

LYG-202 augments tumor necrosis factor- α -induced apoptosis via attenuating CK2-dependent nuclear factor- κ B pathway in HepG2 cells

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Abbreviation: TNF- α , tumor necrosis factor- α ; NF- κ B, nuclear factor- κ B; I κ B, NF- κ B inhibitory protein; IKK, I κ B kinases; CK2, Casein Kinase 2; COX-2, Cyclooxygenase-2; MMP, matrix metalloproteinase; PARP, poly-ADP-ribose polymerase; DAPI, 4',6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay; CHIP, Chromatin immunoprecipitation; TNFR1, TNF receptor 1; SODD, silencer of death domains; TRADD, TNFR-associated death domain protein; TUNEL, TdT-mediated dUTP nick end labeling; PDB, Protein Data Bank.

Abstract

Tumor necrosis factor- α (TNF- α) is being used as an antineoplastic agent in treatment regimens of patients with locally advanced solid tumors, but TNF- α alone is only marginally active. Clinically, it is usually combined with other chemical agents to increase its tumor response rate. Our previous studies reported that LYG-202, a synthesized flavonoid with a piperazine substitution, has anti-proliferative, anti-angiogenic and pro-apoptotic activities in multiple cancer cell lines. Here we evaluated the anti-neoplastic effect of TNF- α and analyzed the mechanism underlying its combination with LYG-202. Our results indicated that LYG-202 significantly increased the cytostatic and pro-apoptotic activity of TNF- α in HepG2 cells, and heightened the protein level of apoptosis-related genes including caspase-3, 8/9, cleaved PARP and Bid. The facts that LYG-202 had a similar fitness score with CK2 inhibitor CX-4945 implied us it may serve as a potential candidate of CK2 inhibitor, and the kinase activity assay suggested that LYG-202 significantly inhibited CK2 activity. Moreover, the EMSA and luciferase assays showed that LYG-202 blocked TNF- α -induced NF- κ B survival signaling pathway primarily by inactivating protein kinase CK2. In murine xenograft models, we also found that LYG-202 enhanced TNF- α antineoplastic activity, inhibited CK2 activity and NF- κ B-regulated anti-apoptotic gene expression. All these results showed that LYG-202 enhanced TNF- α -induced apoptosis by attenuating CK2-dependent NF- κ B pathway, and probably served as a most promising agent in combination with TNF- α .

Introduction

Tumor necrosis factor- α (TNF- α) is a multifunctional cytokine playing roles in apoptosis, cell survival, inflammation and immunity by triggering distinct signal (Balkwill, 2009; Pfeffer, 2003; Wallach, 1997). In several preclinical models and clinical trials, it exerts a direct cytotoxic effect to induce tumor regression and now served as an antineoplastic agent for the patients with locally advanced solid tumors (Egberts et al., 2008; Pilati et al., 2008). However, TNF- α alone only shows marginally activity, even administration with high doses through local drug-delivery systems. To increase malignant cell sensitivity to TNF- α , it is combined with conventional antineoplastic agents (e.g. melphalan, paclitaxel, actinomycin-D) in clinical (de Wilt et al., 2000; Manusama et al., 1996; Seynhaeve et al., 2002).

The majority of TNF- α biological activity is initiated via tumor necrosis factor receptor-1 (TNFR-1). The TNF- α homotrimer binding to the extracellular domain of TNFR-1, induces TNF-R1 trimerization, and releases the inhibitory protein silencer of death domains (SODD) from TNF-R1 intracellular DD, then recruits the adaptors including TNFR-associated death domain protein (TRADD), FADD and caspase-8, modulates caspase-9 and caspase-3. The activated caspase-8 and caspase-3 can induce Bid and PARP cleavage, respectively and switch on cell apoptosis programs (Los et al., 2002; Perez and White, 2000). On the contrary, the bond also activates I κ B kinase (IKK) and phosphorylates I κ B leading to its subsequent ubiquitination and proteasomal degradation, thereby allowing activated nuclear factor- κ B (NF- κ B) to translocate into the nucleus where it binds to specific sequences of DNA and activates the expression of some anti-apoptosis genes (Aggarwal, 2003; Sung et al., 2008). And NF- κ B signal is

considered to be the major blocker in TNF- α -induced apoptosis. So researchers explored agents that interfere with NF- κ B survival pathway to reverse the resistance of both solid and hematological malignancies to TNF- α (Braun et al., 2006; Uzzo et al., 2002) and improve the efficacy of apoptosis-inducing cancer therapies (Mocellin et al., 2005; Orlowski and Baldwin, 2002).

Flavonoids refer to a series of naturally occurring, low molecular weight plant products exhibiting a variety of biological activities such as antibacterial, antiviral, anti-inflammatory, anti-allergic and vasodilatory activities (Middleton et al., 2000; Nakamura et al., 2003; Suk et al., 2003). LYG-202, a known synthesized flavonoid with a piperazine substitution (Fig. 1A), showed stronger anti-tumor effects compared with its parent compound (Zeng et al., 2009). Our previous studies showed that it has pro-apoptotic, anti-proliferative and anti-angiogenic activities *in vivo* and *in vitro* (Chen et al., 2010a; Chen et al., 2010b; Liu et al., 2011; Zeng et al., 2009). In the study, we investigated the effect of LYG-202 on NF- κ B signaling and apoptosis in response to TNF- α . These results demonstrated that LYG-202 could augment TNF- α -induced apoptosis by blocking NF- κ B pathway. However, the exact molecular mechanism remains to be fully elucidated. Therefore, the inverse-docking approach was performed to screen potential protein targets of LYG-202 on NF- κ B pathway (e.g. CK2, MEKK1, MEKK3, TAK1, PKC- θ).

Casein kinase 2 (CK2), a highly conserved and ubiquitous serine/threonine protein kinase that participates in the transduction of signals that promote cell growth and survival, plays a critical role in various steps of NF- κ B signal activation induced by TNF- α (Litchfield, 2003). Firstly, it activates IKK β kinase and phosphorylates I κ B α on

Ser32/Ser36 and COOH-terminal proline-glutamic acid-serine-threonine residues (Schoonbroodt et al., 2000), and then phosphorylates the Rel A on Ser529, and this has a direct effect on the amplitude of transactivation (Wang et al., 2000). Hence, agents that can suppress CK2-induced NF- κ B activation and enhance TNF- α -induced cell apoptosis could significantly improve the anti-tumor activity of TNF- α (Siddiqui-Jain et al., 2010).

In the present study, we evaluated the antineoplastic effect of TNF- α with or without LYG-202 in human hepatocellular carcinoma HepG2 cells which are not sensitive to TNF- α (Kusaba et al., 2007; Sugimoto et al., 1999). Our results indicated that LYG-202 weakened CK2 activity and enhanced TNF- α -induced apoptosis by attenuating NF- κ B signaling pathway *in vitro* and *in vivo*.

Materials and methods

Materials

LYG-202 was synthesized by Dr. Zhiyu Li (China Pharmaceutical University, China), dissolved in DMSO as a stock solution (10 mM), and stored at -20°C, freshly diluted with RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) to the final concentration. The control groups were always treated with DMSO (0.1%, v/v). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Fluka chemical corp (Ronkonkoma, NY, USA) and dissolved in 0.01 M PBS. Recombinant human TNF- α was purchased from Peprotech company (Rocky Hills, NJ, USA), and recombinant mouse TNF- α was purchased from ProSpec-Tany TechnoGene Ltd (Ness Ziona, Israel). Z-VAD-FMK (a caspase inhibitor) was purchased from Beyotime Institute of Biotechnology (Nantong, Jiangsu, China). 5-(3-Chlorophenylamino benzo [c] (Aggarwal, 2003) naphthyridine-8-carboxylate (CX-4945) was obtained from

Biochempartner Co Ltd (Shanghai, China). Primary antibodies of caspase-3, caspase-8, caspase-9, Bid, GFP, Survivin, XIAP, c-Myc, MMP-9, p65, I κ B α , p-I κ B α , β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Bcl-2, Bcl-xL, Histones were obtained from Bioworld (St. Louis Park, MN, USA). Antibodies against PARP, COX-2, Cyclin D1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-CK2 α/α' antibody was purchased from BD Biosciences (San Jose, CA, USA). The plasmids of CK2 α and CK2 β were kindly gifted by Dr. Stefania Sarno (University of Padova, Italy). CK2 α siRNA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines and cell culture

The human hepatoma HepG2, SMMC-7721, BEL-7402 cell lines, the mouse hepatoma H₂₂ cell line, the liver L02 cell line and the colon carcinoma HCT-116 cell line were originally obtained from the Cell Bank of Shanghai Institute of Cell Biology. HepG2 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated calf serum (Sijiqing, Hangzhou, China). SMMC-7721, BEL-7402, L02 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Hangzhou, China). HCT-116 cells were cultured in McCoy's 5A supplemented with 10% heat-inactivated fetal bovine serum. All media were also supplemented with 100 U/ml penicillin (Beyotime, Nantong, China) and 100 μ g/ml streptomycin (Beyotime, Nantong, China), and maintained in a humidified atmosphere of 95% air/5% CO₂ at 37°C.

Animals

Male Kunming mice with body weight ranging from 18 to 22 g were provided by the animals facility at China Pharmaceutical University (No. SCXK (Su) 2002-0011). Animals were maintained in a pathogen-free environment ($23 \pm 2^{\circ}\text{C}$, $55 \pm 5\%$ humidity) on a 12 h light/12 h dark cycle with food and water supplied ad libitum throughout the experimental period.

Cell viability assay

Cells were plated at density of $10^5/\text{ml}$ per well in 96-well plates. After overnight growth, cells were exposed to medium containing LYG-202 for 6 h and then treated with or without 50 ng/ml TNF- α for another 18 h at 37°C . Then the cell viability was determined with the MTT method according to previous description (Chen et al., 2010a).

Combined effect evaluation

Drug interaction between LYG-202 and TNF- α was assessed at a fixed concentration ratio of 1:1. The combination index (CI) was calculated as follows:

$$\text{CI} = (\text{D})_1 / (\text{D}_\text{X})_1 + (\text{D})_2 / (\text{D}_\text{X})_2$$

where $(\text{D}_\text{X})_1$ and $(\text{D}_\text{X})_2$ are the doses for LYG-202 and TNF- α in a combination that inhibits 50% cell growth, and $(\text{D})_1$ and $(\text{D})_2$ are the doses for each drug alone that inhibit 50% cell growth. $\text{CI} < 1$, $\text{CI} = 1$, and $\text{CI} > 1$ indicated synergistic, additive and antagonistic effects, respectively (Chou, 2006). Data analysis was performed by the Calcsyn software (Biosoft, Oxford, UK).

Cell morphology

HepG2 cells were seeded at 5×10^5 cells/well in 6-well plates and grown for 24 h to attach themselves to the surface of the plates completely. Briefly, cells were incubated with 2 μ M LYG-202 for 6 h and then treated with or without 50 ng/ml TNF- α for another 18 h at 37°C, and live cells were taken a picture under the inverted light microscope. And their nuclei were photographed under fluorescent microscopy (Olympus, Japan) with a peak excitation wave length of 340 nm after they were stained with DAPI as described previously (Zhang et al., 2010).

Apoptosis assessment

The apoptosis induced by TNF- α alone or with LYG-202 was detected by Annexin V-FITC apoptosis detection kit (BioVision, CA, USA) according to the manufacturer's protocol. Briefly, cells were pretreated with LYG-202 2 μ M for 6 h, treated with 50 ng/ml TNF- α for 18 h. Then the cells were collected, resuspended in binding buffer (pH 7.5, 10 mM HEPES, 2.5 mM CaCl₂ and 140 mM NaCl), and incubated with Annexin V-FITC and then PI for 10 min in the dark at room temperature, cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA) and a computer station running Cell-Quest software (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis

HepG2 cells were incubated with 2 μ M LYG-202 for 6 h and then treated with or without 50 ng/ml TNF- α for another 18 h at 37°C and cells were collected. Western blot analysis for proteins was performed according to our previous methods (Chen et al., 2010b).

Electrophoretic mobility shift assay (EMSA)

To assess the effect of LYG-202 on TNF- α -induced NF- κ B activation, electrophoretic mobility shift assay (EMSA) was performed with Chemiluminescent EMSA Kit (Beyotime, Nantong, China) following the manufacture's protocol. Briefly, nuclear extracts (5 μ g/sample) were incubated with biotin-labeled oligonucleotides, 5'-AGT TGA **GGG GAC TTT CCC** AGG C-3' and 3'-TCA ACT **CCC CTG AAA GGG** TCC G-5' (Boldface indicates NF- κ B binding sites) in reaction buffers, for 30 min at room temperature. DNA-protein complex was separated from free oligonucleotide on 6% native polyacrylamide gels. The gels were visualized with Bio-Rad Infrared system and quantitated using Image labTM software (Bio-Rad, Berkeley, CA, USA).

Cell transfection and luciferase reporter assay

The effect of LYG-202 on NF- κ B-dependent reporter gene transcription induced by TNF- α was analyzed by luciferase reporter gene assays. HepG2 cells (5×10^5 cells/well) were plated in 6-well plates and transfected transiently with the pNF- κ B-luc (Beyotime, Nantong, China) containing four NF- κ B binding motifs (GGGAATTTC) using Lipofectamine 2000TM reagent (Invitrogen, CA) according to the manufacture's instruction. The pCDNA3.2 plasmid was added to make the total amount of DNA equal (4 μ g/well in a 6-well plate) while GFP served as normalization control. And then the cells were treated for 6 h with LYG-202 and then stimulated with TNF- α (50 ng/ml) for another 18 h. Luciferase assays were performed with the Luciferase Reporter Gene Assay kit (Promega, Madison, WI, USA) and detected using Luminoskan ascent (Thermo,

Waltham, MA, USA).

Chromatin immunoprecipitation (CHIP) assay

CHIP assay generally followed the recommendations as previous description with some modifications. HepG2 cells were incubated with 2 μ M LYG-202 for 6 h and then treated with 50 ng/ml TNF- α for the indicated time. Cells were then cross-linked with formaldehyde, quenched with glycine, sonicated on ice and centrifuged at 4°C. Mixtures were incubated with anti-p65 or pre-immune IgG with rotation at 4°C overnight and then incubated with Protein A+G Agarose at 4°C for 6 h. Finally, immune complexes were captured by Protein A+G Agarose and eluted with elution buffer (1% SDS, 0.1 M NaHCO₃) at 37°C for 30 min. Cross-linking was reversed at 65°C for 4 h in a high salt buffer (0.2 M NaCl, 50 mM Tris pH 6.5, 10 mM EDTA, 0.2 mg/ml Proteinase K). Extracted and dissolved immunoprecipitated DNA was quantified by Real-time PCR with primers encompassing the NF- κ B binding sites. Primers for COX-2 promoter quantification were 5'-TCT GGC GGA AAC CTG TGC GCT GG-3' and 5'-AAA TTG CGT AAG CCC GGT GGG-3' (forward and reverse, respectively), and for MMP-9 promoter quantification 5'-CAG TGG AAT TCC CCA GCC TTG CCT-3' and 5'-CCA CAC TCC AGG CTC TGT CCT C-3'. An equal volume of non-precipitated (input) genomic DNA was used to correct for the differences in PCR amplification efficiencies and amounts of DNA. The PCR analyses were performed by Real-time PCR kit (TaKaRa Biotechnology Co. Ltd., Dalian, China).

Immunocytochemical localization of NF- κ B/p65 subunit

The effect of LYG-202 on the nuclear translocation of p65 was examined by immunocytochemistry as previously described. Briefly, cells were pretreated with 2 μ M LYG-202 for 6 h following seeded on a gelatin-coated glass, after stimulation with or without 50 ng/ml TNF- α for 30 min, fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. After washing with PBS, the fixed cells were blocked with 3% bovine serum albumin for 1 h and then incubated with rabbit polyclonal anti-p65 antibody (1:50) overnight at 4°C, washed with PBS and incubated with anti-rabbit IgG-FITC (1:100) for 1 h, and counterstained for nuclei with fluorochrome dye DAPI (Santa Cruz Biotechnology, USA). Stained slides were observed under a fluorescence microscopy (Olympus IX51, Japan) with a peak excitation wave length of 340 nm.

IKK β activity assay

The effect of LYG-202 on IKK β activity was performed using the HTScan IKK β kinase assay kit (Cell Signaling Technology, Beverly, MA) as previously described (Sun et al., 2010).

Molecular Docking

We used our protein-ligand docking software package GOLD to dock LYG-202 into many potential drug targets which could activate IKK β activity (Hayden and Ghosh, 2004; Schmid and Birbach, 2008). Molecular docking was performed following the Sun's method (Sun et al., 2010). We then ranked each protein according to the GOLD fitness score. Inhibitors of the top targeted protein were considered to have the similar effects of LYG-202 on TNF- α -induced apoptosis, which could be detected in the biological test.

Previous studies show that binding site was defined by analyzing the protein-ligand interactions of co-crystal structures which were deposited in PDB (<http://www.rcsb.org/pdb>). Molecular interactions were observed using Discovery Studio (Accelrys, SD, USA). In the study, LYG-202 was detected for its interaction with major binding sites of selected proteins.

CK2 activity Assay

The CK2 activity treated with TNF- α alone or in combination with LYG-202 was detected with the CK2 assay kit (MBL International, Woburn, MA, USA) according to manufacturer's instruction (Sun et al., 2010).

Down-regulation of CK2 by short interfering RNA

HepG2 cells were plated at 5×10^5 cells/well in 6-well plates and allowed to adhere for 24 hours. siRNA transfections were performed according to the manufacturer's instructions of Lipofectamine 2000TM reagent (Invitrogen, CA). After that, HepG2 cells preincubated with 2 μ M LYG-202 for 6 h and then treated with or without 50 ng/ml TNF- α for another 18 h at 37°C were recovered and used for appropriate determinations.

Real-time PCR analysis

Total RNA was extracted using TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany), and then amplified by polymerase chain reactions (PCR). The primer sets used in the PCR amplification were as follows: CK2 α (forward: 5'-TCA CAG CAG CAT GGG AAT TAT GCA C-3', reverse: 5'-AGC AAC TCG GAC ATT ATA TTC

TTG G -3'). The relative expression of CK2 α was analyzed using quantitative RT-PCR with GAPDH as an internal control as previously described (Lu et al., 2012).

Murine xenograft tumor assay

Murine hepatoma 22 (H₂₂) cells were diluted with icy 0.9% saline and inoculated subcutaneously at right axilla of mice (5 \times 10⁶ viable cells/ml) (Wang et al., 2008; Zhao et al., 2009). Twenty-four hours after inoculation, mice were divided randomly into eleven groups (with 10 mice/group): saline tumor control group; TNF- α 1.5 μ g/day group; LYG-202 750, 500, 250 and 125 mg/kg; LYG-202 + TNF- α combination group; HCPT 30 mg/kg group. Mice were administered orally by LYG-202 or HCPT and injected subcutaneously by TNF- α , which were given once every two days. From the third day after treatment, the tumors were measured continuously. Tumor volume (TV) was calculated using the following formula:

TV (mm³) = $d^2 \times D/2$; where d and D were the shortest and the longest diameter, respectively.

At the seventh day after treatment, the mice were sacrificed and tumors were ablated carefully and weighed after washing off any remaining blood with PBS. Tumor inhibitory ratio was calculated by the following formula:

$$\text{Tumor inhibitory ratio (\%)} = [(W_{\text{Control}} - W_{\text{Treated}})/W_{\text{Control}}] \times 100\%$$

W_{Treated} and W_{Control} were the average tumor weight of the treated and control mice respectively. This study was approved in SPF animal laboratory of China Pharmaceutical University.

The coefficient of drug interaction (CDI) was used to analyze effects of drug

combinations (Cao and Zhen, 1989). CDI is calculated as follows: $CDI = AB/(A \times B)$.

According to the tumor weight of each group, AB is the ratio of the combination groups to control group; A or B is the ratio of the single agent group to control group. When $CDI < 1$, synergism is indicated. When $CDI = 1$, summation is indicated. When $CDI > 1$, antagonism is indicated. CDI less than 0.75 indicates that the drugs are significantly synergistic.

Histology

Hepatoma tumors were fixed in 10% formalin and processed routinely for paraffin embedding. Tissue sections were prepared with a microtome and placed on glass slides. TUNEL assay was performed to detect apoptotic cells using the TUNEL detection kit (KeyGEN, Nanjing, China) according to the manufacture's instructions.

Statistical analyses

All quantitative data are presented as the mean \pm SD from at least three samples per data point. Statistical analyses were performed using an unpaired, two-tailed Student's t-test. All comparisons are made relative to untreated controls and significance of difference is indicated as $*P < 0.05$ and $**P < 0.01$.

Results

LYG-202 enhances TNF- α -induced growth inhibition and apoptosis

The inhibitory effects of LYG-202 and TNF- α on cell viability in HepG2 cells were

assessed after incubation with increasing concentrations of LYG-202 and TNF- α for 24 h. Results indicated that LYG-202 and TNF- α showed inhibitory effect in a concentration-dependent manner (Fig. 1B) and the IC₅₀ of LYG-202 and TNF- α was 262.02 ± 12.52 ng/ml and 5.98 ± 0.65 μ M, respectively. To investigate the inhibitory effect of the combination treatment, cells were exposed to LYG-202 for 6 h and then incubated with TNF- α for 18 h at a fixed ratio (LYG-202 IC₅₀: TNF- α IC₅₀ ratios were 1:1) (Chou, 2006). Data in Fig. 1C showed that the CI values were <1 when the values of fraction affects (Fa) were smaller than 0.409 (at the point, the concentration of LYG-202 and TNF- α was about 2.05 μ M and 88.91 ng/ml, respectively), which indicated that the combination of TNF- α and LYG-202 exerted a synergistic inhibitory effect on cell proliferation in HepG2 cells at concentrations smaller than the threshold values (drug concentrations when CI = 1).

To further determine the effect of LYG-202 on TNF- α -induced growth inhibition of HepG2 cells, we treated HepG2 cells with different concentration of TNF- α , LYG-202 alone or in combination. Our results indicated that it had little effect on the growth of HepG2 treated with TNF- α alone while moderate inhibitory effect with LYG-202 alone, and in the presence of a moderate inhibitory dose of TNF- α (50 ng/ml) or LYG-202 (2 μ M), the combination was highly effective on HepG2 cells growth (Fig. 1D). Similar effects were observed in SMMC-7721 (Fig. 1E) and BEL-7402 cells (Fig. 1F).

The cell viability of TNF- α alone (50 ng/ml), LYG-202 alone (2 μ M), and the combination was 98.3%, 95.6%, and 65.5%, respectively in HepG2 cells. These results suggested the significant synergy of TNF- α and LYG-202 on the inhibition of HepG2 cell proliferation. Similar results were also found in LYG-202 combination with TNF- α in

different hepatocellular carcinoma cell lines (Fig. 1G).

Then we analyzed the effect of LYG-202 on TNF- α -induced apoptosis, which is one of major ways for cell death (Bosman et al., 1996), with DAPI and Annexin V/PI double staining. Results indicated that the live cells were obviously decreased (Fig. 1H, top row), and the number of cells in which the chromatin condensed increased (Fig. 1H, bottom row). Moreover, Annexin V/PI double staining analysis indicated that LYG-202 potentiated TNF- α -induced early apoptosis from 0.72% to 19.43% and late apoptosis strikingly from 2.01% to 40.62% (Fig. 1I and J), suggesting a synergistic effect of LYG-202 with TNF- α in inducing cell apoptosis.

LYG-202 potentiated TNF- α -induced caspase activation, Bid and PARP cleavage in HepG2 cells

To understand the role of caspases in TNF- α -mediated apoptosis with or without LYG-202, Western blot was performed. We found that TNF- α alone had a minimal effect on the protein level of cleavage caspase-8 and -3/9, their activated form, and no effect on procaspase-8 and -3/9. In the presence of LYG-202, however, the cleaved products markedly accumulated while the level of procaspase-8 and -3/9 decreased in a time-dependent manner (Fig. 2A).

Bid is a specific proximal substrate of caspase-8 and both of their cleavage can be found as the feature of TNF- α lethality (Li et al., 1998; Perez and White, 2000). The proteolytic cleavage of PARP, a substrate of caspase-3, has been considered as a hallmark of apoptosis (Los et al., 2002). So the cleavages of Bid and PARP were also tested. Western blot analysis suggested that either of TNF- α and LYG-202 had no effect on Bid

and PARP cleavage but they together were very effective in inducing cleavages of Bid and PARP (Fig. 2A).

These results indicated that LYG-202 can facilitate the activation of caspase and cleavages of Bid and PARP in TNF- α -induced apoptotic pathway.

LYG-202 suppressed the expression of NF- κ B-regulated gene products in HepG2 cells

We know that TNF- α triggers both the caspase-protease pathway and the NF- κ B pathway, and the balance of these two pathways is critical for the ultimate fate of a cell: death or survival (Brown et al., 2010). These findings prompted us that LYG-202 could enhance TNF- α -induced apoptosis by attenuating the NF- κ B pathway.

To examine the effect of LYG-202 on the NF- κ B pathway, we analyzed the expression of its downstream genes involved in anti-apoptosis (Survivin, XIAP, Bcl-2 and Bcl-xL), proliferation (Cyclin D1, COX-2 and c-Myc) and invasion (MMP-9). As shown in Fig. 2B, in the absence of LYG-202, TNF- α induced the expression of survival and invasion genes including Survivin, XIAP, Bcl-2, COX-2, MMP-9 in a time-dependent manner. Interestingly, the level of Cyclin D1 and c-Myc increased for the first 12 h but decreased at 24h while that of Bcl-xL had little change. However, in the presence of LYG-202, the increase of these gene expressions was weakened, even reversed. Pretreatment with Z-VAD-FMK, a caspase inhibitor, could hardly block the down-regulation of NF- κ B-mediated gene expression, suggested that the down-regulation of various target proteins was not caspase-dependent and may be upstream of the apoptotic pathway.

These results indicated that LYG-202 inhibited the target gene expression of NF- κ B, implying that it facilitated TNF- α -induced apoptosis by blocking NF- κ B signaling.

LYG-202 inhibited TNF- α -dependent NF- κ B activation in HepG2 cells

To further understand the role of NF- κ B in TNF- α -induced apoptosis of HepG2 cells treated with LYG-202, the DNA-binding activity of NF- κ B complex in nuclear extracts was evaluated by EMSA. As shown in Fig. 3, LYG-202 alone had minimal effect on NF- κ B activation at different doses and time-courses (Fig. 3A, left column) while significantly inhibited TNF- α -induced NF- κ B activation in dose and time-dependent manners (Fig. 3A, right column).

LYG-202 repressed TNF- α -activated NF- κ B-dependent gene transcription

To determine the effect of LYG-202 on NF- κ B-dependent gene transcription directly, the p65-luciferase reporter gene assay was conducted out. Results indicated that LYG-202 inhibited TNF- α -induced p65 reporter gene activity in a dose-dependent manner (Fig. 3B).

To further confirm this data, we performed CHIP assay to test the activity of NF- κ B binding to promoters of COX-2 and MMP-9, two NF- κ B target genes. Results showed that TNF- α induced this activity in a time-dependent manner while LYG-202 abolished it (Fig. 3C).

These findings suggested that LYG-202 strongly inhibited TNF- α -induced NF- κ B-dependent gene transcription by interfering the binding of NF- κ B to its target gene promoters in HepG2 cells.

LYG-202 inhibited TNF- α -induced nuclear translocation of p65

The nucleus localization of p65, a subunit of NF- κ B complex, is essential for the

transcriptional activity of NF- κ B (Mocellin et al., 2005), so nuclear and cytoplasmic extracts were performed to examine the nuclear translocation of p65. Results showed that TNF- α -induced nuclear translocation of p65 was detected in 15 minutes and lasted for more than 1 h (Fig. 4A, left column) while was inhibited by LYG-202 (Fig. 4A, right column). And the similar data was also observed in the immunofluorescence assay (Fig. 4B). These results suggested that LYG-202 interferes with TNF- α -induced p65, a subunit of NF- κ B, nuclear translocation in HepG2 cells.

LYG-202 inhibited TNF- α -mediated I κ B α phosphorylation, degradation and IKK β activity

NF- κ B activates the transcription of target genes by binding with a consensus sequence in the nucleus, and the translocation of NF- κ B to the nucleus is preceded by the phosphorylation, ubiquitination and proteolytic degradation of I κ B (Karin and Ben-Neriah, 2000). We determined whether LYG-202 inhibitory activity was due to inhibition of I κ B α phosphorylation and degradation. Western blot analysis indicated that TNF- α induced the phosphorylation of I κ B α within 15 min while LYG-202 at 2 μ M blocked its phosphorylation (Fig. 4C, the top row) and suppressed its degradation induced by TNF- α (Fig. 4C, the middle row).

The facts that TNF- α -induced I κ B α phosphorylation requires IKK β (Bocker et al., 2008; Hayden and Ghosh, 2004; Schmid and Birbach, 2008) and is inhibited by LYG-202 prompted us to determine the effect of LYG-202 on IKK β activation. Results from the IKK β kinase assay showed that LYG-202 obviously suppressed IKK β activity in a dose-dependent manner (Fig. 4D).

These results demonstrate that LYG-202 inhibited TNF- α -induced I κ B α phosphorylation, degradation and IKK β activity.

Docking analysis

After docking LYG-202 to each structure of the PDB, we ranked 11 target proteins that could activate IKK β activity according to the Gold fitness score (Table. 1). Among these proteins, the crystal structure of human Casein Kinase 2 (CK2) holoenzyme (PDB entry: 1ZOG) was the highest-ranked human protein with the fitness score of 47.8621, compared to the similar score of 48.2899 of potent CK2 inhibitor CX-4945 (Pierre et al., 2010; Siddiqui-Jain et al., 2010). The results demonstrated that CX-4945 could enhance TNF- α -induced apoptosis (Supplemental Fig. 1B) and inhibit NF- κ B activation (Supplemental Fig. 1C). These effects of CX-4945 are similar to the corresponding characteristic effects of LYG-202, implying that CK2 may be a promising target of LYG-202 in blocking NF- κ B signaling activation.

Previous studies show that binding site was defined by analyzing the protein-ligand interactions of all 23 CK2 co-crystal structures which were deposited in PDB. All the ligands in the complex structures showed the hydrophobic interactions with Val53, Val66 and Ile174, which play a crucial role in CK2 inhibition. Additionally, Arg47, Lys68, Phe113, Val116, Asn118, Met163 and Asp175 are also very important in the binding interactions of CK2 and its ligands. Docking study showed that LYG-202 formed two hydrogen bonds with ASP175 and Asn118, the two key residues located in the polar region and hinge region, respectively, which were essential for CK2 activity. Additionally, LYG-202 showed favorable hydrophobic interaction with ARG43 in the binding pocket of CK2 (Fig. 5A). The results indicated that LYG-202 could be considered as a novel

CK2 inhibitor which should be confirmed in the further biological test.

Effect of LYG-202 on TNF- α -induced CK2 activity

Protein kinase CK2 is a Ser/Thr kinase involved in various steps of TNF- α -induced NF- κ B activation process. We investigated that whether LYG-202 directly inhibits TNF- α -induced CK2 activity. Results from the CK2 kinase assay demonstrated that TNF- α induced the activation of CK2 and LYG-202 suppressed TNF- α -mediated CK2 activity compared with CX-4945 (Fig. 5B). Neither TNF- α nor LYG-202 affected the expression of CK2 protein subunits (Fig. 5C).

Transfection of protein kinase CK2 reverses the effect of LYG-202

It was reported that CK2 could modulate IKK β -mediated phosphorylation I κ B (Yu et al., 2006). And molecular docking analysis showed that LYG-202 had hydrogen bonds with ASP175 and Asn118, which were essential for CK2 activity (Sun et al., 2010), in the binding pocket of CK2; moreover, it inhibited TNF- α -induced CK2 activity but had no effect on the expression (Fig. 5B and C).

To explore the potential role of CK2 in NF- κ B suppression induced by LYG-202, we detect the expression of CK2 in L02, HepG2, HCT116 cell lines and transiently transfected CK2 α (the catalytic subunit) and CK2 β (the regulatory subunit) into cells to determine whether the elevated CK2 activity affects the activity of LYG-202. The results in Fig. 6A show that the expression of CK2 in L02 and HepG2 cells is lower than HCT116 cell with known high CK2 protein level, suggesting that we can transfect the HepG2 cells with CK2 α /CK2 β plasmids to increase CK2 α activity.

As shown in Fig. 6B, transfection of either CK2 α or CK2 β subunits or both give rise to

the expected patterns with the anti-Myc antibody. A 75%, 20% and 102% increase of CK2 activities from HepG2 cells that were transfected with CK2 α , CK2 β and both was observed when compared with control (Fig. 6C). Results in Fig. 6D shown that the IKK β kinase activity had a little change in cells transfected with CK2 β alone, but transfection of CK2 α or both led to a 30% and 54% increase of IKK β kinase activities. These data provide evidence that both are required for NF- κ B activation. In the following experiment, both of them were transfected to activate NF- κ B.

Then we tested the effect of LYG-202 on the NF- κ B-dependent gene transcription and TNF- α -induced apoptosis in the absence or presence of CK2 α and CK2 β . Data indicated that the co-transfection of two subunits reversed, at least partially, the inhibitory effect of LYG-202 on TNF- α -induced NF- κ B-dependent gene transcription (Fig. 6E), consequently diminished the effect of LYG-202 on TNF- α -induced apoptosis (Fig. 6F and G). These results implied that CK2 was involved in LYG-202 suppression of TNF- α -induced NF- κ B activation in HepG2 cells. The results in Supplemental Fig. 2A show that CK2 siRNA down-regulated the mRNA of CK2 for nearly 75% compare with scrambled control. The data in Supplemental Fig. 2B and C indicated that transfection of CK2 siRNA in HepG2 cells exhibiting a significant decrease in CK2 kinase activity displayed lower level of IKK β kinase activity compared with control cells.

Furthermore, transfection of CK2 siRNA mimics the effect of LYG-202 on NF- κ B activation (Supplemental Fig. 2D) and TNF- α -induced cell apoptosis (Supplemental Fig. 2E and F). These findings indicated that CK2 is a promising target of LYG-202 in suppression of NF- κ B activation and plays a crucial role in the signal transduction that promotes cell growth and survival in HepG2 cells.

LYG-202 inhibits hepatoma tumor growth in the H₂₂ murine tumor model by suppressing CK2 activity and NF- κ B-mediated anti-apoptosis protein expression in the presence of TNF- α *in vivo*

Base on the results above, we proposed that LYG-202 sensitized hepatoma to TNF- α by attenuating CK2 and NF- κ B activity. To demonstrate this hypothesis, we examined the effects of LYG-202 on the growth of H₂₂ murine solid tumor and cell apoptosis and CK2-dependent NF- κ B activation with or without the administration of TNF- α *in vivo*.

The results showed a decrease in tumor volume (Fig. 7A) and an increased inhibitory effect (Fig. 7B and Table. 2) in combination with LYG-202 (750, 500, 250 and 125 mg/kg) and TNF- α compared to LYG-202 or TNF- α alone, suggesting that treatment with LYG-202 and TNF- α together had more potential than that alone in a mouse hepatoma H22 solid tumor model. As shown in Table. 2, the CDI of combination-treated groups of LYG-202 (125 mg/kg) was smaller than 0.75, whereas the CDI of LYG-202 (750, 500 and 250 mg/kg) combined with TNF- α were between 0.75 and 1, indicating that the combination of LYG-202 (125 mg/kg) and TNF- α have significantly synergistic effect.

To further uncover whether LYG-202 potentiates cancer cell apoptosis induced by TNF- α *in vivo*, we stained the solid tumor sections with TUNEL apoptosis staining kit. As shown in Fig. 7C, application of LYG-202 in mouse tumor model significantly increased TNF- α -induced apoptosis in a dose-dependent manner. Furthermore, the expression levels of the NF- κ B regulated anti-apoptosis genes including Survivin, XIAP, Bcl-2 and Bcl-xL were significantly decreased (Fig. 7D) *in vivo*. And the decrease of CK2 activity (Fig. 7E) suggested that LYG-202 also had an inhibitory effect on CK2 activity *in vivo*.

These results indicated LYG-202 sensitized mouse hepatoma to TNF- α in mouse H₂₂ hepatoma tumor model.

Discussion

Tumor necrosis factor-alpha (TNF- α) is being utilized as an anticancer agent for the locoregional treatment (Balkwill, 2009; Mocellin et al., 2005). However, its role in cancer therapy is debated for its paradoxical activity: the caspases cascade for apoptosis induction and NF- κ B signal for cell survival promotion (Mocellin et al., 2005; Sethi et al., 2008; van Horssen et al., 2006). So it is a good way to explore agents that enhance TNF- α induced apoptosis by attenuating NF- κ B activation. Molecular docking analysis and biological test showed that LYG-202, a known synthesized flavonoid derivative, probably attenuated NF- κ B signal via the inhibition of CK2 activity and may serve as a candidate compound for the combination with TNF- α . In this study, we tested whether LYG-202 could sensitize hepatoma cancer to the treatment of TNF- α .

In human hepatoma HepG2 cells, which are reported to be resistant against TNF- α (Sugimoto et al., 1999), TNF- α alone had little effect on inhibition of proliferation and induction of apoptosis. Intriguingly, we even noticed that the cell fragment percentage slightly decreased compared with control group. However, combination with LYG-202 significantly enhanced its anti-proliferation and pro-apoptosis activities (Fig. 1). Previous studies showed that NF- κ B is the major survival factor in preventing TNF- α -induced apoptosis, and inhibition of this transcription factor may improve the efficacy of apoptosis-inducing cancer therapies (Balkwill, 2009; Horssen, 2006; Mocellin et al., 2005; Orłowski and Baldwin, 2002; Pilati et al., 2008). Our results indicated that the protein

expression of NF- κ B target genes including Survivin, XIAP, Bcl-2/Bcl-xL, c-Myc, COX-2, Cyclin D1 obviously decreased in the presence of LYG-202 (Fig. 2B), and the further findings demonstrated that LYG-202 suppressed the NF- κ B transcription activity by disturbing bond to its response elements (Fig. 3). We also found that LYG-202 inhibited TNF- α -induced phosphorylation and degradation of I κ B α (Fig. 4C), a critical step for NF- κ B translocation into nucleus (Zandi et al., 1997), by attenuating IKK β activity (Fig. 4D). All these observations suggested that LYG-202 enhanced TNF- α -induced apoptosis by weakening NF- κ B activation.

CK2 is a highly conserved and ubiquitous serine/threonine kinase with a wide range of substrates involved in carcinogenesis and tumor progression (Litchfield, 2003; Yu et al., 2006). And CK2 has also been found to be increased protein expression levels and nuclear localization in cancer cells compared with their normal counterparts. These findings promote CK2 as an unfavorable pronostic marker in several cancers and consequently as a relevant therapeutic target (Prudent et al., 2009; Sun et al., 2010; Trembley et al., 2010). Recent studies report that CK2 contributes to the aberrant NF- κ B activation though enhancing IKK β activity for the phosphorylation of S32/S36 NH₂ terminus of I κ B α , a novel and distinct function from that of CK2 as a known COOH-terminal IKK β (Brown et al., 2010; Yu et al., 2006). Inverse docking indicated that CK2 may be a potent target of LYG-202 in blocking NF- κ B activation. Molecular docking analysis showed that LYG-202 formed hydrogen bonds with ASN118 and ASP175, the key residues located in the hinge region and polar region respectively in the active site of CK2, indicating that LYG-202 could bind stably with CK2 (Fig. 5A). These results indicated that LYG-202 could be a potent and selective CK2 inhibitor and

probably attenuate CK2-mediated NF- κ B signal. Here, we found that LYG-202 inhibited CK2 activity while had no effect on its protein level (Fig. 5B and C) and overexpression CK2 partially reversed the LYG-202 inhibitory effect on the TNF- α -induced DNA-binding activity of NF- κ B in HepG2 cells (Fig. 6E). Moreover, the apoptotic cell percentage of HepG2 treated with TNF- α and LYG-202 reduced in the presence of CK2 α and CK2 β subunits which continuously active CK2 activity (Fig. 6F and G).

However, our studies showed that LYG-202 had little effect on NF- κ B activation and apoptosis, when CK2 expression was almost completely inhibited by transfecting with CK2 siRNA (Supplemental Fig. 2A). The transfection of CK2 siRNA inhibited TNF- α -induced DNA-binding activity of NF- κ B (Supplemental Fig. 2D), induced HepG2 cells apoptosis and mimicked the apoptotic potential of LYG-202 combined with TNF- α (Supplemental Fig. 2E and F). These evidences suggest that CK2 played a crucial role in signal transduction that promotes HepG2 cells growth and survival, and may serve as a target of LYG-202 for the promotional effect on TNF- α -induced apoptosis. Apigenin and other common flavonoids which have been reported to be selective CK2 kinase inhibitors suppress proliferation in a wide variety of solid tumors and hematological cancers (Davies et al., 2000; Zhao et al., 2011), show significant inhibitory effect on CK2 activity and NF- κ B activation (Farah et al., 2003). However, the possibility that LYG-202 could be a potent CK2 kinase inhibitor requires further assessment.

Increased tumor vessels permeability is currently believed to be critical for TNF- α anti-tumor activity. TNF- α improves the pharmacokinetic profile of co-administered drugs by increasing the permeability of tumor vessels and lowering interstitial pressure within the diseased tissue (Lejeune, 2002), which in turn augments drug concentration

within the tumor microenvironment (de Wilt et al., 2000; van der Veen et al., 2000). We found that LYG-202 exerted anti-angiogenic activity both in vitro and in vivo (Chen et al., 2010b), which could be another explanation for the synergy between LYG-202 and TNF- α . Whether the effective contribution of TNF- α known to target tumor vasculature could promote the pro-apoptotic activity of LYG-202 needs to be investigated to fully realize its clinical potential.

Reactive oxygen species (ROS) generation in mitochondria is believed to play a key role in TNF- α -induced apoptosis (Goossens et al., 1995; Schulze-Osthoff et al., 1992). Previous studies have reported that LYG-202 induced HepG2 cells apoptosis through accumulation of ROS and its downstream signaling pathway (Chen et al., 2010a). Thus, the role of generation of ROS in HepG2 cell death induced by TNF- α combined with LYG-202 is worth further investigation.

A number of studies have shown that levels of TNF- α are elevated in the plasma of patients with alcoholic hepatitis (Bird et al., 1990; Miro et al., 1999). We found that LYG-202 with the combination treatment of TNF- α effectively suppressed H₂₂ murine solid tumor growth, induced tumor apoptosis, down-regulated anti-apoptosis genes in xenograft mouse models (Fig. 7). These results suggested that LYG-202 could be an effective agent in the treatment of liver cancer patients with the high level of TNF- α secretion. However, whether the combination effect of LYG-202 with TNF- α *in vivo* mainly via inhibiting CK2 activity would be further investigated, probably using a CK2 α knock-out mouse model.

In this study, we demonstrated that LYG-202 enhanced TNF- α -induced apoptosis via attenuating CK2 activity, which lead to the abrogation of NF- κ B activation in vitro and in

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vivo. These results extended our understanding on the molecular mechanisms underlying the improvement of TNF- α anti-tumor activities by LYG-202.

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Authorship Contributions

Participated in research design: Chen, Lu, and Guo

Conducted experiments: Chen, Lu, Zhang, Zhao, He and Sun

Contributed new reagents or analytic tools: Li and Sun

Performed data analysis: Chen, Lu, Zhang, Zhao, You and Guo

Wrote or contributed to the writing of the manuscript: Chen, Lu, Zhang and Guo.

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Footnotes

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F.C. and N.L. contributed equally to this work.

Legends for figures:

Fig. 1. LYG-202 accelerates TNF- α -induced apoptosis. (A) Molecular structure of LYG-202 (C₂₄H₂₉N₂O₅, MW=438.22). (B) Inhibitory effect of LYG-202 and TNF- α on cell proliferation in HepG2 cells. HepG2 cells were treated with various concentration of LYG-202 or TNF- α alone for 24 h. Cell viability was determined by the MTT method. (C) Inhibitory effect and combination index of the combination treatment of LYG-202 and TNF- α . (D) LYG-202 enhances TNF- α -induced cell growth inhibitory in HepG2 cells. TNF- α slightly inhibits cell growth in a dose-dependent manner and this inhibitory effect was significantly increased by 2 μ M LYG-202 (left). And LYG-202 inhibits cell growth in a dose-dependent manner in the presence of TNF- α at a non-growth inhibitory dose (50 ng/ml) (right). Cell viability was analyzed by the MTT method. (E-F) Potentiation of TNF- α -induced cell growth inhibitory in SMMC-7721 and BEL-7402 cells. Cell growth was analyzed by the MTT method. (G) The treatment effect of LYG-202 combined with TNF- α on cell proliferation in different hepatocellular carcinoma cell lines. The cells were pretreated with LYG-202 for 6 h, and then incubated with TNF- α for 18 h. Cell growth was then evaluated by the MTT method. (H) LYG-202 enhances TNF- α -induced apoptosis. The cells were pretreated with 2 μ M LYG-202 for 6 h and then incubated with 50 ng/ml TNF- α for 18 h. The morphology of HepG2 cells was observed by the inverted light microscope (400 \times), stained with the DAPI reagent and then analyzed under a fluorescence microscope (400 \times). (I) The cells pretreated with 2 μ M LYG-202 for 6 h and then incubated with 50 ng/ml TNF- α for 18 h were examined by Annexin V/PI double-staining assay. Y axis showed PI labeled population and X axis shows FITC-labeled Annexin V positive cells. (J) The apoptotic rate of HepG2 cells caused by

LYG-202, TNF- α and their combination treatment. Values are means \pm SD for at least three independent experiments performed in triplicate (* P < 0.05 and ** P < 0.01 compared with vehicle control).

Fig. 2. LYG-202 potentiated TNF- α -induced caspase activation and suppressed NF- κ B-regulated gene expression. The HepG2 cells were treated with 2 μ M LYG-202 for 6 h and then incubated with 50 ng/ml TNF- α for the indicated times. (A) Augmentation of TNF- α -induced caspase activation, Bid and PARP cleavages by LYG-202. Whole-cell extracts were prepared and analyzed by Western blot using the indicated antibodies. (B) LYG-202 represses TNF- α -induced NF- κ B-dependent expression of anti-apoptosis-(Survivin, XIAP, Bcl-2, Bcl-xL), proliferation-(Cyclin D1, c-Myc, COX-2) and metastasis-(MMP-9) related gene products in the absence and presence of 20 μ M Z-VAD-FMK. The results shown are representative of three independent experiments.

Fig. 3. LYG-202 inhibited TNF- α -induced NF- κ B activation. (A) Dose and Time-dependent effect of LYG-202 on TNF- α -induced NF- κ B activation. The HepG2 cells were pretreated with the indicated concentrations and time of LYG-202 and incubated with 50 ng/ml TNF- α for 30 min, and then subjected to EMSA to test for NF- κ B activation. (B) LYG-202 inhibited TNF- α -induced NF- κ B dependent reporter gene expression. The HepG2 cells were transiently transfected with a NF- κ B luciferase reporter gene. After transfection, cells were pretreated with the indicated concentrations of LYG-202 for 6 h, and then incubated with 50 ng/ml TNF- α for an additional 18 h. Cell supernatants were collected and assayed for luciferase activity as described. (C)

LYG-202 inhibited the binding of NF- κ B to the COX-2 and MMP-9 promoters. The HepG2 cells were pretreated with 2 μ M LYG-202 for 6 h and treated with 50 ng/ml TNF- α for the indicated time. The proteins were cross-linked with DNA by formaldehyde and then subjected to CHIP assay using an anti-p65 antibody with the COX-2 and MMP-9 primers. Values are means \pm SD for at least three independent experiments performed in triplicate (* P < 0.05 and ** P < 0.01 compared with HepG2 cells treated with TNF- α).

Fig. 4. LYG-202 inhibits TNF- α -mediated p65 nuclear translocation, I κ B α phosphorylation and degradation and IKK β activation. (A) Effect of LYG-202 on TNF- α -induced p65 nuclear translocation. Cells were incubated with 2 μ M LYG-202 for 6 h and treated with 50 ng/ml TNF- α for the indicated times. Cytoplasmic extracts (CE) and nuclear extracts (NE) were prepared, fractionated on SDS-PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using the indicated antibodies. (B) Immunofluorescence analysis of p65 localization. Cells were incubated with 2 μ M LYG-202 for 6 h and then treated with 50 ng/ml TNF- α for 30 min. Cells were subjected to immunocytochemical analysis. (C) Effect of LYG-202 on the phosphorylation and degradation of I κ B α by TNF- α . Cells were preincubated with 2 μ M LYG-202 for 6 h and then treated with 50 ng/ml TNF- α for 30 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using the anti-I κ B α and anti-p-I κ B α antibodies. (D) LYG-202 inhibits exogenous IKK β activity. The results shown are representative of three independent experiments.

Fig. 5. LYG-202 reduced TNF- α -induced CK2 α kinase activity. (A) The binding mode, docking score of LYG-202 on CK2 α . (B-C) The effect of LYG-202 on TNF- α -induced CK2 activity and expression in HepG2 cells. Data are presented as mean \pm SD for at least three independent experiments performed in triplicate (* P < 0.05 and ** P < 0.01 compared with vehicle control).

Fig. 6. Transfection of CK2 subunits diminished TNF- α -induced apoptosis. (A) The expression of CK2 in L02, HepG2 and HCT116 cells. (B) Transient overexpression of CK2 subunits in HepG2 cells. HepG2 cells were transfected with empty vector, Myc-His-tagged CK2 α , Myc-His-tagged CK2 β , or co-transfected with Myc-His-tagged CK2 α plus Myc-His-tagged CK2 β . Cells lysates were examined by Western Blot using the monoclonal anti-Myc antibody. (C-D) Transient overexpression of CK2 subunits increases CK2 and downstream IKK β activity. (E) Overexpression of CK2 subunits increased the NF- κ B-dependent reporter gene expression induced by LYG-202. The HepG2 cells were transiently co-transfected with the CK2 subunits plasmids along with a NF- κ B luciferase reporter gene. (F) Co-transfection of CK2 subunits reduced the TNF- α -induced apoptosis. (G) The apoptotic rates of transfected HepG2 cells induced by TNF- α and its combination with LYG-202. Values are means \pm SD for at least three independent experiments performed in triplicate.

Fig. 7. LYG-202 suppresses H22 solid tumor growth, induces apoptosis, inhibits CK2 activity and NF- κ B-regulated anti-apoptotic gene expression with the presence of TNF- α in vivo. (A) LYG-202 inhibited H22 solid tumor growth in xenograft mouse model in the

doses of 750, 500, 250, 125 mg/kg with the administration of TNF- α (1.5 μ g/day). Tumor volume significantly decreased in combination treatment with LYG-202 and TNF- α compared to control group. (B) The inhibitory effect of LYG-202 alone or together with TNF- α on the growth of H₂₂ murine solid tumor. Column, mean; bar, SD (n = 3, **P* < 0.05 and ***P* < 0.01 versus control). (C) LYG-202 facilitated TNF- α -induced tumor apoptosis in xenograft mouse model. Solid tumor were fixed and embedded with paraffin. The 0.5 mm sections were stained with specific TUNEL staining kit (arrowheads indicate the TUNEL signals, magnification, $\times 100$). (D) LYG-202 suppressed the expression levels of NF- κ B-regulated anti-apoptotic gene activated by TNF- α . (E) LYG-202 quenched CK2 activity in absence or presence of TNF- α in vivo. The results shown are representative of three independent experiments. Column, mean; bar, SD (n = 3, **P* < 0.05 and ***P* < 0.01 versus control).

Fig. 8. A schematic diagram of mechanism shows that LYG-202 enhances the anti-tumor activity of TNF- α by attenuating CK2-dependent NF- κ B activation in HepG2 cells. Theoretically, LYG-202 or TNF- α alone can induce HepG2 cell death via a caspase-dependant apoptotic pathway. The apoptotic effect of TNF- α is blocked due to NF- κ B-mediated anti-apoptosis gene expression. LYG-202 can suppress NF- κ B activation via its inhibition of CK2 activity and therefore enhances TNF- α -induced apoptosis.

Table 1. The 11 kinase structures on the upstream of IKK β activation targeted by LYG-202 according to inverse docking. LYG-202 was docking to the structures from the PDB. These kinase structures with the most favorable docking scores are listed in descending order.

PDB ID	Kinase	Score	Rank
1ZOG	CK2	47.8621	1
1HE8	PI3K	39.0231	2
3EQF	MEKK1	38.9389	3
2EVA	TAK1	34.8353	4
3CQW	AKT	32.9431	5
2DYL	MAP3K7	31.5247	6
1F3V	TRAF-2	27.3698	7
1XLD	PKC- θ	26.9243	8
3V55	MALT1	26.5272	9
2G5X	RIP1	25.8782	10
2PPH	MEKK3	22.9584	11

(The Gold score fitness of the CK2 inhibitor CX-4945 is 48.2899 with the PDB ID 1JWH.)

Table 2. Summary of inhibitory effects of TNF- α and LYG-202 on H₂₂ murine solid tumor. IR: inhibitory ratio of tumor weight; CDI: coefficient of drug interaction.

Groups	Incipient-body weight (g)	The number of animals	Tele-body weight (g)	The number of animals	Tumor weight (g)	IR%	CDI
Control	19.9 \pm 0.9	10	22.5 \pm 1.2	10	1.550 \pm 0.110		
TNF- α 1.5 μ g/day	19.3 \pm 0.5	10	23.8 \pm 1.5	10	1.476 \pm 0.252	4.78	
LYG-202 750 mg/kg	20.9 \pm 1.0	10	23.0 \pm 1.0	10	0.718 \pm 0.178**	53.65	
LYG-202 500 mg/kg	20.7 \pm 0.9	10	22.7 \pm 0.9	10	0.748 \pm 0.195**	51.71	
LYG-202 250 mg/kg	18.5 \pm 0.6	10	21.8 \pm 1.0	10	1.053 \pm 0.135*	32.04	
LYG-202 125 mg/kg	19.5 \pm 1.0	10	23.5 \pm 1.3	10	1.416 \pm 0.228	8.61	
LYG-202+TNF- α (750 + 1.5)	19.7 \pm 1.2	10	22.9 \pm 1.2	10	0.621 \pm 0.174***	59.95	0.907 Synergistic
LYG-202+TNF- α (500 + 1.5)	18.8 \pm 0.6	10	24.0 \pm 1.2	10	0.631 \pm 0.155***	59.29	0.885 Synergistic
LYG-202+TNF- α (250 + 1.5)	21.0 \pm 1.2	10	22.7 \pm 0.9	10	0.820 \pm 0.095***	47.09	0.818 Synergistic
LYG-202+TNF- α (125 + 1.5)	21.3 \pm 0.9	10	24.5 \pm 1.0	10	0.926 \pm 0.125***	40.28	0.686 Synergistic
HCPT 30 mg/kg	20.3 \pm 1.0	10	23.9 \pm 1.1	10	0.478 \pm 0.175**	69.18	

***P < 0.01, #P < 0.05 combination compared with the LYG-202 (750, 500, 250 and 125 mg/kg group).

* P < 0.05 compared with control.

** P < 0.01 compared with control.

Figure 1

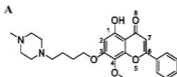
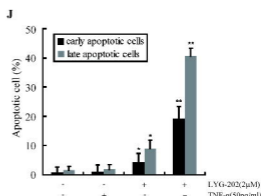
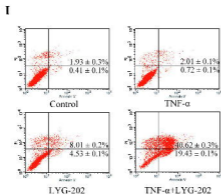
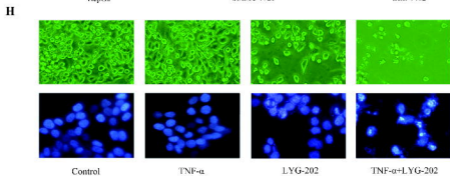
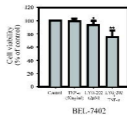
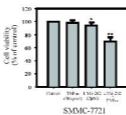
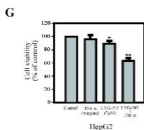
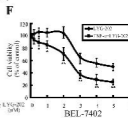
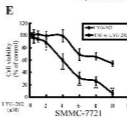
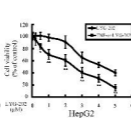
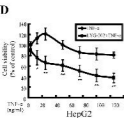
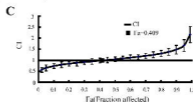
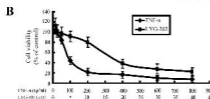
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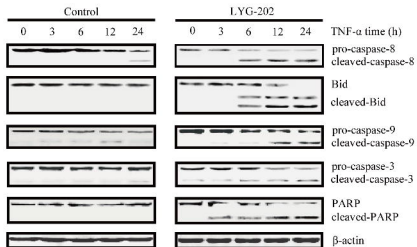
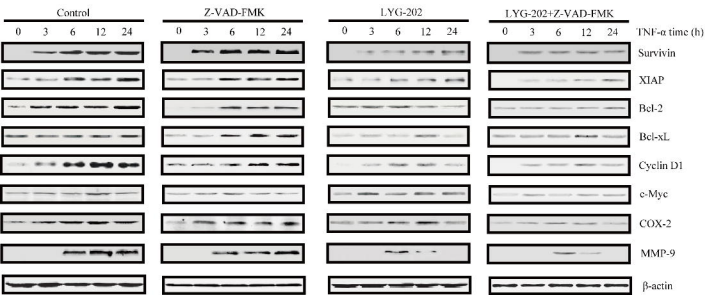
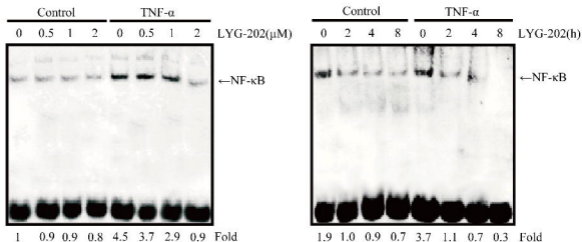
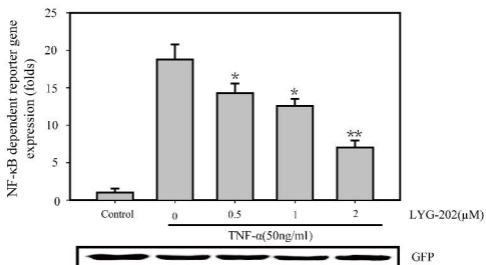
Figure 2**A****B**

Figure 3

A



B



C

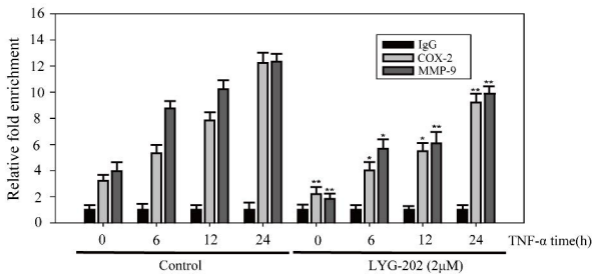


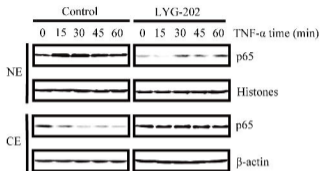
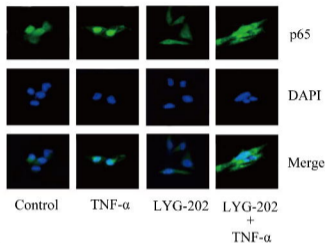
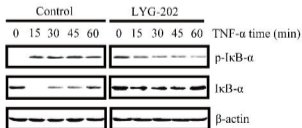
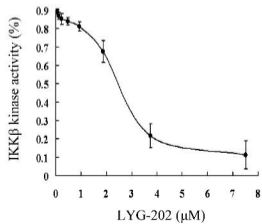
Figure 4**A****B****C****D**

Figure 5

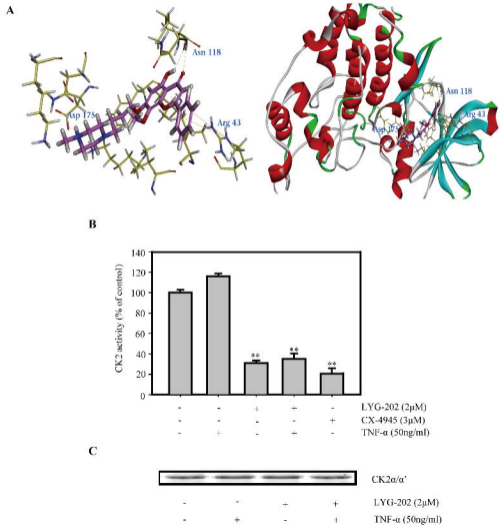


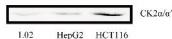
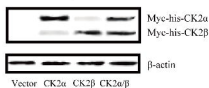
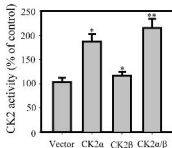
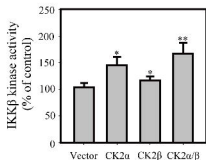
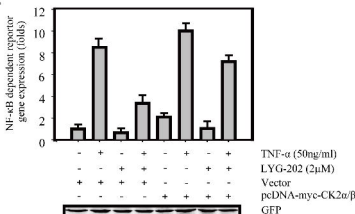
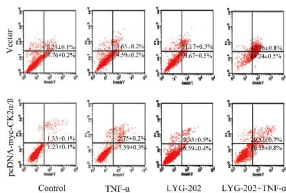
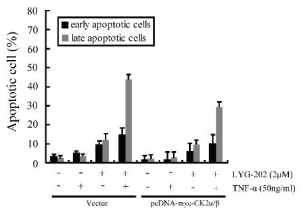
Figure 6**A****B****C****D****E****F****G**

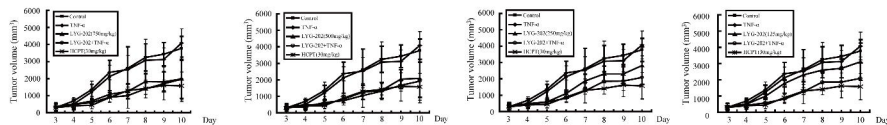
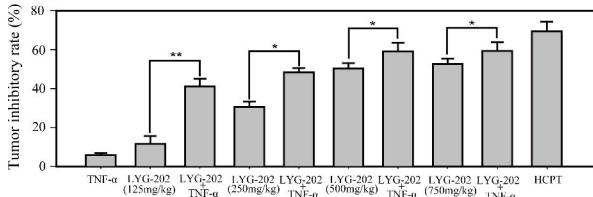
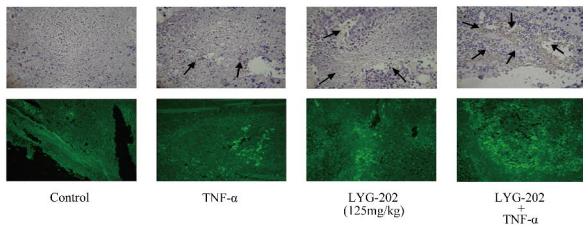
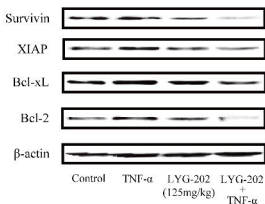
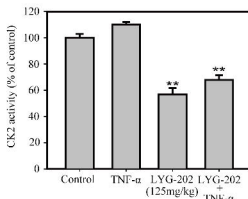
Figure 7**A****B****C****D****E**

Figure 8

