[44,45], amounting to 14% and 25% of DMSO in the preincubation mix. In the plate incorporation assay the initial concentration of DMSO is 3.7% (100 μl per 2.7 ml softagar) but will decrease upon diffusion into the bottom agar.

However, DMSO has been demonstrated to have a marked effect on the activities of CYP enzymes [46]. At concentrations of just 1% (v/v), DMSO can cause up to 75% inhibition of some CYP enzymes. Thus, pushing concentrations of test chemical to very high levels may yield diminishing returns with regard to the generation of potential reactive mutagenic metabolites due to the inhibitory effect of the delivery solvent.

The working group recommended limiting the DMSO concentration to a maximum of 1% for mammalian cell *in vitro* test systems. In the Ames test, the maximal volumes of DMSO should be 100 μl/plate for the plate incorporation but lower in the preincubation test version (31.6 μl/plate of DMSO produced little poisoning of metabolic activation for a number of promutagens [Gocke, personal communication]).

2.1.5. Issue of substrate saturation

The use of very high concentrations of test compound may also prove fruitless if the enzyme kinetics are such that saturation and even substrate inhibition occur. For drugs, it is typical to observe K_M values of below 100 μM. Testing compounds at concentrations higher than this (i.e. up to 10 mM) will generally not yield any greater quantity of metabolites because saturation of the enzyme will already have occurred at lower concentrations. Additionally, it is quite common to observe reductions in the amounts of metabolite at high (compared to low) concentrations due to substrate inhibition of CYP reactions at very high concentrations. If it is desired to generate metabolites that arise via a series of sequential reactions, it is likely that a very high concentration of parent compound in the incubation will take up all of the enzyme, effectively inhibiting downstream metabolism. Finally, to achieve high concentrations of compounds will usually require the use of delivery solvents, which can be inhibitory, as described above. No consensus was reached for recommending a general reduction of the maximal concentration of test compound to values below 10 mM for tests including S9.

2.1.6. Metabolite case examples

Appendix A provides short synopses of case examples shared with the working group where diverse aspects of drug metabolism precipitated further investigations. The examples address situations of unique human metabolites and inefficient metabolism by standard S9 preparations, in some cases because of extrahepatic metabolism or, in others, for unknown reasons. Remedies for solving the issues included direct testing of the metabolite in question or the use of human S9. The extended testing provided assurance that the initially 'missed' metabolites did not pose undue risks. Other cases involved parent molecules, which showed genotoxic activities in the standard studies with or without S9 and bacterial-specific activation steps leading to considerations on potential activation by intestinal bacteria. Problematic aspects of metabolism were recognized in some cases in early, preclinical metabolism studies. In other cases, identification of human specific metabolism was realized in human clinical ADME studies. While the working group was unable to define a minimally essential and/or most appropriate course of action for the individual cases, the examples illustrate well the diversity of issues arising and might serve as a basis for defining expedient approaches in analogous situations.

2.2. Current status of possible remedies

2.2.1. Use of genetically modified bacteria, mammalian cells and animal models for studying the activation and inactivation of genotoxins

To be detected as mutagens, some genotoxic metabolites have to be formed within the target cell by enzymes that are not represented in standard *in vitro* test systems, as exemplified in the introduction for *N*-sulfooxy-2-AAF. SULT-dependent activations are not uncommon. Using genetically modified target cells, activation by SULTs has been demonstrated for more than 100 chemicals, including various carcinogens (such as tamoxifen, cyproterone acetate, safrole, nitrofen and some nitrotoluenes) that are missed in conventional test systems [31–33]. Depending on the compound, varying SULT forms were required for the activation. Like *N*-sulfooxy-2-AAF, several other several sulfo conjugates [e.g. furfuryl sulfate and 1-(α-sulfooxyethyl) pyrene] had to be formed within the target for a positive test result. Other reactive sulfo conjugates underwent spontaneous substitution reactions with components of the culture medium, such as chloride anions, leading to the formation of secondary, membrane-penetrating active species [47]. Moreover, cDNA-mediated expression of organic anion transporters in target cells enhanced the genotoxic effects of some reactive sulfuric acid esters externally added [48]. Such uptake mechanisms might play a role in the organotropism of reactive species, but should not be relied on when testing new compounds.

Other conjugating enzymes (some UGTs, GSTs and NATs) have also been expressed in target cells.

Activation of promutagens by UGTs in such models has not yet been reported (and not been studied). However, co-expression of human UGT1A1 provided protection against the mutagenicity and cytotoxicity of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in CHO-derived cells engineered for expression of CYP1A2 [49]. Human GST T1, expressed in *Salmonella typhimurium*, strongly enhanced the mutagenicity of various dihalogenated alkanes as well as diepoxybutane [50]. The activation of some of these agents could also be demonstrated using external glutathione (GSH)-conjugating systems [51], but extent of uptake and its dependence on the structures of the reactive GSH conjugates are largely unexplored. Heterologous expression of GSTs in mammalian cells conferred resistance against various alkylating agents; in some cases, this protection was enhanced by, or even strictly dependent on, the co-expression of an export pump (MRP-1 or MRP-2) [52]. The expression of endogenous acetyltransferases (termed OAT) in *Salmonella* may be a reason for the high mutagenic activity observed in the Ames test with many amino- and nitroarenes, whose final activation step often is an *O*-acetylation. *Salmonella* strains are available in which OAT has been replaced by a mammalian NAT [33,53], which differ in substrate specificity. Thus, various aromatic hydroxamic acids are activated to mutagens by human NATs, but not by OAT. Such differences may sporadically lead to misleading results when standard bacterial strains are used. Unlike typical phase-2 metabolites, acetyl conjugates are uncharged. Nevertheless the site of their formation can strongly affect the outcome of mutagenicity experiments. Thus, 2-hydroxylamino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole shows much higher mutagenicity in *S. typhimurium* TA98 compared to an OAT-deficient variant of this strain; however, purified OAT in the presence of its cofactor acetyl-CoA drastically reduced its bacterial mutagenicity (although it strongly enhanced the covalent binding to naked DNA) [54]. Various standard mammalian target cells, including most sublines of V79 cells, do not express any endogenous NAT. Heterologous expression of human NATs in these cells strongly enhanced the genotoxic effects of many nitro- and aminoarenes [33,55]. For example, induction of gene mutations by 3-nitrobenzanthrone required 100 times lower substrate concentrations in NAT2-expressing compared to control V79 cells. The isomer, 2-nitrobenzanthrone, was mutagenic in cells engineered for expression of human SULT1A1, but not in control cells (Glatt et al., unpublished result). 2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) induced gene mutations in V79 cells co-expressing human NAT2 or NAT1 together with human CYP1A2, even at a concentration of 0.01 and 1 μM, respectively, but was inactive (even at 30 μM) in cells expressing only CYP1A2 [33,55].

Liver S9 is useful as a source of diverse CYP activities in genetic toxicology studies. CYP forms have been individually expressed in bacterial and mammalian target cells primarily with the aim to identify the forms that can activate a given promutagen [56,57]. It was noticed, however, that this approach can drastically increase the test sensitivity, especially for mammalian cell models. For example, the concentration of classical carcinogens (such as benzo[*a*]pyrene, dibenzo[*a*,*l*]pyrene, aflatoxin B_1 and *N*-nitrosodimethylamine) could be decreased by a factor of 700–25,000 in the V79/*hprt* test, when the appropriate CYP was expressed compared with a standard protocol using S9 (Glatt et al., unpublished results). Several factors may contribute to these differences in sensitivity:

- Some promutagens are preferentially activated by a CYP form that is low in standard S9 (e.g. CYP1B1 or 2E1).
- Diffusion pathways are longer for externally generated active metabolites resulting in more opportunities for alternative chemical reactions (e.g. with components of S9 or cell membranes) than for metabolites formed within the target cell.
- Since S9 is not stable and toxic to cells upon prolonged exposures, incubations with this preparation have to be limited to 2–4 h, which is much less than the replication time of a mammalian cell; in contrast, there is no real time limit for exposing metabolically competent cells (24 h exposures are typical, and 72 h is unproblematic for the V79/*hprt* assay).
- Competing pathways, leading to inactive metabolites, may be more prevalent with S9 than with more specific enzyme systems (this may be the least important point, as in many cases a major portion of the test compound remains unchanged during the incubation time).

The decrease in the substrate concentration required by CYP-expressing cells has some technical advantages, e.g. concentrations of organic solvents can be decreased, and poorly soluble compounds can be tested appropriately. Thus, picene and 3,4,3′,4′-tetrachlorobiphenyl, which are negative in standard *in vitro* tests, were clearly mutagenic (even at a concentration of 100 nM) in cells engineered for expression of CYP1B1 (Glatt et al., unpublished results). It may be speculated that such sensitive systems will produce many false positive results. However, the available results do not justify this fear.

The systems are not only highly sensitive, but also highly specific, with regard to the enzyme system required for the activation of a given compound as well as the compounds activated by a given metabolic system. Moreover, effects occurring at low substrate concentrations *in vitro* are more likely to be relevant for humans at realistic (low) exposures than *in vitro* effects produced at heroic concentrations in standard systems, where promiscuous activation may occur even with carcinogens; this can lead to an apparently correct result via an incorrect mechanism. The fact that appropriate metabolically competent cells readily detect classical carcinogens at very low concentrations (usually in the nM range) would provide a rationale for reducing the maximal concentration of the test compound to a biochemically sound level (for example, in the range of 100 μM).

At present genetically engineered cells are primarily used as an analytical tool for elucidating activation and inactivation mechanisms. In general, only one to three foreign factors (enzymes or transporters) have been expressed in a given cell line. For screening purposes, this number would have to be increased in order to keep low the total number of cell lines required. This is technically feasible. Indeed, a human cell line (MCF-5) exists in which five enzymes have been overexpressed using gene transfer techniques [58]. The working group has not further explored the strengths and weaknesses of this cell line, as not much data has been published for this cell line and no member had practical experience with it.

An important aim of genetic toxicology studies is the detection and assessment of carcinogenic risks to humans. In practice, results of animal carcinogenicity studies represent the "gold standard" used for validating *in vitro* test systems. However, carcinogenicity results from studies in rats and mice are frequently discordant; even larger differences may be expected for human, a phylogenetically remote species. This species-dependence of carcinogenicity is often due to differences in biotransformation. Indeed, cDNA-expressed orthologous enzymes from different species have demonstrated pronounced differences in substrate and product specificity. Likewise they can substantially differ in their regulation and tissue distribution. For example, the expression of most SULT forms is focused on the liver (and is usually sex-specific in adult animals) in the rat; in contrast, several human SULTs are highly expressed in extrahepatic tissues but low or absent in liver, with only minute sex-dependent differences [59]. In recent years, various genes of human xenobiotic-metabolizing enzymes, including their regulatory sequences, have been introduced into mouse models. In some cases, e.g. with human SULT1A1 and 1B1, this resulted in the expression of the human enzyme with a human-like tissue distribution in the mouse (Meinl et al., in preparation). Similar approaches have been used for some CYPs [60]. It will be interesting to explore these models in carcinogenicity and genotoxicity investigations.

2.2.2. *Utility of S9 from human, and potential utility of "humanized" test systems*

In practical terms, there are three possible ways to apply human S9 in genotoxicity testing systems:

(1) As an *in vitro* metabolic activation/detoxification system for hazard identification of unique or major human metabolites.
(2) As a second tier assay for comprehensive assessment.
(3) For replacing induced rat S9.

Human S9 that has a high level of metabolic enzyme activity or shows a potent genotoxicity for a test agent might be useful (in addition to using pooled human S9) for a retroactive approach (see Section 4) to a metabolism issue. In addition, a potential future testing approach combining multiple endpoints including micronuclei, Comet, and TK6 gene mutations based on human cells with human S9, may be proposed as an *in vitro* humanized test system for a proactive approach (see Section 4) to addressing metabolism issues. The humanized genotoxicity tests are also helpful for elucidating the genotoxic mechanisms in human cells. The current status of possible remedies is discussed below.

In a recent publication there were no practical differences in the mutagenicity of three chemicals in the Ames test using pooled human S9 from three different sources that were commercially available (HAB Biomedical Research Institute, In Vitro Technologies, and Xenotech) [37]. When using a crude human liver S9 in the Ames test, contaminating bacterial colonies might be found and could lead to an increased number of apparent revertant colonies over the normal range on control plates. Therefore, it may be useful to use a purified fat- and bacteria-free human S9, as can be obtained by a simple modification to the crude S9 preparation [39].

A large and extensive body of data on the use of human liver S9 or microsomes in the Ames test has recently been presented [36,40–43]. The mutagenicity of about 60 chemicals was studied using the Ames test in the presence of a selected human S9 with a high metabolic activity and also with pooled human S9. The mutagenicity of chemicals in induced rat S9 and human S9 varied considerably, depending on the chemicals. Most of the mutagens tested (75%) were less mutagenic in the pres-

ence of human S9 than in the presence of induced rat S9. On the other hand, the other compounds (25%), including some aromatic amines, polycyclic azaarenes, and nitrosamines, showed a more potent mutagenicity with human S9 than with induced rat S9.

To examine the inter-individual variation in the mutagenicity of chemicals using a variety of human liver S9 samples, the mutagenicity of 3 chemicals was examined in the Ames test using S9 from 18 separate donors and also using a pooled human S9 sample [38]. There was a large inter-individual diversity in the mutagenic response to procarcinogens. The results also suggest that the use of both selected human S9 with high metabolic activity and a pooled S9 could be used as a means to evaluate the inter-individual variability in mutagenic response to chemicals.

The mutagenicity of nine chemicals was assayed by the Ames test using human S9 at varying S9 concentrations (1, 3, 10 or 30% S9 fraction in the S9 mix) (published in part in [37]). Many of the chemicals tested showed higher mutagenic activity at increasing S9 concentrations. A 10% S9 concentration (1 mg S9 protein/plate), which is usually used, may be sufficient to compare the mutagenic activity of chemicals with that produced by induced rat S9, although it may be suggested to use >10% concentrations of human S9 to confirm a negative response. When the mutagenic activity was calculated as induced revertants/μg/plate/pmol total CYP, the data suggest that S9 quality and amount of CYP protein may be probable reasons for the large diversity in the mutagenic activities of the chemicals in the presence of induced rat S9 and human S9. The mutagenic activity varied by as much as 100-fold between induced rat S9 and human S9, depending on the chemicals.

An *in vitro* humanized test system was recently established based on human cells used together with human S9 (Honma et al., in preparation) and 16 chemicals were tested using this assay system. Human lymphoblastoid cell lines WTK-1, which were homozygous p53 mutant cells of TK6, were used as human cell lines. This multiple endpoint genotoxicity test system incorporates the Comet assay, micronucleus test, and the TK gene mutation assay. Human carcinogens (class 1 by IARC), benzidine, cyclophosphamide, and 2-naphthylamine clearly showed genotoxicity with human S9 as well as rat S9. Rodent carcinogens (2A or 2B), benzo[*a*]pyrene, a food mutagen (IQ), and dibutylnitrosamine exhibited no or very weak responses with human S9 although they were extremely genotoxic with rat S9. On the other hand, 2-aminoanthracene, which is non-classified in IARC, yielded stronger genotoxicity with human S9 than with rat S9, suggesting that special attention should be taken for evaluating its human carcinogenicity.

2.2.3. Use of recombinant human CYPs as an external activation source for Ames testing

Experiences using baculovirus-infected insect cell-expressed human CYPs as an exogenous metabolic activation source for *in vitro* bacterial mutagenicity testing were reviewed [61]. The testing of selected model pro-mutagens incorporated the use of Gentest's Supermix™, a mixture of individual cDNA-expressed enzymes (Supersomes™) prepared using a baculovirus expression system. Exogenous glucose-6-phosphate dehydrogenase and NADP were added to the incubation mixtures as co-factors. The enzyme mixture includes CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, which have been demonstrated to be principally responsible for the oxidative metabolism of pharmaceuticals. The mutagenicity results using Supermix™ were compared to results using induced rat and human liver S9 activation sources. 2-Aminoanthracene showed similar mutagenic activity with human S9 and Supermix™ (maximum 40–50-fold increases in revertants above negative controls), with slightly higher activity (maximum 60-fold increases in revertants above controls) noted for induced rat liver S9. The reason for this apparent discrepancy in 2-aminoanthracene response between human and induced rat S9 [36–40] could be related to differences in the amount of enzyme protein or concentration used. In contrast, benzo[*a*]pyrene showed much higher mutagenic activity with induced rat liver S9 (maximum 25-fold increases in revertants above controls) than with Supermix™ or human liver S9 (<5-fold increases), likely attributed to the importance of CYP1A1 in the activation of benzo[a]pyrene. 7,12-Dimethylbenz[*a*]anthracene demonstrated comparable levels of mutagenic activity between induced rat liver S9 and Supermix™ (maximum 14-fold increases). This latter result is somewhat puzzling, as the knockout of CYP1B1 [62,63] or microsomal epoxide hydrolase [64] abolishes the carcinogenicity of 7,12-dimethylbenz[*a*]anthracene in mice. Both enzymes are absent in Supersomes™, and CYP1B1 is absent or extremely low in human S9. Thus, the results with the human enzyme preparations are either false-positive or 7,12-dimethylbenz[*a*]anthracene is carcinogenic in humans via a different activation mechanism than in the mouse models used. Despite such open points, the results confirm and support the utility of alternative exogenous activation sources to complement standard genotoxicity testing with induced rat liver S9. The con-

ditions established to utilize recombinant human CYPs may be important when there are informed data on the route of metabolism and this system (either individual Supersomes™ or Supermix™) may be more proficient at generating a human metabolite of interest from the parent drug in an *in vitro* test system.

2.3. Existing recommendations, guidance or strategies

A position paper was published in 2002 [7] that outlined the pharmaceutical industry views on the qualification criteria for determining whether a metabolite is major or minor or whether it is species specific. This discussion continued with the publication of a draft guidance for comment by the U.S. FDA [10]. Each of these documents discussed the toxicology testing strategy that should be implemented for human specific metabolites. In both cases the recommended strategy includes an initial evaluation of the potential genotoxicity of a specific metabolite in a minimal screen of *in vitro* assays to detect point mutations and chromosomal aberrations. Currently the guidance is undergoing the approval process at the agency.

3. Consensus principles and recommendations

From the review of the information currently available, the working group agreed upon a number of principles that lead to the development of recommendations for a proposed working strategy. Table 2 summarizes the consensus statements that should be integrated into an overall common strategy for genotoxicity testing and risk assessment. The basic elements of the principles center on ensuring that a human metabolite of interest is represented in genotoxicity and eventual carcinogenicity testing. This includes evaluating alternative (and more "competent", i.e. capable of generating the metabolite of interest) metabolic activation or test systems, ensuring the selection of a competent carcinogenicity test species, defining appropriate action triggers based on the extent of human exposures (i.e. "major" or unique), considering any structural knowledge of the metabolite (e.g. evidence of reactivity) and whether any evidence of genotoxicity is apparent in testing with standard metabolic activation (induced liver S9). The working group also emphasized the need to consider these points in relation to the timing of human ADME studies in the case of pharmaceutical development, and proposed both proactive and retroactive strategies to assess metabolite genotoxic potential (below). Lastly, the potential genotoxicity of a metabolite (in either case

Table 2
Consensus principles and recommendations as derived from discussions in the IWGT metabolic considerations subgroup

Rodent species used for carcinogenicity testing can generate more, less or different metabolites from humans. The potential exists for generating false positives/negatives. The following should be considered to reduce this gap
Use of an alternative metabolic activation system for genotoxicity testing that is capable of generating the human metabolite of interest
Assure that a carcinogenicity test species can generate the metabolite of interest
The triggers for safety follow-up may include: precedented clinical exposures to a metabolite, a unique human metabolite, structural knowledge of the metabolite (e.g. evidence of reactivity) or any preclinical evidence of genotoxicity in standard testing of the parent chemical agent (e.g. genotoxic effects seen only in the presence of liver S9)
The timing of the human ADME studies (for pharmaceutical development) is a critical trigger for considering additional safety assessment
Consider information that point to the potential for a unique human metabolite to determine if human ADME studies should be conducted earlier
There is a need for the development of a more proactive but practical approach for genetic toxicology screening applied to metabolites
This strategy should consider structural knowledge of the potential metabolite in the design of an appropriate genotoxicity test system
Based on structural knowledge, consider using proficient tester strains/cells or more appropriate exogenous activation sources (alternative microsomes, S9). Notable examples include
Use of CYP2E1 proficient systems for small molecules
Use of SULT proficient systems for amino, amido and nitroarenes
For pharmaceuticals, there is a need to develop a retroactive but practical strategy to respond to documented human metabolite exposures from human ADME studies
Consider structural knowledge of the metabolite of interest in designing an appropriate genotoxicity test strategy
In evaluating the extent of metabolite exposures generated via external metabolic activation systems from testing the parent chemical agent (or drug), it is inappropriate to extrapolate risk through the use of safety margins comparing concentrations generated in *in vitro* hazard tests with *in vivo* human exposures
If a human metabolite of interest cannot be generated under standard conditions of metabolic activation, exploring a more optimized system to generate the metabolite of interest is an option to consider
Consider the use of rat liver S9 induced by alternatives to Aroclor 1254 or phenobarbital/5,6-benzoflavone, or adjusting enzyme cofactors
Certain types of active metabolites (including many short-lived phase-2 metabolites) will not sufficiently penetrate cell membranes. Therefore, it is not recommended to routinely add cofactors for phase-2 enzymes to detect possible bioactivation by these enzymes
Consider alternative systems/tissues/species. This could include liver S9 from human or other animal species (e.g. hamster), alternative cells & tissues, genetically engineered systems, and

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

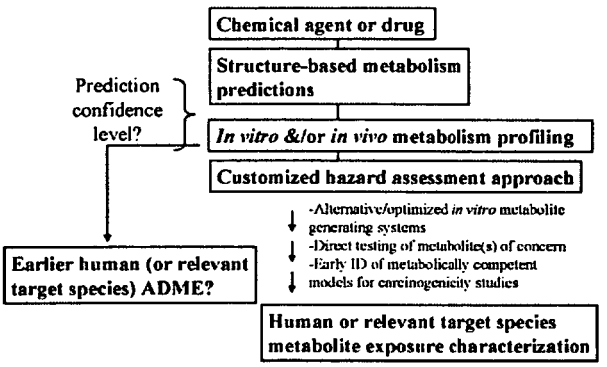

Fig. 1. Proactive-predictive strategy for potential human metabolites.

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

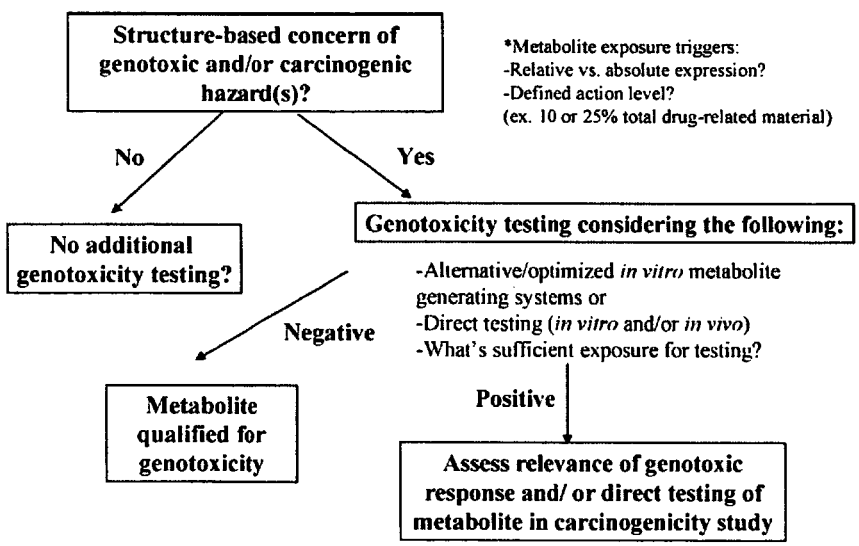

Fig. 2. Retroactive-risk management strategy for precedented unique or "major" human metabolites*.

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.

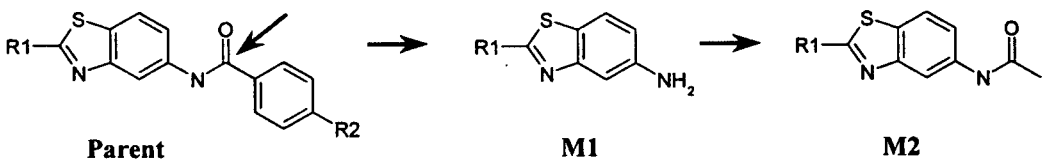
Example 9: A pharmaceutical was found to be negative in the Ames and *in vivo* micronucleus test, but showed a thresholded, high toxicity related clastogenic effect in the *in vitro* chromosome aberration test with human lymphocytes (±S9). *In vivo*, four main metabolites (no human specific one) were 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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Environmental and Molecular Mutagenesis 48:248–257 (2007)

Research Article

3′-Azido-3′-deoxythymidine Induces Deletions in L5178Y Mouse Lymphoma Cells

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3′-Azido-3′-deoxythymidine (AZT), a nucleoside analogue used for the treatment of acquired immunodeficiency syndrome (AIDS), induced a significant dose-related increase in the thymidine kinase (*Tk*) mutant frequency (MF) in L5178Y/$Tk^{+/-}$ 3.7.2C mouse lymphoma cells. Treatment with 1 mg/ml (3,742 μM) AZT for 24 hr resulted in a MF of 407 × 10^{-6} compared to a control MF of 84 × 10^{-6}. The MFs of the large and small colony mutants resulting from AZT exposure were 142 × 10^{-6} and 265 × 10^{-6}, respectively. One hundred and fifty mutants from the 1 mg/ml (3,742 μM) AZT-treated culture and sixty-nine mutants from independent untreated cultures were isolated and analyzed. LOH analysis using a heteromorphic microsatellite locus located in the *Tk* gene was performed to determine the presence or absence of the Tk^{+} allele. Eight other microsatellite markers spanning the entire mouse chromosome 11 also were examined for heterozygosity to determine the extent of LOH. In addition, *Tk* gene dosage analysis was conducted using Real-Time PCR in those mutants showing LOH at the *Tk* locus. The presence of only one *Tk* allele based on Real-Time PCR indicated that the mutant resulted from deletion while the presence of two alleles was consistent with a recombination event. More mutants from the AZT-treated culture showed *Tk* LOH than did independent mutants from the untreated cultures (91% vs. 64%) and the induced mutants also showed distinct chromosome 11 LOH patterns. The mutation spectrum of mutants from AZT-treated cells was also significantly different from that of spontaneous mutants. More deletions and fewer intragenic mutations were observed in the mutants from the AZT-treated culture than independent mutants from the untreated control. Our data indicate that AZT primarily induced LOH mutations in L5178Y mouse lymphoma cells and a large number of LOH mutations resulted from deletions. Environ. Mol. Mutagen. 48:248–257, 2007. Published 2007 Wiley-Liss, Inc.†

Key words: 3′-azido-3′-deoxythymidine; mouse lymphoma assay; loss of heterozygosity; copy number; mutation spectrum

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) has become a serious global problem. For instance, 800,000 children were newly infected in 2004 and most of the infections were due to vertical transmission (from the mother to her newborn child) [UNAIDS, 2005]. 3′-Azido-3′-deoxythymidine (AZT, zidovudine) is an antiretroviral thymidine analogue. It was the first FDA-approved anti-HIV drug and has been the most widely-used drug in the prevention and treatment of AIDS. In one study, it reduced the incidence of transplacental and perinatal transmission of HIV from 22.6 to 7.6% [Connor et al., 1994]. AZT is regularly used in the prevention of vertical transmission and the recommended regimen includes three steps: administration to the mother starting in the 14th week of pregnancy, treatment of the mother during labor, and treatment of the infant for the first 6 weeks after birth [NIH, 2004].

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Invited article on the genotoxicity of perinatal NRTI therapy.

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AZT can incorporate into proviral DNA and cause DNA chain termination, which is one of its therapeutic mechanisms. Unfortunately, several studies have shown that AZT also can be incorporated into mammalian nuclear DNA, leading to genetic toxicity [Agarwal and Olivero, 1997; Zhu et al, 2000]. The nature of AZT's genotoxicity is of special interest because during the prophylactic administration to prevent vertical transmission, uninfected infants are exposed transplacentally and perinatally. This also applies to the prophylactic use in health care workers who are accidentally exposed to HIV [Kennedy and Williams, 2000].

Several studies have demonstrated that AZT is a transplacental and perinatal carcinogen in mice and rats. When given orally to CD-1 mice during the last third of gestation, AZT increased the incidence of tumors in the lung, liver, ovary, mammary gland, and seminal vesicles of the offspring [Olivero et al., 1997]. Also, AZT administrated to neonatal mice on postnatal days 1–8 produced a dose-related increase in lung and liver tumors [Diwan et al., 1999]. It also induced vaginal epithelial cell tumors in adult CD-1 mice and CD rats given this drug for 22 and 24 months, respectively [Ayers et al., 1996], as well as dose-related increases in vaginal squamous cell carcinomas in CD-1 mice exposed in utero and then between 35 days and 24 months after birth [Ayers et al., 1997].

There is also substantial evidence indicating that the tumorgenicity of AZT is closely related to the incorporation of AZT triphosphate into cellular DNA [Vazquez-Padua et al., 1990; Sussman et al., 1999; Meng et al., 2000a]. Transplacental exposure of mice and monkeys to AZT results in AZT incorporation into the genomic DNA of many fetal tissues, including brain, lung, liver, kidney, skin, heart, and placenta [Olivero et al., 1997; Poirier et al., 1999]. Several in vitro systems have demonstrated the incorporation of AZT into cellular DNA [Vazquez-Padua et al., 1990; Darnowski and Goulette, 1994; Olivero et al., 1994]. Olivero et al. [1999] found that the majority of leukocyte samples from the peripheral blood of AZT-treated adults or the umbilical cord blood of infants exposed *in utero* had detectable AZT-DNA levels.

Various in vitro and in vivo studies have demonstrated the genotoxicity of AZT. AZT induced significant increases in chromosome aberration and sister-chromatid exchange (SCE) in human lymphocytes and CHO cells [Gonzales-Cid and Larripa, 1994]. Agarwal and Olivero [1997] showed that AZT induced chromosomal aberrations and micronuclei in human T-lymphocytic H9 cells. The hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) gene MF was increased 1.8-fold by AZT in the *TK6* human lymphoblastoid cell line [Sussman et al., 1999]. A subsequent study measured the *TK* gene MF in *TK6* cells, and the results indicated that AZT increased the *TK* MF to a much greater extent than the *Hprt* MF, and the increases were consistent with AZT-DNA incorporation [Meng et al., 2000a]. Similar results were obtained in in vivo studies. In B6C3F1/$Tk^{+/-}$ mice, AZT induced a significant increase in the *Tk* MF in spleen lymphocytes, but the MF of the *Hprt* gene was not increased [Von Tungeln et al., 2002]. Recently Von Tungeln et al. [2007] demonstrated that transplacental exposure of neonatal offspring of B6C3F1/$Tk^{+/-}$ mice to AZT resulted in increases in micronuclei and *Tk* MF. These results suggest that AZT mainly induces large-scale genetic alterations; this effect is consistent with its action as a DNA chain terminator. Because the *Hprt* gene is located on the X chromosome, the *Hprt* assay is not efficient in the detection of large deletions, nor does it detect recombination events requiring the presence of two chromosomes. The *Tk* gene is located on an autosomal chromosome (chromosome 11 in the mouse, chromosome 17 in humans), and the *Tk* assay can detect both large and small genetic alterations and also recombination [Liber et al., 1989; Moore et al., 1989].

Several studies have demonstrated that AZT induces a high frequency of LOH, both in vitro and in vivo. Using Southern blot analysis, Meng et al. [2000a] found that 84% of AZT-induced mutants in TK6 cells displayed LOH at the *TK* gene. They also found that AZT significantly induced LOH at the adenine phosphoribosyl transferase (*APRT*) locus in the AZH1 human lymphoblastoid cell line [Meng et al., 2000b]. Von Tungeln et al. [2002] found that AZT induced LOH in 61% of *Tk* mutant lymphocyte clones in B6C3F1/$Tk^{+/-}$ mice. However, LOH analysis using only the *Tk* locus does not evaluate the extent of the chromosome damage. Mittelstaedt et al. [2004] analyzed the LOH pattern of chromosome 11 in spleen lymphocyte *Tk* mutants from B6C3F1/$Tk^{+/-}$ mice using allele-specific PCR methods based on microsatellite polymorphisms. Their results showed that the LOH pattern of mutants from AZT-treated mice differed significantly from the control mice and were consistent with mutation induction by chromosome loss, mitotic recombination, and deletion.

However, microsatellite analysis alone cannot distinguish between deletion and recombination. To do so, a gene dosage analysis or cytogenetic analysis is required. Such analysis is possible using mouse lymphoma cell *Tk* mutants, which are relatively easy to isolate, culture, and analyze. For this study, a Real-Time PCR method was developed to detect the *Tk* gene copy number. Therefore, recombination and deletion were distinguished and a more detailed AZT-induced mutation spectrum was determined.

MATERIALS AND METHODS

Cell Culture and *Tk* Mutation Assay

Stock cultures of L5178Y/$Tk^{+/-}$ mouse lymphoma cells were cultured in suspension using Fischer's medium for leukemic cells of mice (Quality Biologicals, Gaithersburg, MD) supplemented with 10% heat-inactivated horse serum, 200 μg/ml sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.05% (v/v) pluronic F68 (Invitrogen, Carlsbad, CA). The cultures were incubated at 37°C in an atmosphere of 5% CO_2 with saturated humidity and maintained in logarithmic growth.

The protocol for the microwell version of the mouse lymphoma assay was described by Chen and Moore [2004]. 3′-Azido-3′-deoxythymidine (AZT) was obtained from Cipla (Mumbai, India), through its U.S. supplier (Bryon Chemical, Long Island City, NY), and dimethylsulfoxide (DMSO) and trifluorothymidine (TFT) were obtained from Sigma (St. Louis, MO). Briefly, cells were centrifuged and resuspended at a concentration of 0.2 × 10^6 cells/ml in 50 ml of medium in 75-cm^2 polystyrene flasks. AZT was dissolved in DMSO. Different concentrations of AZT were added to the cell cultures in a final volume of 200 μl (control cells were exposed to 200 μl DMSO), and the final concentrations of AZT ranged from 100 to 1,000 μg/ml (374–3,742 μM). The flasks were incubated at 37°C in an atmosphere of 5% CO_2 with saturated humidity for 24 hr. The cells were then centrifuged, washed with fresh medium twice, and resuspended in fresh medium. The cells were transferred to new 75-cm^2 flasks and maintained in logarithmic growth for a 2-day expression period. Then the cells were cloned in 96-well plates in medium containing 3 μg/ml TFT for selection and medium without TFT for the measurement of cloning efficiency. All plates were incubated in an atmosphere of 5% CO_2 with saturated humidity at 37°C for 12 days. The colonies were counted and the colony-size was determined by eye using a Quebec dark field colony counter. Total, small colony (SC), and large colony (LC) *Tk* MFs were determined and the relative total growth (RTG) value that measures cytotoxicity was calculated according to the published protocol [Chen and Moore, 2004].

Tk Mutant Isolation and DNA Extraction

One hundred and fifty mutants were randomly selected and isolated from the culture treated with the highest test dose of AZT (1 mg/ml, 3,742 μM). Sixty nine spontaneous mutants were isolated from independent untreated cultures over a period of approximately 2 months (one large and one small colony mutant were randomly isolated from each independent culture). All of the isolated mutant colonies were grown in medium containing 3 μg/ml TFT to confirm their phenotype.

Genomic DNA was extracted from 3 × 10^6 cells of each *Tk* mutant clone using the Qiagen DNeasy tissue kit (Valencia, CA; protocol version: March 2004) and stored at −20°C.

Tk Gene LOH Analysis

Tk gene LOH analysis was conducted using the microsatellite PCR described by Liechty et al. [1996], with some modifications. Briefly, the microsatellite locus *D11Agl2* that resides in the *Tk* gene was amplified using primers Agl2.frw and Agl2.rev [Liechty et al., 1996]. PCR amplifications were conducted in a total volume of 20μl: 2 μl of the extracted mutant DNA were mixed with 10 μl PCR Master Mix (Promega, Madison, WI), primers, and water. The final amount of each reagent was: 1× Taq polymerase reaction buffer, 10 pmol of each primer, 12.5 nmol of each dNTP, and 0.25 U Taq DNA polymerase. The PCR was performed in 96-well plates using a PCR System 9700 (Applied Biosystems, Foster City, CA), with the following touch-down method: a 3 min denaturation at 95°C; followed by 2 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 72°C, and 30 sec extension at 72°C. The annealing temperature was then decreased by 1°C for each additional two cycles until 65°C was reached. Then 20 additional cycles were performed at the 65°C annealing temperature, followed by a final extension at 72°C for 7 min. The reaction products were separated by 2% agarose gel electrophoresis, stained with 1 μg/ml ethidium bromide, and visualized with a UV transilluminator.

Chromosome 11 LOH Analysis

In addition to microsatellite marker *D11Agl2*, 8 other informative microsatellite loci on mouse chromosome 11 (*D11Mit 42, 59, 36, 29, 22, 20, 19,* and *74*) were identified from the mouse genome data base (Jackson Laboratory, Bar Harbor, ME, http://www.informatics.jax.org). The 9 microsatellite loci are almost evenly distributed along the length of the chromosome (with locations at 78.0, 72.0, 58.5, 47.6, 40.0, 25.0, 20.0, 13.0, and 0.0 cM, respectively). LOH analysis was performed at each microsatellite locus using allele-specific PCR.

The same PCR amplification conditions were used for the 8 loci. The conditions varied from those used for *D11Agl2* in the cycling parameters: a 5 min denaturation at 95°C; followed by 40 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 59°C, and 45 sec extension at 72°C; and a final extension of 10 min at 72°C. The reaction products were separated and visualized as above.

Tk Gene-Dosage Analysis

For those mutants showing LOH at the *Tk* locus, *Tk* gene copy number (CN) was further evaluated using a Real-Time PCR method. A 170-base DNA fragment in intron 2 of the *Tk* gene and a 192-base fragment in exon 3 of an unrelated reference gene (*H-2K*) on chromosome 17 were amplified separately and simultaneously [Honma et al., 2001]. The fragment in the *H-2K* gene was used as an endogenous reference for PCR quantification based on the fact that the amplification efficiencies are approximately equal for the *H-2K* and *Tk* fragments (the absolute value of the slope of the relative efficiency plot for the two amplification reactions, <0.1).

The PCR conditions for the two fragments were the same. Real-Time PCR amplification was conducted in a total volume of 50 μl; 2 μl of the extracted mutant DNA was mixed with 25 μl iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), primers, and water. The final amount of each reagent was: 1× Taq polymerase reaction buffer, 20 pmol of each primer, 10 nmol of each dNTP, 1.25 U iTaq DNA polymerase, and 0.5 pmol SYBR green fluorescein. The PCR reaction was performed in 96-well plates using an iCycler iQ Real-Time PCR detection system (Bio-Rad), with the following cycling parameters: a 5 min denaturation at 95°C; followed by 40 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 59°C, and 30 sec extension at 72°C; and a final extension of 10 min at 72°C.

When the amount of amplification product reached a given threshold in the exponential phase, the cycle number of the two amplification reactions was measured using the iCycler iQ optical system software (version 3.0a, Bio-Rad). Each quantitative PCR reaction was repeated three times and the mean cycle number was used for the calculation. The *Tk* gene CN for each of the mutants was calculated as follows:

1. Because the amplification efficiencies were almost the same for the two PCR reactions, the relative cycle number of wild-type *Tk* amplification ($C_{W\text{-}Tk\text{-}Relative}$) was adjusted by the *H-2K* amplification (comparative ΔC_T method):

$$C_{W\text{-}Tk\text{-}Relative} = C_{W\text{-}Tk} + (C_{M\text{-}H2K} - C_{W\text{-}H2K})$$

[$C_{W\text{-}Tk}$ is the cycle number of *Tk* gene amplification using DNA isolated from wild-type ($Tk^{+/-}$) mouse lymphoma cells; $C_{M\text{-}H2K}$ is the cycle number of *H-2K* gene amplification using DNA isolated from the *Tk* mutant; $C_{W\text{-}H2K}$ is the cycle number of *H-2K* gene amplification using DNA isolated from wild-type ($Tk^{+/-}$) mouse lymphoma cells.]

2. The cycle number difference (CD) of the *Tk* gene amplification between the *Tk* mutant and wild-type ($Tk^{+/-}$) cells was calculated as:

$$CD = C_{M\text{-}Tk} - C_{W\text{-}Tk\text{-}Relative}$$

($C_{M\text{-}Tk}$ is the cycle number of *Tk* gene amplification using DNA isolated from the *Tk* mutant.)

3. The *Tk* gene CN of each mutant was then calculated based on the fact that $Tk^{+/-}$ cells have two copies of the *Tk* gene.

$$CN = 2^{1-CD}$$

For example, if CD = 1, then CN = 1: the *Tk* gene was hemizygous (1 copy); if CD = 0, then CN = 2: there were 2 copies of the *Tk* gene.

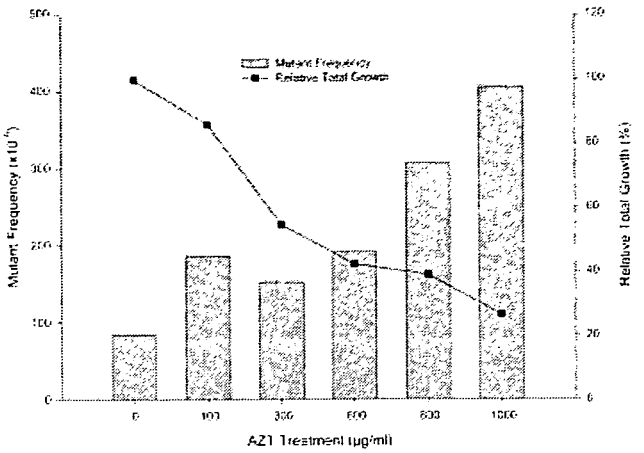


Fig. 1. Tk mutant frequency and relative total growth of L5178Y/$Tk^{+/-}$ mouse lymphoma cells treated with AZT. The doses in molarity are 0, 374, 1,123, 2,245, 2,994, and 3,742 μM, respectively.

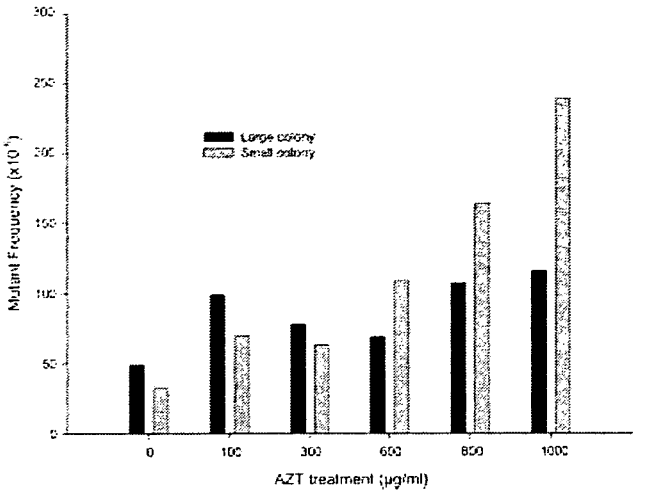


Fig. 2. Large and small colony *Tk* mutant frequencies of L5178Y/$Tk^{+/-}$ mouse lymphoma cells treated with AZT. The doses in molarity are 0, 374, 1,123, 2,245, 2,994, and 3,742 μM, respectively.

According to the criteria set by Honma et al. [2001], the CN of *Tk* mutants was classified as <1.2, 1.2–1.8, and >1.8, which sets the ranges for the hemizygous, mosaic, and homozygous states of the *Tk* gene, respectively [Honma et al., 2001].

Statistical Analysis

The computer program written by Cariello [1994] for the Monte Carlo analysis developed by Adams and Skopek [1987] was used to compare the chromosome 11 LOH patterns and mutation spectra between different groups of mutants.

Weighted sums of the number of LC and SC mutants were used in the comparison of LOH patterns and mutation spectra between different groups considering the proportion difference of LC and SC mutants between the selected mutants and mutants in the original culture (the proportion of SC mutants was 65 and 40% in the AZT-treated culture and control, respectively).

For each class of mutants, the total number including both LC and SC mutants was calculated as the weighted sum of the number of LC and SC mutants:

1. For the mutants from the AZT-treated culture

$$\text{Weighted sum} = 150 \times (35\% \times \text{Number of LC mutants}/55 + 65\% \times \text{Number of SC mutants}/95)$$

2. For the spontaneous mutants

$$\text{Weighted sum} = 69 \times (60\% \times \text{Number of LC mutants}/36 + 40\% \times \text{Number of SC mutants}/33)$$

RESULTS

Tk Mutation Assay

AZT induced dose-related cytotoxicity and mutagenicity responses in L5178Y/$Tk^{+/-}$ mouse lymphoma cells (Fig. 1). AZT (0.8 and 1 mg/ml) (2,994 and 3,742 μM) produced clear increases in *Tk* MF over that of the vehicle control. The results were evaluated using the new criteria developed by the Mouse Lymphoma Expert Working Group of the International Workshop for Genotoxicity Testing (IWGT) [Moore et al., 2006]. For a response to be positive for the microwell version of the assay, these guidelines require an induced MF of at least 126 × 10^{-6} [the Global Evaluation Factor (GEF)] in one or more treated cultures while showing cytotoxicity ≥10% RTG. A positive dose response must also be observed. AZT induced more small colony mutants than large colony mutants (Fig. 2).

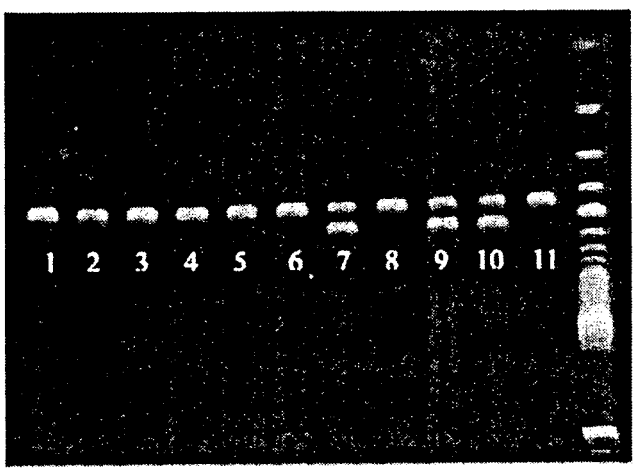
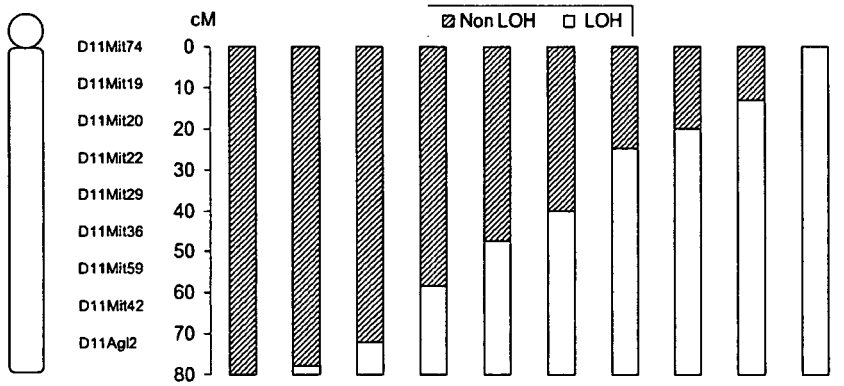


Fig. 3. PCR analysis of the loss of heterozygosity (LOH) at the D11Agl2 microsatellite locus that resides in the *Tk* gene of L5178Y mouse lymphoma cells. Note that mutants 1–6, 8 and 11 show LOH (the 523 bp product is lost).

LOH Analysis

Nine polymorphic microsatellite markers (*D11Agl2*, and *D11Mit 42, 59, 36, 29, 22, 20, 19,* and *74*) that were almost evenly distributed along the full length of chromosome 11



	Microsatellites	R	Mutants showing LOH that extends to								D-LOH
			Agl2	*42*	*59*	*36*	*29*	*22*	*20*	*19*	*74*	
Mutants from AZT-treated culture*	LC (55)	14	6	10	4	6	4	1	0	3	7	0
		25%	11%	18%	7%	11%	7%	2%	0%	5%	13%	0%
	SC (95)	0	7	38	8	9	6	1	3	6	14	3
		0%	7%	40%	8%	9%	6%	1%	3%	6%	15%	3%
	Total** (150)	13	13	49	12	15	10	2	3	9	21	3
		9%	9%	33%	8%	10%	7%	1%	2%	6%	14%	2%
Spontaneous mutants	LC (36)	21	3	1	0	2	2	2	0	0	5	0
		58%	8%	3%	0%	6%	6%	6%	0%	0%	14%	0%
	SC (33)	1	3	11	2	0	3	0	3	1	9	0
		3%	9%	33%	6%	0%	9%	0%	9%	3%	27%	0%
	Total** (69)	25	6	10	2	2	5	2	3	1	13	0
		36%	9%	14%	3%	3%	7%	3%	4%	1%	19%	0%

Fig. 4. Loss of heterozygosity (LOH) analysis of chromosome 11 in mouse lymphoma *Tk* mutants from the AZT-treated culture and untreated independent control cultures (LC: large colony; SC: small colony; R: retain heterozygosity; D-LOH: discontinuous LOH).*The culture that the analyzed mutants were isolated from was treated with 1 mg/ml (3,742 μM) AZT for 24 hr.**Total is the weighted sum of the number of LC and SC mutants considering the proportion difference of LC and SC mutants between the selected mutants and mutants in the original culture (the proportion of SC mutants was 65 and 40% in the AZT-treated culture and control, respectively). The discontinuous LOH patterns of three SC mutants from the AZT-treated culture are shown in Table I.

were identified and used to investigate the extent of chromosome 11 LOH. One hundred and fifty mutants (55 LC and 95 SC mutants) from the 1 mg/ml (3,742 μM) AZT-treated culture and sixty-nine independent spontaneous mutants (36 LC and 33 SC mutants) were analyzed (Figs. 3 and 4). All (95 of 95) of the SC mutants from the AZT-treated culture showed LOH at the *Tk* gene. All but one (32 of 33) of the SC mutants from the untreated control cultures also showed LOH at the *Tk* gene. The primary difference in *Tk* LOH between the mutants from untreated and AZT-treated cultures occurred with the LC mutants. *Tk* LOH was observed in 41 of 55 LC mutants (75%) from the AZT-treated culture but in only 15 of 36 LC mutants (42%) from the untreated control. In total, 91% of the mutants from the AZT-treated culture had LOH at the *Tk* locus while 64% of the spontaneous mutants had *Tk* LOH.

Figure 4 shows the number of mutants having each of the various LOH patterns. For instance, 10 LC mutants from the AZT-treated culture had LOH at both the *Tk* and Mit42 loci. These 10 mutants were heterozygous at all of the rest of the 7 microsatellite markers. Only three SC mutants (all from the AZT-treated culture) showed discontinuous LOH patterns (Table I).

TABLE I. Discontinuous Loss of Heterozygosity (LOH) Patterns of Chromosome 11 in Three Mouse Lymphoma *Tk* Mutants from the AZT-Treated Culture[a]

	Microsatellites								
	Agl2	*42*	*59*	*36*	*29*	*22*	*20*	*19*	*74*
Mutant 1	○	○	○	○	●	○	○	●	●
Mutant 2	○	○	●	●	●	●	●	●	○
Mutant 3	○	○	●	●	○	●	●	●	●

(●: retain heterozygosity; ○: LOH).

[a]The culture that these three mutants were isolated from was treated with 1 mg/ml (3,742 μM) AZT for 24 hr. Microsatellite marker D11Agl2 was identified by Liechty et al. [1996]; all the other markers were selected from the mouse genome data base (Jackson Laboratory, Bar Harbor, ME, http://www.informatics.jax.org).

The LOH patterns of mutants from the AZT-treated culture were significantly different from those of spontaneous mutants isolated from independent cultures ($P < 0.01$). This difference was primarily due to the difference between the LC mutants from the AZT-treated culture and the spontaneous LC mutants ($P < 0.01$). The SC mutants from the AZT-treated culture and spontaneous SC mutants did not show significant difference in their LOH patterns ($P >$

TABLE II. *Tk* Gene Copy Number of Mouse Lymphoma Mutants from the AZT-Treated Culture and Independent Untreated Control Cultures. Data are Presented to Show Both *Tk* Gene Copy Number and the Extent of Chromosomal LOH as Measured by Microsatellite Analysis

Source of mutants	Clone size (No.)	CN of *Tk* gene	LOH extends to								
			Agl2	*42*	*59*	*36*	*29*	*22*	*20*	*19*	*74*	D
Mutants from AZT-treated culture[a]	LC (55)	<1.2	4	1	1	2	3			1	1	
		1.2–1.8	1	3	1	1						
		>1.8	1	6	2	3	1	1		2	6	
	SC (95)	<1.2	5	23	3	4	1	1		1	2	
		1.2–1.8		6	1	2	1				2	
		>1.8	2	9	4	3	4		3	5	10	3
Spontaneous mutants	LC (36)	<1.2										
		1.2–1.8	1									
		>1.8	2	1		2	2	2			5	
	SC (33)	<1.2	1	5			3				1	
		1.2–1.8	2	3							1	
		>1.8		3	2				3	1	7	

CN: copy number; LOH: loss of heterozygosity; LC: large colony; SC: small colony; D: discontinuous LOH.
[a]The culture that the analyzed mutants were isolated from was treated with 1 mg/ml (3,742 μM) AZT for 24 hr. Microsatellite marker *D11Agl2* was identified by Liechty et al. [1996]; all the other markers were selected from the mouse genome data base (Jackson Laboratory, Bar Harbor, ME, http://www.informatics.jax.org).

0.05). In addition, the LOH patterns of LC and SC mutants were distinct from each other, both for the mutants from the AZT-treated culture and the spontaneous mutants ($P < 0.01$).

Tk Gene Dosage Analysis

The CN of the *Tk* gene in mutants showing LOH at the *Tk* gene was measured using a Real-Time PCR method and classified according to the criteria set by Honma et al. [2001]. The hemizygous state of the *Tk* gene (CN < 1.2) indicates a deletion of the Tk^+ allele or a complete chromosome loss; the homozygous state of *Tk* gene (CN > 1.8) indicates that the mutant resulted from recombination or chromosome duplication after chromosome loss. The mutants showing an intermediate CN (1.2–1.8) may be mosaic or the result of complex rearrangements [Honma et al., 2001, 2003].

Table II shows the combined results of the chromosome 11 microsatellite LOH analysis and the *Tk* gene dosage analysis of 150 *Tk* mutants from the 1 mg/ml (3,742 μM) AZT-treated culture and 69 independent mutants from untreated controls. Table III shows the mutation type classification based on the chromosome 11 microsatellite LOH pattern and the *Tk* gene CN, and Table IV shows the summary of the mutation spectra of mutants from the AZT-treated culture and the independent spontaneous mutants. The mutation types are: (1) intragenic mutation (retains heterozygosity at the *Tk* locus; includes point mutation, frame shifts, and small intragenic deletions), (2) deletion (LOH at the *Tk* locus with *Tk* gene CN < 1.2, and retaining heterozygosity of at least one microsatellite marker), (3) chromosome loss (*Tk* gene CN < 1.2, and all microsatellite markers showing LOH), (4) recombination (LOH at the *Tk* locus with *Tk* gene CN > 1.8, and retaining heterozygosity of at least one microsatellite marker), (5) chromosome duplication after chromosome loss (*Tk* gene CN > 1.8, and all markers showing LOH), and (6) other events, including mosaic events or complicated rearrangements (LOH at *Tk* locus with *Tk* gene CN between 1.2 and 1.8).

TABLE III. Classification of Mouse Lymphoma *Tk* Mutants Based on Chromosome 11 Microsatellite LOH Pattern and *Tk* Gene Copy Number

Mutation type	LOH analysis	*Tk* gene copy number
Intragenic mutation	Retain heterozygosity at the *Tk* locus	NA
Deletion	LOH at the *Tk* locus, retain heterozygosity of at least one microsatellite marker	<1.2
Chromosome loss	All markers show LOH	<1.2
Recombination	LOH at the *Tk* locus, retain heterozygosity of at least one microsatellite marker	>1.8
Chromosome duplication	All markers show LOH	>1.8
Mosaic/Complex	LOH at the *Tk* locus	1.2–1.8

NA: Not analyzed.

The mutation spectrum of mutants from the AZT-treated culture was significantly different from the spectrum of independent spontaneous mutants ($P < 0.01$). A lower proportion of intragenic mutations and a higher proportion of deletions were observed in the mutants from the AZT-treated culture. The difference in the mutation spectrum between the spontaneous mutants and mutants from the AZT-treated culture was primarily due to a difference between the LC mutants ($P < 0.01$). The SC mutants did not show any significant difference in their mutation spectrum ($P > 0.05$). In addition, the LC and SC mutants had distinct mutation spectra, both for the mutants from the AZT-treated culture and for the untreated control ($P < 0.01$).