Published in final edited form as:

Free Radic Biol Med. 2013 April ; 57: 162–175. doi:10.1016/j.freeradbiomed.2013.01.002.

Effects of Iodonium-Class Flavin Dehydrogenase Inhibitors on Growth, Reactive Oxygen Production, Cell Cycle Progression, NADPH Oxidase 1 Levels, and Gene Expression in Human Colon Cancer Cells and Xenografts

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Abstract

Iodonium-class flavoprotein dehydrogenase inhibitors have been demonstrated to possess antiproliferative potential and to inhibit reactive oxygen production in human tumor cells, although the mechanism(s) that explain the relationship between altered cell growth and the generation of reactive oxygen species (ROS) remain an area of active investigation. Because of the ability of these compounds to inhibit the activity of flavoprotein-containing epithelial NADPH oxidases, we chose to examine the effects of several iodonium-class flavoprotein inhibitors on human colon cancer cell lines that express high, functional levels of a single such oxidase (NADPH oxidase 1 [Nox1]). We found that diphenylene iodonium (DPI), di-2-thienyliodonium (DTI), and iodoniumdiphenyl inhibited the growth of Caco2, HT-29, and LS-174T colon cancer cells at concentrations (10–250 nM for DPI, 0.5–2.5 μ M for DTI, and 155 nM to 10 μ M for iodoniumdiphenyl) substantially lower than for DU145 human prostate cancer cells that do not possess functional NADPH oxidase activity. Drug treatment was associated with decreased H₂O₂ production and diminished intracellular ROS levels, lasting up to 24 hr, following short-term (1-hr) exposure to the iodonium analogs. Decreased tumor cell proliferation was caused, in part, by a profound block in cell cycle progression at the G₁/S interface in both LS-174T and HT-29 cells exposed to either DPI or DTI; and the G₁ block was produced, for LS-174T cells, by upregulation of p27 and a drug concentration-related decrease in the expression of cyclins D1, A, and E that was partially prevented by exogenous H₂O₂. Not only did DPI and DTI decrease intracellular

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ROS, they both also significantly decreased the mRNA expression levels of Nox1, potentially contributing to the prolonged reduction in tumor cell reactive oxygen levels. We also found that DPI and DTI significantly decreased the growth of both HT-29 and LS-174T human tumor xenografts, at dose levels that produced peak plasma concentrations similar to those utilized for our in vitro experiments. These findings suggest that iodonium analogs have therapeutic potential for NADPH oxidase-containing human colon cancers in vivo, and that at least part of their antineoplastic mechanism of action may be related to targeting Nox1.

Keywords

NADPH oxidase; Nox1; diphenyleneiodonium; colon cancer; gene expression

Introduction

Six members of the epithelial NADPH oxidase (Nox) family (Nox1, 3, 4, and 5; Dual oxidase 1 and 2) that are distinct from the leukocyte NADPH oxidase (Nox2) have been discovered over the past decade; each protein demonstrates substantial sequence homology to the white blood cell oxidase; however, the physiological functions of these epithelial enzymes are only now being clarified [1]. Reactive oxygen species (ROS) are generated by the epithelial Nox proteins in a wide variety of organs and tissues [2]. In addition to an established function in host defense [3, 4], recent studies have indicated that Nox-related ROS formation plays an essential role in the proliferative and invasive capacity of several types of epithelial malignancies, as well as the ability of certain tumors to enhance angiogenesis [5–9]. Studies from our laboratory have revealed that members of the Nox family are distributed in an organ-specific fashion in human tumors, and that the expression of functional Nox proteins in cancer cells can be significantly induced by pro-inflammatory cytokines [10, 11]. These experiments suggest that small molecule inhibitors directed against members of the Nox family might have substantive therapeutic activity against human tumors, if compounds with an appropriate therapeutic index and specificity could be developed [12–16].

Nox1 is expressed in a variety of tissues including vascular smooth muscle, osteoclasts, and normal gastric and colonic mucosa [17]; there is also increasing evidence suggesting that Nox1 is overexpressed in many human colonic adenocarcinomas [10]. The catalytic subunit of Nox1 contains NADPH and FAD binding sites, and associates with p22^{phox} as well as homologues of the granulocyte oxidase complex, p47^{phox} (Nox1 organizer; NoxO1) and p67^{phox} (Nox1 activator; NoxA1), at the plasma membrane together with the GTPase Rac1. Superoxide production, following cytokine or growth factor stimulation, for example, is the result of electron transfer from intracellular NADPH to the Nox1 heme moieties [18]. Regulation of the catalytic function of Nox1, in addition to that provided by the required assembly of the individual components of the Nox1 complex, has recently been shown to be related to the phosphorylation of NoxA1 at serine 282 and serine 172 controlled by the MAPK cascade [19, 20]; in colon cancer cells, changes in Rac1 GTP that directly affect Nox1 activity are a consequence of c-Src tyrosine kinase activity [21]. These recent studies suggest that Nox1 activity might also be sensitive to the levels of protein phosphatases that function interactively with these kinases to maintain phosphorylation homeostasis.

Iodonium-class flavoprotein dehydrogenase inhibitors have been employed to block the activity of NADPH oxidases since the demonstration by Cross and colleagues of the capacity of these compounds to inhibit the oxidative burst of leukocytes $\approx$ 25 years ago [22]. Early mechanistic studies revealed that diphenyleneiodonium (DPI) is activated to a radical intermediate following interaction with flavin-containing components of Nox2 (probably

FAD) [23], leading to the formation of relatively stable covalent adducts that block electron flow from NADPH to molecular oxygen [24]. In particular, it has been suggested that at low nanomolar concentrations DPI directly affects the heme component of gp91^{phox} [25]. Thus, both DPI, as well as di-2-thienyliodonium (DTI), have been utilized to investigate the functions of a variety of different flavoproteins, including the Nox family oxidases, for many years [26–28]. However, in most such studies, DPI has been employed at concentrations 5 μ M to inhibit Nox-dependent reactive oxygen production [29]. Unfortunately, at such high concentrations, DPI can increase, rather than inhibit, oxidative stress by altering components of the pentose phosphate shunt, leading to diminished intracellular reduced glutathione pools and a subsequent decrease in the capacity to detoxify hydrogen and lipid peroxides [30]. Furthermore, DPI can potentially alter mitochondrial electron transport at concentrations 1 μ M [30, 31]. Non-flavin dehydrogenase-dependent cell systems (such as ion channels) are also inhibited by high levels of DPI through mechanisms that are poorly understood [26, 32].

In light of these observations, it is not surprising that DPI has been shown to possess antitumor activity in vitro [33, 34]. We sought, in a recent study, to develop a broader understanding of the potential antiproliferative mechanisms of action of iodonium-class dehydrogenase inhibitors [35]. In that work, DPI and DTI produced unique patterns of tumor growth inhibition across a panel of 60 human tumor cell lines; furthermore, DPI was highly active at nanomolar levels of potency, concentration levels that did not alter mitochondrial reactive oxygen production [24, 35]. The expression of one member of the Nox family (Nox1) was found to be related to growth inhibition by DPI; however, the levels of expression of the entire Nox gene family across the NCI-60 tumor cell panel were relatively low, making it difficult to define Nox1 as a definitive antiproliferative molecular target for iodonium-class molecules.

For the present investigation, we examined the effects of DPI and DTI on proliferation, reactive oxygen production, cell cycle progression, and gene expression in human colon cancer cell lines that possess high levels of functional Nox1. We also evaluated the antitumor efficacy of both DPI and DTI on human colon cancer xenografts in immunocompromised mice, as well as the pharmacokinetics of these agents in vivo. Our results demonstrate that iodonium compounds produce significant growth inhibition, both in vitro and in vivo, that is at least in part due to a ROS-related block in cell cycle progression across the G₁ boundary. Furthermore, we found that both DPI and DTI inhibit not only the oxidase function of Nox1 but also its expression at the RNA level in human colon cancer cells, at drug concentrations that approximate those that can be achieved in vivo.

Materials and methods

Materials

Diphenyleneiodonium sulfate (DPI) and di-2-thienyliodonium chloride (DTI) were obtained from Colour Your Enzyme (Ontario, Canada); they were also produced by the Developmental Therapeutics Program of the National Cancer Institute. Diphenyliodonium chloride and internal standard (I.S.) were purchased from Aldrich Chemical Co. (Milwaukee, WI); iodonium diphenyl (ID) was also purchased from Sigma (St. Louis, MO). Acetonitrile and methanol were of HPLC-grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (ACS grade) was from J.T. Baker (Phillipsburg, NJ). Trifluoroacetic acid, catalase, and the L- and D-isomers of NG-nitroarginine methyl ester (L-NAME, D-NAME) were purchased from Sigma (St. Louis, MO). Water was purified using the Millipore Milli-Q system (Milford, MA). Mouse plasma for preparation of standards and quality controls (QC) was obtained from the City of Hope National Medical Center Animal Resource Center. Pharmacokinetic studies and the experiments to evaluate of

the therapeutic effect of the iodonium analogs in athymic mice were reviewed and approved by the City of Hope Animal Care Committee. Archival tumor tissue frozen following surgery from patients with primary colon cancers and corresponding normal tissues, as well as from colon cancers metastatic to the liver were obtained from the City of Hope Comprehensive Cancer Center Pathology Core Facility after appropriate review and approval of this study by the City of Hope Human Research Committee.

Cell culture

Tumor cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were propagated in the media and under the conditions recommended by the ATCC, as previously described [10]. Culture media and supplements were purchased from Irvine Scientific (Santa Ana, CA).

RNA isolation, cDNA preparation, and reverse transcription PCR

RNA was isolated from tissues after disruption with a Polytron homogenizer using RNA-zol B (TEL-TEST Inc., Friendswood, TX) according to the manufacturer's instructions. Genomic DNA contamination was removed with DNase I treatment (Ambion, Austin, TX). RNA from cell lines was isolated with an RNAqueous-4PCR kit (Ambion, Austin, TX). The quality of isolation was tested on 1% agarose gels (SeaKem, FMC, Rockland, ME). The RNA concentration and A_{260}/A_{280} ratio were measured with a UV spectrophotometer.

cDNA was prepared from 3–4 μ g RNA in a 20 μ l volume, using MMLV reverse transcriptase enzyme and random hexamers as primers (Invitrogen, Carlsbad, CA). cDNA synthesis was performed for 45 min at 42 °C, followed by 5 min denaturation at 75 °C. The reaction was boosted by addition of 1000 units MMLV reverse transcriptase enzyme and repeating the 42 °C step for another 60 min. The enzyme was inactivated for 5 min at 95 °C, and the cDNA was stored at –20 °C until used. Quantification of the level of gene expression was carried out from the cDNA samples with a real-time PCR method.

RT-PCR was performed in a 20 μ l final volume adding 1 μ l cDNA from each sample, using TaqMan Universal PCR mix (Applied Biosystems, Foster City, CA). The PCR amplification was performed on 384 well plates using the default cycling conditions and was detected by an ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The sequences of the primers and probes utilized for the quantitation of members of the NADPH oxidase gene family have been previously described [10]. Primers were also obtained for the quantitation of relative mRNA expression as Taqman Gene Expression Assays (ABI) for the following genes: CAT (Hs00156308_m1), GPX1 (Hs00829989_gH), CCNA2 (Hs00153138_m1), CCNB1 (Hs00259126_m1), CDC25A (Hs00153168_m1), FEN1 (Hs00748727_s1), HDAC4 (Hs00195814_m1), PIK3R1 (Hs00236128_m1), CDKN1A (Hs00355782_m1), PDK4 (Hs01037712_m1), CTGF (Hs00170014_m1), DUSP4 (Hs01027785_m1), CYP1A1 (Hs01054797_g1), PPARA (Hs00947539_m1), and PPARG (Hs01115513_m1). For the absolute calibration curve of the target genes and a housekeeping gene (18S or β -actin), serial dilutions of the plasmids (10^7 to 1 copy range) containing the gene insert were used. NOX1 plasmid was kindly provided by Drs. B. Banfi and K-H. Krause (Geneva, Switzerland); the 18S gene was cloned into the pCRII TA cloning vector in our laboratory (Invitrogen, Carlsbad, CA). Relative gene expression was determined as the ratio of the gene of interest to the internal reference gene expression based on the standard curves.

Northern analysis

Northern analysis was performed with 25–30 μ g total RNA loaded on 1% denaturing (glyoxal) agarose gels (run for 2–3 hours at 50V) using a Northern MaxGly kit according to

the manufacturer's instructions (Ambion, Austin, TX). After electrophoresis, the RNA was transferred to the BrightStar-Plus membrane with semi-dry electroblot system (BioRad, San Diego, Ca) for 90 min at 250 mA. We prepared the probes with asymmetric amplification (StripeZTM PCR Kit, Ambion, Austin, TX) according to the manufacturer's instructions using linearized NOX1 (kindly provided by Drs. B. Banfi and K-H. Krause, Geneva, Switzerland) and 18S plasmids as targets and the PCR primers in a concentration of 0.2 $\mu\text{mol/l}$ (antisense) and 0.002 $\mu\text{mol/l}$ (sense). We labeled the reaction with 5 μCi [$\alpha^{32}\text{P}$] dATP (Perkin/Elmer, Boston, MA). The non-incorporated labeled nucleotides were removed with a QIAquick Nucleotide Removal Kit (Qiagen, Valencia, CA). The membrane was hybridized at 42 °C overnight and washed once with Wash Buffer I for 30 min at room temperature and twice with Wash Buffer II for 30 min at 55 °C. The membrane was exposed to film (−80 °C) or phosphorimager screen (RT) overnight. The screening was done on the Typhoon 9410 scanner (Amersham Biosciences Corp. Piscataway, NJ) and analyzed with ImageQuant software.

Cell proliferation

The effect of iodonium analogs on the growth of human cancer cells was examined using the HT-29, LS-174T, Caco2, and DU145 human tumor lines, representing three colon cancers that express Nox1 at high level, and one prostate cancer line (DU145) that demonstrates minimal expression of any Nox homolog [10]. The cells were treated with DPI, DTI, or iodoniumdiphenyl (ID) over a range of concentrations spanning several orders of magnitude (1 nM to 50 μM). After 48 hr of drug treatment, the cells were stained with trypan blue and counted in a hemocytometer; the data have been presented as the concentration of iodonium analog that produced $\approx 50\%$ inhibition of tumor cell growth relative to untreated control cells (IC_{50}).

Extracellular H₂O₂ production

Tumor cells in logarithmic-phase growth, usually 2–3 days after plating, were harvested, washed with phosphate-buffered saline (PBS), and counted. $1\text{--}2 \times 10^6$ cells were treated with the indicated concentrations of DPI, DTI, or media for 1 hr; untreated cells were collected and washed once with Ringer's solution. Measurement of the effect of iodonium analogs on levels of extracellular H₂O₂ was performed with the Amplex Red[®] Hydrogen Peroxide Assay Kit (A-12212) (Molecular Probes, Eugene OR) using a microtitre plate reader equipped with a fluorescence spectrometer. The assay is based on the detection of H₂O₂ using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red[®] reagent), a highly sensitive and stable detector of H₂O₂. In the presence of horseradish peroxidase, the Amplex Red[®] reagent reacts with H₂O₂ in a 1:1 stoichiometry to produce highly fluorescent resorufin. Stock solutions were prepared according to the manufacturer's protocol. The reaction mixtures were prepared by adding 25 μl of Amplex Red[®] stock solution and 50 μl horseradish peroxidase stock solution to 10 ml of Krebs-Ringer buffer. Into individual wells of the microtitre plate were added 100 μl of the diluted standard, controls, and PBS-washed cell suspensions (10^5 cells). The reaction was started by adding 100 μl of the Amplex Red[®]/horseradish peroxidase reaction mixture; the fluorescence was recorded for 30 min at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Extracellular H₂O₂ production was calculated using an H₂O₂ standard curve from fluorescence data measured at 30 min in the presence or absence of DPI, DTI, or ID. Control experiments demonstrated that H₂O₂ production was linear over the range of 10^5 to 10^6 cells per well, and from 0 to 60 min.

Detection of reactive oxygen species by flow cytometry

Tumor cells were prepared in a fashion similar to that used for determination of H₂O₂ levels; 1.5 to 2×10^6 cells were treated with the indicated concentrations of DPI, DTI, other

respiratory or nitric oxide inhibitors, or media for 1 hr; untreated cells were collected and washed once with Ringer's solution. Cell pellets were resuspended in 1 ml of Ringer's solution, and the fluorescent probe CM-H₂DCF-DA (5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, Molecular Probes, Eugene, OR) was added to the cells in a final concentration of 1 μ M for 5 min. Care was taken to shield cells from ambient light. The samples were analyzed immediately, or at the time intervals indicated following careful washing in Ringer's solution, with a MoFlo MLS flow cytometer (Cytomation, Fort Collins, CO) using an excitation wavelength of 488 nm and an emission wavelength of 515–540 nm. Control tumor cell samples were also analyzed without exposure to CM-H₂DCF-DA. Detection of ROS by this method relies on the oxidation of the intracellular dye by a variety of reactive species, which enhances fluorescence intensity. Results that have been presented are indicative of the data from at least three independent experiments.

Effect of DPI and DTI on oxygen consumption by HT-29 human colon cancer cells

The effects of DPI and DTI on oxygen consumption by intact HT-29 cells were examined with a Strathkelvin model 928 six-channel oxygen system equipped with an RC650 respirometer and model 1302 electrodes (Strathkelvin Instruments, North Lanarkshire, Scotland, UK). The final, 2-ml reaction system contained 5×10^6 HT-29 cells/ml in PBS with 10 mM glucose. Buffers were bubbled with air at 37 °C prior to use. DPI, DTI, or an equal volume of buffer were added at the indicated final concentrations to the temperature-controlled respirometer cells containing the HT-29 cells for 30 min at 37 °C; immediately following pre-incubation, dissolved oxygen concentrations were measured every 20 sec for 20 min. The data represent the mean $\pm$ SD of 3–5 independent determinations for each DPI or DTI concentration.

Cell cycle analysis and TUNEL assay for apoptosis

Tumor cells in logarithmic-phase growth in T75 flasks were synchronized by thorough washing in PBS, replating in serum-free medium, and growth for 24 hr in the absence of serum. Following 24 hr of serum starvation, zero time cells were harvested with trypsin and counted. For cells to be examined subsequently, media was replaced by complete media with serum; after 3–4 hr of growth in serum, DPI, DTI, or media were added at the indicated concentrations for 24 hr. Tumor cells were then harvested for analysis, counted, and $2\text{--}5 \times 10^6$ cells were suspended in 0.5 ml of complete media. Cells were added drop-wise into 5 ml of ice-cold 1 % paraformaldehyde, and incubated on ice for 15 min. The cells were then pelleted, washed in cold PBS, re-pelleted, and finally resuspended in 5 ml of cold 70 % ethanol, and stored for 1–4 days at –20 °C. Biotin-dUTP was incorporated into 3'-end strand breaks to detect apoptotic cells in a TdT-mediated dUTP nick end labeling (TUNEL) reaction. Cells were labeled with avidin-FITC for visualization by flow cytometry, and with propidium iodide for concomitant cell cycle analysis. Flow cytometry was performed at the City of Hope Cancer Center Cytometry Core Facility on a MoFlo MLS flow cytometer. Data was acquired using dual laser excitation. Scatter signals were acquired with a HeNe laser (Melles Groit, Carlsbad, CA). All fluorescence excitation was performed at 488 nm using an Innova-90 Argon laser (Coherent, Santa Clara, CA) at 500 mW. FITC emission was measured through a 530DF30 filter. Propidium iodide incorporation was measured through a 640EFLP filter. The two fluorescent signals were separated with a 580DRLP and a 630DRLP dichroic filter. All filters were purchased from Omega Optical (Brattleboro, VT). Data was acquired and analyzed with Summit software (DAKO Cytomation).

Western analysis

Tumor cells were collected and washed once with PBS. Lysates were prepared by homogenizing cells in a lysis buffer containing 10 mM Tris (pH 8.0), 140 mM NaCl, 0.025% NaN₃, 1mM EDTA, 1mM phenylmethylsulfonylfluoride (PMSF), 1% triton X-100,

and 50 U/ml protease inhibitor cocktail. The lysates were left on ice before centrifugation at $10,000 \times g$ for 30 min. Protein was measured using the method of Lowry. Whole cell lysates (20 μ g protein per lane) were denatured by boiling in 2 $\times$ Laemmli sample buffer and resolved by 10.0 % SDS-PAGE. The proteins were transferred to PVDF membranes (Amersham, Arlington Heights, IL) by electroblotting using Tris buffer containing 10 % methanol. The blots were blocked with 20% horse serum and probed with the indicated antibodies obtained from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA) for the following proteins: cyclin A (sc-751), cyclin E (sc-247), cyclin D1 (sc-450), p21 (sc-6246), p27 (sc-528), and β -actin (sc-1616). Blots were then incubated with HRP-linked secondary antibody followed by ECL detection. β -actin was measured as the protein loading control. The blots were quantified using ImageQuant software (Molecular Dynamics).

Microarray analysis

Samples of total RNA from tumor cells treated with iodonium analogs or solvent were analyzed on Affymetrix HG:U133A 2.0 arrays at the DNA & Protein MicroArray Facility, University of California, Irvine following prescribed protocols in the Affymetrix GeneChip[®] Expression Analysis Technical Manual. Quality assessment, preprocessing, and statistical analyses of gene expression data were performed using the R statistical language and environment (<http://www.r-project.org>) and Bioconductor packages (<http://www.bioconductor.org>). Raw data was processed by using the Robust Multi-Chip Average (RMA) method to obtain gene expression values. Genes with an expression value < 80 in all samples were excluded from further analysis. The Bioconductor package “limma” was used to identify the genes differentially expressed between the control and iodonium-treated samples. The R-contributed libraries “limma” (<http://bioinf.wehi.edu.au/limma/>) provided the necessary routines for fitting linear models to microarray data that incorporate appropriate empirical Bayes smoothing of variances and for making multiple testing adjustments using the false discovery rate (FDR). We used FDR < 0.05 and fold change > 1.5 to identify differential expression. Genes not satisfying these criteria were excluded. Hierarchical clustering using average linkage with the Pearson correlation as similarity metric was applied to differentially expressed gene expression data using the clustering features in GeneSpring v7.2 (Agilent, Palo Alto, CA).

Pharmacokinetics of DPI and DTI after intraperitoneal administration in the mouse

LC-MS/MS analysis was performed using an Agilent Technologies LC 1100 series system (Palo Alto, CA, USA) interfaced with a Micromass Quattro Ultima Triple Quadrupole Mass Spectrometer (Micromass, Inc., Beverly, MA, USA). HPLC separation was achieved using gradient elution across a Phenomenex MercuryMS Aqua C₁₈ analytical column (3 μ , 20 $\times$ 2.0 mm) and C₁₈ guard column (Torrance, CA, USA). The column temperature was maintained at 25 °C and the autosampler temperature was kept at 4 °C. Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The linear gradient program went from 0–100% A over 5.5 min with a total run time of 12 min. Electrospray ionization was carried out in positive ion mode and quantitation was performed using multiple reaction monitoring (MRM). The precursor→product ion combinations chosen for quantitation of DPI, DTI, and I.S. were 279.16→152.17 m/z, 293.04→209.98, and 281.05→154.16, respectively. The flow rate of nitrogen was 190 l/hr for the cone gas and 500 l/hr for the desolvation gas, respectively. The capillary voltage was set to 1.1kV, and the cone voltages and collision energies were optimized to 58 V and 29 eV for DPI, 36 V and 21 eV for DTI, and 32 V and 14 eV for I.S. The source temperature was 125 °C and the desolvation temperature was 320 °C. MassLynx version 3.5 software was used for data acquisition and processing.

For preparation of plasma samples, a volume of 25 μ l plasma was spiked with 5 μ l of I.S. (50 ng/ml in water) and protein was precipitated with 100 μ l of methanol. After thorough mixing and centrifugation in an Eppendorf microcentrifuge at 14,000 rpm for 3 min, 100 μ l of supernatant was removed to a new 0.6-ml tube and mixed with 35 μ l of 0.25% trifluoroacetic acid. The mixture was then centrifuged at maximum speed and 4 °C for 10 min. Finally, the resulting supernatant was transferred to an auto-injector vial and 20 μ l was injected onto the column. The average recoveries across the range of the standard curves were 64.8% and 59.8% for DPI and DTI, respectively. The lower limit of quantitation (LLOQ), defined as S/N = 3, was 0.05 ng/ml for both DPI and DTI. The precision of the assay at the LLOQ was 3.3% for DPI and 9.8% for DTI, and the accuracy was 96.1% and 103.4% for DPI and DTI, respectively. Ion suppression and matrix effects were determined to be negligible, and short-term stability studies demonstrated that DPI and DTI were stable in plasma and plasma extract for more than 24 hr at 4 °C.

It should be pointed out that the levels of protein binding of either DPI or DTI to mouse plasma were not determined in these experiments, and have not been published previously based on a review of the current literature. The recovery data reported above are a characteristic of the assay and are not related to protein binding per se. We report here the total drug concentrations in plasma only.

Mouse pharmacokinetic experiments were performed in duplicate for each compound and the combined plasma concentration-versus-time data from the two studies were analyzed using non-compartmental methods to determine the following mean population pharmacokinetic parameters: maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), area under the concentration-versus-time curve to the last measured time point (AUC_{0-t}), elimination rate constant (K_{el}), area under the concentration-versus-time curve extrapolated to infinity (AUC_{0-inf}), and elimination half-life ($T_{1/2}$). For both colon cancer xenograft models, mice were treated with either 2 mg/kg DPI or 6 mg/kg DTI by single intraperitoneal injection and groups of 3 animals were sacrificed at each time point for serial collection of plasma. These pharmacokinetic experiments were reviewed and approved by the City of Hope Animal Care Committee.

Effect of DPI and DTI on the growth of HT-29 and LS174T human tumor xenografts

Human tumor xenografts were established by subcutaneous injection of one million LS174T or 2.5×10^5 HT-29 cells bilaterally in the flanks of 6- to 8-week old male athymic mice (Charles River Laboratories, Inc. Wilmington, MA). Treatment with an NADPH-oxidase inhibitor was initiated three days following tumor implantation and continued on a daily times five schedule, five days out of every seven. DPI and DTI were delivered by intraperitoneal injection at doses of 2 mg/kg and 6 mg/kg, respectively. Animals bearing HT-29 xenografts were studied for a total of 26–28 days; animals bearing LS-174T xenografts were studied for a total of 18 days. All mice were euthanized when evidence of skin breakdown over the xenografts became apparent. Control animals received an equal volume of 0.9% sodium chloride solution. Tumor volumes were calculated from bi-dimensional measurements, and average tumor volume was calculated using the formula $0.5 \times L \times W^2$ and plotted vs. time. Results for the HT-29 xenografts were determined from 6–8 tumors per time point; data for LS-174T xenografts was collected from 16 tumors per time point. All xenograft studies were reviewed and approved by the City of Hope Animal Care Committee.

Statistical analysis

Two-tailed Student's *t*-tests were performed; values of $P < 0.05$ were considered significant; NS designates values that were not significant. Xenograft data were analyzed using a two-way ANOVA. All values presented are the mean $\pm$ SEM or SD of at least three experiments.

Results

Growth-retarding effects of iodonium-class flavin dehydrogenase inhibitors in cancer cells that possess Nox1

To develop a suitable model for examination of the therapeutic activity of three iodonium flavin dehydrogenase inhibitors (Fig. 1A), we first evaluated a series of human tumor cell lines for their Nox1 expression levels. As shown in Fig. 1B, four human colon cancer lines (HT-29, LS-174T, LS-180, and Caco2) but not other human tumor cell lines, including breast cancers (COH-BR6, BT474, MDA-MB468, and MCF-7) and malignant melanoma cells (A2058, SK-MEL5), expressed Nox1 at the mRNA level. In this context, we also demonstrated Nox1 expression (Fig. 1C) in primary colon cancers (patients 5, 7, and 9) and in some colorectal cancer metastases to the liver (patients 2, 3, and 6). Our previous study demonstrated that HT-29, LS-174T, and Caco2 colon cancer lines do not express other members of the NOX gene family [10]. The three iodonium analogs were then examined for their ability to inhibit tumor cell growth in vitro (Table 1). The IC_{50} values for DPI were in the nanomolar range for all three colon cancer cell lines. On the other hand, for DU145 prostate carcinoma cells that do not express any Nox isoform [10], $>2.5 \mu\text{M}$ DPI concentrations were required to produce a 50% inhibition of tumor cell growth. DTI and iodoniumdiphenyl were both less potent inhibitors of tumor proliferation than DPI, but in every case all three colon cancer lines were more sensitive to these agents than the DU145 prostate cells.

DPI and DTI decrease steady-state reactive oxygen production in human colon cancer cells

To determine whether DPI- and/or DTI-induced alterations in reactive oxygen production accompanied the growth inhibitory activity of the iodonium flavin dehydrogenase inhibitors, the effects of DPI and DTI on H_2O_2 flux were evaluated in three colon cancer lines (Table 2). Because of its relative lack of potency as an inhibitor of cell growth, iodonium diphenyl was not investigated in these experiments; furthermore, the modest baseline H_2O_2 production by DU145 cells (at the lower limit of detection of the assay) suggested that additional studies with this cell line would be unrevealing. As shown in Table 2, both DPI and DTI produced a concentration-related inhibition of H_2O_2 formation in all three human colon cancer cell lines, with DPI or DTI significantly decreasing H_2O_2 production at 100 nM or 1–10 μM concentrations, respectively. At these concentrations, at least for HT-29 cells, neither DPI nor DTI significantly diminished whole cell oxygen consumption rates, which primarily represent the rate of mitochondrial respiration (Supplemental Figs. 1A and B).

To evaluate the effects of the iodonium analogs using a different measure of reactive oxygen status, we determined whether a 1-hr exposure to either DPI or DTI altered net intracellular DCF fluorescence. We found that both agents decreased reactive oxygen production by ≈ 25 –50% in LS-174T and HT-29 colon cancer cells (Figs. 2A–C). Consistent with our measurements of H_2O_2 flux, intracellular ROS levels measured by DCF fluorescence were also clearly decreased in HT-29 cells after a 1-hr exposure to lower concentrations of DPI (200 nM) or DTI (5 μM) (Supplemental Fig. 1C). Furthermore, when the recovery of intracellular ROS levels was evaluated (following washing of the cells to remove DPI or DTI in the media) at time intervals up to 24 hr following the 1-hr exposure to the iodonium

analogs, we found that both compounds produced prolonged inhibition of ROS in both cell lines (Figs. 2B and C). For HT-29 cells exposed to DPI and LS-174T cells exposed to DTI, DCF fluorescence had returned to control values 24 hr following completion of drug exposure.

Because both DPI and DTI have previously been reported to alter the activity of a variety of flavin dehydrogenases (including nitric oxide synthase [28], elements of the mitochondrial electron transport chain [36], and xanthine oxidase [37]), we examined whether inhibitors of these dehydrogenases affected ROS levels in LS-174 and HT-29 cells. As shown in Figs. 3A and C, neither L-NAME, antimycin A, nor allopurinol changed steady-state levels of DCF fluorescence in the two colon cancer cell lines. Furthermore, the nitric oxide synthase inhibitor L-NAME, unlike the iodonium compounds, had no effect on the growth of LS-174T or HT-29 cells following 48 hr of drug exposure (Fig. 3B).

DPI and DTI block cell cycle progression across the G₁ boundary

To elucidate potential mechanisms responsible for the growth inhibitory action of DPI and DTI, we evaluated the effects of these agents on cell cycle progression and the induction of apoptosis using drug concentrations consistent with those required to diminish H₂O₂ production. We found that a 24-hr exposure to DPI or DTI in either LS-174T or HT-29 cells produced a significant G₁ block in cell cycle progression (Figs. 4A and B). For LS-174T cells exposed to DPI but not to DTI, the G₁ block was associated with a substantial increase in apoptosis (TUNEL positivity) originating primarily from cells at the G₁/S interface. HT-29 cells, on the other hand, were more resistant to apoptosis, which was only modestly increased following DTI exposure (Fig. 4B).

To better understand the altered cell cycle progression produced by iodonium analogs, the expression of selected cell cycle proteins was analyzed in LS-174T cells following 24-hr drug treatment. DPI, at nanomolar concentration levels, dramatically decreased the expression of cyclin D₁, cyclin E, and cyclin A; the inhibitory effect of DTI on cell cycle protein expression was clear for cyclin A (Fig. 4C). Both compounds also significantly increased p27 expression, which could also have contributed to the block at G₁ we observed [38]. Under similar experimental conditions, when LS-174T cells were exposed to H₂O₂ (75 μM) for 24 hr, in the presence of DPI or DTI, we found that the inhibition of cyclin A, D₁, and E expression produced by DPI was partially (cyclin A and cyclin D₁) or completely (cyclin E) prevented (Fig. 4D). The co-administration of H₂O₂ also appeared to reverse the reduction in cyclin A expression produced by DTI. To evaluate further the role of H₂O₂ levels on the expression of cell cycle-associated proteins that regulate the G₁/S interface, we exposed both LS-174T and HT-29 cells to extracellular catalase for 48 hr (Supplemental Fig. 2). We found that the presence of extracellular catalase, in a concentration-dependent manner, decreased the expression of cyclins A, D₁, and E in both cell lines, in a fashion quite similar to that produced by the iodonium analogs. Furthermore, catalase exposure was also associated with a marked increase in p27 (and to a lesser extent p21) expression in both cell lines. Heat-inactivated catalase did not produce similar effects.

Effects of DPI and DTI on Nox1 expression, as well as the expression of antioxidant and proliferation-related genes in colon cancer cells

While the covalent binding of an activated radical intermediate of DPI to flavin-containing components of Nox2 was described almost two decades ago [23], there has been relatively little further work to define the mechanism(s) by which iodonium analogs alter the function of other, recently-discovered members of the Nox family [39]. Because steady state ROS levels after a 1-hr exposure to DPI or DTI in both LS-174T and HT-29 cells had not fully recovered for several hr following drug treatment, we considered the possibility that

mechanisms other than direct, inhibitory flavoprotein binding might be involved in decreased ROS production. Under experimental conditions similar to those employed to evaluate drug-related alterations in cell cycle progression and the expression of cell cycle proteins involved in the control of the G₁/S interface, we found that both DPI and DTI significantly decreased the mRNA expression of Nox1 itself, as well as several of the essential members of the Nox1 complex (NoxO1, NoxA1, p22^{phox}) in LS-174T cells (Fig. 5). At the same time, neither iodonium analog altered the expression of catalase or glutathione peroxidase 1, suggesting that our results were not simply due to non-specific, toxic effects of the agents. In related experiments under identical conditions, we found that DPI and DTI also inhibited, by 80% and 40%, respectively, the expression of Nox1 in HT-29 cells.

Because the observed changes produced by the iodonium compounds on Nox1 expression levels were unexpected, we chose to perform a broader analysis of the effects of DPI and DTI on gene expression in LS-174T cells using mRNA expression arrays. As demonstrated in Supplemental Tables 1 and 2 (which provide the detailed description of all the gene expression changes of at least 1.5-fold in magnitude, both up and down, that accompanied drug exposure) and in Table 3 (where some of the alterations in the expression of members of major gene families have been confirmed by real time RT-PCR), the iodonium compounds produce complex alterations in several molecular pathways that are essential for cell proliferation. The microarray results that have been confirmed by RT-PCR reveal, in addition to the downregulation of Nox1 mRNA by both DPI and DTI, that these compounds inhibit the expression of critical signaling pathways (including CDC25A), cell cycle proteins (cyclin A2 and B1), and genes essential for cellular proliferation (HDAC4) and DNA repair (FEN1). Exposure of LS-174T cells to DPI and DTI also upregulates the cell cycle regulatory protein p21, DUSP4 (a critical phosphatase that negatively modulates MAPK signaling), as well as several genes essential for glucose and lipid metabolism (PDK4, PPAR α , and PPAR γ).

Pharmacokinetics of DPI and DTI in non-tumor bearing mice

The mean population pharmacokinetic parameters and plasma concentration versus time curves for DTI and DPI are shown in Table 4 and Fig. 6, respectively. The mean C_{max} for DPI was 166 ng/ml (597 nM) and the T_{max} occurred at 10 min following the intraperitoneal dose (Fig. 6A). The mean elimination T_{1/2} for DPI was 2.0 hr, significantly slower than for DTI. As a result, DPI was detectable in plasma for up to 8 hr after the dose, with a mean plasma concentration at 8 hr of 1.0 ng/ml (3.6 nM). The AUC_{0-t} and AUC_{0-inf} for DPI were 107 (384) and 110 (395) ng/ml (nM) x hr, respectively. DTI reached an average C_{max} of 953 ng/ml (3260 nM) within 1 min following intraperitoneal injection. Subsequent elimination of DTI from plasma was rapid with a mean elimination T_{1/2} of 4.8 min. As a result, DTI fell below the LLOQ within 1 hr after the dose. The AUC_{0-t} and AUC_{0-inf} for DTI were 82 (280) and 86 (294) ng/ml (nM) x hr, respectively.

DPI and DTI decrease tumor growth in human colon cancer xenografts

Preliminary experiments revealed that the maximally tolerated doses of DPI and DTI in athymic mice were 2 mg/kg and 6 mg/kg, respectively, when the agents were administered intraperitoneally on a daily for five days in a row schedule (five days out of every seven). At this treatment level, animals lost < 10% of their lean body mass after several weeks of dosing and exhibited no abnormal signs or symptoms suggesting that these compounds were inducing any severe adverse effects. As shown in Fig. 7, administration of either DPI (Fig. 7A and C) or DTI (Fig. 7B and D) to athymic mice bearing HT-29 or LS-174T xenografts produced a significant ($p < 0.0001$) decrease in tumor growth. In these experiments, the therapeutic effects of DPI and DTI were tested in vivo against colon cancer xenografts

generated from cell lines known to express high levels of Nox1 in vitro. It should be pointed out, however, that the expression of other Nox homologues has been shown to be upregulated by pro-inflammatory cytokines [40]; hence, it is possible that Nox1 expression in vitro might not be completely predictive of Nox1 expression in a tumor xenograft, a phenomenon that, if demonstrated, could enhance the therapeutic potential of the iodonium flavoprotein inhibitors.

Discussion

In this study, we evaluated the growth inhibitory effects of DPI and DTI in cell culture and xenograft models. Our goals for these investigations were to elucidate potential anticancer mechanisms of action for the compounds and to evaluate the possibility that reactive oxygen species, possibly generated by the Nox1 complex, contributed to the proliferative capacity of human colon cancer cells. We found that both DPI (in the nanomolar concentration range) and DTI (in the low micromolar range) effectively diminished the growth of colon cancer cell lines that expressed Nox1; on the other hand, a human prostate cancer line that is devoid of Nox expression [10] was much less sensitive to any of the iodonium analogs evaluated. Of perhaps greater interest, we found that both DPI and DTI possess potent antiproliferative activity in two colon cancer xenograft models (Fig. 7). This observation is important because our pharmacokinetic studies revealed that the peak plasma concentrations of both DPI (166 ng/ml or ≈ 600 nM) and DTI (953 ng/ml or ≈ 3.3 μ M) after a single intraperitoneal injection approximate the concentrations of these agents that produced a prolonged decrease in intracellular ROS levels in vitro (Fig. 2). It should be acknowledged, however, that in the absence of data on the binding of DPI or DTI to mouse or bovine plasma the actual free drug concentrations that were effective in decreasing ROS production or tumor growth in vitro or in vivo are not unequivocally known.

Although both DTI and DPI are rapidly cleared from plasma (DTI faster than DPI; Table 4), the concentrations achieved in vivo—if associated with iodonium drug activation and covalent binding to flavoproteins—could be sufficient to produce the prolonged inhibition of oxidase function we observed. Our pharmacokinetic results may also help to explain how anticancer activity for these agents could have been produced following administration on a schedule that employed a single daily dose. In this regard, the unexpected observation that both DPI and DTI significantly decreased the expression of Nox1 in LS-174T and HT-29 cells may provide a second mechanism by which these compounds diminished steady state ROS levels in tumor cells, contributing to a decrease in their proliferative potential.

Growth inhibition by DPI and DTI in tissue culture was accompanied by a block in cell cycle progression at the G₁/S interface. Accumulation of cells in G₁ following DPI exposure has been described previously in human fibroblasts following exposure to low nanomolar DPI concentrations [41]. In our experiments, both growth inhibition and the G₁ block for human colon cancer cells occurred at drug levels known to be achievable in vivo. Furthermore, at nanomolar concentrations of DPI and low micromolar concentrations of DTI, we demonstrated that mitochondrial respiration (measured as whole cell oxygen consumption in glucose) was not altered by either compound (Supplemental Fig. 1). These data are consistent with those of Hancock and Jones who found that the IC₅₀ concentrations for inhibition of the rat peritoneal macrophage respiratory burst were submicromolar for both DPI and DTI, whereas inhibition of macrophage mitochondrial respiration occurred at 13 or 21 μ M, respectively, for the two compounds [24]. We also found that an inhibitor of nitric oxide synthase had no effect on either ROS levels or tumor cell growth in HT-29 or LS-174T cells. Taken together, these results indicate that both Nox1 (in colon cancer cells) and Nox2 (in macrophages) are significantly more sensitive to the inhibitory effects of the iodonium analogs than are components of the mitochondrial electron transport chain,

suggesting that the use of these compounds at low concentrations provides a degree of specificity for certain flavin dehydrogenases in cell culture that has not been regularly appreciated.

The underlying mechanism(s) causing the G₁ block produced by iodonium analogs appear to be associated with a drug-related downregulation of cyclins D₁, E, and A, as well as the activation of p27 [42]. However, understanding the mechanisms by which altered ROS levels regulate cyclin or p27 expression remains an area of active current investigation [43]. In findings that are similar to those presented herein, Menon and colleagues demonstrated that exposure of mouse embryo fibroblasts to the thiol-containing antioxidant, N-acetyl-L-cysteine, produced a profound block in cell cycle progression at the G₁ interface that was temporally associated with a significant decrease in ROS and cyclin D₁ levels, and a marked increase in p27 expression [38]. They postulated the presence of a ROS-sensitive G₁ checkpoint that might be related to redox-sensitive cysteine moieties in both p27 and cyclin D₁. Other investigators have suggested that ROS play an essential role in the late G₁ phase of the cell cycle by altering the degradation of cyclin A associated with the anaphase promoting complex [44].

Co-administration of H₂O₂ with the iodonium analogs partially prevented inhibition of cyclin expression or p27 activation (Fig. 4D), supporting a relationship between a DPI- or DTI-related decrease in ROS levels and diminished cyclin expression. However, these experiments do not explain how decreased ROS levels actually lead to altered cyclin expression. In a recent study [35], we demonstrated that exposure of HT-29 cells to low nanomolar concentrations of DPI produced an increase in protein tyrosine and serine/threonine phosphatase activity, and a consequent decrease in signal transduction through the MAPK pathway. The expression of cyclin D₁ is under the control of ERK [45], the activation of which is substantially decreased following DPI exposure and up-regulation of protein phosphatase activity levels in HT-29 cells [35]. Thus, it can be hypothesized that down regulation of phospho-ERK, which could be a consequence of diminished steady state ROS levels, might explain our results. This supposition is consistent with a substantial body of literature supporting the oxidant sensitivity of the MAPK pathway [46]; however, our current study does not provide direct evidence for this or other potential oxidative mechanism(s) that might be involved in producing a DPI- or DTI-induced G₁ arrest in human colon cancer cells.

Because of the prolonged inhibition of steady-state ROS levels in iodonium-treated colon cancer cells, we sought to determine whether mechanisms in addition to covalent drug binding to flavoproteins might contribute to decreased oxidant tone. We unexpectedly found that expression of components of the Nox1 complex were significantly inhibited by DTI and DPI. The lack of an effect of these compounds on the expression of antioxidant genes in these cells suggests that this was not a non-specific toxicity of iodonium exposure. A recent investigation utilizing smooth muscle cells suggested that Nox1 expression is regulated by NF- κ B [47], which itself is under oxidant control [48]. Nox1 expression also appears to be under the redox-dependent control of NF- κ B and the level of reduced protein tyrosine phosphatases in hepatocytes [49]. If the Nox1 that is expressed in human colon cancer is under similar regulatory control, it is possible that decreased ROS levels could play a direct feedback role in modulating the inhibition of Nox1 expression we observed. However, additional studies will be required to define the molecular pathways that are involved in the down regulation of Nox1 by iodonium analogs in colon cancer cells.

Using microarray analysis and real time RT-PCR, we also found that the expression of growth-related genes unrelated to the Nox family is altered by iodonium analog exposure. For some of these genes, such as p21, there is evidence that intracellular ROS levels regulate

their expression [50]. However, we also found that the expression of additional genes, such as the phosphatases DUSP4 and CDC25A, which play important roles in cell signaling, were altered by DPI and DTI; their regulation by ROS has not been well-documented previously. Our current results, however, are consistent with a prior bioinformatic investigation suggesting that DPI and DTI could modulate several aspects of tumor cell signal transduction through the MAPK pathway [35]. In any case, the data presented in Table 3 and Supplemental Table 1 will provide a new focus for further investigation of the mechanism of action of the iodonium analogs.

In summary, our experiments suggest that the iodonium analogs DPI and DTI have therapeutic potential, both in cell culture and in vivo, particularly for colon cancer cells that produce substantial levels of ROS. When DPI is utilized at low nanomolar concentrations, it appears to provide a more specific degree of Nox inhibition than has commonly been appreciated; furthermore, both iodonium analogs not only inhibit the oxidase activity of Nox1, but also its mRNA expression. As modeled in Fig. 8, we propose that a decrease in the steady state level of ROS, produced by DPI or DTI, plays an important role in activating p27 and decreasing the expression of critical cyclins that control the G₁ interface, leading to a significant inhibition of tumor cell proliferation. More broadly, it seems clear that DPI and DTI, at least in human colon cancer cells, initiate a range of heretofore unexplored antiproliferative effects and alterations in critical signaling pathways that deserve further investigation. Finally, the anticancer activity of the parent DPI and DTI molecules in vivo suggests that a search for more potent and ever more specific analogs would be appropriate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported with federal funds from the Center for Cancer Research and the Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institutes of Health. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

Abbreviations used in the text

Nox	NADPH oxidase
ROS	reactive oxygen species
DPI	diphenyleneiodonium
DTI	di-2-thienyliodonium
ID	iodoniumdiphenyl

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- DPI and DTI potently block growth and ROS production in human colon cancer cells
- Growth inhibition is associated with a G₁ cell cycle block and upregulation of p27
- Iodonium analogs also downregulate Nox1 mRNA expression in colon cancer cells
- Plasma concentrations of DPI and DTI in vivo are correlated with growth arrest
- Both DPI and DTI are active therapeutic agents in colon cancer xenografts

Fig. 1.

Chemical structures of iodonium analogs and expression of Nox1 mRNA in human tumor cells and colon cancer. (A) Structures of diphenyleneiodonium, di-2-thienyliodonium, and iodoniumdiphenyl. (B) The mRNA expression of Nox1 was examined in 10 human tumor lines; Nox1 mRNA is detectable in the four colon cancer cell lines but not in the four breast cancer or two melanoma lines examined. (C) Nox1 is expressed in surgically-resected primary colon cancers (shown for patients 5, 9, and 7; T represents 'tumor' and N stands for adjacent 'normal' colonic tissue). Nox 1 was also detected in colon cancers metastatic to the liver for patients 2, 3, and 6; Nox1 expression was also found in adjacent, non-involved liver for patient 3.

Fig. 2.

Diphenyleiiodonium (DPI) and di-2-thienyliodonium (DTI) decrease steady-state levels of ROS following a 1-hr exposure in LS-174T and HT-29 human colon cancer cells. (A) After a 1-hr exposure to 1 μ M DPI or to 1–10 μ M DTI, intracellular ROS, measured by the fluorescence of the dye DCFH-DA following conversion to DCF, was diminished by 25–40%. These data are representative of 3–8 experiments with similar results for both compounds and cell lines; $P < 0.05$ for 1 μ M DPI or 10 μ M DTI for both cell lines. Panels (B) and (C) demonstrate the levels of ROS in LS-174T and HT-29 cells following a 1-hr exposure to either 1 μ M DPI (B) or 10 μ M DTI (C), represented as the 0 min data points, and then subsequent changes in ROS levels 60, 120, and 180 min and 24 hr following removal of the iodonium analogs. The data are representative of several studies; relative quantitation of DCF fluorescence for each time point compared to control cells is provided by the bar graphs.

Fig. 3.

Inhibitors of nitric oxide synthase, xanthine oxidase, and mitochondrial electron transport do not change steady state ROS levels in colon cancer cell lines. Panels (A) and (B) Treatment for 1 hr with the nitric oxide synthase inhibitor L-NAME did not alter steady state ROS levels in either LS-174T or HT-29 cells; furthermore, a 48 hr exposure to L-NAME at concentrations from 0.5 to 4 mM had no effect on tumor cell growth. (C) A 1-hr exposure to the xanthine oxidase inhibitor allopurinol (1 mM) or to the mitochondrial electron transport chain inhibitor antimycin A (1 μg/ml) did not alter steady state ROS levels in LS-174T or HT-29 cells. These results are representative of three similar experiments.

Fig. 4.

DPI and DTI block cell cycle progression at the G₁/S checkpoint in both LS-174T and HT-29 cells. In panels (A) and (B), a 24-hr exposure to DPI (250 nM) or DTI (5 μM) produced a block at G₁ in the cell cycle that only for DPI-treated LS-174T cells was associated with an increase in apoptosis. (C) Western analysis of cell cycle regulatory proteins in LS-174T cells treated with various concentrations of either DPI or DTI for 24 hr; β-actin has been used as the loading control. (D) Western analysis of cell cycle regulatory proteins in LS-174T cells exposed to DPI, DTI, or an iodonium analog plus H₂O₂ (75 μM) for 24 hr; β-actin is the loading control. These data are representative of a minimum of three independent experiments.

Fig. 5.

DPI and DTI exposure decreases the mRNA expression of Nox1 and components of the Nox1 oxidase complex. LS-174T cells were treated with DPI (250 nM) or DTI (10 μM) for 24 hr, and the expression levels of Nox1, NoxO1, NoxA1, p22^{phox}, catalase, and glutathione peroxidase 1 were measured by real time RT-PCR. The data represent the % of untreated control expression ± SE of three separate experiments; * = $P < 0.05$.

Fig. 6. Plasma pharmacokinetics of (A) DPI and (B) DTI after intraperitoneal injection of 2 mg/kg or 6 mg/kg DPI or DTI, respectively. The individual time points represent the mean $\pm$ SD of triplicate determinations on duplicate plasma samples of the levels of DPI or DTI.

Fig. 7.

DPI and DTI significantly decrease the growth of HT-29 and LS-174T colon cancer xenografts in athymic mice. In these experiments, mice were treated with 2 mg/kg DPI or 6 mg/kg DTI by intraperitoneal injection on a daily x five schedule for five days out of every seven. Matched control mice bearing xenografts received an equal volume of saline on the same treatment schedule. (A) Four mice were injected subcutaneously with 2.5×10^5 HT-29 cells on each flank (bilaterally); points represent the average tumor volume $\pm$ SE of 8 tumors. (B) In similar fashion, three mice were injected bilaterally with HT-29 cells; points represent the mean tumor volume for 6 tumors. Panels (C) and (D) Eight mice in each group were injected bilaterally with 10^6 LS-174T cells by the subcutaneous route; points represent the mean tumor volume $\pm$ SE of 16 tumors. Significance was determined using a two-way analysis of variance (ANOVA).

Fig. 8. Hypothetical model to describe the effects of DPI and DTI on LS-174T and HT-29 colon cancer cells. We propose that decreased steady state ROS levels, produced by DPI or DTI, play an essential role in regulating p27 activation and decreasing the expression of critical cyclins that control the G₁ interface, leading to a significant inhibition of colon cancer cell proliferation.

Table 1

Effect of iodonium flavin dehydrogenase inhibitors on tumor cell growth

Tumor cell line	Diphenyleneiodonium	Di-2-thienyliodonium	Iodoniumdiphenyl
Caco2	≈10 nM ^a	≈500 nM	≈155 nM
HT-29	≈100 nM	≈2500 nM	≈5800 nM
LS-174T	≈250 nM	≈2500 nM	≈10000 nM
DU145	>2500 nM	≈3500 nM	>10000 nM

^aIC₅₀ = concentration of agent producing 50% inhibition of tumor cell growth.

Table 2

Effect of iodonium flavin dehydrogenase inhibitors on extracellular H₂O₂ flux produced by human cancer cell lines

Treatment	Tumor Cell Line			
	DU145	LS-174T	Caco2	HT-29
Control	0.2 ± 0.1 ^a	1.8 ± 0.1	2.2 ± 0.1	5.0 ± 0.1
Diphenyleneiodonium				
0.1 μM		83 ± 8 ^b	ND ^c	82 ± 2 ^d
1.0 μM		84 ± 5 ^d	53 ± 6 ^d	87 ± 4 ^d
10 μM		67 ± 5 ^d	33 ± 3 ^d	44 ± 2 ^d
Di-2-thienyliodonium				
1.0 μM		92 ± 7	40 ± 4 ^d	48 ± 3 ^d
10 μM		57 ± 3 ^d	12 ± 4 ^d	20 ± 2 ^d

^aPicomoles H₂O₂ /10⁵ cells/min; mean ± SE; n = 6.

^bPercent of control (mean ± SE).

^cND = not determined.

^dP < 0.05 when compared to untreated control cells.

Table 3

Gene expression determined by real time RT-PCR in LS-174T cells treated with DPI (250 nM), DTI (10 μ M), or solvent control for 24 hr^a

Genes	Control	DPI	DTI
<i>Down-regulated</i>			
CCNA2	229,175 $\pm$ 20,970	168,846 $\pm$ 31,730	143,270 $\pm$ 58,490 ^b
CCNB1	222,979 $\pm$ 8,680	126,082 $\pm$ 18,584 ^b	127,258 $\pm$ 12,117 ^b
CDC25A	11,659 $\pm$ 1,798	6,189 $\pm$ 1,422 ^b	8,917 $\pm$ 1,188
FEN1	304,774 $\pm$ 23,404	156,881 $\pm$ 33,640 ^b	353,223 $\pm$ 22,497
HDAC4	23,417 $\pm$ 4,026	10,522 $\pm$ 2,009 ^b	18,126 $\pm$ 3,650
PIK3R1	263,381 $\pm$ 16,943	154,788 $\pm$ 17,233 ^b	266,554 $\pm$ 31,914
<i>Up-regulated</i>			
CDKN1A (p21)	1,179,200 $\pm$ 329,190	2,981,866 $\pm$ 778,541 ^b	2,383,641 $\pm$ 459,889 ^b
PDK4	2,028 $\pm$ 388	11,830 $\pm$ 3,704 ^b	4,633 $\pm$ 1750
CTGF	6,368 $\pm$ 1,419	15,662 $\pm$ 3,725 ^b	12,161 $\pm$ 820 ^b
DUSP4	89,057 $\pm$ 7,559	187,672 $\pm$ 27,917 ^b	141,381 $\pm$ 13,613 ^b
CYP1A1	4,260 $\pm$ 2,417	146,373 $\pm$ 45,343 ^b	434,337 $\pm$ 15,663 ^b
PPARA	25,079 $\pm$ 946	46,329 $\pm$ 4,192 ^b	358,983 $\pm$ 4,388 ^b
PPARG	349,525 $\pm$ 26,858	653,104 $\pm$ 65,604 ^b	577,444 $\pm$ 34,455 ^b

^a Gene expression calculated as a ratio of the target gene expression to β -actin $\times 10^{-6}$; data represent the mean $\pm$ SE of 6–8 independent experiments.

^b $P < 0.05$ when compared to untreated control cells.

Table 4

Average plasma pharmacokinetic parameters

Parmameter	DTI	DPI
C _{max} (ng/ml)	953	166
T _{max} (hr)	0.017	0.17
AUC _{0-t} (ng/mlxhr)	82	107
K _{el} (hr ⁻¹)	9.1	0.36
AUC _{0-inf} (ng/mlxhr)	86	110
T _{1/2} (hr)	0.08	2.0