Induction of apoptosis by phenylisocyanate derivative of quercetin: involvement of heat shock protein

Bin Ye[a,c], Jin-liang Yang[a], Li-juan Chen[a], Xian-xue Wu[b,d], Han-shuo Yang[a], Ju-mei Zhao[a], Zhi-ping Yuan[a], Jiong Li[a], Yan-jun Wen[a], Yong-qiu Mao[a], Song Lei[a], Bing Kan[a], Lin-yu Fan[a], Wen-xiu Yao[a], Rui Wang[a], Guo-qing Wang[a], Xiao-bo Du[a], Huan-yi Liu[a], Hong-bing Wu[a], Jian-rong Xu[a], Hong-xia Li[a], Wei Zhang[a], Xia Zhao[a], Yu-quan Wei[a] and Li Cheng[a,b]

Quercetin, a widely distributed bioflavonoid, inhibits the growth of various tumor cells. The present study was designed to investigate whether a novel quercetin derivative [phenylisocyanate of quercetin (PHICNQ)] exerts antitumor activity against K562 and CT26 tumor cell lines by inducing apoptosis, and to examine the possible mechanism in the phenomenon. The cell proliferation assay of K562 and CT26 tumor cells was determined by the trypan blue dye exclusion test. Apoptosis of PHICNQ-treated cells was determined by morphological analysis, agarose gel DNA electrophoresis and quantitated by flow cytometry after staining with propidium iodide. Cell cycle was evaluated by flow cytometry. The expression of heat shock protein 70 was checked by Western blot analysis. Our results showed that PHICNQ inhibited the proliferation of K562 and CT26 cells in a dose-dependent and time-dependent manner. PHICNQ was 308- and 73-fold more active on CT26 and K562 cells than quercetin, respectively. In addition to this cytostatic effect, treatment of K562 and CT26 tumor cells with PHICNQ induced apoptosis. PHICNQ treatment downregulated the expression of heat shock protein 70 more dramatically than quercetin treatment. These results suggest that PHICNQ is a more powerful antiproliferative derivative than quercetin, with cytostatic and apoptotic effects on K562 and CT26 tumor cells. PHICNQ may trigger apoptosis in tumor cells through inhibition of heat shock protein 70 synthesis and expression. *Anti-Cancer Drugs* 18:1165–1171 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2007, **18:**1165–1171

Keywords: apoptosis, heat shock protein 70, phenylisocyanate of quercetin, quercetin, structure modification

[a]State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, [b]Key Laboratory of Drug Targeting of Ministry of National Education, West China School of Pharmacy, Sichuan University, [c]Chengdu 6th People's Hospital, Chengdu, Sichuan and [d]Department of Chemistry and Environment, Yuxi Teacher's College, PRC

Correspondence to Professor Li Cheng, MS, MD, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, Guo Xue Xiang No. 37, Chengdu, Sichuan 610041, PRC
Tel: +86 28 85250731; fax: +86 28 85250731;
e-mail: chlscup@sina.com, yuquawei@vip.sina.com

Received 14 March 2006 **Revised form accepted** 17 November 2006

Introduction

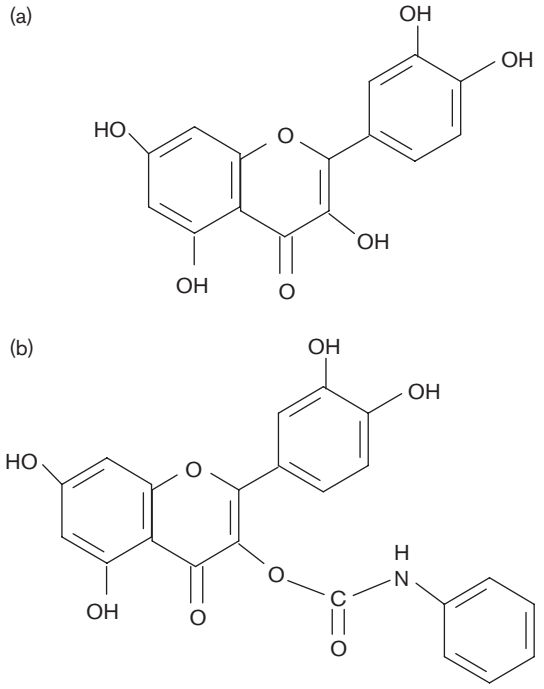
Quercetin (Que), 3,3′,4′,5,7-pentahydroxy flavone (Fig. 1a), an efficient antioxidant [1,2] bioflavonoid is one of the most widely distributed bioflavonoids in the plant kingdom [3–8], and has antitumor activity in various tumor cells from leukemia, colon carcinoma, ovarian carcinoma, breast cancer and lymphoma cells [3,9–14]. The poor solubility in pharmaceutically acceptable solvents and poor pharmacokinetic properties that led to poor absorption/availability after oral administration of Que badly affected the clinic development of quercetin. Therefore, it is significant to design and synthesize quercetin derivatives with enhanced water solubility and better pharmacokinetic properties by structure modification on Que. A novel Que derivative [phenylisocyanate of Que (PHICNQ), Fig. 1b], with enhanced water solubility (the solubility in water is approximately 71.08 μg/ml), was synthesized using isocyanate as an intermediate in the phase-transfer catalyst. In this study, we try to investigate whether PHICNQ exerts antitumor activity by inducing apoptosis and to examine the possible mechanism in the phenomenon.

Materials and methods

Phenylisocyanate of quercetin

Nuclear magnetic resonance (^{1}H-NMR) spectra were measured on Varian$^{\text{UNITY INOVA}}$ spectrometers operating at 400 MHz (Varian, USA). ^{13}C-NMR spectra were measured on AC-E 200 MHz Bruker spectrometers operating (GM, Bruker). IR spectra were measured on a VECTOR22, Bruker (GM, Bruker). Mass spectroscopy spectra were measured on an API3000 (Abi, USA). High-performance liquid chromatography spectra were measured on an Shimadzu LC-10AT (J.P.).

Fig. 1



Structures of quercetin and phenylisocyanate of quercetin: (a) quercetin and (b) phenylisocyanate of quercetin.

PHICNQ was a light yellow crystal m.p. 153–156°C. IR (KBr) υ_{max}: 3344 (br), 1745, 1655, 1600, 1535, 1501, 1450, 1175, 751,701; ^{1}H-NMR ($CDCl_3$, 400 MHz). δ (p.p.m.): 6.77 (d, 1H, H_6), 6.89 (d, 1H, H_8), 7.11 (d, $J = 8.8$ Hz, 1H, $H_{5'}$), 7.37 (td, $J = 7.6$ Hz, $J = 1.6$ Hz, 2H, $H_{3''}$, $H_{5''}$), 7.48 (d, $J = 1.6$ Hz, 1H, $H_{2'}$), 7.55 (dd, $J = 7.2$ Hz, $J = 2$ Hz, 2H, $H_{2''}$, $H_{6''}$), 7.87 (d, $J = 7.2$ Hz, 1H, $H_{6'}$), 8.02 (td, $J = 8.8$, $J = 2.4$, 1H, $H_{4''}$), 8.23 (s, 1H, N–H), 12.13 (s, 1H, 5-OH); ^{13}C-NMR ($CDCl_3$, 50 MHz) δ (p.p.m.): 109.39 (C-8), 114.39 (C-6), 120.25 (C-10), 124.36 (C-3), 127.07 (C-2′), 128.39 (C-5′), 128.53 (C-6′), 128.58 (C-2″, C-6″), 128.89 (C-4″), 130.15 (C-3″, C-5″), 130.51 (C-1′), 131.65 (C-1″), 138.01 (C-2), 142.79 (C-4′), 144.97 (C-3′), 154.54 (-HN–CO-), 163.79 (C-9), 163.49 (C-5), 165.84 (C-7), 171.03 (C-4,C = O); electrospray ionization-mass spectroscopy (+): 445.1 [M + Na^+].

Cell cultures and phenylisocyanate of quercetin treatment

The K562 human chronic myeloid leukemia cell lines and CT26 mice colorectal carcinoma cell lines were obtained from American Type Culture Collection. The cells were grown in RPMI-1640 medium (Gibco), containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, in a humid chamber at 37°C under 5% CO_2. Que was purchased from Sigma. PHICNQ was obtained from the Central Teaching Laboratory for Modern Pharmacy, West China Pharmacy School, Sichuan University. The purity of PHICNQ was above 95%. The solubility in water of PHICNQ was 71.08 μg/ml. Que or PHICNQ was dissolved in dimethylsulfoxide (final concentration 1%) to 10 mmol/l, and then was subsequently diluted in culture medium at a final concentration of 40–500 μmol/l (Que) or 0.5–10 μmol/l (PHICNQ). K562 and CT26 cells were treated with 1% dimethylsulfoxide for 24 h, resulting in 6.067 ± 0.874 and 5.667 ± 0.929% sub-G_1 cells, respectively.

Exponentially growing cells at 2×10^5/ml were exposed to varying concentrations of Que or PHICNQ for varying time intervals. The control culture was left untreated.

Cell proliferation studies

The number of viable cells was determined by a trypan blue dye exclusion test and the percentage of viable cells corresponds to a ratio (total cells–blue cells)/total cells [15,16]. The analysis of IC_{50} values was performed by Bliss software.

Morphological analysis

For the identification of apoptotic cells, cells were resuspended in hypotonic propidium iodide (PI) solution containing 50 μg PI/ml in 0.1% sodium citrate plus 0.1% Triton X-100 and examined by fluorescence microscopy [3].

Agarose gel DNA electrophoresis

The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described in [17]. Briefly, cells (3×10^6) were lysed with 0.5 ml lysis buffer containing 5 mmol/l Tris–HCl (pH 8), 0.25% Nonidet P-40 and 1 mmol/l ethylenediaminetetraacetic acid, followed by the addition of RNase A (Sigma) at a final concentration of 200 μg/ml and incubated for 1 h at 37°C. Cells were then treated with 300 μg proteinase K/ml for an additional 1 h at 37°C. After additions of 4 μl loading buffer, 15 μl samples in each lane were subjected to electrophoresis on 1.5% agarose at 50 V for 3 h. DNA was stained with ethidium bromide. The molecular size of the marker bands is from 200 to 3000 bp.

The quantitative assessment of apoptosis and analysis of cell cycle specificity

Flow cytometric analysis was performed to identify sub-G_1 cells/apoptotic cells and to measure the percentage of sub-G_1 cells after PI staining in hypotonic buffer as described in [1,18,19]. Briefly, cells were suspended in 1 ml hypotonic fluorochrome solution containing 50 μg PI/ml in 0.1% sodium citrate plus 0.1% Triton X-100 and the cells were analyzed by the use of a flow cytometer (ESP Elite; Coulter). Apoptotic cells appeared in the cell cycle distribution as cells with a DNA content of less than that of G_1 cells and was estimated with Listmode software. In addition, cell cycle analysis was performed simultaneously with Multicycle software.

Western blot analysis

Western blot analysis was performed as described in [20]. CT26 cells were treated by 2.5 μmol/l PHICNQ or 100 μmol/l Que at 37°C for 6 h. A total of 1×10^7 cells were lysed in 100 μl lysis buffer. Lysates were centrifuged at 12 000*g* for 5 min and the supernatant was harvested. Protein concentration was determined by the bicinchoninic acid protein assay reagent. Samples (equivalent protein concentration) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transblotted onto a polyvinylidine diflouride membrane (Bio-Rad, Hercules, California, USA). The membrane was blocked by TTBS (including 0.1% Tween 20 in Tris-buffered saline) with 5% nonfat milk and later it was probed with anti-heat shock protein (HSP) 70 mouse monoclonal antibody (Zhongshan Goldenbridge Biotechnology, Beijing, PRC) diluted 1 : 200 at 4°C overnight. Blots were then incubated with 1 : 5000 horseradish peroxidase-conjugated antimouse immunoglobulin (Zhongshan Goldenbridge Biotechnology). Protein bands were visualized by the chemiluminescence (enhanced chemiluminescence) reagent kit (Pierce Biotechnology, Rockford, Illinois, USA).

Statistical analysis

The percentage inhibition measurements of the cells and the flow-cytometric studies were performed in triplicate. The statistical differences between the means of the experimental groups were evaluated by Student's *t*-test; differences were considered significant when P values were below 0.05.

Results

Antiproliferative effect of phenylisocyanate of quercetin and quercetin

The antiproliferative activities of PHICNQ were tested against the K562 human chronic myeloid leukemia cell lines and CT26 mice colorectal carcinoma cell lines. PHICNQ inhibited the proliferation of K562 cells and CT26 cells in a dose-dependent and time-dependent manner. From the dose–response curves, we observed that CT26 cells were much more sensitive (308-fold) to PHICNQ ($IC_{50} = 1.177$ μmol/l) than to Que ($IC_{50} = 362.456$ μmol/l) (Fig. 2c and d) and that K562 cells were more sensitive (73-fold) to PHICNQ ($IC_{50} = 1.852$ μmol/l) than to Que ($IC_{50} = 135.624$ μmol/l) (Fig. 2a and b). PHICNQ inhibited cell proliferation at lower doses than Que. PHICNQ treatment not only inhibited cell proliferation, but also caused cell death when determined by the trypan blue dye exclusion test.

Induction of apoptosis by phenylisocyanate of quercetin treatment

Treatment with PHICNQ of tumor cells resulted in morphological changes characteristic for apoptosis: brightly red-fluorescent condensed nuclei (intact or fragmented) shown by fluorescence microscopy of PI-stained nuclei, reduction of cell volume, condensation of nuclear chromatin, nuclear fragmentation and apoptotic bodies (Fig. 3). By contrast, the untreated cells showed red, diffusely stained intact nuclei. Agarose gel electrophoresis of PHICNQ-treated cells demonstrated a ladder-like pattern of DNA fragments consisting of multiples of approximately 180–200 bp, consistent with internucleosomal DNA fragmentation (Fig. 4). Tumor cells treated with Que at the same concentration for the same time did not show a ladder-like pattern of DNA fragments (data not shown).

By the use of flow cytometry, we could assess the number of sub-G_1 cells (apoptotic cells) (Fig. 3c).

Kinetics of induction of apoptosis and analysis of cell cycle

The quantitative assessment of sub-G_1 cells by flow cytometry was used to estimate the number of apoptotic cells. The apoptosis-inducing effect of PHICNQ was dose-dependent and time-dependent, being observed at 0.5 μmol/l and reached a maximum at 10 μmol/l when analyzed by flow cytometry. The increased number of apoptotic cells was detected after 12 h of continuous PHICNQ treatment, reaching a maximum by 48 h (Fig. 3d and e). Similar results were also found in CT26 cells. For example, CT26 cells treated with 5 and 10 μmol/l PHICNQ for 48 h resulted in 55.633 ± 3.721 and 73.467 ± 4.159% sub-G_1 cells. By contrast, tumor cells treated with Que at the same concentration for the same time did not show any apparent apoptosis-inducing effect (Fig. 3d and e).

Next, a cell cycle affected by PHICNQ in cancer cells was analyzed. When tumor cells were treated with PHICNQ at a concentration from 0.5 to 10 μmol/l for up to 48 h, there was a significant decrease in the number of G_0/G_1-phase and S-phase cells, and a slight decrease in the number of G_2/M-phase cells, at the same time that sub-G_1 cells appeared (data not shown).

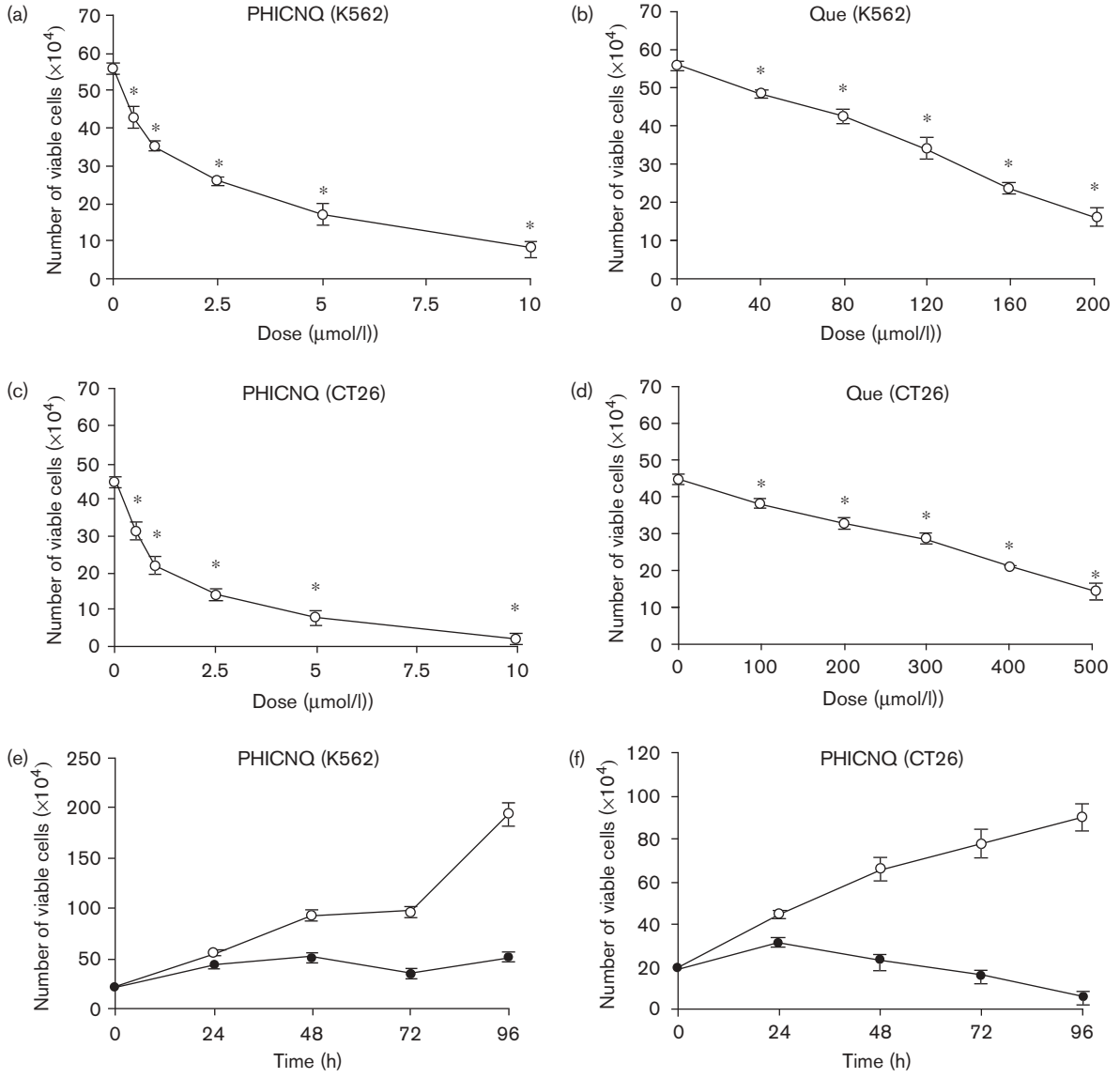
HSP70 analysis by Western blot

Tumor cells (K562 or CT26) were treated with 2.5 μmol/l PHICNQ or 100 μmol/l Que for 6 h. The proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidine diflouride membranes. A distinct band of about 70 kDa, corresponding to the size of HSP70, was visualized in the control (untreated) cells and PHICNQ treatment down-regulated the expression of HSP70 more dramatically than Que treatment (Fig. 5).

Discussion

Although Que has been advocated to have potential as a starting material in drug development programmes, its synthetic studies toward the bioflavonoid antioxidant are

Fig. 2

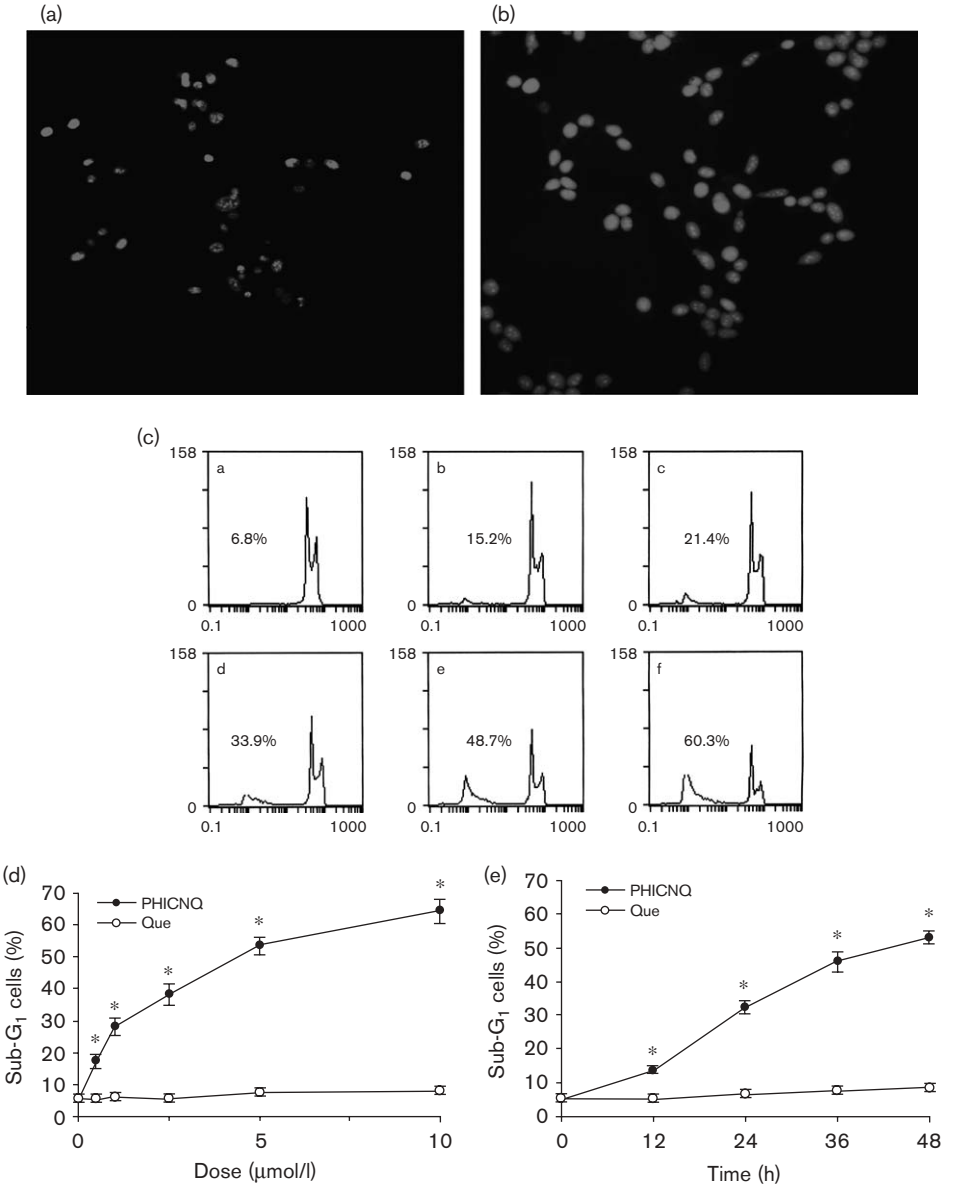


(a–d) Dose-dependent growth inhibition of K562 cells (a and b) and CT26 cells (c and d) by phenylisocyanate of quercetin (Que) (PHICNQ) (a and c) or Que (b and d) treatment. K562 cells and CT26 cells were treated with various doses of PHICNQ or Que for 24 h. *$P<0.05$, when PHICNQ was compared with Que. PHICNQ reduces cell proliferation at lower doses than Que. (e and f) Time-dependent growth inhibition of untreated K562 and CT26 cells by PHICNQ. K562 and CT26 cells were treated with 0.5 μmol/l PHICNQ for various time intervals. Results are expressed as means of triplicate samples; bars, SD. *$P<0.05$, when PHICNQ-treated cells were compared with control (untreated) cells.

rather limited [1,21]. Our work aimed at synthesis of Que derivatives used as potential anticancer drug candidates with enhanced water solubility. Strategies for construction of Que phenylisocyanate were comparatively researched to give a better one as a final choice. It was easy to get isocyanate from quercetin by couple reaction of phenylisocyanate derivatives in the phase catalyst transfer. Through it, a novel Que derivative (PHICNQ), with enhanced water solubility (the solubility in water is 71.08 μg/ml), was synthesized.

In this study, several observations have been made concerning the apoptosis-inducing effect of PHICNQ. This study is the first, to our knowledge, to demonstrate that PHICNQ triggers apoptosis in K562 and CT26 tumor cell lines. We compared the antiproliferative effect of Que and PHICNQ on K562 and CT26 tumor cell lines, and found that PHICNQ had higher antiproliferative and apoptotic effects than Que. PHICNQ is, respectively, 73- and 308-fold more efficient than Que in inhibiting the proliferation of K562 and CT26 tumor cells. The

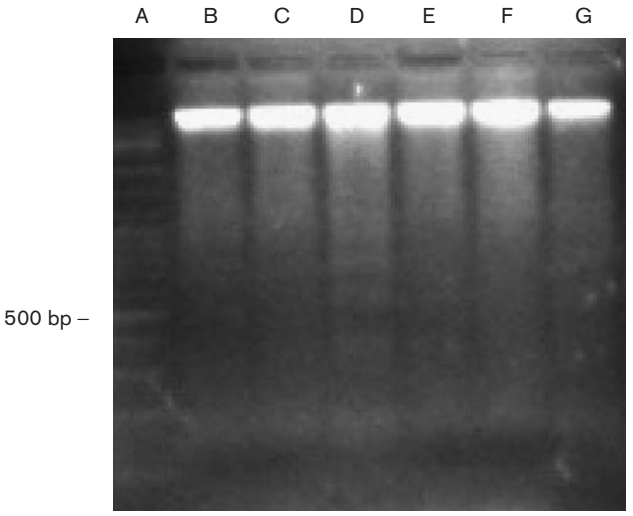
Fig. 3



(a and b) Fluorescence microscopic appearance of propidium iodide (PI)-stained nuclei of phenylisocyanate of quercetin (Que) (PHICNQ)-treated tumor cells. CT26 cells were treated (a) or untreated (b) with 2.5 μmol/l PHICNQ for 24 h, stained with PI and examined under a fluorescence microscope (×200). (c) Representative DNA fluorescence histograms of PI-stained cells. K562 cells were untreated or (b–f) treated with 0.5, 1, 2.5, 5 and 10 μmol/l PHICNQ for 24 h, with 6.8, 15.2, 21.4, 33.9, 48.7 and 60.3% sub-G_1 cells (apoptotic cells), respectively, as assessed by flow cytometry. (d and e) Kinetics of induction of apoptosis by PHICNQ treatment. (d) The dose–response curve of PHICNQ-induced apoptosis. K562 cells were untreated or treated with 0, 0.5, 1, 2.5, 5 and 10 μmol/l PHICNQ for 24 h. (e) The time–response curve of PHICNQ-induced apoptosis. K562 cells were treated with 2.5 μmol/l PHICNQ for 0, 12, 24, 36 and 48 h. The numbers of sub-G_1 cells/apoptotic cells were evaluated by flow cytometry. Results are expressed as means of triplicate samples; bars, SD. *$P<0.05$, PHICNQ-treated cells were compared with control (untreated) cells. Similar results were also found in CT26 cells.

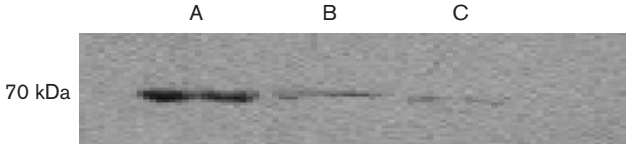
K562 and CT26 tumor cells treated with PHICNQ displayed PI-stained condensed nuclei and nuclear fragmentation, a ladder-like pattern of DNA fragments consistent with internucleosomal DNA fragmentation, and a sub-G_1 DNA peak of PI-stained nuclei analyzed by flow cytometry. These findings are consistent with the characteristics of apoptosis induced by other agents [19,22–27]. Apoptosis has been described as multiple pathways converging from numerous different initiating events and insults [28,29]. It has been reported that the treatment with Que induces cell death by triggering apoptosis [3], Que inhibits the growth of malignant cells through various mechanisms: inhibition of glycolysis, macromolecule synthesis and enzymes; freezing cell

Fig. 4



Agarose gel electrophoretic patterns of DNA isolated from phenylisocyanate of quercetin (Que) (PHICNQ) (2.5 μmol/l, 24 h)-treated and PHICNQ-untreated tumor cells. Lane A, marker. Lane B, untreated CT26. Lane C, Que-treated CT26. Lane D, PHICNQ-treated CT26. Lane E, untreated K562. Lane F, Que-treated K562. Lane G, PHICNQ-treated K562. Three independent experiments were performed.

Fig. 5



Expression of heat shock protein 70 protein determined by Western blot. Lane A, control (untreated CT26) cells. Lane B, CT26 cells were treated with 100 μmol/l quercetin (Que) for 6 h. Lane C, cells were treated with 2.5 μmol/l phenylisocyanate of Que (PHICNQ) for 6 h. Three independent experiments were performed.

cycle; interaction with estrogen type-II binding sites [4–8], and inhibiting the synthesis of HSP70 [3–5]. HSP70 is known to play an important role in the protein metabolism and survival of cells [3,30]. In our study, we found that PHICNQ treatment downregulated the expression of HSP70 more dramatically than Que treatment. It seems likely that HSP70 may be involved in the apoptosis induced by PHICNQ and this may explain why PHICNQ exerts a higher cytotoxicity than that of Que.

It has been reported that Que may cause apoptosis mainly in cells at G_1 and S phases [3]. In this study, however, no specific sensitivity of any phase of the cell cycle is found. Thus, the apoptosis induced by PHICNQ may not result from the freezing cell cycle.

Previous studies have demonstrated that apoptosis may be involved in the cell death induced by chemotherapeutic agents, including cisplatin, cytarabine, etoposide, camptothecin, etc. [18,19]. Accumulating evidence exists that the efficacy of antitumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis [3,18,19,28].

Apoptosis plays an important role in cancer occurrence and progression. Most if not all neoplasms exhibit one or more abnormalities in steps of the apoptotic process governing the ultimate growth of neoplasms in the host. Therefore, induction of apoptosis may be a strategy for the treatment of tumors.

Our present results showed that PHICNQ is a more powerful antiproliferative derivative than Que, with cytostatic and apoptotic effects on K562 and CT26 tumor cells, and enhanced water solubility. These findings may be of practical significance for the further exploration and promotion of the clinical use of Que.

Acknowledgements

This work was supported by National Basic Research Program of China (2001CB510001, 2004CB518800), the projects of National Natural Science Foundation of China and National 863 Program.

References

1 Pietta PG. Flavonoids as antioxidants. *J Nat Prod* 2000; **63**:1035–1042.
2 Wu XX, Cheng L, Xiang D, Wei YQ. Syntheses of carbamate derivatives of quercetin by reaction with amino acid ester isocyanates. *Lett Org Chem* 2005; **2**:535–538.
3 Wei YQ, Zhao X, Kariya Y, Fukata H, Teshigawara K, Uchida A, *et al.* Induction of apoptosis by quercetin: involvement of heat shock protein. *Cancer Res* 1994; **54**:4952–4957.
4 Scambia G, Ranelletti FO, Panici PB, Piantelli M, De Vincenzo R, Ferrandina G, *et al.* Quercetin induces type-II estrogen-binding sites in estrogen-receptor negative (MDA-MB231) and estrogen-receptor-positive (MCF-7) human breast cancer. *Int J Cancer* 1993; **54**:462–466.
5 Hosokawa N, Hirayoshi K, Kudo H, Takechi H, Aoike A, Kawai K, *et al.* Inhibition of the activation of heat shock factor *in vivo* and *in vitro* by flavonoids. *Mol Cell Biol* 1992; **12**:3490–3498.
6 Hosokawa N, Hirayoshi K, Nakai A, Hosokawa Y, Marui N, Yoshida M, *et al.* Flavonoids inhibit the expression of heat shock proteins. *Cell Struct Funct* 1990; **15**:393–401.
7 Scambia G, Ranelletti FO, Benedetti Panici P, Piantelli M, Bonanno G, De Vincenzo R, *et al.* Inhibitory effect of quercetin on primary ovarian and endometrial cancer and synergistic activity with cis-diamminedichloroplatinum(α). *Gynecol Oncol* 1992; **45**:13–19.
8 Verma A, Johnsons JA, Gould MN, Tanner MA. Inhibition of 7,12-dimethylbenz(*a*)anthracene and *N*-nitrosomethylurea induced rat mammary cancer by dietary flavonol quercetin. *Cancer Res* 1988; **48**:5754–5758.
9 Larocca LM, Teofili L, Sica S, Piantelli M, Maggiano N, Leone G, *et al.* Quercetin inhibits the growth of leukemik progenitors and induces the expression of thansforming growth factor-β_1 in these cells. *Blood* 1995; **85**:3654–3661.
10 Ranelletti FO, Ricci R, Larocca LM, Maggiano N, Capelli A, Scambia G, *et al.* Growth-inhibitory effect of quercetin and presence of type-II estrogen-binding sites in human colon-cancer cell lines and primary colomrectal tumors. *Int J Cancer* 1992; **50**:486–492.
11 Osman Y, Moriyama Y, Shibata A. Enhanad elimination of Ph^+ chromosome cells *in vitro* by combined hyperthermia and other drugs (AZT, IFN-α, TNF, and quercetin): its application to autologous bone marrow transplantation for CMI. *Exp Hematol* 1995; **23**:444–452.

12 Prajda N, Singhal RL,Yeh YA, Olah E, Look KY, Weber G. Linkage of reduction in 1-phosphatidylinositol 4-kinase activity and inositol 1,4,5-trisphosphate concentration in human ovarian carcinoma cells treated with quercetin. *Life Sci* 1995; **56**:1587–1593.
13 Avila MA, Velasco JA, Cansado J, Notario V. Quercetin mediates the down-regulation of mutant p53 in the Human breast cancer cell lines MDA-MB468. *Cancer Res* 1994; **54**:2424–2428.
14 Lavoie HA, Witorsch RJ. Investigation of intracellular signals mediating the anti-apoptotic action of prolactin in Nb_2 lymphoma cells. *Proc Soc Exp Boil Med* 1995; **209**:257–269.
15 Wei YQ, Zhao X, Kariya Y, Teshigawara K, Uchida A. Inhibition of proliferation and induction of apoptosis by abrogation of heat-shock protein (HSP) 70 expression in tumor cells. *Cancer Immunol Immunother* 1995; **40**:73–78.
16 Malherbe S, Crepin M, Legrand C, Wei MX. Cytostatic and pro-apoptotic effects of a novel phenylacetate–dextran derivative (NaPaC) on breast cancer cells in interactions with endothelial cells. *Anticancer Drugs* 2004; **15**:975–981.
17 Li Q, Wei YQ, Wen YJ, Zhao X, Tian L, Yang L, *et al*. Induction of apoptosis and tumor regression by vesicular stomatitis virus in the presence of gemcitaibne in lung cancer. *Int J Cancer* 2004; **112**:143–149.
18 Gorczyca W, Gong J, Ardelt B, Traganos F, Darzynkiewicz Z. The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res* 1993; **53**:3186–3192.
19 Barry MA, Reynolds JE, Eastman A. Etoposide-induced apoptosis in human HL-60 cells is associated with intracellular acidification. *Cancer Res* 1993; **53**:2349–2357.
20 Wu Y, Yang L, Hu B, Liu JY, Su JM, Luo Y, *et al*. Synergistic anti-tumor effect of recombinant human endostatin adenovirus combined with gemcitabine. *Anticancer Drugs* 2005; **16**:551–557.
21 Formica JV, Regelson W. Review of the biology of quercetin and related bioflavonoids. *Food Chem Toxicol* 1995; **33**:1061–1080.
22 Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 1993; **362**: 847–849.
23 Walton MI, Whysong D, O'Connor PM, Hockenbery D, Korsmeyer SJ, Kohn KW. Constitutive expression of human Bcl-2 modulates nitrogen mustard and camptothecin induced apoptosis. *Cancer Res* 1993; **53**: 1853–1861.
24 Forbes IJ, Zalewski PD, Giannakis C, Cowled PA. Induction of apoptosis in chronic lymphocytic leukemia cells and its prevention by phorbol ester. *Exp Cell Res* 1992; **198**:367–372.
25 Evans DL, Dive C. Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. *Cancer Res* 1993; **53**:2133–2139.
26 Eastman A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. *Cancer Cells* 1990; **2**:275–280.
27 Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991; **139**: 271–279.
28 Eastman A, Rigas JR. Modulation of apoptosis signaling pathways and cell cycle regulation. *Semin Oncol* 1999; **26**:7–16.
29 Nguyen JT, Wells JA. Direct activation of the apoptosis machinery as a mechanism to target cancer cells. *Proc Natl Acad Sci USA* 2003; **100**:7533–7538.
30 Jaattela M, Wissing D. Heat-shock proteins protect cells from monocyte cytotoxicity: possible mechanism of self-protection. *J Exp Med* 1993; **177**:231–236.