Phenoxodiol, a Novel Isoflavone, Induces G1 Arrest by Specific Loss in Cyclin-Dependent Kinase 2 Activity by p53-Independent Induction of p21WAF1/CIP1

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Abstract
Phenoxodiol, an isoflavone derivative of genistein with unknown mechanism of action, is currently being evaluated in early human cancer clinical trials. To determine the mechanism of antiproliferative effects of phenoxodiol, we examined its effects in a battery of human cell lines. Although we observed caspase-dependent apoptosis in HN12 cells as early as 24 hours after exposure, clonogenic death occurred only after 48-hour exposure despite caspase blockade by the general caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (ZVAD)-fmk. Moreover, clear evidence of cell death as determined by nuclear morphology and plasmatic membrane damage occur despite ZVAD, suggesting that another mechanism besides caspase-dependent apoptosis is required for clonogenic death induced by phenoxodiol. In search for other potential antiproliferative effects, we assessed the effects of phenoxodiol in the cell cycle progression of human carcinoma cell lines. A significant G1-S arrest was observed by 12 hours of exposure in HN12 cell lines at concentrations ≥5 μg/mL. Cell cycle arrest occurred several hours (∼12 hours) before induction of apoptosis. Analysis of in vitro purified cyclin-dependent kinase (cdk) activity showed that phenoxodiol did not inhibit cdk activity. In contrast, cellular cdk2 activity obtained from HN12 cell lines exposed to phenoxodiol for 12 hours decreased by 60%, whereas cdk6 activity remained unaltered, suggesting that the loss of cdk2 activity was specific. Loss in cdk2 activity was preceded by the accumulation of the endogenous cdk inhibitor p21WAF1.

To assess the role of p21WAF1 induction by phenoxodiol, we used HCT116 isogenic cell lines and showed that phenoxodiol induced G1 arrest together with p21WAF1 expression in wild-type clones. In contrast, p21−/− variants failed to show G1 arrest. Finally, induction of p21 by phenoxodiol is p53 independent, as phenoxodiol induced p21 in HCT116 lacking p53. These data therefore indicate that phenoxodiol promotes G1-S arrest by the specific loss in cdk2 activity due to p53-independent p21WAF1 induction. This novel feature of phenoxodiol may have clinical implications, as the majority of human malignancies have aberrations in cell cycle progression regulation.

Introduction
Despite the significant lessons learned within the last 10 years from the sequencing of the human genome and the elucidation of signaling pathways required for proliferation, differentiation, angiogenesis, cellular survival, and apoptosis, the prognosis of advanced epithelial malignancies has not significantly improved (1). Although several novel small molecules and other therapeutics approaches are being tested in the clinic (2), there is still a significant need to determine novel targets for cancer therapy and, based on this information, to develop novel small molecules that are relevant for the mechanism of cellular transformation, also known as “targeted therapy” (2–5).

A family of natural compounds, the flavonoids, has been studied for the prevention and therapy of cancer (6–8). One example in this family, genistein, has several interesting properties, including the promotion of a differentiation phenotype, cell cycle arrest, and induction of apoptosis (7, 9–13). Initial clinical trials with genistein have been published recently, demonstrating good tolerability; however, the clinical development of this agent has been compromised by the inability to achieve adequate plasma concentrations in humans (14–16). Phenoxodiol, a synthetic analogue of genistein, was selected for its improved anticancer potency/efficacy in preclinical models along with a significantly lower susceptibility to metabolism compared with genistein (17). Currently, several phase 1 trials with phenoxodiol are being conducted worldwide (18). Initial mechanistic studies with phenoxodiol showed induction of apoptosis in ovarian cancer cells and in vivo antitumor activity in breast murine models (19, 20). However, the question whether other mechanisms besides apoptosis are relevant for the antiproliferative effects of this agent remains unanswered.

In this study, we show that the antiproliferative effects of phenoxodiol are mediated at least in part by arrest of cell cycle progression at G1-S. This arrest results from the specific loss of cyclin-dependent kinase (cdk) 2 activity by induction of p21WAF1/CIP1. Indeed, p21WAF1/CIP1 is crucial for the cell cycle arrest induced by phenoxodiol, as HCT116 cells lacking p21 are refractory to the G1 accumulation promoted by this agent.

Materials and Methods

Cell culture. Exponentially growing head and neck squamous cell carcinoma (HNSCC) cells (HN12, HN17, and HaCaT) were grown in DMEM containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in a 5% CO2 humidified atmosphere at 37°C as described previously (21, 22). HCT116 colon carcinoma cell lines of various genetic backgrounds were a kind gift from Dr. Bert Vogelstein (Johns Hopkins, Baltimore, MD) and maintained as described elsewhere (23).

Drugs and chemicals. Phenoxodiol (provided by Marshall Edwards, North Ryde, New South Wales, Australia) was resuspended in DMSO (stock...
concentration of 10 mg/mL. Flavopiridol (provided by the Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD) was resuspended in DMSO (10 mmol/L stock solution). The general caspase inhibitor benzoxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone (ZVAD) was obtained from Enzyme Systems Products (Livermore, CA) and was added to cells 30 minutes before phenoxodiol, with a final concentration of 50 μmol/L. ZVAD was added every 12 hours to cells to minimize possible loss of enzymatic activity. Genistein was obtained from Biomol (Plymouth Meeting, PA). All pharmacologic inhibitors were prepared in DMSO at a concentration of ≥1,000-fold. The final concentration of DMSO in the culture medium was at all times ≤0.1%.

Antiproliferative effect of phenoxodiol in head and neck squamous cell carcinoma cells. Cell proliferation studies were done by cell counting as described (24). Briefly, HNSCCs were exposed to phenoxodiol, genistein, or flavopiridol as described in the figure legends, washed briefly in ice-cold PBS, and fixed in 70% ethanol overnight. DNA content was obtained by incubating cells in PBS containing propidium iodide (PI; 50 μg/mL) and RNase A (1 mg/mL) for 1 hour at 37°C. Fluorescence was measured and analyzed in a FACSCalibur using CellQuest software for acquisition (Becton Dickinson Immunocytometry Systems, San Jose, CA) and ModFit software for analysis (Verity Software, Topsham, ME), respectively.

Assessment of S phase as determined by bromodeoxyuridine/7-amino-actinomycin D analysis. Exponentially growing HN12 and HCT116 clones were exposed to phenoxodiol as described in the figure legends and S-phase determination was obtained using the FITC-bromodeoxyuridine (BrdUrd) kit (BD Biosciences, Bedford, MA). Briefly, 1 hour before harvesting, BrdUrd at a final concentration of 10 μmol/L was added to the cultures. One hour later, cells were washed, trypsinized, and resuspended in Cytofix/Cytoperm buffer followed by incubation with DNase, FITC-BrdUrd antibody, and 7-amino-actinomycin D as recommended by the manufacturer. Fluorescence was acquired and analyzed in a FACSCalibur.

Detection of apoptosis by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay. HN12 cells were exposed to phenoxodiol for different times and concentrations as described in the figure legends. Then, cells were cytopun in the presence of 4′,6-diamidino-2-phenylindole (DAPI), where the morphologic effects of phenoxodiol were visualized by fluorescence microscopy. Apoptosis was detected by measuring DNA fragmentation using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (Roche Applied Science, Indianapolis, IN) following the manufacturer's recommendations.

Detection of apoptosis by Annexin V assay. After exposure to phenoxodiol, HN12 cells were harvested, washed with PBS, and washed with Annexin V binding buffer following the manufacturer's recommenda (BD PharMingen). Cells were then centrifuged and resuspended in 100 μL binding buffer with 5 μL Annexin V-FITC. After incubation on ice for 15 minutes, cells were incubated with 400 μL of 0.5 μg/mL PI. Analysis was done on a flow cytometer. A dot plot was set up with the FL1 (red) for PI staining and the FL3 axis as FL-1 (green) for Annexin V.

Clonogenic assays. HN12 cells were exposed to increasing concentrations of phenoxodiol for increasing times. Following treatment, cells were washed with PBS, trypsinized, and seeded in a 24-well plate at 100 cells per well. To determine whether caspase blockade prevented clonogenic cell death, ZVAD (50 μmol/L) was added 30 minutes before phenoxodiol and every 12 hours for the duration of phenoxodiol incubation. Following 2 weeks, HN12 cells were washed with PBS, fixed with methanol overnight, and stained with 4% methylene blue. Colonies (≥50 cells) were counted manually and expressed as percentage of vehicle-treated control.

Results

Potent antiproliferative effects of phenoxodiol in human head and neck cell lines. To understand the mechanism by which phenoxodiol exerts its antiproliferative effect, we initially tested this novel isoflavone against a battery of human head and neck cell lines (HNSCC). Exponentially growing cell lines were exposed to increasing concentrations of phenoxodiol for 24 hours (Fig. 1, left) and 48 hours (Fig. 1A, right) and cells were enumerated by automated cell counting. A significant decrease in cell number was observed in all cell lines tested at concentrations ≥5 μmol/L for 24 hours, reaching IC50 concentrations of 0.3 (1.2 μmol/L), 1 (3.5 μmol/L), and 6.25 (26.2 μmol/L) μg/mL for HaCaT, HN17, and HN12 cell lines, respectively. Moreover, longer exposures...
Although short-term hours of exposure in HN12 cell lines, clinical trials (27) are necessary for antiproliferative effects are easily achieved in human hours (Fig. 1A, left). Of note, phenoxodiol concentrations necessary for antiproliferative effects are easily achieved in human clinical trials (27).

Clonogenicity cell death induced by phenoxodiol requires 48 hours of exposure in HN12 cell lines. Although short-term assays of cell killing may reflect the cellular sensitivity to chemotherapeutic agents, the ability of cells to undergo unlimited proliferation as tested by their ability to form colonies ("clonogenic assay") is considered the "gold standard" for assessment of cellular sensitivity to cytotoxic agents (28). To determine the effects of phenoxodiol on clonogenicity, we exposed HN12 cells to increasing concentrations of phenoxodiol for increasing times and clonogenic potential was determined as described in Materials and Methods. When HN12 cells were exposed for 1, 6, or 12 hours, no clonogenic death was observed (data not shown). Similar lack of clonogenic death was observed when HN12 cells were exposed to 10 μg/mL phenoxodiol for 24 hours (Fig. 1B, left). In contrast, exposure to ≥5 μg/mL for 48 hours clearly induced significant clonogenic cell death (Fig. 1B, right). Thus, the more protracted exposures of phenoxodiol (i.e., ≥48 hours) promoted clonogenic cell death.

Induction of caspase-dependent apoptosis by phenoxodiol is not required for the clonogenic death induced in HN12 squamous carcinoma cell lines. Phenoxodiol is known to induce apoptosis in ovarian cancer models (19). To explore whether apoptosis occurs in HN12 cells and to determine whether the induction of apoptosis can explain the clonogenic death observed in HN12 cells after 48 hours of phenoxodiol, we exposed HN12 cells to phenoxodiol for increasing times and did TUNEL assays for apoptosis detection. Minimal induction of apoptosis was observed by 12 hours in this p53-defective cell line (data not shown). However, exposure for 24 hours (Fig. 2A) showed a significant induction of apoptosis (5 μg/mL ~ 2.8 fold and 10 μg/mL ~ 5 fold over control, respectively). To examine the role of caspases in the induction of apoptosis by this agent, we preincubated HN12 cells with a general caspase inhibitor, ZVAD, and exposed cells for 24 hours. As shown in Fig. 2A, ZVAD prevented caspase-dependent apoptosis as measured by TUNEL, suggesting that the induction of apoptosis by this agent is caspase dependent and occurs by 24 hours of exposure.

To determine whether inhibition of apoptosis induced by phenoxodiol prevents clonogenic cell death, HN12 cells were exposed to either 5 or 10 μg/mL phenoxodiol for 48 hours (a time and concentration required for clonogenic death, see Fig. 1B) in the presence or absence of ZVAD, a general caspase inhibitor. Of note, to minimize a possible intracellular degradation of ZVAD, we added ZVAD every 12 hours for the duration of phenoxodiol incubation. As shown in Fig. 2B, the loss of clonogenicity observed with either 5 or 10 μg/mL occurred despite the presence of caspase inhibition, suggesting that other mechanisms of cell death may be responsible for the clonogenic death induced by this agent.

Of note, inspection of HN12 cells exposed to phenoxodiol showed that a significant number of cells have typical morphologic criteria for cell death as determined by DAPI staining (Fig. 2C, arrowheads). However, many of these "dead cells" were not stained by TUNEL(Fig. 2C, bottom right, arrows). Although ZVAD blocked apoptosis as determined by TUNEL staining, these morphologically dead cells persisted in the presence of ZVAD (data not shown). Again, these results suggest that other mechanisms of cell death besides caspase-dependent apoptosis may be relevant for phenoxodiol. To explore this hypothesis and to have an independent confirmation that this compound induces cell death by different mechanisms, we exposed HN12 cells to phenoxodiol in the presence (and absence of) ZVAD and assessed apoptosis by another methodology, Annexin V, a known assay to determine early apoptosis among (i.e., 48 hours) showed an even more potent effect in all cell lines tested (Fig. 1A, right). Of note, phenoxodiol concentrations necessary for antiproliferative effects are easily achieved in human clinical trials (27).
with PI, a method to assess membrane permeability. As shown in Fig. 2D, phenoxodiol induced apoptosis as measured by Annexin V staining (PXD, top right and bottom right quadrants), confirming the results obtained with TUNEL assay (Fig. 2A). Moreover, HN12 cells exposed to phenoxodiol also showed loss in plasma membrane integrity as measured by increased PI staining (PXD, top left and bottom left quadrants). When HN12 cells were preincubated with ZVAD (Fig. 2D, third panel), as expected from Fig. 2A, there was a significant decrease in Annexin V–positive cells (bottom right quadrant, single arrow). However, the increase in membrane permeability induced by phenoxodiol was not altered by ZVAD pretreatment (top right quadrant, double arrows), suggesting that loss in membrane permeability and morphologic cell death induced by phenoxodiol are not caspase dependent. To extend these data, we used the vital stain trypan blue, a gold standard assay for short-term viability. Again, phenoxodiol promotes loss of viability at concentrations ≥5 μg/mL (Fig. 2E, lanes 2 and 4). The loss of viability induced by phenoxodiol was not prevented by ZVAD.

Taken together, the fact that clonogenic death induced by phenoxodiol requires a protracted exposure (≥48 hours) and seems to be caspase independent suggests that phenoxodiol-induced apoptosis may not be the single explanation to the clonogenic death observed with this agent. Other mechanisms are probably also involved.

Antiproliferative effects of phenoxodiol are associated with accumulation of cells at G1. To explore potential mechanisms other than caspase-dependent apoptosis responsible for the antiproliferative effects of phenoxodiol, we sought to investigate whether phenoxodiol may be targeting the cell cycle regulatory...
mechanisms of HNSCC cells by determining the DNA content of HNSCC cells after exposure to phenoxodiol. To this end, HN12 cells were exposed to increasing concentrations of phenoxodiol for 12 hours and subsequently processed for cell cycle analysis. As illustrated in Fig. 3A, a significant decrease in S phase occurred when HN12 cells were exposed to $\geq 5 \mu$g/mL (concentrations necessary for the loss of clonogenicity promoted by this agent). S phase, as measured by BrdUrd, was significantly decreased from 34% (vehicle control) compared with 7% (5 $\mu$g/mL phenoxodiol). Interestingly, exposure to 1 $\mu$g/mL, a dose that does not induce apoptosis or clonogenic death, promoted increase in S phase (Fig. 3A, upper middle, 53%). To test whether this G1 arrest occurs in other cell lines, we tested several head and neck cell lines, such as HN4, HN6, HN17, HN30, and HN31. Indeed, a consistent G1 arrest was noted in all cell types tested at concentrations $\geq 5 \mu$g/mL phenoxodiol (data not shown).

To better understand the onset of G1-S cell cycle arrest in these cells, time course studies in HN12 cells were done up to 24 hours. G1 accumulation occurred after phenoxodiol treatment for 9 hours, reaching maximal G1 accumulation by 12 hours (see Fig. 3B). The data indicate that phenoxodiol at similar concentrations necessary for antiproliferative effects (as detected by cell counting and clonogenic potential) promotes G1 arrest in all cell lines tested thus far.

We then compared the effects of two known structurally related flavonoids, genistein and flavopiridol. As observed in Fig. 3C, genistein, as reported previously (8), promotes the accumulation of cells at the G2-M transition. In contrast, the known cdk inhibitor flavopiridol promotes a dual arrest of cells at both G1 and G2-M, suggesting that the effect of phenoxodiol in cell cycle progression seems distinct from other known flavonoids.

Taken together, these data suggest that cell cycle arrest is an important mechanism for the antiproliferative effects of phenoxodiol.

Phenoxodiol promotes the specific loss in cellular cyclin-dependent kinase 2 activity in HN12-treated cells. As phenoxodiol affected cell cycle progression at G1-S, we next sought to investigate whether cdks, serine/threonine kinases responsible for cell cycle progression, were targets for inactivation. Initially, we asked whether phenoxodiol modulates the activity of cdks by direct interaction with the catalytic subunit of these kinases. To this end, lysates obtained from exponentially growing HN12 cells were immunoprecipitated for in vitro kinase reactions, and properly activated cdk2 and cdk6 were incubated with increasing concentrations of phenoxodiol in the kinase reaction. As positive controls for the cdk2 and cdk6 kinase studies, we used flavopiridol, a known direct inhibitor of cdks (25–31). As shown in Fig. 4A, no effect was observed on either G1 cdk kinase activity up to 10 $\mu$g/mL, whereas 300 nmol/L flavopiridol, under similar experimental conditions, showed a characteristic inhibition of cdk activity (Fig. 4A, lane 5).

To determine whether the activity of cdks from intact cells already exposed to phenoxodiol were affected, we took two independent approaches: (a) we determined whether phenoxodiol treatment promoted the loss in the phosphorylation of Rb, a known endogenous substrate of G1 cdks, as monitored by phosphospecific antibodies that measure the phosphorylation of Rb at the cdk2 (Rb Ser780; refs. 25, 32), and (b) we asked whether the activity of cdk kinase complexes obtained from HN12-treated cells were affected by phenoxodiol.

To determine the cellular effects of phenoxodiol on G1, cdks, we exposed HN12 cells to increasing concentrations of phenoxodiol for 12 hours and tested the phosphorylation status of the endogenous Rb protein particularly for cdk2 phosphorylation sites (Thr356 and Tyr602) and cdk6 phosphorylation sites (Ser780; ref. 32). As shown in Fig. 4B, cdk2 phosphorylation sites on Rb were strongly inhibited by phenoxodiol at concentrations $\geq 5 \mu$g/mL. Quantitation of relative expression of phospho-Rb 356 showed a significant reduction of $\sim 42\%$ and $56\%$ with 5 and 10 $\mu$g/mL, respectively.

Figure 3. Phenoxodiol arrests HNSCC cells at G1-S transition. A, bivariate distributions (scatter grams) illustrate BrdUrd incorporation (S phase) versus DNA content as described in Materials and Methods from exponentially growing HN12 cells exposed for 12 hours to increasing concentrations of phenoxodiol. Representative of two independent experiments that show similar results. B, HN12 cells were exposed to 10 $\mu$g/mL phenoxodiol for increasing times. G1-phase percentage values over control were assessed. Relative values of G1 DNA content. Columns, mean of two independent experiments; bars, SE. C, DNA distribution histogram using PI labeling (X axis) and total number of cells in each channel (Y axis) from exponentially growing HN12 cells exposed to phenoxodiol (10 $\mu$g/mL), flavopiridol (300 nmol/L), and genistein (100 $\mu$g/mL) for 12 hours. Representative experiment. Nearly identical results were obtained in two additional experiments.
Induction of p21$^{WAF1/CIP1}$ and its association with cyclin-dependent kinase 2 in phenoxodiol-treated head and neck squamous cell carcinoma cells results in loss of cyclin-dependent kinase 2 activity. The loss of cdk2 activity by phenoxodiol can result from loss in G1 cyclins (positive cofactors for cdk activity) or an increase in endogenous cdk inhibitors, such as p21$^{WAF1}$. To address the molecular mechanism of G1 arrest following phenoxodiol, we analyzed the expression levels of known cell cycle regulators (Fig. 5A). After 12 hours of treatment with phenoxodiol (row 1, left), when cells were growth arrested in G1, a significant increase in p21 occurred at 5 and 10 μg/mL concentrations (3.5- and 4.3-fold increase over control, respectively). Similar increase in p21 was observed at 24 hours (Fig. 5A, row 1, right). In contrast, the expression of p27$^{KIP1}$ and other G1 cell cycle relevant proteins, such as cdk2, cyclin E (Fig. 5A), or cyclin D1 (data not shown), remained unaltered. As stated elsewhere (21), HN12 cells lack normal p53 function; thus, the p21$^{WAF1}$ induction observed seems to be related to a p53-independent pathway. To examine the onset of p21 induction by this agent, time course studies using 10 μg/mL phenoxodiol were determined. As shown in Fig. 5B, as soon as 3 hours, clear induction of p21 was observed. To further examine the role of p21 induction in the activity of cdk2 from cells exposed to phenoxodiol, we conducted time course experiments and showed a significant loss in Rb Thr$^{356}$ (cdk2 site) by 6 hours, whereas Rb Ser$^{795}$ (cdk6 site) remain unaltered (Fig. 5C). Together, the data suggest that phenoxodiol induces p21$^{WAF1}$ in HN12 cells. This elevation precedes and leads to the specific loss in cdk2 activity, thereby accumulating cells at G1 phase.

To further characterize the effects of phenoxodiol on p21$^{WAF1}$ induction and to determine if the increased p21$^{WAF1}$ associates to the cdk2 complexes, cdk2 immunoprecipitates from HN12 cells exposed previously with phenoxodiol were immunoblotted against p21 and cdk2. As shown in Fig. 5C, exposure of cells to phenoxodiol for ≥5 hours resulted in increased association of p21$^{WAF1}$ to cdk2.

Figure 4. HN12 exposed to phenoxodiol leads to loss of cdk2 kinase activity in HNSCC cells. A, aliquots (300 μg) of total cell lysate from exponentially growing HN12 cells were immunoprecipitated with antibodies against cdk6 and cdk2. Immunoprecipitates were washed extensively, and Rb (cdk6) or histone H1 (cdk2) kinase reaction assays were done as described before in the presence of increasing concentrations of phenoxodiol (0-10 μg/mL). As positive control, immunoprecipitates were exposed to 300 nmol/L flavopiridol (Flavo). Reactions were resolved in SDS-polyacrylamide gels, dried, and autoradiographed. Representative of three independent experiments. B, HN12 cells were exposed to increasing concentrations of phenoxodiol for 12 hours and Western blots were done to assess the phosphorylation of Rb in intact cells exposed to phenoxodiol. As loading controls, lysates were immunoblotted against total Rb and actin. Rb 795, Rb Ser$^{795}$ (cdk6 site); Rb 356, Rb Thr$^{356}$ (cdk2 site). Two additional experiments yielded comparable results. C, aliquots (300 μg) of total cell lysate were obtained from parallel samples from (B) and immunoprecipitated with cdk2 antisera. Immunoprecipitates were washed extensively, and histone H1 (cdk2) kinase reaction assays were done as described. Reactions were resolved in SDS-polyacrylamide gels, dried, and autoradiographed. Representative of three independent experiments. D, aliquots (300 μg) of total cell lysate were obtained from parallel samples from (B) and immunoprecipitated with cdk6 antisera. Immunoprecipitates were washed extensively, and Rb (cdk6) kinase reaction assays were done as described. Reactions were resolved in SDS-polyacrylamide gels, dried, and autoradiographed. Top, cdk2 activity measurement by H1 kinase activity; bottom, expression of cdk2 mass from parallel Western blots to confirm equal protein loading.
whereas levels of immunoprecipitated cdk2 protein remained unaltered. Collectively, the data suggest that the specific loss in cdk2 activity provoked by phenoxodiol may result from the induction and increased association of p21WAF1 to cdk2, which would explain the accumulation of cells at G1 phase.

Induction of p21 is p53 independent and p21 presence is required for phenoxodiol-induced G1-S arrest in HCT116 cell clones. To assess the contribution of p21WAF1 to the cell cycle effects of phenoxodiol, we took advantage of the availability of isogenic HCT116 colon carcinoma variant cells (wild-type, p53+/−/− and p21+/−/−; ref. 33). As expected and shown in Fig. 6A and B, exponentially growing wild-type HCT116 cell lines exposed to phenoxodiol showed significant decrease in S phase (control 49% versus phenoxodiol 29%). Similar arrest was observed in the p53+/−/− clones (Fig. 6A and B). Thus, G1-S arrest by phenoxodiol seems to be p53 independent, as p53−/− clones were sensitive to this compound.

To establish conclusively the role of p21WAF1 in the cell cycle effects of phenoxodiol, we also tested the effects of phenoxodiol on the cell cycle progression of HCT116 p21−/− clones. Interestingly, phenoxodiol failed to arrest these clones (control 38% versus phenoxodiol 30%), suggesting that G1-S arrest induced by phenoxodiol requires p21. Collectively, we can conclude that phenoxodiol blocks cell cycle progression at G1 by the accumulation of p21WAF1.

To determine the mechanism responsible for p21 induction by this agent, we tested whether p53, a known transcriptional activation of p21, was necessary for this induction. To this end,

Figure 5. G1 arrest and loss of cdk2 activity provoked by phenoxodiol requires the accumulation and association of p21WAF1 to cdk2 complexes. A, lysates (25 μg) obtained from HN12 cells exposed to increasing concentrations of phenoxodiol for 12 hours (left) and 24 hours (right) were resolved in SDS-polyacrylamide gels for Western blots with the indicated antibodies and detected by ECL. B, lysates (25 μg) obtained from HN12 cells exposed to 10 μg/mL phenoxodiol for increasing times were resolved in SDS-polyacrylamide gels for Western blots with the indicated antibodies and detected by ECL. C, proteins (300 μg) obtained from time course studies of HN12 cells exposed to phenoxodiol (10 μg/mL) were immunoprecipitated with cdk2 and immunoblotted with specific antisera to p21WAF1 and cdk2. Representative of three independent experiments.

Figure 6. G1 arrest promoted by phenoxodiol requires the p53-independent induction of p21WAF1. A, exponentially growing HCT116 clones [wild-type (WT), p53−/−, and p21−/−; ref. 33] were exposed to 5 μg/mL phenoxodiol for 8 hours. Bivariate distributions (scatter grams) illustrate BrdUrd incorporation (S phase) versus DNA content as described in Materials and Methods. B, S phase from (A) obtained from gated events in region R1 (R1). Representative of three independent experiments. C, parallel lysates also were resolved in denaturing polyacrylamide gels, Western blotted with antisera recognizing human p21WAF1, p53, and β-actin, and detected using an HRP-conjugated secondary antibody and ECL. Representative experiment. Nearly identical results were obtained in two additional experiments.
we exposed wild-type, p53+/−, and p21−/− clones to phenoxodiol and determined the expression of p21 and p53. As expected, phenoxodiol promotes the induction of p21 in wild-type clones (Fig. 6C, left, lane 1). Similar p21 induction occurred in the p53+/− clones, suggesting that the induction of p21 is p53 independent. Moreover, the expression of p53 in wild-type clones on phenoxodiol is unaltered. Of note, as shown previously in HN12 cells (Fig. 5A), the expression of the endogenous cdk inhibitor p27KIP1 was not altered in these clones (data not shown).

In summary, the antiproliferative effects of phenoxodiol are mediated at least in part by the induction of p21 by a p53-independent mechanism, thereby leading to specific loss of cdk2 activity and G1-S arrest.

**Discussion**

In this report, we show for the first time that phenoxodiol, a novel isoflavone, promotes clonogenic death when given in a protracted fashion. Clonogenic death by this agent occurred in the presence of caspase blockade, suggesting that other mechanisms besides caspase-dependent apoptosis are important for phenoxodiol action. Searching for other mechanisms relevant for the antiproliferative effects of phenoxodiol, we determined that phenoxodiol induces cell cycle arrest in HN12 cells at the G1-S boundary. G1-S arrest occurs as a consequence of the specific loss in cdk2 activity because of the up-regulation and increased association of p21 to cdk2 kinase. The requirement for p21 is supported by the fact that HCT116 isogenic clones lacking p21 are refractory to the cell cycle effects promoted by this agent. Finally, induction of p21 by this compound is p53 independent, as phenoxodiol induces p21 in HN12 (p53 mutant) and HCT116 p53+/− (p53 null) cell lines.

There is a great interest in developing flavonoids for the prevention and therapy of human neoplasms (6, 7, 34, 35). The first flavonoid in clinical trials for cancer therapy, flavopiridol, is a direct inhibitor of cdks, thereby promoting cell cycle arrest, apoptosis, transcriptional modulation, etc. (21, 24, 25, 31, 36). Efforts to develop flavonoids for cancer therapy prompted the preclinical development of genistein and phenoxodiol (7, 12, 13). Several phase I clinical trials are being conducted with phenoxodiol (18).

In this report, we assessed the mechanism by which phenoxodiol exerts its antiproliferative effects in *in vitro* models. We showed that phenoxodiol induces apoptosis (caspase-dependent and caspase-independent and clonogenic cell death). Moreover, we also showed that G1-S arrest proceeds these processes.

It is known that phenoxodiol promotes caspase-dependent apoptosis in *in vitro* ovarian cell lines (19). In this report, we confirmed this effect in HN12 cell lines. However, we also observed that many HN12 cells exposed to phenoxodiol had evidence of short-term cell death as determined by nuclear morphology or loss in cellular membrane integrity in cells that failed to stain with traditional apoptosis assays. Furthermore, inhibition of caspases did not block cell death induced by phenoxodiol as measured by short-term methods, such as DAPI, PI, or long-term methods (e.g., clonogenic assays), suggesting that caspase-dependent apoptosis is not the only relevant mechanism of cell death by this agent.

It has recently become clear that cell death can occur by different mechanisms, such as apoptosis (caspase-dependent and caspase-independent mechanisms), autophagy, paraptosis, and necrosis (37–40). It is also clear that chemotherapeutic agents can induce apoptosis or necrosis depending on cellular environment conditions (37, 41). Moreover, exposure of tumor cell lines with antitumor agents, such as camptothecin, etoposide, or dexamethasone, will in the presence of caspase inhibitors switch from apoptosis to necrosis. Thus, different chemotherapeutic agents may cleave simultaneously (37). The most relevant end point of cell death following cytotoxic therapy is loss of reproductive ability as measured by a colony-forming assay, an assay that will take into account all types of cell death (28, 42). The fact that the induction of apoptosis by phenoxodiol occurred early (in contrast to the delayed effect on clonogenic survival) and that caspase blockade did not prevent loss in cell viability as measured by trypan blue staining (short-term cell death assay) and clonogenic cell death (long term cell death assay) strongly suggests that other important mechanisms besides caspase-dependent apoptosis are relevant for the antiproliferative effects of phenoxodiol.

To investigate other mechanisms involved in the antiproliferative effects of phenoxodiol, we tested whether phenoxodiol has the capacity to block cell cycle progression. Clear accumulation of cells with G1 DNA content was observed in all cell types tested with as little as 9 hours of exposure to phenoxodiol. In contrast, the other known flavonoids, flavopiridol and genistein, promoted a “mixed” G1 and G2-M arrest. This novel information suggests that G1 arrest induced by phenoxodiol may play a significant role in the antiproliferative effects of this agent.

Cell cycle arrest occurs by loss in the activity of cdks (43–45). Inactivation of cdks by small molecules may occur by two main mechanisms: (a) by direct interaction of small molecules with the ATP binding site of cdks or (b) by indirectly modulating the upstream pathways that govern the expression of cyclins, cdks, or endogenous cdk inhibitors, such as p21WAF1 (30, 31, 36, 44, 46). In this report, we showed that phenoxodiol did not inhibit the activity of cdks when added to the kinase assays. In contrast, the activity of cdk2, the main cdk responsible for the G1-S transition, was significantly diminished when cdk2 activity was obtained from HN12 cells exposed to phenoxodiol. This result suggests that the specific loss of cdk2 activity by phenoxodiol is the result of modulation of upstream pathways that modulate cdk/cyclin/endogenous cdk inhibitory complexes (47). To further examine this mechanism, we assessed the effect of this agent in the expression of relevant G1-S cell cycle proteins. A prominent induction of p21 occurred at concentrations and times where clear G1 arrest was observed. In contrast, no alterations in cyclins, cdks, or other endogenous inhibitors were observed. Furthermore, examination of the molecular complexes containing cdk2 revealed a significant increase in p21 association to cdk2, suggesting that the induction of p21 by this agent could result in increased association and inhibition of cdk2.

The endogenous cdk inhibitor p21WAF1 belongs to the CIP/KIP family of endogenous cdk inhibitors (p21, p27, and p57), which regulates directly the activity of cdk (48–51). Furthermore, biochemical and genetic studies showed that p21 family members at low concentrations may promote the assembly and activation of the D-type cyclin kinase while at higher concentrations are able to suppress cdk activity (52, 53). Of note, HN12 cells exposed to 1 μg/mL phenoxodiol (a concentration that does not cause cell death) showed a significant increase in the number of cells in S phase compared with control cells. Similar effects were observed in HCT116 wild-type and p53+/− (data not shown). Thus, it seems that S-phase increase at “subtherapeutic concentrations” is independent of p53, as it occurs in HN12 (p53-defective) and not only in HN12 cells.
HCT116 p53\(^{-/-}\) clones. Of interest, HCT p21\(^{-/-}\) failed to show this increase (data not shown). These results suggest two possible explanations: (a) that the minimal increase of p21 by 1 μg/mL concentrations may promote the assembly and activation of D-type cyclins and G\(_1\) cdk4, leading to increase in S phase, or (b) that the accumulation of p21 may lead to an initial slower S-phase progression. However, at higher phenoxodiol concentrations (≥5 μg/mL), higher p21 concentrations will lead to loss in cellular cdk2 activity. In theory, subtherapeutic phenoxodiol concentrations might promote an increase in cellular proliferation as manifested by increase in S phase; this effect may be detrimental for patients with neoplastic diseases. On the other hand, this phenomenon (increase in S phase with subtherapeutic phenoxodiol doses) could be useful when phenoxodiol is combined with other chemotherapy and/or radiation therapy, as many therapies work preferentially in S-phase cells. These theoretical detrimental and/or beneficial possibilities should be tested in future clinical trials with this agent.

To determine the exact role of p21\(^{WAF1}\) in the cell cycle effects of phenoxodiol, we used the isogenic HCT116 cell lines (33). As expected, phenoxodiol promoted G\(_1\) accumulation in wild-type clones. This effect was accompanied by induction of p21\(^{WAF1}\). However, cells lacking p21\(^{WAF1}\) (HCT116 p21\(^{-/-}\)) failed to accumulate cells in G\(_1\) on phenoxodiol treatment. Together, these data provide the first evidence that phenoxodiol can arrest cells at the G\(_1\) transition by induction of p21\(^{WAF1}\), thereby leading to loss in cdk2 activity.

Initial studies to determine the mechanism by which phenoxodiol promotes p21 involved the use of p53 mutant (HN12) or p53 null cells (HCT116 p53\(^{-/-}\)), as the tumor suppressor gene p53 is a known transcriptional regulator of p21 (48–50). When p53-deficient HN12 cells were exposed to phenoxodiol, clear induction of p21 was observed, suggesting that the induction of p21 is p53-independent. Moreover, when wild-type HCT116 cells were exposed to this agent, no p53 induction is observed, again suggesting that p53 seems to be not operative in the p21 induction by this agent. To confirm the p53 independence of p21 induction, HCT116 p53\(^{-/-}\) cells were exposed to phenoxodiol; a clear p21 induction was observed, demonstrating that p21 induction by phenoxodiol is p53-independent. Further studies are being undertaken in our laboratory to understand the exact mechanism by which this agent induces p21 in a p53-independent manner.

In summary, our data indicate that phenoxodiol accumulates cells at G\(_1\) phase by induction of p21. The accumulation of p21 promotes its association to cdk2, leading to loss of cdk2 activity. Moreover, cells lacking p21 are refractory to the G\(_1\) arrest induced by this agent. Finally, induction of p21 by this agent is p53-independent, as phenoxodiol induces p21 in HCT116 p53\(^{-/-}\) cells. These novel cell cycle and clonogenic effects suggest that phenoxodiol may represent a promising candidate for further evaluation in the treatment and/or prevention of a variety of human neoplasms.

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References


Phenoxodiol, a Novel Isoflavone, Induces G$_1$ Arrest by Specific Loss in Cyclin-Dependent Kinase 2 Activity by p53-Independent Induction of p21$_{WAF1/CIP1}$

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