Table 2 (*Continued*)

in the future more humanized metabolism-competent animal strains (e.g. SULT1A1/2 models)
The potential genotoxicity of a metabolite (in either case of employing an alternative/optimized *in vitro* metabolic activation system or direct testing of metabolite) should be assessed using *in vitro* tests to detect both point mutations and chromosomal aberrations

of employing an alternative/optimized *in vitro* metabolic activation system or direct testing of metabolite) should be assessed using *in vitro* tests to detect both point mutations and chromosomal aberrations.

The working group also identified specific areas where there was insufficient understanding, experience or scientific basis to achieve full consensus (Table 3). The definition of a quantitative human metabolite exposure as a trigger for safety assessment requires broader discussions and debate to reach consensus. This is best exemplified by the differences in which this has been proposed to be defined in a previous industry position [MIST, 7-9], the recently developed FDA guidance draft [10] and a proposed alternative approach that places emphasis on absolute (rather than relative) metabolite abundance [65]. The working group did express the desire to consider further an absolute exposure definition in order to better support risk assessment, analogous to the threshold of toxicological concern (TTC) concept introduced in procedures and guidance to manage genotoxic impurities [66,67]. The working group could not define a universal recommendation for the timing of human ADME studies in the case of pharmaceutical development, and as stated above, requires consideration of certain triggers in deciding whether this assessment should be conducted earlier. Regarding the use of structural knowledge and/or physicochemical properties (e.g. *in silico* systems, literature or expert analysis), the group could not reach consensus in supporting its use as a metabolite "qualification" approach (i.e. by demonstrating the absence of genotoxic alerting features) in lieu of genotoxicity testing. Additional case examples using this approach would be desirable to review. However, the group did see value in applying structural knowledge to prioritize level of concern and design an appropriate follow up test design. Lastly, the group could not define discrete triggers for when one should conduct direct testing of a metabolite versus using a "competent" alternative activation system to generate it. This was largely driven by the inability of the group to define a universal metabolite exposure level that was considered "sufficient" to characterize potential genotoxic hazards when generated by an alternative activation system. The review of more case experiences adopting alternative activation systems for genotoxicity assessment of a human metabolite would be desirable.

Table 3
Points of discussion where consensus could not be reached, but should be revisited in the future when more data and experience have been gained

Use of relative vs. absolute abundance of a circulating or excreted metabolite as an exposure trigger for safety assessment
Requires broader discussions to reach consensus
It is desirable to consider a dose-based approach (e.g. the threshold of toxicological concern approach for genotoxic impurities) for metabolites in supporting risk assessment
Timing of human ADME studies for drugs (at projected pharmacological dose) prior to Phase 3 vs. new drug application (NDA) filing
Cannot derive a universal recommendation for all cases
Needs to consider unique nature of human metabolite and/or structural concerns (similarity to parent drug, presence of potential reactive substituents)
Use of structural knowledge (structure–activity relationships) and physicochemical properties (*in silico* systems, literature, expert analysis) as a stand alone qualification approach to negate need for further genotoxicity testing of a metabolite
There is value in the use of structural knowledge to prioritize the level of concern in planning follow up of a metabolite of interest
Discrete triggers for testing a metabolite directly vs. using a "competent" alternative activation source
Need to acknowledge difficulty in generating unstable/reactive metabolites and/or assuring access to nuclear DNA if generated extracellularly
Inability to define sufficient metabolite exposures when using alternative activation systems to fully assess potential genotoxic hazards
When to consider use of activation systems vs. testing metabolite directly based on the level of concern raised by review of structural knowledge?

4. Proposed strategies

The working group proposed two strategies to consider, a more proactive approach, which emphasizes early metabolism predictions to drive appropriate hazard assessment, and a retroactive approach to manage safety risks of a unique or "major" metabolite identified and quantitated from human ADME studies.

The proactive-predictive approach (Fig. 1) proposes a forward thinking strategy to challenge the adequacy of the current genotoxicity testing paradigm using induced liver S9 as a default metabolic activation source. Instead, this strategy emphasizes the need for development of a more customized testing approach based upon knowledge of human metabolism generated prior to traditional human ADME studies. Obviously, this approach is

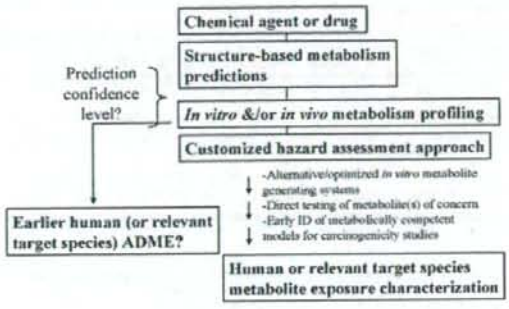

Fig. 1. Proactive-predictive strategy for potential human metabolites.

highly dependent on the confidence level of available metabolism prediction tools, whether structure-based (*in silico* or expert-based) metabolism predictions or those made through *in vitro* and/or *in vivo* (animal) metabolism profiling. Efforts are currently being made to develop *in silico* methods for predicting metabolite profiles of drugs in human [68,69], however such methods are not yet mature enough to ensure high confidence that the correct human metabolite structures would be tested for genotoxicity if applied prospectively. Hence, if applied, there would be some cases in which structures that are not human metabolites would be subjected to genotoxicity testing and others where an actual human metabolite would not be tested. The use of human *in vitro* metabolite profile data (e.g. liver microsomes, hepatocytes) could also be used prospectively. While more advanced than *in silico* methods, *in vitro* approaches may still not cover all human *in vivo* metabolites, and even if correctly identifying human metabolites, *in vitro* methods will not provide a quantitative metabolite profile that is the same as *in vivo*. Thus, based on the current state-of-science, further progress for supporting this proposed proactive strategy in a practical sense needs to be revisited.

A retroactive-risk management strategy (Fig. 2) is proposed primarily to address the unique challenges in the pharmaceutical industry where, although definitive human ADME data are generated as part of clinical trials, there is a recognized need to ensure representation of metabolites of interest in non-clinical safety assessments (including genotoxicity and carcinogenicity testing) to bridge concerns on clinical safety risks. Applying a proactive-predictive strategy to this situation was considered to require further advancements and experiences (i.e. future predictive human ADME capabilities and their application) to ensure that only those metabolites with demonstrated human exposures later in a product's development are addressed for safety. The retroactive-risk management approach considers the fact that definitive and quantitative human ADME best characterizes the unique or "major" nature of any identified metabolites. Once a unique or "major" human metabolite is identified, a structure-based analysis of genotoxic and/or carcinogenic potential should be conducted and considered in assessing the strength of developing a qualification argument based on available knowledge and literature. Though the absence of structural knowledge for concern was found desirable to support no further genotoxicity testing, full consensus on this approach could not be reached at this time. If structural knowledge points to potential hazards, further genotoxicity testing should be considered. This perhaps may first be assessed by considering testing the parent chemical agent

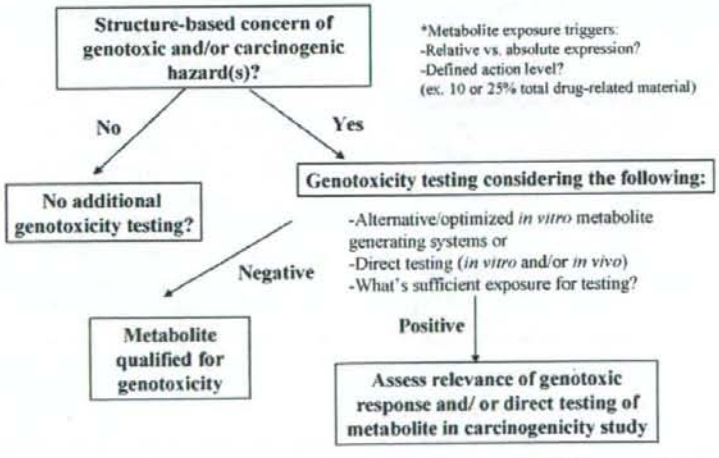

Fig. 2. Retroactive-risk management strategy for precedented unique or "major" human metabolites*.

using alternative or optimized *in vitro* metabolite generating systems (to standard induced liver S9). Direct testing of the synthesized metabolite could be considered if this former approach is deemed unsuitable. Once the appropriate genotoxicity testing is conducted, a metabolite would be considered qualified if no effects were observed. Further actions would have to be considered if effects were observed, including the development of an appropriate carcinogenicity qualification approach. Certain points did not reach consensus, namely exposure triggers for safety assessment, the use of structural knowledge alone to "qualify" a metabolite in lieu of testing and what constitutes sufficient human exposure of a metabolite for testing. These points should be revisited when more data and additional experiences are gathered.

5. Future considerations

The working group recognized the need to address the limitations of current *in vitro* testing protocols that can perturb or reduce the level of metabolic activity. This includes seeking to reduce the level of the common solvent DMSO used to solubilize test articles [46], or seek alternative transition solvents which may be more compatible with preserving enzyme activity in the S9 activation system [70]. Based on the potential for substrate inhibition effects that can occur in S9 incubations where high substrate (i.e. test article) concentrations are used for testing, the working group recognized the need (and recommends in future) to re-evaluate the use of the OECD and ICH guidance limit dose of 10 mM, in particular for tests with metabolic activation.

The group also considered it timely to challenge through broader investigations the suitability of the current induced liver S9 activation source and to consider alternatives that could eventually be incorporated as replacements in standard testing paradigms. This may include the incorporation of S9 from standardized or pooled human hepatocytes or humanized animal systems. This could be an important complementary approach to the proactive-predictive strategy proposed.

Numerous findings have demonstrated the exceptional utility of genetically engineered bacteria, mammalian cells and animal models in elucidating mechanisms of bioactivation/inactivation and detecting mutagenic activity of various carcinogens that are negative in standard *in vitro* tests. It became evident from these studies that several enzymes that are largely ignored in standard *in vitro* tests are relatively frequently involved in the activation of progenotoxins, for example human CYP1B1, CYP2E1, NAT2, SULT1A1, SULT2A1 and GSTT1 (or functionally similar rodent forms). It is therefore necessary to incorporate these activities into test systems, at least if structural alerts indicate a possible role of these enzymes and if no functionally similar enzymes are present. Structural alerts include, for example:

- A small size of the molecule for CYP2E1.
- An allylic or benzylic hydroxyl group (present in the parent molecule or possibly formed by phase-1 metabolism) for SULT1A1 and SULT2A1.
- An aryl amino or nitro group for NAT2 and SULT1A1.
- Two vicinal alkyl halogen substituents for GSTT1.

The enzymes may be taken into account by determining a genotoxic effect in a primary cell or in an established cell line that has retained the corresponding activity, by using a modified external activating system (if it can be assumed that the active metabolite can penetrate cell membranes) or by using genetically engineered target cells.

While the recommendations described in the preceding paragraph are driven by structural alerts or knowledge of biotransformation pathways of the test compound, traditional genotoxicity testing largely ignores such information. Whether such blind testing is sound is debatable. However, in practice it may be difficult to predict the various possible activation pathways for complex molecules. Thus a radical change to a fully customized test strategy for each individual compound is presently not realistic. This raises the question whether a standard activating system could be devised that is superior to S9, either by expressing a small number of further enzymes in the target cells or by replacing S9 solely by expressed enzymes. Would it be possible to express all the activities desired in a single target cell or would many target cells be required? It is probable that a handful of enzymes or two in the S9 mediate nearly all activations observed. This might be technically feasible, but with great effort, by genetic engineering. However, it would be difficult to express a significant number of additional enzymes. Another medium- to long-term option would be the use of a large battery (dozens or hundreds) of target cells (probably bacteria), each engineered for a small set of enzymes from humans or standard laboratory animals, combined with a high throughput endpoint (e.g. a reporter gene). Information from such a system may be useful for further designing an appropriate metabolite testing strategy, selecting an appropriate animal model (e.g. humanized for critical enzymes) and a first prediction of tissues at high risk.

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Appendix A. Synopses of drug metabolite case examples

A.1. Regulatory examples

The FDA Center for Drug Evaluation and Research receives approximately 120 protocols for proposed carcinogenicity studies a year. Out of these, two or three cases involve unique and/or major metabolites. In some instances, a metabolite not present in rats or mice represented as much as seventy percent of the metabolite profile seen in humans. Solutions to this type of problem have been individualized and depend largely on the sponsor's input and choice. In general, either the metabolite alone is included as one arm of the carcinogenicity study or the drug is spiked with the metabolite in all arms. The genotoxic potential of the metabolite is taken into consideration in determining the appropriate concentration of the metabolite to use in the carcinogenicity study.

Examples of designs of carcinogenicity studies involving unique or major metabolites are given below.

Example 1: The pharmaceutical formed a metabolite in humans that was not detected in rodents. Thus, standard genetic toxicology tests *in vivo* in rodents or *in vitro* using a rat S9 metabolic activation system would not adequately characterize the genotoxic or carcinogenic potential of the drug. Genetic toxicology testing of the synthesized unique metabolite gave negative results. In the carcinogenicity study, a second high dose arm spiked with 25-fold the human exposure of the metabolite was included to explore the carcinogenic potential of the unique human metabolite.

Example 2: The pharmaceutical formed a unique metabolite that had structural alerts for genotoxicity and carcinogenicity and was positive in two *in vitro* genetic toxicology assays. The FDA proposed the addition of the metabolite at an equivalent human exposure and at the metabolite maximum tolerated dose to two arms of a rat carcinogenicity study of the pharmaceutical. The sponsor chose to conduct an independent carcinogenicity study of the metabolite alone.

Example 3: The pharmaceutical formed a unique metabolite in the presence of nitrates and acidic conditions in the human stomach. The sponsor and the FDA agreed on genetic toxicology testing of the metabolite and inclusion of the metabolite in an arm of the carcinogenicity study.

Example 4: The pharmaceutical produced two major human metabolites (approximately 30% and 15%) not seen in rats. One was seen in mice. Both metabolites formed glucuronic acid conjugates. No additional testing of the metabolites was deemed necessary.

A.2. Industry examples

Example 1: Metabolite of alerting structure found in abundance in humans. In this example, a quinoline containing structure was identified in the urine of human and animal in radiolabel ADME studies. The parent molecule contained a tetrahydroquinoline moiety that underwent multiple metabolic transformations to yield the quinoline metabolite. On a percentage of total dose basis, the metabolite was more predominant in human urine (30%) than in urine from rat and mouse (5–11%), however since the dose normalized for body weight in humans was substantially lower than that used in animals, the amount of metabolite in animals was actually greater. Nevertheless, while this was deemed sufficient to consider that the animals had been suitably exposed to the metabolite for risk assessment, the *in vitro* genotoxicity tests may not have had acceptable metabolite generation for proper hazard identification. In general, metabolites arising via multiple sequential reactions will not be observed in simple *in vitro* systems, and this had also been the case in this example. It was therefore decided, based on the structural alert of a quinoline moiety and the lack of generation of this metabolite in the Aroclor induced rat liver S9 fraction, that the metabolite would be tested directly in the Ames test. The result was negative, and the issue was considered closed.

Example 2: Unique metabolite in human circulation structurally proximate to an electrophile. In this example, the radiolabel human ADME study revealed a major (65% of total) oxidative circulating metabolite that had not been observed in animals *in vivo*. Furthermore, the structure was such that one more oxidation step would yield a quaternary alicyclic iminium ion, a structure known to be electrophilic and capable of covalently binding proteins. Interestingly, despite being one step away from the iminium ion, this metabolite was not identified as a structure alert in the DEREK program. Efforts were made to determine if Aroclor induced rat liver S9 fraction was capable of generating this metabolite, with the intent that if it were generated to an appreciable extent in these incubations, then the previously run negative Ames test on the parent drug would have provided suitable hazard identification. The concentrations sought in these incu-

bations represented a debatable point: i.e. what multiple of human exposure would be 'adequate coverage' for the metabolite. The outcome was such that small amounts of the metabolite were generated, however these were well below circulating concentrations in humans. Therefore a direct test of the metabolite in the activated Ames test was undertaken, and the result was negative. Nevertheless, it could be questioned as to whether the cationic iminium ion, if generated, would have been able to traverse the cell membrane, a necessary step in exhibiting mutagenicity.

Example 3: Abundant, unique, non-alerting metabolite in human circulation. In this example, an alkyl amine drug was sequentially *N*-dealkylated and oxidized in several steps to a metabolite possessing a structure analogous to salicylic acid. The metabolite was first discovered in the human radiolabel ADME study, and represented 56% of circulating radioactivity. As in example 1, generation of a metabolite that is four sequential metabolic steps to any great extent in the Ames test represents a demanding order from a simple *in vitro* system. In this example, the decision was made to go straight to direct testing of the metabolite in the Ames test, without any investigation of whether the Aroclor induced S9 system would have generated it in the standard Ames test of the parent compound. As a stable structure it was, as expected, negative in the *in vitro* genotoxicity test. Subsequently, although not observed in the single dose radiolabel ADME studies in rats and mice, it was found that exposure to this metabolite in animals was high after repeated administration, as measured using a standard non-radiometric bioanalytical assay, thus providing adequate coverage to human exposure.

Example 4: For a pharmaceutical the standard genotoxicity test battery for a pharmaceutical was performed without adverse findings. A major *in vivo* metabolite (hydrolysis of nitrile to carboxylic acid observed in rat urine) was found to be absent in *in vitro* metabolism experiments with microsomes or S9. Kidney slices were capable of metabolism. In rats >20% of parent was converted to this metabolite. The metabolism step did not generate structural alerts. Prior to entry into man the carboxyl metabolite was tested in the Ames and *in vitro* chromosome aberration (±S9) assays without adverse findings. Another multistep metabolite (intramolecular cyclization, oxidation, cleavage) without structural alerts was observed as major plasma metabolite (30–60% of parent) in experimental animals but only as minor S9 metabolite (uninduced or induced rat S9). Since this compound had already been tested as a synthesis intermediate in the Ames and the *in vitro* chromosomal aberration tests without adverse findings no additional testing was performed.

Example 5: The standard genotoxicity test battery was performed for a pharmaceutical without adverse findings. Metabolism/pharmacokinetic (PK) studies provided evidence of major human specific metabolite, produced by a two-step oxidative metabolism (alcohol, carboxylic acid). No evidence of further metabolisation of this metabolite was obtained. Rodent metabolism did not form this metabolite efficiently. The metabolite was not structurally alerting. The metabolite was included in general toxicity studies in rodents and *in vitro* genotoxicity studies were performed. A marginal positive effect was seen in both the Ames test (strain TA102) and the *in vitro* chromosomal aberration assay, but only with S9 activation. However, inactivated S9 was also capable of producing the effect, so a non-enzymatic activation was indicated. Further investigations suggested that the effect was due to auto-oxidation, leading to disulfide dimer formation and concomitant H_2O_2 generation. This activity was not considered relevant for whole organism conditions. *In vivo* micronucleus and *ex vivo* unscheduled DNA synthesis (UDS) tests performed with the metabolite confirmed an absence of genotoxicity *in vivo*.

Example 6: A pharmaceutical was tested negative in Ames, V79/*hprt*, *in vitro* chromosome aberration, *in vitro* UDS, and *in vivo* (mice) micronucleus assays. A major human metabolite (alcohol, formed by hydrolysis of ester side chain) was not generated in mice, and also not seen in metabolism studies with rat liver homogenate. The alcohol was not structurally alerting. The Ames, V79/*hprt*, *in vitro* chromosome aberration and *in vivo* micronucleus assays were performed with the metabolite in late phase of development. No adverse effects were observed.

Example 7: The standard genotoxicity test battery was performed for a pharmaceutical without adverse findings. In early metabolism studies one carboxylic acid metabolite (formed by two-step oxidative metabolisation at a methyl group), was generated by human liver microsomes and hepatocytes but not by those of rats. In rats, the metabolite was detectable in feces/bile but not in serum. The metabolite was not structurally alerting. It was decided to await the clinical trials data before further genetic toxicology investigations would be undertaken. Human volunteers had plasma levels of around 3% at the maximum dose, not detectable at lower doses. This was considered sufficiently low to forego further testing.

Example 8: A pro-drug (*N*-oxide) showed a weak effect in Ames test only in presence of S9. Other tests (mouse lymphoma, *in vitro* and *in vivo* micronucleus, *ex vivo* UDS) were negative. The active drug was negative

in standard battery (Ames, mouse lymphoma, micronucleus test in rat). A potential hydroxylamine metabolite of the pro-drug (hypothesized to represent the mutagenic metabolite) was synthesized and found negative in Ames test. There were no qualitative differences between human and rat metabolism and *in vitro* versus *in vivo* metabolism. Nevertheless, an Ames test with the pro-drug was performed with human S9 (pooled from six donors) and proved to be completely negative despite good metabolic capacity of the human S9. No 'rat S9' specific metabolite could be detected by analytic means in the *in vitro* mixes. The results were considered to provide adequate evidence that the isolated positive finding with the pro-drug was of no relevance to the human exposure situation.

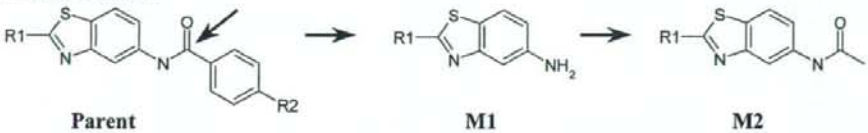
Example 9: A pharmaceutical was found to be negative in the Ames and *in vivo* micronucleus test, but showed a thresholded, high toxicity related clastogenic effect in the *in vitro* chromosome aberration test with human lymphocytes (±S9). *In vivo*, four main metabolites (no human specific one) were identified (mono hydroxy- and keto analogues). There was a request to study the metabolites in general toxicity to increase exposure. Testing for genotoxicity was also requested. Ames and *in vitro* chromosome aberration tests were performed with a mixture of the four metabolites in a ratio conforming to human C_{max} values without S9, only. The Ames test was negative, the *in vitro* chromosome aberration test yielded toxicity-related and threshold effects at higher concentrations than previously observed with the parent drug. The effective concentrations were many orders of magnitude higher than human C_{max} values. It was concluded that the results of the studies with the metabolites did not generate data that changed the risk/benefit situation for the drug.

Example 10: AMP397 was a drug candidate developed for the oral treatment of epilepsy, i.e. not a life-threatening disease. The molecule contains an aromatic nitro group, which obviously is a structural alert for mutagenicity. However, any attempt to replace the group resulted in the loss of desired pharmacological properties. Accordingly, the chemical was mutagenic in *Salmonella* strains TA97a, TA98 and TA100, all without S9, whereas the ICH standard battery mouse lymphoma *tk* and mouse bone marrow micronucleus tests were negative, although a weak high toxicity-associated genotoxic activity was seen in a micronucleus test in V79 cells [71]. In nitroreductase-deficient *Salmonella* strains TA98 NR and TA100 NR the mutagenic activity was abolished, and interestingly the amino derivative of AMP397 was not mutagenic in wild type TA98 and TA100. To exclude that a potentially mutagenic metabolite is released by intestinal bacteria, a Muta™ Mouse study was done with five daily treatments at the MTD, and sampling of 3, 7 and 21 days post-treatment. No evidence of a mutagenic potential was found in colon and liver. Likewise, a comet assay did not detect any genotoxic activity in jejunum and liver of rats, after single treatment with a roughly six times higher dose than the transgenic study, which reflects the higher exposure observed in mice. Also, a radioactive DNA binding assay did not find evidence for DNA binding in the rat liver. Thus, in conclusion, AMP397 was considered to be safe for entering clinical trials in the foreseen indication, because it was hypothesized that the positive Ames test was due to activation by bacterial nitroreductase, while practically all mammalian assays including four *in vivo* assays were negative, and no evidence for activation by mammalian nitroreductase or other enzymes were seen. Finally, no evidence for excretion of metabolites mutagenic for gut cells by intestinal bacteria was found.

Example 11: The drug candidate was developed for chronic treatment in a not life-threatening indication. *In silico* SAR using DEREK and MCASE did not detect a structural alert, and a five-strain Ames test was negative. In a micronucleus and a comet test using V79 cells, genotoxicity was seen only in the presence of Aroclor-induced rat liver S9. However, the ICH *in vitro* chromosome aberration test in human lymphocytes and a rat bone marrow micronucleus test were negative. Metabolism data showed that the compound is metabolized by CYP1A2 (liver) and CYP1A1 (lung). The metabolic pattern changed strongly with CYP1A-induced rat liver microsomes, such as after Aroclor 1254-induction, and there was direct evidence for the formation of reactive metabolites *in vitro* (rat, human) and indirect (glutathione conjugates) *in vivo* (rat), which was corroborated by the significant quenching of covalent binding by glutathione *in vitro* (rat, human). As a consequence, a number of exploratory Comet tests were conducted in order to clarify the genotoxicity. In human lymphocytes, a positive result was obtained with Aroclor-induced rat liver S9, and also in rat hepatocyte cultures with S9. In rats, no DNA damage induction was in blood, liver and kidney cells *in vivo*; however, after pre-treatment with Aroclor, Comets were induced in lung, liver and kidney, 3 and 24 h sampling after a single dose. Therefore, the compound was considered not to be safe for human use, as genotoxic effects were seen in mammalian cells *in vitro* and *in vivo*, which were considered to be due to formation of reactive intermediates. Further, the data obtained after Aroclor induction may be valid for humans because CYP1A can be induced by

food components, smoking, etc. Finally, the data indicate that GSH plays a key role in the deactivation of the reactive metabolites, so that GSH depletion may pose a significant risk.

Example 12: A company had recent experience with a number of molecules containing amide links that have been cleaved to generate potentially genotoxic aromatic amines; this has also been seen for two structures containing sulfonamide links. For reasons that are not understood, cleavage of these amides/sulfonamides is negligible in S9 or microsomal systems. In some cases, cleavage occurs *in vivo* and in hepatocytes *in vitro* but in others, it is clearly demonstrable only *in vivo*. Although some of the resulting aromatic amines have been tested and shown no evidence of bacterial mutagenicity, others have been shown to be genotoxic as illustrated by the structures below.



Parent **M1** **M2**

Five different molecules in this series were tested in the SOS/*umu* bacterial DNA repair test [72] with negative results. Subsequently, metabolism was shown to occur in rat hepatocytes via amide hydrolysis (M1), and then *N*-acetylation (M2) followed by GSH conjugation. Hydrolysis, but not glutathione conjugation, was seen in dog, minipig and human hepatocytes; it could not be demonstrated in rat S9 or microsomal systems.

The heterocyclic amines, M1, from three different parent molecules were then found to be positive in the SOS/*umu* test in the presence of S9 but, interestingly, the *N*-acetylated derivatives were negative with or without S9. Finally, one of two parent molecules was found to be positive in a Comet assay using hepatocytes *in vitro*; the second was negative, but solubility limited the maximum testable concentration. No *in vivo* genotoxicity data are available for this chemical series. The series was subsequently dropped from development.

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Non-homologous end-joining for repairing I-SceI-induced DNA double strand breaks in human cells

Masamitsu Honma, Mayumi Sakuraba, Tomoko Koizumi, Yoshio Takashima, Hiroko Sakamoto, Makoto Hayashi*

Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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ABSTRACT

DNA double strand breaks (DSBs) are usually repaired through either non-homologous end-joining (NHEJ) or homologous recombination (HR). While HR is basically error-free repair, NHEJ is a mutagenic pathway that leads to deletion. NHEJ must be precisely regulated to maintain genomic integrity. To clarify the role of NHEJ, we investigated the genetic consequences of NHEJ repair of DSBs in human cells. Human lymphoblastoid cell lines TSCE5 and TSCE105 have, respectively, single and double I-SceI endonuclease sites in the endogenous thymidine kinase gene (*TK*) located on chromosome 17q. I-SceI expression generated DSBs at the *TK* gene. We used the novel transfection system (Amaxa Nucleofector) to introduce an I-SceI expression vector into the cells and randomly isolated clones. We found mutations involved in the DSBs in the *TK* gene in 3% of TSCE5 cells and 30% of TSCE105 cell clones. Most of the mutations in TSCE5 were small (1–30 bp) deletions with a 0–4 bp microhomology at the junction. The others consisted of large (>60) bp deletions, an insertion, and a rearrangement. Mutants resulting from interallelic HR also occurred, but infrequently. Most of the mutations in TSCE105, on the other hand, were deletions that encompassed the two I-SceI sites generated by NHEJ at DSBs. The sequence joint was similar to that found in TSCE5 mutants. Interestingly, some mutants formed a new I-SceI site by perfectly joining the two original I-SceI sites without deletion of the broken-ends. These results support the idea that NHEJ for repairing I-SceI-induced DSBs mainly results in small or no deletions. Thus, NHEJ must help maintain genomic integrity in mammalian cells by repairing DSBs as well as by preventing many deleterious alterations.

1. Introduction

DNA double strand breaks (DSBs) are the most dangerous form of DNA damage. They can be caused by ionizing radiation (IR) or radiometric chemicals, and they can occur spontaneously during DNA replication. Other DNA damage, such as single strand breaks, easily convert to DSBs when a replication fork encounters them [1,2]. The non- or misrepair of DSBs can cause cell death or neoplastic transformation [3,4], so the accurate repair of DSBs is important for maintaining genomic integrity [5]. DSBs are generally repaired through non-homologous end-joining (NHEJ) or homologous recombination (HR) [6,7]. NHEJ joins sequences at the broken ends, which have little or no homology, in a non-conservative manner, and some genetic information is lost. HR, on the other hand, requires extensive tracts of sequence homology and is

* *Corresponding author*. Tel.: +81 3 3700 9847; fax: +81 3 3700 2348.
E-mail address: honma@nihs.go.jp (M. Honma).
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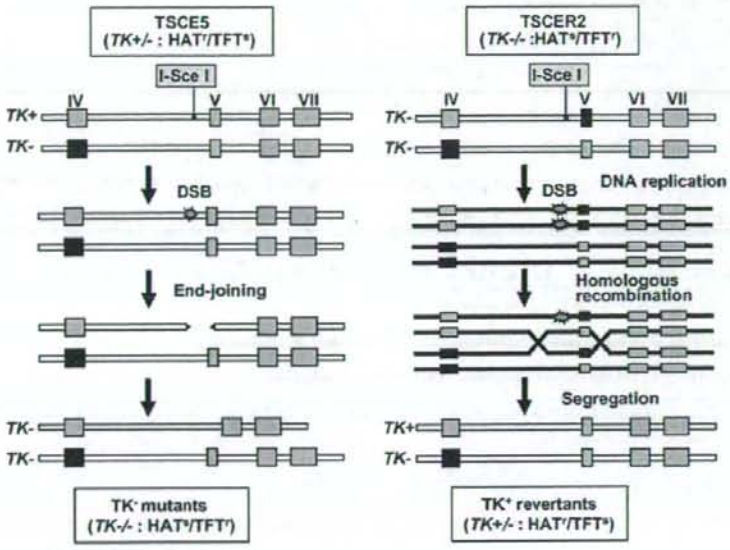

Fig. 1 – Schematic representation of the experimental system. Shadowed and closed rectangles represent the wild type and mutant exons of the TK gene, respectively. In TSCE5 cells, when a DSB at the I-SceI site is repaired by NHEJ and causes an exon 5 deletion, TK-deficient mutants are selected in TFT medium. In TSCER2 cells, when a DSB at the I-SceI site is repaired by HR, TK-proficient revertants are selected in HAT medium.

basically error-free [8]. HR is the primary DSB repair pathway in yeast and prokaryotes, but NHEJ is believed to be the primary pathway in mammalian cells [9]. HR is preferable to NHEJ because it is error-free, but NHEJ may have a different way to maintain genomic integrity.

We previously developed a human cell system to trace the fate of a DSB occurring in an endogenous single copy gene (Fig. 1) [10]. The human lymphoblastoid cell line, TSCE5, is heterozygous (+/−) and TSCER2 is compound heterozygous (−/−) for the thymidine kinase gene (*TK*), and both have an I-SceI endonuclease site in intron 4. DSBs can be generated at the I-SceI site by the introduction of an I-SceI enzyme expression vector. When DSBs occur at the TK locus, NHEJ in TSCE5 cells produces TK-deficient mutants, while HR between the alleles produces TK-proficient revertants in TSCER2 cells. Positive–negative drug selection for the TK phenotypes permits the distinction between NHEJ and HR repair mechanisms. Using the same system, we previously found that almost all I-SceI-induced DSBs in human cells are repaired by NHEJ and result in mainly 100–4000 bp deletions [10]. Drug selection, however, does not recover cells with genetic changes that are too small to influence TK function, and the resulting spectrum of mutations and reversions may be biased quantitatively as well as qualitatively.

To better understand the fate of DSBs in human cells, we randomly isolated non-selected clones after introducing DSBs and directly analyzed their DNA. A novel transfection system (Amaxa Nucleofector™) can introduce the I-SceI expression vector into most of cell population [11] and efficiently produces DSBs at the *TK* gene. With this improved method, we were able to detect cells with deletions at DSBs without drug selection and to trace the fate of DSBs without bias. We also developed a new cell line that has two I-SceI sites in the *TK* gene and can be used as a model for clustering DSBs. DNA sequence analysis of the mutants in this strain revealed that both single and double DSBs were repaired predominantly by NHEJ, producing only small genetic changes, or none. We discuss how NHEJ maintains genomic integrity.

2. Materials and methods

2.1. *Human cell lines for detecting NHEJ and HR induced by a single DSB*

Human lymphoblastoid cell lines TSCE5 and TSCER2 were previously created from TK6 cells [10], which are heterozygous for a point mutation in exon 4 of the *TK* gene (*TK*+/−) (Fig. 1). TSCE5 has a 31bp DNA flagment containing the 18 bp I-SceI site inserted 75bp upstream of exon 4 of the *TK*+ allele and retains TK function. TSCER2 is a TK-deficient mutant spontaneously arising from TSCE5. It has a point mutation (G:A transition) at 23bp of exon 5 of the *TK*+ allele of TSCE5. TSCER2 is compound heterozygote (*TK*−/−) for the *TK* gene. NHEJ for a DSB occurring at the I-SceI site results in TK-deficient mutants in TSCE5 cells, while HR between the alleles produces TK-proficient revertants in TSCER2 cells.

2.2. *I-SceI expression and isolation of mutant clones*

We introduced the I-SceI expression vector (pCBASce) by suspending 5×10^6 cells in 0.1 ml Nucleofector solution V (Amaxa

Biosystem, Koeln, Germany) with 50 μg of uncut pCBASce vector (or without the vector as a control), following the manufacturer's recommendations. We then plated the cells into 96-microwell plates at 1 cell/well. Two weeks later, we randomly isolated single colonies and independently expanded them for DNA analysis.

We maintained the cell culture for 3 days and then seeded them into 96-microwell plates in the presence of 2.0 μg/ml trifluorothymidine (TFT) for isolating TK-deficient mutants or HAT (200 μM hypoxanthine, 0.1 μM aminopterin, 17.5 μM thymidine) for isolating TK-proficient revertants. We counted the drug-resistant colonies 2 or 3 weeks later [12] and calculated the mutation and revertant frequencies according to the Poisson distribution [13].

2.3. *Creating a cell line containing two I-SceI sites*

The targeting vector, pTK10, which we had used to make TSCE5 cells, consists of about 6 kb of the original *TK* gene encompassing exons 5, 6, and 7 and an I-SceI site in intron 4 [10]. We constructed pTK13 by inserting an additional 21 bp DNA fragment containing the 18 bp I-SceI sequence into pTK10 at the Nco I site in intron 5 (152 bp down stream of exon 5) using site-directed mutagenesis (GeneTailer, Invitrogen) (Fig. 4a). To obtain TK-revertant clones with two I-SceI sites in the *TK* gene, we transfected TSCER2 cells (5×10^6) with 20 μg of linearized pTK13 vector using the Nucleofector system. After 72 h, we seeded the cells into 96-microwell plates containing HAT. We identified one revertant clone, TSCE105, as correctly targeted and confirmed its molecular structure by DNA sequencing.

2.4. *DNA analysis*

To analyze mutations in the isolated TSCE5 and TSCE105 clones, we amplified the part of the *TK* gene containing the I-SceI sites by PCR, labeling forward primers with a fluorescent dye. We used the following primers for the I-SceI site in intron 4: forward (166F), 5′-TGG GAG AAT TAA GAG TTA CTC C-3′; reverse (196R), 5′-AGC TTC CAC CCC AGC AGC AGC T-3′. We used the following for the I-SceI site in intron 5: forward (251F), 5′-GGA TGG GCA CAG AGA CAC CA-3′; reverse (241R), 5′-CTG ATT CAC AAG CAC TGA AG-3′. For TSCE105 clones, we used 166F and 241R to amplify the regions containing both I-SceI sites. Amplification was performed by denaturation at 96 °C for 5 min, followed by 25 cycles of 96 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and extension at 72 °C for 10 min. We analyzed the PCR products using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and sequenced them with an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA).

3. Results and discussions

3.1. *Efficiency of the system for detecting NHEJ and HR repair of chromosomal DSBs using Amaxa nucleofection*

The lymphoblastoid cell lines, TSCE5 and TSCER2, which we previously developed, can trace the genetic consequences of chromosomal DSBs in the human genome. NHEJ for a DSB occurring at the I-SceI site results in TK-deficient mutants in TSCE5 cells, while HR between the alleles produces TK-proficient revertants in TSCER2 cells (Fig. 1) [10]. To introduce the I-SceI expression vector into the cells, we now used the Amaxa nucleofection system. The Amaxa Nucleofector™ can directly transfer DNA into the nucleus of the cells at high efficiency. It was designed for primary cells and hard-to-transfected cell lines such as the human B-cell lymphoblastoid [11,14]. Twenty-four hours after the nucleofection, approximately 65% of the transfected TSCE5 cells expressed the I-SceI enzyme, suggesting that DSBs were efficiently introduced into the cells (data not shown; Takashima et al., under submission).

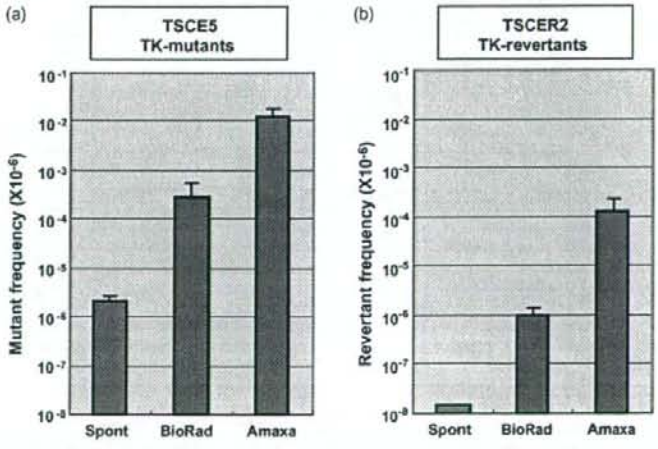

Fig. 2 – Detection of NHEJ and HR repaired DSBs using Amaxa nucleofection or BioRad electroporation. (a) Transfection of TSCE5 with the I-SceI expression vector using Amaxa nucleofection increased the TK-deficient mutant frequency more than 40-fold compared with BioRad electroporation. (b) Transfection of TSCER2 with the I-SceI expression vector using Amaxa nucleofection increased the TK-proficient revertant frequency more than 100-fold compared with BioRad electroporation.

Following Amaxa nucleofection, the mean TK mutant frequency in TSCE5 cells was 1.21%, which was more than 40-fold higher than the frequency we observed with the transfection system we had used previously (BioRad electroporation) (Fig. 2a), and the mean TK-proficient revertant frequency in TSCER2 cells was 1.22×10^{-4}, which was more than 100-fold higher than we observed previously (Fig. 2b). These results demonstrate that the Amaxa nucleofection system effciently introduced the expression vector and generated DSBs with high efficiency in the TSCE5 and TSCER2 cell lines. The relative contribution of NHEJ and HR for reparing the DSBs was 100:1. The value may be biased, however, because the drug selection assay recovers certain classes of NHEJ and HR.

3.2. *Genetic consequences of a chromosomal DSB in non-selected clones*

Because the I-SceI site is inserted into intron 4 of the functional TK allele 75 bp upstream of exon 5, any small deletions caused by NHEJ that do not affect TK function will not be recovered as TFT-resistant mutants in the TSCE5 assay. Similarly, in the TSCER2 assay, short tract gene conversion events that do not extend to exon 5 will not be recovered as TK revertants. Thus, recovery of TK mutants and revertants by drug selection may be biased. Because nucleofection can efficiently generate DSBs at the I-SceI site, however, the system enables detection of deletions and recombination in the TK gene without drug selection. We randomly isolated 926 transfected clones without TFT selection and directly analyzed DNAs from them. We observed that 29 (3.13%) of them had an I-SceI mutation; these

Table 1 – Analysis of non-selected TSCE5 clones after I-SceI expression

Total clones	Mutant clones	Mutants (%)
926	29 (Total)	3.13
	23 (Small deletion, insertion, rearrangement; <60 bp)	2.48 (79.3)
	5 (Large deletion; >60 bp)	0.54 (17.2)
	1 (Gene conversion)	0.11 (3.4)

were usually small (<60 bp) deletions, insertions, or rearrangements (Table 1). Fig. 3 shows the DNA sequences of 21 mutants with small genetic changes. Three of them (1659, 1841, and 1893) contained a 1 bp deletion at a CCC tract within the I-SceI site. Others had mostly 0–4 bp microhomologies at the junction, suggesting that the NHEJ machinery was involved. The mutant that had a 1 bp insertion at a TT tract within the I-SceI site (2018) might have been generated by misalignment of the cohesive ends. The mutant that exhibited a complicated DNA rearrangement involving a 50 bp deletion combined with a 9 bp inverted sequence that was a part of deleted sequence (1614) was probably the result of sister chromatid fusion and breakage after DNA replication, as described previously [10]. Five of the mutants showed large deletions (17.2%). This fraction may correspond to the TK mutants in the drug selection assay. The large deletions which were commonly detected in the drug selection assay ranged from 1070 to 4030 bp, and had 4–7 bp microhomology at their junctions (data not shown) [10].

One mutant was the product of gene conversion between homologous alleles. It had lost the I-SceI site and retained

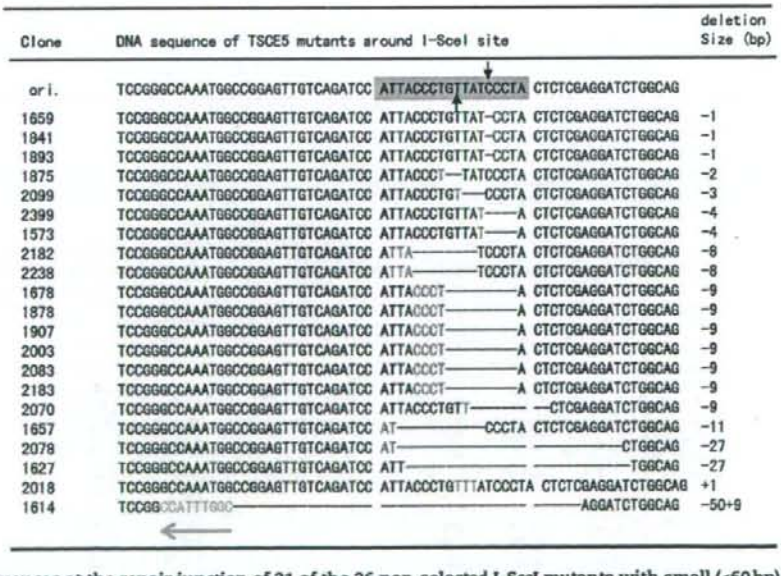

Fig. 3 – DNA sequences at the repair junction of 21 of the 26 non-selected I-SceI mutants with small (<60 bp) genetic changes in TSCE5 cells ("ori." is original sequence). The I-SceI recognition site is highlighted in orange. Arrows indicate I-SceI cleavage sites. The 1 bp deletion in the CCC tract is shown in blue and the 1 bp insertion in the TT tract is shown in green. Microhomologous sequences at junctions are shown in red. The sequence in yellow with a left arrow indicates an inverted sequence from part of a deleted sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

intron 4 of the TK gene that had been originally connected to the I-SceI site. The appearance of HR mutants was infrequent in the non-biased assay, too, suggesting that I-SceI-induced DSBs are mainly repaired by NHEJ, resulting in small deletions [10,15–17]. This does not mean that HR rarely works for DSBs, however, because our I-SceI system does not cover all HR events.

Most I-SceI systems have been developed using artificial reporter substrates based on exogenous drug-resistance or fluorescence genes and are biased in favor of detecting certain classes of deletions and recombination events [18–20]. In the present system, however, we conducted a survey of DSBs occurring in the endogenous single-copy gene, and investigated the consequences of the DSB without selection bias. We first demonstrated the mutational spectrum induced by I-SceI endonuclease in the human genome. However, it does not necessarily reflect the fate of DSBs occurring spontaneously or induced by irradiation, because our I-SceI system does not monitor sister chromatid HR, which must be the major HR pathway in mammalian cells. Other I-SceI systems setting up two tandem copies of the selective gene on the same chromosome can not also evaluate sister chromatid HR quantitatively, because both chromatids are theoretically cleaved during S/G2 phase. We may underestimate the contribution of HR in the I-SceI system.

Although the I-SceI expression vector was introduced into about 65% of the cells, the frequency of mutants at the I-SceI site in the non-selection assay was still only 3.1%. Three possibilities could explain this: (1) only a small proportion of TSCE5 cells expressing the I-SceI vector may undergo a DSB, (2) most cells with DSBs may undergo apoptosis, and (3) some DSBs may go back to their original sequence by perfect joining. The last possibility would be important to the maintenance of genomic integrity following DSB repair, but its demonstration would be difficult because it is impossible to distinguish between non-cleaved and perfectly repaired I-SceI sites.

3.3. *Genetic consequences of two closely separated DSBs*

To efficiently generate DSBs in the genome, we developed a cell line containing two I-SceI sites in the TK gene. We constructed a targeting vector, pTK13, consisting of 6kb of original TK gene including exon 5, 6, 7 and two I-SceI sites flank-

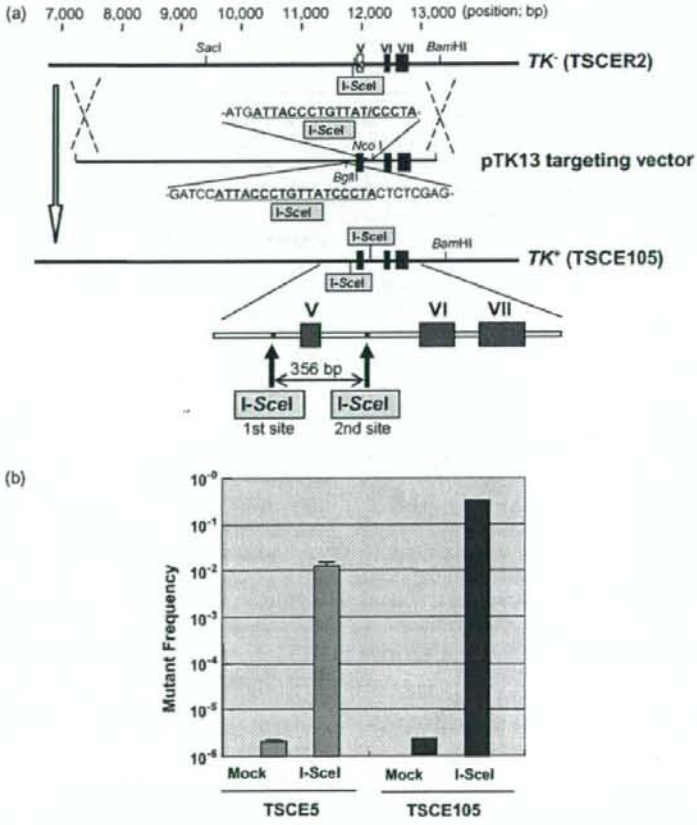

Fig. 4 – (a) Creating the TSCE105 cell line with two I-SceI sites. The functional TK allele in the TSCE105 cell line has two I-SceI recognition sites flanking exon 5, 356 bp apart. (b) The TK-deficient mutant frequency in the TSCE105 cells after introduction of DSBs by Amaxa nucleofection. The mutant frequency was 30-fold higher in TSCE105 than in TSCE5.

Table 2 – Analysis of TSCE105 mutants after I-SceI expression

Total Mutants	Type of Mutation	356bp (1st 2nd)	Number of Mutants(%)
125	Only 1st I-SceI		1 (0.8)
	Only 2nd I-SceI		47 (38)
	Both, independent		6 (4.8)
	Both, combined		70 (56)
	Perfect joining		4
	Joining with small deletion (<60bp)		31
	Joining with large deletion (>60bp)		29
	Joining with rearrangement		6
	Recombination		1 (0.8)

ing exon 5, and transfected it to TSCER2 cells (Fig. 4a). One HAT-resistant recombinant, TSCE105, had another I-SceI site at intron 5 of the TK gene in addtion to the original I-SceI site in TSCE5. The two I-SceI sites are 356 bp apart, flanking exon 5 (Fig. 4a). TSCE105 was also a TK heterozygote and was TFT-sensitive. When we nucleofected the I-SceI expressing vector into TSCE105, the TK-deficient mutant frequency by the TFT selection assay, surprisingly, was extremely high (31.3%) (Fig. 4b). We also examined non-selectecd clones after nucleofection. Among 283 non-selected clones, 83 (29.3%) of them had a deletion mutation involving one or both I-SceI sites. This mutation frequency was about the same as the TK-deficient mutation frequency in the TFT selection assay, suggesting that most mutations in TSCE105 were deletions involving coding sequence of the TK gene.

To investigate the genetic changes induced by the two DSBs, we analyzed 125 mutants (42 TFT-selected and 83 non-selected) and classified them into 4 types depending on whether they occurred (1) only at the first I-SceI site, (2) only at the second I-SceI site, (3) independenly at both I-SceI sites, or (4) at the combined first and second I-SceI sites (Table 2). The majority (56%) were the last type. Interestingly, four of them joined the two I-SceI sites perfectly, creating a new I-SceI site. Fig. 5 shows the DNA sequences around the joint sites of 26 of the 31 mutants that had small deletions. Almost all of them had a 0–4 bp microhomology at the junction, and the sequences were similar to those found around single DSB repair sites (Fig. 3).

While a single DSB in TSCE5 cells caused predominantly small deletions, two closely occuring DSBs in TSCE105 cells were not repaired independently and caused large deletions involving the two I-SceI sites, indicating that multiple DSBs enhance genetic changes qualitatively as well as quantitatively. Mammalian cells may have difficulty retaining small DNA fragments generated by multiple DSBs. High doses of ionizing irradiation, too, not only increase mutation frequency but also change the mutation type to predominantly large deletions [21,22]. The genomic changes observed in TSCE5 and TSCE105 may reflect a dosage-effect, bringing about different numbers of DSBs. In both cases, however, NHEJ is involved and injury is minimized.

The mutants with perfect joining were generated by NHEJ without exonuclease processing in which the cleaved two flanking I-SceI ends simply join. Most of I-SceI-induced DSBs in TSCE5 and TSCE105 cells may be perfectly joined and create a new I-SceI site. Because the I-SceI enzyme is continuously expressed for at least 48 h after nucleofection (Takashima et al., under submission), the new I-SceI sites generated by perfect joining are cleaved again and again. When the DSBs are occasionally joined after exonuclease processing, they accumulate as deletional mutations and are not cleaved any more (Fig. 6). Thus, the perfect joining by NHEJ is important for repairing DSBs, at least endonuclease-induced DSBs. The perfect joining by NHEJ was also reported in other I-SceI-induced DSB systems [23,24]. Van Heemst et al. demonstrated that a blunt DSB induced by the *E. coli* transposon Tn5 were repaired without loss of nucleotides in Chinese hamster cell lines, suggesting that compatible ends precisely join without deletions [25]. The efficiency or accuracy of precise NHEJ was reduced in Ku80, DNA-PK, XRCC4, or p53 deficient cells [23–26].

NHEJ in mammalian cells involves seven components—Ku70, Ku80, DNA-PKcs, Artemis, XRCC4, Cernunnos/XLF, and Ligase IV [4,7,27–29]. Although the exact role of these proteins remains unknown, three steps have been suggested: (1) end-binding, (2) terminal processing, and (3) ligation [9]. Karanjawala et al demonstrated that defects in Artemis and DNA-PKcs, which are key components in step 2 and possess substantial nucleolytic activity, do not cause severe phenotypes or genomic instability [30]. On the other hand, deficiency of Ku (step 1) or Ligase IV (step 3) confers severe radiosensitivity or lethality [30]. Thus, the second step may not be essential in NHEJ of DSBs, especially of endonuclease-induced DSBs, because the cleaved DNA ends are ligatable

Clone	DNA sequence of TSCE105 mutants around junction site	Deletion Size (bp)
	1st site 2nd site	
perfect	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATCCCTA GGTCTGTGCAGACTGC	
2412	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356 (0)
2429	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356 (0)
2445	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356 (0)
2465	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356 (0)
2703	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTAT-CCTA GGTCTGTGCAAACTGC	-357 (-1)
2650	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGT——CCCTA GGTCTGTGCAAACTGC	-359 (-3)
2393	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTG——CCCTA GGTCTGTGCAAACTGC	-360 (-4)
2453	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTA————TCCCTA GGTCTGTGCAAACTGC	-364 (-8)
2434	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC AT————ATCCCTA GGTCTGTGCAAACTGC	-365 (-9)
2345	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT————A GGTCTCTGCAAACGGC	-365 (-9)
2689	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT————A GGTCTCTGCAAACGGC	-365 (-9)
2714	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT————A GGTCTCTGCAAACGGC	-365 (-9)
2764	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT————A GGTCTCTGCAAACGGC	-365 (-9)
2444	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT————A GGTCTCTGCAAACGGC	-365 (-9)
2446	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT————A GGTCTGTGCAAACTGC	-365 (-9)
2424	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT————A GGTCTGTGCAAACTGC	-365 (-9)
2304	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC AT————CCCTA GGTCTGTGCAAACTGC	-367 (-11)
2442	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGT———— ————GCAAACTGC	-372 (-16)
2443	TCCGGGCCAAATGTCCGGAGTTGTCAGATCC ————TA GGTCTGTGCAAACTGC	-372 (-16)
2402	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTAT—— ————AACTGC	-372 (-16)
2425	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATC—— ————TGC	-374 (-18)
2435	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTT———— ————GC	-378 (-22)
2713	TCCGGGCCAAATGGCCGGAGTTGTC———— ———— ——TGTGCAAACTGC	-384 (-28)
2735	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ———— ————CTGC	-378 (-22)
2405	TCCGGGCCAAATGGCCG———— ———— ——TCTGTGCAAACCGC	-392 (-36)
2437	TCCGGGC———— ———— ————AAACTGC	-409 (-53)

Fig. 5 – DNA sequences at the NHEJ repair junction around the I-SceI junction site in TSCE5 cells. "Perfect" is the DNA sequence when two I-SceI sites join perfectly and create a new I-SceI site (highlighted in orange). Sequences in black are upstream of the first I-SceI site and those in blue are downstream of the second I-SceI site. A total of 26 TSCE105 mutants with deletions combining two I-SceI sites are shown. Underlining indicates a new I-SceI recognition sequence produced by error-free NHEJ. Red indicates microhomologous sequences at junctions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and do not require terminal processing. Perfect joining by NHEJ probably skips the second step. Naturally occurring DSBs produced by oxidative stress, ionizing radiation, and DNA-damaging agents, however, do not have directly ligatable DNA ends and need some form of nucleolytic processing [7,9]. Their repair by NHEJ results in deletions, even if it works properly. In the present study, the size of the deletions caused by NHEJ, however, were relatively small. No recovered TSCE5 or TSCE105 mutants exhibited large deletions or translocations similar to those frequently observed

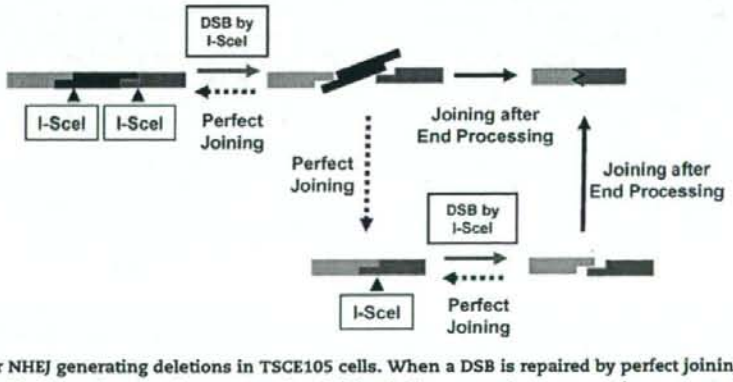

Fig. 6 – A model for NHEJ generating deletions in TSCE105 cells. When a DSB is repaired by perfect joining, an I-SceI site newly generates and is cleaved again. The rare DSB that is joined after exonuclease processing converts to a deletional mutation and accumulates in the cell population.

at the chromosome level in cancer cells [4]. This suggests that NHEJ helps maintain genomic integrity in mammalian cells by repairing DSBs as well as by preventing many deleterious alterations.

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Potassium bromate treatment predominantly causes large deletions, but not GC > TA transversion in human cells

Yang Luan [a,b,c], Takayoshi Suzuki [a], Rajaguru Palanisamy [a,d], Yoshio Takashima [b], Hiroko Sakamoto [b], Mayumi Sakuraba [b], Tomoko Koizumi [b], Mika Saito [b,e], Hiroshi Matsufuji [e], Kazuo Yamagata [e], Teruhide Yamaguchi [a], Makoto Hayashi [b], Masamitsu Honma [b,*]

[a] *Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[c] *Center for Drug Safety Evaluation, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 294 Tai-Yuan Road, Shanghai 200031, China*
[d] *Department of Biotechnology, School of Engineering and Technology, Bharathidasan University, Palkalaiperur, Tiruchirappalli 620024, India*
[e] *Department of Food Science and Technology, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa-shi, Kanagawa 252-8510, Japan*

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Abstract

Potassium bromate ($KBrO_3$) is strongly carcinogenic in rodents and mutagenic in bacteria and mammalian cells in vitro. The proposed genotoxic mechanism for $KBrO_3$ is oxidative DNA damage. $KBrO_3$ can generate high yields of 8-hydroxydeoxyguanosine (8OHdG) DNA adducts, which cause GC > TA transversions in cell-free systems. In this study, we investigated the in vitro genotoxicity of $KBrO_3$ in human lymphoblastoid TK6 cells using the comet (COM) assay, the micronucleus (MN) test, and the thymidine kinase (*TK*) gene mutation assay. After a 4 h treatment, the alkaline and neutral COM assay demonstrated that $KBrO_3$ directly yielded DNA damages including DNA double strand breaks (DSBs). $KBrO_3$ also induced MN and *TK* mutations concentration-dependently. At the highest concentration (5 mM), $KBrO_3$ induced MN and *TK* mutation frequencies that were over 30 times the background level. Molecular analysis revealed that 90% of the induced mutations were large deletions that involved loss of heterozygosity (LOH) at the *TK* locus. Ionizing-irradiation exhibited similar mutational spectrum in our system. These results indicate that the major genotoxicity of $KBrO_3$ may be due to DSBs that lead to large deletions rather than to 8OHdG adducts that lead to GC > TA transversions, as is commonly believed. To better understand the genotoxic mechanism of $KBrO_3$, we analyzed gene expression profiles of TK6 cells using Affymetrix Genechip. Some genes involved in stress, apoptosis, and DNA repair were up-regulated by the treatment of $KBrO_3$. However, we could not observe the similarity of gene expression profile in the treatment of $KBrO_3$ to ionizing-irradiation as well as oxidative damage inducers.

Keywords: Potassium bromate ($KBrO_3$); *TK*-mutation; Loss of heterozygosity (LOH); 8-Hydroxydeoxyguanosine (8OHdG); Gene expression profile

* Corresponding author. Tel.: +81 3 3700 1141x435; fax: +81 3 3700 2348.
E-mail address: honma@nihs.go.jp (M. Honma).

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1. Introduction

Potassium bromate ($KBrO_3$) is used as in bread making a flour improver and in the production of fish-pastes. The EU countries now prohibit its use as a food additive because of its carcinogenicity. Japan and the USA, however, permit its use in bread making on the condition that it never remains in the final product. $KBrO_3$ causes tumors, especially in kidney, in rats, and mice after long-term oral administration in drinking water [1–3]. $KBrO_3$ is also genotoxic. It is positive in in vitro genotoxicity tests – including the bacterial reverse mutation assay [1], the chromosomal aberration test conducted in Chinese hamster cells [4], and the mouse lymphoma assay [5] – and in vivo in the micronucleus test (MN) [6,7].

It has been proposed that $KBrO_3$ induces tumors through the production of oxidative damage to DNA. Oxidative DNA damage can cause mutations that contribute to the activation of oncogenes and/or the inactivation of tumor suppressor genes, thereby leading to tumorigenesis [8,9]. 8-Hydroxydeoxyguanosine (8OHdG) is the main form of oxidative DNA damage induced by $KBrO_3$ [10]. It primarily causes GC > TA transversions (as a result of the pairing of 8OHdG with A) and is believed to be responsible for mutagenesis, carcinogenesis, and aging [11,12]. $KBrO_3$ increases 8OHdG DNA adducts in vivo and in vitro [13–15]. However, $KBrO_3$ induces mutations weakly in microbial mutation assays and the *Hprt* mutation assay in mammalian cells, while it induces chromosome aberrations strongly both in vivo and in vitro [1,16,17]. These findings raise the question of whether 8OHdG is required for the mutagenic process involved in $KBrO_3$-induced carcinogenesis.

In the present study, we examined the genotoxic properties of $KBrO_3$ using the comet assay (COM), the MN test, and thymidine kinase (*TK*) gene mutation assays in human lymphoblastoid TK6 cells [18]. Unlike the X-linked hemizygous *HPRT* gene mutation assay, the *TK* mutation assay can detect not only point mutations, but also large scale chromosomal deletions, recombinations, and aneuploidy [19–21]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumed relevant to carcinogenesis. We analyzed the *TK* mutants induced by $KBrO_3$ at the molecular level and investigated what kind of mutation predominated. We also profiled global gene expression in TK6 cell exposed to $KBrO_3$ using Affymetrix GeneChip® Expression analysis to understand the genotoxic mechanism of $KBrO_3$.

2. Materials and methods

2.1. Cell culture, chemicals, and treatment

The TK6 human lymphoblastoid cell line has been described previously [22]. Cells were maintained in RPMI 1640 medium (Gibco-BRL, Life Technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JR Biosciences, Lenexa, KS), 200 μg/ml sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were incubated at 37 °C in a 5% CO_2 atmosphere with 100% humidity. $KBrO_3$ (CAS No.7758-01-2) was purchased from Wako Pure Chemical Co. (Tokyo) and dissolved in RPMI medium just before use.

We prepared 20 ml aliquots of cell suspension at a concentration of 5.0×10^5 cells/ml in 50 ml polystyrene tubes. Different concentrations of $KBrO_3$ were added to the tubes, which were then placed on a platform shaker and incubated at 37 °C for 4 h with gentle shaking. At the end of the treatment period, the cell cultures were centrifuged, washed once, and re-suspended in fresh medium. We cultured them in new flasks for the MN assay and *TK* gene mutation assay, or diluted them for plating for survival estimates.

2.2. Genotoxicity assays

After treating cells with $KBrO_3$, we prepared slides for conducting the alkaline and neutral COM assay. The alkaline COM assay was performed as previously reported [23]. For the neutral COM assay, the slide was electrophoresed with chilled neutral solution (pH 8) containing of 90 mM Tris, 2 mM Na_2EDTA, and 90 mM boric acid according to the method by Wada et al. [24]. The COM slides were stained with SYBER green (Molecular Probes, Eugene, OR) and observed by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and tail length of the comet was measured. The relationship between $KBrO_3$ treatment and migration was statistically analyzed by the Dunnett test [25].

We prepared the MN test samples 48 h after treatment, as previously reported [23]. Briefly, approximately 10^6 cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold methanol containing 25% acetic acid, then re-suspended in methanol containing 1% acetic acid. A drop of the suspension was placed on a clean glass slide and air-dried. The cells were stained with 40 μg/ml acridine orange solution and immediately observed with the aid of an Olympus model BX50 fluorescence microscope equipped with a U-MWBV band pass filter. At least 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test [26].

We prepared the *TK* gene mutation assay samples 3 days after treatment. We seeded cells from each culture into 96-well plates at 40,000 cells/well in the presence of 3.0 μg/ml trifluo-

rothymidine (TFT). We also plated 1.6 cells/well without TFT to determine plating efficiency. All plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 14 days, we scored colonies on the PE plates and the normal-growing (NG) *TK* mutants on the TFT plates, then re-fed the plates containing TFT with fresh TFT, incubated them for an additional 14 days, and scored them for slow-growing (SG) *TK* mutants. Mutation frequencies, relative survival (RS), and relative suspension growth (RSG) were calculated as previously described [23]. The data of mutant frequencies were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson–Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [27].

2.3. LOH analysis of TK mutations by polymerase chain reaction (PCR)

To avoid analyzing identical mutants, we performed an additional *TK* mutation assay and isolated *TK* mutants from independent culture after a 4 h treatment with 2.5 mM $KBrO_3$. We confirmed the phenotype of the *TK* mutant clones by re-challenging them with TFT medium. We also determined the growth rate of the clones and confirmed whether they were NG or SG mutants.

Genomic DNA was extracted from the *TK* mutant cells and used as a template for PCR. We conducted the PCR-based LOH analysis of the human *TK* gene as described previously [28]. A set of primers was used to each amplify the parts of exons 4 and 7 of the *TK* gene that is heterozygous for frame shift mutations. A third primer set for amplifying parts of the β-globin was also used as the internal control. We applied quantitative-multiple PCR for co-amplification of the three regions. The PCR products were analyzed with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and were classified into "no LOH", "hemizygous (hemi-) LOH", or "homozygous (homo-) LOH". To determine the extent of the LOH, we analyzed 10 microsatellite loci on chromosome 17q by PCR-based LOH analysis [28]. The results were processed by GenoTyper™ software (PE Biosystems, Chiba, Japan) according to the manufacturer's guidelines.

2.4. Gene expression analysis

Total RNA was isolated from the TK6 cells after 4 h treatment with 2.5 mM $KBrO_3$ and was purified by RNeasy columns (Qiagen, Valencia, CA). We conducted a single cDNA synthesis, cRNA labeling, and cRNA fragmentation according to the manufacturer's recommendations (Affymetrix Inc., Santa Clara, CA) and employed Affymetrix GeneChip Expression analysis. The hybridization mixture for each sample was hybridized to an Affymetrix U133A human genome array. We processed the scanned data using Microarray Suite Software Version 5.0 (Affymetrix Inc., Santa Clara, CA) and imported the data into GeneSpring software (Silicon Genetics, Redwood City, CA). Signal intensity was normalized by per-gene and per-chip, and the ratios were calculated by normalizing $KBrO_3$ sample to the corresponding control sample. We used intensity-dependent (step-wise) selection of significant changes with higher cut-off value for lower signal intensity (1.75-, 2.0-, 2.25-, 2.5-, and 3.5-fold for genes intensity range of >1000, 500–1000, 100–500, 50–100, and 10–50, respectively), and up-regulated genes with a presence call in $KBrO_3$ sample, whereas down-regulated genes with a presence call in the control sample.

3. Results

3.1. Cytotoxicity and genotoxicity of $KBrO_3$

$KBrO_3$ exerted strong and concentration-dependent cytotoxicity in TK6 cells (Fig. 1). It induced approximately 50% cytotoxicity (51% RSG and 44% RS) at 2.5 mM. To investigate whether $KBrO_3$ directly causes DNA damage, we conducted the COM assay. Induction of COM tail after the treatment of in alkaline version was statistically significant 2.5 and 5 mM. In the neutral COM assay, the induction was observed form the lower concentration (Fig. 1). Because the neutral COM is thought to be associated with DNA double strand breaks (DSBs) [29], this result indicates that $KBrO_3$ directly causes DNA damage including DSBs. $KBrO_3$ also induced MN and *TK* mutation in a concentration-dependent manner and their inductions were statistically significant (Fig. 1). At the maximum concentration, it induced both MN and *TK* mutation frequencies about 30 times the control values. Two distinct phenotypic classes of *TK* mutants were generated: NG mutants grew at the same rate as the wild type (doubling time 13–17 h), and SG mutants grew at a slower rate (doubling time > 21 h). NG mutants result from intragenic mutations, while SG mutants result from gross changes (extending beyond the *TK* gene) [20]. $KBrO_3$ predominantly induced SG mutants (Fig. 1), implying that $KBrO_3$ treatment predominantly causes gross structural changes, but not small genetic alterations such as point mutations.

3.2. Molecular analysis of TK mutants

The *TK* mutants were randomly isolated from independent cultures treated with 2.5 mM $KBrO_3$ for 4 h. Table 1 shows the cytotoxicity (RSG), mutation frequency, and proportion of SG mutants induced by $KBrO_3$. We subjected 40 induced mutants to LOH analysis. Of those, 32 (80%) were SG mutants, which corresponded closely to the percentage of SG mutants induced in the assay (74.1%), indicating that the result of LOH analysis reflected the character of the induced