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The use of *in vitro* testing to refine cumulative assessment groups of pesticides: The example of teratogenic conazoles

Angelo Moretto [a,b,*], Francesca Di Renzo [c], Erminio Giavini [c], Francesca Metruccio [b], Elena Menegola [c]

[a] Department of Biomedical and Clinical Sciences, University of Milano, Italy
[b] International Centre for Pesticides and Health Risks Prevention (ICPS), Luigi Sacco Hospital, Milano, Italy
[c] Department of Biosciences, University of Milano, Italy

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ABSTRACT

The most relevant issues in cumulative risk assessment (CRA) are the identification of cumulative assessment groups and the hypothesis of dose-additivity, at relevant human exposures. *In vitro* methods can provide meaningful data to help solving those issues. Integration of *in vitro* studies, selected *in vivo* studies, and PBPK modeling for teratogenic conazoles confirmed that *in vitro* studies may give results in a cheaper and faster fashion. In particular, *in vitro* studies with explanted rat embryos provided support for dose-additivity for conazoles causing cranio-facial malformations. Although this could not be immediately quantitatively transferred to the *in vivo* situation, they provided indication on how to conduct targeted *in vivo* studies. In addition, by means of PBPK modeling, it was possible to estimate the dose in humans associated with a defined teratogenic risk and also to conclude that for cumulative risk assessment only exposures occurring within a short period of time (a day or less) need to be cumulated. Although PBPK modeling cannot be widely applied, at least in the short term, it should be considered if available. It is recommended to incorporate *in vitro* testing and PBPK modeling, whenever available and feasible in the process of risk assessment, particularly of CRA.

1. Introduction

As society progresses through the second decade of the 21st century, there is increased need to develop new ideas and new information in the practice of toxicology and risk assessment. In addition, there is societal pressure to reduce the use of animals; hence greater emphasis is put on alternative methods, including *in vitro* methods, to assist in making decisions regarding risk assessment. One particular issue is that related to the identification of chemicals that need to be grouped and considered in a cumulative assessment group. In some cases, based on exposure assessment and/or other considerations such as communication and perception of a particular risk, or risk managers requests, more detailed toxicological information is currently lacking. Several bodies and committees have provided suggestions on how to proceed in the identification of the so called Cumulative Assessment Groups (CAG) of chemicals that should be put through the process of cumulative risk assessment (CRA) (EFSA 2008, 2009a; Meek et al., 2011; EPA, 1999, 2000, 2002; NAS, 2007; EFSA, 2013a, b; SCHER/SCENIHR/SCCS, 2011).

The issue of taking into account exposure early in the process is not addressed here. The focus in on toxicological considerations that can be made in the cases when refinement of hazard characterization of combined exposures needs to be made. In particular, among the several toxicological issues that need to be addressed regarding CRA, the most critical ones appear to be: (i) the toxicological criteria to define the CAG, (ii) the assumption that combined exposure does not result in deviations from the dose-additive effect at human relevant doses (i.e. at doses around or below the no-observed-adverse-effect level, NOAEL).

In general experimental studies conducted for the purpose of risk assessment, use high doses resulting in considerable uncertainty when attempting to extrapolate the effects observed in animals to humans, especially when humans are experiencing much lower environmental exposures, as already noted three decades ago (NAS,

Abbreviations: ADME, adsorption, distribution, metabolism and excretion; AOP, adverse outcome pathway; BMD, BenchMark Dose; BMR, BenchMark Response; CAG, cumulative assessment group; CRA, cumulative risk assessment; IC, index compound; LOAEL, Lowest Observable Adverse Effect Level; NOAEL, No Observable Adverse Effect Level; PBPK, Physiologically-Based PharmacoKinetic; RPF, relative potency factor.

* Corresponding author. Address: c/o ICPS, Ospedale Sacco, via GB Grassi 74, 20157 Milano, Italy. Tel.: +39 02 3568 6650.
E-mail address: angelo.moretto@unimi.it (A. Moretto), francesca.direnzo@unimi.it (F. Di Renzo), erminio.giavini@unimi.it (E. Giavini), francesca.metruccio@icps.it (F. Metruccio), elena.menegola@unimi.it (E. Menegola).

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1983). Besides the difficulties linked to the extrapolation to the human situation of the effects observed with single chemicals at such high doses, combined exposures have been shown to result in deviations from expected outcome: i.e. dose-additivity for compounds sharing a common mode of action, or response-additivity for compounds having different mode of action and/or target organs (see EFSA, 2008 for a summary). However, conducting experiments with animals at such low doses poses statistical difficulties, since the number and size of observable effects will be greatly reduced, unless the number of animals is greatly increased. In addition, the definition of adequate control groups or means of identifying the expected outcome of combined exposures is not always readily evident. Together with the need for toxicity evaluations for the large number of chemicals in commercial use, new *in vitro* and *in silico* technologies and computational systems biology to complement, and eventually replace, whole animal testing need to be introduced or, when already in place, their use greatly increased (Thomas et al., 2013; Andersen and Krewski, 2010; Blaauboer, 2010; Judson et al., 2011). *In vitro–in vivo* extrapolation is necessary to express the dose–response for *in vitro* data on a similar dose scale as the *in vivo* data. In order to do this, the application of Physiologically-Based PhamacoKinetic (PBPK) modeling when enough data are available can be very useful (Tan et al., 2011; McLanahan et al., 2012; Wetmore et al., 2012a, b) to extrapolate from the *in vitro* to the *in vivo* situation in animals, and possibly from animals to humans. However, in the context of CRA, *in vitro* studies can be useful for refinement of grouping and provide an additional advantage because they allow an increase in the number of the possible observations and hence an increase in the dose and mixture combinations. In this way they may more quickly and extensively provide data to support biological reasoning regarding the essentially unanswerable questions such as the assumption of dose addition at low doses, and clear definition and characterization of the dose–response curve at low doses. They may also provide support to considerations regarding time of exposure, especially the dynamic characteristics of the chemicals under study and their relationship with exposure pattern, e.g. intermittent vs. continuous and chronic.

Although, it is suggested that exposure considerations should take priority when formulating the problem for CRA (NAS, 2009; EFSA, 2009a; Meek et al., 2011), there will be situations where chemicals should be screened for grouping according to toxicological characteristics, even beyond chemical similarity. In fact, there might be the same molecular or cellular target or there might be effects on the adverse outcome pathway (AOP) that could lead to a cumulative effect (additivity) (NAS, 2009; Kortenkamp et al., 2009). In addition *in vitro* studies might help in identifying toxicological characteristics for data-poor compounds.

The aim of this paper is to describe the lessons learned from using an *in vitro* method for detecting malformations as applied to the teratogenic potential of certain conazoles, in order to confirm that some of them should be included in a CAG, and to test the assumption of dose-additivity. Published data will be discussed and combined with unpublished data obtained within the ACROPOLIS project.

2. In vitro studies with conazoles

Details of methodology and most of the experimental results are reported elsewhere (Menegola et al., 2000, 2001, 2013; Giavini et al., 1992). In brief, explanted rat embryos at day 9.5 post-coitum (corresponding to the embryonic early neurulation stage) have been incubated with several conazoles either alone or in combinations that differed for compounds and doses, in order to define the *in vitro* dose (concentration) – response curves for individual compounds and the *in vitro* dose (concentration) – response curves for mixtures. The Chi-square test was applied in order to compare the percentage of affected embryos on exposed groups vs. controls.

In order to compare data obtained with different mixtures, it is important to normalize the doses (concentrations) of each individual compound according to their potency; one way is to identify an Index Compound (IC) against which to normalize the potency of the others. However, as indicated in Table 1 (modified from Menegola et al., 2013), the relative potency factors may vary according to the point of departure chosen. In the case of CRA, since exposure of individual compounds are expected to be at or below, sometimes well below, the effective doses, the points of departure are more reasonably chosen at or below the NOAEL or, if using the BenchMark Dose (BMD) at the lowest BenchMark Response (BMR) that provides low dependency on the model used (EFSA 2009b; EFSA, 2011). It should be noted that when using the NOAEL, the relative potency factor is also dependent on the dose-spacing chosen for the experiments and this is unlikely to be proportionally related to the toxicity of each compound.

When performing studies with mixtures from which dose (concentration)-additivity is expected, doses can be (i) around the NOAEL, for all mixture components as reported in Table 2 (Menegola et al., 2013) or (ii) from below the NOAEL to the Lowest-Observed-Adverse-Effect Level (LOAEL), increasing the concentration of one compound at a time (i.e.: comparing the shape of the dose (concentration) – response with or without the presence of a another compound belonging to the same CAG) (Table 3). Also the latter approach suggests that there are no significant deviations from concentration-additivity.

3. In vivo studies with conazoles

Key experiments have been conducted *in vivo* to confirm some of the conclusion of the *in vitro* studies. Pregnant mice ($n = 10$ per group) were administered by gavage on day 8 post-coitum a single dose of triadimefon or flusilazole (dissolved in acetone: corn oil 1:9, volume of treatment 0.1 mL/10 g body weight), either alone or in combination. The time of treatment corresponds in mice to the embryonic early neurulation stage (the same stage selected as starting period for the rat embryo culture). At termination of pregnancy (day 18 post-coitum), females were sacrificed and fetuses were weighed and morphologically examined in order to detect external cranio-facial malformations, including cleft palate. Foetuses from different groups were similar for developmental degree. No general abnormalities were recorded. By contrast, cleft palate was detected as a specific malformation in effective concentration groups. The Chi-square test was applied in order to compare the percentage of affected embryos on exposed groups vs. controls. Particularly relevant is the issue of confirming dose-additivity for the compounds identified *in vitro* as requiring inclusion in the CAG. The dose–response data obtained when the compounds were administered alone or in combination are reported in Table 4. Based on the results of the studies in which the compound had been administered alone, the studies with the combination of both compounds have been performed. As can be seen from Table 4, the dose–response of both triadimefon (fixed dose) + flusilazole (increasing doses), or flusilazole (fixed dose) + triadimefon (increasing doses) suggest dose-additivity.

4. Quantitative extrapolation of *in vitro* studies: PBPK modeling

Quantitative extrapolation from *in vitro* data to the *in vivo* condition is essential to progress from merely qualitative information