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ACROPOLIS

Aggregate and Cumulative Risk Of Pesticides: an On-Line Integrated Strategy

SEVENTH FRAMEWORK PROGRAMME

Deliverable 4.2 A scientific paper describing the *in vitro* experiment of conazoles

Deliverable 4.2 and 4.4 were strongly related and therefor the results were combined in one article.

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The use of in vitro testing to refine CAG of pesticides: the example of teratogenic conazoles

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Abstract

The most relevant issues for toxicology in cumulative risk assessment are the adequate identification of cumulative assessment groups of chemicals and the confirmation of the hypothesis of dose-additivity, at relevant human exposures (i.e.: at or below the NOAEL). In vitro methods can greatly contribute to provide meaningful data to help solving those issues. In the ACROPOLIS project, integration of in vitro studies, selected in vivo studies, and PBPK modeling for teratogenic conazoles (either pesticides or drugs) confirmed that in vitro studies may give results in a cheaper and faster fashion. In particular, in vitro studies with explanted rat embryos provided evidence of dose-additivity for conazoles causing cranio-facial (branchial arch) malformations. Although this could not be immediately transferred to the in vivo situation in a quantitative way, they provided indication on how to conduct targeted in vivo studies, that confirmed qualitatively the in vitro data. 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 test and PBPK modelling, whenever available and feasible in the process of risk assessment, and of CRA in particular.

Key Words: risk assessment, extrapolation, quantitative, modeling, grouping

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 are required to be developed. 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 la., 2011; EPA 199; 2000; NAS, 2007).

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 observable 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 been noted three decades ago (NAS, 1983). Besides the difficulties linked to the extrapolation to the human situation of the effects observed with single chemicals at such high doses, combined exposure 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 of observations 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 et al., 2010; Blaauboer et al., 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 PBPK modeling when enough data are available can be very useful (Tan et al., 2011; McLanahan et al., 2012; Wetmore et al., 2012a; 2012b) 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, since they allow increasing the number of the possible observations and hence increasing the dose and mixture combinations, 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. These may also provide support to considerations regarding time of exposure, especially regarding the dynamic characteristics of the chemicals under study and their relation 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 cumulative risk assessment (NAS, 2009; EFSA 2009; 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 might 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 compound.

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 considered as a CAG, and to test the assumption of dose-additivity. Published data will be discussed and combined with a unpublished data obtained within the Acropolis project.

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 have been incubated 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.

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 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 No Obsevable Adverse Effect Level (NOAEL) or, if using the BenchMark Dose (BMD) at the lowest BenchMark Response (BMR) that provides low dependency on the model used (EFSA 2009b; 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 that is unlikely to be proportionally related with the toxicity of each compound.

Table 1.
Comparison between in vitro NOAEL- and BMD-derived RPFs, using triadimefon (RPF=1) as IC (From Menegola et al, 2013, modified)

Compound	**NOAEL based RPF**	**BMD0.5 based RPF**	**BMD5 based RPF**	**BMD10 based RPF**
Fluconazole	0.2	0.3	0.3	0.3
Triadimenol	1.0	1.3	0.6	0.9
Cyproconazole	0.8	2.1	0.5	0.8
Tebuconazole	0.4	0.4	0.45	0.4
flusilazole	4.0	6.7	6.6	3.3

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 on table 2 (Menegola et al., 2013) or (ii) from below the NOAEL to the LOAEL, increasing the concentration of one compound at 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 this approach suggests that there are no significant deviations from concentration-additivity.

Table 2.
Effect of combined in vitro exposures of explanted rat embryos to conazoles; observed malformations vs malformations expected from BMD modelling. Index compound: Triadimefon. (from Menegola et al 2013, modified)

	Compounds	Concentration (µM)	Ratio observed/expected
1.	Triadmefon	12.5	2.1
	Imazalil	5.0	
2.	Triadimefon	12.5	
	Imazalil	5.0	1.7
	Fluconazole	62.5	
3.	Triadimefon	2.55	
	Imazalil	0.84	
	Fluconazole	1.3	
	Triadimenol	5.07	3.7
	Cyproconazole	0.27	
	Tebuconazole	1.62	
	Flusilazole	0.55	

Table 3.
% Malformations after incubation of explanted rat embryos with Flusilazole or Trichlorfor either alone or in combination (Menegola et al., 2013 in preparation)

Tri-chlorfon (µM) \ Flusilazole (µM)	0	1.57	3.13	6.25	9.38
0		0	18	47	57
6.25	0	1	41	51	
12.5	11	17	52	50	67
25	28	31	56	67	
50	58		72		97

In vivo studies with conazoles

Key experiments have been conducted in vivo to confirm some of the conclusion of the in vitro studies. Pregnant rats (n= 10 per group) were administered by gavage on day 9 post-coitum a single dose of triadimefon or flusilazole, either alone or in combination. At termination of pregnancy, fetuses have been observed for cranio-facial malformations. Particularly relevant is the issue of confirming dose-additivity for the compounds identified in vitro as requiring inclusion in the CAG. The compounds chosen for confirmation were triadimefon and flusilazole. The dose response data obtained when the compounds have been administered alone or in combination, are reported on table 4. On the basis of the results, the

studies with the combination of both compounds have been performed As it 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.

Table 4.
% Fetuses with craniofacial malformations after in vivo treatment with Flusilazole of Trichlorfon either alone or in combination (Menegola et al., 2013, unpublished)

Tri chlorfon (mg/kg) \ Fusilazole (mg/kg)	0	18.8	37.5	75	225
0	0	0	0	0	47
37.5	0	0			
75	0		11	3	
150	0		4		
300	43		41	72	

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 to quantitative application of the results. This is particularly complex in the case of mixtures, because normalization of potency of the different compounds in the CAG cannot be based solely on in vitro data. In fact, the in vivo situation may be significantly different as shown in table 5, where the in vitro and in vivo relative potency factors for several conazoles are compared and shown to be quite different. Moreover, in the case of CRA, timing of combined exposure, especially for effects possibly occurring after a single exposure or repeated exposures over a short period of time, is an information necessary in order to decide on the correct time-frame of exposure it is appropriate to carry out the assessment. Physiologically-Based PhamacoKinetic (PBPK) modeling is a tool that could help in such endeavor (McLanahan et al., 2012, NRC 2007). PBPK modeling was applied to a conazole, tebuconazole, for which we had in vitro teratogenicity data and using a PKPB model with reverse dosimetry, the dose for humans corresponding to a given risk level, as based on the vitro concentrations, could be estimated (van Eijkeren et at, 2013 in preparation). In this specific case, the it was found that at a risk level of 0.0075% for malformations the estimated dose for a pregnant woman would be 1.7 (CI: 0.18-5.4) mg/kg bw which is about 55 times higher that the existing acute reference dose (ARfD) established in Europe for tebuconazole. In addition, PBPK modeling indicated that the elimination of tebuconazole in humans is quick (half-life of a few hours) after a single oral dose, suggesting that only simultaneous (or occurring in a short period of time, less than 24 hours) co-exposures would be relevant for CRA.

Table 5.
Comparison between in vivo (EFSA, 2009a) and in vitro (our data) data for selected azoles

Compound	In vivo		In vitro (µM)	
	BMD5 (mg/kg)	RPF	BMD5 (µM)	RPF
Flusilazole	232	1	2.1	1
Cyproconazole	104	2.2	25	0.2
Triadimefon	198	1.2	13.8	0.3

Discussion

Our in vitro studies with combined exposure to conazoles indicated that, in fact, it is correct to group compounds based on a specific effect such as cranio-facial (branchial arch) malformation. In fact, the incidence of this specific malformation increased in an additive manner after co-exposure. Concentration additivity was hypothesized not based on the characteristics of the apical end-point, but also by some toxicological considerations that tentatively link such malformations to tissue and time specific inhibition of the retinoic acid metabolism (Di Renzo et al., 2009; 2011). However, since there are no methods to measure retinoic acid levels such hypothesis cannot be experimentally confirmed. In any case, our results show that the effects of the conazoles do add up. In addition, when doses are around the NOAEL, there are minor deviations from the dose additivity hypothesis, as shown by the data reported on table 2 and table 3 where different approaches to combined exposure have been taken. In fact, in both the fixed ratio approach (table 2) and the approach of table 3 where the concentration of one compound was kept fixed while those of the other compound were increased, the deviation from the additivity hypothesis was not significant as pointed out in other cases (Boobis et al., 2011, ECETOC, 2012). A limited number of in vivo confirmatory studies was performed. However, since no quantitative extrapolation from in vitro to in vivo data was possible, a dose response curve had to be determined in vivo before performing the combined in vivo exposure studies. The data on table 4, while showing a not unexpected higher variability of in vivo results, indicate that at the NOAEL and to a certain extent also at higher doses, no significant deviation from dose additivity occurs. In essence, there was a qualitative concordance between in vitro and in vivo data in terms of appropriateness of including those conazoles in the same CAG, and of dose additivity. In addition, previous studies (Di Renzo et al., 2009) with the same in vitro method, showed that the window of sensitivity to malformations is very narrow (a few hours), and this was confirmed also in vivo since malformations have been obtained with a single administration on day 9.5 of gestation.
However, as indicated by the different RPFs obtained from in vitro vs. in vivo studies (table 5), a direct quantitative extrapolation from the in vitro concentrations to the in vivo doses, is not possible. Such quantitative extrapolation can be obtained by PBPK modeling. In our project, this was applied to tebuconazole, and by means of reverse dosimetry that allows the estimation of the human systemic dose

corresponding to a given in vitro concentration causing a predefined effect. This was possible on the basis of adsorption, distribution, metabolism and excretion (ADME) data in rabbits that have been extrapolated to humans based on comparison of physiological parameters (van Eijkeren et al., 2013, in preparation). PBPK modeling also provided additional information related to the time frame of combined exposures. In fact, the rapid elimination of tebuconazole after oral administration indicates that dose additivity likely occurs if co-exposures occur within a narrow time windows (few hours, in correspondence of the initial separation of the branchial arches) since the presence of the compound in the body is quite rapid. Hence, considering together the narrow window of sensitivity (a few hours in the rat, possibly slightly longer in humans) and the rapid ADME, it can be confidently concluded that co-exposure to conazoles likely needs to be added up only if occurring in a narrow (up to a day) time-frame.

Conclusions

The results obtained from the studies with selected conazoles proved the usefulness of in vitro studies to provide information relevant for cumulative risk assessment, in a quicker and less resource intensive way than in vivo studies. Our experiments included a large number of data points that have been obtained with significantly less human, economic and logistic resources than those that would have been required by in vivo studies. In fact, only limited in vivo studies have been performed to confirm the in vitro data regarding grouping of compounds and the dose (concentration) additivity assumption.

The information provided by in vitro studies became quantitatively more relevant by extrapolation to the in vivo situation by the application of PB-PK modelling. As it has been shown by the tebuconazole example, PBPK modeling provides a less uncertain indication on the margin of exposure between the expected effect and the actual or estimated exposure/exposure limit (reference point).

In general, it can be concluded that in vitro test systems can be very helpful in the process for definition of common assessment groups, especially when a weight-of-evidence approach is needed for inclusion or exclusion of pesticides or chemicals in a group. Also, in certain instances, like the one addressed with conazoles and also with neurotoxic compounds within ACROPOLIS (Menegola et al., 2013; Heusinkveld et al., 2012; Heusinkveld and Westerink, 2013), the dose addition assumption can be tested, more extensively than with classical in vivo studies with apical endpoints.

A number of additional issue can be potentially addressed by in vitro studies, that are not specific to conazoles. In can be envisaged that in vitro studies might prove useful to quickly identify compounds that can/should be excluded from CAG, even if belonging to the same chemical class, because not acting on the relevant target, either cellular or molecular. On the other hand, in vitro studies might indicate that compounds belonging to different chemical classes should be included in a same CAG, because they act on the same molecular or cellular target in a similar way.

Regarding the extrapolation to the in vivo situation, PBPK modeling proves to be a powerful tool; however,

it cannot be widely applied, at least in the short term, because a lot of compound specific data on ADME are needed. Therefore, while it should be applied if available, and developed in specific cases that need a better hazard characterization, it is envisaged that in a not so far future this approach will become easier and quicker (Andersen et al., 2010; Judson et al., 2011; Meek et al., 2013; Tan et al., 2011; Wetmore et al., 2012a).

It is certainly recognized that exposure considerations may drive the request for CRA or may indicate that there is no need to conduct a CRA, at least for certain compounds (EPA, 2007). In recent times, the risk assessment community has been advocating for a greater emphasis on exposure assessment early in the process of risk assessment (NAS, 2009; EFSA 2009; Meek et al., 2011), and this is particularly relevant for CRA. However, in several cases refinement of toxicological hazard characterization would be needed and in vitro tests and PBPK modeling may prove of utmost importance, for the reasons described above. For instance, large preliminary common assessment groups may be narrowed down to a manageable number of compounds by means of in vitro test systems or other non-animal tests, which may also help in understanding the mode of action, including relevance of time and duration of exposure. Therefore, it is recommended to incorporate such tests, whenever available and feasible in the process of risk assessment, and of CRA in particular.

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