Modulation of Orphan Nuclear Receptor Nur77-Mediated Apoptotic Pathway by Acetylshikonin and Analogues

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Abstract

Shikonin derivatives, which are the active components of the medicinal plant Lithospermum erythrorhizon, exhibit many biological effects including apoptosis induction through undefined mechanisms. We recently discovered that orphan nuclear receptor Nur77 migrates from the nucleus to the mitochondria, where it binds to Bcl-2 to induce apoptosis. Here, we report that certain shikonin derivatives could modulate the Nur77/Bcl-2 apoptotic pathway by increasing levels of Nur77 protein and promoting its mitochondrial targeting in cancer cells. Structural modification of acetylshikonin resulted in the identification of a derivative 5,8-diacetoxyl-6-(1′-acetylxy-4′-methyl-3′-pentenyl)-1,4-naphthaquinones (SK07) that exhibited improved efficacy and specificity in activating the pathway. Unlike other Nur77 modulators, shikonins increased the levels of Nur77 protein through their posttranscriptional regulation. The apoptotic effect of SK07 was impaired in Nur77 knockout cells and suppressed by cotreatment with leptomycin B that inhibited Nur77 cytoplasmic localization. Furthermore, SK07 induced apoptosis in cells expressing the COOH-terminal half of Nur77 protein but not its NH2-terminal region. Our data also showed that SK07-induced apoptosis was associated with a Bcl-2 conformational change and Bax activation. Together, our results show that certain shikonin derivatives act as modulators of the Nur77-mediated apoptotic pathway and identify a new shikonin-based lead that targets Nur77 for apoptosis induction. [Cancer Res 2008;68(21):8871–80]

Introduction

Nur77 (also known as TR3 or NGFI-B), an orphan member of the steroid/thyroid/retinoid receptor superfamily and an immediate-early response gene, plays a critical role in the regulation of differentiation, proliferation, apoptosis, and survival of many different cell types (1, 2). Although the growth-promoting effect of Nur77 has been described in various tumors (3, 4), Nur77 expression also mediates the killing effects of a variety of apoptotic stimuli such as synthetic retinoids, calcium ionophores, etoposide, phorbol ester, chenodeoxycholic acid derivatives, di-n-butyltin dichloride, histone deacetylase inhibitors, cadmium, and 1,1-bis(3′-indolyl)-1-(p-substituted phenyl)ethanes in cancer cells (5–9). The apoptotic effect of Nur77 seems to be clinically relevant as the expression of the Nur77 subfamily member Nor-1 is positively correlated with survival in diffuse large B-cell lymphoma patients treated with chemotherapeutic drugs (10), and down-regulation of Nur77 is associated with the metastasis of primary solid tumors (11).

One of the mechanisms that regulate the opposing biological activities of Nur77, survival and death, is through its subcellular localization. Nur77 confers its growth-promoting activities through its action in the nucleus (12, 13), which requires its DNA-binding and transactivation. In contrast, the apoptotic effect of Nur77 is transcription-independent and occurs in the absence of its DNA-binding domain (5, 8, 14–16). In this case, Nur77 initiates apoptotic cascades by migrating to the mitochondria where it interacts with the Bcl-2 apoptotic machinery by converting Bcl-2 from a protector to a killer (16, 17). Such a Nur77/Bcl-2 apoptotic pathway has been used by a variety of apoptosis-inducing agents in prostate cancer, lung cancer, colon cancer, ovarian cancer, and gastric cancer cells (2, 8, 14, 15, 17, 18). In this regard, Nur77 and Bcl-2 are often overexpressed in cancer cells, providing an excellent opportunity to preferentially induce apoptosis of these cells by inducing Nur77 migration and Bcl-2 conversion (1, 17). Such an approach may be highly effective in inducing cancer apoptosis because it engages a simultaneous suppression of the survival function of Nur77 and Bcl-2, and activation of their proapoptotic potential (1, 17). Thus, agents that specifically activate the Nur77-mediated apoptotic pathway may have therapeutic potential.

In an effort to identify new modulators of the Nur77/Bcl-2 apoptotic pathway, we have undertaken the screening of a natural product library prepared from Chinese herbal medicines. Here, we report that certain shikonin derivatives, active components of the Chinese medicinal plant, Lithospermum erythrorhizon (19, 20), could activate the Nur77-mediated apoptotic pathway in cancer cells. Further evaluation of acetylshikonin analogues showed that 5,8-diacetoxyl-6-(1′-acetylxy-4′-methyl-3′-pentenyl)-1,4-naphthaquinones (SK07) was more active than its parental compound in inducing Nur77 expression and apoptosis. Our mechanistic studies revealed that SK07 increased the levels of Nur77 protein in cancer cells through its posttranscriptional regulation and that it killed cancer cells by inducing Bcl-2 conformational change and Bax activation in a Nur77-dependent manner. Together, our studies identify new modulators of the Nur77/Bcl-2 apoptotic pathway and a potential target-based anticancer lead.

Materials and Methods

Reagents. Lipofectamine 2000, Vybrant apoptosis assay kit no. 2, and Trizol LS from Invitrogen, enhanced chemiluminescence reagents, goat

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Lithospermum erythrorhizon

Shikonin derivatives, which are the active components of the medicinal plant Lithospermum erythrorhizon, exhibit many biological effects including apoptosis induction through undefined mechanisms. We recently discovered that orphan nuclear receptor Nur77 migrates from the nucleus to the mitochondria, where it binds to Bcl-2 to induce apoptosis. Here, we report that certain shikonin derivatives could modulate the Nur77/Bcl-2 apoptotic pathway by increasing levels of Nur77 protein and promoting its mitochondrial targeting in cancer cells. Structural modification of acetylshikonin resulted in the identification of a derivative 5,8-diacetoxyl-6-(1′-acetylxy-4′-methyl-3′-pentenyl)-1,4-naphthaquinones (SK07) that exhibited improved efficacy and specificity in activating the pathway. Unlike other Nur77 modulators, shikonins increased the levels of Nur77 protein through their posttranscriptional regulation. The apoptotic effect of SK07 was impaired in Nur77 knockout cells and suppressed by cotreatment with leptomycin B that inhibited Nur77 cytoplasmic localization. Furthermore, SK07 induced apoptosis in cells expressing the COOH-terminal half of Nur77 protein but not its NH2-terminal region. Our data also showed that SK07-induced apoptosis was associated with a Bcl-2 conformational change and Bax activation. Together, our results show that certain shikonin derivatives act as modulators of the Nur77-mediated apoptotic pathway and identify a new shikonin-based lead that targets Nur77 for apoptosis induction. [Cancer Res 2008;68(21):8871–80]
anti-rabbit and anti-mouse secondary antibody conjugated to horseradish peroxidase from Thermo Fisher Scientific, Inc., anti-mouse IgG conjugated with Cy3 from Chemicon International, polyclonal anti-Nur77 (sc-5569), anti-Hsp60 (sc-7150), anti–Bcl-2 (sc-309), anti-Bax (sc-493), anti-Bax (6A7; sc-23959), FITC-labeled anti-rabbit IgG from Santa Cruz Biotechnology, anti-poly(ADP-ribose) polymerase (PARP; 566494), and anti-cytochrome c (556432) from BD Biosciences, monoclonal anti-β-actin antibody from Sigma, monoclonal anti-Nur77 antibody from R&D Systems, anti–Bcl-2 BH3 (AP1303a) from Abgent, anti–cleaved caspase-3 (Asp175) from Cell Signaling Technology, polyvinylidene difluoride membranes from Millipore, and a cocktail of proteinase inhibitors (80-6501-23) from Amersham were used in this study. All other chemicals were used commercially purchased.

Preparation of acetylshikonin and analogues. L. erythrohorizgon grown in Liao-ning Province in Northeast China were extracted with hexane by Soxhlet apparatus and subjected to purification for shikonin derivative compounds (19, 20). Acetylshikonin (SK03) and two isomers of SK03 analogues, SK06 [5,8-diacetoxyl-2-(1′-acetoxy-4′-methyl-3′-pentenyl)-1,4-naphthaquinones] and SK07 [5,8-diacetoxyl-6-(1′-acetoxy-4′-methyl-3′-pentenyl)-1,4-naphthaquinones], were synthesized (Fig. 1A). Molar structures were identified using spectroscopic techniques including electron ionization mass spectrometry, Fourier transform IR spectroscopy, and nuclear magnetic resonance analysis.

Cell culture. NIH-H460 lung cancer and HeLa cervical cancer cells were cultured in RPMI 1640, whereas HEK293T human embryonic kidney, mouse embryonic fibroblasts (MEF) and MEF Nur77−/− cells were maintained in DMEM containing 10% fetal bovine serum in a humidified atmosphere containing 5% CO2 at 37°C. Subconfluent cells with exponential growth were used throughout the experiments. Cell transfections were carried out by using Lipofectamine 2000 according to the instructions of the manufacturer.

Cell lysis and fraction. Cells lysates were prepared by lysing cells with modified radioimmunoprecipitation assay buffer containing 50 mmol/L of Tris-HCl (pH 7.4), 150 mmol/L of NaCl, 5 mmol/L of EDTA, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS with a cocktail of proteinase inhibitors on ice for 30 min. For cellular fractionation, cells were lysed in cold buffer A [10 mmol/L HEPES-KOH (pH 7.9) at 4°C, 1.5 mm MgCl2, 10 mmol/L KCl, 0.5 mmol/L DTT] with a cocktail of proteinase inhibitors on ice for 10 min. The cytoplasmic fraction was collected by centrifuging at 6,000 rpm for 30 s. Pellets containing nuclei were resuspended in cold high-salt buffer C [20 mmol/L HEPES-KOH (pH 7.9), 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 0.5 mmol/L DTT], with a cocktail of proteinase inhibitors on ice for 30 min. Cellular debris was removed by centrifugation at 12,000 rpm at 4°C for 15 min. Protein concentrations were determined using the Bradford method according to the instructions of the manufacturer (Bio-Rad).

Western blotting. Equal amounts of the lysates were electrophoresed on an 8% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes, which were then blocked with 5% nonfat milk in TBST [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 0.1% Tween 20] for 1 h, incubated with various primary antibodies for 2 h and detected with either anti-rabbit (1:5,000) or anti-mouse (1:5,000) secondary antibodies for 1 h using an enhanced chemiluminescence system. The dilutions of the either anti-rabbit (1:5,000) or anti-mouse (1:5,000) secondary antibodies for

Results

Acetylshikonin and analogues increase levels of Nur77 protein through posttranscriptional regulation. To identify new modulators of the Nur77-mediated apoptotic pathway (17), we screened a natural product library consisting of 3,000 extracts and pure compounds prepared from known Chinese herbs with therapeutic indications (21). Our results show that several shikonin derivatives could increase the levels of Nur77 protein and apoptosis in various cancer cell lines, including NIH-H460 lung cancer, HeLa cervical cancer, HepG2 liver cancer, MCF7 breast cancer, and MGC-803 gastric cancer cells (data not shown). We report here our characterization of the acetyl derivative of shikonin, SK03 (Fig. 1A). Treatment of NIH-H460 and HeLa cells with SK03 resulted in the strong induction of Nur77 protein levels (Fig. 1B and C). In NIH-H460 cells that express high basal Nur77 protein levels, we consistently observed that SK03 could increase Nur77 levels by ~3-fold to 5-fold in a concentration range between 5 and 10 μmol/L for 12 or 24 hours (Fig. 1B), whereas the effect of SK03 was even more significant in HeLa cells that express low basal Nur77 levels (Fig. 1C). Thus, acetylshikonin SK03 can enhance the levels of Nur77 protein in cancer cells.

In order to identify more effective and selective modulators of the Nur77-mediated apoptotic pathway, two acetylshikonin derivatives, SK06 and SK07, were synthesized (Fig. 1A). SK06 and SK07...
were triacetate shikonins that were designed to explore whether modification of pharmacophore quinine, which is the common moiety of various shikonin derivatives, could improve their regulation of Nur77 activities. Analysis of their effect on levels of Nur77 protein in NIH-H460 (Fig. 1B) and HeLa (Fig. 1C) cells showed that the ability of SK03 in increasing Nur77 protein levels was retained in SK07 but impaired in SK06. Thus, the pharmacophore quinine in shikonins is an important determinant for their modulation of Nur77 protein levels in cancer cells.

The induction of Nur77 protein levels by SK07 was dose-dependent and time-dependent (Fig. 1D). Treatment of NIH-H460 cells with SK07 at 1, 5, and 10 μmol/L for 6 hours did not show any effect on Nur77 expression. However, the levels of Nur77 were increased when cells were treated for 12 hours. Although 10 μmol/L was required for optimal induction of Nur77 expression when cells were treated for 12 hours, 1 μmol/L of SK07 was sufficient for the maximal increase of Nur77 proteins when cells were treated with the compound for 24 hours. Such a time-dependent effect of SK07
on Nur77 expression was interesting because induction of Nur77 by growth factors and apoptotic stimuli, such as 3-Cl-AHPC, is often rapid and transient (22). We therefore determined whether shikonins could regulate Nur77 expression at the transcriptional level. NIH-H460 cells were treated with vehicle or with SK03, SK06, or SK07 at 5 µmol/L for 3 hours and their effect on Nur77 transcript was examined by reverse transcription-PCR (RT-PCR; ref. Fig. 1B). As a positive control, cells were treated with phorbol ester 12-O-tetradecanoyl-13-phorbol acetate (TPA; 100 ng/mL), which is known to induce Nur77 expression in NIH-H460 cells (4). Consistent with previous reports, TPA could significantly up-regulate Nur77 mRNA expression. However, we did not detect clear changes in Nur77 mRNA level when cells were treated with SK03, SK06, or SK07 (Fig. 1B), suggesting that the induction of Nur77 protein by SK03 and SK07 is not due to their transcriptional regulation of Nur77 expression.

**Role of Nur77 in shikonin-induced apoptosis.** We next determined whether Nur77 expression played a role in shikonin-induced apoptosis. NIH-H460 cells were treated with 5 µmol/L of SK03, SK06, SK07, or vehicle for 24 hours and apoptosis was analyzed by DAPI staining (Fig. 2A). Although apoptosis was seldom seen in untreated cells (7%), a significant number of cells displayed apoptosis when treated with SK03 (22%) or SK07 (33%). In contrast, SK06 exerted much less effect on apoptosis (14%). Thus, the apoptotic effect of shikonins correlated with their induction of Nur77 protein in cancer cells, suggesting that Nur77 expression contributes to their death effect.

The apoptotic effect of SK07 was also shown by its induction of PARP cleavage, a sensitive apoptotic marker occurring early in the apoptotic response (23). In NIH-H460 cells, SK07-induced PARP cleavage was visible at 6 hours post-treatment, which was increased upon longer treatments (Fig. 2B). Hela cells were more sensitive to SK07 as PARP cleavage occurred when cells were treated with SK07 for 3 hours, with the optimal effect seen between 12 and 24 hours (Fig. 2B). We also examined whether SK07 treatment activated caspase-3, a major executioner caspase known to cleave PARP (23). Immunostaining using an antibody specifically against the active caspase-3 showed that SK07 treatment of NIH-H460 cells resulted in the activation of caspase-3, as a significant amount of SK07-treated cells displayed strong immunostaining, whereas control cells did not (Fig. 2B).

The role of Nur77 in mediating the apoptotic effect of SK07 was also examined by studying the effect of Nur77 transfection in HEK293T cells that are resistant to SK07 (data not shown). Treatment with 5 µmol/L of SK07 did not induce a clear apoptosis of HEK293T cells. However, SK07 induced an extensive apoptosis in HEK293T cells transfected with GFP-Nur77 (~32%; Fig. 2C). SK07 also induced the apoptosis of HEK293T cells transfected with the COOH-terminal region of Nur77 (C-Nur77) but not the NH2-terminal Nur77 region (N-Nur77). The fact that the COOH-terminal E/F domain of Nur77 is sufficient for mediating the apoptotic effect of SK07 suggests that DNA binding and transactivation of Nur77 are not required for mediating the apoptotic effect of SK07.

To further determine the role of Nur77 expression, we evaluated the death effect of SK07 in MEF and Nur77 knockout MEF (MEF Nur77−/−) cells by flow cytometry for Annexin V and PI staining. Annexin V staining serves as a measure of phosphatidylserine externalization and cells that are Annexin V+/PI− represent early apoptotic cells (24). A representative experiment (Fig. 2D) showed that early apoptotic cells (Annexin V+/PI−; 14.66% and 57%) were seen in MEF treated with 1 and 10 µmol/L of SK07, respectively. In contrast, Annexin V+/PI+ staining was significantly reduced in MEF Nur77−/− cells (3.38% with 1 µmol/L and 23.7% with 10 µmol/L SK07 treatment). These results show that the apoptotic effect of SK07 largely depends on Nur77 expression, although other protein factors may mediate its effect when a high concentration of SK07 is used. Interestingly, SK03 treatment (10 µmol/L) resulted in a significant amount of late apoptotic cells (Annexin V+/PI+) in more than half of both MEF cells (54.6%) and Nur77−/− cells (66.8%). In addition, SK03 caused significant necrotic cell death in both MEF cells (31%) and Nur77−/− cells (11.9%), suggesting that SK03 may induce cell death mainly through Nur77-independent pathways.

**SK07 induces Nur77 nuclear export and mitochondrial targeting.** To determine whether acetylsikokin and its analogues could induce cytoplasmic localization of Nur77, the hallmark of the Nur77-mediated apoptotic pathway (17), we examined their effect on the subcellular localization of Nur77 in NIH-H460 cells. As shown in Fig. 3A, Nur77 immunostaining was significantly enhanced in cells treated with SK03 and SK07, but not with SK06, consistent with their induction of Nur77 protein level. For comparison, TPA also strongly induced Nur77 immunostaining. However, TPA-induced Nur77 immunostaining was confined to the nucleus, as reported previously (4). In contrast, most of the cells treated with SK03 and SK07 displayed a diffused distribution of Nur77. Predominant cytoplasmic localization of Nur77 could be seen in some treated cells, which was more apparent when treated with SK07 than with SK03. These results suggested that SK03 and SK07 induced the cytoplasmic localization of Nur77. Cytoplasmic localization of Nur77 observed in cells treated with SK07 might be due to its induction of Nur77 nuclear export, as leptomycin B (LMB), which is known to block CRM1-dependent nuclear export (25), significantly inhibited SK07-induced Nur77 cytoplasmic accumulation. To confirm the effect of shikonins on inducing cytoplasmic localization of Nur77, cytosolic and nuclear fractions of cells treated with SK03, SK06, or SK07 were prepared and analyzed for levels of Nur77 protein by immunoblotting. As shown in Fig. 3B, Nur77 was predominantly nuclear (92%) in control cells. However, in cells treated with SK03 and SK07, a significant amount (65% and 85%, respectively) of Nur77 protein was cytoplasmic. In contrast, only 30% of Nur77 protein resided in the cytoplasm when treated with SK06. Consistent with immunostaining results, pretreatment of cells with LMB reduced the amount of SK07-induced cytoplasmic Nur77 from 83% to 42%. Thus, SK07 is more effective than SK03 and SK06 in inducing cytoplasmic localization of Nur77.

To determine whether SK07-induced cytoplasmic Nur77 could associate with mitochondria, NIH-H460 cells treated with SK07 or vehicle were immunostained with anti-Nur77 antibody and antibody against Hsp60, a mitochondria-specific protein. Confocal microscopy analysis showed that the distribution of Nur77 extensively overlapped with that of Hsp60 when cells were treated with SK07 (Fig. 3C). To confirm the ability of SK07 to induce Nur77 mitochondrial targeting, an expression vector encoding GFP-Nur77 was transfected into NIH-H460 cells and its subcellular distribution in cells treated with or without SK07 was examined. As shown in Fig. 3D, GFP-Nur77 was exclusively nuclear in untreated cells, but it was found in the cytoplasm and colocalized with Hsp60 when cells were treated with SK07. Thus, SK07 can induce Nur77 mitochondrial targeting.

**SK07-induced apoptosis is dependent on cytoplasmic localization of Nur77.** To study whether the cytoplasmic localization of Nur77 was required for the killing activity of SK07,
Figure 2. Induction of apoptosis by SK07 and the role of Nur77. A, DAPI staining. NIH-H460 cells cultured in serum-free medium were treated with SK03, SK06, SK07 (5 μmol/L), or vehicle for 24 h and subjected to DAPI staining. Apoptotic cells were compared between different treatments. *, P < 0.05 (vs. control); **, P < 0.01 (vs. control); ##, P < 0.01 (vs. SK03 or SK06). B, PARP cleavage and caspase-3 activation. NIH-H460 or HeLa cells were treated with 5 μmol/L of SK07 in serum-free medium for the indicated times. Total cell lysates were subjected to Western blotting assay for PARP cleavage using anti-PARP antibody (top). For the analysis of caspase-3 activation (bottom), NIH-H460 cells were treated with shikonins as described above and stained with an antibody recognizing the cleaved caspase-3. Nuclei were visualized by costaining with DAPI. 

C, transfection of Nur77 mediates the apoptotic effect of SK07. HEK293T cells were transfected with full-length Nur77, N-Nur77 (A/B domain), or C-Nur77 (E/F domain) and subjected to SK07 treatment (5 μmol/L) or vehicle in serum-free medium for 8 h. Apoptotic cells examined by DAPI staining were compared between transfected cells carrying different mutants. **, P < 0.01 (vs. control). D, the apoptotic effect of SK07 is impaired in Nur77 knockout MEFs. MEF cells or MEF Nur77−/− cells were treated with vehicle or the indicated concentrations of SK07 or SK03 for 24 h and stained with PI/Annexin V. Apoptosis was analyzed by fluorescence-activated cell sorting analysis.
NIH-H460 cells were treated with 10 μmol/L of SK07 and subsequently stained with anti-Nur77 antibody and DAPI. Microscopic analysis showed that treatment of cells with SK07 resulted in a significant amount of cells displaying cytoplasmic localization of Nur77, many of them showing nuclear morphology typical of apoptosis (~45%; Fig. 4A). However, when cells were cotreated with LMB, SK07-induced Nur77 protein mainly resided in the nucleus, and cells displayed normal nuclear morphology. The inhibitory effect of LMB on SK07-induced apoptosis was confirmed by Annexin V staining (Fig. 4B). Flow cytometry analysis showed that NIH-H460 cells treated with 5 μmol/L of SK07 resulted in extensive early apoptosis (37.9% Annexin V+/PI− cells). This level of apoptosis was dramatically reduced (4.9% Annexin V+/PI− cells) when cells were cotreated with LMB. We also determined the effect of LMB on SK07-induced cytoplasmic localization of GFP-Nur77 and apoptosis. NIH-H460 cells transfected with GFP-Nur77 were exposed to 10 μmol/L of SK07 for 24 hours, and apoptosis was examined by DAPI staining. In the absence of treatment, GFP-Nur77 was found in the nucleus and no apparent apoptotic cells were detected (Fig. 4C). However, GFP-Nur77 could be found in the cytoplasm of cells treated with SK07, which was coincident with extensive nuclear condensation and fragmentation with ~80%...
apoptosis. In the presence of LMB, SK07-induced apoptosis was decreased to ~16%. Again, inhibition of apoptosis by LMB was accompanied by its inhibition of the cytoplasmic localization of GFP-Nur77. Together, these results show that the cytoplasmic localization of Nur77 plays a critical role in mediating the apoptotic effect of SK07.

**SK07 induces Bcl-2 conformational change.** Cytoplasmic Nur77 is known to bind Bcl-2, inducing a Bcl-2 conformational change that is proapoptotic (16, 17). To determine whether cytoplasmic Nur77 could colocalize with Bcl-2, NIH-H460 cells treated with SK07 or vehicle were immunostained with anti-Nur77 and anti–Bcl-2 antibodies. Microscopic analysis showed that the distribution pattern of SK07-induced cytoplasmic Nur77 overlapped extensively with that of Bcl-2 (Fig. 5A). Cotreatment with LMB inhibited the colocalization of Nur77 and Bcl-2 by preventing Nur77 cytoplasmic localization. In transfection experiments, we also observed that SK07-induced cytoplasmic GFP-Nur77 colocalized with Bcl-2 expression (data not shown). The colocalization of cytoplasmic Nur77 with Bcl-2 suggests that SK07-induced Nur77 might interact with Bcl-2.

Interaction of Nur77 with Bcl-2 induces a Bcl-2 conformational change with its BH3 domain exposed (17). Such a Bcl-2 conformation is proapoptotic and can be detected by anti–Bcl-2 antibody against the BH3 domain of Bcl-2, anti–Bcl-2(BH3), (17). To

![Figure 4.](image)

**Figure 4.** SK07-induced apoptosis is dependent on Nur77 expression and Nur77 nuclear export. A, Nur77 cytoplasmic localization and apoptosis. NIH-H460 cells were treated with 10 μmol/L of SK07 in serum-free medium in the presence or absence of LMB (10 ng/ml) for 24 h. Subcellular localization of endogenous Nur77 was examined by immunostaining using anti-Nur77 antibody (R&D) and apoptosis was determined by DAPI staining. B, analysis of the effect of LMB on SK07-induced apoptosis by PI/Annexin V staining. NIH-H460 cancer cells were treated with SK07 (5 μmol/L) in the presence or absence of LMB (10 ng/ml) for 24 h. Apoptosis was determined by PI/Annexin V staining. C, cytoplasmic localization of transfected Nur77 and apoptosis. NIH-H460 cells transfected with GFP-Nur77 expression vector were treated with 10 μmol/L of SK07 in serum-free medium in the presence or absence of LMB (10 ng/ml) for 24 h. Subcellular localization of GFP-Nur77 was examined by fluorescence microscopy and apoptosis was examined by DAPI staining. Quantification was conducted in nontransfected and transfected cells, respectively. **, *P < 0.01 [vs. control or LMB (+)].
SK07 induces Bcl-2 conformational change and Bax activation. An unresolved issue in the Nur77/Bcl-2 apoptotic pathway is whether Bcl-2 conformational change is sufficient to induce apoptosis or whether the exposure of BH3 domain of Bcl-2 acts as a BH3-only protein that induces apoptosis through activation of proapoptotic Bcl-2 family members and/or inhibition of antiapoptotic members (17, 26). Thus, we determined whether Bax activation was involved in SK07-induced apoptosis by immunostaining NIH-H460 cells with anti-Bax(6A7) antibody that recognizes activated Bax. Untreated NIH-H460 cells were not immunostained by the anti-Bax(6A7) antibody, suggesting that Bax was inactive in the cells (Fig. 5B). However, cells treated with SK07 displayed strong immunostaining with the anti-Bax(6A7) antibody, demonstrating that SK07 could activate Bax. Our observation that SK07-treated cells stained by both anti-Bcl-2(BH3) and anti-Bax(6A7) antibodies suggests that SK07-induced Bcl-2 conformational change may play a role in Bax activation.

Bax resides predominantly in the cytosol before its activation and apoptosis induction. During early apoptosis, Bax undergoes conformational change and oligomerization, resulting in its translocation from the cytosol to the mitochondria (27). We then examined whether Bax could associate with mitochondria in SK07-treated cells. Microscopic analysis showed an extensive colocalization of active Bax with mitochondrial-specific protein Hsp60 (Fig. 5C). Furthermore, cells with active Bax immunostaining often displayed nuclear condensation (Fig. 5C), suggesting that SK07-induced apoptosis might involve Bax activation. To further confirm this association, NIH-H460 cells were costained with anti-Bax(6A7) and anti–cytochrome c antibodies. In control cells, cytochrome c displayed a punctate immunostaining pattern, consistent with its mitochondrial localization (Fig. 5D). However, cytochrome c was diffusely distributed in SK07-treated cells with active Bax immunostaining. Thus, SK07-induced Bax activation was associated with cytochrome c release. LMB cotreatment, which prevented Nur77 nuclear export and Bcl-2 conformational change, almost completely inhibited SK07-induced Bax activation and cytochrome c release (Fig. 5D).

SK07 does not affect Bcl-2 and Bax expression. Because it has been widely reported that the ratio between Bcl-2 and Bax determines cell survival or death (26), we next examined whether the apoptotic effect of SK07 was due to its effect on Bcl-2 and Bax expression. The result showed that SK07 caused no apparent change in level of either Bcl-2 or Bax for treatment time ranging from 3 to 24 hours (Fig. 6). Thus, SK07 induces apoptosis through the induction of Bcl-2 conformational change and Bax activation rather than affecting Bcl-2/Bax ratio.

Discussion

Traditional Chinese herbal medicines, which constitute numerous chemically unique and biomedically powerful secondary
metabolites, are a rich source of therapeutic leads (21). Recent examples include the discoveries of paclitaxel and camptothecin (28, 29). Shikonin derivatives, mainly contained in the roots of the Chinese plant L. erythrorhizon, are the naturally occurring naphthoquinones that embody numerous active compounds with a unique pharmacophore quinone moiety (19). These compounds are promising drug candidates as they have been reported to have multiple anticancer effects in vitro and in animals (19, 20). However, a rational design of shikonin analogues is necessary in order to reduce the nonspecific toxicity of shikonins, which has prevented them from clinical development.

Nur77 is a unique orphan member of the nuclear receptor superfamily (1). It is perhaps the most potent proapoptotic member in the nuclear receptor superfamily (16, 17). Recently, we showed a new paradigm in cancer cell apoptosis through Nur77 mitochondrial targeting (17, 30), which was later confirmed by other groups (15, 16, 18). We showed that apoptosis of cancer cells induced by certain apoptotic agents requires Nur77 expression and its translocation from the nucleus to mitochondria where Nur77 interacts with Bcl-2, resulting in a Bcl-2 conformational change that converts it into a cytotoxic molecule (17). These findings suggest that agents which induce cytoplasmic localization of Nur77 and its interaction with Bcl-2 may preferentially kill cancer cells with elevated Bcl-2 levels.

In the course of identifying new agents that modulate the Nur77-mediated apoptotic pathway, we report here our finding that certain shikonin derivatives could activate the Nur77 pathway through their induction of Nur77 expression, nuclear export, mitochondrial targeting, Bcl-2 conformational change, and Bax activation. These results are consistent with previous report that shikonin-like compounds can induce apoptosis through mitochondria and caspase pathway with unknown mechanisms (31). Our current finding that certain shikonins could regulate the Nur77-mediated apoptotic pathway sets the stage for rational design of shikonin analogues with improved anticancer efficacy and specificity. It is generally believed that the nonspecific toxicity of shikonins is caused by the electronic transfer of hydroxyl groups and C-11 positions and subsequently occurring alkylation (20). Our current study indicates that certain esterification of 1-α carbonyl group of acetylshikonins such as SK03 can enhance apoptosis by promoting Nur77 expression and migration. We found that the introduction of acetyl groups into the pharmacophore quinoid moieties of SK03 exerted a great effect on its modulation of Nur77 activity. Induction of Nur77 protein levels by SK03 in cancer cells was retained in SK07 (Fig. 1B and C). Interestingly, the ability of inducing Nur77 cytoplasmic localization by SK03 was significantly enhanced in SK07 but was reduced in SK06 (Fig. 3A and B). The distinct abilities to induce Nur77 expression and Nur77 cytochrome c release from the mitochondria shown in different shikonin derivatives (SK07 and SK06) suggest that quinoid modification is an important determinant for their modulation of Nur77 activities, including its stability and nuclear export.

Our results showed that an increase of Nur77 protein levels and its cytoplasmic localization were associated with the apoptotic effect of shikonins. Treatment of NIH-H460 cells with SK07, which induced Nur77 expression and nuclear export, resulted in cytochrome c release from the mitochondria (Fig. 5D), caspase-3 activation, and PARP cleavage (Fig. 2B). SK07-induced apoptosis was dependent on Nur77 expression level as its killing effect was largely impaired in MEF Nur77<sup>−/−</sup> cells when compared with MEF cells (Fig. 2D) and enhanced by Nur77 transfection (Figs. 2C and 4C). The apoptotic effect of SK07 could be observed in other cancer cells such as HeLa cells (Figs. 1C and 2B; data not shown), consistent with the role of Nur77 expression in different types of cancer cells (8, 14, 15, 18). Our results also showed that SK07-induced apoptosis was associated with cytoplasmic localization of Nur77 because cytoplasmic accumulation of both endogenous Nur77 and transfect Nur77 was accompanied with nuclear condensation and fragmentation (Fig. 4A and C). In addition, LMB blockage of Nur77 cytoplasmic localization significantly inhibited SK07-induced Bax activation, cytochrome c release (Fig. 5D), and apoptosis (Fig. 4). Consistently, deletion of the DNA-binding domain from Nur77 did not affect the apoptotic effect of SK07 in HEK293T cells (Fig. 2C).

In studying the mechanism by which cytoplasmic Nur77 induced apoptosis, we found that SK07-induced cytoplasmic Nur77 targeted mitochondria (Fig. 3C and D) probably through its interaction with Bcl-2 (Fig. 5A), leading to Bcl-2 conformational changes that exposes its BH3 domain (Fig. 5B). These results confirmed our previous finding that cytoplasmic Nur77 could target mitochondria and induce Bcl-2 conversion (17). Interestingly, our results showed that SK07-induced Bcl-2 conformational change was associated with Bax activation (Fig. 5B). This observation is significant, as it would suggest that the converted Bcl-2 requires Bax activation for apoptosis induction. Although it is unclear how converted Bcl-2 activates Bax, it can be envisioned that the converted Bcl-2, with its BH3 domain exposed, may act directly or indirectly on Bax, resulting in Bax activation and apoptosis.

Besides its improved efficacy in inducing apoptosis, SK07 may have improved selectivity towards the Nur77-mediated apoptotic pathway. Shikonins are known to modulate multiple signal transduction pathways, including inhibition of DNA topoisomerases (31), induction of reactive oxygen species release (32), and inhibition of survival pathways involving extracellular signal-regulated kinase, Akt, and nuclear factor κB activities (33). Interestingly, the apoptotic effect of SK07 was largely impaired in Nur77 knockout cells (Fig. 2D). By contrast, SK03, the parent compound of SK07, induced a significant amount of late apoptotic and necrotic death cells in both MEF and MEF Nur77<sup>−/−</sup> cells (Fig. 2D), suggesting that its biological activities largely depended on Nur77-independent pathways. Improved Nur77 selectivity, as that observed with SK07, may be therapeutically desirable as Nur77 is often overexpressed in cancer cells compared with normal cells. Such a tumor selectivity can be further ensured by the fact that the Nur77-mediated apoptotic pathway targets Bcl-2, the expression of which is also often elevated in cancer cells (17). Thus,

![Figure 6](https://www.aacrjournals.org/8879/CancerRes.2008;68:21.11.01.2008)

**Figure 6.** SK07 does not affect Bax and Bcl-2 expression levels. Cells were treated with vehicle or 5 μmol/L of SK07 for the indicated periods of time. Total cell lysates were prepared and examined for Bcl-2 and Bax expression by Western blotting analysis. β-Actin expression was used as a loading control.
SK07 may represent a new drug lead that induces cancer cell death by targeting the Nur77/Bcl-2 apoptotic pathway.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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