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Assessment of genotoxicity of herbal medicinal products: A co-ordinated approach

Olaf Kelber [1], Barbara Steinhoff [*,1], Karin Kraft [1]

Kooperation Phytopharmaka GbR, Plittersdorfer Str. 218, D-53173 Bonn, Germany

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ABSTRACT

The submission of data on genotoxicity is a precondition for marketing authorisation respectively registration of herbal medicinal products (HMPs) with well established or traditional use in some countries. In European regulatory guidelines prepared by the Committee on Herbal Medicinal Products (HMPC) of the European drug regulatory agency EMA, a test strategy is defined giving a pragmatic framework adapted to the assessment of the potential genotoxicity of HMPs. It describes a stepwise approach, including the possibility to reduce the number of extracts of a herbal drug to be tested by the use of a bracketing and matrixing approach. According to this strategy, Kooperation Phytopharmaka, a scientific society in the field of HMPs, has so far coordinated the conduction of genotoxicity tests for 30 herbal drugs within the frame of a joint project of several manufacturers of HMPs. Results are delivered to the cooperation partners for use in regulatory applications.

Regulatory situation and impact on the assessment of HMPs

During the past few years the discussions on safety of HMPs have particularly focused on the issue of genotoxicity. The "Guideline on Non-Clinical Documentation for Herbal Medicinal Products in Applications for Marketing Authorisation (Bibliographical and Mixed Applications) and in Applications for Simplified Registration" adopted in 2006 (EMEA/HMPC/32116/2005) states in general that for many herbal preparations in traditional or well-established use, an adequate safety profile is confirmed already by their long-term use. However, additional preclinical testing e.g. on genotoxicity would be required for specific herbal preparations, if published literature on this subject is not available or insufficient. This may be a pre-condition for registration/marketing authorisation. For the performance of tests, the guideline refers to the stepwise approach described in the respective ICH step 5 guidelines on genotoxicity testing (CPMP/ICH/174/95; CPMP/ICH/141/95).

More detailed guidance on the assessment of genotoxicity was provided by the "Guideline on the Assessment of Genotoxicity of Herbal Substances/Preparations" published in 2008 (EMEA/HMPC/107079/2007). It includes practical approaches on how to perform the tests and to interpret the results and describes a stepwise testing strategy, starting with the Ames test. In case of positive results, this should be followed by a mammalian cell assay and, in case of a still positive result in that assay, by *in vivo* genotoxicity tests. If the respective step yields negative results, progressing to the next test step is not required. This guideline also mentions the option to extrapolate the results obtained with a specific preparation to closely related preparations such as extracts prepared with ethanol/water mixtures of different, but similar concentrations – the so-called "bracketing and matrixing" concept. Using such an approach to the test materials means that a representative range of materials is tested rather than requiring individual manufacturers to undertake their own testing on all their specific preparations. This reduced test design assumes that the genotoxic potential of any intermediate preparation is represented by the test results of the extremes tested.

In order to propose possible approaches for reduced testing designs following this idea, a test strategy was developed in cooperation between Kooperation Phytopharmaka, a German scientific organisation in the field of HMPs and German regulatory authorities. This proposal was implemented in a further guidance document (EMEA/HMPC/67644/2009) (Fig. 1). It provides examples for a standard range of test materials, which might be considered representative for commonly used preparations of an herbal substance. The use of this approach within collaborative projects performed by applicants offers a strategy to lower the number of test materials used, and thus to lower the burden for manufacturers to perform their own investigations on each individual preparation of an herbal substance.

* Corresponding author. Tel.: +49 228 365640; fax: +49 228 351390.
E-mail address: koop.phyto.bonn@t-online.de (B. Steinhoff).
URL: http://www.koop-phyto.org (B. Steinhoff).
[1] On behalf of the Working Group "Efficacy and Safety" of Kooperation Phytopharmaka, Bonn, Germany.

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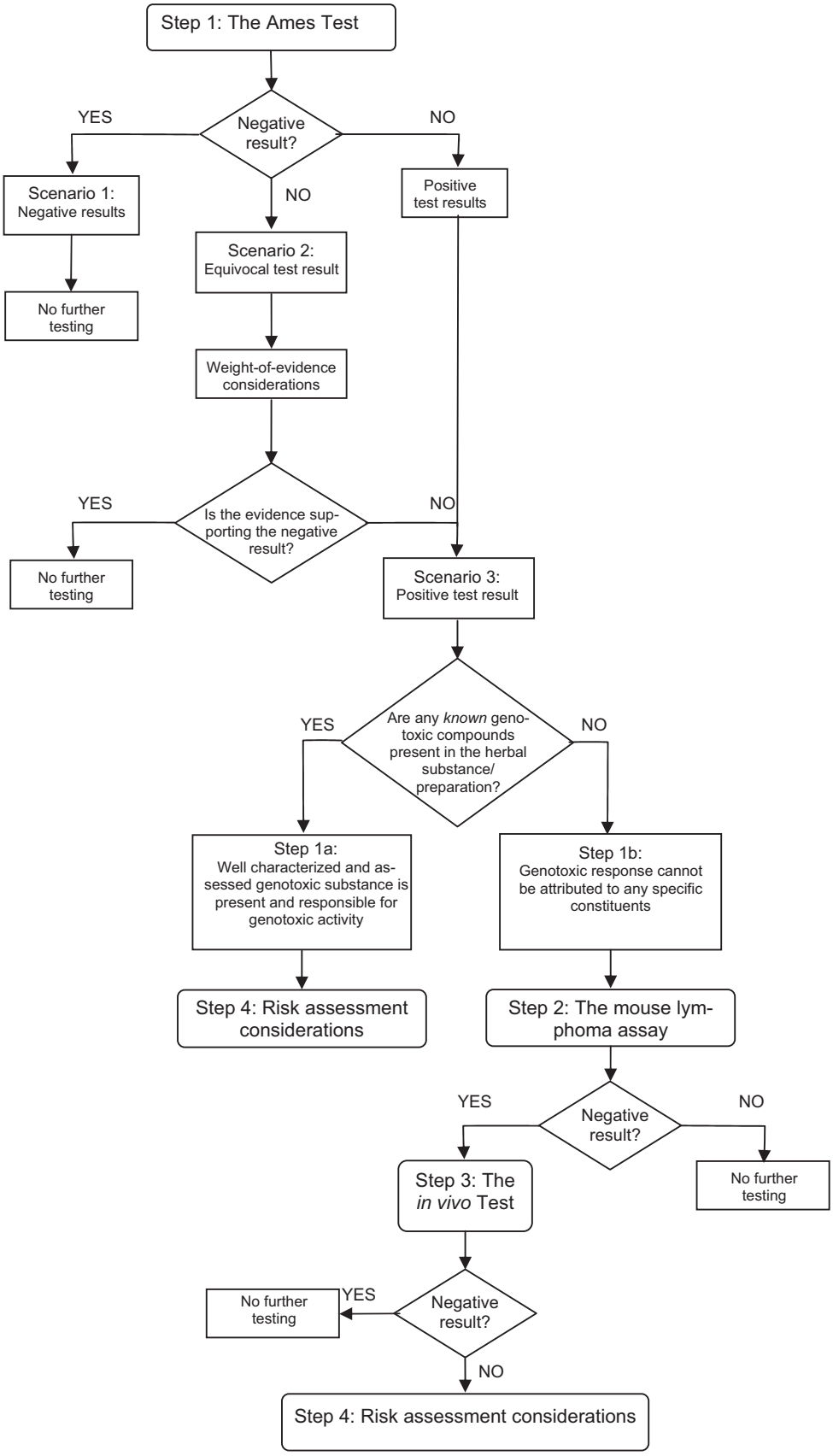

Fig. 1. Decision tree on the assessment of genotoxicity of herbal preparations (from EMEA/HMPC/67644/2009).

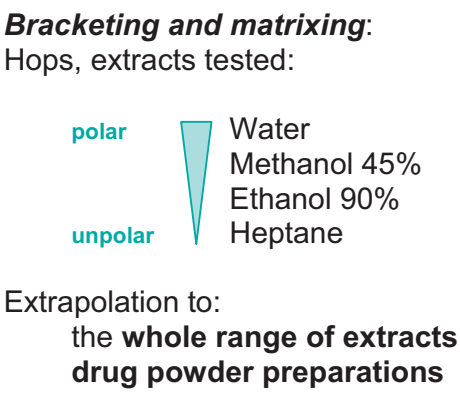

Fig. 2. Bracketing and matrixing, example: preparations from hops.

More recently, representatives of a German regulatory authority (Wiesner and Knöss 2010) stated that, although the set of tests to be performed in the context of preclinical testing can be reduced by this procedure, the results, especially those on genotocixity, still are to be discussed critically by the respective applicant. Additional questions which might arise should be addressed case by case, and the inevitable gap between regulatory requirements and sound experimental methodology should be bridged within the preclinical expert report by means of a qualified discussion and interpretation of data.

Example for a co-ordinated approach

Already in 2007, Kooperation Phytopharmaka started a collaborative model project following the "bracketing and matrixing concept" mentioned above. For each of the herbal drugs tested, several extracts were included covering the whole polarity range of relevant extraction solvents from non-polar to polar ones. This approach allowed the extrapolating of the results to preparations, which had not been tested, and even more the detection of a possible genotoxicity of medicinal drug powder preparations otherwise not easily accessible to *in vitro* methods. It also is in line with the HMPC guidance document on the selection of test materials (EMEA/HMPC/32116/2005). In these cases the guideline recommends test materials which cover the entire spectrum of phytochemical constituents, including polar and non-polar constituents, i.e. the whole phytochemical profile. The choice of solvents should be justified, and inclusion of a mid-range polarity of solvent (e.g. 50% water) is regarded as useful.

For testing, extracts characterized according to their individual specifications were provided by the cooperating pharmaceutical companies. Tests were conducted by GLP-conform laboratories according to the current guidelines, including those of OECD, ICH, and EMA. They also included validation of test results by independent testing in two laboratories. Tests were started with the first step of the test strategy, the Ames test, in five bacterial strains. Examples of the testing strategy and the results are given in Figs. 2 and 3.

Results

The project has already yielded data on preparations of many of the most important herbal drugs in Europe and worldwide. They are listed in Table 1.

For some of these herbal drugs, the availability of results has already been published earlier (Gaedcke et al. 2009a,b).

Table 1
Herbal drugs for which genotoxicity data from the joint project of Kooperation Phytopharmaka is already available (nomenclature according to the European Pharmacopoeia).

Allii sativi bulbus
Althaeae radix
Berberidis cortex
Betulae folium
Cardui mariae fructus
Carvi fructus
Crataegi folium
Crataegi fructus
Curcurbitae oleum
Cynarae folium
Dulcamarae stipites
Ginkgo folium
Ginseng radix
Harpagophyti radix
Hippocastani semen
Hyperici herba
Liquiritiae radix
Lupuli flos
Matricariae flos
Melissae folium
Passiflorae herba
Pini aetheroleum
Primulae radix
Rosmarini folium
Serenoae repentis fructus
Thymi herba
Urticae folium
Urticae herba
Valerianae radix
Visci albi herba

Specific issues related to the test systems

It should always be kept in mind that results from toxicological test models have to be discussed with respect to their scientific relevance for the respective product. In case of positive results, the guideline EMEA/HMPC/107079/2007 gives detailed advice of how to interpret the results or to continue in the test procedure. Such guidance, however, is missing for the case of negative results. Irrespective of that, the adequacy and suitability of the respective standard test systems and procedures always should be addressed in the application for a specific HMP (Wiesner and Knöss 2010; Veit and Klüting 2010).

In this context it is known for example, that the Ames test can give positive results with drugs with a high content of flavonoids, which is due to quercetin (Podginsky et al. 1988). This finding does not correlate with the lacking carcinogenicity of these drugs in animal testing (Okpanyi et al. 1990; Rietjens et al. 2005; Harwood et al. 2007) and therefore does not indicate a risk in drug safety evaluations. On the other hand, some substances were tested to be an-eugenic in mammalian cell cultures, but they did not give positive results in the Ames test. For example, taxol is known since 1971 for its ability to disrupt cell division only in eukaryotic organisms (Wani et al. 1971). However, it could never be established whether it is a relevant carcinogen (Bartsch 2004). In HMPs used in Europe, substances with a comparable mechanism of action have not been identified since then.

Another question can arise from substances, which underly metabolic activation, such as some phenylpropanes e.g. safrol (Wislocki et al. 1977) or methyleugenol (Miller et al. 1983). For both compounds, a phase II reaction (sulfation) of a side-chain hydroxylated metabolite has been observed. The resulting DNA-reactive intermediates have been described in toxicological models and may be mimicked insufficiently by the S9 mix (OECD 1997) used in many *in vitro* models, as this may have only a low activity of the phase II metabolic enzymes. However, more recent studies have questioned the relevance of this type of metabolism for doses

Study Name: 1238600
Experiment: 1238600 HV2 Pre
Assay Conditions:

Study Code: 1238600
Date Plated: 27/01/2009
Date Counted: 30/01/2009

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 98	St John's Wort	3 µg	23.0	1.0	1.0	24, 22, 23
	Dry Extract	10 µg	24.0	4.4	1.0	22, 29, 21
	(3-6:1)	33 µg	22.7	3.5	1.0	23, 19, 26
		100 µg	23.3	6.5	1.0	23, 17, 30
		333 µg	28.0	8.7	1.2	38, 22, 24
		1000 µg	20.7	5.0	0.9	26, 20, 16
		2500 µg	17.3	2.3	0.7	16, 16, 20
		5000 µg	26.3	3.1	1.1	27, 29, 23
		10 µgP1	51.3	8.1	2.2	42, 56, 56
	DMSO (solvent)		23.7	2.1		22, 26, 23
	Untreated Control		31.0	3.0		34, 28, 31
TA 100	St John's Wort	3 µg	127.0	4.4	0.9	129, 130, 122
	Dry Extract	10 µg	128.3	6.0	1.0	134, 129, 122
	(3-6:1)	33 µg	121.0	22.0	0.9	121, 143, 99
		100 µg	120.3	7.4	0.9	112, 126, 123
		333 µg	124.3	19.4	0.9	137, 134, 102
		1000 µg	91.0	24.4	0.7	80, 119, 74
		2500 µg	87.3	7.1	0.6	86, 95, 81
		5000 µg	79.7	14.7	0.6	85, 91, 63
		10 µgP1	147.7	8.1	1.1	157, 143, 143
	DMSO (solvent)		135.0	16.1		120, 152, 133
	Untreated Control		155.0	13.5		159, 140, 166
TA 98	4-NOPD	10 µg	501.0	17.1	21.2	503, 517, 483
TA 100	NaN3	10 µg	2229.3	28.0	16.5	2238, 2198, 2252

Key to Positive Controls
4-NOPD 4-nitro-o-phenylene-diamine
NaN3 sodium azide

Study Name: 1238600
Experiment: 1238600 HV2 Pre
Assay Conditions:

Study Code: 1238600
Date Plated: 27/01/2009
Date Counted: 30/01/2009

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 98	St John's Wort	3 µg	30.0	1.0	0.9	30, 29, 31
	Dry Extract	10 µg	27.3	4.0	0.8	28, 23, 31
	(3-6:1)	33 µg	37.7	6.7	1.2	30, 41, 42
		100 µg	34.7	7.0	1.1	34, 28, 42
		333 µg	40.7	11.9	1.3	37, 31, 54
		1000 µg	38.3	3.8	1.2	40, 34, 41
		2500 µg	40.3	8.3	1.2	43, 47, 31
		5000 µg	40.7	10.2	1.3	45, 29, 48
		10 µgP1	61.3	5.7	1.9	63, 66, 55
	DMSO (solvent)		32.3	4.0		28, 33, 36
	Untreated Control		42.3	5.0		47, 43, 37
TA 100	St John's Wort	3 µg	129.7	20.6	0.9	106, 140, 143
	Dry Extract	10 µg	160.7	10.2	1.1	165, 168, 149
	(3-6:1)	33 µg	150.0	8.5	1.0	149, 142, 159
		100 µg	148.3	19.7	1.0	126, 163, 156
		333 µg	142.7	24.0	1.0	156, 115, 157
		1000 µg	140.7	15.4	1.0	148, 151, 123
		2500 µg	138.3	15.1	1.0	121, 149, 145
		5000 µg	122.3	20.3	0.8	136, 132, 99
		10 µgP1	192.3	22.4	1.3	197, 168, 212
	DMSO (solvent)		144.3	5.5		148, 147, 138
	Untreated Control		174.3	10.6		184, 176, 163
TA 98	2-AA	2.5 µg	1478.7	320.8	45.7	1849, 1286, 1301
TA 100	2-AA	2.5 µg	2051.3	109.2	14.2	2037, 1950, 2167

Key to Positive Controls
2-AA 2-aminoanthracene

Fig. 3. Negative results from the AMES Test, showing data from two *Salmonella typhimurium* strains (TA 98 and TA 100) and controls (P1 = quercetin). These negative results have been validated by independent testing in a second laboratory.

taken up with herbal products, as the relative contribution of the 1′-hydroxylation-sulfation pathway seems to diminish at lower, more clinically relevant substrate concentrations (Smith et al. 2002). In addition, the matrix of herbal products has turned out to be able to decrease the toxic potential compared to isolated substances (Jeurissen et al. 2008). Altogether, it seems to be unlikely that relevant genotoxic effects result from exposure to HMPs containing natural phenylpropanes.

The intestinal deconjugation of glycosides resulting in genotoxic aglycons also might be a mechanism leading to carcinogenic effects *in vivo*, which may be not sufficiently mimicked by *in vitro* genotoxicity testing. This has been suggested after the discovery of cycasin from Cycas in 1979 (Matsushima et al. 1979). However, in recent experiments on the cleavage and metabolism of glycosides during intestinal transit it was shown, that the majority of glycosides passes the small intestine without relevant deglycosylation. Only in the colon, they are deglycosylated by the intestinal microbiota. This step is followed by a rapid metabolism of the resulting aglycons, so that the majority of many aglycons is not available for absorption (Keppler and Humpf 2005; Hasslauer et al. 2010; Vissienon et al. in press). If aglyca are absorbed, they are rapidly conjugated in the intestinal mucosa immediately after absorption (Donovan et al. 2006). Thus the *in vivo* relevance of the potential mutagenicity of aglycons has to be questioned.

Last but not least it is very likely in a test strategy based on a bracketing and matrixing concept, that glycosides are partly present also as aglyca in some of the various extracts tested. This is known e.g. for extracts from *Hypericum perforatum*, which contain various glycosylated forms of quercetin, but also the aglycon (Schütt and Schulz 2010). However, most herbal drugs have been subject to extensive deconjugation of glycosides by glycosidases. These are ubiquitous enzymes in plants (Hegnauer and Hegnauer 2001) being active during processing and drying of the herbs or still even during the extraction process. In addition, the uptake of glycosides from herbal medicines can be expected to range far below their uptake from food. Fruits, vegetables, spices and leisure tea drugs e.g. contain amounts between 10 mg/kg and more than 900 mg/kg of flavonol glycosides, other polyphenol glycosides, glycosylated plant acids and various other glycosides (Crozier et al. 2006; Veit and Klüting 2010). In comparison, the amounts taken up with HMPs are usually negligible (Veit and Klüting 2010).

It can therefore be concluded that cases, where the suitability of the established *in vitro* tests for the determination of genotoxicity, including the Ames test and *in vitro* mammalian cell tests in combination with metabolic activation (OECD 1997), can be questioned, are not likely to occur with medicinal herbal extracts.

Altogether, the issue of suitability of the test system and particularly of the activation system is essential for the assessment of the results of genotoxicity testing and has to be addressed individually for each tested product. In consequence, in the expert report for each HMP these issues are to be explained and discussed, and the results must be assessed in product-specifically.

Availability of the results

It might be of interest for companies, who have not yet participated in these tests, to obtain the results in order to provide information on potential genotoxicity within their application for marketing authorisation or registration of HMPs, respectively. However, publication of the results or their submission to the HMPC as a support for the preparation of Community herbal monographs and list entries would give unlimited access to pharmaceutical manufacturers who have not participated in the tests. This would be a disadvantage for the participants, thereby endangering future continuation of such helpful projects. However, if the companies involved agree to the publication of results of the project for selected herbal preparations, Kooperation Phytopharmaka will make them publically available.

Conclusions

The present project did not only broaden the knowledge on the safety of medicinal important herbs in well established or traditional use in Europe, allowing to meet current regulatory requirements, but also showed that the safety profile of some of them, previously having been under discussion (Podginsky et al. 1988), has to be reassessed when tested by modern validated methods. Thus this project has turned out to be an important step in the continuous process of updating the safety profile of modern phytomedicine, which is already now well documented.

Dedication in Memoriam

This article is dedicated to Prof. Dr. Hilke Winterhoff, who passed away on May 9, 2010, in Münster, Germany, in honour of her important services to the Kooperation Phytopharmaka, as the head of its scientific working group on efficacy and safety, and of her exemplary contributions to the study of the pharmacology and toxicology of herbal medicines.

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