解析し、毒性値を計算した。最終濃度方による毒性値(EC50 値)の再計算結果は下記の通りであった。

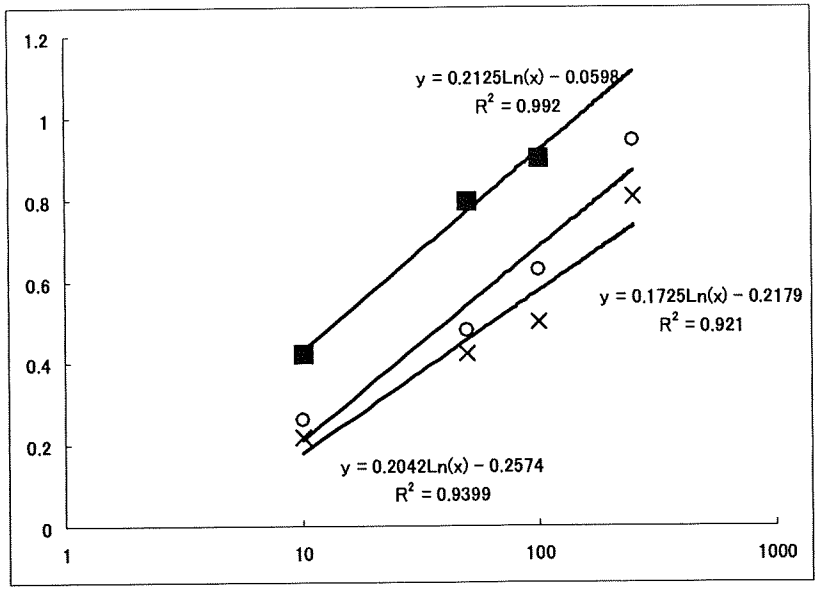
o-Aminophenol	y = 0.2125Ln(x) - 0.0598	14 mg/L
m－Aminophnol	y = 0.2042Ln(x) - 0.2574	41
p－Aminophenol	y = 0.1725Ln(x) - 0.2179	64

ただし、OECD-TG201(2006)の妥当性基準は満たしていないこと(試験期間中に現存量の増加は16倍未満であった、繰り返しが少ないく測定値の変動が小さいことを示せない)から試験の信頼性は高くない。その結果、o-異性体の毒性が他の２つの異性体に較べてやや強いことが分かった。

(表2) Huang, J.C., and E.F. Gloyna(1967)の試験データ

曝露濃度(mg/L)	o-Aminophenol			m－Aminophnol			p－Aminophenol		
	0日	1日	2日	0日	1日	2日	0日	1日	2日
Control	10.4	24.6	76.8	10.4	24.6	76.8	10.4	24.6	76.8
10	10.4	12.8	44.4	10.4	24	60	10.4	22.8	56.8
50	10.4	8.8	15.6	10.4	18.4	44.4	10.4	16.8	40
100	10.4	3.6	7.6	10.4	10	38.5	10.4	9.6	28.8
250	10.4	2.8	2.4	10.4	9.6	14.8	10.4	5.6	4.4
500	10.4	2.8	0	10.4	8.8	8	10.4	0	0
1000	10.4	－		10.4	6.4	4.4	10.4	－	－

試験開始後2日までの藻類密度の変化をクロロフィル濃度の測定値でしめしている。



（図４）生長阻害率（縦軸）とばく露濃度（横軸、対数）との関係
■：o-アミノフェノール　×：m-アミノフェノール　○：P-アミノフェノール

⑦環境省は、m-および p-アミノフェノールを単細胞の緑藻の 1 種 *Pseudokirchneriella subcapitata* を使った OECD-試験ガイドライン 201、藻類生長阻害試験を GLP 基準を遵守して実施した。被験物質は試験条件で安定ではなかったものの p-アミノフェノールの場合分析の結果、試験終了時でも低濃度区で 50%、高濃度区で 25%ほど残存していることが確認されており、毒性値の信頼性を疑うほどではなかった。また m-アミノフェノールではばく露濃度は p-異性体よりも高かったものの濃度はほぼ維持され被験物質は水中で安定であった。試験結果は p-アミノフェノールが m-異性体よりもはるかに毒性が高い結果となった。

8.3.3.4　原生生物への影響

⑧Pauli et al. 1993 および Pauli & Berger, 1992[10]は、繊毛中類のテトラヒメナを用いた m-および p-アミノフェノールの短期毒性試験を行った。この試験の毒性指標は２つあったが忌避行動については必ずしも毒性を示しているものでないので試験の信頼性は低いとし、生長を指標とした毒性値のみを妥当であると判断した。試験方法は、藻類生長阻害試験に準じてテトラヒメナの生長（増殖）を影響指標に 48 時間後の到達密度を計測して、対照区の増殖率から各濃度区の生長阻害率を算出し、設定濃度との関係から 48hEC50 値および NOEC 値を算出している。ばく露濃度区は等比級数で設定しているものの、公比が大きいことから、毒性値そのものの信頼性は高くないが、m-異性体と p-異性体の毒性の比較には十分な情報が得られると判断した。

算出された毒性値を較べると、m-異性体よりも p-異性体の方が 10 程度毒性が高い結果であったが、NOEC 値はまったく同じであったことから、p-異性体の方が毒性が高い傾向にあるものの、藻類ほどには大きく変わるものではないと判断された。

8.3.3.5　水生生物の慢性毒性影響について

９）環境省は、オオミジンコの繁殖試験を m-および p-アミノフェノールについて実施している。OECD 試験ガイドライン 202、Part 2 の試験法で、GLP 基準を遵守して実施した。両物質ともに流水式ばく露方式を採用していることから安定したばく露が実現できたと考えられる。試験法からの逸脱事項はなく、試験の信頼性は高いと判断された。

得られた毒性値は、m-異性体よりも p-異性体が 10 倍ほど毒性が高い結果となった。

⑨環境省は、p-アミノフェノールのメダカを用いた初期生活段階試験を実施した。試験は OECD 試験ガイドライン 210 に従い、GLP 基準を遵守して実施した。試験法からの逸脱はなく、得られたばく露濃度―反応関係は予測の範囲であり、得られた毒性値の信頼性を高いと判断された。ただし、他の種類の異性体の試験結果は入手できないために、毒性の傾向の解析には使用できない。

８．４．リードアクロスの適用可能性の検討

8.4.1　魚類急性毒性

ゼブラフィッシュの胚毒性試験値は魚類の急性毒性試験のよい近似値となることを根拠に、ゼブラフィシュを用いた魚類胚毒性試験が OECD 試験ガイドラインとして提案されている。ところが Sun et al.(2004)の結果は、メダカの急性毒性試験結果とまったく逆の傾向を示している。そのため、ここでは魚類胚毒性と魚類急性毒性とは別のものとして判断する必要がある。メダカの o-アミノフェノールの毒性情報を入手することがますます重要となった。そのために、他の類似の化合物の毒性値も

含めた検討が必要である。

ただし、この３つの異性体間でその毒性が大きくことなることは確実であり、また、その場合の合理的なパラメータとしては、生体膜透過性の指標である LogKow や水中でのばく露の可能性を示す水溶解度などではこのように大きな毒性の違いを説明できないので、別の指標とすべきである。

8.4.2　ミジンコ急性毒性

m-異性体よりも p-異性体の毒性が高い傾向が示されてはいるものの、実験的な誤差を考慮すると必ずしも毒性に差があるとは言えない。したがって、o-異性体の毒性は m-および p-異性体程度の毒性であることになる。ただし、他の生物分類群では際だった毒性の違いが見られていることからその結果には留意する必要がある。

8.4.3　藻類への影響

Pseudokirchneriella subcapitata（ミカヅキモの１種）とクロレラの２種類の緑藻について複数の異性体の毒性値が得られているが、その毒性傾向はまったく異なるものであった。クロレラに関しては大きな各異性体間の感受性差は小さいものの、*P. subcapitata* の場合は m-異性体と p-異性体で毒性値は1000倍以上の差がある。この違いは決して実験誤差とは考えられない。この緑藻２種の間の毒性傾向の差の理由に関しては情報がないこと o-異性体の毒性値をリードアクロスの手法で推定する場合は LogKow をパラメータとすることでは対応できない可能性が高い。

8.4.4　微生物への影響

バクテリアを用いた生態毒性試験に関しては発光バクテリアを用いた試験法以外には情報がなかったが、毒性が高いとする情報も得られていないことから３種の異性体それぞれについて試験を実施することが望ましい。カテゴリーアプローチによるデータフィリングが有効かどうか判断は困難である。

原生生物である繊毛虫類のテトラヒメナの場合は、生長を毒性指標とした EC50 値からは m-アミノフェノールが p-アミノフェノールよりも毒性が高いとの結果であったが、本化学物質の特性の１つとして、試験環境中で被験物質がオリゴマーを形成した場合には毒性の緩和が起こる可能性があり、ばく露濃度の高い時ほど高分子化が進行すると考えられ、しかも試験の実験誤差が大きくなると予測される。テトラヒメナでは NOEC の比較では m-異性体、p-異性体の値は同じであり、異性体間に毒性に関する差は小さいとも考えられる。このように、２つの逆の可能性があることから、毒性傾向を推測するには至っていない。

8.4.5　水生慢性毒性値

ミジンコ繁殖を影響指標とするNOEC値がm-およびp-異性体でえられている。m-異性体よりもp-異性体の方が毒性が高いこと、そしてその傾向はミジンコ遊泳阻害試験の結果と同じ傾向であることから、この毒性傾向は確かなものと見られた。しかしながら、o-異性体の毒性値をm-およびp-異性体の毒性値から推定する手法については、従来のLogKowのような生体膜透過性の指標をパラメータにしては、異性体のように物理化学的性状の類似した化学物質で毒性値がこのように異なる場合には成功しないことは明らかである。

８．５．結論と考察

アミノフェノールの３種異性体の入手可能な環境毒性情報についてその利用可能性について整理した。LogKow と化学物質感受性の相関関係についてはすでに多くの知見があり、実践的にも QSAR（量的構造活性相関）式が LogKow をパラメータとして開発・利用されてきている。

一方、アミノフェノールが属する１）芳香族

アミンは、ミジンコへに高い毒性を有するなど、一般的に生理活性が高いこと、2）同じく芳香族アミンは急性ー慢性毒性比が例外的に高い化学物質が見られること（繁殖影響が著しい）の特徴を有することが知られている。さらに3）フェノール類は、魚類の初期生活段階（胚期、仔魚期）に濃度依存性が弱いものの致死的な影響が見られるという特徴がある。

さらに、アミノフェノールは、試験環境中で高分子化するとの報告があり、このことは、ばく露濃度の高い濃度区の方が高分子化率が高くなることから毒性に関与する単分子（場合によってはオリゴマー）の濃度が、高濃度区と低濃度区が同程度もしくは逆転することもあり得る。この事は、ばく露方式の選択にとっても重要である。具体的には流水式ばく露方式を選択した場合に高濃度の保存溶液を準備するが、この保存溶液の中で高分子化が進むことがなかったか確認する必要がある。今回入手した試験結果においては、流水式試験のすべては被験物質濃度を実測し、その実測濃度に基づいて毒性値を算出しているか、もしくは十分に低い濃度での試験であったために試験の信頼性に疑問のある試験はなかった。止水、半止水式ばく露試験をおこなった試験においても、設定濃度に基づいて毒性値を算出している場合には、常に高分子化に伴う毒性緩和が起きていないか疑問が残る。既存情報の場合にはその確認ができないため、試験結果をどのように受け入れるべきか判断が難しい。

このように、アミノフェノール類の試験結果は通常の物質に較べて試験手順による試験結果の変動が大きいという特徴がある。その場合、試験手順が十分に吟味されており、またその詳細が記述されている試験結果だけを用いて毒性を評価することが必要である。その点では、今回は信頼性の確認の段階で多分に広めに利用できるものとして整理した。

集約した試験結果は、用いた生物種、成長段階によって異なる毒性傾向がみられている。種によるターゲット部位が異なるための違いであるのか、代謝酵素系の有無による差なのか、毒性メカニズムそのものの検討は行っていないためここでは解明できなかった。今後はそのような方向からの検討が必要である。

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研究成果による特許権等の知的財産権の出願・登録状況

1. 特許取得

(該当なし)

2. 実用新案登録

(該当なし)

3. その他

(該当なし)

Ⅱ．研究成果の刊行に関する一覧表

別添４

Ⅱ．研究成果の刊行に関する一覧表

書籍

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Ⅲ．研究成果の刊行物・別刷

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The Mouse Lymphoma Assay Detects Recombination, Deletion, and Aneuploidy

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The mouse lymphoma assay (MLA) uses the thymidine kinase (*Tk*) gene of the L5178Y/*Tk*$^{+/-}$-3.7.2C mouse lymphoma cell line as a reporter gene to evaluate the mutagenicity of chemical and physical agents. The MLA is recommended by both the United States Food and Drug Administration and the United States Environmental Protection Agency as the preferred *in vitro* mammalian cell mutation assay for genetic toxicology screening because it detects a wide range of genetic alterations, including both point mutations and chromosomal mutations. However, the specific types of chromosomal mutations that can be detected by the MLA need further clarification. For this purpose, three chemicals, including two clastogens and an aneugen (3′-azido-3′-deoxythymidine, mitomycin C, and taxol), were used to induce *Tk* mutants. Loss of heterozygosity (LOH) analysis was used to select mutants that could be informative as to whether they resulted from deletion, mitotic recombination, or aneuploidy. A combination of additional methods, G-banding analysis, chromosome painting, and a real-time PCR method to detect the copy number (CN) of the *Tk* gene was then used to provide a detailed analysis. LOH involving at least 25% of chromosome 11, a normal karyotype, and a *Tk* CN of 2 would indicate that the mutant resulted from recombination, whereas LOH combined with a karyotypically visible deletion of chromosome 11 and a *Tk* CN of 1 would indicate a deletion. Aneuploidy was confirmed using G-banding combined with chromosome painting analysis for mutants showing LOH at every microsatellite marker on chromosome 11. From this analysis, it is clear that mouse lymphoma *Tk* mutants can result from recombination, deletion, and aneuploidy.

Key Words: **mouse lymphoma assay; mutation type; loss of heterozygosity; cytogenetics; copy number; 3′-azido-3′-deoxythymidine; mitomycin C; taxol; thymidine kinase.**

The mouse lymphoma assay (MLA), using the thymidine kinase (*Tk*) gene of the L5178Y/*Tk*$^{+/-}$-3.7.2C mouse lymphoma cell line as a reporter gene of mutation, is preferred by a number of international regulatory agencies, including the United States Food and Drug Administration and the United States Environmental Protection Agency, as the *in vitro* mammalian mutation assay in the genetic toxicology screening battery. The decision to prefer the MLA was based on previous research demonstrating that the assay detects most of the mutational events known to be associated with the etiology of cancer and other human diseases, including point mutations and a number of different types of chromosomal mutations (Applegate *et al.*, Blazak *et al.*, 1989; Chen *et al.*, 2002; Clive *et al.*, 1990; 1990; Honma *et al.*, 2001; Hozier *et al.*, 1981, 1992; Liechty *et al.*, 1998; Moore *et al.*, 1985; Zhang *et al.*, 1996).

It is important to determine the various mutation types that can be detected by this assay, so that MLA data can be properly interpreted. Since the development of this assay, quite a number of studies have been conducted to understand the types of mutations that can be detected by the MLA. Several cytogenetic studies have been conducted to analyze *Tk* mutants induced by various mutagens (Blazak *et al.*, 1986, 1989; Hozier *et al.*, 1981; Moore *et al.*, 1985; Zhang *et al.*, 1996). It is clear that many small colony (SC) mutants have recognizable chromosome rearrangements involving chromosome 11, which contains the *Tk* gene. The *Tk*$^{-}$ and *Tk*$^{+}$ chromosomes can be distinguished by a centromeric heteromorphism: the *Tk*$^{+}$ chromosome has a bigger centromere (Hozier *et al.*, 1982; Sawyer *et al.*, 1985). This finding greatly helps the cytogenetic characterization of chromosome aberrations in *Tk* mutants. However, without the help of molecular genetics, small chromosome changes cannot be detected using cytogenetic methods alone; mitotic recombination cannot be identified because the karyotype of the mutant would be normal. Applegate *et al.* (1990) identified a *Nco I* restriction fragment length polymorphism that distinguishes the *Tk*$^{+}$ and *Tk*$^{-}$ alleles. Several studies using Southern blot analysis to determine the status of the *Tk*$^{+}$ allele were subsequently conducted (Applegate *et al.*, 1990; Clive *et al.*, 1990). The

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presence of the Tk^+ allele suggests an intragenic mutation, while the loss of the Tk^+ allele indicates a chromosome mutation. Most large colony (LC) mutants induced by point mutagens, such as ethyl methanesulfonate, retain the Tk^+ allele; while most SC mutants induced by clastogens, such as bleomycin, lose the Tk^+ allele. An allele-specific PCR technique was developed to identify the presence or absence of the Tk^+ allele. Furthermore, based on microsatellite polymorphisms, the loss of heterozygosity (LOH) pattern of the entire chromosome 11 can be investigated (Liechty *et al.*, 1994, 1996, 1998). This labor-saving approach allows many mutants to be analyzed; however, without the detection of *Tk* gene copy number (CN), one cannot distinguish between a deletion and a recombination event. Southern blot analysis can be used to detect *Tk* gene CN (Applegate *et al.*, 1990), but it is resource intensive, requires a relatively large amount of DNA, and the measurement is not precise (Joseph *et al.*, 1993). Therefore, we developed a real-time PCR method to detect the CN of the *Tk* gene (Wang *et al.*, 2007). By combining LOH analysis of chromosome 11 and cytogenetic analysis, we were able to distinguish between deletion and recombination events.

Another important issue for the MLA is whether the assay can detect aneuploidy. Aneuploidy plays a significant role in many adverse human health conditions, including spontaneous abortions, birth defects, and cancer (Aardema *et al.*, 1998; Duesberg *et al.*, 1999; Oshimura and Barrett, 1986; Sen, 2000). Aneugens can interact with the spindle apparatus or impair its function, thereby inducing aneuploidy due to nondisjunction mechanisms. Several aneugens are carcinogens (Cimino *et al.*, 1986; Oshimura and Barrett, 1986). Although the MLA has been shown to detect aneugens, its ability to adequately detect aneuploidy is unclear. Applegate *et al.* (1990) reported that at least some *Tk* mutants show aneuploidy. Honma *et al.* (2001) evaluated two aneugens, colchicine, and vinblastine, using the MLA. The two chemicals did not induce a significant mutant frequency (MF) increase after the regular 3-h treatment. Although they did show positive responses after the long-term (24 h) treatment, the increase of MF was not high. Therefore, many of the mutants that were isolated and analyzed from the chemical-treated cultures would be spontaneous rather than induced mutants.

Overall, the specific types of chromosomal mutations that are detected by the MLA need further clarification. In this study, *Tk* mutants from cultures treated with different chemicals, including two clastogens and an aneugen (3′-azido-3′-deoxythymidine [AZT], mitomycin C and taxol), were used. A combined strategy of both molecular genetic and cytogenetic methods was used to analyze the mutants. LOH analysis of chromosome 11 was conducted first, and then the *Tk* mutants showing LOH involving a large portion of chromosome 11 were selected for further cytogenetic analysis and *Tk* CN detection. It should be noted that it was not our intent to provide a complete analysis of the types of mutational events induced by these three chemicals; rather, we used these chemicals to give us mutants for our analysis that would be expected to include deletions, mitotic recombination, and aneuploidy.

MATERIALS AND METHODS

Cell culture. The L5178Y/$Tk^{+/-}$ -3.7.2C 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 unit/ml penicillin, 100 μg/ml streptomycin, and 0.05% (vol/vol) pluronic F68 (Invitrogen, Carlsbad, CA). The cultures were incubated at 37°C in an atmosphere of 5% CO_2 and saturated humidity and maintained in logarithmic growth.

Tk mutant induction. *Tk* mutants isolated from AZT-treated and taxol-treated cultures were from previous studies using the microwell version of the assay (Moore *et al.*, 2005; Wang *et al.*, 2007). For the induction of *Tk* mutants using mitomycin C (obtained from Sigma, St Louis, MO), the protocol for the microwell version of the mouse lymphoma assay described by Chen and Moore (2004) was followed. 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. Mitomycin C dissolved in dimethyl sulfoxide (DMSO) was added from a stock solution to the cell cultures. Additional DMSO was added to give a final volume of 100 μL The flasks were incubated for 4 h, and then the cells were centrifuged, washed twice, and resuspended in fresh medium. The cells were transferred to new 75-cm^2 flasks and cultured for 2 days for mutant expression with cell counts and cell density adjustment made after 1 day. Then the cells were cloned in 96-well plates in medium containing 3 μg/ml trifluorothymidine (TFT) for selection and medium without TFT for the measurement of cloning efficiency. After 12 days, the 96-well plates were evaluated by eye using a Quebec dark field colony counter to determine the presence or absence of colonies in each well and to enumerate the number of small and LC mutants. Total MF, SC MF, and LC MF were determined, and the relative total growth values that measures cytotoxicity were calculated according to the published protocol (Chen and Moore, 2004). Mutant colonies were randomly selected and isolated from the cultures treated with the highest test dose of AZT (1 mg/ml), mitomycin C (0.4 μg/ml), or taxol (1 μg/ml), respectively.

DNA extraction. The isolated mutant colonies were cultured in fresh medium for several days to obtain sufficient cells for subsequent analysis. Genomic DNA was extracted from 3×10^6 cells of each *Tk* mutant clone using the Qiagen DNeasy tissue kit (Valencia, CA) and stored at − 20°C.

Tk gene LOH analysis. *Tk* gene LOH analysis was conducted using the allele-specific PCR described by Liechty *et al.* (1996), with some modifications (Wang *et al.*, 2007). Basically, the microsatellite locus *D11Agl2* that resides in the *Tk* gene was amplified using a touchdown PCR method. The PCR was performed in 96-well plates using a PCR System 9700 (Applied Biosystems, Foster City, CA). The reaction products were separated by 2% agarose gel electrophoresis, stained with 1 μg/ml ethidium bromide, and visualized with a UV light box.

Chromosome 11 LOH analysis. In addition to microsatellite marker *D11Agl2*, eight other microsatellite loci on mouse chromosome 11 (*D11Mit 42, 59, 36, 29, 22, 20, 19,* and *74*) were used (Wang *et al.*, 2007). The nine 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 0.0cM, respectively). LOH analysis was performed at each microsatellite locus using allele-specific PCR as previously described (Wang *et al.*, 2007). The PCR products were separated and visualized as above.

Tk gene dosage analysis. For those mutants showing LOH involving a large portion of chromosome 11 (at least including microsatellite markers *D11Agl2* and *D11Mit 42*), *Tk* gene CN was evaluated using the real-time PCR $2^{-\Delta\Delta Ct}$ method described previously (Wang *et al.*, 2007). Briefly, a fragment of the *Tk* gene and a fragment of an unrelated gene (*H-2K*) on chromosome

17 were amplified simultaneously. The *H-2K* gene fragment was used as an endogenous reference for PCR relative quantitation.

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.

G-banding analysis. For the mutants showing LOH involving a large portion of chromosome 11 (at least including microsatellite markers *D11Agl2* and *D11Mit 42*), G-banding analysis was performed to examine the alteration of chromosome 11. The protocol established by Sawyer *et al.* (1985) was followed with some revision. Briefly, a 12-ml cell culture (approximately 8–10 × 10^6 cells) was centrifuged at 200 × g for 10 min. The supernatant was discarded, and the cell pellet was resuspended with gentle agitation in a hypotonic solution (10 ml 75mM KCl). Then 120 µl of 10 µg/ml colchicine (Sigma) was added. The cell suspension was then incubated at 37°C for 20 min. Five drops of fixative (5:2, methanol:acetic acid) were added at the end of incubation with gentle agitation to avoid cell clumping. Then the cells were centrifuged, and the supernatant was discarded leaving approximately 0.5 ml of solution over the pellet. The pellet was then gently agitated, and 10 ml of fixative was added. After gentle mixing, the cell suspension was incubated at room temperature for 20 min. The cell suspension was centrifuged, and the fixative was changed twice to eliminate cell debris and to ensure good spreading and staining of chromosomes. The cell pellet was resuspended in about 1 ml fixative to make the slides. For karyotypic analysis, cells were dropped onto precleaned glass slides (soaked in 95% ethanol overnight) and air dried. Trypsin treatment before Giemsa staining was used to sharpen bands and increase contrast. At least 10 cells were examined per clone.

Chromosome painting analysis. Chromosome 11 painting analysis was performed as described by Zhang *et al.* (1996) with some modification. Briefly, colchicine was added to a 12-ml cell culture (approximately 8–10 × 10^6 cells) at a final concentration of 0.5 µg/ml. Cells were incubated for 1 h at 37°C in an atmosphere of 5% CO_2 and saturated humidity. Then the cell culture was centrifuged at 200 × g for 10 min and resuspended in hypotonic solution (75mM KCl), followed by incubation in a water bath at 37°C for 20 min. After that, five drops of fixative (3:1, methanol:acetic acid) were added with gentle agitation. The rest of the cell fixation was accomplished using the procedure described in the G-banding analysis section above.

A whole-chromosome painting probe specific for mouse chromosome 11 labeled with biotin was purchased from Cambio (Cambridge, UK). The chromosome painting procedure described in the manufacturer's instructions was followed. Briefly, chromosomal DNA on slides was denatured with 2× sodium chloride-sodium citrate buffer (SSC)-70% formamide solution (1× SSC is 0.15M NaCl + 0.015M sodium citrate) at 70°C for 2 min followed by dehydration through an ethanol series (70%, 85%, and 100%). Probes were added to the metaphase preparations. After overnight hybridization at 37°C, the slides were washed three times with 2× SSC-50% formamide solution and three times with 2× SSC at 45°C. The slides were then incubated with fluorescent avidin-DCS (Vector Laboratories, Burlingame, CA) solution at 37°C for 30 min and washed with 2× SSC containing 0.1% Tween 20. The slides were then incubated with biotinylated goat anti-avidin DCS (Vector Laboratories) at 37°C for 30 min followed by a second fluorescent avidin-DCS treatment. The slides were washed, counterstained with propidium iodide solution, and mounted. At least 10 metaphases from each slide were examined with fluorescence microscopy.

RESULTS

The *Tk* MFs for the AZT-, mitomycin C–, and taxol-treated cultures used for mutant analysis are shown in Table 1. The complete MF data for AZT can be found in Wang *et al.* (2007), for taxol in Moore *et al.*, (2005), and for mitomycin C in Figure 1. At the highest dose tested, all three chemicals induced high *Tk* MFs (4.8-, 16.6-, and 7.3-fold over the concurrent negative control for AZT, mitomycin C, and taxol, respectively). This indicated that most of the analyzed *Tk* mutants were chemical-induced mutants rather than spontaneous background mutants.

TABLE 1
***Tk* Gene MFs in Mouse Lymphoma Cells Treated with AZT, Mitomycin C, and Taxol (at the Highest dose Tested)**

			Tk MF (× 10^{-6})	
Chemical	Concentration	SC[a] (%)	Control	Treated culture
AZT[b]	1 mg/ml	65%	84	407
Mitomycin C	0.4 µg/ml	44%	56	928
Taxol[c]	1 µg/ml	41%	50	366

[a]SC mutants, as a percent of all *Tk* mutant colonies obtained.
[b]Data from Wang *et al.* (2007).
[c]Data from Moore et al. (2005).

For our detailed analysis to determine if mutants can result from deletion, mitotic recombination, and aneuploidy, we needed mutants with large chromosomal alterations. Therefore, a number of mutants were first screened using nine microsatellite markers spanning chromosome 11. Mutants showing partial chromosome 11 LOH including at least microsatellite markers *Agl2* and *Mit42* were selected for further analysis using G-banding. In addition, *Tk* gene CN of these mutants was evaluated. Chromosome painting analysis was performed for some of the mutants showing LOH at every chromosome 11 microsatellite marker.

Fifteen mutants from the 1-mg/ml AZT–treated culture (Wang *et al.*, 2007) showing LOH including at least microsatellite markers *Agl2* and *Mit42* were analyzed using G-banding analysis. Nineteen mutants from the 0.4-µg/ml mitomycin C–treated culture were analyzed using chromosome 11 LOH analysis; then four mutants showing partial chromosome 11 LOH were analyzed using *Tk* gene dosage analysis and G-banding analysis, and two mutants showing LOH at

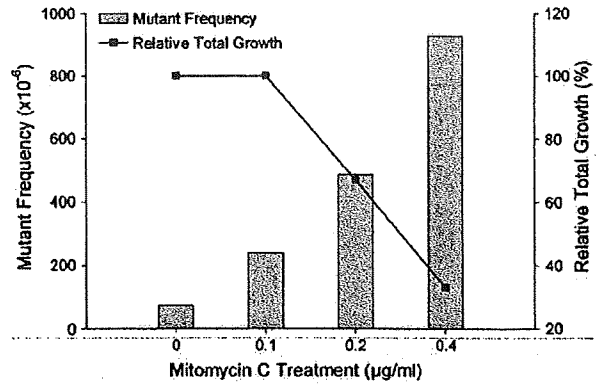


FIG. 1. *Tk* gene MFs and relative total growth values of mouse lymphoma cells treated with mitomycin C.

TABLE 2
The Number of Mutants that were Analyzed Using G-banding Analysis or Chromosome Painting Analysis

Treatment	LOH pattern	Number of mutants analyzed using G-banding analysis	Number of mutants analyzed using chromosome painting analysis
1 mg/ml AZT	Partial LOH including at least microsatellite markers *Agl2* and *Mit42*	15	
0.4 μg/ml mitomycin C	Partial LOH including at least microsatellite markers *Agl2* and *Mit42*	4	
	Complete LOH		2
1 μg/ml taxol	Complete LOH		7

every microsatellite marker on chromosome 11 were analyzed using chromosome painting analysis. Twenty mutants from the 1-μg/ml taxol–treated culture were isolated for chromosome 11 LOH analysis, and seven mutants showing LOH at every microsatellite marker on chromosome 11 were analyzed using chromosome painting analysis. The number of mutants that were analyzed using the combined strategy is summarized in Table 2.

The results of the G-banding analysis can be classified as normal chromosome 11, visible deletion, or complex chromosome alterations. The number of chromosome 11 revealed by the chromosome painting analysis can be classified as one (indicating chromosome loss), two (indicating chromosome duplication after loss), or more than two (including either aneuploidy or polyploidy). The numbers of metaphase spreads showing different numbers of chromosome 11 as revealed by chromosome painting are shown in Table 3. The combined results of the chromosome 11 LOH analysis, *Tk* CN detection, and G-banding/chromosome painting analysis are shown in Table 4.

TABLE 3
The Number of Metaphase Spreads Showing Different Numbers of Chromosome 11 in the *Tk* Mutants Isolated from Mitomycin C– or Taxol-Treated Cultures as Revealed by Chromosome Painting

	Number of chromosome 11				
Mutant[a]	1	2	3	4	>4
ML7	1	9	0	0	0
MS3	3	4	2	1	0
TL2	2	8	0	0	0
TL4	0	9	0	1	0
TL8	3	7	0	0	0
TS2	1	7	0	1	1
TS4	0	8	1	1	0
TS6	0	2	5	0	3
TS7	2	8	0	0	0

[a]The first letter of the mutant name indicates the chemical exposure (M, mitomycin C; T, taxol); the second letter indicates colony size of the mutant (L, large; S, small). All the mutants showed LOH at every microsatellite marker on chromosome 11. Ten metaphase spreads were counted for each *Tk* mutant.

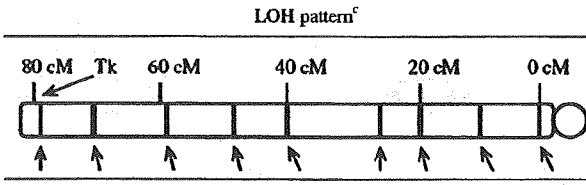
In total, 28 mutants showing partial or complete chromosome 11 LOH were analyzed using this combined strategy. Among them, nine mutants (A1B4, A1B5, A2D4, A3C2, A5B3, A6A2, ML4, ML10, and MS6) retained a normal karyotype, while at least three microsatellite markers showed LOH. With a *Tk* CN of 2, the mutation type of these mutants can be identified as recombination. A representative metaphase cell is shown in Figure 2. Five mutants (A1C6, A3A4, A5C2, A5D6, and A6C2) were identified to be deletions. They all have partial chromosome 11 LOH patterns, one copy of the *Tk* gene, and chromosome 11 showing visible deletions or deletions combined with translocation (representative metaphase cell shown in Fig. 2). Five mutants (A5C6, A5D2, A7B5, A7C1, and MS8) showed complex chromosome alterations. They all have partial chromosome 11 LOH patterns, with more than one copy of the *Tk* gene. G-banding analysis showed that the Tk^- chromosome appears to be normal, while the Tk^+ chromosome is abnormally long (Fig. 3). It is speculated that this complex alteration was the result of multiple events: deletion, duplication (aneuploidy), and translocation. First, the Tk^+ chromosome was partially deleted, which resulted in the LOH pattern; then the Tk^- chromosome was duplicated and translocations occurred. The duplicated Tk^- chromosome was translocated directly to the damaged Tk^+ chromosome or other chromosomes. Mutants A5D2 and MS8 have *Tk* CNs between 1 and 2, indicating that they are mosaic mutants. They may be a mixture of cells with duplicated Tk^- chromosome after the partial deletion of the Tk^+ chromosome or the Tk^- chromosome was not duplicated. For those mutants that showed complete LOH of chromosome 11 (ML7, MS3, TL2, TL4, TL8, TS2, TS4, TS6, and TS7), chromosome painting analysis indicated that they were all mosaic mutants, and overall the major mechanism appears to be the Tk^- chromosome duplication after the loss of the Tk^+ chromosome. Representative photos for chromosome loss and chromosome duplication after loss are shown in Figure 4.

DISCUSSION

A complete evaluation of genetic toxicology data includes an analysis of the mode of action by which the test chemical induced mutation (Dearfield and Moore, 2005). Because there are a number of genotoxicity tests and different tests detect different types of genotoxic damage, they may give a mixture of both positive and negative results. Therefore, it is important to identify the types of mutations detected by the different assays in order to evaluate the data properly.

As mentioned before, it has been established that the MLA can detect both point mutations and chromosomal mutations

TABLE 4
Combined Results of the LOH Analysis, *Tk* Gene CN Detection, and G-Banding/Chromosome Painting Analysis for the *Tk* (Mutants Isolated from AZT-, Mitomycin C–, or Taxol-Treated Cultures



Mutants[a]	L/S[b]	*Agl2*	*42*	*59*	*36*	*29*	*22*	*20*	*19*	*74*	*Tk* CN[d]	G-banding/chromosome painting[e]
A1B4	L	○	○	○	○	○	●	●	●	●	>1.8	Recombination
A1B5	L	○	○	○	○	●	●	●	●	●	>1.8	Recombination
A1C6	L	○	○	●	●	●	●	●	●	●	<1.2	Deletion
A2D4	L	○	○	○	●	●	●	●	●	●	>1.8	Recombination
A3A4	S	○	○	●	●	●	●	●	●	●	<1.2	Deletion
A3C2	S	○	○	○	○	○	●	●	●	●	>1.8	Recombination
A5B3	S	○	○	○	○	○	●	●	●	●	>1.8	Recombination
A5C2	S	○	○	●	●	●	●	●	●	●	<1.2	Deletion
A5C6	S	○	○	○	●	●	●	●	●	●	>1.8	Deletion with aneuploidy
A5D2	S	○	○	○	●	●	●	●	●	●	1.2–1.8	Deletion with/without aneuploidy
A5D6	S	○	○	○	○	●	●	●	●	●	<1.2	Deletion with translocation
A6A2	S	○	○	○	●	●	●	●	●	●	>1.8	Recombination
A6C2	S	○	○	○	●	●	●	●	●	●	<1.2	Deletion
A7B5	S	○	○	○	●	●	●	●	●	●	>1.8	Deletion with aneuploidy
A7C1	S	○	○	○	●	●	●	●	●	●	>1.8	Deletion with aneuploidy
ML4	L	○	○	○	○	●	●	●	●	●	>1.8	Recombination
ML10	L	○	○	○	●	●	●	●	●	●	>1.8	Recombination
MS6	S	○	○	○	●	●	●	●	●	●	>1.8	Recombination
MS8	S	○	○	●	●	●	●	●	●	●	1.2–1.8	Deletion with/without aneuploidy
ML7	L	○	○	○	○	○	○	○	○	○	NA[f]	Chromosome loss/duplication
MS3	S	○	○	○	○	○	○	○	○	○	NA	Chromosome loss/duplication
TL2	L	○	○	○	○	○	○	○	○	○	NA	Chromosome loss/duplication
TL4	L	○	○	○	○	○	○	○	○	○	NA	Chromosome duplication
TL8	L	○	○	○	○	○	○	○	○	○	NA	Chromosome loss/duplication
TS2	S	○	○	○	○	○	○	○	○	○	NA	Chromosome loss/duplication
TS4	S	○	○	○	○	○	○	○	○	○	NA	Chromosome duplication
TS6	S	○	○	○	○	○	○	○	○	○	NA	Chromosome duplication
TS7	S	○	○	○	○	○	○	○	○	○	NA	Chromosome loss/duplication

●, retain heterozygosity; ○, LOH.

[a]The first letter of the mutant name indicates the chemical exposure. A, AZT; M, mitomycin C; T, taxol. For mutants from AZT-treated culture, LOH analysis and *Tk* gene CN detection was done in a previous study (Wang *et al.*, 2007).

[b]L, large colony mutant; S, small colony mutant.

[c]*D11Agl2*, *D11Mit 42*, *59*, *36*, *29*, *22*, *20*, *19*, and *74* are nine microsatellite markers that are almost evenly distributed along the length of chromosome 11.

[d]The *Tk* gene CN analysis was performed for mutants showing microsatellite LOH for at least markers *Agl2* and *Mit 42* but also retaining microsatellite heterozygosity for at least marker *Mit 74* (partial LOH of chromosome 11).

[e]G-banding analysis was performed for the mutants showing LOH at least at microsatellite markers *Agl2* and *Mit 42* but also retaining heterozygosity at least at microsatellite marker *Mit 74* (partial LOH of chromosome 11). Chromosome painting analysis was performed for the mutants showing LOH at every microsatellite marker on chromosome 11 (complete LOH of chromosome 11).

[f]Not analyzed.

(Applegate *et al.*, 1990; Chen *et al.*, 2002; Honma *et al.*, 2001; Hozier *et al.*, 1981; Liechty *et al.*, 1998; Moore *et al.*, 1985). A large experimental trial including 45 labs (Honma *et al.*, 1999) suggested that the MLA and the *in vitro* chromosome aberration test were basically "equivalent" as to their ability to give positive responses for clastogens. It is important to recognize that the MLA detects small-scale alterations that are too small to be detected by cytogenetic assays. In addition, the MLA detects recombination, which may lead to recessive mutations and plays an important role in the deactivation of