A Unique Pharmacophore for Activation of the Nuclear Orphan Receptor Nur77 In vivo and In vitro

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Abstract

Nur77 is a steroid orphan receptor that plays a critical role in regulating proliferation, differentiation, and apoptosis, including acting as a switch for Bcl-2 function. We previously reported that the octaketide cytosporone B (Csn-B) is a natural agonist for Nur77. In this study, we synthesized a series of Csn-B analogues and performed a structure-activity analysis that suggested criteria for the development of a unique pharmacophore to activate Nur77. The components of the pharmacophore necessary for binding Nur77 included the benzene ring, the phenolic hydroxyl group, and the acyl chain of the Csn-B scaffold, whereas the key feature for activating the biological function of Nur77 was the ester group. Csn-B analogues that bound Nur77 tightly not only stimulated its transactivation activity but also initiated mitochondrial apoptosis by means of novel cross-talk between Nur77 and BRE, an antiapoptotic protein regulated at the transcriptional level. Notably, the derivative n-amy1 2-[3,5-dihydroxy-2-(1-nonanoyl)phenyl]acetate exhibited greater antitumor activity in vivo than its parent compounds, highlighting particular interest in this compound. Our findings describe a pathway for rational design of Csn-B–derived Nur77 agonists as a new class of potent and effective antitumor agents.

Introduction

The mouse Nur77 orphan receptor (also called NGFI-B in rats or TR3 in humans) is a member of the steroid/thyroid/retinoid nuclear receptor superfamily. It participates in a variety of biological processes, including T-cell development (1, 2), inflammatory responses (3–5), steroid hormone synthesis (6–8), and hepatic glucose metabolism (9). Nur77 also influences programmed cell death in response to apoptotic stimuli (10–12). In one of its roles, Nur77 functions as a transcription factor by binding to its response elements and negatively or positively regulating the transcription of its apoptosis-associated target genes (13, 14). Additionally, Nur77 acts as a regulatory factor by translocating from the nucleus to the mitochondria, where it interacts with and conformationally converts Bcl-2 to an apoptosis-promoting trigger (15).

A variety of agents have been used to induce Nur77-mediated apoptosis in different cell types, such as 9-cis-retinoic acid, 1-di(3-indoly)-1-(4-X-phenyl)methanes (16, 17), etoposide (18), 5,8-diacetoxyl-6-(1′-acetoxy-4′-methyl-3′-pentenyl)-1,4-naphthaquinones (19), and 12-O-tetradecanoylphorbol-13-acetate (20, 21). However, those compounds regulate Nur77 biological activity through an indirect mechanism. We found that 9-cis-retinoic acid induces heterodimerization of its receptor retinoic receptor α (RXRα) with Nur77, which facilitates its nucleocytoplasmic translocation to the mitochondria. Upon reaching the mitochondria, Nur77 alone initiates apoptosis (10). Thus, apoptotic stimuli seem to trigger Nur77-mediated apoptosis in an indirect, multicomponent fashion.

To date, no physiologic ligand for Nur77 has been identified. The synthetic Nur77 agonists 1,1-di(3-indoly)-1-(p-substituted-phenyl)methanes (DIM-arenes) have been shown to affect Nur77 activation in a structure-dependent manner. Interestingly, DIM-arenes also act as transcriptional agonists for PPARγ and RXRα (22), suggesting that they are not specific agonists for Nur77. We recently found that the natural product cytosporone B (Csn-B), an Nur77 agonist isolated from Dothiorella sp. HTF3 (23), has the ability to directly bind to and activate Nur77, leading to induction of apoptosis via translocation of Nur77 to the mitochondria (24). Moreover, Csn-B does not have any effect on the transcription of reporter genes for the retinoic acid receptor response element, the estrogen receptor response element, or the thyroid receptor response element (24), thereby showing the specificity of Csn-B
for Nur77. However, as is the case with many natural products, the lack of availability of significant quantities of Csn-B due to the low concentrations of active principles extracted from bacterial fermentation limits its further use.

Structural modification of natural products can result in molecules with improved properties, such as adequate production quantity, reduced toxicity, or enhanced efficacy. For example, milataxel, a novel paclitaxel analogue in clinical trials, is 140 times more effective than paclitaxel (25). In an attempt to find a molecule possessing similar functions to Csn-B but with enhanced Nur77-binding and activating properties, a series of novel analogues was synthesized. After evaluating the Csn-B analogues, we identified a unique pharmacophore capable of binding and activating Nur77. Furthermore, we found that Csn-B analogues promote Nur77 antitumor activity through a novel cross-talk between Nur77 and brain and reproductive organ-expressed protein (BRE). BRE, a death receptor–associated protein, specifically downregulates death receptor–mediated apoptosis by inhibiting activation of the mitochondrial apoptotic pathway (26, 27). The Csn-B analogues prepared in this study repress the transcriptional activity of BRE through recruitment of corepressor N-CoR. Importantly, this event is mediated by Nur77. Our results also show that n-amy1 2-[3,5-dihydroxy-2-(1-nonanoyl)phenyl]acetate, the most effective derivative, exhibits remarkable anticancer activity in a tumor xenograft model, thus suggesting the potential of our novel Nur77 agonists as therapeutic agents for cancer treatment.

Materials and Methods

Cell lines and knockout mice. Human gastric cancer BGC-823 cells purchased from the Institute of Cell Biology of China were maintained in RPMI 1640. To establish stable BGC-823 cell lines that expressed siNur77 or pNurRE-Luc, plasmids of siNur77 or pNurRE-Luc together with pCDNA3.1 vector (Invitrogen) were transfected into cells using FuGENE HD (Roche). The stably transfected cells were selected by G418 (300 μg/mL, Sigma). Nur77−/− C57BL/6 mice were purchased from Jackson Laboratory.

Molecular docking. The three-dimensional geometry data file for Nur77 ligand-binding domain (LBD) was obtained from the Protein Data Bank (PDB code 2QW4). The initial structure of Csn-B analogues was generated by ACYLDBS 12.0 and minimized by MolSoft ICM mолекулярных механических (Molsoft LLC) with the Cartesian Merck Molecular Force Field. The MolSoft ICM 3.5a program was selected for the docking of Csn-B analogues to Nur77(LBD).

Synthesis of Csn-B analogues. To study the structure-activity relationships, different series of cytosporane analogues were designed and synthesized (Supplementary Fig. S1). All synthetic compounds were fully characterized by 1H nuclear magnetic resonance (NMR), 13C NMR, mass spectrometry, and elemental analysis. The detailed synthetic approaches and spectroscopic data are outlined in Supplementary Data. All compounds were dissolved in DMSO and stored at −80°C.

Construction of plasmids. The region of the BRE promoter spanning bp −1,800 to +134 (containing a NBRE consensus AAAGGTCA) was subcloned into the pGL2 luciferase reporter vector (Promega) to generate pLUC-BRE, pLUC-BREMUT (NBRE point mutation; AAAGAACGA) was obtained by using the QuikChange II site-directed mutagenesis kit (Stratagene).

To silence the expression of Nur77, the oligonucleotide siNur77 (5′-ACG TGC CAG CCA TGC TCC T-3′) was inserted into the pSuper vector (OligoEngine).

Fluorescence-activated cell sorting analysis. Cells were fixed with 70% ethanol, treated with RNase A (0.25 mg/mL, Roche), and stained with propidium iodide (0.02 mg/mL, Sigma). Samples were analyzed using a FACScan system (BD Biosciences).

Confocal microscopy. Staining procedures were performed as previously described (28). Antibodies including Nur77 (rabbit polyclonal antibody, Santa Cruz Biotechnology), cytochrome c (mouse monoclonal antibody, Santa Cruz), AIF (rabbit polyclonal antibody, Sigma), and Hsp60 (mouse monoclonal antibody, Santa Cruz) were used. The images were captured using a Leica SP laser scan microscope system.

Preparation of nuclear, cytosolic, and mitochondrial fractions and Western blotting. The preparation of nuclear, cytosolic, and mitochondrial fractions and Western blotting were performed according to the methods previously described (29).

Electrophoretic mobility shift assay and chromatin immunoprecipitation assay. Electrophoretic mobility shift assay (EMSA) was carried out using a LightShift Chemiluminescent EMSA kit ( Pierce Chemical Co.) with biotin-labeled oligonucleotides (Invitrogen; ref. 28). Chromatin immunoprecipitation (ChIP) assay was performed as described previously (29).

Xenograft experiment. Five-week-old male athymic BALB/c nude mice were purchased from the Laboratory Animal Center. BGC-823 cells were injected s.c. into the right flank of each mouse (106 cells per 50 μL PBS). The Csn-B analogues dissolved in DMSO and then mixed with 50 μL corn oil were injected into the tumor areas every other day at a dose of 10 mg/kg body weight, whereas the negative control group was injected with vehicle. Synthesized Csn-B (compound 10a) was used as a positive control. Tissues were further examined by immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays as detailed previously (24).

Circular dichroism spectroscopy and fluorescence quenching assay. As previously described (24), circular dichroism (CD) measurements were carried out using a Jasco J-810 spectropolarimeter (JASCO). The α-helical contents of unbound and bound proteins were calculated according to the formula (30).

For fluorescence quenching, a HITACHI fluorescence spectrophotometer (model F-4500, Hitachi) was used. The dissociation constants were determined by fluorescence quenching as previously described (31, 32).

Luciferase assays. The relative NurRE luciferase activity was measured as described previously (33). The luciferase assay of the BRE promoter was conducted as mentioned above. To detect the in vivo transcriptional activity, pluc-BRE (700 ng/g body weight) was mixed with 1 mL of reagents from the hepatocytes transfection kit (Mirus Bio Corp.) and injected into the tail vein of C57BL/6 mice (34).

Measurement of mitochondrial membrane potential. Cells (2 × 105 per chamber) were harvested and incubated with JC-1 (5 μg/mL, Sigma) at 37°C for 10 minutes and then analyzed using a FACScan unit (BD Biosciences).
Results

Determination of the critical components of Csn-B analogues for Nur77 targeting. Recently, we described how Csn-B acts as an agonist by binding specifically to the LBD of Nur77 to activate its transactivating function (24). The structure of Csn-B (in Fig. 1A, synthetic Csn-B is named as 10a) features a core benzene ring functionalized by two hydroxyl groups, an acyl group and an ester group. However, the roles these moieties play in the binding of Csn-B to Nur77

Figure 1. Determination of the key components of Csn-B analogues for Nur77(LBD) binding. A, docking suggesting the poses adopted by Csn-B analogues in the Nur77(LBD); B, Kd values with their corresponding acyl chain lengths.
remain unknown. To evaluate the structural features of Csn-B that are required for interaction with Nur77, we synthesized five series of cytosporone analogues with the aid of molecular docking that predicted the Csn-B analogues capable of binding to Nur77. First, we identified a hydrogen bond (indicated by an arrow) that formed between the hydroxyl group ortho to the acyl group of Csn-B and Y122 of Nur77(LBD) \( \text{eq} = Y453 \) in full-length Nur77; ref. 24; Fig. 1A, ethyl 2-[3,5-dihydroxy-2-(1-octanoyl)phenyl]acetate (10a). The docked pose for 10a suggests that, when the hydroxyl group was shifted meta to the acyl group, the hydrogen bond could also form {Fig. 1A, ethyl 2-[4-hydroxy-2-(nonanoyl)phenyl]acetate (22)}. When this 3-hydroxyl group was replaced by a 2-acetamido group {Fig. 1A, ethyl 2-[3-acetamino-2-(octanoyl)phenyl]acetate (35)}, another conformation was observed that would not permit hydrogen bond formation. Deletion of the 5-hydroxyl group located para to the acyl group {Fig. 1A, ethyl 2-[3-hydroxy-2-(octanoyl)phenyl]acetate (29)} did not affect the docked pose for 29, so that hydrogen bond between the 3-hydroxyl group and the Y122 hydroxyl group could be maintained. The dissociation constant for 29 was decreased by only 0.2 \( \mu \text{mol/L} \). These results support our previous hypothesis that Y453 of full-length Nur77 \[ \text{i.e., Y122 of Nur77(LBD)} \] is important for the protein’s capacity to interact with Csn-B by what seems to be the formation of a hydrogen bond (24).

Next, we investigated the roles of the other two functional groups present on the benzene ring by deleting either the acyl group or the alkyl ester. When glutathione S-transferase (GST)–Nur77(LBD) protein was incubated with increasing amounts of 1-[3,5-dihydroxyphenyl]octan-1-one (14), the fluorescence intensity of the complex gradually decreased; however, when the protein was incubated with ethyl 2-[3-dihydroxy-2-(octanoyl)phenyl]acetate (29), no such decrease was observed (Supplementary Fig. S2). Molecular docking suggested that the aforementioned hydrogen bond was present in the complex of Nur77 with 14, but not with 8a (Fig. 1A). Given that 14 only has an acyl group while 8a only possesses the acetate moiety, it is likely that the acyl group coupled with the benzene ring is responsible for Nur77 binding. Importantly, when the carbonyl group was transformed into a hydroxyl group, the benzene ring was positioned away from Y122 of Nur77(LBD) {Fig. 1A, 1-[3,5-dihydroxyphenyl]-1-octanol (15)}. In this orientation, the hydroxyl group of 15 on the benzene was unable to form a hydrogen bond with Y122 of Nur77(LBD), suggesting that the trigonal planar electron domain (sp2) geometry of the carbonyl group orients its alkyl chain for maximal interaction with hydrophobic groups in the binding pocket of Nur77(LBD).

To further explore the importance of the acyl group in the Csn-B analogues for Nur77 binding, a series of Csn-B analogues that varied only in the length of the acyl group was synthesized. The values of their respective dissociation constants are reported in Fig. 1B. The compound with a decanoyl group {ethyl 2-[3,5-dihydroxy-2-(1-decanoyl)phenyl]acetate (10j)} exhibited stronger affinity for GST-Nur77(LBD) than one with a nonanoyl group {ethyl 2-[3,5-dihydroxy-2-(1-nonanoyl)phenyl]acetate (10f)}, which in turn had a greater affinity than the compound with an octanoyl group (10a). However, ethyl 2-[3,5-dihydroxy-2-(1-undecanoyl)phenyl]acetate (10e), which possesses an undecanoyl group, exhibited no better binding than 10i. This is probably due to the folding of the alkyl tail in 10e, which compensates for its additional two carbons by assuming a cisoid conformation (Supplementary Fig. S3). Thus, various acyl groups may govern the efficiency of the association of Csn-B analogues with Nur77.

The alkyl ester is responsible for activation of Nur77. Three additional series of compounds were further synthesized based on the structures of compounds 10a, 10f, and 10j: the acyl group was fixed for each series, but the ester alkyl chain was lengthened (Fig. 2). Comparison of the dissociation constants reveals that increasing the number of carbons of the ester alkyl chain also resulted in tighter binding compounds, among which n-amyl 2-[3,5-dihydroxy-2-(1-nonanoyl)phenyl]acetate (10i) exhibited the highest affinity (Fig. 2).
We next explored the structure-activity relationship of the various alkyl esters. The transactivation activity induced by Csn-B analogues was first evaluated by a luciferase assay. When BGC-823 cells stably expressing a reporter gene for Nur77 (NurRE) were treated with various compounds, the transactivation activity of Nur77 increased in response to most of the analogues that exhibited high affinities for Nur77. We noted that induction followed a regular pattern with respect to altering the length of the ester alkyl chain (Fig. 3A). As expected, the three compounds (8a, 15, and 35) that lacked affinity for Nur77 had no effect on the transactivation activity. These data suggest that the ability of Csn-B analogues to activate Nur77 transactivation is associated with their affinity for Nur77 in a structure-dependent manner, with the ester moiety playing a particularly important role.

We also examined cell viability in response to these compounds. Compounds 8a, 15, and 35 were not toxic to BGC-823 cells. However, other compounds caused cell death to varying degrees (Fig. 3B). Intriguingly, many round, suspended cells were observed in the culture medium treated with the Csn-B compounds (data not shown), suggesting that an apoptotic event had occurred. Thus, we determined whether the Csn-B analogues activate Nur77 transactivation is associated with their affinity for Nur77 in a structure-dependent manner, with the ester moiety playing a particularly important role.

We also examined cell viability in response to these compounds. Compounds 8a, 15, and 35 were not toxic to BGC-823 cells. However, other compounds caused cell death to varying degrees (Fig. 3B). Intriguingly, many round, suspended cells were observed in the culture medium treated with the Csn-B compounds (data not shown), suggesting that an apoptotic event had occurred. Thus, we determined whether the Csn-B analogues inhibited the growth of gastric cancer cells through an apoptotic mechanism in BGC-823 cells. Compounds 8a, 15, and 35 did not induce apoptosis, whereas ~10% to ~60% of the cells were apoptotic after treatment with the other compounds (Fig. 3C). This result was consistent with the cell viability assay and showed that Csn-B analogue-induced cell death occurs via apoptosis. Interestingly, although 14 did activate Nur77 transactivation to some degree, it exerted no apoptotic effects in BGC-823 cells, further supporting the conclusion that the ester moiety is indispensable for the apoptosis-inducing function of Nur77.

The low-energy binding conformation of Csn-B analogues facilitates Nur77 activation. To evaluate how the ester moiety of Csn-B analogues influences their function, we chose 10f and 10i for further analysis because they harbor the same acyl group but have different ester alkyl chain lengths. Compound 10a was used as a positive control. Docking revealed that these three compounds may bind to Nur77 (LBD) in similar ways by targeting the Y122 residue (Supplementary Fig. S4). In addition, CD spectroscopic assays verified that 10a, 10f, and 10i specifically bound to GST-LBD; however, as a negative control, the CD spectra of GST proteins remained unchanged when GST proteins were incubated with 10i (Fig. 3D). Upon binding, the three compounds caused the conformation change of GST-Nur77(LBD) as indicated by the changes in ellipticity values of 36.5%, 32.2%, and 30.0% in bound protein forms 10a-GST-LBD, 10f-GST-LBD, and 10i-GST-LBD, respectively, compared with 55.4% obtained from the unbound GST-LBD protein form.

We asked why 10i is the strongest activator of Nur77 among the three compounds. Molecular docking data indicated that this series of compounds may achieve a relatively...
Figure 4. Csn-B analogues induce mitochondrial apoptosis through Nur77. A, Csn-B analogues induce apoptosis. Wild-type and stably expressing siNur77 BGC-823 cells were exposed to 10i (10 μmol/L, 16 h) and then stained by PI. The apoptotic cells were counted by flow cytometry. B, Csn-B analogues reduce mitochondrial membrane potential. Cell lines and treatment conditions were the same as above. Mitochondrial permeability was measured using JC-1 fluorescence as a probe. C, compound 10i induces the translocation of Nur77, Cyt c, and AIF. After treatment with 10i (10 μmol/L, 24 h), BGC-823 cells were immunostained with anti-TR3, anti-Cyt c, or anti-AIF antibodies, followed by the corresponding FITC-conjugated secondary antibody to show endogenous proteins, respectively (green); anti-Hsp60 was used followed by Texas Red–conjugated antibody to mark mitochondrial areas (red). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Stained cells were examined with a confocal microscope (top). The nuclear, cytosolic, and mitochondrial fractions were prepared as described, and the expression of involved proteins was determined by Western blot assay (bottom). D, Csn-B analogues induce cleavage of caspases and PARP. BGC-823 cells were treated with the Csn-B analogues as indicated (10 μmol/L, 24 h), and the protein expression levels were determined by Western blotting.
liver lysates were determined (right). Data were obtained from three independent experiments and presented as the means ± SEM.

**ChIP assay**.

D, Csn-B analogues regulate recruitment of cofactors to the NBRE promoter. BGC-823 cells were transfected with BRE promoter construct and β-galactosidase expression vector. The β-galactosidase activity was used to normalize for transfection efficiency. After treatment with 10i (6 h), the activities of the reporter gene were determined by a luciferase assay (left). The same reporter and β-galactosidase were injected into the tail vein of Nur77+/+ and Nur77−/− C57BL/6 mice (n = 5 for each group). After 5 h, mice were given i.p. 50 μL vehicle (DMSO) or 10i (50 mg/kg body weight). At 24-h posttransfection, the luciferase activities in liver lysates were determined (right). Data were obtained from three independent experiments and presented as the means ± SEM. **, P < 0.01; D, Csn-B analogues regulate recruitment of cofactors to the BRE promoter. BGC-823 cells were transfected with Flag-SRC-2 or HA-N-CoR, followed by treatment of Csn-B analogues as indicated (5 μmol/L, 5 h). The recruitment of cofactors to the NBRE on the BRE promoter was analyzed by ChIP assay.

**Figure 5.** Csn-B analogues regulate BRE. A, Csn-B analogues reduce the BRE gene expression level. BGC-823 cells were treated with Csn-B analogues as indicated (10 μmol/L, 5 h), and the mRNA level was measured by RT-PCR. B, Nur77 targets to the BRE promoter assayed by EMSA. GST-Nur77 was incubated with biotin-labeled oligonucleotide NBRE present in the BRE promoter or with its mutant form. C, effect of 10i on the transcriptional activity of the BRE promoter in vitro and in vivo. BGC-823 cells were transfected with BRE promoter construct and β-galactosidase expression vector. The β-galactosidase activity was used to normalize for transfection efficiency. After treatment with 10i (6 h), the activities of the reporter gene were determined by a luciferase assay (left). The same reporter and β-galactosidase were injected into the tail vein of Nur77+/+ and Nur77−/− C57BL/6 mice (n = 5 for each group). After 5 h, mice were given i.p. 50 μL vehicle (DMSO) or 10i (50 mg/kg body weight). At 24-h posttransfection, the luciferase activities in liver lysates were determined (right). Data were obtained from three independent experiments and presented as the means ± SEM. **, P < 0.01; D, Csn-B analogues regulate recruitment of cofactors to the BRE promoter. BGC-823 cells were transfected with Flag-SRC-2 or HA-N-CoR, followed by treatment of Csn-B analogues as indicated (5 μmol/L, 5 h). The recruitment of cofactors to the NBRE on the BRE promoter was analyzed by ChIP assay.

low-energy conformation when docked into the available crystal structure of Nur77(LBD). Comparison of the docking solutions arising from the highest predicted binding affinities for each compound revealed that 10i achieved the best docking scores, followed by 10f and then 10a. With respect to the ICM scores, 10i exhibits the lowest energy (~81.5), followed by 10f (~71.5), and then 10a (~65.4). The hydrophobicities of the compounds (represented as log P) were ranked from greatest to least: 10i (log P, 7.33), 10f (log P, 5.74), and 10a (log P, 5.21). The log P value for 8a, which failed to bind Nur77, was 1.04. Clearly, greater hydrophobicity of Csn-B analogues facilitates Nur77 binding.

**Nur77 is required for Csn-B analogue–induced apoptosis.** Among these three Csn-B analogues (10a, 10f, and 10i), compound 10i is the most potent for inducing apoptosis in BGC-823 cells. To thoroughly investigate whether Nur77 is required for apoptosis induced by 10i, we stably knocked down the expression of Nur77 in BGC-823 cells with a specific siRNA against Nur77. Silencing the expression of Nur77 greatly reduced the apoptosis induced by 10i from 49.04% to 10.8% (Fig. 4A). Similarly, the mitochondrial membrane potential was attenuated by treatment with 10i in a time- and dose-dependent manner (Supplementary Fig. S5).

In contrast, in the presence of 10i, the mitochondrial membrane potential remained unchanged compared with the control group when Nur77 was knocked down (Fig. 4B). Thus, these data indicate that 10i induces apoptosis through a Nur77-mediated process.

Because Csn-B analogues decrease the mitochondrial membrane potential, and given that mitochondrial localization of Nur77 causes a mitochondrial permeability transition (15), we suspected that 10i may cause mitochondrial localization of Nur77 and then initiate apoptosis. To test this hypothesis, we examined the localization of Nur77 and several other mitochondrial apoptosis-related proteins such as cytochrome c (35) and AIF (36) in BGC-823 cells. Nur77 was indeed translocated from the nucleus to the mitochondria after 10i treatment (Fig. 4C, top). As a consequence, mitochondrial Cyt c was released into the cytoplasm and mitochondrial AIF was redistributed in both cytoplasm and nucleus. Consistent with this result, Western blot analysis of subcellular fractionated samples from BGC-823 cells also showed a similar pattern of Nur77, Cyt c, and AIF redistribution (Fig. 4C, bottom). Moreover, the critical apoptotic events of cleavage of caspase-9, caspase-3, and poly(ADP-ribose) polymerase (PARP) were also induced (Fig. 4D). These results clearly show that localization
of Nur77 to the mitochondria is required to trigger Csn-B analogue–induced apoptosis.

**Csn-B analogues repress the transcriptional activity of BRE through Nur77.** To identify novel apoptotic genes specifically responsible for Nur77 regulation, we performed a Gene Chip analysis and found that a novel downstream gene, BRE (brain and reproductive organ-expressed protein), was dramatically repressed by Csn-B. Although BRE is a known antiapoptotic protein that functions by inhibiting the mitochondrial apoptotic pathway (26, 27), a regulatory mechanism involving BRE and Nur77 is unknown. Reverse transcription–PCR (RT-PCR) analysis confirmed that endogenous BRE mRNA levels were indeed reduced by three compounds, 10a, 10f, or 10i (Fig. 5A). Sequence analysis of the BRE promoter revealed a potential monomeric Nur77 binding site (NBRE) located between −1,602 and −1,595 bp, suggesting that Nur77 may bind to the BRE promoter and regulate its transcription. We therefore evaluated the DNA-binding properties of Nur77 by examining its ability to bind the BRE promoter using EMSA. As shown in Fig. 5B, Nur77 directly bound to the NBRE of the BRE promoter, and the binding was specifically reversed by competing with cold oligonucleotides. The NBRE mutation within the BRE promoter completely abolished the formation of DNA-protein complexes. A luciferase assay was performed to further study the BRE promoter activity regulated by Nur77 in response to 10i. Consistent with the result shown above, the transcriptional activity of BRE in BGC-823 cells was decreased with 10i treatment. However, when NBRE in the BRE promoter was mutated, inhibition of BRE transcriptional activity by 10i was not observed (Fig. 5C, left). Furthermore, we used Nur77+/+ and Nur77−/− C57BL/6 mice to analyze the effect of 10i on the transcriptional activity of BRE in vivo. The reporter gene for BRE was injected along with transfection reagent into the tail vein of both groups of mice. Similarly, the transcriptional activity of BRE was significantly repressed by 10i in hepatocytes of Nur77+/+ mice (P < 0.01); by contrast, 10i failed to exert such an inhibitory effect in hepatocytes of Nur77−/− mice (Fig. 5C, right). These data consistently indicate that Nur77 is involved in 10i-regulated BRE transcriptional activity.

Nur77 requires cofactor binding to positively or negatively modulate the transactivation of its target genes, including coactivator SRC-2 for transcriptional activation (24, 37, 38) and corepressor N-CoR for transcriptional repression (39, 40). To test whether Csn-B analogues have the ability to regulate the recruitment of cofactors to the BRE promoter, we performed ChIP assays in BGC-823 cells. As shown in Fig. 5D, all three compounds, 10a, 10f, and 10i, promoted Nur77 and recruited N-CoR but retarded recruitment of SRC-2 to the BRE promoter. This result supports the notion that Nur77 inhibits transcription of BRE by regulating cofactor binding.

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3 Our unpublished data.
recruitment, which may lead to a repressed initiation of transcription.

Csn-B analogues inhibit tumor growth in vivo. We finally assessed therapeutic effects of 10i in a xenograft tumorigenesis model, in which BGC-823 cells were used to generate tumors in the nude mice. After injection of BGC-823 cells into the mice, 10i (10 mg/kg body weight) was given to the mice by intratumoral injection every other day for a total of 11 cycles. Three weeks later, the tumors were dissected and weighed. Compound 10a was used as a positive control. As shown in Fig. 6a and B, tumor xenograft growth was significantly reduced in volume upon treatment with 10a and 10i. Tumor weight inhibitions of 68.7% \((P < 0.01)\) for 10i and 38.0% \((P < 0.05)\) for 10a were obtained.

Immunohistochemistry was performed to further examine the localization of Nur77 in the xenograft tumor. Nur77 was predominantly located in the cytosol in the 10i-treated tissue sections, whereas Nur77 was primarily located in the nucleus in the control group (Fig. 6C, top). Moreover, Western blotting indicated the cleavage of caspase-9, caspase-3, and PARP accompanied increasing Nur77 protein levels in the 10i-treated group (Fig. 6D). TUNEL assays consistently indicated a large number of apoptotic cells in the 10i-treated tissue section (Fig. 6C, bottom). These data further support the claim that translocation of Nur77 is required for Csn-B analogues to induce apoptosis in vivo.

Discussion

Nur77 is expressed in various types of cells and tissues, and it executes a complex series of biological functions. Recent studies have illustrated that Nur77 is an important target for cancer therapy (16, 17, 24). This communication has explored the structure-activity relationship of Csn-B analogues and Nur77 by analyzing a series of chemically synthesized derivatives. The small library of compounds helped us to establish a core pharmacophore for the activation of Nur77 by a direct compound-protein interaction.

The pharmacophore contains three key structural features that are required for Csn-B analogues to bind and activate Nur77. These features may help guide the further improvement of the current compounds. First, the presence of a hydroxyl group para to the acyl group on the benzene ring may be required. This hydroxyl group may closely interact with the receptor molecule through formation of a hydrogen bond with Y122 of Nur77 (LBD). Second, the presence of a carbonyl group in the acyl group may be necessary to make maximal interaction with hydrophobic groups in the binding pocket of Nur77 (LBD) through its trigonal planar electron domain (sp2) geometry. Third, the length of the acyl group contributes to the binding affinity. It seems to be capable of interacting with hydrophobic residues that line the interior pocket surface of Nur77 (LBD), thereby providing an appropriate balance between steric hindrance and hydrophobicity. In addition, the ester chain may serve to allow the synthesized Csn-B analogues to induce the biological activity of Nur77 when the acyl group is present.

Theoretically, the binding affinity of the ligand to the receptor should be governed by both the intrinsic binding energy of the ligand-receptor bond and the structure of the ligand (41). Because receptor stability is determined by ligand binding, which reduces the flexibility of the receptor, a minimal energy surface of ligand with a unique and stable native complex may be an important prerequisite for receptor-specific binding affinity (42). In this sense, the fact that the derivative n-amyl 2-[3,4-dihydroxy-2-(1-nonanoyl)phenyl]acetate (10i) exhibited binding affinity superior to its parent compounds and much more effective antitumor activity in both cell culture and xenograft may be attributed to its having the lowest binding energy. Certainly, extensive physiology tests must still be performed to fully address the relationship between the energy properties of Nur77 agonists and their biological function.

BRE is an important antiapoptotic gene that acts by inhibiting the mitochondrial apoptotic machinery without translocating to the mitochondria (26), and it may act downstream of Nur77 as an effector. Mechanistic analysis indicated that Csn-B analogues may trigger the binding of Nur77 to the BRE promoter, which contains a conserved Nur77 response element (NBRE). Promoter binding of Nur77 consequently negatively or positively regulates the recruitment of cofactors, for example, by discarding SRC-2 and recruiting N-CoR, thereby downregulating the transcriptional activity of the BRE promoter in both mouse and cell models. Remarkably, the inhibition of BRE transcriptional activity by Csn-B analogues was accompanied by changes in the expression profiles of several mitochondria-related apoptotic proteins, including caspase-9, caspase-3, and their substrates, PARP, Cyt c, and AIF. Interestingly, other groups have also reported a correlation between BRE and the expression levels of these proteins in the apoptotic pathway (26). Accordingly, our data suggest a novel nuclear-mitochondrial cross-talk in Nur77-BRE apoptosis, during which the antiapoptotic function of BRE is diminished by Nur77 at the transcriptional level in response to Csn-B analogues.

In summary, this study describes the identification of a new pharmacophore based on the structural features of Csn-B analogues that may serve as a foundation for designing and evaluating the next generation of Csn-B derivatives for clinical applications. Moreover, the discovery that Csn-B analogues induce apoptosis through Nur77 mediation offers evidence that Csn-B analogues represent a novel class of anticancer drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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