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Review

Applicability of a Host-mediated *In Vivo*/*In Vitro* Model in Screening for the Carcinogenic Potential of Chemicals

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Abstract. *Based on a publication of Tomasetti and Vogelstein in 2015, in which the risk of cancer development is postulated to be just one-third caused by genetic predisposition and environmental factors, it seemed worth focusing again on the value of test systems for screening chemicals for their carcinogenicity. This review aims to firstly summarize data on a host-mediated in vivo/in vitro assay developed by our working group to screen the tumorigenic potential of chemicals. Subsequently, in this article the importance and advantages of host-mediated in vivo/in vitro assays in general have been compared with in vivo and in vitro tests. The applicability of the host-mediated in vivo/in vitro assay system within broad screening strategies is discussed. The main intention of this review is to stimulate developments of newer approaches in the field of carcinogenic testing.*

The data of Tomasetti and Vogelstein (1) may essentially help oncology research in bridging a gap within the mechanistic explanation of cancer development. Beside genetic predispositions, cancer has been understood to be a multistage process induced by different carcinogenic agents of either physical (radiation), chemical (chemical carcinogens) or biological nature (tumor-inducing viruses). The multistage origin of cancer and dose-dependency of chemical carcinogenicity has been proven by the vast majority of model systems (2-7). Within the multistage, multimechanistic process of carcinogenesis, mutagenesis, cytotoxicity, and epigenetic alterations of gene expression are observed during the characteristic initiation, promotion and progression stages. Mutations as result of errors in both DNA repair and DNA replication play a critical role in the initiation step of human carcinogenesis. The rate-limiting step in multistage carcinogenesis is the amplification of the cell initiated during epigenetic tumor-promoting events (4, 8). In stem cells, the initiation step probably stops terminal differentiation and allows accrual of more gene or chromosomal mutations and, during the promotion stage, more epigenetic alterations (4, 8).

Nevertheless, it is still not clear why individuals with identical carcinogen exposure risks have different rates of cancer. It can be assumed that cancer is not the result of a 'one-hit' process. Exposure doses, individual genetic backgrounds, duration of exposure, developmental state, confounding factors and synergistic or antagonistic mixtures of endogenous or exogenous agents also need to be considered (4, 8). Tomasetti and Vogelstein's pioneering study (1) suggests that just one-third of the variation in cancer risk among tissues is attributable to environmental factors or inherited predispositions. According to their work, the majority of variation is due to 'bad luck', *i.e.* random mutations arising during DNA replication in normal, noncancerous stem cells. The frequency of these mutations depends on the frequency of stem cell divisions. However, the existence of such mutations does not seem to be the whole answer to understanding the question of cancer risk (4, 8).

As Tomasetti and Vogelstein assigned both environmental and inherited predispositions to contributing to approximately

Dedicated to Professor Robert C. Gallo on his 85th Birthday

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one-third of all cancer (1), the proportion of cancer induced by environmental carcinogens alone is probably high and demands more attention.

Due to recent public debate and lawsuits on the carcinogenic risk of the broad-spectrum herbicide glyphosate, interest in the evaluation of the carcinogenic potential of substances has increased again. The International Agency for Research on Cancer (IARC) classifies glyphosate as "probably carcinogenic to humans" (Group 2A) (5). This classification was based on limited evidence of cancer in humans (gained from real exposures) and sufficient evidence of cancer in experimental animals (gained from studies with pure glyphosate). The IARC also concluded that there was strong evidence for genotoxicity, both for pure glyphosate and for glyphosate formulations.

Against this background, it seems worthwhile to address the different published models of chemical carcinogenesis once again. This may help in choosing the appropriate model system for targeted screening of actual chemicals of interest for carcinogenicity.

This review focuses on a host-mediated *in vivo*/*in vitro* model assay system developed by our group (9). A major advantage of host-mediated models compared to solely *in vitro* studies is that they allow researchers to monitor the biotransformation of chemical mutagens through metabolic activation or detoxification and elimination. This in turn means that several other pharmacokinetic parameters can be analyzed. Studies performed solely with *in vitro* test systems may provide false-negative results when a chemical requires metabolic activation, or disproportionately higher false-positive results if it undergoes detoxification or excretion. Metabolism as well signal transduction is only partially represented in *in vitro* systems. Additionally, these systems give no information with respect to the incorporation of the chemical, or tissue-specific effects, and only limited information with respect to biological availability. Host-mediated *in vivo*/*in vitro* models use the transforming ability of *in vivo* systems, and in a second step, transformed cells are gained from these systems and stable cell lines derived which can then be analyzed *in vitro* using state-of the art molecular analyses. In vivo-models alone require a disproportionally high effort concerning their organization and costs compared with the value of the data they produce. Therefore, a combination of both test systems in theory can provide the perfect basis for studying the transforming effects of chemical mutagens.

The host-mediated assay developed by our group is based on the detection of carcinogen-induced transformation of murine peritoneal macrophages. Directly as well as indirectly acting carcinogens can be examined using this system. A comparison of our data on carcinogen-induced transformation with data on mutagenicity/genotoxicity published so far, as well as with carcinogenicity classification of the IARC seems reasonable and we are convinced that this assay is broadly applicable for screening of transforming potency of chemicals.

Table I. *Definition of clone size classes (6). The size of the clone (no. of cells) is represented by factors, with factor 1 being a total of 15-19 cells. Factors were calculated by dividing the mean cell number of a clone class by the mean cell number of the first significant class. Classes C0-C2 were not designated as significant and do not contribute to the transforming potential (factor=0). Class C3 with 15-19 cells per clone, found in only 25% of the control animals, was designated as the first significant class.*

Class	Number of cells per clone		
	Total	Mean	Factor
C0	0-4	2	0.0
C1	5-9	7	0.0
C2	10-14	12	0.0
C3	15-19	17	1.0
C4	20-24	22	1.3
C5	25-29	27	1.6
C6	30-49	40	2.4
C7	50-69	60	3.5
C8	70-99	85	5.0
C9	≥100-150	125	7.4

In this review, the ability of our *in vivo*/*in vitro* assay system (9) to detect the transforming potential of chemicals was compared to that of established genotoxicity and mutagenicity test systems, such as the Ames test (6), micronucleus assay (10) and Comet assay (7, 11). Additionally, a comparison of literature carcinogenicity data and current classifications by the IARC with respect to carcinogenic potential was made.

For this review, 11 articles on the host-mediated *in vivo*/*in vitro* assay system, published by our working group in the period between 1990 and 2006, were used (9, 12-21).

Host-mediated *In Vivo*/*In Vitro* Assay and Transforming Potential

The transforming potential of potential carcinogens according to our *in vivo*/*in vitro* test system is described as follows. After *in vivo* application of the candidate carcinogens to NMRI mice, macrophages were recovered by peritoneal lavage and cultured in soft agar. Macrophage clones were thus obtained. The frequency of the different clone sizes was used a measure for the extent of transformation of single cells (Table I). Table II presents the different clonal distribution patterns [large clones (type a), high number of small clones (type b) or both (type c)] under treatment with the compounds and their respective transforming potentials. Table III summarizes the transforming potentials of different compounds using our system compared with genotoxicity as well as mutagenicity data of these compounds as derived from the Ames test, micronucleus test and COMET assay, reported in literature

Table II. *Substances examined, clonal pattern and transforming potentials as determined in the host-mediated in vivo/in vitro assay.*

Substance	Amount administered with 100 ng TPA	Type	Mean frequency of clones		Transforming potential
			C3+C4	C5+C6+C7+C8	
4-Nitroanisole	2.8 mg*	a	0.4	1.0	0.0
5,5-Diphenylhydantoin	600 μg	a	1.7	0.0	2.4
2,3,7,8-Tetra-bromodibenzo-*p*-dioxin	195 ng	b	1.7	0.02	1.0
Benzene	300 μg	b	0.0	0.8	2.0
2-Nitrophenol	5.2 mg*	b	0	6.6	4.75
17α-Ethinylestradiol	27 mg	c	3.5	1.4	2.0
2-Nitroanisole	5.2 mg*	c	1.2	4.2	3.75
α-Naphthylamine	500 μg	c	1.5	0.5	4.7
2,3,7,8-Tetra-chlorodibenzo-*p*-dioxin	125 ng	c	4.3	0.5	7.2

TPA: 12-O-Tetradecanoyl-phorbol-13-acetate. *10% of the 50% lethal dose. Type a: Large clones; type b: high number of small clones; type c: both types a and b present. The transforming potential was based on the frequency of clones as mentioned in (6).

(6, 12-21). Data on the current IARC classification of these compounds are also included in the table.

The dose–response effects with respect to the transforming potential for different carcinogens, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (12, 13), nitro-musk derivatives (19) and 2-nitroanisole (20) is apparent in our system. Low doses of diphenylhydantoin, which exhibited no transforming potential when used alone in the *in vivo*/*in vitro* assay, gained a high oncogenic potential when simultaneously administered with low doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (12, 13).

Immortalized Cell Lines

In our *in vivo*/*in vitro* assay, different immortalized peritoneal macrophage cell lines with tumorigenic characteristics were established: TBrDD cells [after administration of 2,3,7,8-tetrabromodibenzo-*p*-dioxin (12)], Aona cells [after administration of 10% of the median lethal dose 2-nitroanisole (20)] and EED cells [after administration of 17α-ethinylestradiol (21)]. All immortalized cell lines revealed similar characteristics:

Cells were larger in size than normal macrophages. Additionally, they exhibited a diminished adherence in culture. Macrophage characteristics of EED and Aona immortalized cell lines were proven by Fc receptor III expression, which was reduced compared to normal macrophages (20, 21) (Table IV). MAC-1 is macrophage-specific and represents a complement receptor (CR3), consisting of integrin αM and integrin β2. MAC-1 antigen expression in transformed cell lines EED and Aona is not altered compared to normal macrophages (14, 15). Detection of unspecific esterase expression is used as additional proof of a monocytic cellular origin. EED cells expressed 32% and Aona cells 35% of non-specific esterases found in normal macrophages (20, 21). Whereas normal macrophages stop growing under serum-free conditions, and addition of serum leads to their transition from the G_0 to the G_1 phase of the cell cycle, EED and Aona cells still exhibited proliferation (20, 21). This loss of dependency on growth factors is characteristic of transformed cells.

Transformation-specific Marker Proteins

In Aona, EED and TBrDD cells, compared to normal macrophages, pronounced expression of additional proteins was detected, especially in the low-molecular weight range (10-20 kDa) (20, 21). 2D-Polyacrylamide gel electrophoresis analysis showed a high similarity of protein expression patterns for EED and TBrDD cells (15, 16, 21) (Figure 1). The appearance of additional proteins expressed only in transformed cell lines (Aona cells, EED cells and TBRDD cells) reflects the carcinogenic activity of these compounds which was detected in our system.

Activity of Proto-oncogenes

In the context of cellular growth and proliferation disturbances during chemical carcinogenesis, transcription factors c-JUN, c-MYC and c-FOS play an important role. Dysfunction in their regulation has been shown for several tumor types, such as lymphoma and sarcoma (22, 23). In Aona cells, nuclear c-FOS and c-MYC oncoproteins were found to be down-regulated, c-JUN on the other hand was clearly overexpressed. In EED cells, c-MYC was down-regulated whilst c-FOS was not altered significantly; c-JUN was overexpressed in a concentration-dependent manner (20, 21).

Oncogenic Potential

Injection of EED cells, Aona cells, or TBrDD cells at two different locations into the back skin of nude mice (nu/nu)

Table III. *Substances examined and transforming potentials as determined in the host-mediated assay, literature mutagenicity compared with literature carcinogenicity data and their International Agency for Research on Cancer (IARC) classification.*

Chemical	Transforming potential by host-mediated assay system	Mutagenicity/genotoxicity literature data			Carcinogenicity		
		Ames assay	Micronucleus test	Comet assay	Literature data		IARC classification
					Animals	Man	
α-Naphthylamine	>2.5	–	?	?	I	I	3
5,5-Diphenylhydantoin	>2.5	–	+/–	+	L	I	2B
Benzo(a)pyrene	>2.5	+	+	+	S	ND	1
N-Nitrosodimethylamine	>2.5	+	+	+	S	ND	2A
Ethidium bromide	>2.5	+	?	–	ND	ND	ND
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	>2.5	+	?	?	S	L	1
Aflatoxin B1	>2.5	+	+	+	S	S	1
Dieldrin	>2.5	+	+	+	L	I	3
1-Methyl-3-nitro-1-nitrosoguanidine	>2.5	+	+	+	S	I	2A
N-Methyl-N-nitrosourea	>2.5	+	+	+	S	ND	2A
9,10-Dimethyl-1,2-benzanthracene	>2.5	+	+	+	S	I	2A
3-Methylcholanthrene	>2.5	+	+	?	S	ND	ND
Hexachlorobenzene	>2.5	–	+	+	S	I	2B
Hexachlorocyclohexane	>2.5	+	+	+	S	I	2B
Benzene	1.5-2.5	–	+	+	L	S	1
2-Naphthylamine	1.5-2.5	+	+	+	S	S	1
Suramin	1.5-2.5	+	?	?	?	ND	ND
Trichloroethylene	1.5-2.5	+	+/–	+/–	S	L	1
Tetrachloroethylene	0.5-1.5	+	+	+	S	L	2A
Chlorambucil	0.5-1.5	+	+	+	S	S	1
Aflatoxin G_2	None	?	?	?	I	S	1
Toluene	None	–	+	+	I	I	3
Azidothymidine	None	–	+	+	ND	ND	ND

Mutagenicity/genotoxicity: +: positive test result; –: negative test result; ?: inconclusive result. ND: No adequate data, I: inadequate evidence, L: limited evidence; S: sufficient evidence; International Agency for Research on Cancer (IARC) classification: 1: carcinogenic to humans, 2A: probably carcinogenic to humans, 2B: possibly carcinogenic to humans, 3: no classifiable carcinogenicity to humans.

led to tumor growth (Figure 2, Aona cells). Histological characterization of tumors by hematoxylin-eosin-staining revealed atypical chromatin-rich nuclei with increased mitotic frequency and alterations of the nucleus–plasma relation (Figure 3). The tumor cells induced by EED and Aona cells microscopically resemble mesenchymal tumor cells with multiple mitoses, spindle-shaped cells, and chromatin-rich nuclei with enlarged nucleoli (20, 21) as characteristic of morphological transformation.

Table IV. *Analysis of Fc receptor expression by binding of opsonized and non-opsonized sheep red blood cells (SRBC) by 2-nitroanisole-transformed cells (Aona), 17α-ethinylestradiol-transformed cells (EED), and normal macrophages.*

Cells	SRBC	Erythrocytes bound per cell, %			
		0	1-4	5-9	>10
Aona	Opsonized	18.8	47.8	24.8	8.8
	Non-opsonized	35.4	61.3	3.3	0
EED	Opsonized	21.0	41.0	28.0	10.0
	Non-opsonized	52.0	28.0	20.0	0
Normal macrophages	Opsonized	7.6	32.9	23.5	36
	Non-opsonized	24.3	48.5	21.3	5.9

Discussion

Cancer is the leading cause of death in most industrialized countries. It is promoted by numerous factors including lifestyle, diet and environmental agents. Carcinogenesis can be characterized as a complex process. Multistage alterations in the genetic makeup of normal cells, provoked by carcinogens, or by the mismanagement of cellular DNA repair systems are involved (4, 8). During promotional stages, inflammatory responses play a crucial role (24, 25). In this context, host-mediated responses to carcinogens, and on the other hand, to therapeutic approaches, must also be considered (26-28).

More than 100 different short-term test systems have been established for the detection of carcinogenic activity of

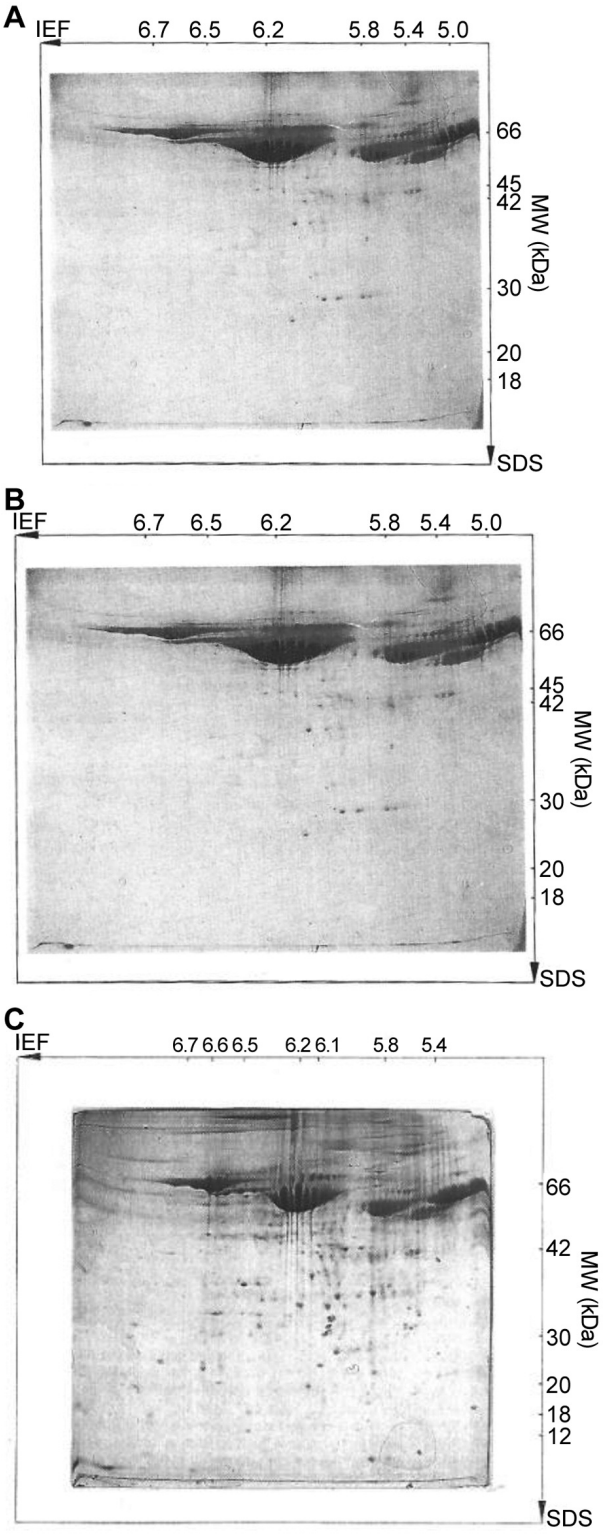

Figure 1. *Transformation-specific low-molecular-weight proteins in normal macrophages (A), 17α-ethinylestradiol-transformed cells (EED) (B) and 2,3,7,8-tetrabromodibenzo-p-dioxin-transformed cells (TBrDD) (C) as determined by 2D- sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.*

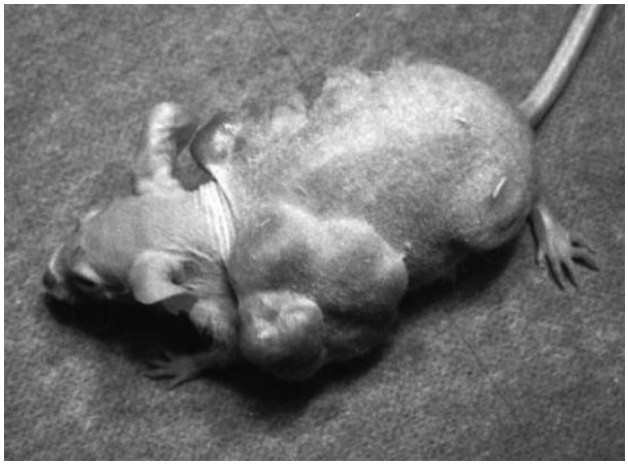

Figure 2. *Tumor induction in nude mice 6 weeks after subcutaneous injection of 1×10^6 Aona cells at two different sites.*

chemicals based on their mutagenic effects [reviewed in (5)]. Systems based on *in vivo* detection of carcinogenicity are not only subject to complex application procedures but are also technically and organizationally demanding. *In vitro* systems on the other hand, use either microorganisms (6) or mammalian cells (29). They are subject to certain methodological limitations which restrict transferability of data to humans. Additionally, *in vitro* systems often lack the metabolic pathways which are necessary to convert a chemical into an active species which may be carcinogenic (30). For this reason, these systems must be supplemented with metabolizing systems, such as the externally added rat liver S9 fraction (6). Compared to *in vitro* assays, host-mediated systems (31, 32) have the advantage that they derive from an intact organism. Therefore, they come closest to the intact organism, which in the case of the risk assessment, is a clear advantage over other test systems. The host-mediated assay attempts to bridge the gap between *in vitro* studies and definitive tests in mammals.

This review compares the data on the transforming potential of compounds obtained by our *in vivo*/*in vitro* assay with published classifications of compounds by established genotoxicity and mutagenicity assay systems. In contrast to the Ames test, our test system detected a transforming potential for α-naphthylamine, 5,5-diphenylhydantoin and hexachlorobenzene, which for the latter two compounds is in full agreement with the classification by IARC (5). For all other compounds tested, the transforming potentials confirmed by our system are in good agreement with the results obtained by the Ames test. The lack of carcinogenicity for toluene found using our system is supported by the findings reported in the Ames test (5).

In our opinion, the host-mediated *in vivo*/*in vitro* assay developed by our group offers several advantages over

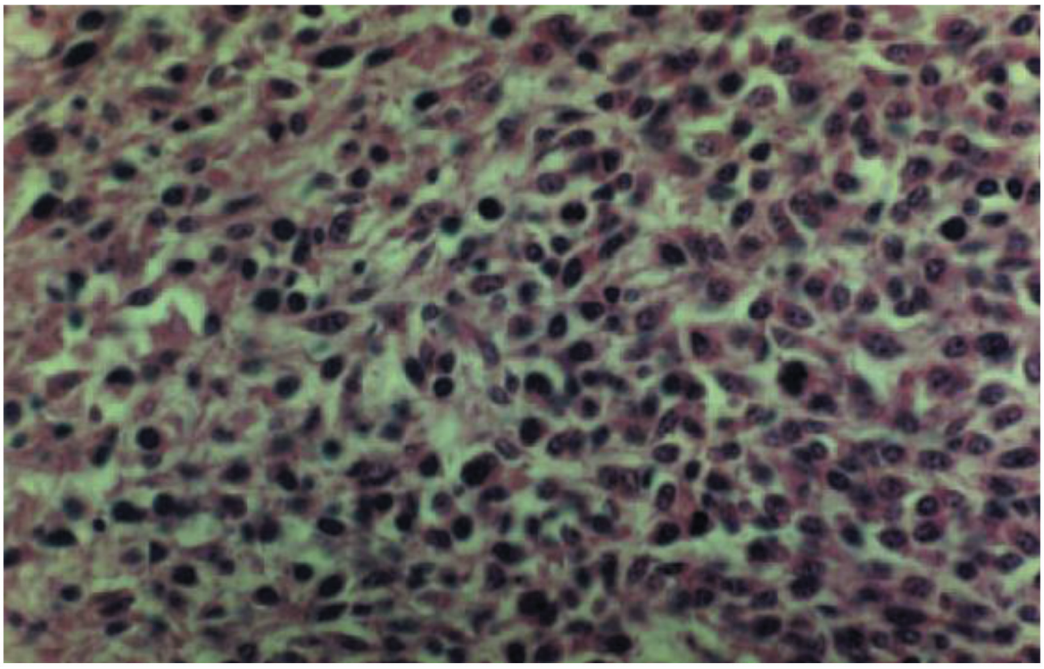

Figure 3. *Hematoxylin and eosin staining of a tumor induced by Aona cells in a nude mouse (magnification, ×1,000).*

currently used standard carcinogenicity and mutagenicity screening assays. Firstly, a specific animal-derived peritoneal macrophage cell population can be isolated very easily. Secondly, carcinogens administered intraperitoneally are not only submitted to the metabolic influence of the animal but also to the specific oxidative routes found in activated macrophages, such as prostaglandin-synthetase-mediated production of active metabolites (33). Thirdly, directly as well as indirectly acting carcinogens can be analyzed in our system without confounding factors (9).

Several older approaches have been mentioned for the screening of carcinogenicity (34-45), only our system covers the key problems related to carcinogenicity of chemicals (9, 12-21).

Recent data published using those assays were mostly derived from infectology studies either with the aim of mechanistically understanding pathogen interactions or bioactivation of pharmaceutical compounds by metabolizing pathways in *in vivo*/*in vitro* assays (46-48). This confirms the significance of host-mediated *in vivo*/*in vitro* assays in also evaluating the effects of chemical agents on biological systems with respect to molecular mimicry in intact organisms as underlined above.

In 2006, the European Parliament and Council passed the REACH regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals) to simplify and harmonize directives on chemicals within the European Union (49). In this process, regulatory strategies for human carcinogenicity were diversified and alternative approaches to replace *in vivo* rodent tests were developed. The mandate of the EU Reference Laboratory for alternatives to animal testing is specified in Directive 2010/63/EU on the protection of animals used for scientific purposes (50) and includes a number of aspects to advance replacement, reduction and refinement of animal procedures. Against this background, carcinogenicity test systems available so far, are being reviewed again systematically. The aim is to exploit recent advances in test methods and identification of assessment approaches to move away from the 2-year cancer bioassay in rodents (51).

In this context, our host-mediated *in vivo*/*in vitro* assay system can be modified for application in other test strategies. Admittedly, a broad-scale use of *in vivo*/*in vitro* assays in systemic screening of chemicals for carcinogenic effects is too elaborate; nevertheless, this assay system may play a decisive role in future applications for testing the carcinogenicity of chemicals.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Kathrin Schlatterer and Prakash Chandra wrote the article and revised the article. Anoosh Esmaeili, Thomas Massa and Savvas Apostolidis reviewed the article and made several additional changes. All Authors read the final article and approved its publication.

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