



Editorial

The 10 basic requirements for a scientific paper reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances in in vitro experiments and animal studies in vivo

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Abstract

There is increasing evidence that chemicals/test substances cannot only have adverse effects, but that there are many substances that can (also) have a beneficial effect on health. As this journal regularly publishes papers in this area and has every intention in continuing to do so in the near future, it has become essential that studies reported in this journal reflect an adequate level of scientific scrutiny. Therefore a set of essential characteristics of studies has been defined. These basic requirements are default properties rather than non-negotiables: deviations are possible and useful, provided they can be justified on scientific grounds. The 10 basic requirements for a scientific paper reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances in in vitro experiments and animal studies in vivo concern the following areas: (1) Hypothesis-driven study design; (2) The nature of the test substance; (3) Valid and invalid test systems; (4) The selection of dose levels and gender; (5) Reversal of the effects induced by oxidants, carcinogens and mutagens; (6) Route of administration; (7) Number and validity of test variables; (8) Repeatability and reproducibility; (9) Statistics; and (10) Quality Assurance.

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The study of the toxicology of bioactive substances from plants and food ingredients has continued to gather momentum over the last 5 years. There has been

Abbreviations: ANOVA, analysis of variance; EMEA, European Agency for the Evaluation of Medicinal Products; GLP, good laboratory practice; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; OECD, Organisation for Economic Cooperation and Development; PAHs, polycyclic aromatic hydrocarbons; PAS-SCLAIM, Process for the Assessment of Scientific Support for Claims on Foods; QSAR, quantitative structure–activity relationship; TBARS, thiobarbituric acid-reactive substances

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a shift in the focus of many papers submitted to this journal in recent years. This is because of the recognition that chemicals/test substances cannot only have adverse effects, but that there are many substances that can (also) have a beneficial effect on health. There are many ingredients, from plants in general and food plants in particular, that are now associated with possible beneficial effects (Dragsted et al., 1993; Ferguson, 1994; Rice-Evans et al., 1997; Verhagen et al., 1997; Handelman, 2001). Terms such as nutraceuticals, herbal extracts, bioactive dietary constituents, phytochemicals and similar have become widely used. The market is being increasingly flooded with functional foods and dietary supplements. As this journal regularly publishes

papers in the area of potentially beneficial effects of dietary factors and has every intention in continuing to do so in the near future, it has become essential that studies reported in this journal reflect an adequate level of scientific scrutiny. Some overall information on the submission of papers is covered in the Instructions to Authors. This editorial particularly is written for envisaged publication of papers that may contribute to the overall dossier on beneficial effects of compounds and therefore form an important piece of evidence. However, even a paper that has gone through peer review, which is the basic requirement for scientific quality, in practice is only judged by three independent people (two referees, one editor). Since the composition of such a judgement team varies from paper to paper, it is deemed necessary to set some more formal guidance as to the general standard of papers. This guidance will add to the overall consistency across papers in this journal and perhaps may also be beneficial for other journals. Most important is that studies submitted should be based on sound scientific thinking, preferentially using updated and state-of-the-art methodology carefully selected for the special case.

At a general level, as well as at a specific level, the scientific validity of the associations between the test substances and efficacy must be taken into consideration. At the **general level** the validity of statements (“claims”) has been the subject of many a code of practice (e.g. reviewed in Richardson et al., *in press*). In this respect, presently the EU project PASSCLAIM (Process for the Assessment of Scientific Support for Claims on Foods; <http://europe.ilsa.org/passclaim>) is extending the currently available science in seven areas, namely: diet-related atherosclerosis; bone health and osteoporosis; physical performance and fitness; insulin sensitivity and diabetes risk; diet-related cancer; mental state and performance; gut health and immunity. One of the essentials to come out of this project will be the achievement of a series of criteria for health claims on foods. As concerns the validity of statements in practical situations with humans, there are several caveats to be taken into account as described previously (Verhagen et al., 1997; Verhagen, 1998; Knasmüller et al., 2002). Three caveats related to dose (the threshold concept, beware of toxicity, the matrix) were identified and four caveats related to effect (assessment of chemopreventive potential, the underlying mechanism, the fact that [anti]carcinogens are not always [anti]mutagens and vice versa, and finally the weight of the evidence).

At the **specific level**, “significant scientific agreement” needs to be based on the adequacy of individual supportive data. As a consequence, the totality of the evidence cannot be better than the adequacy of the individual studies. Hence, if the totality of evidence needs to be improved, this also sets requirements for individual studies. This is far from new, since toxicology

has been working along similar lines for decades. This has resulted in a large series of officially endorsed guidelines for toxicity tests such as issued by the OECD, ICH, EMEA (OECD, 1998; Carere et al., 1995). For diet-related diseases, however, it is necessary to address the effects in the actual target tissue of disease occurrence, a prerequisite that obviously needs to be taken more into account. Moreover, nutrition-derived components will need to be judged on the basis of balanced/non-balanced diets and on the complex interactions arising from physiological metabolites or from cellular responses (Ebert et al., 2001).

This editorial provides practical guidance to researchers who intend to publish new studies in the field of chemoprotection (antioxidant, antimutagenic, anticarcinogenic, etc. effects) in the future. This is an important issue, since experience shows that many papers have been rejected or refused on the basis of shortcomings in the experimental design. We have therefore defined a set of essential characteristics of studies to be reported in this journal. The basic requirements discussed are default requirements rather than non-negotiables. As in toxicology, deviations from study designs as outlined in the guidelines are possible and useful, provided they can be justified on scientific grounds. A survey of these basic requirements is provided in Table 1, and is described in detail below.

1. Hypothesis-driven study design

A priori, a hypothesis should be given including primary and secondary variables. This is a severe problem, as very often scientists just measure a lot of things and set up vague hypotheses, but do not define primary and secondary variables. This is now necessary in clinical trial. Hence, studies should be designed according to a hypothesis rather than be based on the mere availability of test methods and/or test substances. As a consequence, the results should be explained by making reference to a presumed mechanism of action by additional experimental work aimed at explaining the molecular mode of action. The outcome should be properly discussed with reference to the scientific literature on both the model and the test compound. Routine studies on the antioxidant, antimutagenic or anticarcinogenic potential of test substances in *in vitro* experiments and animal studies *in vivo* reported solely for the purpose of data generation are not acceptable.

However, studies that yield results which are not in agreement with a specific hypothesis may also deserve publication. Studies that do not confirm an existing paradigm could yet be submitted for publication in order to publish important scientific information which may lead to the revision of existing hypotheses and would otherwise be lost. As such, these studies may be

Table 1
Survey of 10 basic requirements for a scientific paper reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances

No.	Area	Requirement	Notes	Applicable for in vitro	Applicable for in vivo
1	Hypothesis-driven	A plausible mechanism and supportive hypothesis	No testing because of mere availability	✓	✓
2	Nature of the test substance	Standardise by analytical technical means or by biological effects	Mere literature data are insufficient. In case the manufacturer provides analytical data or specifications these can be used.	✓	✓
3	Valid and invalid test systems	Only use valid test systems; intelligent use of appropriate controls	Reluctance in the use of outdated test systems	✓	✓
4	Selection of dose levels and gender	Minimum three dose levels, adequately spaced, based on knowledge of toxic dose range; two genders only if gender effects expected	Dose-response data needed; Reduce no. of animals	✓	✓
5	Reversal of the effects induced by oxidants, carcinogens and mutagens	One dose level acceptable	Dose level may be substantiated from literature data	✓	✓
6	Route of administration	In line with anticipated human route of exposure (but depending on hypothesis)	Adverse effect inducer can be via another route	–	✓
7	Number and validity of test variables	Substantial amount of data appropriate to the hypothesis tested	Single parameter studies acceptable for QSAR-studies	✓	✓
8	Repeatability and reproducibility	In vitro studies need data from at least three independent replicates; in vitro results need to be reproducible	In vivo studies need not be repeated but use a sufficient number of animals to achieve statistical relevance	✓	✓
9	Statistics	Adequate statistics pre-study and post-study	ANOVA for multiple comparisons; parametric tests whenever possible	✓	✓
10	Quality assurance	GLP and ISO 17025 not required but an advantage	Essentials of GLP and ISO 17025 needed	✓	✓

✓ yes, needs to be considered for a paper reporting antioxidant, antimutagenic or anticarcinogenic potential of a test substance.

– Not applicable.

hypothesis-generating rather than hypothesis-based. In particular, the rapidly evolving area of the “omics” (genomics, proteomics, transcriptomics and metabolomics) is considered to be hypothesis-generating rather than hypothesis-solving. These new high-throughput technologies provide a holistic view on processes at the level of gene expression, protein expression and cell/organism physiology (Williams, 1999; Daniel, 2002). It is envisaged that in toxicology and functional food research, application of these tools will provide new insights into mechanisms, efficacy and safety and will result in improved testing protocols (Guengerich, 2001; Verrips et al., 2001; Simmons and Portier, 2002).

2. The nature of the test substance

The test substance can be either a single chemical or an extract. For a single chemical, the source and its purity need to be known, viz. the nature of impurities needs to be identified. As a general rule, at least 99% of a test substance needs to be known. In case the manufacturer provides analytical data or specifications these can be used. A typical example for a problematic compound whose impurities might lead to inadequate results is chlorophyllin, which has been used in numerous chemoprotection studies. Dashwood (1997) has recently emphasised that the purity of commercially available chlorophyllin is very poor and that no firm conclusions can be drawn from experiments with such preparations.

For an extract it is necessary to standardise it, either by chemical analysis of a least one typical constituent (in particular the proposed active components) and/or by a characteristic biological effect. For example the bacteriocidal activities of *Allium* extracts might serve as a means of standardisation. These experiments should be integrated in the work described in the article and not by mere referring to literature data. This is essential, as it should enable other researchers to reproduce the data. Also, extraction procedures need to be described in sufficient detail to be reproduced by others or referred to as a literature reference giving similar detail. Hence it is not acceptable to state that, for example, “an extract was prepared from a plant bought on a local market or picked in the woods and identified by Professor X”.

3. Valid and invalid test systems

A study should not be based merely on a design or set of variables that have been used by others in the past. As an extension of this, many variables have become outdated and should be omitted from new studies. This applies, among others, to the measurement of lipid peroxidation in vivo using TBARS (Griffiths et al.,

2002). For antioxidant studies in vitro, several methods are described in the literature to evaluate the efficacy of food and biological antioxidants (Frenkel and Meyer, 2000; Aruoma, 1996, in press; Griffiths et al., 2002). Where possible, use of two or more of the methods is encouraged. The characteristics of the test compounds, substrates and expected outcome and potential application of the data need to be clearly defined.

During the last decades numerous antimutagenicity studies with bacterial and mammalian indicator cells have been performed. Experiments in vitro are of value for the understanding of in vivo mechanisms (Glei et al., 2002). However, these experimental models do not usually reflect the complex metabolism of genotoxins and carcinogens in mammals and man, and fail to detect important protective mechanisms such as induction of detoxifying enzymes. Also, false positive results may be obtained when putative antimutagens interact with activating enzymes in the exogenous enzyme mix that is added in experiments with promutagens (Schwab et al., 2000; Knasmüller et al., 2001). Putative protective compounds that cause pronounced shifts of the molarity and/or of the pH value of the enzyme mix will inactivate it and therefore mimic antimutagenic effects. However, simple binding mechanisms (e.g. the inactivation of amines and/or PAHs by fibres, pigments and lactobacilli) can be detected with in vitro experiments, and it has been shown that these dietary components are also active under in vivo conditions in laboratory animals and humans (Wolowski et al., 1999; Schwab et al., 2000). In other words, the question of whether the results of in vitro experiments can be extrapolated to laboratory animals and humans depends on their mode of action. Therefore, it is of crucial importance to include additional experiments to in vitro antimutagenicity studies aimed at identifying the mechanisms involved, and to critically consider whether such mechanisms are also operative under in vivo conditions.

Wherever possible, a positive control substance with established efficacy should be incorporated. The latter will be mandatory for in vitro tests. This is consistent with established practice in short-term genotoxicity tests. In antioxidant in vitro experiments, this is frequently done by comparing activities with vitamin C, vitamin E or Trolox. When comparing activities, this should be done on a molar basis or, if that is not feasible, on a (dry) weight basis. Antioxidant activity in vitro is particularly dependent on the test system applied, and many systems have no relevance to any biological system (e.g. food, blood, tissue). The rationale for the use of such systems must be presented and the limitations of the interpretation discussed thoroughly. A general statement like ‘We conclude therefore that antioxidant A is more potent than antioxidant B’ is therefore not acceptable since it adds only to the confusion regarding biological relevance in this field.

An intelligent inclusion of the appropriate controls, needed for example for evaluating combination experiments, is a requirement that should go beyond regulatory procedures. Concomitant assessment of cytotoxicity, including the data points of the combination treatment groups, is required.

4. The selection of dose levels and gender

In toxicology, dose is a very basic concept. It was Paracelsus (1493–1541), the godfather of toxicology, who said “All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy.” From this beginning, dose–response relationships have become essential in toxicology. In general, at least three doses are tested in which the highest dose gives a clear response, the lowest is ineffective, and the intermediate dose levels give intermediate responses. This knowledge should also be incorporated in studies, to establish beneficial effects. As such, one dose level is usually insufficient to reach a conclusion, and there needs to be a range of doses tested, especially where one is examining the potential benefits of plant extracts. The dose levels should be adequately spaced and the spacing should depend on the study objectives. It is recommended to use a factor of about 3 between doses to look for linearity. A spacing of 5-fold or even 10-fold is appropriate if one wants to span the range from the highest tolerated level to a no-effect level.

The choice of the highest dose levels could be based on knowledge of the relative toxicity of the test substance (e.g. IC_{50}) under those experimental conditions which are used to define a beneficial effect, thus allowing an adequate comparison of beneficial and toxic properties.

Since many articles are aimed at identifying dietary constituents that are protective in humans, it is important that the concentrations used in the experimental trials reflect realistic human exposure levels. In *in vivo* experiments, the doses administered to laboratory animals should not be several orders of magnitude higher than those consumed by humans in the diet. A typical example are isothiocyanates, which have been used in animal experiments in concentrations which are substantially higher than the exposure levels that can be expected in humans after consumption of cruciferous vegetables. This contrasts with the basic toxicology in which doses are taken as high as possible (hazard identification) and subsequently doseresponse data are generated (hazard characterisation) in order to achieve the assessment of essentially safe doses for human exposure (risk assessment) (Smith, 2002). It is also important to note that dietary mixtures that possess antimutagenic and/or anticarcinogenic properties may cause other (adverse) health effects. A typical example would be

coffee constituents, which protect against chemical induction of DNA damage and cancer in animals but may cause hypercholesteraemia in humans. In such cases attempts have to be made to define the dose range in which beneficial effects can be expected (Cavin et al., 2002).

In line with toxicological studies *in vitro* and *in vivo*, it is desirable, though not required, that the actual dose levels be confirmed by chemical analysis of the animal feed, drinking water or dosing solutions or of the culture media for *in vitro* studies.

For studies with experimental animals, unless there are data to indicate the contrary (e.g. in the case of compounds causing hormonal effects), it is assumed that males and females respond similarly. Hence, testing *in vivo* should be done in one gender only. This will concomitantly reduce the number of animals used for testing.

5. Reversal of the effects induced by oxidants, carcinogens and mutagens

The design most often used is administration of a known compound that will induce the desired toxic effect, i.e. oxidative stress, mutagenic effect or a carcinogenic effect, and to examine the effect on the toxic response by administration of a test compound. A reduction in the toxic response from pre-, co- or post-administration of the test compound will be regarded as an indicator of a potential beneficial effect. The compound that induces the desired toxic effect is often well known and only two dose levels need to be used in such a case, the one level being zero. The requirements for using only one dose level above zero are: (1) the dose level is substantiated by studies by the authors or data in the literature; (2) there is concomitant testing of a negative control usually the vehicle minus the toxin; (3) the toxic effect is demonstrated by measurement. An alternative to the design with induced toxic reaction is to use the spontaneous occurrence of oxidative stress, mutagenic events or carcinogenic events. Such a design is demanding on time and resources, and may present problems of statistical strength, but is of course acceptable.

6. Route of administration

This paragraph applies to *in vivo* studies only. Test substances investigated for possible beneficial effects will ultimately serve for the development of recommendations for human exposure. Therefore, the anticipated route of administration will be the oral one, in particular instances perhaps dermal. This pre-empts the use of other routes of administration in experimental studies, such as intravenous or intraperitoneal, unless there is a particular hypothesis to do so which then provides

for the inherent scientific justification. Administration of a positive control substance or an unstable metabolite of the test compound via non-parenteral routes is acceptable though. Also, in studies of bioavailability and kinetics of the test compound and other specialised studies, the use of non-parenteral routes of administration can be justified.

7. Number and validity of test variables

An experiment should be based on a substantial amount of data appropriate to the hypothesis tested. It should be noted that this requirement does not aim at adding extra data for the sole purpose of data generation. It is difficult to set general guidance for this for both *in vitro* and *in vivo* studies. In line with test guidelines for toxicity testing, multiple variables are generally necessary (e.g. for *in vitro* multiple strains, multiple enzyme activities, multiple treatment schedules/exposure duration; for *in vivo* histology and clinical variables).

The subdivision of study data over many individual papers (“salami publishing”) is not appreciated, since it distorts the impact of the study and obscures the overall message. As a result, when a study is reported, additional data on the same study may be requested. It is conceivable though that occasionally a single study may yield data on two (or more) different issues, each of which could include adequate information to warrant the publication of two separate papers since the topics could be very much apart. For example: some indole derivatives, formed by metabolic breakdown of certain glucosinolates found in cruciferous vegetables, have been shown to bring about beneficial effects that are probably unrelated to one another. (1) They act as antioxidants, thus affording a potential anticarcinogenic effect through a preventive action *in vivo* (Ge et al., 2000); (2) They have been shown to induce apoptosis in cultured human cancer cells, which indicates a possible therapeutic effect (Ge et al., 1996, 1999). In the case of such studies, which are relatively rare, publication of more than one paper is justified.

Alternatively, the use of a single parameter could be justified for comparing a large series of test substances for efficacy such as in (quantitative) structure–activity relationship experiments. The number of test substances should then be substantial, but the actual number depends on the class of compounds and no firm number can be given.

8. Repeatability and reproducibility

In *in vitro* experiments at least three replicates should be used, preferentially using a model system that is well

validated under strict quality control in the laboratory, and provides a sufficient amount of historical control data. The reason for that is that a statistical evaluation of the data will necessitate results from at least three independent replicates if the effects, e.g. of anti-genotoxicity, are large. For smaller effects, more replicates will be needed. Thereupon, the results obtained in an *in vitro* experiment should be reproducible, either in the same test system or in additional *in vitro* test systems.

For *in vivo* experiments a sufficient number of test animals per dose level should be used in order to reach sufficient statistical power.

Finally (but beyond the scope of this paper), evidence for protective effects seen in bacterial assays and with human cells could be further supported by additional *in vivo* animal experiments. Thus, upon showing reproducibility, it may be equally important to reproduce a protective effect in another model with a different endpoint. Also, in many cases, ethical permission can be obtained to demonstrating protective properties of food components in humans by performing dietary intervention studies. In this case the utilisation of biomarkers measuring the same variables as those of the *in vitro* studies (preferentially using the same types of cells, e.g. peripheral lymphocytes) will be the most superior way of evaluating specific effects of particular dietary ingredients (Pool-Zobel et al., 1997, 1998; Gleis et al., 2002; Griffiths et al., 2002).

9. Statistics

Any study should be appropriately analysed with statistical methods both in the planning phase before doing the experiments as well as after having obtained the results.

In the design phase a study should have a primary variable well-defined pre-trial. Statistical planning should also include power analysis by an observed, estimated or guessed variability of that primary variable. Only one or two primary variables should be stated. For animal experiments randomisation is essential.

In the analysis phase, when multiple comparisons are made analysis of (co)variance should precede the assessment of statistical significance. Unless there are reasons to do otherwise, statistical tests should be parametric by nature and the prerequisites for parametric testing, e.g. homogeneity of variances and normal distribution, should be adequately tested and reported. The level of significance chosen (e.g. 0.05, 0.01 etc.) should be in line with predefined expectations, viz. it is not acceptable to set a level of significance at 5% for each of 20 different variables, in which case by chance one variable will be expected to show statistically significant differences, while in fact it is without biological relevance. If so, additional data should substantiate the relevance of the finding.

In the reporting phase, data should be reported within the limits of statistical significance, viz. when the coefficient of variation of analytical data is in the order of several%, which is nearly always the case, it is only possible to supply two significant digits (e.g. 43 ± 2 , 6.3 ± 0.7). Data should preferably be reported as means (or median where appropriate) and 95% confidence limits in tables. Graphs should be drawn using means with standard deviations not standard errors (or range).

10. Quality assurance

In regulatory toxicity testing, Good Laboratory Practice (GLP) is a *conditio sine qua non*. For scientific toxicity studies this is not the case. In practice very few basic research groups will have GLP status. It is regarded as an advantage to have a study done under GLP and that should be indicated if it is the case. However, it shall not be a prerequisite for the acceptance of manuscripts. Moreover, it is noted that GLP is an administrative procedure unlike a guarantee for scientific adequacy. The same applies to quality control, a system indicating the technical adequacy of analytical measurements. Appropriate validation of analytical methodology is assumed and should be indicated, but an official ISO 17025 statement will not be required. However, we would like to refer to some papers dealing with analytical validation in the area of bioavailability and kinetics (Shah et al., 1992; Bressolle et al., 1996) and to websites for chemical societies and (inter)national committees for analytical validation (see e.g. www.aoac.org; www.nmkl.org; www.ich.org).

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