15:00 Investigating DNA repair as the genotoxic threshold mechanism for DNA reactive compounds

George E. Johnson (Swansea University, U.K.)

15:30 – 16:00 ****** 休 憩 ******

16:00 – 17:30 セッション 3 座長 青木 康展, George S. Bailey

16:00 Enhanced genotoxicity under the phase II drug-metabolizing enzyme deficient condition

青木 康展（国立環境研究所）

16:30 Toxicity testing strategy based on the concept of the threshold of toxicological concern (TTC)

広瀬 明彦（国立医薬品食品衛生研究所・総合評価研究室）

17:00 閉会の辞

福島 昭治（日本バイオアッセイ研究センター）

Abstracts

Genotoxic thresholds: identification of mutations *in vivo* and mechanistic studies *in vitro*

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Currently, genotoxic carcinogens are regulated based on an assumption that carcinogens with genotoxic properties have no thresholds for their risk on humans. However, there are several issues to be discussed on the policy. The first is a practical issue: how to identify genotoxicity of chemicals. At present, bacterial reverse mutation assay (Ames test) is most widely used for identification of chemicals that interact with DNA and induce mutations. However, there is a gap between results of in vitro and in vivo mutagenicity tests. The second is a mechanistic issue. Organisms including humans possess defense mechanisms against toxic compounds including genotoxic chemicals. Therefore, the defense mechanisms may reduce the level of mutations induced by the chemicals to the level of spontaneous mutations, thereby generating "practical thresholds" for genotoxic compounds. We address these two issues by in vivo and in vitro approaches.

In in vivo studies, we have established transgenic mice and rats, i.e., gpt delta mice and rats, for identification of mutagenicity of chemicals in targets organs of carcinogens. These transgenic rodents allow to detect mutagenicity of chemicals in any organs of mice and rats and the mutations can be identified at sequence levels. We present basic features of gpt delta mice (C57BL/6J background) and rats (Sprague Dawley and Fischer 344 background), and discuss their significance in regulatory toxicology.

In in vitro studies, we have established human cells expressing genetically modified specialized DNA polymerases (Pol), i.e., Pol ζ and Pol κ, involved in translesion DNA synthesis (TLS).TLS is a process where Pols continue DNA synthesis across DNA lesions. The process may be error free or error prone. In either case, TLS rescues damaged cells from genotoxic effects of chemicals by continuing chromosome replication. The established human cells expressing modified specialized DNA polymerases displayed different sensitivity to mutagenicity and cytotoxicity of chemical carcinogens such as benzo[a]pyrene diolepoxide or hydrogen peroxide. We are examining the possibility whether TLS is involved in generation of "practical thresholds" against genotoxic carcinogens. In addition, we report an endeavor to introduce single DNA adduct, i.e., 8-oxo-7,8-dihydro-2'-deoxyguanosine , into a specific site of human chromosome. These experiments could answer the question of whether formation of DNA adducts in the chromosome inevitably leads to induction of mutations or not.

Threshold of genotoxic carcinogens: it is central concerns of carcinogenic risk assessment

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The presence or absence of a carcinogenic threshold will determine the reliability of risk assessment of chemical carcinogen when extrapolated from high dose rodent testing. Therefore, it is essential to verify scientifically whether the non-threshold concept is valid, especially for genotoxic carcinogens. Herein, we present low-dose carcinogenicity data based on medium-term rat liver bioassays for 3 genotoxic heptocarcinogens: 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 2-amino-3-methylimidazo [4,5-f]quinoline (IQ), heterocyclic amines contained in seared meat and fish; N-nitrosodiethylamine (DEN), a N-nitrosocompound synthesized in the stomach through the reaction of secondary amines and nitrites. Very low doses of MeIQx induced formation of DNA-MeIQx adducts; somewhat higher doses caused elevation of 8-hydroxy-2'-deoxyquanosine (8-OHdG) levels, gene mutations and initiation activity; and the more higher dose induced formation of glutathione S-transferase placental form (GST-P) positive foci in the liver, a well-known preneoplastic lesion marker in rat hepatocarcinogenesis. Similarly, only the higher doses of IQ caused an increase in the number of GST-P positive foci in the liver, the lower doses had no effect. Furthermore, the finding that $p21^{Cip/WAF1}$ was significantly induced in the liver at doses well below those required for IQ mediated carcinogenic effects, suggests that induction of $p21^{Cip/WAF1}$ is one of the mechanisms responsible for no-effect doses for IQ carcinogenicity. We also demonstrated that low doses of DEN did not induce either GST-P positive foci formation or gene mutation in the liver. Moreover, concurrent administration of low doses of MeIQx with DEN that had no effects on GST-P positive foci formation still did not increase the GST-P positive foci formation compared to the MeIQx alone, DEN alone and non-treatment control groups, while concurrent administration of high dose of MeIQx with DEN showed at least an additive effect. Based on the above findings of existence of no-effect doses for markers that cells typically acquire as they move through the initiation and promotion stages of carcinogenesis, we argue strongly for the existence of a threshold, at least a practical threshold, for the carcinogenic effects of these three genotoxic carcinogens in the rat.

Lessons learned from 40,000-animal cancer dose-response studies

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We have conducted two 40,000-animal cancer dose-response studies, one with dibenzo(def,p)chrysene (DBC, formerly called dibenzo(a,l)pyrene DBP) and a more recent study with aflatoxin B_1 (AFB_1). These experiments used rainbow trout, an animal model well suited to ultra low-dose carcinogenesis research, to explore dose-response down to a targeted 10 excess liver tumors per 10,000 animals (ED_{001}). In study one, 42,000 trout were fed 0-225 ppm DBC for four weeks, sampled for biomarker analyses, and returned to control diet for nine months prior to gross and histologic examination. Suspect tumors were confirmed by pathology, and resulting incidences were modeled and compared to the default EPA LED_{10} linear extrapolation method. The study provided observed incidence data down to 2 above-background liver tumors per 10,000 animals at lowest dose (that is, an un-modeled ED_{0002} measurement). Among nine statistical models explored, three were determined to fit the liver data well - linear probit, quadratic logit, and Ryzin-Rai. None of these fitted models is compatible with the LED_{10} default assumption, and all fell increasingly below the default extrapolation with decreasing DBC dose. Low-dose tumor response was also not predictable from hepatic DBC-DNA adduct biomarkers, which accumulated as a power function of dose (adducts = $100*DBC^{1.31}$). Two-order extrapolations below the modeled tumor data predicted DBC doses producing one excess cancer per million individuals ($ED_{10\text{-}6}$) that were 500-1500-fold higher than that predicted by the five-order LED10 extrapolation. Study two was of similar design, but using AFB_1. Analysis of the results is underway, and complicated by several differences from study 1, especially presence in some quartiles and treatment groups of a fatty liver syndrome. Preliminary logistic regression analysis excluding fish with this syndrome did not support the EPA linear default assumption (i.e., logistic slope 1.0), rather indicated a sublinear dose-response with slope of 1.42 (95% CI 1.23 – 1.61), and an extrapolated $ED_{10\text{-}6}$ that is 32-fold greater than the LED_{10} default extrapolation. Inclusion of all fish also yielded a sublinear dose-response, with slope 1.31 (95%CI 1.13 – 1.50), and an extrapolated $ED_{10\text{-}6}$ 17-fold greater than the LED_{10} default extrapolation. Thus two genotoxins with differing biological properties yielded ultra-low dose-response curves in the same animal model that are not compatible with the linear default assumption. These results are considered specific to the animal model, carcinogen, and protocol used. They provide the first experimental estimations in any model of the degree of conservatism that may exist for the EPA default linear assumption for a genotoxic carcinogen.

Urinary Bladder Carcinogenesis by DNA Reactive and Non-Reactive Chemicals: Non-linearity's and Thresholds

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Cancer is due to multiple alterations of DNA occurring in a single stem cell. Chemicals can increase cancer risk by directly damaging DNA (DNA reactivity) or by increasing cell proliferation (DNA replications), increasing the number of opportunities for spontaneous DNA damage. Both types of chemicals have been shown to induce urinary bladder cancer in animal models and in humans. For DNA reactive carcinogens, such as aromatic amines like 4-aminobiphenyl (ABP), the dose response for carcinogenesis is non-linear because of the interaction of DNA reactive effects and cytotoxicity with regenerative proliferation at higher doses. The synergy between DNA reactivity and cell replication occurs commonly, such as in response to cigarette smoking. The DNA reactive effect operates through formation of DNA adducts, and the dose response can be linear or non-linear, depending on metabolic activation processes. For DNA reactive carcinogens, the distinction between threshold and level of detection in the assay system needs to be distinguished. In contrast, for non-DNA reactive carcinogens, a threshold is present. Increased cell proliferation can occur either by cytotoxicity and regeneration, by direct mitogenesis, or by decreasing cell death (e.g. inhibiting apoptosis or differentiation). In animal models, cytotoxicity can be produced by formation of urinary solids or generation of reactive metabolites which are excreted and concentrated in the urine. In humans, arsenic is an example of a bladder carcinogen which acts by formation of reactive metabolites. The threshold is dependent on the presence in the urine of a cytotoxic concentration of the metabolite(s). A defined true threshold is involved in the formation of the urinary solids, dependent on the physical-chemical property of solubility. Melamine is such an example. DNA reactive carcinogens have a non-linear dose response with respect to carcinogenicity and frequently have non-linear responses for DNA effects due to competing metabolic and repair processes, some of which are saturable. In contrast, non-DNA reactive carcinogens induce cancer as a consequence of a precursor toxic biologic effects which have thresholds.

A threshold for the murine T-cell lymphoma induction by N-ethyl-N-nitrosourea and/or radiation.

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Current strategy for estimating the risks of genotoxic substances at low-dose is a linear extrapolation from the effects observed at high doses. Since cancer is a genetic disease with stepwise accumulation of multiple mutations, it is generally considered that genotoxic carcinogens have no threshold in exerting their potential for cancer induction. However, a few recent animal studies reported the existence of a threshold for carcinogenicities. We previously observed that there existed a threshold for the N-ethyl-N-nitrosourea (ENU) and fractionated X-rays in induction of T-cell lymphoma in B6C3F1 mice, and that its magnitude was modified by their combined exposure; threshold for ENU was reduced by co-exposure with sub-carcinogenic dose of X rays.

In order to determine the contribution of mutation induction to threshold dose of ENU lymphomagenesis, we examined the mutation frequency and its spectra in the thymic cells, focusing on the point mutations, of B6C3F1 gpt-delta mice after exposure to ENU or the co-exposure with X rays. First, we found that ENU even below threshold dose for lymphomagenesis increased point mutation frequency significantly in a dose dependent manner, and that threshold dose for mutation induction was smaller than that for lymphoma development. However, mutant cells developed by ENU below threshold dose could progress into malignant lymphoma cells after co-exposure with sub-carcinogenic dose X rays, which may be ascribed to the expansion of mutant cells during the regeneration process. As a result, threshold dose of ENU lymphomagenesis decreased. In contrast, X irradiation below threshold dose for lymphomagenesis decreased the frequency of point mutations in cells of untreated cells, suggesting anti-mutagenic effect. X irradiation below threshold dose also reduced point mutation in the cells of ENU-treated mice, but that above threshold dose increased. This suggests that threshold of ENU mutagenesis could be influenced by the dose of co-exposed X rays.

In summary, threshold of ENU for lymphomagenesis is determined not by failure to induce mutation, but by the condition of mutant cells to progress into malignancy.

Exposure to ethylating agents: Where do the thresholds for mutagenic/clastogenic effects arise?

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The presence of EMS (ethyl methanesulfonate) in tablets of a HIV medication triggered non-clinical studies into the dose response for mutation analysis after chronic dosing. Although there is a multitude of in vitro and in vivo studies on the genotoxic activity of EMS, no lifetime carcinogenicity studies, repeat dose mutation data or exposure analysis are available to serve as solid basis for risk assessment. For alkylators like EMS it is generally assumed that the dose response for mutagenicity (and by default for carcinogenicity) is linear – indicating that no 'safe' dose does exist. A recent in vitro genotoxicity study provided evidence, however, that the dose response curve for mutagenic and clastogenic activity was thresholded. We sought to verify the existence of thresholds for mutagenic and clastogenic activity in vivo. Dose levels ranging from 1.25 to 260 mg/kg/day were applied for up to 28 days. The studies were further supported by in depth metabolism and exposure analyses and a general toxicity study in rats.

Our studies provided unambiguous evidence that daily doses of up to 25 mg/kg/day did not induce any increase of mutations in the lacZ gene in the three organs tested (bone marrow, liver, GI tract, liver) or of micronuclei in bone marrow. Only at higher dose levels the genotoxic activity of EMS became apparent. Toxicokinetic assessment of the threshold doses showed AUC and Cmax values which were orders of magnitude higher than the maximal exposure of the patients and, therefore, it could be concluded that the ingestion to the genotoxic contaminant did not confer any genotoxic/carcinogenic risk to the patients.

Further, these studies showed that ethylation of cellular molecules (proteins, DNA) increased approximately linear with dose. We calculated that each liver cell experienced 380'000 ethylations per day at the threshold dose, indicating that the absence of clastogenic/mutagenic effects up to this dose must be due to error-free repair of vast numbers of DNA lesions rather than scavenging of the reactive molecules prior to reaching the DNA target.

Our investigations unambiguously demonstrated thresholded dose relations for mutagenic/clastogenic effects by EMS but gave no evidence of a threshold after exposure to the ethylating agent ENU (ethylnitroso urea), which was included into our studies for correlation purposes. As this observation is conceptually difficult to interpret it was important that subsequent studies with in depth analysis of the very low dose region revealed the likely presence of a threshold also for ENU.

These findings have important implications for the risk assessment of low dose exposures to genotoxic agents, and should impact on impending new regulation, e.g. on the limitation of PGI's (potentially genotoxic impurities) in pharmaceuticals.

Oxidative stress-induced tumorigenesis in the small intestine of *Mutyh*-deficient mice: the effect of low-level exposure to $KBrO_3$

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Oxygen radicals are produced through normal cellular metabolism, and the formation of such radicals is further enhanced by exposure to either ionizing radiation or various chemicals. The oxygen radicals attack DNA and its precursor nucleotides, and consequently induce various oxidized forms of bases in DNA within normally growing cells. Among such modified bases, 8-oxo-7, 8-dihydroguanine (8-oxoG) and 2-hydroxyadenine (2-OH-A) are highly mutagenic lesions, if not repaired. MUTYH is a DNA glycosylase that excises adenine or 2-OH-A incorporated opposite either 8-oxoG or guanine, respectively, thus considered to prevent G:C to T:A transversions in mammalian cells. The Mutyh-deficient mice showed a marked predisposition to spontaneous tumorigenesis in various tissues when examined at 18 months of age. The incidence of adenoma/carcinoma in the intestine significantly increased in Mutyh-deficient mice, as compared with wild-type mice. This high susceptibility of the intestinal tumor-development was well correlated with the condition observed in MAP (MUTYH-associated polyposis) patient. The intestinal tumor susceptibility of Mutyh-deficient mice was further enhanced by treatment with $KBrO_3$, a known oxidative renal carcinogen associated with 8-oxo-G accumulations. Oral administration of $KBrO_3$ at a dose of 0.2% in drinking water dramatically increased the formation of intestinal tumors in Mutyh-deficient mice.

Using this experimental system, we have been investigating the tumorigenic effect of $KBrO_3$. With relevance to the assessment of health risks, the exposure to lower dose in the range of 0.05 % to 0.1% of $KBrO_3$ reduced the frequency of intestinal tumor formation in Mutyh-deficient mice. These results suggest that cells are able to correctly repair oxidative DNA lesions resulting from exposures to a certain level of low doses of endogenous and exogenous chemicals with oxidizing property, and thus are less likely to be transformed to the neoplastic phenotype.

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How do thresholds for mutagenicity and clastogenicity arise for DNA damaging agents?

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There has been a recent shift by the scientific and regulatory community, towards accepting genotoxic thresholds. Nevertheless, there are still many unanswered questions. Such as, what is the biological basis for thresholded responses to genotoxic agents? The mechanisms responsible for 'genotoxic tolerance' at low doses are wide ranging but poorly understood. However, this information is essential for hazard and risk assessment in order to fully accept the concept that genotoxic thresholds exist. For DNA reactive genotoxins, non-linear dose responses can arise from many different biological mechanisms. These include lack of bioavailability and nuclear exclusion, detoxification/activation, DNA repair and other homeostatic defence enzymes. Our recent work has been to investigate the roles of DNA repair in genotoxic thresholds for alkylating agents and pro-oxidant chemicals. Specific DNA repair enzymes have been shown to be up-regulated by low dose alkylating agents, and knocking down specific DNA repair enzymes in vitro alters the shape of the dose response e.g. to EMS. Conversely, for pro-oxidants, we have recently shown that antioxidant defences and specifically the presence of Glutathione, are perhaps more important in genotoxic tolerance at low doses of pro-oxidants. Other mechanisms that impact on the dose response are linked to secondary effects such as dose fractionation and metabolic activity.

Health risk assessment of air pollutants: Air pollutant genotoxicity and its enhancement on suppression of phase II drug-metabolizing enzymes

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In health risk assessment, carcinogenic chemicals are generally categorized according to whether or not they are genotoxic. For example, when the risk assessment value of a carcinogenic hazardous air pollutant is calculated, the air pollutant is generally assigned to one of the following three categories: Category-1, in which the carcinogenicity of the chemical involves genotoxicity; Category-2, in which the involvement of genotoxicity in the carcinogenicity of the chemical is uncertain; and Category-3, in which the carcinogenic chemical is not genotoxic. In Category-1, the genotoxic chemical is judged to be carcinogenic without a threshold, and the risk assessment value is determined from the unit risk. On the other hand, if the carcinogenicity has a threshold, as in Category-3, the assessment value is determined from No-Observed Adverse Effect Level (NOAEL). Weak genotoxic chemicals may have a practical threshold and be categorized in Category-2.

Genotoxic potency is a factor in judging whether there is a threshold in the carcinogenicity; it is determined by not only the reactivity of the chemical to DNA but also the capability of the system that protects the body against chemical toxicity. This protective system is governed by processes such as phase I and II drug-metabolism, excretion, and DNA repair. We asked how the genotoxicity of an air pollutant is affected when part of this protective system is suppressed. Our research focused on phase II drug-metabolizing enzymes, whose constitutive and inducible gene expression is regulated by the essential transcription factor Nrf2. We hypothesized that, in Nrf2-knockout (KO) mice, if the levels of phase II drug-metabolizing enzymes and antioxidant proteins were suppressed, genotoxicity of air pollutants would be enhanced.

We examined the genotoxic potency of air pollutants under Nrf2-deficient conditions by using diesel exhaust (DE) and benzo[a]pyrene (BaP) as model pollutants. After exposing mice to DE for 4 weeks, the levels of bulky-DNA adduct and 8-OHdG in the lungs of Nrf2-KO mice were higher than those in the lungs of Nrf2-bearing control mice. Intratracheal administration of BaP elevated the in vivo mutation frequency (MF) in the lungs of both Nrf2-KO and Nrf2-bearing control mice, but the increase in MF induced by BaP was enhanced in Nrf2-KO mice. These results indicate that the level of phase II-drug metabolizing enzymes is a determinant of the genotoxic potency of air pollutants such as DE and BaP. A possible application of genotoxic potency (in vivo mutagenicity) data for predicting the carcinogenicity of chemicals will be discussed.

Toxicity testing strategy based on the concept of the threshold of toxicological concern (TTC)

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The Threshold of Toxicological Concern (TTC) is a principle that refers to the establishment of a generic human exposure threshold value for groups of chemicals below which there would be no appreciable risk to human health. The concept proposes that such values can be identified for a group of chemicals, including those of unknown toxicity when considering their chemical structures. As for the risk assessment of chemicals used for plastics products of Food Containers, Packaging and Apparatus, most of toxicological profiles of those chemicals have not been evaluated yet. Although, ideally, toxicity testing should be conducted based on the migration levels derived from plastics, it is not realistic to assess fully the potential risks of a large amount of chemicals. Some regulatory authorities (i.e. FDA, EFSA) had developed the comprehensive safety assessment guideline of food-contact materials prior to the application, using tiered toxicity testing strategy based on the migration levels. In Japan, although there is no official comprehensive guidance, several industry associations have introduced independently the self-regulated guidelines like that of FDA or EFSA. The frame works of all guidelines are similar, and necessary set of toxicity tests is required stepwisely depending on a few thresholds of the migration levels. The lowest threshold of 0.5 ppb (1.5 μg/person) was developed by only FDA in 1995. This lowest threshold had been derived from the carcinogenic potency database, and greatly discussed so far at that time. However, scientific bases of higher thresholds (i.e. 50 ppb or 1 ppm) in these guidelines are unclear. For example, only genotoxicity tests are required for the case of for the migration level below 50 ppb for safety evaluation. In order to assure the safety of exposure levels corresponding to below 50 ppb, the comparison of the threshold for all non-genotoxic endpoints with this exposure level should be discussed. Meanwhile, the concept of TTC has been examined and expanded for more general toxicity endpoints (Kroes et al. 2000, 2004) than carcinogenic endpoint. The concept is considered to be helpful for establishment of the threshold corresponding to the specific toxicity risk. Therefore, we examined the possibility for the replacement of these higher thresholds in these guidelines to the exposure threshold derived from the concept of TTC. These works support to establish the scientifically more transparent schema of toxicity testing for the risk assessment for plastics products of Food Containers, Packaging and Apparatus.

第二回 遺伝毒性発がん物質の閾値に関する国際シンポジウム

International Symposium on Genotoxic and Carcinogenic Thresholds

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Application of chemical reaction mechanistic domains to an ecotoxicity QSAR model, the KAshinhou Tool for Ecotoxicity (KATE)

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The validity of chemical reaction mechanistic domains defined by skin sensitisation in the Quantitative Structure–Activity Relationship (QSAR) ecotoxicity system, KAshinhou Tools for Ecotoxicity (KATE), March 2009 version, has been assessed and an external validation of the current KATE system carried out. In the case of the fish end-point, the group of chemicals with substructures reactive to skin sensitisation always exhibited higher root mean square errors (RMSEs) than chemicals without reactive substructures under identical C- or log *P*-judgements in KATE. However, in the case of the *Daphnia* end-point this was not so, and the group of chemicals with reactive substructures did not always have higher RMSEs: the Schiff base mechanism did not function as a high error detector. In addition to the RMSE findings, the presence of outliers suggested that the KATE classification rules needs to be reconsidered, particularly for the amine group. Examination of the dependency of the organism on the toxic action of chemicals in fish and *Daphnia* revealed that some of the reactive substructures could be applied to the improvement of the KATE system. It was concluded that the reaction mechanistic domains of toxic action for skin sensitisation could provide useful complementary information in predicting acute aquatic ecotoxicity, especially at the fish end-point.

Keywords: KATE; chemical reaction mechanistic domain; ecotoxicity; applicability domain

1. Introduction

Risk management of new and existing chemical substances is a significant issue in the construction of an environmentally sustainable society. To resolve unsustainable patterns of consumption and production, in 2002 the World Summit on Sustainable Development [1] adopted a plan, 'aiming to achieve, by 2020, that chemicals are used and produced in ways that lead to the minimization of significant adverse effects on human health and the environment'. In June 2007 a new chemical substances regulation – Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) – was implemented by the European Union [2]. Moreover, in Japan the Chemical Substances Control Laws (CSCLs) have very recently been amended to produce a comprehensive system for the control of chemicals [3]. Under the requirements for risk management, the [Quantitative] Structure–Activity Relationship ([Q]SAR) plays an important role in filling the gaps in knowledge for chemicals so far untested in the laboratory, and may yield the information

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for prioritising the chemicals to be tested [4]. For instance, the usefulness of QSAR in aquatic toxicity has been reviewed, particularly in the context of the REACH legislation [5].

The principles of (Q)SAR have also been discussed by the Organisation for Economic Co–operation and Development (OECD) [6], and one of its five validation principles is that a (Q)SAR should have a defined domain of applicability. A definition of "applicability domain" has been provided by Netzeva et al. [7]: 'The applicability domain of a (Q)SAR is the response and chemical structure space in which the model makes predictions with a given reliability'. Consequently the applicability domain may in practice guarantee the performance of toxic predictions. For example, various response domain spaces with more than one descriptor have recently been introduced and utilised [7–11]. Furthermore, on the basis of the descriptor, chemical structural space and so on, QSAR software that includes aquatic toxicity prediction capability, such as TIssue MEtabolism Simulator (TIMES) [12,13], provides an indication of whether a predicted chemical is in-domain or out-of-domain based on the descriptor and chemical structural space. The KAshinhou Tool for Ecotoxicity (KATE) system [14,15], which provides ecotoxicity QSAR models for 96-h fish LC_{50} and 48-h *Daphnia* EC_{50} end–points, also has domains for evaluating the predicted results. Model users can confirm the reliability of a prediction by the domain information, in addition to the QSAR statistical data.

We have investigated the efficiency of the additional domains for fish and *Daphnia* end-points in the current KATE (March 2009 version) system. In particular, chemical reaction mechanistic domains defined by skin sensitisation [16] were targeted in order to enhance reliability. Skin sensitisation, and aquatic reactive toxicity, i.e. fish, are different end-points, but the protein-binding reaction mechanisms show similar behaviour; consequently dependence on electrophilic reactivity is found for both end-points [17,18]. Moreover, research into the prediction of skin sensitisation potency has been very active over the past few years [16,19–24]. Aptula et al. have examined the relationship between the electrophilic reactivity of a chemical and its skin sensitisation potential [16,19]. Recently, Enoch et al. [25] have used SMiles ARbitrary Target Specification (SMARTS®) [26] patterns to describe structural fragments (substructures) on the basis of information provided by Aptula et al. [16]. In the system of Enoch et al. [25], their set of patterns was assessed for its ability to correctly identify potential mechanisms within the 208 training dataset; by using a knowledge of organic chemistry, the applicability domain within each mechanism was expanded as far as possible.

Note that the SMARTS patterns allow the specification of substructures by using rules that are straightforward extensions of the Simplified Molecular Input Line Entry Specification (SMILES®) notation [27] for chemical substances. Because KATE uses the same SMILES linear notation to predict aquatic toxicity, the SMARTS patterns can easily be applied to detect the reactive substructures of chemicals without the need for stereochemical information.

2. Methodology

2.1 *Definition of reaction mechanistic domains*

We have used the reactive patterns in Table 2 of the paper by Enoch at al. [25] to define chemical reaction mechanistic domains (described in the present article simply as "reactive domains"). We were fortunate in being able to obtain the raw data used by Enoch et al. in

their study [25] and translate the original SMARTS patterns into the classification algorithm, Fragment Identification by Tree Structure (FITS), developed by Yoshioka [28–30]. FITS is able to detect the substructure in the SMILES strings and is the main engine of the current KATE system for the classification rule and the definition of structure domains (i.e. *C*-judgement, Section 2.2 in the present article [14]).

We shall concentrate here on the "reactive" compounds with the patterns of the following reactions [16]: Michael-type addition (MA), pro-MA, aromatic nucleophilic substitution (S_NAr), aliphatic nucleophilic substitution (S_N2), Schiff base formation (SB), pro-SB and acylation. Unfortunately, the pro-S_N2 SMART patterns of the Enoch paper [25], expressing anthracene and phenanthrene substructures, are not definable, for technical reasons associated with the current FITS. Notably, the pro-MA, pro-SB and pro-S_N2 patterns indicate the substructures convertible to those of the corresponding parent electrophilic compounds by metabolism [31,32]. Moreover, substructures designated "reactive" signify only the reactive domain of the defined SMARTS patterns. The designation "non-reactive" denotes compounds without the pattern defined by the above domains.

2.2 *KATE and its domains*

This section provides a review of the KATE system. KATE was developed using the database on aquatic toxicity results gathered on fish (*Oryzias latipes*) and *Daphnia* (*Daphnia magna*) by the Japanese Ministry of the Environment (MoE) [33], and the United States Environmental Protection Agency (US EPA) fathead minnow (*Pimephales promelas*) database [34,35]. In order to construct QSAR models for toxicity prediction, the chemicals in the reference dataset were classified by their substructures, which meant that the classification rules could define the structural spaces of each QSAR model. The equations of the models were linearly correlated with the octanol–water partition coefficient (log *P*) values, with the exception of the equation for the *neutral organics* class, which was an aggregate of the chemicals in the defined classes. These classification rules and equations have appeared in a previous article [14]. In addition, internal validation had earlier shown that acceptable results were given by QSAR models of KATE with more than a 0.5 root mean square error (RMSE), a squared correlation coefficient (r^2) of up to 0.7, and more than five reference data ($n > 5$) [14]. Since acute aquatic QSAR had been developed over more than 30 years, some of the classification rules for the QSAR models in KATE followed similar concepts to those of other aquatic QSAR models based on chemical substructures [5,35–44].

Log *P*- and *C*-judgements are also key features of the KATE system. The former judgement is based on the descriptor log *P* range defined by the reference data of the class (shown in Tables 1 and 2 of Furuhama et al. [14]) to which the chemical belongs. The latter is categorised as a structural domain and is defined by the substructures (specified functional groups) shown in Appendix 3 of Furuhama et al. [14]. The definition of the *C*-judgement is as follows: *C(1)* is the in-domain of the *C*-judgement, defined as all substructures of a test chemical found in reference chemicals in the class. *C(2)* is the in-domain of the *C*-judgement, defined as all substructures of a test chemical found in reference chemicals either in its class or in the class, *neutral organics*. External validation indicated that the group of chemicals in the in-domain of the *C*-judgement had lower RMSE than the group without considering the *C*-judgement [14].

Table 1. Numbers of chemical compounds evaluated.

		KATE domain					
End-point		*all*	*C(2)*	*C(1)*	*log P*	*log P C(2)*	*log P C(1)*
Fish	*Chemicals*	57	39	32	44	33	26
	Predicted	64	44	36	49	38	30
Daphnia	*Chemicals*	78	49	35	52	35	26
	Predicted	86	54	40	58	40	31

Note: *Chemicals*: Number of chemicals in the training set with toxicities to be predicted by KATE in the present study.
Predicted: Total number of predicted toxicity values. Because some chemicals belonged to more than one class, the *Predicted* category was larger than *Chemicals*. When a chemical was found to belong to more than one QSAR class, all the predicted data were adopted. If only the name of the class was available, such data were omitted.
All: Both in-domain and out-of-domain data for log *P*- and log *C*-judgements are included.
log P: In-domain of log *P*-judgement.
C(2): In-domain of *C*-judgement is defined as all the substructures of a test chemical in reference chemicals in either the *neutral organics* class or the class of the test.
C(1): In-domain of *C*-judgement is defined as all the substructures of a test chemical found in reference chemicals in the class.
The domain notation is also defined in the text and in Table 3 of a previous study [14].

2.3 *Test dataset for the present study*

As test sets for our study (Table 1), we selected the Japan MoE dataset of chemicals available after March 2009. The test sets therefore did not include any of the reference data in the March 2009 version of KATE. The ecotoxicity test sets (96-h LC_{50} fish and 48-h EC_{50} *Daphnia*) discussed here have already been published on the Japanese MoE website [33]. The input log *P* values for the toxicity calculation were obtained from the US EPA KOWWIN® [45]. SMILES notations of the predicted chemicals were defined by the system in the Estimation Program Interface (EPI) Suite® [46] and the stand-alone version, KATE on PAS. The predicted toxicity value, log *P*-judgement and *C*-judgement were obtained from the internet version, KATE on NET (March 2009 version) [15].

3. Results and discussion

The predicted toxicities, the log *P* and *C*-judgements and the reactive domains from the skin sensitisation information are summarised in Tables 2 (fish) and 3 (*Daphnia*). When test chemicals are allocated to two or more classes, all the classes have been evaluated during the study. Similarly, when a chemical is categorised as having more than one reactive domain, all reactive mechanisms were listed. For example, 3-formyl-6-*iso*-propylchromone (Figure 1) has two reactive substructures, and is listed as "SB, Michael" in the right column of Table 2.

In the current test dataset, chemicals with in-domain of log *P* and *C(1)* judgements tended to be classified as "non-reactive". If these judgements are ignored, in the predicted fish toxicity values 27 and 37 chemicals were categorised as "reactive" or "non-reactive", respectively, and the equivalent numbers for *Daphnia* were 29 and 57. When only the

Table 2. Results of KATE-based predictions, with CAS number, chemical name, toxicity of chemicals to fish as measured and as predicted by KATE, log *P*- and *C*-judgements (KATE domains), and "reactive" mechanistic domains (Enoch et al. [25]) for the 57 compounds in the test set.[1]

CAS	*Chemical name*	*log P Pred.*	*KATE class name*	*log (1/LC50 [mM])*		*Domain*		*'reactive'*[4]
				Pred.	*Meas.*	*log P*[2]	*C*[3]	
286–62–4	Cyclooctene oxide	2.64	Epoxides (Clog P)	1.91	−0.20	O	*C (1)*	S_N2
2425–79–8	1,4-Bis (2,3-epoxypropoxy)butane	−0.15	Epoxides (Clog P)	1.01	1.19	X	*C (1)*	S_N2
111–30–8	Glutaraldehyde	−0.18	Aldehydes	0.19	1.06	O	*C (1)*	SB
1540–36–9	2,4-Pentanedione, 3-butyl-	1.94	Conjugated systems2	1.75	−0.13	O	*C (1)*	SB
487–68–3	2,4,6-Trimethylbenzaldehyde	3.35	Aldehydes	1.90	1.09	X	*C (1)*	SB
4067–16–7	Pentaethylenehexamine	−3.67	Hydrazines (Clog P)	0.30	0.04	X	X	pro-S_N2
6117–91–5	2-Buten-1-ol	0.63	Conjugated systems2	1.18	1.26	O	*C (1)*	pro-Michael
20103–09–7	2,5-Dichloro-1,4-benzenediamine	0.90	Amines aromatic or phenols1	2.67	0.91	O	*C (1)*	pro-Michael
101–96–2	1,4-Benzenediamine, *N,N'*-bis (1-methylpropyl)-	3.50	Amines aromatic or phenols1	2.65	2.77	O	X	pro-Michael
51963–82–7	Benzenamine, 2,5-diethoxy-4-(4-morpholinyl)-	2.01	Amines aromatic or phenols1	2.66	1.08	O	X	pro-Michael
40220–08–4	Tris(2-hydroxyethyl)isocyanuric acid acrylate	3.75	Amides and imides	1.77	1.79	O	X	Michael, acylating
40220–08–4	Tris(2-hydroxyethyl)isocyanuric acid acrylate	3.75	Acrylates	2.09	1.79	X	X	Michael, acylating
100–43–6	4-Vinylpyridine	1.71	Hydrocarbons aromatic	0.19	2.02	O	*C (1)*	Michael
1855–63–6	1-Cyclohexene-1-carbonitrile	2.04	Neutral organics	0.04	0.42	O	*C (1)*	Michael
1335–46–2	Methylionone	4.84	Conjugated systems2	3.01	1.84	X	*C (1)*	Michael
1855–63–6	1-Cyclohexene-1-carbonitrile	2.04	Nitriles aliphatic	0.56	0.42	O	*C (2)*	Michael
406–86–0	4,4,4-Trifluorocrotonitrile	1.04	Halides2	0.54	2.78	O	*C (2)*	Michael
98–81–7	α-Bromostyrene	3.29	Unclassified	1.55	3.09	O	X	Michael
117–80–6	2,3-Dichloro-1,4-naphthoquinone	2.65	Halides1	2.00	3.86	O	X	Michael
483–63–6	*N*-Ethyl-*N*-crotonoyl-2-toluidine	2.73	Amides and imides	1.01	0.43	O	X	Michael
569–64–2	CI Basic Green 4	0.80	Amines aromatic or phenols5	−0.18	3.42	O	X	Michael
16669–59–3	*N*- (Isobutoxymethyl)acrylamide	0.84	Amides and imides	−0.40	0.42	O	X	Michael
110–26–9	*N, N'*-Methylenediacrylamide	−1.52	Amides and imides	−2.16	−0.19	X	X	Michael
117–80–6	2,3-Dichloro-1,4-naphthoquinone	2.65	Conjugated systems1	4.08	3.86	X	X	Michael

(*Continued*)

Table 2. Continued.

CAS	Chemical name	log P Pred.	KATE class name	log (1/LC50 [mM]) Pred.	log (1/LC50 [mM]) Meas.	Domain log P[2]	Domain C[3]	'reactive'[4]
868–63–3	2-Propenamide, *N*,*N*′-(1,2-dihydroxy-1,2-ethanediyl)bis-	−3.69	Amides and imides	−3.78	0.26	X	X	Michael
89–40–7	4-Nitrophthalimide	1.12	Nitrobenzenes	0.07	1.24	O	X	acylating
22509–74–6	*N*-Carboethoxyphthalimide	2.33	Amides and imides	0.71	2.19	O	X	acylating
78–51–3	Tri-*n*-butoxyethyl phosphate	3.00	Esters phosphate	1.96	1.28	O	*C (1)*	
88–05–1	2,4,6-Trimethylaniline	2.72	Primary amines	0.82	0.39	O	*C (1)*	
88–75–5	*o*-Nitrophenol	1.91	Amines aromatic or phenols4	0.61	0.34	O	*C (1)*	
89–64–5	4-Chloro-2-nitrophenol	2.55	Unclassified	1.00	1.20	O	*C (1)*	
91–17–8	Bicyclo[4.4.0]decane	4.20	Hydrocabons aliphatic	1.88	2.57	O	*C (1)*	
91–17–8	Bicyclo[4.4.0]decane	4.20	Neutral organics	1.86	2.57	O	*C (1)*	
95–87–4	2,5-Xylenol	2.61	Amines aromatic or phenols4	1.11	1.33	O	*C (1)*	
108–87–2	Methylcyclohexane	3.59	Hydrocabons aliphatic	1.42	1.67	O	*C (1)*	
108–87–2	Methylcyclohexane	3.59	Neutral organics	1.35	1.67	O	*C (1)*	
111–78–4	1,5-Cyclooctadiene	3.73	Hydrocabons aliphatic	1.52	0.92	O	*C (1)*	
111–78–4	1,5-Cyclooctadiene	3.73	Neutral organics	1.47	0.92	O	*C (1)*	
120–95–6	2,4-Di-*tert*-pentylphenol	6.31	Amines aromatic or phenols4	3.80	2.91	O	*C (1)*	
504–29–0	2-Aminopyridine	0.53	Amines aromatic or phenols5	−0.32	0.93	O	*C (1)*	
615–58–7	2,4-Dibromophenol	3.29	Amines aromatic or phenols4	1.61	1.84	O	*C (1)*	
634–93–5	2,4,6-Trichloroaniline	3.01	Amines aromatic or phenols3	1.59	1.57	O	*C (1)*	
716–79–0	1H-Benzimidazole, 2-phenyl-	3.00	Hydrocarbons aromatic	1.01	1.35	O	*C (1)*	
764–13–6	2,5-Dimethylhexa-2,4-diene	3.95	Hydrocarbons aliphatic	1.69	1.63	O	*C (1)*	
764–13–6	2,5-Dimethylhexa-2,4-diene	3.95	Neutral organics	1.65	1.63	O	*C (1)*	

873–32–5	*o*-Chlorobenzonitrile	2.18	Amides and imides	0.60	0.57	O	*C (1)*
948–65–2	2-Phenylindole	3.82	Hydrocarbons aromatic	1.52	2.85	O	*C (1)*
2219–82–1	6-*tert*-Butyl-*o*-cresol	3.97	Amines aromatic or Phenols4	2.10	1.58	O	*C (1)*
2243–62–1	1,5-Naphthalenediamine	1.34	Amines aromatic or Phenols3	1.23	0.97	O	*C (1)*
3295–94–1	Allyl *n*-hexyl ether	3.37	Conjugated systems2	2.37	1.46	O	*C (1)*
100–55–0	3-Hydroxymethylpyridine	−0.11	Hydrocarbons aromatic	−0.95	−0.93	X	*C (1)*
462–08–8	3-Aminopyridine	−0.11	Amines aromatic or phenols5	−0.67	1.04	X	*C (1)*
504–24–5	4-Aminopyridine	−0.11	Amines aromatic or phenols5	−0.67	1.44	X	*C (1)*
89–63–4	4-Chloro-2-nitroaniline	2.66	Amines aromatic or phenols3	1.51	1.01	O	*C (2)*
96–96–8	2-Nitro-*p*-anisidine	2.10	Amines aromatic or phenols3	1.39	0.61	O	*C (2)*
140–66–9	4-*tert*-Octylphenol	5.28	Amines aromatic or phenols4	3.05	2.76	O	*C (2)*
827–52–1	Cyclohexylbenzene	4.81	Hydrocarbons aromatic	2.15	2.13	O	*C (2)*
843–55–0	1,1-Bis (4-hydroxyphenyl)-cyclohexane	5.48	Amines aromatic or phenols4	3.19	2.17	O	*C (2)*
2100–42–7	2-Chlorohydroquinone dimethylether	2.80	Unclassified	1.19	0.79	O	*C (2)*
100–63–0	Hydrazine, phenyl-	0.79	Hydrazines (Clog P)	2.16	3.83	O	X
123–46–6	Ethanaminium, 2-hydrazino-*N,N,N*-trimethyl-2-oxo-, chloride	−5.29	Hydrazines (Clog P)	−0.37	−2.02	X	X
497–18–7	Carbonohydrazide	−3.73	Hydrazines (Clog P)	0.28	0.24	X	X
19715–19–6	3,5-Di-*tert*-butylsalicylic acid	6.06	Unclassified	3.61	1.97	X	X
90–30–2	1-(*N*-phenylamino)-naphthalene	4.47	Amines aromatic or phenols5	1.82	2.50	X	X

Note: [1]When a chemical belongs to more than one QSAR class, all the predicted data are adopted.
[2]O: In-domain of log *P*-judgement; X: out-of-domain of log *P*-judgement.
[3]*C (1)*: In-domain of *C*-judgement, defined as: (1) all substructures of a test chemical are found in reference chemicals in the class.
C (2): In-domain of *C*-judgement, defined as: (2) all substructures of a test chemical are in reference chemicals in either *Neutral organics* or the class.
X: Out-of-domain of *C*-judgement.
[4]Names of the reactive mechanistic domains are given in the text.