Selective and irreversible cell cycle inhibition by diphenyleneiodonium

Robin M. Scaife

Laboratory for Cancer Medicine, Western Australian Institute for Medical Research, Centre for Medical Research, The University of Western Australia, Western Australia, Australia

Abstract

Because cell proliferation is subject to checkpoint-mediated regulation of the cell cycle, pharmacophores that target cell cycle checkpoints have been used clinically to treat human hyperproliferative disorders. It is shown here that the flavoprotein inhibitor diphenyleneiodonium can block cell proliferation by targeting of cell cycle checkpoints. Brief exposure of mitotically arrested cells to diphenyleneiodonium induces a loss of the mitotic cell morphology, and this corresponds with a decrease in the levels of the mitotic markers MP-MZ and phospho-histone H3, as well as a loss of centrosome maturation, spindle disassembly, and redistribution of the chromatin remodeling helicase ATRX. Surprisingly, this mitotic exit resulted in a tetraploidization that persisted long after drug release. Analogously, brief exposure to diphenyleneiodonium also caused prolonged arrest in G1 phase. By contrast, diphenyleneiodonium exposure did not abrogate S phase, although it did result in a subsequent block of G2 cell cycle progression. This indicates that diphenyleneiodonium selectively targets components of the cell cycle, thereby either causing cell cycle arrest, or checkpoint override followed by cell cycle arrest. These irreversible effects of diphenyleneiodonium on the cell cycle may underlie its potent antiproliferative activity. [Mol Cancer Ther 2005; 4(6):876–84]

Introduction

Cell cycle driven cell proliferation is the principal hallmark of all eukaryotic life, and its regulation is of critical relevance to human health. The cell cycle is under stringent checkpoint-mediated control, and its deregulation is central to the uncontrolled growth of cancerous cells (1). Conversely, many anticancer strategies are aimed at inhibiting cell proliferation by blocking cell cycle progression (2). Because mitotic spindle function is required to bypass the metaphase checkpoint (3), microtubule drugs, which block mitotic spindle function, arrest cells in mitosis by prolonged activation of the metaphase checkpoint (4). This antimitotic activity of microtubule drugs has seen extensive clinical application in anticancer treatments (5). In light of this, one of the principal aims of current cancer research is the discovery of additional pharmacophores that target the cell cycle.

In addition to effects on the actin cytoskeleton (6), the flavoprotein inhibitor diphenyleneiodonium (7) has a potent antiproliferative activity on both normal and transformed cells (8). As a result of its inhibitory effect on NAD(P)H oxidase-mediated generation of reactive oxygen species (ROS), diphenyleneiodonium may affect signaling pathways involved in mitogenesis and cell proliferation (9–12). Diphenyleneiodonium has recently been reported however to affect the cell cycle-mediated proliferation of cells in a ROS-independent manner (13). Indeed, exposure of Rat1 fibroblasts to diphenyleneiodonium causes an accumulation of cells in the G2 phase of the cell cycle that is not mirrored by the ROS inactivator N-acetylcysteine. Furthermore, exposure of mitotically arrested cells to diphenyleneiodonium causes a down-regulation of cyclin B, with a concomitant loss of the mitotic cell morphology (13). However, this apparent mitotic exit does not seem to involve cell cleavage as the cells remain tetraploid and undergo micronucleation.

Inhibition of key cell cycle–signaling mechanisms, such as Cdk1- and Aurora-mediated phosphorylation, has been reported to similarly lead to tetraploidization and micronucleation (14, 15). Inhibition of Cdk1, or Aurora, thereby leads to mitotic catastrophe and apoptosis (14, 15). The effect of diphenyleneiodonium on mitotic cells was therefore investigated in detail, to determine the response of cells to the diphenyleneiodonium-induced mitotic exit. In particular, the effect of diphenyleneiodonium on chromatin and cytoskeletal remodeling was determined. Furthermore, the long-term effect of diphenyleneiodonium on cell cycle progression was assayed. These results indicate that whereas diphenyleneiodonium causes mitotically arrested cells to progress to an interphase-like state, its subsequent inhibitory effect on the cell cycle precludes resumption of mitotic cell division.

Materials and Methods

Cell Culture and Cell Cycle Synchronization

MCF7 mammary epithelial adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM (Life Technologies,
Mount Waverley, Victoria, Australia) containing 10% FCS (Life Technologies) and 2 mmol/L L-glutamine (Life Technologies) at 37°C and 5% CO2. Cells were synchronized in mitosis by treatment with paclitaxel (0.4 ng/mL, Sigma Chemical Co., St. Louis, MO) or nocodazole (0.1 ng/mL, Sigma Chemical) for at least 20 hours. Cells were arrested at the G2-S boundary by addition of 2 mmol/L hydroxyurea to the medium for at least 16 hours and in G0 by incubation in serum-free medium for 20 hours.

**Immunofluorescence and Phase-Contrast Microscopy**

For immunofluorescence microscopy cells were seeded onto coverslips coated with polylysine (0.1 mg/mL). Following fixation of the cells in −20°C methanol and a 4% paraformaldehyde post-fix, coverslips were rinsed with PBS and incubated with anti-phospho-histone H3 (1:200; Cell Signaling, Beverly, MA; monoclonal antibody 6G3), anti-AuRxA2 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-tubulin antibodies (1:1000 YL/1/2 rat monoclonal antibody), anti-γ-tubulin antibodies (3.5 μg/mL, Sigma monoclonal antibody), anti-phospho-Aurora 2 (Thr288) antibodies (1:200 diluted, Cell Signaling) at 37°C for 60 minutes in PBS containing 2.5 mg/mL bovine serum albumin. Following a PBS wash, the coverslips were incubated with 5 μg/mL biotin-SP conjugated goat anti-mouse (The Jackson Laboratory, Bar Harbor, MI) or 7.5 μg/mL biotin-conjugated goat anti-rabbit (Vector, Burlingame, CA) at 37°C for 60 minutes in PBS containing 2.5 mg/mL bovine serum albumin. Biotin-labeled antigen-antibody complexes were then visualized by incubation for 60 minutes with PBS containing 2.5 mg/mL bovine serum albumin and 2 μg/mL Alexa 488-conjugated streptavidin (Molecular Probes, Eugene, OR), and nuclei were stained with either Hoechst 33342 or propidium iodide. For antibody double labeling, rabbit secondary antibody was employed as described above, whereas mouse antigens were detected using 546-nm Alexafluor-conjugated anti-mouse serum (Molecular Probes). Following a PBS rinse, coverslips were mounted with anti-fade reagent (Cytoskeleton, Denver, CO). Images of representative fields were obtained with Comos and Confocal Assistant software (Bio-Rad, Hercules, CA) following capture on a Nikon Diaphot 300 microscope equipped for UV laser scanning confocal microscopy (Bio-Rad, MRC 1000/1024).

Phase contrast microscopy of cells was done using a 20× objective lens and an Olympus IX71 microscope. Images were captured using an Olympus DP70 camera and Olympus DP software.

**Flow Cytometry**

The DNA content of cells was determined following fixation of the cells in 90% methanol/PBS at −20°C after trypsin-mediated detachment from the culture substrate. Subsequent to permeabilization with 0.2% Triton X-100 and addition of 2 μg/mL DNase-free RNase, the cells were stained with 2 μg/mL propidium iodide. The MP2 content of cells was determined by incubating the fixed cells with anti-phospho-serine/threonine-Pro MP2 antibody (Upstate, Lake Placid, NY; monoclonal antibody) and 488-nm Alexafluor-conjugated anti-mouse secondary antibody (Molecular Probes) before the propidium iodide staining. The propidium iodide and MP2 signals were collected using a Beckman Coulter (Fullerton, CA) Epics XL-MCL machine. Dot plots and histograms of the MP2 or propidium iodide signal were obtained using Coulter ExpoV2 software.

**Cell Lysis, Fractionation, and Western Blotting**

Cells were washed with PBS and lysed in ice-cold 20 mmol/L Tris-HCl buffer (pH 8.0) containing 1% SDS, 10 mmol/L Na2VO4, 10 mmol/L NaF, 10 mmol/L β-glycerophosphate, and protease inhibitors. Following sonication, cell lysates were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Amersham, Piscataway, NJ). Membranes were blocked with 10% nonfat milk (Carnation, Las Vegas, NV) in TBS containing 0.5% Tween 20 and probed with antibodies directed against phospho-histone H3 (Cell Signaling), Erk1/mitogen-activated protein kinase (Santa Cruz Biotechnology), and IAK1/Aurora-A kinase (BD Transduction Laboratories, Lexington, KY) followed by horseradish peroxidase–conjugated secondary antibody (Silenus, Boronia, Victoria, Australia). Antigens were then visualized by enhanced chemiluminescence (Amersham) using Hyperfilm MP (Amersham).

**Results**

**Diphenyleneiodonium-Specific Morphologic Changes in Paclitaxel-Arrested Cells**

To observe the effect of diphenyleneiodonium on the morphology of mitotic cells, paclitaxel-treated MCF7 cells were monitored continuously for several hours by phase-contrast microscopy following addition of diphenyleneiodonium to the culture medium. These live cell recordings clearly show, as indicated previously (13), that exposure of the cells to diphenyleneiodonium causes a loss of the round and translucent mitotic phenotype. At the time of diphenyleneiodonium addition to the culture medium, the paclitaxel-induced mitotic arrest had caused a high proportion of the MCF7 cells to assume the round and translucent mitotic phenotype. The time of diphenyleneiodonium addition to the culture medium, the paclitaxel-induced mitotic arrest had caused a high proportion of the MCF7 cells to assume the round and translucent mitotic phenotype.

Diphenyleneiodonium is a well-characterized flavoprotein inhibitor (7) that targets NAD(P)H oxidase (8). The effect of diphenyleneiodonium, a related inhibitor of NAD(P)H oxidase (9; ref. 16; Fig. 1C), on mitotic cells was therefore assayed. Unlike the case for diphenyleneiodonium, exposure of paclitaxel-treated cells to diphenyleneiodonium did not lead to a loss of cells with a mitotic morphology (Fig. 1D).
Diphenyleneiodonium Induces Exit from Mitosis

To assay whether the loss of the round and translucent mitotic morphology of diphenyleneiodonium-treated cells is due to exit from mitosis, the effect of diphenyleneiodonium on the morphology and the abundance of the mitosis-specific marker MPM2 (17) was examined after collecting mitotic paclitaxel-arrested cells by mitotic shake-off. In the absence of diphenyleneiodonium, the mitotic cells retained their round and translucent mitotic morphology (Fig. 2A, 1). By contrast, incubation of the mitotic cells with diphenyleneiodonium caused a pronounced conversion of the cells to the flat and refractory morphology characteristic of interphase cells (Fig. 2A, 2). Propidium iodide flow cytometry indicated that essentially all of the paclitaxel-treated cells had a 4N DNA content, and anti-MPM2 flow cytometry indicated that the majority of these cells were also positive for this mitotic marker (Fig. 2B and C). Exposure of paclitaxel-treated cells to diphenyleneiodonium did not affect the 4N DNA, although exposure to diphenyleneiodonium did cause these cells to lose most of their anti-MPM2 signal (Fig. 2B and C).

Diphenyleneiodonium Causes a Loss of Mitotic Chromatin Markers in Paclitaxel-Treated Cells

The diphenyleneiodonium-induced decrease in MPM2 levels indicates that it causes cells to exit mitosis, as previously indicated by the chromatin decondensation and micronucleation that occurs in paclitaxel-arrested cells that are exposed to diphenyleneiodonium (13). To establish to what extent this diphenyleneiodonium-induced mitotic exit involves reversion of mitotic signaling mechanisms, the level of phospho-histone H3 (14) was determined for MCF7 cells treated with paclitaxel or paclitaxel plus diphenyleneiodonium. Anti-phospho-H3 immunofluorescence microscopy revealed that exposure of paclitaxel-treated cells to diphenyleneiodonium caused a loss of mitotic chromatin markers in MCF7 cells.

Figure 1. Diphenyleneiodonium (DPI)-specific morphological changes in Taxol-arrested cells. A, MCF7 cells were treated for 20 h with Taxol and observed continuously by phase-contrast microscopy upon addition of 10 µM/L diphenyleneiodonium. Images taken at 0, 30, 60, 90, 120, and 180 min after diphenyleneiodonium addition. B, high-magnification images of the boxed area indicated in A for 60, 180, and 360 min time points. C, chemical structures of diphenyleneiodonium and diphenyliodonium. D, phase-contrast images of MCF7 cells treated for 20 h with paclitaxel followed by 6-h incubation without (1) and with (2) 10 µM/L diphenyleneiodonium. Bar, 100 µm.

Figure 2. Diphenyleneiodonium (DPI) induces exit from mitosis. A, paclitaxel-arrested mitotic MCF7 cells were observed by phase-contrast microscopy following a 6-h incubation without (1) and with (right) 10 µM/L diphenyleneiodonium (2). B, paclitaxel-arrested mitotic MCF7 cells were subjected to flow cytometry of anti-MPM2 and propidium iodide (PI) staining following a 6-h incubation without (left) and with (right) 10 µM/L diphenyleneiodonium. Two-dimensional dot plots of the anti-MPM2 and propidium iodide signal (B) and histogram plots of anti-MPM2 signal (C).
cells to diphenyleneiodonium resulted in a marked decrease in H3 phosphorylation (Fig. 3A). Anti-phospho-H3 Western blotting of lysates from paclitaxel-treated cells confirmed that addition of diphenyleneiodonium to the culture medium resulted in a pronounced loss of phospho-histone H3 signal (Fig. 3B).

The diphenyleneiodonium-induced loss of mitotic chromatin structure was further evidenced by examination of the intracellular distribution of the chromatin remodeling helicase ATRX (18). In contrast with the lack of distinct anti-ATRX staining in mitotic paclitaxel-arrested cells (Fig. 3C, 1), exposure of paclitaxel-arrested mitotic cells to diphenyleneiodonium resulted in a clear punctate nuclear ATRX staining of the multinucleated post-mitotic cells (Fig. 3C, 2) that was essentially identical to the ATRX staining of heterochromatin and PML bodies in interphase cells (18).

Diphenyleneiodonium Reverses Centrosome Mitotic Maturation and Mitotic Spindle Assembly

To assess the degree to which diphenyleneiodonium can reverse the mitotic phenotype, structural components of the mitotic spindle were examined by immunofluorescence microscopy following exposure of paclitaxel-arrested cells to diphenyleneiodonium. Assembly of the mitotic spindle at the onset of mitosis corresponds with centrosome maturation (19) and a pronounced phosphorylation of the centrosome-associated kinase Aurora-A (20–22). Paclitaxel-arrested mitotic cells therefore contained a pair of centrosomes that were strongly labeled by anti-phospho-Aurora (Fig. 4A, 1). Exposure of paclitaxel-arrested cells to diphenyleneiodonium abolished this centrosomal anti-phospho-Aurora staining in the post-mitotic cells (Fig. 4A, 2). Aurora kinases are regulated by both phosphorylation at Thr588 and by APC-mediated proteasomal degradation (23, 24). The levels of Aurora-A following treatment of mitotic cells with diphenyleneiodonium were hence assayed by Western blotting. Compared with untreated control cells, Aurora-A levels were greatly increased in paclitaxel-treated cells (Fig. 4B).
Addition of diphenyleneiodonium to paclitaxel-treated cells led to a pronounced down-regulation of Aurora-A levels (Fig. 4B), indicating that diphenyleneiodonium may trigger proteasomal Aurora-A degradation.

The presence of phosphorylated Aurora-A during the G2-M phase transition seems an important stage of centrosome maturation, permitting assembly of the microtubule network into a spindle (21). The effect of diphenyleneiodonium on spindle microtubule organization was therefore examined by anti-α-tubulin immunofluorescence microscopy. Whereas the microtubules in paclitaxel-arrested mitotic cells were arranged into distinct asters (Fig. 4C, 1), following exposure to diphenyleneiodonium, these microtubule asters were rearranged into the radial microtubule network characteristic of interphase cells (Fig. 4B, 2).

Cell Cycle Progression beyond Metaphase Precludes Diphenyleneiodonium-Induced Tetraploidization and Micronucleation

These results clearly establish that diphenyleneiodonium perturbs mitotic cells, causing cells arrested in prometaphase to exit mitosis. To determine whether diphenyleneiodonium can perturb the latter stages of mitosis, MCF7 cells were exposed to diphenyleneiodonium following progressively longer periods of nocodazole release. Upon nocodazole release, MCF7 rapidly reassembled mitotic spindles (Fig. 5A, 1). This resulted in a progression of the mitotic cells from a >95% prometaphase population following a 30-minute release (Fig. 5A, 1), to a population of mostly metaphase and anaphase cells following 120 minutes of nocodazole release (Fig. 5A, 2). At 180 minutes of nocodazole release, anti-α-tubulin and Hoescht fluorescence microscopy indicated that the majority of the mitotic cells had reached telophase or cytokinesis (Fig. 5A, 3). Propidium iodide flow cytometry indicated that following 180 minutes of nocodazole release, a significant proportion of the cells had also attained a 2N DNA content, as a result of having fully completed mitosis (Fig. 5B). Similar to the effect of diphenyleneiodonium addition in the presence of nocodazole, addition of diphenyleneiodonium within 60 minutes of nocodazole release resulted in a subsequent tetraploidization (i.e., failure to revert to a 2N DNA content) and micronucleation (Fig. 5C and D). However, longer periods of nocodazole release (i.e., ≥120 minutes), before the addition of diphenyleneiodonium, precluded subsequent tetraploidization and micronucleation (Fig. 5C and D).
Diphenyleneiodonium Causes Irreversible Tetraploidization following Nocodazole Release

Because diphenyleneiodonium causes cells to exit mitosis without undergoing chromosome segregation and cell fission, it was important to determine the long-term consequences of this mitotic failure. Nocodazole-arrested cells were released from the mitotic block and cell cycle progression was determined by propidium iodide flow cytometry. As expected, cells released from nocodazole alone, readily returned to a mostly 2N DNA population (Fig. 6A). This contrasts with the situation for cells that were exposed to diphenyleneiodonium following the nocodazole arrest. In this case, the cells failed to progress through the cell cycle and mostly retained a 4N DNA content for many days after removal of both nocodazole and diphenyleneiodonium from the culture medium (Fig. 6A). In accordance with Annexin V staining (data not shown), the appearance of cells with sub-2N DNA content indicates that the diphenyleneiodonium treatment also led to a modest and transient increase in apoptotic cells (Fig. 6A). The failure of cells treated with both nocodazole and diphenyleneiodonium to exit the tetraploid state is not due to a persistent inability to assembly the microtubules required for spindle assembly, because anti-α-tubulin immunofluorescence indicates that following drug release, these cells reassembled microtubules similar to cells released from nocodazole alone (Fig. 6B). Failure of the diphenyleneiodonium-treated cells to exit the tetraploid state was further evidenced by exposure of these cells to pharmacologic agents that arrest the cell cycle. Following 1 week of release of the cells from nocodazole and diphenyleneiodonium, these tetraploid cells were exposed for 24 hours to either hydroxyurea or paclitaxel. Exposure to hydroxyurea arrests cells at the G1-S boundary due to inhibition of DNA synthesis. Treatment of nocodazole- and diphenyleneiodonium-released cell for 24 hours with hydroxyurea did not however yield any cells that had reverted to a 2N DNA content (Fig. 6C) as a result of having undergone mitosis. Paclitaxel blocks cells in mitosis after they have undergone DNA replication during S phase. Treatment of nocodazole- and diphenyleneiodonium-released cells for 24 hours with paclitaxel did not however yield a significant number of cells with an 8N, or hyperploid, DNA content (Fig. 6C) as a result of having undergone endoreduplication of their DNA.

Diphenyleneiodonium-Mediated Cell Cycle Arrest Occurs at Specific Stages of the Cell Cycle

In light of the ability of diphenyleneiodonium to block cell cycle progression following tetraploidization, its effect on other stages of the cell cycle was examined. Similar to nocodazole, the hydroxyurea-mediated cell cycle arrest of cells at the onset of S phase was fully reversible upon drug release, as 2N DNA cells released from hydroxyurea into paclitaxel accumulated quantitatively with a 4N DNA content (Fig. 7A). Interestingly, cells released from hydroxyurea and diphenyleneiodonium into paclitaxel were also capable of progressing in the cell cycle following drug release. Upon removal of the hydroxyurea and diphenyleneiodonium from the culture medium, the cells initiated S phase, and a substantial proportion of the cells attained a 4N DNA content 24 hours following drug release (Fig. 7A). Nonetheless, although exposure to diphenyleneiodonium did not prevent these cells from undergoing S phase, the cells subsequently arrested in the G2 phase of the cell cycle. Indeed, whereas release of cells from hydroxyurea into paclitaxel-containing medium resulted in a pronounced accumulation of mitotic cells in G0/G1 phase (Fig. 5B), the release of cells from diphenyleneiodonium into paclitaxel following nocodazole arrest led to a sequential accumulation of cells in G2 phase, followed by a population of cells with an 8N DNA content (Fig. 7A), consistent with the notion that cells with a tetraploid DNA content that accumulated following nocodazole arrest were unable to progress through S phase.
that stain for anti-phospho-histone H3 (Fig. 7B, 1), release from hydroxyurea plus diphenyleneiodonium into paclitaxel-containing medium prevented accumulation of the anti-phospho H3–positive mitotic cells (Fig. 7B, 2).

In light of this differential effect of diphenyleneiodonium on cell cycle progression out of a mitotic block versus cell cycle progression out of the S-phase arrest, the effect of diphenyleneiodonium on the G1 phase of the cell cycle was examined. In the absence of FCS, MCF7 cells fail to accumulate as a 4N DNA cell population following addition of paclitaxel to the culture medium, whereas addition of FCS to serum-starved cells induces cell cycle progression and accumulation of mitotic cells with 4N DNA content (Fig. 7C). By contrast, exposure of serum-starved cells to diphenyleneiodonium precluded subsequent cell cycle progression. There was no accumulation of cell with >2N DNA content, following addition of FCS and paclitaxel to the culture medium of serum-starved cells that had previously been exposed to diphenyleneiodonium (Fig. 7C).

Discussion

The results presented here show that diphenyleneiodonium induces mitotic exit and causes irreversible cell cycle arrest. The reversion of round and translucent mitotic cells to flat and refractory cells of the monolayer, by diphenyleneiodonium, was monitored by live cell imaging. These live cell recordings of diphenyleneiodonium-induced changes to the morphology of mitotic cells provide direct confirmation of the previously reported diphenyleneiodonium-induced loss of mitotic cells following paclitaxel arrest (13). The pronounced loss of mitotic cells is therefore due to reversion of the mitotic cell morphology, rather than an experimental artifact such as loss of the loosely adherent mitotic cells due to their detachment from the culture substrate. The induction of mitotic exit by diphenyleneiodonium was also evidenced by the loss of molecular markers of mitosis. Loss of the round and translucent mitotic morphology by exposure of a purified population of mitotic cells to diphenyleneiodonium was accompanied by a pronounced decrease in the levels of the mitotic marker MPM2. These results therefore indicate that the diphenyleneiodonium-induced change in morphology of paclitaxel- or nocodazole-arrested cells is due to mitotic exit rather than alteration of the actin cytoskeleton (6) and consequent flattening and readhesion of mitotic cells. The diphenyleneiodonium-induced loss of MPM2 signal is however not accompanied by a change in the 4N DNA content of the cells, indicating that diphenyleneiodonium induces a type of mitotic catastrophe.

It is however not clear whether the diphenyleneiodonium-induced mitotic exit represents a reversion to G2 phase, or progression to a pseudo-G1 state. Continuation of the cell cycle following mitotic exit to a pseudo-G1 state, (e.g., by inhibition of Aurora B; ref. 14), results in hyperploidy or aneuploidy (25). By causing an irreversible cell cycle arrest, the diphenyleneiodonium-induced mitotic exit is however not followed by endoreduplication.

Furthermore, because diphenyleneiodonium causes a pronounced down-regulation of cyclin B levels in paclitaxel-arrested cells (13), the diphenyleneiodonium-induced mitotic exit most likely represents a progression to a pseudo-G1 state rather than a reversion to G2. Indeed, a reversion to G2, as occurs by DNA damage during the spindle assembly checkpoint (26), is associated with elevated cyclin B levels.

The findings presented here therefore show that diphenyleneiodonium can cause an override of the paclitaxel, or nocodazole, mitotic arrest, although this diphenyleneiodonium-induced mitotic exit does not entail chromosome segregation and cell fission. Rather, the cells fail to undergo a normal mitosis, becoming tetraploid and micronucleated. This effect of diphenyleneiodonium on mitotic cells is specific for the early stages of mitosis, as diphenyleneiodonium (DPI) – mediated cell cycle arrest occurs at specific stages of the cell cycle. MCF7 cells were treated with hydroxyurea for 16 h followed by a further 4-h incubation without (1) or with (2) 10 μmol/L diphenyleneiodonium. The cells were then released into DME + 10% FCS containing paclitaxel and either subjected to propidium iodide flow cytometry at the times indicated following removal of hydroxyurea and diphenyleneiodonium (A) or anti-phospho-histone H3 and Hoechst fluorescence microscopy at 24 h after hydroxyurea and diphenyleneiodonium release (B). Bar, 25 μm. Serum-starved MCF7 cells were treated for 4 h without (left) and with 10 μmol/L diphenyleneiodonium (right). Following removal of diphenyleneiodonium from the culture medium, the cells were incubated in the presence of paclitaxel, in the absence and presence of 10% FCS, as indicated. After 24 h, the cells were subjected to propidium iodide flow cytometry (C).

Figure 7. Diphenyleneiodonium (DPI) – mediated cell cycle arrest occurs at specific stages of the cell cycle. MCF7 cells were treated with hydroxyurea for 16 h followed by a further 4-h incubation without (1) or with (2) 10 μmol/L diphenyleneiodonium. The cells were then released into DME + 10% FCS containing paclitaxel and either subjected to propidium iodide flow cytometry at the times indicated following removal of hydroxyurea and diphenyleneiodonium (A) or anti-phospho-histone H3 and Hoechst fluorescence microscopy at 24 h after hydroxyurea and diphenyleneiodonium release (B). Bar, 25 μm. Serum-starved MCF7 cells were treated for 4 h without (left) and with 10 μmol/L diphenyleneiodonium (right). Following removal of diphenyleneiodonium from the culture medium, the cells were incubated in the presence of paclitaxel, in the absence and presence of 10% FCS, as indicated. After 24 h, the cells were subjected to propidium iodide flow cytometry (C).
Diphenyleneiodonium also has a potent and prolonged inhibitory effect on cell cycle progression of the MCF7 cells in the G1 phase, and this distinct cell cycle block is clearly different from the reversible effect of anti-oxidants on the progression from G1-to-S phase (11). The diphenyleneiodonium-induced G1 cell cycle arrest also occurs with Rat 1 fibroblasts, because diphenyleneiodonium fails to cause the entire population of these cells to accumulate with a 4N DNA content (13). Interestingly, the cell cycle inhibitory effect of diphenyleneiodonium does not occur at all stages of the cell cycle, as exposure of hydroxyurea-treated cells to diphenyleneiodonium does not preclude their subsequent progression to G2 phase upon removal of the drugs. Nonetheless, although brief exposure to diphenyleneiodonium does not prevent hydroxyurea-released cells from undergoing S phase, these cells subsequently fail to complete G2 phase, as they do not reach mitosis when released into paclitaxel-containing medium. Thus, despite the absence of diphenyleneiodonium in the culture medium, the cells arrest in G2 phase of the cell cycle due to the prior exposure to this drug. This diphenyleneiodonium-induced G2 arrest of MCF7 cells, which seems masked in a nonsynchronous population of cells due to potent G2 arrest, is reminiscent of the previously reported diphenyleneiodonium-mediated G2 arrest of Rat1 fibroblasts (13). Unlike the G2 arrest of Rat1 cells, the G2 arrest of MCF7 cells seems irreversible. HepG2 cells also seem to undergo irreversible cell cycle arrest by diphenyleneiodonium.2 It will therefore be interesting to determine whether these cell typespecific differences correlate with transformation of the cells.

Diphenyleneiodonium has been well characterized as a flavoprotein inhibitor, and its anti-proliferative activity has been interpreted in terms of its ability to inhibit NAD(P)H oxidase–mediated generation of mitogenic reactive oxygen species (9–12). However, the related flavoprotein inhibitor diphenyliodonium (16) was without effect on the cell cycle, indicating that diphenyleneiodonium may be a novel antimitotic pharmacophore. The ability of diphenyleneiodonium to abrogate cell cycle progression out of G1 further underscores its potent cell cycle inhibitory activity. In light of its ability to cause prolonged inhibition of cell cycle progression following drug-release of the cells and the precedent of its in vivo administration (28, 29), the application of diphenyleneiodonium, or related antimitotic pharmacophores, in anticancer strategies therefore warrants evaluation.

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Robin M. Scaife


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