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A Bioassay for the Cytotoxicity of Gemcitabine Using the Marine Ciliate *Euplotes vannus*

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Abstract This study investigated the cytotoxicity of gemcitabine using the marine ciliate *Euplotes vannus* as the test organism. The median lethal concentrations (LC_{50} values) were determined using acute toxicity tests within an exposure time of 30 min with 0, 6, 12, 24, and 48 mg mL^{-1} gemcitabine. The median inhibition effect (IC_{50} value) on the growth of the ciliate cells was examined using chronic toxicity tests within 5 days (120 h) after exposure for 30 min with 0, 0.7, 3.5, 7, and 14 mg mL^{-1} gemcitabine. The 30-min LC_{50} value was 10.66 mg mL^{-1}. The LC_{50} values decreased with increasing exposure times and well fitted to the toxicity curve equation $LC_{50} = 10.93 + 28.4e^{-0.19t}$ ($R^2 = 0.93$; $P < 0.05$, t = exposure time). The IC_{50} value for growth rates was 7.05 mg mL^{-1}, and the inhibition effect on growth rates well fitted to the model equation $r_{\%} = 0.8681e^{-0.0782C\text{gem}}$ ($r_{\%}$ means growth rate with inhibition by gemcitabine, C_{gem} means concentrations of gemcitabine, $R^2 = 0.99$ and $P < 0.05$). The LC_{50} values of a wide range of gemcitabine concentrations could therefore be predicted for any given exposure time. These results suggest that the clinical dose of gemcitabine (20 mg mL^{-1}) was higher than the 30-min LC_{50} value, which was almost the same as the 6-min LC_{50} value (19.88 mg mL^{-1}) for *E. vannus* cells. The results also demonstrate that *E. vannus* can be used as a robust test organism for bioassays of chemotherapeutic drugs during short exposure periods.

Key words bioassay; cytotoxicity; *Euplotes vannus*; gemcitabine; toxicology

1 Introduction

Gemcitabine is a chemotherapeutic drug that kills cells during their division process (Zhang *et al*., 2017). This drug is used to kill cancer cells that show rapid division rates. However, it also target several essential cells that can divide, *e.g*., the cells in the skin, scalp, and lining of stomach, testes, and bone marrow (Rachel, 2009). Therefore, gemcitabine has numerous adverse side effects, including suppression of bone marrow function; loss of white blood cells, platelets, and red blood cells; and harming the sperm (Siddall *et al*., 2017; Zhang *et al*., 2017). Nevertheless, although gemcitabine has been used clinically via intravenous delivery or direct perfusion, there are only a few reports regarding its cytotoxicity (Krown, 2011; Dubey *et al*., 2016; Birhanu *et al*., 2017; Dyawanapelly *et al*., 2017; Pishvaian and Brody, 2017).

Ciliated protozoa have been widely used as test organisms in bioassays of chemical drugs as they have several advantages such as ease of culture, short generation time, and simple developmental stages (Gray and Ventilla, 1973; Dive and Leclerc, 1975; Rogerson, 1983; Moreno-Garrido and Canavate, 2001; Xu *et al*., 2004). Furthermore, as they are separated from their environment only by a cell membrane, they respond rapidly to changes in their surroundings and are hence used as bioassay tools for assessing cytotoxicity (Dive and Leclerc, 1975; Bearden *et al*., 1999; Girling *et al*., 2000; Herllung-Larsen *et al*., 2000; Seward *et al*., 2001; Fuma *et al*., 2003; Xu *et al*., 2004).

In this study, the model ciliated protozoan *Euplotes vannus* was used as a test cell to determine the lethal and threshold concentrations of gemcitabine. The aims of this study were to provide experimental evidence regarding the cytotoxicity of gemcitabine and to evaluate the potential of *E. vannus* as a model organism in bioassays of chemical drugs.

2 Materials and Methods

2.1 Cultivation of *E. vannus* and Preparation of Gemcitabine Solutions

The marine ciliate *E. vannus* was obtained from the Laboratory of Protozoology, Ocean University of China, Qingdao, China, and was identified based on a combination of morphological and molecular data (Chen and Song, 2002; Hong *et al*., 2017). Clonal and mass cultures of *E.*

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vannus were grown at 25℃ in sterilized artificial marine water comprising 28 g of NaCl, 0.8 g of KCl, 5 g of $MgCl_2{\cdot}6H_2O$, and 1.2 g of $CaCl_2$ per 1000 mL of distilled water (salinity 28).

Ammonia stock solutions (salinity 28, temperature 25℃) were prepared by adding gemcitabine to artificial marine water. Test solutions with different concentrations of gemcitabine were prepared by mixing the stock solution with artificial marine water in appropriate proportions.

2.2 Acute Toxicity Test

For the acute toxicity test, 1 mL of gemcitabine solution was added to each well of a 48-well cell culture plate. To each well, ten *E. vannus* cells were added. The acute toxicity test was carried out in the dark at 25℃. The 30-min experiments were designed as five treatments (including one control), with each treatment as three independent lines (replicates). The concentrations of gemcitabine for the five treatments were 0, 6, 12, 24, and 48 mg mL^{-1}, respectively. For each line, the number of dead cells, including those unable to swim or creep on the bottom of the well, was recorded every 6 min *in vivo* under a stereomicroscope.

The median lethal concentrations (LC_{50}) for 6, 12, 18, 24, and 30 min were calculated using the probit-regression routine of the program IBM SPSS Statistics version 22.0, International Business Corp, USA. The toxicity curve equation was determined using the program SigmaPlot 12.5, Systat Software Inc, USA.

2.3 Chronic Toxicity Test

The inhibition effect of gemcitabine on the growth of *E. vannus* was also investigated in 48-well culture plates at 25℃. To each well, 1 mL of test solution and ten *E. vannus* cells were added. The concentration of bacteria in each well was maintained at 10^8 cells mL^{-1} by adding *Vibrio* sp. The tests were performed using five different concentrations of gemcitabine, 0, 0.7, 3.5, 7, and 14 mg mL^{-1}, with three replicates of each concentration. The ciliate cells in each well were enumerated *in vivo* every 12 h under a stereomicroscope for up to 120 h.

The increase of the number of ciliate cells over the total experimental period was evaluated to ascertain whether it is fitted to the following logistic model:

$$N_t = N_{\max} / [1 + e^{(a-rt)}],$$

where N_t, the density of ciliate cells at time t; $N_{\max}$, the estimated maximum density; r, the growth rate; a, the coefficient constant of initial density; and T_{50}, the time to 50% $N_{\max}$.

All parameters were estimated using the program SigmaPlot. Fitness tests were conducted to determine whether the growth curve fits to the logistic model at the 0.05 significance level (Zhang *et al*., 2012).

The median inhibition concentration (IC_{50}) was computed using regression analysis of the relationship between the concentration of gemcitabine and the treatment/control ratios ($r_{\%}$) of growth rates using SigmaPlot 12.5 (Zhang *et al*., 2012).

3 Results

3.1 Acute Toxicity and Toxicity Curves

Based on the probit-regression analysis of log-dose and mortality data at exposure times of 6, 12, 16, 24, and 30 min, the LC_{50} values were calculated and are shown in Fig.1. The LC_{50} values decreased with increasing duration of exposure. The regression analysis demonstrated that the toxicity curve significantly fitted to the following equation:

$$LC_{50} = 10.93 + 28.4e^{-0.19t},$$

where $R^2 = 0.93$; $P < 0.05$, t = exposure time (Fig.1).

Using this model equation, it is possible to predict LC_{50} values of gemcitabine with different concentrations at a wide range of exposure times.

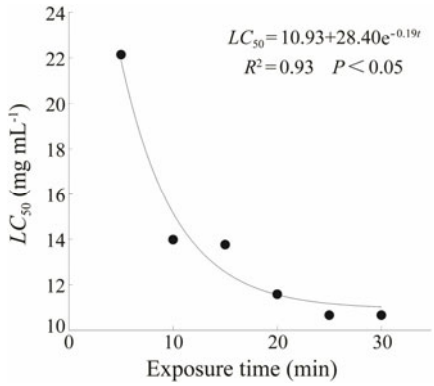

Fig.1 Toxicity curve of gemcitabine for *Euplotes vannus* up to 30 min of exposure.

3.2 Inhibition Effect of Gemcitabine on Cell Growth

The inhibition effect of gemcitabine on the population growth of *E. vannus* for up to 120 h is depicted in Fig.2. Regression analyses revealed that the growth curves of the four treatments (except 14 mg mL^{-1}) significantly fitted to the logistical model equation ($R^2 > 0.95$; $P < 0.05$). The data pertaining to the treatment with 14 mg mL^{-1} of gemcitabine were omitted due to the absence of growth.

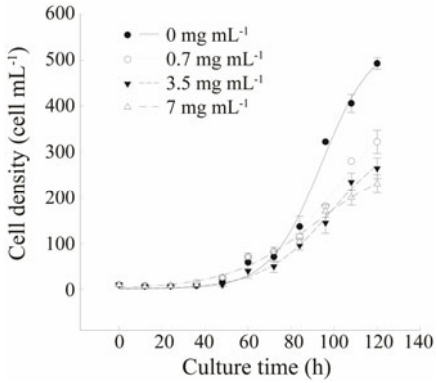

Fig.2 Growth curves showing the inhibition effect of gemcitabine on individual growth of *Euplotes vannus* after an exposure time of 30 min.

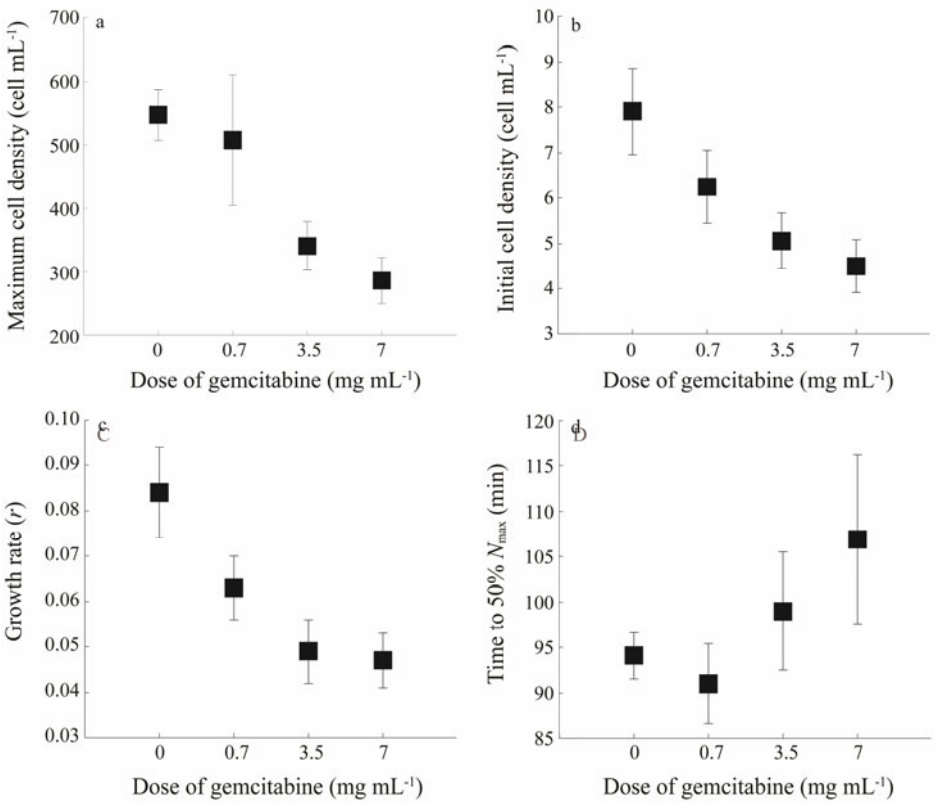

Fig.3 Effects of gemcitabine on the maximum cell density (a), initial cell density (b), growth rate (c), and the time to 50% maximum cell density (d) of *Eulpotes vannus*.

Based on the model equation, the estimated maximum density, the initial density, the growth rate, and the time to reach 50% of maximum density of the ciliate cells were obtained (Fig.3). The maximum density values of gemcitabine with concentrations of 3.5 and 7.0 mg mL^{-1} were significantly lower than those of the control ($P < 0.05$) (Fig.3a), whereas in terms of growth rate, the values at all gemcitabine treatments were significantly lower than those of the control ($P < 0.05$) (Figs.3b and c).

3.3 Median Inhibition Concentration on Growth Rate

The relationship between gemcitabine concentration and the treatment/control ratios ($r_{\%}$) of growth rates is depicted in Fig.4. The regression analysis showed that the dose-$r_{\%}$ significantly fitted to the following equation:

$$r_{\%} = 0.8681\mathrm{e}^{-0.0782C_{\mathrm{gem}}},$$

where $r_{\%}$ is the growth rate with inhibition of gemcitabine, and C_{gem} is the concentration of gemcitabine; $R^2 = 0.99$ and $P < 0.05$.

Based on this equation, the IC_{50} value was obtained, *i.e.*, when $r_{\%} = 50\%$, $C_{\mathrm{gem}} = 7.05$ mg mL^{-1}.

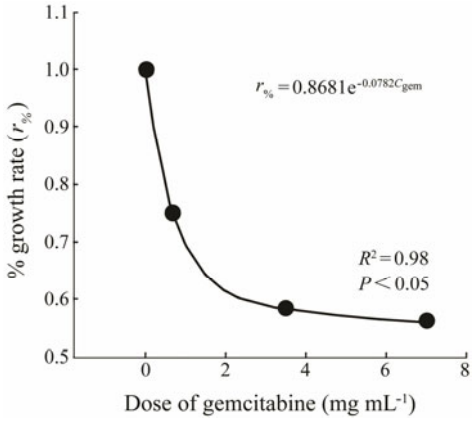

Fig.4 Relationships between dose of gemcitabine and growth rates of *Eulpotes vannus*.

4 Discussion

E. vannus is a well-known model ciliate that has traditionally been used as a bioassay organism in ecotoxicological studies (Coppellotti, 1998; Fernandez-Leborans, 2000; Xu *et al*., 2004; Zhou *et al*., 2011; Li *et al*., 2014; Hong *et al*., 2015). In the present study, *E. vannus* exhibited a measurable dose-response to gemcitabine and hence may be applied as a useful model organism in bioassays of cytotoxicity of this chemotherapeutic drug.

Owing to its hydrophilic properties, gemcitabine is transported into cells *via* molecular transporters for nucleosides and is pharmacologically active as gemcitabine triphosphate after the attachment of a phosphate ion to the gemcitabine molecule (Alvarellos *et al*., 2014). The thrice-phosphorylated gemcitabine molecule can masquerade as cytidine and thus be incorporated into DNA. This allows a native (or normal) nucleoside base to be added next to it and leads to 'masked chain termination' by creating an irreparable error that leads to inhibition of further DNA synthesis, thereby resulting in cell death (Alvarellos *et al*.,

2014). Furthermore, the twice-phosphorylated gemcitabine can inhibit the enzyme ribonucleotide reductase and thus can drive the cell to uptake more gemcitabine from outside the cell (Cerqueira *et al*., 2007; Alvarellos *et al*., 2014).

Gemcitabine is clinically used to treat bladder cancer by irrigation of bladder with a dose of 20 mg mL^{-1} for 30 min (Alvarellos *et al*., 2014). In the present study, the 30-min LC_{50} value was 10.66 mg mL^{-1} (temperature, 25℃), and the LC_{50} values decreased with increasing exposure time following the toxicity curve equation

$$LC_{50} = 10.93 + 28.4e^{-0.19t},$$

where $R^2 = 0.93$, $P < 0.05$, and t is exposure time. Therefore, from this equation, we predict that the clinical dose of gemcitabine (20 mg mL^{-1}) is higher than the 30-min LC_{50} value and almost the same as the 6-min LC_{50} value (19.88 mg mL^{-1}) for *E. vannus* cells. This implies that 20 mg mL^{-1} gemcitabine kills 50% of *E. vannus* cells within 6 min. Based on these results, the IC_{50} value for inhibiting the growth of *E. vannus* cells was 7.05 mg mL^{-1}. This finding suggests that the model ciliate is sensitive to gemcitabine.

It should be noted that to test the cytotoxicity of gemcitabine to the human bladder, mammalian (preferably human) bladder cell lines would be expected to better reflect the cytotoxic response. However, till date, there have been only a few reports on the successful cultivation of bladder cells *in vitro* (Burrows *et al*., 2017). In comparison, the ciliate *E. vannus* has several advantages in bioassays. For example, it can be maintained more easily and at less cost than human body cell lines. Furthermore, the preparation of test solutions for cytotoxicity tests is simpler as *E. vannus* lives in water rather than in a complex physiological solution (Dayeh *et al*., 2005). On the other hand, *E. vannus* is a free-living marine ciliated protozoan and exhibits a higher degree of complexity of cell structure and function than mammalian tissue cells (Zhang *et al*., 2015). Additional tests are therefore needed to determine whether *E. vannus* cells have the same sensitivity to gemcitabine as mammalian cells in general, and bladder cells in particular. Nevertheless, the present findings suggest that *E. vannus* is a useful test organism in bioassays of the cytotoxicity of gemcitabine.

5 Conclusions

The model ciliate species *E. vannus* exhibited a sensitive dose-dependent response to gemcitabine and can be used as a robust test organism in toxicology bioassays for short duration of exposure. The LC_{50} values decreased with increasing exposure time and well fitted to the toxicity curve equation. These results show that the clinical dose of gemcitabine (20 mg mL^{-1}) is higher than the 30-min LC_{50} value and is almost the same as the 6-min LC_{50} value for *E. vannus* cells.

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