Ann. Ist. Super. Sanità
Vol. 25, N. 4 (1989), pp. 605-610

REGULATORY ASPECTS OF CHEMICAL MUTAGENESIS IN ITALY AND IN THE EUROPEAN COMMUNITY

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Summary. *The first part of this paper outlines the guidelines on mutagenicity testing recommended in Italy by the National Advisory Committee on Toxicology. Thereafter, there is a description of the current Italian situation regarding the mutagenicity testing requirements for new chemicals of different use, which also takes into account the European Community approach and its legal implements (directives and recommendations).*

Riassunto (Aspetti regolativi della mutagenesi chimica in Italia e nella Comunità Europea). - *La prima parte di questo lavoro riassume le linee guida della mutagenesi raccomandate dalla Commissione Consultiva Tossicologica Nazionale (CCTN). Dopo di ciò, viene riassunta la situazione italiana riguardante le richieste di test di mutagenesi per nuove sostanze chimiche di usi diversi, tenendo conto anche dell' approccio della Comunità Europea, con i suoi strumenti legislativi (direttive e raccomandazioni).*

Introduction

In the field of genetic toxicology, Italy has been one of the most active countries both in the formulation of guidelines on mutagenicity testing of new chemicals and in legal actions aimed at reducing or even banning genotoxic agents present in the market. A booklet containing the guidelines for carcinogenesis, mutagenesis and teratogenesis was produced in 1977 [1] by a Committee of experts for carcinogenesis, mutagenesis and teratogenesis (CMT) appointed by the Italian Ministry of Health. Since 1985, CMT has been integrated in the wider National Advisory Committee on Toxicology (Commissione Consultiva Tossicologica Nazionale, CCTN). At the end of 1987, CCTN produced a new booklet containing updated guidelines on carcinogenesis, mutagenesis and teratogenesis [2]. The main aspects of the guidelines on mutagenicity testing, recommended by CCTN, are outlined in the next paragraph. Concerning the legislation on old chemicals, a particularly interesting item is the Italian Decree of 18 June, 1976 (followed by a second decree on 7 March, 1979), banning the use of ten components of permanent hair dyes, namely 2,4-diaminoanisole, 4-nitro-o-phenylendiamine, 2-nitro-o-phenylendiamine, 2,5-diaminoanisole, 2-amino-5-nitrophenol, m-phenylendiamine, o-phenylendiamine, 2-amino-4-nitrophenol, 2,5-diaminotoluene and 2,4-diaminotoluene.

Regarding to the European Community, for the past twenty five years legal implements have been developed to ensure an adequate safety level with respect to chemical agents. The Commission, in cooperation with other international organizations (e.g. WHO, OECD) has examined the toxicological test methods available and has contributed to their validation. More recently, reference to methods for genotoxicity testing has been introduced in community directives (e.g., new chemicals and new pharmaceuticals); the possibility of classifying and labelling mutagenic substances also exists now. In particular, the philosophy and approach for mutagenicity testing of new chemicals under the 6th amendment will be critically considered.

Guidelines for mutagenesis recommended by the National Advisory Committee on Toxicology (Commissione Consultiva Tossicologica Nazionale, CCTN)

According to CCTN, mutagenicity and other closely related short-term tests are considered important, notwithstanding obvious limitations and exceptions, for the contribution they give to:

a) identification and evaluation of chemicals which are potentially capable of inducing transmissible genetic damage in offspring as a consequence of their effects at the germ cell level;

b) identification and evaluation of chemicals potentially capable of inducing malignancies as a consequence of their genetic effects at the somatic cell level;

c) quantitative assessment of genetic risk.

This last item is the most problematic, as is also the estimation of the proportion of genetic diseases in man related to specific exposures; nevertheless, these problems cannot be ignored.

Concerning a strategy for mutagenicity testing, CCTN is aware that, notwithstanding the tremendous amount of experimental data now available, a simplyfied, rational and universally acceptable strategy for the definition of test batteries is not yet available. In this context however, CCTN recognizes that the minimal test battery and criteria defined in the framework of the European community directives on classification, packaging and labelling of dangerous substances EEC (Directives 79/831 and 84/449) [3, 4] as well as those recommended by the Organization for Economic Cooperation and Development (OECD) [5] are substantially valid. The recent adoption of new approaches such as the computerized systems CPBS (carcinogenicity prediction and battery selection) and CASE (computer automated structure evaluation) developed by Rosenkranz *et al.* [6-8] or the sophisticated, non-traditional statistical methods used by Benigni and Giuliani [9, 10] offer the possibility for a more rational use of the current data bases and for a more clear picture concerning the real performances of the individual test systems. It is likely that in the near future it will be possible to formulate test batteries on scientific grounds which are more rational and precise than now. Meanwhile, according to CCTN, a clear distinction can be made at present only between *in vitro* and *in vivo* assays, the former being more sensitive and less specific than the latter. On this basis, in the case of chemicals for which limited human exposure is expected, in general, CCTN considers two *in vitro* assays to be sufficient: one at the gene level (e.g., a bacterial test system such as the Ames test) and one at the chromosomal level in mammalian cell cultures. On the other hand, in the case of chemicals for which significant human exposure is foreseen, CCTN recommends the following battery of four *in vitro* test systems:

a) one bacterial assay for gene mutations;

b) one eukaryotic assay for gene mutations;

c) one cytogenetic assay for chromosomal effects;

d) one assay for DNA damage/repair (e.g., UDS or SCE).

In case of clear-cut negative results, a part from exceptional cases, further testing *in vitro* and/or *in vivo* is considered unnecessary. On the other hand, in case of clear-cut positive results of the *in vitro* phase, testing is to be continued with *in vivo* assays at somatic and/or germ cell levels; the extent of the *in vivo* phase (number and types of assays) will depend on the type of results of the *in vitro* phase as well as on other relevant information (e.g., metabolic and pharmacokinetic data, evidence of interactions with germ cells, etc.).

For a quantitative assessment of heritable effects, CCTN refers to *in vivo* assays such as the specific locus mutation and the heritable translocations in mice. However, since these assays are complex, costly and require the use of large numbers of animals, their utilization should be strongly justified.

Concerning the type of assays to be conducted, CCTN, in general, refers to those recommended by OECD and adopted by EC directives; however, CCTN is aware of the limitations of the *in vivo* assays (e.g., target specificity; lack of adequate assays for gene mutations), as well as of the lack of validated assays for genomic mutations.

Furthermore, according to CCTN, mutagenicity testing should be carried out in compliance with so-called "good laboratory practices" (GLP). As far as the interpretation of concerned test results, CCTN emphasizes the following main aspects:

a) an overall estimation is needed in each case and not simply a consideration of the ratio between "positive" and "negative" results; such an evaluation must consider the nature (strength and quality) of each result;

b) generally, results of *in vivo* assays deserve more attention than those obtained *in vitro*; however, this is not necessarily so when the latter but not the former, are positive. In such cases, one should try to clarify the reasons for discrepancy;

c) clear-cut positive results even in only one single type of *in vivo* test system, either at the somatic or germ cell level, should be generally regarded as suggestive of potential mutagenicity in man;

d) *in vitro* studies should be generally regarded as "positive" only when a dose-response is demonstrated, whereas *in vivo* studies may be regarded as "positive" even when carried out at one dose level only;

e) the interpretation and evaluation of results should be done by experts who must also take into consideration other relevant information (e.g., toxicology, metabolism, pharmacokinetics).

Concerning the classification of mutagens, CCTN is aware that genotoxic agents should be regarded as potentially harmful both from a genetic and carcinogenic point of view, and that a sharp boundary between these two types of risk cannot be easily defined. On this basis, CCTN prefers to avoid an absolute distinction between them and therefore, for simplicity and practical reasons, has proposed a general classification of mutagens in five categories, referring to both heritable genetic damage and potential carcinogenicity, and without a specific orientation to labelling purposes under the 6th amendment [2].

Category 1. Compounds for which there is sufficient evidence of heritable genetic damage in man on the basis of epidemiological studies.

Category 2. a) Compounds shown positive in at least one *in vivo* assay at the germ cell level; *b)* compounds shown positive in at least one *in vivo* assay at the somatic cell level.

Category 3. Compounds shown positive in at least two *in vitro* assays and negative *in vivo*.

Category 4. Compounds shown positive in at least one *in vitro* assay and negative *in vivo*.

Category 5. Compounds shown negative after adequate testing.

Currently, CCTN is in the process of proposing a second classification scheme of mutagens in order to give guidance also for labelling purposes under the 6th amendment. In the new proposal, still under discussion, categoty 2 should include: a) *in vivo* somatic mutagens (in the absence of interaction with germ cells); b) clear-cut *in vitro* mutagens, for which *in vivo* studies are either lacking, incomplete or inadequate.

Pharmaceuticals

Two decrees from the Italian Ministry of Health (DM 28th July, 1977 and DM 25th August 1977) gave the Istituto Superiore di Sanità the responsibility of evaluating chemical, toxicological (including mutagenesis) and pharmacological data in view of the pilot clinical trial in man with new pharmaceuticals. According to them, the following three types of assays were required:

a) a bacterial test for gene mutations *in vitro* and *in vivo*;

b) a eukaryotic test for gene mutations *in vitro* and *in vivo*;

c) a DNA-damage repair test (e.g.: UDS).

Italy is now changing the legislation on new pharmaceuticals according to the following scheme:

A) Before the pilot clinical trial

Two *in vitro* assays with and without metabolic activation:

a) a bacterial assay for gene mutations;

b) an assay for chromosomal aberrations in mammalian cells.

B) Before the enlarged clinical trial

In addition to the mutagenicity tests required before the pilot clinical trial the following two types of assays are required:

c) an *in vitro* eukaryotic test for gene mutations;

d) an *in vivo* assay (e.g. micronucleus or metaphase analysis) or an *in vitro* DNA damage/repair (e.g.: UDS or SCE).

This last proposal is very similar to the recommendations made by the European Council in 1984 [11] concerning the mutagenicity tests required for new pharmaceuticals before marketing. According to them, the following four types of assays should be carried out:

a) gene mutations in bacteria;

b) chromosomal aberrations in mammalian cells *in vitro*;

c) gene mutations in a eukaryotic system;

d) an *in vivo* test for genetic damage (the most validated are those at the chromosome level; sufficiently validated is the "mouse spot test").

Pesticides

The Italian DPR from 3rd August, 1968, n. 1255 "Regolamento concernente la disciplina della produzione, del commercio e della vendita dei fitofarmaci e dei presidi delle derrate alimentari immagazzinate" foresees, without giving details, mutagenicity studies before the registration of a new pesticide. Since May 1979, the Public Health Ministry, even in the absence of a new specific law, decided to adopt, for the registration of new pesticides for agricultural use, the guidelines recommended in 1977 by CMT: i.e., two test systems for gene mutations (one in bacteria and one in eukaryotic cells), two tests for chromosomal effects (one *in vitro* and one *in vivo*) and one test for DNA damage-repair.

An analogous approach is being followed in the case of pesticides of domestic, veterinary and civil use.

Since 1988, the mutagenicity testing approach for new pesticides that has been followed is the one recommended by CCTN in the updated guidelines published in 1987 [2] and which is based on a battery of four *in vitro* assays.

Concerning the EC approach, one has to mention that in 1984 the Council of Europe [12] recommended five types of tests: two at the gene level (one in prokaryotic and one in eukaryotic cells), two at chromosomal level (one *in vitro* and one *in vivo*) and one DNA damage-repair test.

New chemicals

With a Presidential Decree (DPR n. 927, 24 November, 1981) Italy drafted the Council Directive 79-831-EC, amending for the sixth time the EC Directive 67/548 concerning the classification, packaging and labelling of dangerous substances.

This directive envisages a tiered scheme of tests for evaluating risks from new chemicals in accordance with the entity of the foreseeable exposure. In this way, test entity and nature is flexible, and not all new chemicals must necessarily undergo the entire battery of tests.

In particular, if a chemical is placed on the market at less than 1 ton per year, the mini-notification required does not include mutagenicity testing. However, also in this case it would be advisable to require at least an *in vitro* assay. This should be a bacterial one (e.g. the Ames test), because of its simplicity, rapidity and sensitivity to a large number of carcinogens. When a substance reaches the so-called level 0, i.e., it is put on the market at more than 1 ton per year but less than 10 tons per year, a "base set" of two tests is required: one for gene mutations in a bacterial system (*S. typhimurium* or *E. coli*) and one for chromosomal aberrations (*in vitro* mammalian cytogenetic test or an *in vivo* assay such as the micronucleus test or the metaphase analysis). The experimental protocols of the five methodologies for the base set are described in the Commission Directive 84-449. For level 0 compounds, for which the present EEC guidelines require two types of assays, it would be highly advisable that, parallel to the gene mutation bacterial assay, an *in vitro* chromosomal assay (instead of an *in vivo* one), be carried out. This because it would save animals, and would have a higher probability of detecting the genotoxic potential of the chemical under test. As a matter of fact, it is well-known that *in vitro* assays generally have a higher sensibility in comparison with the *in vivo* ones, and that most of the compounds which resulted positive *in vivo* are also positive *in vitro*, while the reverse is not true [13]. When a substance reaches level 1, i.e., it is put on the market at 10 tons per year or at a total of 50 tons, the directive requires two additional mutagenicity tests; however, this requirement is optional and becomes obligatory in the case of 100 tons per year or for a total of 500 tons. In general, the two assays which complement

dossier assays (level 0) are chosen in such a way that the final package is composed of four types of tests: two gene mutation *in vitro* assays (one bacterial and one eukaryotic), and two chromosomal assays (one *in vitro* and one *in vivo*).

In any case, at least two other tests are required whenever a positive result has been obtained, even if only in one of the two tests of the Base Set. At this level, a long-term carcinogenicity study is required only in case there are positive results in at least one of two additional assays. When a substance reaches a value of 1,000 tons per year or a total amount of 5,000 tons, a long-term carcinogenicity bioassay is obligatory.

Table 1 shows the fifteen types of mutagenicity test systems adopted by the European Community and for which experimental protocols have been defined. One may note the lack of assays specific for genomic mutations, cell transformation and tumor promotion as well as the limitations of the *in vivo* assays both of germ and somatic cells levels.

According to the 6th amendment, a manufacturer or importer of a new substance has to propose a classification and label based on the results of the experimental studies and according to the categories of danger reported in Annex VI. The procedure for establishing the classification of carcinogens, mutagens and teratogens is set out in the 5th adaptation of the Directive (83/467/EEC of 29.7.83), recently drafted also by the Italian legislation. One has to mention that Annex I of the 6th amendment foresees that even substances already on the market have to be classified according to the same categories.

As shown in Table 2, mutagenic substances are classified into three categories for which specific risk phrases (R46 for categories 1 and 2, and R40 for category 3) and symbols are applied. One may note that while categories 1 and 2 seem to exclusively focus on inherited genetic damage, it cannot be avoided that the mutagenicity data concern the carcinogenic hazard as well, and this applies particularly to category 3. Indeed, for this category, one has to apply the same risk phrase (R40: possible risk of irreversible effects) as for category 3 of carcinogens. After several years of applying of the 6th amendment, the most controversial points are those related to the criteria to be followed for further testing and classification of substances presenting one or more positive findings.

In view of its great relevance, both from sanitary and economical perspectives, efforts should be made to harmonize the different views regarding this problem.

Table 1. - *Organization for economic cooperation and development (OECD). Guidelines on genetic toxicology testing, 1986. General classification of tests*

I) Assays for gene mutations
1. *S. typhimurium* reverse mutation assay (GEN. 471)
2. *E. coli* reverse mutation assay (GEN. 472)
3. Gene mutation in mammalian cells in culture (GEN. 476)
4. Drosophila sex-linked recessive lethal test (GEN. 477)
5. Gene mutation in *S. cerevisiae* (GEN. 2.84)
6. Mouse spot test (GEN. 85.6)

II) Assays for chromosomal mutations
7. *In vitro* cytogenetic assay (GEN. 473)
8. *In vivo* cytogenetic assay (GEN. 475)
9. Micronucleus test (GEN. 474)
10. Dominant lethal test (GEN. 478)
11. Heritable translocation assay (GEN. 7.85)
12. Mammalian germ cell cytogenetic assay (GEN. 5.85)

III) Assays for DNA effects
13. DNA damage and repair: UDS *in vitro* (GEN. 4.85)
14. Mitotic recombination in *S. cerevisiae* (GEN. 3.84)
15. *In vitro* sister chromatid assay (GEN. 1.84)

Table 2. - *Adaptation of the Directive 67/548/EEC (83/467/EEC). Criteria for classification and labelling of carcinogens, mutagens and teratogens*

Mutagens

Category 1. Substances known to be mutagenic for man. There is sufficient evidence to establish a causal association between human exposure to a substance and heritable genetic damage.

Category 2. Substances which should be regarded as if they were mutagenic to man. There is sufficient evidence to provide a strong presumption that human exposure to a substance may result in the development of heritable genetic damage, generally on the basis of: - appropriate animals studies; - other relevant information.

Category 3. Substances which cause concern for man owing to possible mutagenic effects, but in respect of which the available evidence does not satisfactorily demonstrate heritable genetic damage. There is evidence from appropriate mutagenicity studies, but this is insufficient for placing a substance in category 2.

Specific risk phrases and symbols

Categories 1 and 2: can induce heritable genetic effects (R46)
Category 1: toxic
Category 2: harmful
Category 3: possible irreversible effects (R40).
Category 3: harmful

Single cell proteins (feed additives)

Italy has drafted the EC Council Directive 82/471 [14] which requires ".... studies on the potential mutagenicity due to contaminants (e.g.: mycotoxins and bacteria) or residues of the product (substrates, media, solvents) by using *in vitro* tests with and without metabolic activation". No further details are given.

Feed additives

Italy has drafted the EC Council Directive 87/153 [15] according to which four types of tests are requested, namely: two assays for gene mutations (one in a bacterial system and one in a eukaryotic system) and two assays for chromosomal effects (one *in vitro* and one *in vivo*).

Food additives and colourings

In general, Italy follows the recommendations of the EC Scientific Committee for Human Food [16]. According to them, the following battery of four assays should be used:

a) a test for gene mutations in bacteria;

b) an *in vitro* test for chromosome aberrations in mammalian cell cultures or in human lymphocyte cultures;

c) a test for gene mutations in a eukaryotic system;

d) an *in vivo* test for chromosome aberrations.

If required by the circumstances, additional tests might be required.

Cosmetics

In general, Italy follows the recommendations made at the European Community level [17, 18] according to which the mutagenicity testing should include assays at the gene and chromosome level as well as *in vitro* and *in vivo* assays.

Food packaging materials

A document from European Council [19] recommends mutagenicity testing without further details.

In the absence of more precise indications, the Istituto Superiore di Sanità usually recommends two types of assays: one at gene level (e.g. Ames test) and one at chromosomal level (preferentially *in vitro*).

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