CHAPTER II

Materials and Methods

2.1 Chemicals and Reagents

The details of chemical and reagents are shown in Appendix

2.2 Green tea flavonoids

Green tea flavonoids were generously given by Dr.Hirota Fujiki. The dominant species of green tea flavonoids are (+)-catechin, (-)-Epicatechin, (-)-Epigallocatechin, (-)-Epicatechin gallate and (-)-Epigallocatechin gallate. The green tea flavonoids were dissolved in DMSO and divided into 50μl aliquots stored at -20 °C untill use.

2.3 Cell culture

KB carcinoma cell lines were provided by Dr. Michael Gottesman (National Cancer Institute, Besthesda, MD, USA), which are KB-3-1, the drug sensitive parental cell line and KB-V1, the multidrug resistance subline. Both KB-3-1 and KB-V1 were cultured in DMEM with 4.5 g of glucose/l, 5 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin and supplemented with 10% fetal bovine serum. 0.5 μg/ml of vinblastine was supplemented only in KB-V1 culture medium. The cell lines were grown as monolayer at 37°C under 5%CO_2 atmosphere.

2.4 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [92]

Principle

The MTT assay measures the conversion of the tetrazolium 3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to insoluble purple formazan in living cells. The reaction is catalyzed by mitochondrial succinate dehydrogenase and requires NADH which has to be supplied by the living cells, thus providing an indication of cell viability. The formazan crystals are then solubilized with solvents, such as dimethyl sulfoxide (DMSO). The solubilized formazan product is spectrophotometrically measured using an ELISA plate reader.

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Procedure

MTT assay was used to detect cytotoxicity of green tea flavonoids and their influence on cytotoxicity of vinblastine to cervical carcinoma cell lines. The cell lines, KB-3-1 and KB-V1 were placed in flat-bottomed 96 well-plate. The cell concentration was $2x10^3$ and $3x10^3$ cells/well respectively and then cultured overnight. Various concentrations of green tea flavonoid dissoved in 100 µl medium were added and incubated for 48 hours. Then 100µl of medium were removed and 20 µl of MTT dye (Sigma) were added, then incubated for another 4 hours. When the incubation was ended, the supernatant from each well was suctioned off, leaving the purple formazan crystals. A volume of 200 µl DMSO was added to each well to dissolve the formazan crystals and incubated for 10 min. The optical density was measured by ELISA reader at 540 nm with a reference wavelength of 630 nm. In order to detect green tea flavonoids influence on cytotocixity of vinblastine, fresh medium containing 50 or 100 µM of each green tea flavonoids and various concentrations of vinblastine were added. The subsequent procedure was the same as described above.

Percentage of cell survival was calculated by the following formula

$$\%\text{Cell survival} = \frac{\text{Absorbance of treated well}}{\text{Absorbance of control well}} \times 100$$

2.5 Accumulation and efflux of Rhodamine123

Rhodamine123 (Rh123) accumulation was performed as previously described [93]. Briefly, KB-3-1 or KB-V1 at 500,000 cells per tube were incubated with 1µg/ml of Rh123 and various concentrations of green tea flavonoids for 60 min at 37°C in dark. The final concentration of 0.4%DMSO (v/v) was used for all experiments and controls. Rh123 accumulation was stopped by washing twice with ice cold Hank's balance salt solution (HBSS) without phenol red, then placed in 10%FBS in HBSS and analyzed on FACScan flow cytometer (Becton-Dickinson) with a 488 nm argon laser. The green fluorescence of Rh123 was measured by a 530 band pass filter (hLi). Samples were gated on forward scatter and side scatter to exclude debris and clumps.

For determination of Rhodamine efflux, the cells were incubated with 1µg/ml of Rh123 and various concentrations of green tea flavonoids for 60 min at 37°C, then the cells were washed with ice cold HBSS without phenol red, then the medium was replaced with Rh123 free medium

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containing green tea flavonoids. After incubation at 37^{0}C for 30 min, the cells were washed twice and the fluorescence of Rh123 was measured by FACScan flow cytometer. As measured by trypan blue exclusion, the cells remained viable during the Rh123 accumulation and efflux studies.

2.6 Accumulation and efflux of ^{3}H-vinblastine

The effect of green tea flavonoids on drug transport was confirmed by monitoring the intracellular radiolabeled drug accumulation. The method was modified from Plouzek et al [94]. KB-3-1 or KB-V1 were seeded at 500,000 cells per well in 6-well plates and incubated overnight. The cells were treated with various concentrations of green tea flavonoids and 0.05 μCi (^{3}H)-vinblastine/ml (specific activity : 58.00 mCi/mmol) for 60 min at 37^{0}C. The cells were then harvested by centrifugation at 10,000 rpm at 4^{0}C for 3 min. Cells were dissolved in 200 μl of 3N NaOH and incubated for 30 min, then neutralized with 100 μl of 6N HCl. Cell lysate, 250 μl was pipetted into 3 ml of scintillation fluid. The radioactivity was counted by the β-counter. The protein concentration was determined by Bradford method using 10 μl of cell lysate in 96 well plate. The amount of intracellular radioactivity (counting unit) was calculated in the term of percentage of vehicle control.

For determination of drug efflux [95], cells were plated out as described for drug accumulation experiments. Cells were incubated for 60 min at 37^{0}C with (^{3}H)-vinblastine/ml and 10 μM cyclosporineA, the specific inhibitor of Pgp, in order to load the cells with radiolabeled drug. Then the cells were washed with PBS pH7.4 and the medium containing green tea flavonoids was added. After incubation at 37^{0}C for 15 min, cells were washed with ice cold PBS (pH7.4) and harvested. The amount of intracellular radioactivity was determined by scintillation counter.

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2.7 Plasma membrane preparation [96]

After removal of the medium, the monolayer of confluent cells was washed and detached with ice-cold phosphate buffer saline (PBS pH7.4). The cells were collected by centrifugation at 500 g for 5min and then washed twice with cold PBS. The cell pellets were resuspended in hypotonic buffer and kept on ice box for 30 min, then homogenized by using a glass homogenizer for 30 strokes and the cell homogenate was centrifuged at 4,000 g for 10 min. The supernatant was centrifuged at 100,000 g for 1 hr at 4^{0}C. The membrane pellet was resuspended in Leammli's buffer

[97] and divided into 50 μl aliquots, then stored at -20^{0}C. The membrane protein concentration was measured by the Folin-Lowry's method [98].

2.8 Protein determination

The membrane protein concentration was measured by the Folin-Lowry's method.The basic of the method is the reaction of protein with copper (II) ion under alkaline conditions and the Folin-Ceocalteau phosphomolybdicphosphate acid reduction to heteropolymolybdenum blue by the copper catalyzed oxidation of aromatic amino acids [98]. This method is sensitive to low concentration of protein. The suggested concentration was 0.005-0.10 mg/ml.

Procedure

Protein standard curve was constructed by preparing BSA in various concentrations from stock 1 mg/ml BSA as shown in Table 8. Twenty μl of protein samples were dilute with 490 μl of distilled water. Alkaline copper solution, Reagent C, 2.5 ml was added and mixed. After standing at room temperature for 10 min, 250 μl of Folin-phenol reagent was added, mixed gently and allowed to stand for 30 min at room temperature. The absorbance of 750 nm of standard BSA and tested sample were determinded by spectrophotometer.

Table 8. Preparation of bovine serum albumin standard solution

BSA concentration (μg/tube)	Stock BSA (μl)	Distilled water (μl)
0	0	500
25	25	475
50	50	450
75	75	425
100	100	400
125	125	375
150	150	350
175	175	325
200	200	300

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2.9 Western blot analysis and ECL detection

The cell membrane proteins (20 μg/lane) were separated on a 7.5%SDS-polyacrylamide gel electrophoresis (SDS-PAGE) which were formed by the copolymerization of acrylamide monomer with cross-linking agent, N,N'-methylene bisacrylamide (Bis). Gel polymerization is usually initiated by ammonium persulphate and N,N,N'N'-tetramethylethlenediamine (TEMED). A particularly important procedure is polyacrylamide gel in the presence of the anionic detergent, sodium dodecyl sulphate (SDS). The proteins bind 1.4g SDS per 1g protein, effectively masking the anionic charge of the polypeptide chains, so that net charge per unit mass becomes approximately constant. The subsequent electrophoretic separation depends only on the molecular size [99].

The electricity used in the SDS-PAGE was 100 volts for 2.5 h in electrode buffer. Then the separated proteins on the gel were transfered to the nitrocellulose membrane by using 30 volts of electricity overnight in blotting buffer. The membrane was incubated sequentially with 5% skim milk in PBS (blocking solution) for 4 h at room temperature for blocking non-specific binding, then incubated with primary mouse monoclonal anti-Pgp cloneF4 [100] at 1:5,000 dilution in blocking buffer for 1 h at room temperature. Then the membrane was washed by washing solution buffer (0.1%PBS-tween) for 6 times, 5 min per each for removing excess antibodies. The next step, the membrane was incubated with goat anti-mouse IgG linked with peroxidase at a 1:10,000 dilution in blocking buffer for 1 h at room temperature. The membrane was washed by washing buffer for 6 times, 5 min per each. Finally, bound proteins were detected by using the SuperSignal protein detection kit (enhanced chemiluminescence, ECL), then exposed to Kodak X-Omat film (approximately 1-5 min) and quantitated by scanning density of the bands. A membrane containing molecular weight marker (high range, BIO-RAD) was stained by amido black.

The principle of ECL detection is as follows: peroxidase substrate such as luminol has been used for chemiluminescence reaction in Western blot detection. In the presence of hydrogen peroxide, luminol decomposes through intermediates, emitting light as it decomposes (Figure11). The light produced by this enhanced chemiluminescent reaction peaks after 5-20 min and decayed slowly with a half-life of approximately 60 min. The maximum light emission at 425 nm wavelength can be detected by a short exposure to blue-light sensitive autoradiography film.

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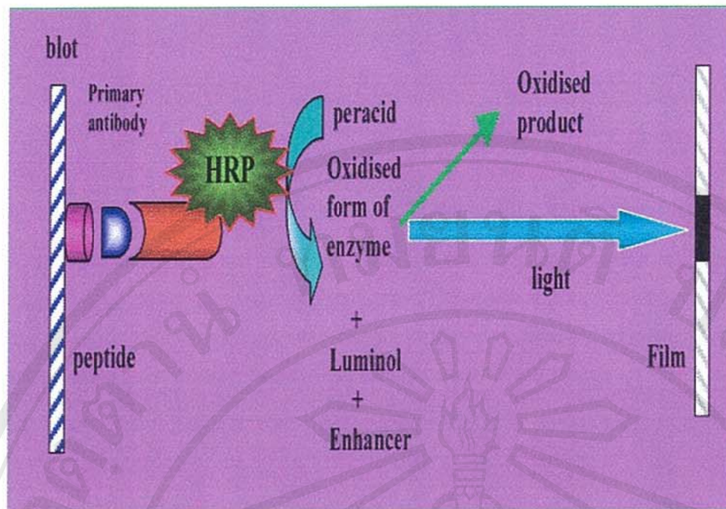

Figure 11. The principle of Enhanced Chemiluminescence (ECL) system

2.10 Statistical Analysis

Data were the mean ± standard deviation of mean from triplicate samples of three independent experiments. Differences between the means were analyzed by one-way analysis of variance. Statistical significance was considered when $P < 0.05$ or $P < 0.01$.

2.11 Cytotoxicity of green tea flavonoids in KB-V1 and KB-3-1 cell lines

To determine the cytotoxicity of green tea flavonoids in KB-V1 and KB-3-1 cell lines, cell were plates ($2x10^3$ and $3x10^3$ cells/well) in 100 μl medium for overnight and then 100 μL of medium containing green tea flavonoids were added and incubated for 48h. The final concentrations of green tea flavonoids were 0-300 μM. The cell viability in each well was determined by MTT assay as described in Section 2.4.

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2.12 Effect of green tea flavonoids on Pgp mediated drugs transport in KB-V1 and KB-3-1 cell lines

2.12.1 Effect of green tea flavonoids on Rh123 accumulation and efflux

KB-3-1 or KB-V1 at 500,000 cells per tube were incubated with 1μg/ml of Rh123 and 0-300 μM of green tea flavonoids for 60 min at 37°C in the dark. The final concentration of 0.4%DMSO (v/v) was used for all experiments and controls. Rh123 accumulation was stopped by washing twice

with ice cold Hank's balance salt solution (HBSS) without phenol red, then placed in 10%FBS in HBSS and analyzed on FACScan flow cytometer as described in Section 2.5

For determination of Rhodamine efflux, the cells were incubated with 1μg/ml of Rhomamine123 and various concentrations of green tea flavonoids for 60 min at 37°C, then the cells were washed with ice cold HBSS without phenol red, then the medium was replaced with Rh123 free medium containing green tea flavonoids. After incubation at 37^0C for 30 min, the cells were washed twice and the fluorescence of Rh123 was measured by FACScan flow cytometer as described in Section 2.5

2.12.2 Effect of green tea flavonoids on radiolabeled drug accumulation and efflux

KB-3-1 or KB-V1 were seeded at 500,000 cells per well in 6-well plates and incubated overnight. The cells were treated with 0-300 of green tea flavonoids with 0.05 μCi (^{3}H)-vinblastine/ml (specific activity : 58.00 mCi/mMol) for 60 min at 37^0C. The cells were then harvested by centrifugation at 10,000 rpm at 4^0C for 3 min. Cells were dissolved with 200 μl of 3N NaOH and incubated for 30 min, then neutralized with 100 μl of 6N HCl. Cell lysate, 250μl was pipetted into 3 ml of scintillation fluid. The radioactivity was counted by β-counter. The protein concentration was determined by Bradford method using 10 μl of cell lysate in 96 well plate. The amount of intracellular radioactivity (counting unit) was calculated in the terms of percentage of vehicle control as described in Section 2.5

For determination of drug efflux, cells were plated out as described for drug accumulation experiments. Cells were incubated for 60 min at 37^0C with (^{3}H)-vinblastine/ml and 10 μM cyclosporineA, the specific inhibitor of Pgp in order to load the cells with radiolabeled drug. Then the cells were washed with PBS pH 7.4 and the medium containing green tea flavonoids was added with a final concentration at 0-300 μM. After incubation at 37^0C for 15 min, cells were wased with ice cold PBS (pH7.4) and harvested. The amount of intracellular radioactivity was determined by scintillation counter.

2.13 Effect of green tea flavonoids on Pgp expression (protein level) in KB-V1 cell line

To investigate the effect of green tea flavonoids on Pgp expression, KB-V1 cells were treated with 0-300 μM of green tea flavonoids for 2 h. In another experiment, cells were treated with 50

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and 100 μM of green tea flavonoids for 48 h. After the end of incubation times, the cells were then harvested as described in Section 2.6. Membrane fraction was prepared and 20 μg of the protein was seperated by SDS-PAGE and analyzed by Western blot and ECL detection as described in Section 2.8 and 2.9, respectively.

2.14 Effect of green tea flavonoids on cytotoxicity of chemotherapeutic drugs (MDR phenotype) in KB-V1 and KB-3-1 cell lines

2.14.1 Effect of co-incubation of green tea flavonoids on cytotoxicity of chemotherapeutic drugs

KB-V1 and KB-3-1 cell lines, cell were plates ($2x10^3$ and $3x10^3$ cells/well) in 100 μl medium for overnight and then 100 μl of medium containing green tea flavonoids and various con centrations of each chemotherapeutic drugs which are vinblastine, doxorubicin, colchicine and paclitaxel were added and incubated for 24 or 48 h. The cell viability in each well was determined by MTT assay as described in Section 2.4.

2.14.2 Effect of pre-incubation of green tea flavonoids on cytotoxicity of chemotherapeutic drugs

KB-V1 cells were plated ($1x10^3$cells/well) in 100μL medium overnight and then 100 μl of the medium containing green tea flavonoids were added and then incubated for 48 h. After 48 h, the cells were washed by fresh medium and incubated for another 48 h with various concentrations of vinblastine. Then the cell viability was determined by MTT assay as described in Section 2.4.

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