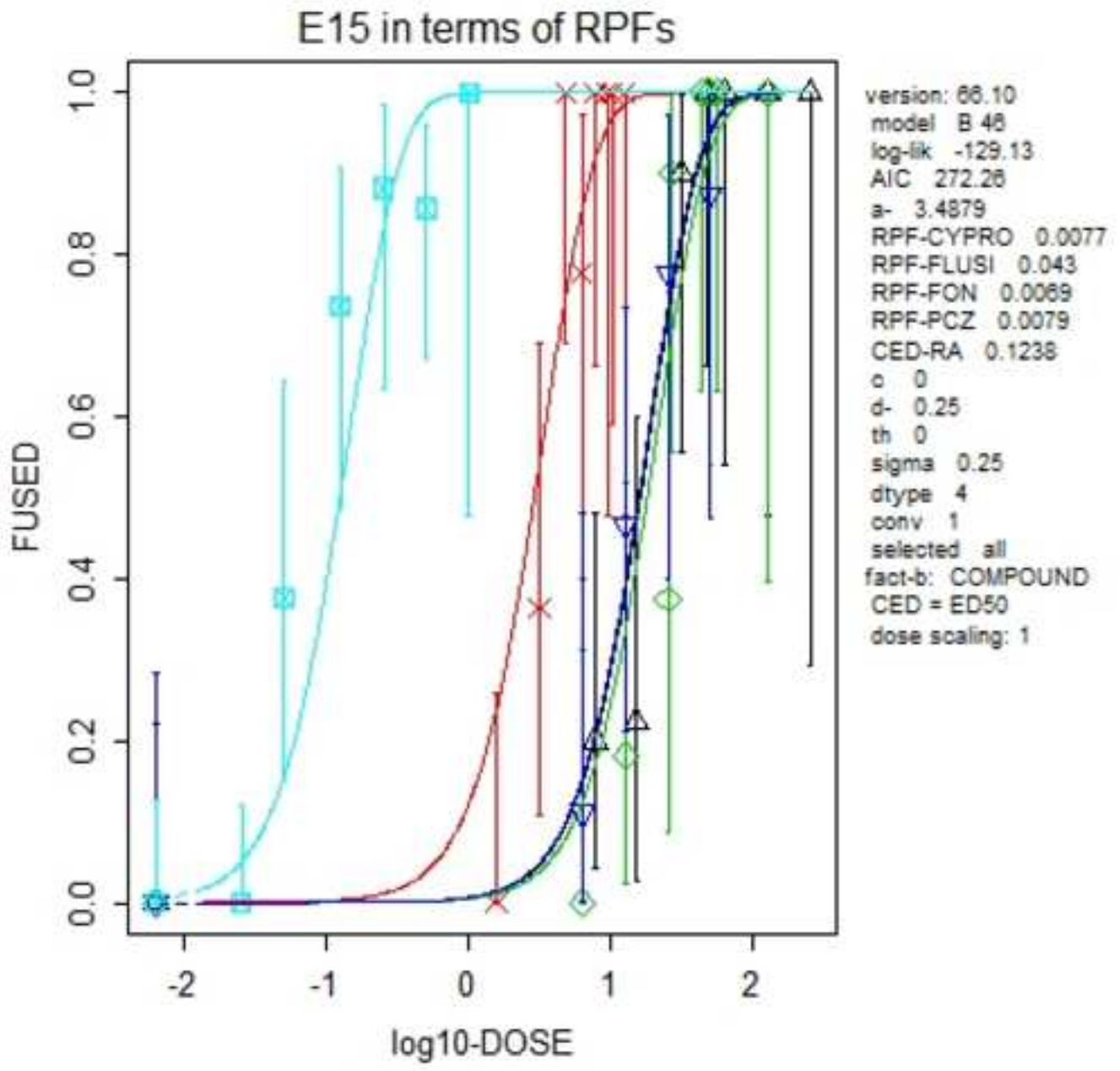
Figure

Click here to download high resolution image



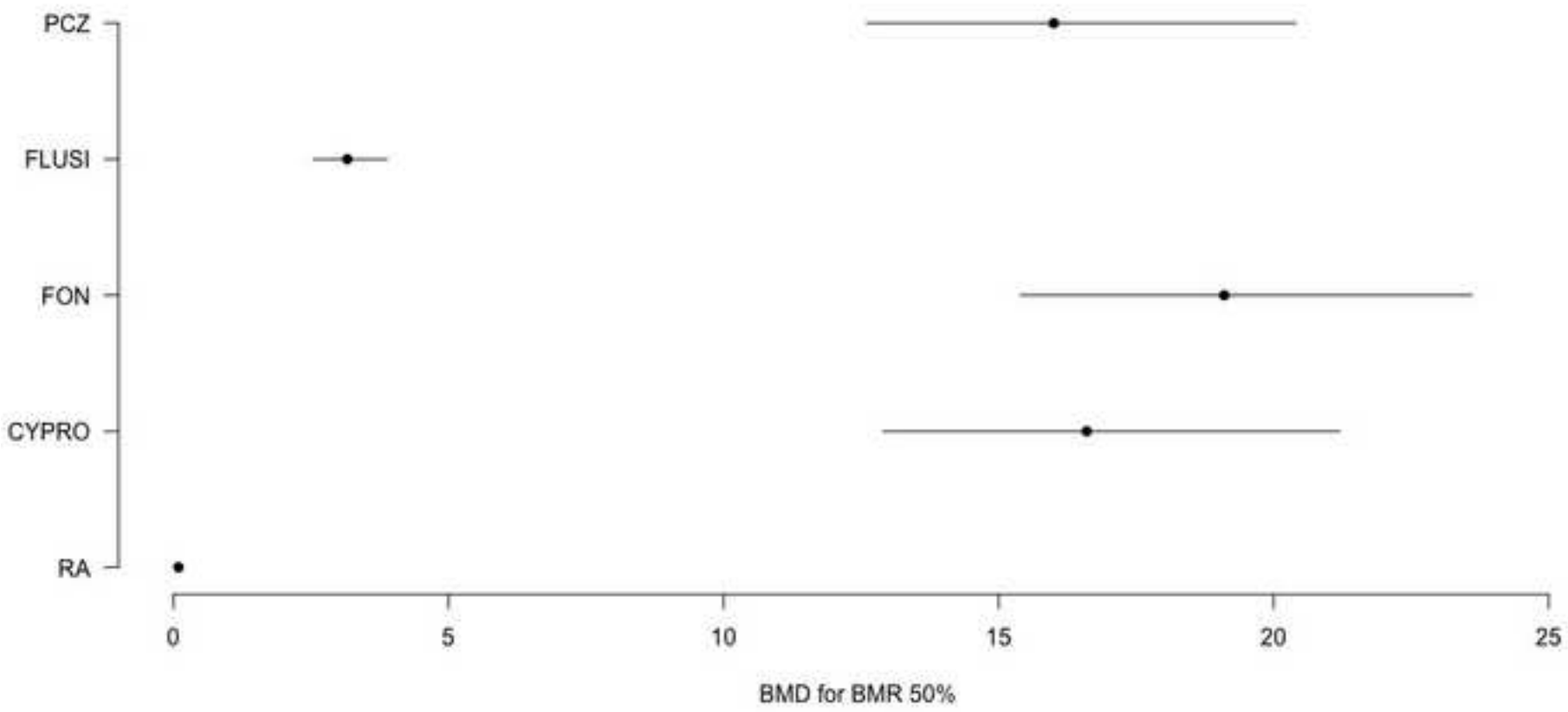
Figure

Click here to download high resolution image



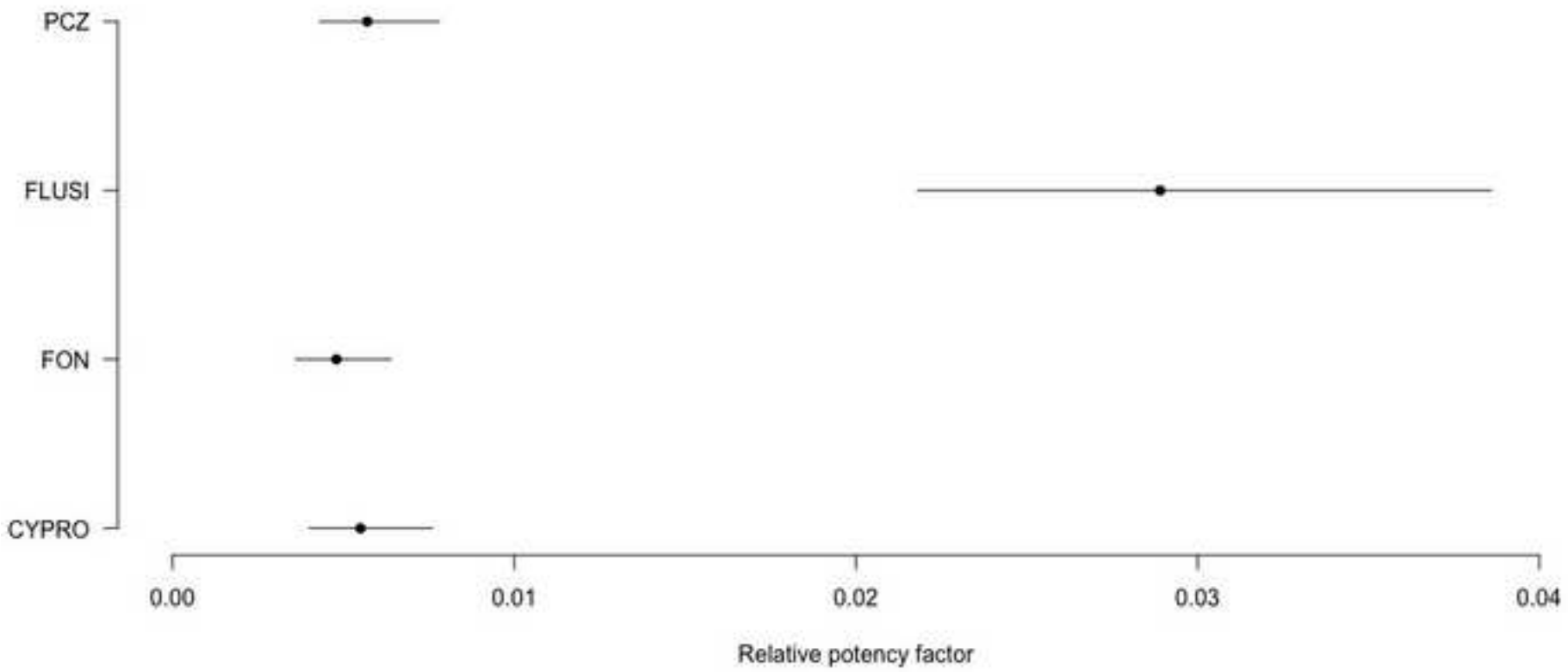
Figure

Click here to download high resolution image



Figure

Click here to download high resolution image



***Conflict of Interest Statement**

The work reported in the manuscript had only financial support from the European Union Horizon 2020 Framework Program under Grant Agreement 633172 (European Test and Risk Assessment Strategies for Mixtures, EuroMix). No other support or financial relationship relevant to the work done has been provided to any of the authors.