The genotoxic potential of azidothymidine (Zidovudine, AZT), chosen as a model compound for nucleotide analogs, was comprehensively assessed in vivo for gene mutation, clastogenicity, and DNA breakage endpoints. Male Wistar rats were treated by oral gavage over 7 days with AZT at dose levels of 2 × 0 (control), 2 × 250, 2 × 500, and 2 × 1000 mg/kg/day with a final single dose given on day 8. DNA damage was then evaluated with the comet assay in liver, stomach, and peripheral blood and with the micronucleus test in bone marrow and peripheral blood (by flow cytometry) in the same animals. After a treatment-free period of up to 42 days, the Pig-a gene mutation assay was performed in peripheral blood of the high-dose animals. In the comet assay as well as the micronucleus test, AZT caused a considerable dose-dependent increase in DNA damage in all tissues evaluated and was highly cytotoxic to bone marrow and peripheral blood cells. These data are well in line with published results. Surprisingly, AZT did not significantly increase the number of Pig-a mutant cells. We speculate that two factors likely contributed to this negative result: a predominance of large deletions caused by AZT, and the relatively low statistical power of the first-generation scoring method used for this study.

Key Words: azidothymidine; 3′-azido-3′-deoxythymidine; comet assay; Pig-a assay; micronucleus test; rat; Zidovudine.

Nucleoside analogs are well known to be genotoxic. The most prominent effects have been observed in clastogenicity assays (Olivero, 2007; Olivero et al., 2004, 2009; Tomicic et al., 2002) often attributed to an imbalance of nucleotide precursor pool, respective interference with DNA synthesis. Incorporation into DNA and direct misconding or chain termination are other genotoxic modes of action potentially leading to point mutations, small or large deletions, and DNA breakage. Compared with reports on clastogenicity (i.e., micronucleus test), much fewer studies on gene mutation induction (e.g., MutaMouse) and DNA breakage (i.e., comet assay) have been published.

In order to comprehensively assess the genotoxic potential of nucleoside analogs in vivo, azidothymidine (AZT, 3′-azido-3′-deoxythymidine) was chosen as a model compound and endpoint for gene mutation, clastogenicity, and DNA breakage was investigated. We employed the micronucleus test using the standard microscopic evaluation in bone marrow and the flow cytometry method in peripheral blood, the comet assay in liver, stomach, and blood cells, and the Pig-a gene mutation assay in peripheral blood.

To get more insight into the exact genotoxic mechanism of AZT, we assessed the capability of AZT to elicit the various genotoxic effects under different in vitro testing conditions (Zeller et al., 2013).

AZT is a widely used HIV-1 nucleoside reverse transcriptase inhibitor (NRTI), which acts by chain termination and blocking the nucleotide-binding site of the HIV-1 reverse transcriptase (Furman et al., 1986; Olivero, 2007). Before being incorporated into viral DNA, AZT requires subsequent phosphorylation to the triphosphate by cellular kinases of the host cell (St Clair et al., 1987). NRTIs are also known to be affine toward eukaryotic polymerases and thus are also incorporated into host nuclear DNA and mitochondrial DNA (Lee et al., 2003; Olivero, 2007; St Clair et al., 1987). Incorporation into DNA results in an arrest of the DNA chain prolongation during replication.

Several in vitro and in vivo experiments with AZT reported the induction of micronuclei (Dertinger et al., 1996; NTP, 1999; Phillips et al., 1991), sister chromatid exchange, and chromosomal aberrations (González Cid and Larripa, 1994). Furthermore, mutations in mammalian cells were reported at different loci, such as APRT, HPRT, and TK genes (Meng et al., 2000a,b; Sussman et al., 1999). In the Ames test, AZT was found to be mutagenic only in TA102 without metabolic activation (Ayers, 1988; Ballardin et al., 2005; NTP, 1999). Our in vitro experiments confirmed these results (Zeller et al., 2013). Recently, AZT was reported to induce the formation of nuclear buds (Dutra et al., 2010), structures containing “undesirable” DNA that is expected to be expelled. Some evidence that...
these buds are precursors of micronuclei containing specific chromosomes was reported (Fenech et al., 2011; Shimizu, 2011; Shimizu et al., 1998). Furthermore, it was demonstrated that AZT exposure can result in centrosemal amplification, leading to chromosome misaggregation and thus aneuploidy (Borrojerdi et al., 2009; Momot et al., 2010).

In the present study, we comprehensively assessed genotoxic and mutagenic endpoints of the nucleoside analog AZT in vivo. Male Wistar rats were treated orally (by gavage) over 7 days with AZT at dose levels of 2 × 0 (control), 2 × 250, 2 × 500, and 2 × 1000 mg/kg/day with a final single dose given on day 8. DNA damage was detected in different organs in the comet assay (liver, stomach, and blood) and in the micronucleus test (bone marrow, peripheral blood by flow cytometry). In addition, following a treatment-free period of up to 42 days, the Pig-a assay was performed in peripheral blood.

MATERIALS AND METHODS

Chemicals and dose volumes. AZT (CAS 30516-87-1) was purchased from Lianhe Chemical Technology Co., Ltd (Zhejiang, China) with a purity of 98.6%. The test item formulation was prepared twice daily shortly before application by dissolving AZT in distilled water. The positive controls ethyl methanesulfonate (EMS, CAS 62-50-10) and cyclophosphamide monohydrate (CP, CAS 6055-19-2) were purchased from Sigma-Aldrich. CP was dissolved in 0.9% NaCl and aliquots from the stock solutions were stored frozen at about −20°C. The aliquots were thawed on the day of treatment. EMS was diluted in water at the day of the administration. Distilled water was administered as vehicle control (VC). Test items were dosed at a volume of 20 ml/kg (AZT), 3 ml/kg (CP), and 3 or 5 ml/kg (EMS) body weight, based on most recent recorded body weight.

Animal husbandry, treatment, and dose levels. Male Wistar rats (stock HanRCCWIST(SPF)), aged of about 8 weeks purchased from Harlan, Netherlands, were acclimatized for around 1 week. The study was performed in conformity with the Swiss Animal Welfare Law (Tierschutzgesetz) and in accordance with Roche internal standard operating procedures and guidelines for care and use of laboratory animals. Rats were housed at the good laboratory practice–certified animal unit. The test facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Care International. Two males per cage had ad libitum access to pelleted standard rodent diet and tap water from the domestic supply. Animals were kept in an air-conditioned animal room under periodic bacteriological control, at 22°C ± 2°C with monitored 40–80% humidity, a 12-h light/dark cycle, and background radio coordinated with light hours. They were assigned randomly based on body weight and identified by cage card, color code for group, and individually by tail tattoo. Clinical signs, body weight, and food consumption were continuously monitored for any abnormalities.

AZT was administered per oral (gavage) to six males per dose group over seven consecutive days twice daily with an interval of approximately 6h between two administrations at dose levels of 2 × 0, 2 × 250, 2 × 500, and 2 × 1000 mg/kg/day body weight/day. In order to assess the AZT-induced DNA damage in the comet assay, a final single dose was administered on day 8 around 3h prior necropsy (Fig. 1). As a positive control for the micronucleus test and comet assay, a single oral dose of 24 mg/kg CP followed by a single oral dose of 200 mg/kg EMS was administered to three additional males at 24 and 3h prior necropsy, respectively.

For the Pig-a assay, satellite animals were treated over seven consecutive days with 2 × 0 or 2 × 1000 mg/kg/day AZT to four and six males, respectively, followed by a treatment-free period (Fig. 1). As a positive control for the Pig-a assay, animals were treated over seven consecutive days with 120 mg/kg/day EMS.

Tissue collection. Blood was sampled sublingually into EDTA tubes from rats under light isoflurane anesthesia at approximately 3h after the first daily dose on days 1, 2, 4, and 8 as well as during the treatment-free period on days 11, 16, 23, 36, and 50 (Fig. 1). For the micronucleus test, around 100 μl of blood was transferred into ultracold methanol and stored at around −70°C until analyzed. For the analysis of Pig-a mutation, blood samples were collected on days 23 and 50, processed according to instructions in the Prototype MutaFlow kit, and immediately shipped refrigerated to Litron Laboratories (Rochester, NY). Blood (25 μl) for the comet assay (days 4 and 8 only) was diluted with PBS and immediately embedded into agarose and further processed for the assay.

For the determination of absolute reticulocyte (RET) counts, around 300 μl of blood was used.

Animals were sacrificed with carbon dioxide and exsanguinated at approximately 3h after the last dose on day 8. Bone marrow (femur) for the micronucleus test as well as liver and stomach for the comet assay was sampled as described below.

Comet assay. The standard protocol for sampling of liver tissues, preparation of a single-cell suspension and slides, followed by DNA unwinding, electrophoresis, and staining of slides, being used follows recommendation given by Burlinson et al. (2007), Hartmann et al. (2003), The comet assay JaCVAM (2013), and Tice et al. (2000).

For the comet assay, liver and stomach were sampled in Hank’s balanced salt solution (1× HBSS), containing 25 mM EDTA and 10% dimethyl sulfoxide, and stored on ice until a single-cell suspension was prepared (liver: mincing

![Figure 1](https://academic.oup.com/toxsci/article-abstract/135/2/309/1655890)
AZT IN THE COMET, MICRONUCLEUS, AND PIG-A ASSAY

with a pair of tweezers, stomach: scraping of cleaned mucosa). Isolated cells were embedded into agarose on a slide, lysed overnight, which was followed by alkaline unwinding for 20 min. Electrophoresis was performed at around 26 V (corresponding to 1 V/cm), 300 mA for 40 min (liver, blood), or 20 min (stomach) at around 4°C. Coded slides were analyzed by Metafer/Relosy 4 (Metasystems, Germany). A total of 150 comets per slide were measured. After artifacts were rejected, the median of the first 100 cells per slide was calculated. Slides with less than 75 comets per slide were excluded from the analysis. Two slides per animal and tissue were analyzed. The mean value of two replicates was calculated for each animal and from this, the mean value of the dose group was calculated. The number of “clouds” or hedgehogs (a morphology indicative of highly damaged cells often associated with severe cytotoxicity, necrosis, or apoptosis) out of 100 cells was scored manually for each replicate. Animals with >30% clouds were excluded from data analysis in order to avoid any interference due to excessive cytotoxicity.

Micronucleus test (bone marrow, blood). For the micronucleus assay in bone marrow, the test methodology was in accordance with requirements of the OECD Guideline 474 (OECD and 474, 1997) and current literature (Hayashi et al., 1994; MacGregor et al., 1987; Mavournin et al., 1990). In brief, bone marrows were flushed with 2 ml fetal bovine serum (FBS, 100%) containing 25mM EDTA. About 1 ml of the cell suspension was loaded onto a cellulose column (Frieauff and Romagna, 1994) and washed with 1.5 ml HBSS. The eluted cells were collected in 1.5 ml of a gradient solution containing 35% Percoll and subsequently centrifuged. The pellet was resuspended in about 10 ml HBSS. An aliquot of 0.2 ml was used to determine the number of cells using a couler counter and the remaining suspension was centrifuged again. The pellet was resuspended in FBS supplemented with 50mM EDTA at a concentration of about 8.5 x 10⁷ cells/ml and two probes were prepared for the separated cells using a Shandon Cytospin centrifuge on poly-L-lysine-coated slides. The slides were fixed and stained with a modified May-Grünwald-Giemsa (Romagna and Staniforth, 1989). A total of 4000 polychromatic erythrocytes (PCEs) per animal were evaluated for the occurrence of micronuclei and the relative proportion of PCE in relation to normochromatic erythrocytes (NCEs). Blinded slides were analyzed using the Metafer/Relosy 4. Statistical evaluation was performed for the frequency of micronucleated (MN)-PCE. The dose groups and the negative controls were compared with each other simultaneously by means of an ANOVA. In addition, each dose group is compared with the negative controls using ANOVA with the same factors. By error, bone marrow from positive control animals (24 mg/kg CP) was not sampled. The Rat MicroFlow Plus kit from Litron Laboratories was used to perform the micronucleus test in peripheral blood via flow cytometry. Blood was sampled and stored as described above (Litron Laboratories, 2003). After at least 1 week, methanol was removed by washing with PBS buffer followed by centrifugation at 400 x g for 10 min. After removal of the supernatant, cells were resuspended and labeled with antibodies against CD71 and platelets. RNA was degraded enzymatically and DNA stained with propidium iodide. Flow cytometric measurement was performed on a Becton-Dickinson FACS Canto II flow cytometer using FACS Diva software. Where possible, 20,000 RETs were analyzed for the presence of micronuclei. Results are given as percentage of RETs and NCEs containing micronuclei (MN-RET, MN-NCE). All steps were performed on kit-supplied negative, positive, and calibration controls (i.e., calibration via malaria-infected rodent blood samples).

Statistical analysis was done for the frequency of MN-RET by performing a one-way ANOVA using Dunnett’s test. Pig-a assay (blood, flow cytometry). A Prototype Rat MutaFlow (Pig-a mutation, first generation) kit from Litron Laboratories was used to perform the test and the provided instructions were followed. Blood was sampled and stored as described above (Litron Laboratories, 2009). Briefly, approximately 50 μl of blood from each animal was collected into EDTA tubes. Leukocytes and platelets were depleted by density-gradient centrifugation with Lympholyte-Mammal solution (Cedarlane Laboratories, Canada) at room temperature. The remaining erythrocyte suspension was shipped at approximately 3°C to Litron Laboratories, where antibody labeling and flow cytometric analysis were performed. Briefly, the cells were incubated with an anti-CD59-PE followed by a washing step and addition of a nucleic acid dye solution. Two separate flow cytometric measurements with different threshold settings were performed with each sample in order to acquire approximately 10⁶ red blood cells (RBCs) and at least 300,000 gated RETs. Statistical analysis was done by log transformation of the data and performing a one-way ANOVA using Dunnett’s test.

RET count. Blood was sampled and stored as described previously. Approximately 300 μl blood was used for the determination of absolute RET counts with the ADVIA120 analyzer (Siemens Healthcare Diagnostics, Germany).

RESULTS

There were no abnormal clinical signs and no effects on body weight or food consumption in animals dosed with AZT at any dose level.

Comet Assay (Liver, Stomach, Blood)

A strong and dose-dependent increase in the tail intensity (°TI) values was observed in liver, stomach (day 8), and blood (days 4, 8) (Fig. 2). In the liver, an increase in °TI from 5.1 ± 1.5 (VC) to 61.4 ± 3.2 (2×10⁰ mg/kg/day) was observed. In the stomach, an increase in °TI from about 4.1 ± 1.4 (VC) to 24.8 ± 6.0 (2×10⁰ mg/kg/day) was observed. The number of hedgehogs or clouds, indicative for cytotoxic effects such as necrosis or apoptosis, was elevated in liver and stomach only at the high dose (liver: VC: 0.2 ± 0.3; 2×10⁰ mg/kg/day: 23.1 ± 4.3; stomach: 2.3 ± 1.4 vs. 2×10⁰ mg/kg/day: 12.3 ± 3.8). None of the individual slides from both tissues showed more than 30% of hedgehogs. In blood, an increase in °TI from 0.4 ± 0.9 (VC) to 19.2 ± 9.1 (2×10⁰ mg/kg/day) and 0.9 ± 0.8 (VC) to 9.3 ± 13.9 (2×500 mg/kg/day) was observed on days 4 and 8, respectively. On day 8, two out of six animals treated with 2×500 mg/kg/day as well as all animals treated with 2×1000 mg/kg/day were excluded from the comet analysis, due to the high number of hedgehogs.

For liver and stomach, all °TI values of the VC animals were within the range of negative control data from previous in-house studies. For blood, slightly higher values than in the previous in-house studies were observed. The positive control EMS caused a considerable increase in the °TI in liver (%TI: 18.07 ± 3.24, n = 3), stomach (%TI: 24.37 ± 8.22, n = 3), and blood (day 4: %TI: 56.28 ± 2.46, n = 3), demonstrating the sensitivity of the test system. Due to coagulation of the blood of the positive control CP/EMS sampled on day 8 during necropsy, these slides could not be analyzed.

Micronucleus Test in Bone Marrow and Peripheral Blood

In the bone marrow (day 8), treatment of AZT caused a strong dose-dependent increase in the number of MN-PCE from 0.23 ± 0.07 (VC) to 6.12 ± 1.65 (2×10⁰ mg/kg/day) (Fig. 3A). Even at the lowest dose of 2× 250 mg/kg/day, a statistically significant increase (MN-PCE: 1.68 ± 1.05%;...
p < 0.01) was observed. In parallel, a considerable decrease in the % PCE (VC: 60 ± 12.4% vs. 2 × 1000 mg/kg/day: 5% ± 2.7) was observed. The spontaneous frequency of MN-PCE was in the normal range of the in-house historical controls.

By comparing the micronucleus results obtained in bone marrow and peripheral blood on day 8 (Figs. 3A and 3B), a similar incidence of MN cells, as well as the relative proportion of PCE and RET (compared with concurrent VCs) were observed, showing the sensitivity and comparability of both endpoints. Statistical significant increases for MN-RET were observed on days 2 and 4 for all dose levels and on day 8 for the mid and high dose (p < 0.05).

In blood, a substantial, time- and dose-related increase in the number of MN-RET was observed following treatment with...
From day 2 to day 8, a dose-dependent increase in the number of MN-RET was observed. During the treatment-free period, a gradual decrease of MN-RET was observed. Throughout the duration of the experiment, the number of MN-NCE was comparable with the negative control value (data not shown).

**Determination of Number of RETs**

No acute effect on the proportion of RETs was observed on day 1 (3 h postdose). From day 2 onward, a dose- and time-dependent decrease in % RET relative to VC was observed, reaching its maximum effect on day 4 (Fig. 4A). For the high dose of $2 \times 1000$ mg/kg/day, the proportion of RETs was decreased to less than 5% when compared with control values. From day 4 to day 8, a recovery or compensation of these effects were noted in all dose groups. Although the increase at $2 \times 1000$ mg/kg/day was only minor on day 8, by day 23 the % RET was fully recovered and comparable with day 1.

**Pig-a Assay**

Blood for the analysis in the Pig-a assay was taken on days 23 and 50 of the study. A slight increase in the frequency of mutated RBCs (CD59−) from $0.5 \pm 1.0$ (VC) to $1.3 \pm 1.0$ (2×1000 mg/kg/day) and $0.3 \pm 0.5$ (VC) to $1.3 \pm 1.4$ (2×1000 mg/kg/day) was observed on days 23 and 50, respectively (Table 1).
However, the effects were within the 95% confidence interval of the laboratory’s historical negative control range for the first-generation method used for this study (RBC, CD59: 0.86–1.34; RET, CD59: 0.78–1.66). In addition, no statistically significant (p ≤ 0.05) increase was detected in an ANOVA using Dunnett’s test, being performed on log transformed data. The positive control EMS, administered at 120 mg/kg/day over seven consecutive days, showed a significant increase in both the number of mutant RBCs (14.3 ± 5.1) and RETs (30.7 ± 19.7). The % RET was comparable between AZT and vehicle-treated animals on days 23 and 50, which is in line with results obtained using the ADVIA120 (Fig. 4A). Thus, it can be concluded that AZT induces at most a weak effect in the Pig-a assay in blood.

**DISCUSSION**

The genotoxic potential of the nucleoside analog AZT was assessed in male Wistar rats using several methods and in various tissues (blood, liver, stomach, bone marrow). Treatment with AZT over seven consecutive days with a final dose on day 8 caused a strong and dose-dependent effect in the comet assay and in the micronucleus test in all tissues analyzed but did not result in a statistically significant increase in Pig-a mutant erythrocytes. The response in the blood micronucleus test was stronger than in the different tissues in the comet assay (liver stronger than peripheral blood and stomach). Although AZT was reported in several publications (Aruna and Jagetia, 2001; Dutra et al., 2010; González Cid and Larripa, 1994; Olivero, 2007) to cause various types of DNA damages, we were especially interested in a comprehensive assessment of AZT as a model compound for nucleoside analogs, involving different endpoints simultaneously in the same animals.

In the comet assay, a linear dose-dependent increase in the DNA damage was observed in all tissues analyzed. Overall treatment with AZT resulted in highly damaged comets with TI values of up to 60% (liver), which is even higher than those caused by the commonly used positive control EMS at 200 mg/kg. As shown by blood samples analyzed on days 4 and 8, the DNA damage increased over the treatment period.

Based on the established chain termination mode of action of AZT, it is tempting to attribute the dramatic increase of TI to single-strand gaps arising as a direct consequence of chain termination or exonucleolytic repair of incorporated AZT. However, this hypothesis would argue that cells in S phase should show much higher TIs than cells in G0, G1, or G2 phase. The majority of the liver cells in 8-week-old rats would not be expected to pass through a round of replication during the relatively short treatment period of 4 or 8 days, and thus, AZT incorporation should not occur by scheduled DNA synthesis. Nevertheless, we rather find high levels of DNA breakage in all cells, i.e., a fairly homogeneous distribution of TIs. Therefore, we conclude that other mechanisms than chain termination or exonucleolytic repair must be responsible.

Escobar et al. (2007) demonstrated that in the in vitro alkaline comet assay with cells from the lymphoblastoid cell line H9, the incorporation of AZT in DNA is highly correlated with the increase in DNA damage. Exposure of cells to AZT for 24 h led to an accumulation of the cells in S phase, probably in consequence of AZT incorporation and chain termination during replication. Thus, their observation of a direct correlation between AZT incorporation and an effect in the comet assay is well explained. Less certain is their suggestion that the DNA breakage is due to alkali-labile sites, not direct single-strand breaks. In another comet assay in vivo study using the DNA repair enzymes Endo III and Fpg, the authors argued that oxidative DNA damage was induced as a consequence to AZT exposure (Slamenova et al., 2006). However, oxidative DNA damages are repaired relatively fast (within 1–2 h) as shown by DNA rejoining in the comet assay (Collins and Horvathova, 2001). In contrast, in our in vitro studies (Zeller et al., 2013), the DNA damage induced by AZT did not reach background level within 24 h after AZT treatment and thus does not support this hypothesis.

As discussed above, we conclude that the strong effects in the largely noncycling liver cells cannot be explained by formation of strand breaks as a direct consequence of incorporation of AZT into DNA—unless incorporation in background unscheduled (repair) DNA synthesis would be sufficient.

The induction of micronuclei following AZT treatment was demonstrated by several in vitro and in vivo experiments (Dertinger et al., 1996; Phillips et al., 1991; Tikoo et al., 2008). In bone marrow as well as peripheral blood, a considerable dose-dependent increase in the number of micronuclei, together with a strong decrease in the number of PCEs and RETs, was observed. Bone marrow–specific cytotoxicity of AZT is described to be caused by an incomplete maturation process of cells from the bone marrow into the peripheral blood (Thompson et al., 1991). Other studies suggest that AZT leads to an active proliferation and specific downregulation of transcription factors in the
bone marrow (Olivero, 2007). In peripheral blood, the number of MN-RETs starts to increase from day 2 onward, which is in line with previous studies that have been performed in order to assess the time course of MN induction in RET (Mughal et al., 2010). With AZT being eliminated quickly from the body, the induced MN-RETs are expected to decrease within a few days because only “penultimate” erythroblasts prior to their final cell division can give rise to MN-RETs. If the damage occurs earlier during their maturation, they will be eliminated and not proceed to the peripheral circulation system.

As determined by the Pig-a gene mutation assay, AZT did not cause a statistical significant increase in the number of mutant phenotypes in RBCs or RETs. As expected (Dobo et al., 2011; Keysar and Fox, 2009), the positive control EMS (120mg/kg/day) induced gene mutations in both RBC and RET. AZT was shown to be mutagenic in the HPRT test in vitro, although the effect was rather low (Sussman et al., 1999). Even though in both assays, mutations are based on X-linked chromosome, the HPRT was recently found to be more responsive to radiation, whereas Pig-a was more responsive to cisplatin (Dobrovolsky and Heflin, in preparation), suggesting that even among X-chromosome reporter genes, differences can exist for detecting certain lesions, perhaps especially gross structural changes as would be expected for AZT. The strong hematotoxicity of AZT observed in our study as well as by others (Lee et al., 2003; Veal and Back, 1995) supports this hypothesis. The hematotoxicity of AZT has been established in animals as well as in patients and is known to be fully reversible (Thompson et al., 1991) as was shown in this study. Furthermore, the statistical power of the Pig-a assay as it was used in the current study is not sufficient to detect modest increases over baseline values and this may have contributed to the negative result.

In our studies with the Ames test (Zeller et al., 2013), an increase in the number of revertant colonies in TA102 and other strains containing a TAA ochre stop codon as mutational site was observed. We suggest that mutations arise through in-frame small deletions (3–6bp), which have been reported for strain TA102 (Levin et al., 1982). In contrast, mutations described in mammalian cells at the HPRT locus are possibly due to large deletions, whereas the Pig-a system does not appear to be capable to detect large deletions. Because AZT is incorporated into the DNA, leading in the first instance to an arrest of DNA chain prolongation, the induced mutations are rather large and thus would support the theory that the Pig-a assay might not be sensitive in detecting this kind of mutations. Pig-a mutations have been reported to be frameshift mutations or base-pair substitutions, but very few intragenic or whole gene deletions have been reported in the Pig-a genes (Mortazavi et al., 2003; Nafa et al., 1998). Recent analysis of the statistical power of the Pig-a assay has indicated that the first-generation approach that analyzes 10⁶ RBCs and 300,000 RETs is of insufficient statistical power to reliably detect two- to threefold increases in mutant cells (Lynch et al., 2011). This limited sensitivity has led to a newly developed immunomagnetic method (Dertinger et al., 2011). The immunomagnetic separation of mutated RBC or RET has been introduced as an additional step in the Pig-a protocol, a modification that allows significantly more cells to be evaluated in about one third of the time, thereby substantially increasing the sensitivity of the assay (Dertinger et al., 2011). Thus, it cannot be excluded that with this more powerful Pig-a method, the relatively weak mutagenic potential of AZT could be observed in vivo as well.

REFERENCES


