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The transgenic mouse assay as an alternative test method for regulatory carcinogenicity studies—Implications for REACH

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ABSTRACT

REACH, an EU regulation that requires the submission of safety data in support of the protection of human and environmental health, mandates that registration should be achieved with the minimum amount of animal testing possible. Under REACH, a two-year carcinogenicity assay may be required for certain chemicals produced at >1000 metric tonnes per year. In addition, some chemicals that are found to be genotoxic will also require testing. Alternative methods have been explored in an attempt to improve the predictivity of this bioassay as well as to reduce the number of animals used for such testing. This research has focused on the use of transgenic/knockout mouse models. Study results from selected models indicate that they are useful in hazard identification, even if they are not entirely suitable for risk assessment on their own. Carcinogenic hazard assessment can be greatly enhanced and animal use reduced if the traditional two-year rat bioassay is combined with a well conducted transgenic mouse assay. Importantly, the use of transgenic animals to supplement a traditional two-year carcinogenicity study may help reduce the number of false negatives, one of the unstated goals of REACH via the precautionary principle.

1. Introduction

Under REACH, the EU initiative that requires the Registration, Evaluation, Authorization and Restriction of Chemicals, the need for a two-year carcinogenicity assay for chemicals produced at >1000 metric tonnes per year will be determined on a case-by-case basis. For each of these high production volume (HPV) chemicals, widespread use and frequent or prolonged human exposure combined with evidence of mutagenicity or induction of hyperplastic/preneoplastic lesions in repeat-dose toxicity studies will trigger the need for an assessment of carcinogenic potential (European Union, 2007). Because REACH mandates that registration should be achieved with the minimum amount of animal testing possible, all available information that can reasonably inform this assessment—from (Q)SAR models, grouping and read-across predictions to *in vitro* studies such as cell transformation assays to short- or medium-term *in vivo* assays such as the repeat-dose toxicity study and the neonatal mouse model—are to be evaluated prior to conducting new studies. If the available information suggests the potential for carcinogenicity, and if a two-year study is not available through any members of the Substance Information Exchange Forum (SIEF), the registration dossier must include a testing proposal drafted by the group to fill this data gap. The two-year carcinogenicity assay is to be performed only if no other options for fulfilling the data requirement are available.

REACH also requires mutagenicity testing for all chemicals manufactured or imported in quantities greater than 1 tonne per year. Those that are identified as Class 1 or Class 2 mutagens (substances known to or presumed to cause heritable genetic defects in humans, respectively) will not require carcinogenicity testing, regardless of the tonnage produced, while HPV chemicals that are classified as Class 3 mutagens (substances for which there is insufficient evidence to be classified as Category 2 mutagens) may be subjected to such testing (European Union, 2007).

The REACH Regulation mandates registration of all phase-in HPV chemicals (i.e., substances manufactured or imported in quantities greater than 1000 metric tonnes per year) by 1 December 2010. Draft decisions from the European Chemicals Agency (ECHA; the regulatory body responsible for administering REACH) on testing proposals for these chemicals are to be prepared by 1 December 2012.

2. The two-year rodent carcinogenicity bioassay

Recently, the European Council adopted the "REACH Test Methods Regulation" (Council Regulation (EC) 440/2008), which pre-

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sents the testing guidelines for studies to be used to fulfill information requirements (European Union, 2008). Generally, these protocols are similar or identical to established OECD methods. The guidelines for carcinogenicity testing of chemicals as set forth in OECD Test Method 451 indicates that "a compound of unknown activity should be tested on two animal species", and that "only negative findings in all species tested (at least two) can be regarded as adequate negative evidence" (OECD, 1981). However, no such declaration is made in the REACH Test Methods Regulation in terms of carcinogenicity (Sections B.32 and B.33).

The two-year rodent carcinogenicity bioassay is generally conducted with at least 400 animals per assay, with rats and mice being the preferred species for the assays (OECD, 1981). The guidelines presented by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) indicate that of the two carcinogenicity assays that it requires, only one traditional rodent bioassay is obligatory. The second traditional bioassay may be replaced by an alternative approach to hazard identification, such as a short- or medium-term *in vivo* rodent assay in transgenic or neonatal mice (ICH, 1997). The US Food and Drug Administration (FDA) has listed this and other the ICH Guidelines concerning carcinogenicity testing on its website as "Guidance for Industry" (www.fda.gov, 2008). The European Medicines Agency (EMEA) also lists these ICH guidelines on its web site under "Scientific Guidelines for Human Medicinal Products" (www.emea.europa.eu, 2008).

ICH stipulates that in the absence of clear evidence favoring one species, the rat should be the species of choice for the primary bioassay performed for the assessment of carcinogenicity. The Agricultural Chemical Safety Assessment (ACSA) Technical Committee of the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) has proposed a tiered testing approach to systemic safety testing of agricultural chemicals in which it recommends eliminating the mouse carcinogenicity study from the list of bioassays performed (Doe et al., 2006a). The rationale behind this recommendation is that the mouse study adds very little to the knowledge of the compound and that a cost/benefit analysis in terms of animal welfare would not support its use (Doe et al., 2006b). In contrast, REACH legislation gives no species preference for the one traditional carcinogenicity bioassay that it recommends for chemicals requiring such evaluation.

The European Commission Joint Research Commission (JRC) published an addendum to a 2003 paper (Pederson et al., 2003) that calculated the approximate costs of animal testing under REACH (van der Jagt et al., 2004). The addendum estimates the number of animals that will be required to implement the REACH Regulation as well as the potential "savings" of animals if methods such as (Q)SAR, risk based testing and intelligent testing strategies are employed. It acknowledges that the impact of these strategies is reduced in cases where no alternative methods exist for certain endpoints where the bioassay requires the use of large numbers of animals, citing two-generation reproductive toxicity, *in vivo* mutagenicity and developmental toxicity studies as examples. It indicates that these three types of studies will account for 72% of the animals required for testing under REACH. However, the addendum does not discuss the two-year carcinogenicity bioassay in this regard.

The JRC specifically mentions that developmental toxicity studies are assumed to be performed in two species, but does not indicate that the same is true for carcinogenicity studies. REACH legislation refers only to "a" carcinogenicity study in Annex X, Section 8.9.1 (European Union, 2007); the flow chart that presents the integrated testing strategy for carcinogenicity also indicates that under the appropriate circumstances, "a" classical two-year study be considered (ECHA-1, 2008).

The JRC estimates that less than 10% of all currently existing chemicals will require new or supplemental carcinogenicity testing (van der Jagt et al., 2004). However, it estimates that 22% of all chemicals will require "further" mutagenicity testing, meaning that preliminary *in vitro* tests have produced results that trigger an *in vivo* mutagenicity test. If this test is positive, then carcinogenicity testing for the chemical may be required (ECHA-1, 2008). The current JRC document does not appear to take this additional potential testing requirement into account in the calculation of the figures that it presents.

In contrast to the <10% estimate for carcinogenicity testing proposed by JRC, a Business Impact Study on REACH commissioned by the European Commission and performed by RPA & Statistics Sweden estimates that of approximately 2700 HPV chemicals, 50% will require carcinogenicity testing (Pederson et al., 2003). In a theoretical extrapolation of the maximum number of animals that might be required for carcinogenicity testing under REACH (assuming 2600 chemicals tested and no reduction of animal use as mandated by REACH), the Bundesinstitut für Risikoberwertung or BfR (the Federal Institute for Risk Assessment in Germany) estimates that as many as 1,040,000 animals could be used for this endpoint alone (Höfer et al., 2004).

The European Chemicals Bureau estimated that of 2465 HPV chemicals in the International Uniform Chemical Information Database (IUCLID) as of December 1998, 14% had base-set hazard information in the data base, 65% had less than base-set information, and 21% had no information at all (Allanou et al., 2003). Information on the availability of carcinogenicity data, which are not part of base-set data, was not included in this report. The figure of 2465 chemicals is comparable to those estimated by the BfR and by RPA & Statistics Sweden. The current HPV chemicals list of the European Substances Information System contains 2782 substances (European Research Center, 2008).

The recent Guidance on Information Requirements and Chemical Safety Assessment (IR/CSA) recommends that chronic toxicity be investigated whenever a carcinogenicity study is conducted (ECHA-1, 2008). To follow the OECD guideline for combined chronic toxicity/carcinogenicity studies (OECD 453), this will require additional animal use—at least 20 animals per sex for the high dose satellite group and 10 animals per sex for the control satellite group in addition to the 400 + animals needed for the traditional carcinogenicity assay. If one or more interim sacrifices are required by the study protocol, then additional animals must be allotted to these groups as well (OECD 453, 1981). While the IR/CSA Guidance documents indicate that the rat is preferred species for this bioassay, neither these documents nor the OECD guideline preclude the use of another species. Indeed, both documents go on to provide information on study duration and group survival rates for assays conducted in mice and hamsters.

3. Transgenic mouse models as alternatives to the two-year carcinogenicity bioassay

REACH legislation stipulates that "Implementation of this Regulation should be based on the use of alternative test methods, suitable for the assessment of health and environmental hazards of chemicals, wherever possible. The use of animals should be avoided by recourse to alternative methods validated by the Commission or international bodies, or recognized by the Commission or Agency as appropriate to meet the informational requirements under this Regulation". (European Union, 2007). According to the IR/CSA tiered strategy, the use of *in vitro* assays or alternative carcinogenicity protocols (including the use of transgenic animals) can be sufficient for fulfillment of the information requirements on carcinogenicity under REACH if such studies give adequate