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CHMP SAFETY WORKING PARTY

CHMP SWP CONCLUSIONS AND RECOMMENDATIONS ON THE USE OF GENETICALLY MODIFIED ANIMAL MODELS FOR CARCINOGENICITY ASSESSMENT

1 INTRODUCTION

The Note for Guidance ICH S1B Testing for Carcinogenicity of Pharmaceuticals (CPMP/ICH/299/95) offers the option to use short or medium-term in vivo rodent test systems, such as transgenic and knockout animal models, in place of a second 2-year rodent bioassay. Ideally, such studies should supplement the long-term carcinogenicity study and provide additional information that is not readily available from the long-term assay. In the spring of 2002, the ILSI/HESI Alternatives to Carcinogenicity Testing Project comprising 21 selected substances was finalised. From the regulatory point of view, the most crucial factor in considering the use of transgenic/knockout mice is how well the now available experience supports regulatory acceptance. For this reason, an ad hoc-group consisting of four assessors from EU regulatory authorities has reviewed the data generated by the ILSI/HESI project.

2 GENERAL CONCLUSIONS AND RECOMMENDATIONS

2.1 Regulatory acceptance

In the TgrasH2 as well as in the p53 model, all 21 ILSI/HESI compounds have been studied. Although some individual studies showed equivocal responses, the outcome as a whole suggests that these two models can be accepted for regulatory purposes and are likely to have an additive value to carcinogenicity assessment if studies are properly designed.

The TG.AC reacted inconsistently and incompletely to known human carcinogens. Although developed to be responsive at the site of application (skin), the ILSI/HESI evaluation of the studies with human carcinogens included responses also at other sites, in order to "suit" the profile of the compound. Nevertheless, the model is considered to be useful for screening the carcinogenic properties of dermally administered pharmaceuticals. The TG.AC model cannot be recommended for oral studies with the forestomach as "reporter site" .

The XPA-/- and XPA/p53 assays appear to be promising models, but more data from studies using acceptable protocols are needed. For the XPA-/-, studies of existing human carcinogens should be completed in order to allow for a reliable evaluation of the sensitivity. With respect to the XPA/p53 model, the data are based on single-dose experiments only and are therefore considered to be very preliminary.

2.2 The use of the transgenic/knockout mice for the assessment of carcinogenic potential

The models identified as acceptable for general regulatory use, i.e. TgrasH2 and p53+/-, can be used as alternatives to the mouse long-term study together with long-term rat study and genotoxicity studies. The available data do not suggest that one model is more appropriate than the other for a particular class of compounds, particular mechanisms of tumourigenic activity, or other specific conditions.

2.3 The use of transgenic animals in the case of equivocal genotoxicity results

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The standard testing battery is usually sufficient for the evaluation of genotoxicity. If the standard battery gives equivocal results, a number of additional genotoxicity tests are available to clarify the equivocal outcome. In general, this will allow for a definite assessment. Transgenic models can also be used as an additional component of the assessment of potential genotoxic carcinogenicity (more so than a classical long-term mouse study). However, the outcome of an experiment with transgenic animals should not be considered as THE decisive factor in the assessment of genotoxicity, but rather as part of the weight of evidence. Results of a transgenic assay may be used to prove or to disprove a hypothesis derived from genotoxicity data rather than to decide whether or not a compound is genotoxic.

2.4 Recommendations on study design

- The number of animals per group used in the ILSI/HESI studies is too small, which sometimes hampers the interpretation of the study. An increase in group size to 20-25 animals per group per sex is recommended.
- If a positive study outcome is defined on the basis of "rare tumour criteria" only, a repetition of the study should be considered (especially when the historical control data on which the definition of a "rare tumour" is based are relatively limited)
- Wild type animals should be used for all studies (except for TG.AC studies) as the difference in response will add information to the outcome of a study.
- The SWP has re-evaluated this issue in february 2004 and came to the following conclusion: In studies using p53 or TgRasH2 mice the inclusion of groups of wild type animals can be helpful to show the influence of the genetic change if in a particular study a positive response is observed in the genetically modified animals only. However, recent data indicate that the chance for a positive response is relatively small. So, the addition of groups of wild type animals is left to the responsibility of the sponsor.
- At present, positive controls should be included in studies with p53 and TgRasH2 mice. In future, verification of the genotype of the test animals may be an acceptable alternative.

3 C57BL/6 (N5) – TRP53 KNOCKOUT

3.1 Introduction

The wild-type p53 protein suppresses cancer in humans and rodents. As a transcription factor, p53 regulates the activity of a variety of genes involved in cell cycle arrest, apoptosis, anti-angiogenesis, differentiation, repair, and genomic stability. Using the AB1 (129Sv) embryonic stem cell line, a functional Trp53 allele was replaced with an inactivated allele termed the null Trp53 allele. This was incorporated into blastocysts and implanted in C57BL/6 female mice. After further backcrossing with C57BL/6 mice the line was introduced into research.

3.2 General characteristics

3.2.1 Spontaneous Tumour Incidences

The overall spontaneous tumour incidence in studies of 26 weeks duration was low, 2.8% in males (n=283) and 6% in females (n=284) in studies without transponders (microchip implants for identification), and 8% in males (n=150) and 11.3% in females (n=150) in studies with transponders. Lymphomas, subcutaneous sarcomas and osteosarcomas were the three most common tumours. Other tumours had a much lower incidence (0.0-0.2%).

Implantation of transponders results in particular in a higher incidence of subcutaneous sarcomas with up to 6.7% in female mice (as compared to 1.4% in females without biochips). The use of this method is therefore not recommended. It has also been suggested that displacement of the transponder can be induced by handling, which may result in confounding tumours at a site distant from that of the implantation site.

3.2.2 Performance of positive control compounds

The response to the most commonly used (19 studies) positive control substance p-cresidine (400 mg/kg, i.g.) was highly variable - the incidence of bladder tumours induced by p-cresidine was between 30 and 80%. In 1/19 studies the outcome was negative. Males were more sensitive (18/19 studies positive) than females (15/19 studies positive).

Actual incidence values were not used to determine sensitivity, but only for a yes-or-no decision whether the animal breeding at that moment had been able to produce animals with the right genetic set-up. The check of the genetic set-up by individual genotyping is suggested and might replace the use of the positive control.

3.3 Overview and discussion of the results

All 21 selected substances were studied. With respect to human carcinogens, p53+/- mice showed a relatively good response, 3/5 gave a positive result, 1/5 an equivocal result and 1/5 a negative result. The 'false negative' phenacetin is clearly a weak human carcinogen and the negative outcome with phenacetin in p53+/- may actually support the usefulness of this model. Since the human carcinogen cyclosporin A induced lymphomas also in wild type animals, the additional value of a similarly positive result in p53+/- animals is low.

The non-genotoxic rodent carcinogens/human non-carcinogens (based on epidemiology) were all negative in p53+/- mice, which is an important result in view of the initial goals of the ILSI/HESI project to identify models detecting only relevant human carcinogens.

From the non-genotoxic rodent carcinogens/human non-carcinogens (based on mechanism) all but the phthalate DEHP were negative. The result obtained with DEHP was classified as 'equivocal' based on the criterion of induction of a rare tumour, i.e. the induction of one renal transitional cell papilloma associated with other nephrotoxic effects. Together with the fact that the positive control was negative, the outcome of this study was classified as 'equivocal'. The other peroxisome proliferators were negative in the p53+/- mouse.

Results with non-genotoxic non-carcinogens were negative.

As regards the ability of the p53+/- mouse to provide additional mechanistic insight into the development of tumours, it is important to compare the outcome in the knockout to the wild-type response with a substance such as cycloclosporin A. (see recommendations). The introduction of a null-allele has been explained as a first genotoxic event in Knudson's two-hit hypothesis for the induction of cancer. However, based on the genotyping of the cells in the tumours, the concordance with the induction of Loss-of-Heterozygosity (LOH) is low. Recent data indicate that acceleration of tumorigenesis in p53 knockout strains may be due to a gene dosage effect and a haploinsufficient phenotype such that a second event (LOH) is not required.

3.4 Data from non-ILSI/HESI studies

Studies of a relatively large number of other compounds in p53+/- mice have been published. Most studies did not include wild-type animals. Genotoxic carcinogens have generally induced a positive response. A few genotoxic carcinogens, e.g. glycidol, gave negative results The reasons for these "false negative" results are unclear.

Phenolphtalein has been reported to induce lymphomas in female mice and this induction of tumours was associated with LOH (loss of heterozygosity), even at dosages that did not induce tumours. Remarkably, other p53 knockout strains (derived from CBA and a C57BL/6_{CIEA} mice) have been reported to be less sensitive (or even insensitive) to phenolphtalein.

3.5 Regulatory acceptance

Based on the high number of compounds tested in the ILSI/HESI project, together with the number of studies with other substances reported in the literature, there is relatively extensive experience with the use of the C57BL p53+/- mouse. In addition, in recent dossiers of several human pharmaceuticals studies with the p53+/- mouse have been included. The p53+/- model may be of additional value to the classical long-term carcinogenicity studies in rats and mice. However, it is too early to decide whether

this model has any advantage over the other transgenic models. A specific sensitivity to genotoxic compounds is clear.

4 CB6F1-TG-RAS H2

4.1 Introduction

The TgrasH2 (CB6F1-Tg-ras H2) transgenic mouse carries the human prototype c-Ha-ras gene. It is produced by microinjection of a human hybrid c-Ha-ras gene construct that has a point mutation in the last intron resulting in the increased expression of the transgene. There are 5 or 6 tandem copies of the transgene per genome. The mice have also been described as hemizygous Tg mice carrying 3 copies of the prototype human c-Ha-ras gene with its own promotor integrated into the genome in a tandem array. The total amount of c-Ha-ras gene product, p21, detected by immunoblot, is reported to be 2 –3 times higher in Tg than in non-Tg mice.

The animals used in carcinogenicity studies are the F1 progeny of transgenic male C57BL/6J and female BALB/c ByJ. The colony up to generation N19 has been reported as stable. Instability of the integrated transgene does not appear to be a significant problem. Body weights of transgenes are 80-90% of corresponding non-Tg, but organ to body weight ratios seem comparable to non-Tg. The survival rate at 77 weeks is 53% and 32% for male and female transgenes, respectively. Corresponding numbers for wild type are 96 and 97%.

Activation of ras oncogenes by point mutation has been reported in about 30% of human cancers. The relevance of the integration of 5 to 6 copies of the c-Ha-ras gene into the CB6F1 mouse to the carcinogenic process in humans is uncertain. Mutations of the transgene are not a prerequisite for tumour development. The mechanism of the enhanced carcinogenic response in the TgrasH2 transgenic mouse is unknown. It has been suggested that the transgene may enhance susceptibility by e.g. mutagenesis at specific activating codons in one of the copies of the transgene. Alternatively, co-operativity between the transgene-mediated overexpression of normal human H-ras and second “hits” by the test material on endogenous mouse genes (myc or p53) could result in transformation. Given the enhanced expression of Ha-ras in multiple tissues and the possibility of enhanced tumour susceptibility by more than one mechanism, Tgras H2 mice were expected to exhibit a relatively high site concordance. Expectations of site concordance are confounded by the existence of 3 ras protooncogenes, the K-ras and N-ras being more frequently involved in human carcinogenesis.

Transgene mutations are not always related to the induction of spontaneous tumours, such as forestomach and skin tumours, while ras gene mutations have been more consistently observed in lung adenocarcinomas. The regulation of transgene expression may be age-dependent and the expression of the transgene can be altered by infection or by a removal of a pathogen from the colony.

4.2 General characteristics

4.2.1 Spontaneous tumour incidences

By 18 months of age, 50% of the animals develop spontaneous tumours. At 6 months, i.e. the study duration used in the ILSI/HESI studies the spontaneous tumour incidence in TgrasH2 mice seems low. The highest incidences of spontaneous tumours included lung adenomas (6.7%), hemangiosarcomas of the spleen (3.9%) and papillomas of the forestomach (2.8%). Other spontaneous tumours in 180 male and 178 female compared with 179 male and 180 female wild-type mice used in the same studies were as follows. In males, lung adenoma (1.6%), liver hemangiosarcoma (1.6%), spleen hemangiosarcoma (1.6%) and skin squamous cell papilloma (1.1%) reached incidences above 1%. Wild-type males showed only lung adenoma and carcinoma, as well as nervous system lipoma (incidence 0.6%) and hemangiosarcomas in the epididymides (0.6%). In females, lung adenoma (1.6%), spleen hemangiosarcoma (1.1%), hematopoietic malignant lymphoma (2.2%), forestomach cell papilloma (1.1%) and Harderian gland adenoma (1.1%) reached incidences above 1%. Wild-type females showed lung adenoma (1.1%) and hematopoietic malignant lymphoma (1.1%).

Higher incidences of spontaneous tumours in TgrasH2 mice, such as lung adenomas, spleen hemangiosarcomas and skin papillomas, ranging from 3 to 8%, have been reported in the literature.

4.2.2 Performance of positive control compounds

In the ILSI/HESI studies, methyl nitrosourea (MNU, 75 mg/kg, i.p.) was used as the positive control. A concurrent positive control was not included for nine of the compounds for which there is data available. These compounds were phenacetin, melphalan, cyclosporin A, 17-β-estradiol, reserpine, methapyrilene, haloperidol, metaproterenol and sulfisoxazole. A positive control is considered particularly important in case of a negative study. Target organs for the positive control, MNU, were the haematopoietic system, thymus, forestomach/stomach (70-100%), skin and Harderian gland. Most of the mice died within 14 weeks due to lymphoma. Overall, tumour frequencies in response to the positive control were consistent with transgenes being more sensitive than wild type.

4.3 Overview and discussion of the results

For the TgrasH2 mouse, data are available for 18/21 of the compounds selected for study. Data are lacking for dieldrin, WY-14643 and D-mannitol. For three of the compounds, melphalan, phenacetin and cyclosporin A, no sponsor was available and the studies were conducted under the auspices of Central Institute for Experimental Animals (CIEA). The CIEA protocol differed from the ILSI/HESI protocol in that only two dose groups were included and the doses were based on the NTP 2-year studies. In addition, for haloperidol only two dose groups seem to have been included.

Of the three genotoxic human carcinogens, two were positive (cyclophosphamide and phenacetin), whereas melphalan gave an equivocal response. The human immunosuppressive carcinogen (cyclosporin A) likewise gave an equivocal response. Among the human hormone carcinogens (DES and estradiol), DES was positive and estradiol negative.

Thus, in the ILSI/HESI studies, 50% of the human carcinogens tested were clearly positive. Three of six known human carcinogens were not detected (estradiol, cyclosporin A, melphalan). Equivocal results were reported with melphalan and cyclosporin A in studies conducted using a protocol different from the ILSI/HESI protocol and without the inclusion of a positive control. In the case of melphalan, the high dose likely was close to or higher than the MTD since several females died of abdominal cavity adhesion. In the cyclosporin A study, the incidence of forestomach papillomas was increased in high dose females, but statistical significance was not reached. The negative result with estradiol appears to be more conclusive.

For the non-genotoxic rodent carcinogens (human non-carcinogens based on epidemiology), data are available for four compounds, of which one is positive (clofibrate) and three are negative (phenobarbital, methapyrilene, reserpine). For clofibrate in a second study, an equivocal outcome was reported. Although actual data for dieldrin were not reported the outcome was reported as negative.

For the non-genotoxic rodent carcinogens (human non-carcinogens based on mechanism), data are available for six compounds. These were all negative, except for the peroxisome proliferator DEHP. Data were later provided for the other peroxisome proliferator WY-14643 indicating a positive result (personal communication). TgrasH2 mice are expected to have a low susceptibility to hepatocarcinogens. In non-ILSI/HESI studies, tests with genotoxic compounds such as DEN and vinyl carbamate were negative with respect to liver tumour formation. The positive liver tumour response to peroxisome proliferators was interpreted as the development of liver tumours in TgrasH2 mice being dependent on the mechanism of hepatocarcinogenesis.

Only 3 non-genotoxic/non-carcinogenic compounds were tested. Negative results were reported for 2 of the compounds while data were not completed for the third.

The data available from the ILSI/HESI project indicate that the model may detect transspecies genotoxic carcinogens and some non-genotoxic carcinogens. The results from studies with six human carcinogens showed that the model may detect human genotoxic and hormone carcinogens. In some studies (cyclophosphamide, DES, clofibrate), tumours were noted in wild-type target tissues. Site concordance in other studies, primarily using genotoxic compounds, seemed variable with tumours often seen in organs susceptible to spontaneous tumour development. In 3 of 5 positive ILSI /HESI studies a clear increase also in spontaneous tumours was noted. Overall, it has been concluded that the interpretation is problematic when increases are noted only in incidences of tumours with a high spontaneous rate, but chemicals that induce tumours in lung, forestomach and spleen should be regarded as carcinogens. There was a tendency towards a dose-related response in three of the studies

with a positive outcome (DEHP, phenacetin and cyclophosphamide), but the dose-response was dependent on the type of tumour and the gender.

Molecular analysis of tumours from rasH2 deficient mice was conducted in relation to the observed tumourigenic responses in some studies. Point mutations at the 12^{th} and 61^{st} codon of h-ras were detected in forestomach tumours of 11/11 and 1/9 animals, given methylnitrosurea and ethylnitrosurea, respectively. In this study, the human h-ras gene expresssion was approximately doubled in all forestomach tumours. In other studies, point mutations of transgenes ranged from 10 to 100% in induced tumours and mutations seemed related to certain types of tumours only. In particular it appears that ras gene mutations have been observed in lung adenocarcinomas and it has been suggested the model could be especially useful in study of lung carcinogenesis. However, the mechanistic rationale for an enhanced carcinogenic response remains unclear.

4.4 Data from non-ILSI/HESI studies

In a published study, 14/17 (82%) genotoxic human and/or rodent carcinogens were reported to be positive. The substances included phenacetin and benzene. Glycidol, a mutagen and a rodent carcinogen, has also been reported to be positive in TgrasH2.

In other non-ILSI/HESI studies, thyroid tumours induced by ethylene thiourea and 4,4-thiodianiline occurred at a similar incidence in transgenic and wild-type mice, indicating that the response to hormonal carcinogens might not be enhanced in TgrasH2 mice.

4.5 Conclusions

The ILSI/HESI data available for the TgrasH2 model, although limited, indicate that human carcinogens, both genotoxic and non-genotoxic, produce positive responses. The equivocal results with melphalan and cyclosporin A may at least in part reflect inadequacy in the choice of the high dose and an overall "low" sensitivity (10-15 animals/sex/group). A "low sensitivity" also suggests that a correct selection of the high dose is important for the outcome of the study. Increasing the size of the groups would improve the power of the study. The positive response with diethylstilbestrol was based on an increase in Leydig cell tumours, also seen with wild-type mice but at a lower incidence, indicating that the response did not directly involve the transgene, but is due to hormonal effects. The other hormone tested, 17-β-estradiol, did not induce any tumours either in trangenes or in wild-type.

The rodent carcinogens classified as putative non-carcinogens for humans were mostly negative in the TgrasH2. The positive responses, increases in liver tumour incidences, to peroxisome proliferators may reflect the general sensitivity of the mouse to this particular type of non-genotoxic liver carcinogens. The response seems separate from the transgene. Other data is also available indicating that the transgene may not play a role in hepatic tumours. These results also emphasise the importance of further studies on the mechanism(s) of the enhanced tumourigenic response in TgrasH2.

The criterion for a positive response was defined as "a statistically significant higher tumour incidence in treated group(s) compared with concurrent control/vehicle group". This refers to at least one type of tumour. The data are too limited to allow any conclusion on rare tumours and instances when increases in rare tumours may be considered a positive response. An increase in tumour incidence at sites prone to spontaneous tumour development was also considered a positive response. The relevance of an increased incidence of tumours at any site as a positive response is not clear. In several cases an overall increase in tumour incidence was noted as well as an increase in a specific tumour.

The response to the positive control was in general consistent and clear. Total tumour incidence was higher than in wild-type, with a high incidence of forestomach papillomas and carcinomas in transgenes compared with wild-type, while the incidence of lung tumours was higher in wild-type compared with transgenes. This seems to contradict the suggestion that the model is particularly useful for studies on lung tumour development.

4.6 Regulatory acceptance of TgrasH2

Overall, it may be concluded that the TgrasH2 model is acceptable for regulatory purposes as a medium term test to supplement the long term assay and to be used in a weight of evidence approach for the assessment of carcinogenic potential. The mechanism of tumourigenesis in the model needs to be further investigated.

5 TG.AC

5.1 Introduction

The TG.AC mouse is one of the transgenic mouse lines created in the FVB/N mouse strain by pronuclear injection of a v-Ha-ras oncogene flanked 5' by a mouse zeta-globin promoter and 3' by an SV-40 polyadenylation signal sequence. The transgene is located proximal to the centromere on chromosome 11 with up to 40 copies arrayed in tandem per allele. The v-Ha-ras oncogene confers on the TG.AC mouse the property of "genetically initiated" skin. Transgene expression cannot be detected in untreated skin. Activation of transgene expression results in tumour formation preferentially in the stratified epithelium. Topical application of promoters or complete carcinogens to the shaved back of TG.AC mice induces epidermal squamous cell papillomas or carcinomas.

To date, the induction of either spontaneous or chemically induced skin and forestomach tumours has been shown to require activation of transgene transcription. However, it is not known how the transgene is activated.

Not all chemicals are appropriate for skin administration. The identification of the forestomach epithelium as a potential second reporter site (forestomach squamous cell papillomas and carcinomas) provides an additional route of chemical exposure in TG.AC mice.

The analysis of tumours at the site of application (SOA) is the primary parameter for the determination of whether the response to a topically or orally administered compound is positive or negative. Not only the overall incidence of tumours in a particular treatment group is important, but also the number of papillomas per affected animal (tumour multiplicity). Other evaluation parameters, which can be used in topical studies, include the latency time (time to first tumour appearance) and time to maximal tumour yield.

5.2 General characteristics of the model

5.2.1 Spontaneous tumour incidences

The v-Ha-ras gene confers on TG.AC mice some rare spontaneous tumours not seen in FVB/N mice. Most conspicuous are tooth tumours and squamous cell papillomas at sites of chronic grooming. At the age of 1 year, almost all TG.AC mice exhibit small skin papillomas, thought to be induced by abrasion, in areas of chronic grooming such as the nose, ears, paws, lips and in the external peri-urogenital and anal areas. TG.AC control data (organ sites with lesions occurring >1%) from the ILSI/HESI studies and from the published literature are given in the table below. Initial TG.AC mouse sudies in the ILSI/HESI project used homozygous animals, whereas later studies used hemizygous mice.

TG.AC control tumour data (untreated or negative control mice, 26-week-studies)

TUMOUR	Incidence (%)					
	Homozygous (ILSI/HESI)		Hemizygous (ILSI/HESI		Hemizygous (literature)	
	males	females	males	females	males	females
Forestomach papilloma	80/178 (45%)	90/180 (50%)	13/60 (22%)	15/59 (25%)	12/162 (7.3%)	19/185 (10.3%)
Tooth tumour, combined	46/178 (26%)	50/180 (28%)	16/60 (27%)	11/60 (18%)	21/162 (13%)	32/185 (17.3%)
Skin, combined	23/178 (13%)	31/180 (17%)	0/60 (0%)	1/60 (2%)	6/162 (3.7%)	7/185 (3.8%)
Vagina/vulva/ ano-urogenital	0/178 (0%)	4/180 (2%)	1/60 (2%)	2/60 (3%)	not recorded	not recorded
Erythroleukemia	5/178 (3%)	6/180 (3%)	5/60 (8%)	4/60 (7%)	1/162 (0.6%)	4/185 (2.2%)

TUMOUR	Incidence (%)					
	Homozygous (ILSI/HESI)		Hemizygous (ILSI/HESI		Hemizygous (literature)	
	males	females	males	females	males	females
Salivary gland carcinoma	5/178 (3%)	3/180 (2%)	1/60 (2%)	0/60 (0%)	2/162 (1.2%)	4/185 (2.2%)
Lung AB adenoma/ carcinoma	2/177 (1%)	4/180 (2%)	3/60 (5%)	1/60 (2%)	7/165 (4.3%)	4/185 (2.2%)
Lymphoma	3/178 (2%)	1/180 (1%)	1/60 (2%)	0/60 (0%)	0/165 (0%)	2/185 (1.1%)

Although there seems to be a good correlation between organ sites and tumours in the ILSI/HESI studies and other published data, much higher incidences of forestomach tumours, particularly in homozygous mice, were reported in the ILSI/HESI studies. The higher incidence of forestomach tumours in homozygous mice probably reflect a "gene dosage" effect. However, the multiplicity of forestomach tumours in untreated or negative control TG.AC mice is stated to be low; usually only one or two papillomas were observed. Skin tumours were also more common in homozygous mice than in hemizygous mice (again probably due to a "gene dosage" effect). As with forestomach papillomas, the multiplicity of skin papillomas in control TG.AC mice in the ILSI/HESI studies is stated to be low, usually only one papilloma per mouse, which appeared toward the end of the 26-week studies. There are no indications that the "reporter sites" in TG.AC mice (skin and forestomach) are important tumour sites in wild-type FVB/N mice.

5.2.2 Performance of positive control compounds

Positive control groups treated with TPA (12-O-tetradecanoyl-phorbol-13-acetate, 1.25 or 2.5 µg per animal 3 times/week) were included in most topical ILSI/HESI studies. DMVC (dimethylvinyl chloride, 100 mg/kg 5 days/week) was used as a positive control agent in about half of the oral (gavage or dietary) studies. Both positive controls induced relatively robust papilloma responses within 26 weeks. With respect to the response to TPA, hemizygous mice developed skin papillomas at an earlier time, but the final 26-week incidence of papillomas was higher in homozygous mice (response in hemizygous mice 33-100%, in homozygous mice 60-100%). The response to DMVC was 53-100% in hemi- and 78-100% in homozygous mice.

5.3 Overview and discussion of the results

Of the 21 compounds selected by ILSI/HESI, 13 were studied after topical administration (accepting ethinyl estradiol as a "substitute" for 17β-estradiol, 14/21 substances were studied dermally). For 12 of these 14 compounds, the oral (gavage or diet) response was also studied. For the following ILSI/HESI substances no TG.AC data are available: dieldrin, haloperidol, chlorpromazine, chloroform, metaproterenol, mannitol, ampicillin.

Human genotoxic carcinogens

Based on SOA papilloma induction, none of the three studied human genotoxic carcinogens (phenacetin, cyclophosphamide, melphalan) was positive in topical studies and only one of them (melphalan) induced forestomach papillomas after oral administration.

Human immunosuppressive carcinogen

Cyclosporin A induced SOA papillomas when administered topically, but failed to do so when given in the diet.

Human hormone carcinogens

Both diethylstilbestrol and ethinyl estradiol induced skin papillomas when administered topically, but did not induce forestomach papillomas after i.g. administration.

Non-genotoxic rodent carcinogens, human non-carcinogens (epidemiology).

Only one of the three compounds studied topically, clofibrate, induced skin papillomas. Topical reserpine and methapyrilene did not induce skin papillomas. Only reserpine was studied orally and did not induce SOA tumours.

Non-genotoxic rodent carcinogens, human non-carcinogens (mechanism)

None of the three studied substances (WY-14643, DEHP, sulfamethoxazole) induced SOA tumours at either dermal or oral administration.

Non-genotoxic non-carcinogen

Only sulfisoxazole was studied. No papillomas were found after dermal or i.g. administration.

With one exception (topical clofibrate) TG.AC mice did not respond to non-genotoxic rodent carcinogens that are putative human non-carcinogens or to the single non-genotoxic non-carcinogen that was studied. The TG.AC response to human carcinogens (genotoxic, immunosuppressive or hormonal) is somewhat confusing and, in the case of genotoxic compounds, unexpected. A clear SOA positive response was seen only with topical cyclosporin, topical diethylstilbestrol, topical ethinyl estradiol and i.g. melphalan. The most surprising result is the failure of TG.AC mice to respond with SOA tumours to genotoxic human carcinogens administered dermally (phenacetin, cyclophosphamide, melphalan) or orally (phenacetin, cyclophosphamide).

For the majority of the compounds the results were evaluated as negative or positive with respect to SOA tumours. However, several substances were evaluated as equivocal or positive (by the TG.AC Assay Working Group) due to the induction of tumours at other sites than the SOA:

- Cyclophosphamide, topical, equivocal (dose-related increase in skin tumours at other sites, no SOA tumours)
- Cyclophosphamide, i.g., positive (dose-related increase in vulva papillomas, no SOA tumours)
- Melphalan, topical, equivocal (dose-related increase in vulva papillomas, no SOA tumours)
- Melphalan, i.g., positive (increased incidences of skin tumours at other sites including vulva papillomas (dose-related), dose-related increased incidence of lung tumours in males, also SOA tumours)
- Cyclosporin A, dietary, equivocal (skin tumours at other sites, lymphoma/leukemia, no SOA tumours)
- WY-14643, dietary, equivocal (hepatocellular adenoma, no SOA tumours)

The criteria for evaluating a non-SOA response as ‘positive’ were not defined. It is not clear to which extent tumours at other sites than the SOA are related to the activation of transgene expression, i.e. a result of the particular genetics of the TG.AC mouse. In the case of skin tumours at "non-SOA", these generally occurred at sites of chronic grooming, indicating that the test substance shortened the latency of spontaneous skin tumours in the TG.AC mouse. In untreated TG.AC mice, these spontaneous skin tumours usually appear late in life and would seem to be preceded by activation of the transgene.

Since a few studies have been conducted with the oral route of administration, the ILSI/HESI-studies contribute to the evaluation of the forestomach as reporter site. No SOA (forestomach papillomas) were observed in oral studies with the single non-genotoxic/non-carcinogenic substance studied. No non-genotoxic rodent carcinogens (putative human non-carcinogens) gave a positive result with respect to SOA tumours. The two human hormone carcinogens gave a negative forestomach response although topical studies were positive. Only one human genotoxic carcinogen (melphalan) induced a positive SOA response. All other human carcinogens were negative as regards SOA tumours, although orally administered cyclophosphamide and cyclosporin were evaluated as "positive" by the TG.AC Assay Working Group due to the induction of "non-SOA" tumours.

High incidences of forestomach tumours were often seen in negative control mice in oral studies. Possibly, these high control incidences may have obscured a positive result. However, it was stated that the multiplicity of forestomach tumours in negative controls was low.

5.4 Data from non-ILSI/HESI studies

A relatively large number of other substances have been studied in TG.AC mice. Generally, a positive response was seen after topical administration of genotoxic carcinogens, such as benzene, p-cresidine and 7,12-DMBA, whereas most topically tested genotoxic non-carcinogens were negative (e.g. 2-

chloroethanol, p-anisidine, 8-hydroxyquinoline, 1-chloro-2-propanol and 2,6-diaminotoluene). Both positive (e.g. TCDD and pentachlorophenol), and negative (e.g. ethyl acrylate and pyridine) responses have been reported for non-genotoxic carcinogens. Topical administration of non-genotoxic non-carcinogens (e.g. phenol and acetone) did not induce skin tumours in TG.AC mice.

5.5 Conclusions

The purpose of all carcinogenicity bioassays is to identify potential human cancer hazards. However, no consistent, positive result was seen with the human carcinogens selected by ILSI/HESI. A positive SOA response to human carcinogens was limited to melphalan-induced forestomach papillomas in males at oral administration and skin papillomas in response to topical cyclosporin (females), ethinyl estradiol (both sexes) and diethylstilbestrol (both sexes). According to the TG.AC Assay Working Group review decisions, several more ILSI/HESI studies with human carcinogens, both dermal and oral, were evaluated as equivocal or even positive due to "non-SOA" responses. This may be regarded either as a sign of "post hoc rationalisation" of generated results or, alternatively, that there is a clear need for a better definition of what constitutes a positive response in the TG.AC model.

In the case of well-established rodent carcinogens, which for different reasons are considered putative human non-carcinogens, the TG.AC model was acceptably successful, since it generated only one "false positive" result (topical clofibrate) and one "false equivocal" result (dietary WY-14643). Dietary WY-14643 induced liver adenomas in HD male TG.AC mice, a response that was, however, demonstrated to be independent of transgene activation.

5.6 Regulatory acceptance

The TG.AC reacted inconsistently and incompletely to known human carcinogens. Although developed to be responsive at the site of application (skin), the evaluation of the dermal studies with human carcinogens included responses also at other sites, in order to "suit" the profile of the compound. Nevertheless, the model may be considered to be useful for screening the carcinogenic properties of dermally administered pharmaceuticals. The TG.AC model cannot be recommended for oral studies with the forestomach as "reporter site".

6 XPA-/- KNOCKOUT MICE

6.1 Introduction

XPA-/- mice (C57Bl/6J-TgH(XPAIm)55CCmg) are almost completely nucleotide excision repair (NER)-deficient (2 identical null allelles with a deletion spanning exons 3 and 4). They are therefore expected to respond selectively to genotoxic carcinogens (or more exactly to those compounds that induce DNA lesions that are linked to the NER pathway).

6.2 General characteristics of the model

6.2.1 Spontaneous tumor incidences

The spontaneous tumor incidences in XPA-/- mice after the 9-month experimental period was 5% in females (n = 178) and 7% in males (n = 180). The most common spontaneous tumours (> 1%) appear to be lymphoma (females), bronchioalveolar adenoma (males), adrenocortical adenoma (males) and adrenal phaeochromocytoma (females). The spontaneous incidence and spectrum are comparable to those of wild-type C57BL/6 mice and is considered to be acceptably low.

6.2.2 Performance of positive control compounds

The three positive controls used, BaP (benzo(a)pyrene) (4 studies), p-cresidine (3 studies), and 2-AAF (2-acetylaminofluorene) (1 study) gave positive responses with the exception of p-cresidine, which was negative in 1 out of 3 studies. Compared with other models, such as p53+/- or TgrasH2, there is only limited experience with positive controls in the XPA-/- model. Moreover, p-cresidine and, in particular, BaP were found to be severely toxic in XPA-/- mice which may adversely affect the outcome of the positive control. The XPA-/- Assay Working Group therefore recommends 2-AAF as positive control for future studies, although insufficient experience is available. Overall, an appropriate positive control for the XPA-/- model is yet to be identified.

6.3 Overview and discussion of the results

Of the 21 ILSI/HESI compounds, 13 were tested in the XPA-/- model. The only genotoxic carcinogen tested, phenacetin, was found to be negative. Positive tumor responses were observed for three compounds, the immunosuppressant cyclosporin A, the hormone carcinogen DES, and the peroxisome proliferator WY-14,643, all classified by ILSI/HESI as non-genotoxic. Negative results were obtained with seven other non-genotoxic rodent carcinogens (estradiol, phenobarbital, clofibrate, reserpine, haloperidol, DEHP, sulfamethoxazole), and two non-genotoxic non-carcinogens (ampicillin, D-mannitol).

In light of the expectations related to the underlying mode of action of the XPA-/- model (responsiveness to genotoxic carcingens only) as well as the expectations related to the classification of the selected compounds (genotoxic vs. non-genotoxic), the outcome of the ILSI/HESI studies with the XPA-/- model is somewhat confusing. There was no correct positive result, one false-negative (phenacetin), 3 false positives (non-genotoxic human and/or rodent carcinogens) and 9 correct-negatives for non-genotoxic compounds. The discussion of the "false" responses by the XPA-/- Assay Working Group focussed on two possible explanations: (1) misclassification of some of the validation compounds (genotoxicity of phenacetin?, non-genotoxicity of DES, WY 14,643?) or (2) that mechanisms others than genotoxicity may be involved in XPA-/- mouse tumor induction. This discussion highlights the main problems that hamper a sound evaluation of the XPA-/- model, i.e. deficiencies in selecting appropriate test compounds (some of the results need a "post-hoc rationalisation") as well as insufficient knowledge of the true biological nature of the model including the carcinogenic mechanisms involved. Both make it difficult to put the data into a proper perspective and to definitely define the potential selectivity of the XPA-/- model for genotoxic compounds. Nevertheless, the available data suggest that XPA-/- mice respond to human carcinogens (irrespective of their mechanism of action), whereas the majority of rodent carcinogens classified as putative human non-carcinogens were negative. The data thus appear to indicate that this assay provides more relevant and targeted data compared to the standard 2-year mouse bioassay.

An issue of concern is the fact that several positive findings (even with positive controls) were defined on the basis of the "rare tumor criteria" only. This is considered to be a less certain/reliable criteria compared to statistical significance (in combination with dose-response), particularly when considering the relatively limited historical control data on which the definition of a rare tumour is based. In order to get more clear-cut results and to develop greater confidence in a positive finding it may be advisable to increase the group size.

In addition, the use of wild-type animals (control and high-dose group) is highly recommended. The comparison of tumour findings in wild-type and XPA-/- mice will indicate whether the observed effects are linked to the DNA repair deficiency, which, in turn, may provide additional information of value for understanding the mechanism of action.

6.4 Data from non-ILSI/HESI studies

Some data are available from studies that were not conducted as part of the ILSI/HESI project. Positive tumour responses were obtained with the well-known genotoxic carcinogens UVB (dermal), 7,12-DMBA (topical), and PhIP and a negative response with a non-carcinogen (p-anisidine). It should be noted, however, that the protocols used in some of these studies differed considerably from the standard protocol used in the ILSI/HESI project.

6.5 Regulatory acceptance

The ILSI/HESI database with the XPA-/- model is too limited (only 13 of 21 ILSI/HESI compounds tested, none was tested in duplicate) to draw a final conclusion on the usefulness of the model. In particular, two of the three genotoxic human carcinogens, mephalan and cyclophosphamide, have not been tested. Although the model appears to be promising, the overall set of data is insufficient and therefore the use for regulatory purposes cannot (yet) be recommended.

7 XPA-/-/P53+/- DOUBLE KNOCKOUT MICE

7.1 Introduction

XPA-/- /p53+/- mice are DNA repair (NER)- deficient and carry only one functioning p53 gene. These mice are expected to respond selectively to genotoxic carcinogens and to display an enhanced tumour response as compared to XPA-/- mice.

7.2 General characteristics of the model

7.2.1 Spontaneous tumor incidences

The spontaneous tumour incidences in XPA/p53 mice after the 9-month experimental period was 13% in females (n = 132) and 9% in males (n = 134). This background incidence is higher than in XPA-/- mice but is still in an acceptable range. Some tumour types additional to those seen in XPA-/-, such as the sarcomas related to the p53+/- genotype, were noted.

7.2.2 Performance of positive control compounds

The three positive controls used, BaP (3 studies), p-cresidine (2 studies), and 2-AAF (1 study) gave consistently positive responses. However, the low numbers of studies indicate that there is not much experience with positive controls. The reliability of these compounds as proper positive controls with good reproducibility of clear positive effects still has to be determined.

7.3 Overview and discussion of the results

Of the 21 ILSI/HESI compounds, only 10 were tested in the XPA/p53 model. The only genotoxic carcinogen tested, phenacetin, was found negative. Positive tumor responses were observed for 3 compounds, the immunosuppressant cyclosporin A, the hormone carcinogens DES and estradiol (males), all classified by ILSI/HESI as non-genotoxic. Negative results were obtained with 5 non-genotoxic rodent carcinogens (phenobarbital, reserpine, haloperidol, DEHP, sulfamethoxazole), and one non-genotoxic non-carcinogens (D-mannitol).

Results with XPA/p53 mice show a quite similar pattern of sensitivity/specificity as XPA-/- mice. The XPA/p53 model gave in general a more robust response (higher tumour incidences) than the XPA-/- model indicating a higher sensitivity, which most likely is due to a synergistic effect of the double knock-out. The only qualitative difference was found with estradiol (negative in XPA-/- mice but positive in the double knock-out mice). This finding further confirms the higher (over?)sensitivity of the XPA/p53 model as compared to the XPA-/- model. As already discussed for the XPA-/- assay data, the positive results with the putative non-genotoxic carcinogens are difficult to interpret, but may indicate that neither model distinguishes definitely between genotoxic and non-genotoxic carcinogens.

7.4 Data from non-ILSI/HESI studies

There are no other data available.

7.5 Regulatory acceptance

The database with the XPA/p53 model is very limited. Only 10 of the 21 ILSI/HESI compounds were tested and none of the compounds was tested in duplicate. The limited use of the model inevitably results in a small historical control database, which may increase the risk of misclassification of "rare tumors" used as criteria for determination of a positive result. Moreover, in almost all XPA/p53 studies only one (high) dose group (instead of 3 groups in all other ILSI/HESI studies) was used, which further limits the experiences. Compared to the other models, the XPA/p53 model gives some (theoretical) concern with respect to exaggerated sensitivity (synergism of the 2 genes affected and/or prolonged study duration of 9 months). The positive result with estradiol may be interpreted as due to exaggerated sensitivity, but due to the limited database this issue cannot be resolved.

Overall, the use of the XPA/p53 model for regulatory testing purposes is considered premature and cannot be recommended at present. Further studies, in particular with weak genotoxic or non-genotoxic carcinogens, are required in order to increase the (historical) data base and to get a better understanding of the sensitivity, specificity and reproducibility of the assay.