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Activity of ginsenoside Rh_2 on the growth of mice splenic lymphocytes investigated by microcalorimetry and factor analysis

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Abstract The power–time curves of mice splenic lymphocytes growth at 37 °C affected by ginsenoside Rh_2 were determined by microcalorimetry using a 3114/3236 TAM air bioactivity monitor with ampoule mode. Then, the minimal inhibitory concentration (MIC) of Rh_2 on splenic lymphocytes growth was determined by serial dilution method. From factor analysis (FA) on six quantitative thermokinetic parameters from the power–time curves, the activity of Rh_2 on splenic lymphocytes could be quickly evaluated by analyzing the changes in the two main parameters: growth rate constant *k,* and maximum heat-output power, P_m. The results showed that Rh_2 had strong inhibitory activity on splenic lymphocytes growth, and this inhibitory activity was strengthened with increasing concentration of Rh_2 in the concentration range of 1.0–32.0 μg mL^{-1}. This strong inhibitory also could be confirmed from the MIC of 50.0 μg mL^{-1} of Rh_2 on splenic lymphocytes growth in RPMI-1640 culture medium. This study illustrated that microcalorimetry could not only offer a useful method for evaluating the activity of drugs, but also serve as a quantitative, sensitive, and simple analytic tool for the evaluation of drugs on cell growth.

Keywords Microcalorimetry · Ginsenoside Rh_2 · Splenic lymphocytes · Inhibitory activity · Factor analysis

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Introduction

Ginsenosides are considered to be the main bioactive constituents in the traditional phytomedicine "ginseng." Ginsenoside Rh_2, a trace component isolated from red ginseng (Radix Ginseng Rubra), which is the steamed root of Panax ginseng C.A. Meyer [1–3], belongs to the family of protopanaxadiols. It possesses an aglycone comprising a dammarane skeleton with a glucose unit conjugated at the C-3 position. Ginsenoside Rh_2 mostly exhibits anti-tumorous activity through induction of apoptosis by different means [4–6]. Based on its distinct pharmacological-based anticancer effective characteristic, Rh_2 has been commercially used as a health product in the North American and Asian markets, and it is also being developed as a new Category I drug in China. For the large-scale use of Rh_2 in most markets, its pharmacological actions as a drug should be sufficiently evaluated. It is known that the activity of Rh_2 on the growth of mice splenic lymphocytes has not been studied from then on.

Splenic lymphocytes are the important cells of immunologic system and play a significant role in a host of acute or chronic diseases. Within the cell growth, the various metabolic events are all heat-producing reactions. Using a sensitive calorimeter, the heat-producing and cell growth progress can be recorded. Further, to find the activity of other materials in the cell growth, microcalorimetry is a very useful tool for performing fundamental studies of the growth metabolism of a cell, which has been used extensively to investigate the state of interaction between drug and cell with a lot of useful information to be furnished [7–14]. Thus, by monitoring the heat effect with a sufficiently sensitive microcalorimeter, the metabolic processes of living cells can be studied in both qualitative and quantitative ways [15, 16]. By analyzing the

information, the activity and potency of drugs on cell growth can be studied.

In this article, a 3114/3236 TAM air bioactivity monitor was used to determine the power–time curves of mice splenic lymphocytes growth under the action of Rh_2. By analyzing these metabolic curves and some quantitative thermokinetic parameters through factor analysis (FA), the activity of Rh_2 on the cell growth was investigated. Meanwhile, the serial dilution method was used to determine the minimal inhibitory concentration (MIC) of Rh_2 on mice splenic lymphocytes growth, which was regarded as the check experiment. This study showed that Rh_2 had an inhibitory effect on mice splenic lymphocytes growth.

Materials and methods

Instrument, chemicals, reagents, and animals

A 3114/3236 TAM air bioactivity monitor (Thermometric AB, Sweden) was used to determine the metabolic power–time curves of splenic lymphocytes. It is an eight-channel heat conduction calorimeter used for heat flow measurements in the milliwatt range under isothermal and thermostat-controlled conditions in the range of 5–60 °C, with a limit of detectability of 2 μW. All calorimetric channels are of twin type, consisting of a sample and a reference vessel. Each vessel is connected to the surrounding heat sink by a Peltier module, and when heat is produced or consumed because of any process, the temperature of the sample vessel is to be changed. The baseline drift was less than 20 μW over 24 h.

Ginsenoside Rh_2 was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing). Its structure is given in Fig. 1, and its purity is more than 98%. RPMI-1640 culture medium and fetal serum were purchased from Gibico Company, USA. Water was purified using a Milli-Q water purification system (Milipore, Bedford, MA). All other chemicals are of analytic purity.

Male balb/c mice, Specific pathogen-Free (SPF) grade, weighing from 20 to 22 g were provided by the Animal Center of National Institute for the Control of Pharmaceutical and Biological Products (Certificate No: SCXK11-00-0010). All the animals were kept under the same laboratory conditions of temperature from 20 to 22 °C and were given access to standard laboratory chow and tap water. The procedures involving animals and their care conform to the Guiding Principles for the Care and Use of Laboratory Animals of China.

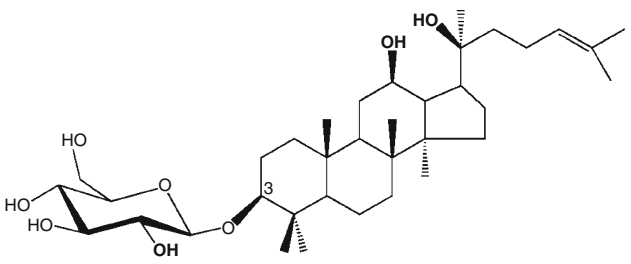

Fig. 1 Chemical structure of ginsenoside Rh_2

Obtainment of mice splenic lymphocytes

BALB/c mice, approximately 8 weeks old, were euthanized by cervical dislocation. Spleens were aseptically removed and were placed in cold Hanks solution and teased apart with a pair of forceps and a needle. A single cell suspension from the teased tissue was obtained by passing it through a 200-mesh by the buffer solution containing 1 mmol L^{-1} Tris–HCl and 1% NH_4Cl (PH 7.2). Cells were washed twice with RPMI-1640 culture medium and subsequently suspended in complete RPMI-1640 culture medium.

Microcalorimetric determination

At the beginning of the experiments, mice splenic lymphocytes (5×10^6 cells mL^{-1}) were prepared and transferred into each ampoule at the same volume. The freshly prepared Rh_2 solution (culture medium as solvent) of each concentration was added into the cell suspension. The microcalorimeter was thermostated at 37 °C, and the ampoule method was adopted in this study. Ampoules, filled with Rh_2 and cell suspension, were sealed with wax and put into the eight-channel calorimeter block. All procedures were completely sterilized. After about 30 min (the temperature of ampoules reached 37 °C), the thermogenic power–time curves of splenic lymphocytes were recorded until they returned to the baseline. All data were collected continuously by using the dedicated software package.

Measurement of MIC

The MIC was determined by serial dilution of Rh_2 solution (0–100 μg mL^{-1}) in test tubes using fresh RPMI-1640 culture medium. Each test tube was inoculated with cell suspension containing 10^5 cells mL^{-1} and incubated at 37 °C for 24 h. The lowest dilution that showed no visible cell growth compared to drug-free culture medium inoculated with cell suspension was considered as the MIC.

Results

Thermogenic curves of splenic lymphocytes growth at 37 °C affected by Rh_2

The thermogenic power–time curves of mice splenic lymphocytes growth in RPMI-1640 culture medium at 37 °C

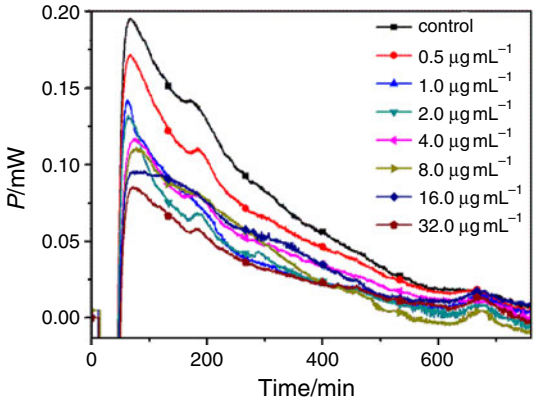

Fig. 2 Power–time curves of splenic lymphocytes growth at 37 °C affected by different concentrations of Rh_2. The concentrations are listed in this figure showing the metabolic profile of splenic lymphocytes growth at 37 °C affected by different concentrations of Rh_2 monitored by a 3114/3236 TAM air bioactivity monitor

affected by different concentrations of Rh_2 were shown in Fig. 2. They showed the total metabolism profile of splenic lymphocytes and could be divided into three phases: a balance phase; the quick growth phase; and a decline phase [17]. As the concentration of Rh_2 increased, the height of the highest peak decreased in a regular fashion, showing that the cell growth was inhibited. However, the shapes of growth metabolism curves were basically the same either in the presence or absence of Rh_2, i.e., the three phases still existed.

Thermo-kinetic data for splenic lymphocytes growth

In the quick growth phase of cell, the power–time curves obeyed the following equation:

$$\ln P_t = kt + \ln P_0 \quad (1)$$

where P_0 represents the heat-output power at the beginning, and P_t represents that at time t. According to Eq. 1, growth rate constant k (shown in Table 1) of splenic lymphocytes can be obtained by fitting $\ln P_t$ and t to a linear equation. The growth inhibition ratio R_i (%) is calculated on the basis of k and is defined as

$$R_i(\%) = (k_0 - k_c)/k_0 \times 100$$

where k_0 is the growth rate constant of splenic lymphocytes without ginsenoside (the control), k_c is the growth rate constant in the exponential phase of splenic lymphocytes affected by inhibitor at concentration c. When the R_i is 50%, the corresponding concentration of inhibitor is called half-inhibitory concentration, IC_{50}. Then, the generation time (t_G), being equal to (ln 2)/k, was also obtained and is shown in Table 1. Also, the maximum heat-output power. P_m, and the appearance time, t_m, of the highest peak were obtained. By integrating the peak areas under the curve, the total heat output Q_t in the whole growth progress

Table 1 Thermokinetic parameters for splenic lymphocytes growth affected by different concentrations of Rh_2

c/μg mL^{-1}	k/min^{-1}	R^a	t_G/min	R_i/%	P_m/mW	t_m/min	Q_t/J
0	0.15523	0.9787	4.46	0	0.1956	68.0	3.01
0.5	0.13818	0.9846	5.02	10.98	0.1718	68.0	2.49
1.0	0.12165	0.9678	5.70	21.63	0.1423	62.3	1.59
2.0	0.11161	0.9737	6.21	28.10	0.1324	64.3	1.44
4.0	0.09748	0.9681	7.11	37.20	0.1170	74.7	1.75
8.0	0.08888	0.9659	7.80	42.74	0.1113	77.7	1.48
16.0	0.07486	0.9269	8.47	51.75	0.0960	78.0	1.63
32.0	0.05673	0.9630	10.39	63.45	0.0857	71.7	1.24

[a] Correlation coefficient

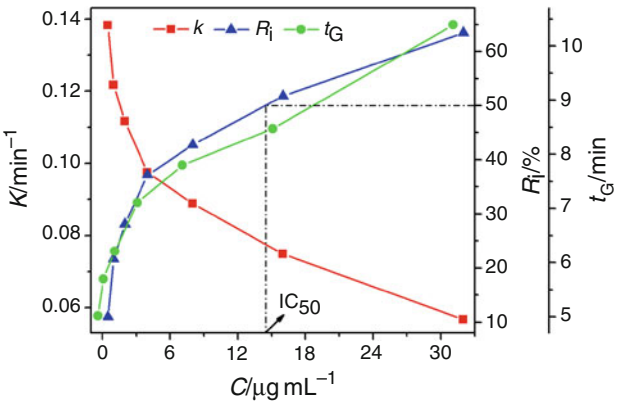

Fig. 3 Relationships among k, R_i, t_G, and c

of splenic lymphocytes affected by Rh_2 was then obtained and shown in Table 1.

Relationships among k, t_G, R_i, and concentration c

To evaluate the activity of Rh_2 on splenic lymphocytes growth swiftly, the relationships among k, t_G, R_i, and c were obtained as shown in Fig. 3 in the concentration range of 0.5–32.0 μg mL^{-1}. The k–c equation was $k = 0.11832–0.00219c$, $R = -0.8980$; the t_G–c equation was $t_G = 5.86874 + 0.15148c$, $R = 0.9486$; the R_i–c equation was $R_i = 23.77397 + 1.40838c$, $R = 0.8980$. From these three equations, it could be found that the linear relationship between t_G and c was the best with the largest R of 0.9486. Further, from the relationship of R_i and c, the 50% inhibitory concentration (IC_{50}) of Rh_2 was 14.92 μg mL^{-1}.

Relationships among P_m, t_m, Q_t, and concentration c

Then, the relationship between P_m and c was also obtained—shown in Fig. 4—in the concentration range of 0.5–32.0 μg mL^{-1}. The P_m–c equation was $P_m = 0.14121–0.00208c$, and $R = -0.8148$. However, the relationships among t_m Q_t, and c could not be linearly calculated.

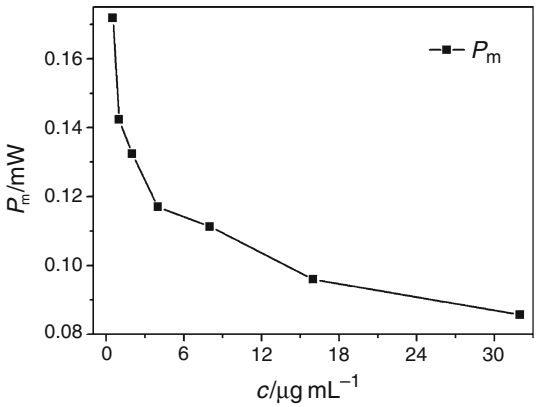

Fig. 4 Relationship between P_m and c

These complicated and irregular changes caused some difficulty when investigating the activity of Rh_2 on splenic lymphocytes growth. So, the FA method is introduced in the next section.

Factor analysis

Factor analysis (FA) [18] is a useful analytical method widely used for reducing the dimensions of multivariate problems. It reduces the dimensionality of the original data set by explaining the correlation among a large number of variables in terms of a smaller number of underlying factors (Fs), without losing much of the information. In this study, FA was performed to find out the values of the six quantitative parameters k, t_G, R_i, P_m, t_m, and Q_t (Table 1) and find out the main parameters using software of SPSS 18.0 (SPSS Inc., USA).

The results of FA showed that the first two Fs (F1 and F2) contained 96.61% of the information of the original four parameters and the equation of F1 and F2 were:

$$F1 = -0.513k - 0.198t_G - 0.203R_i - 0.402P_m - 0.133t_m + 0.154Q_t$$

$$F2 = -0.443k - 0.112t_G - 0.173R_i - 0.398P_m - 0.093t_m + 0.202Q_t$$

The absolute values of the coefficient before these parameters represented the contribution rate of the parameter to F1 and F2. It could be concluded from the two equations that the parameters k and P_m contributed relatively more to the two Fs than the rest of the six parameters, and they are the main parameters required to investigate the activity of Rh_2 on splenic lymphocytes growth. From the values of k and P_m in Table 1, we could quickly and accurately conclude that Rh_2 showed strong inhibitory activity on splenic lymphocytes, and this inhibitory activity was enhanced with increasing concentration of Rh_2.

MIC

The results of repeated serial dilution experiments showed that Rh_2 in RPMI-1640 culture medium had strong inhibitory activity on splenic lymphocytes with MIC of 50 μg mL^{-1}.

Discussion

Microcalorimetry, as a non-invasive physicochemical method, was successfully used to study the activity of ginsenoside Rh_2 on mice splenic lymphocytes. Considering the power–time curves of splenic lymphocytes growth affected by Rh_2 and the relationships among k, t_G, R_i, P_m, and c coupled with FA, one could find that Rh_2 had strong inhibitory activity on splenic lymphocytes growth, and this inhibitory activity was strengthened with the increase of the concentration of Rh_2 in the concentration range of 1.0–32.0 μg mL^{-1}. This strong inhibitory activity could also be confirmed from MIC of 50.0 μg mL^{-1} of Rh_2 on splenic lymphocytes growth in RPMI-1640 culture medium. These results were probably related with the chemical structure of Rh_2. The substitute groups on the dammarane skeleton influenced the hydrophilic character of the compounds required to interact with cell membrane function [19]. One glucose and multiple hydroxies on the chemical mother nucleus enhanced the hydrophilicity of Rh_2, which resulted in a higher affinity of this compound to the cell and its stronger inhibitory activity on splenic lymphocytes. This study provides some references for the reasonable and efficacious use of Rh_2 in clinic.

Furthermore, this study also showed that the microcalorimetric method could be useful as an analytic tool for the characterization of cell growth process and the estimation of the drugs' efficiency. The thermodynamic and kinetic information obtained in this study, which could not be obtained by the conventional evaluation techniques, proved to be significant while investigating the activity of drugs and other substances.

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