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Original Article

The *in vivo* Pig-a gene mutation assay is applied to study the genotoxicity of procarbazine hydrochloride in Sprague-Dawley rats

Jiang Pu[1,2,*], Yuanyuan Deng[1,3,*], Xiaoyan Tan[1,2], Gaofeng Chen[1,2], Cong Zhu[1,2], Naisong Qi[1], Hairuo Wen[1], Jun Guo[1], Xin Wang[1], Yuwen Qiu[2], Jinqiang Liang[2], Xinlu Fu[2], Yanping Hu[1], Jie Song[1], Xingchao Geng[1], Chao Wang[1], Lin Zhang[1], Zhiying Huang[2], Bo Li[1] and Xue Wang[1]

[1]National Centre for Safety Evaluation of Drugs, National Institutes for Food and Drug Control, A8 Hong Da Zhong Lu Road, Beijing Economic-Technological Development Area, Beijing, 100176, China
[2]Sun Yat-sen University, No. 132, East Outer Ring Road, Guangzhou University City, Guangzhou, 510006, China
[3]National Centre for Safety Evaluation of Drugs, No. 28 Gao Peng Avenue, Hitech Development Zone, Chengdu, Sichuan, 610041, China

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ABSTRACT — The Pig-a gene is well-known to encode a key enzyme essential in the biosynthesis of glycosylphosphatidylinositol (GPI), which attaches CD molecules (Cluster differentiation), such as CD55 and CD59, to red blood cells (RBCs) membranes. In this study, the blood was marked with the special antigen CD45-PE (Phycoerythrin) to separate the erythrocytes from the leukocytes, then the Pig-a mutant frequency (MF) of RBCs could be investigated without pivotal antigen CD59-FITC (Fluorescein isothiocyanate), and the optimal ENU concentration was determined to be 100 mg/kg/day. The optimal cell number to count and the stability of specimens were determined. At last, the *in vivo* Pig-a gene mutation assay was utilized to detect the potential genotoxicity of Cis-Dichlorodiammineplatinum (DP), Procarbazine Hydrochloride (PH), and Triptolide (TP). The results indicated that the Pig-a gene mutation obviously occurred in the PH treatment group. In the DP treatment group, an irregular shape with a slightly serrated border in erythrocytes was observed in the blood smear. However, no obvious Pig-a gene mutations were detected in the DP treatment and TP treatment groups under the experimental conditions. In conclusion, this primary *in vivo* Pig-a gene mutation assay will provide an easy protocol to use in screening potential genotoxic compounds.

Key words: Pig-a, ENU, RBCs, Genotoxicity, Procarbazine Hydrochloride

INTRODUCTION

As a type of variation, mutations occur during the process of cell replication. Inheritable and advantageous variations are thought to be a potent evolutional force. However, genetic instabilities are also strongly related to the genetic susceptibility of cancer. In addition, the mutant frequency (MF) has also been deemed as a biological parameter for any cell population (Peruzzi *et al.*, 2010). Once measured, it can provide safety data for clinical therapies.

The Pig-a gene is a well-defined gene that encodes a key enzyme essential in the biosynthesis of glycosylphosphatidylinositol (GPI) (Bessler *et al.*, 1994). The Pig-a gene is X-linked, and mutational inactivation of several exons in somatic cells triggers the inhibition of GPI biosynthesis, blocking the ability of CD molecules attaching to the membrane (Lucio, 2006); this inactivation leads to two relative phenotypes. A previous study has shown that CD55 and CD59 are expressed on the surface of nearly all blood cells through linkage with GPI (Ruiz-Argüelles and Llorente, 2007). The two phenotypes can be distin-

Correspondence: Xue Wang (E-mail: wangxuepaper@sina.com)
Bo Li (E-mail: libonifdc@163.com)
*These authors equally contributed to this work.

guished and detected by indirect immunofluorescence staining (Kimoto *et al.*, 2012). Presently, the Pig-a gene has been utilized successfully to investigate the MF in many studies (Bhalli *et al.*, 2011; Dobrovolsky *et al.*, 2012; Dertinger *et al.*, 2011). The literature data indicated that the absence of GPI is the leading cause of paroxysmal nocturnal haemoglobinuria (PNH) (Phillips *et al.*, 2001).

As an ideal sentinel gene, the Pig-a gene has shown many biological advantages and has been widely used in the study of genotoxicity. Firstly, the Pig-a gene exhibits mono-allelic mutations that are detectable, and the mutant phenotype can be distinguished from wild-type cells by flow cytometry. Secondly, several lines of evidence indicate that no clear or strong selection for or against the two phenotypes exists *in vivo* (Araten *et al.*, 2005; Rosti *et al.*, 1997; Tremml *et al.*, 1999; Ware *et al.*, 1998; Yamamoto *et al.*, 2002). Thirdly, the mutant and wild-type cells can circulate for at least one month in peripheral blood in rats (Miura *et al.*, 2009). Fourthly, Pig-a mutant RBCs accumulate in the peripheral blood after repeated dosing (Miura *et al.*, 2009). Fifth, the Pig-a gene has a high potential of cross-species expression (Peruzzi *et al.*, 2010; Phonethepswath *et al.*, 2008). Thus, experimental data could provide more information and instructions for clinical treatment. The last and the most important factor was that the rate of GPI deletions is consistent with the rate of glycosylphosphatidylinositol anchored protein (GPI-AP) expression loss (Miura *et al.*, 1974). Therefore, the MF can be accurately investigated by flow cytometric analysis.

A genotoxicity test is significant in the non-clinical safety evaluation of new compounds. A standard genetic toxicology battery has been recommended for the prediction of the carcinogenicity by ICH and OECD. However, *in vivo* experiments were mostly designed to predict chromosome damages caused by compounds. Few *in vivo* assays have been performed to detect relevant genetic changes. One study suggests that the *in vivo* Pig-a gene mutation assay would meet these expectations when conducted along with a repeat-dosing experimental design (Dobrovolsky *et al.*, 2010). Thus, multiple time points could be detected in peripheral blood to reflect the mutations in the Pig-a gene.

This study was designed to develop a fast and simple *in vivo* Pig-a mutation assay in Sprague Dawley rats. Then, the methodology was validated and optimized in several aspects. Afterwards, it was employed to study the genotoxicity of Cis-Dichlorodiammineplatinum (DP), Procarbazine Hydrochloride (PH), and Triptolide (TP).

MATERIALS AND METHODS

Reagents

N-Ethyl-N-nitrosourea (ENU; CAS no. 759-73-9), dimethylbenzanthracene (DMBA, Lot: D3254, Sigma, Shanghai, China), ethylenediamine tetraacetic acid dipotassium salt dehydrate (EDTA2K; Lot: 20060222, Beijing Chemical Works, Beijing, China), procarbazine hydrochloride (Procarb, CAS no. 366-70-1; China Langchem Inc., Shanghai, China), triptolide (TP; Lot: 111567-200603, National Institutes for Food and Drug Control, Beijing, China), corn oil (Yihai Group Goldensea Industry, Beijing, China) and propanediol (Lot: 046K0100, Sigma) were obtained from their respective companies. Anti-rat-CD59-FITC (Lot: 550976) and Anti-rat-CD45-PE (Lot: 554878) were purchased from BD Biosciences (Beijing, China).

Animals, treatments, and blood collection

All experimental procedures involving animals were at the oversight of the Experimental Animal Ethics Committee from the National Center for Safety Evaluation of Drugs in China. Male Sprague Dawley rats were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Rodents were allowed to acclimate for approximately one week before treatment. Water and food were available *ad libitum* throughout the acclimation and experimental periods. All compounds were administered as previously described, and sterile water was used as the vehicle for drug dissolution.

The methodology test was launched and included a positive compound selection test, ENU dose screening test and an animal age selection test. For the positive compound selection test, DMBA was administered for three consecutive days at a dose of 40 mg/kg/day by oral gavage, and ENU was administered for three consecutive days at a dose of 100 mg/kg/day by intraperitoneal injection. ENU dose level screening test was conducted involving three dose levels by intraperitoneal injection, that's 40 and 100 mg/kg/day for three consecutive days at intervals of approximately 24 hr, or 160 mg/kg/day once. The animal age selection test was performed in five- and nine-week–old rats, and the treatment was 100 mg/kg/day ENU for three consecutive days. All blood samples were collected on days -1, 7, 14, and 28 after the last exposure to compounds. Blood samples were acquired via tail vein with a syringe. Approximately 20 μL of blood was collected in a BD Microtainer Tube with K2E (EDTA2K), and euthanasia was performed on day 28. Immediately upon collection, all blood samples were leukodepleted through the usage of lymphocyte separation liquid, and

the samples were then stored at 4°C.

The flow cytometric analysis method was optimized by assessing the stability of stained cells, the influence of storage time for anticoagulating blood, and the optimal cell number for flow cytometry detection. For the stability of stained cells, two specimens were observed three times at 0.5 hr whereas the other two were detected at 3.5 hr. the influence of storage time for anticoagulating blood was investigated with relation to time; the storage time of anticoagulant blood samples were 0.5 hr, 8 hr and 24 hr, and blood samples were labelled and analysed as described in "Cell staining". The best cell number for flow cytometry analysis was verified by the detection of one blood sample from the positive control (ENU) on day 28 and the detection of each testing point five times; the cell numbers for detection were set at 1.0×10^5, 3.0×10^5, 5.0×10^5, and 1.0×10^6 total RBCs.

The application experiments were performed to detect the genotoxicity of cis-dichlorodiammineplatinum (DP), procarbazine hydrochloride (PH), and triptolide (TP). For DP, the rats were treated with a single high level of DP (5 mg/kg/day) or with a low-dose (2 mg/kg/day) of DP for five repeated days basing on clinical usage. DP was administered by intraperitoneal injection for each DP treatment group. The blood was collected from the tail vein on days -1, 7, 14, and 28 as described above. The rats were treated with PH (100 mg/kg/day) by oral gavage for three times in succession at intervals of approximately 48 h or with TP (0.45 mg/kg/day) by oral gavage for seven consecutive days. The positive group was treated with 100 mg/kg/day of ENU (5 mL/kg) for three consecutive days. The blood was collected from the tail vein on day -1, 7, 14, 28, 35, and 56 as described above.

Blood samples were collected in a BD Microtainer tube with K2E (EDTA2K) as soon as possible. Animals were killed by CO_2 asphyxiation after the last time point. Immediately upon collection, all blood samples were instantly leukodepleted with lymphocyte separation liquid (within 2 hr), and the samples were then stored at 4°C.

Cell staining

All erythrocyte-enriched samples were washed twice with phosphate-buffered saline (PBS). Approximately 1.5 μL of the blood-EDTA mixture was transferred to a 200-μL PBS tube for each sample. Then, 2.5 μL of anti-CD45-PE and 1 μL of anti-CD59-FITC were added to each specimen, and all specimens were incubated with saturating antibodies for 20 min. After incubation, the stained cells were washed with PBS and collected by centrifugation. The cells were immediately resuspended in 0.5 mL of PBS in an FC tube, and the stained cells were interrogated by flow cytometry within approximately 3 hr.

Acceptable criterion

A self-regulating Instrument Calibration Standard (ICS) was performed before each analytical run. As shown in Fig. 1, two aliquots with 3 μL of homogeneous blood samples were transferred from the same rat of a vehicle control group to a 200 μL PBS tube. To one aliquot, 5 μL of anti-CD45-PE and 2 μL of anti-CD59-FITC were added, and to another only anti-CD45-PE was added. Both were incubated for 20 min. Then, the samples were washed and mixed together; thus, the mixture was able to provide sufficient numbers of RBCs, and a full range of FITC fluorescence intensities were useful for optimizing PMT voltages and fluorescence compensation settings (Dertinger *et al.*, 2011). Moreover, the accuracy of flow cytometry detection was assessed by evaluating the linear correlation between the expected CD59-negative value and the theoretical value; the measurements were set to 1.0×10^5, 2.0×10^5, 3.0×10^5, 4.0×10^5, 5.0×10^5, 6.0×10^5, 7.0×10^5, 8.0×10^5, 9.0×10^5 and 1.0×10^6 total RBCs.

Flow cytometric analysis

A flow cytometer (BD, FACSCalibur) was used to investigate the Pig-a mutant RBCs. This device was equipped with 488 nm and 535 nm lasers, and CELLQuest 5.2 software was used for data acquisition. Anti-CD59-FITC and anti-CD45-PE staining were detected in the FL1 and FL2 channels, respectively. The PMT and compensation parameters were determined with a preliminary Instrument Calibration Standard. The cell number for detection was based on 1.0×10^6 total RBCs per sample. Erythroid cells were first gated on a FSC-SSC dot plot. Erythroid cells were precisely distinguished further by gating anti-CD45-PE positive cells on a FSC-CD45 dot plot. Then, the wild-type cells were separated on a dot plot using anti-CD59-FITC fluorescence from the mutant cells. The frequency of the Pig-a gene mutant per million total RBCs was statistically calculated by evaluating the total RBCs that were CD59-positive and CD59-negative.

Calculations and statistical analysis

The frequency of Pig-a mutant cell phenotype per total RBCs was calculated and expressed as number per 10^6 cells. A t-test was used for comparisons between the vehicle control group and the compound control group at each time point. Levels of significance were set at $P < 0.05$ and $P < 0.01$. The mean value, standard error, and correlation coefficient (r^2) calculations were performed using Excel

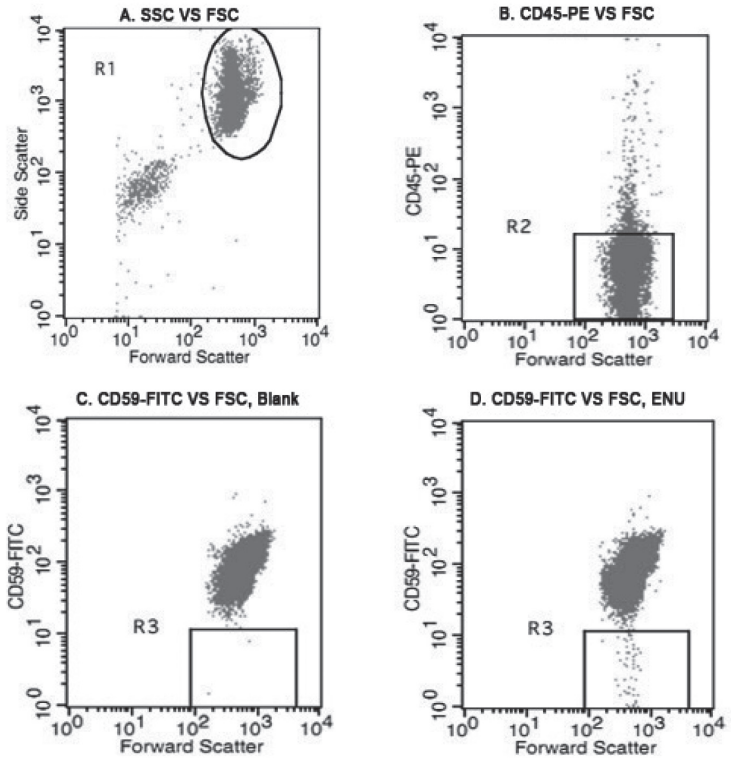

Fig. 1. Four bivariate dot plots explain the gating logic for the evaluation of RBC^{CD59-}. R1 was set to exclude the platelets and cell fragments (Panel A). An FSC-CD45 dot plot was set to collect cells from the R1 region; anti-CD45-PE distinguished the RBCs from the leukocytes. R2 was set to collect one million RBCs in total (Panel B). Panel C and Panel D were set to collect the RBCs from the R2 region, respectively; cells in the R3 region were RBC^{CD59-}; Panel C demonstrated the RBC^{CD59-} value from the vehicle control group; Panel D revealed the strong RBC^{CD59-} value from the ENU-treated control group.

2007 (NCSED, Beijing, China).

RESULTS

Method establishment

Screen for the optimal positive compound

Severe reduction of mean body weight was observed in both the ENU treatment group and the DMBA treatment group. Compared with the vehicle control group, the mean body weight of ENU and DMBA treatment groups were reduced to almost 79.9 and 79.6%%, respectively, of the vehicle control group on day 28; then, the weights recovered gradually, with the mean weights of the ENU and DMBA treatment groups back to 84.1 and 86.3%% on day 56.

The RBC^{CD59-} (RBCs with negative CD59 expression) value was measured on days -1, 7, 14, 28, 35, and 56, as shown in Fig. 2. The frequency of RBC^{CD59-} for the ENU treatment group was statistically significant compared with the vehicle control group after administration. The ENU treatment group exhibited a 7.7-fold increase compared with the vehicle control group on day 7, a 12.6-fold increase on day 14, a 17.2-fold increase on day 28, a 12.1-fold increase on day 35, and a 37.4-fold increase on day 56. Moreover, the RBC^{CD59-} value increased in a time-related manner during the test period. The increased values of mutant erythrocytes induced by DMBA were as follows: 2.1-fold on day 7, 6.1-fold on day 14, 8.8-fold on day 28, 5.9-fold on day 35, and 25.8-fold on day 56. Except for the predosing time point and day 7, the Pig-a mutant frequency (MF) of all points was statistically significant compared with the vehicle control group.

ENU dose level screening

Mean body weight 28 days after the last exposure was different between three protocols of ENU treatments. For the repeated low-dose group (40 mg/kg/day), the mean weight reduced to 83.1%% compared with the vehicle control group. The mean weight of the high level treatment (160 mg/kg/day) decreased to 78.3%% of the vehi-

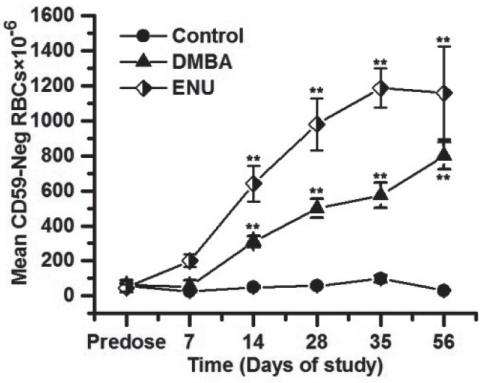

Fig. 2. The RBC^{CD59-} values for ENU and DMBA treatments in peripheral blood ($\times 10^{-6}$), T-test, $P < 0.01$, (n = 4 animals per group).

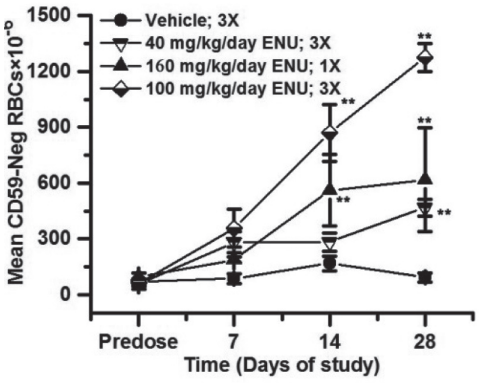

Fig. 3. The RBC^{CD59-} values for three protocols of ENU delivery at different times ($\times 10^{-6}$), T-test, $P < 0.01$, 3X indicates that drugs are administered for three consecutive days, (n = 4 animals per group).

cle control group. The medium-dose treatment (100 mg/kg/day for three days) decreased the mean weight to 63.7%% compared with the vehicle control group on day 28.

The elevated tendency of the RBC $^{CD59-}$ frequency occurred in all three ENU treatment protocols, as shown in Fig. 3. The repeated low-dose group that received doses at 24-hr intervals increased the proportion of Pig-a mutant erythrocytes by 2.2-fold on day 7, by 0.6-fold on day 14 and by 4.1-fold on day 28 compared with the level of the vehicle control group. The medium-dose group that received treatment for three consecutive days exhibited an increase in the proportion of Pig-a mutant erythrocytes by 3.0-fold on day 7, by 4.0-fold on day 14 and by 12.9-fold on day 28. The treatment group treated with ENU once at 160 mg/kg showed a 1.1-fold increase of Pig-a mutant erythrocytes on day 7, a 2.3-fold increase on day 14 and a 5.7-fold increase on day 28. A steep increase of mutant erythrocytes among all ENU treatments groups was observed on day 14. Then, the frequency increased slowly to the peak on day 28 for all groups. In summary, all three treatment protocols showed statistically significant increases after the 7th day post the last administration.

Animal age selection

The animal's age had no effect on the mean RBC^{CD59-} frequency, as shown in Fig. 4. For the five-week-old group and the nine-week-old group, no significant difference was found between the two groups despite that the MF of the five-week old group increased higher than the nine-week old group, by 0.1-fold on day 7 and by 0.65-fold on days 14 and 28. Similarly, both groups expressed

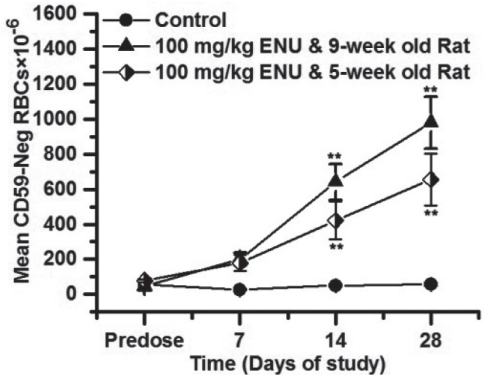

Fig. 4. The RBC^{CD59-} values for different ages in SD Rat peripheral blood ($\times 10^{-6}$), T-test, $P < 0.01$, (n = 4 animals per group).

a time-dependent increase of mutant erythrocytes. All increases were statistically significant compared with the vehicle control group. The background RBC^{CD59-} presented a smooth tendency during the study, and its range was approximately 12-95 $\times 10^{-6}$.

Condition control

Stability of stained cells

Two samples were chosen from the same ENU treatment and vehicle control sample, and each was divided into two specimens. The two specimens were stained for half an hour and analysed three times. Meanwhile, the other two were analysed in parallel three times 3.5 hr after staining. The s deviation (STDEV) and variable coefficient (CV) are listed in Table 1. A t-test was used to compare the mean RBC^{CD59-} values. The difference was not

statistically significant ($P < 0.01$). Therefore, the stained cells are stable for 3.5 hr.

The influence of storage time for anticoagulating blood

Following the validation test, one ENU sample and vehicle sample were analysed twice at 0.5 hr, 8 hr, and 24 hr, to investigate the influence of storage time when the blood samples were kept for at four degrees centigrade. Results indicated that the mean RBC^{CD59-} value increased in a time-dependent manner, as shown in Table 2.

The optimal cell number for flow cytometry detection

The final step was to determine the optimal cell number for flow cytometry analysis. Statistical results in Table 3 showed that the mean RBC^{CD59-} value would be more accurate if the FACSCalibur detected one million erythrocytes. The variable coefficient was inversely proportional to the cell number for flow cytometry analysis. Notably, the variable coefficient was only 1.90% if the FACSCalibur counted 1.0×10^6 erythrocytes; thus the error was allowable.

Method application

cis-Dichlorodiammineplatinum (DP)

All groups that were treated with DP were observed to exhibit severe toxicity. One animal from the low-dose administration group died on the thirteenth day after the last delivery, and the other two animals lost more than 20.0% of their weight, resulting in the immediate removal of DP to meet the animal welfare. The two animals were judged to be suffering from engorgement and prominence of mesenteric vessels by a pathologist, featured with acute atrophy of the thymus gland, and its erythrocytes were observed to be undergoing haemolysis, as shown in Fig. 5.

The mean body weight of the single high level group decreased compared with the vehicle control group. It exhibited approximately 40.4% of the vehicle control group on day 28, whereas the positive group exhibited 91.1% of the control group's weight. The blood samples of all groups were evaluated with no obvious rise in RBC^{CD59-} value except the positive group, as shown in Fig. 6.

Procarbazine hydrochloride (PH) and triptolide (TP)

An obvious adverse reaction was observed during the PH treatment study; the rats showed a loss of hair on their neck after the exposure to PH and recovered on day 21. The mean body weight was reduced to 87.1% of the vehicle control group on day 28. The body weights declined to 68.7% on day 56. No obvious abnormal reaction occurred between the TP experimental group and the vehicle control group. In contrast to the vehicle control group, the ENU control group had an evident reduction in weight of approximately 8.4% on day 28 and 26.8% on day 56.

Both the PH treatment group and the ENU control group animals were evaluated to have severe genotoxic-

Table 1. The RBC^{CD59-} of original samples and parallel samples ($\times 10^{-6}$).

Sample ID	RBC^{CD59-} Value			Mean Value (RBC^{CD59-})	STDEV
Vehicle sample (0.5 hr)	60	60	83	68	13.28
Vehicle sample (3.5 hr)	39	39	49	42	5.77
ENU sample (0.5 hr)	1008	1043	1025	1025	17.50
ENU sample (3.5 hr)	1142	1104	1130	1125	19.43

Table 2. The RBC^{CD59-} values coming from blood samples with different storage times ($\times 10^{-6}$).

Sample Type	0.5 hr		8 hr		24	
	1	2	1	2	1	2
Vehicle sample	60	60	118	352	318	566
ENU sample	1008	1043	1392	1495	1920	1786

Table 3. The RBC^{CD59-}value when counting different numbers of erythrocytes ($\times 10^{-6}$).

Number of erythrocytes	1.0×10^5	3.0×10^5	5.0×10^5	1.0×10^6
RBC^{CD59-} value (X ± SD)	856 ± 76	870 ± 102	865 ± 35	842 ± 16
Variable coefficient	8.87	11.7	4.04	1.9

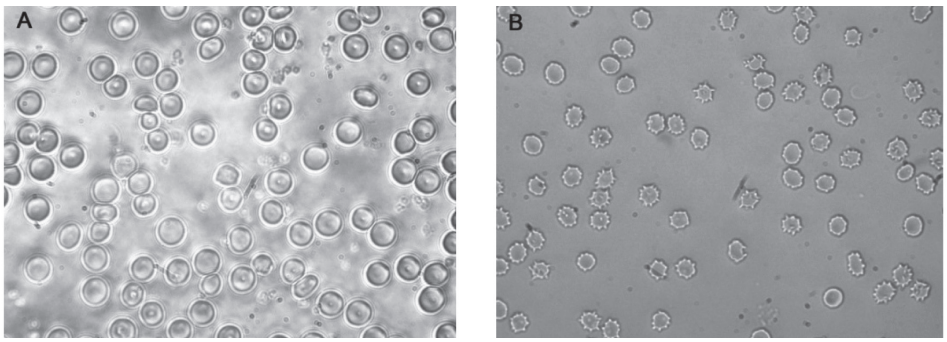

Fig. 5. A: erythrocytes presented a normal rounded shape in the left picture. B: In the DP treatment group, erythrocytes presented an irregular shape with a slightly serrated border in the blood smear.

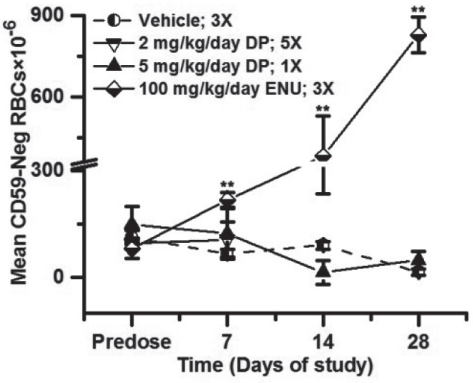

Fig. 6. The RBC^{CD59-} for two protocols of DP delivery at different time points ($\times 10^{-6}$), T-test, $P < 0.01$, One animal from the low-dose administration group died on the thirteenth day after the last delivery, and other two animals were sacrificed by euthanasia due to the severe loss of body weight immediately, (n = 3 animals per group).

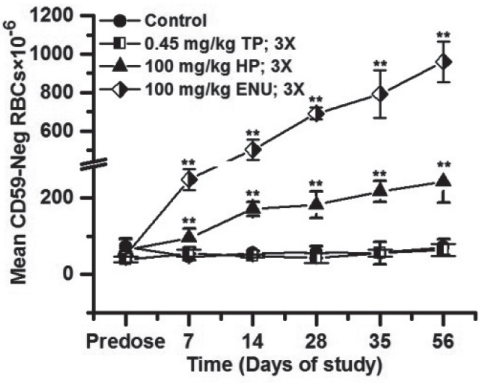

Fig. 7. The RBC^{CD59-} for PH and TP treatment at different periods of time ($\times 10^{-6}$), T-test, $P < 0.01$, (n = 4 animals per group).

ity. As shown in Fig. 7, compared with the vehicle control group, treatment with 100 mg/kg/day PH achieved a statistically significant increase on the proportion of mutant erythrocytes by 1.1-fold on day 7, by 2.1-fold on day 14, by 2.1-fold on day 28, by 2.9-fold on day 35 and by 2.4-fold on day 56. The RBC^{CD59-} frequency of the TP experimental group went smoothly during the test period, and no statistically significant difference was found compared with the vehicle control group. Treatment with 100 mg/kg/day of ENU showed a 4.4-fold increase of mutant erythrocytes on day 7, an 8.1-fold increase on day 14, a 10.9-fold increase on day 28, a 13.2-fold increase on day 35 and a 12.5-fold increase on day 56. These increases were statistically significant compared with the vehicle control group.

DISCUSSION

Method establishment

As a commonly used mutagen, DMBA has been frequently used in establishing experimental animal models. It was reported to damage the Tp53 and Ki-Ras genes (Jean-Marc *et al.*, 2004). We compared the RBC^{CD59-} value in rats treated with ENU and DMBA, and no significant difference was observed between the ENU control group and the DMBA control group. Because the mean value of mutant erythrocytes in ENU-treated group was higher than that of the DMBA group, ENU would be a better selection as a positive compound.

Repeated dose in suitable level should be the critical factor to investigate the genotoxicity of compound. Three methods of administration in this study had achieved positive results that increased with the time. Treatment with a medium dose for three consecutive days increased the

fastest, with a more than one-fold increase over the other methods of administration on day 28. Importantly, however, cumulative growth of mutant erythrocytes had existed in all three treatment protocols.

The SD rats with an age of approximately 2-8 weeks are young adults. Thus, the most sensitive responses to compound could be initiated in this period. However, No significant difference of the Pig-a mutant frequency was detected between the five-week-age group and the nine-week-age group. At last, the spontaneous Pig-a mutant frequency of erythrocytes in rats was approximately 12-95 $\times 10^{-6}$ and was primarily concentrated in a range of 40-60 $\times 10^{-6}$. Dobrovolsky *et al.* (2010) reported that the present spontaneous Pig-a mutant frequency of erythrocytes in SD rats was less than 5×10^{-6} via high throughput *in vivo* Pig-a gene mutation assay. Admittedly, the simple *in vivo* Pig-a gene mutation assay could not detect a low positive mutagen due to its low sensitivity.

Then, a stable detection method was established and optimized, including several conditions. First, five-week-old SD rats should be considered to increase the detection rate of RBC^{CD59-}. For positive compounds, ENU should be the optimal choice. The method of administration should be better executed at a 100 mg/kg/day of ENU for three consecutive days.

Method optimization

It was very important that the experiment was performed in its optimal conditions. Therefore, this methodology was optimized on several aspects. High stability of stained cells was validated after 3.5 hr, and no statistical significance was found between the staining timeframes of 0.5 hr to 3.5 hr. A time-dependent increase in RBC^{CD59-} value was found when the anticoagulation blood samples were kept at four degrees; therefore, the best solution is to process the anticoagulation blood sample within 1 hr. The optimal cell number for flow cytometry detection was 1.0×10^6 cells, with a theoretically allowable margin of error.

Method application

cis-Dichlorodiammineplatinum (DP) exhibited a cumulative toxicity. Treated animals were found dead during the trial, however, no obvious Pig-a mutant erythrocytes were detected during this study. Under the microscope, the erythrocytes in the DP-treated group exhibited an irregular shape with a slightly serrated border in a blood smear. A strange shape may have an influence on flow cytometric analysis. Despite all this, Stephen D *et al.* (Dertinger *et al.*, 2014) found out the genotoxicity of DP using a 0.1 mg/kg/day treatment for consecutive 28 days. Therefore, a strong detection capability was rested with a perfect treatment.

As an antineoplastic agent, many metabolites of PH were severely toxic *in vivo*. Moreover, its metabolites had a similar mechanism of action as alkylating agents. After administration of PH via oral gavage, the erythrocytes were found with Pig-a mutations in SD rats. A significantly different RBC Pig-a MF was detected compared with the vehicle control group at all five time points. Moreover, the RBC^{CD59-} value had a tendency to increase smoothly in a time-dependent manner over four weeks. The results clearly revealed that PH exhibited genotoxicity, leading to Pig-a mutation. No *in vivo* Pig-a mutant erythrocytes was detected for the Triptolide (TP) in the range of experimental doses.

In summary, the *in vivo* Pig-a mutation assay was conducted via flow cytometric analysis of erythrocytes in male SD rats; the blood samples were stained with two types of fluorescent markers. Erythrocytes with Pig-a gene mutations were investigated because GPI-anchored proteins are expressed on blood cells in all mammals. The antigen CD45 is expressed on leukocytes; therefore, leukocytes were excluded from the erythrocytes by marking the pivotal antigen CD45-PE. Then, the Pig-a mutation erythrocytes were evaluated by identifying the erythrocytes without antigen CD59-FITC. ENU was used as a recognized positive compound in the *in vivo* Pig-a gene mutation assay. This method was one of the most *in vivo* Pig-a gene mutation assays; the protocol is very easy to understand and distribute. Obvious *in vivo* genotoxicity could be detected in this short-term test. As an example, PH, DMBA, and ENU were detected to have apparent genotoxicity. Compared with the high-throughput *in vivo* Pig-a gene mutation assay, this simple assay has immeasurable value of its application in multiple fields.

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