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ORIGINAL PAPER

3-Nitro-naphthalimide and nitrogen mustard conjugate NNM-25 induces hepatocellular carcinoma apoptosis via PARP-1/p53 pathway

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Abstract Hepatocellular carcinoma (HCC) is one of the main causes of death in cancer. Some naphthalimide derivatives exert high anti-proliferative effects on HCC. In this study, it is confirmed that 3-nitro-naphthalimide and nitrogen mustard conjugate (NNM-25), a novel compound conjugated by NNM-25, displayed more potent therapeutic action on HCC, both in vivo and in vitro, than amonafide, a naphthalimide drug in clinical trials. More importantly, preliminary toxicological evaluation also supported that NNM-25 exhibited less systemic toxicity than amonafide at the therapeutic dose. The antitumor mechanism of conjugates of naphthalimides with nitrogen mustard remains poorly understood up to now. Here, we first reported that apoptosis might be the terminal fate of cancer cells treated with NNM-25. Inhibition of p53 by siRNA resulted in a significant decrease of NNM-25-induced apoptosis, which corroborated that p53 played a vital role in the cell apoptosis triggered by NNM-25. NNM-25 inhibited the PARP-1 activity, AKT phosphorylation, up-regulated the protein expression of p53, Bad, and mTOR as well as down-regulating the protein expression of Bcl-2 and decreasing mitochondrial membrane potential. It also facilitated cytochrome *c* release from mitochondria to cytoplasm, activated caspase 8, caspase 9, and caspase 3 in HepG2 cells in vitro, as also authenticated in H22 tumor-bearing mice in vivo. Collectively, the conjugation of naphthalimides with nitrogen mustard provides favorable biological activity and thus is a valuable strategy for future drug design in HCC therapy.

Keywords Hepatocellular carcinoma · PARP-1 · Naphthalimide · Alkylating agent · Apoptosis

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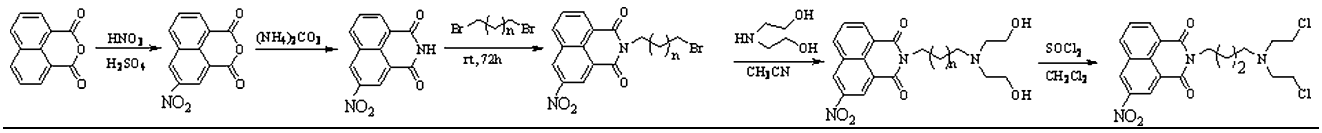
Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer in terms of incidence and the third most common cause of death in cancer in the world [1]. The therapeutic success of chemotherapeutic drugs is often limited by severe adverse effects. Therefore, it is essential to develop novel chemotherapeutic agents, which exert greater efficacy to HCC with none or restricted toxicity to normal cells [2]. Indeed, naphthalimides have shown potent anticancer activity against a variety of murine and human cancer cell lines, some of which, such as amonafide and mitonafide, have been evaluated in clinical trials. However, most of tested naphthalimides have been abandoned because of poor therapeutic index and hematotoxicity [3]. In recent years, we found that some naphthalimide derivatives displayed potent inhibitive effects on HCC. For example, the naphthalimides conjugated with polyamines have been designed to improve their efficacy against HCC with reduced adverse effects [4, 5]. Previous report demonstrated that the conjugates of naphthalimide and alkylating agent were more effective than their respective naphthalimide and alkylating agent in S-180 and Ehrlich ascites carcinoma models [6]. However, the detailed molecular mechanism of their antitumor activity remains dimness up to now.

4-Amino-1,8-naphthalimide (4-AN) exerts potent anti-proliferative effects via poly(ADP-ribose) polymerase 1 (PARP-1) inhibition [7]. PARP-1 plays a pivotal role in DNA damage and repair, post-translational modification, etc. [8]. Several drugs, which were designed to inhibit PARP-1 activity, are currently in clinical development [9]. Furthermore, the drug combination of PARP-1 inhibitors and alkylating agents is also currently being investigated in clinical trials for cancer therapy [10].

On account of these merits of naphthalimides and nitrogen mustards, a series of conjugates were synthesized as potential HCC therapeutic agents in our laboratory. After screening, we found that 3-nitro-naphthalimide and nitrogen mustard (NNM-25), a novel conjugate of 3-nitro-naphthalimide and nitrogen mustard, exerted favorable anti-proliferative activity in hepatoma cells. The purpose of the present study includes the evaluation of HCC inhibition in vivo and in vitro, the preliminary systemic toxicity, and the potential molecular mechanism of NNM-25. As reported herein, our results indicated that NNM-25 could potently inhibit HCC growth in vivo and in vitro via apoptosis. Encouragingly, preliminary toxicological evaluation suggested that NNM-25 had no obvious systemic toxicity at the therapeutic dose.

The synthetic route of 3-nitro-naphthalimide and nitrogen mustard conjugate (NNM-25)



Materials and methods

Cell lines and reagents

Three well-characterized human liver cancer cell lines (HepG2, SMMC7721, and BEL7402), one mice liver cancer cell line (H22), and one human liver non-tumoral cell line (QSG7701) were maintained as an adherent monolayer in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (20% for QSG7701 cells), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma) and incubated at 37°C in 5% CO_2. RNase A, rhodamine-123 (Rh-123), 3-(4,5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide (MTT), Hoechst33342, and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640 and fetal calf serum (FCS) were purchased from Gibco (Grand Island, NY, USA). Annexin V-FITC apoptosis detection kit was purchased from Beyotime (Shanghai, China). Primary antibodies against: Bcl-2, Bad, caspase 9, caspase 3, caspase 8, PARP-1, cytochrome *c*, p53, *p*-AKT, *p*-mTOR, as well as horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were all obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). All other chemicals used in the experiments were commercial products of reagent grade.

Tumor studies in vivo

Kunming male mice (6–8 weeks old) were purchased from the laboratorial animal center of Henan (Zhengzhou, China). All animal procedures were performed following the protocol approved by the Institutional Animal Care and Use Committee of Henan University. For solid tumor model, the mice were injected sub-cutaneously in the right flank with 0.2 mL of cell suspension containing 5×10^7 of H22 cells. Tumors were allowed to grow for approximate 7 days before drug treatment. Tumor-bearing mice were randomly assigned to one of the following three treatment groups ($n = 10$ mice per group): control (physiologic saline), NNM-25 (1.5 mg/kg) and amonafide (5 mg/kg). NNM-25 (purity 99.7%) was synthesized in our laboratory and the structure was shown in Fig. 1a. The doses were chosen according to their acute toxicity (1/2 LD_{10}). For ascites tumor model, the mice were injected in the abdominal cavity with 0.2 mL of cell suspension containing 5×10^7 of H22 cells. Next day, the tumor-bearing mice were randomly assigned to one of the following three treatment groups ($n = 20$ mice per group): control (physiologic saline), NNM-25 (1.5 mg/kg) and amonafide (5 mg/kg). Drug treatments were administered through tail vein injection for 7 consecutive days. For the solid tumor study, the mice were killed by aether anesthesia after 24 h of drug withdrawal. Solid tumors were removed and weighed. The inhibitory rate was calculated as follows: inhibitory rate (%) $= [(\text{weight}_{\text{control}} - \text{weight}_{\text{drug}})/\text{weight}_{\text{control}}] \times 100$. After being weighted, the tumors were fixed in formalin and stained with hematoxylin and eosin. Immunohistochemistry (IHC) analysis was used to detect PARP-1 protein. Rabbit anti-mouse PARP-1 polyclonal antibody (1:200 dilution)

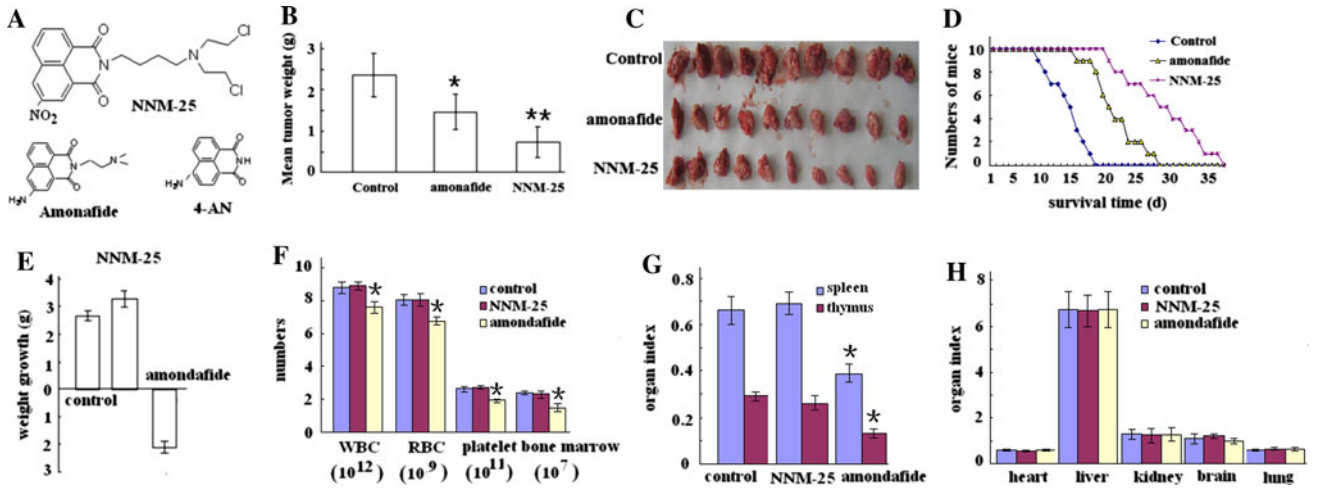


Fig. 1 Antitumor activity and systemic toxicity of NNM-25 were evaluated in vivo. **a** The chemical structure of NNM-25. **b** Mean tumor weight after treated with NNM-25. **c** Specimen of subcutaneously H22 xenograft tumor after treated with NNM-25 at the end of the experiment. **d** Survival time of bearing H22 ascitic mice treated with NNM-25, $n = 10$. **e** The weight change after treatment with NNM-25. **f** Platelet, RBC, and WBC numbers were detected after treatment NNM-25. **g**, **h** The organ index was evaluated after NNM-25 treatment. Compared with control $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 10$

was used in standard indirect immunoperoxidase procedures [11]. For each treatment group in ascites model, ten mice were used for mean survival time (MST) analysis. The extended lifespan rate was calculated as follows: extend rate (%) = ($MST_{drug}/MST_{control}$) × 100 [12]. The remaining ten mice in each treatment group were used for apoptotic mechanism study. The ascites of H22-bearing mice were collected for apoptosis, MMP, intracellular fluorescence intensity and western blotting assay.

Systemic toxicity evaluation

To study the toxic effects of NNM-25, as indicated by loss of body weight, food consumption, hematology, organ weight and macroscopic evaluation, 30 mice without tumors were randomly assigned to one of three treatment groups ($n = 10$ mice per group): control (physiologic saline), NNM-25 (1.5 mg/kg) and amonafide (5 mg/kg). The weight of mice was recorded daily before tail vein injection. The experiment was ended on day 8. Blood and bone marrow were collected, and hematological profiling was undertaken by determining platelet, red blood cell (RBC), white blood cell (WBC) and bone marrow cells counts using a Coulter Counter T-890 (Coulter Electronics, USA). The organ index was investigated for systemic toxicity evaluation. Organ index (%) = (Organ weight/body weight) × 100.

Cell proliferative assay in vitro

The anti-proliferative ability of NNM-25 was evaluated in HepG2 cells, BEL7402 cells, SMMC7721 cells, H22 cells, and QSG7701 cells by the conversion of MTT to a purple formazan precipitate as previously described [13]. Briefly, cells were seeded into 96-well plates at 5×10^3 cells/well. After 12 h, various concentrations (0.1, 1, 10 30, and 50 μM) of NNM-25 were subsequently added and incubated for 48 h and then clearage by dimethyl sulfoxide. The inhibitory rate was calculated from plotted results using untreated cells as 100%.

DNA binding assay

Solutions of ct DNA in 20 mM Tris–HCl buffer (pH 7.4) gave a ratio of UV absorbance at 260 and 280 nm of 1.81:1, indicating that the DNA was sufficiently free of protein. The concentration of ct DNA was determined spectrophotometrically assuming that the molar absorption is 6,600 M/cm (260 nm). NNM-25 was then added into the mixture of ct DNA and ethidium bromide (EB), and the final concentrations of NNM-25 were 1, 2, 3, and 4 μM, respectively. The final concentrations of DNA and EB were 11.3 and 47.8 μM, respectively. All the above solutions were shaken for 30 min at room temperature. Fluorescence wavelengths and intensity areas of samples were measured at following conditions: excitation 356 nm, emission 595 nm [4].

PARP-1 activity assay in cell-free system

Poly(ADP-ribose) polymerase 1 activity was assessed using a commercially available kit (Trevigen, Gaithersburg, MD), based on the measurement of radiolabeled NAD^+ incorporation. The assay was performed using purified PARP-1 protein, untreated or in the presence of graded concentrations of NNM-25. The enzymatic reaction was carried out by

incubating purified PARP-1 with 2 μCi ^{32}P-NAD$^+$ (Amersham, Milan, Italy), 10 μM NAD$^+$, 1 × PARP-1 buffer, 10 μg of nuclease-treated salmon testes DNA, 10 μg of histones, according to the manufacturer's instructions [14].

Intracellular fluorescence intensity assay

The intracellular fluorescence intensity of NNM-25 was detected by high content screening (HCS) analysis (Thermo Scientific Cellomics ArrayScan Vti, Cellomics, Pittsburgh, PA). Briefly, after cells were seeded into 96-well plates at 5,000 cells/well for 24 h, the cells were stained with acridine orange (AO) (50 μg/mL) for 15 min, then washed three times with PBS. NNM-25 (1.0, 10 or 30 μM) was added into 96-well plate and then the intracellular fluorescence intensity of NNM-25 for 30 min was detected using HCS. For H22 cells, which were collected from the ascites of mice bearing H22 cells after treatment with NNM-25, after AO staining, the intracellular fluorescence intensity of NNM-25 was detected using HCS. Cell numbers were determined by AO staining and intracellular fluorescence intensity of NNM-25 was detected at excitation 350 nm and emission 460 nm, AO was detected at excitation 492 nm and emission 526 nm [13].

Cellular apoptotic evaluation

Cell apoptosis was evaluated by Annexin V-FITC apoptosis detection kit using FACScan (Becton–Dickinson, USA). Briefly, HepG2 cells were seeded in 6-well plates, and exposed to NNM-25 for 48 h, then harvested and stained according to manufacturer's protocol. For H22 cells, on day 8 after treatment with NNM-25, amonafide or physiologic saline, the H22 cells were extracted from abdominal cavity of H22-bearing mice and then washed with PBS for apoptosis, mitochondrial membrane potential (MMP) and western blotting assay. Data acquisition and analysis were controlled by CellQuest software [13]. In addition, cell apoptosis was also detected by AO/EB double staining. Briefly, after collected, cells were seeded into 6-well plates and stained with AO (0.15 μM)/EB (0.15 μM) for 30 min. After washing three times with PBS, the fluorescent micrographs were taken using HCS.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was assessed by the retention of Rh-123, a membrane-permeable fluorescent cationic dye. The uptake of Rh-123 by mitochondria is proportional to the MMP. Briefly, cells were incubated with Rh-123 (0.1 μg/mL) in the dark at room temperature for 20 min. After being washed with PBS, cells were counterstained with Hoechst 33342 (1 μM) for 15 min at dark. The change of MMP was analyzed by HCS [13].

Western blotting

After treatment, HepG2 cells and H22 cells were harvested and washed with PBS. Cytosolic and mitochondrial fractions were prepared as previously described [13]. Total cellular protein was isolated using the protein extraction buffer (containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS). Protein concentration was determined using the protein assay kit (Biyuntian, China). Equal amounts of proteins (50 μg/lane) were fractionated using 8 or 12% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with primary antibodies. After washing with PBS, the membranes were incubated with corresponding peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody, followed by enhanced chemiluminescence staining through the enhanced chemiluminescence system. β-Actin (45 kDa, cytosolic protein) was used to normalize for protein loading.

siRNA studies

HepG2 cells were seeded into 6-well plates at a density of 5×10^5 cells per well in RPMI-1640 medium supplemented with 10% fetal bovine serum and grown to 90% confluent overnight before transfection control siRNA (100 nM, CST 6568, Cell Signalling Technology) or p53 siRNA (100 nM, CST 6562, Cell Signalling Technology) was transfected using lipofectamine 2000 (Invitrogen) in 2 ml serum-free medium per well according to the manufacturer's instructions. Knock-down of p53 expression was confirmed by western blot after 72 h transfection. For apoptotic evaluation, the p53 siRNA-transfected HepG2 cells were co-incubated with NNM-25 or amonafide for another 48 h, and then cell apoptosis was evaluated by Annexin V-FITC apoptosis detection kit by FACScan (Becton–Dickinson, USA).

Data analysis

All data are presented as mean ± SD and analyzed using Students t test or analysis of variance (ANOVA) followed by q test.

Results

Antitumor effect and systemic toxicity of NNM-25 in vivo

3-Nitro-naphthalimide and nitrogen mustard conjugate significantly suppressed tumor growth in comparison to

control mice in solid tumor model (Fig. 1b, c). The tumor inhibitory ratio was 40.3% (1.41 ± 0.43) g for amonafide and 68.6% (0.74 ± 0.38) g for NNM-25, as compared to control mice (2.36 ± 0.52) g, respectively. The MST of the control mice was (12.9 ± 3.2) days in ascites model. After treatment with NNM-25 or amonafide, the lifespan was increased by 2.2-fold (28.5 ± 6.5) days and 1.6-fold (20.6 ± 4.3) days, respectively (Fig. 1d).

We did not observe weight loss in the NNM-25-treated mice, whereas treatment with 5 mg/kg amonafide was associated with weight loss, indicating the dose toxicity to some extent (Fig. 1e). Moreover, we found that the food consumption of tested mice was decreased after treatment with amonafide, while NNM-25 treatment had no obvious effect on the food consumption of mice in contrast with control (data not shown). In addition, amonafide resulted in a significant decrease in platelet, RBC, and WBC numbers compared to control, while NNM-25 did not. At the same time, NNM-25 had no obvious influence on bone marrow cell numbers (Fig. 1f), suggesting that NNM-25 possessed no evident hematotoxicity in this test. Additively, amonafide down-regulated spleen and thymus indexes more potently than NNM-25, indicating that NNM-25 had no influence on immune function but amonafide inhibited immune function apparently (Fig. 1g). Finally, no significant difference in the organic factors of heart, liver, kidney, brain, lung, and spleen was observed for both tested drugs and control (Fig. 1h).

Anti-proliferative effects and tumor cell selectivity of NNM-25

Previous data demonstrated that some conjugates of nitrogen mustard and naphthalimide, such as napromustine, only exerted potent antitumor effect in vivo but not in vitro [6]. So we further evaluated whether NNM-25 exerted anti-proliferative activity in vitro. Four HCC cell lines (HepG2, Bel-7402, SMMC7721, and H22) and one normal hepatocyte line (QSG7701) were selected to assess the anti-proliferative activity and tumor selectivity of NNM-25. The IC_{50} value after 48 h treatment was (130.2 ± 8.4), (1.9 ± 0.4), (2.8 ± 0.9), (2.5 ± 0.7) and (3.4 ± 0.9) μM for QSG7701 cells, HepG2 cells, SMMC7721 cells, H22 cells and Bel-7402 cells, respectively. This result implied that NNM-25 exerted HCC selectivity (Fig. 2a). Accordingly, the intracellular fluorescence intensity of NNM-25 was higher in liver cancer cells than in normal hepatocyte (Fig. 2b). Similarly, NNM-25 was also efficiently uptake by H22 cells in vivo (Fig. 2c). These results suggested that the selectivity was nicely correlated with the intracellular contents of NNM-25.

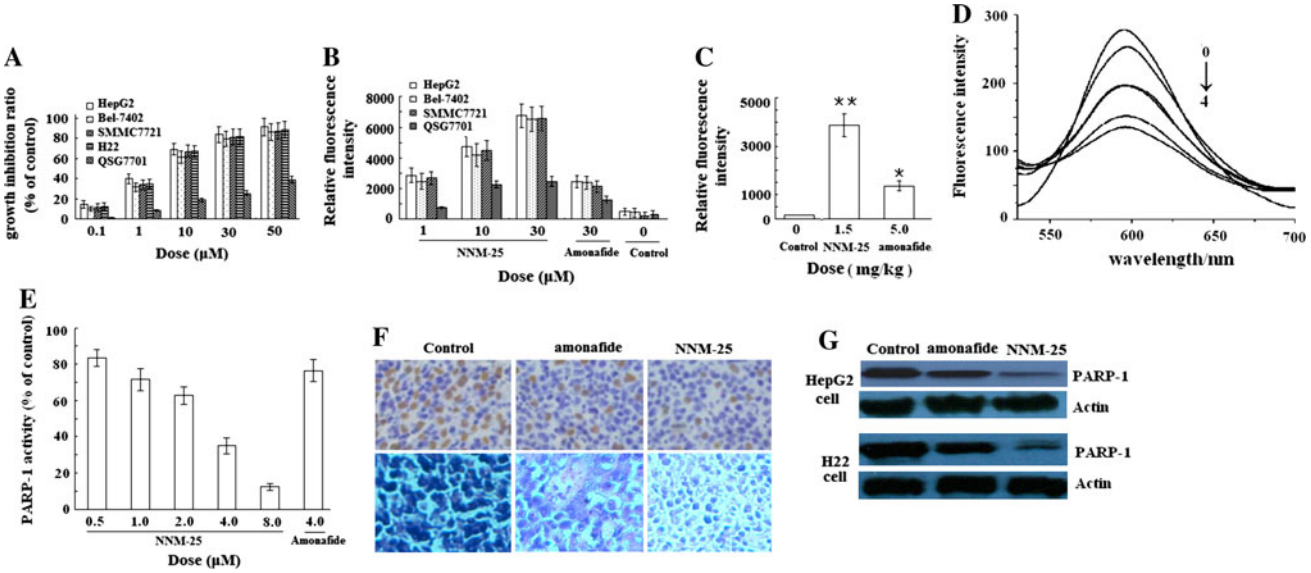


Fig. 2 The growth inhibitory effects, intracellular fluorescence intensity, DNA binding and PARP-1 inhibitory activity were evaluated after treatment with NNM-25. **a** Growth inhibitory effect of NNM-25 after 48 h treatment. *Each point* represents the means ± SD from four independent experiments. **b** The intracellular fluorescence intensity of NNM-25 was detected by HCS after treatment with NNM-25 for 30 min. *Each point* represents the means ± SD from four independent experiments. **c** The intracellular fluorescence intensity of NNM-25 was detected by HCS after treatment with 7 consecutive days in H22 tumor-bearing mice. Compared with control $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 10$. **d** Fluorescence spectra of EB with herring sperm DNA. *Numbers 0–4* indicated the NNM-25 concentration: 0, 1, 2, 3, and 4 μM. **e** Activity of isolated, recombinant PARP-1 was inhibited by NNM-25. *Each point* represents the means ± SD from four independent experiments. **f** Immunohistochemistry analysis was used to detect PARP-1 protein expression in xenografts tumor (*Up*, 400×), histological examination and morphological analysis of tumoral xenografts (*Down*, H&E, 400 ×). **g** The protein expression of PARP-1 was detected by western blotting in HepG2 cell in vitro and H22 cell in vivo

DNA intercalating and PARP-1 inhibitive activity of NNM-25

Previous studies demonstrated that naphthalimides exert potent DNA intercalating properties and are DNA-damaging agents [3]. To verify the hypothesis that NNM-25 is also a DNA intercalating agent, we tested its DNA intercalating ability using DNA-EB assay. In a cell-free system, the fluorescence intensity of DNA-EB was gradually attenuated accompanied by the increasing concentration of NNM-25 (Fig. 2d), indicating that NNM-25 was a more powerful DNA intercalating agent than EB.

Some naphthalimides, such as 4-AN, exert potent antitumor effect via inhibiting PARP-1 activity [8]. To directly assess the inhibitory effect of NNM-25 on PARP-1 activity, we detected the activity of PARP-1 using recombinant PARP-1 in a cell-free assay. As expected, PARP-1 activity was markedly reduced by NNM-25 in a dose-dependent manner (Fig. 2e). To provide evidences that the inhibition of tumor growth by NNM-25 was related to PARP-1 in vivo, a special study was accomplished to analyze PARP-1 in tumor tissues by IHC analysis. PARP-1 protein expression was evidently reduced after NNM-25 treatment (Fig. 2f, up). Histological examination demonstrated that the tumor cells were obviously decreased after treatment with NNM-25 (Fig. 2f, down). Furthermore, the protein expression of PARP-1 was also significantly down-regulated in H22 cells in vivo and in HepG2 cells in vitro (Fig. 2g).

Cell apoptosis and related protein expression in vivo and in vitro

Poly(ADP-ribose) polymerase 1 plays a critical role in DNA damage and repair processes [8]. As NNM-25 exerted potent DNA intercalating and PARP-1 inhibiting activity, we further detected whether NNM-25 could induce cell apoptosis. Compared with control cells, the percentage of annexin V-positive cells was increased after treatment with NNM-25 in HepG2 cells in vitro and in H22 cells in vivo (Fig. 3a). This apoptotic effect was also authenticated by AO/EB staining (Fig. 3b). These data suggested that NNM-25 could induce liver cancer cells apoptosis in vitro and in vivo.

Cell apoptosis is a complicated dead phenomenon and many signal pathways are involved in this process. To elucidate the molecular mechanism of NNM-25-mediated cell apoptosis, we first detected the mitochondrial apoptotic pathway. The MMP of HepG2 cells and H22 cells was decreased significantly after treatment with NNM-25 in vitro and in vivo (Fig. 3c, d), and accordingly facilitated cytochrome *c* release from mitochondria to cytoplasm,

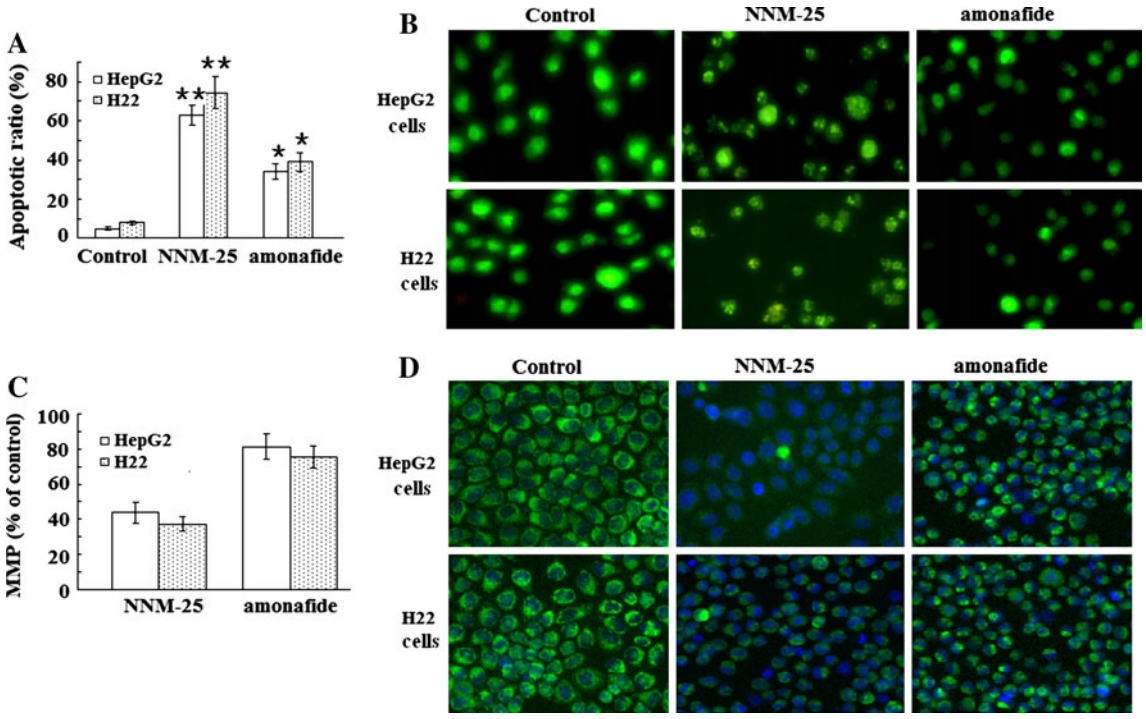


Fig. 3 The apoptosis and MMP were investigated in HepG2 cells in vitro and H22 cells in vivo. **a** The apoptotic ratio was detected using FACScan in HepG2 cells and H22 cells. Compared with control $*p < 0.05$, $**p < 0.01$. **b** The cellular morphous was detected with AO/EB staining using HCS in HepG2 cells and H22 cells (400×). **c**, **d** The MMP was detected with Rh-123/Hoechst33342 staining using HCS in HepG2 cells in vitro and H22 cells in vivo

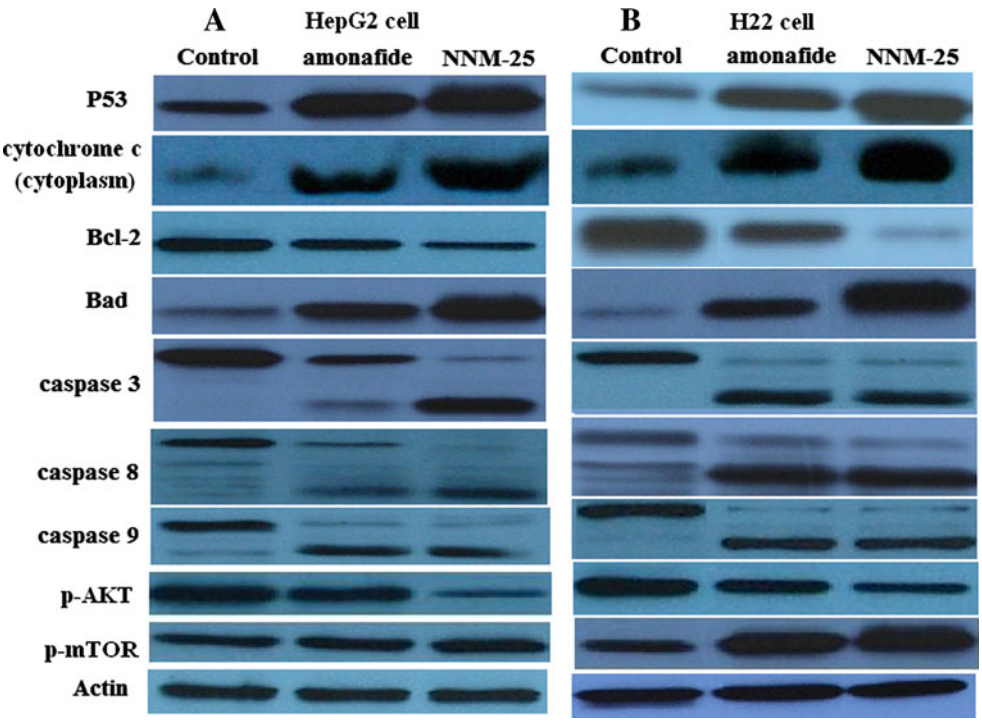


Fig. 4 The protein expression was detected by western blotting in HepG2 cells after 48 h treatment in vitro and H22 cells after 7 consecutive day treatment in vivo. **a** The protein expression was detected by western blotting in HepG2 cells in vitro. **b** The protein expression was detected by western blotting in H22 cells after 7 consecutive days treatment in vivo

respectively (Fig. 4a, b). Furthermore, the protein expression of Bad, p53, *p*-mTOR was up-regulated after treatment with NNM-25. NNM-25 also induced the down-regulation of Bcl-2 and *p*-AKT protein expression (Fig. 4a, b). These results indicated that mitochondria and Bcl-2 were involved in NNM-25-mediated cell apoptosis in HepG2 cells and H22 cells in vitro and in vivo. NNM-25 also activated caspase 8, caspase 9, and caspase 3, suggesting that the proteins of caspase family were involved in NNM-25-mediated cell apoptosis.

p53 is necessary for NNM-25-induced apoptosis

p53 has a major function in transducing stress to the apoptotic machinery of the cell, consistent with the important p53 status as a determinant of cellular response to DNA-damaging drug [15]. To examine the role of p53 in the control of NNM-25-induced apoptosis, we selected a specific p53 small interference RNA (siRNA) to block p53 translation. Following the administration of this p53 siRNA for 72 h, the protein expression of p53 was almost completely inhibited (Fig. 5a). Next, we examined whether the p53 siRNA affects apoptosis. HepG2 cells were incubated with p53 siRNA (100 nM for 72 h) prior to NNM-25 or amonafide treatment, cell apoptosis was then assessed by flow cytometry. Inhibition of p53 with the specific siRNA led to a significant drop in apoptotic ratio after NNM-25 treatment, suggesting that p53 is necessary for NNM-25-induced HepG2 cell apoptosis. However, the apoptotic ratio was only moderately decreased after amonafide treatment (Fig. 5b). Those data demonstrated that the NNM-25 and amonafide-induced apoptotic mechanism is not identical.

Discussion

The combinative administration of PARP-1 inhibitors and alkylating agents has been proved to have synergistic antitumor activities in many preclinical and clinical studies [10]. Indeed, previous work [6] and present data of NNM-25 confirmed that the conjugate of naphthalimides with alkylating agent can also display better antitumor activity than amonafide or mitonafide, naphthalimide derivatives without nitrogen mustard, in the trials of the S-180 and HCC tumoral xenograft growth inhibition and the life span in vivo. Interestingly, the antitumor effect of NNM-25 is obviously different from that of napromustine in vitro, another conjugate of nitrogen mustard and naphthalimide. NNM-25 is potent in tumor growth inhibition both in vivo and in vitro, whereas napromustine is only effective in vivo but not in vitro in the screen of several human cell lines [6].

Although amonafide is effective in clinical trials, it also suffers from the dose-limited bone marrow toxicity [16]. Encouragingly, preliminary toxicological evaluation demonstrated that NNM-25 had improved indices in terms of systemic toxicity, especially hematotoxicity at the therapeutic dose. In addition, the observed minor body weight gains of the mice treated with NNM-25, contrary to weight losses by amonafide treatment compared with the control,

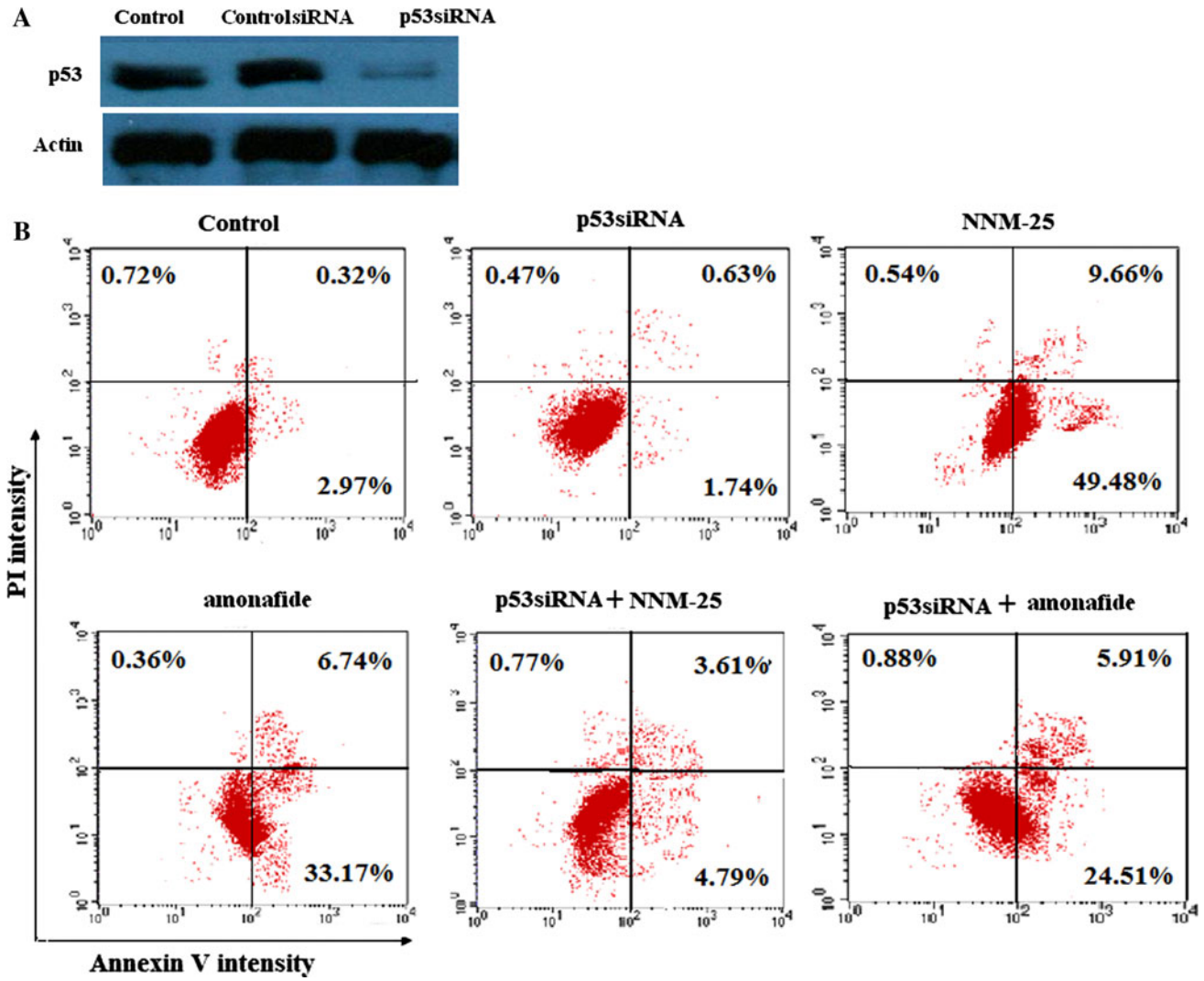


Fig. 5 p53 siRNA inhibited the protein expression of p53 and NNM-25-mediated apoptosis in HepG2 cells. **a** The protein expression was detected by western blotting in HepG2 cells in vitro. **b** The apoptotic ratio was detected using FACScan in HepG2 cells

indicated that NNM-25 displayed activity without obvious dose-limited toxicity. Although amonafide resulted in the less food intake of tested mice, the difference in weight variations might be also illustrated by the better cancerous and normal cell selectivity of NNM-25 than that of amonafide.

As far as we know, the apoptosis produced by the conjugate of naphthalimides with alkylating agents, and the related apoptotic mechanism are not reported, which are important to elicit how the conjugation between naphthalimides and alkylating agents results in the synergistic cell killing effect on tumors. Indeed, obvious apoptosis is observed for both NNM-25 and amonafide. Interestingly, NNM-25 with better bioactivity and less toxicity triggered more potent apoptosis than amonafide. It is therefore necessary to establish the relevant apoptotic mechanism.

We previously found that AKT/mTOR signaling pathway is involved in the naphthalimides, such as NPC-16, induced apoptosis [13]. As expected, NNM-25 also inhibited AKT phosphorylation and promoted mTOR phosphorylation, and caspases 3, 8, and 9 were involved in this pathway. However, it seems that the differences between NNM-25 and amonafide on *p*-AKT are not proportional to that of apoptotic content, especially for H22 cells. Since naphthalimides and alkylating agents can target DNA, it is rational to speculate that DNA might be at least one of the molecular targets of NNM-25. Unsurprisingly, NNM-25 could displace EB, one known DNA intercalator, from DNA-EB complex in the fluorescence experiments as previously reported [4]. This result is also in consistent with that of naphthalmustine [17].

Poly(ADP-ribose) polymerase 1 is a nuclear enzyme that plays a critical role in DNA damage response

processes. PARP-1 inhibition has been successfully employed as a novel therapeutic strategy to enhance the cytotoxic effects of DNA-damaging agents [18]. Because PARP-1 inhibitor can enhance the antitumor effect of alkylating agents and 4-AN is a PARP-1 inhibitor [19], so we supposed that the encouraging biological results of NNM-25, with the similar naphthalimide segment to 4-AN, are also related to its PARP-1 inhibitive capacity. Thus, the PARP-1 protein expression and activity were detected using a cell-free assay, and IHC analysis, western blotting in vivo and in vitro. Our results demonstrated that NNM-25 significantly inhibited PARP-1 protein expression in H22 xenograft tumor and ascites H22 cell in vivo, and also inhibited PARP-1 activity in a cell-free system in vitro. In addition, NNM-25 inhibited the activity and protein expression of PARP-1 more effectively than the control drug amonafide, which is in accordance with the more potent inhibition of NNM-25 against HCC in vitro and in vivo than amonafide. Huang et al. [20] discovered that PJ34, an inhibitor of PARP-1, exerted suppressive HepG2 cell growth in vivo and in vitro, and also induced cell apoptosis via caspase pathways. The observed H22 cell apoptosis in vivo and HepG2 cell in vitro significantly induced by NNM-25 are in accordance with above reports.

DNA damage-triggered signaling and execution of apoptosis depend on the p53 status, death-receptor responsiveness, and most importantly, DNA repair capacity [21]. p53 could repair the impaired DNA to protect cells from DNA damage, and also facilitate cell apoptosis once the DNA damage could not be soundly repaired [22]. In addition, p53 participates directly in the intrinsic apoptosis pathway by interacting with the multidomain members of the Bcl-2 family to induce mitochondrial outer membrane permeabilization [23]. Bcl-2 family proteins are also often involved in mitochondria-mediated apoptosis [13]. Accordingly, we turned our attention to Bad and Bcl-2, which are two classical pro-apoptotic and anti-apoptotic Bcl-2 family members, respectively. Our results demonstrated that NNM-25-mediated apoptosis was associated with these facilitative and inhibitive apoptotic factors. Indeed, NNM-25 increased the expression of p53 and Bad, decreased the expression of Bcl-2, degraded MMP and promoted cytochrome c release from mitochondria to cytoplasm, and activated caspase-3, -8, and -9. Furthermore, inhibition of p53 by specific siRNA led to a significant decrease in apoptotic ratio after NNM-25 treatment, suggesting that p53 is a vital factor for NNM-25-induced HepG2 cell apoptosis. However, the comparatively mild effect of p53 siRNA on amonafide triggered apoptosis indicated that the introduction of alkylating agents to naphthalimides did change the apoptotic mechanism.

Collectively, this study showed that NNM-25 exerted potent effects on HCC in vivo and in vitro compared to amonafide. At the same time, the improved systemic toxicity at the therapeutic dose added more evidences that the conjugation of naphthalimides with nitrogen mustards provides favorable biological activity and thus is a valuable strategy for future drug discovery in HCC therapy. To our knowledge, the NNM-25 induced HCC cell apoptosis, which is different from that of amonafide, is first reported. And the present results help to establish a complex signaling pathway of apoptosis involving AKT/mTOR and PPAR-1/p53, which gives a clue for further exploration of the detailed molecular mechanism of conjugates formed by naphthalimides with nitrogen mustards.

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Conflict of interest None.

References

1. Alves RC, Alves D, Guz B, Matos C, Viana M, Harriz M (2011) Advanced hepatocellular carcinoma. Review of targeted molecular drugs. Ann Hepatol 10:21–27
2. Duffy A, Greten T (2010) Developing better treatments in hepatocellular carcinoma. Expert Rev Gastroenterol Hepatol 4:551–560
3. Ingrassia L, Lefranc F, Kiss R, Mijatovic T (2010) Naphthalimides and azonafides as promising anti-cancer agents. Curr Med Chem 16:1192–1213
4. Tian ZY, Xie SQ, Du YW, Ma YF, Zhao J, Gao WY, Wang CJ (2009) Synthesis, cytotoxicity and apoptosis of naphthalimide polyamine conjugates as antitumor agents. Eur J Med Chem 44:393–399
5. Tian ZY, Xie SQ, Mei ZH, Zhao J, Gao WY, Wang CJ (2010) Conjugation of substituted naphthalimides to polyamines as cytotoxic agents targeting the Akt/mTOR signal pathway. Org Biomol Chem 7:4651–4660
6. Pain A, Samanta S, Dutta S, Saxena AK, Shanmugavel M, Kampasi H, Quazi GN, Sanyal U (2003) Synthesis and evaluation of substituted naphthalimide nitrogen mustards as rationally designed anticancer compounds. Acta Pol Pharm 60:285–291
7. Horton JK, Stefanick DF, Zeng JY, Carrozza MJ, Wilson SH (2011) Requirement for NBS1 in the S phase checkpoint response to DNA methylation combined with PARP inhibitory. DNA Repair 10:225–234
8. Powell C, Mikropoulos C, Kaye SB, Nutting CM, Bhide SA, Newbold K (2010) Pre-clinical and clinical evaluation of PARP inhibitors as tumour-specific radiosensitisers. Cancer Treat Rev 36:566–575
9. Rodon J, Iniesta MD, Papadopoulos K (2009) Development of PARP inhibitors in oncology. Expert Opin Investig Drugs 18:31–43
10. Heacock ML, Stefanick DF, Horton JK, Wilson SH (2010) Alkylation DNA damage in combination with PARP inhibitory results in formation of S-phase-dependent double-strand breaks. DNA Repair 9:929–936
11. Wang S, Jia L, Zhou H, Shi W, Zhang J (2009) Knockdown of caveolin-1 by siRNA inhibits the transformation of mouse hepatoma H22 cells in vitro and in vivo. Oligonucleotides 19:81–88

12. Kuang DM, Peng C, Zhao Q, Wu Y, Chen MS, Zheng L (2010) Activated monocytes in peritumoral stroma of hepatocellular carcinoma promote expansion of memory T helper 17 cells. Hepatology 51:154–164
13. Xie SQ, Li Q, Zhang YH, Wang JH, Mei ZH, Zhao J, Wang CJ (2011) NPC-16, a novel naphthalimide-polyamine conjugate, induced apoptosis and autophagy in human hepatoma HepG2 cells and Bel-7402 cells. Apoptosis 16:27–34
14. Alano CC, Kauppinen TM, Valls AV, Swanson RA (2006) Minocycline inhibits poly(ADP-ribose) polymerase-1 at nanomolar concentrations. Proc Natl Acad Sci USA 103:9685–9690
15. Bergamaschi D, Gasco M, Hiller L, Sullivan A, Syed N, Trigiante G, Yulug I, Merlano M, Numico G, Comino A, Attard M, Reelfs O, Gusterson B, Bell AK, Heath V, Tavassoli M, Farrell PJ, Smith P, Lu X, Crook T (2003) p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis. Cancer Cell 3:387–402
16. Van Quaquebeke E, Mahieu T, Dumont P, Dewelle J, Ribaucour F, Simon G, Sauvage S, Gaussin JF, Tuti J, El Yazidi M, Van Vynckt F, Mijatovic T, Lefranc F, Darro F, Kiss R (2007) 2,2,2-Trichloro-*N*-({2-[2-(dimethylamino)ethyl]-1,3-dioxo-2,3-dihydro-1*H*-benzo[de]isoquinolin-5-yl}carbamoyl)acetamide (UNBS3157), a novel non-hematotoxic naphthalimide derivative with potent antitumor activity. J Med Chem 50:4122–4134
17. Pain A, Samanta S, Dutta S, Saxena AK, Shanmugavel M, Kampasi H (2003) Evaluation of naphthalmustine, a nitrogen mustard derivative of naphthalimide as a rationally-designed anticancer agent. J Exp Clin Cancer Res 22:411–418
18. Sandhu SK, Yap TA, de Bono JS (2010) Poly(ADP-ribose) polymerase inhibitors in cancer treatment: a clinical perspective. Eur J Cancer 46:9–20
19. Löser DA, Shibata A, Shibata AK, Woodbine LJ, Jeggo PA (2010) Chalmers, sensitization to radiation and alkylating agents by inhibitors of poly(ADP-ribose) polymerase is enhanced in cells deficient in DNA double-strand break repair. Mol Cancer Ther 9:1775–1787
20. Huang SH, Xiong M, Chen XP, Xiao ZY, Zhao YF, Huang ZY (2008) PJ34, an inhibitor of PARP-1, suppresses cell growth and enhances the suppressive effects of cisplatin in liver cancer cells. Oncol Rep 20:567–572
21. Fiorillo C, Ponziani V, Giannini L, Cecchi C, Celli A, Nassi N, Lanzilao L, Caporale R, Nassi P (2006) Protective effects of the PARP-1 inhibitor PJ34 in hypoxic-reoxygenated cardiomyoblasts. Cell Mol Life Sci 63:3061–3071
22. Pu T, Zhang XP, Liu F, Wang W (2010) Coordination of the nuclear and cytoplasmic activities of p53 in response to DNA damage. Biophys J 99:1696–1705
23. Smith ML, Kumar MA (2010) The "two faces" of tumor suppressor p53-revisited. Mol Cell Pharmacol 2:117–119