

Research Paper

Iodine Alters Gene Expression in the MCF7 Breast Cancer Cell Line: Evidence for an Anti-Estrogen Effect of Iodine

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The protective effects of iodine on breast cancer have been postulated from epidemiologic evidence and described in animal models. The molecular mechanisms responsible have not been identified but laboratory evidence suggests that iodine may inhibit cancer promotion through modulation of the estrogen pathway. To elucidate the role of iodine in breast cancer, the effect of Lugol's iodine solution (5% I₂, 10% KI) on gene expression was analyzed in the estrogen responsive MCF-7 breast cancer cell line. Microarray analysis identified 29 genes that were up-regulated and 14 genes that were down-regulated in response to iodine/iodide treatment. The altered genes included several involved in hormone metabolism as well as genes involved in the regulation of cell cycle progression, growth and differentiation. Quantitative RT-PCR confirmed the array data demonstrating that iodine/iodide treatment increased the mRNA levels of several genes involved in estrogen metabolism (CYP1A1, CYP1B1, and AKR1C1) while decreasing the levels of the estrogen responsive genes TFF1 and WISP2. This report presents the results of the first gene array profiling of the response of a breast cancer cell line to iodine treatment. In addition to elucidating our understanding of the effects of iodine/iodide on breast cancer, this work suggests that iodine/iodide may be useful as an adjuvant therapy in the pharmacologic manipulation of the estrogen pathway in women with breast cancer.

Key words: Iodine, Gene Expression, Breast Cancer, Estrogen, Hormone Metabolism

Introduction

The high rate of breast disease in women with thyroid abnormalities (both dietary and clinical) suggests a correlation between thyroid and breast physiology [1-3]. In addition, women with breast cancer have larger thyroid volumes than controls [2]. Multiple studies suggest that abnormalities in iodine metabolism are the likely link [4-7]. Additionally, the impact of iodine therapy for the maintenance of healthy breast tissue has been reported in both animal [4-7] and clinical studies [8, 9] yet the mechanisms responsible remain unclear.

Iodide (I⁻) uptake is observed in approximately 80% of breast cancers as well as fibrocystic breast disease and lactating breasts; however, quantitatively, no significant iodide uptake is reported in normal, non-lactating breast tissue [10]. Clinical trials have demonstrated that women with cyclic mastalgia [9] or fibrocystic disease [8] can have symptomatic relief

from treatment with molecular iodine (I₂). Iodine deficiency, either dietary or pharmacologic, can lead to breast atypia and increased incidence of malignancy in animal models [11]. Furthermore, iodine treatment can reverse dysplasia which results from iodine deficiency [5]. Rat models using *N*-methyl-*N*-nitrosourea (NMU) and dimethyl-benz[*a*]anthracene (DMBA) to induce dysplasia and eventually carcinogenesis have shown that the presence of molecular iodine in the animal's diet can prevent tumor formation; yet, when iodine is removed from the diet, these animals develop tumors at rates comparable to those of control animals [5, 7]. These data suggest that iodine diminishes early cancer progression through an inhibitory effect on cancer initiating cells.

Evidence indicates that the impact of iodine treatment on breast tissue is independent of thyroid function. For example, iodine deficient rats given the thyroid hormone thyroxine (T₄) did not achieve reduced tumor growth following NMU treatment sug-

gesting that the effect of iodine on tumor growth is independent of the thyroid gland or thyroid hormone [7]. Additionally, Eskin *et al* and others have reported that administration of molecular iodine has a greater impact on tumor growth than the equivalent dose of iodide [5-9]. Since the thyroid primarily utilizes iodide as opposed to iodine [5], this data supports the hypothesis that iodine is not acting through the thyroid.

In addition to differences in the metabolism of iodine, the mechanisms of iodine and iodide uptake appear to differ. While iodide uptake is essentially via the Sodium-Iodide Symporter (NIS) in the thyroid, data suggests that iodine uptake in the breast may be NIS-independent, possibly through a facilitated diffusion system [12]. Together this data indicates that the effect of iodine on breast cancer progression is in part independent of thyroid function and suggests that iodine's protective effect on breast cancer progression is elicited through its direct interactions with breast cancer cells.

One proposed mechanism by which iodine may influence breast physiology and cancer progression is through an interaction with estrogen pathways. Qualitative changes in the estrogen receptor have been found in the breasts of iodine deficient rats compared to normal euthyroid animals suggesting that the iodine pathway may augment the synthesis of the estrogen receptor α (ER α) [13]. Furthermore, when estrogen-responsive and estrogen-independent tumors were transplanted into mice, estrogen-responsive tumors had higher radioactive iodine uptakes than estrogen-independent transplants [14]. Additionally, iodine deficiency induced atypia is worsened by estrogen addition [15]. Together, this data supports the hypothesis that an interaction exists between iodine and estrogen within the breast [16]. However, the precise molecular mechanisms responsible for this interaction remain unknown. We hypothesize that iodine effects breast physiology through an interaction with the estrogen pathway.

To test our hypothesis, we analyzed the effects of Lugol's iodine solution (5% I₂, 10% KI) on global gene expression in the estrogen responsive MCF-7 breast cancer cell line. Analysis of the gene expression profile was used to evaluate potential mechanisms of action of iodine.

Results

1mM iodide/iodine does not impact cellular proliferation or viability at 48 hours

Lugol's iodine solution, which contains 5.0% Iodine and 10% Iodide, was used to adjust standard RMPI 1640 medium to a concentration of either 1 mM or 5 mM Iodine/iodide. Medium was supplemented

with all-trans-retinoic acid (tRA) and 17 β -Estradiol (E2) for 24 hours prior to iodine treatment. Our data in figure 1 shows that at 48 hours, 1 mM iodine/iodide had no effect on cell proliferation or viability, relative to control cells. However, treatment with 5 mM iodine/iodide was toxic to the cells, inhibiting cell proliferation and reducing cell viability to less than 5% of control cells ($P < 0.01$). Since no significant change in proliferation or viability was observed with 1 mM iodine/iodide, this concentration was used for the gene array studies.

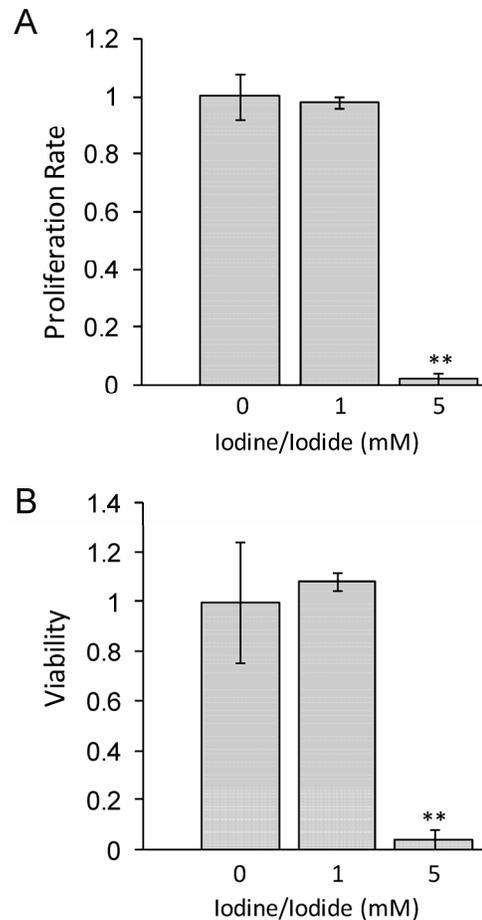


Figure 1: 1 mM iodine/iodide does not impact cell viability or proliferation at 48 hours. MCF-7 cells were grown in RPMI-1640 supplemented with 1 μ M tRA and 1 nM estradiol (control medium) or control medium supplemented with Lugol's iodine solution (5% iodine, 10% iodide) to a concentration of 1 mM iodine (1.0 mM iodine/iodide) or 5 mM iodine (5 mM iodine/iodide) for 48 hours and the effect on cell proliferation (A) and cell viability (B) was analyzed. Significant decrease in proliferation and viability was observed in the 5 mM iodine/iodide condition. Relative change in cell proliferation (A) and relative change in viability (B) for the control condition was set to one. Standard deviation is shown. ** denotes $P \leq 0.01$

Interestingly, it has been reported that iodine alone can induce apoptosis at concentrations as low as

1 μ M [14], however we did not see cytotoxicity even at significantly higher (1mM) doses. Although the reasons for this difference remain unknown, several possible explanations exist. First, it may reflect differences in the cell lines used. Second, the presence of all-*trans*-retinoic acid, and/or 17 β -estradiol may protect the MCF-7 cells from the cytotoxic effects of iodine previously reported. Finally, since previous studies have all used iodine alone while we used a combination of iodine and iodide, it is possible that the presence of iodide protects the MCF-7 cells from the detrimental effects of iodine. Indeed, it has been reported that iodide up to 5mM did not have a cytotoxic effect on MCF-7 cells [14]. Furthermore, this may explain why breast cancers, which have increased NIS expres-

sion [17, 18] and increased iodide [18] uptake do not undergo apoptosis.

Gene Expression Profiling

Gene expression profiling was performed in triplicate on MCF-7 control cells and MCF-7 cells treated for 48 hours with medium supplemented with Lugol's Iodine solution to 1mM iodine/iodide. As described in the methods, all cells were pretreated for 24 hours with tRA and 17 β -estradiol prior to beginning iodine treatments. Data normalization and analysis are described in the Methods and Material section. Common genes with a mean change greater than two fold were considered significantly changed. Twenty-nine genes were up-regulated (Table 1A) and fourteen genes were down regulated-regulated (Table 1B).

Table 1. MCF7 cells were treated with Lugol's iodine solution or vehicle alone for 48 hr (see Methods and Materials for details). RNA was isolated and subjected to Microarray Analysis (see Methods and Materials for details). 29 genes were upregulated \geq 2.0-fold (A) and 14 genes were down regulated \geq 2.0-fold (B) in response to treatment. Genes were then clustered into functional categories using the DAVID Bioinformatics Database Gene Functional Classification Tool (NIAID/NIH). The fold change in expression is relative to control cells. Bold genes were verified by qRT-PCR (Figure 2).

A. 29 Genes that are Up-Regulated ¹ in Response to Iodine Treatment ²			
Cell Cycle/Proliferation			Fold Change
NM_001673	ASNS	asparagine synthetase	4
L24498	GADD45A	growth arrest and DNA-damage-inducible, alpha³	2
Steroid Metabolism			
NM_000104	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1³	11.3
NM_001353	AKR1C1	aldo-keto reductase family 1, member C1³	6
NM_000499	CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1³	2.5
Transcription			
NM_003900	SQSTM1	sequestosome 1	2.8
AB025432	TSC22D3	delta sleep inducing peptide, immunoreactor	2.6
DNA repair			
NM_019058	DDIT4	DNA-damage-inducible transcript 4	2.2
L24498	GADD45A	growth arrest and DNA-damage-inducible, alpha³	2.0
Lipid Metabolism			
NM_000693	ALDH1A3	aldehyde dehydrogenase 1 family, member A3	3.2
NM_004315	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	2.2
tRNA synthesis			
NM_004184	WARS	tryptophanyl-tRNA synthetase	3
NM_002047	GARS	glycyl-tRNA synthetase	2.3
NM_003680	YARS	tyrosyl-tRNA synthetase	2
Other			
NM_021158	TRIB3	tribbles homolog 3 (Drosophila)	4
NM_005218	DEFB1	defensin, beta 1	3.3
NM_033197	C20orf114	chromosome 20 open reading frame 114	2.7
NM_004753	DHRS3	dehydrogenase/reductase (SDR family) member 3	2.7
NM_002083	GPX2	glutathione peroxidase 2 (gastrointestinal)³	2.5
NM_006636	MTHFD2	methylene tetrahydrofolate dehydrogenase	2.5
AF104032	SLC7A5	solute carrier family 7, member 5	2.4
NM_004864	GDF15	growth differentiation factor 15	2.2
NM_000416	IFNGR1	interferon gamma receptor 1	2.1
NM_002356	MARCKS	myristoylated alanine-rich protein kinase C substrate	2.0
Function Unknown			
AK001064	LOC86026	hypothetical protein DKFZp434P055	3.5
AK054816	ORAOV1	oral cancer overexpressed 1	3.0
NM_006470	TRIM16	tripartite motif-containing 16	2.4
AF245505	MXRA5	matrix-remodelling associated 5	2.3
AL162069	LOC144501	hypothetical protein LOC144501	2.2
BE884686	LTB4DH	leukotriene B4 12-hydroxydehydrogenase	2.1

B. 14 Genes that are Down-Regulated ⁴ in Response to Iodine Treatment ²			
Estrogen responsive genes			Fold Change
NM_003225	TFF1	trefoil factor 1 (estrogen-inducible sequence) ³	-2.3
NM_003881	WISP2	WNT1 inducible signaling pathway protein 2 ³	-2.4
Cell Cycle genes			
NM_003258	TK1	thymidine kinase 1, soluble	-3.7
NM_002305	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1)	-2.6
NM_007019	UBE2C	ubiquitin-conjugating enzyme E2C	-2.5
NM_001071	TYMS	thymidylate synthetase	-2.3
NM_053056	CCND1	cyclin D1 (PRAD1: parathyroid adenomatosis 1) ³	-2.0
NM_002466	MYBL2	v-myb myeloblastosis viral oncogene homolog like 2 ³	-2.2
Cell Growth/Proliferation Genes			
NM_005264	GFRA1	GDNF family receptor alpha 1 ³	-2.5
NM_007161	LST1	leukocyte specific transcript 1	-2.5
Ion Transport Genes			
NM_004669	CLIC3	chloride intracellular channel 3	-2.2
Chromatin Organization			
NM_002105	H2AFX	H2A histone family, member X	-2.2
Function Unknown			
NM_007173	PRSS23	protease, serine, 23	-3.7
NM_006408	AGR2	anterior gradient 2 homolog (Xenopus laevis)	-2.1

¹ Increase ≥ 2.0-fold relative to control

² Accession number is to left, followed by gene symbol, name, and fold change.

³ Verified by QRT-PCR

⁴ Decrease ≥ 2.0-fold relative to control

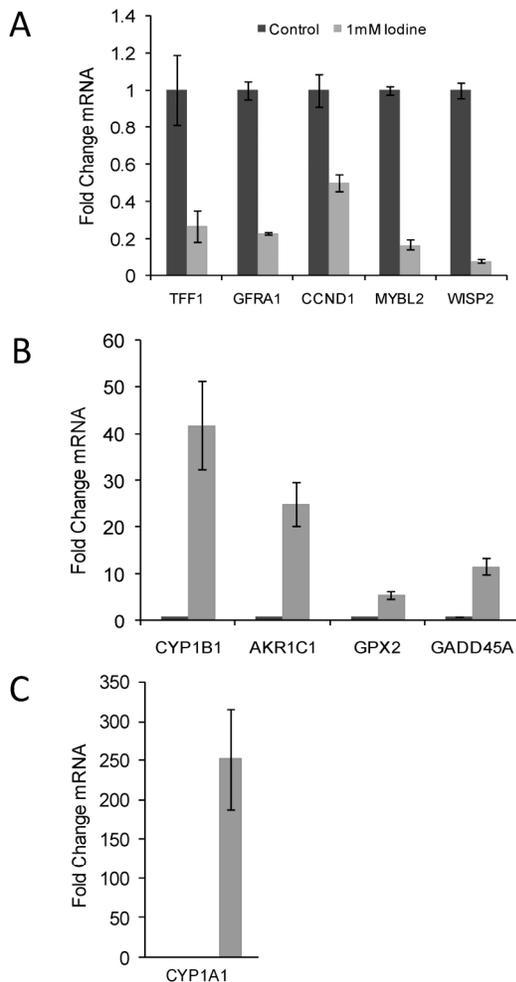


Figure 2: Quantitative RT-PCR confirmed the changes in gene expression identified by microarray analysis. RNA was isolated from control cell and cells grown in the presence of 1 mM iodine for 48 hours. QRT-PCR analysis of genes predicted by microarray analysis to be down-regulated (A) and up-regulated (B & C) in response to iodine treatment. Cyclophilin A was used as control for normalization. All 10 genes showed significant changes ($P < 0.03$) in response to iodine treatment in concordance with the array data. The mRNA level in the control samples was set to 1 and the fold change is shown. In panel C control bar is not visible. Dark bars represent control samples while grey bars represent samples treated with 1.0 mM Iodine. Standard Deviation bars are shown.

Included in the down-regulated genes were genes involved in cell cycle (LGALS1, UBE2C, TYMS, MYBL2, and CCND1) [19-25], cell growth/differentiation (GFRA1) [26], nucleotide synthesis (TK1 and TYMS) [27, 28], and ubiquitination and cyclin destruction (UBE2C) [19]. Up-regulated genes include genes involved in estrogen metabolism (CYP1A1, CYP1B1, AKR1C1) [29, 30], DNA repair (GADD45A and DDIT4) [31, 32], cell cycle and proliferation (ASNS and GADD45A) [33, 34], tRNA synthesis (WARS, GARS, YARS) [35-37] and transcription (SQSTM1 and DSIPI) [38, 39].

The list was compared to a set of genes with experimentally or computationally determined estrogen responsive elements (EREs) in their promoter region [40]. Nine (32%) of our up regulated genes (GADD45A, CYP1B1, TSC22D3, DDIT4, ASAH1, YARS, DHRS3, SLC7A5 and MARCKS) and five (38%) of our down

regulated genes (TFF1, WISP2, TYMS, CCND1, and H2AFX) have putative EREs in their promoter region further supporting an interaction between iodine/iodide and the estrogen pathway.

Quantitative RT-PCR confirmation of array data

10 genes of interest (5 up-regulated gene and 5 down-regulated genes) were chosen for quantitative RT-PCR confirmation. Up regulated genes included CYP1B1, AKR1C1, CYP1A1, GPX2, and GADD45A and the down regulated genes included GFRA1, TFF1, MYBL2, WISP2, and CCND1. Quantitative RT-PCR was run in triplicate and normalized to cyclophilin A. All ten genes demonstrated significant changes in steady state mRNA ($p < 0.03$) in response to 48 hr treatment with 1 mM iodine/iodide, confirming the accuracy of the array data (Figure 2). The two estrogen responsive genes (TFF1 and WISP2) showed a significant decrease in mRNA expression levels (Figure 2A) while the estrogen metabolism genes (CYP1A1, CYP1B1, and AKR1C1) demonstrated a significant increase in mRNA levels (Figures 2B and C).

Discussion

As the body of evidence builds, the importance of iodine on the maintenance of healthy breast tissue and its role in carcinogenesis becomes clearer. Unveiling iodine's mechanism of action is of crucial importance. Verifying the protective effects of iodine in breast cancer pathways will improve our understanding of breast cancer physiology and potentially lead researchers toward the development of novel treatments or enhancements of current therapies. In this study we provide the first gene array profiling of an estrogen responsive breast cancer cell line demonstrating that the combination of iodine and iodide alters gene expression. Among the list of altered genes were several genes documented to be estrogen responsive such as TFF1 and WISP2. Furthermore, the list contained several genes involved in the estrogen response including Phase I estrogen metabolizing enzymes (CYP1A1 and CYP1B) and Cyclin D1, a competitive inhibitor of BRCA1 [41].

Consistent with our initial hypothesis that iodine/iodide interacts with the estrogen pathway, we found that iodine/iodide altered mRNA expression of several genes involved in the estrogen pathway and down-regulated several estrogen responsive genes. Furthermore, many of the genes identified contain putative Estrogen Responsive Elements in their promoter region. One potential mechanism in which iodine/iodide can repress the estrogen effect on cellular metabolism is through alterations in the Cytochrome P450 pathway. Our data shows that treatment with iodine and iodide increases the mRNA levels of Cy-

tochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1), two estrogen phase I estrogen metabolizing enzymes that oxidizes 17 β -estradiol to 2-hydroxyestradiol (2-OH-E₂) and 4-hydroxyestradiol (4-OH-E₂), respectively. These catechol estrogens can be further oxidized to quinones. 3,4-estradiol quinone, a metabolite of 4-OH-E₂, has been shown to react with DNA forming de-purinating adducts resulting in genotoxicity [42], while data suggests that 2-OH-E₂ can be metabolized to 2-methoxyestradiol, an estrogen metabolite with anti-proliferative effects [43]. The observed increase in the CYP1A1/CYP1B1 ratio may shift the direction of estrogen metabolism favoring 2-OH-E₂ which may either directly affect proliferation through increasing 2-methoxyestradiol, decreasing 3, 4-estradiol quinone or indirectly via the inactivation of E₂. The importance of the CYP1A1/CYP1B1 ratio in-vivo is evident in the increased presence of 4-OH-E₂ in breast cancer tissue compared to non breast cancer controls [44]. However, the regulation and interplay between CYP1A1, CYP1B1, other Phase I and II enzymes and estrogen is complex, being influenced by multiple factors and multiple polymorphisms, thus more data is required to illuminate the importance of these changes in response to iodine.

In addition to affecting estrogen metabolism, iodine/iodide may also inhibit estrogen induced transcription via increased BRCA1 activity. BRCA1 is a known inhibitor of ER α transcription while Cyclin D1 is thought to enhance the estrogen response via a competitive inhibition with BRCA1 [41]. Our data demonstrates decreased Cyclin D1 mRNA which could result in decreased competitive inhibition of BRCA1 allowing BRCA1 to inhibit estrogen induced transcription. Increased transcription of GADD45A, CYP1B1, and CYP1A1 are consistent with increased BRCA1 activity [34, 45].

Either through its interactions with estrogen or through estrogen independent mechanisms, the combination of iodine and iodide seems to have an impact on genes involved with cell growth (GFRA1 and GDF15), cell cycle (LGALS1, UBE2C, MYBL2, TYMS, CCND1, ASNS, GADD45A), and differentiation (GFRA1, GDF15). Of particular interest is the down-regulation of GFRA1. GFRA1 has been shown to increase NUMB expression which in turn degrades NOTCH; the net result of decreased GFRA1 expression is increased NOTCH. NOTCH has been implicated in stem cell differentiation during breast development, preventing uncontrolled basal cell proliferation during alveolar development [46]. As such, iodine may play a crucial role during periods of breast maturation during puberty and pregnancy.

Finally, this data further supports the need for clinical studies involving the use of iodine/iodide in conjunction with current estrogen modulation therapies. Despite the efficacy of current treatments of breast cancer with medications such as Tamoxifen, the development of resistant cancers remains critically important. It has been found that CCND1 over-expression plays an important role in the development of Tamoxifen resistant breast cancer [25, 47-49]; our data shows that iodine/iodide treatment can decrease mRNA levels of CCND1. This provides two potential mechanisms by which iodine/iodide could enhance the efficacy of Tamoxifen therapy: 1) having an additive effect on estrogen inhibition and 2) inhibiting the expression of CCND1 thus preventing or slowing the development of Tamoxifen resistance.

The results presented in this paper build on the substantial epidemiologic, clinical and cellular data regarding the actions of iodine in breast physiology. We suggest that the protective effects of iodine/iodide on breast disease may be in part through the inhibition or modulation of estrogen pathways. Data presented suggests that iodine/iodide may inhibit the estrogen response through 1) up-regulating proteins involved in estrogen metabolism (specifically through increasing the CYP1A1/1B1 ratio), and 2) decreasing BRCA1 inhibition thus permitting its inhibition of estrogen responsive transcription. These data open the way for further defining pathways impacted by the essential element, iodine, in the cellular physiology of extra-thyroidal tissues, particularly the breast.

Materials and Methods

Cell lines.

Human MCF-7 breast cancer cells (ATTC, Manassas, VA) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Gemini Bio-Products, West Sacramento, CA) and incubated at 37°C with 5% CO₂.

Iodine and estrogen treatments.

24 hours prior to iodine treatment cells were grown in control medium consisting of RPMI 1640 supplemented with 1 μ M all-*trans*-retinoic acid (tRA) in DMSO vehicle and 1 nM 17 β -estradiol (Sigma-Aldrich, St. Louis, MO) in EtOH vehicle. DMSO and EtOH concentrations did not exceed 0.1% (v/v). Lugol's iodine solution (Sigma-Aldrich, St. Louis, MO) containing 5% I₂ and 10% KI was added to the experimental medium to a concentration of 1mM iodine/iodide. Cells were grown for an additional 48 hours.

Proliferation and Viability Assays.

To evaluate the effects of iodine on proliferation, MCF-7 cells were plated on 96 well plates. Cells were pretreated with tRA and estradiol control medium and treated with iodine as described above. MTT proliferation assay (ATTC, Manassas, VA) was performed in accordance with the manufacturer. To test cell viability, cells were grown on a six well plates, trypsinized, stained using ViaCount® Reagents (Guava Technologies, Hayward, CA) and analyzed using flow cytometry (Guava EasyCyte Mini.)

RNA isolation.

Following iodine treatment, total RNA was isolated using RNAsy mini kits (Qiagen, Valencia, CA) according to the manufacturer. RNA was quantified using a Bio-Mini DNA/RNA/Protein Analyzer (Shimadzu Scientific Instruments, Inc.).

Microarray Analysis.

Microarray slides were provided by the Genomic Facility at Drexel University College of Medicine containing 23,000 human 70mer oligos (Human Genome Oligo Set Version 2.0) on a glass slide. RNA amplification and labeling was performed using the MessageAmp™ aRNA Amplification Kit (Ambion, Austin, TX). Equal micrograms of fragmented, labeled aRNA was hybridized to a cDNA microarray using Slide-Hybe Buffer #1 (Ambion, Austin, TX). After 24 hours of hybridization microarray slides were washed and scanned on Axon 4000B Dual Laser Slide Scanner (Molecular Devices, Sunnyvale, CA.). Data was analyzed using GenePix (Molecular Devices, Sunnyvale, CA.) disregarding signals with a signal to noise ratio < 1 and a sum of the means <600. Three biological duplicates were then analyzed using the GenePix Auto-Processor (GPAP) program. Pre-processing and normalization of data was accomplished using R-project statistical environment (<http://www.r-project.org>) and Bioconductor (<http://www.bioconductor.org>) through the GPAP website (<http://darwin.biochem.okstate.edu/gpap>). The resulting data was annotated and analyzed using the DAVID Bioinformatics Database Gene Functional Classification Tool (NIAID/NIH). Genes with a greater than 2 fold change in 2 or more arrays were considered significant.

Quantitative RT-PCR.

Primer probe sets were purchased from Applied Biosystems (Foster City, CA). Probes and Assay ID included Cytchrome P450 1A1 (CYP1A1; Hs00153120_m1), Cytochrome P450 1B1 (CYP1B1; Hs00164383_m1), Aldo-keto Reductase 1C1 (AKR1C1; Hs00413886_m1), Glutathione Peroxidase 2 (GPX2;

Hs00702173_s1), Growth Arrest and DNA-Damage-Inducible α (GADD45A; Hs00169255_m1), trefoil factor 1 (TFF1; Hs00170216_m1), GDNF family receptor alpha 1 (GFRA1; Hs00237133_m1), Cyclin D1 (CCND1; Hs00277039_m1), v-myb myeloblastosis viral oncogene homolog (avian)-like 2 (MYBL2; Hs00231158_m1), and WNT1 inducible signaling pathway protein 2 (WISP2; Hs00180242_m1). Quantitative RT-PCR was performed using Brilliant QRT-PCR Master Mix kits (Stratagene, La Jolla, CA) and analyzed using the Mx3000P real-time PCR machine (Stratagene, La Jolla, CA). Cyclophilin A was used for normalization.

Conflict of interest

The authors have declared that no conflict of interest exists.

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