Marchantin C, a novel microtubule inhibitor from liverwort with anti-tumor activity both in vivo and in vitro

Yan-qiu Shi a, Chang-jun Zhu b, Hui-qing Yuan b, Bo-qin Li b, Jie Gao a, Xian-jun Qu a, Bin Sun a, Yan-na Cheng a, Song Li a, Xia Li a, Hong-Xiang Lou a,∗

a School of Pharmaceutical Sciences, 44 Wen Hua Xi Road, Jinan, Shandong 250012, China
b School of Medicine, Shandong University, 44 Wen Hua Xi Road, Jinan, Shandong 250012, China

ARTICLE INFO

Article history:
Received 27 September 2008
Received in revised form 1 November 2008
Accepted 4 November 2008

Keywords:
Marchantin C
Macrocyclic bisbibenzyl
Microtubule inhibitor
Cell cycle arrest
Tumor cell apoptosis

ABSTRACT

Microtubules are long-standing targets in cancer chemotherapy. Previously, we reported that marchantin C triggers apoptosis of human tumor cells. We show here that marchantin C induced cell cycle arrest at G2/M phase in A172 and HeLa cells. In addition, marchantin C decreased the quantity of microtubules in a time- and dose-dependent manner in these cells. Exposure of purified bovine brain tubulin to marchantin C inhibited polymerization of gross tubulin in vitro. Moreover, marchantin C potently suppressed the growth of human cervical carcinoma xenografts in nude mice. Marchantin C-treated xenografts showed decreased microtubules, Bcl-2 and increased cyclin B1, Bax, caspase-3, indicating that marchantin C possess the same ability to induce microtubules depolymerization and tumor cell apoptosis in tumor-bearing mice as in vitro. In conclusion, marchantin C is a novel microtubule inhibitor that induces mitotic arrest of tumor cells and suppresses tumor cell growth, exhibiting promising antitumor therapeutic potential.

© 2008 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Microtubules are a major dynamic components of the eukaryotic cytoskeleton, which participate in a variety of cellular functions, including the development and maintenance of cell shape, reproduction and division, signaling, intracellular transportation and cell movement [1,2]. Importantly, microtubules constitute the mitotic spindle apparatus during cell division, which is critical for cellular proliferation. This vital role of microtubules has made them a general target of numerous antitumor drugs [3,4]. Currently, microtubule-targeted drugs comprise some of the most effective and potent agents in cancer chemotherapy [5]. However, non-specific cytotoxicity of these drugs has required the continued investigation and characterization of potent and tumor-specific compounds.

The overwhelming majority of microtubule-targeting drugs are naturally derived products with a diversity of chemical structures, produced as self-defensive agents by a large number of plants and animals. Most microtubule-targeted agents inhibit the dynamics of microtubules by destabilizing or hyperstabilizing microtubules. In this way, the microtubule-targeted agents can be further classified into two groups. One group binds to the tubulin dimers and inhibits the polymerization of microtubules, and includes compounds such as the vinca alkaloids, cryptophycins, and colchicines. The other group binds to microtubule polymers and promotes the polymerization of microtubules, and includes compounds such as the taxanes (taxol and taxotere), epothilones,
eleutherobins, laulimalide and discodermolide. These drugs suppress microtubule dynamics by binding to different sites on tubulin heterodimer and then disturb the assembly of the mitotic spindle apparatus and arrest cell cycle progression through M-phase, which leads to the eventual cell apoptosis [6,7].

Macrocyclic bisbibenzyls, a class of secondary metabolites produced exclusively in liverworts, belong to the family of phenolic compounds of stilbenoids [8,9]. This class of compounds has been demonstrated to possess a wide range of biological activities including cytotoxicity, antibacterial and antifungal activity [10–14]. Marchantin C, a macrocyclic bisbibenzyl, has been demonstrated to exhibit strong antioxidant activity to free radicals [15]. inhibitory effects on inducible nitric oxide synthase (iNOS) in macrophages [16], as well as the antimicrobial activity against the Gram-positive bacterium Bacillus subtilis and cytotoxic activity against KB cell reported previously [11]. Recently, we found that marchantin C induces apoptosis of various tumor cells, such as A172 cells, by up-regulation of Bax and down-regulation of Bcl-2 expression [17]. However, no macrocyclic bibenzyl family compounds have previously been demonstrated to affect mitotic mechanisms.

In this study, we demonstrated that marchantin C is a potent anti-microtubule agent that arrests mitotic cells in the G2/M phase, by depolymerizing microtubules both in cultured cells and in vitro. In vivo experiments in nude mice proved that marchantin C was not only able to remarkably inhibit the growth of the human cervical tumor xenografts through down-modulating anti-apoptosis proteins Bcl-2 expression, but also able to decrease the amount of microtubules in tumor tissues, demonstrating that marchantin C is a powerful inhibitor of microtubule polymerization. Our results provide insight that marchantin C may hold potential as an antitumor candidate with promising and potent microtubule-targeting activities.

2. Materials and methods

2.1. Chemicals and antibodies

Paclitaxel, vincristine, mouse anti-α-tubulin antibody were purchased from Sigma (St. Louis, USA). Mouse anticyclin B1 antibody was purchased from Cell signal Technologies, Inc. (Beverly, USA). Polyclonal rabbit anti-Bcl-2, rabbit anti-Bax and mouse anti-caspase-3 antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Tubulin for the polymerization assay was purchased from Cytoskeleton Inc. (Denver, USA), and the SABC kit used for immunohistochemistry assay was purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, USA).

The structure of marchantin C, isolated from Dumortiera angust was identified by interpretation of spectral data (MS, 1H NMR, 13C NMR, 2D NMR) as described previously [18]. 10 mM marchantin C dissolved in dimethyl-sulfoxide (DMSO) was stored at −20 °C as stock solution and suitable dilution was used according to experimental requirements.

2.2. Cell culture

The human tumor cell lines A172 (glioblastoma cells) and HeLa (cervical carcinoma) were obtained from American Type Culture Collection (ATCC) and cultured at 37 °C, 5% CO2 in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin, respectively.

2.3. Flow cytometry assay

Adherent cells were detached with trypsin and collected by centrifugation, followed by washing, fixation, and PI staining. The cell cycle distribution was examined by a FACScan flow cytometry (Becton–Dickinson, USA), and then data was analyzed using the Modfit program (Becton–Dickinson, USA).

2.4. Transmission electron microscopy observation

Cells were fixed with 4% glutaraldehyde for 1 h at room temperature and centrifuged at 3000 rpm. Cells were subsequently treated with 0.15 M phosphate buffer with 3% OsO4 for 1 h, followed by a rapid wash in the same buffer. Afterwards, specimens were dehydrated with alcohol-water solutions of varying concentrations and 100% propylene oxide. Araldite embedding comprised of initial treatment with a 1:1 mixture of propylene oxide/araldite (v/v) for 1 h, followed by 1:3 mixture overnight at room temperature.

After an additional treatment in undiluted resin for 1 h, polymerization was performed at 60 °C for 3–4 days. Thin sections stained with 1% toluidine blue were mounted on copper grids. Observation under transmission electron microscope (JEM-1200EX, Japan) was generally performed at 60 KV. The quantitative analysis on microtubules in tumor cells was performed using Image-ProPlus 5.0 software from Media Cybernatics, Inc. (Bethesda, USA).

2.5. Immunofluorescence microscopy observation

Cells seeded on 12-mm round glass cover-slips and placed at the bottom of 12-well plates. After experimental treatment, cells on glass cover-slips were fixed with cold methanol/acetone (1:1) for 5 min followed by immunostaining for α-tubulin and actin using mouse anti-α-tubulin antibody and phalloidin as described previously [19]. DNA was counterstained with DAPI (1 μg/ml) for 5 min at room temperature. The samples were mounted on microscope slides with mounting medium and analyzed by fluorescence microscopy. The fluorescence images were processed using AutoQuant X 2.1 software from Media Cybernatics, Inc. (Bethesda, USA).

2.6. Tubulin polymerization assay

Bovine brain tubulin (>97% pure tubulin) was suspended with 10 ml of G-PEM buffer (80 mM PIPES, 2 mg MgCl2, 0.5 mM EGTA, 1.0 mM GTP, pH 6.9) plus 5% glycerol in 0.1% DMSO at 4 °C, with and without test compound, using the tubulin polymerization assay kit (Cat. BK004)
according to manufacturer’s protocol (Cytoskeleton Inc., Denver, USA) Next, the sample mixture was transferred to the pre-warmed 96-well plates, and polymerization of tubulin was measured by the change in absorbance at 340 nm every 1 min for 1 h using a spectrophotometer (Perkin–Elmer/Wallac Inc., Freiburg, Germany).

2.7. Human tumor xenografts

Female BABL/c mice, purchased from SLAC laboratory animal Co. Ltd. (Shanghai, China), 6–8 weeks old, were housed in stainless steel cages and raised in alternating 12 h light and 12 h dark cycles, supplied with sterile food and autoclaved water. All procedures complied with the protocols approved by the Institutional Animal Care Committee. HeLa cells (3 × 10⁶) suspended in 200 μL of saline were administered to left flank regions of mice via s.c. injection [20]. Seven days later, lateral tail vein injections of marchantin C at 10 or 20 mg/kg, or sterile 0.9% NaCl were used as vehicle control. Ten days later, developed tumors from different groups were obtained and evaluated in terms of weight. Following the analysis of tumor mass, 20 mg/kg of marchantin C-treated tumor samples (n = 6) and control group were chosen randomly for Western blot analysis and immunohistochemistry assay.

2.8. Western blotting assay

The cell lysates were resolved by 12% SDS–PAGE and electrophoresed onto a nitrocellulose membrane, followed by incubation with corresponding primary antibodies, including Bcl-2, Bax and cyclin B1 at 1:500 dilution overnight at 4 °C. Secondary antibodies were used at 1:1000 dilutions for 2 h at room temperature. Visualization was performed using the chemiluminescence method as described previously [21]. β-Actin was used as internal reference.

2.9. Immunohistochemistry assay

Frozen tumor tissue section slices were incubated with primary antibody against α-tubulin (1:500) or Bcl-2 (1:100) and biotinylated secondary antibody IgG, respectively, followed by visualized using SABC kit according to its protocol (Boster Biological Technology Co., Ltd., Wuhan, China). The slide was observed under microscope at 100 and 400× magnifications.

2.10. Statistical analysis

All experiments were done at least three times. Statistical analysis was performed using Student’s t-test and statistical significance was expressed as P < 0.01.

3. Results

3.1. Marchantin C induces cell division cycle arrest at G2/M phase

In our previous study, we found that marchantin C was capable of inhibiting the proliferation of human glioma tumor cells (A172) by inducing apoptosis [17]. To survey the detailed mechanism, we examined the effects of marchantin C on cell cycle progression using a flow cytometry procedure. Both A172 (human glioma cell line) and HeLa (human cervical adenocarcinoma cell line) cells were used in our study to explore the general effects of marchantin C on human tumors. A172 cells were cultured with 8, 12 or 16 μM marchantin C, respectively, for 24 h and then collected for flow cytometry assay. The same amount of DMSO, the solvent of marchantin C, was used as control. The results showed that as marchantin C concentration increased from 8 to 16 μM, there was an increasing population of cells at G2/M phase, as well as an increase in the sub-G1 population (Fig. 1A). Immunoblotting of treated cell lysates displayed an increase of cyclin B1 protein (Fig. 1B). These results indicate that marchantin C induced A172 cell cycle arrest at G2/M phase and apoptosis in a concentration-dependent manner. Next, we analyzed the cell cycle profile of HeLa cells treated with 16 μM marchantin C for 6, 16, 24 and 48 h, respectively, using the flow cytometry assay. As shown in Fig. 1C, in the absence of marchantin C, there were 12~20% of cells in G2/M phase from 6 to 48 h. In contrast, significant accumulation of HeLa cells in G2/M phase were observed after marchantin C treatment for 6 (28.7%), 16 (47.1%) and 24 (39.3%) h. However, a sharp decline in G2/M population occurred after marchantin C treatment for 48 h (6.6%), concomitant with a dramatic increase in sub-G1 population, indicating increased cell death. This result suggests that the marchantin C temporarily arrested cells in G2/M phase, followed by a resulting increase in cell death. Collectively, our results demonstrated that marchantin C induced cell cycle arrest at G2/M phase and apoptosis in a dose- and time-dependent manner.

3.2. Marchantin C inhibits microtubule polymerization in cultured cells

In order to determine the mechanism underlying the G2/M arrest in cells treated with marchantin C, we used immunofluorescent staining techniques to analyze the effect of marchantin C on cytoskeleton network. First, HeLa cells were cultured for 1 h in the absence or presence of 8, 12 and 24 μM of marchantin C, respectively, and were then fixed with cold methanol/acetone, followed by immunostaining for α-tubulin. The cellular microtubule networks were then visualized by immunofluorescence microscopy. As shown in Fig. 2A, intact microtubules arrays could be observed in untreated cells. However, during treatment with increasing dosages of marchantin C, microtubules networks were decreased and short microtubules fragments were observed in the cytoplasm. We next treated A172 cells with 8, 12, 24 μM of marchantin C overnight and then immunostained for tubulin and actin. Similar results to HeLa cells were observed in A172 cells. In addition, 24 μM marchantin C treatment resulted in loss of cellular microtubule networks in the cytoplasm, accompanied by short microtubule fragments and tubulin aggregations similar to that observed with 1 μM vincristine (VCR) treatment. We further observed simultaneous formation of actin stress fibres in marchantin C-treated cells, presumably as a result of the changes in cellular microtu-
bule networks (Fig. 2B). Remarkably, we observed multiple spindle pole formation and/or multinucleation in cells treated with marchantin C for longer time points (data not shown), which is a characteristic phenotype observed during disturbed microtubules dynamics in dividing cells. These results indicate that marchantin C can affect cellular microtubules dynamics, particularly inhibiting cellular microtubule assembly and promoting cellular microtubule depolymerization.

To further confirm marchantin C’s ability to depolymerize microtubules in cultured cells, microtubules in A172 cells were quantitatively assessed at high resolution by transmission electron microscopy (TEM). Mitotic A172 cells treated with 8, 12, 16 μM of marchantin C for 24 h were observed under transmission electron microscopy. Cells treated with the same amount of DMSO or 1 μM of vincristine were used as controls. A single microtubule in the cross-section of mitotic cells can be viewed as a circle of the tubulin heterodimers packed around a central hole in the electron micrographs (Fig. 3A). The numbers of microtubules from the same size of area in micrographs were analyzed with Image-Pro Plus 5.0 software. As shown in Fig. 3B, there were an average of 2655 ± 286, 1900 ± 182, 1256 ± 102, 273 ± 54 or 1200 ± 98 (mean ± SD) microtubules in the same size of mitotic cells treated with DMSO (control), 8, 12, 16 μM of marchantin C and 1 μM of vin-
cristine, respectively. Therefore a dramatic decrease of microtubule numbers was observed in higher concentration of marchantin C-treated cells. These results also showed that decreasing microtubules was dependent on the concentration of marchantin C. Together these data suggest that marchantin C can efficiently depolymerize microtubules and result in disassembly of the mitotic spindle, arresting cultured tumor cells in G2/M phase.

3.3. Marchantin C inhibits microtubule polymerization in vitro

Given the microtubule depolymerization activity of marchantin C in cultured cells, we next examined the effect of marchantin C on microtubules in vitro. Purified unpolymerized tubulin was mixed with various concentrations of marchantin C at 4, 8, 16 μM. One micromolar of
vincristine, 10 μM of paclitaxel and same amount of DMSO were used as controls. The polymerization of tubulin was measured by spectrometer at the absorbance of 340 nm every minute for 1 h. Compared to DMSO-treatment alone, paclitaxel increased the absorbance, indicating that tubulin polymerization was enhanced. In contrast, vincristine de-
increased the absorbance, indicating that tubulin polymerization was inhibited. Treatment of marchantin C decreased the absorbance in a dose-dependent manner (Fig. 4). Therefore, marchantin C decreased the polymerization rate of gross tubulin, similar to microtubule depolymerizor vincristine. These results clearly show that marchantin C can
significantly inhibit polymerization of tubulin in vitro and provides direct evidence of the inhibitory activity of marchantin C on the assembly of microtubules and microtubule dynamics in cells.

3.4. Marchantin C inhibits the growth of human cervical tumor xenografts in vivo

Marchantin C can depolymerize microtubules in cultured cells and in vitro with similar efficacy as vincristine, which is one of the most widely used anti-tumor drugs. We next examined the antitumor activity of marchantin C in human cervical tumor xenografts in nude mice. HeLa cells ($3 \times 10^6$) were implanted into 7-week-old female athymic nude mice and grown as s.c tumors. When the tumors reached $\sim 1$ cm$^3$, the mice were i.v. treated daily with either vehicle control or marchantin C at 10 or 20 mg/kg. On day 10, the mice were sacrificed to obtain the tumors for analysis. Both the body weight of the mice and the tumor were measured as reference. The results showed that after 10 days of treatment with marchantin C, no significant variation in body weight was detected (Fig. 5A), suggesting that marchantin C did not produce significant non-tumor toxicity in tumor-bearing mice. However, the average weight of tumors treated with marchantin C at 10 or 20 mg/kg were 0.94 g and 0.48 g, respectively, versus 1.17 g of control tumors, which corresponds to 20% and 59% of inhibition ($P < 0.01$, Fig. 5B). In addition, the size of tumors treated with marchantin C also significantly decreased when compared with non-treated (data not...
These results suggest that marchantin C was able to effectively inhibit tumor growth in vivo.

In our previous study, we observed that marchantin C triggered cultured tumor cell apoptosis by stimulating the mitochondria-mediated intrinsic apoptotic pathway. Therefore, we analyzed whether inhibition of tumor growth in vivo by marchantin C was achieved through the same pathway. The levels of Bax, Bcl-2 and caspase-3 proteins in control- or marchantin C-treated tumor samples (20 mg/kg) were evaluated using Western blot analy-

Fig. 6. Immunohistochemistry assay of the tumor tissues from nude mice. The tumor tissues from nude mice treated without (upper) or with (lower) marchantin C (MC) were prepared as tissue sections, assessed by immunohistochemistry assay using mouse anti-Bcl-2 (A) or mouse anti-α-tubulin (B) antibodies. The results are representative of three independent experiments.
sis. The results showed significant increases of caspase-3 and Bax, but decrease of anti-apoptosis protein, Bcl-2, in tumor tissues from marchantin C-treated mice compared with controls (Fig. 5C). In addition, immunohistochemistry staining showed that Bcl-2 protein expression was significantly down-regulated in the tumors treated with marchantin C (Fig. 6A). These results indicate that marchantin C induces apoptosis in vivo cellular apoptosis by modulating the activity of proteins associated with apoptosis. Interestingly, high levels of cyclin B1 protein were also found in the extracts of marchantin C-treated tumors (Fig. 5C), evidence that further supports the arrest at G2/M phase induced by marchantin C in tumor cells. The in vitro experiments showed that marchantin C-induced microtubule depolymerization was a direct cause for G2/M phase arrest. To further validate this mechanism in vivo, immunohistochemical analysis was conducted to evaluate the level of microtubules in tumor tissues from the nude mice treated with or without marchantin C. As shown in Fig. 6B, marchantin C treatment led to a remarkable decrease of microtubules compared with the vehicle control. These results indicate that marchantin C promotes microtubule depolymerization both in vivo and in vitro. Collectively, our findings showed that marchantin C plays the same role in microtubule depolymerization and apoptotic effects and subsequent anti-tumoral activity in vivo.

4. Discussion

Marchantin C, a member of the bisbibenzyl family, has been verified to boast a wealth of medicinally important activities including antibacterial and antitumor [10,22]. In our previous study, we reported that marchantin C could trigger apoptosis in various cultured tumor cells [17]. Herein, we found that tumor cell apoptosis induced by marchantin C resulted from cell cycle arrest at G2/M phase (Fig. 1). Further investigation demonstrated that marchantin C causes microtubule depolymerization in a concentration-dependent manner both in cultured cells and in vitro tubulin polymerization assay (Figs. 2–4). In addition, experiments on human cervical tumor xenografts in nude mice reflected similar microtubule depolymerization activity and apoptotic induction, as well as subsequent tumor suppression, conferred by marchantin C in vivo as in vitro (Fig. 5 and 6).

Induction of tumor cell apoptosis is the final goal of most anti-cancer drugs, as well as the potential pro-anti-cancer drugs. It has also become the general standard to define or screen the potent novel anti-cancer drugs [23,24]. There are two important pathways involved in cellular apoptosis: the death-receptor-mediated extrinsic pathway and the mitochondria-dependent intrinsic pathway [25]. Previously, we found marchantin C could up-regulate Bax expression, but down-regulates Bcl-2 expression in cultured tumor cells. In this study, in vivo animal experiments confirmed the same effect of marchantin C as in vitro, indicating that marchantin C stimulates tumor cell apoptosis through the mitochondria-dependent intrinsic pathway [26]. Notably, FACS analysis indicated that marchantin C induced cell cycle arrest at G2/M phase in cultured tumor cells. At the same time, immunoblotting assay demonstrated an inappropriate accumulation of cyclin B1 in cultured tumor cells in vitro and tumor xenografts in vivo, indicating G2/M phase arrest of tumor cells treated with marchantin C. Moreover, marchantin C treatment promoted apoptosis after cell cycle arrest at G2/M phase, as confirmed by FACS and immunoblotting analysis. These results suggested that marchantin C-induced apoptosis likely resulted from cell cycle arrest at G2/M phase. The G2/M phase arrest induced by marchantin C was likely due to abnormal mitotic spindle formation and perturbed microtubule dynamics [27]. Consistent with our assumption, immunofluorescent staining assay showed that marchantin C disturbed intact microtubule networks in cultured tumor cells. TEM observation displayed significant microtubule decrease in a marchantin C dose-dependent manner as well. In vitro tubulin polymerization assay presented a direct inhibition of tubulin polymerization by marchantin C. Furthermore immunohistochemistry of tumor tissues in nude mice treated with marchantin C showed lower level of microtubules than that of vehicle control mice. Our data demonstrate that marchantin C possesses potent inhibitory activity for microtubule polymerization, abrogating microtubule dynamics and mitotic spindle formation, resulting in cell cycle arrest at G2/M phase and eventually leading to apoptotic cell death both in vitro and in vivo. More interestingly, similar G2/M phase arrest as well as cellular microtubule depolymerization could be observed in marchantin C-treated hTERT-RPE1 cells derived from normal human retinal pigment epithelial cell and immortalized by hTERT. However, there is no significant cell death in these cells even after 96 h treatment of marchantin C (data not shown). Collectively, these results provide evidence for marchantin C as a potential anti-tumor drug candidate.

Microtubules are crucial components of mitotic spindle in the process of mitosis, which has made it a favorable target of antitumor therapy. Most microtubule-targeted antitumor compounds have been discovered through large-scale screening of natural products, are found either to stabilize or destabilize microtubule polymerization [28,29]. For instance, taxane stabilize microtubules, while vinca alkaloid derivatives and colchines destabilize microtubules [29]. Such compounds interact with three known pharmacologic sites on tubulin: the taxane site, the Vinca domain, and the colchine domain. Marchantin C has the chemical structure of macrocyclic bisbibenzyls composed by two bibenzyl skeletons linked with two ether bonds which is different from classical microtubule inhibitors such as paclitaxel, vinca alkaloids and colchines. However, it is structurally similar to a dihydrated dimer of combretastatin A-4 (CA-4), a proven inhibitor of microtubule polymerization used as a successful tumour vascular targeting agent in recent reports [30,31]. Therefore, the bibenzyl skeleton and methoxy-groups may play an important role to depolymerize microtubules and then exhibit cytotoxic potential which provide insight into synthetic modification of the structure-activity of marchantin C in the future research.

In conclusion, marchantin C is a potent inhibitor of microtubule polymerization that blocks mitosis progress
and induces apoptosis of tumor cell in vivo as well as in vitro. For the first time, our study shows that this compound of the macrocyclic bisbibenzyls family has a novel property of inhibition activity of microtubule polymerization but is structurally distinct from classical microtubule inhibitors including colchicine, vinblastine and paclitaxel. These findings indicate that marchantin C is a potential antitumor agent by inhibiting microtubule polymerization.

Conflicts of interest statement

None declared.

Acknowledgements

This work was supported by Grants from National Natural Science Foundation of China (No. 30271537), Shandong Provincial Foundation for Scientific Research (Nos. 2006GG1102023 and 2005GG3202107) to H.X. Lou and National Natural Science Foundation of China (No. 30700396) to C. Zhu.

References