2. Material and Methods

2.1. Materials

2.1.1. Reagents

Salts and buffer reagents were purchased in analytical quality from Sigma (St. Louis, MO) or Roth (Karlsruhe, Germany). Special agents were purchased as listed below:

Alamar Blue solution	Biosource (Nivelles, Belgium)
Alkallive phosphatase steptavidin	Pharmigen (Heidelberg, germany)
Curdlan (β-[1,3]-D-glucan)	Sigma (Steinhein, Germany)
DEAE-Sephacel	Amersham Pharmacia (Uppsala Sweden)
Epoxy-activated Sepharose ™ 6B	Amersham Pharmacia (Uppsala Sweden)
Fluorescein isothiocyanate	Sigma (Steinhein, Germany)
FluoSpheres Fluorescent microspheres	Molecular Probes (Eugene, USA)
Glyceraldehyde 3-phosphate	Sigma (Steinhein, Germany)
GAPDH	Advanced Immunochemical (California, USA)
β-1,3-Glucanase	Interspex (San Meteo, USA)
IL-6	Pharmigen (Heidelberg, Germany)
Laminarin	Sigma (Steinheim, Germany)
LPS (lipopolysaccharide)	Calbiochem (Damstadt, Germany)
LPS-FITC	Molecular Probes (Eugene, USA)
α-Mannan	Sigma (Steinheim, Germany)
Molecular weight standard (for gel electrophoresis)	Sigma (St. Louis, USA)
Molecular weight standard (for gel filtration chromatography)	Bio-rad (Bichmond, USA)
Mouse IgG reagent Grade	Sigma (Steinheim, Germany)
Naphthylethylene-diamine dihydrochloride	Sigma (Steinhein, Germany)
β-Nicotinamide adenine dinucleotide	Sigma (Steinhein, Germany)
Phytohemagglutinin	Sigma (Steinheim, Germany)
Polymyxin B sulfate	Sigma (Steinheim, Germany)
Pronase E	Calbiochem (La Jolla, USA)

Purified mouse IgM	Pharmingen (Heidelberg, Germany)
Resveratrol	Sigma (Steinhein, Germany)
Sephadex G-25F	Amersham Pharmacia (Uppsala Sweden)
Sephacryl S-300HR	Amersham Pharmacia (Uppsala Sweden)
Sephacryl S-400HR	Amersham Pharmacia (Uppsala Sweden)
SP Sepharose high performance	Amersham Pharmacia (Uppsala Sweden)
Sulfanilamide	Sigma (Steinhein, Germany)

2.1.2. Antibody

Anti-mouse CD3 fluorescein conjugate	Biosource (Nivelles, Belgium)
Anti-mouse CD 14 fluorescein conjugate	Pharmingen (San Diego, USA)
Anti-mouse CD19 fluorescein conjugate	Dianova (Hamburg, Germany)
Anti-mouse CD19 R-phycoerythrin conjugate	Dianova (Hamburg, Germany)
Anti-mouse CD19 Microbeads	Milttenyi Biotec (Auburn, USA)
Anti-mouse CD25R-phycoerythrin conjugate	Dianova (Hamburg, Germany)
Anti-mouse CD71R-phycoerythrin conjugate	Dianova (Hamburg, Germany)
Anti-mouse IgG Biotin conjugated (γ-chain specific)	Sigma (Missouri, USA)
Anti-mouse IgM Biotin conjugated (μ-chain specific)	Sigma (Steinheim, Germany)

2.1.3. Kit

Annexin V apoptosis detection kit	Bender MedSystems (Vienna, Austria)
BCA protein assay reagent kit	Pierce (Rockford, USA)
Dig glycan detection kit	Roche(Mannhein, Germany)
IL-1β Kit	R&D (Minneapoil, USA)
TNF-α Kit	R&D (Minneapoil, USA)

MidiMACS Kit including 2 ml MACS CD19 MicroBeads suspension, LS^+ separation column, a strong magnet and a stand for magnet was bought from Miltenyi Biotec (Suite, USA).

2.1.4. Medium

RPMI 1640 medium	Biochrom (Berlin, Germany)
DME medium	Biochrom (Berlin, Germany)
Penicillin-streptomycin stock solution	Biochrom (Berlin, Germany)
Fetal calf serum	Kraeber (Wedel, Germany)

2.1.5. Crude extracts

The crude extracts LZ, YZ, HSH, HT, LZSBS and HZ were extracted with hot water from the fungi fruit body of *Ganoderma lucidum*, *Coriolus versicolor*, *Hericium erinaceum*, *Grifola frondosa*, spore of *Ganoderma lucidum* and the plant root of *Polygonum cuspidatum*, respectively.

2.1.6. Cell lines

Bon	neuroendocrine, pancreas carcinoid
HTB-26	human fibrosarcoma cell line
J774	mouse mouse macrophage cell line
K562	human chronic myelogenous leukemia cell line
L929	mouse fibrosarcoma cell line
LS180	human colon carcinoma cell line
MCF7	human breast adenocarcinoma, pleural effusion cell line
Sarcoma 180	mouse sarcoma cell line
SW620	human lymph node metastasis, colon carcinoma cell line
U937	human monocyte cell line

All cell lines were obtained from the American Type Culture Collection (Manassas, USA).

2.1.7. Animals

Balb^{c} and C3H/HeJ strain mice were purchased from Zentralinstitut für Versuchstierzucht (Hannover) and bred under normal condition.

2.2. Methods

2.2.1. Cell preparation and cell culture

2.2.1.1. Tumour cell lines

RPMI 1640 medium (Biochrom AG) was supplemented with 0.44 g/l glutamine, 10% heat-inactivated FCS, 100 µg/ml streptomycin and 100 IU/ml penicillin. DMEM medium (Biochrom AG) was supplemented with 0.44 g/l glutamine, 10% heat-inactivated FCS, 100 µg/ml streptomycin, 100 IU/ml penicillin and 0.1 mM Na-pyruvate. K562, SW620, MCF 7, HTB-26, LS180 and U937 cell lines were cultured in RPMI 1640 medium and J774, L929, Bon and Sarcoma 180 was cultured in DMEM medium in a humidified atmosphere of 5% CO_2 at 37°C. Cell lines were subcultured according to the introduction of each cell line.

2.2.1.2. Preparation of lymphocytes from mouse spleens

$Balb^c$ mice, 8-10 week age (ca. 28±1 g), were used for lymphocyte preparation. Spleens were removed after killing the mice by cervical dislocation. The spleens were cut into several pieces, then pressed through a stainless steel mesh (100 mesh) into a culture plate using a sterile syringe plunger. The mesh was rinsed twice with PBS under sterile conditions. The spleen cell suspension was transferred into a new tube and sedimented for 10 min. The supernatant was pipetted into another tube and the cell clumps in the bottom of the tube were discarded. After centrifuged at 300 g for 6 min, cell pellets were washed twice with PBS. 3×10^8 cells were resuspended for 10 min at room temperature in 1 ml Tris-HCl buffer, Tris-HCl buffer adjusted pH to 7.2 by adding 0.83% NH_4Cl solution, [mix 9 volumes of 0.83% (w/v in water) NH_4Cl with 1 volume of Tris-HCl (2.06 W/V in water, pH 7.65), adjust pH to 7.2] in order to lyse red cells. Cells were counted in a Z series Counter (Counter electronics, Miami, USA). The cell suspension was further diluted with five-fold excess of medium. After mixing and centrifugation, the cell pellets were finally resuspended in RPMI 1640 medium containing 10% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin for further experiments. Cell viability was assessed by trypan blue exclusion.

2.2.1.3. Preparation of lymphocytes from mouse spleens of sarcoma-bearing $Balb^c$ mice (TMSLs)

Mice with similar weight (28 ±1g) and ages (8-10 weeks) were selected. Then 1.0×10^6 sarcoma 180 cells in 100 µl PBS were injected into one leg of each mouse. After 15 days, these mice were checked, the mice that had grown tumours were selected and their spleens were removed. Then the suspension of spleen lymphocytes was prepared according to the above method.

2.2.1.4. Separation of B cells from mouse spleen lymphocytes by magnetic cell sorting

Lymphocytes were prepared from mouse spleens. The cells were washed, the supernatant were removed completely and cell pellet resuspended in 90 µl of buffer (PBS with 1% BSA) per 10^7 total cells. 10 µl of magnetic cell sorting (MACS) CD19 MicroBeads were added to per 10^7 total cells. Cell suspension was mixed well and incubated at 6°C for 20 min. Then the cells were washed with 20-fold volume buffer and centrifuged at 300 g for 10 min. The supernatant was removed completely. The cell pellets were resuspended in 500 µl of buffer per 10^8 cells. Then the cell suspension was transferred to LS^+ separation column which had been washed with 5 ml of PBS and placed in the MidiMACS magnet. The cell suspension was run through the column and the effluent was collected as non-B cells. Then the column was rinsed with 3 ml buffer three times and then removed from the magnet separator. Finally, 5 ml of buffer was added to the reservoir of LS column and the B cells were flushed out firmly using a plunger.

2.2.1.5. Preparation of bone marrow-derived macrophages (BMMs)

Bone marrow-derived macrophages were prepared according to the method described by Stanley (1997). Mice with similar weight (28 ±1g) and ages (8-10 weeks) were selected for obtaining bone marrow macrophages at random. Mice were killed by cervical dislocation, and the tibias and the femurs were removed from $Balb^c$ mice by cutting the proximal end of the femur and the distal end of the tibia, leaving the other ends intact. A 23-gauge needle was inserted into the intact ends and bone marrow was flushed out through the cut ends with ice-cold DMEM. The marrow cells were dispensed by three passed through a 22-gauge needle and centrifuged (1200 g, 5 min, 4° C). The cell density was adjusted to 10^6 cell/ml in a complete DMEM medium containing 10% L929 (ATCC, Rockville, MD, CCL1) conditioned medium. After being incubated at 37°C for 3 days, the non-adherent cells were collected, and the adherent cells were discarded. Then the cells were seeded into bacteria-culture dishes with 10% L929 conditioned medium, and incubated at 37°C for another three days. The non-adherent cells were washed away with sterile PBS. Adherent macrophages were collected for use by scraping with cold PBS, replacing the growth medium with complete medium.

2.2.1.6. Preparation of bone marrow-derived macrophages from sarcoma-bearing $Balb^C$ mice (TBMMs)

Mice with similar weight (28 ±1g) and ages (8-10 weeks) were selected. Then 1.0×10^6 of sarcoma 180 cells in 100 μl of PBS were injected into one leg of each mouse. After 15 days, the mice were checked, those which had grown tumours were selected and their tibias and femurs were removed. The bone marrow-derived macrophages was then prepared according to the above method.

2.2.2. Separation, purification and identification

2.2.2.1. Separation and purification of GLIS from *Ganoderma lucidum* (GL)

The crude extract of *Ganoderma lucidum* (GL) was prepared by extracting fruit body 3 times with boiling water. The extract was dialysed and fractionated over a DEAE-sephacel column (2.6 × 100 cm) applying a linear gradient of NaCl in water (0 to 2 mol/l NaCl within 1500 ml). The fractions were monitored for peptide content by UV absorption at 280 nm, for carbohydrate content by the phenol-sulfuric acid reaction, and for immunostimulatory capacity using an appropriate bioassay. A fraction eluted with about 500 mmol/l NaCl was further fractionated applying gel filtration chromatography on Sephacryl S-300 (Step 2) and S-400 (Step 4) and cation exchange chromatography on SP-Sepharose (Step 3), all from Amersham Pharmacia Biotech (Uppsala Sweden). All the columns were eluted with water. A fraction obtained from the last step, that exhibited the strongest specific stimulation of lymphocyte proliferation, was designated GLIS.

2.2.2.2. Determination of purity and estimation of Mr of the active fraction GLIS by gel-filtration HPLC

For the gel-filtration, GF-450 and GF-250 combined column were balanced with a 0.2 molar sodium phosphate buffer (pH 7.0), 20 µl of standards (Bio-Rad) and the tested samples were injected onto the column separately. Then, compared to the molecular weights of a standard sample, the molecular weight of the sample was calculated. The purity of the tested sample was judged according to the number and shape of the peak.

2.2.2.3. Quantitation of carbohydrate content in active fraction GLIS by DIG Glycan Detection Kit

The carbohydrate content of the eluates from HPLC was measured using DIG Glycan Detection Kit. Five µl of the fraction was transferred onto nitrocellulose membrane. After the membrane was dried, the filter was washed in 50 ml PBS and oxidised with 10 ml of sodium metaperiodate (10 mM) in sodium acetate buffer (pH 5.5) for 20 min in the dark at room temperature. The membrane was washed 3 times, each time for 10 min, and incubated with 1 µl of digoxigenin-3-O-succinyl-E-amino-caproic acid-hydrazide hydrochloride dissolved in 5 ml of sodium acetate buffer (pH 5.5) for 1 h. After the membrane was washed 10 min three times with 50 ml of TBS, it was incubated in the blocking solution for 30 min. Then it was washed 3 times for 10 min with 50 ml of TBS, and incubated with anti-digoxigenin-AP for 1 h. The membrane was then washed 3 times for 10 min each with 50 ml of TBS and stained with the staining solution (10 ml of Tris-buffer with 200 µl of NBT/X-phosphate solution pH 9.5).

2.2.2.4. Polymyxin B treatment of GLIS

The antibiotic polymyxin B, which binds to and inactivates endotoxin (Coyne, 1993), was used to investigate whether GLIS was contaminated with endotoxin. The GLIS suspension was pretreated with 10 µg/ml polymyxin B for 30 min before being added to macrophages. The macrophages were then incubated for 48 h, and the supernatants were detected for NO production as described above.

2.2.2.5. Treatment of GLIS with Pronase E and $NaIO_4$

GLIS was treated with either pronase E or $NaIO_4$ to destroy either protein or carbohydrate residues from glycoproteins, respectively. Briefly, 500 µl of 10 mg/ml GLIS was incubated with 50 µl of 6 mg/ml pronase E (Calbiochem, La Jolla, USA) at 60° C in 0.1 M of Tris-HCl buffer containing 50 mM $CaCl_2$. After 16 h of incubation, another 50 µl of pronase E was added for another 16 h. Then the reaction mixture was heated at 100°C for 10 min to inactivate pronase E and dialysed against PBS. In addition, the samples were incubated with 0.1 M $NaIO_4$ at 25°C for 4 h in the dark, subsequently, 20% ethylene glycol was added before the samples were dialysed.

2.2.2.6. Separation and purification of the active compound from *Polygonum cuspidatum*

The crude extract HZ was re-dissolved in water ad centrifuged at 10000 × g for 30 min, the supernatant was partitionated between n-butanol and water (2:1). The water phase was lyophilized to produce a residue HZ-1. After vacumn evaporation of n-butanol at 60°C, the residue of butanol phase was dissolved in ethanol. According to their solubility in ethanol, the insoluble part (HZ-2) ad soluble part (HZ-3) was divided.

HZ-3 (1 g) was dissolved in 2 ml of ethanol and then 100 ml of H_2O was added. After the solution was mixed ad put in 4°C for one week, it was centrifuged at 10000 × g for 30 min. The supernatant of the solution was lyophilized to produce a residue HZ-3-2. The sediment, which was washed with cold redistilled water for three times and lyophilised, was HZ-3-1. HZ-3-1 was dissolved in ethanol and loaded onto reverse C_{18} column. Five fractions were collected according to the peak. All fractions were detected using TLC.

2.2.2.7. Identification of HZ-3-1-b by thin layer chromatography (TLC)

Preliminary analysis of the fractions and subfractions by thin layer chromatography on silicagel GF_{254} was performed. Pre-coated glass 10 × 10cm F_{254} HPTLC plates (5 µm particle size, 0.2 mm layer thickness, Merck, Darmstadt, Germany) were cut to 2 × 5 cm size TLC plate cutter prior to use. The plates were spotted with 5 µl of the stock solution and developed in $CHCl_3$ / CH_3OH (9:1) systems. The plates were removed from the development chamber when the solvent front had reached the top of the TLC plate (a migration distance of about 4 cm from the point of origin) and dried by flushing the plate under a stream of N_2 at room temperature. Spots were visualised under UV illumination at a wavelength of 254 nm and Rf was measured.

2.2.2.8. Identification of HZ-3-1-b by high performance liquid chromatography (HPLC)

Reversed C_{18} column (Eurospher 100-C_{18}, 4.0 × 25 mm, size 5.0 µm) was used for analysing the standard and HZ-3-1-b. The samples were dissolved in 90% acetonitril. 100 µl of sample was injected into the chromatographic column and the effluent was monitored at 345 nm continuously. The mobil phase system was acetonitril / H_2O (9:1), the flow rate was 1.0 ml/min, and the pressure was 14.9 Mpa. Retention times were determined.

2.2.3. Bioassay

2.2.3.1. Determination of activation by the Alamar Blue Assay

After cell suspensions were adjusted to a suitable concentration, 180 µl of the cell suspension was added to each well of a 96-well microplate, meanwhile 20 µl of various test agents were added in triplicate. After incubation at 37°C in a 5% CO_2 atmosphere for a defined time, 20 µl Alamar Blue was added to each well. After incubation for another 6 h, the change of color of the solution was measured using a micro ELISA autoreader at 570 nm and 600 nm. The activation rate was calculated according to the formula provided by the Alamar Blue Assay (Nivelles, Belgium).

2.2.3.2. Quantification of cytokines IL-1β and TNF-α

The presence of the cytokines IL-1β or TNF-α released from macrophages into the culture media was quantified using a sensitive and specific sandwich ELISAs, according to the manufacturer's instructions (R& D). Macrophages from normal and tumour-bearing mice were prepared according to the above standard methods. Macrophage cell suspensions were adjusted to 1×10^5 cells/ml, and 180 µl of the cell suspension was added to each well of a 96-well microplate. Meanwhile, 20 µl of various test agents were added in triplicate. After incubation at 37°C in 5% CO_2 atmosphere for 24 h, the cell-free supernatants were collected for cytokines measurement. 50 µl of cell-free supernatant, IL-1β or TNF-α standard samples were transferred onto a 96-well microplate coated with polyclonal antibody specific for mouse IL-1β or TNF-α, then 50 µl of assay dilute was added into each well. After the microplate was gently mixed and incubated at 37°C for 2 h, the plate was washed 4 times with washing buffer and 100 µl of horseradish peroxidase. Anti-IL-1β or TNF-α was added to each well and incubated at 37°C for 2 h. After washing 4 times with the washing buffer, 100 µl of chromogen was added and incubated for 30 min. Finally l00 µl of stop solution was added and the absorbance of each well was read at 450 nm with a reference wavelength 570 nm.

2.2.3.3. Measurement of nitric oxide

Production of nitric oxide was detected by measuring nitrite levels with the Griess reaction (Alleva et al., 1994). First, macrophages suspension was adjusted to 1×10^6 cells/ml, and 180 µl of the cell suspension was added to each well of 96-well microplate. Meanwhile, 20 µl of various test agents were added in triplicate. After incubation at 37°C in 5% CO_2 atmosphere for 48 h, the 100 µl of cell-free supernatants was collected for nitrite assessment. The supernatant (100 µl) was mixed with 50 µl of Griess reagent (1% sulfanilamide, 0.1% naphthylethyylene-diamine dihydrochloride, 2.5% phosphoric acid) and incubated for 10 min at room temperature, and the absorbance at 543 nm was measured. The nitrite concentration was determined using sodium nitrite as standard. The standard curve was linear between 0.1 and 100 µM.

2.2.3.4. Determination of immunoglobulin production

IgM or IgG in the culture supernatants of B cells was measured by enzyme-linked immunosorbent assay (ELISA). B cells were incubated with or without agents at the indicated concentration for 1 to 8 days. Aliquots of a suspension of each sample were used for the immunoglobulin ELISA-assay. 100 µl of 0.2 µg/ml purified anti-mouse Ig (Boehringer Mannheim Biochemica Mannheim, Germany) was added to a 96-well microplate overnight at 4°C. Unbound antibodies were removed by washing with PBS containing 0.05% Tween-20 (PBS-Tween) five times. Then the plate was incubated with 1% BSA in PBS at room temperature for 1 h to block the unspecific binding. After the plate was washed five times with PBS-Tween, 100 µl of cell-free supernatant, IgM or IgG standard samples were added and incubated for 2 h. The plate was washed with PBS-Tween five times and 100 µl of biotin-labelled goat anti-mouse IgM or IgG (Amersham Pharmacia Biotech Uppsala, Sweden) in

PBS-Tween containing 1 % BSA were added to the well, and the plate was incubated for 2 h at room temperature. After washing the well with PBS-Tween five times, each well was incubated with 200 µl of alkaline phosphatase-streptavidin (DIANOVA, Hamburg, Germany) for 1 h at room temperature. The plate was washed five times with PBS-Tween, each well was incubated with 200 µl of chromogenic substract solution (1 mg of p-nitrophenylphosphate disodium salt in 1 ml of diethanolamine buffer, pH 9.8) for 30 min, and stopped with 50 µl of 1 M Na_2CO_3. The absorbance was measured at 405 nm using a micro ELISA autoreader.

2.2.4. Phagocytosis assay

The phagocytic activity was assayed by the method described by Krause et al (1998) with small modification. Macrophages were harvested using cold PBS and diluted into 2×10^5 cells/ml. Cells were seeded into 96-well plates at 100 µl per well. Latex beads were washed three times with PBS, and diluted to 1×10^7 per ml in RPMI medium. 80 µl of latex beads and 20 µl of sample were added to the wells and incubated for 24 h at 37°C. After incubation, the cells were washed three times with PBS, and the quantitative evaluations of the phagocytic cell percentages were achieved microscopically.

2.2.5. Macrophages-mediated anti-tumour activity

The assay modified for the measurement of anti-tumour activities of macrophages is based on an assay described previously (Moon et al., 1998). Macrophages were seeded on 96-well plates and attached for 2 h. After incubation, macrophages were co-incubated with L929 tumour cells (at a ratio of 10 macrophages to 1 target cell) in the presence of different concentration of GLIS for 48 h in a 5% CO_2 humidified incubator at 37°C. The cells were then stained with crystal violet containing 11.1% ethyl alcohol and 10% formaldehyde for 30 min. Anti-tumour activity of macrophages was expressed as a cytolysis percentage of tumour cells as follow:

$$\text{Cytolysis (\%)} = \{1- \frac{\text{O.D. of [(target+macrophages)-macrophages]}}{\text{O.D. of target (non-treated)}} \} \text{ X } 100\%$$

2.2.6. Analysis of activation and the change of subpopulation of lymphocytes after stimulation by active fractions using flow cytometry

B cells suspension was obtained according to the method described above. Then 4 ml of cell suspension (2×10^6 cells/ml) were added in 6-well plates with or without test agents. After B cell suspension was obtained according to the method described above. Then 4 ml of cell suspension (2×10^6 cells/ml) was added to 6-well plates with or without test agents. After treatment for 48 h, B cells were centrifuged and washed twice with PBS. The cells were then labelled with special mAb of anti-CD19-FITC/anti-CD25-PE, anti-CD19-FITC/anti-CD71-PE, anti-CD3-FITC/anti-CD25-PE and anti-CD3-FITC/anti-CD71-PE which were optimised by preliminary experiments in PBS for 0.5 h. After staining, the cells were washed three times with PBS, and resuspended in 0.3 ml of PBS. Fluorescence analysis was performed using a FACScan analyser. B cells were gated on the basis of both of forward and side scatter and a

mean of 10000 cells in this gate were analysed. All samples were measured within 2 h. CELLQuest software was used to analyse the data for the calculation of the percentage of different lymphocyte subpopulations.

2.2.7. Response of macrophage β-glucan, mannose receptor antagonist and anti-CD14 Ab to GLIS

Macrophages were incubated with the soluble mannose receptor antagonist α-mannan (0.5 mg/ml, Sigma), β-glucan receptor antagonist laminrin (0.5 mg/ml, sigma) and anti-CD14 (5 μg/ml) for 45 min prior to the addition of GLIS, followed by a subsequent 24 h incubation. Subsequently, the medium was removed and assayed for nitrite.

2.2.8. Measurement of binding of GLIS to macrophage surface

1 mg of GLIS was dissolved in 200 μl of 0.1 M bicarbonate buffer (pH 9). While the sample solution was stirred, 1 μl reactive FITC dye (10 mg/ml) was added and the reaction was stirred at room temperature for 1 h protected from light. After the reaction, 7 μl hydroxylamine was added to the conjugation solution and this solution was stirred at room temperature for 30 min. The sample was then eluted to a PD-10 desalting column to separate the FITC-GLIS to FITC.

The binding of FITC-GLIS on the macrophage surface was measured with fluorescence microscopy and FACScan flow cytometry. Macrophage monolayer in 3-cm culture plates was treated with 2 μg/ml FITC-GLIS or anti-rabbit FITC for 20 h, and washed afterwards 3 times with warm PBS. The cells were then observed under fluorescence microscopy. For FACS analysis, 100 μl 10^6 macrophages or macrophages cell lines were incubated with 2 μg/ml GLIS-FITC in DMEM medium at 37°C for 1 h. After staining, cells were washed three times with PBS, and resuspended in 0.3 ml PBS. All samples were measured within 2 h.

2.2.9. B cell survival assay

B cells were isolated from MSLs or TMSLs according to the method described above. 180 μl of B cell suspension (2×10^6 cells/ml) was added to each well of the 96-well microplate, meanwhile 20 μl of various test agents were added in triplicate. The cells were collected from triplicate wells daily on day 1 through day 8. B cells with or without treatment were centrifuged and washed two times with PBS. The cells were then labelled with 100 μl of 5 μg/ml propidium iodide for 30 min. After being washed with PBS 3 times, the cells were resuspended in 0.3 ml of PBS. Fluorescence analysis was performed using a FACScan analyzer. CELLQuest software was used to analyse the data for calculation of the percentage of dead cells.

2.2.10. B cells co-cultured with macrophages or supernatants (SN) of macrophages

Macrophages were diluted to the different concentrations 10, 10^2, 10^3, 10^4 and 10^5 cells/ml in RPMI 1640 medium. B cells isolated from MSLs were diluted to 2×10^6 cells/ml using the medium which contained different macrophage concentrations. After being stimulated with

different stimuli, the aliquots of a suspension of each sample were used for the immunoglobulin ELISA-assay.

15 ml of macrophages (1×10^5 cells/ml) were cultured in complete PRMI 1640 medium in a 9 cm plate stimulated with 500 µg/ml GLIS or 50 µg/ml LPS. After 24 h, the culture supernatants (SN) were collected and filtered. B cells were diluted to 2×10^6 cells/ml using SN and added to 96-well plates. After being stimulated with different stimuli, the aliquots of a suspension of each sample were used for the immunoglobulin ELISA-assay.

2.2.11. Inhibition of the proliferation of different tumour cell lines

Tumour cells in log phase were harvested and resuspended in appropriate medium. For adhesive cells, PBS-EDTA or 0.1 % trypsin solution was used to make the cells float and the cells were prepared for single cell suspension. After count, cell suspension was diluted to 10000 cells/ml. Then cell suspension was seeded into 96-well microplates (1800 cells/0.18 ml/well) and incubated. After 24 h, different test agents were added into each well and the microplates were incubated for another 72 h. Cells incubated with normal medium only served as controls. Then the inhibition rate of different tumour cells by different agents was measured by the Alamar Blue Assay.

2.2.12. Identification of apoptosis of tumour cells by annexin V-FITC conjugate under fluorescence microscopy

Tumour cells were seeded into a 24-well plate. Into each well was added 1×10^5 cells in 1 ml medium with 10% FBS. After continuous incubation with the agents for a defined time, cells were collected, centrifuged and washed twice with PBS. Then 10^6 cells were resuspended in 100 µl of annexin V incubation reagent (10 µl of 10 × binding buffer, 1 µl of 50 µg/ml annexin V-FITC conjugate, and 79 µl of double distilled water) and incubated for 15 min in the dark at room temperature. Afterwards, the cells were centrifuged and the cell pellets transferred to a glass slide. When the solution was almost dry, a drop of evanol was added onto it and covered with a cover glass. Finally, the glass slides were observed by fluorescence microscopy for identification of apoptosis.

2.2.13. Quantification of apoptosis by flow cytometry using propidium iodide staining method

Tumour cells were seeded into a 6-well microplate. To each well was added 5×10^5 cells in 5 ml medium with 10% FBS. After continuous incubation with the agents for a defined time, the cells were trypsinised, centrifuged and washed twice with PBS. The cells were then permeabilised and fixed with 70% ethanol overnight. After the cell suspension was centrifuged and washed once with PBS, the cell pellets were resuspended in 200 µl of PBS with 50 µg/ml propidium iodide and 25 µg/ml RNase A and incubated for 6 h at 4° C. After the cell were washed once with PBS, quantification of DNA fragmentation of the cells was analysed by FACScan analyser (Becton Dickinson, Eremdodegem, Belgium). Viable cells were gated on the basis of both forward and side scatter. A mean of 10000 cells in this gate

were analysed. Analysis of the DNA content of the cells was done by CELLQuest software (Becton Dickinson, Eremdodegem, Belgium).

2.2.14. Affinity chromatography

The preparation protocol of the resveratrol-sepharose column was according to manufacturer's instructions. Resveratrol was coupled to sepharose by linking hydroxyl groups to epoxy-activated Sepharose 6B, which is a pre-activated gel for the immobilization of various ligands. 0.15 g epoxy-activated Sepharose 6B was suspended in distilled water. The gel swelled immediately and was washed for 1 h on a sintered glass filter. Five mg of resveratrol was dissolved in a coupling buffer [50% ethanol / $NaHCO_3$ buffer (pH 11)] and mixed with the gel. After the pH was adjusted to 12, the solution was incubated overnight. Then the gel was washed with coupling buffer to remove the excess ligand. Any remaining active groups were blocked with 1 M ethanolamine (pH 8.0) for 4 h. The product was washed thoroughly with at least three cycles of alternating pH. Each cycle consisted of a wash with an 0.1 acetate buffer (pH 4.0) containing 0.5 M NaCl, followed by a wash with a 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl.

2 ml of cell lysates of SW620 cell or J774 cell were loaded onto the affinity column and circulated through the column at room temperature for 2 h. After being eluted with 50 ml solubilization buffer, the column was washed with PBS (pH 7.2), glycin-HCl (pH 5.5) and glycin-HCl (pH 3.0) respectively.

2.2.15. Mass spectrometry analysis of the binding protein

The protein, which was isolated from the solubilization of SW620 cells, was separated onto 10% SDS PAGE gel and stained with Coomassie Brilliant Blue (CBB). The band of approximately 35-40 kDa was excised with clean scalpel, cutting as close to the edge of the band as possible. The excised band was chopped into cubes and transferred into 1.5 ml Eppendorf tube. After the gel particles were washed with 100 µl of water, acetonitrile was added for 10-15 min until the gel piece shrunk, became white and stuck together. The gel particles were dried out and expanded in 10 mM dithiotreitol/0.1 M NH_4HCO_3 and incubated for 30 min at 56°C to precipitate protein. After the gel pieces were shrunk with acetonitrile, iodoacetamide solution (55 mM iodoaacetamide/0.1 M NH_4HCO_3) was incubated with the gel for 20 min at room temperature in the dark. After removal of the iodoacetamide solution, the gel particles were washed with 150-200 µl of 0.1M NH_4HCO_3 for 15 min. After the gel particles were dried in a vacuum centrifuge, they were rehydrated in the digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$ and 12.5 ng/µL of trypsin at 4°C for 30-45 min. Then the remaining supernatant was removed and 5-25 µl of the same buffer was added without trypsin to cover the gel pieces and keep them wet during enzyme cleavage. After incubation overnight at 37°C, a small aliquot of supernatant was used for MALDI analysis. The mass spectrometric analysis of proteolytically derived peptides was performed using a Bruker Reflex MALDI-TOF mass spectrometer (Bruker, Bremen, Germany) with α -cyano-4-hydroxycinnimanic acid as a matrix. For the analysis of fragment ions generated by post-source decay, the FAST™ method developed by Bruker was used.

2.2.16. GAPDH activity assay

GAPDH activity was assayed according to a modified previously reported procedure (Tomazela, 2000). Reduced NAPDH was measured spectrophotometrically at 340 nm at 30 second intervals. The reaction medium was 50 mM Tris-HCl buffer (pH 8.6) with 1 mM EDTA, 1 mM β-mercapto-ethanol, 30 mM Ma_2HAs_4, 2.5 mM NAD^+ and 0.3 mM glyceraldehyde-3-phosphate in a total volume of 200 µl. The reaction was initiated by the addition of enzyme.