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Alternative strategies for carcinogenicity assessment: an efficient and simplified approach based on *in vitro* mutagenicity and cell transformation assays

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The need for tools able to predict chemical carcinogens in less time and at a lower cost in terms of animal lives and money is still a research priority, even after several decades of effort in that direction. Now, new regulatory requirements (e.g. the Registration, Evaluation, Authorisation and Restriction of Chemical substances recently implemented in Europe) have even increased the pressure to develop new tools in this field. Drawbacks of the present testing strategies have come to light again recently especially in view of new requirements in worldwide regulations. Among these are (i) the lack of assays able to identify non-genotoxic carcinogens, (ii) the exaggerated rate of misleading (false) positive results of the *in vitro* mammalian cell-based short-term mutagenicity tests and (iii) the extremely low sensitivity of *in vivo* short-term mutagenicity tests. Within this perspective, we analyse the contribution of cell transformation assays (CTAs), and we show that they are a valid complement to tools able to detect DNA-reactive carcinogens. We also show that a tiered strategy, with inexpensive and fast tests in Tier 1 (e.g. the Ames test or structural alerts) and the Syrian hamster embryo CTA in Tier 2, is able to identify up to 90% of carcinogens.

Introduction

The need for tools able to predict chemical carcinogens in less time and at a lower cost in terms of animal lives and money remains a research priority. Historically, the convergence between the basic genetic research on chemically induced mutagenesis, and the Millers' work on the electrophilic, DNA-reactive chemical carcinogens, has stimulated the scientific community to concentrate on mutation-based short-term tests (STTs) over other possible approaches (1,2). Since no single method alone is able to detect all possible genotoxic events, a wide array of test systems have been developed and adopted internationally in regulatory schemes.

These regulatory schemes and strategies vary depending on the types of chemicals and their intended uses (e.g. industrial chemicals, pharmaceutical drugs, food additives or constituents); they also vary from one regulatory authority to another. However, a dominant trend can be recognized: most often a two-tiered integrated testing approach is used.

The first tier (Tier 1) includes *in vitro* assays. In this tier, bacterial mutation assays (such as the Ames test) are used first, followed by *in vitro* tests based on mammalian cells (detecting gene mutations or chromosomal aberrations). The second tier (Tier 2) involves the use of short-term *in vivo* studies (usually a bone-marrow cytogenetics assay) to assess whether any potential for mutagenicity detected at the Tier 1 *in vitro* stage is actually expressed in the whole animal. Thus, negative results *in vitro* are usually considered sufficient to indicate lack of mutagenicity, whereas a positive result is not considered sufficient to indicate that the chemical represents a mutagenic hazard (i.e. it could be a misleading positive). The above approach to mutagenicity testing has a fundamental theoretical unity, and has been recommended internationally as part of the strategy for predicting and quantifying mutagenic and carcinogenic hazard. It should also be emphasized that the various national and international agencies may recommend different implementations according to the type of chemicals, intended use, production levels, etc. (3–8) (see also the Technical Guidance Documents of the European Chemicals Agency: http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_r7a_en.pdf?vers=20_08_08).

Weaknesses of the present testing strategies have already been noted and recently these problems have been re-emphasized because of the new requirements of regulations worldwide. Among these are (i) the lack of assays able to identify non-genotoxic carcinogens, (ii) the exaggerated rate of misleading positive results of the *in vitro* mammalian cell STTs and (iii) the extremely low sensitivity of *in vivo* mutagenicity STTs (2,9). All these difficulties have stimulated the revision and modification of presently available STTs, as well as the consideration of new assays.

A category of STTs not directly based on the concept of genetic mutation are the cell transformation assays (CTAs), which mimic some stages of *in vivo* multi-step carcinogenesis. Cell transformation has been defined as the induction of certain phenotypic alterations in cultured cells that are characteristic of tumorigenic cells (10). These phenotypic alterations can be induced by exposing mammalian cells to carcinogens. Transformed cells that have acquired the characteristics of malignant cells have the ability to induce tumours in susceptible animals (11,12). CTAs have been proposed for assessing carcinogenic potential of chemicals for many years; however, they have undergone different cycles of favour and disfavour among the scientific community and have never been consistently included in regulatory testing schemes. Recently, the Organisation for the Economic Cooperation and Development (OECD) has reconsidered CTAs and published a report that includes both experimental results and data analyses (13).

In this paper, we use the OECD compilation and we present analyses on the ability of the three main CTAs [the Syrian hamster embryo (SHE) cell, the BALB/c 3T3 [Balb] and the

C3H10T1/2 [C3H] assays) to predict chemical carcinogenicity. These results are put in a wider perspective by contrasting them with results from other STTs. Finally, we sketch the role of CTAs in a new testing strategy potentially able to eliminate the serious pitfalls of the present strategies.

Data and methods

Cell transformation systems

The present analysis focuses on the three main *in vitro* CTAs, the SHE, the Balb and the C3H assays. The SHE assay uses primary, karyotypically normal cells and is believed to detect early steps of carcinogenesis. The other two assays are based on immortalised aneuploid cell lines that measure later stages of carcinogenesis.

In vitro transformed cells exhibit morphological changes related to neoplasia. The phenomenon of morphological cell transformation involves changes in behaviour and growth control of cultured cells, such as alteration of cell morphology, disorganised patterns of colony growth and acquisition of anchorage-independent growth (14). Later on, transformed cells become able to grow in semi-solid agar (anchorage-independent growth), produce autocrine growth factors and can evolve to tumorigenicity when injected into appropriate hosts. They acquire the ability to divide indefinitely (immortalised) that is associated with other alterations, such as aneuploid karyotype and altered genetic stability. Accumulated evidence strongly supports the assumption that cellular and molecular processes involved in cell transformation *in vitro* are similar to those of *in vivo* carcinogenesis (for reviews: 14,15).

Data

The CTA results used in this analysis were retrieved from the OECD compilation (13). Many experts have participated in different OECD meetings and have critically reviewed the data before including them in the above compilation. The original experimental results were extracted from published articles and peer review articles, especially those on SHE, Balb and C3H assays (16) and on BALB/c 3T3 (17,18). Regarding SHE, the OECD compilation reports separately the results obtained with the pH 6.7 protocol (SHE-6.7), and with pH 7.0 or higher (SHE-7). We maintain this distinction in this analysis.

The present analysis considers only organic chemicals present in Table 11-1 of the OECD compilation. We did not consider chemicals for which the OECD experts found discordant results (codes D1 and D2). Results obtained only in tumour promotion experiments were not considered either. The number of available experimental results on chemicals for the various CTAs are (i) SHE-7 $n = 141$, (ii) SHE-6.7 $n = 82$, (iii) Balb $n = 129$ and (iv) C3H $n = 83$.

The OECD compilation contains also rodent carcinogenicity results for the chemicals tested with CTAs. Results from the Ames (*Salmonella typhimurium*) mutagenicity assay were retrieved from the ISSCAN v3a database. ISSCAN v3a is a chemical relational database primarily aimed at collecting chemical structures and rodent experimental carcinogenicity results; it is characterised by the high quality of the reported biological calls (19). It also contains Ames test results and is available at http://www.iss.it/ampp/dati/cont.php?id=233&lang=1&tipo=7.

Structural alerts (SAs) for genotoxic and non-genotoxic carcinogenicity were identified in the studied chemicals through the Benigni/Bossa rulebase implemented in the Expert System Toxtree 2.1.0. Toxtree (http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=TOXTREE) is an open-source software application that places chemicals into categories and predicts various kinds of toxic effect by applying various decision tree approaches. Toxtree was developed by IdeaConsult Ltd. (Sofia, Bulgaria) under the terms of an ex-European Chemicals Bureau contract (20). A detailed description of the Benigni/Bossa rulebase is given elsewhere (21).

Results and discussion

The scientific background of genotoxicity testing

The research of the Millers on the mechanisms of chemical carcinogenicity (22,23) interacted with, and was cross-fertilized to a large extent by, concomitant research on chemical mutagenicity. This led to the theory that electrophilic chemicals could be both mutagenic and carcinogenic, with the corollary that mutagenicity could be a crucial step in the carcinogenicity process. It should be emphasized that at the beginning of their research, James and Elizabeth Miller could only say that there were good theoretical reasons why mutagens should be carcinogens, but there were as yet insufficient data to support such a view in a compelling manner. The view that mutagens were carcinogens did not really happen until Heinrich Malling had shown that the S30 liver fraction can metabolically activate many carcinogens into mutagens, and Bruce Ames had used the S9 fraction systematically in a bacterial assay to create a large database of mutagens that were also carcinogens (1).

The application of the Ames test to large numbers of chemicals has shown that this assay has a high positive predictivity for the chemical carcinogens: Ames-test mutagens have a high probability of being carcinogenic, whereas a negative result has no discriminatory value (a chemical negative in the Ames test can be either a non-carcinogen or a non-genotoxic carcinogen, with the same probability) (24,25). The brilliant results obtained by Bruce Ames persuaded the scientific community that the correlation between chemical carcinogenicity and mutation was a general one, and that it was possible to increase the correlation by considering also genetic events different from those at the basis of the Ames test (i.e. base substitutions and deletions/additions). Thus, a myriad of further genotoxicity assays, based on events such as structural chromosome aberrations (breaks and rearrangements) and numerical chromosome aberrations (loss or gain of chromosomes, defined as aneuploidy), were developed. In addition, since the Ames test uses bacteria, tests complementary to the Ames test were sought by extending the experimental systems to *in vitro* mammalian cells, as well as to *in vivo* assays. The *in vivo* assays, because of their absorption, distribution, metabolism and excretion (ADME) characteristics, were considered to be a filter able to discriminate between chemicals that were positive only *in vitro*, and those whose mutagenic potential would be expressed in the whole animal as well.

The performance of the present genotoxicity testing strategy in identifying carcinogens

However, even though several of the 'additional' genotoxicity assays have gained a large popularity and have been adopted in various regulatory settings, rigorous comparisons of real experimental data have pointed to serious weaknesses in the above theoretical scheme (2,9,25–27).

First, a series of analyses of different databases showed that STTs based on different genetic endpoints and/or phylogenetic

characterisation are not complementary in terms of responses to the chemicals. For example, the chromosomal aberration assay in CHO cells responds to arrays of different chemicals in a way much more similar to that of the Ames test (from which it differs both for types of cells—mammalian versus bacterial and genetic endpoint—chromosomal damage versus gene mutation) than to that of the sister chromatid exchange (SCE) assay in CHO cells (same cells and similar endpoint) and of the mouse lymphoma mutation assay (based on mammalian cells, and sharing a partially similar genetic endpoint) (26,28,29).

Second, the National Toxicology Programme (NTP) has directly addressed the issue of the complementarity of *in vitro* STTs for predicting rodent carcinogenicity. In a well-designed comparative study, 114 chemicals—already bioassayed—were studied with four *in vitro* STTs: Ames test, chromosomal aberrations in CHO cells, SCE in CHO cells and mouse lymphoma mutation assay. The main outcome was that only the Ames test has a strong statistically significant association with the rodent bioassay. The chromosomal aberration test in CHO cells has a weaker correlation (and it is not complementary to the Ames test in a battery approach), whereas the two other tests have no correlation with the carcinogenicity results (25,30).

In fact, the experimental results of the NTP study point to (i) a group of chemicals positive in all four *in vitro* assays, most of these chemicals being also carcinogens; (ii) another group of chemicals negative in all the assays and (iii) chemicals negative in the Ames test and positive in one or in all of the three other assays. The mutagenicity results for the latter chemicals do not correlate with their carcinogenicity. Whereas the Ames test has a strong positive predictivity for the rodent bioassay (chemicals positive in the Ames test have a high probability of being also carcinogenic), the three other *in vitro* genotoxicity assays are prone—to a large extent—to generate misleading positive predictions of carcinogenicity.

The overall correlation between the Ames test and rodent carcinogenicity can be appreciated in Table I, which is based on a large database of high-quality results. The data were retrieved from the ISSCAN v3a database. The carcinogens are stratified into the categories of DNA reactive and non-DNA reactive, based on structural considerations: the stratification was performed by identifying the DNA-reactive chemicals with the expert system Toxtree 2.1.0.

The majority of the non-carcinogens are negative in the Ames test, the majority of non-DNA-reactive carcinogens are negative as well, whereas the majority of the DNA-reactive carcinogens are positive in the Ames test. It should be emphasized that Table I also points clearly to the strength of the Ames test: an Ames-positive chemical has ~80% probability of being a carcinogen. Given the intrinsic variability of the biological experimental systems (e.g. the Ames test has 80–84% inter-laboratory repeatability 31), this correlation is quite high, and makes the use of positive Ames information very robust and reliable.

Table I. Rodent carcinogenicity bioassay versus Ames test: comparison of results

Carcinogenicity	Ames test	
	Neg	Pos
Non-carcinogens	**233** *(0.75)*	**76** *(0.25)*
Carcinogens		
Non-DNA reactive	**136** *(0.80)*	**34** *(0.20)*
DNA reactive	**79** *(0.22)*	**277** *(0.78)*

The table contrasts the rodent carcinogenicity and Ames test results for 835 chemicals, as reported in the ISSCAN v3a database. The distinction between DNA-reactive and non-DNA-reactive carcinogens is based on structural considerations. To facilitate the comparisons, the numbers of chemicals (bold) are reported together with the row percentages (italics). Neg, negative; pos, positive.

On the other hand, no mutation-based STTs complementary to the Ames test for identifying the non-DNA-reactive carcinogens have been found: chemicals negative in the Ames test and positive only for other genetic endpoints do not have a significantly high probability of being carcinogenic (9,25,26). Thus, the overall lesson learned from the accumulation of experimental results over the last few decades seems to be that the equation 'mutation = induction of cancer' has a strong validity only in a limited area of the chemical space, i.e. for DNA-reactive chemicals. For the identification of these chemicals, the Ames test is a very sensitive tool. Another useful tool are the SAs for DNA-reactive chemicals. SAs are a distillation of the mechanistic knowledge on chemical carcinogenicity, and are demonstrated to be a valid model of the Ames test itself. The agreement between SAs and Ames test is ~80%, which is closely comparable with the inter-laboratory repeatability of the Ames test (31). Because of their mechanistic and operational overlap, the Ames test and the SAs have comparable agreement with rodent carcinogenicity (~70% accuracy): the Ames test is more specific (generating fewer misleading positives) and the SAs are more sensitive (generating fewer false negatives) (2,32).

The other important pillar of the present testing strategies (i.e. the use of *in vivo* genotoxicity assays as confirmation of *in vitro* positive genotoxicity results) has not lived up to expectations. The underlying rationale is that *in vitro* mutagenicity results should be checked in systems where the ADME characteristics of mammals are operating, thus providing a more realistic experimental setting than the 'simple' *in vitro* assays. In this way, *in vivo* STTs should counteract the excess of misleading positive results generated by mammalian *in vitro* assays. The most commonly used *in vivo* genotoxicity assay to confirm positive *in vitro* results is a test for the detection of damage to chromosomes or the mitotic apparatus, namely the mammalian erythrocyte micronucleus test in mice. However, there is growing appreciation that this assay is quite insensitive, responding negatively to many carcinogens that are able to induce both gene mutations and chromosomal damage (33).

In conclusion, the issue remains of reliably and rapidly identifying non-DNA-reactive carcinogens, for which the general purpose, viable tool is still the rodent bioassay (34). In the next section, we analyse CTAs as alternatives to the animal assay.

CTAs and the prediction of carcinogenicity

In a first analysis, the concordance between the results of CTAs and rodent carcinogenicity bioassays on a series of organic chemicals of very different classes was studied. All experimental results were retrieved from an OECD compilation (13). The predictive abilities of the CTAs are displayed as a receiver operating characteristics (ROC) graph in Figure 1. The comparison shows that SHE-7 performs best in predicting rodent carcinogenity, with the highest sensitivity to carcinogens (sensitivity = true positive rate = 0.88) and a good specificity (false positive rate = 1 – specificity = 0.23). Full details on the performance of SHE-7 are the following: specificity: 0.77, sensitivity: 0.88, accuracy: 0.85, positive predictivity: 0.89 and negative predictivity: 0.75.

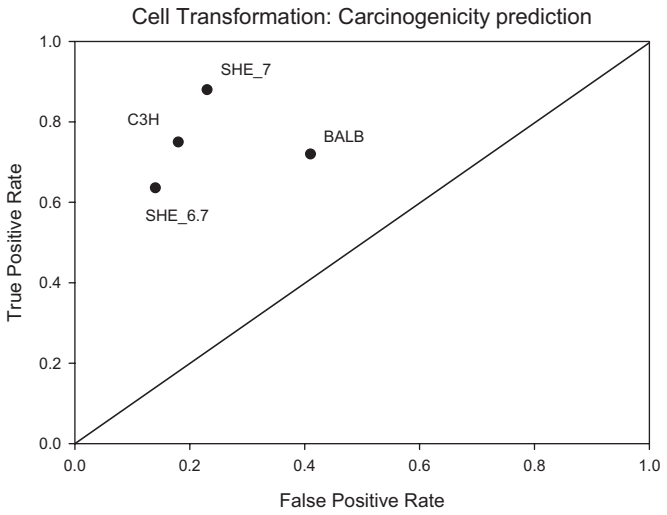

Fig. 1. Cell transformation: carcinogenicity prediction. The ROC graph in the agreement between the CTAs and the carcinogenicity results. An ROC graph has 1 − specificity (false positive rate) on the *x* axis and sensitivity (true positive rate) on the *y* axis.

Table II. Rodent carcinogenicity bioassay versus CTAs: comparison of results

Carcinogenicity	SHE-7		Balb		C3H	
	Neg	Pos	Neg	Pos	Neg	Pos
Non-carcinogens	**33** *(0.75)*	**11** *(0.25)*	**24** *(0.57)*	**18** *(0.43)*	**14** *(0.82)*	**3** *(0.18)*
Carcinogens						
Non-DNA reactive	**6** *(0.18)*	**28** *(0.82)*	**12** *(0.41)*	**17** *(0.59)*	**6** *(0.35)*	**11** *(0.65)*
DNA reactive	**5** *(0.8)*	**58** *(0.92)*	**12** *(0.21)*	**46** *(0.79)*	**11** *(0.22)*	**38** *(0.78)*

The table contrasts the rodent carcinogenicity and CTAs results, as reported in the OECD compilation. The distinction between DNA-reactive and non-DNA-reactive carcinogens is based on structural considerations. To facilitate the comparisons, the numbers of chemicals (bold) are reported together with the row percentages (italics). Neg, negative; pos, positive.

When stratifying the carcinogens into the categories of DNA-reactive and non-DNA-reactive/non-genotoxic chemicals, SHE-7 performs better than the other CTAs both with the DNA-reactive and the non-DNA-reactive/non-genotoxic carcinogens (Table II).

Sketching an alternative strategy for the identification of carcinogens

From the evidence provided in the previous sections, it appears that the Ames test is a very reliable tool for the identification of DNA-reactive carcinogens, and the SHE-7 CTA is able to identify both DNA-reactive and non-genotoxic carcinogens. An additional point is that SAs are a good model for both the DNA-reactive carcinogens and for the Ames test.

We are proposing a tiered testing strategy within this framework. First the Ames test or alternatively SAs are used in Tier 1. Then chemicals negative in Tier 1 are tested with the SHE-7. We have simulated this tiered strategy with SHE-7 data reported in the OECD survey (13) (Figure 2). Also, the carcinogenicity data used to check the strategy are derived from the OECD compilation. The Ames test data were retrieved from ISSCAN v3a. Toxtree 2.1.0 identified the chemicals with SAs. The first line in Figure 2 separates the sample of the chemicals into carcinogens and non-carcinogens.

In one option (left side of Figure 2), the fast and inexpensive SAs are used in Tier 1. We have further distinguished between SAs for DNA-reactive chemicals (the large majority in Toxtree) and SAs for non-genotoxic carcinogens. It appears that 56 chemicals (out of the 141 in the initial sample) do not possess SAs, and so should go to Tier 2 (based on SHE-7). It should be noted that application of SAs reduces the number of carcinogens in the sample from 97 to 30. Then SHE-7 is applied to the 56 chemicals without SAs; out of these, 23 are SHE-7 negative. As shown in the left box at the bottom of Figure 2, the sample of 23 chemicals negative in each of the steps of the tier is composed of 18 non-carcinogens and only 5 unidentified carcinogens. Thus, a considerable reduction of the carcinogens (to a final 5% of the initial number) is operated.

In the second option (right side of Figure 2), the Ames test is used as the first screening tool. Out of the initial 116 chemicals, 65 are negative in the Ames test and go to the next tier. The chemicals negative in SHE-7 are 25, including 8 carcinogens. Thus, also Option 2 generates a remarkable result, with the carcinogens reduced to 10% of the initial number.

For a comparison, we performed a similar exercise on a tiered testing strategy using the more traditional mouse lymphoma mutation assay in Tier 2, instead of SHE-7. Table III gives the overall results: it presents the percentage of chemicals of the initial sample that are negative after the tiered approach. It appears that the strategy of using SHE-7 as Tier 2 is considerably more sensitive (fewer false negatives in the final sample) and more specific (more real negatives in the final sample) than that of using the mouse lymphoma assay.

Conclusions

In the 1970s, the Millers' success in elucidating the mechanisms of action of several DNA-reactive carcinogens and the success of Bruce Ames in showing that many carcinogens were also able to induce gene mutation (by reacting with DNA), stimulated the scientific community to design further mutation-based STTs to complement the Ames test. The hypothesis was that the equation 'mutation = cancer' is generally valid, and that the consideration of different genetic endpoints (gene mutation, chromosomal damage), and different cells (bacterial, mammalian) would cover the spectrum of cancer-relevant factors. The large databases of experiments accumulated during the past decades have shown that this hypothesis—even though originally sensible—has not given the expected result. Overall, it appears that the Ames test and SAs are able to detect efficiently the portion of 'genotoxic' carcinogens that are DNA reactive. On the other hand, the chemicals positive in other mutagenicity-based assays (e.g. chromosomal aberrations, mutations in *in vitro* mammalian systems) but not in the Ames test (or SAs) do not correlate with carcinogenicity (2,9). This reality is now generally recognized. However, different approaches to improve the present testing strategies are possible.

One approach is based on the continued acceptance of the complementarity of STTs in terms of genetic endpoint and phylogenetic position of the assay systems. Operational improvements are sought by trying to manipulate the assay systems (e.g. to reduce their sensitivity 35,36). Other approaches explore completely new tools, such as the tracing

Table III. Tiered testing strategies: a comparison

Strategy (tests)	Chemicals in the initial sample		Chemicals negative in the tier (proportion of the initial sample)	
	Non-canc	Canc	Non-canc	Canc
SAs + SHE-7	**44**	**97**	**18** *(0.41)*	**5** *(0.05)*
Ames + SHE-7	**32**	**84**	**17** *(0.53)*	**8** *(0.10)*
SAs + MLY	**46**	**67**	**14** *(0.30)*	**11** *(0.16)*
Ames + MLY	**46**	**67**	**18** *(0.39)*	**17** *(0.25)*

The table shows the performance of different tiered strategies. The table reports the number of chemicals considered in each exercise, together with the number (and proportion) of chemicals negative after the two tests of each tier. To facilitate the comparisons, the numbers of chemicals (bold) are reported together with the row percentages (italics). Non-canc, non-carcinogens; canc, carcinogens; MLY, mouse lymphoma cells mutation assay.

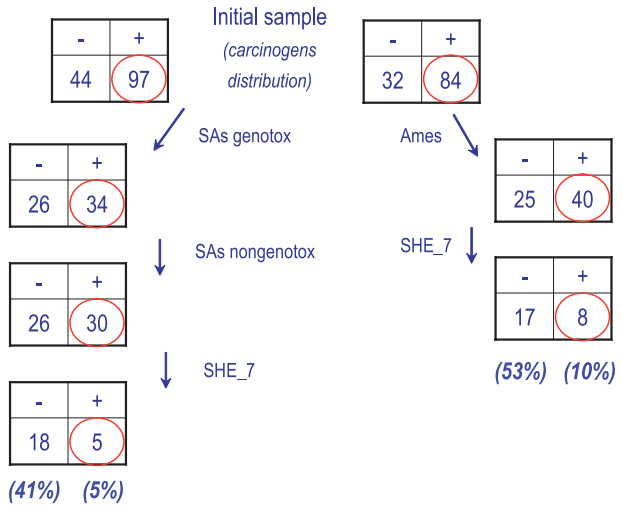

Fig. 2. Tiered strategies. The initial sample of chemicals is subjected to two strategies, one including first the SAs and then SHE-7, and the second one including first the Ames test and then SHE-7. After each step of the tier, the boxes report the chemicals that are negative in the step (see details in the text).

of molecular perturbations related to specific biochemical pathways with the use of various omics technologies. It appears that the latter approach is in its infancy and still needs much work and refinement (37).

Another approach is to accept that the equation mutation = cancer is valid only for DNA-reactive chemicals, and that other tools have to be devised for non-DNA-reactive carcinogens. The evidence provided in this paper shows that a very promising tool is the SHE-7 test, which is sensitive to both DNA-reactive chemicals and non DNA-reactive (supposedly non-genotoxic) carcinogens. The exercise performed in this work indicates that a tiered approach (consisting of Ames or SAs in Tier 1, and SHE-7 in Tier 2) is a powerful screening/priority setting approach: the sample is enriched in 'good' chemicals, and only 5–10% of the initial number of carcinogens go undetected. It should be noted that this tiered approach reduces the use of the SHE-7 test (that requires more time and skill) to only those chemicals that were negative in the more economical and quick approaches (SAs or Ames test).

Overall, the experience with the mutagenicity STTs confirms the notion that the gap between *in vitro* assays and their *in vivo* counterparts is difficult to fill. This is true also outside of the carcinogenicity prediction field. For example, a recent review by the European Food Safety Authority on the replacement, reduction and refinement of *in vivo* assays for the whole array of toxicological tests has pointed out that only skin irritation and corrosion testing in animals can be confidently replaced by *in vitro* alternatives. For a number of other endpoints (acute toxicity, eye irritation, genotoxicity, carcinogenicity), *in vitro* assays can help to direct the animal testing, thus contributing to diminishing the number of animals used. On the other hand, the development of alternative methods has shown to be more difficult for a range of other toxicological endpoints, such as toxicokinetics, skin sensitisation, acute systemic and local toxicity, repeated dose toxicity, reproduction and developmental toxicity studies. The same applies for ecotoxicity endpoints, such as acute and chronic toxicity in fish and birds, and bioconcentration in fish (38).

It is worth trying to understand the reason for the relative success of the *in vitro* assays considered in this paper, i.e. Ames test and the SHE-7 transformation assay. The CTA detects phenotypic alterations, which are characteristic of tumorigenic cells. It should be emphasised that *in vitro* cell transformation can be produced by a plethora of different molecular mechanisms that do not include the induction of mutations (39). Among other mechanisms, these transformation assays are models of cell–cell and cell–stroma communication phenomena typical of cancer; this cannot be seen as a 'single-cell' phenomenon, but is linked to modifications of the relations among cells in tissues (40). This interpretation is favoured by the growing interest in the related fields of tumour microenvironment (41) and cell adhesion mechanisms (42). On the other hand, the Ames test is sensitive to a very large number of carcinogens that are able to interact with DNA according to various molecular mechanisms (e.g. direct or indirect alkylation, acylation, intercalation, formation of amino-aryl DNA adducts). Thus, it appears that efficient stand-alone assays are characterised by a remarkable degree of 'aspecificity', and are not confined to the detection of very narrow sections of biological mechanisms or biological pathways.

In conclusion, the road to define alternatives to the rodent bioassay is paved with both successes and failures. Overall, a number of reliable tools is already available (e.g. the Ames test, SAs, the SHE-7 transformation assay). We think that it is possible to improve considerably the overall performance further by building on this core of tools. In particular, we see as particularly useful the expansion of structure–activity relationship concepts by including more SAs for non-genotoxic carcinogens, and the enlargement of the database of chemicals tested in SHE-7.

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