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Research article

Acute toxicity and genotoxicity studies on new melatonergic antidepressant GW117

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ABSTRACT

GW117, a novel derivate compound of agomelatine that acts as both a 5-HT2C receptor antagonist and a MT1/MT2 receptor agonist, likely underlines the potent antidepressant action with less hepatotoxicity than agomelatine. We evaluated the acute toxicity of GW117, and the genotoxicity of GW117 using bacterial reverse mutation test, mammalian chromosomal aberration test in Chinese hamster lung cells (CHL) and mouse bone marrow micronucleus test. The acute toxicity test results showed that maximum tolerated dose (MTD) of GW117 was 2000 mg/kg, under which mean C_{max} and $AUC_{0\rightarrow t}$ was 10,782 ng/mL and 81,046 ng/mL × h, respectively. The result of bacterial reverse mutation test showed that the number of bacterial colonies in each dose group of GW117 did not increase significantly compared with that in the solvent control group with or without S9 metabolic activation system. *In vitro* chromosome aberration test of CHL cells, the chromosome aberration rate of each dose group of GW117 did not increase with or without S9 metabolic activation system. In mouse micronucleus test, the highest dose was 2000 mg/kg, the micronucleus rate did not increase significantly. Under the conditions of this study, the MTD of a single GW117 administration was 2000 mg/kg, there was no genotoxicity effect of GW117.

1. Introduction

Depression is a common mood disorder syndrome, and biological, psychological and social environmental factors are involved in the pathogenesis of depression [1–5]. Most patients are accompanied by low mood, insomnia, sadness, hopelessness, and an inability to enjoy life, furthermore, severe patients can develop suicidal tendencies. Depression has become a worldwide problem [6–9]. Although psychotherapy can be used for patients with mild depressive disorder, drugs are still an important treatment for moderate and severe patients. At present in clinical practice, the commonly used antidepressant drugs include selective serotonin reuptake inhibitors (SSRIs), noradrenaline reuptake inhibitors (NARIs), serotonin and noradrenaline reuptake inhibitors (SNRIs), and tricyclic antidepressants (TCAs), etc. [10–13], however, these drugs have adverse reactions, such as the risk of weight gain, hepatotoxicity and sexual dysfunction, so the patients have to stop the medication if necessary [14–17]. Therefore, it is imperative to develop new and effective antidepressants in order to achieve good clinical results. The melatonin analog agomelatine, both the first melatonin (MT1

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and MT2) receptor agonist and 5-HT2C receptor antagonist, it is mainly used in the treatment of adult depression [18,19]. It was approved by the European Medicines Agency in 2009 and is the only antidepressant targeting melatonin to date [20]. However, the repeat dose toxicity studies of agomelatine in rats and monkeys both showed that liver was the target organ of toxicity [21], and its clinical application was also limited due to its hepatotoxic risk [22–24].

GW117 is the structural optimization of agomelatine with the same targets screened according to receptor binding test. It is a novel compound that acts as both a 5-HT2C receptor antagonist and a MT1/MT2 receptor agonist, which showed a potent antidepressant effect in multiple animal models of depression [25]. According to the behavioral test, GW117 reduced the rest time in the forced swimming test in rats and tail suspension test in mice, and it had a better antidepressant effect than agomelatine. Qiushi Yang et al. [26] conducted a 28-day repeated dose toxicity study of GW117 and agomelatine via oral gavage in Sprague Dawley rats, the results showed that agomelatine can increase alanine aminotransferase (ALT), total bilirubin (TBIL), alkaline phosphatase (ALP), while GW117 only slightly elevated ALP. Pathology examination of liver revealed agomelatine caused mild to moderate hepatocyte and hepatobiliary injury, while GW117 only caused a mild hepatobiliary injury, indicating that the degree of damage caused by GW117 was less severe than that caused by agomelatine. In addition, we conducted 28-day repeated toxicity study with GW117 in beagle dogs, the results also showed a higher safety of GW117 [27]. Therefore, the development of GW117 has great social and economic significance. Genetic toxicity research is an important part of the non-clinical safety evaluation of drugs, which is closely related to carcinogenicity and reproductive toxicity, and is an important link for drugs to enter clinical trials and marketing. Previous study showed in a standard battery of *in vitro* and *in vivo* genotoxicity studies, no mutagenic or clastogenic potential of agomelatine was found, and single dose toxicity studies showed a rather low acute toxicity profile (LD50 $\geq$ 100 times the human dose) [21]. In this study, according to ICH M3 (R2), ICH and NMPA guidelines [28–31], three standard test combinations including Ames test, *in vitro* mammalian cell chromosome aberration test and *in vivo* micronucleus test were selected to evaluate the genotoxicity of GW117, and its oral acute toxicity was also evaluated, in order to provide reference for safe clinical medication. All studies were conducted in compliance with the FDA and NMPA Good Laboratory Practice for Nonclinical Laboratory Studies [32,33].

2. Materials and methods

2.1. Test article

GW117 were supplied by Beijing GreatWay Pharmaceutical Technology Co. Ltd.

2.2. Chemicals and reagents

Sodium azide was purchased from Shandong West Asia Chemical Co. Ltd, 4-nitroquinoline-*N*-oxide, 2-aminofluorene, 1,8-dihydroxyanthraquinone and cyclophosphamide were purchased from Sigma Aldrich, Mitomycin C was purchased from Roche, Hepatic microsomal enzymes (S9) induced by Aroclor 1254 in male SD rats was purchased from Molecular Toxicology, Inc.

2.3. Animals

Protocol for the acute oral toxicity and the micronucleus test was approved (IACUC-2018-104, IACUC-2018-132) by the Institutional Animal Care and Use Committee in accordance with Guide for the Care and Use of Laboratory Animals, 8th edition and Good Practice Guide [34,35]. 6-Week-old Sprague Dawley (SD) rats (36 females and 36 males, 206 g ~ 242 g) and 6-week-old Kunming (KM) mice (30 females and 30 males, 26.08 g ~ 33.15 g) were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. During the entire period of the experiments, the animals were raised to the ambient temperature of 20 °C–26 °C [34], relative humidity of 40%–70%, the light and dark alternation time was 12 h/12 h. Diet and water were supplied *ad libitum* to animals (except for special study requirements). They were acclimatized for 5 days before administration [36].

2.4. Acute oral toxicity test in rats

Four groups were designed in this study, including vehicle control group and GW117 200, 600 and 2000 mg/kg groups, with 9 rats/sex/group (among which 4/sex were used for toxicokinetics sampling). Before dosing, animals were fasted overnight with free access to water, food was given at approximately 2 h post-dose. Animals were orally administered with a single dose of vehicle [0.5% (w/v) carboxymethylcellulose sodium aqueous solution (CMC-Na)] or GW117 using a dose volume of 20 mL/kg followed by 14-day observation. Animals were under close observation for 4 h after dosing, cage-side observation once daily during the recovery phase, and detailed clinical observation on Day 2 and Day 9. Body weight was recorded on Day 1 (pre-dose), Day 3, 7, 11, 14 and 15 [40]. Food consumption was measured on Day 2, 6, 10 and 13. Blood samples were collected from rats in toxicokinetics satellite groups at pre-dose (0 h) and approximately 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h post-dose for toxicokinetics analysis. All animals were euthanized for gross observation at the end of recovery phase.

2.5. Bacterial reverse mutation test (Ames test)

According to the guidelines [29,31], "For bacterial cultures, precipitating doses are scored provided precipitate does not interfere with scoring, toxicity is not limiting, and the top concentration does not exceed 5 mg/plate. If dose related cytotoxicity or mutagenicity

is noted, irrespective of solubility, the top dose scored should be based on cytotoxicity, but without exceeding a top dose of 5 mg/plate".

In the presence and absence of S9, the dosages of test article GW117 was designed at 5 mg/plate, 2.5 mg/plate, 1.25 mg/plate, 0.625 mg/plate, 0.3125 mg/plate and 0.15625 mg/plate, respectively. In addition, groups of negative control, vehicle control (DMSO) and positive control was included. The concentration of S9 in S9 mixture was 10% (V/V). As for the positive control group, in the absence of S9 mix, the dosage of 4-nitroquinoline *N*-oxide was 0.5 μg/plate for TA97a and TA98; the dosage of sodium azide was 1.5 μg/plate for TA100 and TA1535; the dosage of mitomycin C was 0.5 μg/plate for TA102. In the presence of S9 mix, the dosage of 2-Aminofluorene was 10 μg/plate for TA97a, TA98 and TA100; the dosage of 1,8-Dihydroxyanthraquinone was 50 μg/plate for TA102; the dosage of Cyclophosphamide was 200 μg/plate for TA1535.

The plate incorporation method was adopted in the study. Added 0.1 mL of fresh bacterial culture, 0.1 mL of test article or 0.1 mL DMSO (0.5 mL of S9 mix was added when the activation system was required) to the 2.0 mL minimal agar (flask). Added corresponding bacterial culture to the negative control group. After well mixing, it was quickly poured onto the minimal agar plate. Rotated the plate to distribute the overlay agar uniformly on the minimal agar. Placed the plates flat and solidify the medium. Inverted the plates and incubated at 37 °C for 48 h before counting [37,38].

2.6. *In vitro chromosomal aberration test*

According to the guidelines [29,31], "For *in vitro* cytogenetic assays for metaphase chromosome aberrations, cytotoxicity should not exceed a reduction of about 50% in cell growth." The highest dosage will be 0.5 mg/mL when the test article produces no cytotoxicity. If the cytotoxicity is induced, the highest dosage will be determined based on relative population doubling (RPD) [39,40]. A preliminary cytotoxicity test was performed to determine the toxicity of GW117 by calculating the RPD in GW117-treated cultures. The RPD value was calculated using the following formula.

$$\text{RPD}\ (\%) = (\text{Population doubling of treatment group/Population doubling of control group}) \times 100$$

$$\text{Population doubling (PD)} = [\log(\text{Processed number of cells/Initial number of cells})] \div \log 2$$

According to the guidelines, a limit of about 50% cell growth is considered sufficient to be the maximum dose level. Therefore, in the 6 h-exposure groups, the dosages of GW117 were designed at 32 μg/mL, 16 μg/mL, 8 μg/mL, 4 μg/mL and 2 μg/mL in the absence and presence of S9. In the 24 h-exposure groups, the dosages of GW117 were designed at 28 μg/mL, 14 μg/mL, 7 μg/mL, 3.5 μg/mL and 1.75 μg/mL in the absence of S9. In the 48 h-exposure groups, the dosages of GW117 were designed at 32 μg/mL, 16 μg/mL, 8 μg/mL, 4 μg/mL and 2 μg/mL in the absence of S9. In addition, the groups of vehicle control (DMSO, no more than 0.5% of the total volume), negative control (RPMI 1640 medium with 10% fetal bovine serum) and positive control were designed in the presence and absence of S9.

The day before the experiment, a certain number of CHL cells were inoculated into culture flask (9.25×10^5 cells were inoculated, with the density of 1.85×10^5 cells/mL, 5 mL/flask), then placed in a 5% CO_2 incubator at 37 °C.

After the 6 h-exposure, the medium in 6 h groups were refreshed, washed twice with incomplete culture medium, then added with 10 mL of new medium and culture till 24 h. Groups for 24 h and 48 h were not processed. Colchicine (at the final concentration 0.4 μg/mL) was added to each group at 3–4 h prior to the harvest. When harvesting cells, the cell medium was decanted and washed with incomplete culture medium twice. In the process of cell dissociation using trypsin, the complete medium was added to terminate the trypsinization and the cells were counted when cells fell off from the flask wall. The cell suspensions were transferred into centrifuge tubes, mixed completely and centrifuged at 1000 r/min for 5 min to remove the supernatant.

A volume of 7 mL potassium chloride solution (0.075 M) was added into the centrifuge tubes. The cells were gently mixed by pipetting up and down to disperse cells and achieve the suspension. The centrifuge tubes were placed in the water bath at 37 °C and taken out after 10 min. Then, 2 mL of stationary liquid (methanol: glacial acetic acid = 3:1) [40,41] was added to the tubes and the tubes were centrifuged at 1000 r/min for 10 min to remove the supernatant. And then, a volume of 7 mL stationary liquid was added and mixed completely. After approximately 20 min, the tubes were centrifuged at 1000 r/min for 10 min to remove the supernatant. The above procedures were repeated once to fix the samples and remove the supernatant. Several drops of stationary liquid were added (which was adjusted based on the cell amount), mixed completely to obtain the cell suspension. A number of slides were prepared and were put into the jar containing Giemsa application solution for 15 min. Then they were taken out, washed with water and dried in the air.

At least 300 well-dispersed metaphase cells were observed for each concentration under the microscope with oil immersion lens. The number of cells with chromosome structural aberrations and type of chromosome structural aberrations were recorded. Chromatid-type and chromosome-type aberrations as well as their subtypes (break, chromatid exchange, etc) were separately recorded. Gap was documented separately but excluded from calculating the aberration rate. Numerical aberrations of polyploid and endoreduplication were recorded.

2.7. *In vivo mouse micronucleus test*

Totally 60 KM mice confirmed to be qualified in the acclimation phase were randomly assigned to groups based on their body weight, including vehicle control (0.5% CMC-Na), GW117 250 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 mg/kg and positive control (40 mg/kg Cyclophosphamide) groups. There were 5 animals per sex in each group. Animals in vehicle control and test article groups were

administered once daily for three consecutive days via oral gavage, while animals in positive control group were only administered on Day 3 via intraperitoneal injection [42]. The sampling was performed within 18–24 h after the last dosing. Mice were anesthetized with 1% pentobarbital sodium (approximately 90 mg/kg), euthanized and sampled for sternum to prepare the slides. Place the slides in methanol solution for 10 min. The fixed slides were put into the jar containing Giemsa application solution for 15min. Then they were taken out, washed with tap water immediately to remove the staining solution on the slide in a gentle manner. Then slides were washed with purified water, dried in the air and covered.

Count at least 500 bone marrow polychromatic erythrocytes for each animal to calculate the proportion of immature erythrocytes (polychromatic erythrocytes, PCE) among total erythrocytes (immature erythrocytes and normochromatic erythrocyte, NCE). Meanwhile, count at least 4000 immature erythrocytes per animal and record the incidence of micronucleated immature erythrocytes, results should be presented as the micronucleated polychromatic erythrocytes (MNPCE).

2.8. Statistical analysis

Statistical analysis of quantitative data was performed by SPSS software. Quantitative parameters (body weight, the number of revertant colonies, PCE/(PCE + NCE) ratio and MNPCE) were statistically analyzed using one-way analysis of variance (ANOVA). When the difference was statistically significant ($P < 0.05$), Dunnett test method was used. In the case of heterogeneity of variances by the Levene's test ($P < 0.05$), Kruskal-Wallis (K–W) H test was used for statistical analysis. Mann-Whitney ($M - W$) U test was used for pairwise comparison if K–W H test showed statistically significant differences ($P < 0.05$). The chromosome aberration rate was analyzed using Fisher's exact test (EXACT). $P > 0.05$ indicates no statistically significant difference, and $P < 0.05$ indicates a statistically significant difference.

3. Results

3.1. Acute oral toxicity test in rats

When SD rats were orally administered with a single dose of GW117 at 200, 600 and 2000 mg/kg, no animal death occurred, and no test article-related abnormalities were noted in clinical signs, such as appearance and signs, general behavioral activities, mental state, gland secretion, respiratory state and fecal characteristics. No test article-related abnormalities were noted in body weight (Fig. 1), food consumption or gross observation of animals in each group. The maximum tolerated dose (MTD) of GW117 for a single dose in SD rats was 2000 mg/kg, under which mean maximum concentration (C_{max}) and area under the plasma concentration-time curve ($AUC_{0\rightarrow t}$) was 10,782 ng/mL and 81,046 ng/mL × h (Table 1), respectively.

3.2. Bacterial reverse mutation test (Ames test)

The result of the revertant colony count is presented in Table 2.

In the presence and absence of S9 mix, milky white overlay agar was observed in 1.25 mg/plate, 2.5 mg/plate and 5 mg/plate after treatment, while precipitate was observed in 0.625 mg/plate, 1.25 mg/plate, 2.5 mg/plate and 5 mg/plate. No obvious abnormalities were noted in the vehicle control group.

In the presence and absence of S9 mix, no significant differences were noted in the number of revertant colonies of vehicle control group when compared with the negative control group, while the number of revertant colonies in tester strains induced by the positive control was 2-fold higher than that in the vehicle control, with significant differences ($P < 0.05$). These suggested the test system was reliable. In the absence of S9 mix, decreased number of revertant colonies was noted in TA97a 0.625 mg/plate and 1.25 mg/plate when

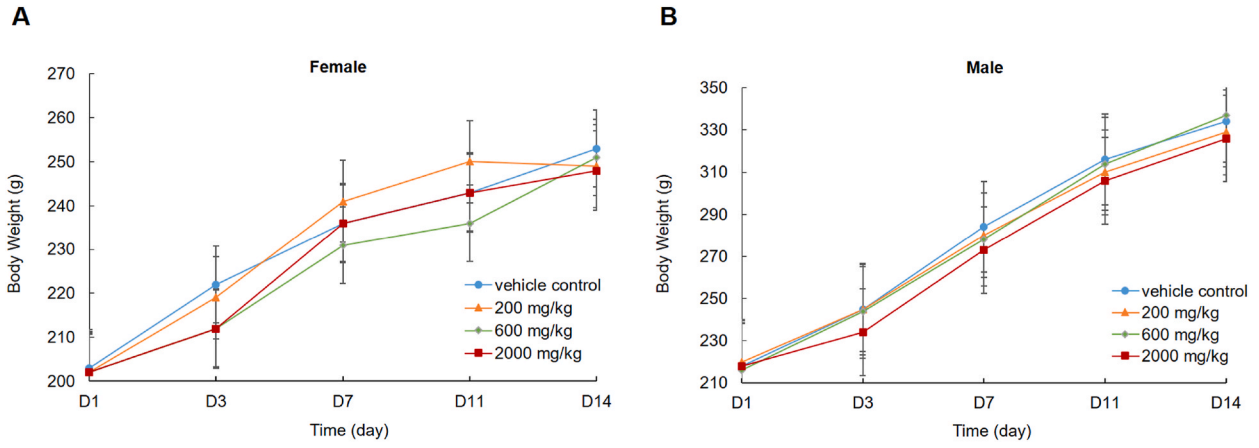

Fig. 1. Effect of GW117 on body weight of female (A) and male (B) rats in acute oral toxicity test. (n = 5 rats/sex/group).

Table 1
Toxicokinetics Parameters in rats orally administered with GW117.

Group	Sex	Parameter		
		T_{max} (h)	C_{max} (ng/ml)	$AUC_{0\rightarrow t}$ (ng/mL × h)
200 mg/kg dose group	F	1.8 ± 0.5	5059.7 ± 1642.7	30009.9 ± 8114.1
	M	1.8 ± 0.5	5271.9 ± 2050.7	24965.6 ± 12211.2
600 mg/kg dose group	F	4.5 ± 2.5	11068.0 ± 2377.4	82693.9 ± 10974.3
	M	3.8 ± 3.1	5527.5 ± 2391.8	43419.7 ± 17227.6
2000 mg/kg dose group	F	4.5 ± 2.5	12682.2 ± 975.4	101674.9 ± 24754.8
	M	3.3 ± 3.2	8881.9 ± 2331.7	60417.4 ± 1646.0

Note: Data are presented as the mean ± SD (n = 4). T_{max}, time of the maximum concentration; C_{max}, maximum concentration; $AUC_{0\text{-}t}$, area under the plasma concentration-time curve.

Table 2
Effect of GW117 on revertant colonies.

Group	Revertant colonies									
	TA97a		TA98		TA100		TA102		TA1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Negative control	130.0 ± 19.2	149.3 ± 18.5	22.0 ± 1.0	23.7 ± 1.2	119.0 ± 13.1	143.3 ± 6.4	238.3 ± 19.6	263.0 ± 14.5	9.7 ± 2.1	17.0 ± 2.6
Vehicle control	125.0 ± 13.5	145.3 ± 11.7	22.0 ± 2.6	22.3 ± 3.2	104.3 ± 9.1	125.3 ± 9.5	243.3 ± 21.2	271.3 ± 18.2	9.3 ± 1.5	14.0 ± 2.6
0.15625 mg/plate	139.0 ± 11.8	139.3 ± 21.6	23.7 ± 2.1	27.0 ± 3.0	110.7 ± 14.2	135.0 ± 10.1	238.7 ± 14.4	266.3 ± 22.1	11.7 ± 2.1	13.3 ± 2.1
0.3125 mg/plate	126.7 ± 20.1	140.3 ± 21.4	22.3 ± 2.1	23.3 ± 3.5	114.3 ± 16.4	134.7 ± 12.1	236.7 ± 13.2	263.7 ± 20.6	11.7 ± 2.1	15.7 ± 4.2
0.625 mg/plate	92.7 ± 10.2*	108.0 ± 14.4	22.3 ± 1.5	21.3 ± 1.5	94.0 ± 6.1	124.3 ± 8.1	230.3 ± 8.4	235.7 ± 18.8	8.3 ± 2.1	12.3 ± 1.2
1.25 mg/plate	93.7 ± 9.0*	108.3 ± 13.0	18.3 ± 2.1	19.7 ± 4.5	81.3 ± 3.1*	104.7 ± 11.2	217.7 ± 12.2	238.7 ± 15.0	9.0 ± 2.6	15.0 ± 4.4
2.5 mg/plate	99.7 ± 3.2	104.3 ± 15.6*	18.0 ± 4.6	18.7 ± 3.5	80.0 ± 4.6*	79.3 ± 4.0*	212.7 ± 13.3	241.3 ± 17.2	10.7 ± 1.5	9.3 ± 4.2
5 mg/plate	98.7 ± 6.7	105.3 ± 16.5	20.0 ± 2.0	19.0 ± 4.4	70.3 ± 3.1*	57.0 ± 9.8*	208.3 ± 16.3	234.3 ± 16.5	8.0 ± 3.6	9.7 ± 2.5
Positive control	396.7 ± 59.0*①	1360.0 ± 102.1*④	312.7 ± 27.6*①	1402.7 ± 146.7*④	1258.7 ± 108.0*②	1498.7 ± 160.3*④	1368.0 ± 110.9*③	810.7 ± 64.2*⑤	1277.3 ± 60.0*②	254.3 ± 27.5*⑥

Note: Data are presented as the mean number of revertant colonies ± SD (n = 3). *, $P < 0.05$ when compared with the vehicle control; ①4-nitroquinoline *N*-oxide, 0.5 μg/plate; ②Sodium azide, 1.5 μg/plate; ③Mitomycin C, 0.5 μg/plate; ④2-Aminofluorene, 10 μg/plate; ⑤1,8-Dihydroxyanthraquinone, 50 μg/plate; ⑥Cyclophosphamide, 200 μg/plate.

compared with the vehicle control ($P < 0.05$). Decreased number of revertant colonies was noted in TA100 at 1.25 mg/plate, 2.5 mg/plate and 5 mg/plate when compared with the vehicle control ($P < 0.05$). In the presence of S9 mix, decreased number of revertant colonies were noted in TA97a at 2.5 mg/plate when compared with the vehicle control ($P < 0.05$). Decreased number of revertant colonies was noted in TA100 at 2.5 mg/plate and 5 mg/plate when compared with the vehicle control ($P < 0.05$).

Results showed no mutagenic effects were induced by GW117 at 0.15625 mg/plate-5 mg/plate in tester strains TA97a, TA98, TA100, TA102 or TA1535. The result of this reverse mutation assay was concluded to be negative.

3.3. *In vitro chromosomal aberration test*

The result of the chromosome aberration assay is shown in Table 3.

Compared with the vehicle control, the chromosome aberration rate in negative control group had no statistically significant differences when CHL cells were exposed for 6 h, 24 h and 48 h in the absence and presence of S9 metabolic activation system, while statistical significance was noted in the chromosome aberration rate of positive control group ($P < 0.05$), suggesting the test system was reliable.

Results showed no significant increase in the chromosome aberration rate was noted in 2 μg/mL-32 μg/mL 6 h groups (±S9), 1.75 μg/mL-28 μg/mL 24 h groups and 2 μg/mL- 32 μg/mL 48 h groups when compared with the vehicle control, suggesting GW117 could not induce chromosome aberration of CHL cells. The result of this *in vitro* mammalian chromosome aberration test was concluded to be negative.

Table 3
Effect of GW117 on chromosome aberration in CHL cells.

Group	Dosage (μg/mL)	RPD (%)	Numerical Aberration		Structural Aberration							
			Polyploid	Aberration rate (%)	Gap	Chromatid break	Chromosome break	Deletion	Rings	Chromatid exchange	Minute chromosome	Aberration rate (%)
Without S9, 6 h												
Negative control	/	96.90	0	0	1	2	1	1	0	0	0	1.33
Vehicle control	/	100.00	1	0.33	2	1	0	0	1	0	0	0.67
GW117	2	102.49	0	0	3	1	1	2	0	1	2	2.33
	4	104.13	1	0.32	3	0	0	1	0	0	1	0.64
	8	95.28	2	0.66	0	0	0	1	1	0	2	1.32
	16	87.55	0	0	1	0	0	1	1	0	3	1.67
	32	74.42	1	0.33	1	0	0	0	1	0	2	1.00
Mitomycin C	0.15	76.07	0	0	4	16	3	3	1	35	4	19.80*
With S9, 6 h												
Negative control	/	103.38	1	0.33	2	0	0	0	0	0	1	0.33
Vehicle control	/	100.00	1	0.33	0	2	0	0	0	0	1	1.00
GW117	2	102.52	0	0	0	1	1	1	1	0	0	1.32
	4	99.22	0	0	1	3	0	1	0	0	0	1.33
	8	96.82	1	0.33	1	1	0	1	1	0	1	1.32
	16	86.20	1	0.33	1	2	1	1	0	0	0	1.33
	32	72.47	0	0	1	3	1	1	0	0	0	1.67
Cyclophosphamide	10	73.51	2	0.66	1	24	5	7	1	33	1	22.52*
Without S9, 24 h												
Negative control	/	96.56	1	0.33	2	2	0	1	0	0	0	1.00
Vehicle control	/	100.00	0	0	2	1	0	2	0	2	1	2.00
GW117	1.75	98.31	0	0	1	1	0	2	0	0	1	1.33
	3.5	96.78	1	0.33	1	1	0	0	0	1	1	1.00
	7	72.94	0	0	2	2	0	1	0	0	1	1.33
	14	63.17	1	0.33	2	3	0	0	0	0	1	1.32
	24	51.14	0	0	2	1	0	1	0	0	1	1.00
Mitomycin C	0.1	73.97	0	0	4	20	7	13	0	42	2	25.67*
Without S9, 48 h												
Negative control	/	101.04	0	0	2	2	0	1	0	1	0	1.33
Vehicle control	/	100.00	1	0.33	1	1	0	1	0	0	1	1.00
GW117	2	94.06	0	0	2	2	0	3	0	0	1	1.99
	4	91.13	1	0.33	0	2	0	2	0	0	0	1.33
	8	71.79	0	0	1	2	0	1	0	0	1	1.33
	16	62.10	0	0	2	3	0	2	0	0	0	1.63
	32	47.05	1	0.33	2	3	0	1	0	0	0	1.32
Mitomycin C	0.05	77.21	2	0.66	1	25	8	12	0	52	1	30.90*

Note:/, not applicable; *, $P < 0.05$ when compared with the vehicle control. RPD, relative population doubling.

3.4. In vivo mouse micronucleus test

The result of the weight, PCE/(NCE + PCE) and MNPCE and are presented in Tables 4 and 5.

No obvious abnormalities were noted in mice of vehicle control, test article or positive control groups after dose administration.

Compared with the vehicle control group, significantly higher PCE/(PCE + NCE) ratio was noted in male mice of positive control group ($P < 0.05$), besides, MNPCE of female and male mice was both higher than the vehicle control group ($P < 0.05$). These suggested a reliable test system.

Results showed that compared with the vehicle control group, significantly higher PCE/(PCE + NCE) ratio was noted in male mice of GW117 2000 mg/kg group ($P < 0.05$), while no significant differences were noted in the MNPCE. So it can be concluded that GW117 could not induce a higher incidence of micronuclei in bone marrow erythrocytes of mice over the dose range from 250 mg/kg ~ 2000 mg/kg. The result of this *in vivo* bone marrow micronucleus assay was concluded to be negative.

4. Discussion

Genotoxicity is an important part of non-clinical safety evaluation of drugs and plays an important role in drug development, which will affect the process of new drug development to a large extent. Current drug genotoxicity research technical guidelines recommended genotoxicity test combination should include: bacterial reverse mutation test, *in vitro* chromosomal damage test (*in vitro* metaphase chromosome aberration test or *in vitro* micronucleus test), or *in vitro* mouse lymphoma *TK* gene mutation test, and *in vivo* genotoxicity test (e.g., micronucleus test, bone marrow chromosomal aberration test) [29,31]. Therefore, in this study, the genotoxicity of GW117 was evaluated by the combination of Ames test, *in vitro* chromosomal aberration test in CHL cells and micronucleus test in mice.

In Ames test, different strain combinations were used, such as TA97a, TA98, TA100, TA102 and TA1535 combination [43–45], TA97, TA98, TA100, TA102 and TA1535 combination [46], TA98, TA100, TA1535, TA1537 and WP2 uvrA combination [47–49], TA98, TA100, TA102, TA1535 and TA1537 combination [50,51], TA97a, TA98, TA100, TA1535 and WP2 uvrA combination [52], etc. In this study, tester strains of *Salmonella typhimurium histidine auxotrophs* including TA97a, TA98, TA100, TA102 and TA1535 were used as the test system. Tests on histidine auxotrophy, lipopolysaccharide barrier defect, uvrB repair deficiency, R factor, Tetracycline resistance, spontaneous reverse mutation and reaction to positive mutagen were conducted on the tester strains, and only qualified ones could be used for the study. According to the evaluation requirements, a high dose of 5 mg/plate was used, at which GW117 could produce precipitation, but the precipitate does not interfere with the count. In the range of 0.625–5 mg/plate, though the number of revertant colonies of TA97a decreased in the presence and absence of metabolic activation system, the decrease was not dose-related and it was still within the range of laboratory background data, so it was considered to have no biological significance. In the range of 1.25–5 mg/plate (with metabolic activation system) and 2.5–5 mg/plate (without metabolic activation system), the number of revertant colonies of TA100 decreased, which was dose-dependent and beyond the range of laboratory background data, which was determined to be caused by the bacterial toxicity of GW117. The mean number of revertant colonies was not increased of GW117 treated for all test bacterial strains , the results demonstrated that GW117 could not induce gene mutation.

CHL cells, peripheral blood lymphocytes cells, etc. Can be used in chromosome aberration test [48,53]. The number of chromosomes in Chinese hamster lung cells is generally 25 [54], in addition, CHL is easy to establish cell lines *in vitro*, the test conditions are easy to control, and the spontaneous mutation rate is low, which is convenient for chromosome analysis. Therefore, in the present study, CHL cells were selected as the test system. Before treatment, mycoplasma inspection was conducted with fluorescent staining method to verify whether there was mycoplasma contamination, and the aberration rate <5% suggested the cells could be used for the study [55]. In this study, the chromosome aberration rate in the 6 h-, 24 h- and 48 h-treatment groups of GW117 were all less than 5%, and no significant differences in the chromosome aberration rate were noted. Therefore, G117 did not induce chromosome aberration of CHL cells.

In vivo micronucleus test in mice, Kunming mice were selected in this study, which has a lot of biological historical data and is also the commonly used animal species in micronucleus test [56,57]. According to the evaluation requirements, the recommended highest dosage is 2000 mg/kg when the dosing phase is ≤ 14 days, so, dosage of 2000 mg/kg were administered. No clinical abnormal symptoms were observed during the study, and no significant differences were noted in body weight and MNPCE when compared with

Table 4
Effects of GW117 on body weight of mice (g).

Group	The first dosing (Day 1/Day 3)		Sampling (Day 4)	
	F	M	F	M
Vehicle control	28.91 ± 2.26	30.94 ± 1.94	29.89 ± 2.28	33.79 ± 2.39
250 mg/kg	28.89 ± 1.43	31.12 ± 1.37	30.26 ± 1.59	33.73 ± 1.46
500 mg/kg	29.00 ± 1.50	31.17 ± 1.69	30.83 ± 1.41	33.32 ± 1.87
1000 mg/kg	28.71 ± 1.55	30.84 ± 1.70	29.71 ± 1.58	33.44 ± 1.82
2000 mg/kg	28.88 ± 2.13	30.86 ± 1.80	29.21 ± 1.13	31.56 ± 2.78
Positive control	29.53 ± 1.30	32.86 ± 1.44	29.75 ± 1.45	33.77 ± 1.30

Note: Data are presented as the mean weight ± SD (n = 5). Day 1 was the first dosing day for vehicle control and test article groups; Day 3 was the dosing day for positive control group.

Table 5
Results of the bone marrow micronucleus assay for GW117 in mice.

Group	PCE/(NCE + PCE)		MNPCE (%)	
	Female	Male	Female	Male
Vehicle control	0.61 ± 0.02	0.60 ± 0.01	0.13 ± 0.01	0.15 ± 0.02
250 mg/kg	0.62 ± 0.01	0.60 ± 0.03	0.15 ± 0.02	0.16 ± 0.04
500 mg/kg	0.62 ± 0.01	0.62 ± 0.04	0.15 ± 0.02	0.14 ± 0.02
1000 mg/kg	0.62 ± 0.01	0.61 ± 0.03	0.13 ± 0.01	0.12 ± 0.06
2000 mg/kg	0.62 ± 0.01	0.66 ± 0.02*	0.15 ± 0.02	0.14 ± 0.04
Positive control	0.61 ± 0.02	0.63 ± 0.03*	1.45 ± 0.14*	1.35 ± 0.10*

Note: Data are presented as the mean ± SD (n = 5). *, $P < 0.05$ when compared with the vehicle control group. PCE, polychromatic erythrocytes; NCE, normochromatic erythrocyte; MNPCE, micronucleated polychromatic erythrocytes.

the vehicle control. The PCE/(PCE + NCE) ratio of GW117 2000 mg/kg group in male mice was statistically significant, however, it was considered to be of no toxicological significance because the change was small, and it was within the range of laboratory background data. Above, these indicated that GW117 could not induce the cytotoxicity of bone marrows cells and could not induce a higher incidence of micronuclei.

In the three tests, corresponding solvents and positive controls were set up to verify the reliability of the system. For the evaluation of GW117, the maximum dose was used according to the guidelines, in order to evaluate its genotoxicity fully. The results showed that within the experimental conditions and dose range, the Ames test, chromosome aberration test in CHL cells and micronucleus test in mice were all negative, and there was no potential genotoxic effect of GW117.

In the acute toxicity test of rats, GW117 up to 2000 mg/kg was administered, and necropsied after 14-day recovery. No test article-related abnormalities were noted in clinical signs, body weight and food consumption. No abnormal changes related to GW117 were found in the color, morphology, size or texture of organs or tissues in gross necropsy. The proposed clinical starting dose of GW117 is 20 mg/day, and the MTD of a single GW117 administration was 2000 mg/kg, which is approximately 6000 times higher than the clinical dosage, indicating that GW117 is relatively safe.

Thus, it can be concluded that GW117 has no oral acute toxicity at up to 2000 mg/kg. In the Ames test and *in vivo* micronucleus test, GW117 could not result in gene mutations and a higher incidence of micronuclei when doses are at up to 5 mg/plate and 2000 mg/kg. In addition, GW117 could not induce chromosome aberration *in vitro* chromosomal aberration test. As mentioned earlier, GW117 has better antidepressant effect and lower toxicity than agomelatine, all the results indicate that GW117 has high tolerability and safety, and supporting further development as an antidepressant drug.

5. Conclusions

The results of Ames test, chromosomal aberration test in CHL cells and micronucleus test in mice were all negative, indicating no potential genotoxic effect of GW117. Acute toxicity test showed that the MTD of GW117 in rats was 2000 mg/kg, indicating that GW117 was safe in a single dose.

Author contribution statement

Haiji Sun; Daizhou Zhang: Contributed reagents, materials, analysis tools or data; Wrote the paper.

MEI GAO: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Hui Ma: Performed the experiments.

Tianbin Liu; Chong Cao; Zhiyong Zheng; Liansheng Tang: Contributed reagents, materials, analysis tools or data.

Wei Gu: Conceived and designed the experiments.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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Conflict of interest

The authors declare that there are no conflicts of interest.

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