Activation of Nur77 by Selected 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes Induces Apoptosis through Nuclear Pathways*

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Nur77 is an orphan receptor and a member of the nerve growth factor-I-B subfamily of the nuclear receptor
family of transcription factors. Based on the results of transactivation assays in pancreatic and other cancer
cell lines, we have now identified for the first time Nur77 agonists typified by 1,1-bis(3-indolyl)-1-(p-anisyl)meth-
ane that activate GAL4-Nur77 chimeras expressing wild-
type and the ligand binding domain (E/F) of Nur77. In
Panc-28 pancreatic cancer cells, Nur77 agonists activate
the nuclear receptor, and downstream responses in-
clude decreased cell survival and induction of cell death
pathways, including tumor necrosis factor-related apo-
ptosis-inducing ligand (TRAIL) and poly(ADP-ribose)
polymerase (PARP) cleavage. Moreover, the transactiva-
tion and apoptotic responses are also induced in
other pancreatic, prostate, and breast cancer cells that
express Nur77. In Panc-28 cells, small inhibitory RNA
for Nur77 reverses ligand-dependent transactivation
and induction of TRAIL and PARP cleavage. Nur77 ago-

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pathways where Nur77 expression is induced and/or Nur77 protein undergoes intracellular translocation, because ligands for this receptor have hitherto not been reported. This report shows that 1,1-bis(3'-indolyl)-1-(p-substitutedphenyl)methanes containing trifluoromethyl, hydrogen, and methoxy substituents induce Nur77-dependent transactivation in Panc-28 pancreatic and other cancer cell lines. Nur77 agonists also induce typical cellular signatures of apoptosis, including PARP cleavage and induction of TRAIL, and both ligand-dependent transactivation and induction of apoptosis were associated with the action of nuclear Nur77. This study shows for the first time that ligand-dependent activation of the orphan receptor Nur77 induces apoptosis in cancer cells, suggesting that Nur77 agonists represent a new class of anticancer drugs.

MATERIALS AND METHODS

Cell Lines and Reagents—Panc-28, Panc-1, MiaPaCa-2, LNCaP, MCF-7, HT-29, and HCT-15 cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). RKO, DLD-1, and SW-480 colon cancer cells were provided by Dr. S. Hamilton, and KU7 and 253-JB-V-33 bladder cells were provided by Dr. A. Kamat (M. D. Anderson Cancer Center, Houston, TX). C-substituted DIMs were synthesized in this laboratory as previously described (22). Antibodies for PARP (sc8007), Sp1 (sc-59), and TRAIL (sc7877) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Nur77 (IMG-528) from Imgenex (San Diego, CA). The GAL4 reporter containing five GAL4 response elements (pGAL4) was provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). The GAL4-Nur77 (full-length) and GAL4-Nur77 (E/F) chimeras were provided by Dr. Jae W. Lee (Baylor College of Medicine, Houston, TX) and Dr. T. Perlmutter (Ludwig Institute for Cancer Research, Stockholm, Sweden), respectively, and Dr. Lee also provided the Nur77 response element-luciferase (NurRE-Luc) reporter construct. The GAL4-coactivator fusion plasmids pMSRC1, pMSRC2, pMSRC3, pMDRIP205, and pMCARM1 were kindly provided by Dr. Shigeki Kato (University of Tokyo, Tokyo, Japan). For RNA interference assays, we used a nonspecific scrambled (Scr) oligonucleotide as described (23). The small inhibitory RNA for Nur77 (iNur77) was identical to the reported oligonucleotide (16), and these were purchased from Dharmaco (Lafayette, CO). Leprotumycin B (LMB) was obtained from Sigma, and caspase inhibitors were purchased from BD Pharmingen. The following oligonucleotides were prepared by IDT (Corvalle, IA) and were in gel mobility shift assays; NBRE, 5'-GAT CCT CGT GCG AAA AGG TCA AGC GCT A-3'; NurRE, 5'-GAT CCT GAT ATT TAC CTA AGC GTA AAG-3'.

Transfection Assays—Transfection assays were essentially carried out as previously described using Lipofectamine Plus reagent (Invitrogen), and luciferase activities were normalized to β-galactosidase activity. For RNA interference studies, cells were transfected with small interfering RNAs for 36 h to ensure protein knockdown prior to the caspase assays.

Mammalian Two-hybrid Assays—Panc-28 cells were plated at 1 × 10⁶ cells/well in DMEM/F-12 media supplemented with 2.5% charcoal-stripped fetal bovine serum. After growth for 16 h, various amounts of DNA, i.e. Gal4Luc (0.4 μg), β-gal (0.04 μg), VP-Nur77(E/F) (0.04 μg), pMSRC1 (0.04 μg), pMSRC2 (0.04 μg), pMSRC3 (0.04 μg), pMDRIP205 (0.04 μg), and pMCARM1 (0.04 μg), were added for competition experiments and incubated for 5 min; 100-fold excess of unlabeled wild-type or mutant oligonucleotides were added for competition experiments and incubated for 5 min. The mixture was incubated with labeled DNA probe for 15 min on ice. The reaction mixture was loaded onto a 5% polyacrylamide gel and ran at 150 V for 2 h. The gel was dried, and protein-DNA complexes were visualized by autoradiography using a Storm 860 PhosphorImager (Amersham Biosciences).

Quantitative Real-time PCR—cDNA was prepared from the Panc-28 cell line using a combination of oligodeoxythymidylc acid (Oligo-d(T)12), and dNTP mix (Applied Biosystems) and Superscript II (Invitrogen). Each PCR was carried out in triplicate in a 20-μl volume using Sybr Green Mastermix (Applied Biosystems) for 15 min at 95 °C for initial denaturing, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min in the ABI Prism 7700 Sequence Detection System. The ABI Dissociation Curves software was used following a brief thermal protocol (95 °C 15 s and 60 °C 20 s, followed by a slow ramp to 95 °C) to control for multiple species in each PCR amplification. Values for each gene were normalized to expression levels of TATA-binding protein. The sequences of the primers used for reverse transcription-PCR were as follows: TRAIL forward, 5'-CGA GAG TTG CCA CTT GAC TTG-3'; reverse, 5'-ACG GAG TTG CCA CTT GAC TTG-3'; and TATA-binding promoter forward, 5'-TCC ATG GCC ATG AGT GGT-3'; reverse, 5'-TAC ACA GCC CTC CAC CA-3'.

Xenograft Experiment—Male athymic nude mice (BALB/c, ages 8–12 weeks) were purchased from Harlan (Indianapolis, IN). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions. Panc-28 cells were harvested from subconfluent cultures by trypsinization and washed. Panc-28 cells (2 × 10⁶) were injected subcutaneously into each mouse on both flanks using a 30-gauge needle. The tumors were allowed to grow for 11 days until tumors were palpable. Mice were then randomized into two groups of seven mice per group and dosed by oral gavage with either corn oil or DIM-C-pPhOCH₃ every second day. The volume of corn oil was 75 μl, and the dose of DIM-C-pPhOCH₃ was 25 mg/kg/day. The mice were weighed, and tumor areas were also measured every other day. Final body and tumor weights were determined at the end of the dosing regimen, and selected tissues were further examined by routine H & E staining and immunohistochemical analysis for apoptosis using the TUNEL assay.

RESULTS

Nur77 Expression and Structure-dependent Activation by C-substituted DIMs—Studies in this laboratory have been investigating the anticarcinogenic activities of a series of ring-substituted 3,3'-diindolylmethanes (DIMs) and methylene (C-)substituted DIMs, and many of these compounds were active in vivo and in cell culture assays (22, 24–26). Some members of a C-substituted DIMs activated peroxisome

null fractions, or whole cell lysates were obtained at various time points, analyzed by Western blot analysis, and bands were quantitated as previously described (22, 23). Immunocytochemical analysis was determined using Nur77 antibodies as previously reported (23). Gel Shift Assay—Cells were seeded in DMEM/F-12 medium supplemented with 2.5% charcoal-stripped serum and treated with 10 μM DIM-C-pPhOCH₃ for 30 min. Nuclear extracts were obtained using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Co.). Oligonucleotides were synthesized, purified, and annealed, and 5 pmol of specific oligonucleotides was ³²P-labeled at the 5'-end using T₄ polynucleotide kinase and [γ-³²P]ATP. Nuclear extracts were incubated in HEPES with ZnCl₂ and 1 μg of polyoxyethylenyl-dextran and for 5 min; 100-fold excess of unlabeled wild-type or mutant oligonucleotides were added for competition experiments and incubated for 5 min. The mixture was incubated with labeled DNA probe for 15 min on ice. The reaction mixture was loaded onto a 5% polyacrylamide gel and ran at 150 V for 2 h. The gel was dried, and protein-DNA complexes were visualized by autoradiography using a Storm 860 PhosphorImager (Amersham Biosciences).
Nuclear Nur77 Induces Transactivation and Apoptosis

Fig. 1. Nur77 expression and activation in cancer cell lines. A, Nur77 protein expression. Whole cell lysates from 12 different cancer cell lines were analyzed for Nur77, Nurr1, and Nor1 by Western blot analysis as described under “Materials and Methods.” Nor1 protein was not detected in these cell lines. Activation of Gal4-Nur77 (B) and NuRE (C) Panc-28 cells were treated with 10 or 20 μM of the various compounds, transfected with GAL4-Nur77/pGAL4 or NuRE, and luciferase activity was determined as described under “Materials and Methods.” D, Nur77 activation by isomeric DIM-C-PhOCH₃ compounds. Panc-28 cells were treated with 10 or 20 μM of the DIM-C-PhOCH₃ isomers, transfected with GAL4-Nur77/pGAL4 and luciferase activity determined as described under “Materials and Methods.” Results are expressed as means ± S.E. for at least three separate determinations for each treatment group, and significant (p < 0.05) induction is indicated by an asterisk. The compounds in B and C were 1,1-bis(3’-indolyl)-1-(p-substitutedphenyl)methanes and the p-substituent X is shown directly in the figure. D compares the activity of the p-substituted methoxy derivative (DIM-C-PhOCH₃) with the meta (DIM-C-mPhOCH₃) and ortho (DIM-C-oPhOCH₃) isomers.

proliferator-activated receptor γ (PPARγ) but not PPARα, retinoic acid receptor, retinoic X receptor, estrogen receptor α, or the aryl hydrocarbon receptor. Previous studies have linked Nur77 to decreased cell survival and activation of cell death pathways by apoptosis-inducing agents in some cancer cell lines (15–21), and we therefore investigated expression of Nur77 in cancer cell lines and the effects of a series of eleven C-substituted DIMs on Nur77 activation/translocation. Fig. 1A
A compound was active. We also investigated NpPhOCH₃ as a model, and the position of the methoxyl group activation of Nur77 was also investigated using DIM-C-NurRE in MiaPaCa-1 pancreatic, HCT-15 colon, and MCF-7 containing a Nur response element (NurRE) (Fig. 1). Dim-C-pPhOCH₃ or DIM-C-Ph and transfected with pNurRE showed decreased Nur77 protein in whole cell lysates and a nonspecific “scrambled” small inhibitory RNA (iScr), or small inhibitory RNA for Nur77 (iNur77). The results (Fig. 2B) showed decreased Nur77 protein in whole cell lysates and a 90–100% decrease in ligand-induced transactivation over the different concentrations of compounds, thus confirming the role of Nur77 in mediating this response. As noted above, one compound that contained a p-hydroxy substituent (DIM-C-pPhOH) did not induce activity (Fig. 1B) and DIM-C-pPhOH was further investigated as a potential Nur77 antagonist. Panc-28 cells were transfected with GAL4-Nur77/pGAL4 and cotreated with DIM-C-pPhOH and Nur77 agonists DIM-C-pPhCF₃, DIM-C-pPhOCH₃, and DIM-C-pH (Fig. 2C). The results show that DIM-C-pPhOH antagonizes activation of Nur77 by all three C-DIM compounds. The structural specificity of Nur77 antagonists was further investigated using meta-hydroxy (DIM-C-mPhOH) and ortho-hydroxy (DIM-C-oPhOH) analogs. DIM-C-mPhOH (10 or 20 μM) did not inhibit DIM-C-pPhOCH₃ or DIM-C-pPh-induced transactivation (Fig. 2D). DIM-C-pPhOH also did not inhibit Nur77 antagonist activity (Fig. 2E); however, high doses (20 μM) of both Nur77 agonists and DIM-C-pPhOH were toxic. Thus, activation of Nur77 by C-DIMs was E/F domain-dependent and Nur77 activation was inhibited by DIM-C-pPhOH; moreover, both activation and inhibition of Nur77-mediated transactivation was dependent on the structure of the C-DIM compounds.

Nur77 DNA Binding and C-DIM-induced Nur77-coactivator Interactions—Incubation of nuclear extracts from Panc-28 cells treated with Me₂SO or DIM-C-pPhOCH₃ with 32P-labeled NBRE and NurRE (lanes 1 and 2, and 5 and 6, respectively) gave retarded bands in EMSA assays (Fig. 3A). Retarded band intensities were decreased after incubation with 100-fold excess NurRE (lane 3) or NBRE (lane 7) but not by mutant NurRE (lane 4) or mutant NBRE (lane 8) oligonucleotides. These results show that nuclear extracts containing Nur77 bind NurRE and NBRE as dimers and monomers, respectively, and this corresponds to their migration in an electrophoretic mobility shift assay. Extracts from cells treated with Nur77-active C-substituted DIMs gave retarded band intensities similar to those observed for solvent-treated extracts suggesting minimal ligand-dependent loss of nuclear Nur77 in these cells. The retarded band pattern corresponds to that observed in previous studies using nuclear extracts from cells or in vitro translated Nur77 (27, 28).

Ligand-dependent activation of nuclear receptors is dependent on interaction of the bound receptor with coactivators (29–31), and Fig. 3 (B–D) summarizes results of a mammalian two-hybrid assay in Panc-28 cells transfected with VP-Nur77 (ligand binding domain) and GAL4-coactivator chimeras. Ligand-induced Nur77-coactivator interactions were determined using a construct (pGAL4) containing 5 GAL4 response elements. Coactivators used in this study include SRC-1, SRC-2 (TIFII), SRC-3 (AIB1), PGC-1, TRAP220, and CARM-1. A GAL4-repressor (SMRT) chimera was also included in the assay. All three ligands induced transactivation in cells transfected with GAL4-SRC-1, GAL4-PGC-1, and GAL4-TRAP220 chimeras. DIM-C-pPhOCH₃-induced transactivation in cells transfected with GAL4-SRC-3 and GAL4-CARM-1 was slightly activated by DIM-C-pPhOCH₃ and DIM-C-pPhCF₃. The results demonstrate that there were some ligand-dependent differences in transactivation observed for GAL4-SRC-3 and GAL4-CARM-1; however, the most significant interactions between VP-Nur77 and GAL4 chimeras expressing SRC-1, PGC-1, and TRAP220 were induced by all three compounds. Effects of Nur77-active C-DIMs on Cell Survival and Apoptosis and Role of Nuclear Nur77—In several cancer cell lines transfected with Nur77-GFP constructs, treatment with apoptosis and differentiation-inducing agents results in rapid translocation of Nur77 into the cytosol/mitochondria (15–20). Similar results have been observed in BGC-823 human gastric cancer cells where endogenous Nur77 is nuclear and TPA induced Nur77 translocation into the cytosol, and this was accompanied by apoptosis but not by Nur77-dependent transactivation (17). Results summarized in Fig. 4A show immunostaining of Nur77 in the nucleus of Panc-28 cells treated with Me₂SO and Nur77-active DIM-C-pPhCF₃, DIM-C-pPhOCH₃, and DIM-C-Ph for 6 h, and comparable results.
were obtained in Panc-28, MiaPaCa, and LNCaP cells after treatment for 6 or 12 h (data not shown). In all cases, Nur77 remained in the nucleus, and cells exhibited a compacted nuclear staining pattern typically observed in cells activated for cell death pathways. In a separate experiment, Panc-28 cells were treated with 10 or 20 μM DIM-C-pPhCF3, DIM-C-pPhOCH3, and DIM-C-Ph or 10 μM DIM-C-pPhOH for 12 h, and Nur77 protein levels were determined by Western blot analysis of cytosolic and nuclear extracts (Fig. 4B). These results also confirm that Nur77, in the presence or absence of C-substituted DIM agonists, is a nuclear protein and ligand-induced Nur77 translocation from the nucleus is not observed. Sp1 is a nuclear protein and was used as a control to ensure efficient separation of the two extracts, and Sp1 was identified only in the nuclear fraction (Fig. 4B).

Nur77 agonists significantly decreased survival of Panc-28 cells (Fig. 5A), and IC50 values for DIM-C-pPhCF3, DIM-C-pPhOCH3, and DIM-C-Ph were between 1 and 5 μM, whereas DIM-C-pPhOH did not affect cell survival. At longer time points (4 and 6 days), DIM-C-pPhOH slightly inhibited cell proliferation; however, induction of cell death was not observed for this compound at concentrations as high as 20 μM. De-
FIG. 3. DNA binding of Nur77 and ligand-induced coactivator-Nur77 interactions. A, gel mobility shift assay. Cells were treated with Me2SO (DMSO) or Nur77 agonists for 0.5 h, nuclear extracts were incubated with 32P-labeled NurRE and NBRE, and formation of retarded bands was determined in a gel mobility shift assay as described under “Materials and Methods.” Arrows denote the specifically bound bands. GAL4-coactivator interactions with VP-Nur77(E/F) in Panc-28 cells were treated with DIM-C-pPhCF3 (B), DIM-C-pPhOCH3 (C), and DIM-C-Ph (D). Cells were transfected with the pGAL4, VP-Nur77(E/F), and GAL4-coactivator/repressor (chimera) constructs and treated with the Nur77 agonists, and luciferase activity was determined as described under “Materials and Methods.” Significant (p < 0.05) induction of luciferase activity is indicated (*), and results are expressed as means ± S.E. for three separate determinations for each treatment group.
increased cell survival is also observed for agents that induce apoptosis and/or Nur77 nuclear to cytosolic translocation in cancer cells (15–20). Results illustrated in Fig. 5B show that treatment of Panc-28 cells with Nur77 agonists induced cleavage of PARP, whereas the Nur77-inactive DIM-C-pPhOH did not induce this response. PARP cleavage is associated with activation of cell death pathways; however, this was not accompanied by changes in levels of bax (Fig. 5B) or bcl-2 proteins (data not shown). Moreover, treatment of Panc-28 cells with 10 and 20 μM DIM-C-pPhOCH$_3$ for 8 and 12 h showed a time- and dose-dependent increase of annexin V-stained cells using a green fluorescent Alexa Fluor 488 probe (Fig. 5C). The effects of camptothecin (positive control for apoptosis) and DIM-C-pPhOCH$_3$ were comparable. After treatment with DIM-C-pPhOCH$_3$ for 6 h, annexin V-stained cells were significantly increased, plasma membrane blebbing was observed, and there was minimal PI staining. However, after 12 h, PI staining was increased. Induction of PARP cleavage by Nur77 agonists was also observed in other pancreatic (MiaPaCa-2), prostate (LNCaP), and breast (MCF-7) cancer cell lines (Fig. 5D). Induction of PARP cleavage by the Nur77-active compounds in Panc-28 cells was not accompanied by changes in Nur77 expression (Fig. 4B), and this was in contrast to TPA, which activates nuclear pathways by inducing Nur77 expression (21). Using a protocol comparable to that outlined in Fig. 5B, the induction of PARP cleavage by the Nur77 agonists in Panc-28 cells was not affected by the nuclear export inhibitor leptomycin B (LMB) (1 ng/ml) (Fig. 5E). LMB alone slightly induced PARP cleavage and, for some cells cotreated with LMB plus Nur77 agonists, there was enhanced PARP cleavage. In contrast, previous studies showed that LMB inhibits apoptosis in cells treated with apoptosis-inducing agents that activate nuclear-cytosolic/mitochondrial translocation of Nur77 (15, 16). These results demonstrate that activation of nuclear Nur77 by C-substituted DIMs induces apoptosis in Panc-28 and other cancer cell lines; however, evidence for activation of the intrinsic apoptotic pathways was not observed.

Nur77-active C-DIMs Induce TRAIL—In thymocytes, there is evidence that Nur77-induced apoptosis is linked to transcriptional activation (32), and microarray studies in thymocytes undergoing Nur77-dependent apoptosis identified several apoptosis-related genes, including fasL and TRAIL (33). Results in Fig. 6A show that Nur77 agonists that induce PARP cleavage also induce TRAIL (but not fasL) protein expression in Panc-28 cells, suggesting that this response may be a direct or indirect downstream target of Nur77 agonists in cancer cells. The Nur77-inactive DIM-C-pPhOH did not induce TRAIL. In addition, DIM-C-pPhOCH$_3$ or DIM-C-Ph induced TRAIL mRNA levels in Panc-28 cells (Fig. 6B). Because TRAIL activates the extrinsic apoptosis pathway and activation of caspase 8, we also investigated the effect of a caspase 8 inhibitor (z-IETD-fmk) and the pan-caspase inhibitor (z-VAD-fmk) on induction of PARP cleavage by Nur77 agonists (Fig. 6D). The results show that both inhibitors blocked (60–90%) induction of PARP cleavage by Nur77 agonists.

The role of Nur77 in mediating induction of TRAIL and PARP cleavage by DIM-C-pPhOCH$_3$ was further investigated in Panc-28 cells transfected with nonspecific RNA (iScr) and iNur77 (Fig. 6C). Levels of Nur77, PARP cleavage, and TRAIL proteins were determined by Western blot analysis of whole cell extracts, and the results showed that iNur significantly decreased levels of all three proteins. In addition, cotreatment of Panc-28 cells with DIM-C-pPhOH$_3$ or DIM-C-Ph and the Nur77 antagonist DIM-C-pPhOH (Fig. 6E) showed that the latter compound also inhibited induction of PARP cleavage and TRAIL protein expression induced by Nur77 agonists. These results demonstrate that Nur77 agonists induce apoptosis pathways in cancer cells through transcriptional (nuclear)
FIG. 5. Nur77 agonists decrease cell survival and induce apoptosis. **A**, cell survival. Panc-28 cells were treated with different concentrations of C-substituted DIMs for 2 days, and cell numbers were determined as described under “Materials and Methods.” Results are expressed as means ± S.E. for three separate determinations for each treatment group, and a significant (p < 0.05) decrease in cell survival is indicated by an asterisk. DIM-C-pPhOH inhibited cell growth only after treatment for 96 or 144 h; however, this compound did not induce cell death at any time point. **B**, effects of Nur77 agonists on PARP cleavage in Panc-28. Cells were treated with the different compounds alone or with LMB, and PARP cleavage was determined by Western blot analysis of whole cell lysates as described under “Materials and Methods.” Bax and bcl-2 (not shown) protein levels were not affected by treatment and NS (nonspecific) protein served as a loading control. **C**, annexin staining. Panc-28 cells were treated with camptothecin (positive control) or DIM-C-pPhOCH₃ for 6 h, and annexin staining was determined as described under “Materials and Methods.” Approximately 30–40% of cells treated with DIM-C-pPhOCH₃ exhibited annexin staining. Induction of apoptosis in LNCaP, MiaPaCa-1, and MCF-7 cells (**D**) or Panc-28 cells (**E**) treated with Nur77 agonists alone or in combination with LMB, respectively. Cells were treated essentially as described (**A**), and PARP cleavage determined by Western blot analysis as described under “Materials and Methods.”
mechanisms, and at least one of the induced proteins (TRAIL) activates an extrinsic apoptotic pathway. In summary, selected C-substituted DIMs have now been identified as ligands for the orphan receptor Nur77, and activation of this receptor is associated with decreased cancer cell survival, induction of TRAIL, and apoptosis.
mice overexpressing full-length Nur77 (13, 14, 35, 36). Activation of cell death in macrophages is associated with increased expression of Nur77, and decreased cell death was observed in Nur77-deficient macrophages (37). A recent study (38) show that cadmium acetate induced apoptosis in WI-38 human lung fibroblasts and A549 human lung carcinoma cells, and this was also accompanied by induction of Nur77. Moreover, transfection with dominant-negative Nur77 protected the cells against cadmium-induced apoptosis.

Ongoing studies in the laboratory with a series of C-substituted DIMs indicate that these compounds inhibit growth or induce cell death of multiple cancer cell lines, and some of these analogs, including DIM-C-pPhCF3, activate PPARy (22, 26). However, several PPARγ-inactive C-substituted DIMs also decreased cell survival of several cancer cell lines (e.g. Fig. 5A) and inhibited carcinogen-induced mammary tumor growth in female Sprague-Dawley rats (data not shown). Nur77 was considered as a possible target for C-DIM compounds based on results of several studies with retinoids, apoptosis, and differentiation-inducing agents that also inhibit cell growth and activate extranuclear Nur77 (15–20). Initial studies confirmed Nur77 protein expression in 12 prostate, colon, bladder, pancreatic, and breast cancer cell lines (Fig. 1A). Results of screening a panel of structurally diverse C-substituted DIMs shows that DIM-C-pPhCF3 and two PPARγ-inactive analogs, DIM-C-pPhOCH3 and DIM-C-Ph, activate Nur77-dependent transactivation in Panc-28 and other cancer cell lines transfected with GAL4-Nur77 (full-length) or NurRE (Fig. 1, B and C). Moreover, ligand-induced transcriptional activation is observed with GAL4-Nur77(E/F) chimeras (Fig. 2C) in which only the ligand binding domain of Nur77 is expressed. The role of Nur77 in mediating ligand-dependent transactivation was confirmed in studies showing that these responses were inhibited by either iNur77 (small inhibitory RNA) (Fig. 2B) or DIM-C-pPhOH, which exhibited Nur77 antagonist activity (Fig. 2C). Previous studies on the crystal structure of the mouse Nurr1 LBD (39) and the Drosophila Nurr1 homolog DHR38 (40) show that the ligand binding pocket is occupied by bulky hydrophobic amino acid side chains. Moreover, due to the high sequence homology among NGFI-B family proteins, it has been suggested that Nur77, Nurr1, and Nor-1 may represent a class of orphan receptors that function independently of ligand binding (41). In contrast, this study shows that selected C-DIM compounds uniquely induce nuclear Nur77-mediated transactivation; this induction response is observed through the E/F domain of Nur77 (Fig. 1C) and can be inhibited by DIM-C-pPhOH, a Nur77 antagonist (Figs. 2 and 5). Moreover, both activation of Nur77 and Nur77 antagonist activities by C-DIMs were highly structure-dependent (Figs. 1 and 2). These results suggest that the active C-DIM compounds interact with the E/F domain of Nur77 and induce conformational changes, resulting in binding to the ligand binding pocket or other sites within the C-terminal region of Nur77. Currently, we are examining critical ligand interaction sites within the E/F domain of Nur77 by deletion/mutation analysis and by crystallization of the Nur77 LBD in the presence or absence of the C-DIM ligands.

Several studies have reported activation of Nur77-dependent transactivation in different cell lines, and these responses primarily involve the AF-1 domain of Nur77 and activation by kinases (28, 42, 43). For example, induction of Nur77-dependent transactivation was observed for the coactivator ASC-2 in CV-1 and HeLa cells; however, this effect was dependent on calcium/calmodulin-dependent protein kinase IV and did not involve direct ASC-2-Nur77 interactions (42). Transactivation mediated by Nur77 homodimers is enhanced by protein kinase A and SRC1–3 in CV-1 and AtT-20 cells and these responses...
were AF-1-dependent (28). Another report also confirmed that Nur77 transactivation in C2C12 and COS-1 cells was enhanced by SRCs and other coactivators, and involved direct interactions of coactivators with the A/B (and not E/F) domain of Nur77 (43). These observations are consistent with the crystal structure of Nur1, which lacks the “classical binding site for coactivators” (39). However, ligand-dependent activation of Nur77 E/F domain observed in this study (Fig. 1C) should also be accompanied by interactions with some nuclear receptor coactivators/coregulators. Initial studies showed that, in the absence of ligand, VP-Nur77(E/F) did not interact with GAL-4-coactivators (PGF-1, CARM-1, SRC1-3, and TRAP220) or GAL-SMRT chimeras (data not shown); however, DIM-C-pPhCF3, DIM-C-pPhOCH3, and DIM-C-Ph induced interactions between several common nuclear receptor coactivators (PGC-1, SRC-1, and TRAP220) and the LBD (E/F) of Nur77 in mammalian two-hybrid assays (Fig. 3, A–D). These results are consistent with other studies on activation of nuclear receptors by ligands and their interactions with specific coactivators through binding receptor E/F domains. For example, our recent studies with PPARγ-active C-substituted DIMs in colon cancer cells show that ligand-induced PPARγ(E/F domain)-coactivator interactions in mammalian two-hybrid assays primarily involved PGC-1 (26), whereas C-DIM-induced Nur77-coactivator interactions in Panc-28 cells involve multiple coactivators. Although the crystal structure of unliganded Nur1 shows that this receptor does not contain a classic coactivator interaction site in the E/F domain (helix 12), novel coactivator interaction surfaces have recently been identified between helices 11 and 12 in Nur1 (44). This region is similar in human Nur77, and current studies are investigating C-DIM-induced interaction surfaces between the E/F domain of Nur77 and coactivators. In summary, the transactivation and coactivator-Nur77 interactions induced by DIM-C-pPhCF3, DIM-C-pPhOCH3, and DIM-C-Ph are consistent with results obtained for other ligand-activated nuclear receptors suggesting that selected C-substituted DIMs are a novel class of compounds that induce E/F domain-dependent activation of Nur77.

Treatment of Panc-28 cells with Nur77-active C-substituted DIMs agonists decreased cell survival (Fig. 4A) and induced nuclear condensation within 48 and 24 h, respectively, and this is typically observed in cells undergoing cell death. We therefore further examined Nur77-mediated induction of PARP cleavage, which is a well characterized downstream marker of activated cell death pathways. PARP cleavage was induced in Panc-28 cells treated with Nur77 agonists (Fig. 5B), and similar results were observed in other pancreatic, prostate, and breast cancer cell lines (Fig. 5D). Annexin V staining was also observed in Panc-28 cells treated with Nur77-active C-DIMs (Fig. 5C), and these data further confirm induction of apoptosis in these cancer cell lines. Previous studies report that induction of cell death pathways by apoptosis-inducing agents in some cancer cell lines is accompanied by translocation of Nur77 from the nucleus to the cytosol/mitochondria, and this has been linked to cytochrome c release and direct interaction of Nur77 with bcl-2 (15–20). In contrast, we observed that treatment of Panc-28 cells with Nur77-active C-DIMs resulted only in formation of a nuclear complex (Fig. 4, A and B). Moreover, inhibition of nuclear export of Nur77 by LMB did not affect PARP cleavage induced by Nur77-active C-DIMs (Fig. 5E), suggesting that this response is mediated through nuclear Nur77. This nuclear pathway for induction of apoptosis is in contrast to the effects observed for TPA and CD437, which induce nuclear export of Nur77 in cancer cell lines, and inhibition of Nur77 nuclear export by LMB, which inhibits induction of apoptosis (15, 16). These results clearly distinguish between the induction of cell death pathways in cancer cells through ligand-dependent activation of nuclear Nur77 (this study) and through induction of Nur77 nuclear translocation (15–20).

Overexpression of Nur77 in thymocytes induces expression of several genes associated with apoptosis (33), and at least one of the genes, TRAIL, (protein and mRNA), is also induced by Nur77 agonists in Panc-28 cells (Fig. 6, A and B). RNA interference assays with iNur77 (Fig. 6D) and inhibition studies with the Nur77 antagonist DIM-C-pPhOH (Fig. 6E) demonstrate that induction of TRAIL and PARP cleavage by DIM-C-pPhOH and DIM-C-Ph are Nur77-dependent. Thus, the nuclear action of Nur77 agonists in cancer cell lines is comparable to the transcriptionally dependent pathway observed in T-cells overexpressing Nur77 (33). TRAIL typically activates caspase 8, and the extrinsic pathways of apoptosis and the caspase 8 inhibitor z-IETD-fmk significantly blocks (>60%) induction of PARP cleavage by Nur77 agonists (Fig. 5C). The pan-caspase inhibitor z-VAD-fmk blocked >90% of induced PARP cleavage suggesting that, although TRAIL may be a major Nur77-induced gene in Panc-28 cells, other pro-apoptotic genes may also be induced; these are currently being investigated. We also observed in xenograft experiments that DIM-C-pPhOCH3 inhibited tumor growth in athymic nude mice bearing Panc-28 cell xenografts (Fig. 7).

In summary, results of this study have identified a new group of C-substituted DIMs that activate the orphan receptor Nur77 through the E/F domain. These results are in contrast to previous reports showing that kinase/coactivator-dependent activation of Nur77 was primarily AF-1-dependent (23, 42, 43). It has also been reported that nuclear receptor coactivators interact with the N-terminal A/B but not E/F domains of Nur77, and in the absence of C-DIM compounds, coactivator-Nur77(E/F) interactions were not observed in this study. However, DIM-C-pPhCF3, DIM-C-Ph, and DIM-C-pPhOCH3 induced coactivator interactions with the E/F domain of Nur77 (Fig. 3), and this was consistent with Nur77 (nuclear)-dependent transactivation. Activation of Nur77 by selected C-DIMs is associated with decreased cancer cell survival, induction of apoptosis, induced expression of the apoptosis gene/protein TRAIL, and inhibited tumor growth in vivo. These results suggest that C-DIM ligands that activate Nur77 are a potential new class of anticancer agents. Their activities and mechanisms of action in other cancer cell lines are currently being investigated.

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**Nuclear Nur77 Induces Transactivation and Apoptosis**
Activation of Nur77 by Selected 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes Induces Apoptosis through Nuclear Pathways
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